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Original Article

Electrotaxis of alveolar epithelial cells in direct-current electric fields

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ABSTRACT

Purpose: This study aims to elucidate the electrotaxis response of alveolar epithelial cells (AECs) in direct-current electric fields (EFs), explore the impact of EFs on the cell fate of AECs, and lay the foundation for future exploitation of EFs for the treatment of acute lung injury.

Methods: AECs were extracted from rat lung tissues using magnetic-activated cell sorting. To elucidate the electrotaxis responses of AECs, different voltages of EFs (0, 50, 100, and 200 mV/mm) were applied to two types of AECs, respectively. Cell migrations were recorded and trajectories were pooled to better demonstrate cellular activities through graphs. Cell directionality was calculated as the cosine value of the angle formed by the EF vector and cell migration. To further demonstrate the impact of EFs on the pulmonary tissue, the human bronchial epithelial cells transformed with Ad12-SV40 2B (BEAS-2B cells) were obtained and experimented under the same conditions as AECs. To determine the influence on cell fate, cells underwent electric stimulation were collected to perform Western blot analysis.

Results: The successful separation and culturing of AECs were confirmed through immunofluorescence staining. Compared with the control, AECs in EFs demonstrated a significant directionality in a voltage-dependent way. In general, type I alveolar epithelial cells migrated faster than type II alveolar epithelial cells, and under EFs, these two types of cells exhibited different response threshold. For type II alveolar epithelial cells, only EFs at 200 mV/mm resulted a significant difference to the velocity, whereas for, EFs at both 100 mV/mm and 200 mV/mm gave rise to a significant difference. Western blotting suggested that EFs led to an increased expression of a AKT and myeloid leukemia 1 and a decreased expression of Bcl-2-associated X protein and Bcl-2-like protein 11.

Conclusion: EFs could guide and accelerate the directional migration of AECs and exert antiapoptotic effects, which indicated that EFs are important biophysical signals in the re-epithelialization of alveolar epithelium in lung injury.

Introduction

Acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS) is a life-threatening disease with a high fatality rate of 40% – 60%.[1] ALI/ARDS mainly manifests as the acute onset of progressive hypoxemia, dyspnea and bilateral pulmonary edema resulting from uncontrolled inflammatory responses, excessive alveolocapillary permeability, and widespread damage to alveolar epithelial cells (AECs).[2–4] AECs, consisting of type 1 cells (AT1s) and type 2 cells (AT2s), are structurally and functionally important for the integrity of the lung. Upon injury, AT2s migrate directionally into the sites of injury and transdifferentiate into AT1s, which are primary regulators of alveolar immunological activities, and AT1s collaborate with AT2s in epithelial repair and regeneration.[5–8] However, the complicated regulatory mechanism behind this process has not been fully elucidated. Therefore, it is important to further study the mechanisms of pulmonary epithelial repair.

In epithelial tissue, the polarized distribution of ion channels and the subsequent difference in ion transporting capacity lead to the transepithelial potential difference. Upon injury, the damaged

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epithelial barrier creates endogenous electric fields (EFs), the damage center being the cathodal pole, and cells within the fields migrate directionally toward the cathode, which is one of the key steps of re-epithelialization.[9,10] In a previous study on tracheal epithelial injury, the immediate current reached (1.59 ± 0.21) μA/cm^2 upon injury, and the applied EFs guided the directional migration of airway epithelial cells.[11] However, the impact of EFs on AECs remains to be discovered.

In the current study, we successfully extracted AECs from rat lungs and, by analyzing their responses to different voltages of EFs. We found that EFs guided directional migration of AECs and human bronchial epithelial cells, increased their proliferation, suppressed their apoptosis and increased their antiapoptotic ability through activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway. Our study indicated the underlying therapeutic effects of EFs and may shed light on future treatments for ALI/ARDS.

Methods

Purification and culture of rat AECs

The animal study protocols were approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (approval number for production: SCXK (PLA) 20120011; approval number for application: SCXK (PLA) 20120031). AECs were obtained through the detailed procedure reported in our previous study.[12] Sprague–Dawley rats weighing 120 − 150 g and aged 3 − 4 weeks were obtained from the animal center of Daping Hospital, Army Medical University. After euthanasia, pharyngeal skin and tissues were dissected to perform tracheal intubation. The lung samples were obtained en bloc from the rats, and were washed twice with saline before being completely digested, cut into pieces, and filtered. The resulting samples were then centrifuged to obtain the cell precipitate, which was dispersed and blended with anti-rat T1α (Sigma, #1995, St. Louis, USA). The cells were subsequently incubated with anti-rabbit IgG microbeads (Miltenyi Biotec, #130-047-102, Bergisch Gladbach, Germany), and then resuspended and cultured in 8-well plates.

Electrotaxis assay and quantification of cell migration

We applied EFs through agar-salt bridges in Steinberg's solution connected with silver/silver chloride electrodes to pooled medium on either side of the electrotaxis chamber. Cells after 48 h of *in vitro* culture were exposed to direct-current-induced EFs of 0, 50, 100, and 200 mV/mm, and serial time-lapse images were taken. Cellular migration directionality (cos θ, where θ is the angle formed by the EF vector and a straight line connecting the initial and final positions of the cellular movements) and velocity were analyzed using ImageJ software (NIH, Bethesda, Massachusetts, USA) with the MTrackJ and Chemotaxis tool plugins. The trajectories of cells were pooled to composite graphs. A directionality value close to 0 indicated high randomness of cellular movements.

Cells and reagents

The human bronchial epithelial cells transformed with Ad12-SV40 2B (BEAS-2B cells) were provided by Professor Lin P from Southwest University, Chongqing, China, and cultured in RPMI-1640 (HyClone, SH30096.01, Utah, USA) with Earle's salts (Gibco, #11150059, Massachusetts, USA), 2 mmol/L L-glutamine (Gibco, #25030081, Massachusetts, USA), 10% fetal bovine serum (Gibco, #10100147C, Massachusetts, USA), and 100 U/mL penicillin-streptomycin solution (HyClone, #16777−164,Utah, USA) at 37 °C, 5% CO_2 and 90% humidity. Anti-Bcl-2-like protein 11 (Anti-Bim) (#2933S), AKT (#9272S), phosphorylated AKT (#4060S), B-cell lymphoma-extra large (#2764), phosphorylated-myeloid leukemia 1 (#14765), myeloid leukemia 1 (MCL-1) (#94296), Bcl-2-associated X protein (Bax) (#41162), and glyceraldehyde-3-phosphate dehydrogenase (#2118S) antibodies were purchased from Cell Signaling Technology (Massachusetts, USA).

Immunofluorescence staining

Cells were washed with phosphate-buffered saline (PBS), and fixation with 4% paraformaldehyde was performed at room temperature for 30 min. The blockage for nonspecific binding was performed utilizing 10% normal goat serum (Cell Signaling Technology, #5425S, Massachusetts, USA) for BEAS-2B cells and 10% donkey serum (Abcam, ab7475, Cambridge, England) for AECs. Former incubated cells (overnight in PBS with 10% bovine serum albumin at 4 °C) were then further incubated with the primary antibodies, rabbit monoclonal phosphorylated AKT for BEAS-2B cells, aquaporin-5 (AQP5) (Abcam, ab78486, Cambridge, England) for AT1s and pro-surfactant protein C (Abcam, ab40897, Cambridge, England) for AT2s.

After being washed with PBS, the cells were incubated with secondary antibodies at 37 °C for 30 min, Cy3-conjugated goat-anti-rabbit IgG (Bioworld Technology, BS10007, Nanjing, China) and conjugated secondary antibodies donkey-anti-mouse IgG (Bioworld Technology, BS10016, Nanjing, China) for another 1 h at 37 °C. Finally, the cells were counterstained with 4′,6-diamidino-2-phenylindole (Solarbio, C0065, Beijing, China) and viewed through an ImageXpress Micro (Molecular Devices, California, USA) high-throughput imager.

Western blot analysis (WBA)

EF-stimulated cells were collected, washed with PBS, and then lysed for 30 min at 4 °C in radio-immunoprecipitation assay buffer (Thermo Fisher Scientific, #89900, Massachusetts, USA). The lysates were then centrifuged at 12,000×*g* for 20 min at 4 °C, and protein concentration was determined using a bicinchoninic acid assay (Millipore, #71285-3CN, Massachusetts, USA). Equal amounts (25 mg) of determined protein was loaded on electrophoresis gel for 2 h at 110 V, followed by transfer onto polyvinylidene fluoride membranes (90 min, 200 mA) (Millipore, C3117, Massachusetts, USA). Then, blocking was performed using 5% bovine serum albumin for 1 h at room temperature, and incubation with primary antibodies was carried out overnight at 4 °C. After washed, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (Sino Biological, SSA004, Beijing, China) at a concentration of 0.02 μg/mL for 1 h. Visualization was achieved through the Chem-iDoc Touch System (Bio-Rad, #1708370, California, USA).

Statistical analysis

We set the velocity and directionality of cells in EFs of different voltages as two independent targets and compared the significance of their difference respectively among groups. The comparison of difference was conducted firstly between control and experimental groups, which, if significant, were further followed by the comparison among experimental groups. GraphPad Prism 9.0 (GraphPad Software, California, USA) was used for the unpaired 2-sided *t* tests.

Results

AECs were separated from rat lung tissues and identified

AECs were obtained from digested rat lung tissues, and single-cell isolation was successfully performed using magnetic

activated cell sorting (Fig. 1A). AQP5, a specific antigen on the membrane of AT1s, and proSPC, which exists in the cytoplasm of AT2s, were used as cellular markers for the identification of AECs. Immunofluorescent staining (Fig. 1B) illustrated high expression levels of AQP5 in cultured AT1s and high levels of surfactant protein C (SPC) in AT2s; these results indicated the successful separation of rat AECs.

EFs guided and accelerated the directional migration of AECs

After the successful isolation of AECs, the cells were placed into direct-current EFs of different voltages (0, 50, 100 and 200 mV/mm), and cell migration was recorded. The time-lapse figures (Figs. 2A and 3A) showed the cellular activity with and without EFs. In the control groups, 0 mV/mm, through observation for 90 min, the cells exhibited random movements, while cells within EFs demonstrated directed migration from the anode to the cathode (Figs. 2B and 3B, and Appendix A1-4).

Then, we investigated whether the cellular responsiveness was voltage-dependent. We obtained trajectories by placing AECs in EFs of different voltages (0, 50, 100, and 200 mV/mm) and recorded their movements. Without EFs, both AT1s and AT2s migrated randomly. As the voltage of the EFs increased, an increasing number of AECs migrated directionally to the cathode. The directionality of cells within EFs at both 100 mV/mm and 200 mV/mm was significantly higher than that of EFs at 0 mV/mm and 50 mV/mm, suggesting voltage dependency (Figs. 2 and 3). For AT2s, especially, cells within EF at 50 mV/mm demonstrated significantly higher directionality, indicating the difference relating to cell types in response. The impact of EFs on cellular velocity, however, differed. Generally, AT1s moved faster than AT2s, and for AT1s, EFs at both 100 mV/mm and 200 mV/mm resulted in significantly higher speeds than those without EF and at 50 mV/mm. However, for AT2s, only EFs at 200 mV/mm contributed to a significantly higher speed (Figs. 2C and 3C). In conclusion, EFs can guide directional migration

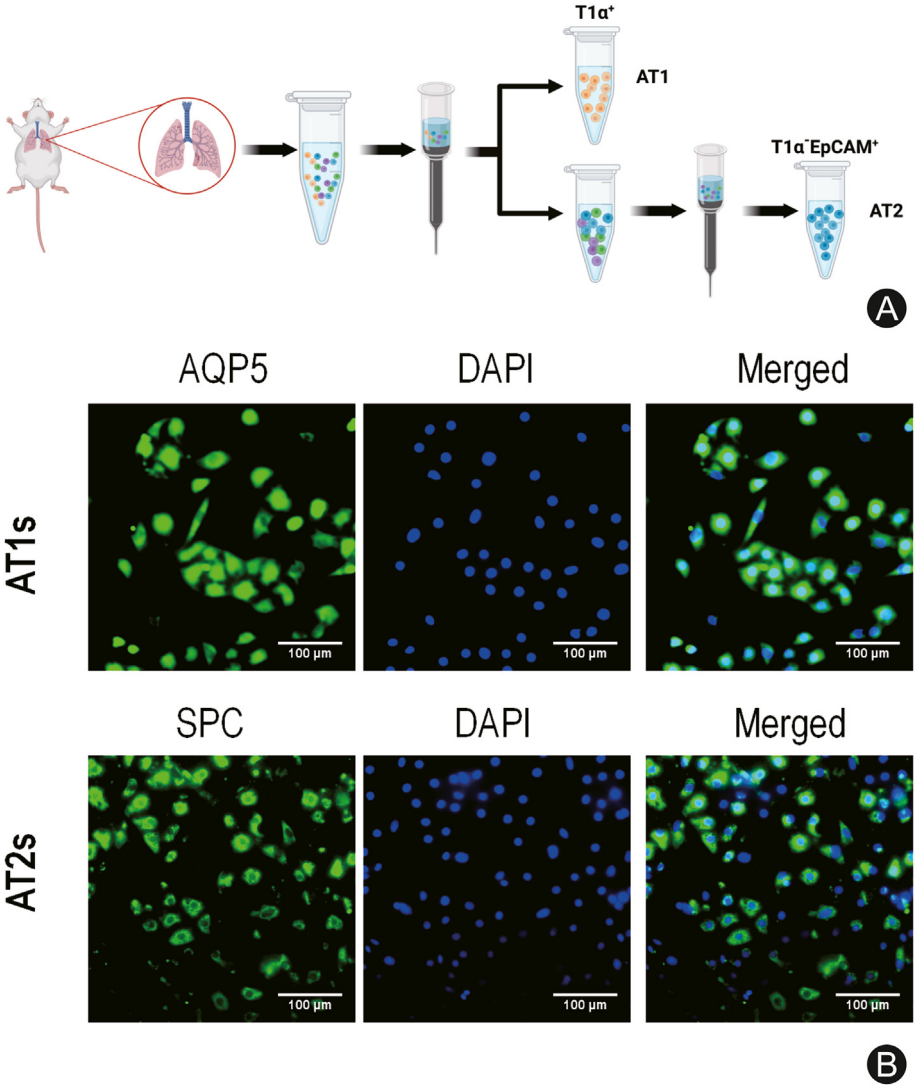

Fig. 1. Purification of rat AECs by MACS and identification. (A) Schematic illustration of purifying AECs through MACS. T1α+ for AT1s and T1α- EpCAM + for AT2s; (B) Immunofluorescent staining of purified AECs. AQP5 for AT1s, SPC for AT2s and DAPI for nuclei.
AECs: alveolar epithelial cells; MACS: magnetic-activated cell sorting; AT1s: type I alveolar epithelial cells; AT2s: type II alveolar epithelial cells; AQP5: aquaporin-5; SPC: surfactant protein C; DAPI: 4′,6-diamidino-2-phenylindole.

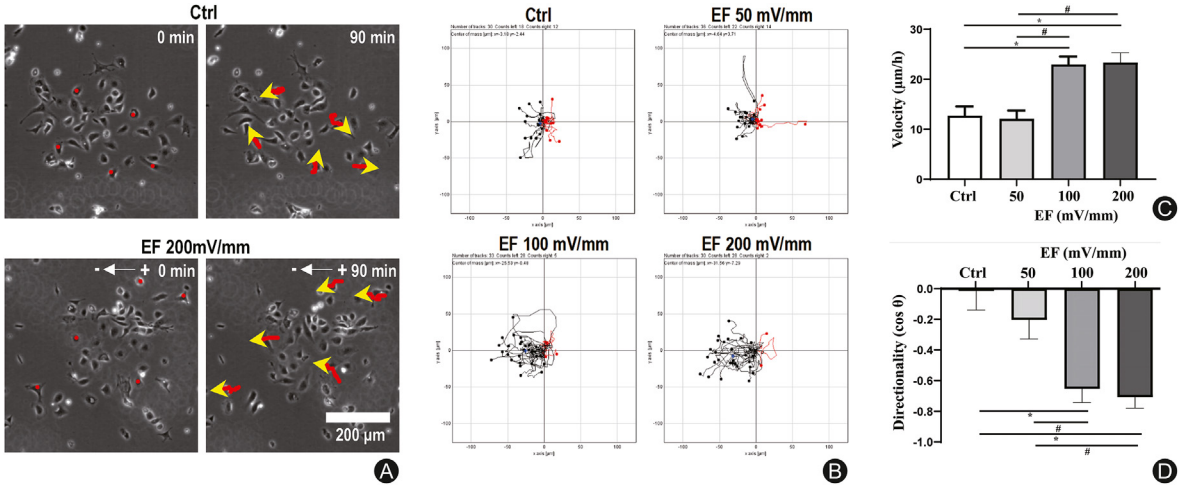

Fig. 2. The responses of AT1s to EFs. (A) Time-lapse photographs of AT1s with and without an EF of 200 mV/mm at 0 min and 90 min, with red line segments and yellow arrowheads representing cell migration paths and their directions, respectively; (B) Migration trajectories of AT1s at an EF of 0, 50, 100 or 200 mV/mm, with black lines as the migrations toward the cathodal pole and red lines toward the anodal pole (or left and right in control, EF of 0 mV/mm); (C) and (D) Migration speeds and directionality of AT1s at different voltage levels.
$^{\#}p < 0.001$ compared with 50 mV/mm.
$^{*}p < 0.001$ compared with the control.
AT1s: type I alveolar epithelial cells; EF: electric field; Ctrl: control, 0 mV/mm.

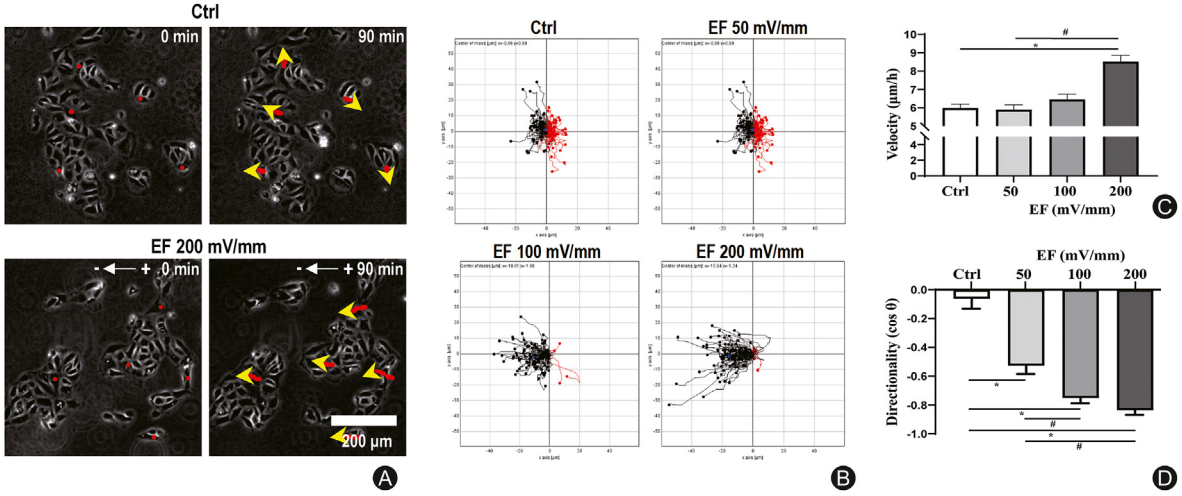

Fig. 3. The migration of AT2s became swifter and more directional in EFs. (A) Time-lapse photographs of AT2s at 0 min and 90 min in the control and 200 mV/mm EF, with red line segments and yellow arrowheads indicating cell migration paths and their directions, respectively; (B) Trajectories of AT2 migration at different voltage levels of EFs, cells migrating toward the cathode being represented by black lines and vice versa by red ones (or left and right in the control, no EF); (C) and (D) The speed of migration and directionality of AT2s at different EFs.
$^{*}p < 0.001$ compared with the control; $^{\#}p < 0.001$ compared with 50 mV/mm.
AT2s: type II alveolar epithelial cells; EF: electric field; Ctrl: control, 0 mV/mm.

of AECs in a voltage-dependent manner and speed up cell migration.

EFs guided the directed migration of BEAS-2B cells and promoted cell survival

Next, we aimed to further determine the impact of EFs on pulmonary tissues. Commercial BEAS-2B cells, epithelial cells isolated from the normal human bronchial epithelium of noncancerous individuals, were cultured and placed in EFs. Time-lapse images showed random migration in the control group and directed migration in the experimental group (Fig. 4A and Appendix A5-6). Cell trajectories in all groups were drafted, and obvious directionality was observed at an EF of 200 mV/mm (Fig. 4B). Applying EFs to BEAS-2B cells significantly affected their migration speed and directionality (Fig. 4C). Then, whether the phosphorylation of AKT played a role in responses to EFs was studied. Through immunofluorescence staining and WBA, we deduced that EFs increased the phosphorylation of AKT and thus participating in the activation of PI3K/AKT pathway. Next, we studied whether EFs influence cellular apoptosis. WBA demonstrated distinctively increased MCL-1, a Bcl-2 family protein that enhances cell survival, and significantly decreased Bim and Bax, which are proapoptotic proteins that promote cell death, indicating the antiapoptotic potential of EFs.

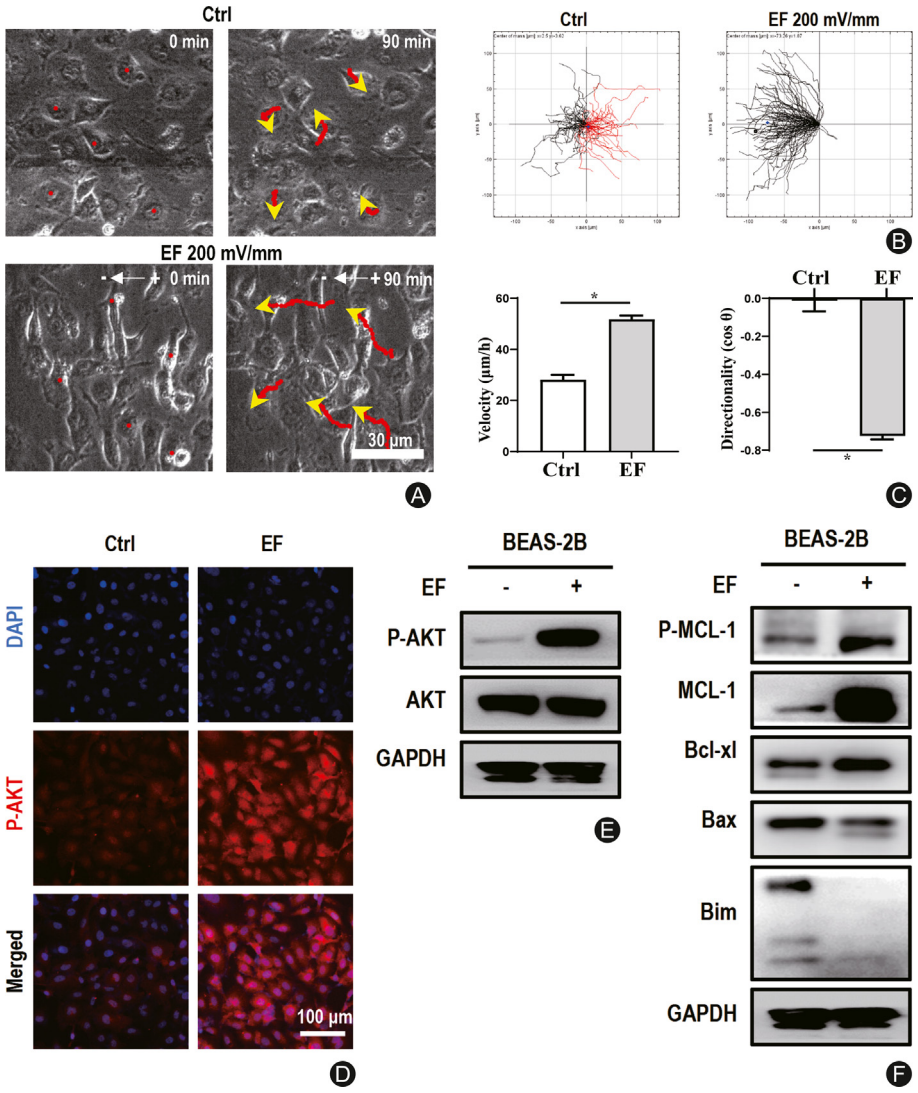

Fig. 4. Electrotaxis of BEAS-2B cells and the antiapoptotic potential of EFs. (A) Time-lapse photographs of BEAS-2B cells in the control group and the experimental group at an EF of 200 mV/mm, with red lines and yellow arrowheads as cell migration routes and their directions; (B) Trajectories of the migration of BEAS-2B cells with EFs at 0 and 200 mV/mm, with black lines as cells migrating toward the cathode and red lines vice versa (or left and right in the control, no EF); (C) Velocity and directionality of cellular migration in EFs. (D) Immunofluorescence staining of BEAS-2B cells with EFs of 0 and 200 mV/mm; (E) WBA showing the difference in the phosphorylation of AKT after application with EFs in BEAS-2B cells; (F) WBA showing the difference in apoptosis-related molecules after application with EFs in BEAS-2B cells.
*$p < 0.001$ compared with the control.
BEAS-2B cells: human bronchial epithelial cells transformed with Ad12-SV40 2B; WBA: western blot analysis; EF: electric field; Ctrl: control, 0 mV/mm; DAPI: 4′,6-diamidino-2-phenylindole; P-AKT: phosphorylated AKT; P-MCL-1: phosphorylated myeloid leukemia-1; Bcl-xl: B-cell lymphoma-extra large; Bax: Bcl-2-associated X protein; Bim: Bcl-2-like protein 11; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Discussion

ARDS is characterized by widespread alveolar epithelial damage. Thus, it is vital to repair the damaged epithelia to help regain pulmonary function.[13] Alveolar epithelial damage triggers the migration and differentiation of AT2s to gain cellular balance in the alveoli.[6] In the current study, by applying EFs of different voltage levels, we found that EFs guided directional migration of AECs, and this electrotactic response is consistent with other epithelial cells, such as skin and cornea.[14] The increased phosphorylation of AKT and MCL-1 and decreased expression of Bcl-2-like protein 11 proved the positive effects of EFs on pulmonary cell proliferation as well. Therefore, EFs could be regarded as a potential therapeutic method for alveolar repair.

In fact, the feasibility of EFs has been proven in some fields. For instance, in the blood samples of COVID-19 patients, the usage of alternating electric fields prevented cytokine storms through the suppression of clonal expansion of the activated lymphocytes.[15] In addition, applying EFs to wounds has been shown to control inflammation, enhance wound blood perfusion, and increase fibroblast migration, and the therapeutic use of EFs in wound and pain management has been listed in some guidelines.[16] Besides, coupling a 15 − 30 V electrical stimulation significantly decreased the *Staphylococcus aureus* biofilm formation, which is promising in fighting infections.[17] Moreover, the addition of intratumoral modulation therapy that uses multiple electrodes to deliver EFs to treat cancer has been proven to improve the survival of certain glioblastoma patients.[18] Interestingly, a study on collective cells suggested that even though the barrier competed with the electric stimulation and recovered quickly, the center cells could memorize the previous command and the intercellular interaction dynamics could remain affected even poststimulation, noting the need for a deeper understanding of the mechanisms.[19]

Nonetheless, the impacts of EFs on lung injury repair are complex. A recent study showed that applying EFs could result in a

transient increase in lung neutrophils and a decrease in eosinophils, accompanied by an increase in IL-6 levels,[20] and an early study demonstrated that neutrophils could help repair airway epithelium by removal of injured epithelial cells.[21] Furthermore, a recent study stated that EFs could guide the directional migration of T cells to the cathode, but significantly decrease T-cell activation, and remarkably dampen CD4 (+) T-cell polarization.[22] Additionally, EFs can significantly strengthen the macrophage phagocytic ability of various targets, including apoptotic neutrophils, enhance PI3K and extracellular signal-regulated kinase activation, and selectively modulate cytokine production.[23] Besides, the EFs have also been proven to modulate the follistatin production of mesenchymal stem cells from human bone marrow, which serves negatively in the activation of transforming growth TGF-β and exerts anti-fibrotic role. Since the activation of TGF-β characterizes the transitional state, EFs may contribute to the early stage of re-epithelialization of ALI repair by promoting AT2s self-renewal.[24,25]

Moreover, upon injury, multiple other factors affect epithelial repair. Researchers found that microvesicles secreted by ALI/ARDS alveolar macrophages suppressed the expression of a subunit of epithelial sodium channels and alveolar fluid clearance, contributing to lung injury and pulmonary edema,[26] and that exposure to microenvironmental IL-37 and HMGB1 induces pulmonary injuries,[27] suggesting multidirectional communication between the cells and the environment. Also, the culture medium for *in vitro* experiments can play a role in the cell fate determination. A549 cells, for example, acquire an AT1 phenotype when grown onto traditional plastic substrates.[28] Thus, a more comprehensive understanding of the factors affecting the re-epithelialization and regeneration of pulmonary tissues is important.

Though we demonstrated that EFs are important bio-physical signals for the alveolar damage repair, some limitations still remained. First, the experiment is conducted at cellular level, convenient but limiting the practicability at tissue and higher levels, which could be further improved by animal experiments. What's more, the experimental EFs were applied horizontally, suitable for attached cells, but not enough for the aim as medical use. More detailed researches into the injury-targeted usage of EFs are needed, the method for EFs generation within alveoli at the center of the damage, for example. Besides, the voltages given were based on, alveolar epithelia having none, the previous measure on the EF changes of damaged airway epithelia that may be inconsistent with the ones in the alveoli, which could be improved by the further researches into the bio-electric features of alveoli.

In conclusion, through extracting AECs from pulmonary tissues and exploring their responses to direct-current EFs, we demonstrated that EFs could guide and accelerate the directional migration of AECs and exert antiapoptotic effects through the activation of PI3K/AKT pathway, suggesting their therapeutic potential in alveolar damage.

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Ethical statement

The animal study protocols were approved by the Laboratory Animal Welfare and Ethics Committee of the Third Military Medical University (approval number for production: SCXK (PLA) 20120011; approval number for application: SCXK (PLA) 20120031).

Declaration of competing interest

Bing Song, Jian-Xin Jiang and Li Li have a patent pending for "A nanomaterial-based magnetoelectric repair system for lung injury" (202310229927.1).

Author contributions

Chao-Yue Yang and Li Li wrote the manuscript; Jian-Hui Sun, Kan Zhu and Juan Du conducted the experiments; Ying Zhang, Cong-Hua Lu, Wen-Yi Liu, Ke-Jun Zhang contributed to the experiments and statistical analysis. Li Li, An-Qiang Zhang, Ling Zeng and Jian-Xin Jiang funded and supervised the work.

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Appendix A. Supplementary data

Supplementary A1-4 related to this article can be found at https://doi.org/10.1016/j.cjtee.2023.03.003.

Supplementary A5-6 related to this article can be found at https://doi.org/10.1016/j.cjtee.2023.03.003.

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