

Panax notoginseng saponins Attenuate Pulmonary Fibrosis by Targeting the miR-16-5p/c-Jun Signaling Axis

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Abstract

Background

This study aims to investigate the mechanism by which panax notoginseng saponins (PNS) ameliorate pulmonary fibrosis by regulating miR-16-5p.

Methods

MiRNA-sequencing was performed on serum exosomes isolated from 4 healthy control subjects and 4 patients with idiopathic pulmonary fibrosis (IPF). Ago2-RIP assay, along with gain and loss-of-function experiments of miR-16-5p in cultured cells, was applied to verify the interaction between miR-16-5p and c-Jun. Furthermore, cellular and in-vivo pulmonary fibrosis models were established. The expression levels of miR-16-5p, its target gene c-Jun, and fibrotic markers were detected following PNS intervention.

Results

MiRNA-sequencing and public database analyses revealed significant downregulation of miR-16-5p in serum exosomes from IPF patients. Bioinformatic tools including miRWalk and miRPathDB predicted the binding interaction between miR-16-5p and c-Jun. Ago2-RNA immunoprecipitation simultaneously enriched miR-16-5p and c-Jun mRNA. Transfection of miR-16-5p mimics reduced c-Jun expression, whereas miR-16-5p inhibitor transfection increased c-Jun abundance. In bleomycin (BLM)-induced cellular and animal pulmonary fibrosis models, PNS treatment markedly upregulated miR-16-5p and suppressed its target c-Jun. Additionally, downstream fibrosis-related effectors mediated by c-Jun, including cellular communication network factor 2 (CCN2), α -SMA, fibronectin 1 (FN1) and collagen type I alpha 1 chain (COL1A1), were inhibited. Rescue experiments further demonstrated that the PNS-mediated mitigation of endothelial-mesenchymal transition was dependent on the miR-16-5p/c-Jun axis.

Conclusion

PNS alleviates pulmonary fibrosis by upregulating miR-16-5p, which consequently represses c-Jun and downstream fibrosis-related molecules CCN2, α -SMA, FN1, and COL1A1.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive and highly fatal interstitial lung disease, representing the most prevalent and severe subtype of pulmonary fibrosis (PF), accounting for approximately 17%–37% of all PF cases[1, 2]. Its pathogenesis is primarily attributed to persistent myofibroblast activation and excessive extracellular matrix (ECM) deposition[3]. Conventionally, alveolar epithelial-mesenchymal transition (EMT) has been considered the major mechanism generating of

fibroblasts[4]. Nevertheless, emerging evidence highlights endothelial-mesenchymal transition (EndMT) as another pivotal mechanism underlying pulmonary fibrosis[5, 6]. Although currently available anti-fibrotic agents, pirfenidone and nintedanib, can partially retard disease progression, their clinical utility is compromised by adverse effects such as gastrointestinal intolerance and hepatic dysfunction[7, 8]. Accordingly, there is an urgent demand for safer and more efficacious therapeutic candidates to slow IPF progression.

Given the limitation of current anti-fibrotic therapies, traditional Chinese medicine represents a potential source of novel anti-fibrotic agents. *Panax notoginseng* (Burkill) F.H. Chen is a perennial medicinal herb of the Araliaceae family that has been widely used in traditional Chinese medicine. Modern pharmacological studies have demonstrated that *Panax notoginseng* exerts multiple biological activities including anti-inflammatory, anti-oxidative and anti-fibrotic effects. It is capable of suppressing aberrant fibroblast activation and effectively suppressing the progression of multi-organ fibrosis[9–11]. *Panax notoginseng* saponins (PNS), the principal bioactive constituents of PNS, have been developed into diverse pharmaceutical preparations[12]. Recent research on PNS has largely focused on its anti-pulmonary fibrosis pharmacological properties. Cumulative reports have validated that PNS alleviates pulmonary fibrotic pathology via multiple pathways, including modulation of the renin-angiotensin system, regulation of autophagy and apoptosis, as well as inhibition of epithelial-mesenchymal transition[13–16]. Studies have demonstrated that multiple miRNAs contribute to the development of the IPF phenotype[17, 18]. However, whether PNS ameliorates PF through regulating miRNA expression remains unreported. The present study aims to systematically explore the mechanistic basis by which PNS mitigates pulmonary fibrosis via targeting miR-16-5p.

2. Materials and Methods

2.1 Ethical Approval and Subject Characteristics

This study was approved by the Ethics Committee of Xi'an Chest Hospital Affiliated to Northwest University (Approval No. R2025-012-01). Prior to participation, all subjects provided written informed consent.. All patients diagnosed with IPF fulfilled the diagnostic criteria outlined in the 2022 ATS/ERS/JRS/ALAT guidelines[19]. Diagnoses were established based on high-resolution computed tomography (HRCT) findings, and patients with other known causes of interstitial lung disease were excluded. A two-phase study design was employed. Consecutive eligible subjects were recruited at Xi'an Chest Hospital between December 2025 and March 2026. In the discovery phase, four patients with IPF and four healthy controls (n = 8) were enrolled for small-RNA sequencing to screen differentially expressed miRNAs. In the validation phase, an independent non-overlapping cohort consisting of 10 patients with IPF and 10 healthy controls (n = 20) was recruited, and quantitative real-time polymerase chain reaction (qRT-PCR) was performed to validate candidate miRNAs. Exclusion criteria for all subjects comprised coexistence of other subtypes of interstitial lung disease, definite obstructive airway diseases, acute or chronic infection, malignant neoplasms, severe hepatic or renal insufficiency, as well

as recent trauma history. Both phases applied identical exclusion criteria, and baseline characteristics were comparable ($P > 0.05$; Supplementary Tables S1 and S2).

2.2 Blood Collection and Serum Preparation

We obtained peripheral venous blood (5 mL) from fasting subjects in the early morning using vacuum blood collection tubes without anticoagulant. Following collection, the blood samples were kept at 24–26°C for 30 min, followed by centrifugation at $3000 \times g$ for 10 min under identical temperature conditions. The upper serum fraction was carefully transferred into new sterile centrifuge tubes without disturbing the intermediate buffy coat. The obtained serum was either immediately used for downstream experiments or aliquoted for storage at -80°C .

2.3 Serum Sample Storage and Pre-processing for EV Isolation

If extracellular vesicle (EV) isolation could not be performed immediately, aliquoted serum was stored at -80°C to avoid repeated freeze-thaw cycles. Prior to EV isolation, frozen serum was slowly thawed on ice. After thawing, samples were centrifuged at $12\,000 \times g$ for 10 min at 4°C to remove residual cellular debris and precipitates, and the supernatant was collected for subsequent EV isolation.

2.4 Library Construction, Sequencing and Bioinformatic Analysis (Discovery phase, $n = 8$)

The PAXgene® Blood RNA Kit (Qiagen, Cat. No. 762174) was utilized to isolate total RNA from whole-blood specimens collected from the eight discovery-phase participants (IPF: $n = 4$; healthy controls: $n = 4$). RNA quality (purity and integrity) was evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific) and the Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. High-quality RNA was used to construct small-RNA sequencing libraries with the VAHTS Small RNA Library Prep Kit for Illumina V2 (Vazyme, Cat. No. NR811), following the supplier's protocols. The Illumina HiSeq platform was employed for single-end 50 bp sequencing. Trimmomatic was applied to the raw reads for the removal of adapters, low-quality bases, and sequences shorter than 18 bp, ultimately yielding clean reads. Clean reads were mapped to the reference genome and annotated for known miRNAs using miRDeep2; miRNAs with mean counts > 2 across samples were retained. Normalization was conducted via DESeq2, followed by differential expression analysis via the binomial test. Differentially expressed miRNAs were identified based on the criteria of $|\log_2\text{FC}| > 1$ and $P < 0.05$. In total, 82 differentially expressed miRNAs were preliminarily identified between the two groups by these criteria.

2.5 Isolation of Exosome-Derived RNA from Serum

Serum samples from 20 subjects in the validation phase (10 IPF patients and 10 healthy controls) were filtered through a $0.8 \mu\text{m}$ filter. An equal volume of Buffer XBP was combined with the filtrate before the mixture was transferred to exoEasy spin columns (QIAGEN, Germantown, MD, USA; Cat. No. 77144). The column was then spun at $500 \times g$ for 1 min. Columns were washed with 3.5 mL Buffer XWP by

centrifugation at $5000 \times g$ for 5 min. Next, the column received 700 μL of QIAzol lysis reagent (QIAGEN). The lysate was collected via a 5-min spin at $5000 \times g$ and subsequently left at room temperature for 5 min. Following the addition of 90 μL chloroform, the sample was vortexed rigorously for 15 s. This was followed by a 2–3 min incubation at room temperature and centrifugation at $12\,000 \times g$ for 15 min (4°C). Approximately 400 μL of the upper aqueous phase was carefully recovered and mixed with twice the volume of absolute ethanol. The mixture was transferred to an RNeasy MinElute column and centrifuged at $\geq 8000 \times g$ for 15 s. Sequential washes were performed with 700 μL Buffer RWT and 500 μL Buffer RPE (repeated once, with a 2-min centrifugation for the second wash). Columns were dried by open-lid full-speed centrifugation for 5 min. RNase-free water (14 μL) was added onto the membrane. Following a 1-min incubation, the RNA was eluted via a 1-min full-speed centrifugation and preserved at -80°C for downstream applications.

2.6 Screening Strategy for Candidate Target Genes

A two-step integrative screening strategy was applied to identify potential target genes of miR-16-5p (Fig. 2A). First, putative targets of miR-16-5p were predicted using two independent databases: miRWalk (version 3.0) and miRPathDB (version 2.0). Only genes predicted by both databases were retained to form the initial candidate gene pool[20]. Based on Gene Ontology (GO) biological process annotations and published literature, candidate genes were further filtered according to two GO terms: TGF- β signal transduction (GO:0007179) and Smad protein signal transduction (GO:0060395)[21, 22]. Genes that satisfied both filtering steps constituted the final set of candidate target genes.

2.7 Cell Culture and PNS Concentration Screening

We maintained human umbilical vein endothelial cells (HUVECs) in M199 medium. This medium contained 1% penicillin-streptomycin-amphotericin B, 20% fetal bovine serum, 0.5% thymidine, 0.1% basic fibroblast growth factor (bFGF), 2% HEPES, and 0.18% heparin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 . Subculturing was performed at intervals of 2–3 days. For the experiments, we utilized cells from passages 3 to 8. For the assays, HUVECs were plated into six-well plates, with each well receiving 1×10^6 cells. To screen PNS concentrations, HUVECs were exposed to 25 $\mu\text{g}/\text{mL}$ BLM along with various PNS concentrations (25, 50, 100, and 200 $\mu\text{g}/\text{mL}$) for a 24-h period. According to the findings, 50 $\mu\text{g}/\text{mL}$ was determined to be the optimal dose for further experiments.

For miR-16-5p intervention assays, three groups were established: the NC group, the NC + BLM group, and mimic + BLM group, with three biological replicates per group. miR-16-5p mimic and negative control (NC) were transfected using Lipofectamine 2000. Six hours after transfection, culture medium of the NC + BLM and mimic + BLM groups was replaced with medium containing 25 $\mu\text{g}/\text{mL}$ BLM, while the NC group received fresh complete medium. Cells were harvested after 24 h incubation. Transfection with miR-16-5p inhibitor and its corresponding inhibitor-NC was performed following the same procedure. For PNS intervention experiments, three groups were included: CON group, BLM group (25 $\mu\text{g}/\text{mL}$ BLM), and PNS + BLM group (25 $\mu\text{g}/\text{mL}$ BLM plus 50 $\mu\text{g}/\text{mL}$ PNS). Cells were collected after 24 h of treatment.

2.8 Rescue Assays

Four experimental groups were established: the NC group, the NC + BLM group (25 µg/mL BLM), the NC + BLM + PNS group (25 µg/mL BLM + 50 µg/mL PNS), and the inhibitor + BLM + PNS group (25 µg/mL BLM + 50 µg/mL PNS). After 6 h of transfection in HUVECs, the NC + BLM group was supplied with medium containing 25 µg/mL BLM; the NC group received fresh complete medium; the NC + BLM + PNS and inhibitor + BLM + PNS groups were cultured in medium containing 25 µg/mL BLM plus 50 µg/mL PNS. Cells were harvested after an additional 24 h of incubation.

2.9 RNA Immunoprecipitation (RIP) Assay

We utilized normal rabbit IgG as a negative control and anti-HuR antibody as a positive control. RIP was performed using the BeyoRIP™ RIP Assay Kit (Beyotime, China, Cat. No. P1805S) according to the supplier's protocol. Briefly, 6×10^6 HUVECs were lysed. Lysates were incubated overnight at 4°C with normal rabbit IgG, anti-HuR antibody (5 µg, Beyotime, Cat. No. P1821S, China), or anti-Ago2 antibody (5 µg, Abcam, USA, Cat. No. ab186733). Protein A/G magnetic beads were added and incubated for 4 h at 4°C. The bead-antigen complexes were divided into two aliquots. RNA was isolated from one aliquot; enrichment was quantified by qRT-PCR with Input normalization and IgG as the negative control. c-Jun mRNA and miR-16-5p were measured in the Ago2-immunoprecipitated fraction, and ACTB was detected in the HuR group as a positive control. The other aliquot was subjected to Western blotting analysis for Ago2 to validate immunoprecipitation efficiency.

2.10 Immunofluorescence Staining

We fixed the cell coverslips in 4% paraformaldehyde, blocked them with goat serum, and rinsed them in PBS. Immunofluorescence staining for α-SMA (CST, USA, Cat. No. 19245; 1:200) and Nuclei were counterstained with DAPI. We then quantified the fluorescence intensity via Image J software, analyzing at least three random fields per group.

2.11 Animal Experiments

We obtained thirty specific-pathogen-free (SPF) male C57BL/6J mice (7 weeks old, 20 ± 2 g) from the Animal Experiment Center of Xi'an Jiaotong University. Mice were housed with ad libitum access to food and water. Animals were randomly assigned to five groups ($n = 6$ per group): control group (CON), pulmonary fibrosis model group (PF), pirfenidone group (PFD, 20 mg/kg, Macklin, China), low-dose PNS group (L-PNS, 30 mg/kg, National Institutes for Food and Drug Control, China), and high-dose PNS group (H-PNS, 120 mg/kg). This animal study was approved by the Animal Ethics Committee of Thoracic Hospital Affiliated to Northwest University. The PF model was induced by intratracheal administration of bleomycin (5 mg/kg). Anesthesia was achieved via intraperitoneal injection of sodium pentobarbital. After fixation and glottis exposure, a 20-G indwelling needle was inserted into the trachea. Upon correct positioning, BLM solution was administered, followed by injection of 0.2 mL air to facilitate drug distribution. Mice were rotated post-administration to achieve uniform drug dispersion. Beginning on day 1 after bleomycin instillation, mice in PNS-treated groups and the model group received once-daily intraperitoneal injection (the model group received an equal volume of normal saline). The PFD group

received intragastric administration every other day. After continuous treatment for 28 d, mice were anesthetized and euthanized, and lung tissues were harvested for downstream analyses.

2.12 Western Blotting

Cell or tissue samples were lysated and centrifuged, then the supernatants were collected. The BCA assay was utilized to quantify total protein content. Equal quantities of protein underwent SDS-PAGE separation and were subsequently blotted onto PVDF membranes. Membranes were sequentially incubated with primary and secondary antibodies. Protein bands were detected via ECL chemiluminescence, and band intensity was quantified with Image J. Primary-antibody information: c-Jun (Santa Cruz, USA, Cat. No. sc-74543; 1:500), p-c-Jun (Santa Cruz, USA, Cat. No. sc-822; 1:500), CCN2 (CST, USA, Cat. No. 3418; 1:1000), Fibronectin (Affinity, China, Cat. No. AF5335; 1:1000), α -SMA (CST, USA, Cat. No. 19245; 1:1000), β -tubulin (Affinity, China, Cat. No. ET1602; 1:20000).

2.13 Quantitative Real-Time PCR (qRT-PCR)

We isolated total RNA from cells and lung tissues using TRIzol reagent. The purification procedure involved chloroform extraction, isopropanol precipitation, and a final wash with 75% ethanol. For mRNA, reverse transcription was conducted using the mRNA reverse-transcription kit (Vazyme, Nanjing, China, Cat. No. R222-01). For miRNA, the commercial kit (Thermo Fisher, USA, Cat. No. 4366596) was applied for reverse transcription. Quantitative PCR amplification relied on the SYBR-Green method with qPCR kits (Promega, USA, Cat. No. A6001; GenStar, China, Cat. No. A304-05). β -actin served as the internal reference. Amplification was performed on a real-time PCR instrument (Tianlong Science and Technology Co., Ltd., Xi'an, China). Relative gene-expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method. Detailed primer sequences are provided in Table S3.

2.14 Histopathological Staining and Analysis

Lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into sections. For Hematoxylin-Eosin (H&E) staining, sections were processed by standard protocols for hematoxylin nuclear staining and eosin cytoplasmic staining. For immunohistochemistry (IHC), antigen retrieval, blocking, primary-antibody and secondary-antibody incubation, DAB chromogenic reaction, and hematoxylin counterstaining were performed sequentially. Masson's trichrome staining was conducted following the manufacturer's instructions (Beyotime, China, Cat. No. C0189S) to visualize collagen-fiber distribution with hematoxylin, Ponceau-acid fuchsin, and brilliant green. All sections were digitally whole-slide-scanned using the ZEISS whole-slide imaging system equipped with ZEN 3.9 software. Image J software was used to quantify the proportion of IHC-positive regions and the percentage of collagen-deposition area from Masson-stained sections.

2.15 Statistical Analysis

Data were analyzed using GraphPad Prism 9.0.0 (GraphPad Software, San Diego, CA, USA) and expressed as mean $\pm$ SD. Normality and variance homogeneity were assessed by Shapiro-Wilk, F-test, and Bartlett's tests. For normally distributed data, two-group comparisons utilized unpaired Student's t-

test or Welch's t-test, while multiple groups were analyzed using one-way ANOVA (Dunnett's or Tukey's) or Welch's ANOVA (Dunnett's T3). Non-normally distributed data were evaluated by Mann-Whitney U test for two groups, or Kruskal-Wallis test with Dunn's post hoc test for multiple groups. A two-tailed $P < 0.05$ indicated statistical significance.

3. Results

3.1 PNS Upregulates miR-16-5p Expression

Accumulating evidence indicates that multiple miRNAs participate in the regulation of EndMT in IPF. For instance, miR-23b-3p targets DPP4 and the miR-200 family targets ZEB1/2, both of which exert inhibitory effects on EndMT[17, 23]. To explore whether altered miRNA expression contributes to IPF-associated cellular phenotypes, bioinformatic analyses were performed on serum-derived exosomes from four control subjects (CON) and four IPF patients. In total, 82 differentially expressed miRNAs were screened. Subsequent intersection analysis against public datasets GSE124787, GSE220107 and GSE221937 yielded 22 highly overlapping miRNAs (Fig. 1A), among which 14 miRNAs reached statistical significance ($P < 0.01$; Fig. 1B). The expression abundance of these miRNAs in both groups was further evaluated based on transcripts per million (TPM) values (Fig. 1C). miRNAs with relatively high abundance in both control and IPF groups were filtered using the cutoff $\text{Log}_2(\text{TPM} + 1) > 3$ and further validated in serum exosome samples. Consistent with the bioinformatic results revealed that miR-27b-3p, miR-26b-5p, miR-409-3p and miR-16-5p were markedly down-regulated in serum exosomes from IPF patients compared with controls (Fig. 1E), implying that these dysregulated exosomal miRNAs may play direct or indirect roles in IPF pathogenesis. Previous studies have demonstrated that PNS alleviates oxidative-stress-triggered cardiomyocyte injury and apoptosis by up-regulating miR-30c-5p in myocardial ischemia-reperfusion injury models. In myocardial-fibrosis models, PNS elicits anti-fibrotic activity by elevating miR-29c to repress collagen and TGF- β 1 expression[24, 25]. Human umbilical vein endothelial cells (HUVECs) represent a well-established cellular model for EndMT research, and BLM stimulation has been reported to recapitulate key molecular signatures of pulmonary-fibrosis-related EndMT in HUVECs[26, 27]. Accordingly, we established an in-vitro fibrotic cell model by challenging HUVECs with bleomycin and detected the expression profiles of the above-identified miRNAs following PNS treatment. PNS intervention significantly increased the expression of miR-16-5p and miR-26b-5p in HUVECs. Nevertheless, in BLM-stimulated HUVECs, PNS could only reverse the BLM-provoked down-regulation of miR-16-5p, but did not restore miR-26b-5p levels (Fig. 1F, G; Figure S1). Collectively, these data confirm down-regulation of miR-27b-3p, miR-26b-5p, miR-409-3p and miR-16-5p in serum exosomes from IPF patients, suggesting their potential involvement in IPF progression. In HUVEC-based cellular assays, PNS selectively rescues BLM-induced reduction of miR-16-5p, pointing to the protective potential of PNS against BLM-mediated pathological alterations

Figure 1 PNS upregulates miR-16-5p expression. (A) Venn diagram showing the overlap among the three public datasets (GSE124787, GSE220107, GSE221937) and the miRNA-seq results from this study. (B) Heatmap of differentially expressed miRNAs ($P < 0.01$). (C) Evaluation of miRNA abundance in serum

exosomes from the CON and IPF groups based on TPM values. (D) qRT-PCR quantification of miRNAs (TPM > 7) in serum exosomes from IPF patients and healthy controls. (E) qRT-PCR detection of differentially expressed miRNAs in HUVECs after treatment with PNS (100 µg/mL) for 24 h. (F) qRT-PCR assessment of miR-16-5p levels in BLM-induced fibrotic HUVECs under different treatment conditions (control, BLM, PNS, and BLM + PNS). Data are presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. BLM

3.2 miR-16-5p Directly targets with c-Jun

Based on the above findings demonstrating the regulatory role of miR-16-5p in IPF, we performed Venn diagram analysis using miRWalk and miRPathDB databases with TGF-β signal transduction and Smad protein signal transduction as functional filtering criteria to predict target genes of miR-16-5p, and c-Jun was identified as a candidate target (Fig. 2A). To validate the interaction between miR-16-5p and c-Jun, HUVECs were transfected with miR-16-5p mimics or inhibitor. The mRNA and protein expression of c-Jun were subsequently quantified by qRT-PCR and Western blotting, respectively. Compared with the control group, transfection of miR-16-5p mimics markedly reduced both mRNA and protein levels of c-Jun, whereas miR-16-5p inhibitor transfection significantly upregulated c-Jun mRNA and protein expression (Fig. 2B–2E). These results indicate that miR-16-5p negatively regulates c-Jun expression. To further verify the physical binding between miR-16-5p and c-Jun, an Ago2-specific antibody-mediated RNA immunoprecipitation (RIP) assay was performed, with IgG serving as the negative control and HuR as the positive control. Western blotting confirmed the specificity of the Ago2 IP (Fig. 2F). qRT-PCR was used to assess RNA enrichment. The results showed that both miR-16-5p and c-Jun mRNA were substantially enriched in the Ago2 immunoprecipitated fraction, with enrichment levels significantly higher than those in the IgG control group (Fig. 2G), confirming that miR-16-5p and c-Jun mRNA are components of the same Ago2-containing RNP complex.

3.3 miR-16-5p Attenuates Endothelial-Mesenchymal Transition via Inhibition of c-Jun

Given that EndMT acts as a major driver of ECM deposition in IPF[27, 28] and that miR-16-5p is downregulated during PF[29, 30], we further investigated whether miR-16-5p exerts anti-fibrotic effects by targeting c-Jun. qRT-PCR results revealed that mRNA levels of c-Jun and fibrotic markers including CCN2, α-SMA, COL1A1 and FN1 were markedly elevated in the NC + BLM group relative to the NC group, indicating that BLM treatment triggers EndMT in HUVECs. Upon transfection with miR-16-5p mimics, overexpression of miR-16-5p significantly suppressed the mRNA expression of c-Jun and the aforementioned fibrotic markers (Fig. 3A, 3C). Meanwhile, miR-16-5p overexpression restored the BLM-repressed expression of endothelial markers CD31, VE-cadherin and VWF (Fig. 3B). Consistent with these findings, immunofluorescence assays demonstrated that transfection of miR-16-5p mimics substantially reduced α-SMA expression in HUVECs, reflected by diminished intracellular red fluorescence intensity (Fig. 3D-E). Western blotting analysis further showed enhanced c-Jun phosphorylation, accompanied by increased protein abundance of mesenchymal markers CCN2, α-SMA

and FN1 in BLM-challenged HUVECs. Transfection of miR-16-5p mimics markedly downregulated all these proteins (Fig. 3F). Collectively, these data indicate that miR-16-5p suppresses EndMT, at least in part, by inhibiting c-Jun, and suggest that elevated miR-16-5p suppresses EndMT in HUVECs through repressing c-Jun.

Figure 3 miR-16-5p mitigates EndMT by suppressing c-Jun. (A-C) qRT-PCR assessment of c-Jun, mesenchymal marker (A, C: CCN2, α -SMA, FN1, and collagen I) and endothelial markers (B: CD31, VE-cad, and VWF) mRNA levels in HUVECs transfected with miR-16-5p mimic (or controls) followed by BLM treatment. (C-D) IF staining and quantitative analysis of α -SMA in each treatment group (scale bar = 50 μ m). (E) Protein levels of p-c-Jun, CCN2, α -SMA, and FN1 determined by Western blotting. Data are presented as mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. NC; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. NC + BLM

3.4 PNS Attenuates Endothelial-Mesenchymal Transition via Regulation of the miR-16-5p/c-Jun Axis

Our preceding experiments verified that PNS upregulates miR-16-5p expression. We first performed a dose-screening assay, and 50 μ g/mL PNS was identified as the optimal concentration for inducing miR-16-5p upregulation, which was therefore adopted as the treatment concentration for subsequent cellular experiments (Figure S2). Previous work has also reported that 50 μ g/mL PNS effectively inhibits epithelial-mesenchymal transition[15].

Subsequently, HUVECs were challenged with bleomycin to establish an in vitro fibrotic cell model, followed by PNS treatment. qRT-PCR and Western blotting results revealed that the expression of c-Jun and mesenchymal markers including CCN2, α -SMA, COL1A1, and FN1 was markedly increased in the BLM group. PNS treatment significantly suppressed the expression of these molecules (Fig. 4A-B, 4E). Immunofluorescence staining further demonstrated that intracellular red fluorescence intensity was substantially reduced in PNS-treated HUVECs, confirming that PNS markedly inhibited α -SMA expression (Fig. 4C-D). Taken together, these results indicate that PNS reverses EndMT in HUVECs, and this protective effect is at least in part, by regulating the miR-16-5p/c-Jun axis.

3.5 Blockade of the miR-16-5p/c-Jun Axis Abrogates the Protective Effect of PNS against Endothelial-Mesenchymal Transition

To verify whether blockade of the miR-16-5p/c-Jun axis could reverse PNS-mediated mitigation of endothelial-mesenchymal transition (EndMT), HUVECs were assigned to different experimental groups and stimulated with BLM, followed by treatment with PNS together with miR-16-5p inhibitor (20 μ M). The mRNA levels of CCN2, α -SMA, COL1A1 and FN1 in each group were quantified by qRT-PCR. The results showed that mRNA expression of c-Jun and mesenchymal markers (CCN2, α -SMA, COL1A1, FN1) was markedly upregulated in BLM-challenged HUVECs. Such upregulation was significantly suppressed upon

PNS intervention, whereas co-treatment with miR-16-5p inhibitor partially restored the mRNA levels of these fibrotic markers (Fig. 5A-E). Consistent with the qRT-PCR findings, Western blotting assays revealed that protein levels of p-c-Jun, CCN2, α -SMA and FN1 were substantially elevated in BLM-stimulated HUVECs. PNS treatment markedly reduced the abundance of these proteins; nevertheless, addition of miR-16-5p inhibitor reversed such PNS-triggered protein downregulation to varying extents (Fig. 5F-G). Collectively, these data demonstrate that inhibition of the miR-16-5p/c-Jun axis partially downregulation the anti-EndMT effect conferred by PNS.

3.6 PNS Alleviates BLM-Induced Pulmonary Fibrosis in Mice via Regulating the miR-16-5p/c-Jun Axis

Having validated in HUVEC-based in vitro assays that PNS mitigates BLM-triggered endothelial-mesenchymal transition through the miR-16-5p/c-Jun axis, we further investigated the in-vivo therapeutic effects of PNS using C57BL/6J mice. Mice were divided into five groups: control group, model group, pirfenidone-treated group, low-dose PNS group, and high-dose PNS group. A schematic illustration of the establishment of a mouse model of PF and the drug administration regimen is shown in Fig. 6A. Lung index was used as an auxiliary indicator for evaluating lung injury and fibrosis. The overall severity of PF was comprehensively assessed by combining lung index, Masson's trichrome staining and IHC analysis[31]. Compared with the BLM model group, the lung index was decreased in the PFD, L-PNS and H-PNS groups (Fig. 6B), indicating attenuated fibrotic lesions in these treatment groups. We next detected miR-16-5p abundance, c-Jun phosphorylation level, as well as the expression of EndMT-related mesenchymal markers CCN2, α -SMA and FN1 in lung tissues. miR-16-5p expression was markedly down-regulated in lung tissues from BLM-challenged mice relative to control animals, whereas treatment with PFD or PNS significantly restored miR-16-5p levels compared with the BLM group (Fig. 6C). Moreover, the phosphorylation level of c-Jun, together with mRNA and protein levels of mesenchymal markers CCN2, α -SMA and FN1, were substantially elevated in the BLM group versus the control group; these parameters were markedly reduced in both PFD-treated and PNS-treated groups (Fig. 6D-6G).

H&E staining revealed that PNS-treated mice retained more intact alveolar architecture and exhibited less inflammatory cell infiltration compared with BLM-model mice, demonstrating that PNS ameliorates BLM-provoked alveolar destruction and inflammatory infiltration. Masson's trichrome staining showed reduced collagen deposition following PNS administration. IHC further confirmed that PNS suppressed α -SMA expression (Fig. 6H). Collectively, these findings suggest that PNS improves BLM-induced PF in mice by modulating the miR-16-5p/c-Jun axis.

4. Discussion

The global incidence of IPF continues to rise. Patients typically exhibit shortened survival and poor prognosis following diagnosis, imposing an increasingly heavy burden on public healthcare systems[32, 33]. Accumulating studies have demonstrated that PNS exhibits therapeutic potential in virious fibrotic diseases. In this study, miRNA sequencing of serum exosomes from IPF patients combined with public

database analyses revealed significant downregulation of miR-16-5p in IPF, which was further validated in both in vitro and in vivo models. Notably, we show that PNS alleviates BLM-induced PF by upregulating miR-16-5p, suppressing c-Jun phosphorylation and the downstream expression of CCN2, α -SMA, FN1 and COL1A1. This protective effect is mediated predominantly through the inhibition of EndMT.

Previous reports have shown that miR-16-5p suppresses asthma-associated PF[17] and cardiac fibrosis[34]. A systematic review[30] categorized miR-16-5p as an anti-fibrotic miRNA commonly downregulated in both COVID-19 and IPF. One prior study[29] also reported marked reductions of serum exosomal let-7d and miR-16 in IPF patients, establishing the first direct link between exosomal miR-16 family members to IPF. In addition, miR-16-5p can mitigate PF by inhibiting pathways including TGF- β 1/Smad3 and WNT5A/WNT10B[35, 36]. Nevertheless, most of these prior works focused on correlative expression changes or single signaling cascades, without establishing a direct mechanistic link between miR-16-5p and specific downstream transcriptional regulators under pharmacological intervention.

The present study delineates a mechanistic axis linking PNS, miR-16-5p, c-Jun and EndMT. As a key downstream transcription factor of JNK, c-Jun heterodimerizes with Fos family proteins to form the AP-1 complex, which activates multiple fibrosis-related genes[35, 37]. In BLM stimulation of HUVECs, c-Jun was phosphorylated and activated, accompanied by decreased expression of endothelial markers CD31, VE-cadherin and VWF, and increased levels of mesenchymal markers α -SMA, FN1, CCN2 and COL1A1. Both PNS treatment or miR-16-5p overexpression markedly reverses these phenotypic alterations. In animal experiments, PNS administration upregulated lung miR-16-5p, inhibited c-Jun and downstream fibrotic mediators, and ameliorated alveolar architectural disruption, inflammatory infiltration and collagen deposition. These findings support the notion that EndMT constitutes an important cellular source of PF[5, 6], and suggest that PNS suppresses EndMT, at least in part, by targeting the miR-16-5p/c-Jun axis.

PNS exerts its effects through coordinated modulation of multiple pathways, including PI3K/AKT/mTOR, MAPK/Snail/TWIST1 and PAR-1[16, 38, 39]. However, the current work focuses specifically on the miR-16-5p/c-Jun axis. PNS did not significantly alter miR-26b-5p expression (Figure S1), indicating that its regulation of miR-16-5p is relatively selective rather than a global, non-specific effect on miRNA expression.

In summary, this study elucidates a novel mechanism by which PNS inhibits EndMT and ameliorates pulmonary fibrosis through the miR-16-5p/c-Jun axis (Fig. 7). This discovery identifies the miR-16-5p/c-Jun axis as a key mediator underlying the anti-fibrotic activity of PNS and provides theoretical support for miRNA-based therapeutic strategies against IPF. Nevertheless, several limitations remain. First, the upstream regulatory mechanisms governing miR-16-5p require further exploration. Second, whether PNS cooperates with other signaling pathways such as the TGF- β /Smad cascade remains to be verified. Future work will investigate upstream regulatory effectors and evaluate the translational potential of this pathway for clinical application.

5. Conclusion

This study elucidates that PNS mitigates PF via the miR-16-5p/c-Jun axis, thereby downregulating the expression of downstream pro-fibrotic proteins (CCN2, α -SMA, FN1, and COL1A1). Moreover, we demonstrate that this effect is mediated through the inhibition of EndMT. These findings reveal a potential anti-fibrotic mechanism of PNS and support the development of miRNA-based therapeutic strategies for IPF. Further studies on the upstream regulation of miR-16-5p and the clinical translation of PNS are warranted.

Abbreviations

PNS Panax notoginseng saponins

PF pulmonary fibrosis

IPF idiopathic pulmonary fibrosis

FN1 Fibronectin

CCN2 cellular communication network factor 2

COL1A1 collagen type I alpha 1 chain

ECM extracellular matrix

EMT epithelial-mesenchymal transition

EndMT endothelial-mesenchymal transition

HUVECs human umbilical vein endothelial cells

NC negative control

PFD pirfenidone

Declarations

Ethics approval and consent to participate:

This study was conducted in accordance with the ethical principles of the institutional and national research committees and the 1964 Helsinki Declaration (including its later amendments or comparable ethical standards). The study protocol (R2025-012-01) received ethical approval from the Xi'an Chest Hospital Ethics Committee (affiliated with Northwest University) on 24 November 2025. The animal component (A2024-001-01) was approved by the same committee on 13 December 2024.

Consent for publication:

Written informed consent was obtained from all study participants, who also provided authorization for publication.

Conflicts of Interest:

The authors declare no conflicts of interest.

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Author Contribution

Conceptualization and design: F.S., G.R., L.L. Experiment: X.G.1, Z.W., J.P. Clinical data collection: J.L.2, T.D., R.W., Y.Z., W.L. Data analysis and visualization: X.G1., J.P., W.Y., J.L.1 Writing – original draft: X.G.1, Z.W. Writing – review & editing: F.S., G.R., L.L., W.Y., X.G.2 Funding acquisition: F.S. Supervision: F.S., G.R., L.L. All authors approved the final version.

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Data Availability

Publicly available datasets: The gene expression profile data analyzed in this study were obtained from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GSE124787, GSE220107, and GSE221937. These datasets are publicly available and freely accessible. Target gene identification for miR-16-5p was performed using the following online databases: miRPathDB (<https://mpd.bioinf.uni-sb.de/>) and miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>). All these resources are publicly accessible. Newly generated data: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

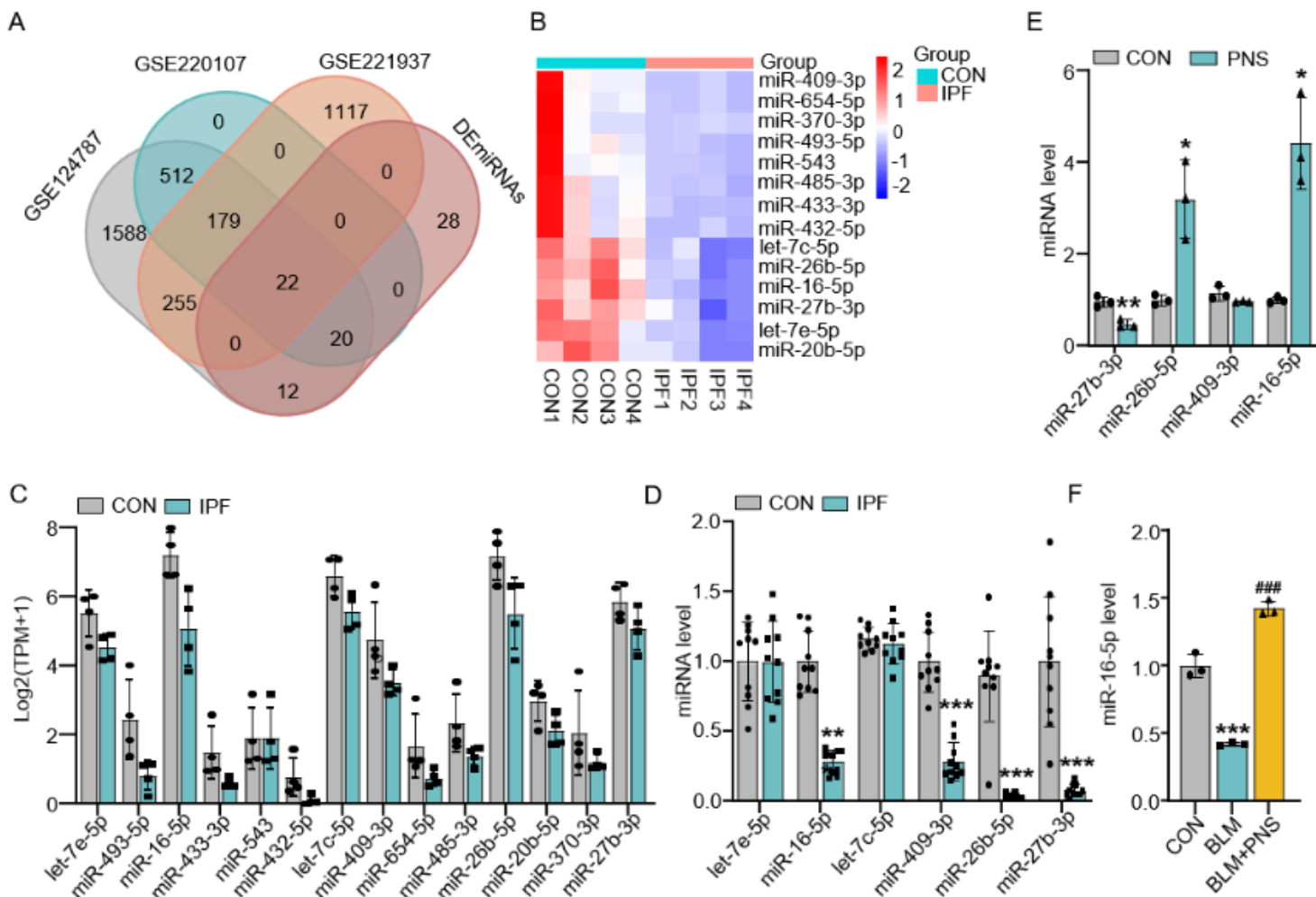


Figure 1

PNS upregulates miR-16-5p expression. (A) Venn diagram showing the overlap among the three public datasets (GSE124787, GSE220107, GSE221937) and the miRNA-seq results from this study. (B) Heatmap of differentially expressed miRNAs ($P < 0.01$). (C) Evaluation of miRNA abundance in serum exosomes from the CON and IPF groups based on TPM values. (D) qRT-PCR quantification of miRNAs ($TPM > 7$) in serum exosomes from IPF patients and healthy controls. (E) qRT-PCR detection of differentially expressed miRNAs in HUVECs after treatment with PNS (100 $\mu\text{g/mL}$) for 24 h. (F) qRT-PCR

assessment of miR-16-5p levels in BLM-induced fibrotic HUVECs under different treatment conditions (control, BLM, PNS, and BLM + PNS). Data are presented as mean $\pm$ SD. * P <0.05, ** P <0.01, *** P <0.001 vs. control; # P <0.05, ## P <0.01, ### P <0.001 vs. BLM

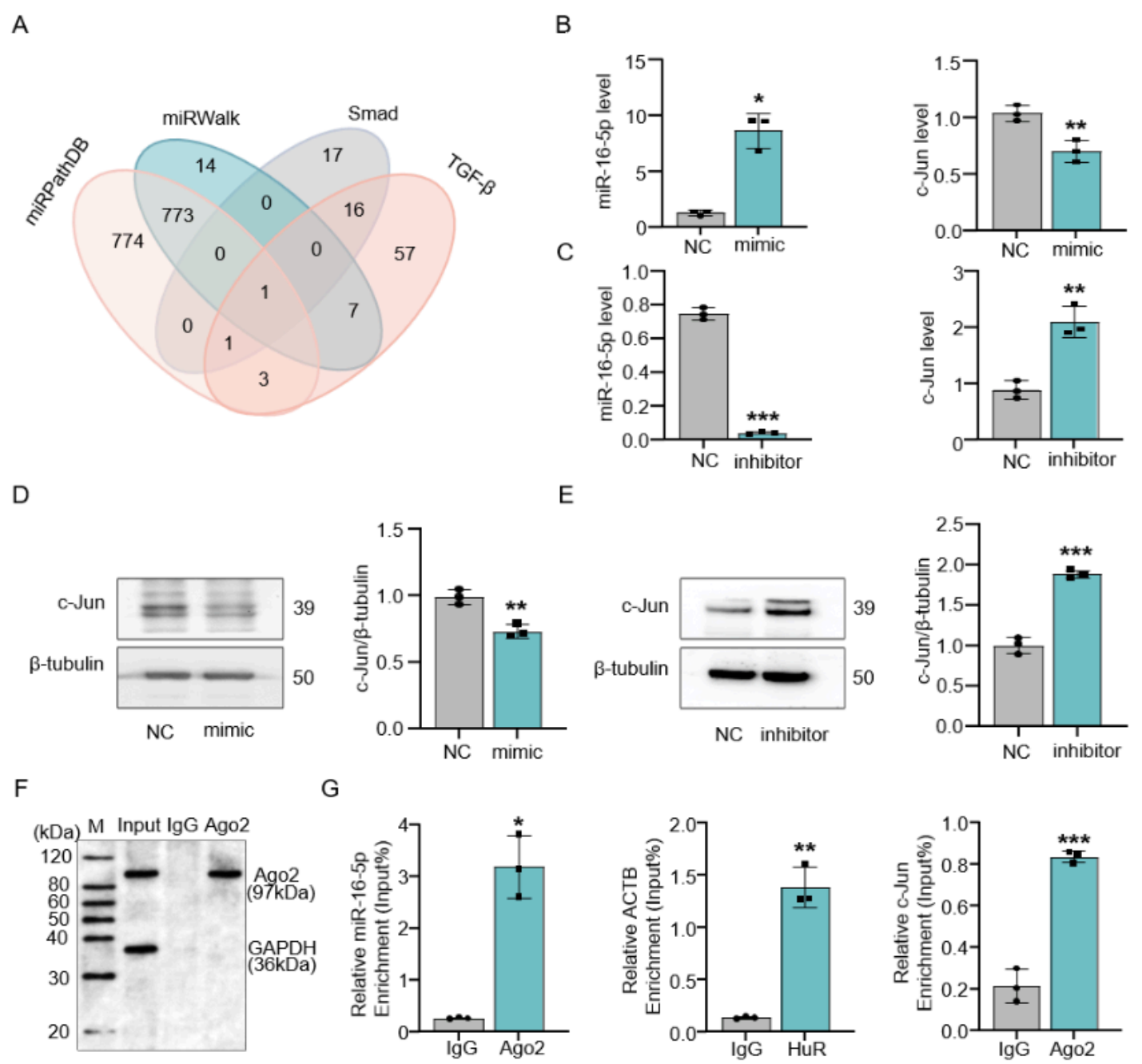


Figure 2

miR-16-5p directly binds to c-Jun. (A) Two-step integrated screening for candidate target genes. Left: overlap of target genes predicted by miRWalk and miRPathDB. Right: genes annotated to TGF- β and Smad3 signaling based on GO biological processes. Their intersection identified c-Jun as the sole candidate target gene. c-Jun emerged as the sole overlapping candidate. (B-C) c-Jun mRNA levels measured by qRT-PCR after miR-16-5p mimic or inhibitor treatment. (D-E) Western blot assessment of c-

Jun protein levels following miR-16-5p overexpression or inhibition. Data are presented as mean $\pm$ SD. * P <0.05, ** P <0.01, *** P <0.001 vs. NC

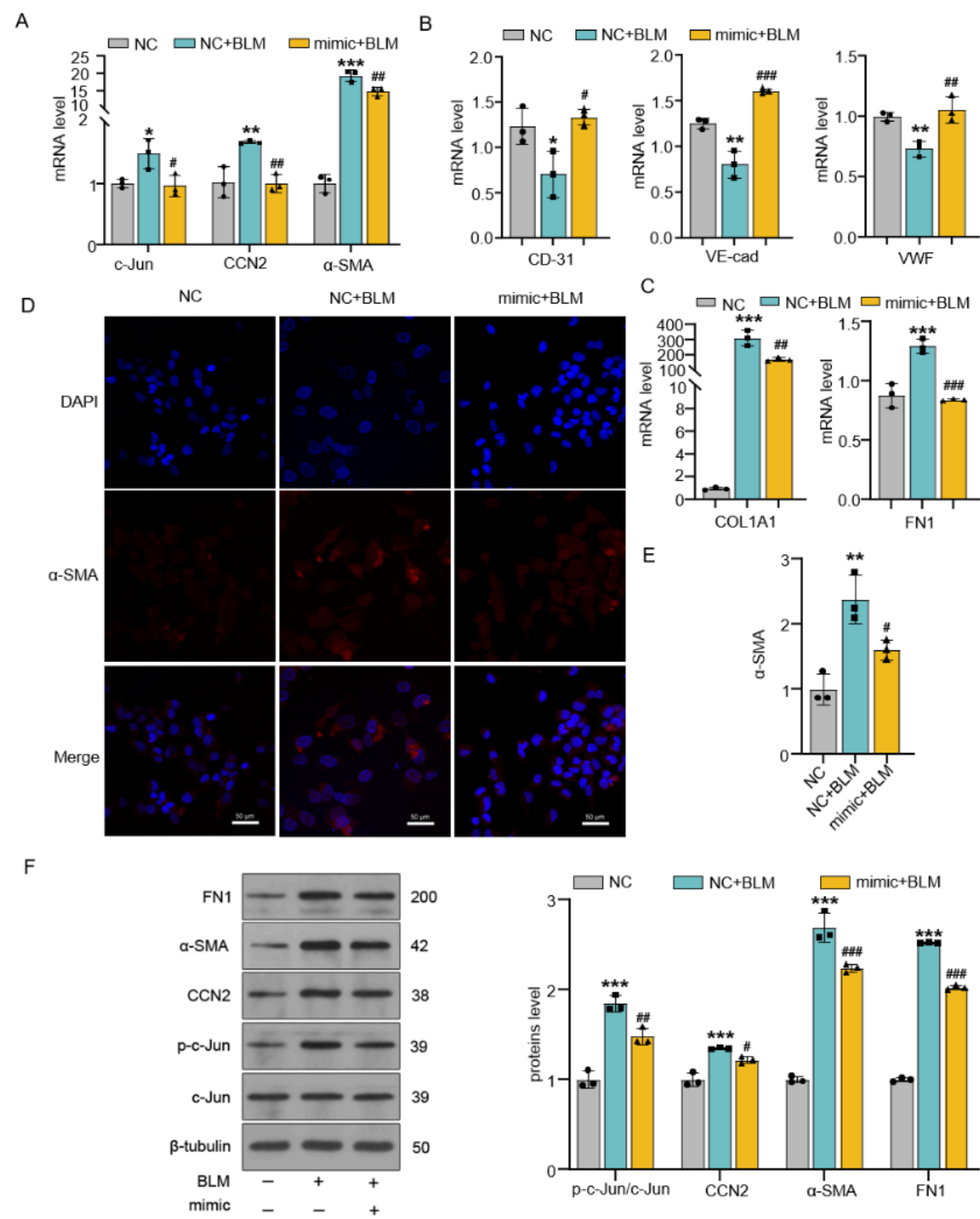


Figure 3

miR-16-5p mitigates EndMT by suppressing c-Jun. (A-C) qRT-PCR assessment of c-Jun, mesenchymal marker (A, C: CCN2, α-SMA, FN1, and collagen I) and endothelial markers (B: CD31, VE-cad, and VWF)

mRNA levels in HUVECs transfected with miR-16-5p mimic (or controls) followed by BLM treatment. (C-D) IF staining and quantitative analysis of α -SMA in each treatment group (scale bar = 50 μ m). (E) Protein levels of p-c-Jun, CCN2, α -SMA, and FN1 determined by Western blotting. Data are presented as mean $\pm$ SD. * P <0.05, ** P <0.01, *** P <0.001 vs. NC; # P <0.05, ## P <0.01, ### P <0.001 vs. NC+BLM

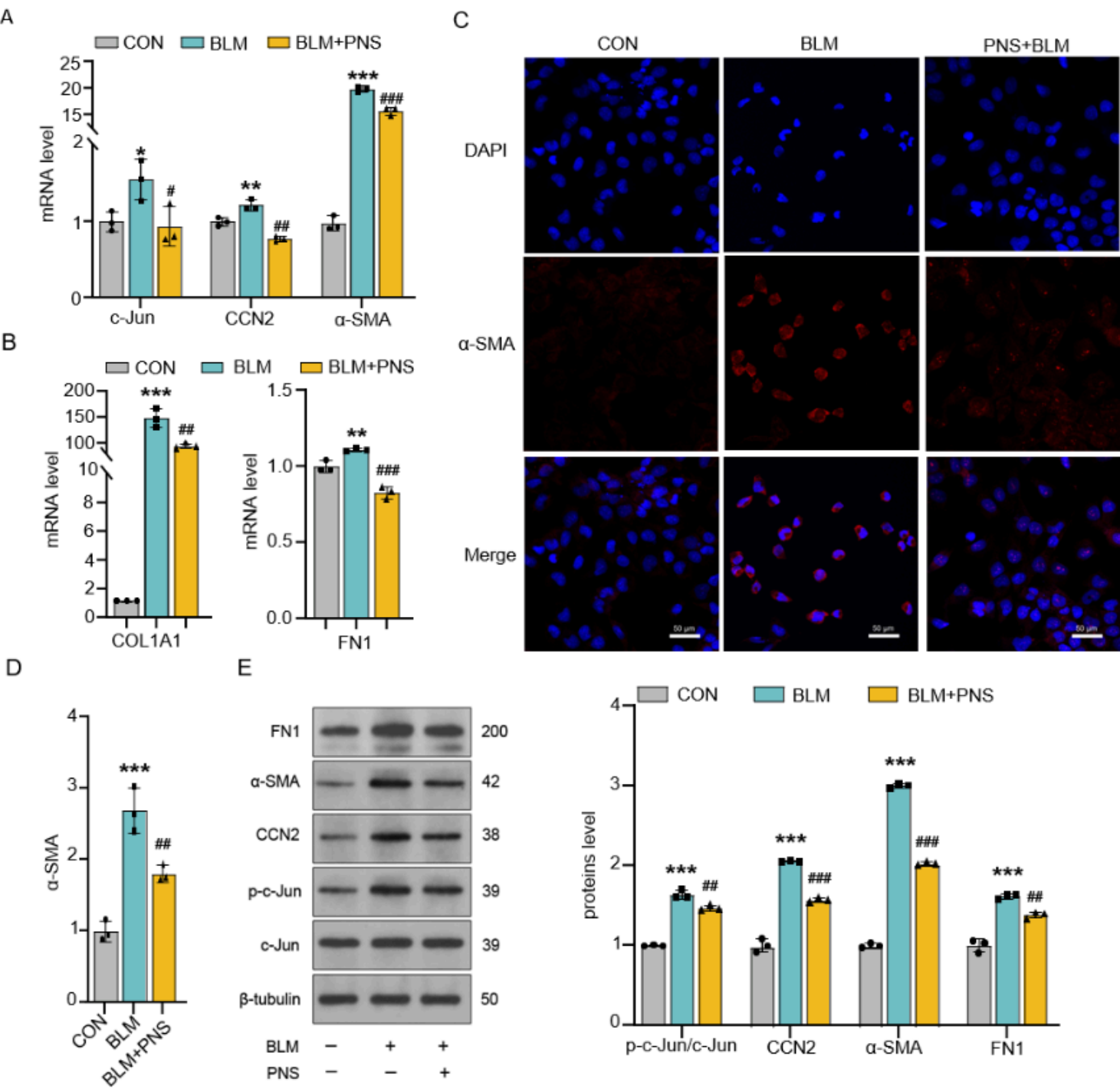


Figure 4

PNS ameliorates EndMT via the miR-16-5p/c-Jun axis. (A-B) qRT-PCR assessment of c-Jun, CCN2, α -SMA, FN1, and collagen I mRNA levels in HUVECs subjected to the indicated treatments. (C-D) IF

staining and quantitative analysis of α -SMA in each treatment group (scale bar = 50 μ m). (E) Protein levels of p-c-Jun, CCN2, α -SMA, and FN1 determined by Western blotting. Data are presented as mean $\pm$ SD. * P <0.05, ** P <0.01, *** P <0.001 vs. control; # P <0.05, ## P <0.01, ### P <0.001 vs. BLM

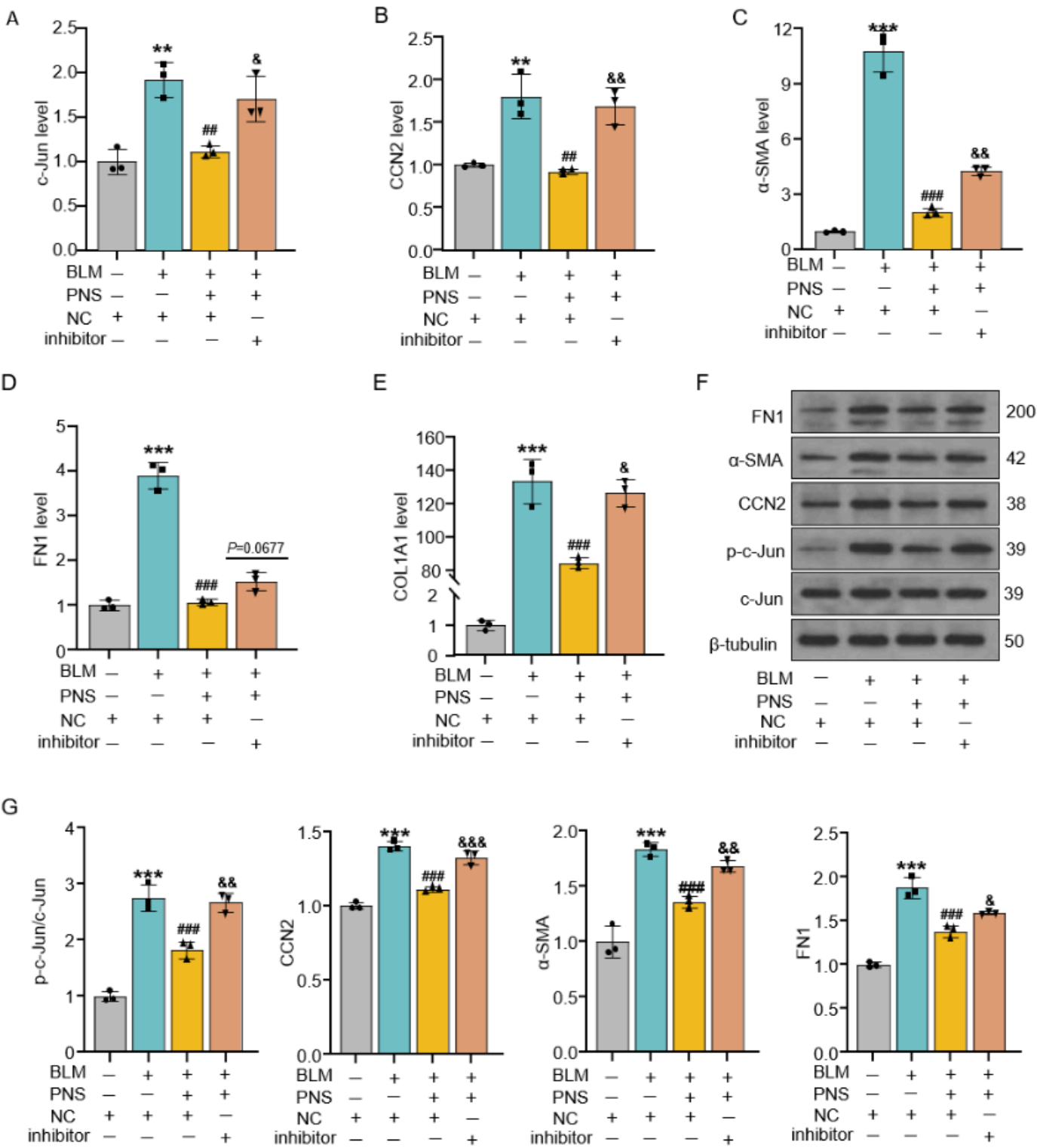


Figure 5

Blockade of the miR-16-5p/c-Jun Axis Abrogates the Protective Effect of PNS against Endothelial-Mesenchymal Transition (A-E) The mRNA levels of CCN2, α -SMA, COL1A1 and FN1 in each group were quantified by qRT-PCR. (F-G) Protein levels of p-c-Jun, CCN2, α -SMA, and FN1 determined by Western blotting. Data are presented as mean $\pm$ SD. * P <0.05, ** P <0.01, *** P <0.001 vs. control; # P <0.05, ## P <0.01, ### P <0.001 vs. BLM; & P <0.05, && P <0.01, &&& P <0.001, vs. BLM+PNS

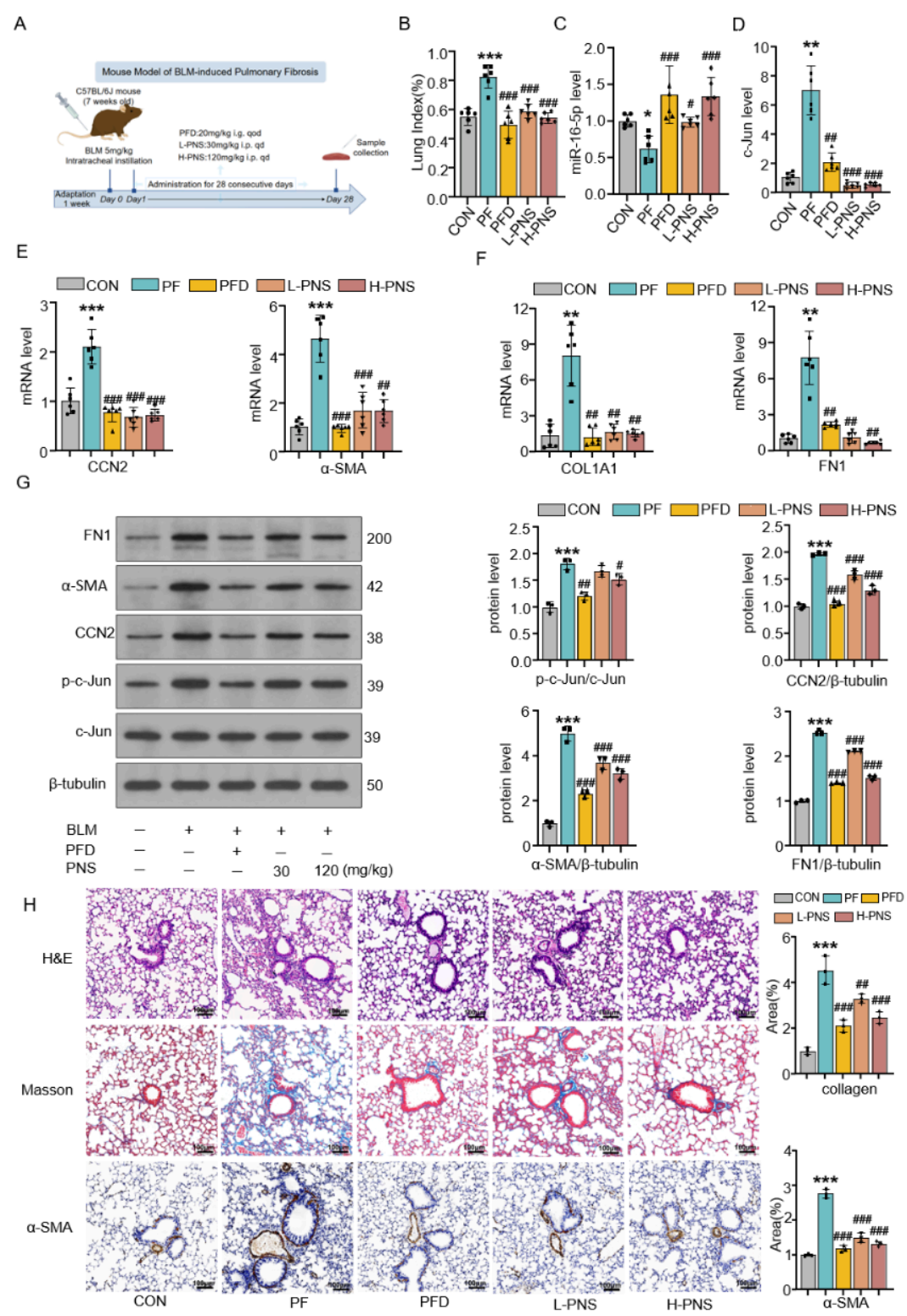


Figure 6

PNS ameliorates BLM-induced pulmonary fibrosis via the miR-16-5p/c-Jun axis in mice. (A) Schematic illustration of BLM-induced pulmonary fibrosis mouse model establishment and drug administration protocol. (B) Lung index (lung weight/body weight ratio) for each group. (C-F) qRT-PCR assessment of miR-16-5p, c-Jun, α -SMA, FN1, and collagen I mRNA levels in pulmonary tissues. (G) Protein levels of p-c-Jun, CCN2, α -SMA, and FN1 detected by Western blotting. (H) Representative images of H&E and Masson's trichrome staining showing histopathological changes in lung tissue, as well as IHC staining for α -SMA (scale bar = 100 μ m). * P <0.05, ** P <0.01, *** P <0.001 vs. control, # P <0.05, ## P <0.01, ### P <0.001 vs. PF.

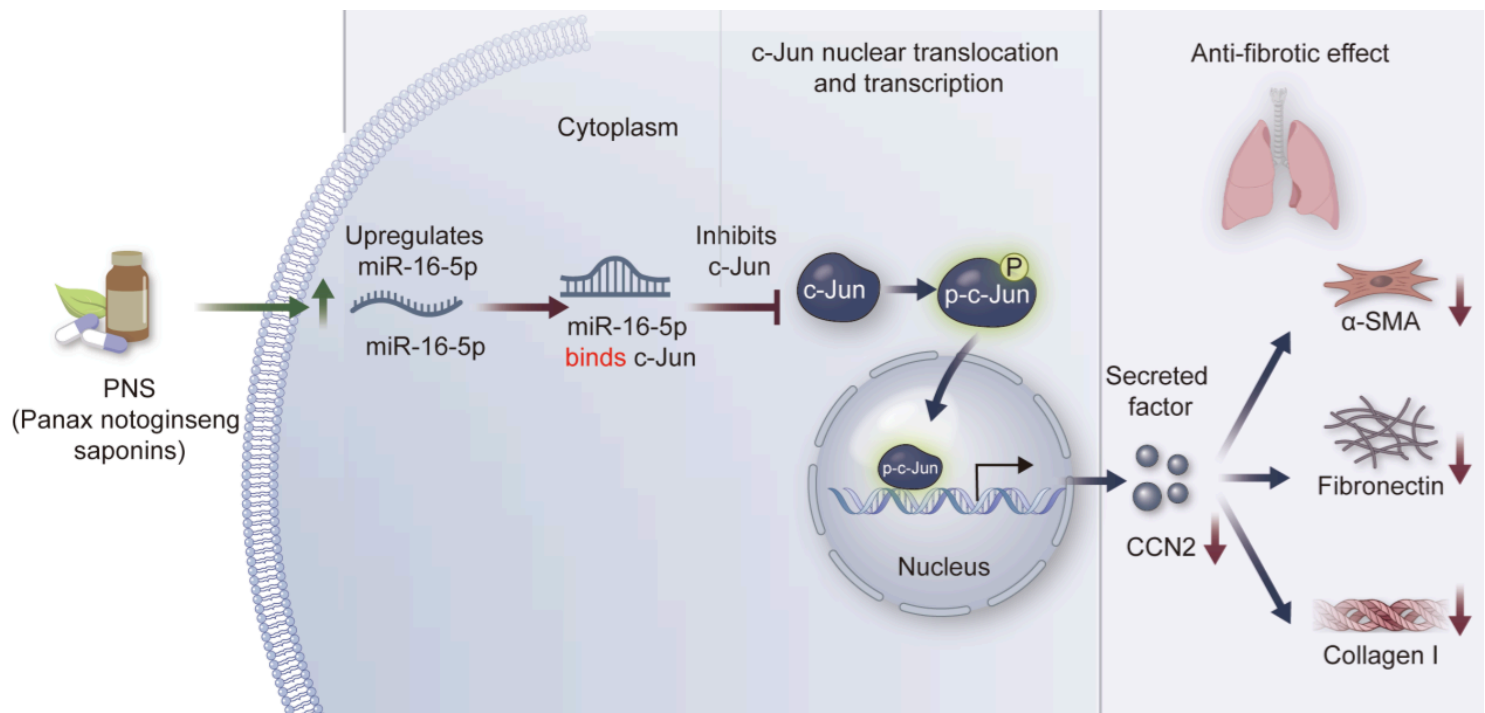


Figure 7

Schematic illustration of the mechanism by which PNS alleviates pulmonary fibrosis. Under pathological stimuli (e.g., bleomycin), resulting in c-Jun phosphorylation. Subsequently, p-c-Jun translocates to the nucleus, which drives CCN2 transcription. Elevated CCN2 upregulates α -SMA, promoting EndMT and pulmonary fibrosis. Notably, Treatment with PNS upregulates miR-16-5p, which directly suppresses c-Jun, thereby blocking the activation of the aforementioned downstream signaling pathway

Supplementary Files

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