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Impaired self-healing capacity in airway epithelia lacking aquaporin-3

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ABSTRACT

In this study, we utilized AQP3-knockout mice as the in vivo model and AQP3-knockdown human bronchial epithelial cells (HBECs) as the in vitro model. Airway injury was experimentally induced by intra-tracheal injection of naphthalene. HE staining, transmission and scanning electron microscope were performed to evaluate self-healing capacity in vivo. Transwell and wound-healing assays were performed to evaluate epithelial cell migration in vitro. We found that both the airway epithelial cells of AQP3-knockout mice and AQP3-knockdown HBECs exhibited an obviously impaired self-healing capacity with defective epithelial cell migration through AQP3-facilitated glycerol transport. In addition, glycerol supplementation could largely correct defective injury healing and epithelial cell migration. For the first time, we found evidence for distinct defects in AQP3-deficient airway epithelial cell migration. Mechanistic analysis showed AQP3-facillitated glycerol transport plays a role in airway epithelial self-healing after injury.

1. Introduction

The lung is constantly subjected to harmful exposures, such as inhaled toxic substances, particulate matter, viral or bacterial infections, and autoimmune reactions that cause injury to the airway and alveolar epithelium. The epithelial barrier plays a significant role in a number of airway diseases, including asthma, where an impaired epithelial barrier function makes the airway susceptible to damage. Currently, existing therapies fail to reverse the course of disease but instead only focus on improving symptoms (Lambrecht and Hammad, 2012). Adjunct therapies targeting repair of airway epithelium are needed.

Aquaporins (AQPs) are a family of transmembrane channel proteins responsible for transporting water and other small solutes between and into cells (Rojek et al., 2008; Verkman, 2005). AQP3 belongs to the so-called aquaglyceroporin group of AQPs, which transport both water and the humectant glycerol (Boury-Jamot et al., 2009). AQP3 is expressed in various tissues such as the skin, kidney, respiratory tract, and gastrointestinal tract. AQP3 is known to be necessary for urine concentration in the kidney. AQP3-facilitated water and glycerol transport is essential for the healing of cutaneous wounds and the hydration and elasticity of skin. Recent data implicate AQP3-facilitated water and glycerol transport in cell migration, proliferation, differentiation, lipid metabolism and barrier formation (Hara-Chikuma and Verkman, 2008a).

In the lung and airways, fluid transport across epithelial and endothelial barriers occurs during alveolar fluid movement, airway hydration, and submucosal gland secretion. AQP3 is localized in basal epithelial cells in large airways and throughout the nasopharnyx (King et al., 1997; Nielsen et al., 1997). Understanding its contribution to the self-healing capacity of airway epithelium following injury is limited. The present study aimed to investigate the involvement and potential mechanism of AQP3 in the self-healing capacity of airway epithelium.

2. Materials and methods

2.1. Transgenic mice and animal husbandry

AQP3$^{-/-}$ mice (BALB/c genetic background) were maintained at pathogen-free facilities in Fudan University, Shanghai, China. Litter mates of six to eight week old AQP3$^{+/+}$ and AQP3$^{-/-}$ mice were used in the experiments. 5% glycerol was used for 3 days before naph-

Abbreviations: AQP, aquaporin; HBEC, human bronchial epithelial cell; SEM, scanning electron microscopy; TEM, transmission electron microscopy; siRNA, small interfering RNA.

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thalene treatment in the glycerol supplement study. This study was approved by Ethic Committee of Zhongshan Hospital, Fudan University and was carried out in accordance with the National Institutes of Health guide for the care of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Naphthalene treatment

At six to eight weeks of age, mice were anesthetized to undergo treatment. Naphthalene (Sigma Chemical Co., St Louis, MO) was dissolved at a concentration of 30 mg/ml and administered to mice (275 mg/kg) by intratracheal injection. Animals were sacrificed on 2 h, 24 h and 7 days following Naphthalene exposure (Van Winkle et al., 2002; Xing et al., 2012).

2.3. Cell culture

Human bronchial epithelial cells (HBECs) were obtained from Lonza (Lonza, Basel, Switzerland). Cells were cultured in Dulbecco's modified Eagle's medium with or without 10% fetal bovine serum (FBS, Hyclone) at 37 °C in a 5% CO2, 95% air environment in humidified incubators.

2.4. Scanning electron microscopy (SEM)

The collected samples were immediately rinsed with jet streams of physiological saline to remove mucus, followed by serial fixation with 3% glutaraldehyde and 1.5% osmium tetroxide. They were then dehydrated in a graded series of alcohol, immersed in amylacetate, and dried with liquid carbon dioxide at the critical point. The dried samples were fixed on the stage under a dissecting microscope and subjected to ion-spattering coating with Au-Pd. Samples were examined and photographed under a scanning electron microscope (Hitachi Ltd, Tokyo, Japan).

2.5. Transmission electron microscopy (TEM)

The samples were double-fixed with 1.5% osmium tetroxide and 3% glutaraldehyde, dehydrated in a graded series of alcohol, embedded in Epon 812, and then cut into 60 nm ultra-thin sections and stained with uranyl acetate and lead citrate. Sections were examined and photographed under a transmission electron microscope (Philips, Hillsbro, USA).

2.6. Immunohistochemistry

The tracheae were fixed in paraformaldehyde for 12 h, and then cut into 4 μm-thick transverse sections. Sections were blocked with 1% BSA for 30 min, incubated in AQP3 primary antibody (Abcam Inc., Cambridge, MA) overnight, and then incubated with secondary antibody (Sigma Chemical Co., St Louis, MO) after three washes. Sections were examined and photographed under a Nikon A1R confocal laser scanning microscope system (Nikon Corp., Tokyo, Japan).

2.7. Small interfering RNA (siRNA) transfection

SiRNA transfections were performed according to the manufacturer's protocol. Briefly, in 6-well plates using Lipofectamine™ 2000 with three different sequences of siRNA duplexes targeting AQP3 (Supplementary Table 1) and RNA negative control (GenePharma, shanghai, China), 3 μl of Lipofectamine™ 2000 and 60 pmol of each siRNA were transfected in triplicate, except for ratio-dependent effect studies where several ratios of Lipofectamine™ 2000/siRNA were tested to optimize the efficacy of transfection. Cells were prepared for experiments either 48 h or 72 h after transfection.

2.8. Migration assay

In the transwell (Corning Inc., Corning, NY) assay, HBECs at the concentration of 5×10^5/ml in serum-free medium were seeded in the upper chamber, while medium containing 10% FBS was added to the lower chamber. Cells migrated through the permeable membrane at 48 h were fixed and stained with Giemsa, and then counted under microscope. In the wounding-healing assay, the wound track was made by scraping the monolayer-adherent cells with a sterilized pin. Cell migration into the cell-free area was assessed using an inverted light microscope and migration distances were measured at 0, 24 and 48 h in the absence or presence of glycerol (20 ng/ml) (Sigma Chemical Co., St Louis, MO).

2.9. Measurement of glycerol and ATP content

Trachea homogenates (3500 g, 10 min, 4 °C) were assayed for glycerol and ATP concentration. For glycerol measurement, we uesd Triglyceride Determination Kit (Catalog Number TR0100, Sigma). As shown in manufacturers' instructions, the procedure of colorimetric assay kit involved enzymatic hydrolysis by lipase of the triglycerides to glycerol and free fatty acids. The glycerol produced was then measured by coupled enzyme reactions. The increase in absorbance at 540 nm was directly proportional to triglyceride concentration of the sample. For ATP measurement, we uesd ATP Bioluminescence Assay Kit HS II (Catalog Number 11699709001, Sigma). As shown in manufacturers' instructions, the determination of ATP used the ATP dependency of the light emitting luciferase catalyzed oxidation of luciferin.

2.10. Statistical analysis

Data was expressed as mean ± standard errors. For the statistical analysis, 0.05 was set as the P value for significance. Normal distribution was assumed. The data of two groups were analyzed by t-test. The data of three groups were analyzed by ANOVA.

3. Results

3.1. Impaired in vivo airway epithelial repair in AQP3$^{-/-}$ mice

Immunohistochemistry showed AQP3 expression in the airway epithelium of wild-type (AQP3$^{+/+}$) mice but not in AQP3$^{-/-}$ mice (Fig. 1A). AQP3 was most abundant in the plasma membranes of basal epithelial cells. We then measured healing after airway injury induced by naphthalene in AQP3$^{+/+}$ and AQP3$^{-/-}$ mice. Fig. 1B shows impaired airway epithelial repair after 7 days of airway injury in AQP3$^{-/-}$ mice. At 2 h after naphthalene treatment, airway epithlial cells had a more rounded appearance in both AQP3$^{+/+}$ and AQP3$^{-/-}$ group. Many of the epithlial cells had exfoliated from the airway basement membrane, leaving extremely attenuated squamated cells. At 7 days after naphthalene treatment, airway was gradually covered by squamated cells to rapidly restore some barrier protection in AQP3$^{+/+}$ group. Newly regenerated epithlial cells comprised most of the airway epithelium in AQP3$^{+/+}$ mice. In contrast, fewer Clara cells regeneration were shown in AQP3$^{-/-}$ mice.

The effects of AQP3 on airway epithelial ultrastructure were assessed by SEM and TEM. At 2 h after injury, SEM showed no morphologic differences in cilia between the AQP3$^{+/+}$ group and AQP3$^{-/-}$ groups (Fig. 2A and B). At 24 h after injury, disruption and deciduation of ciliated epithelial cells were seen in both the AQP3$^{+/+}$ and AQP3$^{-/-}$ groups (Fig. 2C and D). 7 days after injury, repair of the

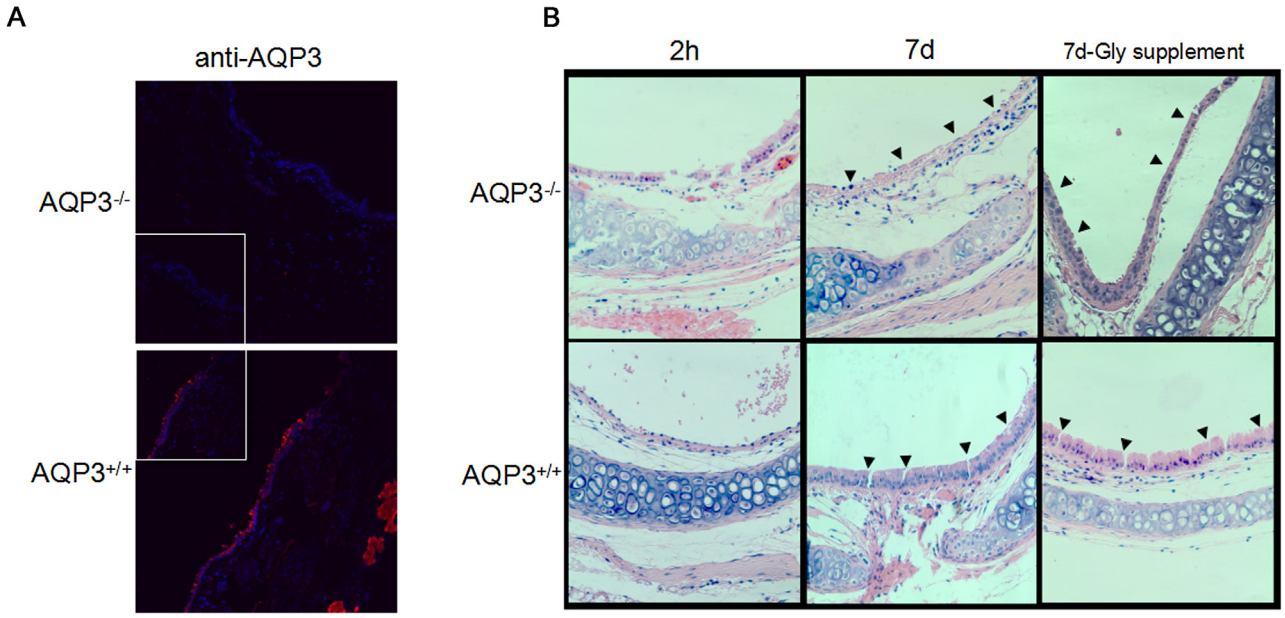

Fig. 1. Airway epithelial repair in vivo after injury induced by toxicant naphthalene. (A) AQP3 protein expression in wild-type mice airway epithelia. (B) Histology showing wounded airway epithelia after 2 h (left column), 7 days (middle column) of healing and 7 days of healing after glycerol supplement (right column). Triangle mark denote for airway epithelium.

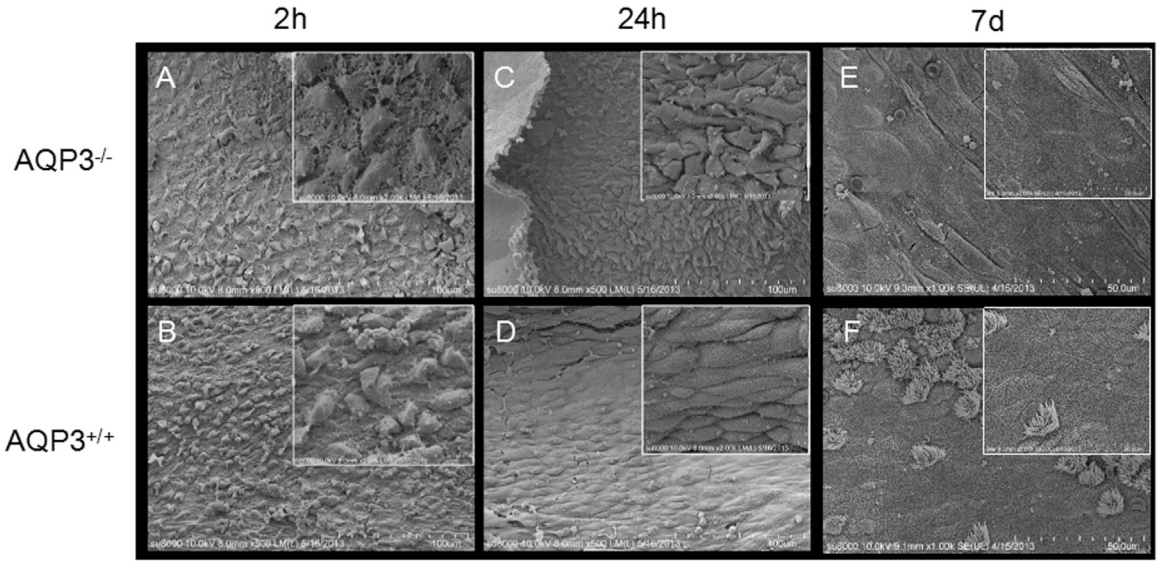

Fig. 2. Scanning electron microscopy showing airway epithelial repair in vivo after injury induced by toxicant naphthalene. Wounded airway epithelia after 2 h (left column), 24 h (middle column) and 7 days (right column) of healing.

ciliated cells had begun in the AQP3$^{+/+}$ group with some regeneration of cilia (Fig. 2F). However, unrepaired ciliated epithelial cells and compound cilia were observed in AQP3$^{-/-}$ group (Fig. 2E), suggesting that the repair process after injury takes more time in AQP3$^{-/-}$ group.

On TEM, no abnormalities were noted in the AQP3$^{+/+}$ group and AQP3$^{-/-}$ group at 2 h after naphthalene treatment (Fig. 3A and B). At 24 h after injury, gaps occurred between ciliated epithelial cells, and basal cells were present and activated below the epithelium (Fig. 3C and D). 7 days after injury in the AQP3$^{+/+}$ group, there was further deciduation of ciliated epithelial cells and migration of inflammatory cells and basal cells during mitosis (Fig. 3E). However, less basal cells were observed in the AQP3$^{-/-}$ group, especially during mitosis (Fig. 3F). These findings suggest impaired repair process in AQP3-null airways may result from defects in airway epithelial cell migration. Therefore, we next measured cell migration to determine the role of AQP3 in airway repair.

3.2. Reduced cell migration in AQP3-konckdown epithelial cells

The role of AQP3 in HBECs migration was investigated by transwell assay. Cells migrated from top-to-bottom through the porous transwell membrane and were stained. The migration was quantified by cell counting. AQP3-knockdown HBECs by si-RNA had significantly reduced capacity of migration toward 10% FBS compared to the control cells ($P < 0.01$, Fig. 4A), while cell adherence was not significantly different compared to the control group (Supplementary Fig. 1).

A standard wound-healing assay was also used to evaluate the role of AQP3 in HBECs migration. Wound closure rates were accel-

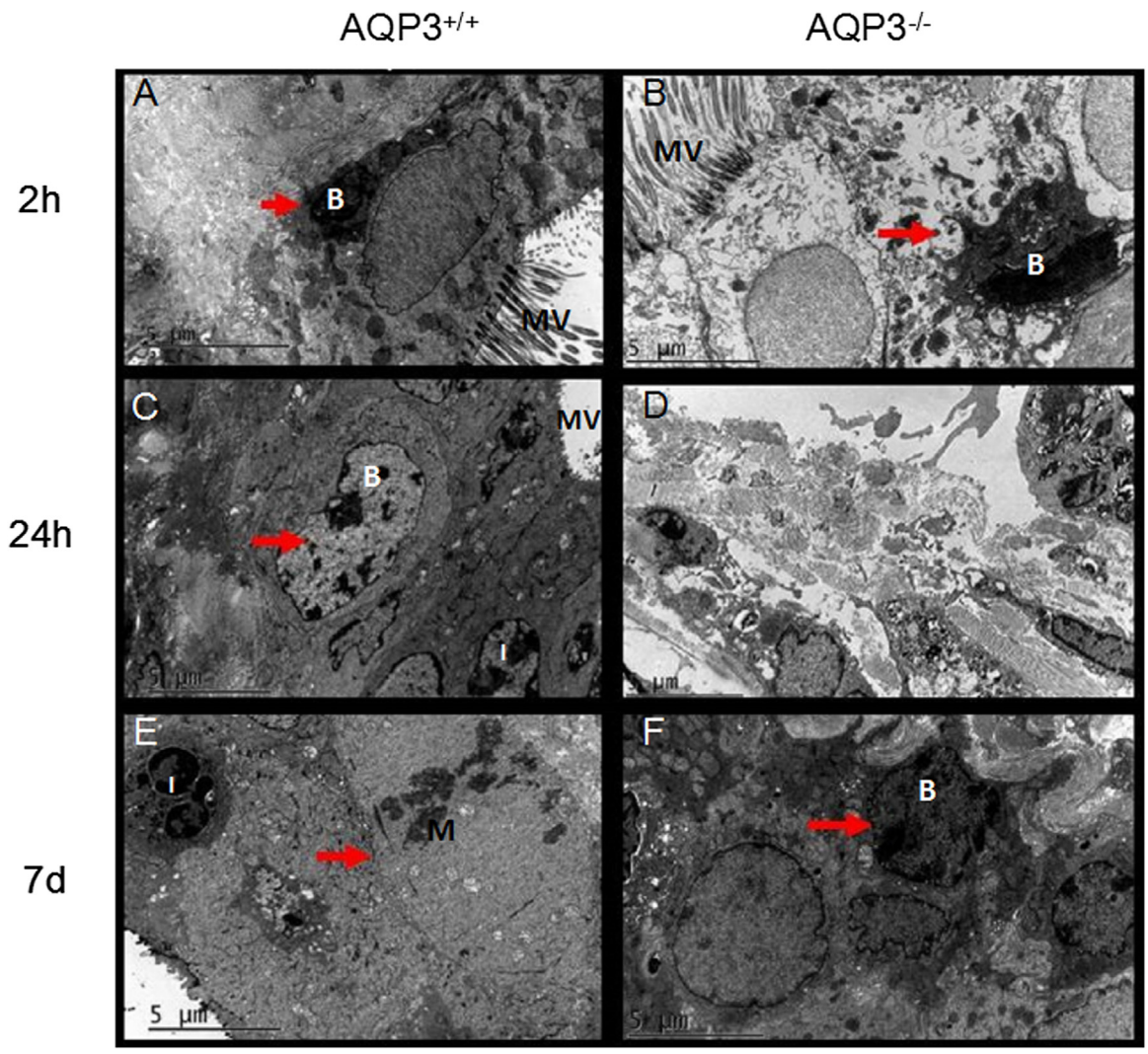

Fig. 3. Transmission electron microscopy showing airway epithelial repair in vivo after injury induced by toxicant naphthalene. Wounded airway epithelia after 2 h (top row), 24 h (middle row) and 7 days (bottom row) of healing. B: basal cell; MV: microvilli; I: inflammatory cell; M: mitotic basal cell. The red arrows indicate the basal cell or mitotic basal cell.

erated in cultures grown on fibronectin-coated wells compared to uncoated plastic wells in accordance with previous studies. In vitro wound healing was thus quantified in fibronectin cultures after a relatively short (24-h and 48-h) healing period to avoid confounding effects of cell proliferation on healing. Fig. 4C shows delayed wound healing in AQP3-knockdown HBECs at 48 h after creation of linear wounds (three wells per group, $P < 0.01$, Fig. 4D), supporting the conclusion from the in vivo part that AQP3-deficiency impairs airway epithelial cell migration after injury.

3.3. Reduced glycerol and ATP contents in AQP3$^{-/-}$ airways

Glycerol is not only one of the energy sources in the mammalian body, but also a critical compound that links the metabolism of sugar, fat and protein. To test the role of AQP3 in airway energy metabolism, we measured glycerol and ATP content in the respiratory tract. As demonstrated in Fig. 5A, the glycerol content was significantly decreased in AQP3$^{-/-}$ airways (43.56 ± 3.18 vs. 73.57 ± 4.11 nmol/mg protein in wild type mice, $P < 0.05$), as was the ATP content (310.54 ± 37.98 vs. 531.15 ± 20.18 nmol/mg protein in wild type mice, $P < 0.05$, Fig. 5B). The results provide evidence for the involvement of AQP3-faciliated glycerol transport in airway energy metabolism.

3.4. Glycerol supplement corrects impaired injury repair and cell migration in airway epithelia

To explore the role of AQP3-facilitated glycerol transport in airway repair after injury in vivo, we tested whether glycerol supplementation might correct impaired airway repair in AQP3$^{-/-}$ mice. Mice were given glycerol orally for 3 days prior to the naphthalene treatment. Light microscopy showed that oral glycerol administration largely improved the speed of airway epithelial repair in AQP3$^{-/-}$ mice compared to the control group but did not completely correct repair (Fig. 1B).

The in vitro study demonstrated that impaired cell migration in AQP3-knockdown HBECs was greatly restored after glycerol supplementation through the transwell migration assay ($P < 0.05$, Fig. 4A, B2 and B3) and the wound-healing assay ($P < 0.05$, Fig. 4C and D). Thus, the present findings support the conclusion that AQP3-facilitated glycerol transport is involved in airway epithelial cell migration after injury.

4. Discussion

This study provides evidence for the involvement of AQP3 in the self-healing capacity of the airway. Defective airway epithelial

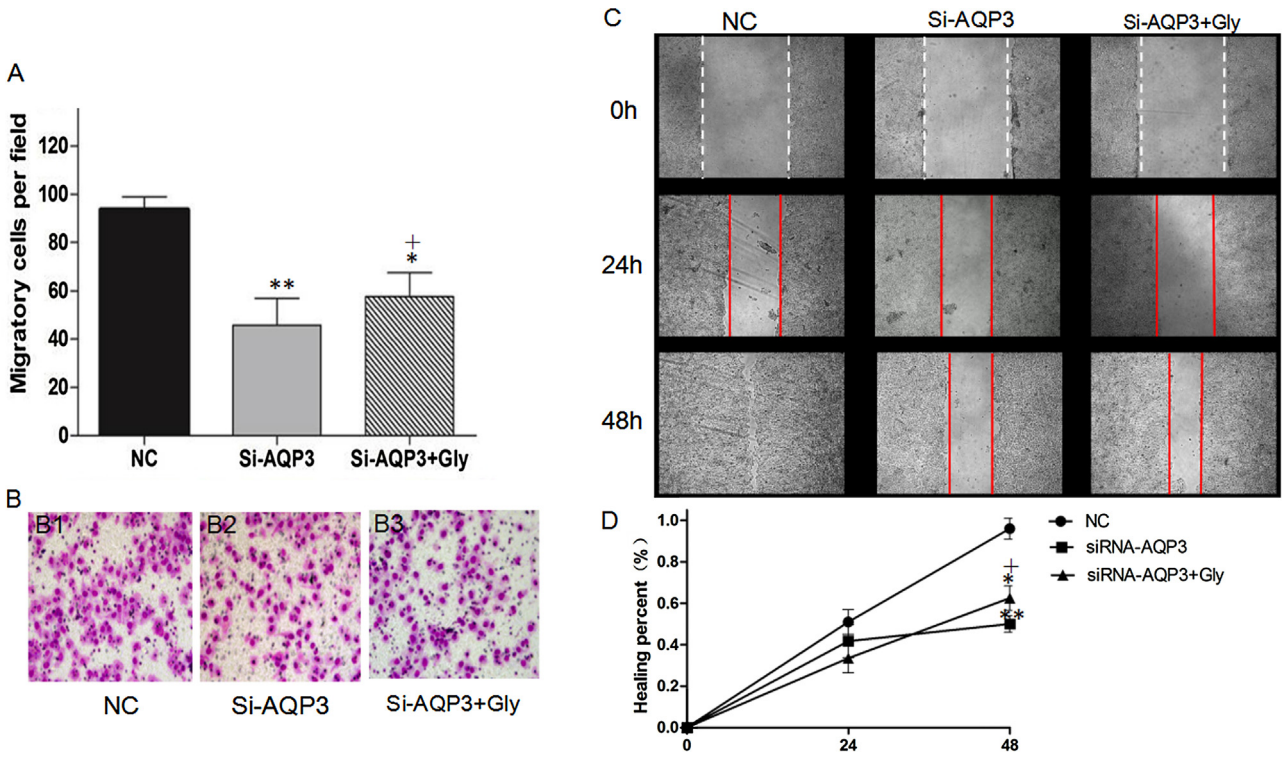

Fig. 4. AQP3 deficiency impairs cell migration in cultures of human bronchial epithelial cells (HBECs). (A) Average cell numbers of migration in three identical experiments by transwell assay. (B) Effect of endogenous AQP3 and exogenous glycerol supplement on HBEC migration. Figures showed Giemsa stained HBECs which migrated through the permeable membrane at 48 h. Migration of AQP3-knockdown HBECs by siRNA toward 10% FBS was significantly reduced compared to the control cells. Glycerol supplement corrected the migration of AQP3-knockdown HBECs. (C) Effect of endogenous AQP3 and exogenous glycerol supplement on HBEC migration by wound-healing assay. Light micrographs of wounded cell monolayers grown on fibronectin-coated wells showing delayed wound closure at 24 and 48 h in AQP3-knockdown HBECs, but accelerated wound closure at 48 h in AQP3-knockdown HBECs after glycerol supplement. (D) Levels of healing percent. HBECs were pretreated with or without glycerol supplement, followed by siRNA transfection. NC: RNA negative control. (* and ** stand for p values less than 0.05 and 0.01, respectively, as compared with control group. +stands for p values less than 0.05, as compared with si-AQP3 group.).

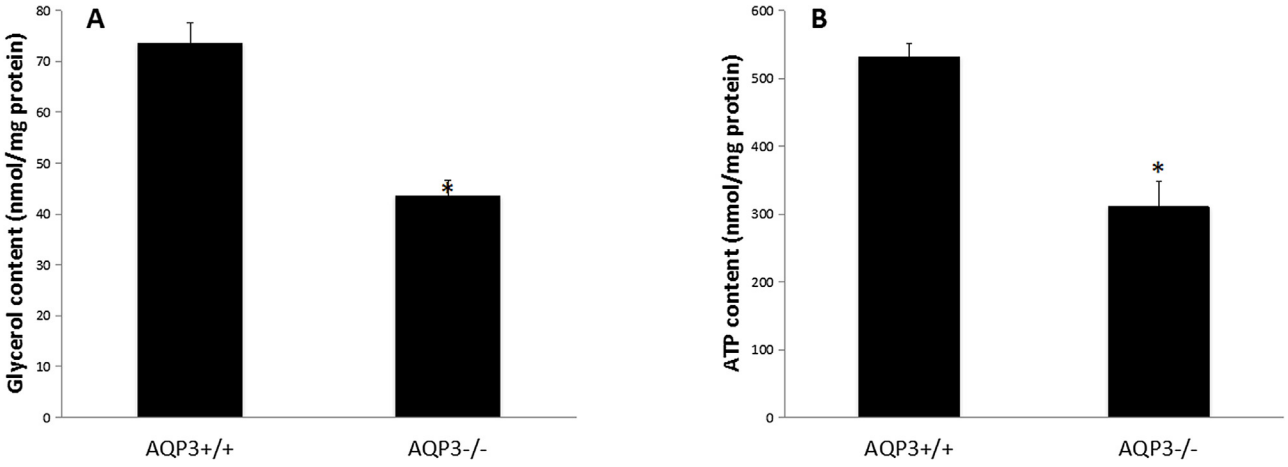

Fig. 5. Airway epithelia glycerol and ATP content. (A) The glycerol content was significantly lower in AQP3$^{-/-}$ respiratory tract [(43.56 ± 3.18) vs. (73.57 ± 4.11) nmol/mg protein in wild type mice]. (B) The ATP content was also significantly lower in AQP3$^{-/-}$ respiratory tract [(310.54 ± 37.98) vs. (531.15 ± 20.18) nmol/mg protein in wild type mice]. (*P < 0.05).

repair after injury implicated a role for AQP3 in airway epithelial migration both in vivo and in vitro, which was proved by experiments using AQP3-null mice and AQP3-knockdown HBECs.

AQP3 has been termed an aquaglyceroporin because it is capable of transporting glycerol as well as water (Johnston et al., 2000; Matsuzaki et al., 1999; Ma et al., 2000). The extensive distribution of AQP3 in small and distal airways has been reported in previous studies (Mobasheri et al., 2005; Liu et al., 2007). In those regions, AQP3 was thought to be important in dealing with the large volume absorption that occurs as a result of the axial movement of water from the alveolus (Nielsen et al., 1997; Lehmann et al., 2008). In our previous study, we have already found that both dexamethasone and ambroxol stimulated the expression of AQP3 at the mRNA and protein levels (Ben et al., 2008). However, our previous study concluded that AQP3 appeared to be of minor importance in the humidification of upper and lower airways, hydration of the airway surface liquid, and isosmolar fluid absorption (Song et al., 2001). As discussed previously (Verkman et al., 2000), the tissue-

specific expression of an aquaporin may indicate its physiological significance. Our identification of discrete AQP3 contributions to the human airways repair after injury may be an initial step toward a further understanding of the mechanistic physiology of AQP3.

Airway epithelial self-healing after injuring is highly regulated (Grainge and Davies, 2013; Puchelle et al., 2006). Migration is of great importance to normal basal epithelial cells and is accelerated during airway injury. There is now good experimental evidence indicating that these airway basal cells can be considered as a population of multipotent stem cells that drive both homeostasis of the normal epithelium and its orderly self-healing after injury (Delplanque et al., 2000). AQP3 is expressed in basal cells of the bronchial and pseudostratified nasal epithelium (Nielsen et al., 1997; Avril-Delplanque et al., 2005). In our study, at 24 h after injury, TEM showed basal cells present and activated underneath the epithelium in both groups. Fewer basal cells, especially during mitosis, were observed in the AQP3$^{-/-}$ group 7 days after injury. These findings suggest an impaired repair process in AQP3$^{-/-}$ airways may result from defects in the airway epithelial basal cell. Clearly, it will be important to try and correlate the role of AQP3 with the multipotent stem cells that drive both homeostasis of the normal epithelium and its orderly self-healing after injury. AQP3 is also expressed on T cells and regulates their traffiking in cutaneous immune reactions(Hara-Chikuma et al., 2012). In our study, we noticed that there was further migration of inflammatory cells in the AQP3$^{+/+}$ group based on TEM image. Further studies are required to elucidate the underlying mechanism in the near future.

In a number of airway diseases, especially asthma, the epithelial barrier is disrupted. Impaired epithelial barrier function makes the airways susceptible to infections and environmental insults (Xiao et al., 2011). In our study, we used a naphthalene-induced murine model of acute epithelial damage and repair, which is characterized by selective cytotoxicity of naphthalene to Clara cells. Disruption of Clara cells results in the formation of lesions in the airway similar to the epithelial damage observed in endobronchial biopsies from asthma patients (Royce et al., 2014). Several distinct mechanisms for the self-healing capacity of epithelial cells have been identified in previous studies. Previous data have shown impaired p38 activation in keratinocytes during skin wound healing, which is in agreement with the role of the p38-MAPK pathway in regulating epidermal cell response to inflammation and stress (Carlin et al., 2000; Itoh et al., 2001; Hara-Chikuma and Verkman, 2008b). Further experiments are needed to investigate the link between p38-MAPK cell signaling and airway epithelial repair.

Oral glycerol supplementation corrected both the impaired airway epithelial cell repair in AQP3$^{-/-}$ mice and the decreased cell migration in AQP3-knockdown HBECs. AQP3-mediated glycerol transport was found previously to be critical for lipid biosynthesis in skin and nerve (Hara- Chikuma and Verkman, 2008a; Sugimoto et al., 2013). Glycerol-3-phosphate, one of the critical metabolic intermediates for ATP production, is metabolized from synthesized glycerol (Brisson et al., 2001). The role of glycerol metabolism in respiratory disease remains unexplored. Currently studies are much needed to elucidate the precise metabolic pathways in airway epithelial cells linking glycerol and ATP generation. Our data here support the requirement of AQP3-facilitated glycerol transport in cell migration during airway repair.

Current standard therapies for airway diseases, such as asthma, only manage symptoms but barely reverse the natural course (Leonardi et al., 2012). It is evident that the self-healing capacity of airway epithelium plays a key role in the natural course of airway diseases, including remodeling and bronchial hyperreactivity (Hallstrand et al., 2012). Thus, strategies focused on the self-healing capacity of the airway epithelial cells may shed new light on altering the natural course of disease (Puchelle et al., 2006; Holgate, 2011).

In conclusion, the present study demonstrates that an impaired respiratory self-healing capacity after injury in vivo and in vitro results from distinct defects in airway epithelial cell migration, markedly related to the impaired AQP3-facilitated glycerol transport. Decreased AQP3-dependent epithelial cell migration may be one of the crucial events in respiratory diseases associated with airway epithelial self-healing capacity. Future strategies for respiratory disease therapy may target pharmacologic modulation of AQP3 function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.resp.2016.08.002.

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