DECONSTRUCTING THE AIR-LIQUID-INTERFACE: THE ROLE OF OXYGEN IN EPITHELIAL CELL DIFFERENTIATION

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Abstract

Current airborne particulate toxicity screens require the culture of a ciliated, pseudostratified epithelium to achieve physiologically accurate cytotoxicity data. Air-liquid interface (ALI) is the *de facto* standard technique that is used to drive differentiation of epithelial cells towards an *in vivo*-like phenotype. This technique consists of exposing the apical side of the cellular surface to ambient incubator air, and the basal surface to culture medium which diffuses through a porous substrate. Microporous filters are most commonly used to achieve ALI for human bronchial epithelial cell (HBEC) culture. However, these filters are expensive and difficult to handle, and it remains unclear which biophysical cues specific to this culture system promote differentiation. Understanding how defined microenvironmental cues drive ALI-related differentiation is the first step towards eliminating the need for this complex culture processing condition. In this thesis, I hypothesize that exposure to increased oxygen levels present on the air-side of the ALI culture would trigger HBEC differentiation. Hence, properly designed hyperoxic culture system would eliminate the need for an ALI in *in vitro* pulmonary health studies. In this thesis, a model human bronchial epithelial cell line was used to confirm that cells were sensitive enough to minute oxygenation differences arising from the addition of an apical media layer during culture. A finite element simulation was designed to model the oxygen diffusion gradient present in apical media during submerged culture. This model aided us in the design of our hyperoxic incubator conditions. HBECs were then cultured in standard/ALI, standard/submerged and hyperoxic/submerged conditions and it was found that hyperoxic/submerged culture surprisingly promoted the highest level of differentiation. This work shows that ALI is not necessary for HBEC differentiation and that other microenvironmental characteristics of the filter culture system work in conjunction with proper oxygenation to allow for HBEC differentiation. A better understanding of these microenvironmental cues gained through these experiments can hence guide the design of novel high-throughput culture systems for airborne particulate toxicity studies.

Résumé

Les études actuelles sur la toxicité des particules nécessitent un épithélium cilié et pseudostratifié pour atteindre des résultats de cytotoxicité pertinents. L'interface air-liquide (IAL) est la technique *de facto* utilisée pour promouvoir la différenciation de cellules épithéliales vers un phénotype ressemblant celui *in vivo*. Cette technique consiste d'exposer le côté apical de la surface cellulaire à l'air ambiant et la surface basale au milieu de culture qui diffuse à travers un substrat poreux. Les filtres microporeux sont traditionnellement utilisés pour atteindre une interface air-liquide durant la culture de cellules épithéliales bronchiques humaines (CÉBHs). Toutefois, ces filtres sont couteux and difficile à manipuler, et ce n'est pas connu quel signaux biophysiques émis par cette méthode de culture promeuvent la différenciation. Comprendre comment les signaux microenvironmentaux reliés à l'IAL influencent la différenciation est la première étape vers l'élimination du besoin de cette méthode de culture complexe. Nous avons comme hypothèse que les concentrations d'oxygène augmentés présents sur the coté d'air de l'IAL déclenchent la différenciation de CÉBHs. Par conséquent, si on conçoit un système hyperoxique idéal, on éliminerait le besoin de IAL dans les études de santé pulmonaire. Dans cette thèse, une lignée de cellules épithéliales bronchiques humaines a été utilisé pour confirmer que les cellules sont assez sensibles aux perturbations d'oxygène résultant de l'addition d'une couche de milieu de culture apical. Une simulation par éléments finis a été créé pour modeler la diffusion d'oxygène à travers cette couche de milieu durant la culture submergée. Ce model nous a aidé à sélectionner les conditions de culture hyperoxiques. Ensuite, des CÉBHs ont été culturés en conditions standard/IAL, standard/submergé et hyperoxique/submergé et on a observé que la culture hyperoxic/submergé entraine le plus haut niveau de différenciation. Ces résultats démontrent qu'une IAL n'est pas uniquement nécessaire pour la différenciation de CÉBHs et suggèrent que d'autres caractéristiques microenvironnementales permettent la différenciation cellulaire en conjonction avec l'oxygénation. Une meilleure compréhension de ces signaux environnementaux obtenue par ces expériences peut, par conséquent, guider le développement de nouveaux systèmes pour les études de toxicité à haut débit.

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Contributions of Authors

The writing on the following thesis was primarily the work of the author and was kindly edited by Dr. Christopher Moraes. The data presented in this work was collected and analyzed solely by the author. Dr. Christopher Moraes supervised the findings of this work.

Julie Goepp and Carolina Martini handled the seeding of the primary bronchial epithelial cell cultures. Julie Goepp, Carolina Martini, Elizabeth Matthes and Dr. John Hanrahan contributed to the interpretation of the results and were involved in planning experiments.

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1. Introduction

Inhalable drug and respiratory toxicity assays are conventionally performed on human bronchial epithelial cells (HBECs). These primary cells are obtained from healthy bronchial explants and expanded in growth medium whilst in their undifferentiated state[1,2]. These cells are then differentiated for several weeks at an air-liquid interface (ALI) to form a pseudostratified epithelium, similar to what is seen *in vivo*, which consists of closely packed ciliated cells, mucus-producing goblet cells and other columnar cells. This differentiated epithelium is then capable of mucociliary clearance[3], one of the most important functions of the human airway epithelium[4] and a desirable culture characteristic for inhalable particle toxicity studies.

Porous filters have been used as ALI culture models in differentiation studies for decades, since they were first shown to promote HBEC differentiation[5]. Cells are traditionally seeded onto the apical compartment of the filter inserts and fed basolaterally with culture media, thus allowing cellular contact with both ambient air and a nutrient supply and partially mimicking *in vivo* conditions. However, ALI filter culture is expensive, labor-intensive, and difficult to scale for high-throughput applications. Moreover, the filter pores hamper cell visibility during Brightfield imaging, making it impossible to obtain morphological data from live samples. It also remains unclear as to which ALI-exclusive biophysical cues prompt differentiation. Understanding these signals could lead to greatly simplified designs of culture platforms optimized for airway studies compatible with high-throughput methods.

The assumption that complete ALI is a uniquely necessary condition for HBEC differentiation has led to filter culture becoming the *de facto* technique for HBEC studies. This assumption was only recently challenged by Gerovac et al., who demonstrated that a thin layer of culture media over lung cells at ALI can further improve differentiation[6]. This suggests that differentiation in submerged culture is not only possible, but that the increased availability of soluble nutrients may enhance differentiation efficiency under appropriate culture conditions. Eliminating the need for ALI could eliminate the need for filter culture platforms and reduce the cost of drug discovery.

Recently, it has been shown that the cellular microenvironment plays an important role in driving cellular function[7-9]. ALI culture, specifically, provides certain biophysical microenvironmental cues related to the filter substrate porosity and stiffness, as well as the oxygenation conditions at the ALI. In submerged culture, limited diffusion of oxygen through culture media causes an oxygen-depleted zone to form at the cell surface. In contrast, ALI provides cells with increased oxygen access. Here, I hypothesize that exposure to increased oxygen levels present on the air-side of the ALI culture triggers differentiation, and that appropriately designed hyperoxic culture conditions would allow differentiation under submerged conditions.

In these studies, I assessed the effects of standard/hyperoxic and ALI/submerged culture conditions (**Figure 1**) on HBEC differentiation and were thus able to re-evaluate the necessity of ALI culture in modern differentiated studies. I first used a model bronchial epithelial cell line to evaluate oxygenation differences in ALI and submerged culture conditions. I did this by confirming that the addition of a thin liquid layer of media above cells grown on porous filters created a steep enough oxygen diffusion gradient to shield these cells from hyperoxic incubator conditions. I then designed a finite element model to simulate the oxygen diffusion gradient in apical medium during submerged culture conditions. This simulation allowed us to determine the appropriate incubator oxygen concentration necessary to achieve normoxic

conditions at the cell monolayer during hyperoxic/submerged culture. Next, I filter-cultured HBECs isolated from three healthy human donors in standard/ALI, standard/submerged and hyperoxic/submerged conditions. Nucleic hypoxia-inducible factor 1-alpha (HIF-1α) expression was evaluated and we established that cells cultured in standard/submerged conditions were being subjected to hypoxia. Transepithelial electrical resistance (TEER) was monitored throughout the culture to confirm epithelial barrier integrity and HBEC differentiation was determined by measuring epithelial layer thickness, ciliated surface area, beat speed and functional ciliation, by particle movement assay. Our studies indicate that an air-liquid interface is not necessary to produce highly-differentiated human bronchial epithelial cells and that increased oxygen availability, coupled with increased nutrient availability can produce the highest rates of *in vitro* epithelial cell differentiation.

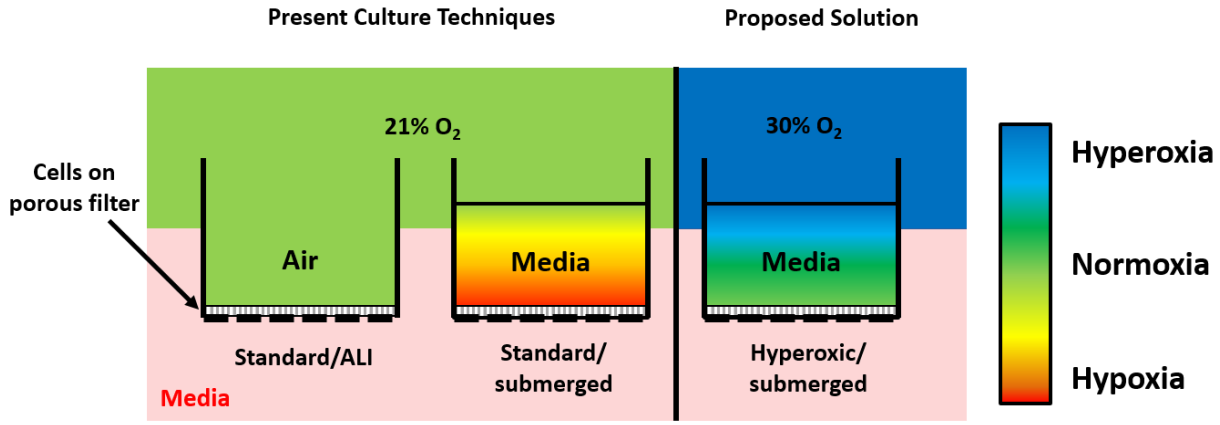

Figure 1. *Schematic of bronchial epithelial cells cultured in the apical compartment of a porous, polyester Transwell® filter insert in three different experimental culture conditions. Both standard/ALI and hyperoxic/submerged cultured provide the cell monolayer with normoxic levels of oxygen, whereas the standard/submerged conditions produce a hypoxic environment for cells.*

2. Background and Literature Review

2.1 Pulmonary Health

In 2010, there were over 100,000 lung disease-related deaths in the United States alone[10]. Lung disease affects people of different gender, race, age and lifestyle. Some of these deaths are caused by environmental factors (cigarette smoke, pollution, occupational dusts and chemicals, wood smoke and other biomass fuel[11]) and others, by a variety of other factors such as the existence of a genetic predisposition to a certain disease.

Pulmonary diseases are categorized as follows: interstitial lung diseases, neonatal lung disease, influenza, pneumonia or diseases affecting the airway and diseases affecting pulmonary circulation. **Figure 2** presents the percentage of total deaths in the U.S. in 2010 for each of these categories. Almost 60% of these deaths are due to lung diseases affecting the pulmonary airways. Some of the most common diseases of this type are asthma, chronic obstructive pulmonary disease (COPD), chronic bronchitis, emphysema and cystic fibrosis [12]. According to the U.S. Department of Health and Human Safety, smoking causes about 90% of all lung cancer deaths in men and women and, more specifically, is the cause of about 80% of deaths from COPD[13].

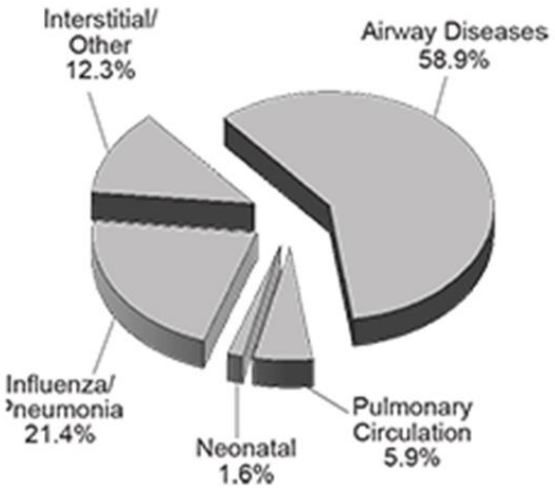

Figure 2. *Deaths from Lung Diseases, U.S., 2010[14]. Total number of deaths in the U.S. from lung disease in 2010 was 234,533, which represents 10% of total human deaths that year.*

Although not as well documented, pollution can also have a negative effect on lung health, especially in children [11,15,16]. Children with underlying respiratory disease are known to be more vulnerable to the adverse effects of air pollution and are prone to asthma exacerbations and to an increase in respiratory infections [15,17]. Another study showed that there is a strong and consistent relationship between the number of children's hospital admissions and outdoor air pollutant levels of particulate matter (PM) [18]. Lastly, recent advancements in nanotechnology have driven an increase in the use of nanoparticles in technological and commercial applications [19]. Because they are still a fairly new technology, the effects that nanoparticles can have on the environment and on human health are still largely debated, driving

the need for reliable pulmonary health models. These complex tissue engineering lung culture models enable the testing of airborne particles in highly controlled conditions, but recreating *in vivo* architecture is a key challenge.

2.2 Lung Epithelium Physiology

The human lung is a complex organ responsible for our body's oxygen uptake. The lung's primary defence against inhalable pollutants is the epithelium which provides a barrier between these particles and the underlying tissue. The human airway epithelium is comprised of 4 major cell types [20-23]:

- **Ciliated cells**, as their name suggests, contain about 200 membrane-bound cilia each and abundant long microvilli on their apical surface [22,24]. These cells comprise about half of the total cells present in the human airway and are partially responsible for mucociliary clearance which occurs when foreign objects enter the airways.
- **Goblet cells** are secretory cells containing large electron-lucent granules that store mucins [22].
- **Basal cells** are small, rounded cells present at the basement membrane. During normal epithelial turnover and repair, these cells differentiate into both ciliated and goblet cells [21].
- **Undifferentiated columnar cells** are similar to basal cells in that they have the possibility of differentiating into either ciliated or goblet cells. The most notable difference is that these cells are columnar in shape and can reach from the basement membrane all the way to the apical surface of the airway epithelial membrane.

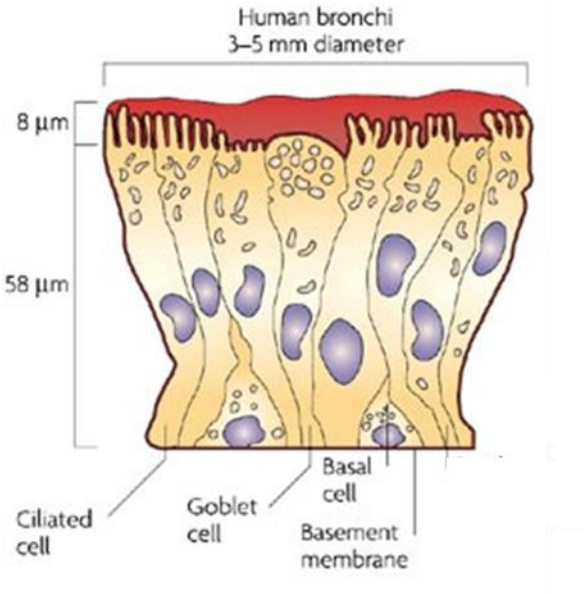

Figure 3. *Human bronchial epithelium[25].*

The primary function of the human airway epithelium is to defend the lungs against inhalable pathogens and particulates. This epithelial barrier is characterized by its tight junctions, hindering the diffusion of airborne particles into the blood stream, as well as its capacity to eject any particles that have settled onto the airways via mucociliary clearance.

Ciliated and goblet cells, specifically, are responsible for mucociliary clearance, which is defined as the self-clearing mechanism of the bronchi. The airways are lined with a thin mucosal membrane between 5 and 100 µm thick which is the result of the accumulation of goblet cell mucin excretions (shown in orange in **Figure 3** above). This mucosal membrane consists of an amalgam of plasma proteins, surfactants, DNA, antibacterial substances and mucins [22,26]. Mucins, which are mostly responsible for the viscoelastic properties of the mucus membrane, are long, thread-like, complex molecules (500 kD) that are bound together to create a gel-like substrate that has the elastic properties of solids and the viscous properties of liquid [22,26]. These properties allow for efficient capture of particles entering the airways. The ciliated cells then displace this mucus-particle mixture and transport it up the airways and towards the pharynx to be expelled.

Noxious gases, airborne particles, disease and other inflammatory mediators can all cause damage to the airways. One of the first signs of epithelial damage is ciliary discoordination. This can eventually progress to a complete loss of ciliary motion which can affect the efficiency of mucociliary clearance. Another gauge for the health of lung epithelium is the membrane mucus layer. The epithelial mucus layer is normally very thin for healthy patients, but increases in thickness in patients suffering from bronchitis, asthma, cystic fibrosis or viruses [22,27]. Mucosal membrane thickening is caused by goblet cell hyperplasia, which is characterized by an increase in the reproduction rate of goblet cells in the epithelium at the expense of the ciliated cells [22,26]. Many diseases and environmental factors can cause such abnormalities; therefore, it is important to have access to a culture model which can aid in the evaluation of the mechanisms resulting in these disturbances.

2.3 Models for Pulmonary health

We have access to a variety of pulmonary health models including epidemiological studies, which consist of evaluating the lung health of populations exposed to certain ambient environmental factors, animal studies, which allow us to test specific particle toxicity in a more controlled setting, and *in vitro* studies which utilize human cells to more accurately predict human lung response.

2.3.1 Epidemiological studies

Epidemiological studies allow us to study the effects of various risk factors on large human populations without the extrapolation of data that is required during animal studies[28]. Many of the social studies attempting to relate pollution to lung disease do not conclusively evaluate the toxicity of certain pollutant particles. This is because exposures to particulate matter consist of a mixture of different types and sizes of particles, making it difficult to determine whether a specific particle type of a particular size is responsible for a unique health effect [29]. Other difficulties that affect the reliability of these studies include the selection of a proper control population and the existence of confounding variables, such as the subjects' smoking habits, dietary habits and physical activity levels.

2.3.2 Animal Toxicity Screens

Traditionally, toxicity screens of inhaled substances are performed using animal test subjects. The testing strategies involve subacute to chronic inhalation of particles over a time span of one to several months, along the guidelines set forth by the Organization for Economic Co-operation and Development

(OECD). The OECD regulations dictate that inhalation testing should be performed up to a maximum concentration of 5 mg/L for aerosols, which is a very unrealistic exposure scenario [30]. Moreover, the animals subjected to these high-concentration testing strategies can often experience distress or pain that can affect the results of these experiments. Finally, another common concern is that these animal models might not accurately predict the responses in humans and therefore might not be a reliable method for toxicity screens [30]. Creating a physiologically relevant human epithelium *in vitro* to perform toxicity screens is one way of bypassing some of these issues. Air-liquid interface culture models have been developed to attempt to replicate the multifaceted human airway epithelium.

2.3.3 In vitro lung models

Human airway cell lines have been developed and characterized for *in vitro* pulmonary health assays[31,32] and have been shown to be relevant preclinical models for the study of the human airway[33]. Cell lines are often used in these studies because of their economic efficiency and ease of handling, but primary cell cultures have been shown to produce more physiologically representative systems. A multitude of *in vitro* models have been developed to represent each section of the human airway, such as the nasal passages[34,35], trachea[36,37], bronchi[38-42] and alveoli[43,44]. These models range from simple 2D biochemically-supplemented cultures to complex organ-on-a-chip systems that can include the co-culture of multiple cell types on various substrates and environments. Lung-on-a-chip systems, specifically, allow us to analyze barrier function, mucociliary clearance, metabolic and biochemical activities within the context of functional human lung tissue[45]. Stucki et al. as well as Huh et al. developed an actuating alveolus-on-a-chip model that mimics the cyclic strain experienced by alveolar epithelial and endothelial cells during breathing[46-48]. Humayun et al. engineered a multi-compartment lung-on-a-chip to model the airway's tissue structure. They differentiated human airway epithelial cells on a suspended hydrogel with smooth muscle cells adhered onto its underside and exposed the cells to an air-liquid interface[49]. These specialized systems allow insight into the physiological phenomena occurring *in vivo*. Many lung-on-a-chip models require air-liquid-interface (ALI) culture for complete epithelial differentiation[50]. Therefore, a better understanding of the differentiation cues present in ALI systems would heighten development of lung culture models.

2.4 Air-Liquid-Interface Culture

Human bronchial basal epithelial cells are obtained from bronchial donor explants, expanded in traditional culture and frozen down for use in later assays. These undifferentiated cells are incapable of differentiating into ciliated and goblet cells or of forming the tight junctions that are characteristic of the airway epithelium whilst in traditional tissue-culture flasks. Performing toxicity assays on these undifferentiated cells would not produce physiological results, therefore, differentiation protocols have been developed.

Culturing HBECs at an air-liquid interface (ALI) has been shown to promote differentiation[51]. This model uses Transwell® filters, which are traditionally used for compartmentalized co-culture systems. The upper and lower compartments are separated by a porous polyester membrane, which allows media to flow freely between the two sections but prevents cells from migrating from one compartment to the other. **Figure 4** shows the culture of primary human bronchial epithelial cells (HBECs) grown on porous culture filters. Initially, cells are seeded on a liquid-liquid interface (**Figure 4**A). Once they reach

confluence, the media is aspirated out of the apical compartment, creating an air-liquid interface (**Figure 4**B). In this environment, cells differentiate into cell ciliated monolayers that more closely resemble the lung epithelium *in vivo* [5,23,52].

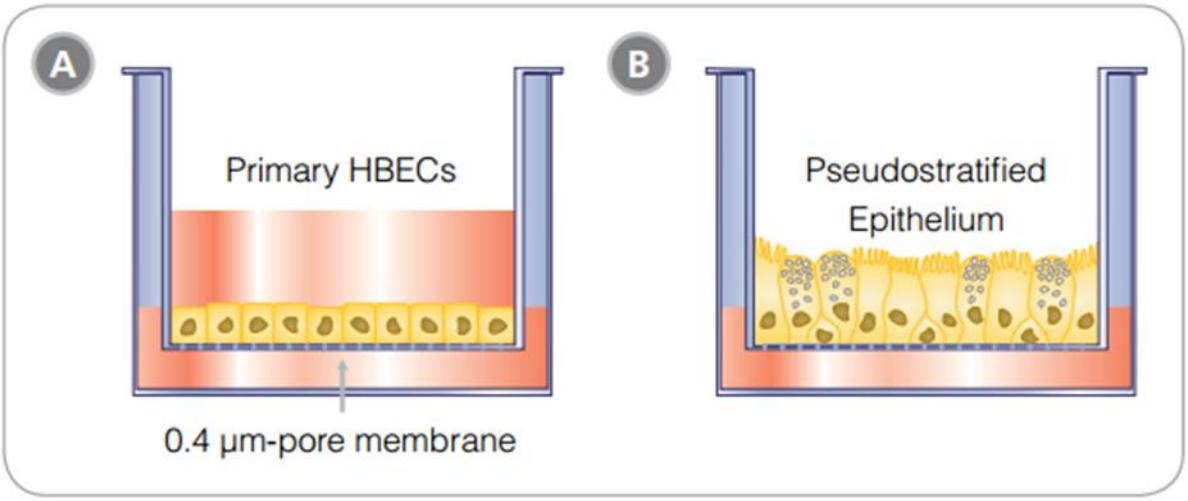

Figure 4. *Air-Liquid Interface Culture Schematic[53]. Schematic of submerged* ***A.*** *and air-liquid interface* ***B.*** *culture of primary human bronchial epithelial cells grown using porous culture filters.*

2.4.1 Epithelial Differentiation Biomarkers

Before proceeding to the cytotoxicity assay, it is important to evaluate the level of differentiation that has occurred in the ALI culture. To do this, several differentiations markers are first evaluated. The most obvious morphological sign of ciliated cell differentiation is the presence of motile cilia which can be observed using Brightfield or SEM microscopy[5] (**Figure 5**).

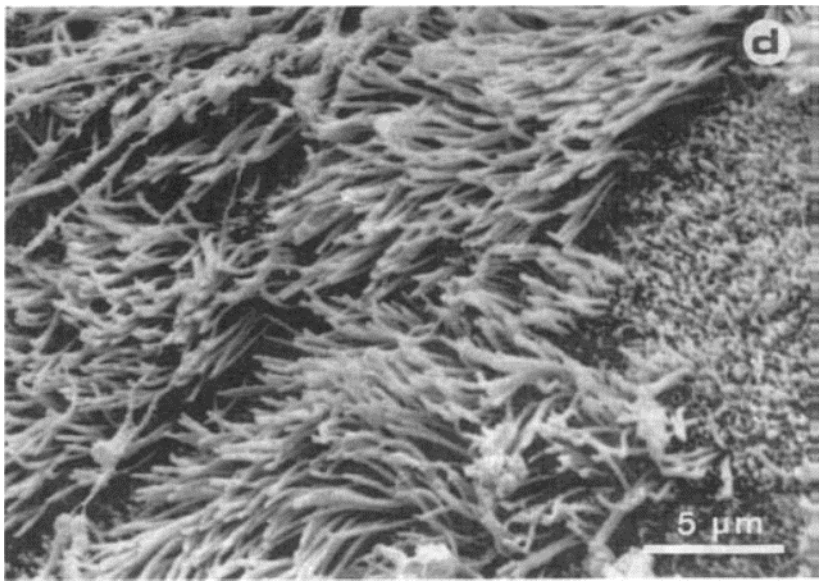

Figure 5. *Scanning electron micrographs of HBEC culture after 21 days at air-liquid interface. The mature cilia reached a length of approximately 5µm [5].*

Another common way to evaluate the differentiation of epithelial cells into ciliated cells is to evaluate the expression of β-tubulin, a protein that makes up the internal structure of cilia [52]. A similar fluorescent stain can be performed to evaluate goblet cell differentiation by staining for mucin 5AC [52]. If there is production of mucin on the surface of the epithelial layer, then it can be concluded that cells have differentiated into secretory goblet cells.

The airway epithelium is characterized by its tight junctions that form a relatively impermeable membrane. The integrity of the epithelial monolayer cultured on ALI can be evaluated using two common methods: staining for the tight junction protein zonula occludens-1 (ZO-1) and monitoring the transepithelial electrical resistance (TEER) of the monolayer [52,54,55]. The latter involves filling the apical Transwell® compartment with media, placing one electrode in the upper compartment and a second electrode in the lower compartment (**Figure 6**). The TEER values of the epithelial layer are measured by applying an alternating current (AC) voltage to the electrodes and measuring the resulting current [54]. The higher the transepithelial resistance, the tighter the junctions of the epithelial monolayer, indicating more physiologically relevant model. TEER measurements are relatively low during the initial submerged culture stage (under 100 $\Omega.cm^2$). This value increases once cells are subjected to the ALI and can achieve values of up to 3000 $\Omega.cm^2$ after about 3 week in culture [54].

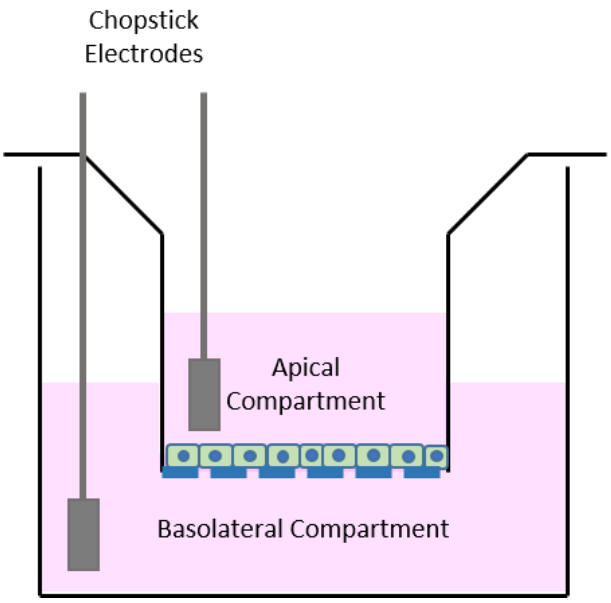

Figure 6. *Transepithelial electrical resistance (TEER) measurement of epithelial cells in Transwell® filter plate.*

2.4.2 Advantages of ALI culture

For a culture system to produce reliable data, the system itself needs to properly represent the *in vivo* environment. When designing ALI culture as a method to induce differentiation in airway epithelial cells, scientist took inspiration from the human lung epithelium which is both in direct contact with ambient air and fed basolaterally from nutrients in the blood. Epithelial cells cultured on ALI have been shown to properly differentiate and to form the tight junctions seen in the actual airway epithelium [5,23,52,56]. It has also been shown that airway epithelial cells (AECs) grown in primary cultures at the air-liquid interface closely recapitulate the transcriptional profile of airway epithelial cells *in vivo* [23]. Therefore, the transcriptional profile of these cells can act as a baseline when evaluating that of diseased cells. It is also important that a cell culture platform be proven to be both robust, making it easily reproducible with consistent results, and sensitive to changes in toxin concentrations. Azzopardi et al. showed that, when primary bronchial epithelial cells were cultured on ALI, they exhibited a dose-dependent toxicity trend and the culture system was able to differentiate between smoking regimens with regard to cytotoxicity [51]. This cell culture platform can potentially be just as reliable for other assay types.

The economic component of toxicity assays must be considered when selecting the optimal culture system for an experiment. Although ALI culture systems are more expensive than traditional 2D culture plates, they are more economically viable than experiments involving particle inhalation using small animals, since animal testing is costly in nature. This system also allows the use of primary human cells, which eliminates the argument that animal subjects might not react similarly to humans to certain toxic substances.

2.4.3 Potential Issues with ALI culture

The use of ALI culture is not yet widespread because of some of the technical requirements, financial commitments and experimental limitations. The average cost of 1st passage airway epithelial cells purchased from a commercial supplier is over US$ 500 per 0.5 x 10^6 cells [57]. Moreover, an important consideration that needs to be taken when working with ALI culture is the passage number of the cells being used, since it has been shown that it can strongly affects the cytotoxicity assay as well as the transcriptional profile [23,51,54]. Another very important setback to ALI culture is that, on average, the cells require 3 weeks of ALI culture to fully differentiate [21,32,58,59]. This, combined with the handling difficulties that come with changing the cell media every second day and having to dissect and mount the filters at the end of culture, makes ALI cytotoxicity assays incompatible with high-throughput testing.

Although there has been much success in ALI culture properly representing the physiological environment of the lung, there have been some cell types cultured on ALI that have only shown to only partially represent the *in vivo* environment [52]. Delgado-Ortega et al. have shown that when newborn pig trachea (NPTr) cells were culture on ALI, the epithelial culture was more similar to a stratified squamous than pseudo-stratified epithelium [52]. A squamous epithelium is characterized as being permeable and occurs when small molecules can pass quickly through the membrane by diffusion. This suggests that airway epithelial cell lines might have different differentiation pathways depending on their genotype.

Unfortunately, the mechanism which drives this epithelial cell differentiation has not been definitively established, which means that optimization of these ALI systems is only possible on a trial-and-error basis.

2.5 Microenvironmental Considerations Affecting Differentiation

Recently, there has been much interest in studying the local cell microenvironment because it has been shown to affect cellular functions and phenomena such as differentiation, proliferation, migration and metabolism[7-9]. External mechanical stimuli, matrix composition, matrix mechanics and topography, as well as surrounding geometry are all biophysical parameters of which the cellular environment is composed[60]. Culture systems have been designed to probe each parameter individually to determine how it affects specific cells and therefore offer insight into how these cells perceive their environment. During ALI culture, there are multiple environmental factors that must be considered when attempting to identify what triggers HBEC differentiation. Three major cues to be considered are oxygen tension, cell culture medium composition, and culture substrate characteristics, both physical and chemical.

2.5.1 Oxygen Tension

When attempting to identify the mechanism which permits the differentiation of human airway epithelial cells during ALI culture, the most obvious environmental cue to research would be the difference in oxygen tension that the cells are exposed to in ALI compared to submerged culture. In submerged culture, oxygen must diffuse through a layer of culture media before reaching the cell surface, which creates an oxygen-depleted zone[61]. The idea of oxygen tension affecting differentiation of epithelial cells is a valid hypothesis given that hypoxia has been proven to cause an arrest in differentiation for several cell types [62-65]. Heinis et al. showed that hypoxia decreases pancreatic beta-cell development *in vivo* by exposing pregnant rats to hypoxic conditions (8% O_2) for 24 hours, then dissecting the embryonic

pancreases and using immunohistochemistry, as well as qPCR, to evaluate the expression of certain transcription and hypoxia inducing factors known to be linked with cell differentiation. Their findings show that hypoxia can downregulate endocrine differentiation not only *in vitro*, but *in vivo* as well [62].

There is an increase in intestinal epithelial cell differentiation rates in ALI culture, similar to that of AECs [66]. Therefore, it appears, that this cell culture system provides environmental cues to the cells that are necessary even for cells that are not subject to a constant ALI *in vivo*. Oxygen tension of the gas in the human gut is normally between 120 and 150 mm Hg [67], similar to ambient air. If it is assumed that the intestinal cells only differentiate when they are at air-liquid interface *in vivo*, it would mean that the intestinal epithelial cells would only be able to differentiate when the intestine is empty. This is unlikely since the intestinal epithelium faces highly variable pressure, mechanical stress and oxygen supply conditions. This does not seem like a plausible assumption, therefore there must be another characteristic of the ALI that allows it to produce cells with similar differentiation tendencies as cells *in vivo*.

The physiology of human lung development also suggests that oxygen tension is not the sole contributor to differentiation. During the first trimester, placental oxygen tension hovers at around 20-40 mmHg and increases to a value of over 60 mmHg during the second trimester [68-73]. What is quite surprising is that, during the third trimester, which is the stage at which the lungs of the fetus fully develop, the intervillous oxygen tension drops back to 40 mmHg, presumably because of increased oxygen demand of the placenta [69,74]. It has also been shown that placentas from high altitudes are exposed to oxygen tensions that peak at around 50-60 mmHg, which could mean that the intervillous oxygen tension in the third trimester could be even lower than the 40 mmHg [68]. The lungs must be fully developed by the time the child takes its first breath, therefore there must be a way for the airway epithelial cells to differentiate without access to ambient oxygen tension and well as the physical presence of an air-liquid interface. These findings lead to the hypothesis that there must be another mechanism that can trigger differentiation of these cells.

2.5.2 Culture medium

Growth mediums and differentiation mediums have been developed for human bronchial epithelial cell *in vitro* ALI culture[75-77]. Throughout the past 3 decades, various growth factors, hormones and serums have been studied to see if they can aid in driving HBEC differentiation. Cozens et al. and O'Boyle et al. showed that supplementing their culture medium with epidermal growth factor and retinoic acid increased differentiation of bovine bronchial epithelial cells and ovine airway epithelial cells, respectively, at ALI[78,79]. Compounds such as insulin, triidothyronine, transferrin, hydrocortisone and retinol have also been shown to regulate HBEC differentiation [80-82], which is why they are often included in ALI medium formulations. However, these ALI medium formulations do not trigger HBEC differentiation alone, given that, when cultured on traditional tissue culture plastic, these cells are incapable of differentiation. Therefore, medium composition and environmental cues from the culture substrate work synergistically to promote HBEC differentiation.

2.5.3 Culture substrate characteristics

The importance of matrix stiffness has come to light in recent years. The human body ranges in stiffness from a couple of hundreds pascals such as the lungs, all the way to a bone stiffness of 3-4 GPa [83]. Therefore, it is only logical that specific cells will exhibit changes in both morphology and biological

function when cultured on matrices of different stiffness. It is for this reason that stiffness tunable substrates have gained popularity in the past few years [83-85].

Culture substrate porosity has also been shown to influence differentiation rates and lineage in human stem and progenitor cells[86-90]. During ALI filter culture, cells obtain nutrients via diffusion of media through the pores of the filter. Therefore, varying filter pore size will alter the rate at which cells absorb nutrients, affecting the cells' metabolic rate and thus differentiation[91-93]. Cozens et al. showed that bovine bronchial epithelial cells (BBECs) grown on high-pore-density filters differentiated into a columnar, pseudostratified epithelium, whereas BBECs grown on low-pore-density filters remained squamous and non-ciliated[78]. A similar trend has been observed for ovine airway epithelial cells[79], although this has not yet been studied for human bronchial epithelial cells. Given that HBECs are not capable of differentiating when cultured on traditional tissue culture plastic, ALI culture might promote differentiation due to the filter porosity, and not due to the presence of an actual air-liquid interface. In addition, substrate functionalization techniques can also influence differentiation rates of epithelial cells. Neugebauer et al. demonstrated that they can achieve ciliogenesis of primary human nasal epithelial cells during submerged culture on ECM-coated tissue culture plastic[94]. Although, it has not yet been shown if this is possible with HBECs, which are known to react much differently to biochemical stimuli[95].

2.5.4 Other microenvironmental factors that might trigger differentiation

As mentioned earlier, there must be another possible pathway that leads to the differentiation of epithelial cells given that an air-liquid interface is not present during fetal development and that intervillous oxygen levels do not normally exceed 80 mmHg, half of the oxygen tension in ambient air. Matrix composition as well as matrix stiffness have recently been shown to affect differentiation, function and morphology rates of many cells lines [83,85,96,97]. A better understanding of the environmental cues affecting epithelial cell differentiation can enable us to create more effective cell culture platforms. These culture systems will, therefore, react in a more realistic way when performing toxicity screens for pollutants, nanoparticles and other inhalable substances. They can also be used in conjunction with diseased cells to better understand the mechanism of action of certain sicknesses. These improvements can further advance the scientific community's knowledge on the importance of environmental cues affecting human epithelial differentiation.

3. Objectives

Better understanding the environmental cues affecting epithelial cell differentiation can enable us to create more effective cell culture platforms. These culture systems will, therefore, react in a more realistic way when performing toxicity screens for pollutants, nanoparticles and other inhalable substances. They can also be used in conjunction with diseased cells to better understand the mechanism of action of certain sicknesses. These improvements can further advance the scientific community's knowledge on the importance of environmental cues affecting human epithelial differentiation. In this work, we will determine the effects of oxygenation on human bronchial epithelial cell differentiation, which will allow us to re-evaluate the necessity of ALI filter culture in differentiated studies and potentially allow the optimization of high-throughput HBEC culture. The main objectives of this project are to:

1. Use a model bronchial epithelial cell line to evaluate oxygen sensitivity and determine whether submersion causes noticeable oxygenation differences to the cells.
2. Design a finite element simulation which allows us to determine oxygen concentrations at the cellular level during submerged culture and use this model for the design of a hyperoxic/submerged culture system which allows normoxic oxygen conditions at the cellular level.
3. Determine the effects of oxygen tension on human bronchial epithelial cell differentiation by characterizing ciliation area, epithelial thickness, intercellular junction integrity and ciliary function.

4. Materials and Methods

Unless otherwise specified, all reagents obtained from Sigma-Aldrich, and equipment obtained from Fisher Scientific.

4.1 CFBE41o- CFTR-WT and HBEC cell culture

The bronchial epithelial model cell line CFBE41o- CFTR-WT was used in preliminary oxygenation experiments. These cells were cultured in growth medium consisting of 1X MEM medium with glutamine (10-010-CV, Fisher Scientific), supplemented with 10% fetal bovine serum (SH3039602, Hyclone™ Fetal Bovine Serum (Canada)), 1% antibiotic/antimycotic (15240-062, Gibco® by Life Technologies) and 1% L-glutamine (25030081, Gibco by Life Technologies). Cell were grown on 25cm^2 cell culture flasks with vented cap (10062-872, VWR) at 37^{o}C and detached from the flask surface by applying a 0.25% solution of Trypsin/1mM EDTA (25200-072, Thermo Fisher) for 8-10 minutes. Trypsin was removed from samples by centrifugation and aspiration and cells were re-suspended in media before being plated. Cells were seeded (density of 250,000 cells/cm^2) onto 1cm^2 polyester Transwell® filters (0.4 µm pore size, 07-200-156, Corning™) that had been functionalized overnight with a protein solution consisting of 49 mL of 1X LHC basal medium (12677027, Thermofisher), 67µL of 7.5% bovine serum albumin solution (A8412-100ML, Sigma Aldrich), 500 µL Pure Col (#5005-100ML, Advanced BioMatrix) and 500 µL human plasma fibronectin (FC010-5MG, Sigma-Aldrich), such as has been shown to allow for adequate adhesion[98]. These cells were not used past passage 12 to avoid passage-dependent variability that occur in long term cell line culture. Cells were cultured with 0.5 mL of media in the apical compartment and 1.5 mL of media in the basolateral compartment, which was replaced 3 times per week. Once cells had reached confluence of the filter surface, they were brought to their designated culture conditions: standard/ALI, standard/submerged, hyperoxic/ALI and hyperoxic/submerged. Cells in standard conditions were cultured at 21% O_2 and 5% CO_2. Cells in high oxygen conditions were cultured in an O_2- and CO_2-controlled incubator (ProOx C21 and C-274, BioSpherix, Parish NY) at 5% CO2 and varying concentrations of oxygen (40%, 60% and 80% O_2). Cells at ALI were cultured without media in the apical Transwell® compartment and cells in submerged conditions were cultured under 0.5 mL of medium.

Passage 2 human bronchial epithelial cells (HBECs) from four healthy lung donor patients (BD00843, BD00218, BD00972, BD00954) from McGill's Primary Airway Cell Biobank were used for differentiation studies. 0.33 cm^2 Transwell® filters were functionalized with 1:10 solution of Collagen type IV (10X, C7521, Sigma Aldrich), one of the main components of the lung basement membrane, in double-distilled water overnight before seeding. Cells were seeded at densities between 70,000 and 120,000 cells/filter. Once seeded on the Transwell® filters, HBECs were cultured in a 50:50 solution of Dulbecco's Modified Eagle Medium and LHC basal medium supplemented with 0.87 µM insulin, 0.125 µM transferrin, 0.1 µM hydrocortisone, 0.01 µM triiodothyronine, 2.7 µM epinephrine, 0.5 ng/ml epidermal growth factor, 5x10^{-8} M retinoic acid, 0.5 µM phosphorylethanolamine, 0.5 µM ethanolamine, 0.5 mg/ml bovine serum albumin, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 0.25 µg/ml amphotericin B. Filters were kept in standard/submerged conditions for the first 4 days in culture with 500µL of medium in the basolateral Transwell® compartment and 100µL of medium in the apical compartment, or until they reached confluence. Culture medium was replaced once a day during this period with the biological safety cabinet lights off, given that the culture media is photodegradable. Once filters reached confluence, apical media was removed from the designated ALI cultures and replaced for the designated submerged

cultures. Plates were placed in their respective oxygenation conditions: either 21% O_2 and 5% CO_2 or 30% O_2 and 5% CO_2 and their cell culture medium was changed 3 times per week.

A single donor (BD00218) patient's cells did not exhibit any ciliation in any of the three culture conditions, including the standard/ALI condition which involves using a well-established HBEC differentiation protocol. Therefore, results obtained from experiments using these cells were excluded from this work.

4.2 Transepithelial Electrical Resistance Measurements

Transepithelial electrical resistance (TEER) measurements of the cells cultured on Transwell® filters were taken using an EVOM2 epithelial voltohmmeter and an STX2 chopstick electrode (World Precision Instruments, Sarasota FL) every 2-3 days to evaluate the strength of cell-to-cell junctions. Prior to each use, the EVOM2 meter was calibrated using the 1000 Ω test resistor it was supplied with and the chopstick electrode was sterilized with a 70% ethanol/water solution, then preconditioned in the appropriate cell culture medium. Before taking TEER measurements, cell culture medium was changed, and the apical side of the Transwell® filters was filled with 100 µL of medium for 0.33 cm^2 filters and 500 µL for 1.12 cm^2 filters. TEER measurements were taken by positioning the shorter electrode into the center of the Transwell® filter without disturbing the cell layer and the longer electrode to the exterior of the filter insert, between the insert and the walls of the well. The resistance measured using the voltohmmeter represents that of the cell layer as well as the resistances of the filter membrane and the cell culture medium, therefore a reference resistance value had to be taken. The TEER reference system consisted of a Transwell® filter with media on the apical and basolateral sides, but without cells cultured on its surface. This value was subtracted from the TEER measurements obtained from the seeded filters.

4.3 Immunostaining

Whole filters were also stained for hypoxia inducible factor 1-alpha (HIF-1α) evaluate levels of hypoxia within the cells. This stain was coupled with ZO-1 and Hoechst nucleic stains. Cells were fixed using the 4% paraformaldehyde in PBS solution for 15 minutes and washed with PBS three times for 5 mins. A blocking buffer solution consisting of 5% goat serum and 0.3% Triton-X100 diluted in 1X PBS was applied for 60 minutes as per manufacturer protocol. The blocking solution was then replaced with a primary antibody solution consisting of a 1:800 dilution of HIF-1α monoclonal antibody (D1S7W, Cell Signaling Technology, Danvers MA) and a 1:50 dilution of ZO-1 monoclonal antibody (mouse host, Invitrogen 339100) in the prepared blocking buffer solution. Filters were incubated at room temperature overnight and washed three times with 1X PBS for 5 minutes each. A 1:1000 dilution of goat anti-mouse IgG (Alexa Fluor 488, Abcam ab150113) and 1:1000 dilution of goat anti-rabbit IgG (Texas Red, Abcam ab 6719) in blocking buffer was pipetted onto the filters and cells were incubated at room temperature for 1.5 hours. After washing 3 times with 1X PBS for 5 mins each, cell nuclei were stained using a 1 µg/ml Hoechst 33258 (Invitrogen) in PBS solution for 1 hour. Filters were cut off polystyrene supports using a scalpel and tweezers and mounted onto glass slides using Fluoromount (aqueous mounting medium, F4680, Sigma-Aldrich).

4.4 HIF-1α expression quantification analysis

HIF-1α is known to regulate cellular adaptation to low oxygen conditions[99] and when present in the nucleus, denotes that the cell is in a hypoxic state. To characterize the amount of HIF-1α present in the nuclei of primary bronchial epithelial cells, they were immunostained for HIF-1α, then nuclear stained (Hoechst 33258, BioReagent) and fluorescently imaged using an Olympus epifluorescent microscope

(Olympus IX73 Inverted microscope). Both nucleic and HIF-1α images were flat-fielded to correct any uneven illumination during image acquisition. To do this, a "pseudo-flat-field" image was generated in ImageJ for each of these images by using the "Gaussian Blur" function with a kernel size of 100 pixels. The mean gray value of these resulting images was measured and noted. The final flat-fielded image was created using the image "Calculator Plus" function and the variables were set as follows: i1: experimental image, i1: flat-field image, Operation: Divide, k1: mean gray value, k2=0. Once all images were flat-fielded, a mask was created by auto-thresholding the nucleic stained images in ImageJ and setting the background to white and the nuclei to black. The area fraction representing the nuclei was measured using the *measure* function on the software. The *image calculator* function was used to multiply the thresholded nucleic images with the HIF-1α stained images to create a 32-bit image. The resulting image consisted of the HIF-1α stain only in the nuclear regions. The mean gray value of each image was calculated using the *measure* function and normalized based on the nucleic area fraction. These gray values were compared to determine any differences between the three culture conditions.

4.5 Histology

To determine epithelial layers thickness and goblet cell counts, cross-sectional slices of the filters were necessary. After 25 days in their respective culture conditions, HBEC filter cultures were fixed with 4% paraformaldehyde (reagent grade, crystalline, Sigma-Aldrich) in PBS (pH 7.4, Sigma-Aldrich) for 15 minutes, washed 3 times with PBS for 5 mins and sent to McGill's Rosalind & Morris Goodman Cancer Research Centre Histology Core Facility for processing. The top half of the Transwell® filters were clipped off using wire cutters to allow for them to fit into the histology cassettes. Once in the cassettes, the samples were left in 70% ethanol overnight to commence the dehydration process. The next day, the cassettes are inserted into an automatic tissue processor (Tissue-Tek VIP ® 6 AI Vacuum Infiltration Processor, Sakuraus, California) which performs the following reagent exchanges at room temperature: 70% EtOH in RO water for 15 mins, 80% EtOH for 15 mins, 90% EtOH for 15 mins, 100% EtOH for 15 mins, another 100% EtOH for 15 mins, xylene for 25 minutes and a final xylene exchange for 25 minutes. The xylene is then replaced with paraffin wax at 63^{o}C. The paraffin wax is changed 3 times in 15-minute intervals and allowed to rest in the final wax solution for 30 minutes. The filters are embedded into a paraffin block using the Leica EG1150 Modular Tissue Embedding Center (Leica Biosystems, Ontario). The Transwell® filters are removed from the cassette, dissected into two half circle pieces and placed cut-side-down onto the bottom of a metal mold filled with liquid paraffin wax. The lid is removed off the histology cassette and the bottom half of the cassette is then placed on top of the metal mold, and enough paraffin wax is poured onto the cassette until the bottom surface is submerged. The wax is allowed to cool until it has solidified and the tissue sample, as well as the cassette piece can be removed from the mold in one piece. This embedded block is them placed onto the block holder of microtome (Leica RM2255 Fully Automated Rotary Microtome, Leica Biosystems, Ontario) and sectioned into 4 µm thick slices which are placed onto charged microscope slides (Epic Scientific). The slides are placed onto a rack and then into a 36^{o}C oven overnight to dry and eliminate any folds. The slides are then stored at room temperature until stained.

4.6 H&E staining of histology slices

Histology cross-sections of HBECs on Transwell® filters were also H&E stained after sectioning at McGill's Rosalind & Morris Goodman Cancer Research Centre Histology Core Facility to allow for easy visualization of cell morphology with a color brightfield microscope. The morning after the filters have been sectioned and dried, the slides are automatically H&E stained using the Leica ST5020 slide stainer

(Leica Biosystems, Ontario). Samples are first rehydrated using xylene and a similar ethanol gradient as previously described in the "histology" section above, then stained with Harris Hematoxylin and Eosin. Samples are then dehydrated once again using ethanol and xylene to prepare for cover slipping onto pre-cleaned glass slides (Leica Biosystems), which is performed by an automatic cover slipper (Leica CV5030 Fully Automated Glass Coverslipper, Leica Biosystems, Ontario) using acrytol mounting media (Leica Biosystems, Ontario).

4.7 Oxygen Diffusion Gradient Finite Element Simulation

The oxygen gradient present during submerged Transwell® culture was simulated in COMSOL. The medium on the apical side of a 0.33 cm^2 (24-well) Transwell® filter was represented by a cylinder of liquid with aqueous properties with a diameter of 6.5 mm and a height of 3 mm. The system was set to a temperature of 37^oC. The diffusivity of oxygen in water at 37^oC was calculated to be 2.616 x 10^{-5} cm^2/s [100]. The boundary conditions were set for each facet of the geometry. For the walls of the Transwell® it was assumed that there was no oxygen transport through the polystyrene plastic of the filter. The bottom of the cylinder represented the interface between the cell culture medium and the HBECs, therefore we assumed a uniform oxygen sink across this surface. The oxygen consumption rate of the NIH-H460 human epithelial lung cancer cell line (30 amol/cell-s) was used as an estimate for the oxygen consumption of HBECs. And it was assumed that the filter surface was populated with 250,000 cells/cm^2. The top surface of the cylinder represented the interface between the incubator air and the cell culture medium. It was assumed that the top surface was oxygen-saturated and therefore contained 6.727 mg/L of dissolved oxygen. It was also assumed that initially, the cell culture medium was equilibrated to the ambient air and was thus oxygen saturated at t=0s. This simulation was run at varying oxygen concentrations to determine at which ambient oxygen percentage the cell layer would be exposed to ambient saturated oxygen conditions.

4.8 Ciliation Analysis and validation

HBEC differentiation was determined by quantifying the percent surface area that is ciliated. After 18 days in standard/ALI, standard/submerged and hyperoxic/submerged conditions, live phase contrast videos (2s, 20 ms exposure time, Olympus IX73 Inverted microscope) were taken of the cell surface and exported as stacks. A z-projection with standard deviation was performed on the stacks in ImageJ to visualize the areas of cilia movement. The brightness areas produced from the z-projection denoted areas with the highest cilia movement. The projection was then thresholded to outline ciliated area and the area fraction was quantified using the *measure area fraction* function.

Our aforementioned ciliation measurement protocol was validated against H&E-stained cross-sectional histology slices of BD00843 HBECs cultured for 25 days on Transwell® filters in standard/ALI, standard/submerged and hyperoxic/submerged conditions. Histology cross-sections were imaged using Brightfield microscopy (Olympus IX73 Inverted microscope). The ciliated surface area percentage was determined by manually measuring the length of the filter surface (blue line, **Figure 16**D) and that of the ciliated surface (green lines, **Figure 16**D) and calculating the ratio between the two.

4.9 Cilia beating measurements

HBEC ciliation beating was analyzed by scattering fluorescent beads on the ciliated epithelial surface. After 19 days in standard/ALI, standard/submerged and hyperoxic/submerged culture conditions, the media in the apical chamber of the Transwells® was aspirated and replaced with a 1:1000 dilution of fluorescently labelled polystyrene microbeads (FluoSpheres™ Carboxylate-Modified Microspheres, 0.2

μm. Red fluorescent (580/605), 2% solids) in PBS. Beads were left to settle onto the surface for 5 minutes before the solution was aspirated to remove excess beads. Live epifluorescent videos (2s, 20 ms exposure time, Olympus IX73 Inverted microscope) were taken of the cell surface covered in fluorescent beads and exported as stacks. Files were uploaded into ImageJ and the *2D/3D particle tracking* function, included in the *MosaicSuite* plugin[101], was used to track the coordinates of each bead in every frame (particle detection radius=16 pixels, cutoff=0.001, per/abs=0.500, link range=2, displacement=30 pixels, Dynamics: Brownian). These set of coordinates were used to calculate the average bead movement speed and the cumulative bead displacement throughout the live stream.

5. Results

5.1 CFBE41o- CFTR-WT filter culture in four conditions

A model bronchial epithelial cell line (CFBE41o- CFTR-WT) with corrected wildtype CFTR gene was used for our initial experiments to determine how epithelial barrier integrity was affected by four different culture conditions. CFBE41o- CFTR-WT cells were cultured in standard/ALI, standard/submerged, hyperoxic/ALI and hyperoxic/submerged conditions and transepithelial electrical resistance (TEER) measurements were taken every 2 or 3 days. Incubator oxygen concentrations of 40%, 60% and 80% were used as hyperoxic conditions to determine at which oxygen concentration cells lose their barrier integrity due to hyperoxia and whether the addition of a media layer above the cells can combat hyperoxia-induced death in these high-oxygen conditions. Interestingly, cells cultured in standard/submerged conditions exhibited at least a 3-fold increase in TEER values when compared to their ALI counterpart, likely due to the increased nutrient availability in submerged culture, which might allow cells to grow on top of each other. In the hyperoxic/ALI conditions, epithelial barrier disruption was observed at 60% O_2 and 80% O_2 when the TEER values drop off towards zero after the second day in culture. However, the addition of a media layer in the hyperoxic/submerged condition prevent cells cultured in a 60% O_2 incubator from hyperoxia-induced death, confirming that a 3 mm liquid diffusion gradient is adequate to alter oxygen conditions at the cellular level and thus influence cell function. Given that these cells are sensitive to these minute oxygenation differences, it is likely that human bronchial epithelial cells cultured in ALI/submerged conditions will be comparatively sensitive. To properly design the oxygenation conditions for these cultures, a finite element simulation of the oxygen diffusion gradient through media was required.

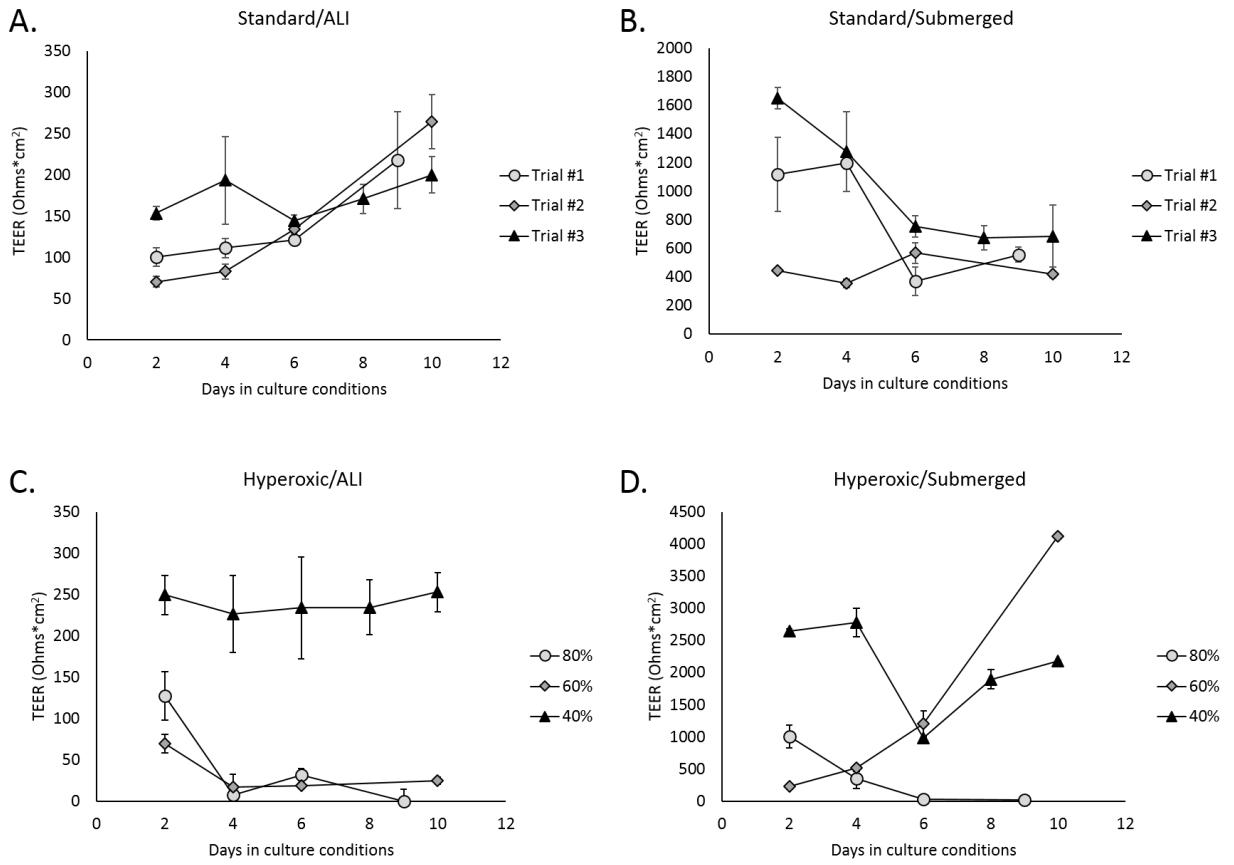

Figure 7. *TEER measurements of CFTR-WT cells on 1.12cm² Transwell® filters (n=3 wells, mean ± SD) in* ***A.*** *standard/ALI,* ***B.*** *standard/submerged,* ***C.*** *hyperoxic/ALI and* ***D.*** *hyperoxic/submerged conditions. For standard culture conditions, the three different curves represent three different experiments seeded at the same time as the associated hyperoxic experiments. Cells cultured at 60% hyperoxia quickly lost epithelial barrier integrity when cultured at an ALI, whereas in submerged culture, their barrier integrity increased with culture time. This suggests that the apical media layer in submerged culture shields cells from hyperoxia-induced epithelial barrier degradation.*

5.2 Finite element simulation of O_2 diffusion in submerged culture

When cells are cultured in standard/ALI conditions, their surface remains moist due to the osmotic transfer of water into the cells and the excretion of mucins onto the cell surface. These cells are therefore directly in contact with a fluid layer that is in equilibrium with 21% O_2 and 5% CO_2 incubator conditions. Henry's law was used to calculate the equilibrium concentration of dissolved oxygen (DO) in this liquid and determined that cells would be exposed to a DO concentration of 6.727 mg/L, or 0.210 mol/m^3. A finite element simulation of oxygen diffusion through culture medium was designed to estimate the incubator oxygen concentration necessary to achieve these normoxic conditions at the cellular level during submerged culture. The simulation was modeled after the 3mm-thick layer of medium present on the apical side of a 24-well Transwell® filter insert and included an oxygen sink at the bottom surface of the liquid representing cellular oxygen consumption. The oxygen concentration in the culture medium was set to be at equilibrium with 21% O_2 in ambient air at t=0s and the top surface of the liquid layer was always assumed to be saturated with respect to the incubator oxygen levels. This simulation was performed at different ambient oxygen concentrations and the relationship between the incubator oxygen concentration and the oxygen concentration at the cellular level was shown to be linear (**Figure 8**A).

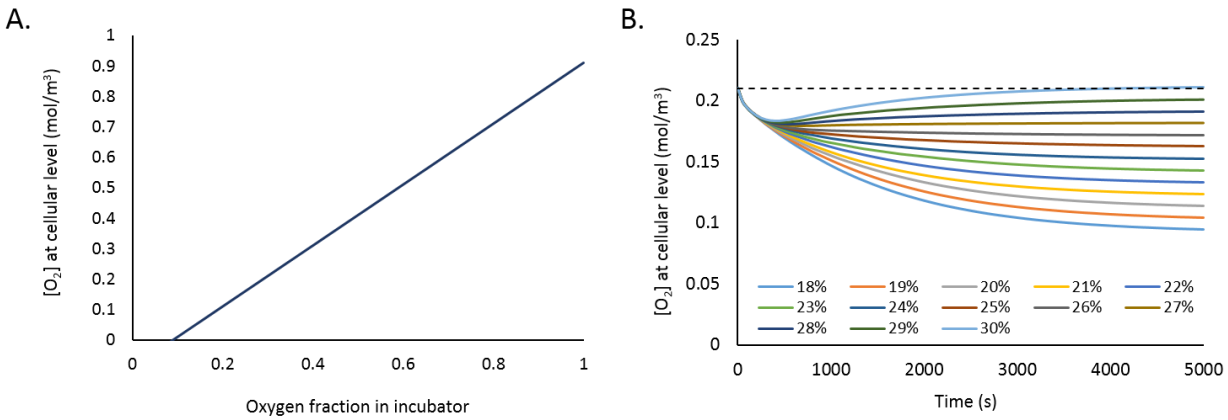

Figure 8. *Finite element model graphs.* ***A.*** *Dissolved oxygen concentration at cellular level (mol/m^3) as a function of the oxygen fraction in the incubator.* ***B.*** *Dissolved oxygen at the cellular layer (mol/m^3) as a function of time for different incubator oxygen concentrations. The black dotted line represents the normoxic condition of 0.21 mol O_2/m^3.*

When cells are cultured under 3 mm of cell culture medium, they are exposed to a DO concentration of 0.12 mol/m^3 which is slightly over half of the aforementioned concentration of oxygen that they would normally have access to in standard/ALI conditions (**Figure 8**A). To compensate for this oxygen limitation, this simulation was repeated using increased incubator O_2 concentrations and it was determined that an incubator oxygen concentration of 30% would allow for a normoxic DO concentration of 0.21 mol/m^3 at the cellular level (**Figure 8**B). This concentration would therefore be used in the subsequent hyperoxic culture experiments (**Figure 9**). The results of this simulation were compared to a simple 1D diffusion problem using Fick's first law of diffusion (**Equation 1**). We found that edge effects in the cylindrical system were negligible when it came to diffusion of oxygen through the medium because concentrations at the cellular level were calculated to be 0.21 mol/m^3 at 30% incubator oxygen concentration, similar to what was observed in the finite element model.

$$J = -D\frac{\partial C}{\partial z} \qquad (1)$$

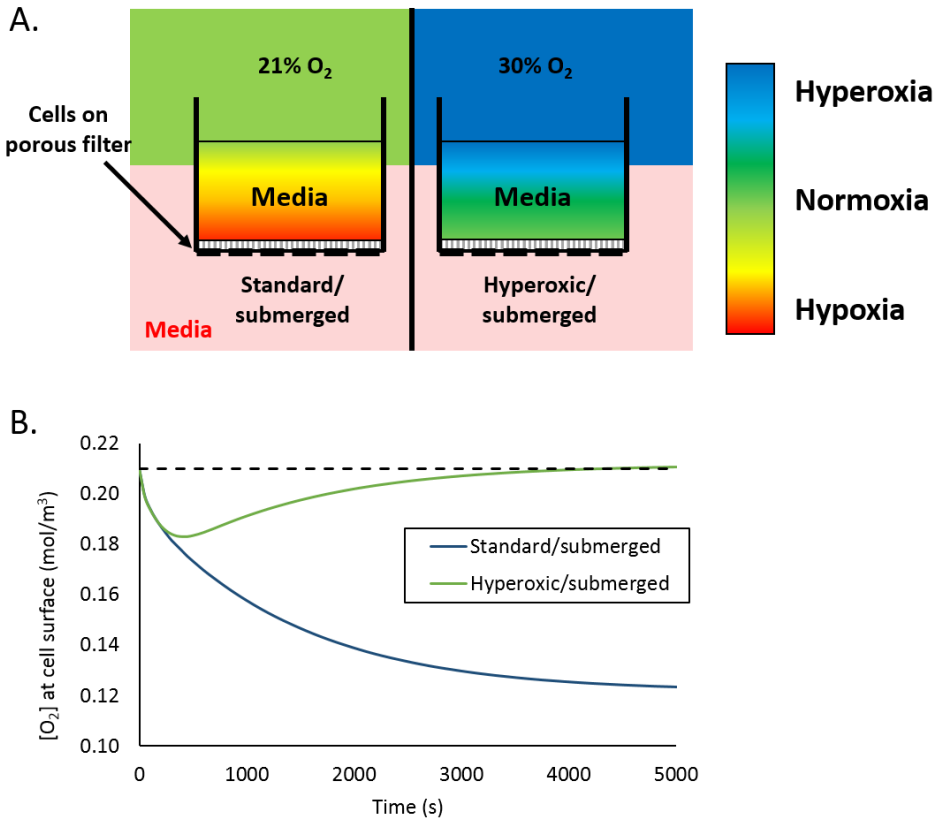

Figure 9. *Comparison of standard/submerged hyperoxic/submerged filter cultures.* ***A.*** *Schematic of oxygen diffusion gradients in standard/submerged and hyperoxic/submerged cultures.* ***B.*** *Finite element model dissolved oxygen concentration at the cellular surface as a function of time for both culture conditions.*

The oxygen diffusion profile of the culture medium during standard (21% O_2) and hyperoxic (30% O_2) submerged cultures was simulated and we found that the systems reach equilibrium after t=5000s (

Figure 10). A parametric sweep varying liquid layer height, oxygen consumption rate of cells and incubator oxygen levels was performed to identify different combinations of these three variables that result in a normoxic DO concentration of 0.21 mol/m^3 at the cellular level (**Figure 11**).

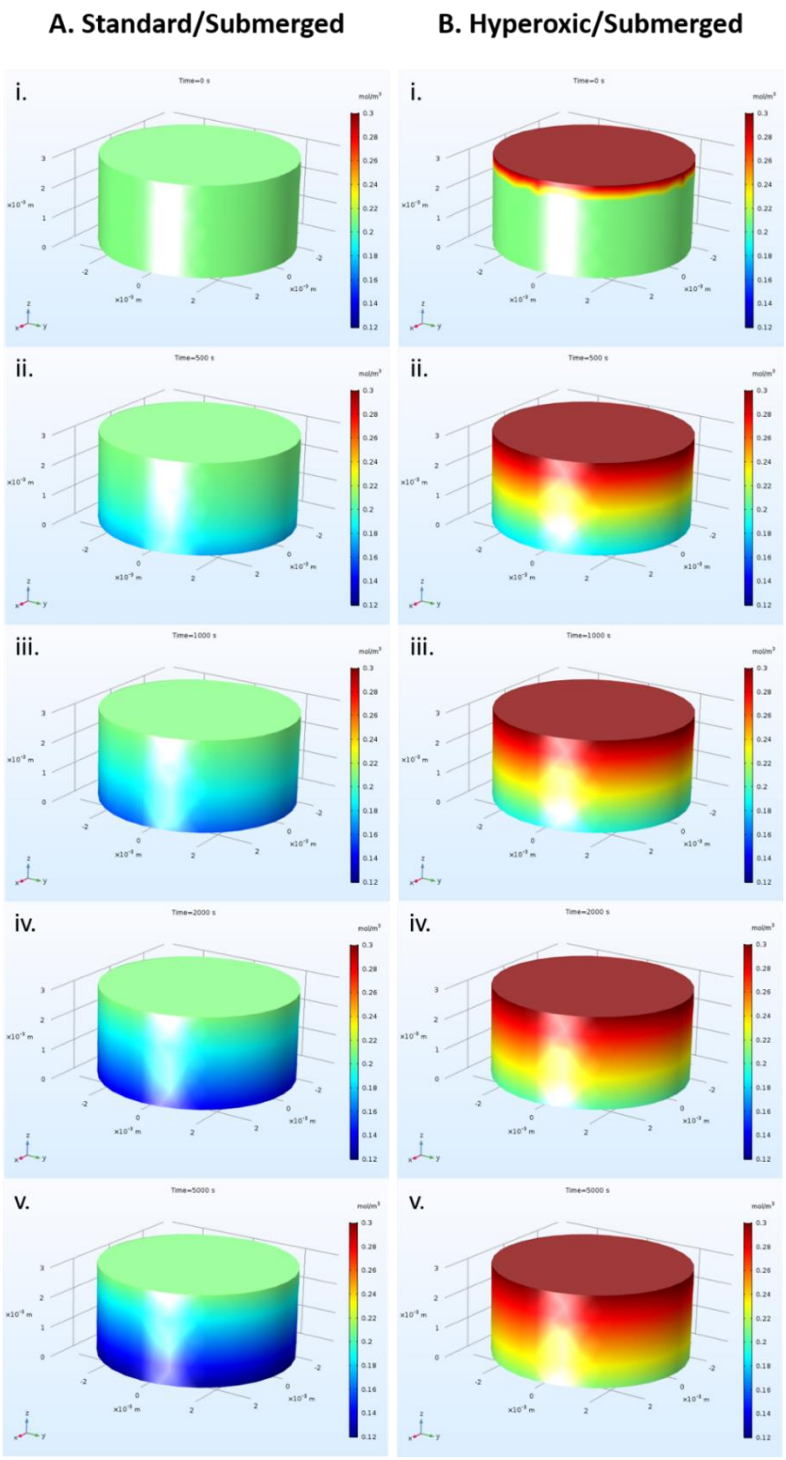

Figure 10. *Surface area plot of oxygen diffusion through apical liquid media layer during* ***A.*** *Standard/submerged and* ***B.*** *Hyperoxic/submerged cultures at (i) t=0s, (ii) t=500s, (iii) t=1000s, (iv) t=2000s and v) t=5000s. Media layer is initially in equilibrium with 21% ambient oxygen conditions and an oxygen sink is set at the bottom of the cylinder to represent the oxygen consumption rate of the cells. Green region represents normoxic conditions (0.21 mol O_2/m^3), red region represents hyperoxic conditions and blue represents hypoxic conditions. At equilibrium (t>5000s), cells in standard/submerged conditions are exposed to 0.12 mol O_2/m^3 and cells in hyperoxic/submerged are exposed to a normoxic concentration of 0.21 mol O_2/m^3.*

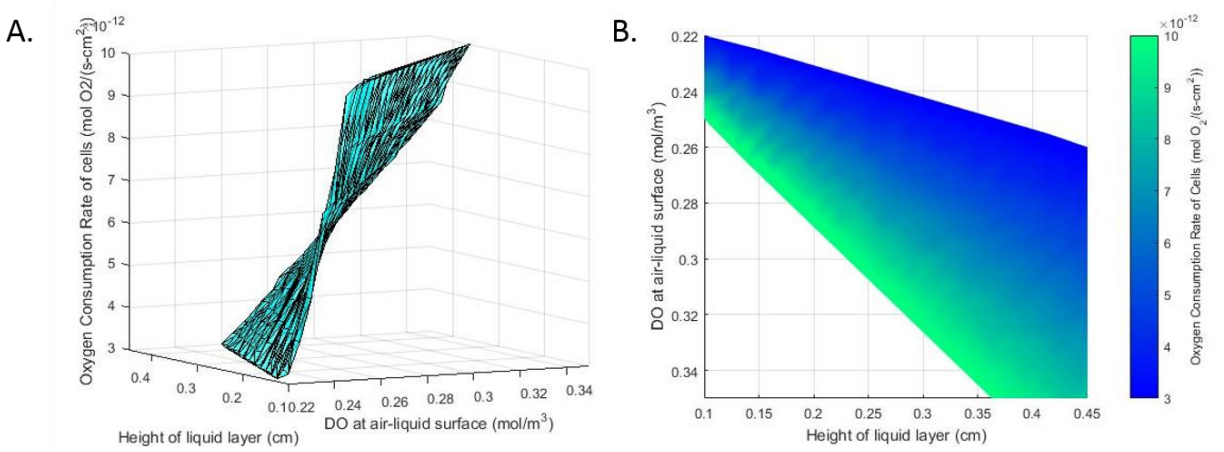

Figure 11. *Multiparametric analysis of oxygen kinetics in submerged culture to identify optimal parameters for normoxic culture conditions.* ***A.*** *3D Surface plot demonstrating the various combination of cellular OCR, apical media height and DO at air-liquid surface that allow for normoxic oxygen concentrations at the cell layer.* ***B.*** *2D representation of the same multiparametric analysis which allows for selection of optimal culture conditions, allowing for normoxia at the cellular level. For example, if we assume that the cellular OCR is 7.4 x 10^{-12} mol O_2/(s-cm^2) and that the height of the media layer above the cells is 3 mm, then we would have to set the incubator oxygen concentration to a value which would allow for the upper media surface to be equal to 0.3 mol O_2/m^3. Using Henry's law, we can determine that an incubator oxygen concentration of 30% O_2 would allow for a dissolved oxygen concentration of 0.3 mol O_2/m^3 in the cell media.*

5.3 HBEC tight junction and cell spread area

Human bronchial epithelial cells from three donors (BD00843, BD00954 and BD00972) were cultured on 0.33 cm^2 Transwell® culture filters in standard/ALI, standard/submerged and hyperoxic/submerged conditions. Hypoxic incubator oxygen concentration was set to 30%, given the results of our finite element simulation. The hyperoxic/ALI condition was not evaluated throughout the primary culture experiments because the cells would be in direct contact with the hyperoxic incubator oxygen concentrations and thus exhibit hyperoxic cell function, which was not within the scope of this project. The culture's epithelial barrier integrity was evaluated by monitoring TEER values throughout the 25-day culture, and by fixing and fluorescently labelling nuclei and ZO-1, a tight junction protein. TEER measurements were similar within all three culture conditions (**Figure 12**A) suggesting that increased nutrient accessibility due to submersion does not drive HBEC cells to proliferate more rapidly and stack on top of each other, in contrast with the results from the CFBE41o- CFTR-WT cell line culture. Moreover, TEER measurements stabilized after 7 days at around 500 Ohms-cm^2 (**Figure 12**A) and cells stained brightly for the tight junction protein ZO-1 in all three culture conditions (**Figure 12**C), which indicates the formation of a healthy, stable epithelial layer. Cell spread area was also evaluated and no statistical differences between the three conditions were observed (**Figure 12**B), supporting that all three conditions allow formation of an equally dense epithelium. These results show that cells are spreading similarly in all three systems and that epithelial barrier integrity is not compromised in any of the three culture conditions. Hence, we can be certain that barrier integrity, cell confluency and/or cell density are not the cause of any subsequently observed differences in HBEC differentiation in standard/hyperoxic and ALI/submerged culture environments.

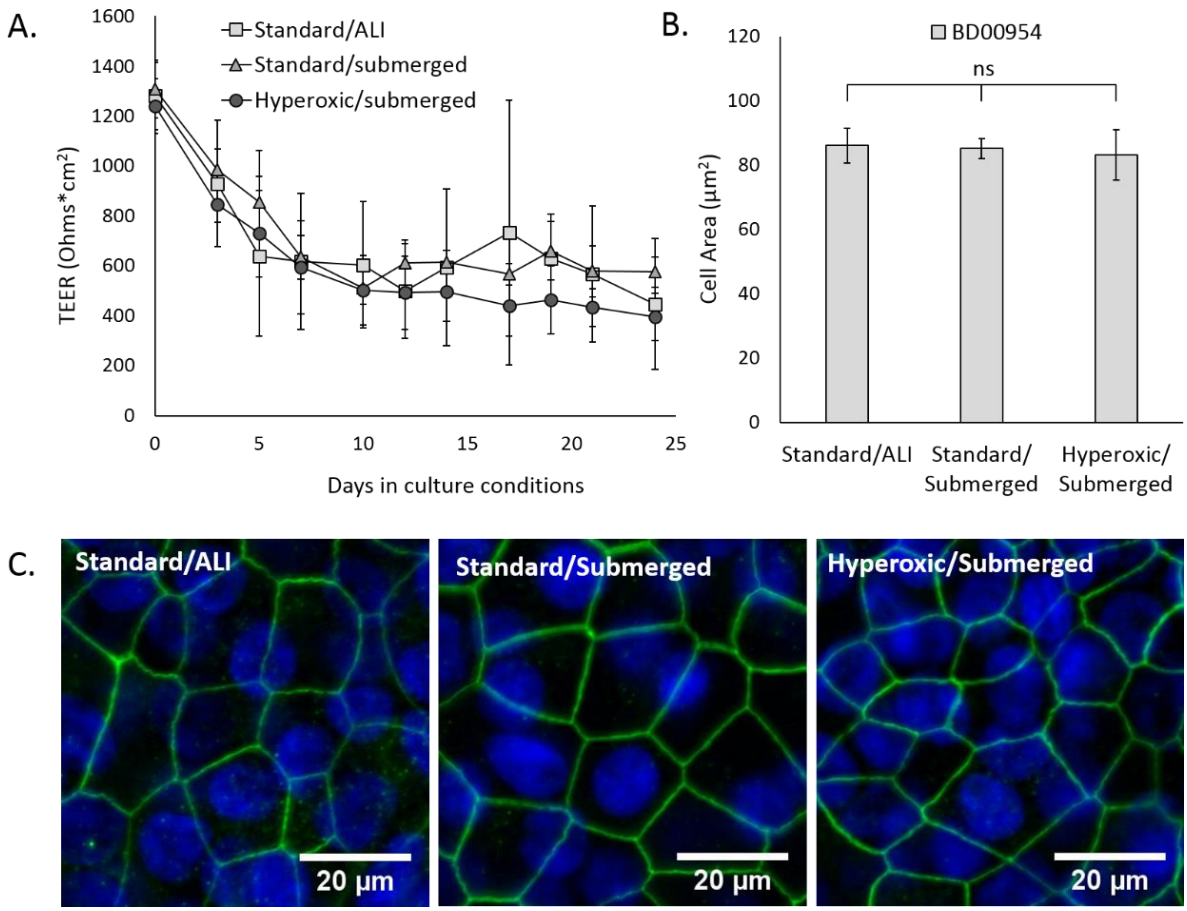

Figure 12. *HBEC intercellular junctions and cell area in standard/ALI, standard/submerged and hyperoxic/submerged culture conditions.* ***A.*** *TEER measurements of HBECs throughout the entire 25-day culture (n=3 donors, mean ± SD). Epithelial barrier integrity seemed to follow a similar trend when HBECs were cultured in all three conditions.* ***B.*** *Average cell area of BD00954 donor cells after 25 days in culture (n=3, mean ± SD, two-tailed t-tests, *p<0.05). There was no significant difference between cell area all three conditions indicating that cells formed equally dense epithelial layers.* ***C.*** *ZO-1 tight junction stain (green) and nucleic stain (Hoechst 33258, blue) of BD00843 cells after 25 days in culture.*

5.4 HIF-1α expression in HBECs cultured in different conditions

Once it was established that cell health and intercellular junction integrity was not compromised during submerged or hyperoxic culture, I sought out to discover whether the cells were sensitive to the oxygen differences they were subjected to. The hypoxia-inducible transcription factor 1a has been shown to be expressed in hypoxic cells' nuclei as the cells adapt to the stressful hypoxic environment[102]. HBECs from donor BD00954 were isolated and cultured on Transwell® filters in standard/ALI, standard/submerged and hyperoxic/submerged conditions for 25 days, and their nucleic HIF-1α levels were evaluated via immunofluorescence. Filters were nucleic acid stained (Hoechst) (**Figure 13**A) as well as HIF-1α immunostained (**Figure 13**B). A mask was then created with the thresholded nucleic stain (**Figure 13**C), applied onto the HIF-1α -stained image (**Figure 13**D) and the mean gray value of the resulting images were measured. Cells cultured in the standard/submerged condition expressed statistically higher level of nucleic HIF-1α than the other two conditions, proving that the oxygen diffusion gradient present in this culture system induces hypoxia in HBECs (**Figure 13**E). Cells cultured in the standard/ALI and hyperoxic/submerged conditions expressed statistically similar levels of HIF-1α, indicating that the increased oxygen levels in the hyperoxic/submerged culture were adequate to compensate for the oxygen diffusion gradient caused by submersion (**Figure 13**E). These results confirm that the thin layer of media does cause a sufficient oxygen depletion zone to influence cell function, and that hyperoxygenation can be used to rescue this phenotype.

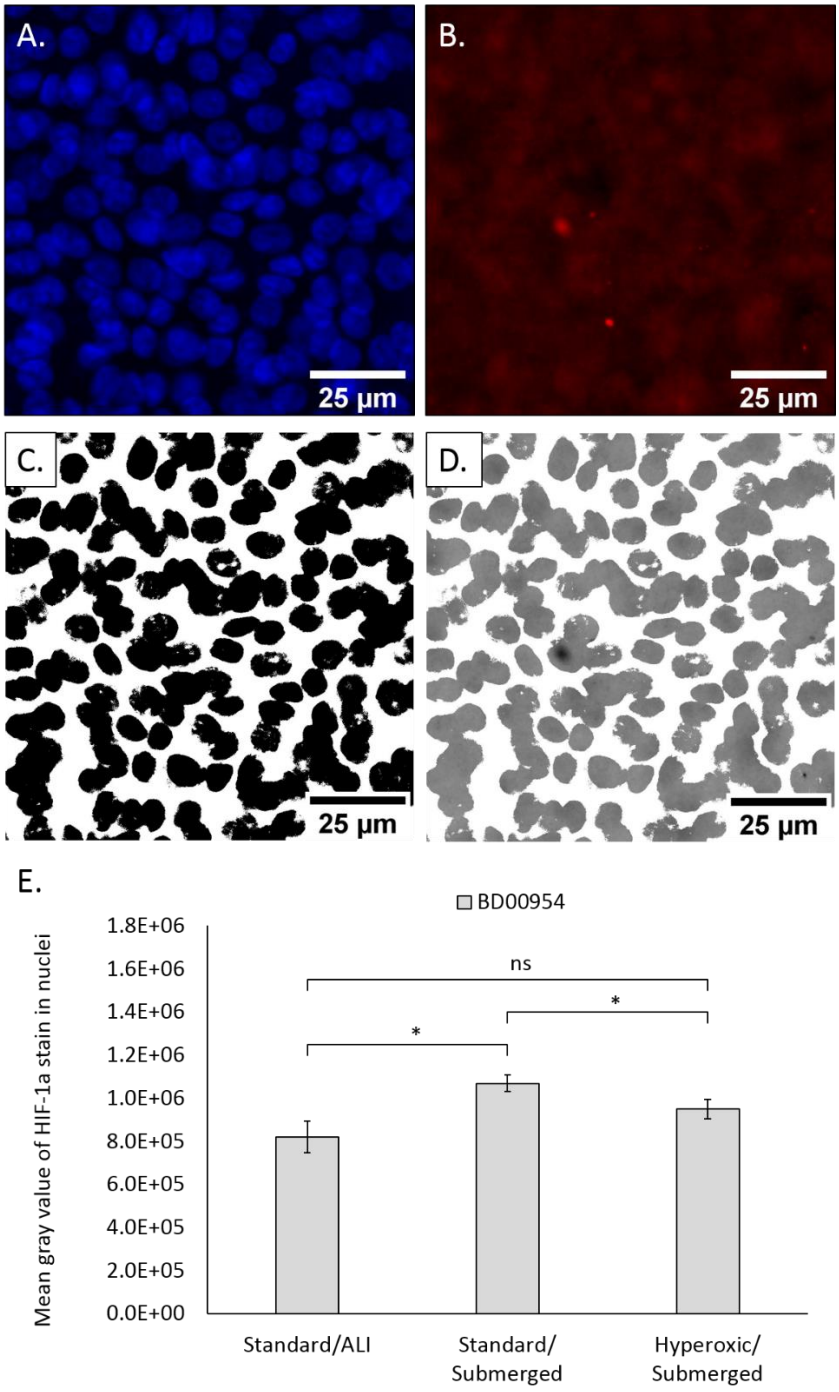

Figure 13. *Hypoxia analysis of cells cultured in three conditions using fluorescently labelled HIF-1α.* ***A.*** *Nucleic stain (Hoechst, blue).* ***B.*** *HIF-1α stain (red).* ***C.*** *A thresholded mask was created from the nucleic images to overlay onto the HIF-1α stain.* ***D.*** *Image multiplication of nucleic mask and HIF-1α stain. The darker areas represent nuclei with higher HIF-1α expression.* ***E.*** *Mean gray value of HIF-1α stain within the nuclei (n=3, mean ± SD, one-tailed t-tests, *p<0.05). Cells cultured in standard/submerged conditions express higher levels of the hypoxia marker HIF-1α, such as expected. Hyperoxic/submerged and standard/ALI conditions resulted in statistically similar amount of nucleic HIF-1α, suggesting that increased incubator oxygen concentrations compensate for diffusion limitations in submerged culture.*

5.5 HBEC layer thickness

A healthy pseudostratified human bronchial epithelium is characterized by a single cell layer consisting of columnar cells connected to the basement membrane. During *in vitro* HBEC culture, it is desirable to obtain an epithelial monolayer close to 25 µm, similar to what can be observed in *ex vivo* bronchial histological slices[103-105]. HBECs from three donors were cultured for 25 days in our three experimental culture conditions, fixed, embedded in paraffin and sliced. Thickness measurements were taken manually from images of the histology cross-sections, and it was shown that for all three donors (BD00954, BD00843, BD00972), cells cultured in hyperoxic/submerged conditions had significantly higher thicknesses (6.19 ± 0.59 µm, 9.38 ± 1.67 µm and 12.90 ± 1.80 µm, respectively) than cells in standard/ALI conditions (5.18 ± 1.12 µm, 6.35 ± 1.63 µm and 8.19 ± 1.29 µm, respectively) (**Figure 14**A,B). Moreover, for two of the donors (BD00954 and BD00843) cells cultured in hyperoxic/submerged conditions had statistically higher thicknesses than cells in standard/submerged (4.77 ± 0.88 µm and 5.16 ± 0.76 µm, respectively) as well (**Figure 14**A). This suggests that the combination of increased nutrient availability present in submerged culture and increased oxygen availability in hyperoxic culture might be driving HBEC differentiation into a columnar form more rapidly than conventional standard/ALI culture techniques.

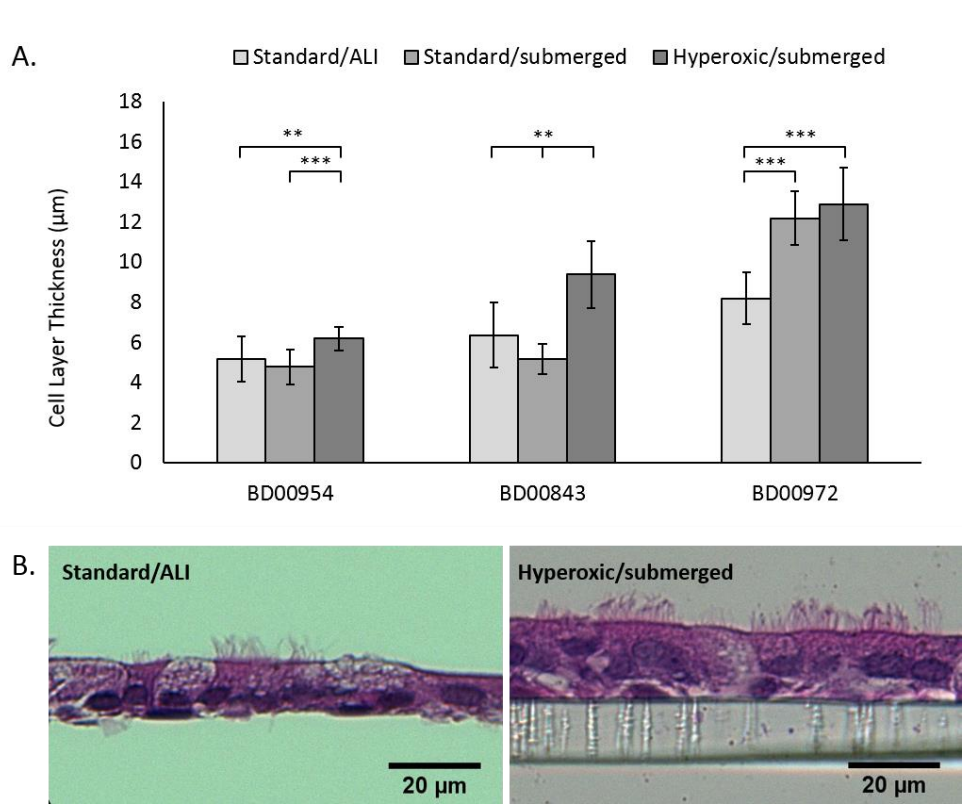

Figure 14. *HBEC thickness analysis.* ***A.*** *Epithelium layer thickness from histology cross-sectional slices for three donor patient cells after 25 days in culture (n=3, mean ± SD, **p<0.01, ***p<0.001). Hyperoxic/submerged conditions universally produced thicker epithelial cell layers, whereas the other two conditions produced variable results, seeming to be donor dependent.* ***B.*** *H&E stained cross-sectional histology slices of BD00843 patient cells cultured in standard/ALI and hyperoxic/submerged conditions for 25 days. In the standard/ALI image, the filter had detached from the epithelial layer during histological processing, whereas the filter remained attached in the hyperoxic/submerged condition. Histology showed higher rates of ciliation, as well as thicker epithelial layers of cells in hyperoxic/submerged conditions.*

5.6 Analysis of HBEC ciliation rates and validation

Ciliation is a primary marker of human bronchial epithelial cell differentiation and is characterized by the maturation of functional, beating cilia on the apical epithelial surface. I evaluated ciliation of HBECs cultured on Transwell® polyester filters in standard/ALI, standard/submerged and hyperoxic/submerged conditions by obtaining 2s phase contrast live streams with a frame rate of 50fps, quick enough to capture the rapid ciliary movement. These files were imported into ImageJ and their standard deviation was computed and auto-thresholded to obtain an area fraction of the surface that produces the most movement, and thus, representing the ciliated surface area (**Figure 15**).

Ciliation increases with culture age, and I confirmed this for BD00843 HBECs in standard/ALI and hyperoxic/submerged conditions by evaluating cell ciliation after 18 (11.87 ± 2.67 % and 24.60 ± 6.59 %, respectively) and 25 days (21.69 ± 1.36 %, and 41.11 ± 3.87 %, respectively) in culture (**Figure 15**A). Although, there was no significant difference between percent ciliated surface at t=18 days versus t=25 days for the standard/submerged condition (18.27 ± 4.27 % vs 26.20 ± 4.85 %) (**Figure 15**A), which suggests that perhaps cells cultured in this condition reach a ciliation plateau earlier than the other two conditions, possibly due to oxygen limitations. More importantly, at both time points, cells exhibited a similar significant difference between ciliation of standard/ALI culture and hyperoxic/submerged culture, indicating that a shorter culture time can produce similar statistical differences between conditions and thus it is not necessary to maintain the culture for an extra 7 days. A similar ciliation analysis was performed on two other donors (BD00972 and BD00954) and it was shown that, when averaging the three donors, the hyperoxic/submerged condition produces epithelial monolayers with significantly increased surface ciliation (22.17 ± 2.11 %) when compared to standard/ALI (9.22 ± 3.31 %) and standard/submerged conditions (12.58 ± 5.22 %).

Given that this technique of measuring ciliation has not been established before, I sought to validate our results using cross-sectional histological slices (**Figure 16**C). The ciliated surface area percentage was determined by measuring the length of the filter surface (blue line, **Figure 16**D) and that of the ciliated surface (green lines, **Figure 16**D) and calculating the ratio between the two. When comparing the ciliation results from the live stream analysis (**Figure 16**E) and the histology analysis (**Figure 16**F), it is clear that they produce similar results and identical statistical differences between standard/ALI and hyperoxic/ALI conditions, validating our ciliation analysis protocol.

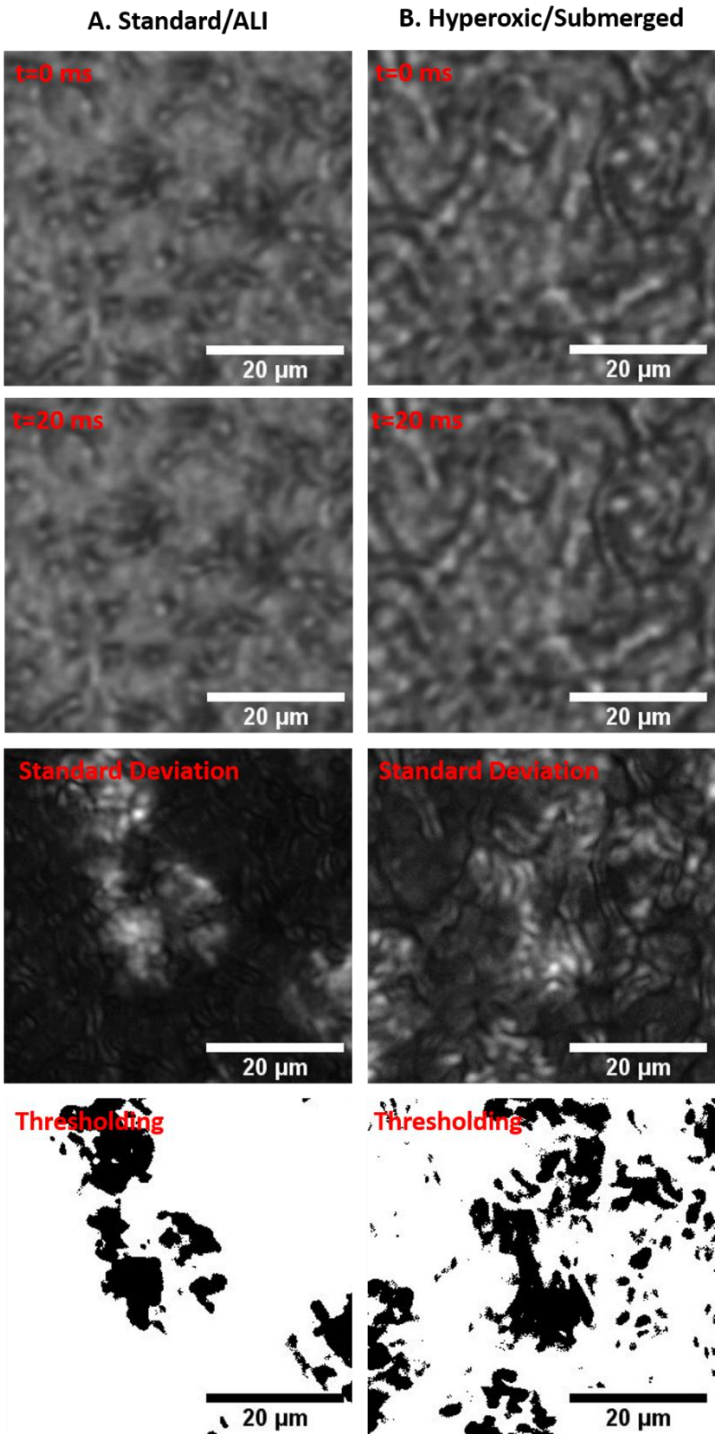

Figure 15. *Ciliation analysis technique for* ***A.*** *Standard/ALI and* ***B.*** *Hyperoxic/submerged cultures. A phase contrast time lapse of the ciliated surfaces was taken for 2 seconds with an exposure time of 20ms. The two first images of this time lapse are presented above, at t=0 s and t=20 ms. There are subtle differences between these two images that can only be identified using image analysis software. The standard deviation between these 2 second time lapses were calculated and resulted in the third images. The brighter sections in this image represent the epithelial surface area with the most ciliary movement. These were then auto-thresholded to obtain an area fraction of ciliation (in black).*

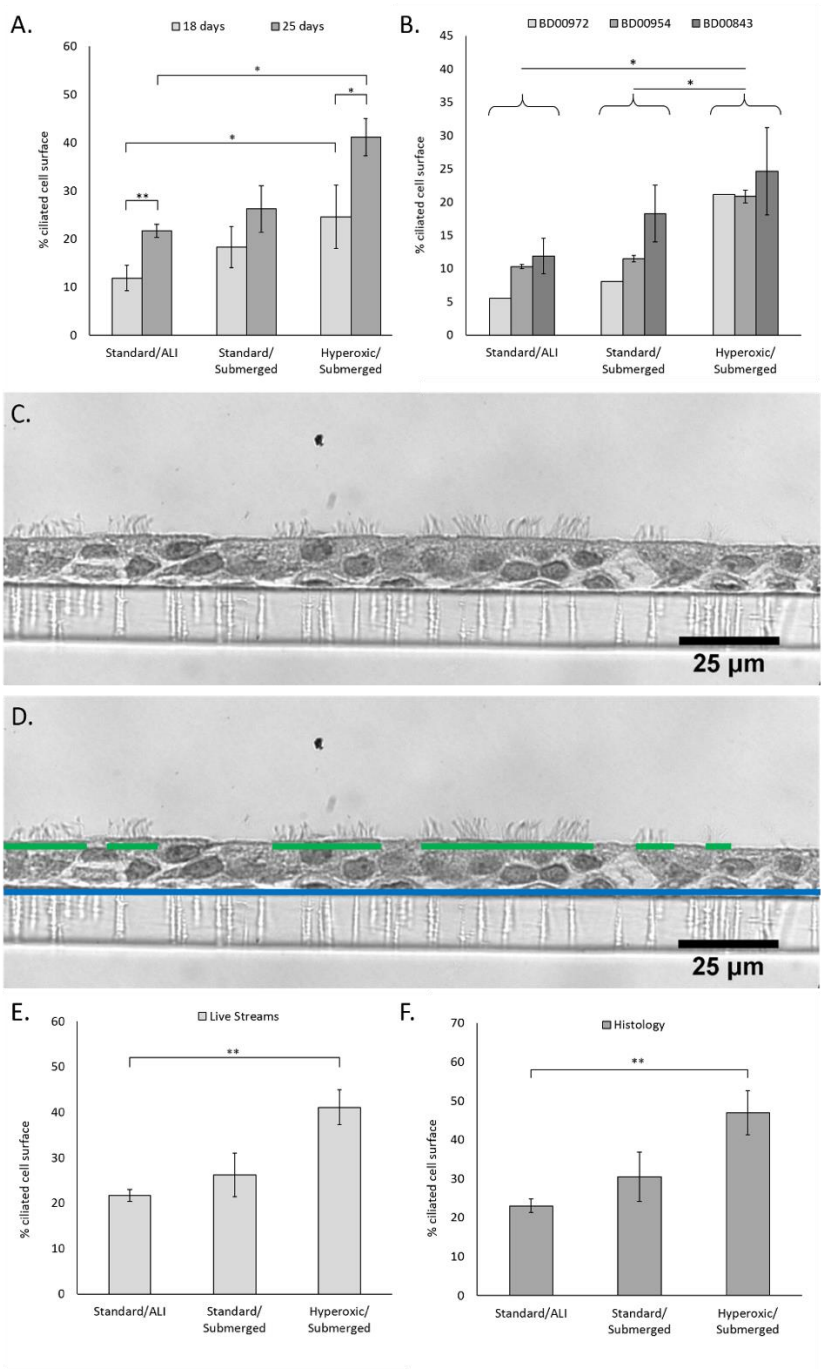

Figure 16. *Ciliation analysis results and validation.* ***A.*** *Percent ciliated surface of BD00843 donor cells cultured in all three conditions for 18 and 25 days (n=3, mean ± SD, two-tailed t-test, *p<0.05), measured using live stream image capture and processing.* ***B.*** *Percent ciliated surface of BD00972, BD00954, BD00843 donor HBECs after 18 days in three culture conditions (n=3, mean ± SD, two-tailed t-test, *p<0.05), measured using live stream image capture and.* ***C.*** *Histology cross-section of BD00843 patient cells cultured in hyperoxic/submerged conditions for 25 days.* ***D.*** *Example of ciliation measurement technique used with histology samples. The blue line indicated the measurement taken for the surface area length and the green lines represent the ciliated surface area length measured.* ***E.*** *Percent ciliated surface of BD00843 donor cells cultured in three conditions and measured using live stream image capture and processing (n=3, mean ± SD, two-tailed t-test, **p<0.01).* ***F.*** *Percent ciliated surface of BD00843 donor cells cultured in three conditions and measured from cross-sectional histology slices (n=3, mean ± SD, two-tailed t-test, **p<0.01). Both ciliation measurement techniques produced similar results and identical statistical differences between culture conditions.*

5.7 Functional analysis of cilia particle transport

Cilia are largely responsible for mucociliary clearance in the lung. This mechanism occurs when debris is inhaled, settles onto the lung's mucus membrane, and is then moved up the respiratory tract by cilia to be expelled through the mouth or swallowed[106]. It is therefore important, when culturing HBECs, that the cilia observed are functional and are capable of moving small particles. To confirm that the movement we observed during our ciliation analysis was indeed ciliary beating, I scattered small 3.2 um fluorescently-labelled beads onto the surface of the cells and recorded high frame-rate time lapses to quantify their displacement (**Figure 17**). The average ciliary beating speed of cells cultured in the hyperoxic/submerged condition (18.14 ± 3.71 μm/s) was statistically higher than that of the cells cultured in the standard/ALI (9.35 ± 2.84 μm/s) condition (**Figure 18**A). This can be explained by the dampening effect caused by the increased mucin accumulation in ALI culture compared to submerged culture, where the mucins are consistently dissolving into the apical medium which is being changed every two days.

The cumulative displacement of each individual bead was also plotted (**Figure 18**BCD) and it can be observed that there are more beads with higher total displacement in the hyperoxic/submerged condition than in standard/submerged condition. This can be explained by the fact that cells in standard/submerged conditions ciliate less. This phenomenon can also be explained by the possibility that the hypoxic conditions in standard/submerged culture are altering the cells' metabolic state and, therefore, affecting certain cellular functions, such as cilia beating frequency.

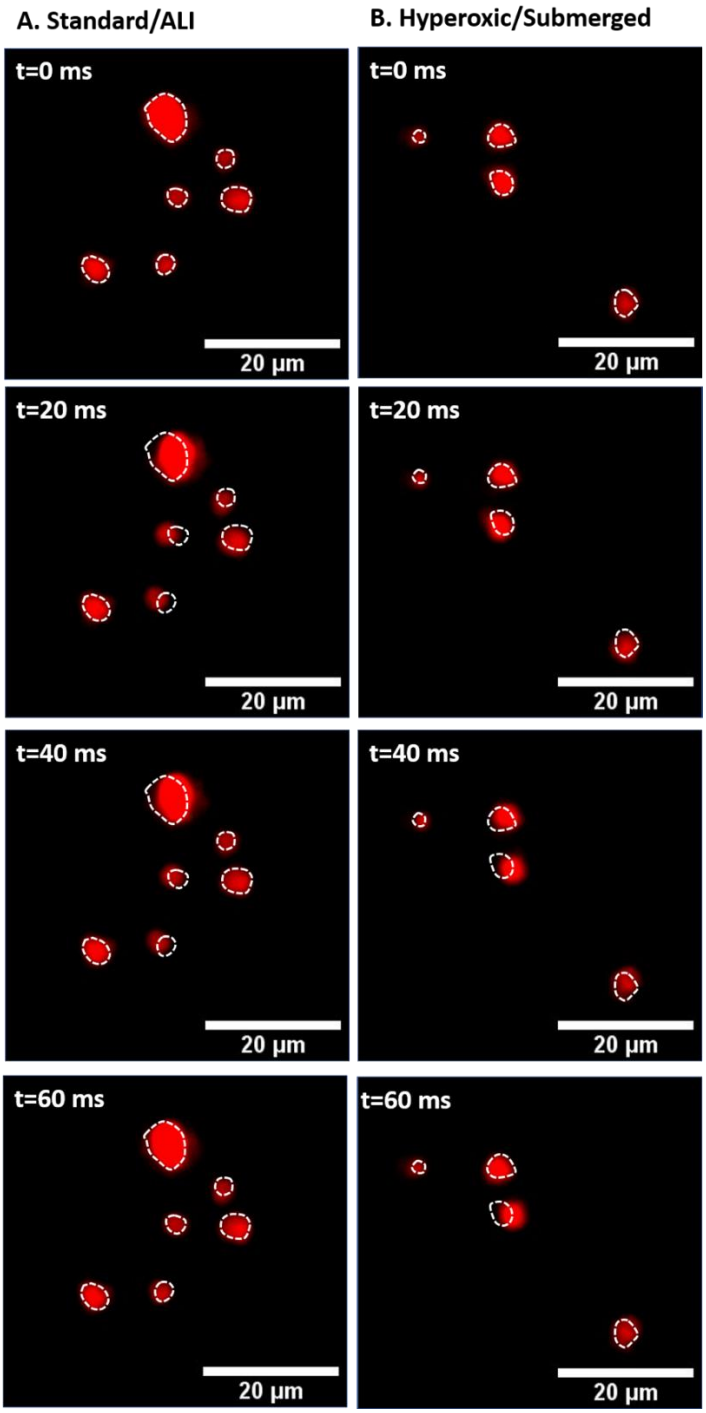

Figure 17. *Time lapse of fluorescently labeled beads scattered atop the ciliated epithelial surface (donor BD00843) of cells cultured in* ***A.*** *standard/ALI and* ***B.*** *hyperoxic/submerged conditions at t=0 s, t=20 ms, t=40 ms and t=60 ms. The white dashed outlines represent the initial positions of each fluorescent bead. As observed above, the beads oscillate back and forth quickly, instead of being transported along the entire epithelial surface. This suggests that the beads likely settled onto the base of the cilia.*

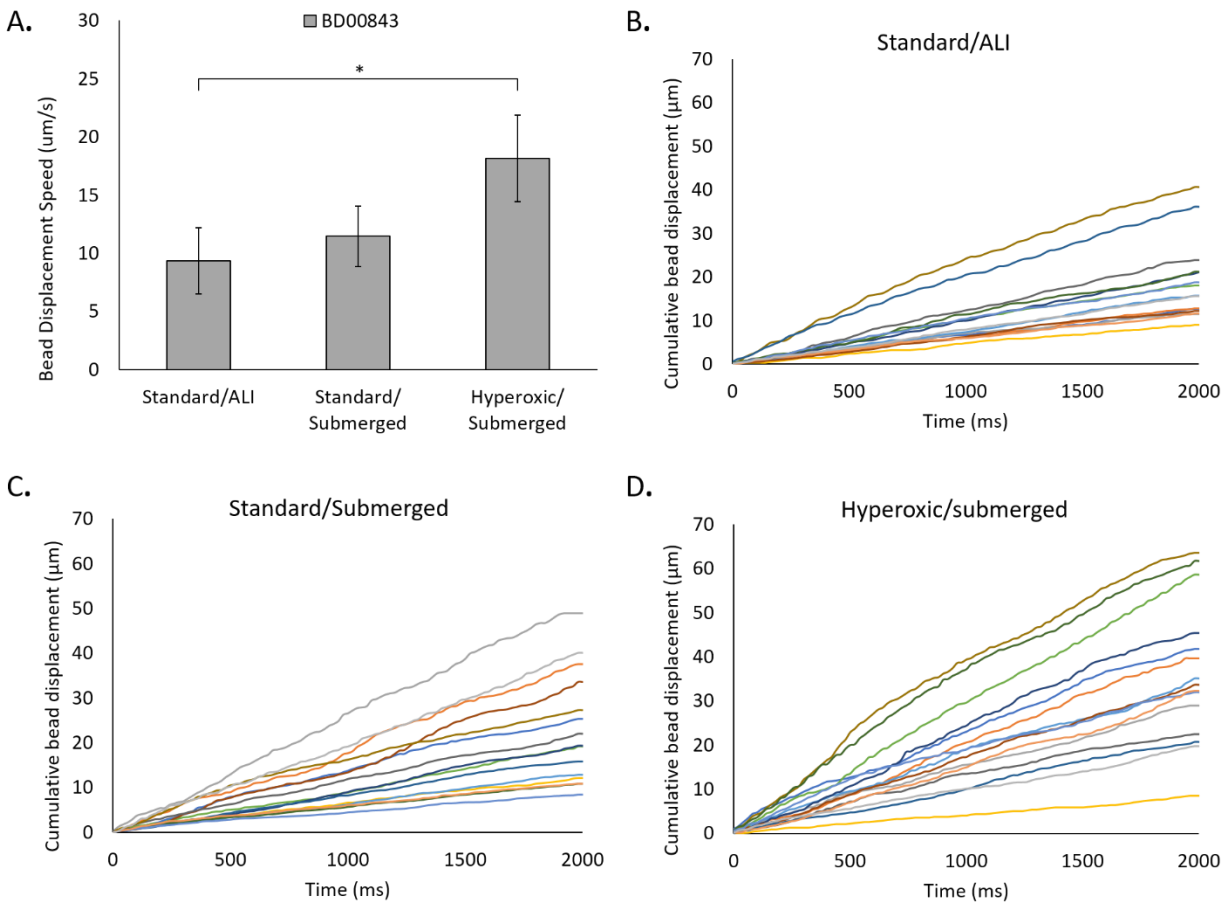

Figure 18. *Cilia speed and fluorescent bead displacement.* ***A.*** *Average displacement speed of beads settled on ciliated epithelial surface of cells (BD00843) cultured in standard/ALI, standard/submerged and hyperoxic/submerged conditions (n=3, mean ± SD, *p<0.05). Hyperoxic/submerged conditions resulted in the highest bead displacement speed, likely due to the absence of accumulated mucins on the epithelial surface which would dampen ciliary beating.* ***B.*** *Cumulative bead displacement in standard/ALI conditions (5 beads/well).* ***C.*** *Cumulative bead displacement in standard/submerged conditions (5 beads/well).* ***D.*** *Cumulative bead displacement in hyperoxic/submerged conditions (5 beads/well).*

6. Discussion

In this study, it was shown that a model bronchial epithelial cell line was sensitive to minute oxygen concentration perturbations by monitoring epithelial barrier integrity throughout standard/hyperoxic and ALI/submerged cultures. Then, a finite element model was designed to simulate the oxygen diffusion gradient throughout apical media in submerged culture conditions. This model allowed us to determine that a 30% incubator oxygen concentration would allow for normoxic oxygen levels at the cellular level during submerged culture under 3 mm of apical media. Human bronchial epithelial cells were then cultured on microporous polyester filters in standard/ALI, standard/submerged and hyperoxic/submerged conditions and their barrier integrity as well as their degree of differentiation was evaluated. It was found that cells cultured in our meticulously designed hyperoxic/submerged system exhibited the highest degree of differentiation compared to the other two conditions, thus challenging the widely-accepted assumption that ALI is uniquely necessary for HBEC differentiation.

The CFBE41o- model cell line with wild-type CFTR is derived from immortalized cystic fibrosis primary bronchial epithelial cells and thus commonly used for epithelial barrier integrity studies[107,108]. This cell line was used for initial oxygenation and submersion experiments to determine if oxygenation differences caused by the addition of apical medium has effects on epithelial integrity. Transepithelial electrical resistance measurements of cells in standard/ALI culture reached values of over 200 Ohms-cm^2 after 10 days in culture, similar to was has been previously reported[109]. Unexpectedly, these cells produced higher TEER values when cultured in submerged conditions, a culture system that has not previously been studied for this cell line. When these filters were fixed and nucleic-acid stained, thicker cell layers were observed throughout fluorescent microscopy. We suspect that increased nutrient availability due to the presence of apical medium in standard/submerged and hyperoxic/submerged conditions allows for cells to proliferate at higher rates and stack on top of each other, resulting in higher resistance values, but not necessarily resulting in tighter junctions. When these cells were exposed to 60% hyperoxic/ALI conditions, their TEER dropped off to zero within 4 days in culture, indicating hyperoxia-induced degradation of epithelial barrier integrity. When apical medium was added to the hyperoxic culture (hyperoxic/submerged condition), junction tightness increased with time, suggesting that the oxygen diffusion gradient created by the media layer had a shielding effect against hyperoxia. Moreover, these differences in barrier function indicate that CFBE41o- cells are oxygen-sensitive enough to distinguish between 40% O_2/ALI and 40% O_2/submerged oxygenation conditions, thus proving that oxygen differences in ALI/submerged culture can affect cellular function. It is therefore possible that HBECs are just as oxygen-sensitive, and that the presence of an oxygen diffusion barrier during submerged culture can alter their differentiation capabilities.

A finite element model was designed to simulate the oxygen diffusion gradient in apical culture medium during submerged filter culture. We assumed diffusion properties of oxygen in water, which was adequate for a first estimation, given that we also assumed oxygen consumption rates based on an undifferentiated model human lung epithelial cell line and not based on differentiated cells which have been shown to have increased oxygen consumption rates[110]. Moreover, we assumed that the oxygen consumption rate of cells did not change as a function of incubator oxygen concentrations, which is unlikely because the metabolic rates of cells are affected as they adapt to their oxygen environment[111]. According to the simulation results, during standard 21% O_2/submerged culture, there is formation of an

oxygen-depletion zone at the cellular level which contains half normoxic oxygen concentration. When incubator oxygen is increased to 30%, normoxic oxygen conditions are achieved. During the first 500s, cellular oxygen consumption (**Figure 8**B) outcompeted oxygen diffusion rates in the media, resulting in a rapid drop in O_2 at the cellular level. The higher incubator oxygen concentration (30%) then allowed media to recover lost oxygen levels as it reached equilibrium at t=5000s. Hyperoxic culture conditions were designed based off the results of this finite element analysis.

HBECs were cultured on porous Transwell® filters in standard/ALI, standard/submerged and hyperoxic/submerged conditions and their barrier integrity was evaluated throughout the culture to assure its health. Contrary to what has been seen in literature, TEER measurements HBECs from our three donors, cultured in all three conditions, dropped from an initial resistance of around 1300 Ohms-cm^2 at day 0 in standard/hyperoxic and ALI/submerged conditions, to a resistance of 500 Ohms-cm^2 at around day 7. In most ALI studies, TEER measurements are shown to start low and increase until they stabilize at roughly 400 Ohms-cm^2, when they have formed a confluent monolayer [112,113]. We suspect that the use of higher seeding densities of undifferentiated HBECs caused an initial proliferation boom, which resulted in cells running out of filter surface area to adhere onto and thus piling on top of each other, increasing initial resistance measurements, such as seen with the CFBE41o- cell line. Presumably, only cells directly in contact with the filter surface were able to maintain their adhesion and differentiate into ciliated and goblet cells, at which point proliferation arrest occurs[114]. The weakly adherent cells were then likely washed away during each media replacement, thus lowering TEER measurements until they plateau at day 7. To confirm that this phenomenon is occurring, separate samples would have to be fixed and histologically sliced at different time points within the first week in culture to observe cell stacking.

HBEC cultures were maintained for 25 days in their given culture conditions to reach confluence and differentiate, before being evaluated for nucleic HIF-1α, an HBEC oxygen-sensitive marker[115]. Cells cultured in standard/submerged conditions exhibited higher expression of nucleic HIF-1α, suggesting that cells were in a more hypoxic state when compared to the other two conditions, as I hypothesized. Moreover, statistically similar quantities of the hypoxia-marker were observed in cells cultured in standard/ALI and hyperoxic/submerged culture, affirming the results of the finite element simulation which showed that a hyperoxic incubator oxygen concentration of 30% created a non-hypoxic cell environment.

Ciliation area, epithelial membrane thickness, mucus secretion and ciliary functionality are all markers of a differentiated human bronchial epithelium[112,116]. In this study, I directly evaluated ciliary functionality, ciliation area and epithelial thickness to assess differentiation levels of healthy donor-derived HBECs. Many techniques have been used to characterize ciliary beating, such as differential dynamic microscopy[117], spectral domain optical coherence phase microscopy[118], Doppler-based optical coherence tomography[119]. Here, I used a simplified approach to assess ciliary functionality of cells cultured in standard/hyperoxic and ALI/submerged conditions. Ciliary beating speed was lowest for cells cultured in standard/ALI conditions. We suspect that this is due to the presence of mucosal layer on the surface of ALI cultures which has a dampening effect on the ciliary beating patterns. The mucus layer is visible as a glossy film on the cell-laden filter surface by the naked eye. Moreover, I observed the movement of mucus "rafts" along the ALI cell surface during high-magnification live phase-contrast imaging, similar to what

has been previously reported in culture[120,121]. During submerged culture, apical media is changed three times per week, therefore any mucins secreted by differentiated goblet cells are likely aspirated away and not able to accumulate to form a mucus membrane. As a result, there is no dampening of ciliary movement and cilia are free to move more rapidly through the non-viscous culture medium instead. HBECs cultured in submerged conditions cannot therefore be used for mucociliary clearance studies unless transferred to ALI culture for a few days, until they have excreted enough mucins to form mucosal layer[3]. When scattering the fluorescently-labelled microbeads on the cell-laden filter surface, the beads likely settle at the base of the cilia and get shuffled back and forth during ciliary beating, therefore the displacement of the beads does not represent the full trajectory of the cilia. Although, this technique still offers insight into the relative ciliary beating speed of cells cultured in our three experimental conditions.

A video-based ciliation area measurement technique was designed and validated using cross-sectional histological filter slices. We found that cells from all three donors exhibited the highest ciliation rates when cultured in hyperoxic/submerged conditions and, surprisingly, the lowest ciliation rates in standard/ALI culture, contrary to the widely-accepted assumption that ALI produces superior airway epithelial cell ciliation[5,122,123]. A similar trend was observed when measuring epithelial thickness, which was designated as the distance between the porous filter surface and the apical surface of the epithelial cells, excluding the cilia. Epithelial thickness measurements of HBEC cross-sections showed that hyperoxic/submerged conditions produced the thickest epithelial layers for all three donors', ranging from 6.19 ± 0.59 µm to 12.90 ± 1.80 µm. In comparison, the human bronchial epithelium measures roughly 25 µm in thickness in healthy human subjects[103-105]. Min et al. showed that normal human bronchial epithelial cells cultured on polyester filter supports differentiated into thicker epithelial membranes when cultured under submerged conditions, in comparison to ALI conditions[75], similar to what we observe here. Differences between epithelial thickness of cells in standard/ALI and standard/submerged conditions seem to be donor-dependent, suggesting that higher oxygen concentrations in ALI culture and more nutrient availability in submerged culture have similar effects on HBEC differentiation.

Several older papers compare ALI to submerged culture[124,125]. de Jong et al. evaluated ciliogenesis of HBECs cultured in submerged conditions on both tissue culture plastic and collagen membranes and compared them to ALI culture on collagen membranes and de-epidermized dermis[5]. In both ALI systems, cells grew mature cilia within 21 days of culture, however, mature cilia were not found in submerged cultures even after 31 days. Kondo et al. showed that dog tracheal epithelial cells cultured on cellulose ester membranes exhibited improved electrical properties and a thicker, more multilayered and ciliated epithelium when cultured at an ALI versus under submerged conditions[126]. Differentiation media formulations have improved substantially over the past three decades[79,127-129], but the necessity of an ALI has not been reassessed using these updated recipes. As a result, although an ALI might have been necessary for HBEC differentiation with initial media, it might no longer be a requirement with these more sophisticated formulations. It was only recently that Gerovac et al. challenged this assumption and showed that the addition of a thin media liquid layer on top of filter-cultured HBECs resulted in better differentiation efficiencies[6].

Our results demonstrate that an ALI is not uniquely necessary for primary human bronchial epithelial cell differentiation. We speculate that both increased nutrient availability and increased oxygen

availability can promote higher rates of differentiation than the current standard differentiation techniques. Although, some might argue that a submerged filter culture system is less physiologically similar to the *in vivo* lung epithelial environment, compared to ALI culture models. This is because, *in vivo*, HBECs are basolaterally fed by nutrients that diffuse through the basement membrane from the blood and are apically in direct contact with ambient air[130]. Therefore, during air-liquid interface filter culture, the collagen IV-coated porous filter simulates the basement membrane, the basolateral media represents the blood and the ambient incubator air acts as the gas entering the lung. However, we must consider that the human lung epithelium undergoes peak differentiation during fetal development[131], not during day-to-day epithelial repair[132,133]. Lung epithelial differentiation first occurs during the canalicular stage of fetal development, between the 16th and 26th week of pregnancy[134]. During this period and throughout the rest fetal development, the lungs are filled with liquid secreted by the pulmonary epithelium[135-137]. Undifferentiated epithelial cells are therefore subjected to a liquid-liquid-interface and are still capable of differentiation. By subjecting healthy donor-derived primary bronchial epithelial cells to a liquid-liquid-interface during submerged filter culture, we hypothesize that we are partially simulating this rapidly differentiating fetal environment.

7. Conclusion

Airborne particle toxicity studies are often performed on differentiated human bronchial epithelial cells. Air-liquid-interface filter culture has been the default culture system used given its success in driving HBECs differentiation into ciliated and mucus-producing cells. However, this culture system is expensive, labor-intensive and incompatible with live microscopy techniques. Developing more simplified culture platforms for HBEC differentiation requires a better understanding of the biophysical cues that drive this cellular phenomenon. In this work, I sought to determine the effects of oxygen and air-liquid-interface on human bronchial epithelial cell differentiation.

A model bronchial epithelial cell line was first used to evaluate oxygen sensitivity. We observed that epithelial barrier integrity was affected by slight oxygenation differences induced by the addition of an apical media layer during culture. Given the similarities between this cell line and primary HBECs, these results suggest that HBECs will likely also be sensitive to these minute oxygenation differences. A finite element model was designed to simulate the oxygen diffusion gradient in apical cell culture medium. This simulation showed that, when submerged under 3 mm of culture medium, cells are exposed to less than half of normoxic oxygen concentrations. Moreover, the results of the simulation showed that a hyperoxic incubator oxygen concentration of 30% would allow for normoxia at the cellular level. A nucleic HIF-1α stain of HBECs cultured in standard/ALI, standard/submerged and hyperoxic/submerged conditions confirmed these results and revealed that controlled hyperoxic ambient conditions can compensate for the oxygen diffusion gradient present in submerged conditions.

Hyperoxic/submerged HBEC culture triggered highest rates of ciliation and allowed for the thickest epithelial layer formation in all three primary donors, which contradicts the widely accepted assumption that ALI is necessary for HBEC differentiation. Both increased ciliation and thicker epithelium can be a result of increased nutrient and oxygen availability in hyperoxic/submerged culture. Ciliary beating was also evaluated, and a lower ciliary beating speed was observed in the standard/ALI condition, which was likely due to a thick mucosal layer on top of the cells, dampening ciliary movement. Throughout submerged culture, these mucins excreted by goblet cells likely dissolve into the surrounding media and thus do not form the viscous dampening layer necessary for mucociliary clearance studies. Although, cells differentiated in submerged conditions could be later moved to ALI to allow for mucosal layer formation.

Interestingly, the ability of these cell types to differentiate at all under hypoxic and hyperoxic submerged conditions suggests that improved media formulations may have already reduced the need for ALI cultures. Although, when HBECs were cultured in similar submersion conditions on collagen-coated coverslips, they completely failed to differentiate, which gives us reason to believe that the intrinsic properties of the porous filter substrate in ALI might be the driving factor in HBEC differentiation. Therefore, novel hyperoxic/submerged culture techniques would likely have to include a porous substrate to allow for complete HBEC differentiation into a pseudostratified epithelium. Lastly, the submerged filter culture conditions might reflect the rapid increase in differentiated lung cells that occurs during the first trimester of fetal development, before ALI conditions exist in the lung[138]. Therefore, not only is ALI not required to provoke this differentiation boom, but submerged culture actually better recapitulates the *in vivo* phenomenon.

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