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Original Article

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) activates p38 to affect pulmonary fibrosis

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ABSTRACT

Objective: We aimed to examine whether heparin-binding epidermal growth factor-like growth factor (HB-EGF) affects the lung fibrosis process through the activation of p38 protein in mitogen-activated protein kinases (MAPK) signaling pathway, as well as the expression of downstream inflammatory factors.

Methods: The expression levels of HB-EGF, collagen type I (COL-I), and hexokinase 2 (HK2) in peripheral blood mononuclear cells (PBMCs) of patients with connective tissue disease-related interstitial lung disease (CTD-ILD) were examined by qPCR, Western blotting and ELISA.

Results: In vitro experiments showed that HB-EGF was increased in almost all subtypes [rheumatoid arthritis (RA), systemic sclerosis (SSc) and idiopathic inflammatory myopathies (IIMs)] as well as in all groups ($P < 0.05$). For embryonic lung fibroblast (A549) cells, the expression levels of HK2 and α-smooth muscle actin (α-SMA) genes were elevated during 0–4 h and then plateaued. Transforming growth factor-β1 (TGF-β1) induced fibrosis in human embryonic lung fibroblasts (MRC-5) cells and A549 for a certain period of time, but the degree of induction varied, which may be related to the redifferentiability of cells at different spatial locations. Moreover, HB-EGF at concentrations above 1 ng/ml stimulation increased COL-I expression ($P < 0.05$), and for α-SMA gene, even 1 ng/ml concentration of HB-EGF had a stimulatory effect, and different concentrations of HB-EGF did activate the expression of p38 in a concentration-dependent manner within a certain concentration range, and by The qPCR results showed that for interleukin 6 (IL-6), an inflammatory factor regulated downstream of p38, the expression was significantly increased in A549 cells compared to control ($P < 0.05$), but tumor necrosis factor-α (TNF-α) expression was downregulated ($P < 0.05$), but for interleukin-1β (IL-1β) gene, there was no significant difference in A549 cells, and expression was downregulated in MRC-5 cells. Therefore, it is suggested that HB-EGF regulates the expression of inflammatory factors through p38 will be differential across cells.

Conclusion: Our study shows that HB-EGF can suppress pulmonary fibrosis through downstream activation of p38/MAPK pathway activity, as well as the expression of various inflammatory factors downstream of it.

1. Introduction

Pulmonary fibrosis is a group of progressive diseases caused by multiple etiologies characterized by airway damage, massive secretion of inflammatory factors, proliferation of mesenchymal cells and abnormal deposition of extracellular matrix (ECM), in which alveolar epithelial cells are tightly attached to adjacent cells or the base to protect the lung from injury and infection. During

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pulmonary fibrosis, multiple cellular components and inflammatory/fibrotic factors interact and influence each other in the development of connective tissue disease-related interstitial lung disease (CTD-ILD) [1,2], with damage to capillary endothelial cells and alveolar epithelial cells, and with the progression of early alveolitis, alveolar structure is progressively destructed and subsequently lost function.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is another HSPG-binding growth factor that has been implicated in various factor-induced lung pathogenesis. HB-EGF is a potent mitogen for lung fibroblasts and epithelial cells and has been shown to stimulate cell migration in other cell systems [3]. EGF has been shown to reduce elastin gene expression in chicken aortic smooth muscle cells and lung fibroblasts. The effect of HB-EGF on elastin gene expression has not been examined. Since its activity is regulated by HSPG and HSPG is a target of elastase therapy, HB-EGF may be an important signaling ligand in elastase-induced injury/repair [4]. However, the transforming growth factor-β (TGF-β)-mitogen-activated protein kinases (MAPK) pathway is considered to be the most important one among the non-TGF-β/Smad pathways [5]. It is now known that MAPK consists of four major members, namely extracellular signal-regulated kinase (ERK1/ERK2), stress-activated protein kinase (SAOK/JNK), p38MAPK and ERK5, of which p38MAPK is closely associated with the pulmonary fibrosis process [6]. Li Fengfei et al. [7] found that stimulation of HBE cells with SiO_2 resulted in decreased levels of E-calmodulin expression. while α-SMA and V-waveform protein expression levels increased, and some cells morphologically showed shuttle-shaped changes, exhibiting distinct mesenchymal cell characteristics, confirming the occurrence of epithelial−mesenchymal transition (EMT). Yamaguchi M et al. [8] showed the opposite results, suggesting that the HB-EGF/EGFR pathway is involved in the development of lung fibrosis, and that dysregulated HB-EGF expression and/or sustained EGFR activation may lead to airway epithelial of abnormal airway repair, thus promoting airway fibrosis. Reprogramming of glucose metabolism is the pathological basis of fibrosis in human tissues and organs. M. Bueno et al. [9] found that activated myofibroblasts in lung tissue of IPF patients also undergo alterations in glucose metabolism patterns. HB-EGF is known to have potential binding sites with HK1, and HB-EGF can regulate the expression and enzymatic activity of HK1 [10]. Kolosova et al. [11] observed morphological and functional changes in lung epithelial cell lines (HAE) after 96 h treatment with TGF-β. They found a decrease in epithelial one-cell contacts, prolongation of epithelial cells, downregulation of the epithelial cell marker E a calmodulin, and mesenchymal cell markers α-actin (α-SMA), fibronectin, and collagen I were upregulated. Doerner et al. [12] found that TGF-α induction of EMT was accompanied by morphological changes, with cells becoming spindle-like and an increase in intracytoplasmic α-SMA. The same was confirmed in many studies.

In summary, studies have confirmed that p38MAPK is closely related to the pulmonary fibrosis process, and that it also affects inflammatory factors as well as glycolytic processes. Then, there is no conclusive conclusion on how HB-EGF affects the pulmonary fibrosis process through p38 protein, and this paper is focused on revealing how HB-EGF indirectly regulates the pulmonary fibrosis process through activation of p38.

2. Materials and methods

2.1. Information data collection

This is a mixed clinical and basic research study. In the clinical study, 30 patients with CTD-ILD were comprehensively evaluated, including 12 diagnosed with RA, 9 with SSc, and 9 with IIMs; 30 healthy controls matched for age and sex; all CTD cases were diagnosed according to the commonly used classification criteria established by the American College of Rheumatology [13]. Cases were collected from January 2021 through December 2022. Data collection: clinical data, laboratory indices (routine blood, blood biochemistry, immunoglobulin and autoantibody tests) were collected from all study subjects. The collected data and indicators were shown in Table 1.

2.2. Cell source

Human embryonic lung fibroblasts (MRC-5) cell line and embryonic lung fibroblast (A549) cell line were purchased from National Central Cell Bank. Passage 9 to 16 cultured cells were used for all experiments listed below. Hepatocyte growth factor (HGF), TGF-β1, and HB-EGF cytokines were purchased from Procell Biotechnology (Wuhan Procell).

2.3. Induction of cells by TGF-β1 and HB-EGF treatment

TGF-β1 and HB-EGF induced the transformation of MRC-5 and A549 fibroblasts into myofibroblasts. MRC-5 cells were grown in 6-well cell culture plates (Wuxi Cell-nest Biotechnology Co., Ltd.) at a cell density of 3×10^4 cells/cm^2. The medium was α-minimal essential medium (α-MEM) containing 10% (v/v) fetal bovine serum (FBS, Gibco). The cells were induced by adding 0, 1, 2, 10 ng/ml of HB-EGF and 5 ng/LTGF-β1 to the medium for 48 h. The normal cultured cells without TGF-β1 and the cells with TGF-β1 were also induced by adding 5 ng/LTGF-β1 to the medium. The normal cultured cells without TGF-β1 and the TGF-β1/HB-EGF induced cells were cultured for 48 h, while the cells without HGF or HB-EGF were used as parallel controls for the whole experiment, respectively. Cells were collected to extract protein and total RNA for Western blot analysis or qPCR analysis.

2.4. qPCR analysis

qPCR was performed to analyze α-SMA, Col(I), HB-EGF, p38, interleukin 6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) expression in cells: Total cellular RNA was isolated from MRC-5 and A549 cells with TRIzol reagent (Takara, Kusatsu, Japan) and then reverse-transcribed into cDNA with PrimeScript® RT reagent kit with gDNA Eraser (Takara) according to the

Table 1
The levels of factors in serum.

Group	Number of cases	HB-EGF/pg/ml	IL-6/pg/ml	Serum ferritin/pg/ml
Control group	30	0.60 ± 2.97	34.24 ± 1.09	12.24 ± 1.65
CTD-ILD patients				
RA typing	12	52.77 ± 7.40*	35.87 ± 4.03	14.67 ± 2.67
SSc typing	9	58.28 ± 7.80**	35.34 ± 5.09	17.87 ± 3.24
IIMs typing	9	78.52 ± 9.79**	43.03 ± 13.58**	19.77 ± 0.38*

CTD-ILD: connective tissue disease-related interstitial lung disease; RA: rheumatoid arthritis; SSc: systemic sclerosis; IIMs: idiopathic inflammatory myopathies.

Table 2
Specific primer sequences in qPCR and amplified fragment lengths.

Gene	Size (bp)	Up-stream primer	Down-stream primer
GAPDH	516	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
CoL(I)	693	GTGCTCGTGGAAATGATGGT	CTGGGGACCTTCAGAGCCT
HB-EGF	454	GGGTTCTTACTGCACGACAG	CAAACAAGTGTAGCACATTC
IL-6	639	AACCTGAACCTTCCAAAGATGG	TCTGGCTTGTTCCTCACTACT
TNF-α	702	TGGCGTGGAGCTGAGAGATA	TGATGGCAGAGAGGAGGAGTTG
α-SMA	810	ATGCCTCTGGACGCACAACT	CCCGGACAATCTCACGCTCA
HK2	745	AACATGATCGTGCGCTCTGCAAGTGCAGC	GCTCAGACCTCGCTCCATTT
p38	1152	GATGACGACGATGATGAC	TGCGCTCTGCAAGTGCAGC
GADPH	470	GGTGA AGGTC GGAGT CAACG	CAAAG TTGTC ATGGA TGHACC

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; COL-I: collagen type I; HB-EGF: Heparin-binding epidermal growth factor-like growth factor; IL-6: interleukin 6; TNF-α: tumor necrosis factor-α; α-SMA: α-smooth muscle actin; HK2: hexokinase 2; GADPH: D-glyceraldehyde-3-phosphate dehydrogenase.

concentration. The condition for PCR was as follow; denaturation at 94 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min (40 cycles). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene mRNA level was used as a control for all qPCR reactions, and image processing of electrophoretic bands as well as grayscale analysis was performed using ImageLab software. The specific primer sequences in qPCR and amplified fragment lengths were shown in Table 2. qPCR was repeated at least three times and gene expressions were calculated based on the $2^{-\Delta\Delta\ Ct}$ method.

2.5. Western blotting

Cell lysates were separated on ristocetin-induced platelet aggregation protein lysate (Beyotime Biotechnology, Inc.). Centrifuging at 12,000 rpm for 20 min at 4 °C. Approximately 30 μg of protein was subjected to SDS-PAGE and then transferred overnight to polyvinylidene fluoride membranes. The membranes were briefly stained in Ponceau S dye (Sigma, St. Louis, MO) to check for equivalent protein loading and transfer. Membranes are then blocked with team-based simulation training (TBST) containing 5% milk (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) in a shaker for 15 min. And then incubated with primary antibody for 2 h at room temperature or overnight in a shaker at 4 °C. After washing the membrane with TBST, secondary antibody is added and incubated at 37 °C for 45 min to visualize specific proteins by chemiluminescence. To detect the blot with secondary antibody. At the end of the secondary antibody incubation, the membrane was rinsed three times for 10 min each using TBST buffer. Liquid A and B in the ECL luminescent solution kit (Tanon) were prepared at a ratio of 1:1 and developed with the help of a chemiluminescent imager. Protein level was evaluated through densitometry using the Image J freeware program (rsb.info.nih.gov/ij).

2.6. Enzyme-linked immunosorbent assay detection of inflammatory factors in serum

The Quansys BiosciencesQ-Plex™ Multifactor Enzyme-linked immunosorbent assay (ELISA) Kit was used for each inflammatory factor analysis (IL-6, iron lactalbumin, etc.) in patient serum, collected patient serum samples and calibrators are added to a 96-well plate coated with antibodies encapsulated so that they are immobilized in the 96-well plate. Unbound proteins are washed away and a specific biotin-labeled multifactor antibody mixture is added. After washing away the unbound biotin-labeled antibodies, horseradish peroxidase is added, followed by another wash. The amount of horseradish peroxidase contained in each well of the 96-well plate is proportional to the amount of marker captured by the encapsulated antibodies.

2.7. Statistical analysis

All data were shown as mean ± standard deviation. The normality of datasets was analyzed with Shapiro–Wilk's test. Statistical analyses included one-way analysis of variance (ANOVA) along with covariance analysis (ANCOVA). SPSS 26.0 software was used for these analyses. P-value <0.05 was considered statistically significant.

3. Results

3.1. Patients' serum levels of IL-6, serum ferritin, sedimentation, CRP, platelets, pulmonary function (carbon monoxide diffusing capacity, DLCO), and complement C3C4 water

Subsequently, we analyzed HB-EGF, IL-6 inflammatory factor and immune-related protein iron-lactalbumin in peripheral blood mononuclear cells (PBMCs) of CTD-ILD patients, and the results showed that HB-EGF as well as HB-EGF were increased in almost all subtypes (RA, SSc and IIMs) compared to controls ($P < 0.05$), This suggests that patients with CTD-ILD IIMs subtypes have different degrees of inflammatory response, and similar results were found in the ferric whey protein content, but there was no continuity between patients, suggesting that both the inflammatory response and the immunity of the patients were affected, which may be linked to the HB-EGF gene. (Table 2).

3.2. In vivo cell model assay

3.2.1. Induced transformation of MRC-5 and A549 embryonic lung fibroblasts into myofibroblasts by TGF-β1

In vitro TGF-β1 stimulation on human fibroblast (MRC5) cells and A549 (human embryonic lung fibroblasts) was used to construct a cell model, and the expression levels of collagen type I (COL-I), HK2, and α-SMA genes were measured after 48 h of stimulation by collecting cells (0 h, 2 h, 4 h, 8 h, 24 h, and 48 h). The qPCR analysis confirmed that the expression of COL-I, HK2 and α-SMA genes were increased to different degrees after TGF-β1 induction within 0–8 h, and (Fig. 1) thus showed that MRC-5 fibroblasts induced by TGF-β1 could be transformed into myofibroblasts with high α-SMA expression. For A549 embryonic lung fibroblasts, the expression levels of HK2 as well as α-SMA genes were elevated during 0–4 h and then plateaued as shown in (Fig. 2). In summary, TGF-β1 can induce fibrogenesis in MRC-5 and A549 for a certain

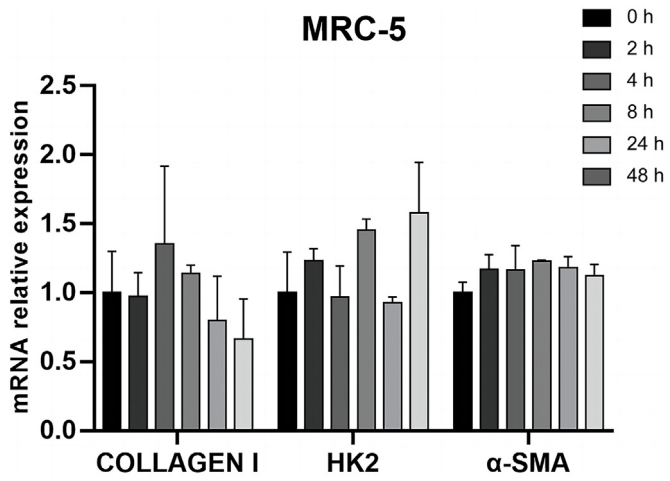

Fig. 1. Expression of COL-I, HK2 and α-SMA genes at different times.

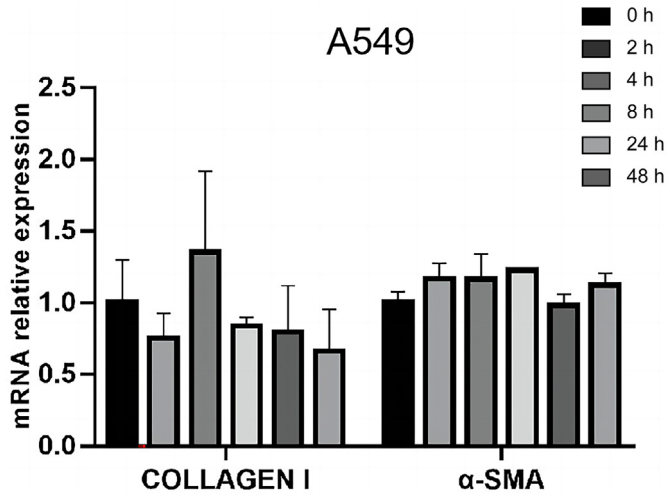

Fig. 2. Expression of COL-I and α-SMA genes at different times.

period of time, but the degree of induction varies, which may be related to the differentiability of cells in different spatial locations.

3.2.2. Expression of COL-I and α-SMA genes after stimulation on MRC-5 with different concentrations of HB-EGF and TGF-β1

In vitro, MRC5 cells were stimulated with different concentrations (0 ng/ml, 1 ng/ml, 2 ng/ml, 10 ng/ml) of HB-EGF and 5 ng/ml of TGF-β1 to detect the expression levels of COL-I and α-SMA genes. qPCR results showed that HB-EGF at concentrations above 1 ng/ml stimulation increased expression of COL-I ($P < 0.05$), and for α-SMA gene, even 1 ng/ml of HB-EGF had a stimulatory effect, but compared with the TGF-β1-induced effect, HB-EGF did not have a strong ability to stimulate fibril formation in MRC-5, and even when the concentration reached 10 ng/ml it was lower than the effect of TGF-β1 as shown in Fig. 3.

3.2.3. HB-EGF affects pulmonary fibrosis through p38 activation

Through what pathway does HB-EGF affect lung fibrosis? Specifically, through what pathway does HB-EGF regulate the expression of COL-I, hexokinase 2 (HK2) and α-SMA genes to affect lung fibrosis? We found that HB-EGF can affect lung fibrosis through the p38MAPK pathway according to previous researches, and then we examined the p38 protein level. As shown in Fig. 4A, HB-EGF at different concentrations could indeed activate p38 expression, but its effect was not as strong as TGF-β1, but HB-EGF at 2 ng/ml seemed to have a somewhat inhibitory effect, suggesting that there may be other factors involved in this regulatory pathway, and the qPCR results were consistent with Western blot results, as shown in Fig. 4B.

3.2.4. HB-EGF regulates inflammatory factor gene expression via p38

Previous studies have shown that HB-EGF can affect pulmonary fibrosis by inhibiting the inflammatory response and apoptosis during pulmonary fibrosis, so could HB-EGF affect pulmonary fibrosis by activating the expression of p38, which in turn regulates the expression of its downstream inflammatory factors. We then examined the expression of p38-regulated inflammatory factors IL-6, TNF-α, and IL-1β, and found that IL-6 expression was significantly increased ($P < 0.05$) but TNF-α expression was down-regulated ($P < 0.05$) in MRC-5 and A549 cells compared to control cells, but for IL-1β gene, its expression was not significantly different in A549 cells, but then down-regulated in MRC-5 cells, so this suggests that our HB-EGF regulation of inflammatory factor expression through p38 would be different from cell to cell, see Fig. 5.

4. Discussion

In this paper, to determine how HB-EGF alone affects lung fibroblasts, we used exogenous addition of different concentrations of TGF-β1 and HB-EGF to induce the transformation of MRC-5 and A549 fibroblasts into myofibroblasts experimentally, cells were treated with HB-EGF for different times or at different concentrations. Our results revealed that HB-EGF at concentrations above 1 ng/ml stimulation increased COL-I expression and even HB-EGF at 1 ng/ml concentrations increased α-SMA gene expression, but compared to the TGF-β1 induction effect, HB-EGF was not as strong for stimulating fibrogenesis in MRC-5. Even 10 ng/ml was lower than the effect of TGF-β1. Previous studies have shown [14–16] that HB-EGF has the ability to affect the pulmonary fibrosis process, but the exact pathway through which it does so has not been elucidated. In this study, we intend to examine whether HB-EGF's are regulated through the JNK and p38/MAPK pathways, phosphorylation-activated p38 protein is associated with pulmonary fibrosis, and p38 phosphorylation is associated with induction of MRC5 fibrosis. HB-EGF was originally identified as a product of cultured human macrophages. It promotes cell survival and protects against cellular hypoxia and intestinal ischemia/reperfusion injury. Recent studies have shown that HB-EGF can reduce the incidence of NEC in a neonatal rat model, in part by reducing apoptosis. Subsequently, we examined the downstream p38 protein expression by western blotting and found that its expression was increased by stimulation of exogenous HB-EGF and showed a dose-dependent relationship within a certain concentration. Moreover, the expression of IL-6 was significantly increased and TNF-α expression was downregulated in MRC-5 cells and A549 cells compared with the control group, so this suggests that the expression of inflammatory factors regulated by HB-EGF through p38 can be cellularly different. This is consistent with the findings of previous studies that HB-EGF can influence pulmonary fibrosis by affecting the inflammatory response and apoptosis during pulmonary fibrosis [17].

The current study found that p38 protein expression was significantly upregulated in HB-EGF-induced MRC-5 human embryonic lung fibroblasts, thus providing a very valuable result that HB-EGF may suppress the lung fibrosis process by activating p38 protein, which also affects the expression of various inflammatory

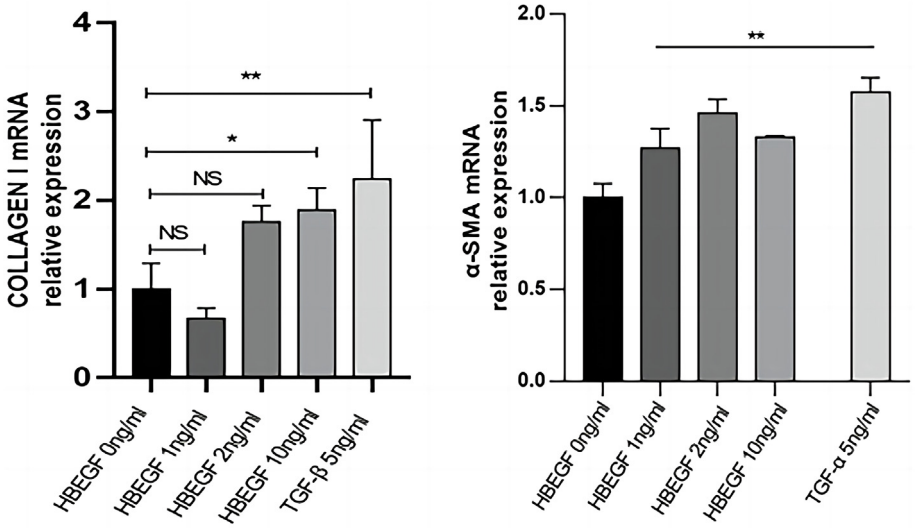

Fig. 3. Expression of COL-I and α-SMA genes in MRC-5 cells.

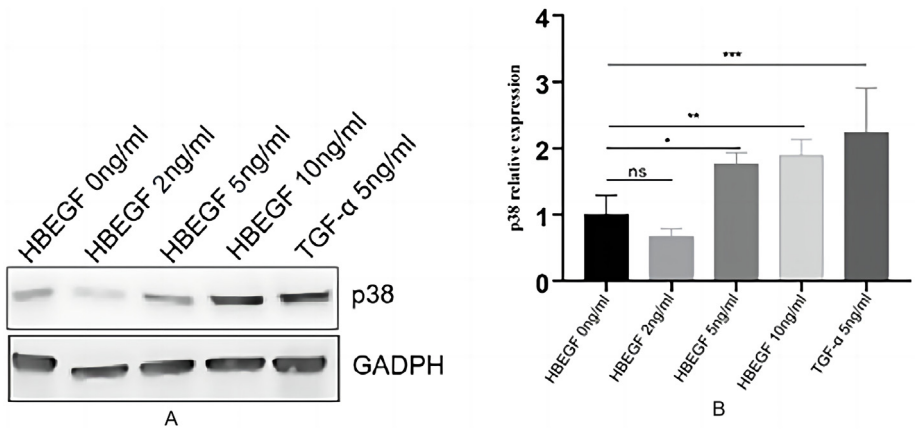

Fig. 4. HB-EGF affects pulmonary fibrosis through p38 activation. A. Western blot was performed to detect p38 protein expression level; B. qPCR was performed to verify p38 protein expression level.

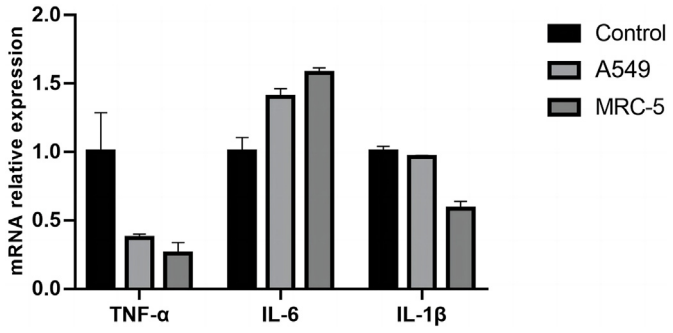

Fig. 5. HB-EGF indirectly regulates the expression level of inflammatory factors (IL-6, TNF-α, IL-1β) through p38 protein activation.

factors. The specific HB-EGF-induced lung normal cells play a very important role in the formation of pulmonary fibrosis, the specific molecular mechanism of p38 protein is not yet clear, and a more detailed study of the above mechanism can provide the basis for the clinical search for new drug targets, and more subsequent studies are needed to explore.

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Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Shandong Provincial Hospital. Informed consent was obtained from all recipients of cells or tissues.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Authors' contributions

Yang QR and Ma ZZ conceived of the study, and An Y, Yan SY, Xu W, Li MQ and Dong RR participated in its design and data analysis and statistics and An Y helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

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