

KGFR promotes Na⁺ channel expression in a rat acute lung injury model

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Abstract

Background: Binding of keratinocyte growth factor (KGF) to the KGF receptor (KGFR) plays an important role in the recovery of alveolar epithelial cells from acute lung injury (ALI).

Objectives: To evaluate the effect of gene therapy via adenovirus gene transfer of KGFR on the treatment of ALI.

Methods: Sprague–Dawley rats were divided into four groups: normal controls, injury controls, normal adenovirus transduced group and injury adenovirus transduced group. The ALI model was induced by lipopolysaccharide (LPS) injection. Recombinant adenovirus (AdEasy-KGFR) was injected via the tail vein. Expression of the sodium (Na⁺) channel in rat alveolar type II (ATII) epithelial cells was determined by PCR, immunohistochemistry and immunoelectron microscopy of rat lung tissues.

Results: Gene expression of the Na⁺ channel and KGFR in ATII cells was higher in the normal adenovirus transduced group than the three other groups; expression of these two genes in the injury adenovirus transduced group was higher than the injury control group. Na⁺ channel protein expression was lower in the injury adenovirus transduced group but higher than the injury control group.

Conclusions: KGFR over-expression induced Na channel expression could potentially be beneficial for ALI therapy.

Keywords Keratinocyte growth factor-receptor (KGFR); sodium (Na⁺) channel; rat model; acute lung injury (ALI); gene therapy

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Introduction

Acute lung injury (ALI) leading to respiratory distress syndrome has a high mortality rate [1], and its treatment with gene therapy is attracting increasing attention [2–4]. It has been found that the epithelial cell-specific keratinocyte growth factor (KGF) can reduce injury to the lung in lipopolysaccharide (LPS)-induced ALI and play a protective role through increasing the proliferation and repair capacity of alveolar type II (ATII) cells [5–7]. However, KGF exerts its effects on epithelial cells in a paracrine fashion through interaction with the KGF receptor (KGFR). Binding to KGFR on the alveolar epithelial cell membrane by KGF expressed by alveolar interstitial cells promotes various biological functions such as regeneration of alveolar epithelial cells, Na⁺

and H₂O transportation, and the synthesis and secretion of surfactant proteins (SPs) [8–10]. KGF binding to KGFR also plays an important role in the recovery of alveolar epithelial cells from ALI [11–13]. The observation that, in the absence of KGFR, KGF has only a preventive role with no therapeutic effect on ALI [14] suggests that a functional KGF–KGFR signaling pathway requires the presence of a sufficient number of KGFRs on the epithelial cell membrane. To restore the ALI-blunted KGF–KGFR signaling pathway and achieve a therapeutic adenovirus transduced effect, we investigated the biological function of a KGFR expressing adenoviral vector, constructed in our previous study, in an animal model of ALI [15]. Our aim was to provide a theoretical basis for further exploration of ALI gene therapy.

Materials and Methods

Animal model

Forty healthy, male Sprague–Dawley rats (SF level) provided by the Center for Experimental Animals, Tongji Medical College of Huazhong University of Science and Technology, weighing 170~225g were divided randomly into four groups: normal control

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($n = 8$); injury control ($n = 10$); normal adenovirus transduced ($n = 10$); and injury adenovirus transduced ($n = 12$).

The animals in the normal and injury adenovirus transduced groups were injected with 1 ml of the KGFR gene expression adenoviral vector (AdEasy-KGFR; 1.5×10^{10} PFU/ml), which was previously constructed by our research group [15], via the tail vein; the normal and injury control groups were injected with same amount of saline. Seventy-two hours after virus injection, the

injury control and adenovirus transduced groups were injected with 5 mg/kg LPS (0.5 mg/ml); the normal adenovirus transduced and control groups were injected with same amount of saline. The rats were sacrificed after 48h and their lung tissues were harvested.

Primers and probes

Primers and probes were designed using ABI Primer Express 3.0 software and synthesized by Takara Biotechnology Co., Ltd (Dalian, China). The sequences are shown in Table 1.

Table 1. Primers and probes used in the study

Primer	Sequence	Probe
Na ⁺ channel α subunit (for real-time PCR)	Forward: 5`-gcg cgg cgg gtc tt-3` Reverse: 5`-acc agt tgg aag ccg atc ttc-3`	5`-(FAM)-tac gcg aca aca atc ccc aag tgg a-(TAMRA)-3`
KGFR (for real time PCR)	Forward: 5`-gcg gtg gga atc gat aag g-3` Reverse: 5`-ctg tgg cgt cat ctt tca aca-3`	5`-(FAM)-cag acc caa gga gca gtc cac cgt-(TAMRA)-3`

Isolation of total RNA from rat lung tissues

Total RNA was extracted using an RNA isolation reagent kit (Takara Biotechnology Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. The total RNA was then dissolved in diethylpyrocarbonate-treated water and preserved at -80°C. The quality of the isolated total RNA was confirmed by measuring the optical density OD₂₆₀/OD₂₈₀ ratio.

amplified separately by PCR and the concentrations of the purified amplification products were measured and converted to DNA copy numbers. The PCR products of the two genes were then diluted by 10⁸, 10⁷, 10⁶ and 10⁵, based on the copy numbers calculated, to form a standard curve. The copy numbers of the Na⁺ channel α subunit and KGFR in each group were calculated automatically using the real-time PCR instrument (ABI 7300, USA).

Real-time quantitative PCR

Differential expression of the Na⁺ channel α subunit and KGFR genes was analyzed by one-step real-time fluorescence quantitative PCR. The 25 μ l PCR reaction system included the following components: 12.5 μ l of 2× One Step RT-PCR buffer III; 0.5 μ l Takara Ex-Taq; 0.5 μ l Primer Script RT Enzyme Mix II; 0.5 μ l forward primer; 0.5 μ l reverse primer; 1 μ l probe; 2 μ l total RNA; and 7.5 μ l RNase free dH₂O. The reaction conditions were as follows: 42°C for 30 min, 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each amplification cycle was measured at 60°C 1 min. The Na⁺ channel α subunit and KGFR genes were

Immunohistochemistry

Lung tissues from each group were frozen for immunohistochemistry, or embedded in paraffin and sectioned for conventional staining to observe the extent of lung injury. Immunohistochemistry was performed according to the manufacturer's instructions using rabbit anti-rat Na⁺-K⁺-ATPase α -1 antibody (Upstate Inc., New York, NY, USA) and the Histostain-Plus Kit (Jingmei Biotech Co., Ltd., Shenzhen, China). Positive unit (PU) values were determined by application of a pathologic imaging analysis system according to the following equation:

$PU = 100 \times |G_a - G_A| / [(1 - A_{Aa}) \times G_{max}]$, where $A_{Aa} = n/(m + n)$.

Immunoelectron microscopy

Rat lung tissue samples ($1 \text{ mm}^2 \times 10 \text{ mm}$) were pre-fixed in 4% paraformaldehyde for 5 min, cut into $1 \times 1 \times 1 \text{ mm}$ pieces, fixed for another 2 hrs, rinsed with phosphate buffered saline (PBS), dehydrated in an acetone gradient and embedded in epoxy resin. The tissue was cut into $20\text{--}40 \mu\text{m}$ thick sections, scooped up with nickel grids, dried and placed in 5% H_2O_2 etching solution for 3 min. The sections were then washed with saline, blocked in 8% bovine serum albumin-PBS solution for 1 hr, and incubated with rabbit anti-rat $\text{Na}^+ \text{-K}^+$ -ATPase α -1 antibody (5 $\mu\text{g}/\text{ml}$) at 4°C for 20 h and at room temperature for another 2 hr. The sections were rinsed with Tris buffered saline (TBS), incubated with biotinylated secondary antibodies at room temperature for 20 min, rinsed with TBS, incubated with SABC reagent at room temperature for 20 min, rinsed with TBS, incubated with diaminobenzidine for 15 min, rinsed with TBS, fixed in 1% osmium tetroxide for 15

min, washed and observed under a transmission electron microscope. Tissue incubated with normal rabbit serum as the primary antibody served as a negative control.

Statistical analysis

Data were analyzed using SPSS 10.0 statistical software and are presented as the mean \pm standard deviation. RT-PCR data were analyzed by completely randomized analysis of variance, the real-time PCR data by a completely randomized non-parametric Kruskal-Wallis test and the immunohistochemistry results by a completely randomized *t*-test of the mean. $P < 0.05$ was considered statistically significant.

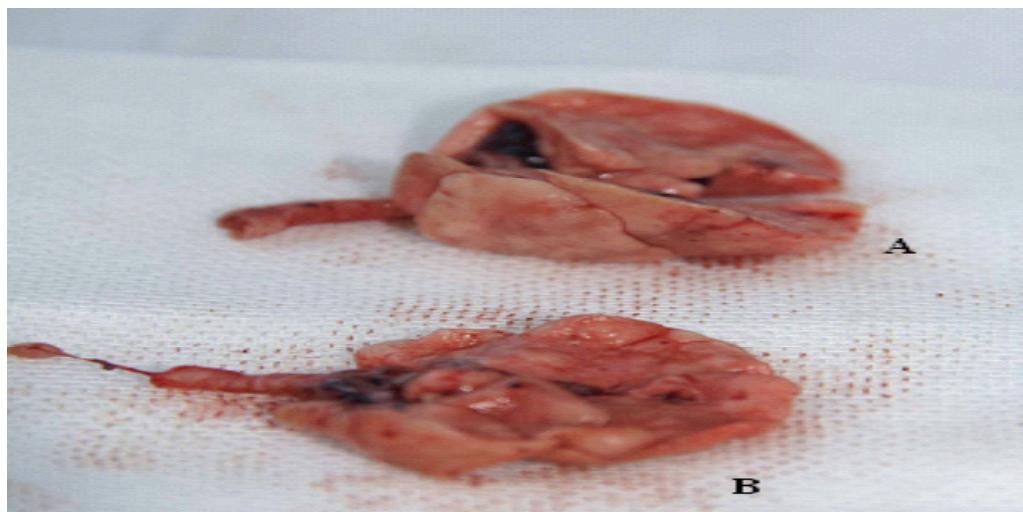
Results

KGFR-adenovirus-transduction reduced damage of the lung injury

To investigate the roles of the KGFR in lung injury, we established a lung injury animal model of four groups as described in material and method. Thoracotomy showed enlargement of and lesions in the lung tissue, indicating successful establishment of an LPS-induced rat ALI model (Fig 1).

Fig. 1. Rat model of ALI induced by LPS. Enlargement of and lesions in lung tissue were more obvious in LPS-treated rats (A) than in normal rats (B).

Fig 1

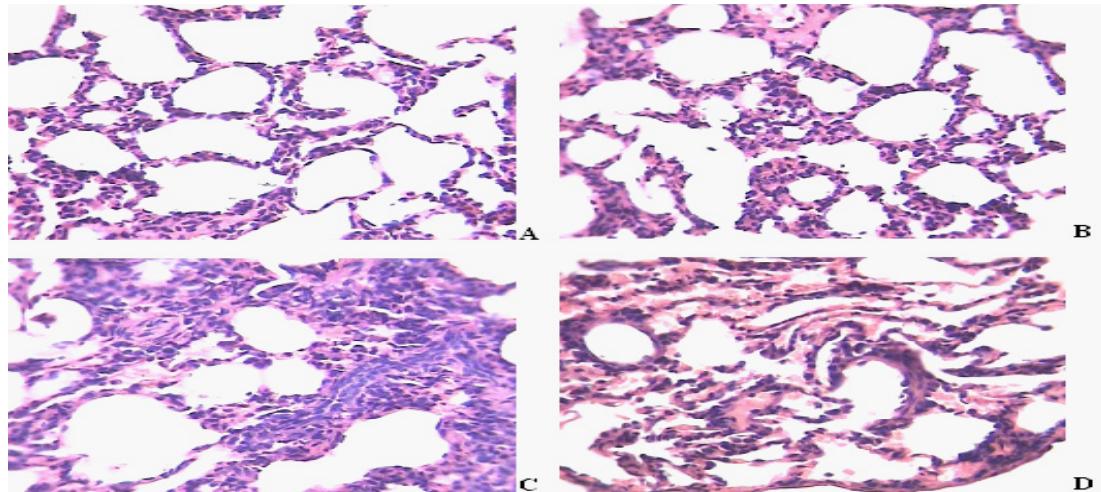


Hematoxylin and eosin staining of lung sections showed that alveolar structures in the normal control and normal adenovirus transduced groups were intact, with no signs of alveolar septum hyperplasia, or cells or exudates in the alveolar space. The alveolar septa in the injury adenovirus transduced group were intact but exhibited hyperplasia; a small amount of phagocytic cells and red blood cells were observed in the alveolar space. The

alveoli in the injury control group were collapsed, with fuzzy alveolar septa, and the alveolar space was filled with phagocytic cells, red blood cells and fluid. Lung tissue damage in the injury control group was markedly more severe than in the other three groups; tissue damage in the injury adenovirus transduced group was markedly less than in the injury control group (Fig. 2). These data suggested that KGFR-adenovirus transduction had an important impact on easing lung injury.

Fig. 2. Routine pathologic sections from the four experimental groups (hematoxylin and eosin staining, $\times 400$). Alveolar structures were intact in the normal control (A) and normal adenovirus transduced (B) groups. Alveolar septa in the injury adenovirus transduced group (C) were intact but exhibited hyperplasia; a small amount of phagocytic cells and red blood cells were observed in the alveolar space. Alveoli in the injury control group (D) were collapsed, with fuzzy alveolar septa, and the alveolar space was filled with phagocytic cells, red blood cells and fluid.

Fig. 2



KGFR-adenovirus transduction promotes Na^+ channel subunit α gene expression

To further investigated the reason why the KGFR plays an important role in the recovery of alveolar epithelial cells in acute lung injury (ALI), we detected Na^+ channel subunit α gene expression in different groups according to the described above. Analysis of the real-time PCR results showed statistically significant differences among the groups ($\chi^2_{\text{Na-channel}} = 35.017$, $P < 0.001$; $\chi^2_{\text{KGFR}} = 34.083$, $P < 0.001$). Gene expression of the Na^+ channel α subunit and KGFR was significantly higher in the normal adenovirus transduced group than in the other

three experimental groups ($P < 0.001$). Expression of the two genes in the injury adenovirus transduced group was significantly higher than the injury control group ($P < 0.001$) and slightly higher than the normal control group, but this difference was not statistically significant ($P = 0.541$). Expression of the two genes in the normal control group was significantly higher than the injury control group ($P < 0.001$). These results indicated that the KGFR-adenovirus transduction in vivo successfully activated the expression of the Na^+ channel effector gene on the surface of ATII epithelial cells. All data are shown in Table 2.

Table 2. Expression of Na^+ channel α subunit and KGFR, as determined by real-time PCR^a

Group	Na^+ channel α subunit (copies/ μl)	KGFR (copies/ μl)
Normal control ($n = 8$)	(4.02 ± 0.90) $\times 10^6$	(5.42 ± 1.84) $\times 10^7$
Injury control ($n = 10$)	(4.03 ± 1.99) $\times 10^4$	(4.12 ± 1.18) $\times 10^5$
Normal transgenic ($n = 10$)	(3.34 ± 0.65) $\times 10^7$	(3.52 ± 0.72) $\times 10^8$
Injury transgenic ($n = 12$)	(6.67 ± 1.89) $\times 10^6$	(7.30 ± 1.69) $\times 10^7$

^aKruskal-Wallis test (mean \pm standard deviation), $\chi^2_{\text{Na-channel}} = 35.017$, $P < 0.001$; $\chi^2_{\text{KGFR}} = 34.083$, $P < 0.001$

KGFR-adenovirus transduction promotes Na⁺-K⁺-ATPase expression in type II epithelial cells of lung tissue

Next, we investigated the Na⁺-K⁺-ATPase expression in type II epithelial cells of lung tissue at protein levels

by Immunohistochemical staining. It was (Figure 3) shown that the Na⁺-K⁺-ATPase was highly expressed in the normal-control group's lung tissue, and almost all the alveolar type II epithelial cells were positively stained.

Fig. 3. Immunohistochemical staining of rat lung tissues from the four experimental groups ($\times 400$). Na⁺-K⁺-ATPase was highly expressed in the normal control (A) and normal adenovirus transduced (B) groups. Na⁺-K⁺-ATPase was expressed in the injury adenovirus transduced group (C), but the number of positively stained cells was relatively lower than the normal adenovirus transduced and control groups. In the injury control group (D), there was almost no positive staining for Na⁺-K⁺-ATPase. Brown-stained positive cells are indicated by arrows.

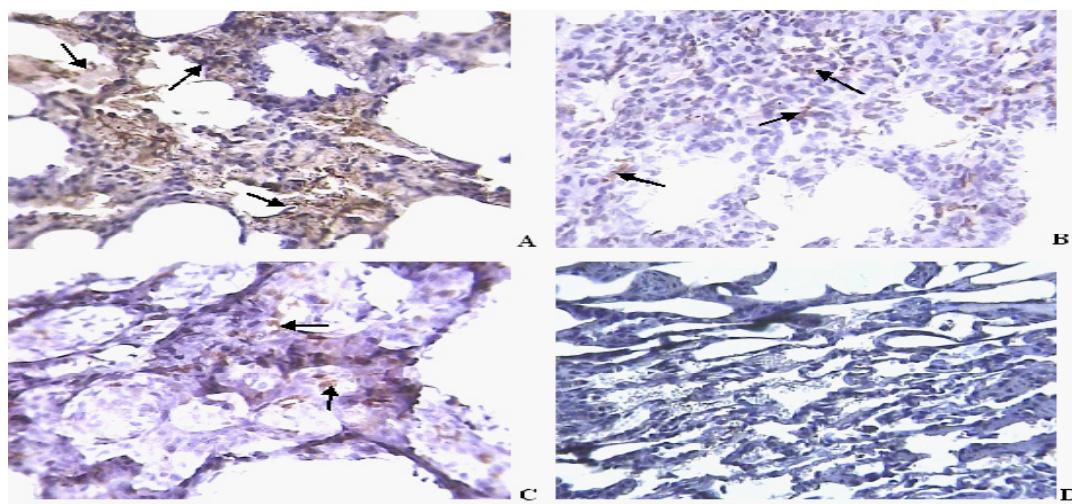


Fig 3

Na⁺-K⁺-ATPase was also highly expressed in the normal adenovirus transduced group, with ATII epithelial cell staining similar to the normal control group. There was no significant difference in the PU values for Na⁺-K⁺-ATPase staining between the normal adenovirus transduced and control groups ($t = 0.376$, $P > 0.05$). There was almost no positive staining for Na⁺-K⁺-ATPase in the injury control group. Although Na⁺-K⁺-ATPase was expressed in the injury adenovirus transduced group, the number of positively stained cells was relatively lower than the normal adenovirus transduced and control groups, and the intensity of the

staining was much lighter. However, the expression of Na⁺-K⁺-ATPase in the injury adenovirus transduced group was higher than the injury control group. When the staining in the injury adenovirus transduced group was compared with the normal control, normal adenovirus transduced and injury control groups, the differences were all statistically significant ($t = 10.601$, $t = 9.134$ and $t = 16.466$, respectively, all $P < 0.001$; Table 3). It was further approved that KGFR promotes sodium channel expression. Interestingly, KGFR could not promote Na⁺-K⁺-ATPase expression to normal levels in injured lung tissue.

Table 3. Expression of Na⁺-K⁺-ATPase, as determined by immunohistochemistry

Group	PU (mean \pm standard deviation)
Normal control ($n = 8$)	47.7 \pm 3.33 ^a
Injury control ($n = 10$)	5.1 \pm 2.3
Normal transgenic ($n = 10$)	46.9 \pm 5.21
Injury transgenic ($n = 12$)	29.19 \pm 4.11 ^b

^aPU for Na⁺-K⁺-ATPase staining compared between normal transgenic and control groups ($t = 0.376$, $P > 0.05$)

^bInjury transgenic group was compared with normal control, normal transgenic and injury control groups ($t = 10.601$, $t = 9.134$ and $t = 16.466$, all $P < 0.001$)

KGFR increased the microvilli and osmiophilic lamellar bodies in type II epithelial cells in lung

We had approved that sodium channel were activated by KGFR, so we wanted to research the roles of KGFR in lung tissues. So we detected alveolar type II epithelial cells of normal-adenovirus transduced groups and normal groups by immune electron microscopy. It was shown that rat alveolar type II epithelial cells from the normal-adenovirus transduced group showed positive reactions with high electron density on the membrane surface and on the microvilli, yet no positive reactions

in the intracellular structures. A few positive reactions with high electron density were observed on the surface of the ATII epithelial cell membrane in the normal control group. In addition, due to the stimulatory effect of the KGFR transduction, there were more osmiophilic lamellar bodies in the normal adenovirus transduced group than the normal control group (Fig. 4). It was confirmed that the AdEasy-KGFR vector not only increased the expression of the Na⁺ channel and Na⁺-K⁺-ATPase, but also increased the secretion of alveolar SPs.

Fig. 4. Immunoelectron microscope observations pre/post-KGFR gene transfer in normal rats. A few positive reactions with high electron density were observed on the surface of the ATII epithelial cell membrane in the normal control group (A). Positive reactions with high electron density were seen on the membrane surface and microvilli, but there were no positive reactions in intracellular structures in the normal adenovirus transduced group (B). The membrane surface and microvilli of ATII epithelial cells are indicated by filled arrows; osmiophilic lamellar bodies are indicated by open arrows.

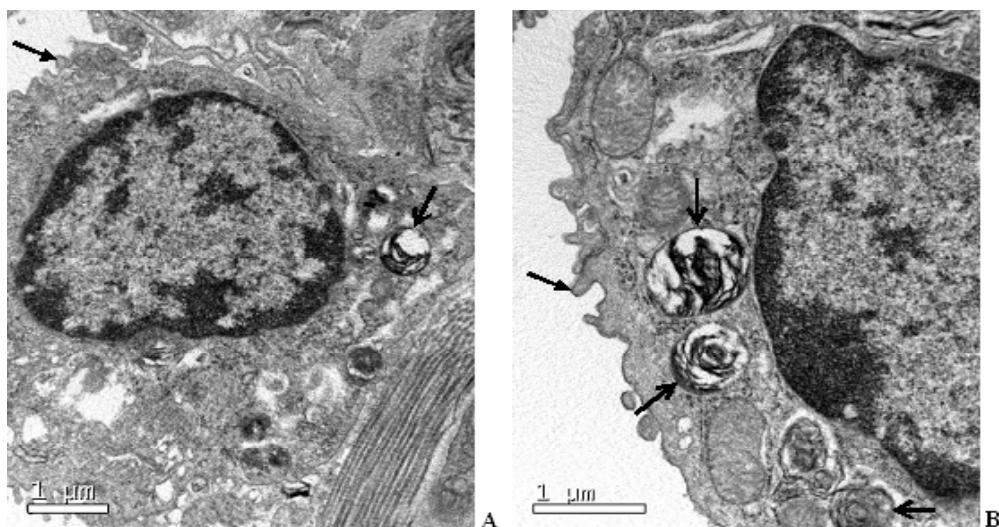


Fig 4

Discussion

Gene therapy has been suggested as a cheaper alternative for many protein replacement therapies, and gene therapy for ALI is gradually becoming recognized in the field [3, 4]. In this study, we used the KGFR gene, which has a protective effect against ALI in alveolar epithelial cells. On binding to KGF, KGFR increases expression of $\text{Na}^+ \text{-K}^+$ -ATPase and the number of Na^+ channels on alveolar epithelial cell membranes, promoting fluid clearance in these cells [16], and induces ATII epithelial cells to accumulate large amounts of immunoactive SP-B and C, which protect these cells from inflammation-induced damage [1, 7]. In addition, binding of KGF to KGFR promotes the regeneration, differentiation and wound-healing abilities of alveolar epithelial cells [3]. Therefore, the repair and regeneration of alveolar epithelial cells are inseparable from the KGF-KGFR interaction. The importance of the KGF-KGFR signaling pathway in the regeneration of alveolar epithelial cells and in the repair of damage caused by ALI suggests KGF and KGFR as candidate genes for ALI gene therapy [12].

During the ALI process, pathologic changes including edema, hemorrhage, neutrophil infiltration, focal atelectasis and hyaline membrane degeneration occur in the alveoli and alveolar septa. The active transportation system for Na^+ and H_2O in alveolar epithelial cells comprises Na^+ channels, $\text{Na}^+ \text{-K}^+$ -ATPase and H_2O channels. Na^+ is transported into the cells through Na^+ channels on the alveolar side and discharged by $\text{Na}^+ \text{-K}^+$ -ATPase on the basolateral side; the latter process is accompanied by passive absorption of H_2O [13]. Therefore, in the present study, the Na^+ channel and $\text{Na}^+ \text{-K}^+$ -ATPase were selected as functional KGFR effectors for determination of the biological activities of the KGFR adenovirus transduction.

The effects of ALI gene therapy in this study were validated at both the gene and protein expression levels. Real-time PCR showed the gene expression of Na^+ channel α subunit and KGFR to be higher in the normal adenovirus transduced group than in the other three groups. The expression of these two genes in the injury adenovirus transduced group was markedly higher than the injury control group, and slightly higher than the normal control group (Table 2). These results indicate that the AdEasy-KGFR vector expressed the KGFR gene *in vivo* in the experimental rats and activated expression of the Na^+ channel effector gene on the surface of ATII epithelial cells, leading to increased expression of the Na^+ channel α subunit and KGFR in the adenovirus transduced groups, even under LPS

challenge. This phenomenon was also confirmed by protein analysis. Pathologic staining of lung sections demonstrated that the extent of lung enlargement, lung tissue edema, hyperplasia of alveolar septa and infiltration of phagocytic cells into the alveolar space were all reduced in the injury adenovirus transduced group, compared with the injury control group.

Immunohistochemistry showed no difference in $\text{Na}^+ \text{-K}^+$ -ATPase expression between the normal control and normal adenovirus transduced groups, implying that AdEasy-KGFR had a minimal effect on protein expression of $\text{Na}^+ \text{-K}^+$ -ATPase under normal conditions (Fig. 3). However, under injury conditions the $\text{Na}^+ \text{-K}^+$ -ATPase expression level in the injury adenovirus transduced group was higher than the injury control group (Table 3), indicating that the therapeutic effect of the AdEasy-KGFR transduction may mitigate alveolar congestion and edema in the injury adenovirus transduced group, compared with the injury control group. These results demonstrate that KGFR increased gene expression of the Na^+ channel and $\text{Na}^+ \text{-K}^+$ -ATPase on alveolar epithelial cell membranes and led to lower levels of lung tissue damage in the injury adenovirus transduced group compared with the injury control group. Immunoelectron microscopy (Fig. 4) confirmed that the AdEasy-KGFR vector not only increased the expression of the Na^+ channel and $\text{Na}^+ \text{-K}^+$ -ATPase, but also increased the secretion of alveolar SPs. This was reflected by the fact that more osmiophilic lamellar bodies were seen in the adenovirus transduced group than the control group. Secretion of SPs to the alveolar surface through exocytosis of osmiophilic lamellar bodies maintains alveolar plasticity and surface tension, which play an active role in protecting lung tissues from ALI.

It has been observed that the ability of the AdEasy-KGFR vector to promote effector gene expression *in vivo* varies. In the present study, the ranking of Na^+ channel expression (RNA level) induced by the KGFR transduction, from high to low, was as follows: normal adenovirus transduced > injury adenovirus transduced \approx normal control > injury control. However, the ranking of $\text{Na}^+ \text{-K}^+$ -ATPase expression (protein level) induced by the KGFR transduction, from high to low, was: normal adenovirus transduced \approx normal control > injury adenovirus transduced > injury control. These findings suggest that, under LPS-induced injury conditions, the ability of the AdEasy-KGFR vector to induce Na^+ channel expression was stronger than its ability to promote the expression of $\text{Na}^+ \text{-K}^+$ -ATPase. However, to properly interpret this conclusion, the

results of our gene and protein analysis must be compared and verified. The universality of the ability of AdEasy-KGFR to promote effector gene expression needs to be explicitly investigated in other animals and tissues, and comprehensive future investigations are required.

The gene transfer efficiency of adenoviral expression vectors is affected by virus inoculation and the immune responses of the host, which can greatly limit both the access of the transduction to target host cells and its biological activity, leading to a relatively low therapeutic effectiveness. We tried three different methods to deliver our recombinant adenovirus into animals: aerosol transduction through the respiratory tract, intraperitoneal injection and intravenous injection through the tail vein; the optimal results were achieved with intravenous injection. This may be because adenovirus infection is common in the upper respiratory tract of both animals and humans, with no pathological response under normal circumstances. Thus, the rats used in our study had probably already acquired adenoviral immunity, and this, along with the blood barrier and other factors, may be responsible for the low efficiency of transduction through the respiratory tract or intraperitoneal injection.

Although quantitative analysis by real-time PCR demonstrated AdEasy-KGFR promoted Na^+ channel gene expression (Table 2), the Na^+ channel gene expression level in the normal adenovirus transduced group was not as high as predicted (two to three times greater than the normal control group). This implies that there was problem with the gene transfer efficiency of the adenoviral vector. However, it is noteworthy that, under injury conditions, a therapeutic effect of AdEasy-KGFR was evident. Expression of the Na^+ channel effector gene in the injury adenovirus transduced group was two times greater than the injury control group (Table 2). These findings, which achieved the study's desired objective, may have resulted from the preventive effect of AdEasy-KGFR, which was injected into the animals and given time to act before the induction of ALI. This investigation of ALI gene therapy under prophylactic conditions thus demonstrated the therapeutic effect of the KGF–KGFR signaling pathway. Our results are consistent with previous reports in which injection of KGF had a protective but not therapeutic effect on lung injury [14]. Improving gene transfer efficiency will be the subject of a future study.

In summary, this study demonstrated that activation of the upstream KGF–KGFR signaling pathway in ATII epithelial cells stimulated downstream expression

of Na^+/K^+ -ATPase, the Na^+ channel and SP genes, it may had some relationship to improving alveolar fluid clearance, anti-inflammatory capacity and repair capacity in the lung. Our data provide valuable information for further investigations of ALI gene therapy.

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Declaration of Interest

The authors declare that they have no financial or personal relationships between themselves and other parties that might bias their work.

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