Local Delivery of Bcar3 siRNA by Nano-Self-Assembly for Treating Pulmonary Fibrosis via Blocking the Interaction of Macrophages and Fibroblasts

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Abstract

Background

Idiopathic pulmonary fibrosis (IPF) is a life-threatening progressive interstitial lung disease with limited therapeutic options, characterized by macrophage infiltration and fibroblast activation in response to chronic lung injury. Specifically, the interaction of macrophages and fibroblasts is critical for facilitating IPF progression. However, no valuable therapeutic strategy has been proposed to inhibit this crosstalk.

Results

In this study, we investigated the role of breast cancer anti-estrogen resistance 3 (Bcar3), an adaptor molecule in the mitogenic signaling pathway, in IPF pathogenesis. We found that Bcar3 expression was upregulated in the lung tissues of both IPF patients and fibrotic mice. Furthermore, Bcar3 facilitated macrophage activation and fibroblast differentiation induced by IL-4 and TGF-β1, respectively. Mechanistically, Bcar3 up-regulation depended on Stat6 in macrophages and TGFβR1/Smad3 in fibroblasts. Furthermore, Bcar3 enhanced IL-4/Stat6 and TGF-β/Smad3 signaling in macrophages and fibroblasts, respectively, establishing a reciprocal activation loop between these two cell types, thus accelerating lung fibrogenesis. Additionally, intratracheal administration of Bcar3 siRNA-loaded liposomes afforded a precise delivery of gene therapeutics to macrophages and fibroblasts in the lung, protecting mice against FITC or bleomycin-induced lung fibrosis, as well as in human precision-cut lung slices stimulated with TGF-β1.

Conclusions

Overall, our study not only highlights the critical role of Bcar3 in the interaction between macrophages and fibroblasts during the progression of pulmonary fibrosis but also demonstrates that strategies aimed at targeting Bcar3 could be considered a promising therapeutic strategy for managing IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and inevitably fatal interstitial lung disease that mainly manifests as exertional dyspnea and cough [1, 2]. Despite extensive research efforts and an increasing incidence of this disorder, effective therapies to improve survival remain lacking, resulting in a 5-year mortality rate of 70%-80% after diagnosis [3]. Therefore, developing an effective and safe therapeutic strategy for IPF is urgently needed. In general, IPF is initiated by repetitive alveolar epithelial cell injury, followed by the infiltration of macrophages and fibroblasts into fibrotic lesions [4, 5]. Previous studies have demonstrated that macrophages, particularly the alternatively activated (M2) macrophages, play a pivotal role in producing fibrotic factors such as TGF-β, IL-17, and AREG, which promote fibroblast transition into myofibroblasts [6, 7]. These myofibroblasts are the main effector cells responsible for
depositing extracellular matrices (ECMs) in the lung interstitium during the progression of IPF [8]. Although interrupting the interaction between fibroblasts and macrophages is considered a theoretically feasible approach for IPF treatment, the lack of ideal targets or applicable drug delivery vehicles has been a limiting factor for its clinical implementation.

Breast cancer antiestrogen resistance 3 (Bcar3), initially identified as a contributor to estrogen resistance in breast cancer cells, belongs to the novel Src homology 2-containing protein family [9]. Functionally, Bcar3 acts as an adapter protein downstream of several growth factor receptors to control tumor cell adhesion, migration and invasion by binding to protein tyrosine phosphatase α and Bcar1 via its N-terminal Src homology 2 domain and C-terminal guanine nucleotide exchange factor (GEF)-like domain [10, 11]. Previous studies illustrated that the ectopic overexpression of Bcar3 remarkably activates Src kinases and FAK kinases [9, 12], both of which are involved in M2 polarization [13] and fibroblast transition [14]. Our deep RNA-sequencing data revealed that both IL-4-induced macrophages and TGF-β1-induced fibroblasts exhibited overexpression of Bcar3, indicating the potential of Bcar3 as a sally port to interrupt the vicious cycle between macrophages and fibroblasts. Nevertheless, the precise role of Bcar3 in the progression of pulmonary fibrosis remains unclear.

In this study, we demonstrated aberrant levels of Bcar3 in the lungs of both IPF patients and mice with pulmonary fibrosis. Furthermore, the level of Bcar3 showed a positive correlation with the expression of various fibrogenic genes. Immunostaining data indicated that Bcar3 was primarily localized in macrophages and fibroblasts. Additionally, Bcar3 knockdown in macrophages and fibroblasts significantly attenuated M2 macrophage polarization and fibroblast differentiation into myofibroblasts. We further elucidated that Bcar3 interfered with the macrophage-fibroblast interaction through the TGF-β signaling pathway. Finally, we proposed a promising administration strategy utilizing cationic liposomes for precise gene therapeutic delivery to macrophages and fibroblasts in the lung nidus. Efficient down-regulation of Bcar3 in these cells effectively interrupted the vicious circle, providing protection against FITC or bleomycin-induced lung fibrosis in mice, as well as in human precision-cut lung slices stimulated with TGF-β1. Collectively, our study suggest that Bcar3 is involved in the interaction between macrophages and fibroblast during the progression of pulmonary fibrosis. Therefore, therapeutic strategies targeting Bcar3 hold promise as viable therapies against pulmonary fibrosis.

**Results**

**Bcar3 expression is upregulated in pulmonary fibrosis and is localized in macrophages and fibroblasts.**

To elucidate the molecular basis of macrophage-fibroblast interactions, we conducted transcriptome analysis using RNA sequencing. We identified 27 abnormally expressed genes in IL-4-treated macrophages and TGF-β1-treated fibroblasts, with 6 genes showing upregulation (Fig. 1A-B) and 21 genes showing downregulation (Figure S1A-B). Among the overexpressed genes, Bcar3 was validated through RT-PCR in IL-4-treated macrophages (Fig. 1C) and TGF-β1-treated fibroblasts (Fig. 1D). To further corroborate these findings, we examined Bcar3 expression in lung tissues from IPF patients (n = 5) and
control subjects (n = 5). Western blot analysis demonstrated a significant upregulation of Bcar3 in IPF patients compared to controls (Fig. 1E). This observation was further validated through coimmunostaining of Bcar3 with CD68 (a macrophage marker, Fig. 1F) and PDGFR-β (a fibroblast marker, Fig. 1G) in lung sections from IPF patients and controls.

Subsequently, we collected lung tissues from mice at different time points (0 d, 7 d, 14 d, 21 d) after intratracheal instillation of BLM to analyze Bcar3 expression. Consistent with the data from IPF patients, Bcar3 exhibited a time-dependent overexpression following BLM administration, accompanied by increased expression of Fibronectin, Collagen 1, and α-SMA, which are recognized markers of pulmonary fibrosis (Fig. 1H). Additionally, Bcar3 mRNA levels positively correlated with Fn1, Col1a1, and Acta2 mRNA expression in fibrotic lungs (Fig. 1I-K). Furthermore, elevated Bcar3 expression was detected in macrophages (Fig. 1L) and fibroblasts (Fig. 1M) in mice with pulmonary fibrosis, as evidenced by the increased numbers of F4/80+Bcar3+ cells and Pdgfr-β+Bcar3+ cells in the lung sections of BLM-treated mice compared to control mice. Collectively, our data provide compelling evidence that pulmonary fibrosis is characterized by induced Bcar3 overexpression in both macrophages and fibroblasts, suggesting its potential involvement in the pathogenesis of the disease.

**Bcar3 facilitates M2 macrophage polarization via Stat6 signaling**

Macrophages, especially M2 macrophages, play a critical role in the pathogenesis of PF [15]. We sought to explore the expression pattern of Bcar3 in response to IL-4 stimulation in macrophages. Notably, Bcar3 expression exhibited dose-dependent (Fig. 2A) and time-dependent (Fig. 2B) upregulation in IL-4-treated macrophages, coinciding with the overexpression of M2 macrophage markers CD206, Ym1, and Arg1. Since phosphorylated Stat6 (p-Stat6) is crucial for maintaining M2 polarization [7], we investigated whether Bcar3 was downstream of p-Stat6. As expected, treatment with AS1517499 (a small molecule inhibitor of Stat6) abolished M2 macrophage polarization, as evidenced by reduced Ym1 and Arg1 expression (Fig. 2C). Notably, the IL-4-induced upregulation of Bcar3 was completely reversed following AS1517499 administration, suggesting that Bcar3 expression is regulated by the IL-4/p-Stat6 signaling pathway during M2 polarization (Fig. 2C).

To assess the functional relevance of Bcar3 overexpression in macrophages, we designed three Bcar3 siRNA sequences and examined their knockdown efficiency in BMDMs using Western blotting (Figure S2A). The knockdown of Bcar3 expression significantly attenuated M2 polarization, as indicated by the reduced expression of Ym1 and Arg1 upon IL-4 stimulation (Fig. 2D). Moreover, Bcar3 siRNA transfection notably reduced M2 macrophage-derived TGF-β1 production (Fig. 2D), a critical factor implicated in fibroblast activation during PF progression. RT-PCR analysis further validated these findings, confirming decreased expression of Arg1, Chil3, Mrc1, and Retnla upon Bcar3 knockdown (Fig. 2E).

Given our previous findings on the importance of p-Stat6 in IL-4-induced M2 polarization [16], we examined the effect of Bcar3 on p-Stat6 levels following IL-4 stimulation. Treatment with IL-4 led to
robust p-Stat6 upregulation, which was significantly prevented by Bcar3 siRNA, suggesting that Bcar3 plays a role in modulating p-Stat6 expression (Fig. 2F). To further verify this, we constructed pCMV-Bcar3 and confirmed Bcar3 overexpression in BMDMs (Figure S2B). Consistently, Bcar3 overexpression significantly enhanced p-Stat6 levels (Fig. 2G) and Arg1 expression (Fig. 2H). Furthermore, inhibiting p-Stat6 with AS1517499 effectively abrogated the upregulation of p-Stat6 and Arg1 induced by Bcar3, supporting the notion that Bcar3-mediated M2 macrophage polarization is dependent on p-Stat6 (Fig. 2G-H).

A positive feedback regulatory loop between TGFβR1-Smad3 signaling and Bcar3 is detected during fibroblast to myofibroblast transition

To investigate the functional role of Bcar3 in fibroblast, primary human lung derived fibroblasts were cultured and stimulated with TGF-β1. Notably, the expression of Bcar3 induced by TGF-β1 was found to be dose- and time-dependent, along with the upregulated expression of fibrotic markers (Fig. 3A-B). These findings suggest that the TGF-β signaling pathway may contribute to the overexpression of Bcar3 during fibroblast differentiation. Therefore, we examined the effect of the canonical TGF-β pathway on Bcar3 expression. Inhibiting TGFβR1 with SB-431542 (Fig. 3C) and p-Smad3 with SIS3-HCl (Fig. 3D) significantly abrogated the upregulation of Bcar3 after TGF-β1 stimulation, indicating that TGFβR1 and Smad3 are essential elements for inducing Bcar3 during myofibroblast formation. Subsequently, we showed that knockdown of Bcar3 dramatically mitigated myofibroblast formation, as indicated by the attenuated expression of fibrotic markers after TGF-β1 stimulation (Fig. 3E). Conversely, upregulating Bcar3 facilitated fibroblast-to-myofibroblast differentiation (Fig. 3F). We then examined the effect of Bcar3 on the canonical TGF-β pathway. Treatment with TGF-β1 strongly enhanced Smad2 and Smad3 phosphorylation, whereas inhibiting Bcar3 expression markedly prevented the upregulation of p-Smad3 but not p-Smad2 (Fig. 3G). Consistently, overexpression of Bcar3 significantly enhanced the levels of p-Smad3 (Fig. 3H). Additionally, inhibition the activation of p-Smad3 abrogated Bcar3 promoted fibroblast transition (Fig. 3I).

Bcar3 suppresses reciprocal macrophage-fibroblast interactions

M2 macrophages are recognized as a principal source of TGF-β1, a key inducer of fibroblast-to-myofibroblast differentiation during the pathogenesis of pulmonary fibrosis [17]. These data prompted us to hypothesize that Bcar3 perpetuates pulmonary fibrosis by TGF-β1-mediated macrophage-fibroblast interactions. To confirm this hypothesis, we stimulated DMSO- or SB431542-treated fibroblasts with Scr- or Bcar3 siRNA-transfected M2 macrophage supernatant (Fig. 4A). As expected, the administration of SB431542 effectively inhibited fibroblast-to-myofibroblast differentiation induced by supernatants from Scr siRNA-transfected M2 macrophages, indicating that TGF-β1 plays a crucial role as one of the major profibrotic factors in the M2 macrophage supernatant (Fig. 4B). Interestingly, when fibroblasts were stimulated with supernatants from Bcar3 siRNA-transfected M2 macrophages, the expression of fibrotic markers was significantly attenuated compared to Scr siRNA-transfected M2 macrophage supernatant.
(Fig. 4B). Moreover, fibroblast differentiation induced by SB431542 treatment was completely abolished after stimulation with supernatants from Bcar3 siRNA-transfected M2 macrophages, suggesting that TGF-β1 is the primary factor responsible for mediating the promotion of fibroblast differentiation by Bcar3 siRNA-transfected M2 macrophages (Fig. 4B).

Characterization of Bcar3 siRNA-loaded liposomes and in vivo biodistribution after intratracheal injection

We next sought to translate these findings to therapeutic benefits for pulmonary fibrosis. First, we generated cationic lipid (C12-200)-based nanoparticles loaded with Bcar3 siRNA (Fig. 5A). The prepared liposomes had a > 95% entrapment efficiency for loading siRNA with a zeta potential of 4.9 mV (Fig. 5B). In addition, the generated liposomes possessed an average diameter of ~ 100 nm (Fig. 5C) and a uniform spherical morphology (Fig. 5D). Furthermore, these liposomes were continuously stable for more than 24 h (Fig. 5E). To examine the biodistribution of the liposomes in vivo, DiR-labeled liposomes were prepared and intratracheally injected into the lung. Images were captured by IVIS at different time points (0 h, 4 h, 3 d, 6 d). The fluorescence signal of DiR-labeled liposomes was mainly concentrated in the lung and gradually declined over time (Fig. 5F). Additionally, similar results were obtained in ex vivo images (Fig. 5G). To further examine the cellular location of liposomes in the fibrotic lung, DiO-labeled liposomes were prepared and intratracheally injected into mice with pulmonary fibrosis for 48 h. Interestingly, immunostaining showed that DiO (green) was mainly located in macrophages (CD68+ cells) and fibroblasts (Vimentin+ cells) (Fig. 5H). Furthermore, we observed similar results by flow cytometry (Fig. 5I). Indeed, low expression of Bcar3 was detected in macrophages (Fig. 5J) and fibroblasts (Fig. 5K) in the lung sections of mice with Bcar3 siRNA-transfection. Above results demonstrated that liposomes could precisely deliver gene therapeutics to macrophages and fibroblasts in the lungs, and efficiently suppress Bcar3 expression, which might contribute to an interruption of the vicious cycle between these two cells. Moreover, a significant decline in Bcar3 expression was detected on Day 3 after the intratracheal administration of Bcar3 siRNA-loaded liposomes, while Bcar3 expression gradually increased and was restored on Day 6 (Fig. 5L), which prompted us to treat mice with liposomes at 14 days and 17 days after BLM induction.

Bcar3 siRNA-loaded liposomes abrogate mice against pulmonary fibrosis and fibrosis-associated phenotypes in hPCLS

To evaluate the therapeutic effects of Bcar3 siRNA-loaded liposomes on pulmonary fibrosis, we first generated a mouse model of FITC-induced pulmonary fibrosis. The fluorescence signal of FITC was significantly reduced in mice treated with Bcar3 siRNA-loaded liposomes compared to control groups (Fig. 6A). Histological analysis revealed that the lungs of FITC-treated and FITC + Scr siRNA-loaded liposome-treated mice exhibited severe lung injury and extensive collagen deposition, while those treated with Bcar3 siRNA-loaded liposomes showed significant improvement in lung injury and fibrosis (Fig. 6A). Additionally, hydroxyproline levels, a marker of collagen deposition, were markedly reduced in the lungs of mice treated with Bcar3 siRNA-loaded liposomes (Fig. 6B). Furthermore, Western blot and RT-PCR analysis demonstrated decreased expression of myofibroblast markers in the Bcar3 siRNA-loaded
liposome-treated groups (Fig. 6C and D). The expression of M2 macrophage markers, CD206, and Arg1, was increased in FITC-treated mice and FITC + Scr siRNA-loaded liposome-treated mice, whereas significant decreases in M2 markers were observed in mice treated with Bcar3 siRNA-loaded liposomes (Fig. 6E). Immunostaining for CD206 also supported these findings (Supplementary Fig. 3A).

Next, we utilized a mouse model of lung fibrosis induced by intratracheal administration of BLM. Similar to the previous results, mice treated with Bcar3 siRNA-loaded liposomes showed improved fibrotic lesions, as evidenced by pathological staining (Fig. 6F), lower Ashcroft scores (Fig. 6G), reduced levels of hydroxyproline (Fig. 6H), and decreased expression of fibrotic markers (Fig. 6I). Furthermore, intratracheal administration of Bcar3 siRNA-loaded liposomes markedly inhibited M2 macrophage polarization, as shown by reduced expression of Arg1 and CD206 (Fig. 6J). Similar results were observed by immunostaining of CD206 (Supplementary Fig. 3B).

Additionally, we investigated the effects of Bcar3 siRNA-loaded liposomes in human precision-cut lung slices (hPCLS), a three-dimensional model of lung tissue. Treatment with Bcar3 siRNA-loaded liposomes significantly reduced collagen accumulation and expression levels of Fibronectin and collagen 1 after TGF-β1 stimulation (Fig. 7A-B). RT-PCR analysis confirmed the consistency of these findings (Supplementary Fig. 4). Moreover, Bcar3 siRNA-loaded liposomes effectively inhibited M2 macrophage polarization in hPCLS (Fig. 7C-D). Overall, these results indicate that Bcar3 siRNA-loaded liposomes hold promise as a potential therapeutic strategy for abrogating pulmonary fibrosis.

**Discussion**

In the current study, we investigated the role of Bcar3 in the interaction between macrophages and fibroblasts during the progression of pulmonary fibrosis. Our findings revealed that Bcar3 expression was upregulated in M2 macrophages and myofibroblasts, and this upregulation was also observed in the lungs of IPF patients and mice with pulmonary fibrosis. Moreover, the up-regulation of Bcar3 significantly facilitated M2 macrophage polarization and fibroblasts-to-myofibroblast differentiation. Mechanistic studies identified the IL-4/Stat6/Bcar3 and TGF-β1/Smad3/Bcar3 positive feedback loops in macrophages and fibroblasts, respectively. Importantly, suppressing Bcar3 expression abrogated macrophage and fibroblast interactions via TGF-β1. Notably, intratracheal administration of Bcar3 siRNA-loaded liposomes significantly reversed pulmonary fibrosis induced by FITC or BLM, as well as hPCLS stimulated with TGF-β1 (Fig. 7E). Collectively, our study not only highlights the critical role of Bcar3 in the interaction between macrophages and fibroblasts during the progression of pulmonary fibrosis but also provides compelling evidence that intratracheal administration of Bcar3 siRNA-loaded liposomes reversed established pulmonary fibrosis, which may be a reliable treatment for pulmonary fibrosis.

Accumulating evidence suggests that macrophages and fibroblasts engage in communication to facilitate the progression of pulmonary fibrosis [6, 8]. Specifically, activated macrophages produce factors such as TGF-β, PDGFs and amphiregulin (AREG), which drive fibroblast differentiation and proliferation. Therefore, impairing the interaction of macrophages and fibroblasts is a worth-adopting
therapeutic strategy for IPF. Nowadays, first-line clinical drugs, pirfenidone and nintedanib partially implement this strategy, abrogating the activation of macrophages and fibroblasts [18]. However, their multiple targets cause severe side effects in IPF patients, limiting their clinical use. In this study, our aim was to identify the coregulatory factors of macrophage activation and fibroblast differentiation. Based on the deep RNA-seq data, we identified 27 altered genes in macrophages and fibroblasts after stimulation, of which 6 genes were upregulated and 21 genes were downregulated. Bcar3, which was one of the 6 overexpressed genes, is a critical adaptor molecule that has feeble and indirect roles in M2 macrophage polarization [13] and fibroblast transition [14]. Therefore, we verified the role of Bcar3 in macrophages and fibroblasts during the progression of pulmonary fibrosis.

To examine the involvement of Bcar3 in macrophages, we measured Bcar3 expression in IL-4-treated macrophages. We found that Bcar3 expression was upregulated during M2 polarization, and this effect was markedly reversed by an inhibitor of Stat6. Thus, we showed that Bcar3 expression was controlled by IL-4/p-Stat6 signaling in macrophages. Moreover, Bcar3 promoted the IL-4-induced M2 program by inducing Stat6 activation. These findings suggest that Bcar3 drives a positive feedback loop during M2 polarization. Blocking Bcar3 inhibited macrophage alternative activation. Additionally, a similar expression pattern of Bcar3 was observed in fibroblasts in a dose- and time-dependent manner after TGF-β1 stimulation. Furthermore, our data showed that the expression of Bcar3 in TGF-β1-treated fibroblasts was dependent on TGFβR1 and Smad3, as shown by the low expression of Bcar3 in SB-431542- or SIS3-HCl-treated fibroblasts after TGF-β1 stimulation. Notably, the gain and loss assays provided strong evidence that the expression of Bcar3 in fibroblasts predisposes the cells to undergo myofibroblast differentiation following TGF-β1 treatment and this process is Smad3 dependent. These data indicated the other positive feedback loop associated with Bcar3 in the fibroblast-to-myofibroblast transition. Thus, we demonstrated that two positive feedback loops associated with Bcar3 facilitate pulmonary fibrosis progression (Fig. 7F).

Given that no effective therapy is currently available for IPF, we then sought to translate these findings into a therapeutic strategy. Compared with conventional drugs, RNA therapy using specific exogeneous nucleotide fragments could provide precise modulation of target cells, including targets that were previously considered to be “undruggable” [19]. Since siRNAs are not stable and are digested by endonuclease [20], an appropriate vector is needed. It is encouraging that the FDA approved patisiran in 2018, which is the first siRNA drug based on liposomes, which have excellent safety and provide controlled drug release [21]. In this study, we used a type of liposome that could be simply prepared and was suitable for convenient clinical application. Intratracheal administration of the prepared liposomes showed excellent biocompatibility, and these liposomes were enriched in the fibrosis zone and could be guided to target macrophages and fibroblasts. The most significant discovery was that intratracheal administration of Bcar3 siRNA-loaded liposomes reversed established pulmonary fibrosis without any side effects (Figure S5).

Our study has some limitations. Since this was the first study to show that Bcar3 modulates macrophage polarization and fibroblast differentiation, the underlying mechanism is far from clear. Although we
showed that Bcar3 could enhance Stat6 and Smad3 signaling in macrophages and fibroblasts, respectively, the detailed mechanism needs to be examined further. In addition, we clarified the function of Bcar3 in both macrophages and fibroblasts. However, Bcar3 is also expressed in other cells, such as epithelial cells and T cells during the process of pulmonary fibrosis. We should further examine the function of Bcar3 in these cells. Finally, we showed that TGF-β1 controlled the Bcar3-mediated fibroblast-macrophage interaction to promote pulmonary fibrosis progression, but we cannot exclude other signals involved in this interaction.

In conclusion, we demonstrate that Bcar3 is involved in the pathogenesis of IPF by enhancing M2 macrophage polarization and fibroblast differentiation. Specifically, Bcar3 was involved in two positive feedback loops in macrophages and fibroblasts. Importantly, inhibiting Bcar3 abrogated the macrophage-to-fibroblast interaction via TGF-β1. Therefore, intratracheal administration of Bcar3 siRNA-loaded liposomes could reverse established pulmonary fibrosis induced by FITC and BLM without any side effects. Taken together, our data show that targeting Bcar3 may be a promising strategy for the treatment of pulmonary fibrosis.

Materials and methods

Human samples

An IPF diagnosis was made according to the consensus diagnostic criteria of the European Respiratory Society (ERS) / American Thoracic Society (ATS) [22]. Lung explant material from IPF patients (n = 5) and resected para-carcinoma lung tissues from non-small cell lung cancer (NSCLC) patients (n = 5) were collected at Tongji Hospital. For Human precision cut lung slice (hPCLS), the lung tissues were also collected from resected para-carcinoma lung tissues of NSCLC patients (n = 3). Written informed consent was obtained from all participants. The study was conducted in compliance with the Declaration of Helsinki and was approved by the Human Assurance Committee of Tongji Hospital (TJ-IRB20220443). Clinical data from IPF patients and control subjects are provided in Supplementary Table 1.

FITC- and BLM-induced pulmonary fibrosis

C57BL/6 mice (male, 6–8 weeks old) were purchased from GemPharmatech Co., Ltd. (Nanjing, China). All mice were housed in a specific pathogen-free (SPF) animal facility at Tongji Hospital with a 12:12 h reversed day-night cycle. The mice were anesthetized with sodium pentobarbital (60 mg/kg) and then intratracheally administered FITC (14 mg/kg; Sigma, MO, USA) or BLM (1.5 mg/kg; MedChemExpress, Shanghai, China) in 50 µl of PBS [23]. Mice in the control group were administered the same volume of PBS. SiRNA-loaded liposomes were intratracheally injected into the anesthetized animals on Days 14 and 17 after FITC or BLM injection. Finally, the mice were euthanized on Day 21 to analyze pulmonary fibrosis. All experimental procedures were approved by the Animal Care and Use Committee of Tongji Hospital (TJH-202105001).

Immunofluorescence staining
Cryosections or paraffin sections of lung tissues from IPF patients and pulmonary fibrosis mice were used for immunofluorescence staining. The primary antibodies used for staining were as follows: rabbit anti-Bcar3 (Proteintech, Wuhan, China, 1:100) and mouse anti-PDGFR-β (Santa cruz, CA, USA, 1:100), mouse anti-α-SMA (Santa cruz, CA, USA, 1:100) and mouse anti-Vimentin (Proteintech, Wuhan, China, 1:100). Alexa 488- or 594-conjugated anti-mouse or anti-rabbit antibodies (Abbkine, CA, USA, 1:400) were used as fluorescent secondary antibodies, and nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI). Images were obtained with a fluorescence microscope (Olympus, Shinjuku, Japan).

**Histological analysis**

The left lungs were removed and fixed in 4% neutral buffered paraformaldehyde for 24 h at room temperature, embedded in paraffin and sectioned. The sections were then stained with hematoxylin and eosin (H&E), Masson's trichrome, and Sirius red as previous reported. Fibrosis was scored on a scale of 0–8 using the Ashcroft scoring method [7].

**Culture and treatment of BMDMs**

Bone marrow-derived macrophages (BMDMs) were isolated from the bone marrow collected of C57BL/6 mouse femurs and tibias. BMDMs were prepared in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Gibco, Shanghai, China), 30 ng/ml macrophage colony-stimulating factor (M-CSF; PeproTech, NJ, USA), and 1% penicillin/streptomycin for 7 days [24]. IL-4 (10 ng/ml, Biolegend, CA, USA) stimulation was performed on Day 7. The cells were then harvested for Western blotting and RT–PCR.

**Culture and treatment of fibroblasts**

Mouse lung fibroblasts were isolated from the resected lung tissues of C57BL/6 mice. Briefly, fibroblasts were generated by mincing the fresh lung tissue into small pieces, which were plated evenly in 10cm plates containing 2ml of medium; the medium was replenished to 10ml after 24h [25]. The cells were cultured in DMEM containing 15% fetal bovine serum (FBS) and penicillin/streptomycin in a 37°C incubator, and only cells between passages 3 and 5 were used. TGF-β1 (MedChemExpress, Shanghai, China) stimulation was performed.

**Macrophage and fibroblast interactions**

To examine macrophage-mediated promotion of fibroblast differentiation, BMDMs were transfected with Scr or Bcar3 siRNA for 48 h. Then, the supernatant of transfected BMDMs was discarded after IL-4 (10 ng/ml) stimulation for 24 h, and new medium was added and incubated for 24 h. Subsequently, fibroblasts were pretreated with DMSO or SB431542 (10 nM) and stimulated with macrophage culture medium for 24 h before being harvested for Western blotting.

**Western blot analysis**

Cells and lung tissues were homogenized in RIPA lysis buffer (Beyotime, Shanghai, China). Western blot was performed according to previously reported protocols [26]. In brief, proteins were separated by 10%
sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes (0.4 µm). The membranes were blocked in 5% milk for 60 min at room temperature and then incubated with primary antibodies at 4°C overnight. The primary antibodies used were anti-Bcar3 (Boster, Wuhan, China, 1:1,000), anti-Fibronectin, anti-Collagen 1, anti-β-actin, anti-Gapdh (Proteintech, Wuhan, China, 1:1,000). HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies were added and incubated for 1 h at room temperature. Detection was performed using a chemiluminescent substrate system (Bio-Rad Laboratories, CA, USA). The gray values were analyzed by ImageJ software.

**RT–PCR analysis**

Total RNA was isolated from lung tissues and cells with TRIzol reagent (Takara, Dalian, China) according to previous reported [139]. RNA quantity and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). Complementary DNA (cDNA) was synthesized using a reverse transcriptase kit (Invitrogen, CA, USA). Quantitative real-time PCR analysis was performed on a CFX96 real-time PCR detection system (Bio-Rad, CA, USA) using SYBR Green mix (Takara, Dalian, China) under the following conditions: 30 s at 95°C for initial denaturation, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The comparative Ct method was used to normalize the data for presentation as the means of the normalized transcript levels. The RNA levels of *Actb* were used to normalize the data. The primer sequences used for RT–PCR are provided in supplementary Table 2.

**SiRNA or plasmid transfection**

Small interfering RNAs (siRNAs) specific for *Bcar3* and a corresponding scrambled siRNA were purchased from RiboBio (Guangzhou, China) and then transiently transfected into fibroblasts using Lipofectamine 3000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Briefly, fibroblasts were seeded in 12-well plates and transfected with siRNA. Forty-eight hours after transfection, the cells were stimulated with murine TGF-β1 (10 ng/ml) for the indicated time points. The *Bcar3*-specific siRNA targeted the following sequence in *Bcar3* mRNA: 5′- CCA CTA CAT GAT CCT TGA T -3′. Transfection efficiency was monitored by RT–PCR or Western blot at 48 h after transfection.

The *Bcar3* plasmid and a vector plasmid were purchased from GeneChem (Shanghai, China), and 0.5 µg of purified DNA was mixed with the transfection reagent (Invitrogen, Shanghai, China) according to the manufacturer’s instructions and applied to fibroblasts. Forty-eight hours after transfection, the transfection efficiency was analyzed by Western blot.

**Preparation and characterization of siRNA-loaded liposomes**

SiRNA-loaded liposomes were prepared as previously described [28]. A lipid solution containing lipidoids, cholesterol, DSPC, and mPEG-DMG at a molar ratio of 50:38.5:10:1.5 dissolved in ethanol and 10 mM sodium citrate was prepared. Then, siRNA was dissolved in 10 mM citrate buffer, and the lipid components were mixed with the dissolved siRNA by vortexing such that the final weight ratio of
lipidoids:siRNA was 1:20. The next step was ultrafiltration centrifugation (3000 rpm × 10 min) to exclude free siRNA. Finally, the siRNA-liposomes were diluted in PBS. The hydrodynamic diameter, polydispersity, zeta potential, and stability of the liposomes were measured by dynamic light scattering (DLS) (Malvern Zetasizer Nano-ZS, UK). A RiboGreen assay was used to calculate the siRNA entrapment efficiency. After being stained with 2% phosphotungstic acid, the liposomes were characterized by transmission electron microscopy (TEM, Jeol, Japan).

**In vivo biodistribution of liposomes**

For the in vivo biodistribution study, the DiR-labeled liposomes were prepared and intratracheally injected into the mice after 14 days of BLM induction. Subsequently, the mice were anesthetized with isoflurane (Sigma–Aldrich, St. Louis, MO, USA) and photographed at 0 h, 4 h, 3 d, and 6 d by an in vivo imaging system (IVIS Lumina XR, SI Imaging, AZ, USA) (excitation: 745 nm, emission: 830 nm). After 6 d, the mice were euthanized, and their lungs, hearts, livers, spleens, kidneys, and intestines were harvested for ex vivo fluorescence imaging.

**Precision Cut Lung Slices (PCLS) Generation and Culture**

The fresh resected para-carcinoma lung tissues from NSCLC patients were slowly infused with 1.5% low melt agarose (Sigma, MO, USA) at 38°C via a visible bronchus and cooled ice water for 20 min, as previous reported [29]. The prepared tissues were sectioned (400 µm) using a Compressstome (VF-300-0Z by Precisionary) at cutting speed of 6 µm/s and oscillation frequency of 5Hz. The hPCLS were cultured in 12 multi-well plates in 1 ml DMEM-F12 no-phenol red containing 0.1% FBS and penicillin/streptomycin in a 37°C incubator. The hPCLS were treated with Bcar3 siRNA-loaded liposomes or Scr siRNA-loaded liposomes for 48 h before TGF-β stimulated for 24 h.

**Statistical analysis**

Comparisons between groups were undertaken using the Prism software (GraphPad Prism 8.Ink, GraphPad Software Inc). Two experimental groups were compared using the two-tailed Student's t-test (data with normal distribution, homogeneity of variance) or two-tailed Mann–Whitney test (data without normal distribution). Once more than two groups were compared, one-way or two-way analysis of variance with Tukey's multiple comparison test (data with normal distribution) or Kruskal-Wallis test with Dunn's post-hoc tests (data without normal distribution) were used. All experiments were conducted with at least 3 independent replications. The data are presented as the mean ± SEM. In all cases, p < 0.05 was considered with statistical significance.

**Declarations**

**Ethics approval and consent to participate**

The study was conducted in compliance with the Declaration of Helsinki and was approved by the Human Assurance Committee of Tongji Hospital (TJ-IRB20220443). All experimental procedures were approved by the Animal Care and Use Committee of Tongji Hospital (TJH-202105001).
Consent for publication

Not applicable

Availability of data and materials

The data and materials used in the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

QW and CXZ performed the experiments. XTJ, XHL and CJW analyzed the data. XNF, WNX and JY provided human lung tissues and clinical data. QZ and YW designed the experiments and supported the preparation of the manuscript. YW led the investigation and wrote the manuscript. All authors read and approved the final manuscript.

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References


Figures
Figure 1

Upregulated Bcar3 expression was detected in IPF patients and mice with pulmonary fibrosis. A-B: A heatmap of the co-overexpressed genes identified by RNA-seq analysis in IL-4-treated macrophages (A) and TGF-β1-treated fibroblasts (B). The color of the heatmap represents the fold enrichment in each sample. C-D: RT-PCR analysis of Bcar3 expression in macrophages (C) and fibroblasts (D) after IL-4 or TGF-β1 stimulation, respectively. E: Western blot analysis of Bcar3 expression in the lung homogenates...
of IPF patients (n = 5) and control subjects (n = 5). F-G: Representative results of coimmunostaining of Bcar3 and CD68 (a macrophage marker, F) or PDGFR-β (a fibroblast marker, G) in the lung sections of IPF patients (n = 5) and control subjects (n = 5). The nuclei were stained blue with DAPI, and the images were taken at an original magnification of ×400. H: Western blot analysis of Fibronectin, Collagen 1, α-SMA and Bcar3 expression in the lungs of mice (n = 5) at different times following BLM induction. I-K: RT-PCR analysis of the correlation between Bcar3 and Fn1(I), Col1a1(J) and Acta2(K) expression after TGF-b1 induction. L-M: Representative coimmunostaining results showing Bcar3 and F4/80 (L) or PDGFR-β (M) in the lung sections of BLM- or PBS-treated mice (n = 5). The nuclei were stained blue with DAPI, and the images were taken at an original magnification of ×400. The data are represented as the means ± SEM of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. IPF: idiopathic pulmonary fibrosis; BLM: bleomycin; α-SMA: α-smooth muscle actin; HPF: high-power field.
Figure 2

Bcar3 promoted M2 macrophage polarization by enhancing p-Stat6 activation. A-B: Western blot analysis of Bcar3, CD206, Ym1 and Arg1 expression in BMDMs after treatment with different doses of IL-4 (A) for different time points (B). C: Western blot analysis of Arg1, Ym1 and Bcar3 expression in AS1517499-treated BMDMs following IL-4 stimulation. D: Knockdown of Bcar3 attenuated IL-4-induced M2 macrophage polarization. Left panel: Western blot analysis of Arg1, Ym1 and Bcar3 expression. Right panel: Western blot analysis of p-STAT6 and STAT6 expression.
panel: Bar graph showing the mean data of each group. **E:** RT-PCR analysis of Arg1, Chil3, Mrcl and Retnla in Bcar3 siRNA-transfected BMDMs after IL-4 stimulation. **F:** Western blot analysis of Stat6 and p-Stat6 expression in Bcar3 siRNA-transfected BMDMs after IL-4 stimulation for 1 h. **G:** Western blot analysis of p-Stat6 and Stat6 expression in Bcar3-overexpressing BMDMs pretreated with AS1517499 following IL-4 induction for 1 h. **H:** Western blot analysis of Arg1 expression in Bcar3-overexpressing BMDMs pretreated with AS1517499 following IL-4 induction for 24 h. The data are represented as the means ± SEM of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. BMDMs: bone marrow-derived macrophages; Arg1: Arginase 1.
Figure 3

**Bcar3 facilitated fibroblast-to-myofibroblast differentiation via the TGF-β1/Smad3 pathway.**

A-B: Western blot analysis of Bcar3, Fibronectin, Collagen 1 and α-SMA expression in fibroblasts after treatment with different doses of TGF-β1 (A) for different times(B). C-D: Western blot analysis of Bcar3, Fibronectin, Collagen 1 and α-SMA expression in SB431542 (C) or SIS3-HCl (D)-treated fibroblasts following TGF-β1 stimulation for 24 h. E: Western blot analysis of Bcar3, Fibronectin, Collagen 1, TGF-β1 and α-
SMA expression in Bcar3 siRNA-transfected fibroblasts after TGF-β1 induction for 24 h. F: Western blot analysis of Fibronectin, Collagen 1 and α-SMA expression in Bcar3-overexpressing fibroblasts. G: Western blot analysis of Bcar3, p-Smad2, p-Smad3 and Smad2/3 expression in Bcar3 siRNA-transfected fibroblasts after TGF-β1 induction for 1 h. H: Western blot analysis of p-Smad2, p-Smad3 and Smad2/3 expression in Bcar3-overexpressing fibroblasts that were pretreated with SIS3-HCl following TGF-β1 induction for 1 h. The data are represented as the means ± SEM of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 4

Bcar3 suppresses reciprocal fibroblast-macrophage interactions. A: Schematic showing the experimental design of macrophage promotion of fibroblast differentiation. B: Western blot analysis of Fibronectin, Collagen 1, and α-SMA expression in fibroblasts treated with the supernatant of BMDMs. The data are represented as the means ± SEM of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.
Biodistribution of Bcar3 siRNA liposomes after intratracheal injection. A: Preparation of Bcar3 siRNA-loaded liposomes. B: The hydrodynamic diameter, PDI, zeta potential and entrapment efficiency of liposomes (empty or Bcar3 siRNA-loaded). C: Representative TEM image of Bcar3 siRNA-loaded liposomes. D: Hydrodynamic diameter distribution of Bcar3 siRNA-loaded liposomes. E: Colloidal stability of Bcar3 siRNA-loaded liposomes in PBS. F: IVIS images of a mouse at different time points after
intratracheal injection of DiR-labeled liposomes. **G:** Ex vivo IVIS images of the major organs of mice. **H:** Immunofluorescence images showing the distribution of DiO-labeled liposomes (green), CD68+ cells (red), and Vimentin+ cells (red) in the lungs of mice. **I:** Flow cytometric analysis of liposome distribution in the lungs. **J:** Immunofluorescent staining of CD68 (green) and Bcar3 (red) in BLM-treated mice followed by Bcar3 siRNA-loaded liposome injection. **K:** Immunofluorescent staining of Bcar3 (green) and α-SMA (red) in BLM-treated mice followed by Bcar3 siRNA-loaded liposome injection. **L:** Western blot analysis of the temporal expression of Bcar3 in BLM-treated mice following Bcar3 siRNA-loaded liposomes injection. Three or five mice were included in each study group. The data are presented as the mean ± SEM. *p < 0.05.
Figure 6

Intratracheal administration of *Bcar3* siRNA-loaded liposomes protected mice from FITC-induced and BLM-induced pulmonary fibrosis. A: The fluorescence signal (top) of FITC, H&E (middle) and Masson (bottom) staining of lung in in the different groups after FITC induction. Images were taken at 200× magnification. B: Bar graph showing hydroxyproline levels in each group after FITC induction. C: RT-PCR analysis of *Fn1, Col1a1, Acta2* and *Bcar3* expression after FITC induction. D: Western blot analysis of
Fibronectin, Collagen 1, α-SMA and Bcar3 expression in FITC-treated mice. E: Western blot analysis of CD206 and Arg1 expression in FITC-treated mice. F: H&E (middle) and Masson (bottom) staining of lung in in the different groups after BLM induction. G: Bar graph showing the semiquantitative Ashcroft scores of the fibrosis severity. Images were taken at 200× magnification. H: Bar graph showing hydroxyproline levels in the lungs after BLM induction. I: Western blot analysis of Fibronectin, Collagen 1, α-SMA and Bcar3 expression in BLM-treated mice. J: Western blot analysis of CD206 and Arg1 expression in each group after BLM-induction. Five mice were included in each study group. *p < 0.05; **p < 0.01; ***p < 0.001

Figure 7
**Bcar3 siRNA-loaded liposomes attenuates fibrosis-associated phenotypes in TGF-β treated hPCLS.** A: Masson staining (top) and Sirius red staining (bottom) of the fibrosis-associated phenotypes in TGF-β treated hPCLS following Bcar3 siRNA-loaded liposomes treatment. B: Western blot analysis of Bcar3, fibronectin and collagen 1 expression in hPCLS following TGF-β and Bcar3 siRNA-loaded liposomes treatment. Left panel: representative western blot results. Right panel: bar graph of the western blot results. C: Western blot analysis of CD206 expression in hPCLS following TGF-β and Bcar3 siRNA-loaded liposomes treatment. Left panel: representative western blot results. Right panel: bar graph of the western blot results. D: Immunofluorescence of CD206 in the section of hPCLS following TGF-β and Bcar3 siRNA-loaded liposomes treatment. E: A diagram of the mechanisms revealing Bcar3 regulation of pulmonary fibrosis. The data are represented as the mean ± SEM of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

**Supplementary Files**

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