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## Method

A novel method for pulmonary research: Assessment of bioenergetic function at the air–liquid interface <sup>☆</sup>

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## ABSTRACT

Air–liquid interface cell culture is an organotypic model for study of differentiated functional airway epithelium *in vitro*. Dysregulation of cellular energy metabolism and mitochondrial function have been suggested to contribute to airway diseases. However, there is currently no established method to determine oxygen consumption and glycolysis in airway epithelium in air–liquid interface. In order to study metabolism in differentiated airway epithelial cells, we engineered an insert for the Seahorse XF24 Analyzer that enabled the measure of respiration by oxygen consumption rate (OCR) and glycolysis by extracellular acidification rate (ECAR). Oxidative metabolism and glycolysis in airway epithelial cells cultured on the inserts were successfully measured. The inserts did not affect the measures of OCR or ECAR. Cells under media with apical and basolateral feeding had less oxidative metabolism as compared to cells on the inserts at air–interface with basolateral feeding. The design of inserts that can be used in the measure of bioenergetics in small numbers of cells in an organotypic state may be useful for evaluation of new drugs and metabolic mechanisms that underlie airway diseases.

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## Introduction

Airway epithelium is a highly complex system integral to lung repair and immune defense. Once thought to act only as a protective, lubricating physical barrier to the external environment, it is now known that the pseudostratified epithelium additionally functions as a mediator of innate and adaptive immune responses, as a mediator of airway homeostasis and as a unique gateway to the external environment. In all species, the majority of cells composing pseudostratified epithelium are basal, ciliated and goblet cells [1].

Airway diseases such as asthma and chronic obstructive lung diseases are characterized by remodeling, or structural changes in airway cells and tissues. Recent studies suggest that the airway epithelium may be a critical effector of remodeling changes [2–5].

**Abbreviations:** ECAR, extracellular acidification rate; iNOS, inducible nitric oxide synthase; MTEC, mouse tracheal epithelial cell; OCR, oxygen consumption rate; TCA, tricarboxylic acid

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The airway epithelium can produce cytokines and reactive oxygen and nitrogen species that initiate and/or maintain airway inflammation and remodeling [6,7]. For example, both human and murine airway epithelium express high levels of the inducible nitric oxide synthase (iNOS), which is increased in asthma. Recent advances in organotypic culture model systems allow the recreation of the airway architecture in an air–liquid interface culture [8–13]. Airway epithelial cells de-differentiate to a flat nonciliated morphology when placed into routine culture under media, but upon lifting cultures to an air–liquid interface that mimics the *in vivo* condition of direct exposure to air and ambient oxygen concentration, the cells differentiate into pseudostratified columnar epithelium that form high-resistance barriers [12,13]. The airway epithelium relies upon discrimination of apical and basal orientation for specialized functions, such as ciliation and directional NO production to the lumen of the airway [14]. In airway epithelial cells, mitochondria are present in both the apical and the basolateral domains, but they are more concentrated in the apical region, in order to provide energy for specialized apical functions, such as ciliary movement [15,16].

Mitochondria are essential to cellular energy production in all higher organisms adapted to an oxygen-containing environment. Cellular respiration consumes oxygen and glucose to make energy-storing molecules of ATP. Under aerobic conditions in most cells,

pyruvate converted from glucose enters the mitochondria to be further metabolized *via* the tricarboxylic acid (TCA) cycle. Under anaerobic conditions, pyruvate is reduced to lactate. Mitochondria are also the primary source of endogenously produced free radicals, generated through oxidative phosphorylation as a byproduct of ATP synthesis, and mitochondria participate in signal transduction processes *via* ROS production [17,18]. Abnormalities of mitochondrial dynamics and function have been implicated in human inflammatory diseases including asthma [19,20]. Thus, the study of cellular metabolism in these cells directly exposed to oxygen in the ambient air may provide insight to normal physiology and pathophysiology of airway diseases. However, there is currently no method to allow for study of metabolism in airway epithelium in air–liquid interface. Here, we engineered a custom insert to differentiate airway epithelial cells at an air–liquid interface cell culture system in order to study metabolism of differentiated airway epithelial cells in a Seahorse Extracellular Flux Analyzer.

## Methods and materials

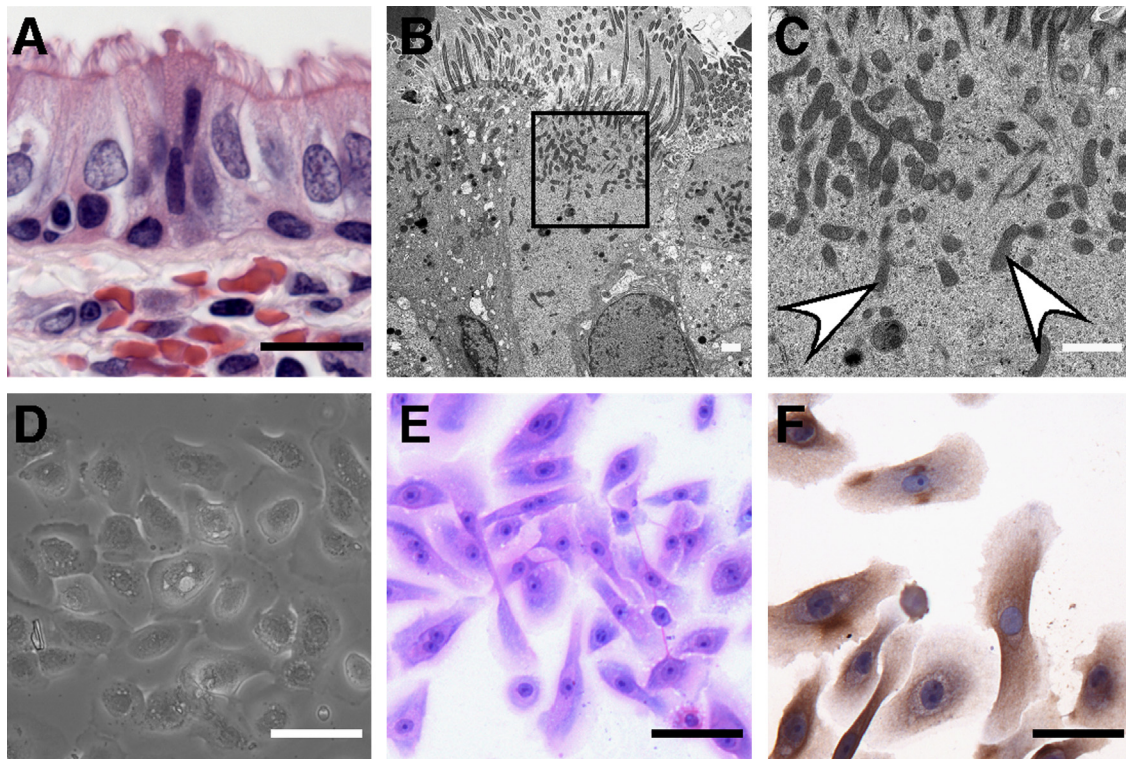
### Animals

Female C57/BL6 mice (6–8 week old) were purchased from the Jackson Laboratory (Bar Harbor, ME). All experiments were approved by the local Institutional Animal Care and Use Committee (IACUC).

### Cell culture

Primary culture of murine airway epithelial cells was prepared as previously reported [11]. Briefly, twelve-well transwell plates

(Fisherbrand, Fisher Scientific, Pittsburgh, PA) were seeded with Madin–Darby Canine Kidney (MDCK) cells to deposit a natural basement membrane. Murine airway epithelial cells were isolated from fresh tracheas harvested from 6–8 week old C57/BL6 female mice (Jackson Laboratory, Bar Harbor, ME), which were sacrificed *via* intraperitoneal injection of pentobarbital. The tracheas were excised, cleaned of adherent tissue and digested overnight at 4 °C in Han's F-12 medium containing 0.15% pronase (Roche, Basel, Switzerland) and 1% penicillin and streptomycin. The tracheas were gently pipetted to loosen epithelial cells that were then collected by centrifugation. The cells were then re-suspended and filtered through a 100  $\mu$ m filter. Epithelial cells were purified by seeding on a 100 mm Petri dish for 3 h in mouse tracheal epithelial cell (MTEC) Basic Media (DMEM/F-12), allowing the fibroblast cells to attach while leaving epithelial cells in suspension. The suspended cells were collected and seeded into the top wells of a 12-well transwell (Fisherbrand) in 500  $\mu$ l MTEC Plus media (MTEC Basic Media with 10  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 0.1  $\mu$ g/ml cholera toxin) (Sigma-Aldrich, St. Louis, MO), 30 mg/L bovine pituitary extract protein (Sigma-Aldrich), 25 ng/ml epidermal growth factor (BD Biosciences, Franklin Lakes, NJ), and 5% FBS plus  $5 \times 10^{-8}$  M retinoic acid (Sigma-Aldrich). The bottom wells contained 1 ml of MTEC Plus media. The epithelial cells from one trachea were used to seed two transwells. Cells were grown at 37 °C in 21% O<sub>2</sub>. Media was changed every other day in both the top and bottom chambers with the conditioned media collected and saved at –20 °C. Six days following seeding, the media type was switched to MTEC Serum-Free media [5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 0.0225  $\mu$ g/ml cholera toxin, 5 ng/ml epidermal growth factor, 30 mg/L bovine pituitary extract protein, and 1 mg/ml bovine serum albumin, fraction V (Fisher Scientific) plus  $5 \times 10^{-8}$  M retinoic acid]. The cultures were “lifted” to the air–liquid interface by adding 500  $\mu$ l media to the bottom chamber only, allowing the airway epithelial cells seeded on the apical chamber



**Fig. 1.** Human Bronchial Epithelial Cells *in vivo* and under submerged culture *in vitro*. (A–C) Human Bronchial Epithelial Cells *in vivo*. Hematoxylin and Eosin (H&E) staining showed ciliary pseudostratified epithelium (A). Electronic microscopy images (B and C) demonstrated ciliated cells (B) and mitochondria (C). Mitochondria distributed predominantly toward the apical domain (arrowheads). Scale bars: 20  $\mu$ m (A) or 1  $\mu$ m (B and C). (D–F) Morphological and immunohistochemistry analysis of human airway epithelial cells under submerged culture *in vitro*. Submerged culture human airway epithelial cells under phase contrast microscopy (A), (H&E) staining (B) and Immunohistochemistry analysis (C). Brown cytoplasm staining indicated Keratin positive. Scale bars: 20  $\mu$ m.

to be exposed to air (Day 0). The cells were allowed to grow for an additional ten days and were harvested for experiments. All other reagents were purchased from Invitrogen (Carlsbad, CA). All experiments were conducted in accordance with IACUC protocols. For Seahorse experiments, murine tracheal epithelial cells (MTEC) were seeded onto our uncoated inserts at the same density as above. Inserts were individually placed into each well of a 24-well Transwell plate. Media (MTEC Plus) was changed every other day in the bottom chamber while the top chamber was left undisturbed to allow cells to adhere. After seven days, cells were lifted similarly to above, with 200  $\mu$ l MTEC Plus in the basal chamber. Submerged cells remained in MTEC Plus (200  $\mu$ l basal/20  $\mu$ l apical) for the duration of the culture period. Media was changed every two days, and the apical surface of lifted cells was washed every two days with PBS. Measures of metabolism were done on Day 8.

Human bronchial epithelial cells were obtained from volunteers who underwent bronchoscopy with a flexible fiberoptic bronchoscope with cytology brushings from second- and third-order bronchi with a 1 mm cytology brush (Microvasive Inc., Watertown, MA) [21]. A549 cells (ATCC, Manassas, VA), an epithelial cell line derived from human lung adenocarcinoma, were cultured in MEM (Invitrogen, Carlsbad, CA) with 10% heat-inactivated FCS, 1% penicillin/streptomycin and 1% L-glutamine. BET-1A cells, a human bronchial cell line transformed by SV40 T antigen, were cultured in serum-free LHC-8 (Biofluids, Inc., Rockville, MD) with the additives 0.33 nM retinoic acid and 2.75  $\mu$ M epinephrine on precoated plates.

#### Histology

The transwell plates containing differentiated airway epithelial cells were placed on ice and rinsed three times with ice-cold PBS. The base of the transwell insert was carefully excised with a scalpel and placed in 4% paraformaldehyde (pH 7.4) for 30 min. The membranes were then transferred to 50% Flex alcohol (Thermo Scientific, Waltham, CA) overnight at room temperature. The samples were sent to the Cleveland Clinic Lerner Research Institute Imaging Core Facility for paraffin embedding and sectioning in 5  $\mu$ m sections. The slides were stained with hematoxylin and eosin.

#### Toluidine blue light microscopy

As previously reported [11], the epithelial cells were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde at pH 7.3 at 4 °C overnight. Sections (1  $\mu$ m) were cut and Toluidine blue (100  $\mu$ l of 4% stain in 1% sodium borate) was applied to each section.

#### Immunohistochemical and confocal analyses

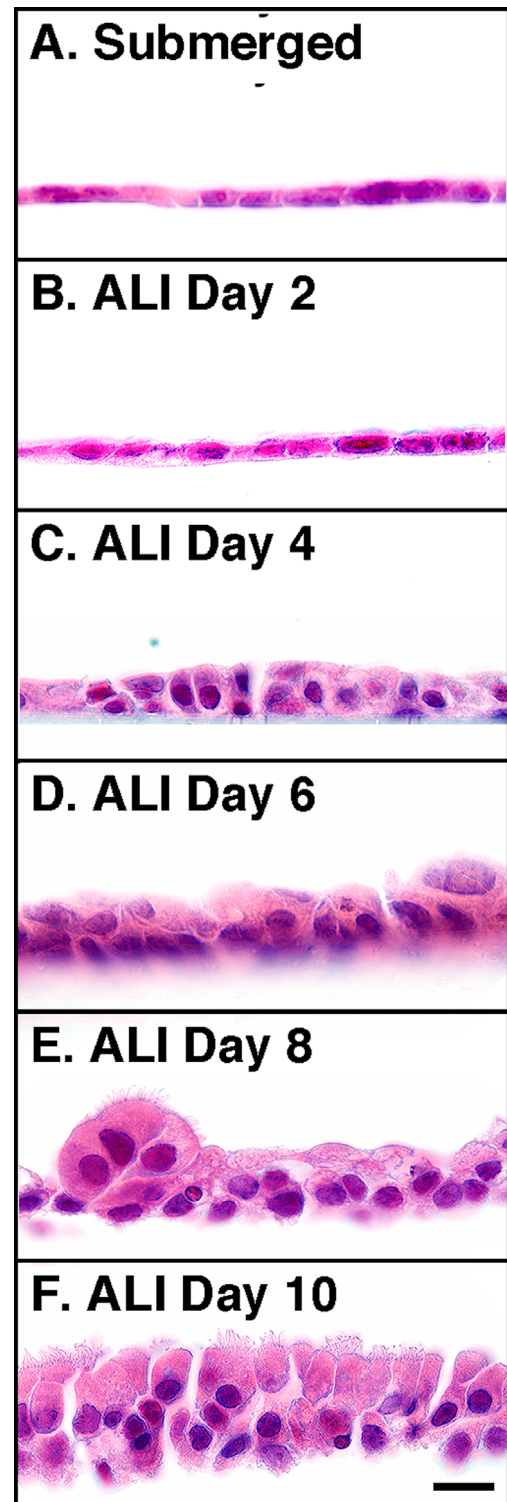
The airway epithelial cells were fixed in 4% paraformaldehyde in PBS at room temperature for 15 min and then 100% ice-cold Methanol for 10 min. Tubulin  $\beta$ 4 antibody (MU178-MC; BioGenex, San Ramon, CA; or NBP1-32205; NOVUS Biologicals, Littleton, CO) and pan-Cytokeratin antibody (H-240; Santa Cruz Biotechnology, Santa Cruz, CA) were applied to cells. The cells were transferred to a glass slide and mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Labs, Burlingame, CA), sealed, and analyzed by confocal laser-scanning microscopy (TCS-40; Leica Microsystems, Cambridge, UK).

#### Ultrastructural analyses

Ultrastructure of airway epithelial cells was studied using FEI Tecnai G2 Spirit BioTWIN Transmission Electron Microscope (FEI Company, Hillsboro, OR). Samples were prepared as previously described [22].

#### Electrophysiological measurements

Experiments of bioelectric properties performed by C. Cotton as previously described [12,13]. Using chambers with sequential drug additions: 30 min basal current, 5 min amiloride, 10 min Forskolin/IBMX. Mean  $\pm$  SE for the basal current and resistance, values with amiloride, delta induced by amiloride (measure of sodium absorption),



**Fig. 2.** Differentiation occurs following exposure to air. Histological analyses of normal differentiation of murine airway epithelial cells in air-liquid interface cultures. Scale bars: 20  $\mu$ m.



values with Forskolin/IBMX, and delta induced by Forskolin/IBMX (measure of CFTR-dependent chloride secretion).

### Production of inserts

A sheet of 1 mm thick clear cell cast acrylic (Plexiglass, ePlastics.com) was placed onto the bed of a CO<sub>2</sub> laser cutter. A 4 mm inner diameter was cut out. Next, a sheet of 0.4 μm PTFE track-etched polyester (PET) membrane (Sterlitech Corporation, Kent, WA) was laid over the acrylic sheet. The 6.25 mm outer diameter was cut from the two layers, simultaneously fusing the PET membrane to the acrylic ring. Rings were examined under the microscope, and only rings with a fully welded perimeter were used. After manufacture, inserts were sterilized using ethylene oxide.

### Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in cells

ECAR and OCR were measured using the Seahorse Extracellular Flux (XF24) Analyzer (Seahorse Bioscience Inc. North Billerica, MA). A Seahorse Islet Plate (Seahorse Bioscience Inc. North Billerica, MA) was prepared by adding our inserts to wells. A549 and BET-1A cells were resuspended in their standard growth media, and 65000 cells were added to the insert or empty wells. Four wells and four inserts remained cell-free to assess whether the inserts alone had any effect on oxygen and pH measures. Cells were maintained in the islet plate for approximately one (BET-1A) or two (A549) days before measurement of metabolism. Seahorse assay media (DMEM without glucose, L-glutamine, phenol red, sodium pyruvate, and sodium bicarbonate (Sigma-Aldrich Corp. St. Louis, MO) supplemented with 6 mM (A549 and BET-1A) or 13.5 mM (air-liquid interface) glucose, 1.85 g/L sodium chloride, 1 mM sodium pyruvate, and 15 mg/L phenol red) was freshly supplemented with 2 mM L-glutamine on the day of the assay and the pH adjusted to 7.35 with sodium hydroxide. For A549

and BET-1A experiments, all wells were washed three times with Seahorse assay media. For air-liquid interface experiments, the basal and apical chambers were washed one time with 200 μl and 20 μl respectively of Seahorse assay medium. Inserts were then removed from the Transwells and placed into a Seahorse islet plate with cells facing up. Final volume in all wells for all experiments was 500 μl. The plate was incubated in a 37° non-CO<sub>2</sub> incubator for 1 h prior to measurement. The plate was then transferred to the Seahorse XF24 Analyzer (Seahorse Bioscience Inc. North Billerica, MA) for analysis. Once in the XF24, A549 cells underwent measurement of basal oxygen consumption and extracellular acidification; BET-1A cells underwent successive treatments with oligomycin A (0.5 μM), FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone) (0.5 μM), and rotenone and antimycin A (1.5 μM); lifted and submerged MTECs underwent basal measurement of oxygen consumption followed by treatment with rotenone and antimycin A (1.5 μM) or basal measurement of extracellular acidification followed by treatment with 2-deoxyglucose (2-DG)(100 mM). All OCR and ECAR measures were done three to five times in a 3–2–3 min mix-wait-measure cycle.

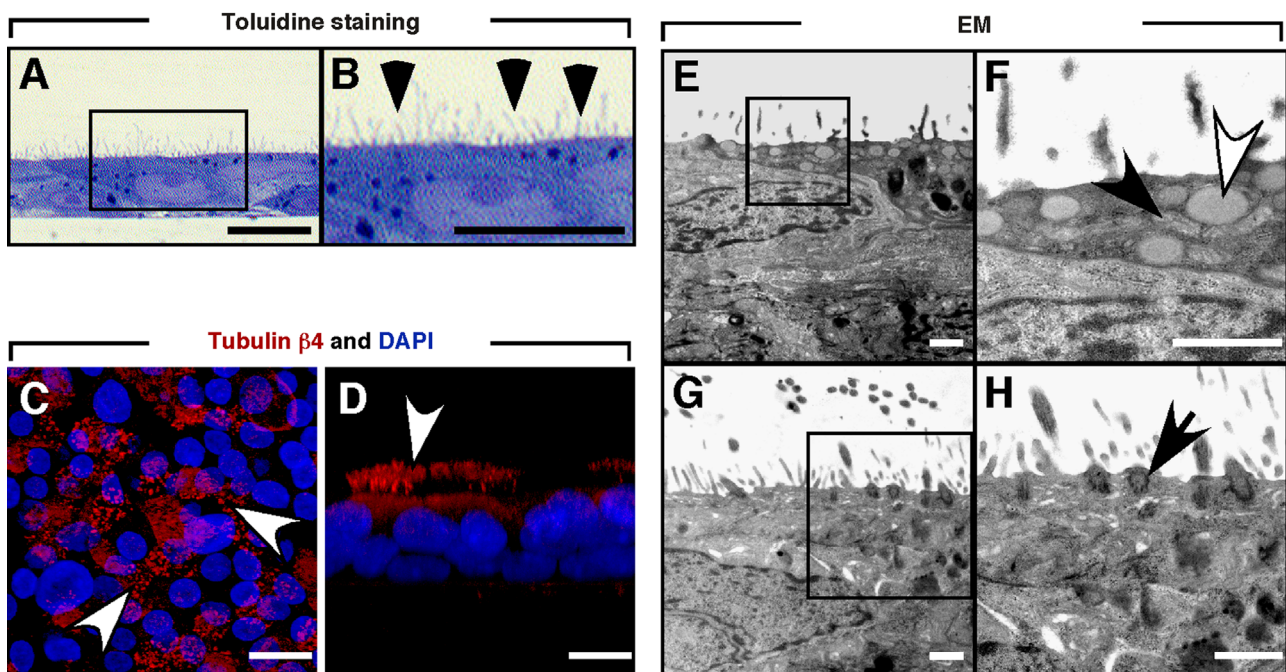
### Statistical analyses

Data are shown as mean ± SE. All statistical comparisons are performed using the Student's *t*-test or one-way ANOVA as appropriate. The level of significance for *P* was chosen at 0.05. All data were analyzed with statistical program JMP Pro 10 (SAS Institute, Cary, NC).

## Results

### Airway epithelial cells differentiation occurs following exposure to air

In order to study differentiation in airway epithelial cells, we utilized an organotypic air-liquid interface cell culture system with



**Fig. 3.** Human airway epithelial air-liquid interface cultures *in vitro*. (A–B) Toluidine staining of human airway epithelial cells at air-liquid interface cultures. Toluidine staining section of human airway epithelial cells at air-liquid interface culture demonstrated histologic appearance of ciliated cells (arrowheads). Scale bars: 10 μm. (C–D) Three-dimensional confocal images of human airway epithelial cells at air-liquid interface. Cilia were highlighted by Tubulin β4 staining (red), and DAPI-stained nuclei were blue. The apical surface of human airway epithelial cells at air-liquid interface had cilia (C; white arrowheads point to cilia). A cross-sectional confocal (D) identified nuclei (blue) at basal regions of cells, and cilia apically [white arrowhead]. Scale bars: 20 μm. (E–H) Ultrastructural analyses of human airway epithelial cells at air-liquid interface cultures. Cells were ciliated. High power (F and H) revealed mitochondrion (black arrowhead in F) and typical mucus product in cells (white arrowhead in F), and cilium (arrow in H). Scale bars: 1 μm. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

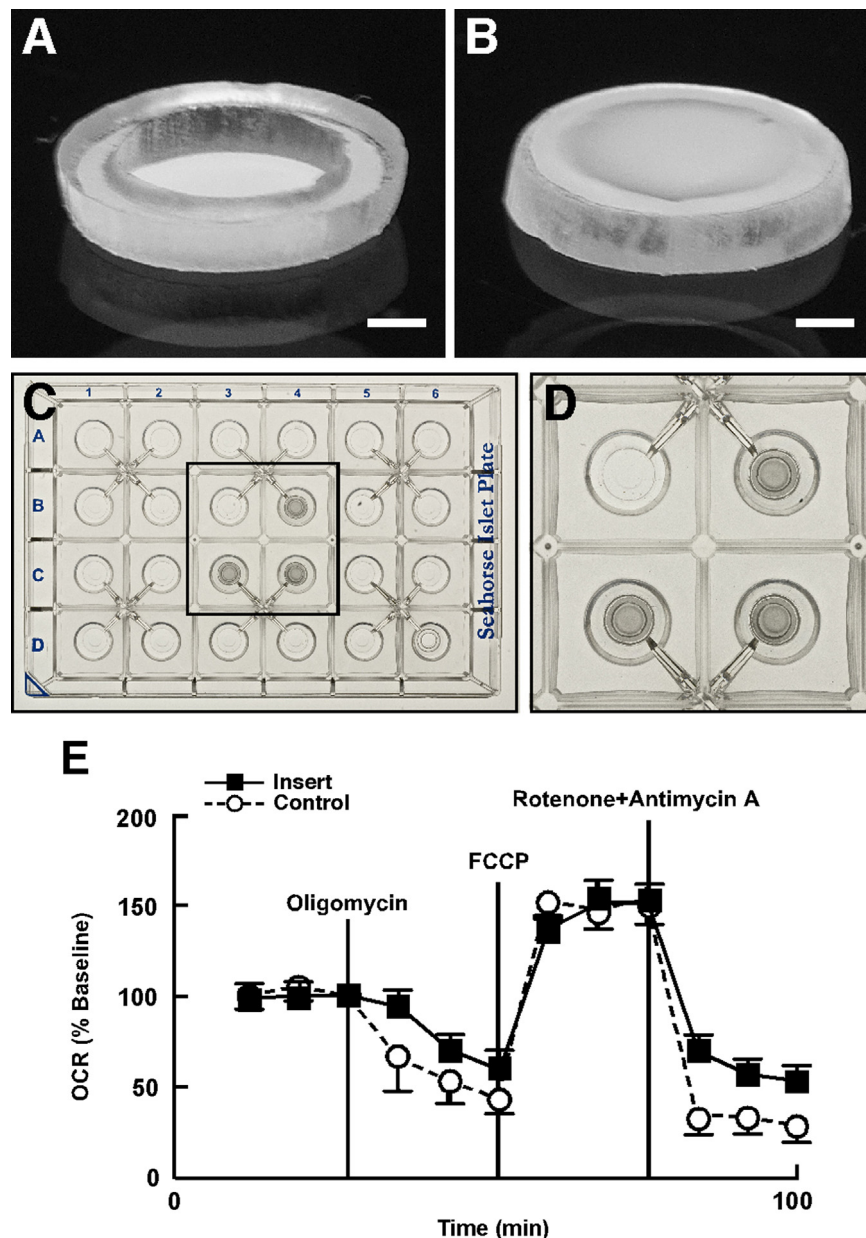
human and murine primary airway epithelial cells. Unlike ciliary pseudostratified columnar epithelium *in vivo* (Fig. 1A–C), human airway epithelial cells under submerged culture were single cell-layered but flat without a pseudostratified appearance and had no cilia (Fig. 1D–F). Mouse airway epithelial cells cultured on air–liquid transmembranes reached confluency at approximately six days from

the day of seeding and were subsequently exposed to atmospheric oxygen levels by “lifting” to air. Cells were significantly taller between Day 0 (submerged) and Day 10 when the cells reached complete differentiation and resembled that of normal airway epithelium ( $P < 0.001$ ) (Fig. 2). Multiple cell types, including goblet cells and ciliated cells, resulting from cell differentiation became

**Table 1**

Bioelectric properties of primary human bronchial epithelial cells in air–liquid interface cultures verified airway epithelial differentiation function.

	Basal	Amiloride	$\Delta$ Amiloride $\text{Na}^+$ Absorption	Forskolin and IBMX	$\Delta$ Forskolin and IBMX $\text{Cl}^-$ Secretion
Short-circuit current ( $\mu\text{Amp}/\text{cm}^2$ )	$23.7 \pm 5.3$	$1.6 \pm 0.4$	$22.1 \pm 4.9$	$5.1 \pm 1.1$	$3.5 \pm 0.8$
Transepithelial resistance ( $\text{k}\Omega\text{-cm}^2$ )	$1.39 \pm 0.05$	$2.28 \pm 0.12$	$0.89 \pm 0.16$	$1.69 \pm 0.08$	$0.63 \pm 0.04$



**Fig. 4.** BET-1A cellular bioenergetics in insert for Seahorse Islet Plate. (A–B) Top and bottom of inserts. Cells were seeded into the top well of the insert formed by the acrylic ring shown in A. Scale bars: 1 mm. (C–D) Inserts fitted into the wells of the Seahorse islet plate. D is a close-up view of C, showing three inserts and one empty well. (E) Similar MitoStress profile in BET-1A cells in engineered inserts ( $n=6$ ) or without ( $n=4$ ) as measured by the Seahorse XF 24 Analyzer. Inhibitors were used to assess mitochondrial function. ATPase inhibitor oligomycin (oligomycin), mitochondrial uncoupler (FCCP) and a cocktail of rotenone (ETC complex I inhibitor) and antimycin A (ETC complex III inhibitor) were injected sequentially at indicated times after basal rates were measured. OCR was normalized to the final basal measurement. Basal OCR was  $112 \pm 10$  in inserts and  $215 \pm 12$  in wells (control). There was no significant difference between inserts and controls with oligomycin or with FCCP (both  $P > 0.05$ ).

evident starting at Day 6. Between Day 6 and Day 10, the number of ciliated surface cells increased from  $12.7\% \pm 6.8$  to  $60.2\% \pm 17.6$ . Ciliated cells and goblet cells were also observed in human airway epithelial cells at air–liquid interface culture (Fig. 3). Toluidine and immunohistochemical staining of human airway epithelial cells at air–liquid interface culture showed histologic appearance of ciliated cells (Fig. 3A–D). Ultrastructure of transmission electron microscopy images demonstrated secretory products in goblet cells and confirmed the appearance of cilia in airway epithelial cells at air–liquid interface culture (Fig. 3E–H). We further validated the functional differentiation of the airway epithelial cultures by electrophysiological measurements of the human air–liquid interface cultures ( $n=3$ ) [11–13]. The cells grown at air–liquid interface formed typical high-resistance epithelial barriers that express both amiloride-sensitive sodium absorption and cAMP-induced chloride secretion (Table 1).

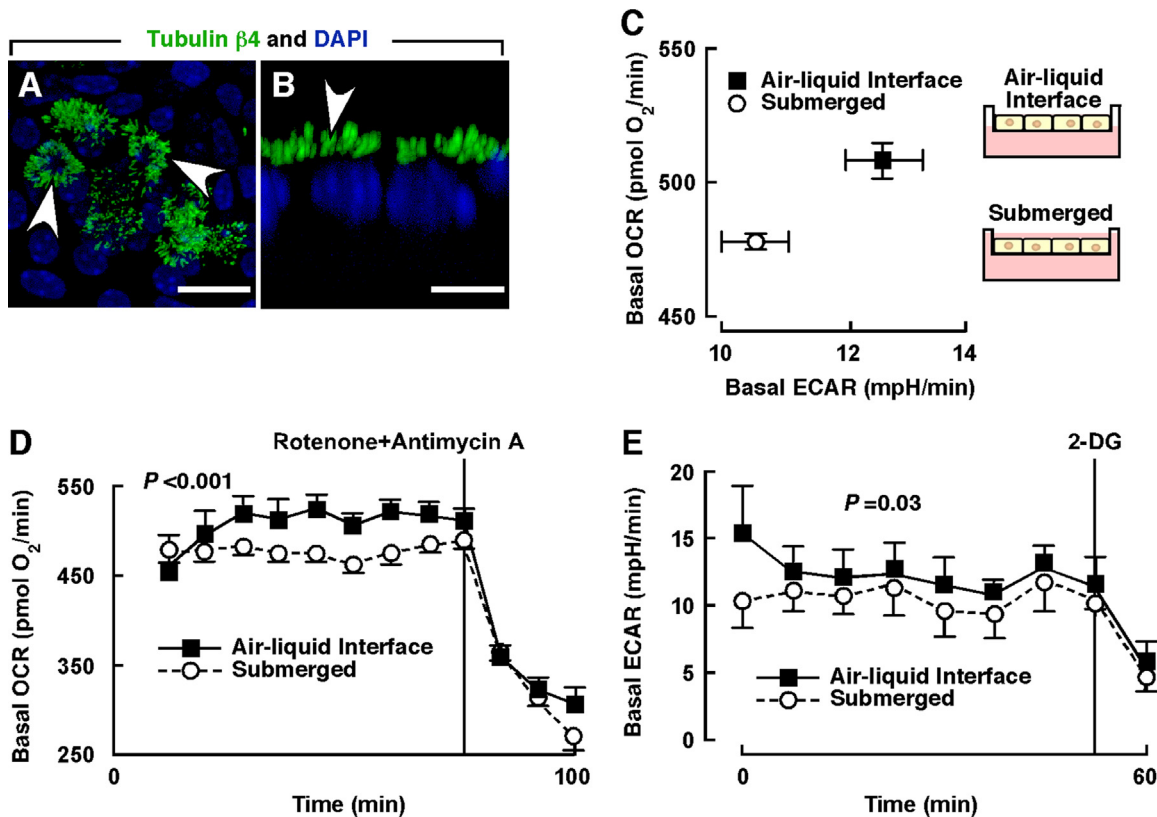
#### Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of cells in engineered insert for Seahorse XF24 Analyzer

To make a means by which we can study the effects of differentiation of airway epithelium in air–liquid interface on glycolysis and mitochondrial respiration, we engineered an insert for the Seahorse XF24 Analyzer (Fig. 4A–D). To assess whether the inserts had an effect on basal oxygen consumption and extracellular acidification, A549, BET-1A cells and murine airway epithelial cells were used. There was no effect on basal oxygen consumption or extracellular acidification of A549 cells in the engineered insert compared to in wells of the Seahorse cell culture plate ( $P > 0.05$ ) (data not shown). Compared to in

wells ( $n=4$ ), BET-1A cells in engineered inserts ( $n=6$ ) had similar mitochondrial stress tests in response to oligomycin and FCCP ( $P > 0.05$ ), but a higher non-mitochondrial oxygen consumption (with rotenone and antimycin A) ( $P < 0.05$ ) (Fig. 4E). Metabolism of mouse airway epithelial cells cultured on our inserts either in air–liquid interface or submerged was also studied ( $n \geq 3$ ) (Fig. 5A–E). The mouse airway epithelial cells under air–liquid interface cultures for 8 days had greater OCR (more respiration) and greater glycolysis (more 2-DG inhibitable ECAR) than cells grown submerged (both  $P < 0.05$ ), suggesting greater glucose oxidation and glucose utilization in cells grown at air–liquid interface.

## Discussion

Human bronchial epithelial cells can be differentiated, polarized and developed into an organotypic pseudostratified bronchial epithelium-like tissue with air–liquid interface culture. They show the typical characteristics of ciliated, goblet, and basal cells, and display the functional bioelectric physiologic properties of the well-differentiated lung bronchial epithelial barrier *in vivo*. These morphological properties support that the organotypic model of cell differentiation for airway epithelial cells is useful *in vitro* to study normal biological processes and disease mechanisms, and to develop new therapies [11,13,23]. The typical epithelial functions, including generation and secretion of mucus and continuous beating of cilia at the apical surface, likely require greater energy by the epithelial cells. In fact, polarized airway epithelium concentrate mitochondria at the apical surface in close proximity to cilia and sites of mucus production. In support of the greater bioenergetic needs in these cells, a prior



**Fig. 5.** Metabolism of mouse airway epithelial cells in air–liquid interface in insert for Seahorse Islet Plate. (A–B) Confocal images of murine air–liquid interface. Cilia were highlighted by Tubulin  $\beta 4$  staining (green, white arrowheads), and DAPI-stained nuclei blue. Scale bars: 20  $\mu\text{m}$ . (C–E) Mouse airway epithelial cells were cultured in air–liquid interface or submerged ( $n \geq 3$ ). Basal OCR and basal ECAR were plotted against each other (C). OCR (D) and ECAR (E) were measured in murine airway epithelial cells grown submerged or differentiated at air–liquid interface for 8 days using our inserts. Data showed the mean  $\pm$  SE. The air–liquid interface cultures had greater basal OCR and basal ECAR (both  $P < 0.05$ ), suggesting greater glucose oxidation and glucose utilization in cells grown at air–liquid interface. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



study suggests that the air-interface differentiated epithelial cells may consume more oxygen than those cultured undifferentiated under media [24]. Methods for metabolic assessment of cells in culture have advanced rapidly, allowing assessment of cell respiration and glycolysis even in small numbers of cells. However, the measure of organotypic cultures of the airway has been precluded due to technical limitations of measurement methods. Here, we describe the air–liquid interface culture of airway epithelium and engineering of an insert that allows evaluation of aerobic and anaerobic glucose metabolism of differentiated cells in culture. This methodology may be helpful to study of the airway epithelium in health, and in airway diseases where disturbances in mitochondrial functions have been described [25–27].

As previously reported [11,13,24], airway epithelial cells at air–liquid interface in this study manifested differentiated functions and structures. To develop methods to study metabolic shifts in the air–interface differentiated cells as opposed to immersion culture, we designed an insert that would allow for air–liquid interface culture and could be fitted directly into wells of the Seahorse islet plate. Importantly, inserts alone did not affect function of the Seahorse XF Analyzer. BET-1A cells in engineered inserts had similar mitochondrial stress tests in response to oligomycin and FCCP as cells on the islet plate ( $P > 0.05$ ). However, the values for non-mitochondrial OCR of cells in inserts were greater than in wells (Fig. 4E). This may be due to the fact that the XF software uses an algorithm that takes into account the volume of oxygen in the well chamber and this volume may be different in the lifted cultures. Alternatively, some of the difference may be due to the different distribution of cells on the membrane as compared to on the Seahorse XF islet plate. In addition, primary airway cells on inserts that were lifted to air also displayed a tendency to less suppression of OCR with rotenone and antimycin A than cells on inserts but not lifted to air (Fig. 5D). This suggests that lifted cultures may use more oxygen in non-mitochondrial respiration functions, or require optimization of compounds specific to lifted cultures in inserts. Overall, comparisons of cells at air–liquid interface on inserts should be performed consistently across cell types and conditions on the inserts for ability to directly compare raw values. Oxidative metabolism and glycolysis in murine tracheal epithelial cells cultured on the inserts under media with apical and basolateral feeding were compared to cells cultured on the inserts with basolateral feeding and air–interface at the apical surface. The cells under media had less glucose oxidation and less glucose utilization, as determined by less oxygen consumption and less extracellular acidification. The results suggest that the cells at air–interface with differentiated functions shift to greater oxidative metabolism. *In vitro* cell culture models of primary airway epithelia have been very useful for study of drug therapies and permeability abnormalities [28]. Our development of an insert to measure metabolism of the airway epithelium in an organotypic state will be useful for evaluation of metabolic mechanisms that may contribute to the pathogenesis of airway diseases.

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