

The profibrotic effect of downregulated Na,K-ATPase β 1 subunit in alveolar epithelial cells during lung fibrosis

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Abstract. Idiopathic pulmonary fibrosis (IPF) is a chronic progressive interstitial lung disease characterized by progressive lung scarring and excessive extracellular matrix deposition. When stimulated, alveolar epithelial cells (AECs) are aberrantly activated, the expression of profibrotic molecules is enhanced, and lung fibrosis is promoted, but the mechanism for this is unclear. It has been reported that a downregulation of the Na,K-ATPase β 1 subunit in renal epithelial cells is involved in renal fibrosis development, but the role of this protein in lung fibrosis remains unknown. In the present study, the expression of the Na,K-ATPase β 1 subunit was revealed to be markedly decreased in AECs of patients with IPF and a bleomycin-induced pulmonary fibrosis mouse model. Treatment with transforming growth factor β -1 led to significantly downregulation of the Na,K-ATPase β 1 subunit in lung adenocarcinoma A549 cells. Furthermore, the knock-down of the Na,K-ATPase β 1 subunit in A549 cells resulted in the upregulation of profibrotic molecules, activation of the neurogenic locus notch homolog protein 1 and extracellular signal-regulated kinase 1/2 signaling pathways and induction of endoplasmic reticulum stress. These findings reveal that the downregulation of the Na,K-ATPase β 1 subunit enhances

the expression of profibrotic molecules in AECs and may contribute to IPF pathogenesis.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a prevalent and progressive fatal fibrotic lung disease with few available effective therapies (1-4). It mainly occurs in elderly adults with a median survival time of 2-3 years (5,6), and the etiology of IPF remains unclear. A prevailing hypothesis for IPF pathogenesis is that abnormal wound healing in response to ongoing alveolar epithelial microinjuries causes fibroblast activation and excess extracellular matrix deposition, ultimately resulting in lung damage (7-9).

Alveolar epithelial cells (AECs) serve a crucial role in IPF pathogenesis (10). Persistent microinjuries to AECs are thought to be a trigger of lung fibrosis. The origins of lung injury are often varied and complex. Exposure to smoke, various types of dust, gastroesophageal reflux and viral infection can induce AEC injury (11-15). Rebuilding AECs is a key component of normal wound healing following injury. This requires a carefully programmed response, including the proliferation and migration of type II AECs. However, type II AECs isolated from the lungs of patients with IPF are aberrantly activated with increased collagen, α -smooth muscle actin (α -SMA) and fibronectin, and decreased expression of E-cadherin (16). The role of epithelial-mesenchymal transition (EMT) in lung fibrosis remains controversial (17,18). Furthermore, AEC senescence, endoplasmic reticulum (ER) stress, fibroblast resistance to apoptosis, insufficient autophagy, ubiquitination dysfunction, abnormal macrophage activation, gene mutation and epigenetic changes are involved in IPF development (19-25).

The Na,K-ATPase β 1 subunit has been reported to be involved in organ fibrosis. Rajasekaran *et al* (26) demonstrated that Na,K-ATPase β 1 subunit expression is significantly decreased in renal fibrotic tissues. The knockdown of this subunit in porcine kidney LLC-PK1 cells induced EMT, as did its downregulation in retinal pigment epithelial cells (27).

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Na,K-ATPase, also known as a sodium pump, transports 3 Na⁺ and 2 K⁺ ions in opposite directions across the cell membrane to maintain osmotic equilibrium. This protein pump is composed of 3 subunits, α , β and γ . The functional α subunit has 4 isoforms (α 1, α 2, α 3 and α 4), whereas the β (β 1, β 2 and β 3) and γ (isoforms 1-7) subunits are regulatory (28). Additional functions of Na,K-ATPase have been identified in the regulation of cell proliferation, cell motility, and apoptosis (29,30).

In the present study, the expression of Na,K-ATPase β 1 subunit was revealed to be decreased in AECs of patients with IPF and in a bleomycin-induced pulmonary fibrosis mouse model. Based on this observation, the role of the downregulation of the Na,K-ATPase β 1 subunit in AECs during lung fibrosis was investigated.

Materials and methods

Tissue samples from patients. Lung tissue samples from 13 patients with IPF and 5 healthy donors (all male; age 51.08±10.03 years) were obtained from the China-Japan Friendship Hospital (Beijing, China) during surgical lung biopsy and lung transplantation for inclusion in the present study. The diagnosis of IPF was based on the 2011 American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Association Guidelines for Diagnosis and Management (5). All patients provided signed consent and the study was approved by the Ethics Committee of the China-Japan Friendship Hospital (approval no. 2017-25-1).

Animal model. C57BL/6N mice (50 mice; male; 7-8 weeks old; 22-24 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were maintained at a controlled temperature of 24±1°C with a 12/12 h light-dark cycle, and were fed a standard diet. Water was freely available. The mice were randomly divided into 2 groups: For the model of pulmonary fibrosis, a dose of 2 mg/kg bleomycin (Nippon Kayaku Co., Ltd., Tokyo, Japan) was intratracheally administered, and the control mice were injected intratracheally with the same volume of saline. The bleomycin and saline were administered only once. The mice were euthanized on day 21 with an intraperitoneal injection of 1% pentobarbital sodium (100 mg/kg animal weight). This study was approved by the Animal Ethics Committee of China-Japan Friendship Hospital (Beijing, China).

Immunohistochemistry and immunofluorescence. The preparation of the human and mouse lung specimens for histology was performed as previously described (23,31). Briefly, the samples were dehydrated, paraffin-embedded, and cut into 4- μ m sections. The tissue sections were deparaffinized and rehydrated. Following a microwave treatment for 20 min in EDTA buffer and subsequent cooling, the endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 15 min in the dark. Following blocking in 5% goat serum (OriGene Technologies, Inc., Beijing, China) for 20 min, the sections were incubated with antibodies against the Na,K-ATPase β 1 subunit (cat. no. ab193669; 1:600 dilution) and fibronectin (cat. no. ab2413; 1:500 dilution) (both Abcam, Cambridge, UK) overnight at 4°C as described previously (32),

the samples were observed using an optical microscope (magnification, x100) and were analyzed using Aperio Imagescope version 12.0 software (Leica Microsystems, Ltd., Milton Keynes, UK).

For immunofluorescence, the mouse lung tissue sections (4 μ m) were de-paraffinized, hydrated using xylene, 100, 95, 85 and 70% ethanol, and PBS solution. The non-specific binding was blocked with 10% goat serum, and the samples were incubated overnight at 4°C with the desired primary antibodies against the Na,K-ATPase β 1 subunit (cat. no. ab2873; 1:500 dilution) and prosurfactant protein C (cat. no. ab90716; 1:4,000 dilution) (both Abcam), and then incubated with a specific fluorescence-conjugated secondary IgG (fluorescein isothiocyanate-conjugated, cat. no. ZF-0311; rhodamine B isothiocyanate-conjugated, cat. no. ZF-0313; both 1:100 dilution; OriGene Technologies, Inc.) for 1 h in a light-protected chamber at room temperature. Subsequently, the sections were counterstained with DAPI (cat. no. P0131; Beyotime Institute of Biotechnology, Haimen, China) at room temperature and immunofluorescence signals were detected immediately using fluorescence microscopy (magnification, x100).

Cell culture and small interfering (si)RNA transfection. Human lung carcinoma epithelial A549 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). A549 is a human AEC line with similar characteristics to type II AECs. It has been used as a stable AEC line in a number of studies (33,34). The cells were maintained in RPMI-1640 medium with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (both Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). Transforming growth factor β -1 (TGF- β 1; 10 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA) was added to subconfluent cultures and the same volume of citric acid was added to the control cells. The cells were maintained in a humidified incubator at 37°C in 95% air (21% O₂) and 5% CO₂.

The A549 cells were seeded in 6-well plates and incubated overnight. Na,K-ATPase β 1 subunit siRNA (5 μ M; sequence, 5'-AAUGUUCUCACCGUACGCCtt-3') and negative control siRNA (5 μ M; cat. no. 4390843; Silencer[®] Select Negative Control; Thermo Fisher Scientific, Inc.) were separately mixed with Lipofectamine[®] 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and Opti-MEM medium (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cells were incubated for 24 h for the measurement of RNA levels and 48 h for cell morphology observation under an optical microscope and protein detection experiments.

RNA purification and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. As previously described (35), total RNA was isolated from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RT was performed on 1 μ g total RNA with oligo(dT) primers in 25- μ l reactions using the Omniscript RT kit (Tiangen Biotech Co., Ltd., Beijing, China) at 37°C for 60 min according to the manufacturer's instructions. The qPCR was performed on an ABI 7500 instrument (Applied Biosystems; Thermo Fisher

Scientific, Inc.) using SYBR-Green PCR reagents (Tiangen Biotech Co., Ltd.). The thermocycling conditions used were as follows: Initial denaturation at 94°C for 2 min and 40 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 20 sec and extension at 69°C for 35 sec. The primers used were: β -actin forward, 5'-AGGCCAACCGTGAAAAGATG-3'; and reverse, 5'-AGAGCATAGCCCTCGTAGATGG-3'; and Na,K-ATPase β 1 subunit forward, 5'-ATGTGCCCAGTGAACCGAAA-3'; and reverse, 5'-TCCAGAGCAATTTCCAGCC-3'. The relative expression of the target gene was calculated using the $2^{-\Delta\Delta C_q}$ method (36), normalized to the levels of β -actin.

Protein extraction and western blot analysis. Total cell lysates were obtained using radioimmunoprecipitation assay buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) containing 1:100 phenylmethylsulfonyl fluoride, phosphatase inhibitors and protease inhibitor. The cell lysates were resuspended in protein loading buffer containing 5% mercaptoethanol. The protein concentration was determined using a bicinchoninic acid assay kit. Western blotting was performed as previously described (23). The denatured proteins (20 μ g per lane) were separated by 10% SDS-PAGE using a Mini-Protein electrophoresis module assembly (both Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 80 mV and transferred to nitrocellulose membranes (Merck KGaA, Darmstadt, Germany) for 100-120 min using the Mini Trans-Blot electrophoresis transfer cell (Bio-Rad Laboratories, Inc.) at 300 mA, according to the molecular weight. The primary antibodies used were anti-human and mouse α -SMA (cat. no. ab124964; 1:5,000 dilution), fibronectin (cat. no. ab2413; 1:2,000 dilution), β -actin (cat. no. ab6267; 1:3,000 dilution), Na,K-ATPase β 1 subunit (cat. no. ab2873; 1:600 dilution) (all Abcam), cleaved Notch1 (cat. no. 4147T), extracellular signal-regulated kinase (ERK)1/2 (cat. no. 9101), phosphorylated ERK1/2 (cat. no. 8544), and immunoglobulin heavy chain-binding protein (BiP, cat. no. 3177T) (all 1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA). The membranes were incubated with the primary antibodies overnight at 4°C and treated with IRDyeCW800 (green)- or IRDyeCW800 (red)-conjugated affinity purified anti-rabbit (cat. no. 925-32211) or anti-mouse (cat. no. 925-32210) IgG (both 1:15,000 dilution; LI-COR Biosciences, Lincoln, NE, USA). The intensity of the bands was evaluated using a LI-COR Odyssey infrared double-fluorescence imaging system (LI-COR Image Studio Software version 4.0; LI-COR Biosciences).

Statistical analysis. Data are expressed as mean \pm standard error of the mean. Two-tailed Student's t-test was performed for the comparison of mRNA and protein expression levels. The statistical analyses were performed using the Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate statistically significant differences.

Results

Na,K-ATPase β 1 subunit expression is downregulated in lung fibrosis. To examine the Na,K-ATPase β 1 subunit expression level in lung fibrosis, immunohistochemistry was performed on

lung tissue sections from patients with IPF. The Na,K-ATPase β 1 subunit was mainly expressed in the cytoplasm of AECs. The staining of this subunit was visibly diminished in the fibrotic and lesion-adjacent areas compared with that in the healthy lung tissue (Fig. 1A). Na,K-ATPase β 1 subunit expression was also investigated in tissue from a bleomycin-induced pulmonary fibrosis mouse model. The results of the western blot and immunohistochemistry analyses demonstrated that Na,K-ATPase β 1 subunit expression was markedly decreased in the lung tissue of the bleomycin group compared with that of control (Fig. 1B and C).

Na,K-ATPase β 1 subunit expression in type II AECs in mouse lung tissue. In contrast to the human lung tissue, the immunohistochemistry of the mouse lung tissue revealed that not all AECs express the Na,K-ATPase β 1 subunit (Fig. 1B). Therefore, immunofluorescence was used to identify which type of AECs express Na,K-ATPase β 1 subunit in mouse lung. As displayed in Fig. 2, the Na,K-ATPase β 1 subunit was primarily expressed in the cell membrane of type II AECs.

TGF- β 1 treatment decreases Na,K-ATPase β 1 subunit expression in A549 cells. TGF- β 1 is an important profibrotic cytokine. To assess the level of Na,K-ATPase β 1 subunit in TGF- β 1-stimulated AECs, total protein was extracted from the cells following treatment with 10 ng/ml TGF- β 1 for 48 and 72 h. The results indicated that the treatment led to a significant decrease in the protein expression of Na,K-ATPase β 1 subunit at the two tested time points (Fig. 3).

Knockdown of Na,K-ATPase β 1 subunit mediates changes in the cell morphology of A549 cells. Due to the observed significant difference between the expression of Na,K-ATPase β 1 subunit in lung fibrosis and normal lung samples, the effects of the downregulation of the Na,K-ATPase β 1 subunit on AECs was explored. The Na,K-ATPase β 1 subunit expression in A549 cells was knocked down using siRNA interference. As demonstrated in Fig. 4A, Na,K-ATPase β 1 subunit mRNA was significantly decreased 24 h post-transfection, as were the protein expression levels at 48 h (Fig. 4B and C). In addition, the knockdown of Na,K-ATPase β 1 subunit resulted in an altered spindle morphology in the A549 cells (Fig. 4D).

Knockdown of the Na,K-ATPase β 1 subunit promotes the upregulation of profibrotic proteins in A549 cells. To further investigate the role of the Na,K-ATPase β 1 subunit in AECs during lung fibrosis, the expression of fibrosis-associated proteins fibronectin, α -SMA and E-cadherin was examined in A549 cells following siRNA silencing of the Na,K-ATPase β 1 subunit. The results revealed that the fibronectin and α -SMA levels were increased, but E-cadherin expression was not significantly altered, compared with that in the cells transfected with NC-siRNA (Fig. 5).

Knockdown of the Na,K-ATPase β 1 subunit activates ERK1/2 and neurogenic locus notch homolog protein 1 (Notch1) signaling and induces ER stress. The downstream signaling pathway of the Na,K-ATPase β 1 subunit was investigated. As observed in Fig. 6A, phosphorylated ERK1/2 and cleaved Notch1 were significantly upregulated in cells with

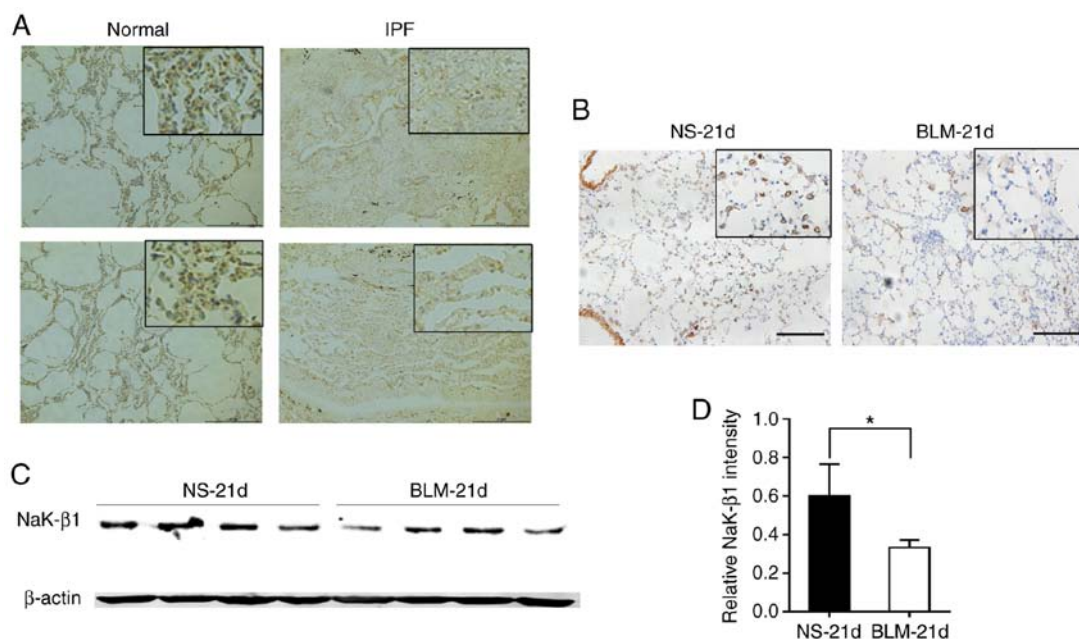


Figure 1. NaK- β 1 expression in lung fibrosis. (A) Representative immunohistochemistry images of NaK- β 1 staining in lung tissue sections from patients with IPF. Original magnification, x100; scale bar, 20 μ m. (B) Representative immunohistochemistry of NaK- β 1 staining in lung tissue samples from a bleomycin-induced pulmonary fibrosis mouse model. Original magnification, 100x; scale bar, 10 μ m; upper right panel scale bar, 1 μ m. (C) Western blot analysis of protein extracted from lung tissue of a BLM-induced pulmonary fibrosis mouse model. (D) Relative quantification of the western blot band intensities. Data are expressed as the mean \pm standard error of the mean (n=4 mice per group). *P<0.05. IPF, idiopathic pulmonary fibrosis; NaK- β 1, Na,K-ATPase β 1 subunit; NS, saline-administered control group; BLM, bleomycin.

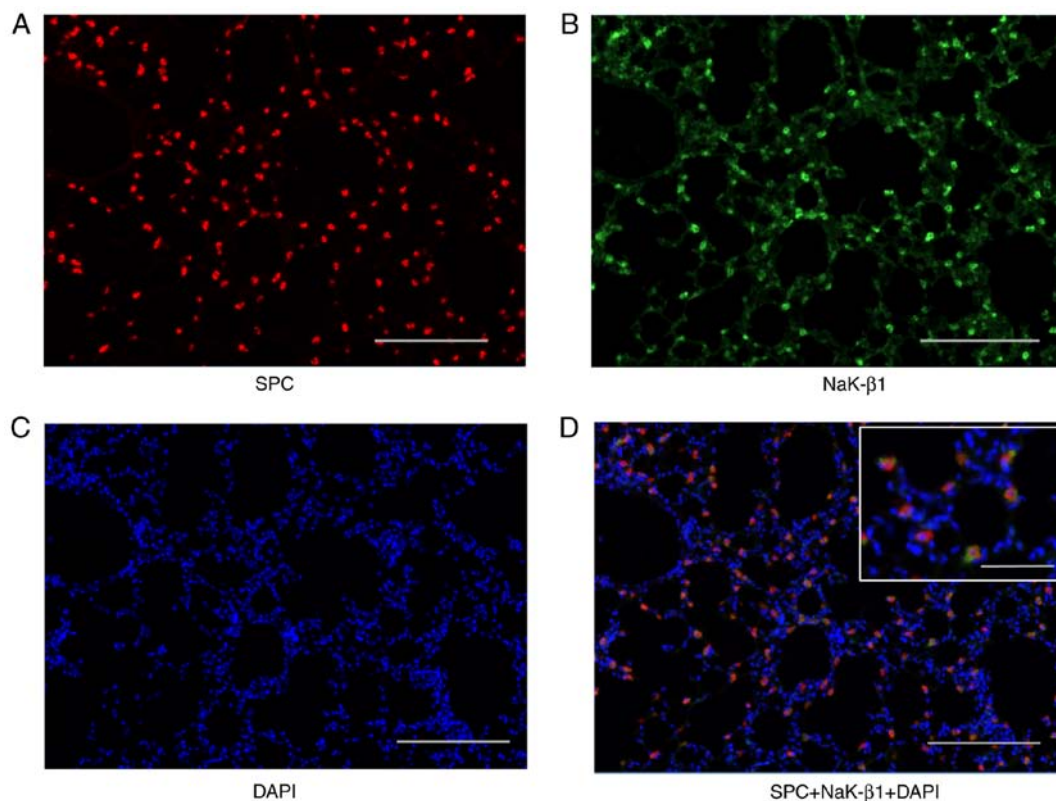


Figure 2. NaK- β 1 expression in type II AECs in mouse lung tissues. Representative immunofluorescence images of (A) SPC (red), (B) NaK- β 1 (green) and (C) nuclei (blue) in mouse lung tissue. (D) Merged image of the three types of staining. Original magnification, x100; scale bar, 10 μ m; upper right panel scale bar, 1 μ m. NaK- β 1, Na,K-ATPase β 1 subunit; SPC, surface protein C.

Na,K-ATPase β 1 subunit silencing, suggesting that a deficiency of this protein may lead to the activation of the ERK1/2 and

Notch1 signaling pathways, contributing to lung fibrosis. In addition, the knockdown of Na,K-ATPase β 1 subunit resulted

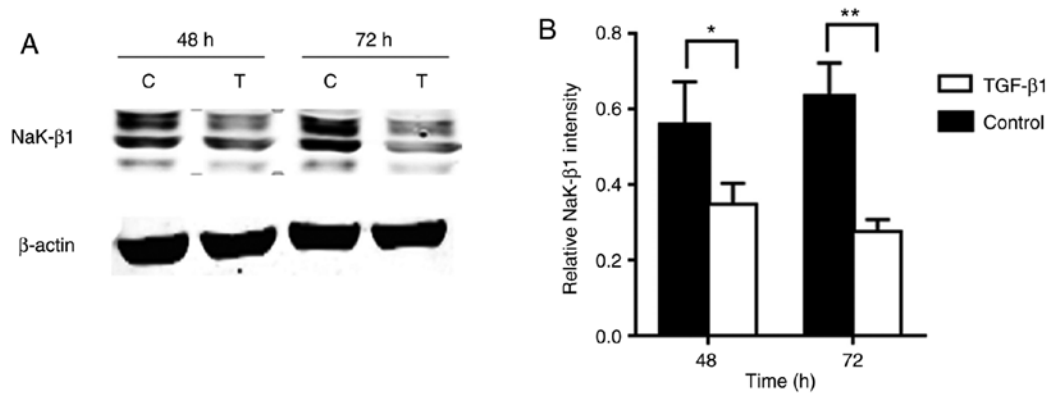


Figure 3. TGF- β 1 treatment decreases NaK- β 1 expression in A549 cells. (A) Representative image of a western blot analysis of NaK- β 1 from A549 cells treated with TGF- β 1 or citric acid for 48 or 72 h. (B) Relative quantification of the western blotting results. The data are expressed as the mean \pm standard error of the mean. *P<0.05; **P<0.01. NaK- β 1, Na,K-ATPase β 1 subunit; TGF- β 1, transforming growth factor β -1.

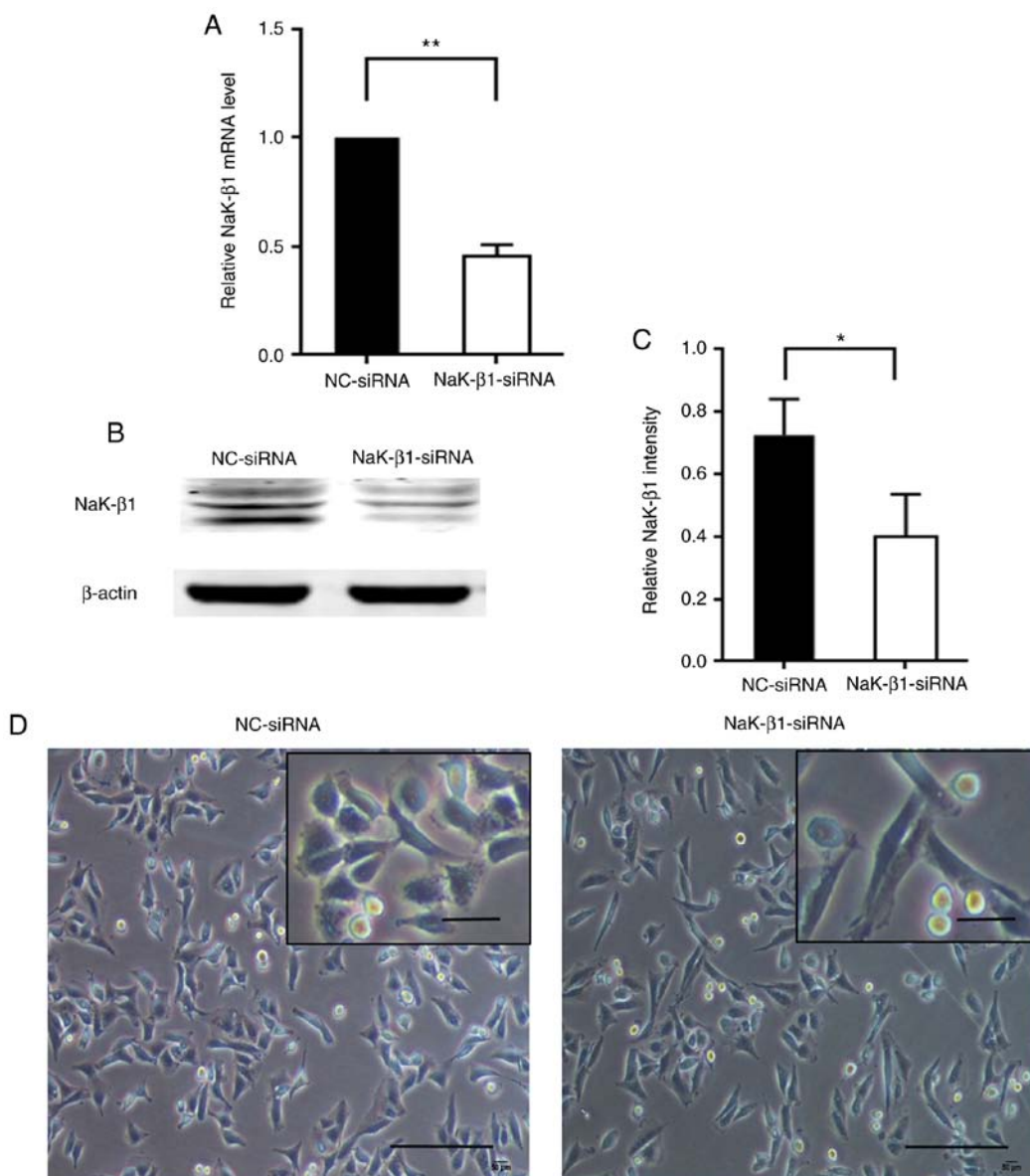


Figure 4. Knockdown of NaK- β 1 mediates spindle morphology changes in A549 cells. (A) Relative NaK- β 1 mRNA levels 24 h post-transfection with siRNA. (B) Western blot analysis of NaK- β 1 protein expression levels 48 h post-transfection. (C) Relative quantification of the western blotting results. The data are expressed as mean \pm standard error of the mean. *P<0.05; **P<0.01. (D) Representative images indicating the morphology of A549 cells transfected with NaK- β 1- and NC-siRNA for 48 h. Original magnification, x20; scale bar, 50 μ m; upper right panel scale bar, 5 μ m. NaK- β 1, Na,K-ATPase β 1 subunit; NC, negative control; siRNA, small interfering RNA.

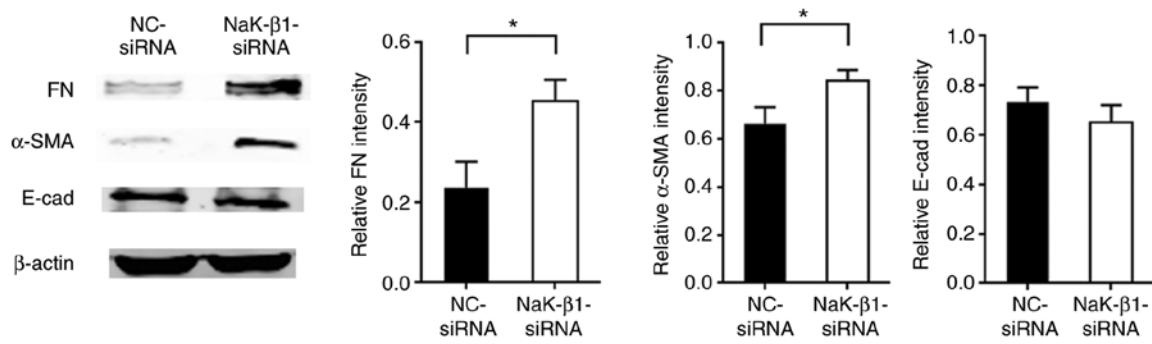


Figure 5. Knockdown of NaK- β 1 upregulates profibrotic proteins in A549 cells. Fibrosis-associated proteins FN, α -SMA and E-cad expression levels were investigated 48 h after A549 cells were transfected with NaK- β 1- and NC-siRNA, using western blot analysis. Representative western blot images and relative quantification of the results are indicated. The data are expressed as the mean \pm standard error of the mean. * P <0.05. NaK- β 1, Na,K-ATPase β 1 subunit; FN, fibronectin; α -SMA, α -smooth muscle actin; E-cad, E-cadherin; NC, negative control; siRNA, small interfering RNA.

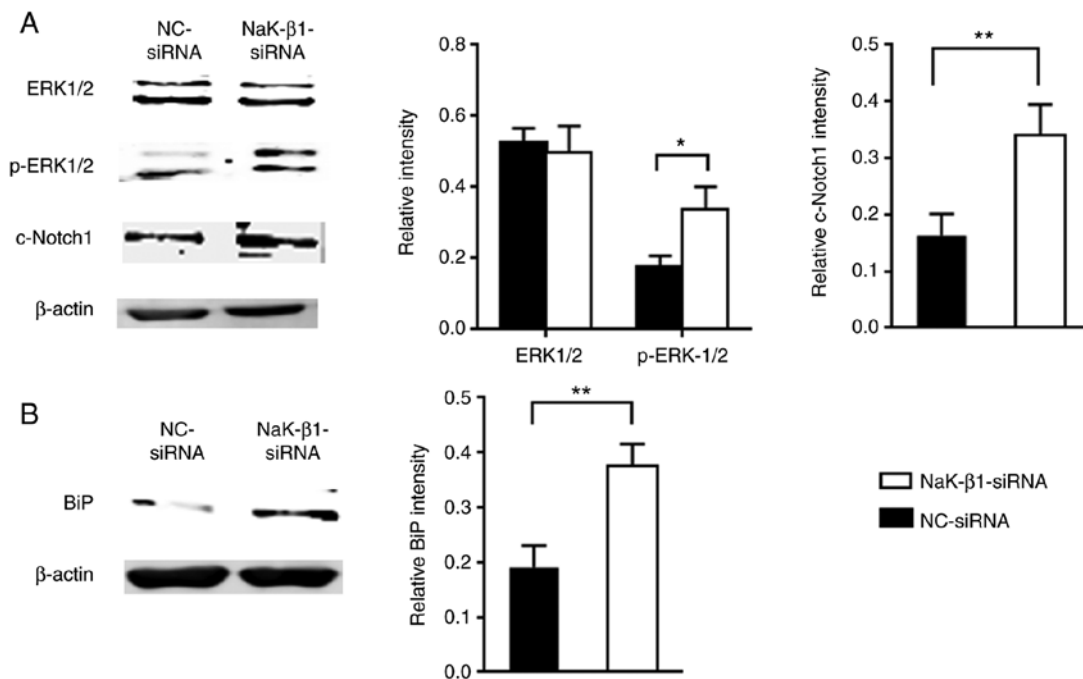


Figure 6. NaK- β 1 knockdown activates ERK1/2 and Notch1 signaling, inducing ER stress in A549 cells. Western blot analysis and relative quantification of (A) ERK1/2, p-ERK1/2 and cleaved Notch1 levels and (B) BiP levels in A549 cells transfected with NaK- β 1- and NC-siRNA. The data are expressed as the mean \pm standard error of the mean. * P <0.05; ** P <0.01. NaK- β 1, Na,K-ATPase β 1 subunit; ERK, extracellular signal-regulated kinase; p-ERK1/2, phosphorylated ERK1/2; Notch1, neurogenic locus notch homolog protein 1; c-Notch1, cleaved Notch1; BiP, immunoglobulin heavy chain-binding protein; NC, negative control; siRNA, small interfering RNA.

in a significant increase in the expression of BiP, an ER-stress protein marker (Fig. 6B), suggesting that downregulation of this ion pump causes ER stress in A549 cells.

Discussion

IPF is a chronic and lethal interstitial lung disease. It is generally accepted that the initial progression of IPF is stimulated by the aberrant activation of AECs in response to repetitive microinjury. In the present study, the Na,K-ATPase β 1 subunit protein expression was revealed to be downregulated in lung fibrosis, mainly in AECs, enhancing profibrotic protein expression, activating the ERK1/2 and Notch1 signaling pathways, and inducing ER stress, consequently leading to lung fibrosis.

The present study has demonstrated that the expression of the Na,K-ATPase β 1 subunit is different in human and mouse lung tissues. It is expressed in type I and II AECs, and located in the cytoplasm of AECs in human lungs. However, in mouse lungs, the Na,K-ATPase β 1 subunit is mainly expressed in type II AECs and is located in the cell membrane. The human Na,K-ATPase β 1 subunit exhibits two bands in immunoblot analyses, where the lower 40-kDa band represents the intracellular immature fraction of the subunit and the higher molecular weight band represents the mature plasma membrane form. However, the mouse Na,K-ATPase β 1 subunit results in only a single 42-kDa band (37).

The Na,K-ATPase β 1 subunit belongs to the N-linked glycoproteins, and as a regulatory subunit, its main function is to assist the folding of the α subunit and its transport from

the ER to the plasma membrane (38). Furthermore, the Na,K-ATPase β 1 subunit is a molecular partner of Wolframin, an ER protein involved in ER stress (39). The results of the present study indicated that the knockdown of this subunit led to the upregulation of BiP, whereas the level of DNA damage-inducible transcript 3 protein was not altered (data not shown). Over the past decades, accumulating evidence has suggested that ER stress serves an important role in the pathogenesis of lung fibrosis, as ER stress markers are highly expressed in AECs in IPF. ER stress in lung fibrosis induces AEC injury and apoptosis, causing inflammation and cell phenotype alteration (20,40-43).

The present data revealed that the knockdown of Na,K-ATPase β 1 subunit led to the enhanced expression of profibrotic proteins fibronectin and α -SMA, but no changes in epithelial marker E-cadherin were observed, suggesting that AECs undergo incomplete activation and partly maintain epithelial characteristics (44). Treatment of A549 cells with TGF- β 1 resulted in a decrease in Na,K-ATPase β 1 subunit expression, which may affect electrolyte metabolism in AECs. Further investigation is required to clarify the role of Na,K-ATPase α 1 subunit-mediated electrolyte metabolism dysfunction in the pathogenesis of lung fibrosis.

Attempts to use plasmids to overexpress Na,K-ATPase β 1 subunit in A549 cells proved unsuccessful in the present study. Ouabain, an inhibitor of Na,K-ATPase, leads to the upregulation of Na,K-ATPase β 1 subunit expression and suppresses EMT (26,45). In addition, a previous study of the present group revealed that ouabain ameliorates bleomycin-induced pulmonary fibrosis (46). Therefore, it can be inferred that this inhibitor suppresses EMT due to the upregulation of Na,K-ATPase β 1 subunit expression, providing direction of subsequent studies.

In conclusion, Na,K-ATPase β 1 subunit expression is down-regulated in clinical human IPF samples, in the lung tissue of a bleomycin-induced pulmonary fibrosis mouse model, and in TGF- β 1-stimulated lung carcinoma A549 cells. Additionally, Na,K-ATPase β 1 subunit deficiency in A549 cells upregulates profibrotic protein expression, activates ERK1/2 and Notch1 signaling pathways and induces ER stress. Therefore, the results of the present study suggest that decreased expression of Na,K-ATPase β 1 subunit in AECs serves a crucial role in the progression of lung fibrosis.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

BL, WN, HD and CW designed the experiments. BL performed the experiments and drafted the manuscript. BL, XX and XH analyzed the data. XX, WN, HD and CW revised the manuscript. All authors have read and approved the final version for publication.

Ethics approval and consent to participate

This study was approved by the Ethics Committee (approval no. 2017-25-1) and the Animal Ethics Committee (approval no. 2017-18-2) of China-Japan Friendship Hospital, Beijing, China.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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