Am J Transl Res 2018;10(7):2047-2054
www.ajtr.org /ISSN:1943-8141/AJTR0074143

Original Article

pre-B cell colony enhancing factor negatively regulates Na^+ and fluid transport in lung epithelial cells

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Received February 6, 2017; Accepted May 6, 2018; Epub July 15, 2018; Published July 30, 2018

Abstract: This study was undertaken to investigate the effect of pre-B cell colony enhancing factor (PBEF) on Na^+ and fluid transport in lung epithelial cells. Methods: Type 1 and 2 cells were isolated from lung epithelium. After hypoxia reoxygenation treatment, the primary cell cultures were transfected with a plasmid over-expressing PBEF. Sodium-potassium ATPase (NKA), epithelial sodium channel (ENaC), type I cell marker rT140, surfactant protein (SP) and PBEF protein were analyzed at mRNA and protein levels using PCR and Western blot analysis. Immunofluorescence assays showed type 1 and 2 cells were successfully isolated. After the transfection with PBEF overexpression vector, PBEF and RTI40 levels were increased, while ENaC and SP as well as NKA, were decreased in both cells. It is clear that PBEF negatively regulates the expression of ENaC and NKA in the Na^+ and fluid transport in lung epithelial cells.

Keywords: Type 1 cell, type 2 cell, pre-B cell colony enhancing factor, epithelial sodium channel (ENaC), sodium-potassium ATPase (NKA)

Introduction

In pulmonary edema, the fluid in pulmonary capillaries infiltrates into and remains in lung interstitium and alveoli, leading to excessive extravascular fluid. It is mainly caused by increased permeability or high blood pressure of alveolar capillaries [1]. Pulmonary edema is the key point leading to acute lung injury (ALI) after surgery in cardiopulmonary bypass (CPB), resulting mainly from reduced fluid clearance and increased permeability. Timely alveolar liquid clearance is the key to prevent pulmonary function disorder, reduce severe complications such as perfusion lung and ALI and improve the prognosis after CPB surgery [2]. Lung epithelium has type 1 and 2 cells. They play a crucial role in regulating the balance of alveolar fluid and the repair of lung injury [3-6]. Epithelial sodium channel (ENaC) is an important part of alveolar sodium transport and is crucial for the clearance of edema fluid, which is the rate limiting step in across-membrane sodium ion transportation and provides driving force for removal of fluid from the alveolar space. ENaC cooperates with sodium-potassium ATPase (NKA) to achieve sodium ion transportation for alveolar fluid clearance [7, 8].

Na^+ and fluid transport is mainly composed of ENaC, NKA and water channel. ENaC is the main driving force for removal of fluid from the alveolar space [9]. ENaC is a transmembrane protein, abundantly expressed in alveolar epithelial cells (AECs) and is important for lung fluid transport [10]. NKA is an membrane-bound ATPase that participates in Na^+ and fluid transport process to regulate the lung fluid clearance [11]. The pre-B cell colony enhancing factor (PBEF) promotes early differentiation of B cells and regulates the permeability of pulmonary vascular endothelial cells and AECs. Therefore, it impacts lung liquid clearance and subsequently ALI [12]. Studies have shown that PBEF promotes the release of inflammatory mediators by inhibiting the apoptosis of neutrophils in the lung, leading to increased cell permeability, pulmonary edema and lung injury [13]. Wang et al showed that insulin can regulate ENaC-mediated alveolar fluid clearance in ALI through the PI3K/Akt pathway [14]. Peng et al found that PBEF induces the translocation of

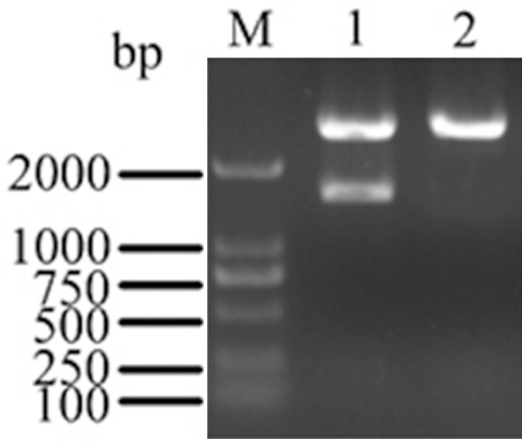

Figure 1. Restriction mapping of pUC57-PCAGGS-PBEF. Lane M: DNA size markers; lane 1: *Bam*HI and *Xba*I linearlized pUC57-PCAGGS-PBEF and lane 2: pUC57-PCAGGS.

insulin receptor in AECs, leading to insulin resistance. PBEF binds and activates insulin receptor to regulate lung fluid clearance [15]. However, little is known about how pulmonary edema and ALI are regulated by PBEF at molecular levels. In this study, we overexpressed PBEF in isolated type 1 and 2 AECs to investigate its impact on Na^+ and fluid transport. The findings would provide new clues for the prevention and treatment of pulmonary edema and ALI.

Materials and methods

Animals

Sprague Dawley rats were purchased from Slack Experimental Animals, Hunan, China and hosted under pathogen-free conditions and had access to standard rodent food and water ad libitum. Experiments were performed on rates between 7 and 10 weeks of age. This study received approval by the Animal Research Ethics Board at the Second Affiliated Hospital of Nanchang University, Nanchang, China.

Reagents and instruments

RPMI 1640 glucose medium (cat no. KGM-31800S-500) was purchased from Sijiqing Biologicals, Hangzhou, China; 0.25% trypsin (containing EDTA, cat no. 20170101) was obtained from KGI Biologicals, Beijing, China; fetal bovine serum (FBS, cat no. 155268) was purchased from BI Biologicals, Beijing, China; Gel Recovery Kit (cat no. DP209-02) was from Tiangen, Beijing, China; rabbit monoclonal anti-PBEF antibody (cat no. PB1069, 1:300 dilution) was obtained from Boster, Beijiang, China; rabbit anti-antibodies against ENaC (cat no. bs-2957R, 1:1000 dilution), SP (cat no. bs-10265R, 1:1000 dilution), SFTPA1 (cat no. bs-10265R, 1:3000 dilution) were from Bioss, Woburn, Massachusetts, USA; rabbit monoclonal anti-RT140 antibody (cat no. ab131216, 1:5000 dilution) was obtained from Abcam, Cambridge, USA; goat anti-rabbit IgG, Cy3 conjugated (cat no. CW0159, 1:200 dilution) was obtained from CWBIO, Beijing; Lipofectamine 3000 Transfection Reagent (cat. No L3000015) was purchased from Invitrogen, USA; ChemiDocTM XRS gel imaging system was from Biorad Laboratories, Shanghai, China; inverted fluorescence microscope (TE2000) was obtained from Nikon, Japan Nikon company); fluorescence microscopy (IX51) was a product of Olympus, Japan. Rat NKA ELISA detection kit (cat no. ml339670) was supplied by Mlbio, Shanghai, China.

Vector construction

The sequence of the PBEF gene was fully synthesized based on mRNA sequence available for the NCBI database (https://www.ncbi.nlm.nih.gov/) at Haijierui, Shanghai, China. *Bam*HI and *Eco*RI sites were added to the sequence to facilitate cloning. The sequence was ligated to vector pGH to generate an expression vector pCAGGS-PBEF. The vector was sequenced before use.

Isolation of AECs

1.4 AECs were isolated as previously reported [16]. Briefly, rats were anesthetized, and the lungs were perfused with sterile PBS to white and injected with 10 ml preheated PBS containing Dispase (10 U/ml) for 40 min. The digested lungs were added with 1 ml dispersion solution (RPMI 1640 medium with 20% FBS and 100 g/ml DNase 1), cut into pieces and filtered through meshes with 100 and 40 mm pore size. The cells were collected by centrifugation and responded for subsequent use.

Cell treatments

Non-treated and freshly isolated type 1 and 2 were used as control. For model, the cells were

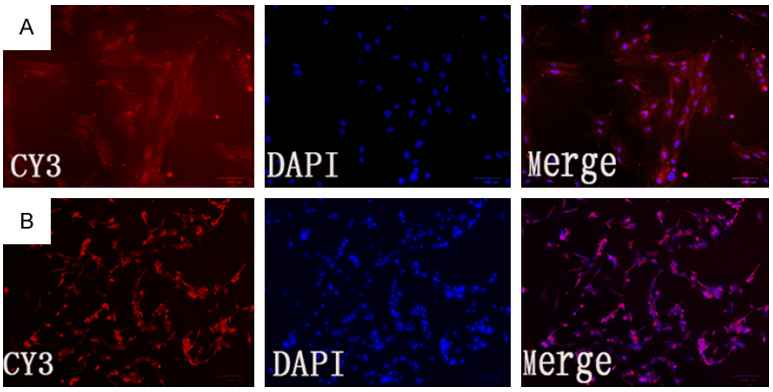

Figure 2. Detection of cell-specific marker CY3 using fluorescent-labelled CY3 antibody. A. Type 1 cell; B. Type 2 cells.

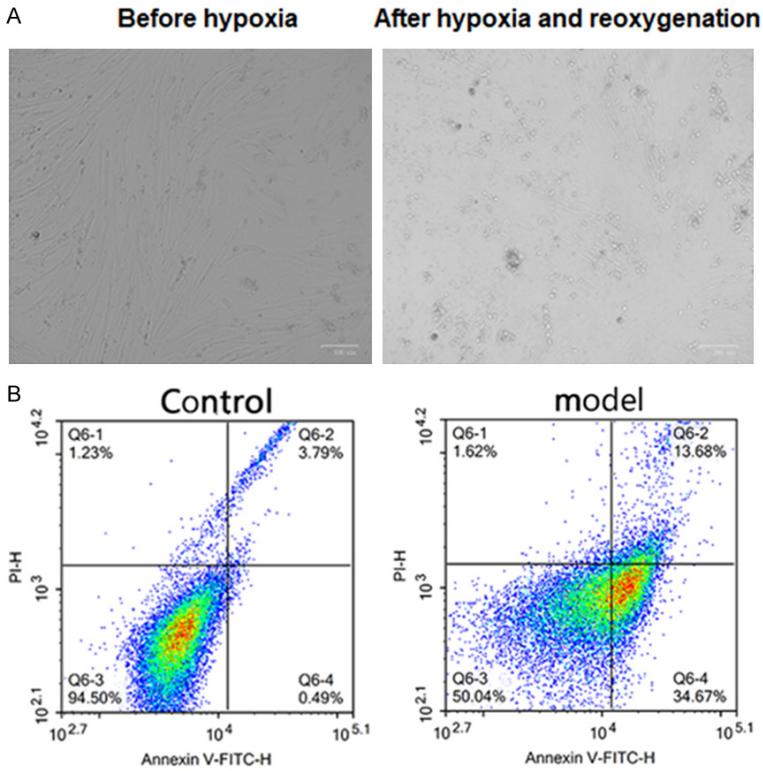

Figure 3. Morphology and apoptosis of cells undergoing hypoxia and reoxygenation treatment. A. Morphology; B. Flow cytometry

cultured in hypoxia condition for 24 h and then reoxygenated for 24 h as described [17]. The cells were then transfected with pCAGGS-PBEF or empty vector. For transfection, cells were cultured to 80% confluency and transfected using Lipofectamine according to the supplier's instructions. Transfected cells were cultured in 5% CO_2 incubator at 37°C for 4 days and then subjected to hypoxic and reoxygenated treatments as described above.

Immunofluorescence assay

The cells were allowed to grow on cover glasses, fixed in 4% paraformaldehyde and then cleared with 0.5% Triton X-100 at room temperature for 20 min. After blocked with normal goat serum at room temperature for 30 min, the slides were added with rabbit anti-SFTPA1 antibody and incubated in the dark at 4° overnight. The slides were then reacted to Cy3 conjugated goat anti-rabbit IgG at 37°C for 1 h and counted stained with DAPI for nuclei in the dark at 37°C for 5 min before image acquisition.

Fluorescent quantitative PCR

RNA was extracted from type 1 and 2 cells 96 h after transfection using Trizon Reagent and reversely transcribed into cDNA using HiFiScript first strand cDNA synthesis kit according to the manufacturer's instructions. A total of 2.5 μl of the resulting cDNA was subjected to pre-amplification using the TaqMan PreAmp Master Mix (Applied Biosystems) in a total volume of 12 μl. Non-fluorescent probes were used at 1X. Pre-amplification cycling conditions were 10 min at 95°C followed by 14 cycles, each one consisting of 15 s at 95°C and 4 min at 60°C. Later on a 1:5 dilution of the pre-amplified cDNA was performed. RT-qPCR was performed on the 7900HT Fast Real-Time PCR system using TaqMan gene expression assays probes (Applied Biosystems). Human glyceraldehyde-3-phosphate dehydrogenase, *GADPH* (Hs03929097_g1), was used as an internal control. The primers used were as follows: PBEF F: 5-CTGTTCCAGGCTATTCTGTTCCA, PBEF R: 5-ACTATCAGGTGTCTCAGGTCTTCA; SP F: 5-ATGT-CATGTGTCTTTGGCCTTC, SP R: 5-TCAAAATTCACAAACAGCCAGCC; RTI40

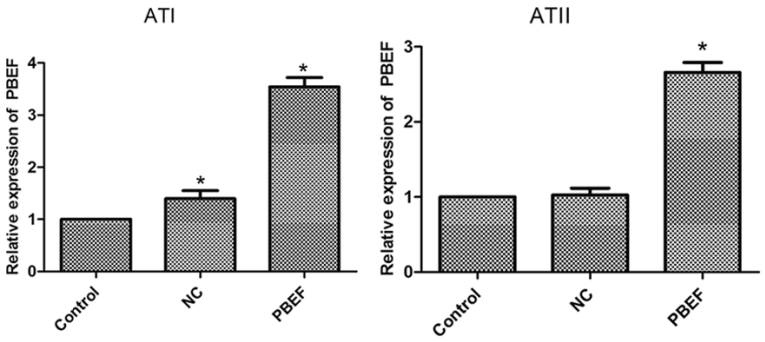

Figure 4. mRNA levels of PBEF in transfected type 1 and 2 cells.

F: 5-CAGTGTTGCTCTGGGTTTTG, RTI40 R: 5-CTATTGGGGTGAGTTGTTTTCT; ENaC F: 5-CCCAGGCACCAACCATT, ENaC R: 5-CCCCATACAAGGACAGCAA; GAPDH F: 5-GGACCTGACCTGCCGTCTAG, GAPDH R: 5-GTAGCCCAGGATGCCCTTGA.

The data were managed using the Applied Biosystems software RQ Manager v1.2.1. Relative expression was calculated by using comparative Ct method and obtaining the fold change value (2-ΔΔCt) according to previously described protocol [18].

Western blot analysis

2×10^7 type 1 and 2 cells were washed twice with cold PBS and lysed with RIPA buffer that containing protease and phosphotase inhibitors cocktail (Roche, UK). The supernatants were collected after centrifugation at 12000 rpm for 20 min. The protein was subjected to polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, and then detected by the proper primary and secondary antibodies before visualization with a chemiluminescence kit. The intensity of blot signals was quantitated using Quantity one software (v.4.62, General Electric, UK).

ATPase activity assay

ATPase activity in cell cultured was measured as described [11] using a NKA ELISA detection kit according to supplier's instruction.

Statistical analysis

All data were expressed as means ± standard error of the mean (SEM) obtained from at least three independent experiments. Statistical comparisons between experimental and control groups were assessed by using the Student's *t*-test. $P<0.05$ was considered statistically significant.

Results

pCAGGS-PBEF construction

Synthesized PBEF coding sequence was ligated to *Bam*HI and *Xba*I liberalized pUC57-PCAGGS and the recombinant plasmid was analyzed. Gel electrophoresis showed that there were two correctly sized fragments of 2710 bp and 1488 bp long, representing vector and the coding sequence, respectively (**Figure 1**). Sequencing also showed the insert sequence is correct.

Characterization of AECs

Isolated AECs were cultured for 36 h and then examined microscopically after incubated with antibody against cell-specific marker CY3. Freshly isolated type 1 and 2 cells were round- or oval-shaped (**Figure 2**) and were reactive to CY3 antibody (**Figure 2**).

Morphology and apoptosis of cell models

Before hypoxia and reoxygenation treatment, the cultured cells were intact and uniform in size. However, after the hypoxia and reoxygenation treatment, damages were seen, such as broken and irregular shape in these cells (**Figure 3A**). Flow cytometry studies showed that after the hypoxia and reoxygenation treatment, the apoptotic rate increased to 48.35% from 4.8% in the control, suggesting that the cells are damaged (**Figure 3B**).

Transfection efficiency

The cells were then transfected with pUC57-PCAGGS-PBEF to over express PBEF. PCR assays showed that after the transfection, the mRNA levels of PBEF were significantly higher in transfected type 1 and 2 cells than in untransfected cells or cells transfected with empty vector (**Figure 4**).

mRNA and protein expression

In model cells, PBEF and RTI40 were significantly up-regulated and ENaC and SP were sig-

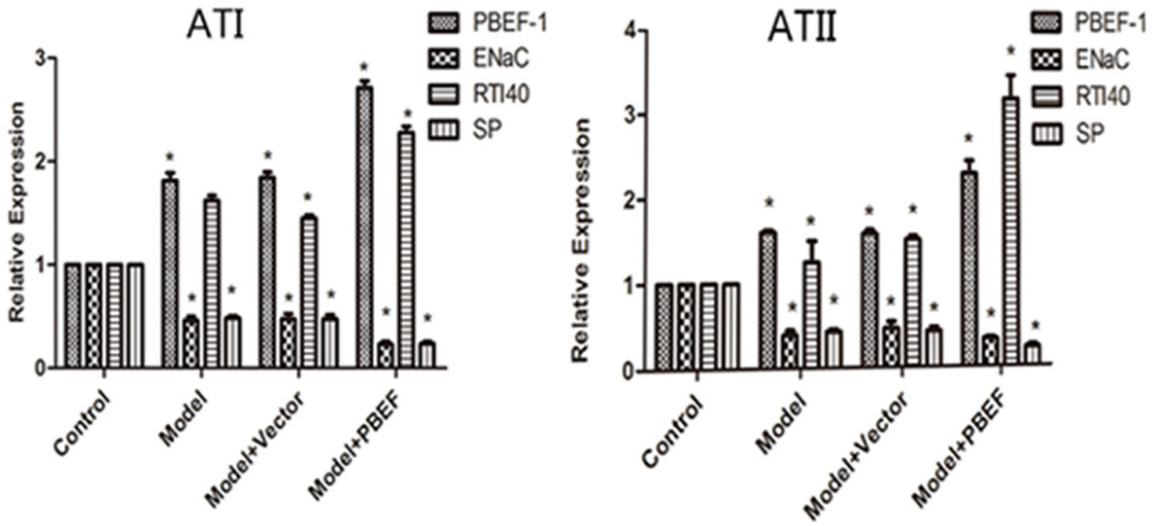

Figure 5. mRNA levels of PBEF, ENaC, RT140 and SP in RLE-6TN and R3/1 cells after transfected with pUC57-PCAGGS-PBEF. * denotes significant difference vs the control.

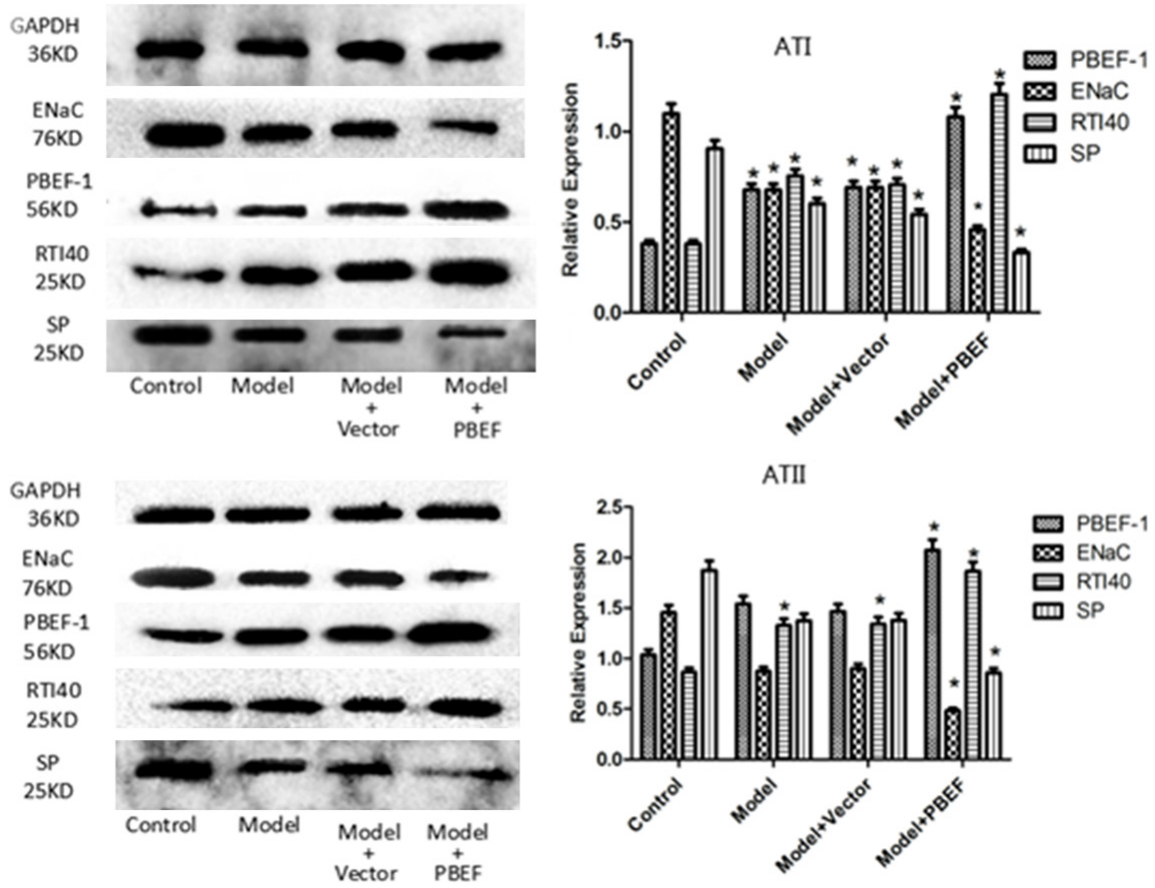

Figure 6. Protein expression of PBEF, ENaC, RT140 and SP in type 1 and 2 cells after transfected with pUC57-PCAGGS-PBEF. Left pane: representative Western blots, right pane: relative protein level. * denotes significant difference vs the control.

nificantly down-regulated (**Figure 5**) as compared with control. After transfection with pUC57-PCAGGS-PBEF, mRNA levels of PBEF and RTI40 (a type I cell-specific protein in the

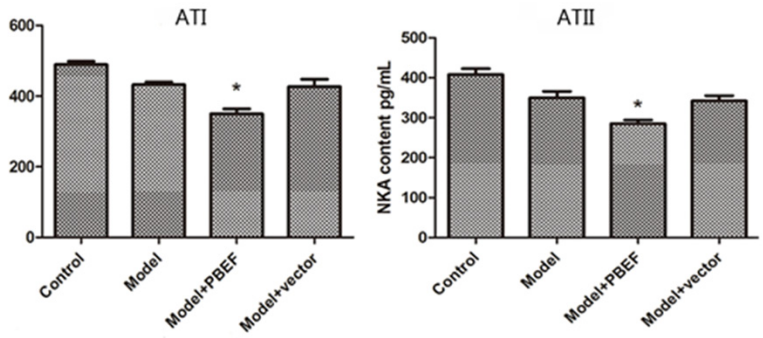

Figure 7. Level of NKA in type 1 and 2 cells after transfected with pUC57-PCAGGS-PBEF. * denotes significant difference vs the controls.

alveolar epithelium of rat lungs with bleomycin-induced fibrosis) were further increased and those of ENaC and SP further (P<0.05) in both type 1 and 2 cells (**Figure 5**). No difference was observed between empty vector-transfected cells and model cells (**Figure 5**). Similar results were observed for protein expression of these genes (**Figure 6**).

ATPase level

As shown in **Figure 7**, compared with the controls, the expression of NKA in type 1 and type 2 cells decreased significantly after the cells were transfected with pUC57-PCAGGS-PBEF (*P*<0.05).

Discussion

Edema, especially pulmonary edema, is increasingly being regarded as a surgery complication. In the postoperative care program, it is usually treated by avoiding excessive liquid drug administration and reducing inflammation and capillary permeability [19]. However, although this scheme has been widely adopted clinically, it is still a big challenge to treat postoperative complications such as ALI. PBEF has been shown to increase the permeability of lung epithelial cells which may lead to pulmonary edema [20, 21]. AECs are mainly composed of type 1 and 2 cells. Previously, type 1 cells are considered to be terminally differentiated cells that are no long capable of proliferation. The cells have few biological markers and are difficult to isolate. Therefore, they are studied using cells differentiated from type 2 cells [22, 23]. However, recent studies have found that although the number of type 1 cells is relatively fewer, they are widely distributed in the lung epithelium [24]. Moreover, both cells have various biological activities, such as ion transport and immune regulation, and play a certain role in the process of damage repair. However, their biological activities are different [25]. Therefore, it is desirable to isolate both cells for comparative study. Studies have shown that hypoxia/reoxygenation can lead to cell death, but the basic regulatory mechanism still exists in the treated cells [26]. The purpose of this work is to study the regulation of pulmonary edema by PBEF. Therefore, we used hypoxic/reoxygenation treatment to mimic lung damage. This would allow us to identify the effect of PBEF in damaged cells as it occurs in clinical conditions. We then overexpressed PBEF in isolated AECs and investigated the expression of Na^+ and fluid transport-related genes to elucidate the effect of PBEF on signal transduction in the Na^+ and fluid transport. Na^+ and fluid transport in AECs is mainly ENaC and the up-regulation of ENaC could reduce pulmonary edema [27]. SP is an alveolar surface protein which specifically binds to the surfactants secreted by AECs to protecting them from injury [28]. NKA is a sodium potassium ATPase located on outside membrane of the alveoli basolateral membrane. It consists of αandβsubunits and pumps sodium and potassium ions across the cells using ATP as energy source [29]. Studies have shown that when AECs are in pathological state, the activity of NKA is decreased and the clearance of pulmonary edema fluid is also blocked. Therefore, reduction of NKA expression is an important mechanism underlying pulmonary edema [30]. PCR and Western blot analyses showed that when PBEF was overexpressed, the expression of ENaC and SP was decreased, suggesting that overexpression of PBEF inhibits the expression of main Na^+ and fluid transporters, affecting the clearance alveolar liquid. The decreased SP expression may compromise its protection for AECs, conferring vulnerability to them to external stimulation. At the same time, ATPase was found to be lower after PBEF overexpression, which may further reduce the removing of alveolar fluid. These results show that PBEF plays a negative regulating role in the clearance of fluid in AECs.

In summary, we show that PBEF expression negatively regulates alveolar Na^+ and fluid transport. These findings provide clues for developing new drugs and therapeutic strategies for treatment of pulmonary edema and may help to improve the quality of life of patients after cardiopulmonary bypass surgery.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant number: 81660073) and Young Scientist Science Fund of Jiangxi, China (grant number: 20161BAB215236).

Disclosure of conflict of interest

None.

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