Bach1 siRNA attenuates bleomycin-induced pulmonary fibrosis by modulating oxidative stress in mice

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Abstract. Oxidative stress plays an essential role in inflammation and fibrosis. Bach1 is an important transcriptional repressor that acts by modulating oxidative stress and represents a potential target in the treatment of pulmonary fibrosis (PF). In this study, we knocked down Bach1 using adenovirus-mediated small interfering RNA (siRNA) to determine whether the use of Bach1 siRNA is an effective therapeutic strategy in mice with bleomycin (BLM)-induced PF. Mouse lung fibroblasts (MLFs) were incubated with transforming growth factor (TGF)-β1 (5 ng/ml) and subsequently infected with recombined adenovirus-like Bach1 siRNA1 and Bach1 siRNA2, while an empty adenovirus vector was used as the negative control. The selected Bach1 siRNA with higher interference efficiency was used for the animal experiments. A mouse model of BLM-induced PF was established, and Bach1 siRNA ($1x10^9$ PFU) was administered to the mice via the tail vein. The results revealed that the Bach1 mRNA and protein levels were significantly downregulated by Bach1 siRNA. Furthermore, the MLFs infected with Bach1 siRNA exhibited increased mRNA and protein expression levels of heme oxygenase-1 and glutathione peroxidase 1, but decreased levels of TGF-β1 and interleukin-6 in the cell supernatants compared with the cells exposed to TGF-β1 alone. Bach1 knockdown by siRNA also enhanced the expression of antioxidant factors, but suppressed that of fibrosis-related cytokines in mice compared with the BLM group. Finally, the inflammatory infiltration of alveolar and interstitial cells and the destruction of lung structure were significantly attenuated in the mide administered Bach1 siRNA compared with those in the BLM group. On the whole, our findings demonstrate that Bach1 siRNA exerts protective effects against BLM-induced PF in mice. Our data may provide the basis for the development of novel targeted therapeutic strategies for PF.

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Introduction

Pulmonary fibrosis (PF) is a c\hronic and progressive disease characterized by diffuse inflammation, interstitial fibrosis and the distortion of the lung architecture, which contributes to extensive damage and dysfunction of the lungs (1). However, the explicit pathogenesis of PF remains inadequately understood, and there is an unmet need for effective treatments. Novel strategies aimed at enhancing the therapeutic effects have gained significant interest. Previous studies have demonstrated that the imbalance of oxidants and antioxidants caused by oxidative stress plays an important role in the development of PF (2-4), including bleomycin (BLM)-induced PF (5,6). Thus, the key components involved in oxidative stress have been receiving increased attention and are considered as promising therapeutic targets for the treatment of PF.

The transcription factor Bach1, which is a member of the cap 'n' collar family of basic leucine zipper, is known to be involved in the regulation of oxidative stress response (7). Bach1 executes transcriptional inhibition via competitive binding to the Maf-recognition element closely related to antioxidant response element (ARE), thus antagonizing the activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) (8,9). Nrf2 functions as one of the most important molecules involved in oxidative stress that promotes the expression of Nrf2-dependent antioxidant genes and proteins (10,11). It has been reported that Nrf2 deficiency is associated with the pathogenesis of lung fibrosis in mice and humans (12,13). Nrf2 agonists protect against PF by regulating the lung oxidant levels in mice with BLM-induced lung fibrosis (14). Furthermore, ARE/Nrf2-dependent antioxidants, including glutathione peroxidase 1 (GPx1), heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase-1 (NQO1) may be critical in pulmonary protection (15-17). Additionally, the expression of Nrf2-dependent antioxidants can be suppressed by the transcriptional induction of Bach1 (8). Although the evidence suggests that Bach1 is a transcriptional repressor of Nrf2, the inhibitory effects of Bachl on Nrf2-dependent antioxidants and fibrotic processes in pulmonary tissue remain still poorly understood. To date, there are no available published studies investigating whether targeting Bachl attenuates BLM-induced lung fibrosis, at least to the best of our knowledge.

As BLM causes cell damage, and the emergence of free radicals and the subsequent induction of oxidative stress ultimately

results in inflammation and fibrosis (18,19), it is currently the most commonly used animal model to investigate PF (20). It has been demonstrated that multiple factors, including tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β1, interleukin (IL)-1 and -6, and connective tissue growth factor (CTGF) are implicated in the development and progression of PF (21,22). A previous study found markedly increased levels of TGF-β1 and IL-6 in both bronchoalveolar lavage fluid (BALF) and in the serum of mice following exposure to BLM (23). TGF-β1 has been identified as a key mediator of lung fibrosis, and it induces the proliferation and migration of lung fibroblasts, as well as the remodeling of the extracellular matrix (ECM) (24,25). In this context, TGF-β1 and IL-6 are considered to be useful parameters for observing the progression of PF.

Small interfering RNA (siRNA), which can attain target-specific gene silencing, may be a potent tool for gene therapy (26,27). In this study, we focused on Bach1, since it is an important mediator of oxidative stress and may be a critical target for PF therapy. Based on these reasons, we hypothesized that adenovirus-mediated Bach1 siRNA may be effective in silencing Bach1 transcripts *in vitro* and *in vivo*, representing a potential means with which to promote the expression of Nrf2-dependent antioxidants and ameliorate fibrotic process in BLM-induced lung fibrosis. For our experiments, Bach1 siRNAs were administered to mouse lung fibroblasts (MLFs) and to mice with BLM-induced PF in order to monitor the antioxidant and anti-fibrotic effects via the measurement of antioxidant factors, fibrosis-related cytokines and histological changes. Taken together, the findings of our study may provide novel insight into the role of Bach1 in the regulation of oxidative stress involved in the pathogenesis of PF, thus leading to the development of promising strategies for the treatment of PF.

Materials and methods

Construction of Bach1 siRNA expression vectors and recombinant adenovirus. Two siRNAs targeting mouse Bach1 mRNA were designed using a software available at www.ambion.com via a cDNA sequence (SEQ ID, XM_006522879), and an empty adenoviral vector was used as a negative control. The specific siRNA coding sequences were designed to contain the target sequences and their reverse complement 19-nucleotide sequences which are separated by 9-nucleotide sequences of stem-loop structure (Table I).

The forward and reverse oligonucleotides were chemically synthesized and annealed to form a duplex. Two siRNA sequences were cloned into the plasmid vector, pGenesil-1. The siRNA expression cassette was subsequently excised from pGenesil-1 using *Eco*RI and *Hind*III, and also ligated into the linearized adenoviral shuttle vector, pShuttle-CMV. The ligation products were transformed into *E. coli* DH5α, and positive clones were selected. The recombinant adenovirus vectors were obtained by homologous recombination with pShuttle-CMV-Bach1-siRNA and the skeleton plasmid of pAdeasy-1 in bacteria BJ5183. The recombinant cosmids titled Ad-siBach1 carrying either Bach1 siRNA or adenoviral vector with green fluorescent protein (GFP) were linearized by *Pac*I digestion and then transfected 293A cells (Wuhan Cell Marker Biotechnology, Wuhan, China) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The success of Bach1 siRNA insertion into adenoviral plasmid was confirmed by DNA sequencing and the titer of each virus stock was determined by plaque assay on 293A cells. Finally, the siRNA adenoviral vector was calculated as $1.1x10^9$ plaque-forming units (PFU)/ml and the empty adenoviral vector was $1.4x10^9$ PFU/ml. The virus was purified by double CsCl gradient ultracentrifugation used for *in vivo* experiments. The transfection efficiency was monitored by fluorescence microscopy and flow cytometry. The silencing efficiency of target gene and protein were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. The selected siRNA sequence with the highest inhibitory effect was used in the animal experiments.

Animal experiments and sample preparation. Seven-week-old female C57BL/6 mice (body weight, 18-20 g) were purchased from Vital River Laboratories (Beijing, China). The animals were maintained under specific pathogen-free conditions and kept for 1 week prior to use. The room temperature was maintained at 22±3˚C, with a 12-h/12-h day/night cycle and relative humidity of 50±10%. All animals had free access to rodent chow and water. The mice were randomly divided into 4 groups as follows: a control with saline (n=5), BLM (n=5), BLM + control siRNA (n=5) and BLM + Bach1 siRNA groups (n=5). For the model of PF, 5 mg/kg BLM (Nippon Kayaku Co., Ltd., Tokyo, Japan) in 50 μl phosphate-buffered saline (PBS) were administered intratracheally to the mice; the control animals were injected intratracheally with the same volume of sterile saline. BLM and saline were administered only once. The mice in the control siRNA and Bach1 siRNA group were injected with control siRNA or Bach1 siRNA, respectively, via the tail vein (one injection every other day), with a total dose of $1x10^9$ PFU viruses per mouse after 2 weeks of BLM administration. At the designated time points (28 days post-BLM administration), the mice were humanely sacrificed by an overdose of the anesthesia (inhalation of ether) for serum, BALF and histological measurements. We lavaged the lungs using 0.9% saline using a tracheal cannula for 3 times and collected 1.5 ml BALF in each mouse. The blood of the experimental mice was collected using a capillary through the conjunctiva and into the orbital sinus. Following centrifugation (2000 x g for 5 min at 4˚C), the serum and supernatants were stored at -80˚C until use. In addition, the lungs were removed and immediately frozen in liquid nitrogen, and then stored at -80˚C until further processing. All procedures involving animals were approved by the Ethics Committee of Capital Medical University, Beijing, China and complied with guidelines on the Use of Experimental Animals.

Lung histopathology. The left lung tissues from the mice were dissected and fixed in 4% paraformaldehyde. After 24 h, the tissues were dehydrated and embedded in paraffin. Paraffin blocks of 3-4 μm thickness were cut and stained with hematoxylin and eosin (H&E) and Masson's reagent (Solarbio, Beijing, China) for the assessment of PF histopathology. The histological severity of pulmonary alveolitis was scored as previously described (28), and PF was scored as described in the study by Ashcroft *et al* (29).

Cell culture and treatment. The MLF cell line (MIC-CELL-0040) was purchased from PriCells Biomedical

Table I. Sequence of forward and reverse strands of the Bach1 shDNA oligonucleotides.

Target	Target siRNA sequences
Bach1 siRNA#1	F: 5'-GCG TAC ACA ATA TCG AGGA TTCAAGACG TCC TCG ATA TTG TGT ACGC TTTTTT-3'
	R: 5'-AAAAAA GCG TAC ACA ATA TCG AGGA CGTCTTGAA TCC TCG ATA TTG TGT ACGC-3'
Bach1 siRNA#2	F: 5'-GAA TCT CAC CTT GTA GACC TTCAAGACG GGT CTA CAA GGT GAG ATTC TTTTTT-3'
	R: 5'-AAAAAA GAA TAT CTC CTT GTA GACC CGTCTTGAA GGT CTA CAA GGT GAG ATTC-3'

siRNA, small interfering RNA.

Technology Co., Ltd. (Hubei, China) and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin and streptomycin. The cells were plated at $1x10^5$ cells/100 mm and cultured at 37˚C in a 5% CO_2 humidified atmosphere. We used 0.25% trypsin for digestion and the cells were filled in 2 culture flasks for passage (1:2). All experiments proceeded using cells between 3 and 4 cell passages. For the induction of fibrosis using the recombinant protein, TGF-β1, the cells were seeded in 12-well plates at a density of $1x10^5$ cells/well and starved for 24 h and then incubated with TGF-β1 (5 ng/ml) in complete medium for 24 h. To knockdown Bach1 expression, the MLFs were infected with the Bach1 siRNA at a multiplicity of infection (MOI) of 50 for 2 h and were then washed to remove the virus. The cells in in the control group (control siRNA) were transfected with empty vector. The cells were then cultured for a further 48 h and then analyzed for their GFP intensity using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and directly observed under a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan).

RT-qPCR. Total RNA was isolated from the mouse pulmonary tissue samples and the MLFs using the simple Total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. RNA was reverse transcribed using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Shanghai, China) and quantitative PCR (qPCR) was performed using the SYBR PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). The initial denaturation step was at 95˚C for 10 min, and then PCR amplification was achieved by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The process of RT-qPCR was implemented by ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). The sequences of the primers used are listed in Table II.

The level of gene expression was quantified using a standard curve and the comparative CT method normalized to GADPH mRNA expression. We used the formula $2^{-\Delta\Delta CT}$ to calculate the relative expression levels of genes. Both samples were examined in triplicate, and the experiment was repeated at least 3 times.

Western blot analysis. Cell pellets or lung tissues were lysed in RIPA lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (Applygen Technologies, Inc., Beijing, China) on ice, and the concentration of the sample proteins was tested using the BCA method before being mixed in 5X SDS loading buffer.

Table II. Sequences of primers used for PCR in this study.

Target	Primer sequence
Bach1	F: 5'-GAACAG GGCTAC TCGCAA AG-3'
	R: 5'-AAAGGG CAGTTG ACGGAA C-3'
HO-1	F: 5'-GACAGAAGAGGCTAAGACCGC-3'
	R: 5'-TGACGAAGTGACGCCATCT-3'
GPx1	F: 5'-GCACATCTACCACGCAGTCA-3'
	R: 5'-AGAGTCTCAAGAACATCGCCT-3'
NQO1	F: 5'-GCTTTAGGGTCGTCTTGGC-3'
	R: 5'-TGGCGTAGTTGAATGATGTCT-3'
GAPDH	F: 5'-AAGACCCAGAAATGAAC-3'
	R: 5'-TCTACACGATAACAACCA-3'

HO-1, heme oxygenase-1; GPx1, glutathione peroxidase 1; NQO1, NAD(P)H:quinone oxidoreductase-1.

Following heat denaturation at 100˚C for 5 min, equal amounts of lysate (60 mg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Applygen Technologies, Inc.). The membranes were blocked with 5% fat-free milk for 1 h at room temperature, followed by incubation with primary antibodies against β-actin (1:500; sc-130301; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Bach1 (1:300; ab49657), HO-1 (1:500; ab13248), GPx1 (1:1,000; ab140883) and NQO1 (1:500; ab28947) (all from Abcam, Cambridge, UK) at 4˚C overnight. The following day, the membranes were incubated with fluorescent-labeled secondary antibodies (1:10,000; 600-101-096; Rockland, Inc., Gilbertsville, PE, USA) for 1 h at room temperature, and the bands were visualized using a double-infrared laser scanning imaging system (LI-COR Biosciences, Lincoln, NE, USA). Protein expression was analyzed and normalized to that of β-actin.

Measurements of cytokine levels using enzyme-linked immunosorbent assay (ELISA). BALF and blood from the mice were sampled at the end of 4 weeks after the initiation of the animal experiment. The supernatants of MLFs were also collected after 48 h of the transfection of siRNA for measurements. We then used the BALF, serum and cell supernatants to analyze the levels of cytokines in lung fibrosis. ELISA was used to detect TGF-β1 and IL-6 using respective ELISA kits (R&D Systems, Minneapolis, MN, USA). The supernatants of MLFs, serum and

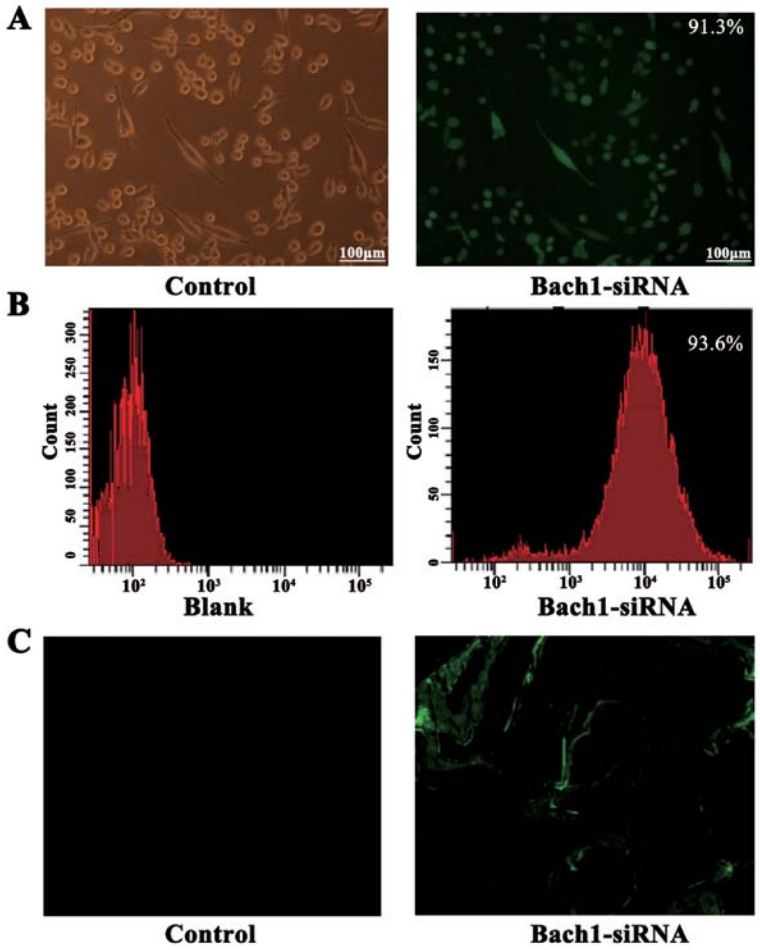

Figure 1. Transfection efficiency of Bach1 small interfering RNA (siRNA) i mouse lung fibroblast (MLFs) and its targeting of the lungs. MLFs were transfected with Bach1 siRNA for 48 h, and the transfection efficiency was determined by (A) under a fluorescence microscope, followed by (B) flow cytometric analysis. (C) The mice were administered an injection of Bach1 siRNA, and GFPs of frozen biopsies were observed under a fluorescence microscope to determine whether the siRNA targeted the lungs.

BALF of mice were diluted according to different proportions along with reagents and standard dilutions prepared before the experiment. Standard, control and samples (100 μl/well) were incubated for 2 h at room temperature. They were then washed with wash buffer 5 times and 100 μl of conjugate was then added for 2 h followed by substrate solution for 30 min away from light. After the addition of 100 μl of stop solution, the sample concentrations were determined to measure the absorbance at a 450 nm using a microplate reader (Bio Rad, Hercules, CA, USA). Both assays were performed in duplicate.

Statistical analysis. Data were analyzed using a statistical software package (SPSS 13.0; SPSS, Inc., Chicago, IL, USA), and expressed as mean ± SD. One-way analysis of variance (ANOVA) was performed for multiple group comparisons. A value of $P<0.05$ was considered to indicate a statistically significant difference and a value of $P<0.01$ was considered to indicate a marked statistically significant difference.

Results

Transfection efficiency and targeting of Bach1 siRNA. The successful construction of Bach1 siRNA adenoviral vectors was determined by gene sequencing. To demonstrate the transfection efficiency of Bach1 siRNA on MLFs and its targeting *in vivo*, we used a fluorescent microscope and flow cytometric analysis. After the Bach1 siRNA was transfected into the MLFs, >90% GFP was expressed in the fluorescence states (Fig. 1A), and the results of flow cytometry also suggested that the transfection efficiency of Bach1 siRNA was 93.6% (Fig. 1B). After 2 weeks of siRNA administration, GFPs were observed in the tissue sections of the lungs of mice, suggesting that Bach1 siRNA targeted the lungs (Fig. 1C).

Confirmation of Bach1 gene knockdown in vivo and in vitro. The MLFs were incubated with recombinant protein TGF-β1 (5 ng/ml) for the induction of fibrosis prior to transfection with control siRNA, or Bach1 siRNA#1 and #2 *in vitro*. Similarly, superior Bach1 siRNA (Bach#1) was injected into the mice with BLM-induced PF *in vivo*. The inhibitory effects of Bach1 siRNA on Bach1 mRNA and protein expression were analyzed by RT-qPCR and western blot analysis. As shown in Fig. 2A, the mRNA level of Bach1 in the TGF-β1 group was significantly increased compared with that of the blank group ($P<0.05$), suggesting that TGF-β1 promoted Bach1 generation. The mRNA expression of Bach1 in the MLFs following transfection with Bach1 siRNA#1 or #2 was significantly decreased compared with that of the TGF-β1 group or the control siRNA

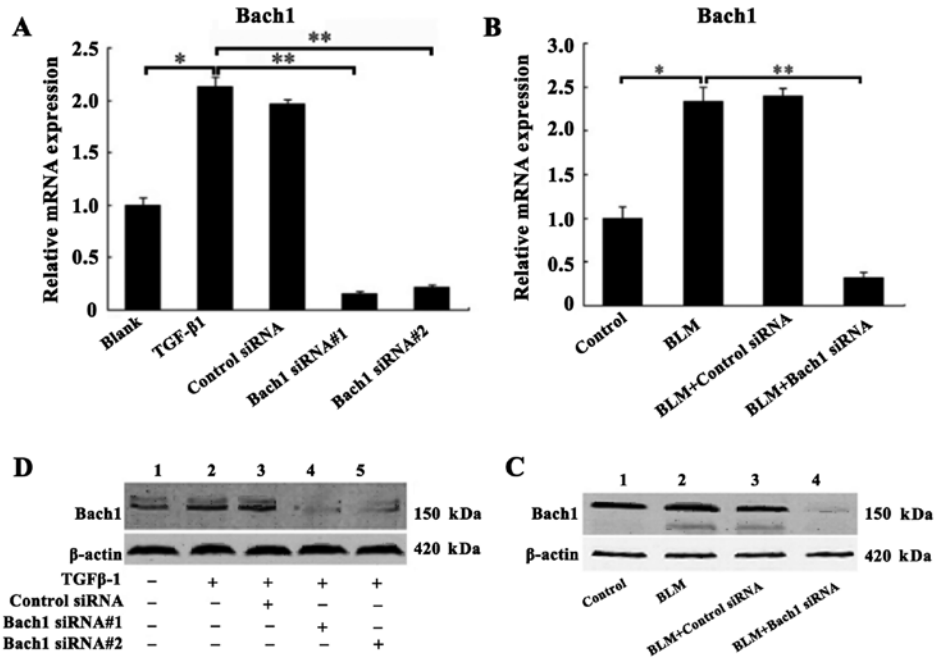

Figure 2. Inhibitory effects of Bach1 small interfering RNA (siRNA) on transforming growth factor (TGF)-β1-induced fibrosis in mouse lung fibroblasts (MLFs) and bleomycin (BLM)-induced pulmonary fibrosis (PF) in mice. (A) Bach1 expression were determined by RT-qPCR following TGF-β1 stimulation and transfection of MLFs with Bach1 siRNA. (B) Bach1 expression were determined by RT-qPCR after the injection of Bach1 siRNA into mice with BLM-induced PF. For (A and B) the Bach1 mRNA levels were normalized against GAPDH. (C) The expression level of Bach1 were detected by western blot analysis in MLFs exposed to TGF-β1 and transfected with Bach1 siRNA. (D) Bach1 expression levels were detected by western blot analysis in lung tissues of mice with BLM-induced PF that were treated with Bach1 siRNA. Data are expressed as the means ± SD. *P<0.05, **P<0.01 vs. TGF-β1 or BLM group.

group (P<0.01), and was also significantly decreased by 15.3 and 22%, respectively (P<0.01) compared with the blank group (P<0.01) (Fig. 2A). Similarly, the results of western blot analysis also revealed that the protein expression of Bach1 in the MLFs following transfection with Bach1 siRNA#1 or #2 was markedly decreased (Fig. 2C). To further characterize Bach1 expression *in vivo*, we detected Bach1 expression in the lungs of mice and found that Bach1 mRNA and protein expression in the mice was decreased significantly following the administration of BLM and Bach1 siRNA, compared with the control group and the control siRNA group (Fig. 2B and D).

Effects of Bach1 knockdown on the expression of antioxidant genes and proteins. To examine the mechanisms through which Bach1 siRNA affects the expression of antioxidant factors, such as HO-1, GPx1 and NQO1, we examined their mRNA and protein expression levels in the MLFs and lungs of mice. The results of RT-qPCR revealed that the mRNA levels of HO-1 and GPx1 were decreased following exposure of the MLFs to TGF-β1 compared with the blank group (both P<0.05; Fig. 3A and B), and the knockdown of Bach1 significantly increased the HO-1 and GPx1 mRNA levels compared with the TGF-β1 group (P<0.01; Fig. 3A and B). The effect of Bach1 siRNA#1 was more prominent than that of Bach1 siRNA#2 in the MLFs. Furthermore, as shown in Fig. 3D and E, we observed a significant increase in the mRNA levels of HO-1 and GPx1 in the lung tissues of mice following treatment with Bach1 siRNA compared with the BLM group (P<0.05 and P<0.01). Concomitantly, the knockdown of Bach1 significantly increased the protein levels of HO-1 and GPx1 in the MLFs in the blank group or TGF-β1 group, and in the lung tissues from mice in the control group or BLM group (Fig. 3G and H). However, Bach1 siRNA did not alter the mRNA and protein levels of NQO1 neither in the MLFs nor in the lung tissues (Fig. 3C and F). The results of protein expression were similar to those of mRNA expression. All these results suggest that Bach1 siRNA promotes the generation of oxidation resistance factors, and it may thus inhibit the oxidative stress induced by TGF-β1 and BLM.

Effects of Bach1 siRNA on the expression of fibrosis-related cytokines. Due to the important role of TGF-β1 and IL-6 in the pathogenesis of PF, we analyzed their concentrations in the supernatant of MLFs, and in serum and BALF from mice. The levels of TGF-β1 and IL-6 in serum and BALF from mice with BLM-induced lung fibrosis were significantly increased compared with the control group (both P<0.01; Fig. 4A-D). Their concentrations in the supernatant of the MLFs following stimulation with TGF-β1 were significantly increased compared with the blank group (both P<0.01; Fig. 4E and F). Additionally, the levels of IL-6 and TGF-β1 in serum and BALF from the mice in the Bach1 siRNA group were significantly decreased compared with those of the mice BLM treated with control siRNA or in the control group (both P<0.01; Fig. 4A-D). The results of the analysis of the expression of IL-6 and TGF-β1 in the supernatant of MLFs were similar to those obtained from the serum and BALF of mice. These results suggest that the use of Bach1 siRNA suppresses the expression of TGF-β1 and that of related cytokines in BLM-induced fibrosis.

Effects of Bach1 siRNA on histopathological changes in mice with BLM-induced fibrosis. For histological analysis, the lung tissues of mice were stained with H&E and Masson's staining.

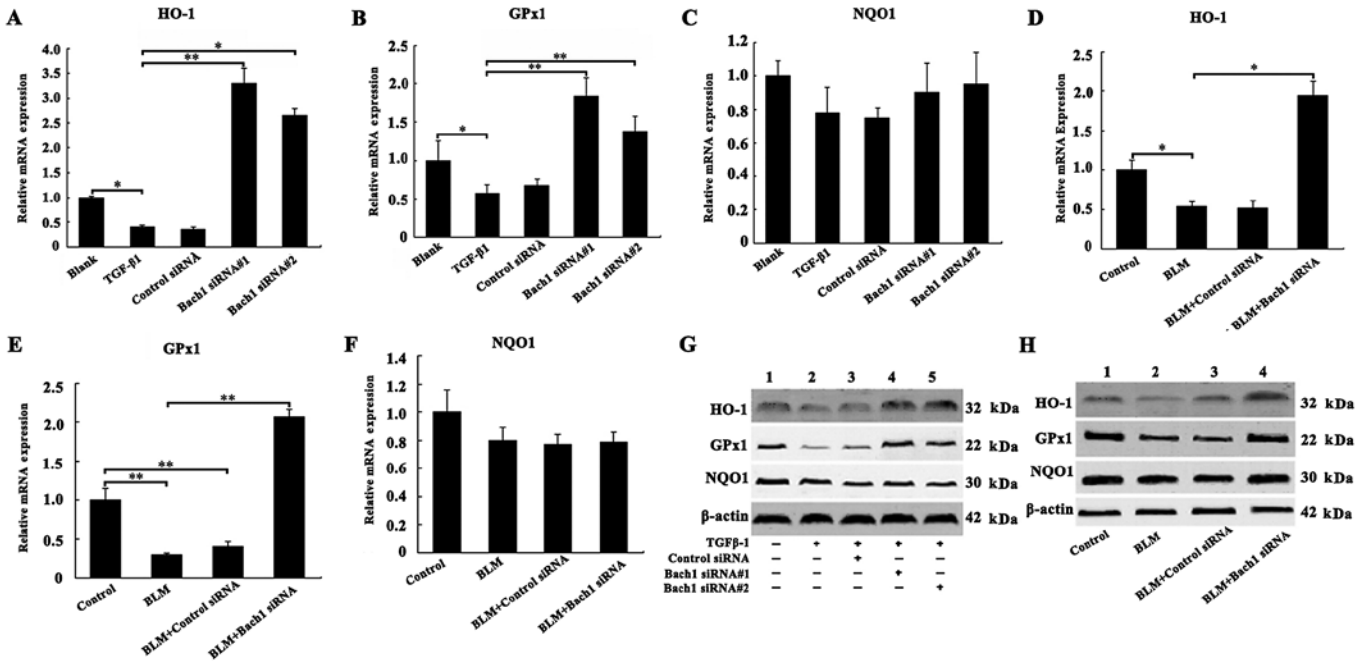

Figure 3. Expression of antioxidant factors in mouse lung fibroblasts (MLFs) following stimulation with transforming growth factor (TGF)-β1 and in mie with bleomycin (BLM)-induced pulmonary fibrosis (PF). MLFs were exposed to TGF-β1 (5 ng/ml) for 24 h prior to transfection with control small interfering RNA (siRNA), Bach1 siRNA#1 siRNA or Bach1 siRNA#2 for 48 h. The mRNA expression levels of heme oxygenase-1 (HO-1), glutathione peroxidase (GPx1) and NAD(P)H:quinone oxidoreductase-1 (NQO1) in the MLFs in the different groups were detected by RT-qPCR. (A-C) Each bar corresponds to the means ± SD of 3 independent experiments. In addition, (D-F) mice with BLM-induced PF were treated with control siRNA or the superior Bach1siRNA, and the mRNA expression levels of HO-1, GPx1 and NQO1 in the lungs of mice were detected by RT-qPCR. (G and H) The protein levels of HO-1, GPx1 and NQO1 were detected by western blot analysis in MLFs and mouse lung tisues. Data are expressed as the the means ± SD. *P<0.05, **P<0.01 vs. TGF-β1 or BLM group.

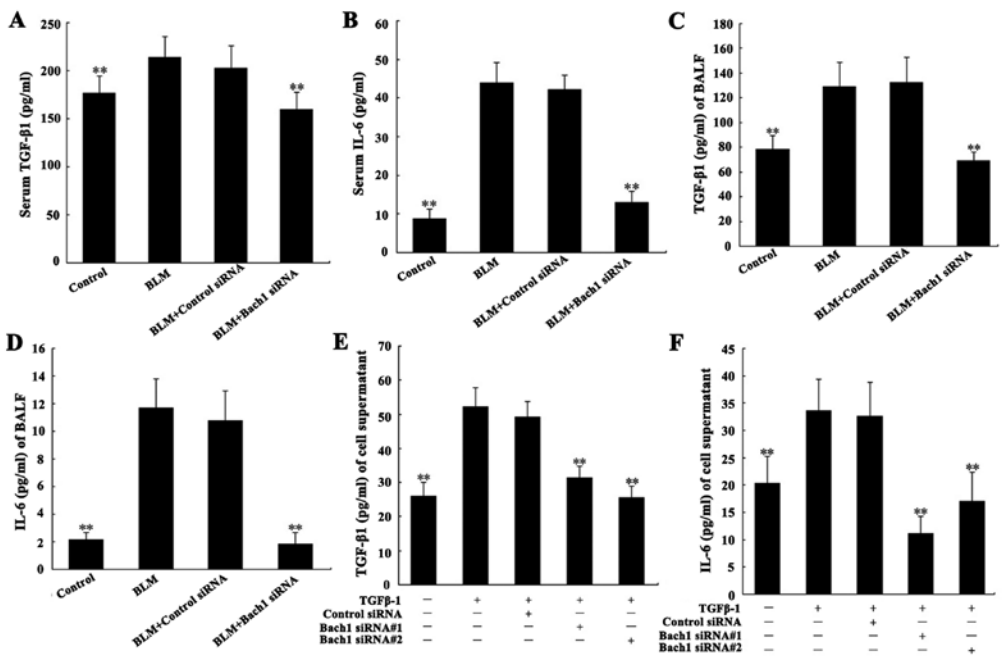

Figure 4. Analysis of transforming growth factor (TGF)-β1 and interleukin (IL)-6 levels in serum, bronchoalveolar lavage fluid (BALF) and supernatant of cells. Cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA). (A and B) Serum levels of TGF-β1 and IL-6 following the administration of bleomycin (BLM) with or without Bach1 small interfering RNA (siRNA) treatment. (C and D) TGF-β1 and IL-6 levels in BALF following the administration of BLM treatment with or without Bach1 siRNA treatment (**P<0.01 vs. BLM group). (E and F) Cytokine levels in supernatants of mouse lung fibroblasts (MLFs) following transfection with Bach1 siRNA compared with the TGF-β1 group (**P<0.01). Data are expressed as the means ± SD.

The degree of lung fibrosis was accessed by alveolitis, fibering and the integrity of the structure. Intact alveoli, a normal interstitium and a few inflammatory cells in the lung tissues were observed in the control group (Fig. 5A). Nevertheless,

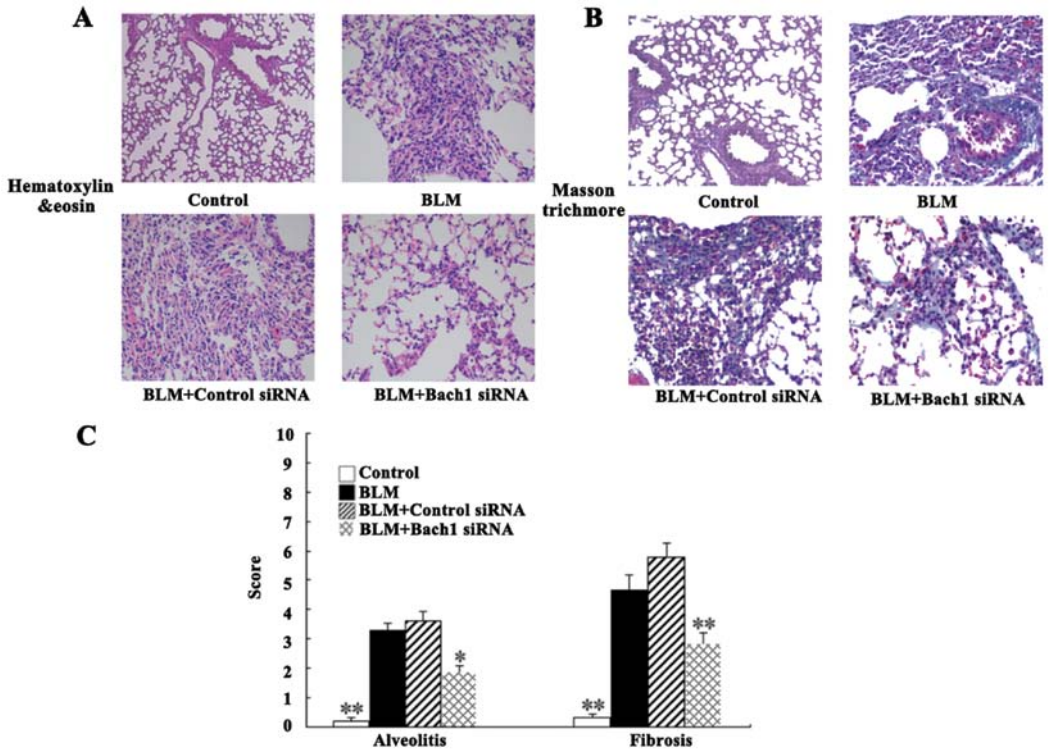

Figure 5. Histopathological changes in the lung tissues in mice in the different groups at the experiment endpoint [hematoxylin and eosin (H&E) staining, x200 magnification]. (A) Lung fibrosis in mice was evaluated by H&E staining (x200 magnification). (B) Degree of fibrosis in lung tissues evaluated by Masson's staining (x200 magnification). (C) Lung fibrosis was evaluated by inflammation and fibrosis scores. The data are expressed as the means ± SD. *P<0.05, **P<0.01 vs. bleomycin (BLM) group.

H&E staining of the BLM-adminstered mice (at 28 days) revealed the extensive destruction of alveoli (collapsed and disappeared), the extensive thickening of the lung interstitium, peribronchial and interstitial infiltrations of inflammatory cells, predominant lymphocytes, and multiple focal fibrotic lesions. There were milder inflammatory infiltrations and the destruction of alveoli following treatment with Bach1 siRNA compared with the BLM group and control siRNA group (Fig. 5A). As shown by Masson's trichrome staining, we observed massive fibrosis (blue dye), the accumulation of inflammatory cells and the extensive destruction of alveoli in the BLM group (Fig. 5B). The same change was detected in the control siRNA group. However, treatment with Bach1 siRNA significantly attenuated these fibrosis-related changes in the mice with BLM-induced fibrosis (Fig. 5B).

The alveolitis and fibrosis scores indicated that the mice in the Bach1 siRNA group had significantly lower alveolitis and fibrosis scores than those in the BLM group and control siRNA group (P<0.05 and P<0.01; Fig. 5C). Therefore, treatment with Bach1 siRNA potentially inhibits the histopathological progress of BLM-induced lung fibrosis in mice.

Discussion

Oxidative stress is considered a prominent mechanism associated with the pathogenesis of PF (30,31). The decline in antioxidant capacity and the elevated oxidant burden contribute to the progression of lung fibrosis via the activation of inflammation and growth regulatory cytokines, the regulation of related enzymes essential to the induction of antioxidants, and the stimulation of the production of myofibroblasts and ECM (32). Bach1 is involved in the induction of oxidative stress by competing with Nrf2 and negatively regulating ARE-mediated gene expression (33,34). Previous studies have demonstrated the importance of Nrf2 and its antioxidant pathway in PF (12-14), suggesting that Bach1 may play a potential role in the pathogenesis of the disease. In the present study, we demonstrated that the expression of Bach1 was markedly increased in both MLF following TGF-β1 stimulation and in the lung tissues of mice with BLM-induced fibrosis. It has been established that fibroblasts are associated with PF due to the conversion from fibroblasts into myofibroblasts, which is one of the principal characteristics of the pathogenesis of PF (35). TGF-β1 stimulation results in the proliferation and differentiation of fibroblasts, and in the increase of matrix synthesis in the lungs related to the subsequent development of PF (36). The use of BLM results in the elevation of TGF-β1 in fibroblasts, advanced inflammation and epithelial-mesenchymal transition (EMT), subsequent progressive fibrosis, and the exacerbated destruction of pulmonary structure in animal models (37). Furthermore, the model of BLM-induced lung fibrosis is rather an acute lung injury with major oxidative and inflammatory responses (18). The results of the present study supported our hypothesis that the overexpression of Bach1 may be associated with the pathogenesis of PF by affecting the antioxidant/oxidant balance.

To suppress a target gene, siRNA is considered a powerful tool due to the same effects as a knockout gene (38). Our data

demonstrated that two adenovirus-mediated Bach1 siRNAs were successfully transfected into MLFs and specifically targeted the lungs of mice by custom designing. More importantly, adenovirus-mediated siRNAs effectively suppressed Bach1 expression in MLFs following TGF-β1 stimulation. For *in vivo* experiments, the superior Bach1 siRNA was systemically administered to mice with lung fibrosis to silence Bach1, and the evidence suggested that the mRNA and protein expression levels of Bach1 were markedly inhibited in the lung tissues of mcie with BLM-induced fibrosis.

A key mechanism in the cellular defense against oxidative stress is mediated by the transcriptional induction of ARE-driven genes that include stress-response genes (e.g., HO-1), direct antioxidants (e.g., GPx) and phase 2 detoxifying enzymes (e.g., NQO1) (13). Nrf2 forms a heterodimer with basic-region leucine zipper (bZIP) transcription factors for ARE binding and ARE-driven gene transcription that may be critical in pulmonary protection (13). Bach1 binds to ARE-like sequences, functioning as a competitive antagonist of Nrf2, thus inhibiting the transcriptional activation of ARE-driven genes (8). The inhibition of Nrf2 and ARE-driven antioxidants enhances the oxidative burden and has been implicated in the pathogenesis of PF (39). The altered expression of ARE-driven antioxidants in PF also suggests that oxidative stress may contribute to pathogenesis of PF. Among these ARE-driven antioxidants, GPx has been regarded as the direct antioxidant whose functional roles in oxidative tissue stress have been widely defined (40,41). The phase 2 detoxifying enzyme, NQO1, contributes to facilitate the excretion of oxidized, reactive secondary metabolites via xenobiotic detoxification (42). In addition, the stress-response protein, HO-1, protects cells from various oxidant insults (39). A previous study demonstrated that the deficiency of ARE antioxidative signaling in mice exacerbates BLM-induced lung injury and fibrosis (15). It has been found that GPx activity and GPx1 expression are decreased in C57BL/6 mice with BLM-induced fibrosis (17). The downregulation of NQO1 and HO-1 have been observed in the lung tissues of BLM-treated animals (43). Furthermore, Nrf2 siRNA has been shown to decrease NQO1 and HO-1 mRNA expression in fibroblasts, and induces the conversion from fibroblasts to myofibroblasts (44). As previously described, our results demonstrated that the downregulation of antioxidants, such as HO-1 and GPx1 was observed in MLFs following TGF-β1 stimulation and in the lungs of C57BL/6 mice following exposure to BLM. We also investigated whether Bach1 siRNA regulates ARE-driven antioxidants involved in lung fibrosis. Whether Bach1 affects the expression of other ARE-dependent genes remains controversial. Previous studies have reported that Bach1 represses the expression of ARE-dependent genes, such as HO-1 and NQO1 (33,45). However, in studies in which the effects of Bach1 siRNA on ARE-dependent gene expression were examined by microarray analysis or qPCR, little or no effect on the expression of genes other than HO-1 was observed (46,47). In this study, our data demonstrated that the suppression of HO-1 and GPx1 in TGF-β1 and BLM-induced lung fibrosis was alleviated by Bach1 siRNA; however, no effect was observed on the expression of NQO1, thereby indicating that Bach1 siRNA participates in the mechanism of protection in TGF-β1 and BLM-mediated oxidative stress in PF. The complete mechanisms involved are not known, although it has been postulated that Bach1 is involved in regulating the expression of antioxidant genes. The present study further supports the notion that Bach1 siRNA alleviates fibrotic processes in pulmonary tissues.

There are several growth factors and fibrogenic cytokines contributing to lung fibrosis, including TGF-β1 and IL-6 (21). These molecules result in the proliferation and differentiation of lung fibroblasts that are responsible for remodeling of the ECM (23). Choe *et al* (23) demonstrated that TGF-β1 and IL-6 were upregulated in BALF and serum following the exposure of mice to BLM. In this study, we provide compelling evidence that BLM induced a marked increase in the levels of TGF-β1 and IL-6 in both BALF and serum of mice. By contrast, Bach1 siRNA inhibited the expression of fibrosis-related cytokines, such as TGF-β1 and IL-6 in the serum and BALF of mice with BLM-induced fibrosis. Moreover, the elevation of TGF-β1 and IL-6 expression was also found in MLFs following TGF-β1 stimulation, and Bach1 siRNA significantly decreased the expression of these two cytokines. To further explore whether Bach1 siRNA exerted anti-fibrotic effects on lung fibrosis, we determined the pathological changes in lung tissues. The results revealed that the administration of BLM led to the thickness of alveolar septa, narrowing of the alveolar space, the extensive infiltration of inflammatory cells, and the accumulation of collagen deposition in the lungs, which is consistent with results of previous studies (48,49). In addition, Bach1 siRNA markedly attenuated inflammatory cell infiltration, the destruction of alveoli and the accumulation of collagen deposition. On the whole, our findings demonstrated that the silencing of Bach1 attenuated the histopathological changes in the lung tissues from mice with BLM-induced fibrosis and suppressed the expression of fibrosis-related cytokines. On the basis of these findings, we believe that Bach1 represents a crucial molecule in the pathogenesis of PF, thereby highlighting Bach1 as a potential therapeutic target in PF.

In conclusion, the present study demonstrated that the silencing Bach1 inhibited TGF-β1 and BLM-induced PF. This anti-fibrotic effect was associated with the expression of antioxidants and the regulation of antioxidant capabilities. Bach1 siRNA may play an important role in the pathogenesis of PF and may have potential for use in the treatment of lung fibrosis. Further studies on the role of Bach1 may provide further insight into the mechanisms responsible for the development of PF.

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