Effect of Jagged1/Notch1 Signaling Pathway on Endothelial-to-Mesenchymal Transition in Idiopathic Pulmonary Fibrosis (Postprint)

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Abstract

Background Idiopathic Pulmonary Fibrosis (IPF) is a worldwide disease with complex pathological mechanisms and no effective treatment currently available. Endothelial-Mesenchymal Transition (EndMT), as the pathological basis of various chronic vascular diseases, has recently been identified as one of the important risk factors for IPF, but its related mechanisms remain unclear. Objective To observe the effect of the Jagged1/Notch1 signaling pathway on EndMT, thereby elucidating the related mechanisms of EndMT in the IPF process. Methods From March to June 2021, Human Pulmonary Artery Endothelial Cells (HPAEC) were cultured in vitro. HPAEC treated with blank serum served as the control group, HPAEC stimulated with 5 mg/L TGF-$ $1 for 72 h served as the model group, and the model group treated with 500 nmol/L Notch signaling inhibitor (DAPT) served as the DAPT group. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to detect the mRNA expression of Notch1, Jagged1, and CBF1; Western Blotting (WB) was used to detect the protein expression of Notch1, Jagged1, and CBF1; Co-immunoprecipitation (Co-IP) was used to detect the binding of Notch1 Intracellular Domain (NICD1) to CBF1; MTT assay was used to detect cell proliferation; Transwell and scratch assays were used to detect cell migration capacity; and immunofluorescence was used to detect the expression of endothelial cell markers Platelet Endothelial Cell Adhesion Molecule-1 (CD31) and Vascular Endothelial Cadherin (VE-cadherin), as well as mesenchymal cell markers Fibroblast-Specific Protein 1 (FSP1) and α-Smooth Muscle Actin (α-SMA). Results The comparisons of Notch1, Jagged1, and CBF1 mRNA and protein expression levels, cell proliferation, and migration capacity among the three groups showed statistically significant differences ($P<0.05$). Specifically, the control group and DAPT group exhibited lower expression of Notch1, Jagged1, and CBF1 mRNA and protein, as well as lower cell proliferation and migration capacity, compared with the model group ($P<0.05$).

Co-IP experimental results showed differences in the binding of NICD1 to CBF1 among the three groups, with the binding being inhibited in the control group and DAPT group, while binding occurred in the model group. Immunofluorescence results showed statistically significant differences in the expression of CD31, VE-cadherin, FSP1, and α-SMA among the three groups (P<0.05); specifically, the endothelial cell markers CD31 and VE-cadherin were expressed at higher levels in the control group and DAPT group than in the model group, while the mesenchymal cell markers FSP1 and α-SMA were expressed at lower levels (P<0.05). Conclusion The Jagged1/Notch1 signaling pathway is involved in the EndMT process during IPF, and blocking this signaling pathway can inhibit EndMT.

Full Text

The Impact of the Jagged1/Notch1 Signalling Pathway on Endothelial-Mesenchymal Transition in Idiopathic Pulmonary Fibrosis

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Abstract

Background Idiopathic pulmonary fibrosis (IPF) is a complex global disease with poorly understood pathological mechanisms and no effective treatment currently available. Endothelial-mesenchymal transition (EndMT), identified as a pathological basis for various chronic vascular diseases, has recently been recognized as a significant risk factor for IPF, though the underlying mechanisms remain unclear. **Objective** To observe the effects of the Jagged1/Notch1 signalling pathway on EndMT and elucidate its related mechanisms during IPF progression. **Methods** From March to June 2021, human pulmonary arterial endothelial cells (HPAEC) were cultured in vitro. A control group was established using HPAEC treated with blank serum, a model group was created by stimulating HPAEC with 5 mg/L TGF-$ 1 $for 72 hours, and a DAPT group received the same TGF- $1 stimulation followed by treatment with 500 nmol/L Notch signalling inhibitor (DAPT). Reverse transcription quantitative polymerase chain reaction (RT-PCR) was employed to measure Notch1, Jagged1, and CBF1 mRNA expression, while Western blotting (WB) assessed protein expression levels. Co-immunoprecipitation (Co-IP) examined the binding of Notch1 intracellular domain protein (NICD1) with CBF1. Cell proliferation was evaluated using the MTT assay, Transwell and scratch assays measured cell migration capacity,

and immunofluorescence detected expression of endothelial markers (CD31 and VE-cadherin) and mesenchymal markers (FSP1 and α-SMA). **Results** Significant differences were observed among the three groups in Notch1, Jagged1, and CBF1 mRNA and protein expression levels, as well as cell proliferation and migration capacity ($P<0.05$). Both the control and DAPT groups showed lower expression of Notch1, Jagged1, and CBF1 mRNA and proteins, along with reduced cell proliferation and migration compared to the model group ($P<0.05$). Co-IP results demonstrated differential binding of NICD1 to CBF1 among groups, with binding inhibited in the control and DAPT groups but occurring in the model group. Immunofluorescence revealed significant differences in CD31, VE-cadherin, FSP1, and α-SMA expression ($P<0.05$), with the control and DAPT groups showing higher endothelial marker expression and lower mesenchymal marker expression than the model group ($P<0.05$). **Conclusion** The Jagged1/Notch1 signalling pathway participates in the EndMT process during IPF, and blocking this pathway can suppress EndMT.

Keywords Idiopathic pulmonary fibrosis; Endothelial cells; Endothelial-to-mesenchymal transition; Jagged1/Notch1 signaling pathway; Human pulmonary artery endothelial cells

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease of unknown etiology with worldwide prevalence [1]. Clinically, patients experience recurrent cough, gradually decreasing exercise tolerance, and progressive respiratory deterioration that may develop into respiratory failure [2]. The median survival after diagnosis is only 3–5 years [3], with increasing incidence and mortality in recent years [4]. Although pirfenidone and nintedanib can partially slow lung function decline, these treatments have tolerance issues and cannot cure the disease [5]. Therefore, investigating the pathogenesis of IPF to develop effective therapeutic strategies is of great significance.

The pathogenesis of IPF is complex, with abnormal extracellular matrix (ECM) deposition promoting fibrous connective tissue hyperplasia and alveolar structural destruction representing a key pathological feature [6]. Aberrant accumulation and activation of fibroblasts constitute the primary source of excessive ECM secretion [7]. While epithelial-mesenchymal transition (EMT) has been established as a major source of fibroblasts [8], emerging research indicates that endothelial-mesenchymal transition (EndMT) also significantly contributes to fibroblast accumulation [9]. EndMT is the process by which endothelial cells transform into mesenchymal cells, leading to dramatic increases in fibroblast numbers and subsequent ECM production [10]. Furthermore, EndMT reduces capillary bed density, causing tissue hypoxia and ischemia that exacerbates IPF [11]. Our previous studies demonstrated that TGF-$ 1 − $induced EndMT in human pulmonary arterial endothelial cells (HPAEC) was associated with elevated expression of binding factor (CBF1), suggesting that the Jagged1/Notch1 signalling pathway may participate in EndMT during

$established fibrotic factor TGF-$1 to induce an EndMT model and used the Jagged1/Notch1 signalling inhibitor DAPT to investigate the pathway's role in EndMT during IPF progression.

Materials and Methods

1.2.1 Cell Line HPAEC were purchased from Guangzhou Genio Biotechnology Co., Ltd. and cultured routinely to passage 4 before subsequent experiments.

1.2.2 Main Reagents and Instruments The following reagents and instruments were used: GAPDH antibody (Zen-bio, USA, Cat# 200306-7E4); Notch1, Jagged1, CBF1, and NICD1 antibodies (Abcam, UK, Cat# ab8925, ab109536, ab113203, ab8925); CD31, VE-cadherin, α-SMA, and FSP1 antibodies (Beijing Biosynthesis Biotechnology, Cat# AO04265948, AC03185478, AG02247616, AC09223656); DAPT (MCE, USA, Cat# HY-13027); TGF-$ 1($Peprotech, USA, Cat#100-21$); $BCA protein assay kit (Beyotime Biotechnology, Cat#P0011$); $anti-fluorescence quenching agent (Beijing Solarbio Technology, Cat#S2100$); $fetal bovine serum, RPMI1640 medi$ $Aldrich, Cat#V900933$); $PVDF membrane (Millipore, USA, Cat#ISEQ00010$); CO_{2} incubator and microplate reader (ThermoFisher, USA); laser confocal fluorescence microscope (Zeiss, Germany); TY-80R decolorizing shaker (Jintan Medical Equipment Factory); SDS-PAGE electrophoresis and wet transfer apparatus (Bio-Rad, USA); chemiluminescence detection system (Shanghai Tianneng Technology).

1.3.1 Cell Model Preparation and Treatment HPAEC were incubated with RPMI-1640 medium containing 0.5% fetal bovine serum for 24 hours, then treated with 5 mg/L TGF-$ $1 for 72 hours. Successful EndMT model establishment was confirmed when cells transformed from tightly adherent cobblestone-like morphology to spindle or elongated shapes with increased intercellular spaces and reduced adhesion.

1.3.2 Experimental Groups and Treatment HPAEC were divided into three groups: a control group treated with blank serum, a model group stimulated with 5 mg/L TGF-$ $1 for 72 hours, and a DAPT group receiving 5 mg/L TGF-$ $1 for 72 hours followed by 500 r$ and blank serum were selected based on previous work by Qu et al. [12].

1.3.3 RT-PCR Detection of Notch1, Jagged1, and CBF1 mRNA Expression After grouping and modeling, HPAEC were cultured for 72 hours. Medium was removed, cells were washed with PBS, and total RNA was extracted using Trizol. RNA concentration and purity were measured, and reverse transcription was performed using the Takara kit. With GAPDH as the internal reference, the TAKARA PrimeScript™ RT reagent Kit with gDNA Eraser was used. The ΔCt method was applied: ΔCt = Ct(target gene) - Ct(reference gene), ΔΔCt = ΔCt(experimental group) - ΔCt(control group), with relative mRNA expression calculated as 2^(-ΔΔCt). Each experiment was repeated three times. Primer

sequences are shown in .

1.3.3 Western Blot Detection of Notch1, Jagged1, and CBF1 Protein Expression After grouping and modeling, HPAEC were cultured for 72 hours. Medium was removed, cells were washed with PBS, and proteins were extracted on ice using a cell scraper with 100 L RIPA+PSFM lysis buffer for 30 minutes. After centrifugation (4°C, 12,000 r/min) for 15 minutes, the supernatant was collected. A small portion was used for protein concentration determination by BCA assay, while the remainder was mixed with RIPA and SDS-PAGE loading buffer, boiled at 95°C for 15 minutes, aliquoted, and stored at -20°C. Proteins were separated on 4-20% SDS-PAGE gels at 150 V for 1 hour, transferred to membranes at 250 mA for 4 hours, blocked with 5% skim milk at room temperature for 1 hour, incubated with primary antibodies (1:1000) overnight at 4°C, washed with TBST, incubated with secondary antibodies (1:5000) at room temperature for 1 hour, washed again, and visualized using chemiluminescence. Gray value analysis was performed using Image J to detect Notch1, Jagged1, and CBF1 protein expression.

1.3.4 Co-immunoprecipitation (Co-IP) Detection of NICD1-CBF1 Binding After grouping and modeling, HPAEC were cultured for 72 hours. Medium was removed, cells were washed with PBS, and lysed on ice. Whole protein lysate concentration was adjusted, with a portion reserved as Input sample. For immunoprecipitation, 50 L Protein A/G beads were added to 500 L whole protein lysate, rotated slowly at 4°C for 10 minutes, centrifuged (4°C, 5,000×g) for 30 seconds, and the supernatant was collected. Five micrograms of CBF1 antibody was added, incubated overnight with slow rotation at 4°C, followed by addition of 50 L Protein A/G beads for 6 hours at 4°C. After centrifugation (4°C, 5,000×g) for 30 seconds and washing three times with wash buffer, 50 L 2× loading buffer was added, mixed gently, boiled at 100°C for 10 minutes, and centrifuged (room temperature, 13,000×g) for 1 minute to obtain IP samples. Both IP and Input samples underwent SDS-PAGE electrophoresis; Input samples were used to detect NICD1 and CBF1 protein expression, while IP samples were used to detect NICD1-CBF1 binding.

1.3.5 Immunofluorescence Detection of CD31, VE-Cadherin, FSP1, and α-SMA Expression After grouping and modeling, HPAEC were cultured for 72 hours. Medium was removed, cells were washed with PBS, fixed with 4% paraformaldehyde for 20 minutes, washed, permeabilized with 0.5% Triton X-100 in PBS for 20 minutes, washed again, and blocked with 3% BSA-PBS at room temperature for 30 minutes. Primary antibodies were incubated overnight at 4°C (CD31 1:50, VE-cadherin 1:200, FSP1 1:100, α-SMA 1:200). After PBS washing, fluorescence-conjugated secondary antibodies were incubated for 1 hour at room temperature in the dark, followed by DAPI (100 mg/L) for 5 minutes in the dark. Anti-fluorescence quenching solution was added, and expression was observed under laser confocal fluorescence microscopy.

1.3.6 MTT Assay for Cell Proliferation HPAEC were grouped and modeled as described, then cultured for 24, 48, or 72 hours. Ten microliters of 5

g/L MTT was added to each well, incubated for 4 hours, medium was removed, 150 L DMSO was added to each well, and absorbance was measured at 570 nm after thorough mixing. Cell growth inhibition rate in the DAPT group was calculated as: (1 - A(DAPT group)/A(model group)) × 100%.

1.3.7 Transwell Assay for Cell Migration HPAEC were collected and seeded in 6-well plates at 5$×10^{5}$ cells/well. After grouping, modeling, and 72-hour culture, cells were resuspended in RPMI-1640 medium containing only 10% FBS, seeded in Transwell upper chambers, and lower chambers were filled with medium containing 10% FBS. After 8 hours, Transwell inserts were removed, unmigrated cells were wiped away, washed with PBS, fixed with methanol at room temperature for 10 minutes, stained with crystal violet at room temperature for 10 minutes, washed, dried, and migrated cells on the lower surface were counted under microscopy.

1.3.8 Scratch Assay for Cell Migration HPAEC were collected and evenly seeded in 6-well plates at 5$×10^{6}$ cells/well. After grouping, modeling, and 72-hour culture, medium was removed, cells were washed with PBS, and three straight lines were scratched using a 200 L pipette tip. After PBS washing and microscopic photography, cells were cultured for an additional 12 and 24 hours before photographing and measuring scratch distances.

1.4 Statistical Methods SPSS 26.0 was used for statistical analysis. Measurement data are expressed as ($\bar{x}\pm s$). One-way ANOVA was used for multi-group comparisons. $P<0.05$ was considered statistically significant.

Results

2.1 Notch1, Jagged1, and CBF1 mRNA Expression Levels Significant differences were observed among the three groups in Notch1, Jagged1, and CBF1 mRNA expression levels ($P<0.05$). The control group showed lower expression of Notch1, Jagged1, and CBF1 mRNA compared to the model group ($P<0.05$). The DAPT group also exhibited lower expression of these mRNAs compared to the model group ($P<0.05$). See .

2.2 Notch1, Jagged1, and CBF1 Protein Expression Levels Significant differences were found among the three groups in Notch1, Jagged1, and CBF1 protein expression levels ($P<0.05$). The control group had lower protein expression than the model group ($P<0.05$), and the DAPT group showed lower expression compared to the model group ($P<0.05$). See [Figure 1: see original paper] and .

2.3 NICD1-CBF1 Binding Co-IP results demonstrated differential NICD1-CBF1 binding among the three groups. Binding was inhibited in the control and DAPT groups, while it occurred in the model group. See [Figure 2: see original paper].

2.4 Cell Proliferation Significant differences in cell proliferation were

observed among groups at 48 and 72 hours ($P<0.05$). The control group showed lower proliferation than the model group at 48 and 72 hours ($P<0.05$). The DAPT group exhibited lower proliferation than the model group at 24, 48, and 72 hours ($P<0.05$), with the inhibition rate increasing over time. See .

2.5 Cell Migration Significant differences were found among groups in the number of cells migrating to the lower surface of Transwell chambers ($P<0.05$). Both the control and DAPT groups showed fewer migrated cells than the model group ($P<0.05$). See [Figure 3: see original paper] and .

2.6 Scratch Assay Results Significant differences in scratch distances were observed among groups at 12 and 24 hours ($P<0.05$). The control group showed greater scratch distances than the model group at both time points ($P<0.05$), and the DAPT group also showed greater distances than the model group ($P<0.05$). See [Figure 4: see original paper] and .

2.7 Expression of Endothelial Markers CD31 and VE-Cadherin Immunofluorescence revealed significant differences in CD31 and VE-cadherin expression among groups. Both the control and DAPT groups showed higher expression of these endothelial markers compared to the model group. See [Figure 5: see original paper] and [Figure 6: see original paper].

2.8 Expression of Mesenchymal Markers FSP1 and α-SMA Immunofluorescence demonstrated significant differences in FSP1 and α-SMA expression among groups. The control group showed lower expression of these mesenchymal markers than the model group, and the DAPT group also exhibited lower expression than the model group. See [Figure 7: see original paper] and [Figure 8: see original paper].

Discussion

IPF is a chronic, progressive interstitial lung disease predominantly affecting middle-aged and elderly populations, posing a significant threat to human life due to the lack of effective treatments [13]. Aberrant fibroblast accumulation represents a crucial pathological process in IPF [14]. Previous studies have shown that bronchial epithelial cells and type II alveolar epithelial cells can transform into fibroblasts through EMT following injury, leading to excessive proliferation, differentiation, and migration of fibroblasts, ECM secretion, alveolar structural destruction, fibrotic scar formation, and irreversible lung function decline [15]. However, recent research indicates that EndMT, as a special form of EMT, also contributes to abnormal fibroblast accumulation [16] and represents an important risk factor for IPF [17]. Under physiological conditions, endothelial cells exhibit a cobblestone-like morphology with tight adhesion molecules and ECM coverage [18]. When stimulated by hypoxia, hypertension, or mechanical injury, they transform from tightly adherent cobblestone shapes to spindle or elongated forms with reduced intercellular adhesion, increased cell gaps, enhanced proliferation and migration capacity, and gradual detachment

from the endothelium. They lose endothelial markers CD31 and VE-cadherin while acquiring mesenchymal phenotypes such as α-SMA and FSP1, proliferate extensively, migrate to the interstitium, and transform into fibroblasts that promote fibroblast focus formation and ECM deposition [19]. EndMT not only underlies various chronic vascular diseases but also promotes multiple fibrotic disorders [20]. Inhibiting EndMT can delay pulmonary fibrosis progression, though the mechanisms remain incompletely understood [21].

The Notch signalling pathway is highly conserved and crucial, consisting primarily of Notch receptors (Notch1/2/3/4), Notch ligands (Delta-like1/3/4, Jagged1/2), CBF1, and other regulatory molecules, participating in cell proliferation, differentiation, and apoptosis [22]. Both Notch receptors and ligands are membrane proteins that mediate intercellular activation effects. After translational modification, the Notch receptor becomes a complex comprising extracellular, transmembrane, and intracellular domains. Upon binding of the extracellular domain to ligands on neighboring cells, the Notch receptor complex undergoes three proteolytic cleavage steps, culminating in γ-secretase-mediated cleavage that releases the transcriptionally active intracellular domain NICD. NICD translocates to the nucleus and binds to the transcription factor CBF1, forming an NICD-CBF1 complex that regulates downstream target genes including Hes1, Hes5, and Hey, thereby activating the Notch signalling pathway. In the absence of NICD, CBF1 remains in an inhibited state and is typically degraded through ubiquitination [23]. Jagged1 is a common Notch ligand that activates fibrotic responses by increasing H3K79me3 enrichment at the Jag1 gene promoter [24]. Notch1 is a key functional receptor in vascular development; upon receiving Jagged1 ligand signals, γ-secretase cleaves the Notch1 receptor complex to release the active intracellular domain protein NICD1, which influences pro-fibrotic protein expression and accelerates pulmonary fibrosis [25]. As mentioned, our previous studies observed elevated expression of Notch1, Jagged1, and CBF1 proteins in TGF-$ 1−
$induced EndMT models, leading us to hypothesize that the Jagged1/Notch1 signalling pathway participates in En$
to establish an EndMT model and used the Jagged1/Notch1 signalling inhibitor DAPT to investigate the pathway's impact on EndMT and further clarify its role in IPF pathogenesis.

TGF-$ 1 $is a well−established fibrotic factor in IPF and an initiator of EndMT, which it induces by promoting Sm$
$dependent Ac−CoA synthesis from pyruvate−derived acetate, which causes acetylation of TGF− 1 receptor ALI$
signaling, creating a positive feedback vicious cycle. DAPT is a commonly used γ-secretase inhibitor that blocks intracellular S3 cleavage of Notch ligands, preventing NICD release and thereby blocking Notch signal transduction from extracellular to intracellular compartments [28]. Therefore, this study used TGF-$ $1 to stimulate HPAEC and establish an EndMT model, then applied DAPT to block the Jagged1/Notch1 signalling pathway to observe its effects on EndMT and further elucidate the pathway's role in IPF.

Our results demonstrate that HPAEC undergo EndMT upon TGF-$ $1 induction, accompanied by activation of the Jagged1/Notch1 signalling

pathway. Blocking this pathway with DAPT inhibited EndMT, suggesting that Jagged1/Notch1 signalling participates in the EndMT process during IPF. In conclusion, EndMT represents an important risk factor for IPF, with Jagged1/Notch1 signalling pathway activation occurring during this process, and blocking the pathway can suppress EndMT progression. However, EndMT is a complex process regulated by multiple factors, and its molecular mechanisms remain poorly understood [11]. Excessive secretion of various cytokines can promote Jagged1 overexpression and activate the Jagged1/Notch1 signalling pathway [29-30], while IPF pathogenesis involves interactions among multiple cell types and signalling pathways [1]. Whether EndMT is also influenced by other signalling pathways requires further investigation.

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Author Contributions: YANG Qifen conceived and designed the study, performed data collection and analysis, created figures and tables, and drafted the manuscript. ZHAO Huiliang and GUO Yongsheng revised the manuscript. QU Jinglian supervised quality control and review and took overall responsibility for the article.

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Note: Figure translations are in progress. See original paper for figures.