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Original Article

Astragaloside IV inhibits idiopathic pulmonary fibrosis through activation of autophagy by miR-21-mediated PTEN/PI3K/AKT/mTOR pathway

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Article Info

Abstract

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As the main active ingredient of Astragalus, Astragaloside IV (AS-IV) can ameliorate pulmonary fibrosis. In this experiment, we studied how AS-IV reduces idiopathic pulmonary fibrosis (IPF). Bleomycin (BLM) or TGF-β1 was treated in mice or alveolar epithelial cells to mimic IPF in vivo as well as in vitro. ASV-IV alleviated levels of inflammatory cytokines and fibrosis markers in IPF model. Through the detection of autophagy-related genes, ASV-IV was observed to induce autophagy in IPF. Besides, ASV-IV inhibited miR-21 expression in IPF models, and overexpression of miR-21 could reverse the protective potential of ASV-IV on IPF. PTEN was targeted by miR-21 and was up-regulated by ASV-IV in IPF models. In addition, levels of inflammatory cytokines and fibrosis markers, autophagy, as well as the PI3K/AKT/mTOR pathway regulated by ASV-IV could be neutralized after treatment with autophagy inhibitors, miR-21 mimics, or si-PTEN. Our study demonstrates that ASV-IV inhibits IPF through activation of autophagy by miR-21-mediated PTEN/PI3K/AKT/mTOR pathway, suggesting that ASV-IV could be acted to be a promising therapeutic method for IPF.

Keywords: Astragaloside IV, Autophagy, Idiopathic pulmonary fibrosis, miR-21, PI3K/AKT/mTOR, PTEN

1. Introduction

As a chronic, progressive, as well as fibrotic interstitial lung disease [1], idiopathic pulmonary fibrosis (IPF) was manifested as diffuse alveolar inflammation, massive proliferation of fibroblasts, massive deposition of extracellular matrix (ECM), as well as collapse of alveolar structure [2]. The cause of IPF may be related to environmental factors, such as dust, metal powder, wood chips, and smoking, with a certain genetic tendency [3]. Autophagy defects can promote myofibroblast metaplasia, accelerate ECM deposition, and enhance the process of fibrosis, so enhancing autophagy activity becomes an important target for the treatment of pulmonary fibrosis [4].

At present, there is no specific treatment for IPF, but traditional Chinese medicine (TCM) has distinct superiorities in IPF therapy, and numerous studies have revealed that TCM can mitigate the symptoms and lung function of IPF patients [5]. Astragalus is one of the most frequently used supplements in the treatment of pulmonary fibrosis [6]. Its main active ingredients include saponins, flavonoids, polysaccharides, alkaloids and so on [7]. Astragaloside IV (AS-IV) belongs to a kind of the major active ingredients of Astragaloside [8]. AS-IV has been proven to delay the progression of IPF by improving immune capacity, redu-

cing inflammatory damage, and regulating TGF-β1/Smads signaling pathway [9]. Literature has found that AS-IV can improve the level of autophagy [10], but whether AS-IV can delay IPF and its target by activating autophagy is unclear. Hence, it is essential to investigate the influence of AS-IV on autophagy in IPF and its regulatory mechanism for the development and exploration of TCM anti-pulmonary fibrosis drugs.

MicroRNA (miRNA) belongs to a kind of endogenous noncoding RNA with a length of around 23 nucleotides and widely exists in eukaryotic cells [11]. Mature miRNA can inhibit translation or lead to degradation by binding to specific sites of target gene mRNA, and miRNA often has multiple target genes [12]. A gene may be negatively regulated by multiple miRNAs, thus forming a complex miRNA-mRNA-protein regulatory network [13]. Aberrant expression of miRNA is implicated in the development of various diseases, and miRNA is of great significance for disease diagnosis and targeted therapy [14]. MiR-21 is a kind of miRNA widely expressed in multiple organs and has a momentous potential in the pathogenesis of pulmonary fibrosis [15, 16]. Literature has found that TGF-β1 elevates miR-21 expression in fibroblasts during the course of inducing pulmonary fibrosis, and overexpression

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of miR-21 can further enhance the pro-fibrotic function of TGF-β1 signaling in cells [17]. However, the relation between AS-IV and miR-21 in IPF remains obscure.

In our study, we majorly investigated the potential together with the possible mechanism of AS-IV in IPF and uncovered the regulatory relationship of AS-IV on miR-21 as well as its downstream signaling.

2. Material and methods
2.1. Ethics statement
The feeding, nursing, as well as experimental programs of all animals, were approved by the Animal Protection and Use Committee of the Second Affiliated Hospital of Shaanxi University of Chinese Medicine. All procedures on the mice were implemented following the guidelines from the National Institutes of Health.

2.2. IPF model and treatment
Forty SPF grade c57bl/6 mice were randomly separated into control group, BLM group, AS-IV group, as well as AS-IV + miR-21-agomir group. After anesthesia, the mice in the BLM group received injection of BLM (10 mg/kg, Nippon Kayaku, Japan) through the trachea to induce IPF, and the control group accepted injection with the same amount of normal saline. From the second day, AS-IV group and AS-IV + miR-21-agomir group were given AS-IV (40 mg/kg/d, Sigma-Aldrich, USA) by gavage for 28 days. During this period, mice in the AS-IV + miR-21-agomir group were injected with miR-21-agomir, (30 mg/kg/day) through tail vein for 28 days. Twenty-eight days later, all mice were sacrificed, and the lung tissues were excised for follow-up analyses.

2.3. Hematoxylin and eosin (H&E) staining
After the routine preparation of paraffin sections of the lung tissue specimens, the paraffin sections were put in an incubator at 30°C and lasted 1 h, followed by dewaxing with xylene as well as grading ethanol. The paraffin sections were coated on a glass slide and stained by hematoxylin for 5 min as well as eosin for 3 min. The specimens were histologically analyzed with a high-resolution image analyzer.

2.4. Masson staining
After the routine preparation of paraffin sections of the lung tissue specimens, the paraffin sections received staining by Masson tricolor blue. The specimens were dyed with hematoxylin for 6 min, Masson compound oil for 5 min, 5% phosphomolybdic acid for 5 min, as well as 2% aniline blue for 10 min. The specimens were placed on a slide followed by routine dehydration. The specimens were histologically analyzed with a high-resolution image analyzer.

2.5. Cell culture and treatment
American Type Culture Collection (ATCC, USA) offered the human type II alveolar epithelial cells (A549), which were cultivated in DMEM (Hyclone, USA) which contained 10% fetal bovine serum (Gibco, USA) at 37°C with 5% CO_2. The addition of 10 ng/L TGF-β1 to A549 cells induced the interstitial transformation of alveolar epithelial cells. The optimal concentration of AS-IV was determined by CCK-8 by adding 0~50 μmol/L AS-IV into the supernatant of cell culture. In addition, 10 mml/L

3-MA was treated in the cells to inhibit the autophagy activity of the cells. In addition, miR-21 mimics purchased from Gene Pharma (Shanghai, China) were treated into the cell culture supernatant to overexpress miR-21 and the expression of PTEN was silenced using si-PTEN (Gene Pharma, China). All these cell transfection was implemented with the help of Lipofectamine™ 3000 reagent (Invitrogen, USA).

2.6. ELISA
The right lung of the mice could be homogenized with 50 mmol/L PBS which included 0.5% hexadecylammonium bromide as well as 5 mmol/L EDTA. Followed by centrifugation of lung extracts for 15 minutes at 4°C, the supernatants could be obtained. TNF-α as well as IL-6 levels were examined with the help of the corresponding ELISA kit (R&D Systems, USA).

2.7. Immunofluorescence
The pulmonary tissues were prepared via soaking in formaldehyde solution (10%) and embedding in paraffin. After the antigen was heated in a water bath in 0.01 mol/L citric acid buffer, 30% H_2O_2 was added to inactivate the endogenous enzyme for 10 minutes. Afterwards, the slides were sealed with 5% BSA solution. After incubating with primary antibodies including α-SMA and collagen I at 4°C overnight, the sections were treated with Alexa 594 secondary antibody at room temperature for 60 min. Nuclei were counterstained with DAPI, and the sections were then captured with a fluorescence microscope.

For in vitro immunofluorescence, the cells were immobilized with 4% paraformaldehyde, permeabilization with 0.1% Triton-X 100, and blocked with 5% BSA for half an hour. After incubation with primary antibodies including α-SMA, collagen I and LC3B at 4°C overnight, cells were rinsed, and then treated with Alexa 594 secondary antibody. DAPI was adopted for dying the nucleus. The images were captured by a microscope.

2.8. Western blot
Lung tissue samples and cells were fully lysed using RIPA lysis buffer. The supernatant was boiled in an SDS sample buffer for 5 min for albuminous degeneration. Subsequently, the protein sample was separated on 10% SDS-PAGE and shifted to polyvinylidene fluoride (PVDF). The membrane was sealed with 5% skimmed milk for 2 h, followed by cultivation with primary antibodies including α-SMA, collagen I, LC3-I/II, Beclin-1, p62, PTEN, p-PI3K, PI3K, p-AKT, AKT, p-mTOR, mTOR as well as GAPDH at 4°C overnight. After 1 h of exposure to the secondary antibody at 37°C, the membrane was detected with an ECL reagent.

2.9. CCK-8
In each group, A549 cells (1×10^4 cells/mL) were planted in a 96-well plate overnight. Afterwards, the prepared 100 μL CCK-8 solution (Dojindo, Japan) was added into cells and cultured for 2 h. The absorbance at 450 nm was examined with a microplate reader (Thermo Fisher Scientific, USA).

2.10. RT-qPCR
Total RNA could be isolated with TRIzol Reagent (Takara, Japan). The PrimeScript™ RT-PCR Kit (Takara, Ja-

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Table 1. Forward and reverse primers of genes.

Genes		Forward (5'-3')	Reverse (5'-3')
miR-21	Human	GCCACCACACCAGCTAATTT	CTGAAGTCGCCATGCAGATA
	Mouse	ACACTCCAGCTGGGTAGCTTATCAGACTGA	TGGTGTCGTGGAGTCG
α-SMA	Human	CATCACCAACTGGGACGACATGGAA	GCATAGCCCTCATAGATGGGGACATTG
	Mouse	GGAACGCTTCACGAATTTG	CACCATCTCCAGAGTCCAGCACAAT
COL1A1	Human	GTGCTCCTGGTATTGCTGGT	GGCTCCTCGTTT TCCTTCTT
	Mouse	GGAGGGCGAGTGCTGTGCTTT	GGGACCAGGAGGACCAGGAAGT
PTEN	Human	TGGATTCGACTTAGACTTGACC	AGGATATTGTGCAACTCTGCAA
	Mouse	CACAGAATTCCAGACATGACAGCCATCATC	GTGGATCCTCTAGGTTTATCCCTCTTG
U6	Human	ATTGGAACGATACAGAGAAGATT	GGAACGCTTCACGAATTTG
	Mouse	GCTTCGGCAGCACATATACTAAAAT	CGCTTCAGAATTTGCGTGTCAT
GAPDH	Human	GCTGGCGCTGAGTACGTCGTGGAGT	CACAGTCTTCTGGGTGGCAGTGATGG
	Mouse	CGACTTCAACAGCAACTCCCACTCTTCC	TGGGTGGTCCAGGGTTTCTTACTCCTT

pan) was adopted for processing first-strand cDNA synthesis. Relative gene levels were assessed with the SYBR Green PCR Master Mix (Takara) on an iCyler iQ Real-Time PCR System (Bio-Rad Laboratories Inc., USA). GAPDH and U6 were adopted as internal controls. The sequences used are indicated in Table 1. The CT values of each gene were calculated following the $2^{-\Delta\Delta Ct}$ method.

2.11. Luciferase reporter assay

The sequence of miR-21 including the wild-type (WT) binding site or the mutated (MUT) binding site of PTEN 3'UTR was cloned into the firefly luciferase PmirGLO reporter vector (Promega, USA). The reporter plasmids were co-transfected into cells together with miR-21 mimics or NC mimics in A549 cells with Lipofectamine™ 3000 reagent. After 48 h transfection, the relative luciferase activities could be examined with the Luciferase Reporter Gene Assay System.

2.12. Statistical analysis

Data were exhibited as mean ± standard deviation. Differences were analyzed with one-way ANOVA with SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ meant statistical significance.
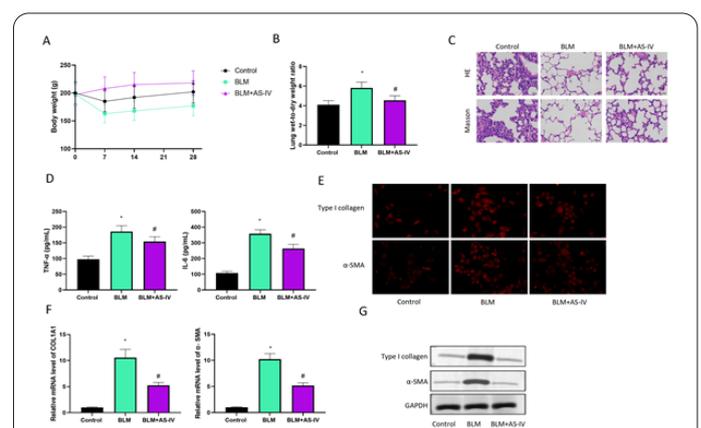
3. Results

3.1. ASV-IV alleviates BLM-induced IPF in mice

After intratracheal BLM injection, obvious weight loss and lung dry-wet ratio increase were seen. But, BLM-stimulated weight loss as well as the elevation in the wet-to-dry weight ratio of lung could be offset after ASV-IV treatment (Figure 1 A and B). H&E together with Masson staining revealed that the BLM group had more diffuse fibrosis, lymphocyte infiltration, along collagen deposition than the control group. Relative to the BLM group, fibrosis symptoms were apparently relieved in the ASV-IV treatment group (Figure 1C). Besides, BLM caused obvious promotion in TNF-α as well as IL-6 levels, whereas ASV-IV addition simultaneously repressed the production of these cytokines (Figure 1D). In view of α-SMA together with collagen I are IPF-related markers, their expression was analyzed using immunofluorescence, RT-qPCR as well as western blot analyses. Immunofluorescence results demonstrated that BLM treatment elevated α-SMA together with collagen I expression, but treatment with ASV-IV lessened their expression (Figure 1E). Similarly, RT-
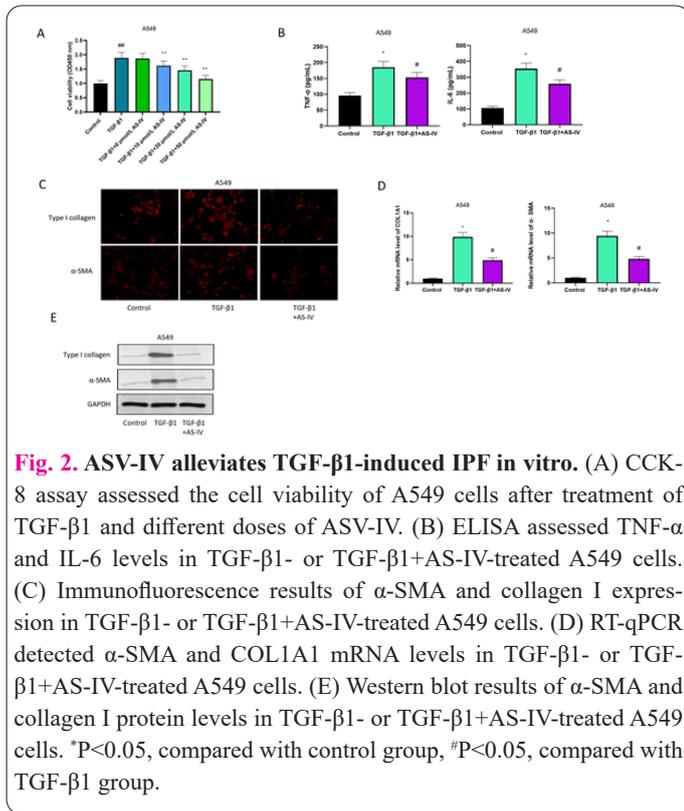
qPCR and western blot analyses displayed that relative to controls, α-SMA and collagen I mRNA as well as protein levels were also promoted upon BLM treatment, but ASV-IV treatment relieved their levels (Figure 1 F and G).

3.2. ASV-IV alleviates TGF-β1-induced IPF in vitro

We then treated 10 ng/L TGF-β1 into A549 cells to induce an in vitro model of IPF, followed by adding 0~50 μmol/L AS-IV into the supernatant of cell culture. CCK-8 results revealed that the cell viability was promoted upon TGF-β1 treatment, and then declined after 0~50 μmol/L AS-IV treatment in a dose-dependent way (Figure 2A). Therefore, we selected 50 μmol/L AS-IV for subsequent analyses. ELISA results displayed that TNF-α as well as IL-6 levels were increased followed by TGF-β1 treatment, but lessened after co-treatment of AS-IV (Figure 2B). Immunofluorescence results revealed that TGF-β1 treatment up-regulated α-SMA together with collagen I expression, but treatment with ASV-IV reduced their expression (Figure 2C). Likewise, it was observed that α-SMA and collagen I mRNA as well as protein levels were also enhanced

Fig. 1. ASV-IV alleviates BLM-induced IPF in mice. Mice were divided into control, BLM and BLM+AS-IV groups. (A) Body weight of mice in different groups. (B) The wet-to-dry weight ratio of lung in different groups. (C) H&E together with Masson staining assessed the pathological changes of tissues in different groups. (D) ELISA assessed TNF-α and IL-6 levels in different groups. (E) Immunofluorescence results of α-SMA and collagen I expression in different groups. (F) RT-qPCR detected α-SMA and COL1A1 mRNA levels in different groups. (G) Western blot results of α-SMA and collagen I protein levels in different groups. *$P<0.05$, compared with control group, #$P<0.05$, compared with BLM group.

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Fig. 2. ASV-IV alleviates TGF-β1-induced IPF in vitro. (A) CCK-8 assay assessed the cell viability of A549 cells after treatment of TGF-β1 and different doses of ASV-IV. (B) ELISA assessed TNF-α and IL-6 levels in TGF-β1- or TGF-β1+AS-IV-treated A549 cells. (C) Immunofluorescence results of α-SMA and collagen I expression in TGF-β1- or TGF-β1+AS-IV-treated A549 cells. (D) RT-qPCR detected α-SMA and COL1A1 mRNA levels in TGF-β1- or TGF-β1+AS-IV-treated A549 cells. (E) Western blot results of α-SMA and collagen I protein levels in TGF-β1- or TGF-β1+AS-IV-treated A549 cells. *P<0.05, compared with control group, #P<0.05, compared with TGF-β1 group.

upon TGF-β1 treatment, and ASV-IV treatment alleviated their levels significantly (Figure 2 D and E).

3.3. ASV-IV induces autophagy in both in vivo and in vitro IPF model

Autophagy has a vital potential in the pathogenesis of pulmonary fibrosis [18], and many reports have indicated that ASV-IV participates in the development of diseases via regulating autophagy [10]. Herein, we also explored whether ASV-IV alleviated IPF via regulating autophagy. Western blot analysis proved that LC3-II and Beclin-1 protein levels were declined after BLM treatment, but enhanced after co-treatment of ASV-IV. Meanwhile, the elevated protein level of p62 caused by BLM treatment was reversed after co-treatment of ASV-IV (Figure 3A). Similarly, in vitro immunofluorescence result of LC3B also indicated that the fluorescence intensity of LC3B was significantly weakened in TGF-β1-stimulated A549 cells, whereas was enhanced after ASV-IV co-treatment (Figure 3B). In addition, LC3-II together with Beclin-1 protein levels in A549 cells declined after TGF-β1 treatment, but enhanced after co-treatment of ASV-IV. Meanwhile, the elevated protein level of p62 caused by TGF-β1 treatment was reversed after co-treatment of ASV-IV (Figure 3C).
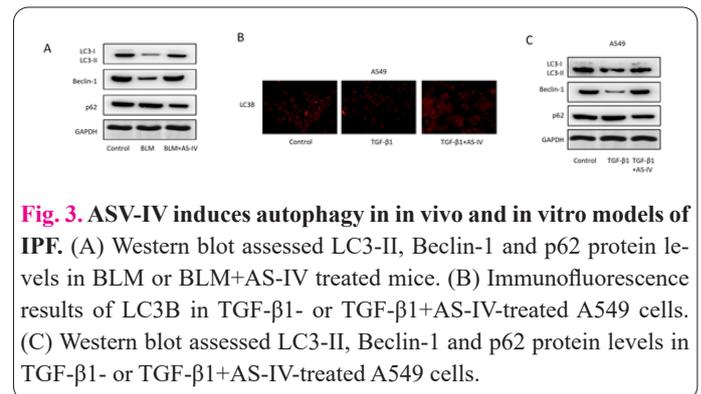
3.4. ASV-IV inactivates the PI3K/AKT/mTOR pathway via miR-21/PTEN axis in IPF

Studies have revealed that TGF-β1 can elevate miR-21 expression in fibroblasts during the induction of pulmonary fibrosis, and overexpression of miR-21 can further enhance the pro-fibrotic function of TGF-β1 signaling in cells [16]. Therefore, we speculated that ASV-IV might alleviate IPF through regulating miR-21. We first examined miR-21 expression in BLM- or TGF-β1-induced IPF models, and discovered that miR-21 expression was elevated after BLM or TGF-β1 treatment, but reduced after ASV-IV co-treatment (Figure 4A). Through bioinformatics analysis, we discovered that PTEN was directly targeted by
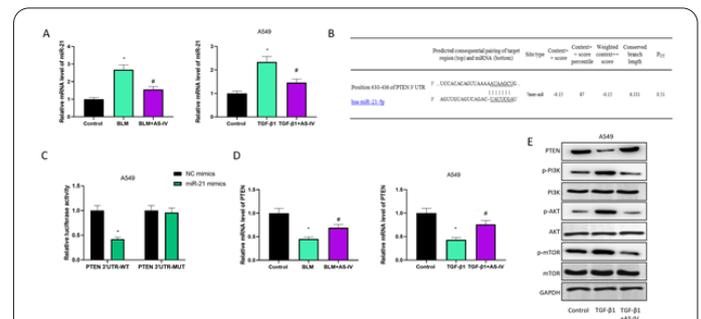
miR-21 (Figure 4B). Luciferase reporter assay further validated that overexpressed miR-21 could reduce the luciferase activity of PTEN 3'UTR-WT, but rarely affected that of PTEN 3'UTR-MUT, further validating the combination between miR-21 and PTEN (Figure 4C). Furthermore, we found that PTEN expression was significantly down-regulated after BLM or TGF-β1 treatment, but up-regulated after ASV-IV co-treatment (Figure 4D). As everyone knows, PTEN is a prime antagonist of PI3K and therefore a negative modulator of the PI3K/AKT/mTOR pathway [19]. We further detected the impact of ASV-IV on the levels of PTEN/PI3K/AKT/mTOR pathway in TGF-β1-stimulated A549 cells. Western blot indicated that TGF-β1 treatment lessened the PTEN protein level while elevating that of p-PI3K, p-AKT, as well as p-mTOR, but these effects were offset after ASV-IV co-treatment (Figure 4E).

3.5. ASV-IV alleviates IPF in vivo through inhibiting miR-21 expression

We further performed rescue assays to investigate whether ASV-IV alleviates IPF in vivo through regulating miR-21 expression. We found that the reduced expression of miR-21 caused by ASV-IV treatment was increased in mice tissues after injecting with a miR-21 agonist through a tail vein for 28 days (Figure 5A). Besides, ASV-IV-caused weight increase and the decrease in the wet-to-dry weight ratio of lung could be counteracted after miR-21

Fig. 3. ASV-IV induces autophagy in in vivo and in vitro models of IPF. (A) Western blot assessed LC3-II, Beclin-1 and p62 protein levels in BLM or BLM+AS-IV treated mice. (B) Immunofluorescence results of LC3B in TGF-β1- or TGF-β1+AS-IV-treated A549 cells. (C) Western blot assessed LC3-II, Beclin-1 and p62 protein levels in TGF-β1- or TGF-β1+AS-IV-treated A549 cells.

Fig. 4. ASV-IV inactivates the PI3K/AKT/mTOR signaling pathway via regulating miR-21/PTEN axis in IPF. RT-qPCR examined miR-21 mRNA levels in BLM or BLM+AS-IV treated mice and TGF-β1- or TGF-β1+AS-IV-treated A549 cells. (B) Binding sites between miR-21 and PETN 3'UTR. (C) Luciferase reporter assay assessed the binding between miR-21 and PETN 3'UTR. (D) RT-qPCR examined PTEN mRNA levels in BLM or BLM+AS-IV-treated mice and TGF-β1- or TGF-β1+AS-IV-treated A549 cells. (E) Western blot assessed protein levels of PTEN and PI3K/AKT/mTOR signaling pathway-related genes in TGF-β1- or TGF-β1+AS-IV-treated A549 cells. *P<0.05, compared with control group, #P<0.05, compared with BLM group or TGF-β1 group.

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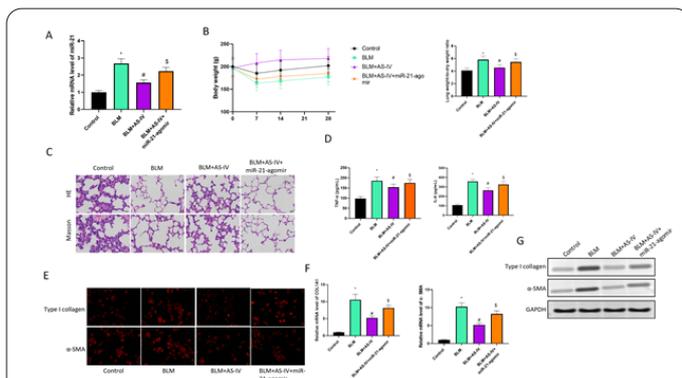Cell. Mol. Biol. 2024, 70(2): 128-136

agonist treatment (Figure 5B). H&E together with Masson staining revealed that the reduced fibrosis symptoms in the ASV-IV treatment group were reversed after miR-21 agonist treatment (Figure 5C). Moreover, the reduced TNF-α and IL-6 levels caused by ASV-IV treatment were neutralized after miR-21 agonist treatment (Figure 5D). Additionally, the outcomes of immunofluorescence, RT-qPCR as well as western blot demonstrated that the lessened levels of α-SMA together with collagen I caused by ASV-IV treatment could be reversed after miR-21 agonist treatment (Figure 5 E-G).

3.6. ASV-IV induces autophagy and affects the PTEN/PI3K/AKT/mTOR pathway in IPF through regulating miR-21 expression
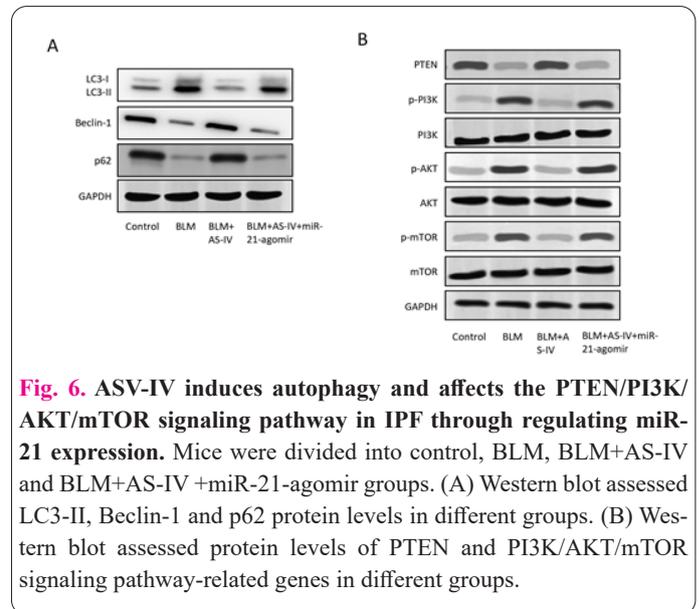
We also assessed whether ASV-IV induced autophagy and affected the PTEN/PI3K/AKT/mTOR pathway in vivo through regulating miR-21 expression. Western blot analysis revealed that the increased LC3-II together with Beclin-1 protein levels as well as the decreased p62 protein level in BLM+ASV-IV group was reversed after miR-21 agonist treatment (Figure 6A). Meanwhile, the increased protein level of PTEN as well as the impaired p-PI3K, p-AKT, along p-mTOR protein levels in BLM+ASV-IV group was reversed after miR-21 agonist treatment (Figure 6B).

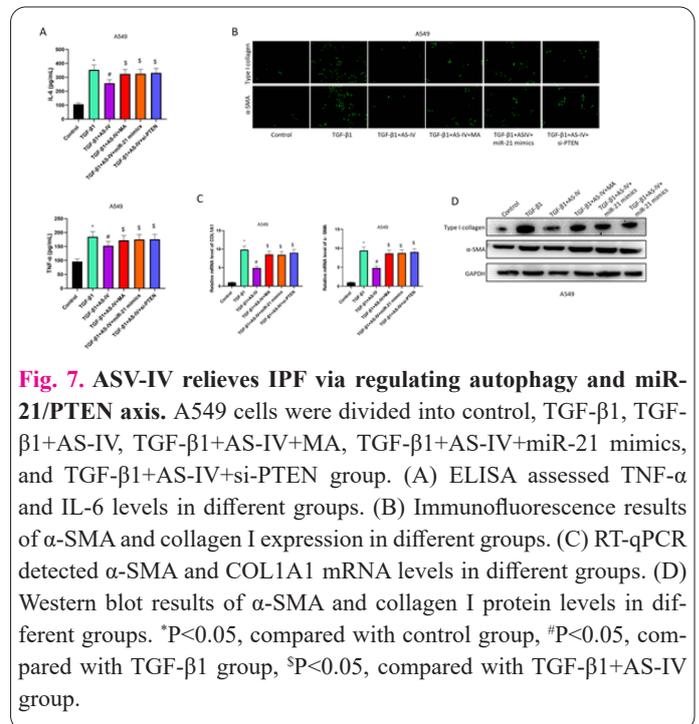### 3.7. ASV-IV relieves IPF via regulating autophagy and miR-21/PTEN axis

Furthermore, we performed in vitro rescue assays to validate whether ASV-IV relieved IPF via regulating autophagy and miR-21/PTEN axis. We discovered that the reduced TNF-α as well as IL-6 levels in TGF-β1+ASV-IV group were neutralized after treatment of autophagy inhibitor, miR-21 mimics, or si-PTEN (Figure 7A). The outcomes of immunofluorescence, RT-qPCR, as well as western blot demonstrated that the lessened α-SMA together

Fig. 5. ASV-IV alleviates IPF in vivo through inhibiting miR-21 expression. Mice were divided into control, BLM, BLM+AS-IV and BLM+AS-IV +miR-21-agomir groups. (A) RT-qPCR detected miR-21 mRNA levels in different groups. (B) Body weight of mice and the wet-to-dry weight ratio of lung in different groups. (C) H&E together with Masson staining assessed the pathological changes of tissues in different groups. (D) ELISA assessed TNF-α and IL-6 levels in different groups. (E) Immunofluorescence results of α-SMA and collagen I expression in different groups. (F) RT-qPCR detected α-SMA and COL1A1 mRNA levels in different groups. (G) Western blot results of α-SMA and collagen I protein levels in different groups. $^*P<0.05$, compared with control group, $^#P<0.05$, compared with BLM group, $^\$P<0.05$, compared with BLM+AS-IV group.

Fig. 6. ASV-IV induces autophagy and affects the PTEN/PI3K/AKT/mTOR signaling pathway in IPF through regulating miR-21 expression. Mice were divided into control, BLM, BLM+AS-IV and BLM+AS-IV +miR-21-agomir groups. (A) Western blot assessed LC3-II, Beclin-1 and p62 protein levels in different groups. (B) Western blot assessed protein levels of PTEN and PI3K/AKT/mTOR signaling pathway-related genes in different groups.

Fig. 7. ASV-IV relieves IPF via regulating autophagy and miR-21/PTEN axis. A549 cells were divided into control, TGF-β1, TGF-β1+AS-IV, TGF-β1+AS-IV+MA, TGF-β1+AS-IV+miR-21 mimics, and TGF-β1+AS-IV+si-PTEN group. (A) ELISA assessed TNF-α and IL-6 levels in different groups. (B) Immunofluorescence results of α-SMA and collagen I expression in different groups. (C) RT-qPCR detected α-SMA and COL1A1 mRNA levels in different groups. (D) Western blot results of α-SMA and collagen I protein levels in different groups. $^*P<0.05$, compared with control group, $^#P<0.05$, compared with TGF-β1 group, $^\$P<0.05$, compared with TGF-β1+AS-IV group.
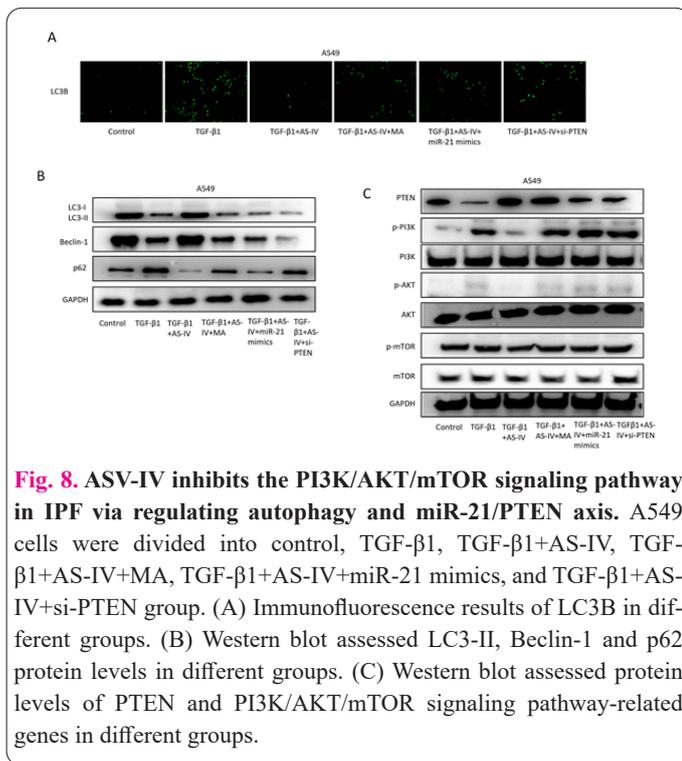
with collagen I levels in TGF-β1+ASV-IV group could be reversed after treatment of autophagy inhibitor, miR-21 mimics, or si-PTEN (Figure 7 B-D).

3.8. ASV-IV inhibits the PI3K/AKT/mTOR pathway in IPF via regulating autophagy and miR-21/PTEN axis

Moreover, it was discovered that the increased LC3B fluorescence intensity, elevated LC3-II and Beclin-1 protein levels as well as the decreased p62 protein level in TGF-β1+ASV-IV group were reversed after treatment of autophagy inhibitor, miR-21 mimics, or si-PTEN (Figure 8 A and B). Simultaneously, the increased protein level of PTEN as well as the lessened p-PI3K, p-AKT, along with p-mTOR protein levels in TGF-β1+ASV-IV group was reversed after treatment of autophagy inhibitor, miR-21 mimics, or si-PTEN (Figure 8C).

4. Discussion

Herein, we uncovered the protective role and regulatory mechanism of ASV-IV on IPF. There are several important

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Fig. 8. ASV-IV inhibits the PI3K/AKT/mTOR signaling pathway in IPF via regulating autophagy and miR-21/PTEN axis. A549 cells were divided into control, TGF-β1, TGF-β1+AS-IV, TGF-β1+AS-IV+MA, TGF-β1+AS-IV+miR-21 mimics, and TGF-β1+AS-IV+si-PTEN group. (A) Immunofluorescence results of LC3B in different groups. (B) Western blot assessed LC3-II, Beclin-1 and p62 protein levels in different groups. (C) Western blot assessed protein levels of PTEN and PI3K/AKT/mTOR signaling pathway-related genes in different groups.

findings of this research. (1) ASV-IV induces autophagy in IPF. (2) ASV-IV improves IPF through inhibiting miR-21 expression. (3) ASV-IV induces autophagy and affects the PTEN/PI3K/AKT/mTOR pathway in IPF through modulating miR-21 expression.

In recent years, the clinical benefits of TCM in preventing and treating IPF have been gradually recognized [20]. AS-IV belongs to the most abundant and active component of Astragaloside, which has the effect of anti-pulmonary fibrosis, liver fibrosis, kidney fibrosis and myocardial fibrosis [21, 22]. The main mechanisms of AS-IV to improve tissue fibrosis include improving the activity of antioxidant enzymes, reducing oxidative stress damage, inhibiting the expression of inflammatory factors, inhibiting cell apoptosis, inhibiting α-SMA and ECM deposition, down-regulating the expression of fibrogenic factors TGF-β1 and VEGF, and regulating multiple cell signaling pathways, etc., which participate in the anti-fibrosis process [23, 24]. Consistently, our study proved that ASV-IV decreased TNF-α and IL-6 levels, as well as α-SMA and collagen I expression in both in vivo as well as in vitro IPF models.

In addition, AS-IV can also mediate the protection of tissues and organs by regulating the expression of miRNA [25]. In addition, AS-IV has been shown to relieve renal fibrosis by repressing miR-21-stimulated podocyte dedifferentiation along with mesangial cell activation [26]. As previously reported, miR-21 is actively implicated in the progression of pulmonary fibrosis [27]. By inhibiting Smad7 protein, miR-21 can reduce its negative regulatory effect on TGF-β/Smads signaling pathway, and promote the reduction of E-cadherin and the increase of α-SMA in epithelial cells, thereby promoting the occurrence of EMT and pulmonary fibrosis [28]. MiR-21 can also activate extracellular regulatory protein kinase (ERK) by inhibiting SPRY and promoting TGF-β1-induced fibrosis [29]. Conversely, inhibition of miR-21 expression can reduce the fibrosis induced by TGF-β1 [30], so miR-21 may be a possible target to improve the progression of pulmonary fi-

brosis. In our study, we further demonstrated that ASV-IV could down-regulate miR-21 expression in both in vivo as well as in vitro IPF models, and performed rescue assays to confirm that ASV-IV alleviated IPF through regulating miR-21, which was consistent with previous study [31].

Autophagy is a lysosomal-dependent self-degradation pathway during the genesis and development of eukaryotes and has a crucial role in maintaining cell homeostasis [32]. The mechanisms of autophagy involvement in pulmonary fibrosis may include: (1) Autophagy defects promote the proliferation along with differentiation of fibroblasts [33]: activated fibroblasts/myofibroblasts have a crucial potential in the formation of pulmonary fibrosis, and autophagy can promote the deposition of ECM, thus forming pulmonary fibrosis. Patel *et al*. [34] found that after silencing LC3B together with Beclin-1 genes, the expression of α-SMA and fibronectin along with other markers of myoblast increased significantly, indicating that inhibition of autophagy activation can promote myoblast proliferation and ECM deposition. Araya *et al*. [35] confirmed through in vitro experiments that the absence of autophagy can promote the phenotypic transformation of fibroblasts into myofibroblasts. (2) Defects in autophagy promote apoptosis and inflammatory response of epithelial cells [36]: alveolar and bronchiolar epithelial cell apoptosis can promote the recruitment of inflammatory cells in the lungs, promote the release of chemokines, and enable neutrophils and monocytes to accumulate in the mesenchymal cell together with alveoli. Neutrophils, in turn, produce ROS, metalloproteins, and pro-inflammatory cytokines that retain epithelial apoptosis and lung inflammation, leading to a positive feedback loop of apoptosis and inflammation [37]. Autophagy may help clear dead cells, thereby affecting inflammatory responses and tissue repair [38]. In the BLM-stimulated pulmonary fibrosis model, activation of autophagy can prevent this positive feedback loop by inhibiting apoptosis and improving the ability to clear dead cells, while blocking autophagy by knocking out ATG4B will increase apoptosis of A549 cells and aggravate pulmonary fibrosis [39]. (3) Autophagy defects promote alveolar epithelial-mesenchymal transition (EMT) [40]: When abnormal lung tissue injury is repaired, alveolar type II epithelial cells can be transformed into fibroblasts and myofibroblasts through EMT, while collagen I together with α-SMA expression in alveolar epithelial cells increases after autophagy is inhibited [41]. Changes in the level of phagocytosis have been found in a number of lung diseases, including a reduction in the number of autophagosomes in the lung epithelial cells and fibroblasts of IPF patients, and in the BLM-stimulated IPF model, it has been found that mice with defects in autophagy genes show more obvious neutrophil aggregation and a significant increase in inflammatory factors [42]. These findings indicate that autophagy can control the progression of IPF by regulating collagen degradation and autophagy-related cell death. Autophagy deficiency is an important pathogenesis of pulmonary fibers, and activation of autophagy can inhibit the occurrence and development of pulmonary fibrosis. Moreover, many reports have unveiled the important role of ASV-IV in improving tissue fibrosis via regulating autophagy. Such as, AS-IV improves renal fibrosis through modulating the SIRT1-NF-κB pathway along with autophagy activation [43]. AS-IV ameliorates cardiomyocyte apoptosis and fibrosis regulating autophagy [44].

In our study, we proved that ASV-IV induced autophagy in IPF through TEM and detection of autophagy-related genes, which was in line with previous study [45].

The regulatory mechanism of autophagy is very complex. The mammalian target of rapamycin (mTOR) belongs to a crucial factor that negatively modulates the formation of autophagy or autophagy, and its activation degree can reflect the level of autophagy [46]. mTOR is a component of phosphatidyl inositol kinase-associated protein kinase (PIKK) [47]. Overexpression of mTOR can lead to inhibition of autophagy activity [48]. Inhibition of mTOR activity by rapamycin is followed by activation of autophagy in this regulatory pathway [49]. Studies have confirmed that mTOR overexpression is implicated in the pathogenesis of diverse fibrotic diseases, containing pulmonary fibrosis [50]. Growth factors such as TGF-β and cytokines convert PIP2 into PIP3 by activating phosphatidylinositol 3-kinase (PI3K) and promote phosphorylation of AKT, thereby activating mTOR and inhibiting autophagy activity [51]. mTOR inhibitors can effectively treat pulmonary fibrosis in animal models [52]. PTEN is a major negative modulator of the cellular pathway triggered by PI3K [53]. By repressing the PI3K/AKT/mTOR pathway, PTEN inhibits TGF-β signaling and the resulting extracellular matrix deposition, activates anti-inflammatory response, activates macrophages, releases the inhibition of mTOR on autophagy, and intervenes in the fibrosis of multiple organs [54]. Downregulation of PTEN expression and overactivation of AKT have been found in alveolar epithelial cells and lung fibroblast foci in patients with IPF [55]. In addition, low PTEN expression promotes abnormal induction of PI3K/AKT/mTOR pathway as well as pathological proliferation of fibroblasts [56]. Therefore, the modulation of PTEN/PI3K/AKT/mTOR signaling is believed to exert a critical role in the pathogenesis of IPF, and this pathway may become a potential therapeutic target for IPF. Notably, our research proved that PTEN was directly targeted by miR-21, and ASV-IV could up-regulate PTEN expression to repress the activities of PI3K/AKT/mTOR signaling, thereby inducing autophagy.

In conclusion, our study demonstrates that ASV-IV inhibits IPF through activation of autophagy by miR-21-mediated PTEN/PI3K/AKT/mTOR pathway, suggesting that ASV-IV could be acted to be a promising therapeutic method for IPF.

Informed Consent
The authors report no conflict of interest.

Availability of data and material
We declared that we embedded all data in the manuscript.

Authors' contributions
LT conducted the experiments and wrote the paper; GX, JR, SY, DY and WF analyzed and organized the data; WY conceived, designed the study and revised the manuscript.

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