• Basic Research •

# Nintedanib regulates miR-23b-3p/TGFBR2 axis and competitively binds to TGFBR2 protein, inhibiting EMT process in human pterygium cells

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Received: 2024-11-11 Accepted: 2025-01-25

## Abstract

● **AIM:** To investigate the effects of nintedanib on epithelial-mesenchymal transition (EMT) in cells derived from pterygium, aiming to explore its potential as a pharmacological intervention for pterygium treatment.

● **METHODS:** Primary human pterygium epithelial cells (hPEC) and human conjunctival epithelial (hCJE) cells were isolated from patients, cultured, and characterized. The impact of nintedanib on transforming growth factor beta (TGF-β)-induced EMT was assessed by examining the expression of EMT markers such as vimentin and E-cadherin. Additionally, the modulation of the miR-23b-3p/transforming growth factor beta receptor 2 (TGFBR2)/Smad2 pathway by nintedanib was investigated to elucidate its potential antifibrotic mechanism.

● **RESULTS:** The expression of miR-23b-3p gene in hCJE cells was significantly higher than that in hPEC cells. Nintedanib effectively mitigated TGF-β-induced EMT in cells derived from pterygium, as evidenced by the downregulation of vimentin and upregulation of E-cadherin. When the nintedanib concentration exceeded 1 μmol/L, it significantly suppressed the proliferation of hPEC cells and significantly inhibited the migration distance of hPEC cells within 48h ($P$<0.01). The immunoprecipitation experiment showed that nintedanib modulated the TGFBR2 protein's response to TGF-β independently of miR-23b-3p. Both nintedanib and transfection with miR-23b-3p mimic significantly inhibited the expression levels of phosphorylated Smad2, snail homolog 1 (Drosophila, SNAIL), and SNAI2 (also known as SLUG, snail family transcriptional repressor 2) proteins.

● **CONCLUSION:** Nintedanib is found to modulate the miR-23b-3p/TGFBR2/Smad2 pathway, suggesting a novel antifibrotic mechanism. These findings collectively highlight nintedanib's therapeutic potential in managing pterygium, marking a significant step toward non-surgical treatment options. Nintedanib may offer a targeted pharmacological treatment that could complement or reduce the need for surgical interventions.

● **KEYWORDS:** pterygium; epithelial-mesenchymal transition; nintedanib; molecular docking; co-immunoprecipitation

**DOI:10.18240/ijo.2025.05.03**

**Citation:** Zhang KK, Li M, Liao YH, Liu XT, Bao YB, Gong Y. Nintedanib regulates miR-23b-3p/TGFBR2 axis and competitively binds to TGFBR2 protein, inhibiting EMT process in human pterygium cells. *Int J Ophthalmol* 2025;18(5):779-791

## INTRODUCTION

Pterygium, a proliferative ocular surface disorder often affecting the conjunctival canthus, is characterized by the hyperplasia of fibroblasts and neovascularization that progressively encroach upon the cornea[1]. Despite the evolution of surgical techniques and adjunctive therapies such as mitomycin, cyclosporine, and bevacizumab, the incidence of pterygium recurrence remains notably high[2-3]. Targeted pharmacological interventions, informed by a deeper understanding of pterygium's pathogenesis, may offer a promising approach to reducing these recurrence rates[3-4]. Recent studies have highlighted the pivotal role of epithelial-mesenchymal transition (EMT) in developing pterygium. EMT is a transformative process where epithelial cells, under pathological stress, adopt characteristics akin to mesenchymal cells, augmenting their migratory, invasive, and extracellular matrix secretion capabilities[5]. The initiation and progression of EMT are intricately linked to the transforming growth factor beta (TGF-β) signaling pathway, a key player in embryonic

development, tumor progression, and tissue fibrosis[6]. In the context of pterygium, the TGF-β signaling pathway appears to be markedly upregulated, with elevated TGF-β levels observed, which are implicated in the excessive fibrotic response within pterygium fibroblasts[7]. This overactivity is also hypothesized to be associated with the recurrence of pterygium post-surgery. Specifically, TGF-$\beta_2$ has been shown to induce the phenotypic transition of pterygium fibroblasts into myofibroblasts[8]. The transforming growth factor beta receptor 2 (TGFBR2), initiating the canonical signaling cascade of TGF-β[9], is responsive to TGF-β, triggering the activation of Smad proteins and culminating in the EMT process[10-11]. In light of these findings, the targeted inhibition of TGFBR2's protein activity is a potential therapeutic strategy for managing the EMT process in pterygium, offering new avenues for treatment and possibly mitigating disease recurrence.

Nintedanib is an innovative small molecule tyrosine kinase inhibitor that exerts its effects by competitively inhibiting the activity of a range of tyrosine kinases, thus impeding the intracellular signaling cascades they initiate[12]. These pathways play a pivotal role in the processes of angiogenesis, as well as in the proliferation and survival of associated cells[13]. Nintedanib has shown efficacy in halting the TGF-β-induced EMT in retinal pigment epithelial cells[14] and in curbing the excessive fibrosis of human Tenon's capsule fibroblasts under TGF-β stimulation, with its primary mechanism being the modulation of Smad proteins and their downstream signaling pathways[15]. These insights suggest that nintedanib can effectively modulate the Smad signaling pathway activated by TGF-β, contributing to inhibiting fibrotic processes. In our research group's prior studies, nintedanib was identified to suppress the proliferation of pterygium cells and to enhance the expression of proteins associated with apoptosis by inhibiting the fibroblast growth factor receptor 2/extracellular signal-regulated kinase signaling pathway[16]. However, the specific role of nintedanib in modulating the fibrotic characteristics of pterygium remains to be fully elucidated. Considering nintedanib's established regulatory influence on the TGF-β/Smad signaling pathway, coupled with the critical role of EMT in pterygium development, we hypothesize that nintedanib may exert its anti-EMT effects by fine-tuning the TGF-β/TGFBR2/Smad signaling axis.

MicroRNA-23b-3p (miR-23b-3p), a small non-coding RNA molecule, is pivotal in regulating cellular differentiation and EMT, both critical in various pathophysiological processes[17-18]. Our research team has analyzed gene sequencing data from pulmonary fibrosis patients treated with nintedanib (GEO ID: GSM3554073). The findings demonstrated that nintedanib significantly enhances the expression of miR-23b-3p in a concentration-dependent manner. In addition, bioinformatics analysis has highlighted that the TGFBR2 is a key target gene of miR-23b-3p in the context of fibrosis. Our study thus proposes that nintedanib potentially modulates the expression of miR-23b-3p, thereby regulating TGFBR2 and curbing the fibrotic process in pterygium cells.

## MATERIALS AND METHODS

**Ethical Approval** The Ethics Committee of Ningbo Eye Hospital has granted formal approval for this human research study, designated with protocol number No.2020-20(K)-C1. Adhering strictly to the ethical principles outlined in the Declaration of Helsinki, this study ensures its conduct meets global ethical standards.

**Cell Culture** Human pterygium epithelial cells (hPEC) and human conjunctival epithelial (hCJE) cells, preserved in our laboratory, are cultivated in strict accordance with the protocols established in prior research[16]. The cells are grown in DMEM/F12 medium and maintained in an incubator at a temperature of 37°C with a 5% $CO_2$ atmosphere to foster optimal cell growth. To identify the characteristics of hPEC and hCJE cells, we utilized immunofluorescence, real-time polymerase chain reaction (RT-PCR), and Western blot to assess the expression levels of key cellular markers, including vimentin and α-smooth muscle actin (SMA). Furthermore, staining with Phalloidin (17466-45-4, MedChemExpress, USA) was employed to visualize the expression of F-actin, offering insights into the cells' morphology and functional status. These methodologies guarantee the standardization and precision of the cell culture process and lay a solid foundation for the reliability and support of the cell models and data for future experimental studies.

**CCK-8 Assay** Plate hPEC and hCJE cells at a density of 5000 cells per well in a 96-well plate and incubate for 24h. Then, introduce nintedanib at different concentrations to the cells, resulting in final concentrations of 0, 0.25, 0.5, 1, 2, 4, and 8 μmol/L, and proceed with incubation for another 48h. Subsequently, add 10 μL of cell counting kit (CCK)-8 solution to each well to evaluate cell viability. Utilize a microplate reader to measure and analyze the absorbance at a wavelength of 450 nm.

**Western Blotting** Cells are lysed using TGFBR radio immunoprecipitation assay (RIPA) lysis buffer supplemented with phosphatase inhibitors to harvest protein samples. Proteins are then separated *via* SDS-PAGE gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane is blocked with 5% non-fat milk solution at room temperature. The blot is incubated overnight at 4°C with the respective primary antibodies: vimentin (ab92547), E-cadherin (ab314063), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ab9485) as a loading control, fibroblast growth factor 7 (FGF7, ab236065), TGFBR2

(ab270440), Smad2 (ab33875), phosphorylated Smad2 (ab188334), snail homolog 1 (Drosophila, SNAIL, ab216347), and SNAI2 (also known as SLUG, snail family transcriptional repressor 2, ab27568). This is followed by incubation with a goat anti-rabbit IgG-horseradish peroxidase (IgG-HRP) secondary antibody (ab6721) at a dilution of 1:2000 for 2h at room temperature. The bands are visualized using an ECL chemiluminescent substrate and imaged with the ChemiDoc-It Imaging System (UVP, USA). All antibodies used in this study were sourced from Abcam China. The band intensities are normalized to GAPDH and quantified using Image J software for statistical analysis.

**TGF-β Treatment of hCJE Cells** In the experimental phase, hCJE cells were incubated with 10 ng/mL of recombinant TGF-β (ab50036, provided by Abcam, China) for 72h. This targeted intervention is crafted to proficiently elicit the phenotypic shifts indicative of the EMT.

**qRT-PCR Assay** The $2\times10^6$ cells from each group and isolate total RNA utilizing the Trizol reagent. MiRNA was transcribed into cDNA using the HiScript IV 1st Strand cDNA synthesis kit (R412, Novozymes, China). Execute quantitative real-time polymerase chain reaction (qPCR) assays with the SYBR qPCR SuperMix Plus Kit (E096, Novoprotein, China) on the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher, USA). Utilize the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression levels of miR-23b-3p, FGF7, TGFBR2, vimentin, and E-cadherin normalized to GAPDH or U6. The primer sequences are as follows: miR-23b-3p forward: 5'-GCC GAG ATC ACA TTG CCA GGG ATT ACC AC-3'; miR-23a-3p forward: 5'-GCC GAG AAT CAC ATT TGC CAG GGA TTT CC -3'; U6 forward: 5'-TCG GCA GGG TGC TCG CTT CGG-3'; universal miRNA reverse primer: 5'-CTC AAC TGG TGT CGT GGA-3'; vimentin forward: 5'-AGG CAA AGC AGG AGT CCA CTG A-3', feverse: 5'-ATC TGG CGT TCC AGG GAC TCA T-3'; E-cadherin forward: 5'-GCC TCC TGA AAA GAG AGT GGA AG-3', reverse: 5'-TGG CAG TGT CTC TCC AAA TCC G-3'; TGFBR2 forward: 5'-GTC TGT GGA TGA CCT GGC TAA C-3', reverse: 5'-GAC ATC GGT CTG CTT GAA GGA C-3'; FGF7 forward: 5'-CTG TCG AAC ACA GTG GTA CCT G-3', reverse: 5'-CCA ACT GCC ACT GTC CTG ATT TC-3'; GAPDH forward: 5'-GTC TCC TCT GAC TTC AAC AGC G-3', reverse: 5'-ACC ACC CTG TTG CTG TAG CCA A-3'.

**Correlation Between miR-23b-3p and TGFBR2** This research initiative began with an exhaustive examination of the dataset within the Gene Expression Omnibus (GEO) database, focusing on the pulmonary fibroblast cells subjected to nintedanib treatment (GSM3554073). The objective was to pinpoint miRNAs that exhibit increased expression in correlation with rising concentrations of nintedanib. The Encyclopedia of RNA Interactomes (ENCORI) database was engaged to identify potential miRNAs targeting the *TGFBR2* gene. Utilizing Venn diagram analysis, we integrated findings from both databases to narrow the list to critical target miRNAs. On the ENCORI platform, we forecasted the target genes for miR-23b-3p, leveraging a composite of insights from six miRNA target prediction databases, namely PITA, miRmap, microT, miRanda, PicTar, and Targetscan. In parallel, the Genecards database furnished us with a curated list of fibrosis-associated genes (TOP 250). Employing Venn diagram analysis again, we juxtaposed the miRNA targets against the fibrosis-related genes to uncover overlapping elements. Armed with the insights derived from the Venn diagram analysis, we proceeded to scrutinize the expression variances of miR-23a-3p, miR-23b-3p, FGF7, and the *TGFBR2* gene in hCJE and hPEC cells after their exposure to nintedanib.

**miR-23b-3p on the Regulatory Effect of Nintedanib** In this research, we conducted a detailed grouping and treatment process for hCJE and hPEC cells as follows: 1) hCJE Group: hCJE cells were subjected to a 72-h induction in the presence of 10 ng/mL TGF-β to mimic the physiological conditions of EMT; 2) miR-23b-3p mimic Group: after transfection with miR-23b-3p mimic, hCJE cells were induced for 72h in the presence of 10 ng/mL TGF-β to study the influence of miR-23b-3p on EMT; 3) Nintedanib(Nin) Group: hCJE cells were incubated for 72h under the combined effect of 10 ng/mL TGF-β and 2 μmol/L nintedanib to evaluate the potential inhibitory effects of nintedanib on EMT; 4) Nin+miR-23b-3p inhibitor Group: hCJE cells were first transfected with miR-23b-3p mimic and then incubated for 72h in the presence of both TGF-β and nintedanib to analyze the role of miR-23b-3p in the EMT process under nintedanib's influence. For hPEC cells, the grouping was analogous to that of the hCJE cells, except for the TGF-β treatment step. Post-grouping and treatment, we utilized quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot to assess the gene and protein expression levels of vimentin and E-cadherin, pivotal biomarkers in the EMT process. Moreover, we performed cell scratch assays to gauge alterations in cell migratory capacity. Cells were treated with TGF-β or nintedanib for 48h, selected for consistency in experimental conditions and to ensure the comparability of results, as the control group cells were observed to have fully covered the culture dishes by 72h.

**Cell Scratch Assay** hPEC and hCJE cells were seeded in a 6-well plate at a density of $5\times10^5$ cells per well. Upon reaching complete confluence, the cells were scraped vertically with a 200 μL pipette tip under microscopic guidance to remove cells along the lines. The medium was refreshed to eliminate any detached cells, and the cells post-scraping were examined and documented at 0h using phase-contrast microscopy. After

that, the cultures were left to incubate, and the migration of cells at the marked sites was monitored under phase-contrast microscopy at 0 and 48h for different treatment groups, including the miR-23b-3p mimic, nintedanib, and the combination of nintedanib with the miR-23b-3p inhibitor. The migration distances were quantified using Image Pro Plus 6.0 software.

**Binding Relationship Between miR-23b-3p and TGFBR2** For cell transfection, an appropriate volume of cells is seeded into 24-well plates to reach a cell density of 50%–60% at the time of transfection. Lipofectamine™ 2000 introduces the miR-23b-3p mimic or inhibitor (100 nmol/L) into 293T cells. Post-transfection, the expression levels of the *TGFBR2* or miR-23b-3p genes are assessed 48h later. Mutant sequences are constructed based on the predicted target binding sites of miR-23b-3p with the *TGFBR2* gene. Using the pIS0 plasmid, we have constructed recombinant plasmids for wild-type or mutant *TGFBR2* with dual-luciferase reporter genes. The pRL-TK plasmid serves as an internal control. The dual luciferase assay system from Promega is conducted using a Luminometer to measure relative luciferase activities, which are expressed as the ratio of firefly luciferase to Renilla luciferase activity.

**Immunofluorescence** Cells are seeded onto a culture medium containing coverslips and are grown to achieve 60%-70% confluence. Once this density is reached, the cells are prepared for immunofluorescence staining on the slides, following protocols established in prior studies[16]. The antibodies utilized in this process include α-SMA, vimentin, E-cadherin, and TGFBR2. For the fluorescent detection, Goat anti-rabbit IgG-FITC (ab6717, Abcam, China) or Goat anti-rabbit IgG-Alexa Fluor® 594 (ab150080, Abcam, China) are employed as secondary antibodies.

**Molecular Docking of Nintedanib and TGFBR2 Protein** The 3D molecular structure of nintedanib can be sourced from the PubChem database, which is accessible *via* their official website (https://pubchem.ncbi.nlm.nih.gov/). For the 3D structure of the TGFBR2 protein, the RCSB Protein Data Bank (https://www.rcsb.org/) is the go-to resource. The primary objective is to substitute the known inhibitor ligand sites of the TGFBR2 protein with nintedanib to simulate the docking structure. Utilizing AutoDockTools version 1.5.6, the coordinates of the ligand binding sites are precisely determined. Subsequently, the potential binding affinity between nintedanib and the TGFBR2 protein is evaluated with the aid of AutoDock Vina software. The culmination of this analysis is the visual representation of the simulated docking site, which is adeptly accomplished using Pymol software.

**Immunoprecipitation Experiment** After transfection of hCJE and hPEC cells with miR-23b-3p mimic or inhibitor, the groups were treated as follows: 1) the control group with either hCJE or hPEC cells; 2) the 23b-mimic group, where hCJE or hPEC cells were transfected with the miR-23b-3p mimic; 3) the Nin group, where hCJE or hPEC cells were exposed to 2 μmol/L nintedanib for 72h; 4) the Nin+miR-23b-3p inhibitor group, where hCJE or hPEC cells were first transfected with the miR-23b-3p inhibitor and then exposed to 2 μmol/L nintedanib for 72h. Cell lysates were prepared using RIPA buffer, and Protein A/G agarose beads coupled with the TGFBR2 antibody (ab259360, Abcam, China) were added. The mixture was incubated at 4°C for 6h to facilitate the binding of immune complexes to the beads. After incubation, the samples were centrifuged at 4°C to pellet the agarose beads. The supernatant was carefully removed, and the beads were washed with lysis buffer to eliminate unbound proteins. The beads were then resuspended in an SDS-containing loading buffer and heated at 100°C for 5min to denature the proteins. Western blot subsequently analyzed the enriched proteins using TGF-β (ab215715, Abcam, China) and TGFBR2 as primary antibodies at a dilution of 1:1000.

**Construction of Cells with Overexpression and Knockdown of TGFBR2** The overexpression vector pCDH-TGFBR2-puro was procured from Zhejiang Ruyao Biotechnology Co., Ltd (Ningbo, China). Recombinant plasmids, along with Δ 8.91 and pVSV-G (at a mass ratio of 10:10:1), were transfected into 293T cells utilizing the cationic lipid-mediated method (X-tremeGENE HP DNA Transfection Reagent, Roche, Switzerland). The supernatant was harvested after 24h, filtered, and then combined with 2 μg/mL polybrene before being added to hCJE or hPEC cells for co-culture. Following a 72-hour infection period, the medium was replaced with complete growth medium containing 2 μg/mL puromycin to select for the transfected cells, which were then cultured for an additional 7-9d. *TGFBR2* gene silencing and overexpression efficiency were ascertained through qRT-PCR.

Cells with manipulated TGFBR2 expression were categorized and treated as follows: 1) control groups with either hCJE or hPEC cells; 2) TGF-β group, where cells were incubated with 10 ng/mL TGF-β for 72h; 3) the Nin group was co-cultured with 10 ng/mL TGF-β and 2 μmol/L nintedanib for 72h; 4) Nin+TGFBR2 group, cells overexpressing TGFBR2 were incubated with 10 ng/mL TGF-β and 2 μmol/L nintedanib for 72h; 5) In the mimic group, cells were transfected with miR-23b-3p mimic before a 72-hour incubation with 10 ng/mL TGF-β; 6) In the Mimic+TGFBR2 group, cells overexpressing TGFBR2 were transfected with miR-23b-3p mimic and then incubated with 10 ng/mL TGF-β for 72h. Protein expression levels of Smad2, phosphorylated Smad2, SNAIL, and SLUG were assessed in all groups, alongside gene expression levels of vimentin and E-cadherin.

**Statistical Analysis** Data were expressed as triplicate measurements' mean±standard deviation (SD). Before *t*-tests and ANOVA analyses, the data underwent normality and equal variance tests. For comparisons between two groups, a *t*-test was employed, while one-way ANOVA was utilized for data involving more than two groups, complemented by Tukey's post hoc test for multiple comparisons where statistical significance was observed. Statistical significance was set at a *P*-value of less than 0.05. All statistical analyses were conducted using GraphPad Prism version 8.0.

## RESULTS

**Nintedanib Inhibits the EMT of hPEC and hCJE Cells Induced by TGF-β** In this study, we successfully isolated and cultured patients' primary cell strains of hCJE and hPEC. Through F-actin staining (Figure 1A, 1B), we observed that hPEC cells exhibited the typical morphology of fibroblastic cells along with a distinctive pattern of directional growth. At the protein expression level, both hPEC and hCJE cells demonstrated positive staining for vimentin and α-SMA (Figure 1C–1E). Further investigation into the effects of nintedanib on these cells revealed that when the drug concentration exceeded 2 μmol/L, it significantly inhibited the normal proliferation of hCJE cells. Similarly, when the concentration exceeded 1 μmol/L, it significantly suppressed the proliferation of hPEC cells ($P$<0.01; Figure 1F). Upon in-depth analysis of protein expression, we noted that treatment with 1 and 2 μmol/L nintedanib significantly reduced the expression levels of vimentin protein in both hPEC cells and TGF-β-induced hCJE cells while also increasing the expression of E-cadherin protein. This indicates that nintedanib can effectively inhibit the EMT process ($P$<0.01; Figure 1G–1L).

**Inhibition of miR-23b Partially Reverse the Inhibitory of Nintedanib on Cell EMT and Migration Ability** This study aimed to verify the impact of nintedanib on the EMT process by regulating miR-23b-3p. To this end, we first conducted qRT-PCR analysis on hPEC and hCJE cells treated with nintedanib and induced by TGF-β to fibrose. The results showed that 1 and 2 μmol/L nintedanib significantly increased the expression level of miR-23b-3p, with a statistically significant difference compared to the TGF-β treated group or the hPEC control group ($P$<0.05; Figure 2A, 2B). In further experiments, we found that the miR-23b-3p mimic significantly enhanced the expression of the *E-cadherin* gene (Figure 2C, 2D) and its protein (Figure 2E–2G) in hPEC and hCJE cells while reducing the expression level of vimentin, with a significant difference compared to the hCJE or hPEC control group ($P$<0.01). Conversely, the transfection of the miR-23b-3p inhibitor significantly reversed the upregulation effect of 2 μmol/L nintedanib on the expression of the *E-cadherin* gene and protein, as well as the inhibitory effect on the expression of the vimentin gene and protein, with a statistically significant difference compared to the nintedanib treated group ($P$<0.01). Additionally, both the miR-23b-3p mimic and nintedanib significantly inhibited the migration ability of cells within 48h, with a significant difference compared to the hPEC or hCJE control group ($P$<0.01). The miR-23b-3p inhibitor, on the other hand, significantly increased the cell migration distance, with a statistically significant difference compared to the nintedanib-treated group ($P$<0.05; Figure 2H, 2I).

**Nintedanib Affects the Expression of *TGFBR2* Gene Through miR-23b-3p** To elucidate the regulatory interaction between miR-23b-3p and TGFBR2, our study commenced by leveraging the GEO database to scrutinize the miRNA expression profiles in pulmonary fibroblasts treated with varying concentrations of nintedanib. Utilizing the ENCORI predictive tool, we identified 20 miRNAs potentially targeting *TGFBR2*. Further investigation into the role of miR-23b-3p in nintedanib-mediated fibrosis regulation involved predicting its target genes, followed by a comparative analysis with the top 250 fibrosis-associated genes listed in the Genecard database. This analysis revealed FGF7 and TGFBR2 as key targets of miR-23b-3p (Figure 3A). The expression of *TGFBR2* gene ($P$<0.05; Figure 3B, 3C) and protein ($P$<0.05; Figure 3D–3F) was significantly downregulated under the influence of miR-23b-3p mimic and nintedanib, while the miR-23b-3p inhibitor significantly reversed the inhibitory effect of nintedanib on *TGFBR2* gene ($P$<0.05). In contrast, FGF7 expression remained largely unaffected ($P$>0.05). In addition, the expression of miR-23b-3p gene in hCJE cells was significantly higher than that in hPEC cells ($P$<0.01; Figure 3G). TGFBR2 protein expression was also robust in hPEC cells and comparatively weaker in quiescent hCJE cells (Figure 3H). Employing a dual-luciferase reporter gene assay, we substantiated the direct regulatory influence of miR-23b-3p on the *TGFBR2* gene. The findings indicated that miR-23b-3p mimic significantly dampened the luciferase activity of the wild-type TGFBR2 promoter ($P$<0.01), with no significant impact on the mutant variant ($P$>0.05; Figure 3I, 3J). In 293T cells, the introduction of miR-23b-3p mimic markedly elevated the expression of miR-23b-3p and concurrently repressed TGFBR2 expression ($P$<0.01; Figure 3K, 3L). Conversely, applying miR-23b-3p inhibitor led to decreased miR-23b-3p expression and a corresponding increase in TGFBR2 expression ($P$<0.01).

**Nintedanib Regulate the Response of the TGFBR2 Protein to TGF-β Independently of miR-23b-3p** As a small molecule compound, nintedanib has the potential to act as either an agonist or an antagonist of proteins, thereby modulating their activity. Preliminary protein-level evidence has shown that nintedanib at concentrations of 0.5, 1,

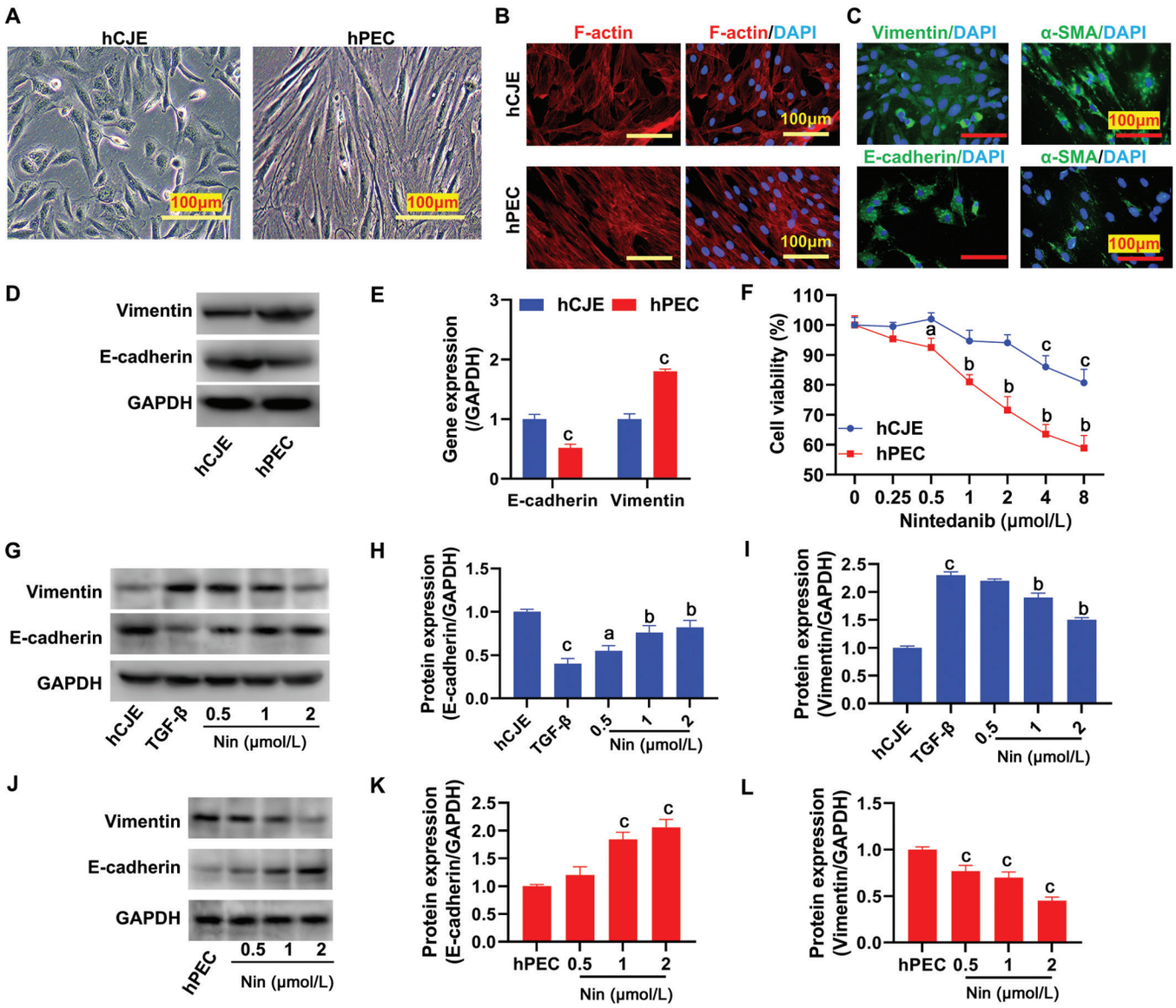


**Figure 1 Inhibition of the EMT process in hPEC and TGF-β-induced hCJE cells by Nin** A: Observation of the morphology of hCJE and hPEC cells under a differential interference contrast microscope; B, C: Immunofluorescence detection of the expression of vimentin and α-SMA proteins; D, E: Western blot analysis of vimentin and E-cadherin protein expression; F: CCK-8 assay to evaluate the influence of nintedanib on cell activity; G–L: Western blot detection of the changes in the expression of vimentin and E-cadherin proteins following nintedanib treatment, along with quantitative statistical analysis of the protein band optical density values. $^{a}P<0.05$, $^{b}P<0.01$ indicate statistically significant differences compared to the 0 μmol/L or TGF-β group, $^{c}P<0.01$ indicates a statistically significant difference compared to the 0 μmol/L or hCJE or hPEC group. hCJE: human conjunctival epithelial cells; DAPI: 4',6-diamidino-2-phenylindole; SMA: Smooth muscle actin; CCK-8: Cell counting kit-8; EMT: Epithelial-mesenchymal transition; Nin: Nintedanib; hPEC: Human pterygium epithelial cells; TGF-β: Transforming growth factor beta.

and 2 μmol/L can significantly inhibit the expression of the TGFBR2 protein, with statistically significant differences compared to the TGF-β or hPEC groups ($P<0.05$; Figures 4A–4D). To elucidate this characteristic, this study investigates the inhibitory ligand binding site of the TGFBR2 protein as a potential binding site for nintedanib. Molecular docking experiments (Figure 4E) indicate that the potential binding energy of nintedanib with the TGFBR2 (PDB ID: 5E8Y) protein is -8.8 kcal/mol, which is more favorable than the binding energy of the known TGFBR2 inhibitor Staurosporin with TGFBR2, at -5.5 kcal/mol. To validate the regulatory effect of nintedanib on the TGFBR2 protein's ability to bind TGF-β, co-immunoprecipitation (Co-IP) experiments were conducted. These experiments demonstrated that the miR-23b-3p mimic does not influence the co-precipitation level of the TGF-β protein with the TGFBR2 protein. Specifically, the ratio of TGF-β to TGFBR2 protein in the nintedanib-treated group was significantly reduced compared to the hCJE or hPEC groups ($P<0.01$). However, the co-treatment of hCJE or hPEC cells with both the miR-23b-3p inhibitor and nintedanib did not significantly alter the ratio of TGF-β to TGFBR2 protein ($P>0.05$; Figures 4F–4L), suggesting that nintedanib

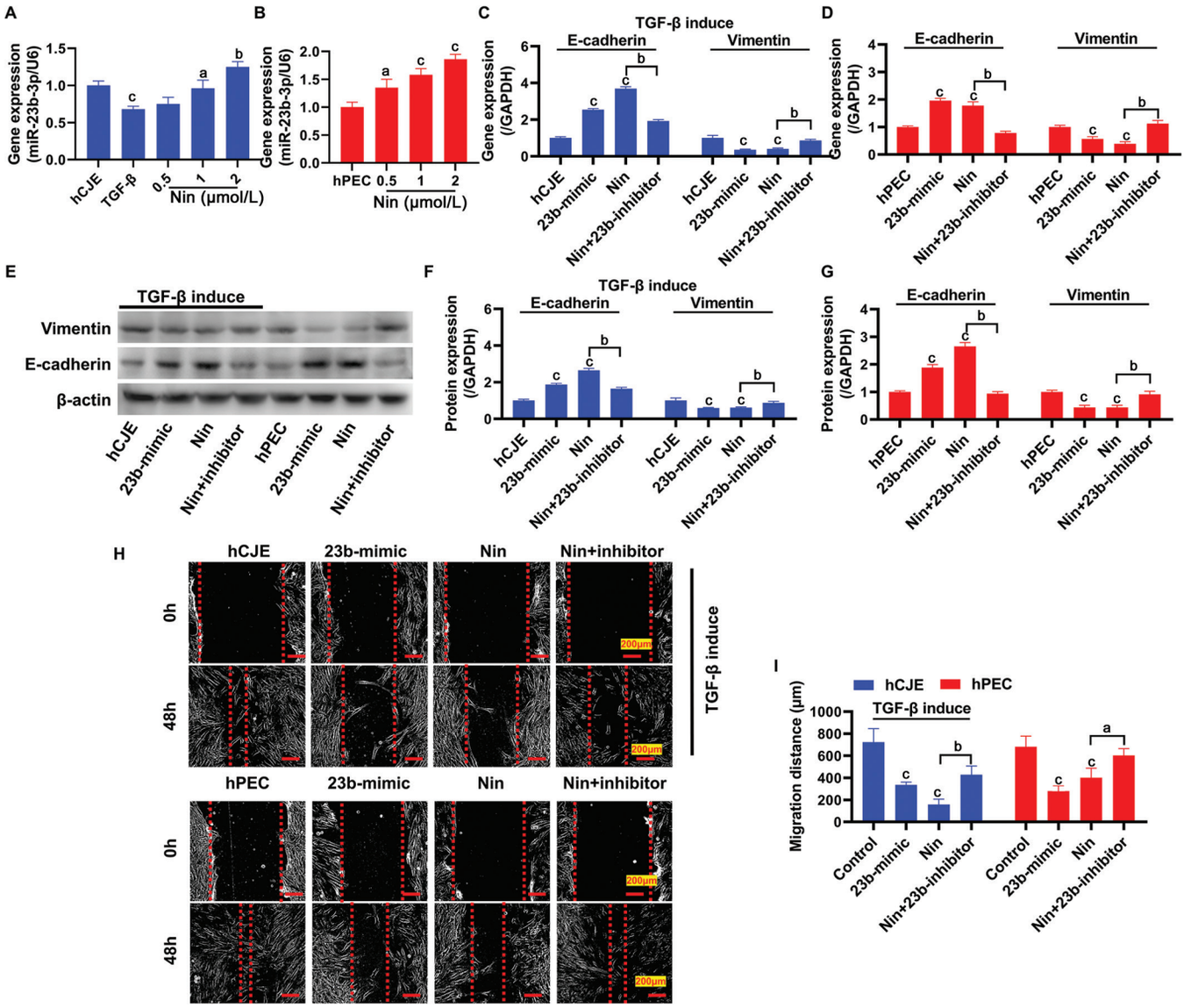


**Figure 2 Nin regulates the EMT and migratory capacity of fibrotic hCJE and hPEC cells *via* miR-23b-3p** A–D: qRT-PCR was used to detect the expression levels of miR-23b-3p, E-cadherin, and vimentin genes.Data are expressed as mean±SD. [a]*P*<0.05, [c]*P*<0.01 compared with hPEC or hCJE groups; E–G: Western blot was conducted to assess the protein expression levels of vimentin and E-cadherin, with quantification of the band optical density values; H, I: A cell scratch assay evaluated the cells' migratory capacity over 48h. [a]*P*<0.05, [b]*P*<0.01, the Nin+23b-inhibitor group compared with the Nin group, [c]*P*<0.01, compared with the hCJE or hPEC group;indicating statistically significant differences. hCJE: Human conjunctival epithelial cells; qRT-PCR: Quantitative real-time polymerase chain reaction; EMT: Epithelial-mesenchymal transition; Nin: Nintedanib; hPEC: Human pterygium epithelial cells; TGF-β: Transforming growth factor beta.

can modulate the TGFBR2 protein's response to TGF-β independently of miR-23b-3p.

**Nintedanib Regulates miR-23b-3p/TGFBR2/Smad2 to Suppress the Expression of EMT-related Transcription Factor Genes and Proteins** To elucidate the influence of nintedanib on the miR-23b-3p/TGFBR2 pathway, in this section, overexpression of the TGFBR2 protein was first induced in both hCJE and hPEC cells. Within the OE-TGFBR2 group, there was a significant increase in TGFBR2 protein levels in hCJE and hPEC cells ($P$<0.01 compared to the OE-NC group; Figure 5A–5D). Further validation of the downstream signaling cascade of TGFBR2 was conducted (Figure 5E–5L), revealing that both nintedanib treatment and miR-23b-3p mimic transfection markedly suppressed the expression of phosphorylated Smad2 protein, as well as SNAIL and SLUG proteins ($P$<0.05 compared to the TGF-β group). Conversely, overexpression of TGFBR2 notably counteracted these regulatory effects on protein expression ($P$<0.01 compared to the Nin group). qRT-PCR data indicated that the downstream transcriptional targets of the Smad2/SNAIL/SLUG pathway, specifically E-cadherin, were significantly upregulated following nintedanib treatment or miR-23b-3p mimic transfection. At the same time, the expression of the vimentin gene was significantly downregulated. These differences were statistically significant compared to the TGF-β group ($P$<0.01). Additionally, the overexpression of TGFBR2

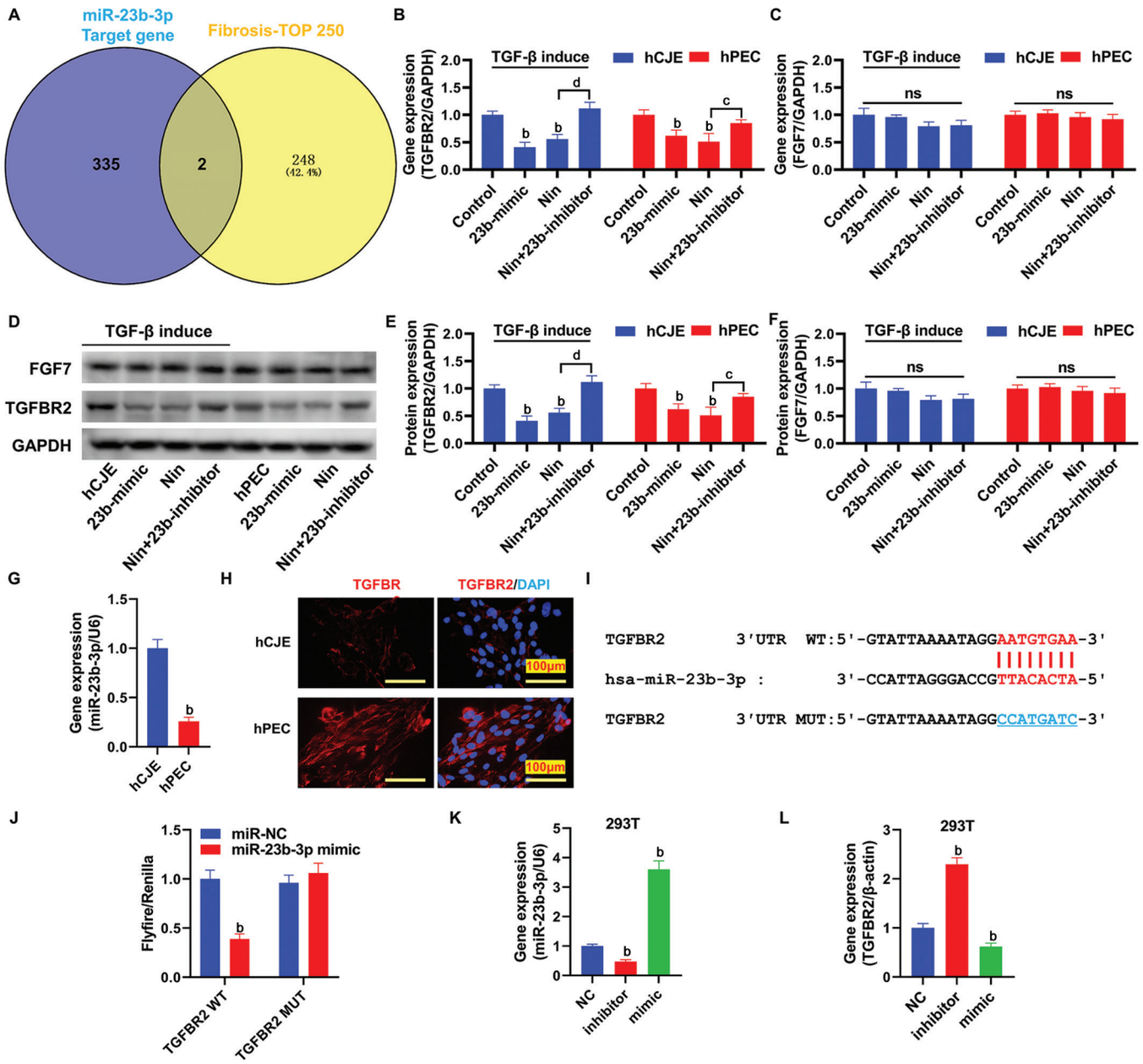


**Figure 3 Nin affects the expression level of the *TGFBR2* gene through miR-23b-3p** A: Venn diagram analysis of miRNAs upregulated by nintedanib in the GEO database (GSM3554073) and the predicted potential miRNAs targeting TGFBR2; B, C: qRT-PCR analysis of the expression changes of the target miRNA after treatment with nintedanib; D–F: Western blot analysis of the common targets of miR-23b-3p and fibrosis; G: qRT-PCR detection of the expression levels of the miR-23b-3p; H: Immunofluorescence is used to detect the expression distribution and intensity of the TGFBR2 protein; I, J: Dual-luciferase reporter assay to test the targeting relationship between miR-23b-3p and the TGFBR2 gene; K, L: qRT-PCR to detect the expression levels of the miR-23b-3p and TGFBR2 genes after cell transfection. $^{b}P<0.01$, compared with the control or NC group; $^{c}P<0.05$, $^{d}P<0.01$, indicating a statistically significant difference between groups; ns: No statistically significant difference between groups. GEO: Gene Expression Omnibus; FGF7: Fibroblast growth factor 7; hCJE: Human conjunctival epithelial cells; NC: Negative control; qRT-PCR: Quantitative real-time polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; Nin: Nintedanib; hPEC: Human pterygium epithelial cells; TGF-β: Transforming growth factor beta; TGFBR2: Transforming growth factor beta receptor 2.

protein significantly reversed the expression modulation effects of nintedanib or miR-23b-3p mimic on these genes (Figure 5M–5P, $P<0.01$ compared to either the Nin or mimic group).

## DISCUSSION

The pathological features of pterygium encompass abnormal cellular proliferation, excessive expression of anti-apoptotic factors, and an enhanced EMT phenotype, among others[5]. Our prior research successfully established a primary culture system for hPEC. We identified several anti-VEGF medications, including ranibizumab, conbercept, and nintedanib, to evaluate their inhibitory impact on hPEC. Our findings indicated that ranibizumab and conbercept exhibited minimal inhibitory

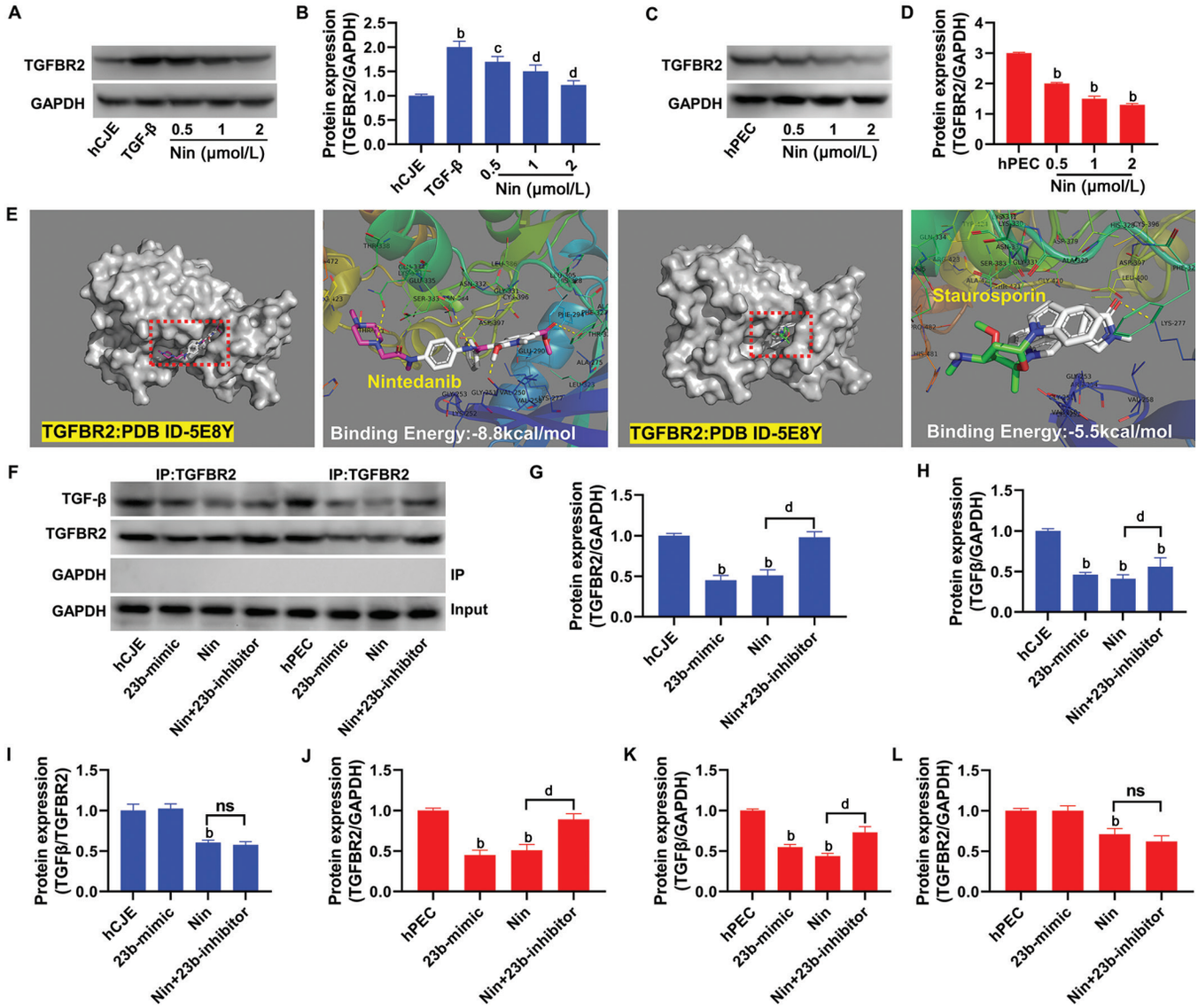


**Figure 4 Nin regulates the response of TGFBR2 protein to TGF-β independently of miR-23b-3p** A–D: Western blot analysis of the effect of nintedanib on the expression levels of TGFBR2 protein in hCJE or hPEC cells, with quantification of the band optical density values; E: Isualization of the potential binding relationship between nintedanib and the TGFBR2 protein using Pymol software; F-L: Co-IP experiments to analyze the binding level of TGFBR2 protein with TGF-β protein and to calculate the ratio of TGF-β to TGFBR2 protein. $^{b}P<0.01$, compared with the hCJE or hPEC group; $^{c}P<0.05$, $^{d}P<0.01$, indicating a statistically significant difference between groups or compared with the hPEC group; ns: No statistically significant difference in group comparison. Nin: Nintedanib; hCJE: Human conjunctival epithelial cells; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IP: Immunoprecipitation; TGFBR2: Transforming growth factor beta receptor 2; hPEC: Human pterygium epithelial cells; TGF-β: Transforming growth factor beta; Co-IP: Co-immunoprecipitation.

effects on hPEC, whereas nintedanib demonstrated a notably significant inhibitory effect[19]. In the therapeutic context of metastatic renal cell carcinoma, nintedanib effectively suppresses tumor growth and metastasis by inhibiting the vascular endothelial growth factor receptor, thus restricting neovascularization and depriving the tumor tissue of essential nutrients and oxygen[20]. Further investigation into the mechanism of action of nintedanib revealed that it targets the fibroblast growth factor receptor 2/extracellular signal-regulated kinase signaling pathway, thereby inducing apoptosis in hPEC cells[16].

In the GEO database, an analysis of miRNA expression in pulmonary fibroblast cells treated with nintedanib revealed a significant upregulation of miR-23b-3p expression, which intensifies with increasing drug concentrations. Concurrently, there was a notable decrease in the expression levels of TGFBR2. Extensive studies have demonstrated that miR-23b-3p can suppress cells' EMT[17,21]. This discovery opens up the possibility for further research into the regulation of miR-23b-3p to control the EMT in hPECs.

In the pathogenesis of pterygium, the EMT induces cellular characteristic changes that may lead to the transformation of

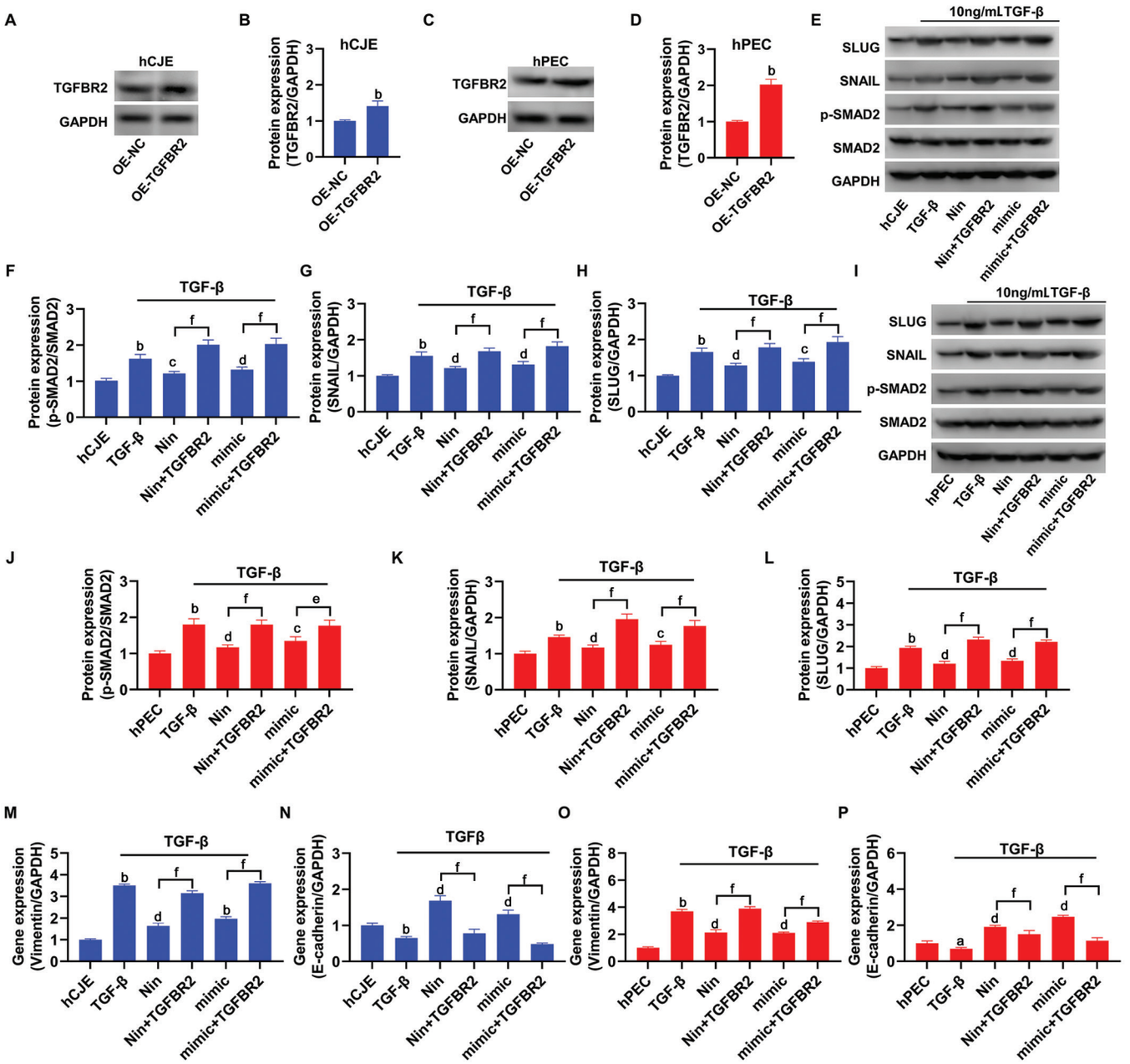


**Figure 5 Nin modulates the miR-23b-3p/TGFBR2/Smad2 axis to inhibit the expression of EMT-related transcription factors and proteins** A–D: Western blot to evaluate the efficiency of TGFBR2 protein overexpression; E–H: Western blot to assess the expression levels of p-Smad, Smad, SNAIL, and SLUG proteins in hCJE cells, with quantitative analysis of the protein band optical density; I–L: Western blot to assess the expression levels of p-Smad, Smad, SNAIL, and SLUG proteins in hPEC cells, with quantitative analysis of the protein band optical density; M–P: qRT-PCR to measure the expression levels of the vimentin and E-cadherin genes. $^{a}P<0.05$, $^{b}P<0.01$, compared with the OE-NC or hCJE or hPEC group; $^{c}P<0.05$, $^{d}P<0.01$, compared with the TGF-β group; $^{e}P<0.05$, $^{f}P<0.01$, indicating statistically significant differences between groups. Nin: Nintedanib; EMT: Epithelial-mesenchymal transition; Nin: Nintedanib; hPEC: Human pterygium epithelial cells; TGF-β: Transforming growth factor beta; hCJE: Human conjunctival epithelial cells; hCJE: Human conjunctival epithelial cells; OE-NC: Over-expression negative control; qRT-PCR: Quantitative real-time polymerase chain reaction; Smad: Drosophila mothers against decapentaplegic; SNAIL: Snail homolog 1 (Drosophila); SLUG: SNAI2 (snail family transcriptional repressor 2); GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TGFBR2: Transforming growth factor beta receptor 2.

epithelial cells into fibroblasts, thereby promoting the synthesis and deposition of the extracellular matrix[22]. This process could potentially accelerate the hyperplasia and invasive progression of pterygium tissue. Inhibiting EMT may help to mitigate or even prevent the advancement of pterygium by reducing the conversion of epithelial cells into fibroblasts[23]. Our preliminary studies have demonstrated that nintedanib can suppress the proliferation of pterygium cells and induce apoptosis. However, its effects on the EMT process in hPECs remain to be elucidated. The experimental outcomes of this

study have unveiled a significant downregulation of the EMT molecular marker Vimentin by nintedanib, suggesting that it may hinder the cells' shift towards a mesenchymal phenotype. Concurrently, nintedanib has been shown to upregulate the EMT molecular marker E-cadherin expression, whose elevation generally implies that cells are maintaining or regaining their epithelial properties, further corroborating the potential of nintedanib to suppress EMT[14,24]. These insights are grounded in the pathological characteristics of pterygium, encompassing excessive extracellular matrix accumulation, cellular proliferation, overexpression of anti-apoptotic factors, and an exacerbated EMT phenotype. The therapeutic mechanisms of nintedanib offer a novel perspective for pterygium treatment and may lay the groundwork for formulating innovative therapeutic approaches.

miR-23b-3p is posited to play a crucial regulatory role in the malignant transformation and metastasis of cancer[25]. An analysis of the GEO database has uncovered that 44 miRNAs exhibit dose-dependent upregulation in response to nintedanib treatment, with miR-23a-3p and miR-23b-3p being identified as the target miRNAs of the TGFBR2 gene. The ENCORI online analytical tool's predictions hint that nintedanib might suppress the activation of Smad2 by modulating the miR-23b-3p/TGFBR2 axis, thereby impeding the onset of EMT[26]. Validation through qPCR has confirmed that nintedanib can elevate the expression levels of miR-23b-3p. Subsequent cellular transfection experiments and dual-luciferase reporter gene analyses have demonstrated that the interaction between miR-23b-3p and TGFBR2 significantly curtails the migratory capabilities of and hCJE undergoing fibrosis induced by TGF-β. Moreover, this interaction also dampens the EMT process, mitigating the intensification of the EMT phenotype. In rescue experiments, the overexpression of TGFBR2 was observed to notably reverse the inhibitory effects of nintedanib on the proteins Smad2, SNAIL, and SLUG within fibrotic cells, a reversal intricately linked to TGFBR2's role in activating the phosphorylation of the Smad2 protein[27]. TGF-β, targeted through Smad signaling mediated by TGFBR2, has demonstrated therapeutic potential across a spectrum of fibrotic diseases[28]. The classical Smad2 signaling pathway is implicated in the ECM deposition and myofibroblast activation associated with pterygium[29-30]. However, the precise mechanism by which nintedanib upregulates miR-23b-3p remains unconfirmed in this study and could involve the stabilization of pri-miR-23b, potentially through mechanisms such as m6A RNA modification or DNA methylation levels. To date, no studies have directly established the regulatory role of nintedanib at the non-coding RNA level, marking a significant avenue for future research endeavors[26].

TGF-β plays a pivotal role in the EMT process of hPEC, initiating a cascade of downstream signal transductions through its interaction with the canonical receptor protein TGFBR2[31]. As a key receptor in this process, the binding affinity of TGFBR2 for its ligand is crucial for signal modulation. In this study, we utilized molecular docking techniques to perform a computational analysis of the interaction between nintedanib and the active site of the TGFBR2 protein. The results indicated that nintedanib's binding capacity to TGFBR2 exceeds its natural ligands, exhibiting a more potent binding energy[32]. This discovery opens up new avenues for therapeutic strategies and research directions. We conducted co-immunoprecipitation experiments to substantiate this finding further and explore the interaction between nintedanib and TGFBR2. The experimental outcomes demonstrated that nintedanib can competitively bind to TGFBR2, which diminishes the overall quantity of TGFBR2 protein and reduces the proportion of TGFBR2 binding to TGF-β, potentially inhibiting the propagation of the TGF-β signal. These insights enhance our comprehension of the mechanisms of action of nintedanib and offer promising prospects for developing novel therapeutic approaches, particularly in treating EMT-associated diseases such as pulmonary fibrosis and certain types of cancer[33]. We anticipate that future research will further elucidate the specific role of nintedanib in modulating the TGF-β signaling pathway and will investigate its potential clinical applications.

Nintedanib has been crucial in curbing EMT and fibrosis associated with pterygium. Our research has uncovered two principal mechanisms by which nintedanib operates: first, by upregulating miR-23b-3p to suppress the expression of TGFBR2 at both the genetic and protein levels; second, by binding directly to the TGFBR2 protein, it inhibits the response of hPEC and fibrotic hCJE cells to TGF-β, thereby restricting the activation of TGFBR2 and its downstream effector Smad2. Collectively, these actions suppress the expression of pro-EMT proteins such as SLUG and SNAIL, reduce the levels of vimentin, and enhance the transcriptional and translational levels of E-cadherin (Figure 6). This study elucidates the cellular mechanisms of nintedanib's action and offers novel targets and strategies for developing analogous drugs. Significantly, the ocular formulation of nintedanib, CBT-001, has demonstrated positive outcomes in clinical studies for pterygium treatment. In Phase IIa clinical trials, CBT-001 notably decreased pterygium-related angiogenesis and lesion length while exhibiting tolerability[34-35]. These clinical findings further bolster the potential of nintedanib as an efficacious medication for preventing pterygium recurrence.

In conclusion, this research has paved the way for developing additional drugs targeting TGFBR2 and Smad2. By thoroughly investigating the mechanisms of nintedanib, we have provided theoretical backing and empirical data for its use in treating

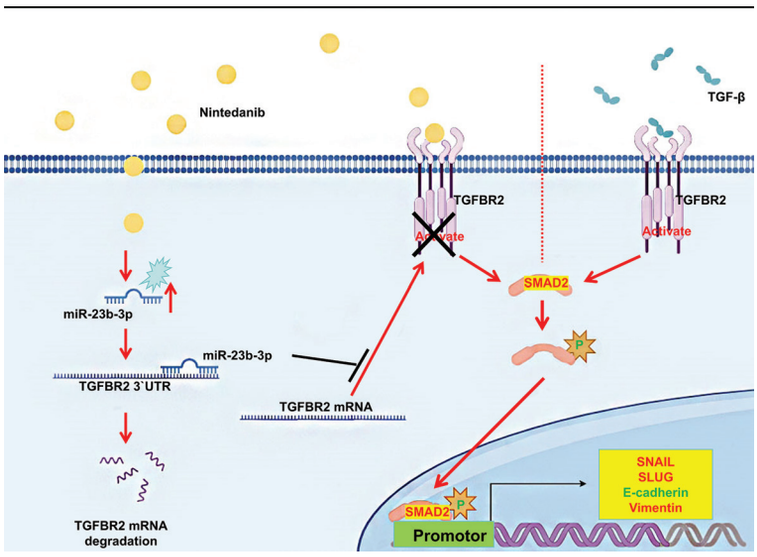


**Figure 6 Nintedanib can stimulate the upregulation of miR-23b-3p expression, thereby increasing the probability of binding to the TGFBR2 3'UTR gene target, inducing the degradation of TGFBR2 mRNA, and thus reducing the amount of TGFBR2 protein corresponding to TGF-β** This leads to the limited activation of TGFBR2/Smad2 and the inhibition of the transcription and translation of SNAIL/SLUG/vimentin, upregulating the expression of E-cadherin; additionally, nintedanib can directly antagonize the active site of the TGFBR2 protein with TGF-β, thereby restricting the expression of downstream EMT-related proteins and genes. TGFBR2: Transforming growth factor beta receptor 2; hPEC: Human pterygium epithelial cells; TGF-β: Transforming growth factor beta; Smad: Drosophila mothers against decapentaplegic; SNAIL: Snail homolog 1 (Drosophila); SLUG: SNAI2 (snail family transcriptional repressor 2); EMT: Epithelial-mesenchymal transition.

pterygium and valuable insights for the future development of innovative antifibrotic drugs that target the TGF-β signaling pathway. In summary, the therapeutic potential of nintedanib in pterygium has been corroborated by both preclinical and clinical research, presenting new horizons and hope for managing related conditions.

## ACKNOWLEDGEMENTS

**Authors' Contributions:** Zhang KK and Li M designed the study. Zhang KK drafted the original manuscript, Zhang KK, Li M, and Liao YH collected the data and reviewed the literature. Liu XT, Bao YB, and Gong Y interpreted the data and critically reviewed the manuscript. All authors have read and approved the final manuscript.

**Data Availability:** The datasets during the current study are available from the corresponding author on reasonable request.

**Foundations:** Supported by the Ningbo Natural Science Foundation (No.2023J212); Zhejiang Medical and Health Technology Program (No.2023KY1141).

**Conflicts of Interest: Zhang KK,** None; **Li M,** None; **Liao YH,** None; **Liu XT,** None; **Bao YB,** None; **Gong Y,** None.

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