

A cell culture model for alveolar epithelial transport

Hui Ren¹ and Vinod Suresh^{1, 2, 3}

¹ Auckland Bioengineering Institute

² Department of Engineering Science

³ Maurice Wilkins Centre for Molecular Biodiscovery

University of Auckland, New Zealand

Abstract

Background

The thickness and composition of the surface liquid lining the human lungs are maintained by a balance between epithelial secretion and absorption of ions and water. An understanding of epithelial transport pathways and the factors that regulate them will provide insight into the development of conditions such as lung edema and guide the development of treatment modalities. Here we report on the development and characterisation of a cell culture model of the alveolar epithelium that will be useful for investigating the components of epithelial transport pathways and interpreting molecular mechanisms involved in transport related diseases.

Methods

An in vitro cell culture model was developed using human alveolar epithelial cell lines NCI-H441 and A549 cultured with the apical surface exposed to air (air-medium) or covered by nutrient medium (medium-medium). Cell monolayer was presented by visualizing cell morphology under microscope. Transepithelial electrical resistance, potential difference and fluorescence permeability measurements were used to assess the formation of a polarised epithelium with functional barrier properties. Expression of tight junction, adherens junction and ion/water transport proteins were examined by Western blot and RT-PCR.

Results

NCI-H441 cells cultured under air-medium conditions exhibited electrical resistance ($258 \pm 28 \Omega \cdot \text{cm}^2$), potential difference ($4.8 \pm 0.1 \text{ mV}$) and strong expression of $\alpha_1\text{-Na}^+\text{-K}^+\text{-ATPase}$ and tight junction protein ZO-1 consistent with the formation of a polarised epithelium. These cultures also expressed the chloride channel CFTR and all four subunits of the sodium channel ENaC. Cells cultured under medium-medium conditions had a 4-fold higher electrical resistance ($1009 \pm 15 \Omega \cdot \text{cm}^2$), but similar level of potential difference ($4.9 \pm 0.2 \text{ mV}$) and weaker expression of $\alpha_1\text{-Na}^+\text{-K}^+\text{-ATPase}$ and ZO-1. The A549 cell line developed low levels of electrical resistance and potential difference and did not express ZO-1. No significant difference in CFTR and ENaC transport protein expression was observed between the cell lines or culture conditions.

Conclusion

The NCI-H441 cell line cultured under air-medium conditions develops into a polarised epithelium with functional barrier properties and expresses transport proteins for sodium and chloride transport. Hence it can serve as a suitable model for investigating water and ion transport in the alveolar epithelium.

Corresponding author contact information

Hui Ren, M.Sc.

Auckland Bioengineering Institute, University of Auckland

51 Address: Level 7, UniServices House, 70 Symonds Street, Auckland 1010, New Zealand
52 Tel: +64-9-373-7599 Ext. 81030;
53 Fax: +64-9-367-7157;
54 E-mail: hren587@aucklanduni.ac.nz

Key words

alveolar epithelial cells, ion and water transport, cell culture model, NCI-H441, A549, epithelial sodium channel, cystic fibrosis transmembrane conductance regulator

Abbreviations

AMC: air-medium culture; HMC: HBSS-medium culture; MMC: medium-medium culture; TEER: transepithelial electrical resistance; TEPD: transepithelial potential difference; Flu-Na: sodium fluorescein; ENaC: epithelial Na⁺ channel; CFTR: cystic fibrosis transmembrane conductance regulator; AQP3: aquaporin 3; AQP5: aquaporin 5; ZO-1: zona occludens protein-1

Introduction

The alveolar surface, which comprises more than 99% of the internal surface area of human lungs, is lined by a continuous epithelium that forms a barrier between the organism and the outside world (Hollenhorst, Richter & Fronius, 2011). The alveolar epithelium is protected by a surfactant lining fluid, which is essential for maintaining efficient gas exchange, surfactant homeostasis, and defense against inhaled toxins and pathogens (Bove et al., 2010; Geiser et al., 2001).

The composition and volume of the overlying liquid are carefully regulated by a balance between secretion and absorption of ions and water by the lung epithelial cells (Matthay, Folkesson & Clerici, 2002). Both paracellular and transcellular pathways are involved in this process. The paracellular pathway provides a route for passive transepithelial transport, with ions moving in either direction driven solely by their electrochemical gradient. The permeability and ion selectivity of the paracellular pathway is critical for establishing or dissipating ion concentration gradients and hence for determining the ionic composition of the apical compartment and net volume flow (Van Itallie & Anderson, 2006). The transcellular pathway comprises the epithelial cells with their distinct apical and basolateral membranes containing channels, transporters, and pumps. Together, they generate active (and sometimes passive) transepithelial ion transport (Flynn et al., 2009). However, the relative contributions of the different pathways and the factors that affect them are not well understood (Strengert & Knaus, 2011).

Studies of epithelial fluid transport have provided important new concepts regarding the resolution of lung edema, a common clinical problem that has direct relevance to the pathophysiology of acute lung injury. Under such disease condition, Na^+ channel activity is

impaired, which leads to the failure in Na^+ reabsorption and fluid clearance (Matthay, Folkesson & Clerici, 2002). Uncovering the mechanisms regulating ion and water transport is crucial for understanding lung fluid balance under both normal and pathologic conditions.

A number of mathematical models incorporating electrophysiological components (anion and cation channels, pumps and co-transporters, water channels) have been developed to describe water and/or ion transport in the epithelia (Novotny & Jakobsson, 1996; Warren, Tawhai & Crampin, 2009). Such models are essential for building a framework to interpret experimental data and explore ‘what-if’ scenarios, but model parameterisation and validation are particular challenges. In existing models parameter values are often taken from studies in different species and validation depends on using model outputs to infer experimentally accessible quantities.

Thus it will be useful to develop a model system in which individual proteins and pathways can be perturbed under characterised and controlled conditions. A variety of models, including whole animal, isolated lung, isolated cell, and cultured cell model systems, has been extensively used in the regulation of ion and liquid transport in different species (Dobbs & Johnson, 2007; Fernandes & Vanbever, 2009). In vitro models offer a useful tool as they bring up fewer ethical questions. Among them, human cells are the most representative of the clinical situation, but they are costly and can only be maintained *in vitro* for a few days. To the best of our knowledge, a single report has been published using primary human alveolar epithelial cells to study ion and water transport (Bove et al., 2010). Therefore, respiratory epithelial cell lines are used extensively as representative models because the experimental conditions are more reproducible and easier to control (Mathias, Yamashita & Lee, 1996). Several cell lines deriving from bronchial epithelial cells, such as Calu-3, BEAS-2B and 16HBE14o⁻, have been shown to be suitable models for studies of airway epithelium

(Mathias, Yamashita & Lee, 1996). However, only H441 and A549 cell lines are derived from alveolar epithelial cells and have been characterised to present alveolar epithelial phenotype (Rehan et al., 2002; Lieber et al., 1976).

In traditional cell culture models, adherent cells are usually immersed in medium. However, this does not reflect the physiological condition of lung epithelial cells which are exposed to air. In many studies for lung epithelial cells, air-liquid interface has been introduced by growing cells on microporous membranes in a two chamber system (Blank et al., 2006). This system has been proved to mimic the physiological conditions for lung epithelial cells and drives differentiation towards a phenotype similar to that reached in vivo (Stewart et al., 2012). In our present study, three different culture conditions were used for alveolar epithelial cell culture. One is air-medium culture (AMC) in which the apical surface of the cells is exposed to air. The second one is medium-medium culture (MMC), which is identical to the traditional culture technique with both chambers filled with identical medium. The last one is HBSS-medium culture (HMC), which is an intermediate state between AMC and MMC conditions with cells covered by Hank's Balanced Salt Solution (HBSS).

The aim of this research is to establish a model system using human alveolar epithelial cell lines H441 and A549 and characterise the transport profile of alveolar epithelial cells under this model. Such a model system is essential in validating theoretical models using direct experimental measurements. It will be useful to determine quantitative contributions of different electrophysiological components to transport under physiological conditions. It will also be helpful to interpret molecular mechanisms of transport related diseases.

Materials and Methods

Materials

Cell culture medium RPMI 1640 and F-12K, FBS, penicillin-streptomycin (P/S) and insulin-transferrin-selenium (ITS) were bought from Invitrogen, New Zealand. Dexamethasone, tri-iodo-thyronine (T3) and sodium fluorescein (Flu-Na), RIPA buffer and protease inhibitor cocktail were bought from Sigma, New Zealand. Antibodies against ZO-1 and E-cadherin were bought from Invitrogen, New Zealand. Antibody against $\alpha_1\text{-Na}^+\text{-K}^+\text{-ATPase}$ was bought from Millipore, USA. Antibodies against $\alpha\text{-ENaC}$, AQP3, AQP5, $\beta\text{-actin}$ and HRP labelled secondary antibodies were bought from Santa Cruz, USA. Antibody against CFTR was from University of North Carolina, USA.

Cell culture and maintenance

NCI-H441 cells (HTB-174) and A549 cells (CCL-185), which are human pulmonary adenocarcinoma cell lines with characteristics of alveolar type II cells, were obtained from the American Type Culture Collection (ATCC, Australia) and grown in T-75 culture flasks in an atmosphere of 5% CO_2 at 37°C. H441 cells and A549 cells were maintained in RPMI 1640 and F-12K medium respectively containing 10% FBS and 1% penicillin-streptomycin. Cells were grown in three different culture conditions, i.e. air-medium culture (AMC), HBSS-medium culture (HMC) and medium-medium culture (MMC). Cells were seeded onto 12-well transwell inserts (Costar 3460, Corning, USA) at a density of 10^5 cells/well for AMC and HMC groups, and 2.5×10^4 cells/well for MMC groups. Cells were allowed to attach for 24 h before 1.5 ml polarization medium were applied into the bottom chamber. Polarization medium was made up of basic medium RPMI 1640 or F-12K containing 4% FBS, 1% P/S and 1% ITS, 200 nM dexamethasone and 10 nM T3. For AMC condition, the upper chambers were left empty. For HMC and MMC conditions, the upper chambers were filled with 0.5 ml

HBSS and polarization medium respectively. Buffers and medium in the three groups were changed every two days.

Transepithelial electrical resistance (TEER) and transepithelial potential difference (TEPD)

Pre-warmed HBSS was added to the apical (0.5 ml) and basolateral (1.5 ml) sides of the cell monolayer. TEER and TEPD values were measured over time using an EndOhm-12 chamber voltohmmeter device (WPI, USA), and were corrected by subtracting the values of a blank insert without cells. Three wells were assigned for measurement and the resistance of the cell monolayer in each of the three wells was measured from day 0 to day 14 of culture period. Final resistance-area products ($\Omega \cdot \text{cm}^2$) are obtained by multiplication with the effective growth area (1.12 cm^2).

Permeability study

Flu-Na (MW = 367 Da) was used to assess paracellular transport as a measure of barrier integrity. Cells were cultured on the transwell inserts under AMC, HMC or MMC conditions till they reached the maximum resistance. To ensure that the integrity of the monolayer was maintained during the course of the experiment, TEER was measured before and after these studies. Before each experiment the culture medium was removed from each compartment and the monolayer was washed twice with warm HBSS (37°C). In the basolateral compartment, 1.5 ml of pre-warmed HBSS was placed and the cells were returned to the incubator at 37°C for 30 minutes to equilibrate. To the apical compartment, 0.5 ml of 10 μM flu-Na solution was added. Samples of 0.1 ml were taken from the basal compartment of each well over 2 hours, with each volume being replaced with equal amount of fresh warm HBSS. The fluorescence of flu-Na was measured in black, 96-well plates using a fluorescence plate

189 reader (Fluoroskan Ascent FL, Thermo Scientific, USA), using excitation and emission
190 wavelengths of 485 and 520 nm respectively. Permeability coefficients P_{app} were calculated
191 using the equation: $P_{app} = ((dQ/dt)V)/(AC_0)$, where Q, V, A and C_0 are the amount of flu-Na
192 permeated across the cell layer, the volume of buffer in the basal chamber, the diffusion area,
193 and the initial donor concentration respectively.

195 **Western Blot**

196 Cells were lysed in RIPA buffer containing protease inhibitor cocktail. Cell lysates were
197 separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes.
198 The membranes were blocked in 5% skimmed milk for 1 hours and then incubated with
199 primary antibodies overnight at 4°C followed by the appropriate HRP-conjugated secondary
200 antibodies for 2 hours at RT. Immuno-reactive bands were visualized using ECL kit (Bio-rad,
201 USA) according to the manufacturer's instructions. The pictures were captured by
202 ImageQuant LAS 4000 (GE Healthcare, UK).

204 **RT-PCR**

205 RNA was extracted by RNeasy Plus Mini kit (Qiagen, Germany) from cells. Human ENaC,
206 CFTR, α_1 -Na⁺-K⁺-ATPase, AQP3, AQP5 and GAPDH primer sequences are listed in detail
207 in the Supplement. Reverse transcription was carried out using High Capacity cDNA Reverse
208 Transcription Kit (Applied Biosystems, New Zealand), and amplified using GeneAmp High
209 Fidelity PCR System (Applied Biosystems, New Zealand) in a mastercycler gradient
210 thermocycler (Eppendorf, Germany). The PCR process involved a single cycle of 95 °C for 2
211 minutes, and then 35 cycles of 94 °C for 30 seconds, annealing temperature (as indicated in
212 the Supplemental S1) for 45 seconds, 72 °C for 60 seconds followed by a single 7-minute

cycle at 72 °C for extension. RT-PCR products were electrophoresed on 2% E-Gel EX Agarose Gels (Invitrogen, New Zealand). GAPDH was used as normalization control.

Inhibitor treatment

H441 cells were grown under AMC conditions till they reached the maximum resistance. Cells with TEER higher than 200 $\Omega \cdot \text{cm}^2$ can be used for further experiment. Cells were treated for 5 minutes (with 100 μM amiloride, 10 μM CFTR inh172, 10 μM NPPB, or 10 μM forskolin) or for 10 minutes (with 1 mM ouabain). TEPD across the cell monolayer was measured before and after treatment. Cells treated with HBSS were used as control.

Statistical Analysis

The results of multiple observations are presented as the means $\pm$ SEM and as a representative result of two or three different separate experiments, unless otherwise stated. Data were analyzed using t test and ANOVA test. Values were considered significant at $P < 0.05$.

Results

Human lung epithelial cells under different culture conditions showed different TEER and TEPD values

The barrier properties of H441 and A549 cells cultured under different growth conditions were determined by measuring TEER and TEPD values. The TEER values of H441 cells were significantly different among cells grown under AMC, HMC and MMC conditions (Figure. 1A). TEER values for H441 cells under the three conditions increased throughout the culture period and reached peaks on day 7. TEER reached the maximum value of $257.6 \pm 27.53 \Omega \cdot \text{cm}^2$ in the AMC group and slightly lower peak values of $182.3 \pm 5.63 \Omega \cdot \text{cm}^2$ were observed

238 in the HMC group. TEER values of AMC and HMC groups maintained around the maximum
239 levels for 3-5 days. For H441 cells grown under MMC condition, the TEER values were
240 almost 4 times higher, i.e., $1009 \pm 14.87 \Omega \cdot \text{cm}^2$ and dropped soon after they reached the
241 maximum. In contrast, TEER values for A549 cell under the three conditions didn't change
242 much over the investigated time period with a peak of around only $25 \Omega \cdot \text{cm}^2$ (Figure. 1B).
243 The TEPD values of H441 cells under AMC and MMC conditions reached the maximum of
244 $4.75 \pm 0.096 \text{ mV}$ on day 11 and $4.9 \pm 0.235 \text{ mV}$ on day 7 respectively (Figure. 1C). The
245 positive TEPD mean the apical surface was more negative than the basolateral surface. In
246 addition, H441 cells under HMC conditions showed much smaller change of TEPD values
247 with the maximum of $-0.65 \pm 0.029 \text{ mV}$. By contrast, no obvious TEPD values from A549
248 cells were observed under all the three conditions. Values were in the range from -0.4 to 0
249 mV (data not shown).

250

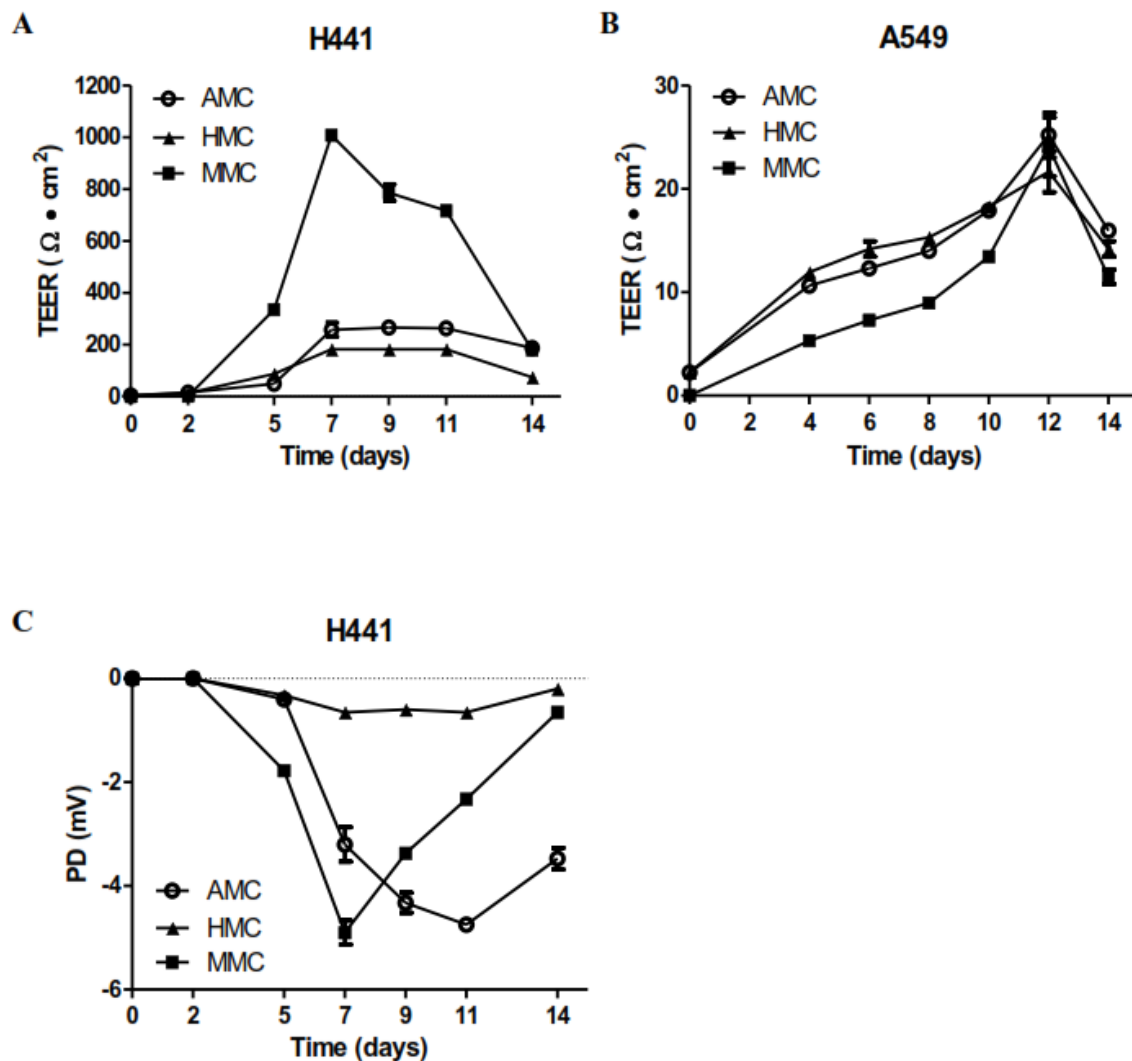


Figure 1. TEER across H441 cells (A) and A549 cells (B) cultured under AMC, HMC or MMC conditions measured in the culture period (0-14 days). (C) TEPD values across the H441 cell monolayer measured in the same period. Data are shown as mean $\pm$ SEM (n=4)

Human lung epithelial cells under different culture conditions showed different permeability

As the maximum TEER values and cell morphology of H441 cells and A549 cells were attained on day 7 and 12 respectively, these time points were selected as being suitable for performing permeability studies. Fitted lines showed flu-Na concentration across the cell

monolayer in the three groups increased proportional over time (Figure. 2A,C). H441cells cultured using HMC and MMC were less permeable to solute flux than cells cultured using AMC (Figure. 2B, $p < 0.0001$). The P_{app} values of the AMC group ($371.4 \pm 1.79 \times 10^{-8}$ cm/s) are about twice that of HMC ($167.2 \pm 2.81 \times 10^{-8}$ cm/s) and MMC groups ($157.3 \pm 1.13 \times 10^{-8}$ cm/s). In contrast, A549 cells cultured under the three conditions showed similar permeability with no significant difference (Figure. 2D). The P_{app} values of A549 cells in AMC, HMC and MMC groups are $356.2 \pm 3.897 \times 10^{-8}$ cm/s, $350.1 \pm 4.382 \times 10^{-8}$ cm/s, $326.4 \pm 19.2 \times 10^{-8}$ cm/s respectively.

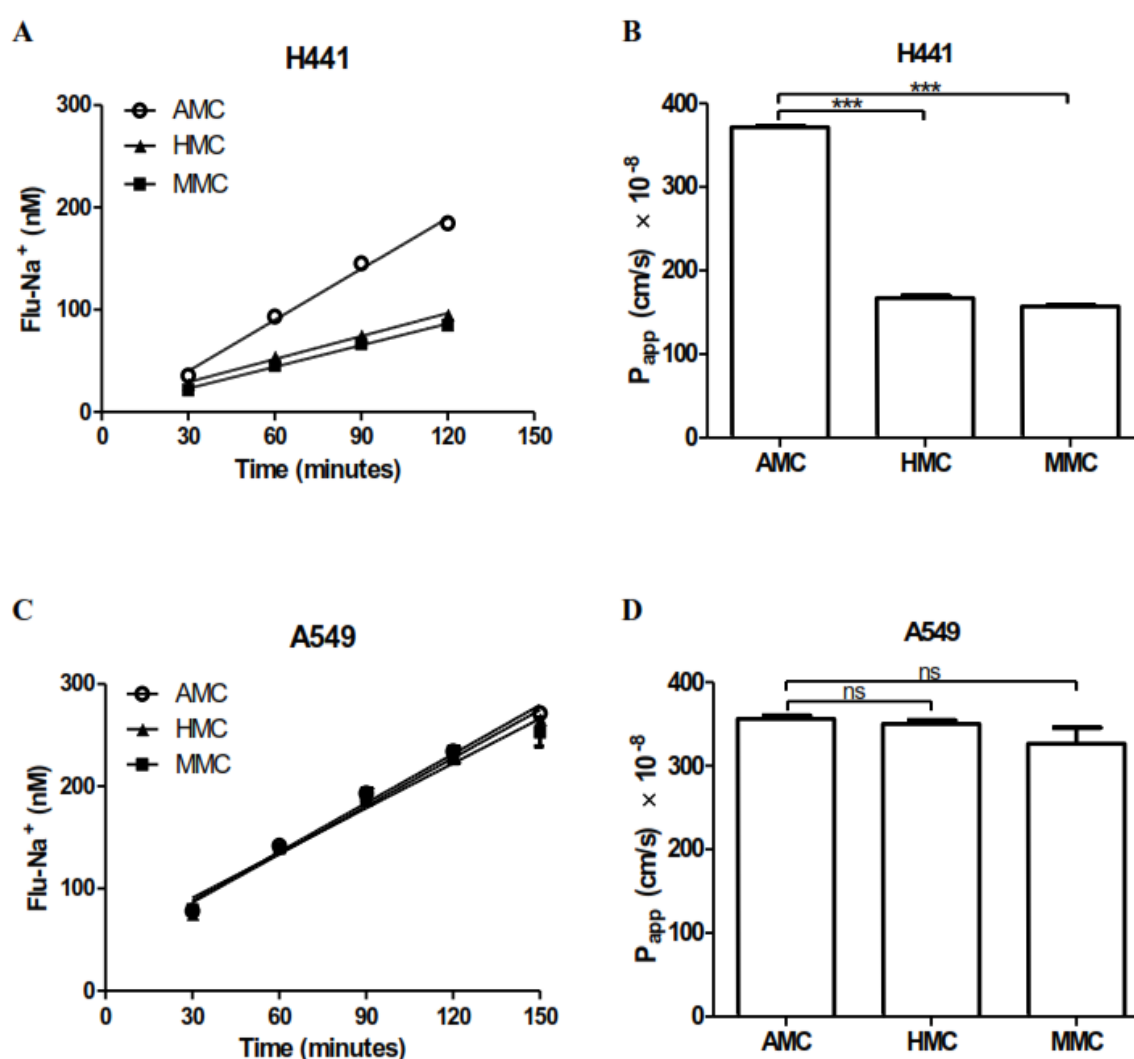


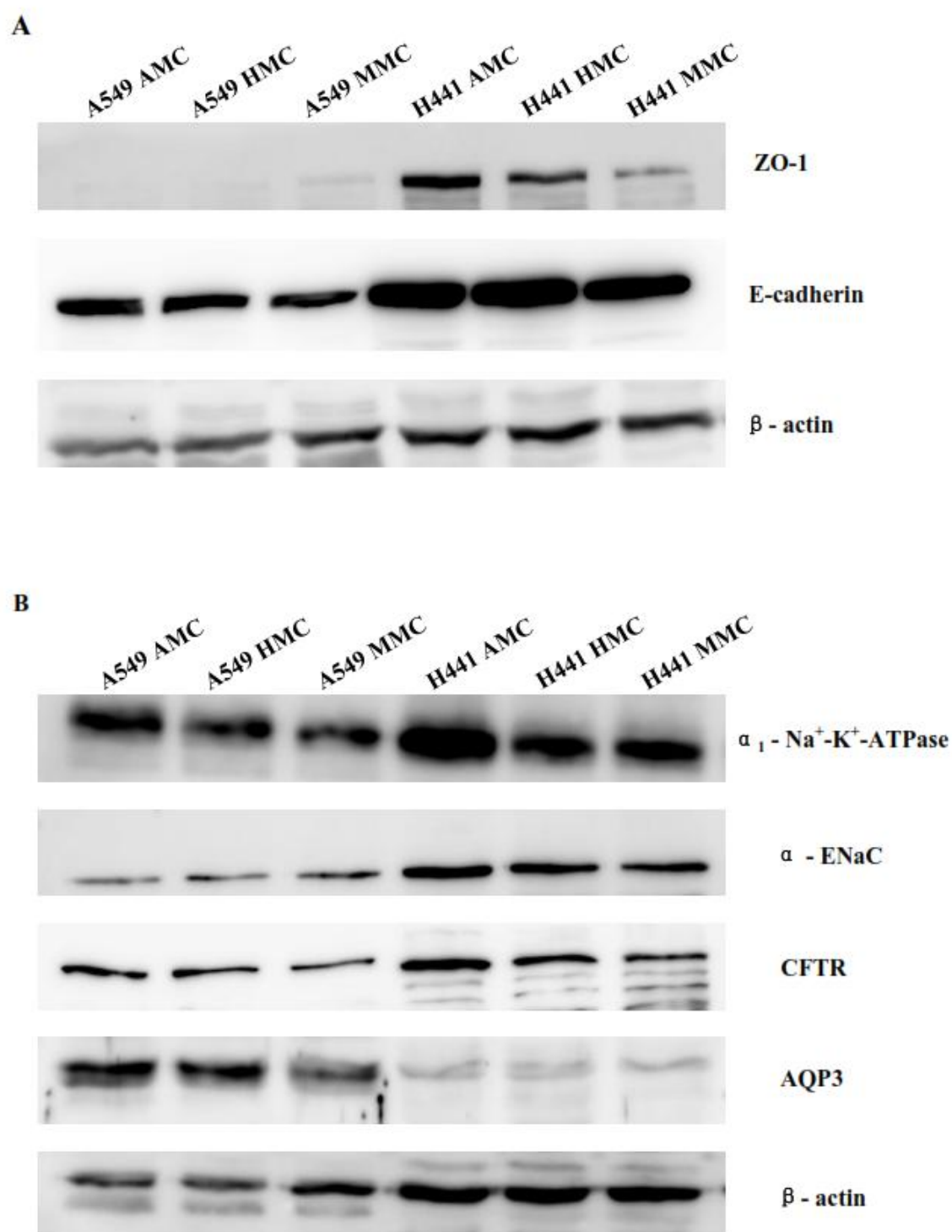
Figure 2. Time course (A) and permeability (B) of flu-Na across H441 monolayers. (C) and (D) show similar data for A549 monolayers. Permeability was measured in the apical-to-

basolateral direction. Data are shown as mean $\pm$ SEM (n=3). (*, $P<0.05$; **, $P<0.01$; ***, $P<0.0001$ by one-way ANOVA)

Protein expression of tight junctions, adherens junction, and ion and water transport proteins

The expression of ZO-1 and E-cadherin were determined in order to assess the formation of a tight, polarised epithelial monolayer. H441 cells cultured using AMC showed the highest expression of ZO-1 (Figure. 3A). Cells in the MMC group had much lower ZO-1 expression compared with AMC and HMC groups. In contrast, none of A549 cells in the three groups showed obvious ZO-1 expression. However, no significant difference in the E-cadherin expression was observed within each of the individual cell lines, H441 and A549 respectively (Figure. 3A).

In addition, the expression of the major proteins that contributes to Na^+ and Cl^- and water transport were identified. α -ENaC, CFTR and AQP3 were expressed in both cell lines under the three culture conditions at similar levels (Figure. 3B). However, $\alpha_1\text{-Na}^+\text{-K}^+\text{-ATPase}$ was expressed at much higher levels in both H441 and A549 cells under the AMC condition. Besides, no expression of AQP5 was found in either cell line (data not shown).



290

291 Figure 3. Protein expression of tight junction, adherens junction, ion and water transport

292 proteins. Expression of tight junction ZO-1, adherens junction E-cadherin (A) and

293 water transport proteins α -ENaC, α_1 - Na^+ - K^+ -ATPase, CFTR and AQP3 (B) were examined

294 by western blot. β -actin was used as internal standard.

295 **mRNA expression of ion and water transport proteins**

296 Furthermore, the expression of ZO-1, E-cadherin, α -ENaC, β -ENaC, γ -ENaC, α_1 -Na⁺-K⁺-
297 ATPase, CFTR, APQ3 and AQP5 at mRNA level was examined. H441 cells and A549 cells
298 under three culture conditions expressed similar levels of ZO-1, E-cadherin, α -ENaC, β -
299 ENaC, γ -ENaC, α_1 -Na⁺-K⁺-ATPase, CFTR and APQ3 (Figure. 4). No mRNA expression of
300 AQP5 was found in either cell line (data not shown).

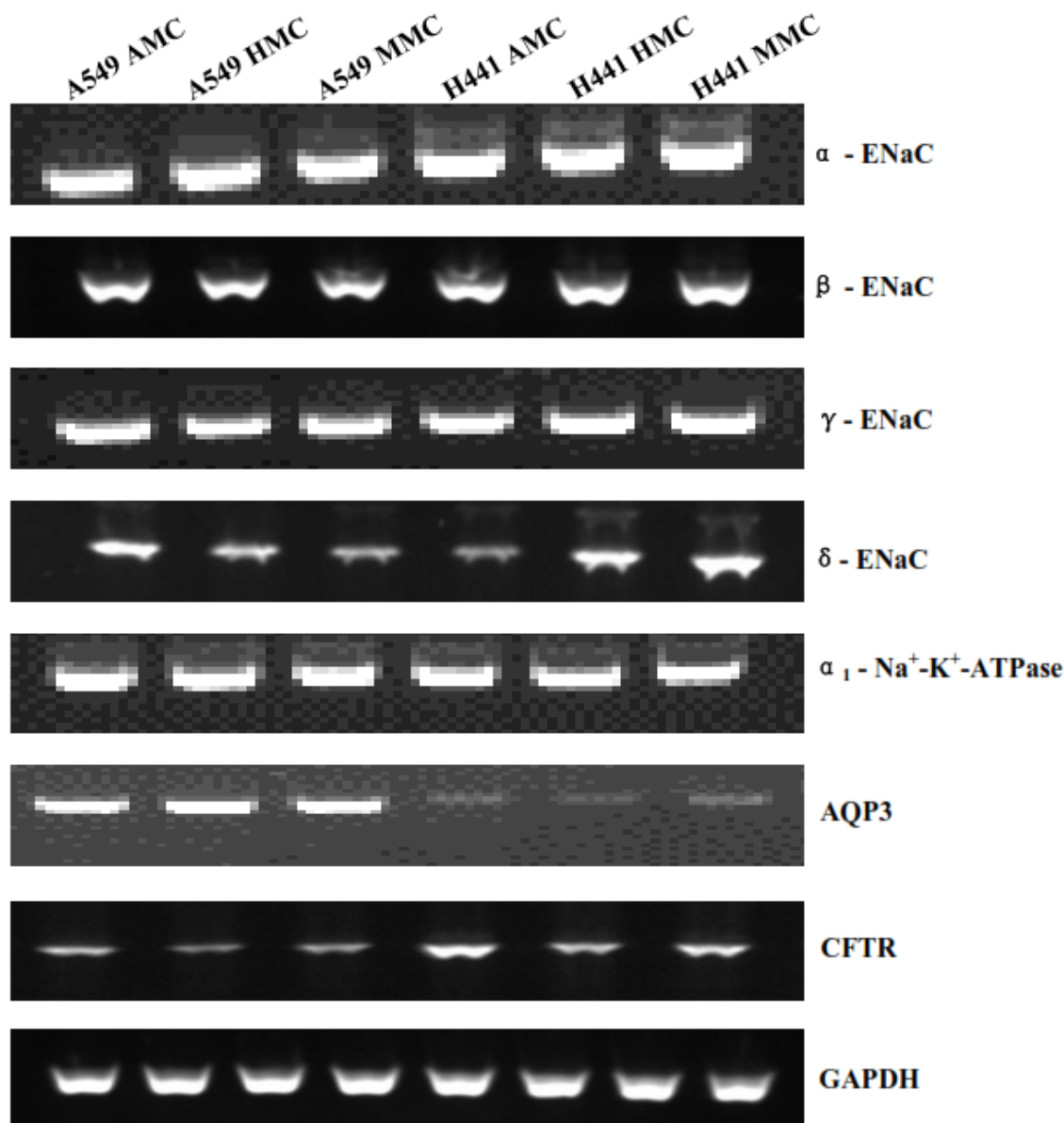


Figure 4. mRNA expression of ion and water transport proteins. H441 cells and A549 cells were grown on transwell filters and cultured under AMC, HMC or MMC conditions till they reached the maximum resistance. mRNA expression of ZO-1, E-cadherin, α-ENaC, β-ENaC,

Effect of inhibitors on the ion transport

Inhibitors were used to determine the role of Na⁺ and Cl⁻ channels in ion transport in H441 cells. TEPD was decreased to 10.75% after treated by 10 μM amiloride, which suggested that the sodium absorption by ENaC was responsible for most of the TEPD (Figure. 5A). This is

similar to what the Boucher group found in the primary human cells (Bove et al., 2010). In the meantime, treatment with 1 mM ouabain could decrease the TEPD to 71.89% (Figure. 5B). As for the Cl^- channels, 10 μM CFTR inhibitor could increase the TEPD by 38.27%, i.e. the apical surface became more negative (Figure. 5C). This indicates that CFTR is absorbing Cl^- at baseline. In contrast, the Boucher group found that CFTR secretes Cl^- in the primary human cells at baseline (Bove et al., 2010). We found other Cl^- channels were expressed at mRNA level in the H441 cells under AMC conditions (data not shown), so a broad spectrum Cl^- channel inhibitor NPPB (10 μM) was used to see if they contributed to Cl^- transport. In this case, TEPD was increased by 25% (Figure. 5D). This indicated that other Cl^- channels might secrete Cl^- . In addition, cells were treated with 10 μM forskolin which activates both ENaC and CFTR. TEPD was increased by 47.75% which indicated that Na^+ absorption increased more than Cl^- absorption (Figure. 5E).

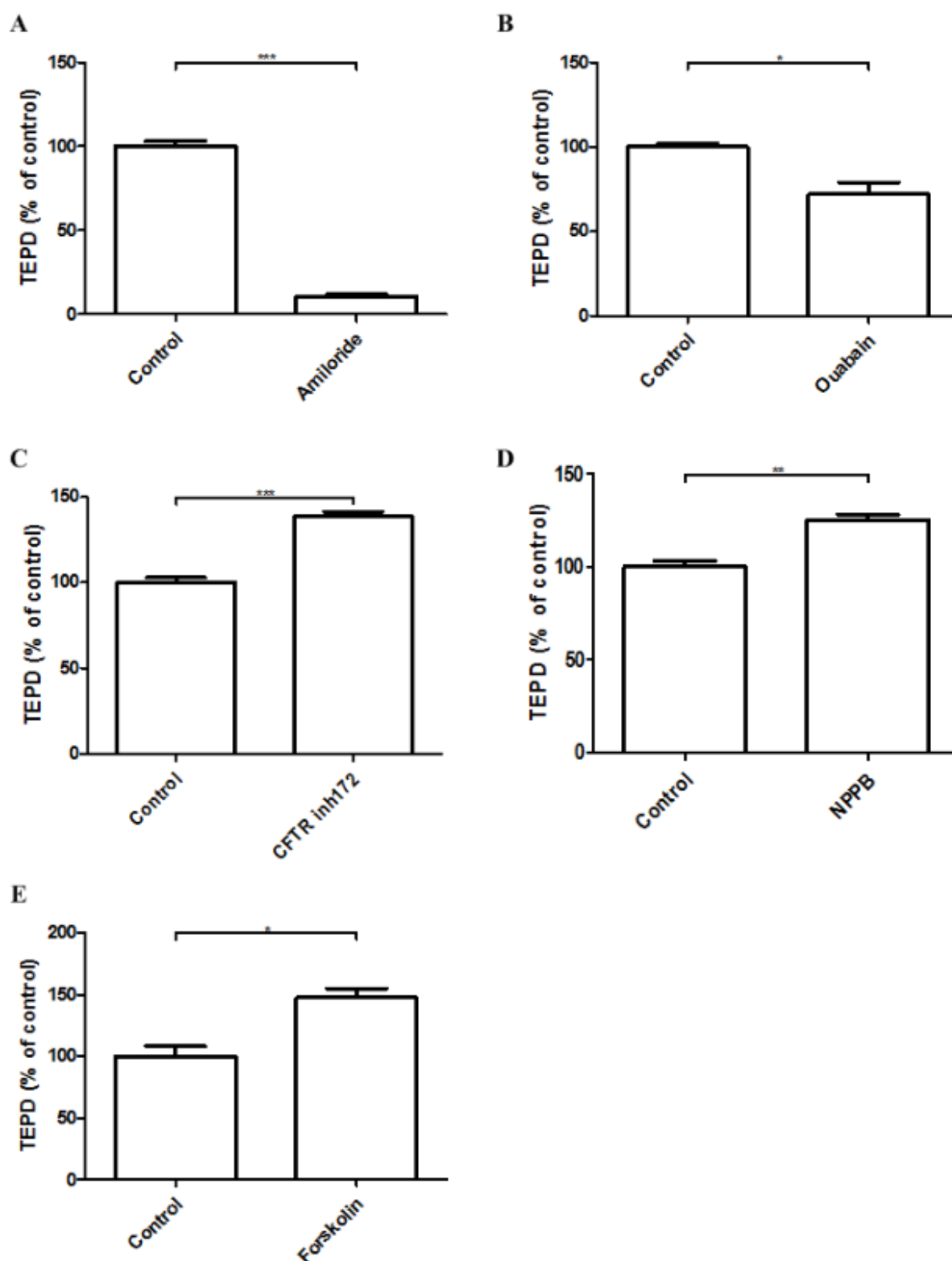


Figure 5. Effect of 100 μM amiloride (A), 1 mM ouabain (B), 10 μM CFTR inh172 (C), 10 μM NPPB (D), 10 μM forskolin respectively on TEPD across H441 monolayers grown under AMC conditions.

Discussion

This study has demonstrated that, by direct comparison, alveolar epithelial cells cultured under different conditions showed starkly different properties. H441 cells cultured using MMC presented the highest transepithelial electrical resistance with the lowest paracellular permeability. However, H441 cells under AMC conditions produced the highest ZO-1 and α_1 -Na⁺-K⁺-ATPase expression. As to the A549 cells, much smaller differences in the TEER, TEPD, permeability and expression of transport related proteins were found among different groups. In addition, compared with A549 cells, H441 cells showed much higher level of TEER, TEPD and expression of ZO-1, E-cadherin and α_1 -Na⁺-K⁺-ATPase.

We used H441 and A549 cell lines as in vitro models for alveolar epithelial cells in our study. H441 cell line originates from a human lung adenocarcinoma has been described to have the features of alveolar type II cells (Rehan et al., 2002; Duncan, Whitsett & Horowitz, 1997). There are also reports indicating that it has the characteristics of bronchiolar (i.e., Clara) epithelial cells (Newton et al., 2006; Zhang, Whitsett & Stripp, 1997). Therefore, studies can be designed to characterise metabolism and transport properties of these two particular cell types (Ehrhardt, Laue & Kim, 2008). In addition, the most frequently used alveolar epithelial model is the A549 cell line derived from a human pulmonary adenocarcinoma. A549 cells also have some morphologic and biochemical features of the alveolar type II cells (Lieber et al., 1976). It has been reported that H441 cells are capable of forming monolayer of polarised cells and exhibiting a significant TEER (Shlyonsky et al., 2005; Woollhead & Baines, 2006). On the contrary, A549 cells lack the ability to form tight monolayer of polarised cells, due to the inability to form functional tight junctions (Blank et al., 2006; Kim, Borok & Crandall, 2001). Consistent results with previous studies were shown in our study. TEER of H441 cell is much higher than that of A549 cells, and the expression of tight junction ZO-1 and adherens

junction E-cadherin in H441 cells are more abundant. TEER and TEPD values of human alveolar type II cells have been previously measured using human primary cells (Bove et al., 2010). TEER under air-liquid interface was $419.3 \pm 168.1 \Omega \cdot \text{cm}^2$ at day 5 in culture, and the TEPD was around -3 mV (Bove et al., 2010). There is also report on the TEER difference of A549 cells under different culture conditions (Blank et al., 2006). TEER values of A549 cells in the air-exposed culture were slightly lower (around $150 \Omega \cdot \text{cm}^2$) than that in the submersed culture (around $160 \Omega \cdot \text{cm}^2$) in the previous study (Blank et al., 2006). However, the difference of TEER and TEPD across H441 cell monolayer has not been demonstrated.

We found that H441 cells cultured under MMC condition showed higher TEER and lower permeability coefficients compared with cells cultured under AMC condition. Similar results have been shown using Calu-3 cells (Stentebjerg-Andersen et al., 2011; Grainger et al., 2006). In addition, the TEPD values of H441 cell monolayer reach the minimum at the pace as TEER values reach the top in the MMC group. Unlike this, TEPD values reach the minimum 2 days after TEER values reach the peak in the AMC group. This indicates that different culture conditions have an impact on the polarization course of lung epithelial cells.

H441 cells cultured under HMC condition were observed to have the lowest TEER (slightly lower than the AMC group) and TEPD (much lower than both AMC and MMC groups). Interestingly, the permeability of HMC group showed similar level to MMC group, which is much lower than the AMC group. Generally speaking, cell monolayer with lower transepithelial resistance, which means it is leaky, has higher permeability coefficients. The relation of low TEER and low permeability and the underlying mechanism appeared in the HMC group needs to be further investigated. In addition, lung epithelial cells cultured under HMC condition can be used to study how the covering liquid affects the functions of alveolar cells under abnormal condition, such as pulmonary edema or lung injury, although more

376 complicated factors like the component and volume of leaking fluid should be taken into
377 consideration.

378

379 Tight junction ZO-1 and adherens junction E-cadherin function in the formation and
380 maintenance of the tight epithelial monolayer and the polarized phenotype of epithelial cells.

381 Zonula occludens, a type of tight junction protein, are intercellular junctional structure that
382 act as scaffolding proteins and interact with many binding partners (Schneeberger & Lynch,

383 1992). They form a selectively permeable occlusion in the paracellular pathway, thereby
384 defining apical and basal compartments, and are thought to be at least partially responsible

385 for the maintenance of polarity and vectorial transport functions of epithelial cells (Shin,
386 Fogg & Margolis, 2006; Cereijido et al., 1998). In the meanwhile, $\text{Na}^+\text{-K}^+\text{-ATPase}$, which is

387 widely used as a marker for epithelial polarity, is localized to the basolateral membrane in the
388 epithelial cells and plays a crucial role in maintaining the intracellular ion homeostasis

389 (Rajasekaran et al., 2001). The transmembrane electrochemical gradients generated by $\text{Na}^+\text{-K}^+\text{-ATPase}$
390 are involved in regulating directional transport of molecules across epithelial

391 cells (Rajasekaran et al., 2007; Rajasekaran & Rajasekaran, 2009). Recent evidence suggests
392 that $\text{Na}^+\text{-K}^+\text{-ATPase}$ might have a more direct or indirect role in transport across the

393 epithelial barrier by regulating tight junction structure and permeability (Rajasekaran &
394 Rajasekaran, 2009). In our study, H441 cells under MMC condition has the highest

395 transepithelial resistance but the lowest ZO-1 expression, which indicates that other tight
396 junctions may play important roles in the barrier formation. In the meantime, H441 cells

397 under AMC condition express the highest ZO-1 and $\alpha_1\text{-Na}^+\text{-K}^+\text{-ATPase}$, so do the A549 cells
398 in the expression of $\alpha_1\text{-Na}^+\text{-K}^+\text{-ATPase}$. This means that lung epithelial cells cultured under

399 AMC condition are easier to polarize, though only moderate transepithelial resistance is
400 observed.

401

402 To regulate and maintain the composition and height of the covering fluid layer, lung
403 epithelial cells are equipped with a wide variety of ion transport proteins, among which Na^+ ,
404 Cl^- , and K^+ channels have been identified to play major roles in the process (Hollenhorst,
405 Richter & Fronius, 2011). Expression of epithelial sodium channel (ENaC) has been detected
406 both biochemically and physiologically in airway and alveolar epithelial cells (Nie et al.,
407 2009). Apical ENaC, combined with basolaterally located $\text{Na}^+-\text{K}^+-\text{ATPase}$, form a major
408 pathway for the vectorial transport of ion and water across the epithelial layer of the lung
409 gas–blood barrier (Nie et al., 2009). It has clearly been demonstrated by the fundamental
410 studies that the deletion of α -, β - and γ - ENaC subunits delays and strikingly reduces fluid
411 clearance from the airspace of knockout mice at birth (Barker et al., 1998; Hummler et al.,
412 1996; McDonald et al., 1999). A large part of the airway chloride secretion in humans is
413 mediated by the apically located cAMP-dependent cystic fibrosis transmembrane
414 conductance regulator (CFTR) channel (Anderson et al., 1991). In addition to its Cl^- channel
415 function, the CFTR has been proposed to be able to regulate other ion channels, such as
416 ENaC (Stutts et al., 1995). Mutations in CFTR cause cystic fibrosis (CF) lung disease
417 featured by defective ion transport and abnormalities in the airway surface liquid (Knowles et
418 al., 1983). The aquaporins (AQPs) are a family of small, integral, hydrophobic membrane
419 proteins that are highly and, in most cases, specifically permeable to water. Under normal
420 physiological conditions, water transport driven osmotically across cell membranes is the
421 principal mechanism of fluid transport (Verkman, Matthay & Song, 2000). It has been
422 reported that AQP3 and AQP5 are expressed in the respiratory tract and provide the principal
423 route for water transport osmotically driven across the alveolar epithelial and endothelial
424 barriers (Ben et al., 2008). Previous study has shown that α -ENaC, β -ENaC, γ -ENaC, CFTR,
425 AQP3, AQP5 are expressed in the human alveolar type II cells in culture (Bove et al., 2010).

426 However, the expression level of the above proteins changes over the culture period (Bove et
427 al., 2010). In our study, we didn't see the expression of AQP5 in H441 and A549 cells. In
428 addition, we didn't find significant difference in the expression of α -ENaC, CFTR, AQP3 in
429 H441 and A549 cells under three conditions at protein and mRNA levels.

430

431 Previous studies have shown that culture conditions have an impact on ion transport in the
432 lung epithelial cells. For example, sodium absorption was enhanced in canine bronchi cells
433 under AMC condition compared with MMC (Johnson et al., 1993). Besides, it has been
434 shown that type II cells under AMC condition predominantly expressed highly-selective
435 sodium channel which was replaced by non-selective cation channel when cultured under
436 MMC (Jain et al., 2001). In our study, no significant expression difference of ENaC subunits
437 was found. Further studies regarding ion transport and protein functions of lung epithelial cell
438 monolayer under different culture conditions need to be investigated.

Conclusion

Lung epithelial cells can present different properties under different cell culture conditions. It is important to characterise cell properties before using a certain cell culture model to carry on further studies. H441 cells and A549 cells cultured under AMC, HMC and MMC conditions were directly compared in the present study. Lung epithelial cells cultured under AMC condition mimics in vivo conditions at the maximum and express the highest level of polarization marker α_1 -Na⁺-K⁺-ATPase and tight junction ZO-1, which makes it more suitable as a cell culture model than the other two conditions. Lung epithelial cell monolayer cultured under AMC condition will provide a platform for the evaluation of transport studies.

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References

- Anderson MP, Gregory RJ, Thompson S, Souza DW, Paul S, Mulligan RC, Smith AE, Welsh MJ. 1991. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science* 253:202-205.
- Barker PM, Nguyen MS, Gatzky JT, Grubb B, Norman H, Hummler E, Rossier B, Boucher RC, Koller B. 1998. Role of gammaENaC subunit in lung liquid clearance and electrolyte balance in newborn mice. Insights into perinatal adaptation and pseudohypoaldosteronism. *J Clin Invest* 102:1634.
- Ben Y, Chen J, Zhu R, Gao L, Bai C. 2008. Upregulation of AQP3 and AQP5 induced by dexamethasone and ambroxol in A549 cells. *Respiratory Physiology & Neurobiology* 161:111-118.
- Blank F, Rothen-Rutishauser BM, Schurch S, Gehr P. 2006. An optimized in vitro model of the respiratory tract wall to study particle cell interactions. *Journal of Aerosol Medicine* 19:392-405.
- Bove PF, Grubb BR, Okada SF, Ribeiro CMP, Rogers TD, Randell SH, O'Neal W,K., Boucher RC. 2010. Human alveolar type II cells secrete and absorb liquid in response to local nucleotide signaling. *The Journal of Biological Chemistry* 285:34939.
- Cereijido M, Valdés J, Shoshani L, Contreras RG. 1998. Role of tight junctions in establishing and maintaining cell polarity. *Annu Rev Physiol* 60:161-177.
- Dobbs LG, Johnson MD. 2007. Alveolar epithelial transport in the adult lung. *Respiratory Physiology & Neurobiology* 159:283-300.
- Duncan JE, Whitsett JA, Horowitz AD. 1997. Pulmonary surfactant inhibits cationic liposome-mediated gene delivery to respiratory epithelial cells in vitro. *Hum Gene Ther* 8:431-438.
- Ehrhardt C, Laue M, Kim K. 2008. In vitro models of the alveolar epithelial barrier. *Drug Absorption Studies*. p 258-282
- Fernandes CA, Vanbever R. 2009. Preclinical models for pulmonary drug delivery. *Expert Opinion on Drug Delivery* 6:1231-1245.
- Flynn AN, Itani OA, Moninger TO, Welsh MJ. 2009. Acute regulation of tight junction ion selectivity in human airway epithelia. *Proceedings of the National Academy of Sciences* 106:3591-3596.

479 Geiser T, Atabai K, Jarreau PH, Ware LB, Pugin J, Matthay MA. 2001. Pulmonary edema fluid from
 480 patients with acute lung injury augments in vitro alveolar epithelial repair by an IL-1 β -dependent
 481 mechanism. *American Journal of Respiratory and Critical Care Medicine* 163:1384.

482 Grainger CI, Greenwell LL, Lockley DJ, Martin GP, Forbes B. 2006. Culture of Calu-3 cells at the air
 483 interface provides a representative model of the airway epithelial barrier. *Pharm Res* 23:1482-1490.

484 Hollenhorst MI, Richter K, Fronius M. 2011. Ion transport by pulmonary epithelia. *Journal of*
 485 *Biomedicine and Biotechnology* 2011

486 Hummler E, Barker P, Gatzky J, Beermann F, Verdumo C, Schmidt A, Boucher R, Rossier BC. 1996.
 487 Early death due to defective neonatal lung liquid clearance in α ENaC-deficient mice. *Nat Genet*
 488 12:325-328.

489 Jain L, Chen X, Ramosevac S, Brown LA, Eaton DC. 2001. Expression of highly selective sodium
 490 channels in alveolar type II cells is determined by culture conditions. *American Journal of*
 491 *Physiology-Lung Cellular and Molecular Physiology* 280:L646-L658.

492 Johnson LG, Dickman KG, Moore KL, Mandel LJ, Boucher RC. 1993. Enhanced Na transport in an
 493 air-liquid interface culture system. *American Journal of Physiology-Lung Cellular and Molecular*
 494 *Physiology* 264:L560-L565.

495 Kim K, Borok Z, Crandall ED. 2001. A useful in vitro model for transport studies of alveolar
 496 epithelial barrier. *Pharm Res* 18:253-255.

497 Knowles M, Stutts M, Spock A, Fischer N, Gatzky J, Boucher R. 1983. Abnormal ion permeation
 498 through cystic fibrosis respiratory epithelium. *Science* 221:1067-1070.

499 Lieber M, Todaro G, Smith B, Szakal A, Nelson-Rees W. 1976. A continuous tumor-cell line from a
 500 human lung carcinoma with properties of type II alveolar epithelial cells. *International Journal of*
 501 *Cancer* 17:62-70.

502 Mathias NR, Yamashita F, Lee VH. 1996. Respiratory epithelial cell culture models for evaluation of
 503 ion and drug transport. *Adv Drug Deliv Rev* 22:215-249.

504 Matthay MA, Folkesson HG, Clerici C. 2002. Lung epithelial fluid transport and the resolution of
 505 pulmonary edema. *Physiol Rev* 82:569-600.

506 McDonald FJ, Yang B, Hrstka RF, Drummond HA, Tarr DE, McCray Jr PB, Stokes JB, Welsh MJ,
 507 Williamson RA. 1999. Disruption of the β subunit of the epithelial Na channel in mice: hyperkalemia
 508 and neonatal death associated with a pseudohypoaldosteronism phenotype. *Proceedings of the*
 509 *National Academy of Sciences* 96:1727-1731.
 510 Newton DA, Rao KMK, Dluhy RA, Baatz JE. 2006. Hemoglobin is expressed by alveolar epithelial
 511 cells. *J Biol Chem* 281:5668-5676.
 512 Nie H, Chen L, Han D, Li J, Song W, Wei S, Fang X, Gu X, Matalon S, Ji H. 2009. Regulation of
 513 epithelial sodium channels by cGMP/PKGII. *J Physiol (Lond)* 587:2663-2676.
 514 Novotny JA, Jakobsson E. 1996. Computational studies of ion-water flux coupling in the airway
 515 epithelium. I. Construction of model. *American Journal of Physiology-Cell Physiology* 270:C1751-
 516 C1763.
 517 Rajasekaran SA, Barwe SP, Gopal J, Ryazantsev S, Schneeberger EE, Rajasekaran AK. 2007. Na-K-
 518 ATPase regulates tight junction permeability through occludin phosphorylation in pancreatic
 519 epithelial cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 292:G124-
 520 G133.
 521 Rajasekaran SA, Palmer LG, Moon SY, Soler AP, Apodaca GL, Harper JF, Zheng Y, Rajasekaran
 522 AK. 2001. Na, K-ATPase activity is required for formation of tight junctions, desmosomes, and
 523 induction of polarity in epithelial cells. *Mol Biol Cell* 12:3717-3732.
 524 Rajasekaran SA, Rajasekaran AK. 2009. Na, K-ATPase and epithelial tight junctions. *Front Biosci*
 525 14:2130-2148.
 526 Rehan VK, Torday JS, Peleg S, Gennaro L, Vouros P, Padbury J, Rao S, Satyanarayana RG. 2002.
 527 1 α , 25-dihydroxy-3-epi-vitamin D3, a natural metabolite of 1 α , 25-dihydroxy vitamin D3:
 528 production and biological activity studies in pulmonary alveolar type II cells. *Mol Genet Metab* 76:46-
 529 56.
 530 Schneeberger EE, Lynch RD. 1992. Structure, function, and regulation of cellular tight junctions.
 531 *American Journal of Physiology-Lung Cellular and Molecular Physiology* 262:L647-L661.
 532 Shin K, Fogg VC, Margolis B. 2006. Tight junctions and cell polarity. *Annu Rev Cell Dev Biol*
 533 22:207-235.

534 Shlyonsky V, Goolaerts A, Van Beneden R, Sariban-Sohraby S. 2005. Differentiation of epithelial Na
 535 channel function an in vitro model. *J Biol Chem* 280:24181-24187.

536 Stentebjerg-Andersen A, Notlevsen IV, Brodin B, Nielsen CU. 2011. Calu-3 cells grown under AIC
 537 and LCC conditions: Implications for dipeptide uptake and transepithelial transport of substances.
 538 *European Journal of Pharmaceutics and Biopharmaceutics* 78:19-26.

539 Stewart CE, Torr EE, Mohd Jamili NH, Bosquillon C, Sayers I. 2012. Evaluation of differentiated
 540 human bronchial epithelial cell culture systems for asthma research. *J Allergy* 2012

541 Strengert M, Knaus UG. 2011. Analysis of Epithelial Barrier Integrity in Polarized Lung Epithelial
 542 Cells. *Permeability Barrier* p 195-206

543 Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, Boucher RC. 1995. CFTR as a
 544 cAMP-dependent regulator of sodium channels. *Science* 269:847-850.

545 Van Itallie CM, Anderson JM. 2006. Claudins and epithelial paracellular transport. *Annu Rev Physiol*
 546 68:403-429.

547 Verkman A, Matthay MA, Song Y. 2000. Aquaporin water channels and lung physiology. *American*
 548 *Journal of Physiology-Lung Cellular and Molecular Physiology* 278:L867-L879.

549 Warren N, Tawhai M, Crampin E. 2009. A mathematical model of calcium-induced fluid secretion in
 550 airway epithelium. *J Theor Biol* 259:837-849.

551 Woollhead AM, Baines DL. 2006. Forskolin-induced cell shrinkage and apical translocation of
 552 functional enhanced green fluorescent protein-human α ENaC in H441 lung epithelial cell monolayers.
 553 *J Biol Chem* 281:5158-5168.

554 Zhang L, Whitsett JA, Stripp BR. 1997. Regulation of Clara cell secretory protein gene
 555 transcription by thyroid transcription factor-1. *Biochimica Et Biophysica Acta (BBA)-Gene*
 556 *Structure and Expression* 1350:359-367.