Genes & Diseases (2017) 4, 204–214

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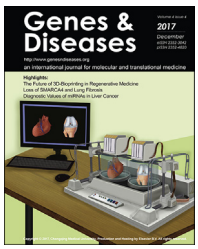

FULL LENGTH ARTICLE

Deletion of SMARCA4 impairs alveolar epithelial type II cells proliferation and aggravates pulmonary fibrosis in mice

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Received 7 October 2017; accepted 11 October 2017
Available online 25 October 2017

KEYWORDS
Cell proliferation;
Pulmonary fibrosis;
SMARCA4;
Transgenic mice;
Type II alveolar epithelial cells

Abstract Alveolar epithelial cells (AECs) injury and failed reconstitution of the AECs barrier are both integral to alveolar flooding and subsequent pulmonary fibrosis (PF). Nevertheless, the exact mechanisms regulating the regeneration of AECs post-injury still remain unclear. SMARCA4 is a part of the large ATP-dependent chromatin remodelling complex SWI/SNF, which is essential for kidney and heart fibrosis. We investigates SMARCA4 function in lung fibrosis by establishing PF mice model with bleomycin firstly and found that the expression of SMARCA4 was mainly enhanced in alveolar type II (ATII) cells. Moreover, we established an alveolar epithelium-specific SMARCA4-deleted *SP-C-rtTA/(tetO)$_7$-Cre/SMARCA4$^{f/f}$* mice (*SOSM4$^{\Delta/\Delta}$*) model, as well as a new SMARCA4-deleted alveolar type II (ATII)-like mle-12 cell line. We found

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Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2017.10.001
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that the bleomycin-induced PF was more aggressive in $SOSM4^{\Delta/\Delta}$ mice. Also, the proliferation of ATII cells was decreased with the loss of SMARCA4 *in vivo* and *in vitro*. In addition, we observed increased proliferation of ATII cells accompanied by abnormally high expression of SMARCA4 in human PF lung sections. These data uncovered the indispensable role of SMARCA4 in the proliferation of ATII cells, which might affect the progression of PF.
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Introduction

Pulmonary fibrosis (PF) is a fatal disease with unclear pathogenic mechanism. Most PF patients show a progressive decline in pulmonary function, which eventually leads to respiratory failure and death.[1] Conventional therapy consisting of glucocorticoids or immunosuppressive drugs has been affirmed ineffective. Currently, lung transplantation is considered to be an effective treatment approach, but only in limited number of patients, with a 5 year post-transplantation survival rate of about 50%. Recently, two novel disease-modifying therapies, nintedanib and pirfenidone, have shown promising results, nonetheless both these drugs are based on pleiotropic mechanisms of action.[2,3] The limited treatment approaches are caused by the elusive pathogenic mechanisms. Therefore, further explorations of the PF pathogenesis are exigent.

As a major component of interstitial lung disease, PF is characterised by repeated damage of alveolar type I (ATI) and type II (ATII) cells. Under normal conditions, when these cells die, the pulmonary stem cells proliferate and differentiate into the functional cells, consequently repairing the injury. Otherwise, they are replaced by abundant fibroblasts[2,4] and extracellular matrix proteins,[5] resulting in destruction of normal pulmonary tissue, thickening of the walls and honeycombing of the lungs.[6] In general, insufficient proliferation and differentiation of pulmonary stem cells into the functional cells following lung injury constitute a predisposition to PF. As the pulmonary stem cell populations, ATII can secrete surfactant and serve as a progenitor for ATI cells in response to injurious stimuli.[7] Therefore, exploring the influences of ATII cell proliferation may be helpful for illustrating the pathogenesis of PF.

In recent years, SMARCA4, a catalytic subunit of the yeast switch in mating type (SWI)/sucrose nonfermentation (SNF) chromatin remodelling complex,[8] was reported to be involved in the progression of fibrosis in many different organ systems.[9–13] In hearts following pathological stress, SMARCA4 and forkhead box M1 (FoxM1) transcription factor complexes regulate the transcription of angiotensin through conversion of enzyme (Ace) and Ace2 by binding to their promoter regions in the coronary endothelial cells. These in turn trigger angiotensin I-to-II conversion, followed by cardiac hypertrophy and fibrosis.[12] Furthermore, SMARCA4 could also be activated by cardiac stresses and could form a complex with histone deacetylase (HDAC) and poly (ADP ribose) polymerase (PARP) to induce a pathological α-myosin heavy chain (Myh6) to β-myosin heavy chain (Myh7) shift in cardiomyocytes, and to promote myocyte proliferation and accelerate cardiac fibrosis.[11] Beside myocardial fibrosis, SMARCA4 has been shown to be abnormally elevated in renal fibrosis following ischaemia-reperfusion injury by binding to the promoters of proinflammatory or profibrotic genes to accentuate their transcription.[13] Moreover, SWI/SNF complex has also been reported to influence the regeneration following liver injury and ear wound,[14] and to influence the proliferation of various cell types.[8,15–17] Nevertheless, very little is known about the role of SMARCA4 in pulmonary fibrosis.

In this study, we established the PF model with bleomycin (BLM) and found elevation of SMARCA4 expression mainly in ATII cells of BLM-induced PF mice. After constructing lung epithelium-specific SMARCA4 depleted mice and SMARCA4-depledted ATII cell line, we found that the proliferation of ATII cells was decreased with the loss of SMARCA4 *in vivo* and *in vitro*. Moreover, the human lung sections from PF patients also confirmed the increased proliferation of ATII cells accompanied by abnormally high expression of SMARCA4, further uncovering the critical role of SMARCA4 in PF pathogenesis.

Materials and methods

Bleomycin—induced lung fibrosis

All the protocols were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (Library of Congress Control Number: 2010940400, revised 2011) guidelines and relevant regulations. A total of 2.5 or 5.0 mg/kg of bleomycin (Sigma, USA) was intratracheally (i.t.) injected in wild-type or in transgenic 8—10 weeks old mice. All mouse experiments were approved by the Ethics Committee of Chongqing Medical University.

Transgenic animals and doxycycline administration

$SMARCA4^{f/f}$[18] and *SPC-rtTA/(tetO)$_7$-Cre* mice[19–21] were crossed to generate homozygotes $SOSM4^{\Delta/\Delta}$ (*SPC-rtTA/(tetO)$_7$-Cre/*$SMARCA4^{f/f}$), heterozygotes $SOSM4^{\Delta/+}$ (*SPC-rtTA/(tetO)$_7$-Cre/*$SMARCA4^{f/+}$) and WT (*SPC-rtTA/(tetO)$_7$-Cre/*$SMARCA4^{+/+}$) mice. To induce expression of the Cre transgene in ATII cells postnatally, 8—10 weeks old mice received doxycycline (Dox, Sigma Aldrich, USA), administered in their drinking water for 1week following treatment.

Primary ATII cells isolation

Primary ATII cells were isolated from adult mice as previously described.[22] Magnetic sorting was used for CD45-negtive and epithelial-cell adhesion molecule (EpCAM)-positive selection. Every ATII cell sample was collected from three different mice sharing the same genotype and administration.

Human lung tissue samples

Human lung tissues were obtained from patients undergoing surgical lung biopsy for idiopathic pulmonary fibrosis (IPF) and from surgical specimens showing normal lung parenchyma distant from the tumour nodules (control). The serial adjacent slides were collected according to previously described method[23–25] and were used for histological staining. All patients read and signed the informed consents. This study was approved by the Research Ethics Committee of Chongqing Medical University and undertaken in accordance with the principles of the revised Declaration of Helsinki.

Statistical analysis

All values are expressed as means ± SEM. Data were analysed using two-way analysis of variance. A student's t test analysis was used for the single-parameter comparisons. Statistical differences were analysed using SPSS software (version 16). $p < 0.05$ was considered statistically significant. Kaplan–Meier survival analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA) and statistical significance was determined by log-rank test.

(Additional data on materials and methods are provided in the "Electronic Supplementary Material")).

Results

ATII cells in the lung tissues of PF mice showed increased SMARCA4 expression

To investigate the function of SMARCA4 in PF, we remodelled the PF procession in mice. Briefly, PF mice models were established by bleomycin intratracheal injection, and were consequently sacrificed 21 days post-bleomycin administration. Lung fibrosis was assessed by H&E, Masson's trichrome staining and the content of acid-soluble lung collagen. Histological results showed thickened alveolar walls, damaged lung structure, formation of fibrous masses and collagen deposition in bleomycin-treated mice. The Ashcroft score was significantly higher in PF models. Furthermore, higher acid-soluble lung collagen was observed in PF models compared to saline-treated mice (Suppl. Fig. S1A–C). Moreover, the survival rate of PF models was lower than that of control group (Suppl. Fig. S1D). To sum up, these results suggested successful establishment of PF models.

Accounting for SMARCA4-positive cells, we found positive cells to be significantly increased in the lung tissues of PF models (Fig. 1A, C); these data were further confirmed by immunoblotting approaches (Fig. 1B, D). Given that many different cell types, i.e. myofibroblasts (marked by smooth muscle-specific α-actin, α-SMA), macrophages (marked by CD11b) and ATII cells (marked by surfactant protein C, SPC), participated in the pathogenesis of PF,[5,23,26,27] we further investigated the SMARCA4 expression using immunohistochemistry of lung serial sections.[23–25] Interestingly, the enhanced SMARCA4 expression was observed in the lung epithelial cells (Supple Fig. S2A, B), while the myofibroblasts and macrophages with aberrance expression of SMARCA4 were rare in lungs of PF models (Suppl. Fig. S2C-F). For more accurate detection, the ATII cells were isolated (Suppl. Fig. S3 and Fig. 1E) by immunomagnetic bead cell sorting and the increase of SMARCA4 in those cells were confirmed by immunoblotting and flow cytometry approach (Fig. 1G and H). Furthermore, higher number of ATII cells recovered from PF mice 21 days post-bleomycin treatment was observed compared to control mice (Fig. 1F). In addition, compared to control group, higher proliferation of ATII cells in PF mice was detected following BrdU incorporation (Fig. 1I), indicating that the elevated SMARCA4 might enhance the ATII cells proliferative capacity during PF procession.

Mice with epithelial SMARCA4 deficiency were viable and healthy

To further explore the function of the elevated SMARCA4 in ATII cells during PF development, ATII cells specific SMARCA4 knock-down mice were bred by crossing *SPC-rtTA/(tetO)$_7$-Cre* mice with *SMARCA4$^{f/f}$* mice (Suppl. Fig. S4A). After genotyping (Suppl. Fig. S4B–D), we fed the mice with doxycycline for one week. Comparing with the WT mice (*SPC-rtTA/(tetO)$_7$-Cre/SMARCA4$^{+/+}$*), only about 40% of SMARCA4 expression was detected in the isolated ATII cells in *SOSM4$^{\Delta/\Delta}$* and *SOSM4$^{\Delta/+}$* mice, while the expression of SPC was not impacted (Fig. 2A). The knock-down efficiency was further confirmed by flow cytometry (Fig. 2B). The residual SMARCA4 expression in the homozygotes might probably occurred due to incomplete excision by SPC-Cre.[7] Moreover, the similarity of SMARCA4 expression between the *SOSM4$^{\Delta/\Delta}$* and *SOSM4$^{\Delta/+}$* was possibly caused by the same reason. Also, *SOSM4$^{\Delta/\Delta}$* and *SOSM4$^{\Delta/+}$* mice were healthy and did not show any signs of polypnea or emaciation until seven months post-doxycycline administration. Furthermore, the histology of the lung tissue of *SOSM4$^{\Delta/\Delta}$* and *SOSM4$^{\Delta/+}$* mice was normal comparing with their littermates (WT) (Fig. 2C and D). To conclude, the obtained data indicated that the SMARCA4 knock-down in ATII cells did not compromise the respiratory function in mice.

Epithelial SMARCA4 deficiency aggravates bleomycin-induced PF

Given that knock-down of SMARCA4 in ATII cells was insufficient to cause PF independently, to further elucidate the function of enhanced SMARCA4 in PF model, we constructed the PF model in *SOSM4$^{\Delta/\Delta}$* mice and their littermates (WT) following feeding with Dox for one week. As high dose of bleomycin (5 mg/kg) would induce severe

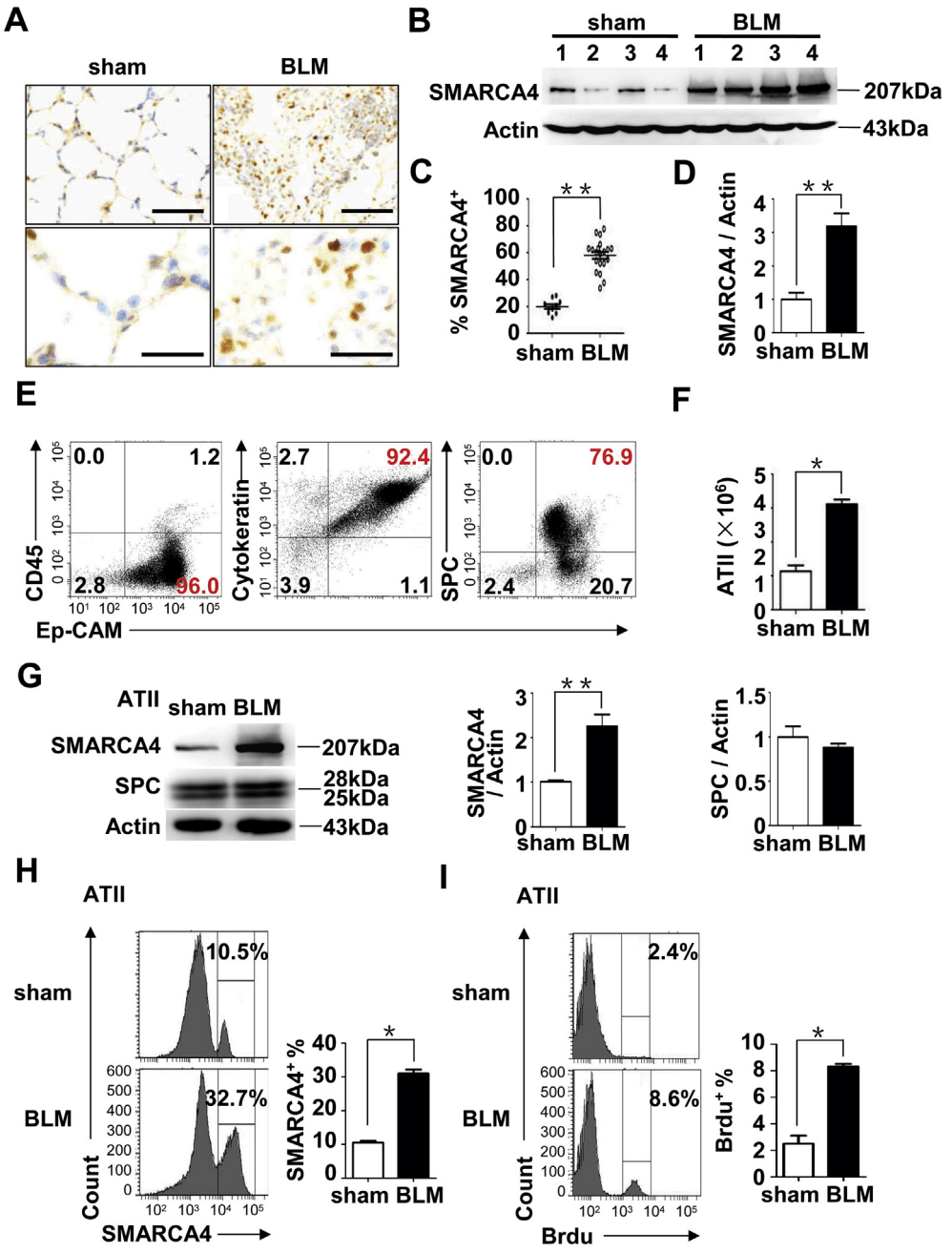

Figure 1 Elevation of SMARCA4 expression in the lung epithelium of BLM induced pulmonary fibrosis mice. (**A**) Representative images of immunohistochemistry for SMARCA4 (brown) of lung sections at 21 days post- saline (sham, $n = 10$) or 5 mg/kg of bleomycin (BLM, $n = 20$) administration. Scale bars: upper panels, 50 μm; lower panels, 20 μm. Quantitative evaluation is shown in (**C**). The number of SMARCA4$^+$ cells were counted, and data were expressed as percentage of SMARCA4$^+$ cells to total number of cells in fifteen random fields. (**B**) The expression levels of SMARCA4 protein were determined by immunoblotting of the whole lung lysates. β-actin was used as a loading control. Quantitative evaluation is shown in (**D**). (**E**) Representative flow cytometry data of isolated ATII cells. Left, co-staining for CD45 and Ep-CAM; middle, co-staining for cytokeratin and Ep-CAM; right, co-staining for SPC and Ep-CAM. (**F**) Number of ATII cells recovered from mice in sham and BLM groups. (**G**) Representative images of SMARCA4 expression in the isolated ATII cells (immunoblotting). Quantitative evaluation is shown on the right. Western blots were cut before antibody exposure and therefore cropped blots are displayed. (**H**) and (**I**) Representative flow cytometry data of SMARCA4$^+$ or BrdU$^+$ cells in the isolated ATII cells. Quantitative evaluations were both shown on the right. For ATII cells, trials repeated three times. Data are presented as mean ± SEM. *$p < 0.05$. **$p < 0.01$.

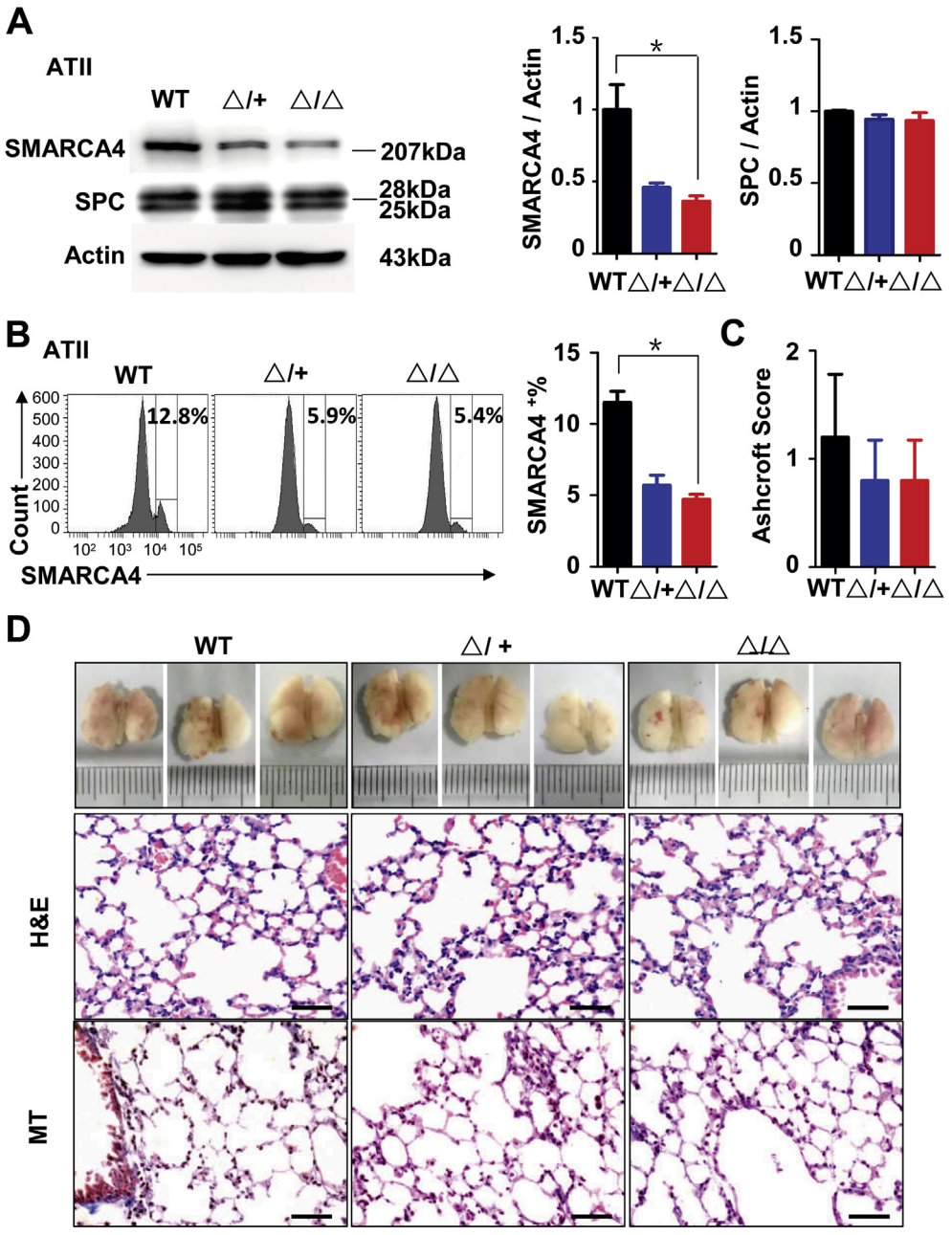

Figure 2 Pulmonary epithelial SMARCA4-deleted mice were viable and healthy. (**A**) The expression levels of SMARCA4 protein were determined by immunoblotting of the isolated ATII cells from mice with indicated genotypes after Dox treatment. β-actin was used as a loading control. Quantitative evaluations were shown on the right. Western blots were cut before antibody exposure and therefore cropped blots are displayed. (**B**) Representative flow cytometry data of SMARCA4^{+} cells in the isolated ATII cells. Quantitative evaluations were both shown on the right. Trials repeated three times. (**C**) Quantitative evaluation of the histological findings by ashcroft score. (**D**) H&E, MT staining of lung sections of *SOSM4*$^{\Delta/+}$ and *SOSM4*$^{\Delta/\Delta}$ mice and their littermates (WT) ($n = 5$ for each group.) Scale bars: 40 μm. Data are presented as mean ± SEM. *$p < 0.05$.

pulmonary fibrosis and lead to death rapidly in both of them, we reduced the dosage to 2.5 mg/kg. Then, the different responses of *SOSM4*$^{\Delta/\Delta}$ and WT mice to bleomycin were distinguishable.

After bleomycin administration, all the mice showed PF in different levels. Also, 60% reduction of SMARCA4 protein in isolated ATII cells lysates were observed in *SOSM4*$^{\Delta/\Delta}$ mice compared to their littermates (WT) (Fig. 3A), which was further confirmed by flow cytometry (Fig. 3B and C). Interestingly, we found that *SOSM4*$^{\Delta/\Delta}$ mice tend to die earlier than their littermates following bleomycin exposing (Fig. 3D). Moreover, the lung tissues of *SOSM4*$^{\Delta/\Delta}$ mice showed augmented fibrosis with histological examination compared with their littermates (Fig. 3F and G). Also, the acid-soluble lung collagen in response to bleomycin was significantly higher in *SOSM4*$^{\Delta/\Delta}$ mice compared to WT mice

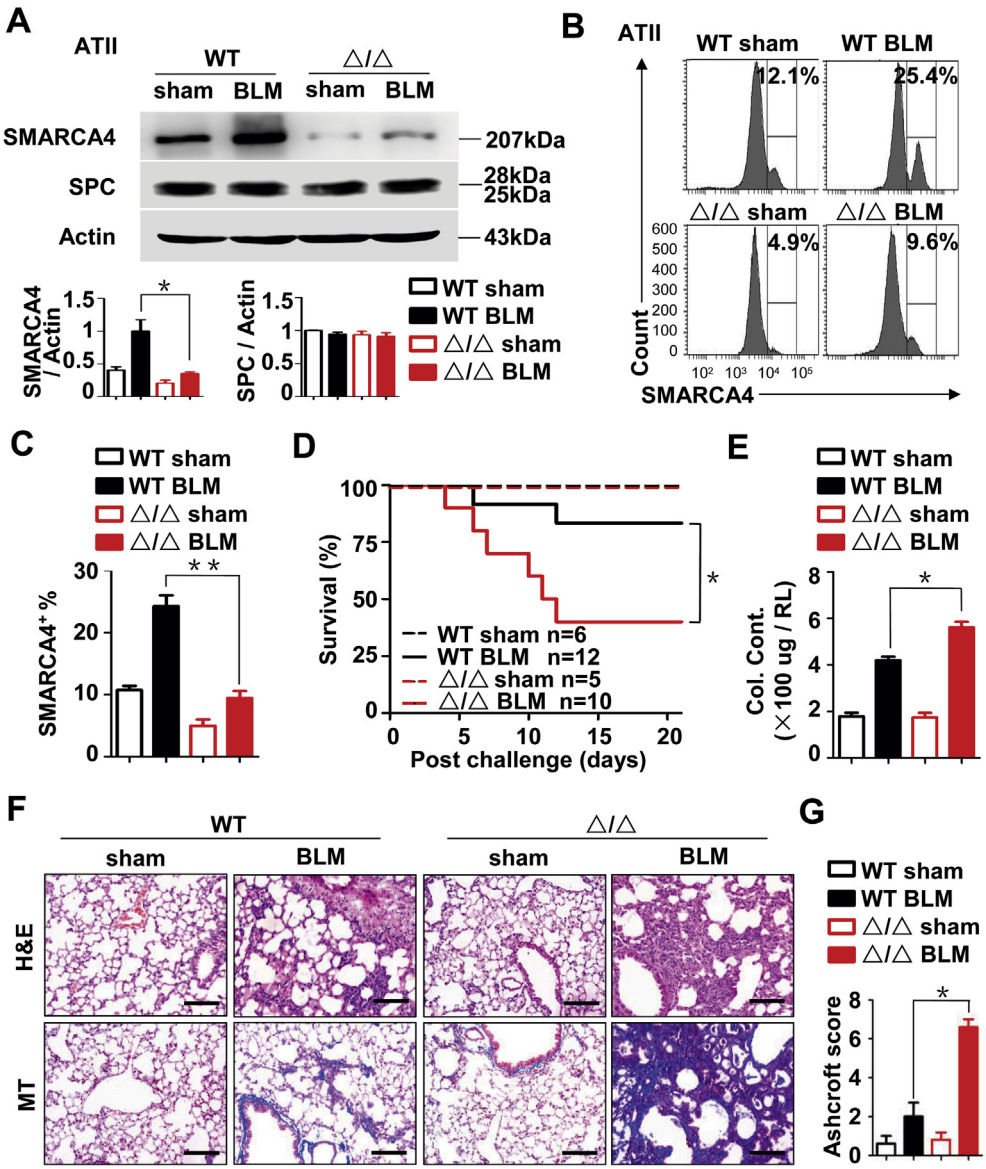

Figure 3 Epithelial SMARCA4 deficiency aggravates bleomycin-induced pulmonary fibrosis. *SOSM4*$^{\Delta/\Delta}$ mice and their littermates (WT) were fed with Dox for one week and then treated with 2.5 mg/kg BLM and sacrificed 21 days post- BLM injury. Mice treated with saline were used as control (sham). (**A**) Immunoblots of SMARCA4 protein in the lysates of isolated ATII cells. β-actin was used as a loading control. Quantitative evaluations were shown below. Western blots were cut before antibody exposure and therefore cropped blots are displayed. (**B**) Representative flow cytometry data of SMARCA4^{+} cells in the isolated ATII cells. Quantitative evaluations were shown in (**C**). Trials repeated three times. (**D**) Kaplan–Meier survival curves for *SOSM4*$^{\Delta/\Delta}$ and WT mice 21 days after saline or BLM intratracheal injection. (**E**) Collagen contents (Col. Cont.) in the right lungs (RL) assessed by Sircol assay. (**F**) Representative pictures of H&E and MT staining. Scale bars: 100 μm. (**G**) Ashcroft score of the H&E and MT staining. ($n = 5$ each group). Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

(Fig. 3E). Ultimately, these data suggested that the deletion of SMARCA4 in ATII cells could exacerbate PF induced by bleomycin in mice.

Loss of SMARCA4 reduced the proliferation of ATII cells

To future unveil the potential mechanism behind this phenotype, we explored the endoplasmic reticulum (ER) stress marked by X-box binding protein 1 (XBP1) mRNA splicing and heavy-chain Ig binding protein (BiP) accumulation, which in turn led to reactive oxygen species production from ATII cells and therefore contributed to aberrant lung remodelling followed by enhanced susceptibility of PF.[28–31] Our data showed that BiP mRNA and XBP1 splicing in whole-lung tissue samples and isolated ATII cells were similar between *SOSM4*$^{\Delta/\Delta}$ mice and their littermates (Suppl. Fig. S5). Furthermore, without bleomycin stimulation, reduction of SMARCA4 in ATII cells did not affect the

expression of SPC (Figs. 2A and 3A) that potentially underwent transcriptional inhibition[32] and was involved in ER stress.[31] Hence, there could be other mechanisms to explain the influence of SMARCA4 on the progression of PF.

It has been shown that SMARCA4 influences cell proliferation in many different diseases.[15,11,33] Considering that proliferation of ATII cells is vital for the progression of PF,[4,34,35] we further investigated the ATII proliferation ability following SMARCA4 knock-down. Notably, lower number of ATII cells was observed in injured lung sections of *SOSM4*$^{\Delta/\Delta}$ mice compared to WT mice (Fig. 4A, F upper panels, 4B, E). While, the accumulation of macrophages and myofibroblasts in impaired lung tissues were not influenced by lung epithelium-specific SMARCA4 deletion (Fig. 4A, F middle and lower panels, 4C, D). To further look insight to the affected cell functions, we detected the apoptosis and proliferation in the lung tissues by TUNEL staining and BrdU incorporation. Finally, no differences in apoptosis were observed between *SOSM4*$^{\Delta/\Delta}$ and WT mice (Suppl Fig. S7A). But, interestingly, the proliferative ability of ATII cells was stronger in *SOSM4*$^{\Delta/\Delta}$ mice compared to WT mice (Fig. 4G), which was re-confirmed by cell proliferation marker, PCNA, as well (Suppl Fig. S6B).

To examine the effect of SMARCA4 on the proliferation of ATII cells, we knocked down SMARCA4 in immortalised ATII cell line mle-12 using small interfering RNA (Suppl. Fig. S8, Fig. 5A). Our data indicated that transient SMARCA4 knock-down slowed the growth of mle-12 (Fig. 5B), possibly caused by the reduced cell proliferation according to BrdU incorporation assay and PCNA protein detection (Fig. 5A, C, D). Additionally, the cells were arrested at G0/G1 following SMARCA4 deletion (Fig. 5E). Nevertheless, no difference in mle-12 cell apoptosis was observed (Suppl. Fig. S7). In general, SMARCA4 was indispensable for the cell proliferation of ATII cells. Considering the protective effect of ATII cells on PF progression, our results indicated that ATII cells specific SMARCA4 deletion might exacerbate PF by resisting ATII cells proliferation.

The ATII cells of human IPF lung tissues showed enhancement of SMARCA4 expression

To determine whether SMARCA4 in PF mouse model is associated with human PF, we detected the expression of SMARCA4 in ATII cells from lung biopsy specimens. Consequently, we observed that SMARCA4 expression was markedly increased in ATII cells from lung sections of the individuals with PF compared to control lung tissues (Fig. 6). These observations were similar to those verified in our mouse models (Suppl. Fig. S2A, B), suggesting that the hyperplastic ATII cells were accompanied with the enhancement of SMARCA4 in lung tissues of IPF patients.

Discussion

Extensive loss of AECs from the alveolar wall leads to proliferation and activation of fibroblasts and excessive collagen deposition, which in turn induce fibrosis.[36] ATII cells, which are progenitor cells of the pulmonary epithelium, have been shown to patch the loss of AECs following lung injury.[7] Therefore investigating the mechanisms underlying the proliferation of ATII cells appears to be the key for understanding the pathogenesis of PF. In this study, we uncovered the pivotal role of epithelial SMARCA4 in PF pathogenesis. Postnatal knock-down of SMARCA4 in ATII cells resulted in exacerbated lung scarring and collagen accumulation post-injury. Furthermore, the impaired lungs of epithelial SMARCA4-knockdown mice exhibited less cell count and decreased proliferation of ATII cells. The suppression of SMARCA4 deletion on cell proliferation was confirmed on mle-12 cell line. We observed that hyperplastic ATII cells were accompanied with enhancement of SMARCA4 in human IPF lungs. In conclusion, our data indicated that epithelial SMARCA4 was essential for ATII cell proliferation following lung injury, which illustrated a novel pathogenic mechanism of PF.

To date, the exact pathophysiology of PF remains unclear. Different studies have reported the implication of epithelial and mesenchymal cells with PF. Myofibroblasts, the activated form of fibroblasts, are believed to be important in wound healing and fibrosis.[37] The extracellular matrix proteins they produce, such as collagen, have been shown to accumulate in tissues, which in turn replace normal structure required for proper organ function.[5] Macrophages, the major elevated inflammatory cells in PF, are also critical in the pathogenesis of fibrosis.[23] The effects of macrophages on PF depend on different subsets. M1 cells produce proinflammatory cytokine in order to exacerbate fibrosis, while M2 cells can ameliorate fibrotic diseases.[38] Repetitively impaired ATII cells lead to uncontrolled recruitment and activation of mesenchymal cells.[36] Furthermore, misfolding of surfactant protein A (SPA) and SPC leads to ER stress and reactive oxygen species production from ATII cells, which contributing to aberrant lung remodelling.[28–31] In the present study, we detected an aberrant expression of SMARCA4 in the lungs of PF mice and assessed the expression of the same among the three distinct cell types. Interestingly, our data indicated enhanced expression of SMARCA4 mainly in ATII cells. However, after SMARCA4 knockdown, the ER stress of ATII cells was not affected. Although the high expression of SMARCA4 in myofibroblasts was independent of PF, depletion of SMARCA4 promoted the differentiation of fibroblasts to myofibroblasts (data not shown).

SMARCA4, an essential catalytic subunit of SWI/SNF chromatin remodelling complex, is involved in many fibrotic diseases. In human hypertrophic hearts, SMARCA4 is highly activated and strongly correlated with disease severity. Mice with endothelial or myocardial SMARCA4 deletion have shown resistance to stress-induced cardiac hypertrophy and fibrosis.[11,12] Renal ischaemia-reperfusion injury has also shown to cause progressive increase and binding of SMARCA4 to proinflammatory/profibrotic genes in renal cortex.[13] Furthermore, SWI/SNF complex participates in liver regeneration and affects the pathogenesis of hepatic fibrosis.[14] However, our results suggested that SMARCA4 could serve as a protective factor in PF.

The mechanism of SMARCA4 on the progression of fibrosis are distinct in different organs. In hearts following pathological stressed, SMARCA4 chromatin remodeler and forkhead box M1 (FoxM1) transcription factor cooperate within coronary endothelial cells to trigger the Ace2-to-Ace enzyme switch, angiotensin I-to-II conversion, and cardiac

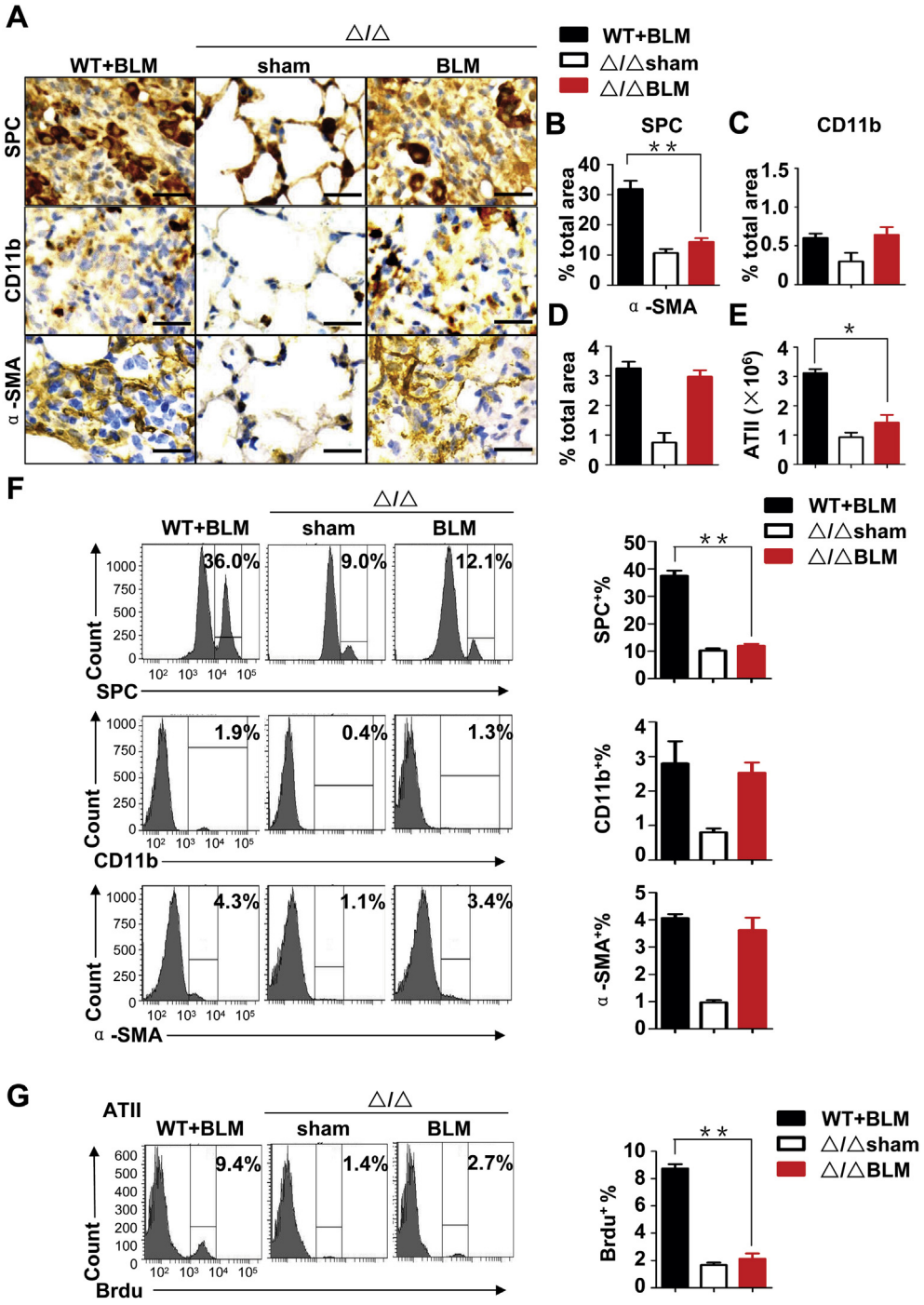

Figure 4 **SMARCA4 depletion attenuates proliferation of ATII cells *in vivo*.** WT and *SOSM4*$^{\Delta/\Delta}$ mice were treated with 5 mg/kg (for WT) or 2.5 mg/kg (for *SOSM4*$^{\Delta/\Delta}$) of bleomycin (BLM) following feeding with Dox for one week. *SOSM4*$^{\Delta/\Delta}$ mice treated with saline (sham) were used as a control. (**A**) Immunohistochemistry for SPC, CD11b and α-SMA of lung sections 21 days post- BLM administration. Scale bars: 20 μm. (**B**)–(**D**) Quantification of SPC positive areas in forty random fields, and CD11b or α-SMA positive areas in fifteen random fields respectively. ($n = 4$ in each group) (**E**) Number of ATII cells recovered from the mice 21 days post-BLM administration. (**F**) Representative flow cytometry data of SPC$^+$, CD11b$^+$ or α-SMA$^+$ cells in the lung tissue homogenates 21 days post- BLM administration. Quantitative evaluations were shown on the right separately. (**G**) Representative flow cytometry data of BrdU$^+$ cells in the isolated ATII cells 14 days post- BLM administration. Quantitative evaluations were shown on the right. Data are expressed as the mean ± SEM of three repeated trials. *$p < 0.05$, **$p < 0.01$.

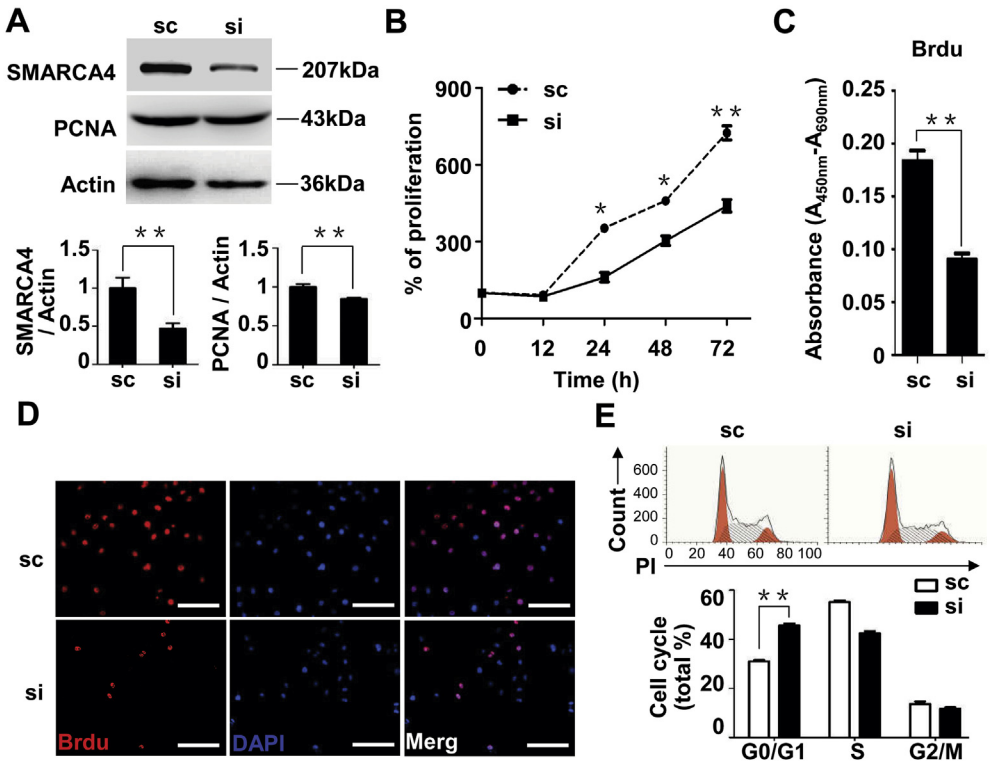

Figure 5 **SMARCA4 depletion attenuates proliferation of AEII cells *in vitro*.** (**A**) The expression of SMARCA4 and PCNA were detected by immunoblotting of cells lysates from mle-12 infected with scRNA or siRNA of SMARCA4 for 48 h. The quantitative comparisons were performed by using β-action as loading control. Western blots were cut before antibody exposure and therefore cropped blots are displayed. (**B**) The normalized proliferation curves of the mle-12 cells infected with scRNA and siRNA of SMARCA4, measured by CCK8 staining. (**C**) The mle-12 cells were transfected with scRNA or siRNA of SMARCA4 for 48 h, their viability was assessed by bromo-deoxyuridine (BrdU) incorporation. Representative BrdU immunofluorescence pictures were shown in (**D**) Scale bars: 100 μm. (**E**) Cell cycle distribution was measured by PI staining followed by flow cytometry. Data are expressed as the mean ± SEM for three trials. $^{*}p < 0.05$, $^{**}p < 0.01$.

hypertrophy and fibrosis.[12] Moreover, in response to angiotensin II stimulation, SMARCA4 and H3K4 methylation complex (Ash2/Wdr5) are recruited to the endothelin (ET-1) promoter region in endothelial cells to enhance the transcription of ET-1, and consequently induce cardiac hypertrophy and fibrosis.[10] Focusing on the cardiomyocytes, SMARCA4 is activated by cardiac stresses and forms a complex with histone deacetylase (HDAC) and poly (ADP ribose) polymerase (PARP) which in turn induces a pathological Myh6 to Myh7 shift, suppress cardiac differentiation and promote myocyte proliferation.[11] In renal ischaemia-reperfusion injury, SMARCA4 binds to the promoters of proinflammatory/profibrotic genes to accentuate the transcription of these genes.[13] In our study, deletion of SMARCA4 impairs ATII cells proliferation and aggravates PF induced by bleomycin, demonstrating a protective role of SMARCA4 in lung fibrosis.

SMARCA4 is essential for cell proliferation. For example, SMARCA4 is indispensable for the proliferation of neuroblastoma cells,[17] while it decreases the proliferation ability of the lung adenocarcinoma NCI-H522 cells.[33] Regarding non-neoplastic disease, it promotes myocyte proliferation and suppresses cardiac differentiation simultaneously.[11] Our research revealed that SMARCA4 was essential for the proliferation ATII cells, which were considered putative alveolar stem cells.[35] Contrary, Chapman et al. have shown that in the severe lung injury caused by bleomycin, integrin α6β4-positive Sftpc-negative cells generated ATII cells are not associated with the proliferation of pre-existing ATII cells.[39] Nevertheless, many different studies have supported the theory that Sftpc-positive ATII underwent clonal proliferation and generated multiple ATI and ATII cells following lung injury.[7,40] The probable mechanisms of that SMARCA4 deletion could affect the cell proliferation were different in disparate cell types. In SMARCA4-null myocardium, the Bmp10 expression was nearly abolished, while $p57^{kip2}$, a cyclin-dependent kinase inhibitor which is suppressed by Bmp10, appeared ectopically and finally caused proliferation reduction.[11] In SMARCA4-deleted neuroblastoma cells, the expression of several key genes related to cell growth and proliferation, such as PI3KCA and BCL2, were impacted.[17] Nonethless, the molecular mechanisms of SMARCA4 deletion in ATII cells that might explain the phenotype of the transgenic mice were not involved in this article and need to be further explored.

In the present study, hyperplastic ATII cells were found in the lung tissues of IPF patients and mice models, as other reports.[36] The hyperplastic ATII cells is often observed in PF

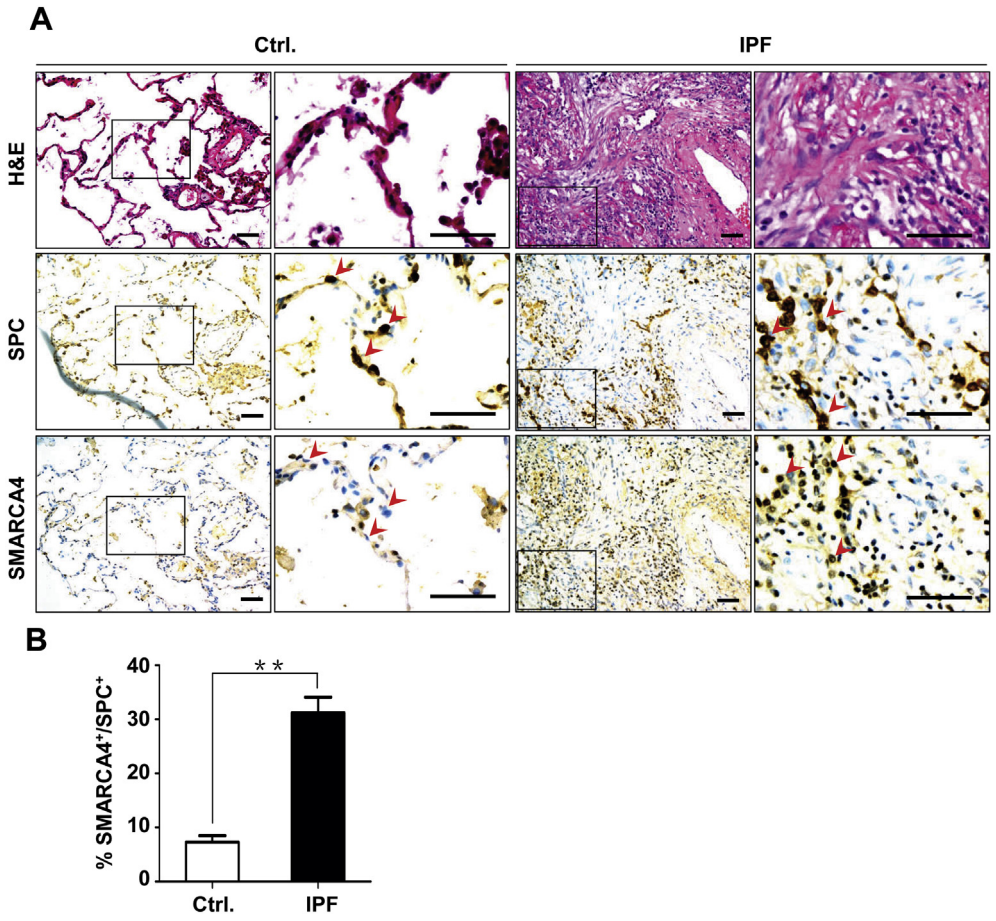

Figure 6 ATII cells of human IPF lung tissues showed enhancement of SMARCA4 expression. (**A**) Representative images of H&E and immunohistochemistry for SMARCA4 and SPC of lung serial sections from donors and IPF patients. Right panels are magnified view of the squares in the left panels. In control tissues (Ctrl.), only subtle expressions of SMARCA4 protein are detected in the SPC^+ cells (panels on the second column, arrows). In IPF, SPC and SMARCA4 double-positive cells are visible (panels on the last column, arrows). Scale bars: 50 μm. (**B**) The ratios of $SMARCA4^+$ in ATII cells (SPC^+) in fifteen random fields were calculated. Data are the mean ± SEM of five patients in each group. $^{**}p < 0.01$.

due to further arrested differentiation to other functional lung cells.[27] A plenty of genes are involved in the process of proliferation and differentiation, and SMARCA4 is one of the vast genes. Increased expression of SMARCA4, as VEGF-A_{165}b,[41] might be compensatory increase during IPF process. The mechanism and function of this compensatory increase in IPF should be further investigated.

Overall, we demonstrated that SMARCA4 in ATII cells was vital for lung fibrosis, by mediating the proliferation of ATII cells, which provides new insights into the pathogenesis of pulmonary fibrosis.

Conflict of interest

The authors declare none competing financial interest.

Acknowledgements

This work was supported by the National Natural Science Foundation Committee of China [grant numbers 81373444, 81570142, 81670018]; the Chinese Ministry of Science and Technology [grant number 2016YFA0101300]; and the Key Grant from the Chongqing Science and Technology Commission [grant number cstc2014yykfC10003].

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.gendis.2017.10.001.

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