Ginsenoside Rg3 Inhibits Pulmonary Fibrosis by Preventing HIF-1α Nuclear Localisation

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Abstract

Introduction: Excessive fibroblast proliferation during pulmonary fibrosis leads to structural abnormalities in lung tissue and causes hypoxia and cell injury. However, the mechanisms and effective treatment are still limited.

Methods: In vivo, we used bleomycin to induce pulmonary fibrosis in mice. IHC and Masson staining were used to evaluate the inhibitory effect of ginsenoside Rg3. In vitro, scanning electron microscopy, transwell and wound healing were used to evaluate the cell phenotype of LL 29 cells. In addition, biacore was used to detect the binding of ginsenoside Rg3 and HIF-1α.

Results: Here, we find that bleomycin induces the activation of the HIF-1α/TGFβ1 signalling pathway and further enhances the migration and proliferation of fibroblasts through the epithelial mesenchymal transition (EMT). Ginsenoside Rg3 can slow down the progression of pulmonary fibrosis by inhibiting the nuclear localisation of HIF-1α. In addition, molecular docking and biacore experiments indicated that ginsenoside Rg3 can bind HIF-1α and restrict the progression of pulmonary fibrosis in animals. Hypoxia can lead to excessive proliferation of lung fibroblasts and further accelerate lung fibrosis.

Conclusions: This finding suggests that early targeted treatment of hypoxia may have potential value in the treatment of pulmonary fibrosis.

Background

Idiopathic pulmonary fibrosis is a severe interstitial lung disease that can cause progressive loss of the lung function and has high lethality[1, 2]. Pulmonary fibrosis is an end-stage change in a large class of lung diseases characterised by abnormal fibroblast proliferation, extracellular matrix accumulation with inflammatory damage and tissue structure destruction. The alveolar tissue of patients is damaged and abnormally repaired, causing scarring[1, 3, 4]. During pulmonary fibrosis, the abnormal proliferation of fibroblasts is similar to the biological behaviour of tumour cells[5–7]. Pulmonary fibrosis severely affects the human respiratory function. The respiratory function of patients continues to deteriorate as the disease and lung injury worsen. Idiopathic pulmonary fibrosis has a higher mortality rate than most tumours and is called a ‘tumour-like disease’[4, 8, 9].

Pulmonary fibrosis and tumours share common characteristics, hypoxia and excessive cell proliferation. Rapid tumour growth causes hypoxia in the internal tissue cells of solid tumours. Stress stimulation of hypoxia in tumour cells imparts some tumour cells with a strong migration ability, eventually leading to distant metastasis[10, 11]. In addition, tumour cells demonstrate vascular mimicry by simulating the structure of blood vessels to provide themselves with purchased nutrients[12, 13]. Similarly, after pulmonary fibrosis, the alveoli are replaced by fibrotic cells, which prevent oxygen from entering the blood and cause a wide range of hypoxia[14]. However, the relationship between hypoxia and fibroblast hyperproliferation remains unclear.

In this study, we found an abnormal expression of HIF-1α in the lung tissue of mice with bleomycin-induced pulmonary fibrosis. HIF-1α can enhance cell migration and proliferation through the epithelial–mesenchymal transition (EMT) pathway. In addition, ginsenoside Rg3 can bind and inhibit the nuclear localisation of HIF-1α. Through in vitro and in vivo experiments, we verified that ginsenoside Rg3 inhibits excessive fibroblast proliferation and pulmonary fibrosis through the HIF-1α pathway. These observations suggest that HIF-1α may serve as a potential molecular target for the hypoxic treatment of pulmonary fibrosis.

Methods

Cell lines

The lung fibroblast cell line LL 29 was obtained from ATCC. The cells were cultured in F12K minimum essential medium containing 15% fetal bovine serum (Gibco, USA) in an environment containing 5% CO_2 at 37 °C.

Pulmonary fibrosis model and experimental design

BALB/c mice weighing 18–22 g was purchased from Charles River Laboratories. The mice were randomly divided into groups with eight mice each. Mice anesthetised with sodium pentobarbital (40 mg/kg) were administered with 5 mg/kg of bleomycin through the trachea. Control mice were treated with an equal volume of saline. A fibrosis model was successfully established after bleomycin treatment for 30 days and pathological examination of the lungs. We continuously administered ginsenoside Rg3 (5mg/kg body weight) to the lung fibrosis mice. After 28 days, the mice were euthanized through the intravenous injection of pentobarbital sodium at a final concentration of 100 mg/kg. The mice were checked, and death was confirmed by observing lack of respiration and cardiac output. Tissues were fixed or frozen for subsequent pathology testing. Euthanasia methods was in accordance to the proper practice of AVMA 2020. All procedures involving animals were performed in accordance with the ethical standards of the Institutional Animal Care and Use Committee (IACUC) at Tianjin International Joint Academy of Biomedicine.

Pathological analysis

After the tissue was cut into 4μm sections and dewaxed, the sections were treated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity. After antigen repair, the sections were incubated with normal goat serum at 37 °C for 10 min. Afterwards, the sections were incubated with the following primary antibodies: SMA (Affinity, Changzhou, China), HIF-1α (Affinity, Changzhou, China), E-cadherin (Affinity, Changzhou, China) and Vimentin (Affinity, Changzhou, China). After 2 h, the sections were washed and incubated with a secondary antibody (Zhongshan Biotechnology Co., Ltd., Beijing, China) at 37 °C for 30 min. After staining with 3,3'-diaminobenzidine, the sections were fixed for observation.

Cell migration assay

The treated cells were seeded onto a 24-well plate and grown to 70% density at 37 °C with 5% CO_2. A straight scratch was then made in the middle of the plate. After 24 h, cell migration and the migration rates were recorded with a microscope.

Cell invasion assay

Matrigel was evenly spread on the bottom of a Transwell chamber. The treated cells were seeded into the Transwell chamber and cultured in a serum-free medium. Then, the 24-well plate was added with 10% FBS medium. After 16 h of incubation in an environment containing 5% CO_2 at 37 °C, the cells inside the small chamber were removed; the cells outside were stained with crystal violet, photographed and analysed for cell invasion ability.

Colony formation assay

The treated cells were uniformly seeded onto a 6-well plate at a final concentration of 1,000 cells per well and then incubated for 2 weeks at 37 °C in an environment saturated with 5% CO_2. After clones were formed, the cells were washed with 1× PBS and subsequently fixed and stained. The number of clones in each group was determined.

Western blot

The Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology Co., Ltd. P0027) was used to extract nuclear and plasma proteins from the tissues and cells. All lysates contained protease inhibitors. The quantified proteins were separated by 10% SDS-PAGE. After transferring the protein to the PVDF membrane and blocking with 5% BSA, the PVDF membrane was blocked at room temperature with the following primary antibodies: HIF-1α (Affinity, Changzhou, China), TGFB1 (Affinity, Changzhou, China), SAMD3 (Affinity, Changzhou, China), p-SMAD3 (Affinity, Changzhou, China) E-cadherin (Affinity, Changzhou, China), Vimentin (Affinity, Changzhou, China), Lamin B (Affinity, Changzhou, China) and GAPDH (Affinity, Changzhou, China). GAPDH and Lamin B were used as loading controls. After 4 h, the excess primary antibody was removed, and the PVDF membrane was incubated with HRP-labelled secondary antibody at room temperature for 2 h. Protein intensity was detected with an Image Lab instrument (Bio-Rad, USA).

Biacore assay

Human HIF-1α cDNA was synthesized by Genwiz (Beijing, China) and cloned into PET-His prokaryotic protein expression plasmid using BamHI and NheI endonucleases. Biacore assay was performed with a Biacore 3000 instrument (GE Healthcare, Piscataway, NJ, USA). 50mM NHS and 200mM EDC were mixed in equal volumes and injected into a closed CMD500M chip (XanTec Bioanalytics) at a rate of 10 μl/min. The purified HIF-1α protein was diluted with sodium acetate buffer pH 5.0 and injected into the chip, and the remaining active groups were blocked with 1M ethanolamine. Subsequently, RG3 was injected into

the CM5 sensor chips at a rate of 30μl/min. Data analysis was conducted using the BIA evaluation software.

Molecular docking

The crystal structure of HIF-1α was downloaded from the PDB database and used to perform molecular docking with Rg3 by using the Sybyl X1.1 software.

Scanning electron microscope inspection

After treatment, the cells were grown on climbing films. After 24 h, the cells were fixed and dehydrated in acetone/isoamyl acetate (1:1) and dried with a gradient concentration of acetonitrile. The cells were then coated with gold and photographed using a scanning electron microscope (LEO 1530 VP, Germany).

Statistical analysis

Data were analysed with the SPSS 18.0 statistics software (SPSS Inc., Chicago, IL, USA) and presented as the mean ± standard deviations of the mean. Significant differences between two groups were compared using a Student's t-test. Comparisons among three or more groups were conducted using ANOVA with post hoc contrasts by Student–Newman–Keuls test. $p<0.05$ was considered to indicate a statistically significant difference.

Results

Ginsenoside Rg3 inhibits bleomycin-induced pulmonary fibrosis

The mice with bleomycin-induced pulmonary fibrosis were treated with ginsenoside Rg3. After euthanasia, mouse lung tissue was obtained for pathological examination and fibrosis analysis. The HE staining results showed that the normal morphology of the lung tissue of the mice treated with bleomycin disappeared, and the alveolar structure of the mice treated with Rg3 was significantly improved (Fig. 1A). Masson staining results indicated that collagenous decreased after Rg3 treatment (Fig. 1B). In addition, the evaluation of the lung coefficients and Ashcroft scores of pulmonary fibrosis mice treated with Rg3 showed that Rg3 improved pulmonary fibrosis (Fig. 1C&1D). Immunohistochemical detection was performed to analyse SMA and HIF-1α expression. The results showed that the expression of SMA was significantly inhibited after Rg3 treatment, and the inhibitory effect of Rg3 on HIF-1α was weaker than that of SMA (Fig. 1E). After analyzing the results of IHC staining, it was found that Rg3 had a greater inhibitory effect on the expression of HIF-1α in the nucleus (Fig. 1F). This finding indicates that the role of Rg3 in pulmonary fibrosis is likely related to hypoxia.

Ginsenoside Rg3 inhibits the bleomycin-promoted migration of fibroblasts by preventing HIF-1α nuclear localisation

To verify how bleomycin inhibits pulmonary fibrosis, we treated LL 29 lung fibroblasts with bleomycin and found that the bleomycin-treated cells had typical EMT characteristics, as revealed by scanning electron microscopy. The cells became spindle-shaped and their adhesion decreased. However, the EMT phenotype was suppressed after treatment with Rg3 (Fig. 2A). Next, we performed transwell and wound healing assays to detect changes in cell invasion and migration capacity after bleomycin or Rg3 treatment. The results showed that bleomycin enhanced the invasion and migration ability of LL 29 cells, whereas Rg3 inhibited this effect (Fig. 2B&2C). Given that the role of HIF-1α in EMT is mainly achieved through transcriptional regulation, we examined the expression of HIF-1α in the nucleus and cytoplasm. Western blot results showed that bleomycin induced the expression of HIF-1α and promoted its nuclear localisation, whereas Rg3 inhibited the nuclear localisation of HIF-1A. We speculate that Rg3 may inhibit the migration and invasion of fibroblasts by inhibiting the nuclear localisation of HIF-1α (Fig. 2D).

Ginsenoside Rg3 binds HIF-1α to inhibit EMT evolution in fibroblasts

To investigate if Rg3 directly binds HIF-1α and thus inhibits its role in fibroblasts, we performed molecular docking to simulate the interaction and found that a strong binding force exists between Rg3 and HIF-1α (Fig. 3A). Subsequent Biacore experiments further verified the binding of Rg3 to HIF-1α (Fig. 3B). These experiments suggest that Rg3 can prevent the entry of HIF-1α and inhibit the cell's EMT process. Given that HIF-1α can promote the transcription of TGFB1, we examined the expression of TGFB1 and EMT markers. The results showed that Rg3 inhibited the expression of TGFB1 and Vimentin but decreased the expression of E-cadherin (Fig. 3C).

Up-regulation of HIF-1α can reverse the inhibitory effect of ginsenoside Rg3 on fibroblasts

To confirm that Rg3 inhibits the EMT evolution of fibroblasts through HIF-1α, we overexpressed HIF-1α in Rg3-treated fibroblasts. We discovered that HIF-1α overexpression reversed the inhibitory effect of Rg3 on fibroblast migration and invasion (Fig. 4A&4B). We also conducted clone formation experiments and found that HIF-1α expression restored the fibroblasts' proliferative ability (Fig. 4C). For the molecular mechanism, we performed Western blot and discovered that HIF-1α expression could resist the inhibitory effect of Rg3 on its nuclear localisation, thereby promoting the occurrence of cell EMT through the TGFB1/Smad3 pathway (Fig. 4D).

HIF-1α counteracts the effects of ginsenoside Rg3 and accelerates bleomycin-induced pulmonary fibrosis

The experiments showed that Rg3 inhibited pulmonary fibrosis by inhibiting the HIF-1α/TGFB1 signalling pathway. Here, we overexpressed HIF-1α by AAV in Rg3-treated lung fibrosis animals. HE and Masson staining revealed that the expression of HIF-1α accelerated the lung fibrosis inhibited by Rg3 (Fig. 5A&5B). The immunohistochemical staining results showed that HIF-1α promoted the expression of TGFB1 and Vimentin and inhibited the expression of E-cadherin (Fig. 5C). All experiments showed that the inhibitory effect of Rg3 on bleomycin-induced pulmonary fibrosis was achieved by preventing the nuclear localisation of HIF-1α, thus inhibiting the TGFB1-mediated EMT process.

Discussion

The pathophysiology of many diseases is accompanied by hypoxia. A hypoxic microenvironment is a double-edged sword; It can cause stress response in some cells and cause damage to human organs[15]. During tumour and lung fibrosis, hypoxia stimulation leads to the excessive proliferation of tumour cells and fibroblasts, which accelerates the progression and deterioration of the disease[16]. HIF-1α plays an important role in the occurrence and development of many diseases as a response factor to hypoxia. In tumours, HIF-1α can trigger the transcription of a series of oncogenes and promote the malignant progression of tumours[17, 18]. HIF-1α is also widely involved in fibrosis in various tissues. In the kidney, the HIF-1α signalling pathway is believed to play a role in the early onset of renal fibrosis[19]. HIF-1α can also participate in liver fibrosis by regulating the function of liver sinusoidal endothelial cells[20]. In this study, we found that bleomycin induction can increase HIF-1α expression and nuclear localization. HIF-1α can activate the TGFβ1/Smad3 signalling pathway to activate EMT in fibroblasts, which ultimately leads to enhanced cell proliferation and migration capacity and excessive fibroblast proliferation. We used ginsenoside Rg3 treatment and found that it can prevent the nuclear localisation of HIF-1α and inhibit the EMT-mediated appearance of cell proliferation caused by activation of the TGFβ1 signalling pathway. At the cellular and molecular level, we confirmed by molecular docking and Biacore experiments that ginsenoside Rg3 can bind HIF-1α and reduce its nuclear localisation.

EMT is a process in which epithelial cells lose their polarity, the epithelial cell markers disappear, the adhesion ability was reduced, and the characteristics of mesenchymal cells are obtained. EMT is widely involved in a series of physiological processes, including development, organ formation and malignant tumour progression[21–23]. Extensive research evidence has confirmed that EMT is vital in fibrosis remodelling [24, 25]. In radiation-induced pulmonary fibrosis, the interaction of Foxm1 and Snail1 activates TGFβ1-induced EMT and promotes pulmonary fibrosis [26]. Similarly, in vitro and in vivo blocking of TGF-β can reduce the progression of bleomycin-induced pulmonary fibrosis[27].

TGFβ1/Smad is a key signal element that regulates EMT. It affects cell proliferation, differentiation, apoptosis and extracellular matrix production to regulate tissue morphogenesis and differentiation[28]. TGFβ1 can increase the expression of the mesenchymal cell marker Vimentin and decrease the expression of the epithelial cell marker E-cad[29, 30]. In this study, we found that bleomycin induced an increase in the nuclear localisation of HIF-1α and activated the TGFβ1/Smad signalling pathway, eventually causing the EMT-mediated hyperproliferation of fibroblasts. After treatment with ginsenoside Rg3, the entry of HIF-1α into the nucleus was reduced, thereby slowing the progression of pulmonary fibrosis.

Conclusion

Overall, ginsenoside Rg3 can inhibit BLM-induced pulmonary fibrosis. The mechanism is attributed to the ability of ginsenoside Rg3 to reduce the nuclear localisation of HIF-1α and inhibit the evolution of EMT-related pulmonary fibrosis mediated by the TGF-β1/Smad3 signalling pathway. This research is expected

to provide experimental evidence for the use of clinical hypoxic treatment methods, such as ginsenoside Rg3, in pulmonary fibrosis.

Abbreviations

Hypoxia inducible factor alpha (HIF-1α), Epithelial mesenchymal transition (EMT), immunochemistry (IHC)

Declarations

Ethics approval and consent to participate: All procedures involving animals were performed in accordance with the ethical standards of the Institutional Animal Care and Use Committee at Tianjin International Joint Academy of Biomedicine followed standard guidelines for the humane care and use of animals in scientific research.

Consent for publication: Not applicable

Competing interests: The authors report no conflicts of interest.

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Authors' contributions: ZF responsible for animal experiments and writing, YX responsible for cell and biological experiments, CC conceived and designed the study. All authors have read and approved the manuscript

Availability of data and materials: The datasets used during the current study are available from the corresponding author on reasonable request.

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Figures

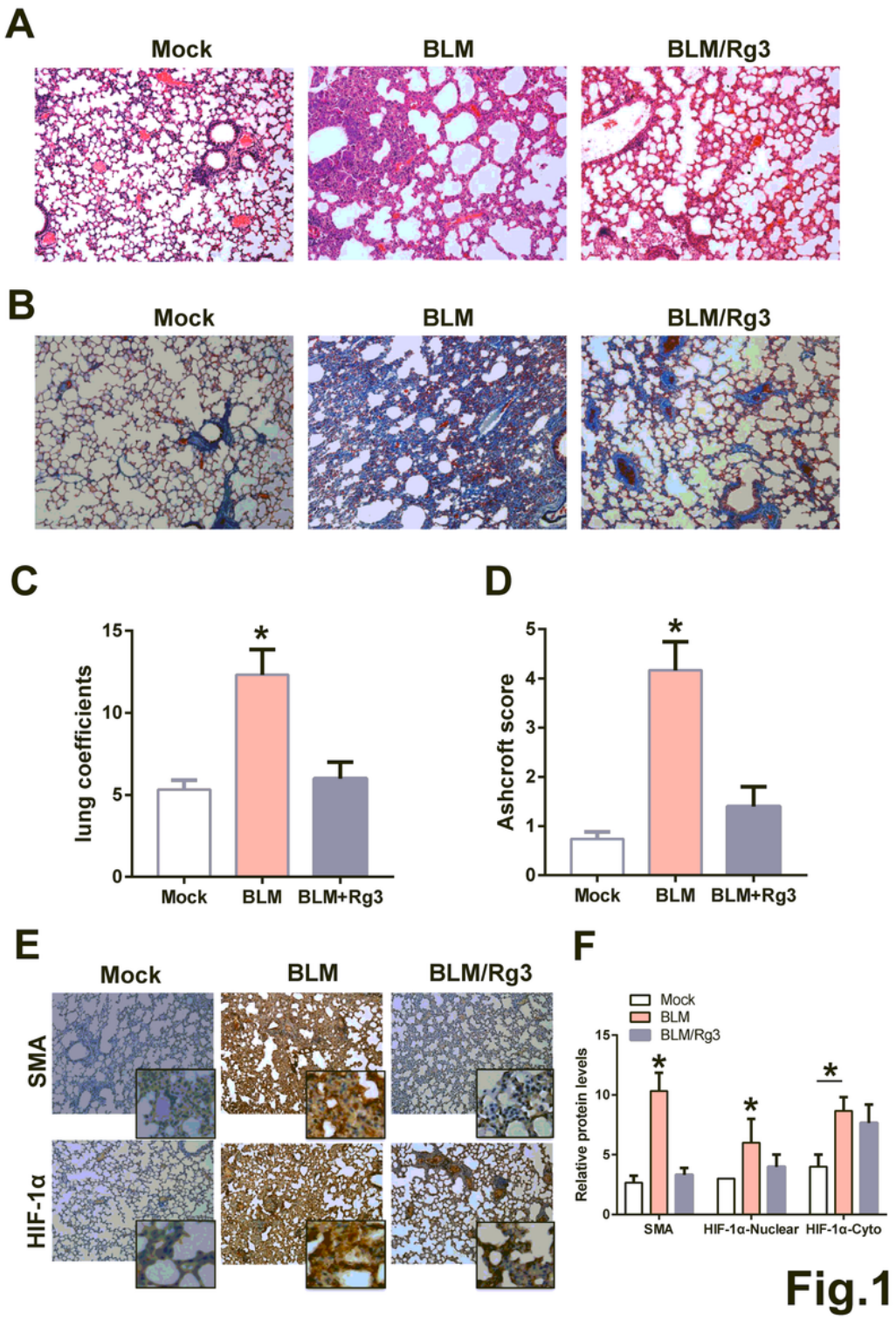

Figure 1

Rg3 inhibits bleomycin-induced pulmonary fibrosis. Potential therapeutic effects of Rg3 in mice with bleomycin-induced pulmonary fibrosis. (A) HE staining was used to detect the degree of pulmonary fibrosis. (B) Masson staining was performed to observe the content of fibroblasts. (C) lung coefficients for evaluating pulmonary fibrosis. (D) Ashcroft score for evaluating pulmonary fibrosis. (E and F) Immunohistochemical detection of SMA and HIF-1α expression.

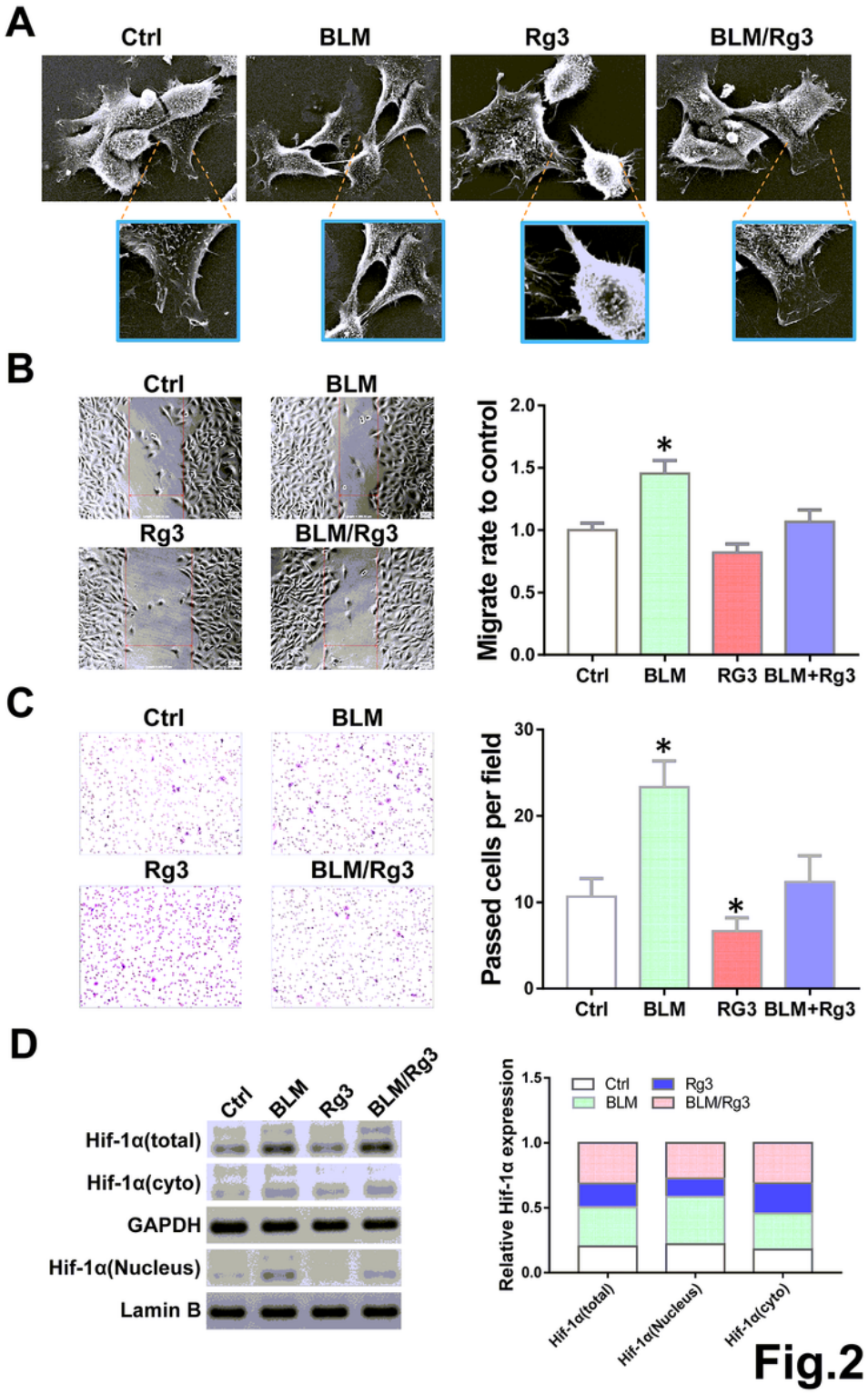

Figure 2

Rg3 inhibits fibroblast migration and invasion. Fibroblasts were treated with bleomycin alone or simultaneously with Rg3. (A) Cell phenotype was detected by SEM. (B) The effect of Rg3 on cell migration ability was detected using wound healing. (C) The effect of Rg3 on cell invasion was detected via Transwell assay. (D) The nuclear–plasma ratio of HIF-1α was detected by Western blot.

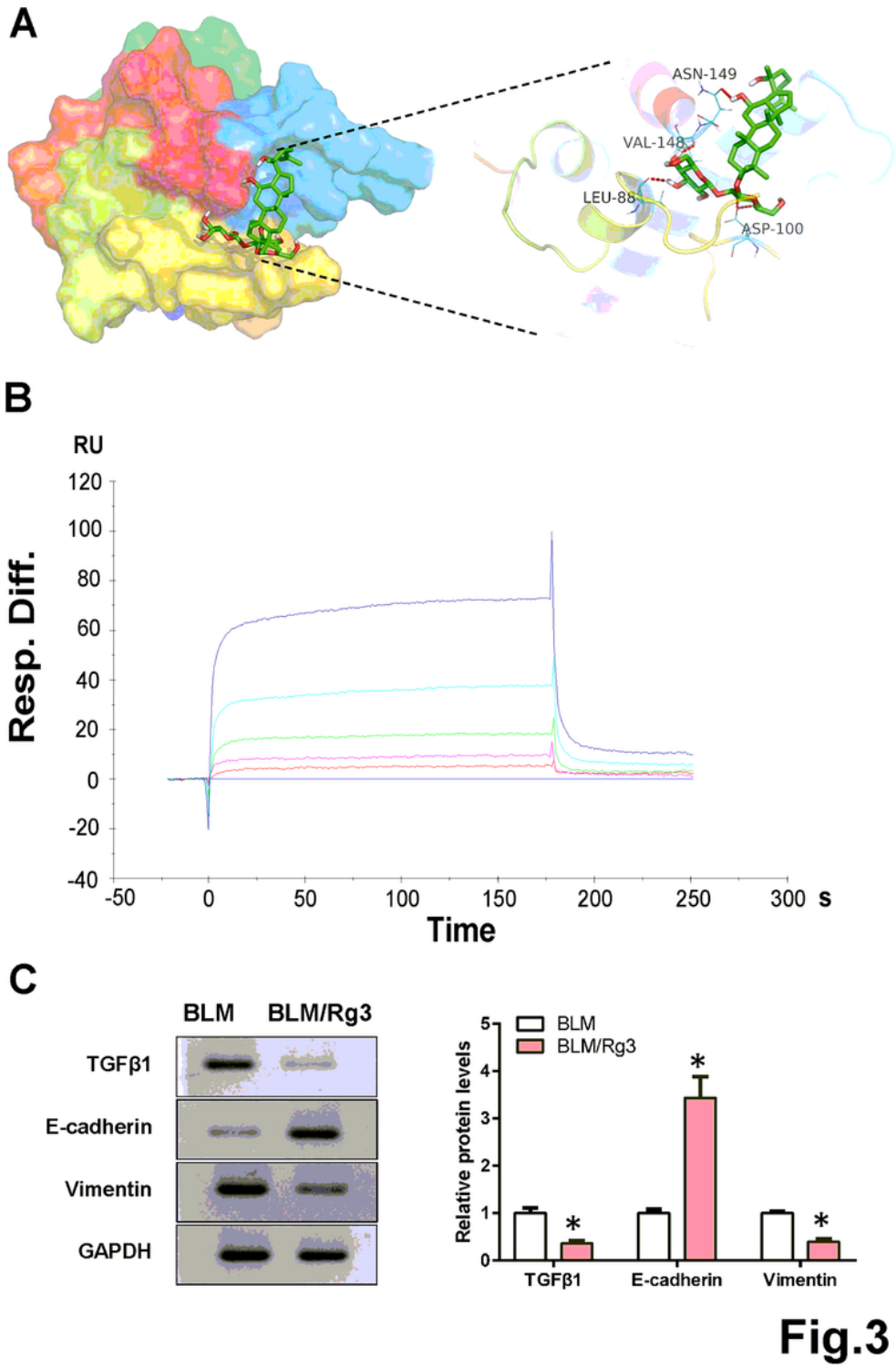

Fig.3

Figure 3

Rg3 can directly bind HIF-1α. (A) Molecular docking model of Rg3 and HIF-1α. (B) Biacore was used to analyse the binding ability of Rg3 to HIF-1α. (C) Western blot was applied to detect the expression levels of TGFB1 and EMT markers.

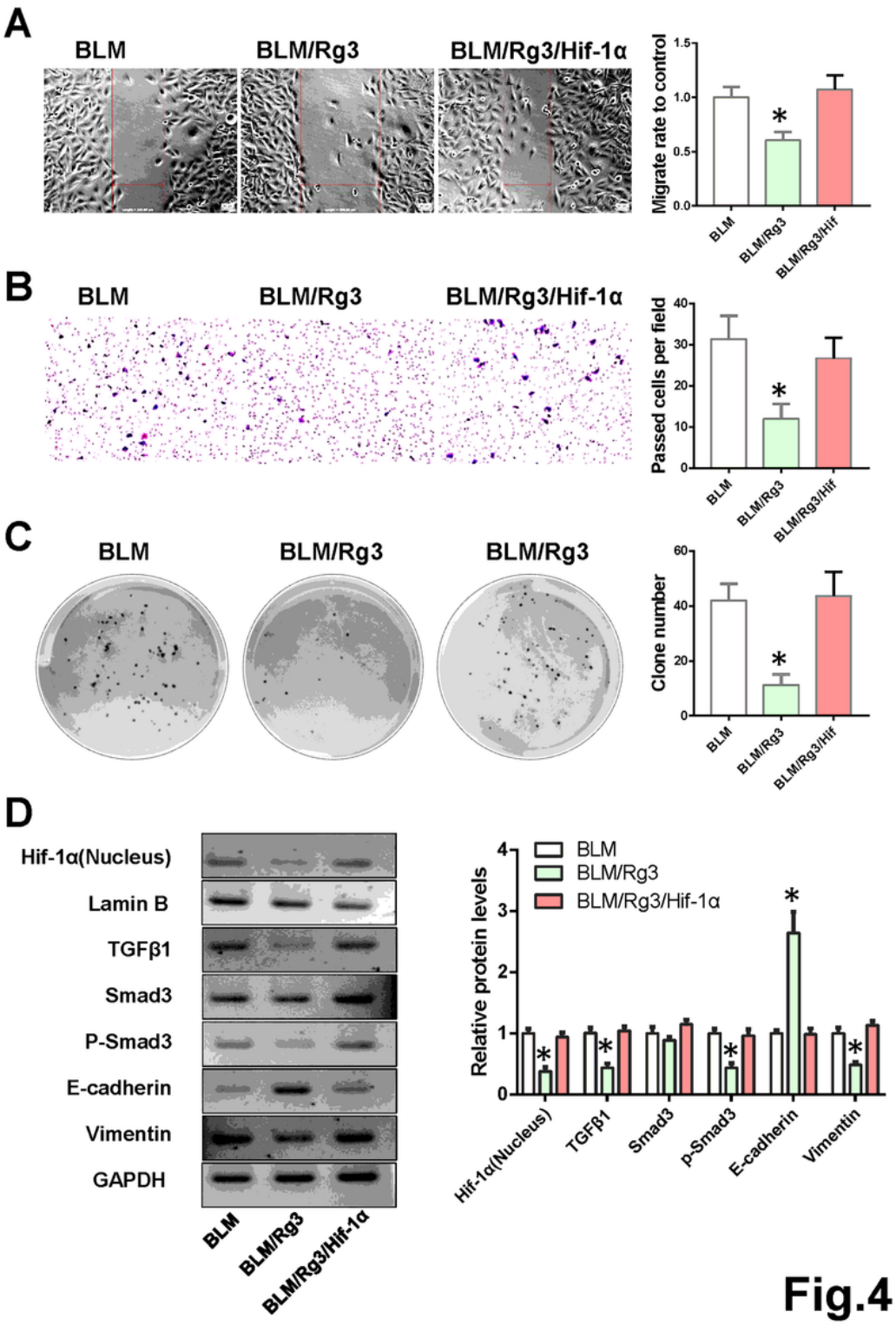

Figure 4

HIF-1α promotes the EMT process of fibroblasts. HIF-1α was overexpressed in Rg3-treated fibroblasts, and EMT-related functions were detected. (A) Cell migration ability was detected via wound healing assay. (B) Transwell assay was used to determine cell invasion. (C) Cell clones were tested through clone formation experiments. (D) Western blot was utilised to detect the expression of the TGFB1/Smad3 pathway and EMT markers.

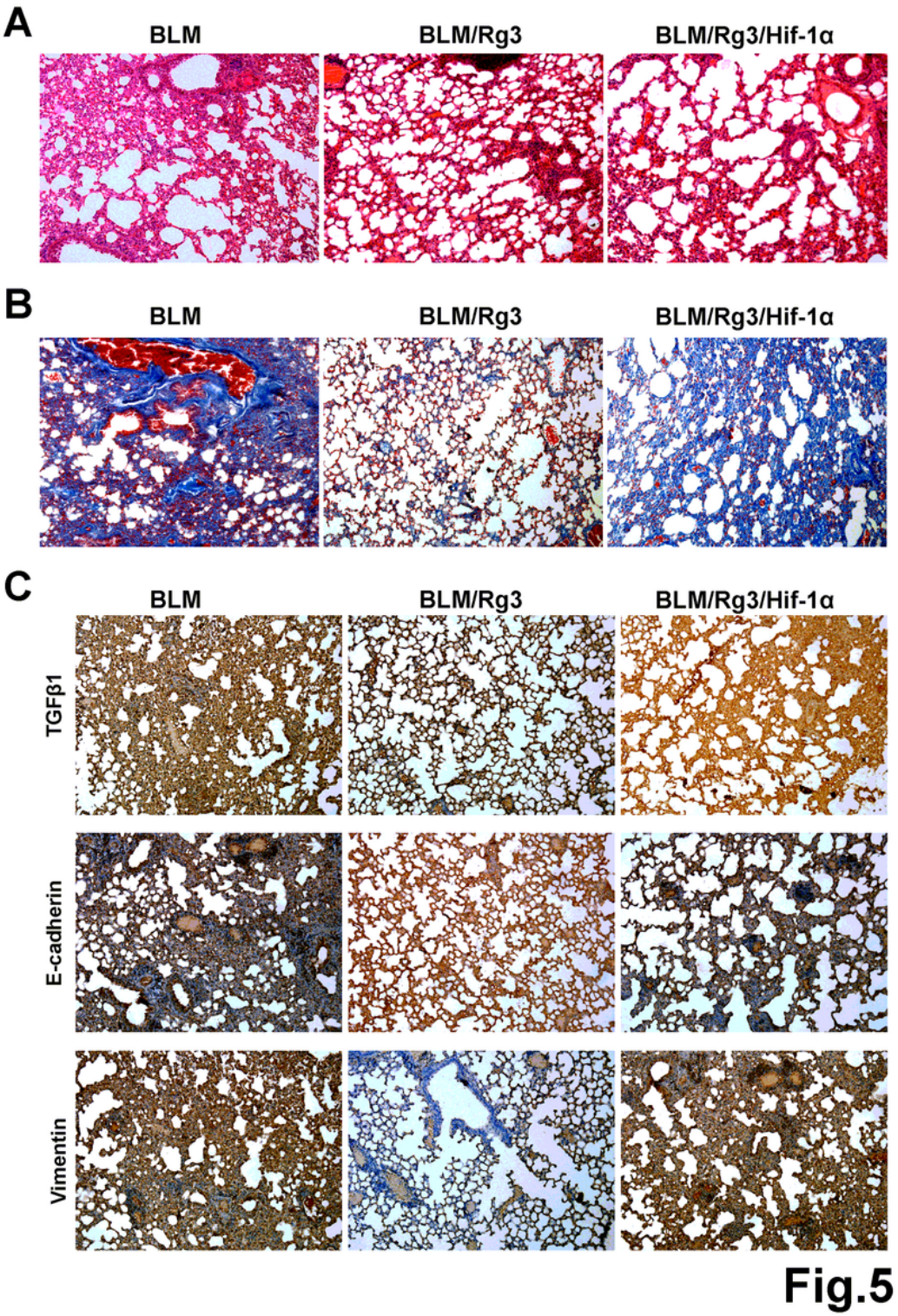

Figure 5

HIF-1α reverses the inhibitory effect of Rg3 on fibrosis. We overexpressed HIF-1α whilst treating with Rg3 in mice with bleomycin-induced pulmonary fibrosis and then evaluated the extent of pulmonary fibrosis. (A) HE staining was performed to detect the morphology of lung tissue. (B) Masson staining was conducted to detect the proportion of fibroblasts. (C) Immunohistochemistry was applied to observe the expression of TGFB1 and EMT markers.

Supplementary Files

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- ARRIVEguidelines2.0English.pdf
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