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Neuropilin-1 Negatively Regulates the Phosphatidylinositol 3-Kinase/Protein Kinase B Pathway to Inhibit Lung Fibroblast Activation and Its Role in Murine Pulmonary Fibrosis: A Postprint Study

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Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is characterized by complex pathogenesis, limited therapeutic options, and poor prognosis, making the identification of safe and effective therapeutic targets crucial.

Objective: To investigate the regulatory effect of Neuropilin-1 (Nrp1) on mouse pulmonary fibroblasts (MPFs) activation via the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway, and to evaluate the therapeutic efficacy and safety of recombinant Nrp1 protein in a mouse model of pulmonary fibrosis (PF).

Methods: Datasets GSE150910, GSE218997, GSE173523, GSE47460, and GSE32537 were downloaded from the GEO database to analyze Nrp1 expression in PF mouse models and IPF patient lung tissues, as well as the correlation between Nrp1 and pulmonary function indices in IPF patients. Forty-five IPF patients diagnosed at the Department of Respiratory and Critical Care Medicine, General Hospital of Ningxia Medical University between 2022 and 2024, and 29 healthy controls from the Health Management Center of the same hospital were enrolled. Cell-free RNA (cfRNA) sequencing was performed to analyze plasma Nrp1 expression levels and its diagnostic efficacy. MPFs were divided into control group, transforming growth factor-$ 1(TGF- 1)group, Nrp1overexpressiongroup, andTGF- 1 + Nrp1overexpressiongroup.TGF- $1 was used to induce MPFs activation, and Nrp1 overexpression was employed to examine its effects on the PI3K/AKT pathway and the expression of α-SMA, Vim, and Fn. Thirty male C57BL/6J mice were randomly allocated into control group, bleomycin (BLM) group, and

BLM+Nrp1 group (n=10 per group). Following establishment of the mouse PF model, the BLM+Nrp1 group received intraperitoneal injection of recombinant Nrp1 protein at 100 g・kg-1・d-1 for 20 consecutive days. Hematoxylin-eosin (HE) and Masson staining were utilized to observe pathological changes in mouse lung tissues. Hepatic and renal function indicators were assessed. Western blotting and immunohistochemistry were conducted to detect the expression of α-smooth muscle actin (α-SMA), Vimentin (Vim), and Fibronectin (Fn) in lung tissues. Enzyme-linked immunosorbent assay (ELISA) was used to measure Nrp1 levels in plasma and bronchoalveolar lavage fluid (BALF).

Results: Dataset analysis revealed that Nrp1 expression was significantly decreased in lung tissues of PF mice and IPF patients compared to normal lung tissues (P <0.05). Nrp1 expression was positively correlated with forced expiratory volume in one second percentage predicted (FEV1%), forced vital capacity percentage predicted (FVC%), and diffusing capacity of the lungs for carbon monoxide percentage predicted (DLCO%). Plasma Nrp1 mRNA expression level was significantly lower in IPF patients than in healthy controls (P <0.05). The area under the receiver operating characteristic curve for plasma Nrp1 in diagnosing IPF was 0.754 (95%CI=0.634~0.874). Compared with the TGF-$ 1*group*, *theTGF*− $1+Nrp1 overexpression group exhibited decreased p-PI3K/PI3K and p-AKT/AKT ratios, along with reduced expression of α-SMA, Vim, and Fn (P <0.05). Following intraperitoneal injection of recombinant Nrp1 protein, pathological features of PF mice were ameliorated. No statistically significant differences were observed in plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine (CREA) levels among the control, BLM, and BLM+Nrp1 groups (P >0.05). Compared with the BLM group, the BLM+Nrp1 group showed decreased α-SMA and Fn expression in lung tissues and increased Nrp1 levels in plasma and BALF (P <0.05).

Conclusion: Nrp1 expression is reduced in lung tissue, plasma, and BALF of IPF patients. Nrp1 inhibits mouse pulmonary fibroblast activation by negatively regulating the PI3K/AKT signaling pathway. Intraperitoneal injection of recombinant Nrp1 can alleviate pulmonary fibrosis in mice with systemic safety.

Full Text

Nrp1 Negatively Regulates PI3K/AKT Pathway to Inhibit the Activation of Lung Fibroblasts and Its Role in Mouse Pulmonary Fibrosis

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Abstract

Background: Idiopathic pulmonary fibrosis (IPF) has a complex pathogenesis, limited treatment options, and poor prognosis, making identification of safe and effective therapeutic targets crucial. **Objective:** To investigate the regulatory effect of Neuropilin-1 (Nrp1) on mouse pulmonary fibroblast (MPF) activation through the PI3K/AKT signaling pathway and evaluate the therapeutic efficacy and safety of recombinant Nrp1 protein in mouse pulmonary fibrosis (PF). **Methods:** Datasets GSE150910, GSE218997, GSE173523, GSE47460, and GSE32537 were downloaded from the GEO database to analyze Nrp1 expression in PF mouse models and IPF patient lung tissues, as well as correlations between Nrp1 and pulmonary function parameters in IPF patients. Forty-five IPF patients diagnosed in the Department of Respiratory and Critical Care Medicine and 29 healthy controls undergoing physical examinations at the health management center of our hospital from 2022 to 2024 were enrolled. Cell-free RNA (cfRNA) sequencing was used to analyze plasma Nrp1 expression levels and diagnostic efficacy. MPFs were divided into control, TGF-\$ $1, Nrp1overexpression, andTGF- 1+Nrp1overexpressiongroups.TGF-$ \$1 was used to induce MPF activation, and the effects of Nrp1 overexpression on the PI3K/AKT pathway and expression of α-SMA, Vim, and Fn were examined. Thirty male C57BL/6J mice were randomly divided into control, bleomycin (BLM), and BLM+Nrp1 groups (n=10 each). After establishing the PF model, the BLM+Nrp1 group received intraperitoneal injections of $100\ \mathrm{g \cdot kg^{-1} \cdot d^{-1}}$ recombinant Nrp1 protein for 20 consecutive days. HE and Masson staining were used to observe pathological changes in lung tissues. Liver and kidney function indicators were evaluated. Western blotting and immunohistochemistry detected α-smooth muscle actin (α-SMA), Vimentin (Vim), and Fibronectin (Fn) expression in lung tissues. ELISA measured Nrp1 levels in plasma and bronchoalveolar lavage fluid (BALF). **Results:** Dataset analysis showed that Nrp1 expression was decreased in lung tissues of PF mice and IPF patients compared to normal lung tissues (P<0.05), and Nrp1 expression was positively correlated with FEV1%, FVC%, and DLCO%. Plasma Nrp1 mRNA expression was lower in IPF patients than in healthy controls (P<0.05), with an AUC of 0.754 (95%CI=0.634-0.874) for diagnosing IPF. Compared with the TGF-\$ $1group, theTGF-$ \$1+Nrp1 overexpression group showed decreased p-PI3K/PI3K and p-AKT/AKT ratios and reduced α-SMA, Vim, and Fn ex-

pression (P<0.05). After recombinant Nrp1 injection, pulmonary pathological features improved in PF mice. No significant differences were observed in plasma ALT, AST, ALP, BUN, and CREA levels among the control, BLM, and BLM+Nrp1 groups (P>0.05). Compared with the BLM group, the BLM+Nrp1 group showed decreased α-SMA and Fn expression in lung tissues and increased Nrp1 levels in plasma and BALF (P<0.05). **Conclusion:** Nrp1 expression is decreased in lung tissues, plasma, and BALF of IPF patients. Nrp1 inhibits mouse pulmonary fibroblast activation by negatively regulating the PI3K/AKT signaling pathway. Intraperitoneal injection of recombinant Nrp1 protein can alleviate pulmonary fibrosis in mice while maintaining systemic safety.

Keywords: Pulmonary fibrosis; Neuropilin-1; Fibroblast activation; Recombinant Nrp1 protein

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal interstitial lung disease characterized by abnormal activation of fibroblasts, excessive extracellular matrix (ECM) deposition, and destruction of lung architecture, with a median survival of only 3–5 years [1-3]. Currently approved clinical drugs (pirfenidone and nintedanib) can only slow the decline in lung function but cannot reverse the fibrotic process, and they have limitations including hepatotoxicity and gastrointestinal adverse effects [4-7]. Therefore, exploring new mechanisms of IPF pathogenesis and identifying safe and effective therapeutic targets is crucial.

Neuropilin-1 (Nrp1) is a transmembrane glycoprotein receptor initially studied for its roles in angiogenesis and axon guidance [8-10]. Recent studies have revealed that Nrp1 promotes fibrosis in the liver, kidney, and myocardium [11], but its role in pulmonary fibrosis (PF) remains incompletely understood. In radiation-induced pulmonary fibrosis (RIPF) models, Nrp1 accelerates fibrosis by promoting M2 macrophage polarization and epithelial-mesenchymal transition (EMT) [12]. She et al. [13] demonstrated that Nrp1 expression increases after ionizing radiation and promotes collagen deposition by regulating arginase 1 (Arg1) and other M2 macrophage markers, while the antagonist EG00229 significantly inhibits fibrosis. Other studies have reported that Nrp1 expression is significantly reduced in lung tissues of IPF patients and PF animal models and is closely correlated with impaired lung function, including decreased percentage of predicted forced expiratory volume in one second (FEV1%), forced vital capacity (FVC%), and diffusing capacity for carbon monoxide (DLCO%) [14]. Shen et al. [15] found that Nrp1 specifically expressed in type 2 innate lymphoid cells (ILC2) in lung tissue is induced by transforming growth factor $ 1(TGF− $1) and promotes fibrosis by enhancing interleukin-33/suppression of tumorigenicity 2 (IL-33/ST2) signaling.

The phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway is a core driver of PF, promoting fibroblast activation, EMT, and inflam-

matory responses that drive IPF progression. Recent studies indicate that Nrp1 overexpression activates the PI3K/AKT pathway, enhancing non-small cell lung cancer resistance to osimertinib [16], and Nrp1 upregulates vascular endothelial growth factor receptor 2 (VEGFR2) expression through the PI3K/AKT pathway to promote angiogenesis and aggravate liver cirrhosis [17], suggesting that Nrp1 can regulate the PI3K/AKT pathway. Based on these findings, this study aimed to validate the expression characteristics of Nrp1 in IPF patients and bleomycin (BLM) mouse models, investigate whether Nrp1 directly regulates lung fibroblast activation through the PI3K/AKT pathway, and evaluate whether exogenous supplementation with recombinant Nrp1 protein can improve fibrosis progression and its safety, providing new insights into IPF pathogenesis and diagnostic research.

Methods

Study Subjects

This study enrolled 45 IPF patients diagnosed in the Department of Respiratory and Critical Care Medicine at the General Hospital of Ningxia Medical University from 2022 to 2024, along with 29 healthy controls who underwent physical examinations at the health management center of our hospital during the same period. The study protocol was approved by the Ethics Committee of Ningxia Medical University (KYLL-2023-0516).

Inclusion criteria for IPF patients: (1) Diagnosis of IPF according to the latest American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Society (ATS/ERS/JRS/ALAT) guidelines [18]; (2) Age >50 years; (3) Exclusion of other known causes of interstitial lung disease, such as environmental exposures (dust, mold), drug toxicity, or autoimmune diseases; (4) Ability to communicate verbally or in writing and complete study-related auxiliary examinations; (5) Signed informed consent.

Exclusion criteria for IPF patients: (1) Other types of interstitial lung disease, such as connective tissue disease-associated interstitial lung disease (CTD-ILD), non-specific interstitial pneumonia, hypersensitivity pneumonitis, or sarcoidosis; (2) Severe systemic diseases, including uncontrolled cardiovascular disease or liver/kidney failure; (3) Pregnancy or lactation; (4) Inability to cooperate with examinations or follow-up.

Inclusion criteria for healthy controls: (1) No history of respiratory or chronic lung disease; (2) Age and gender matched with IPF patients; (3) Voluntary provision of informed consent and compliance with study procedures.

Experimental Animals and Husbandry

Thirty wild-type male C57BL/6J mice (6–8 weeks old, 18–22 g) were purchased from the Laboratory Animal Center of Ningxia Medical University

and housed in SPF-grade animal facilities under controlled temperature (22$±2°*C*), *humidity*(50±$10%), and a 12-hour light/dark cycle with free access to standard rodent chow and sterilized water.

Experimental Cells

Mouse pulmonary fibroblasts (MPFs) were purchased from iCell Bioscience Inc. Construction and identification of the Nrp1 overexpression plasmid were completed by Shanghai GeneChem Co., Ltd.

Reagents

Bleomycin (BLM, IB0871) was purchased from Beijing Solarbio Science & Technology Co., Ltd. Antibodies against Fibronectin (Fn, ab2413), α-smooth muscle actin (α-SMA, ab5694), Vimentin (Vim, ab24525), and Nrp1 (ab81321) were obtained from Abcam. Antibodies against β-actin (10494-1-AP), glyceraldehyde-3-phosphate dehydrogenase (Gapdh, 60004-1-Ig), PI3K (60225-1-Ig), phosphorylated PI3K (p-PI3K, 66444-1-Ig), and AKT (10176-2-AP) were purchased from Proteintech Group. Phosphorylated AKT (p-AKT, T40116M) antibody was obtained from Abmart. Recombinant mouse TGF-$ $1 was purchased from R&D Systems. Recombinant mouse Nrp1-Fc protein was obtained from MedChemExpress. BCA protein assay kit (KGB2101-500) was purchased from KeyGen Biotech. Nrp1 ELISA kit (JL54766) was obtained from Shanghai Jianglai Biotechnology.

Experimental Procedures

Bioinformatics Analysis Gene expression datasets GSE150910, GSE218997, GSE173523, GSE47460, and GSE32537 were downloaded from the Gene Expression Omnibus (GEO) database. Data cleaning, normalization, and differential analysis were performed using R software with DESeq2 and limma packages, followed by data visualization.

Plasma cfRNA Sequencing Fasting peripheral venous blood (5 mL) was collected from IPF patients and healthy controls using EDTA anticoagulant tubes. Plasma was separated by centrifugation at 3,000 rpm for 10 minutes at 4°C (radius 15 cm), followed by a second centrifugation at 16,000×g for 10 minutes at 4°C to remove cellular debris. The supernatant was aliquoted and stored at -80°C before being sent to Shenzhen HaploX Biotechnology for cfRNA sequencing.

TGF-$ $1-Induced MPF Activation MPFs at passages 3–5 were used for activation experiments. Cells were seeded at 3$×10^{5}$ cells/well in six-well plates and cultured for 24 hours until fully adherent. They were then divided into control and TGF-$ 1*groups.Thecontrolgroupreceivedcompletemedium*, *whiletheTGF*− 1*groupwastreate*
for 24 hours [19].

Nrp1 Overexpression Experiment Cells were divided into control, TGF-$ 1, *Nrp*1*overexpression*, *andTGF*− 1+*Nrp*1*overexpressiongroups*, *withthreereplicatespergroup*.*Cellsweres*
*wellplatesonedaybeforetransfectionat*60–80 1+*Nrp*1*overexpressiongroups*, *freshcompletemediumwasadde*
*Nrp*1*overexpressionandTGF*− 1*groupsreceivedcompletemediumcontaining*10*ng*/*mLTGF*− $1, while the control and Nrp1 overexpression groups received complete medium. After an additional 24 hours, cells were collected for RNA and protein extraction.

Recombinant Nrp1 Protein Treatment in Mouse PF Model Thirty male C57BL/6J mice were randomly divided into control, BLM, and BLM+Nrp1 groups (n=10 each). A mouse PF model was established by intratracheal instillation of BLM, with the control group receiving equal volume of 0.9% saline. Starting on day 2 post-modeling, the BLM+Nrp1 group received intraperitoneal injections of 100 $\mathrm{g \cdot kg^{-1} \cdot d^{-1}}$ recombinant Nrp1 protein for 21 consecutive days, while the control and BLM groups received equal volume of 0.9% saline. Mice were sacrificed on day 21, and lung tissues, plasma, and BALF were collected [20]. This experimental protocol was approved by the Animal Ethics Committee of Ningxia Medical University (IACUC-NYLAC-2023-252).

Hematoxylin-Eosin (HE) Staining Lung tissue sections from control, BLM, and BLM+Nrp1 groups were deparaffinized, stained with hematoxylin, differentiated with hydrochloric acid ethanol, blued with bluing reagent, counterstained with eosin, dehydrated with ethanol, cleared with xylene, and mounted with neutral resin.

Masson Staining [21] Lung tissue sections were deparaffinized, immersed in potassium dichromate overnight, washed with tap water, stained with a 1:1 mixture of iron hematoxylin A and B solutions, washed, differentiated, washed, stained with Ponceau acid fuchsin, washed, treated with phosphomolybdic acid, stained with aniline blue, differentiated with 1% acetic acid, dehydrated with two changes of absolute ethanol, cleared with xylene, and mounted with neutral balsam for microscopic examination and image analysis.

Plasma Liver and Kidney Function Indicators Blood was collected from mice in the control, BLM, and BLM+Nrp1 groups. Plasma was separated by centrifugation at 3,000 rpm for 10 minutes at 4°C (radius 5 cm). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine (CREA) levels were measured using an automatic biochemical analyzer.

Immunohistochemistry Lung tissue sections were deparaffinized, subjected to antigen retrieval, blocked for endogenous peroxidase activity, and serum-blocked before overnight incubation with primary antibodies against Nrp1 (1:250) and α-SMA (1:250). Secondary antibodies were applied the following

day, followed by DAB chromogen development, nuclear counterstaining, dehydration, mounting, and analysis using a slide scanner.

Western Blotting Analysis Mouse lung tissues and MPFs were lysed with RIPA buffer, and total protein was collected from the supernatant after centrifugation. Protein concentration was determined by BCA assay. Twenty micrograms of protein were separated by SDS-PAGE, transferred to PVDF membranes, blocked with 5% skim milk, and incubated with primary antibodies at 4°C overnight. After three washes with TBST, membranes were incubated with appropriate secondary antibodies. ECL substrate was applied for protein imaging, and grayscale values were calculated using Image J software to analyze expression levels of Nrp1, α-SMA, Vim, Fn, PI3K, p-PI3K, AKT, and p-AKT.

Quantitative Real-Time PCR (qRT-PCR) RNA was extracted using TRIzol and chloroform, then reverse-transcribed to cDNA. Relative mRNA expression levels were calculated using the 2^(-ΔΔCt) method with Gapdh or β-actin as internal controls. Primers were synthesized by Shanghai Sangon Biotech, and sequences are listed in Table 1 .

ELISA Nrp1 levels in plasma and BALF from control, BLM, and BLM+Nrp1 groups were measured according to the manufacturer' s instructions.

Statistical Analysis

Data were analyzed using GraphPad Prism 9.0 software. Normally distributed continuous data are presented as mean±standard deviation ($\bar{x}\pm s$) and compared between two groups using t-tests. Multiple groups were compared using one-way ANOVA with LSD-t test for pairwise comparisons. Non-normally distributed data are presented as median (P25, P75) and compared using non-parametric tests. Receiver operating characteristic (ROC) curves were plotted to evaluate the diagnostic efficacy of plasma Nrp1 for IPF. Statistical significance was set at $P<0.05$.

Results

Nrp1 Expression in GEO Datasets and Correlation with Pulmonary Function

Analysis of dataset GSE150910 revealed that Nrp1 gene expression was significantly lower in IPF patient lung tissues compared to normal lung tissues ($P<0.05$) (Figure 1 [Figure 1: see original paper]A). Datasets GSE218997 and GSE173523 showed that Nrp1 gene expression was decreased in lung tissues of BLM-induced fibrosis model mice compared to normal controls ($P<0.05$) (Figure 1B, 1C). Analysis of datasets GSE47460 and GSE32537 demonstrated that

Nrp1 gene expression in BALF of IPF patients was positively correlated with DLCO%, FEV1%, and FVC% (Figure 1D–1H).

Comparison of Baseline Characteristics Between IPF Patients and Healthy Controls

The healthy control group comprised 18 males and 11 females with a median age of 63 years, while the IPF group included 27 males and 18 females with a median age of 66 years. No significant differences were observed in gender, age, BMI, or total lung capacity (TLC) between groups (P>0.05). However, IPF patients showed significantly lower FVC, FVC%, TLC%, and DLCO% compared to healthy controls (P<0.05) (Table 2).

Nrp1 Expression in Plasma and Diagnostic Performance in IPF Patients

Analysis of cfRNA sequencing data from IPF patients and healthy controls, using P<0.05 and $|\log_2 FC|>1$ as screening criteria, revealed that plasma Nrp1 mRNA expression was significantly lower in IPF patients (P<0.05) (Figure 2 [Figure 2: see original paper]A, 2B). ROC curve analysis showed that the AUC for plasma Nrp1 in diagnosing IPF was 0.754 (95%CI=0.634–0.874) (Figure 2C).

Effect of Nrp1 Overexpression on Activation-Related Molecules in MPFs

Western blotting and qRT-PCR results demonstrated significant differences in protein and mRNA expression levels of α-SMA, Vim, Fn, and Nrp1 among the four groups (P<0.05). Compared with the control group, the TGF-$ $1 group showed increased α-SMA and Fn expression and decreased Nrp1 expression. The Nrp1 overexpression group exhibited decreased α-SMA, Vim, and Fn expression and increased Nrp1 expression compared to controls. Compared with the TGF-$ 1*group, theTGF−* $1+Nrp1 overexpression group showed significantly reduced α-SMA, Vim, and Fn expression and increased Nrp1 expression (P<0.05) (Figure 3 [Figure 3: see original paper], Tables 3–4 -).

Effect of Nrp1 Overexpression on the PI3K/AKT Pathway in MPFs

To investigate whether Nrp1 regulates MPF activation through the PI3K/AKT pathway, Western blotting was performed to examine the effects of Nrp1 overexpression on PI3K, p-PI3K, AKT, and p-AKT protein levels. Significant differences were observed in p-PI3K/PI3K and p-AKT/AKT ratios among the four groups (P<0.05). The TGF-$ 1*groupshowedincreasedp−PI3K/PI3Kandp−AKT/AKTratioscomparedtocontrols.Nrp1overexpressionalonedecreasedp−PI3K/PI3Kratio.ComparedwiththeTGF−1group,theTGF−* $1+Nrp1 overexpression group showed significantly reduced p-PI3K/PI3K and p-AKT/AKT ratios (P<0.05) (Figure 4 [Figure 4: see original paper], Table 5).

Histopathological Effects of Recombinant Nrp1 Protein in PF Mice

HE staining revealed intact lung architecture in the control group, while the BLM group showed severe alveolar structural damage with inflammatory cell infiltration and thickened alveolar walls. These pathological features were significantly improved after recombinant Nrp1 intervention. Masson staining demonstrated extensive collagen deposition and fused alveolar structures in the BLM group, which were reduced after Nrp1 treatment, indicating that recombinant Nrp1 can ameliorate PF pathology and decrease collagen deposition (Figure 5 [Figure 5: see original paper]).

Plasma Liver and Kidney Function Parameters in Mice

No significant differences were observed in plasma ALT, AST, ALP, BUN, and CREA levels among the control, BLM, and BLM+Nrp1 groups ($P>0.05$) (Table 6).

Effect of Recombinant Nrp1 on α-SMA, Vim, and Fn Expression in PF Mouse Lung Tissues

Immunohistochemistry revealed increased α-SMA in lung tissues after BLM induction, which was reduced by recombinant Nrp1 injection, indicating effective inhibition of myofibroblast activation. Additionally, few Nrp1-positive brown-yellow areas were observed in the BLM group compared to controls, while Nrp1 treatment increased Nrp1-positive expression (Figure 6 [Figure 6: see original paper]).

Western blotting showed significant differences in α-SMA, Vim, Fn, and Nrp1 protein expression among the three groups ($P<0.05$). Compared with controls, the BLM group exhibited decreased Nrp1 expression and increased α-SMA, Vim, and Fn expression. Compared with the BLM group, the BLM+Nrp1 group showed decreased Fn expression ($P<0.05$) (Figure 7 [Figure 7: see original paper], Table 7).

Effect of Recombinant Nrp1 on Nrp1 Levels in BALF and Plasma of PF Mice

Significant differences were observed in Nrp1 levels in both BALF and plasma among the control, BLM, and BLM+Nrp1 groups ($P<0.05$). The BLM group showed decreased Nrp1 levels in both BALF and plasma compared to controls, while the BLM+Nrp1 group showed significantly increased Nrp1 levels compared to the BLM group ($P<0.05$) (Table 8).

Discussion

This study integrated multiple GEO datasets and found that Nrp1 gene expression was downregulated in lung tissues of both IPF patients and BLM-induced mouse PF models, consistent with previous reports [14]. In BALF of IPF patients, Nrp1 expression was positively correlated with pulmonary function parameters (DLCO%, FEV1%, FVC%), suggesting that Nrp1 may influence lung function by regulating lung tissue repair and fibrotic processes. The mechanism requires in-depth analysis combined with cell type specificity. The consistent low expression of Nrp1 in IPF and BLM models reflects its protective role in maintaining lung tissue homeostasis. Zhang et al. [15] found that TGF-$ 1 − Nrp1signalinginlungtissueenhancesIL − 33receptorST2expressionandILC2functiontoacceleratefibrosis, whileNrp1deletioninalveolarepithelialcells dependentfunctionaldivergenceexplainscontradictoryphenomenaindifferentmodels : inRIPFmodels, Nrp1promotesEMTthroughenhancedWnt/ −cateninandTGF− $1/Smads signaling [22], whereas BLM/IPF models are dominated by fibroblast activation where low Nrp1 expression weakens its anti-fibrotic function. The strong correlation between Nrp1 expression and DLCO% is clinically significant, as DLCO% reflects gas exchange efficiency across the alveolar-capillary membrane. As a regulator of angiogenesis, low Nrp1 expression may reduce microvascular density and directly impair gas exchange [23].

High-throughput sequencing of cfRNA from IPF patient plasma revealed downregulated Nrp1 mRNA expression with moderate diagnostic discriminatory ability (AUC=0.754). This finding provides a new potential biomarker for non-invasive diagnosis of IPF and suggests that Nrp1 may participate in the pathophysiological regulatory network of IPF, warranting further investigation of its biological significance and clinical potential. As a receptor for Semaphorin 3A, Nrp1 regulates alveolar epithelial cell migration and injury repair. Reduced expression may impair epithelial barrier repair capacity, leading to persistent micro-injury, which is the initiating factor of IPF [24]. Late-stage IPF is often accompanied by pulmonary vascular destruction and abnormal angiogenesis. As a VEGF co-receptor, Nrp1 downregulation may disrupt normal vascular homeostasis, promote pathological vascular remodeling, and accelerate fibrosis progression [25]. Although the diagnostic efficacy of plasma Nrp1 mRNA (AUC=0.754) is slightly lower than traditional clinical indicators such as KL-6 (AUC 0.85) or HRCT visual scoring, its advantages include easy plasma collection for dynamic monitoring and PCR-based detection that facilitates clinical promotion.

Lung fibroblasts are key effector cells in pulmonary fibrosis, and their proliferation and activation are critical during fibrogenesis. TGF-$ 1isakeypro − fibroticcytokinethatpromotesfibroblastactivation, inducesdifferentiationintomyofibroblasts, andstimulate Nrp1overexpressiongroupsrevealedthatNrp1negativelyregulatesthePI3K/AKTsignalingpathwaytoinhibitr stimulation concurrently downregulated Nrp1 gene and protein expression while activating the PI3K/AKT pathway and upregulating the core fibroblast activation marker α-SMA and the key ECM scaffold protein Fn. Nrp1

overexpression effectively reversed these processes, significantly reducing p-PI3K/PI3K, p-AKT/AKT, and fibroblast marker levels. This finding deepens understanding of ECM remodeling mechanisms in fibrotic diseases and provides new insights for targeted interventions.

Intraperitoneal injection of recombinant Nrp1 protein in PF mice improved pulmonary pathological features, reduced α-SMA and Fn expression in lung tissues, and increased local Nrp1 levels. Notably, plasma liver and kidney function indicators (ALT, AST, ALP, BUN, CREA) showed no significant fluctuations during treatment, while Nrp1 levels in BALF increased, suggesting that exogenous Nrp1 can be effectively delivered to lung tissue to exert anti-fibrotic effects. Combined with the observation that TGF-$ $1 stimulation significantly downregulates Nrp1 expression in MPFs while activating the PI3K/AKT pathway and inducing α-SMA and Fn synthesis, we hypothesize that exogenous Nrp1 may competitively bind TGF-$ $1, thereby blocking downstream PI3K/AKT phosphorylation. This study provides a new intervention strategy for targeting Nrp1 in pulmonary fibrosis, though its mechanisms and clinical potential require multi-dimensional evidence for deeper interpretation.

This study has several limitations. First, the relatively small sample size and lack of population stratification may obscure associations between Nrp1 expression and specific clinical phenotypes. Second, diagnostic specificity was not validated against other interstitial lung diseases (e.g., CTD-ILD), limiting assessment of Nrp1' s diagnostic specificity for IPF.

In summary, this study demonstrates that Nrp1 expression is reduced in lung tissues, plasma, and BALF of IPF patients, suggesting its potential as a biomarker. Nrp1 inhibits lung fibroblast activation by negatively regulating the PI3K/AKT signaling pathway. Furthermore, intraperitoneal injection of recombinant Nrp1 alleviates pulmonary fibrosis in mice while maintaining systemic safety. These findings deepen understanding of IPF heterogeneity and provide experimental evidence for developing precision therapeutic strategies targeting Nrp1. Pulmonary fibrosis management requires multidisciplinary collaboration among respiratory medicine, radiology, pathology, rheumatology (for some CTD-associated cases), and general practitioners. Nrp1 as a novel biomarker and therapeutic target provides new content for multidisciplinary team discussions. Understanding the value of Nrp1-related research helps general practitioners integrate specialist treatment opinions, rehabilitation plans, long-term follow-up monitoring (e.g., periodic plasma Nrp1 trend detection), and symptom management to ensure continuous, integrated care for pulmonary fibrosis patients.

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