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INVITED REVIEW

Invited review: human air-liquid-interface organotypic airway tissue models derived from primary tracheobronchial epithelial cells—overview and perspectives

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Abstract

The lung is an organ that is directly exposed to the external environment. Given the large surface area and extensive ventilation of the lung, it is prone to exposure to airborne substances, such as pathogens, allergens, chemicals, and particulate matter. Highly elaborate and effective mechanisms have evolved to protect and maintain homeostasis in the lung. Despite these sophisticated defense mechanisms, the respiratory system remains highly susceptible to environmental challenges. Because of the impact of respiratory exposure on human health and disease, there has been considerable interest in developing reliable and predictive in vitro model systems for respiratory toxicology and basic research. Human air-liquid-interface (ALI) organotypic airway tissue models derived from primary tracheobronchial epithelial cells have in vivo–like structure and functions when they are fully differentiated. The presence of the air-facing surface allows conducting in vitro exposures that mimic human respiratory exposures. Exposures can be conducted using particulates, aerosols, gases, vapors generated from volatile and semi-volatile substances, and respiratory pathogens. Toxicity data have been generated using nanomaterials, cigarette smoke, e-cigarette vapors, environmental airborne chemicals, drugs given by inhalation, and respiratory viruses and bacteria. Although toxicity evaluations using human airway ALI models require further standardization and validation, this approach shows promise in supplementing or replacing in vivo animal models for conducting research on respiratory toxicants and pathogens.

Keywords Air-liquid-interface (ALI) airway cultures · Exposure system · Inhalation toxicology · Pulmonary drug testing · Pathogen-host interaction

Abbreviations

ALI	Air-liquid-interface
AZM	Azithromycin
CB	Chronic bronchitis
CBF	Cilia beating frequency
CF	Cystic fibrosis
CFD	Computational fluid dynamics
CI	Canadian Intense
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoke
CSC	Cigarette smoke condensate
CSE	Cigarette smoke extract
CXM	Cefuroxime
DA	Diacetyl
DE	Diesel exhaust
ENM	Engineered nanomaterial
GHS	Global Harmonization System
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IL-13	Interleukin-13
ISO	International Organization for Standardization

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LVX	Levofloxacin
MCC	Mucociliary clearance
MCT	Mucociliary transport
MMAD	Mass median aerodynamic diameter
MWCNT	Multi-walled carbon nanotube
MXF	Moxifloxacin
NHBE	Normal human bronchial epithelial cells
NIOSH	National Institute for Occupational Safety and Health
NP	Nanoparticle
OECD	Organisation for Economic Co-operation and Development
PCD	Primary ciliary dyskinesia
P-gp	p-Glycoprotein
QCM	Quartz crystal microbalance
RA	Retinoic acid
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
TEER	Transepithelial electrical resistance
TJ	Tight junction

Introduction

The respiratory system is the key interface between the external environment and systemic circulation. It not only allows O_2/CO_2 gas exchange, but also simultaneously provides a barrier to protect the body from invasion by airborne pathogens and exposure to toxic chemical substances (LeMessurier *et al.* 2020). The respiratory system is composed of numerous specialized cell types (Fig. 1). It has evolved highly elaborate and effective mechanisms for filtering out, inactivating, and removing foreign materials. These defense mechanisms include mucociliary clearance (MCC) and innate immune capabilities, such as secretion of antimicrobial peptides and cytokines by epithelial cells and resident leukocytes in order to recruit and activate the adaptive immune system (LeMessurier *et al.* 2020; Sharma *et al.* 2020). Despite these defense mechanisms, the respiratory system remains highly susceptible to direct effects and/or penetration by potentially toxic pathogens, particles, and gases that are commonly encountered in modern environments. Examples of various sources of potential damage to the respiratory system include air pollution, occupational exposure to chemicals and particles, and tobacco products, as well as chemicals, fumes, particles, and gas exposures from everyday household chemicals, consumer products, and cosmetics. Additionally, respiratory system-specific pathogens (e.g., influenza, tuberculosis, rhinovirus, and coronaviruses) have evolved and adapted to evade the respiratory defense mechanisms (LeMessurier *et al.* 2020; Sharma *et al.* 2020). Infection by respiratory bacteria and viruses sickens and kills millions of people worldwide each year as well as inflicts huge economic burdens.

Given the impact of respiratory exposures to human health and disease, development of model systems for respiratory toxicology and basic research has been an area of longstanding interest. Reliable and predictive models of the human respiratory system continue to be a pressing need. Specific applications of respiratory model systems include regulatory safety and hazard assessment of chemicals and nanoparticles (NPs), tobacco research, infectious respiratory disease, and pulmonary drug development (Lacroix *et al.* 2018).

Animal models, such as mouse, rat, dog, guinea pigs, and non-human primate, have been in widespread use for inhalation toxicology applications for many years. Such approaches have the advantage of identifying systemic effects from inhalation exposure that integrate pharmacokinetic and pharmacodynamic processes in the model animal. However, significant differences in the anatomy, physiology, and breathing patterns between animals and humans have made translation of experimental results obtained in animals to humans problematic. Furthermore, animal models of human respiratory diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis, are not fully equivalent to the human diseases, leading to high failure rates of clinical trials designed using animal data (Shanks *et al.* 2009).

The limited success in translating animal data to human outcomes has become increasingly recognized and appreciated in recent years. Ethical concerns regarding the use of animals in toxicological research also have been raised. The National Research Council Report "Toxicity Testing in the 21st Century: A Vision and a Strategy" recommends the replacement of animal tests with relevant in vitro human-based test systems (National Research Council 2007). Transition from animal- to human-based models is ultimately expected to lead to faster and better predictive toxicity assessments and therapeutic development at lower cost. In line with these goals, US federal regulatory agencies consider the development and validation of alternative in vitro methods for acute toxicity testing, including acute inhalation toxicity testing, to be a high priority (Clippinger *et al.* 2018). Specifically, the US Environmental Protection Agency (EPA) has a stated goal of reducing in vivo acute inhalation testing for pesticide submissions (EPA 2016a; EPA 2016b).

Following the initial development of culture medium capable of supporting in vitro growth of normal human bronchial epithelial cells (Lechner *et al.* 1981), submerged monolayer cultures derived from human respiratory tissues have been used widely as tools for respiratory research and toxicology studies (Berube *et al.* 2010). However, these traditional methods, which promote extensive cell proliferation, do not adequately reproduce the differentiated phenotype of in vivo airway epithelial tissues. To circumvent these limitations, culture methods that result in a better representation of three-dimensional, well-differentiated in vivo respiratory tissues have been developed.

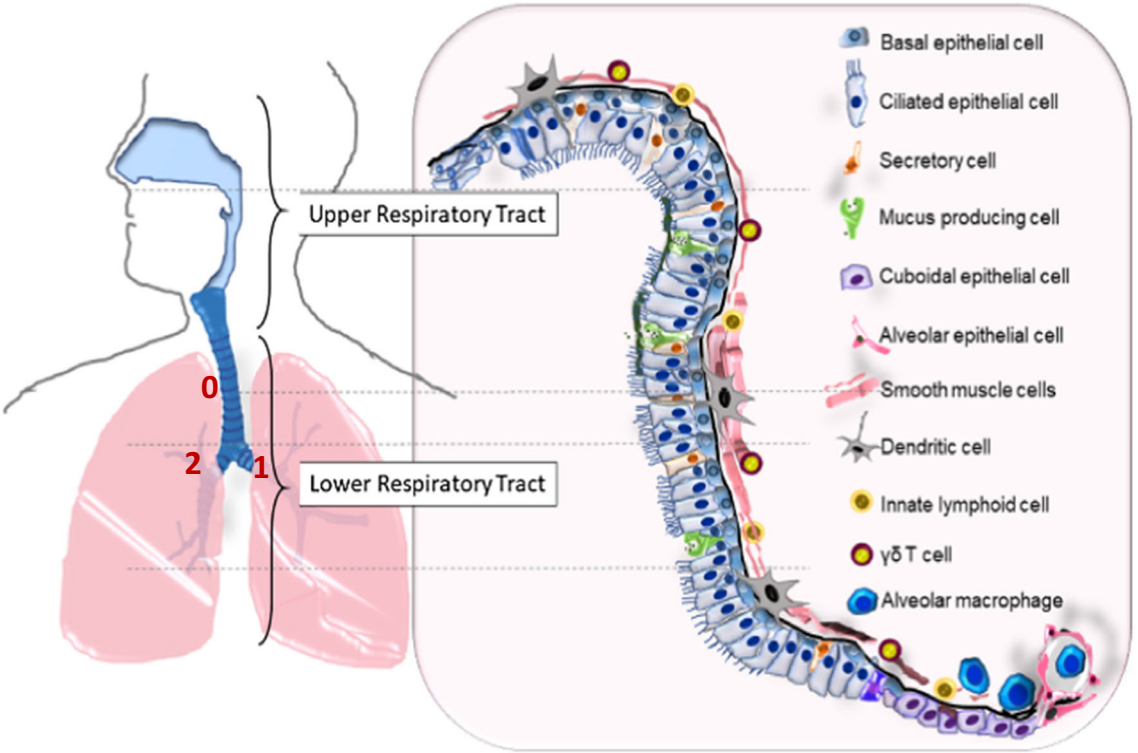

Figure 1. Human respiratory tract is lined with region-specific cell types. Both the upper and lower respiratory tracts are shown in this illustration. The lower respiratory comprises the conducting zone for air passage and respiratory zone for gas exchange between the lung and pulmonary capillaries. The conducting airway consists of the trachea, bronchi, and bronchiole; the respiratory airway consists of the respiratory bronchioles as well as the alveolar ducts and sacs. Each region of the respiratory tract is lined with region-specific cell types as illustrated in this figure. Human airway has 23 generations of dichotomous branching, starting from the trachea as generation 0. The ALI airway tissue models discussed in this review are derived from epithelial cells harvested from generations 0 to 2 (labeled in the *graph*). Minor modification of the drawing published by LeMessurier *et al.* (2020) Front Immunol 11: 3.

Human airway organoids developed from human cell induced pluripotent stem cells, or adult tissue stem cells possess the cell polarity and several of the cell types (e.g., goblet cells, ciliated cells, basal cells, and club cells) found in airway epithelium. Thus, they represent a primary cell–derived in vitro airway epithelial model (van der Vaart and Clevers 2020). Three-dimensional organoids can be maintained successfully under submerged conditions in long-term culture (Sachs *et al.* 2019). Airway organoids have been used for a variety of applications, including drug screening and as disease models (van der Vaart and Clevers 2020). However, they form as spheres with lumens inside and are absent of an air interface. It is, therefore, not possible to directly expose lung organoids to inhaled substances in their native forms, as occurs in vivo. An alternative airway model that can be exposed directly to gases, vapors, and aerosols involves culturing primary airway cells on microporous membrane scaffolds at the air-liquid interface (ALI) (Adler *et al.* 1987; Whitcutt *et al.* 1988).

Compared to submerged monolayer cultures, differentiated, organotypic ALI airway models have a more realistic in vivo–like structure, as well as barrier properties and metabolic functions similar to those found in vivo, and can be dosed in a more human-relevant manner than can submerged or organoid cultures (Wu *et al.* 1986; Yamaya *et al.* 1992; Kaartinen *et al.* 1993). Primary cells that undergo cellular differentiation can reproduce an in vivo–like transcriptional profile similar to that of human airway epithelium (Pezzulo *et al.* 2011) and replicate in vivo toxicity responses, such as cilia dysfunction (Brekman *et al.* 2014), squamous metaplasia, and goblet cell hyperplasia (Bolmarcich *et al.* 2018). Infection by human respiratory pathogens, such as *Bordetella pertussis* (Soane *et al.* 2000), influenzae (Chan *et al.* 2010), and coronavirus (Jia *et al.* 2005), is also highly dependent on the differentiation state of airway epithelium. The use of human cells in ALI airway culture systems also allows studying the etiology of respiratory diseases, such as asthma, COPD, cystic fibrosis (CF), and idiopathic pulmonary fibrosis, as well as variability in population-based responses.

This review will provide a state-of-the-art update on ALI airway tissue models. A survey of ALI exposure systems and key applications, such as inhalation toxicity testing of NPs and chemicals, tobacco research, pulmonary drug testing, and host-pathogen interactions, will be presented. Finally, the strengths and limitations of the current technology, status of using these in vitro systems as a replacement for in vivo

animal models, and recommendations for future directions will also be discussed.

Overview of the Airway ALI System

Organotypic ALI airway tissue models were first successfully developed in the late 1980s by growing primary tracheal epithelial cells from guinea pigs at the air-liquid interface (Alder *et al.* 1987; Whitcutt *et al.* 1988). A chamber with a permeable gelatin membrane was employed to separate the culture environment into two compartments that simultaneously exposed cells to air and supplied culture medium through the membrane. Under such conditions, primary airway cells differentiated into heterogeneous cell populations with a polarized mucociliary phenotype resembling the native tissue from which they were derived. With the advances made in stem cell culture techniques, ALI models have been established from human primary cells of the nasal, proximal, and distal airway epithelium (Fuchs *et al.* 2003; Fulcher *et al.* 2005; Muller *et al.* 2013; Huang *et al.* 2017; Rayner *et al.* 2019). Models simulating the tracheobronchial epithelium of the lung have been most extensively characterized and employed in respiratory research and, therefore, are the focus of this review.

Functions of Airway Epithelial Cells The human tracheobronchial epithelial lining consists of a mixed population of secretory cells, ciliated cells, and basal cells organized into a pseudostratified columnar structure, in which all cells reside on basal lamina. Ciliated cells account for 50–70% of the epithelial cell population (Staudt *et al.* 2014) and each cell has about 200 to 300 cilia of approximately 6 μm in length on its luminal side (Serafini and Michaelson 1977). Cilia are microtubule-based structures. Active ciliary movement depends on both the basal body and the cytoskeleton structures surrounding it and are driven by the energy produced by mitochondria aggregated close to the luminal surface (Harkema *et al.* 1991).

The mucus-producing goblet cells are the major secretory cells found in tracheobronchial airways. These cells contain mixtures of highly glycosylated electron-lucent mucin granules that can be readily released upon stimulation (Jeffery 1983). Together with beating ciliated cells, they form a dynamic mucociliary escalator, which is one of the major defense mechanisms for trapping and clearing inhaled substances out of the airways without inducing inflammatory responses. Obstruction of the airways as a result of secretory cell hyperplasia and metaplasia as well as compromised MCC is common in subjects with chronic inflammatory airway diseases (Hauber *et al.* 2006).

Another major type of airway epithelial cell is basal cells. These are undifferentiated epithelial cells and account for approximately 31% of the airway epithelial cell population (Boers *et al.* 1998). Although a long-held theory is that basal cells are the only progenitor cells that can undergo self-renewal during normal tissue maintenance and terminal differentiation into secretory or ciliated cells in response to tissue injury (reviewed in Berika *et al.* 2014), increasing evidence demonstrates that other cell types also possess cellular plasticity. For instance, stimulated secretory cells were found to transdifferentiate into ciliated cells (Ayers and Jeffery 1988; Rawlins *et al.* 2009). Ciliated cells can de-differentiate into squamous cells soon after the injury to preserve tissue integrity and later re-differentiate into secretory cells and ciliated cells to restore an intact layer of airway epithelium (Park *et al.* 2006). Besides its role as the airway progenitor cells, basal cells have other important structural and regulatory functions. Owing to their central location at the epithelial-mesenchymal trophic unit of the airways, basal cells can interact with various cell types and thus also play a role in regulating inflammatory responses and transepithelial water movement and forming lateral intercellular space along the basement membrane (Evans *et al.* 2001). A balance between mucus secretion and clearance as well as proportions of different epithelial cell types, therefore, is critical for the health of airway epithelium.

Functions of Airway Epithelium Epithelial cells are interconnected through a series of junctional complexes, such as tight junctions (TJs), adherens junctions, and desmosomes (Ganesan *et al.* 2013). Airway epithelium, however, is more than just a physical barrier covering the respiratory tract; it also provides innate immunity crucial for maintaining airway tissue integrity and homeostasis during normal tissue renewal as well as when responding to tissue injuries (Tam *et al.* 2011). The protective functions of airway epithelium are achieved by its unique cellular structures, such as intercellular junctional complexes and ion channels, and mucociliary escalators, as well as its ability to produce antimicrobial molecules, including cytokines, chemokines, and proteases (Godfrey *et al.* 1992; Bals 2000; Fahy and Dickey 2010). The epithelial junctional complexes not only modulate the movement of water, ions, and macromolecules, but also function as signaling platforms and regulate proliferation, apoptosis, and differentiation of the epithelial cells (Balda and Matter 2009). Environmental challenges to these junctional complexes could cause membrane leakage and abnormal tissue repair and regeneration, leading to the development of lung diseases, such as asthma, COPD, and lung cancer.

Defects in barrier function have been reported in the lung of subjects with CF and asthma (Rezaee and Georas 2014). Immunohistochemistry staining of asthmatic bronchial epithelial biopsies revealed a reduction in the expression of α-catenin, E-cadherin, ZO-1, and possibly occludin, accompanied by a reduced transepithelial electrical resistance (TEER) and an increased permeability to dextran of less than 20 kDa (de Boer *et al.* 2008; Xiao *et al.* 2011). In patients with CF,

disorganized TJ strands extend beyond the apical belt (Godfrey *et al.* 1993). Furthermore, mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*) result in dysregulation of the depth and barrier functions of TJs (LeSimple *et al.* 2010). These structural abnormalities impair epithelial functions and significantly increase the susceptibility of CF patients to pathogenic bacteria, such as *Pseudomonas aeruginosa* (Rezaee and Georas 2014), colonization of which in the airways further disrupts the assembly of TJs (Plotkowski *et al.* 1999). Thus, in vitro models capable of reproducing the key structural and functional aspects of the airway epithelium will be powerful tools for assessing respiratory toxicity and evaluating the disease potential of airborne substances.

Characteristics of ALI Airway Tissue Models Primary normal human bronchial epithelial (NHBE) cells isolated from the tracheobronchial region of the airways are de-differentiated cells when they are propagated as monolayers in submerged culture (Wise and Lechner 2002). Although these cells have been used widely for respiratory biology and toxicology studies (Takizawa *et al.* 1999; Kawasaki *et al.* 2001; Fields *et al.* 2005), the presence of a single cell type and the lack of the structural and functional features of the in vivo airway have greatly limited their usefulness as in vitro cell models for toxicity assessment.

The organotypic ALI airway model is generated in situ by differentiating NHBE cells at an air-liquid interface created by a semi-permeable membrane support (e.g., Corning® Transwell®, Millipore® Millicell®, or Greiner Bio-One ThinCert™ culture insert). The apical surface of the ALI culture is exposed to the surrounding air, which oxygenates the epithelial cells and promotes cellular differentiation (Bebök *et al.* 2001). Cells are nourished from medium in the basolateral compartment through the microporous membrane. Under these biphasic culture conditions, NHBE cells proliferate and differentiate into a polarized pseudostratified tissue-like structure resembling that of the airway epithelium, with ciliated cells interspersed with goblet cells facing the apical side and basal cells spreading along the basolateral membrane (Fig. 2) (Yamaya *et al.* 1992; Bals *et al.* 2004). In addition, extracellular matrix is secreted and deposited onto the basolateral side of the epithelium during differentiation (Cozens *et al.* 2018).

Comparison of the transcriptome profiles of the primary cell–based ALI cultures and NHBE cells grown in submerged culture indicates that ALI cultures are much more similar to native epithelial tissues in vivo than are submerged monolayer cultures (Pezzulo *et al.* 2011). However, subtle differences in gene expression patterns exist (Dvorak *et al.* 2011). Compared to human airway tissue, ALI cultures express higher levels of basal cell-related genes as well as genes involved in the cell cycle and proliferation. Such discrepancies in gene expression may reflect the difference in the proportion of epithelial cell types between the in vitro and in vivo airway epithelium. The overall similarity in their gene expression also extends to the expression of essential xenobiotic metabolizing enzymes. A broad panel of the phase I and phase II metabolism genes, including cytochrome P450 (CYP) enzymes, glutathione S-transferases (GSTs), and UDP-glucuronosyltransferases (UGTs), is significantly upregulated during maturation of the ALI cultures and their expression remains elevated over a period of 6 months in culture (Boei *et al.* 2017; Qin *et al.* 2019). In particular, enzymes expressed exclusively in the lungs, such as CYP2A13, CYP2F1, and CYP4B1 (Carr *et al.* 2003; Poch *et al.* 2005; Su *et al.* 2000), are markedly increased upon differentiation (Newland *et al.* 2011; Boei *et al.* 2017; Qin *et al.* 2019), whereas their expression in undifferentiated NHBE cells is marginal. Enzymatic activity assays using CYP-specific substrates confirm the activity of key xenobiotic metabolism enzymes in ALI cultures.

In contrast to the upregulation observed with many *CYP* genes, expression of *CYP1A1* was found to be significantly lower in differentiated ALI cultures compared to undifferentiated NHBE cells (Qin *et al.* 2019). This observation, however, is consistent with the report that expression of *CYP1A1* was inducible by cigarette smoke (CS) and, therefore, only detected in lung tissues from smokers (MeLemore *et al.* 1990). Besides CS, exposure of ALI airway cultures to 2,3,7,8-tetrachlorodibenzodioxin (TCDD), aromatic hydrocarbons, and diesel exhaust (DE) also significantly enhanced the expression of *CYP1A1* (Boei *et al.* 2017). Altogether, these observations suggest that well-differentiated ALI cultures possess metabolic capabilities to biotransform xenobiotics that are absent in conventional submerged primary cultures. Conservation of metabolic activities in the ALI cultures is essential for employing such culture systems in toxicity testing and human risk assessment, especially for test articles that require biotransformation. As an example, Qin and colleagues recently demonstrated that the tobacco-specific genotoxicant and carcinogen, 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK), failed to induce DNA damage in undifferentiated NHBE cells in the absence of an exogenous liver S9 activation system, due to the lack of bioactivation in these cells (Qin *et al.* 2019).

Considerable overlap has been observed in the protein composition of apical secretions from ALI airway cultures and normal induced sputum (Kesimer *et al.* 2009; Baxter *et al.* 2015). In particular, host defense proteins, such as mucins, proteases, and antimicrobial proteins, are found in both in vitro and in vivo secretions, making up 20–30% of their total mass. Although the secretions have overall similarities, the levels of individual protein components vary (Baxter *et al.* 2015). For instance, several studies have reported that MUC5B is present in lower abundance in the apical secretions

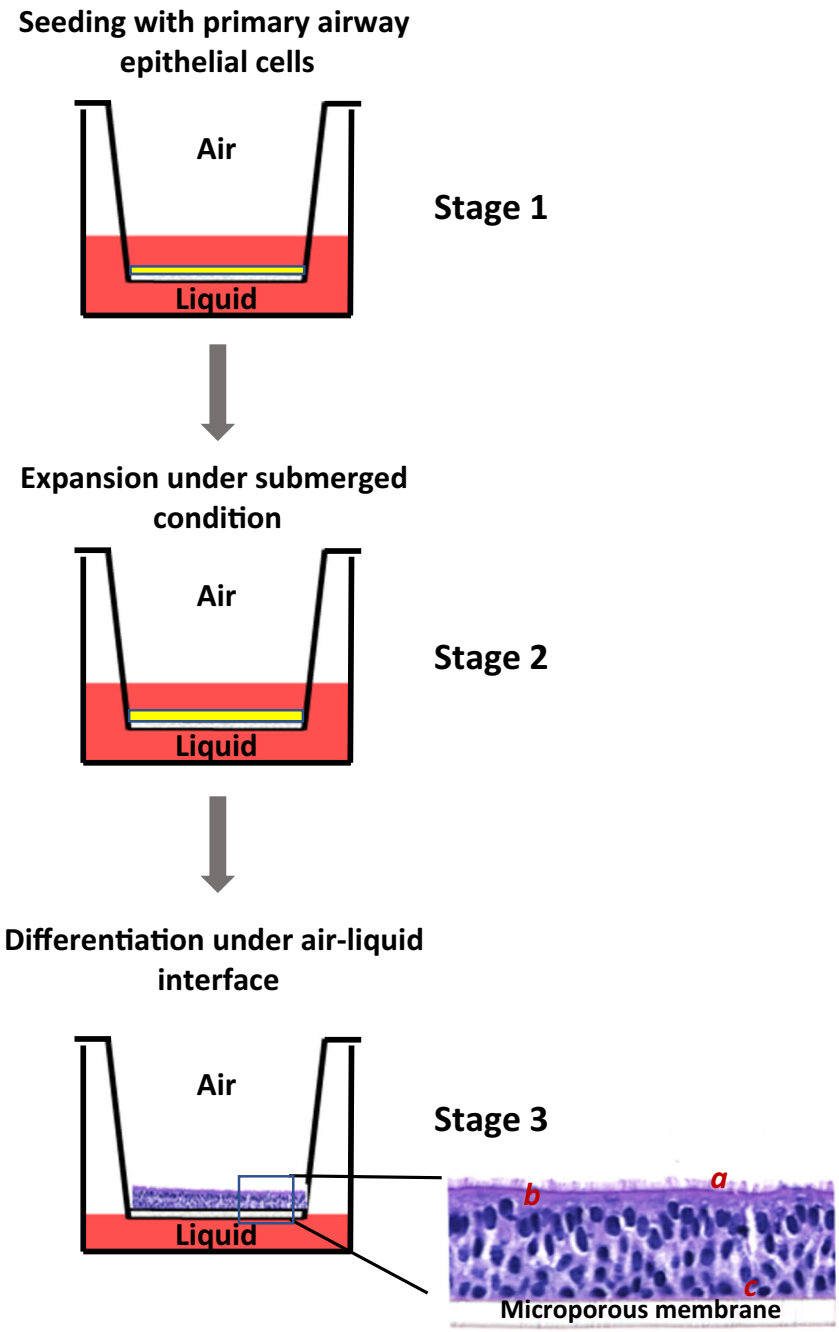

Figure 2. A schematic diagram of the human ALI airway tissue model. The procedure of establishing the ALI airway tissue model is schematically illustrated on the left panel. NHBE cells are seeded onto the microporous membrane pre-coated with extracellular matrix proteins, such as collagen (stage 1). Primary cells continue to proliferate under the submerged condition until they reach complete confluence (stage 2). Differentiation is initiated by lifting NHBE cells to the air-liquid interface and feeding with differentiation medium from the basolateral side (stage 3). NHBE cells are differentiated into a pseudostratified phenotype on a microporous membrane around 4 weeks after air-lift. The apical side of the ALI culture is exposed to air; the basolateral side takes up nutrients through microporous membrane. (*a*) Ciliated cell; (*b*) mucus-producing goblet cells; (*c*) basal cells.

of ALI airway cultures compared to induced sputum (Ali *et al.* 2011; Baxter *et al.* 2015). Differences in the collection, processing, and analytical methods may have accounted for the discrepancies. It is also possible, however, that induced sputum could contain molecules secreted by immune cells and other cell types not present in the ALI cultures. Collectively, these differences may confound experimental findings, leading to equivocal conclusions. Methodologies that take potential variables into consideration, therefore, should be developed.

Fully differentiated ALI airway models retain key functions of the in vivo airway epithelium for maintaining homeostasis and responding to environmental challenges. Intercellular junctions, such as TJs and adherens junctions, and drug transporters, such as p-glycoprotein (P-gp), have been identified at the juxta-apical region of ALI airway cultures (Lin *et al.* 2007; Prytherch *et al.* 2011; Rayner *et al.* 2019). The orientation of these apical structures contributes to the polarized morphology of the ALI cultures (Gaillard *et al.* 2010; Saint-Criq and Gray 2017). Similar to TJs

assembled in vivo, the TJ complexes in ALI cultures consist of desmosomes in the subapical regions (Davis *et al.* 2015). This structural resemblance supports the use of ALI cultures as an in vitro system for investigating the TJ-modulating effects of chemicals, such as interleukin-13 (IL-13), cadmium, and CS (Cao *et al.* 2015; Schmidt *et al.* 2019; Tatsuta *et al.* 2019). Schmidt and colleagues reported that IL-13 altered the expression patterns of claudins, induced the colocalization of TJ proteins with proteasomes, and triggered ubiquitination of TJ proteins, eventually leading to degradation and disruption of the junctional complexes (Schmidt *et al.* 2019). Functional epithelial ion channels, such as the sodium channel (ENaC) and CFTR, also are present in ALI airway cultures (Enuka *et al.* 2012; Rayner *et al.* 2019). These ion channels play essential roles in regulating the height, viscosity, and pH of the airway surface liquid. Conceivably, dysregulation of epithelial ion channels can result in dehydration of the airway surface and impair the function of mucociliary escalators as seen in CF and COPD patients.

Several studies have reported that submerged NHBE cells and differentiated ALI cultures respond differently to environmental challenges. van Wetering and colleagues explored effects of differentiation status on Th2 cytokine-mediated production of eosinophil-attractant chemokines eotaxins in both culture systems (van Wetering *et al.* 2007). Although gene and protein expression of eotaxins was induced in both NHBE cells and ALI cultures, increases in the level of eotaxin-3 were much higher in ALI cultures. Furthermore, the IL-4-mediated secretion of eotaxin-2 was mitigated by TNFα only in submerged NHBE cells. In another study, Ji and colleagues demonstrated that palladium NPs were taken up at a much lower level in ALI cultures than in submerged NHBE cells (Ji *et al.* 2017; Wilkinson *et al.* 2011). Furthermore, the NPs tend to gather at one spot in the ALI cultures, possibly due to the sweeping motion of ciliated cells. Similar differences in response between the two systems also were observed with aldehyde vapors, where significant induction of cytokine release and oxidative stress occurred only in the ALI cultures (Dwivedi *et al.* 2018).

The differential responses observed in submerged NHBE cells and ALI cultures may result from factors other than their differentiation status. Exposures at the air-liquid interface allow direct exposure of the apical side to airborne substances in their native form, simulating in vivo inhalation exposure. Such exposures prevent potential interactions between the test article and medium components and, therefore, facilitates the uptake of aerosolized particles (Loret *et al.* 2016). Hypoxia has been proposed as an additional factor underlying the differential responses between the two systems (Ghio *et al.* 2013). However, this hypothesis needs to be further corroborated using better controlled experimental designs. Nevertheless, these findings suggest that fully differentiated ALI cultures may be more suitable as an in vitro tool for generating toxicity data than traditional submerged cultures. Along with its ability to retain its tissue-specific functions for an extended period, airway ALI tissue models represent a simple and relatively cost-effective and easy-to-use advanced in vitro lung model for acute and sub-acute toxicity evaluations of inhaled substances (Berube *et al.* 2010; Lacroix *et al.* 2018).

Unique Endpoints Developed in ALI Airway Models The structural and functional similarity of ALI airway cultures to in vivo airway epithelium has greatly expanded the possibility of measuring tissue-specific endpoints, such as tissue barrier functions, ion channel physiology, MCC, and tissue morphology, in an in vitro system. Evaluation of tissue responses that are relevant to respiratory diseases is expected to provide valuable information for determining the potential adverse health effects of airborne substances.

Barrier function can be assessed by measuring TEER and paracellular permeability (Strengert and Knaus 2011). TEER measurement is a non-destructive technique that has been widely applied for evaluating epithelial integrity preserved by intact TJs (Srinivasan *et al.* 2015). Either a Ussing chamber or volt ohm meter (e.g., EVOM2, World Precision Instruments, Sarasota, FL) can be used to measure the flow of electrical current across the cell layer of the ALI cultures. When cultures are well-differentiated, they develop barrier functions that typically have a resistance above 200 Ω cm^2. Changes in tissue integrity in response to environmental challenges alter ion flux and can be reflected in electrical resistance or impedance. The tracer flux assay is routinely used for assessing the traverse of aqueous solutes. To conduct this assay, ALI cultures are incubated from the apical side with fluorophore-conjugated probes of different sizes and shapes for a predetermined time. Tracers migrate through the paracellular space and accumulate in medium bathing the basolateral side of the cultures. Concentrations of the tracers in the basolateral medium are measured and an apparent permeability coefficient (P_{app}) of the epithelium calculated (Strengert and Knaus 2011). Besides these quantitative assays, confocal microscopy also has been used as a visual tool for studying the dynamics of TJs (Buckley *et al.* 2018). Depending on the purpose of the studies, these methods can be used in combination to comprehensively assess the tissue barrier properties of the ALI cultures under experimental conditions.

Electrophysiological properties of ion transporters, such as ENaC and CFTR, can be evaluated in ALI cultures using a Ussing chamber designed to fit the configuration of the culture inserts (Fulcher *et al.* 2005; Gianotti *et al.* 2018). ALI cultures are mounted between 2 hemi-chambers; both are filled with buffer solution (e.g., Kreb's bicarbonate Ringer's solution) gassed continuously with a mixture of CO^2 and air (e.g., 5% CO_2–95% air) for maintaining the physiological pH.

Transepithelial electrical resistance, potential difference, and short-circuit current are measured sequentially. By using the Ussing chamber, the role of ion channels in mucosal pathophysiology and effects of test substances on the ion channels can be studied.

The mucociliary escalator is an essential component of the airway innate defense for maintaining the health of the respiratory system (Knowles and Boucher 2002; Fahy and Dickey 2010). It is composed of viscous gel-forming mucins and beating ciliated cells. Airborne substances are first trapped in the mucus and then propelled out of the airways by the coordinated movement of ciliated cells. Compromised MCC results in extended exposure of the airways to inhaled toxicants and contributes to the undesirable health effects associated with diseases, such as CF, COPD, and primary ciliary dyskinesia (PCD) (Rogers 2005; Donaldson *et al.* 2007; Bush and Hugg 2012). Effects on MCC can be studied indirectly in ALI airway cultures by evaluating responses of the individual components of the mucociliary escalator, i.e., ciliary beat frequency (CBF) and mucus production, or directly by measuring mucociliary transport (MCT) rates.

Commercial software (e.g., Sisson-Ammons Video Analysis System, Clio, MI) is available for quantitatively measuring CBF and active ciliary points (Sisson *et al.* 2003). Production and secretion of two major gel-forming airway mucins, MUC5AC and MUC5B, can be analyzed semi-quantitatively using commercial or laboratory-developed ELISA assays. The effects of test articles on MCC can be inferred based on the beating of ciliated cells and mucin production. MCT rates also can be measured directly and quantitatively in ALI cultures by tracking the movement of fluorescent microspheres, usually 1 to 2 μm in diameter, over the mucosal surface (Worthington and Tarran 2011). It should be noted that the ALI culture is a closed system where mucus is not continuously removed as occurs in vivo. Conceivably, accumulation of mucus on the apical surface may affect the MCT rate (Sears *et al.* 2015). For assays assessing barrier functions and MCC, the temperature at which the assays are conducted and the medium formulation may introduce variation into the measurements. Development of standardized protocols, therefore, is critical to ensure the comparability of data from longitudinal studies or between laboratories.

Besides the aforementioned endpoints, tissue morphology has been evaluated using immunohistochemistry staining of formalin-fixed, paraffin-embedded tissue sections (Xiong *et al.* 2018; Xiong *et al.* 2019). These measurements provide valuable insight into the responses of individual cell types as well as signs of early morphological changes that may lead to pathogenesis. For instance, goblet cell morphology can be evaluated using Alcian blue/periodic acid-Schiff staining; its ultrastructure can be assessed using electron microscopy. Treatment-associated apoptosis, mitosis, and tissue degeneration can be detected at much lower levels than possible with biochemistry assays. Pathology observations in conjunction with other measurements can generate a comprehensive picture of adverse tissue responses to external stimulation. The ALI airway culture, therefore, is considered a sophisticated in vitro model for evaluating many of the toxicity endpoints used for the in vivo assessment of airborne substances.

Sources of the ALI Cultures

The airway ALI culture is a significant improvement over monolayer NHBE cells. Owing to its structural and functional similarities to in vivo airway epithelium, the ALI model has gained increasing recognition as a physiologically relevant in vitro culture system for respiratory research (Berube *et al.* 2010). Commercially available culture inserts with semi-permeable membranes effectively support the biphasic culture environment that is required for differentiating the primary cells. In general, three stages are involved in the development of ALI airway cultures: (1) initial expansion of submerged primary cultures on plastic substrata, (2) secondary cell expansion on culture inserts under submerged conditions, and (3) differentiation of primary cells on culture inserts under ALI conditions. Expansion of primary cells in culture dishes not only generates sufficient cells for establishing ALI cultures, but also allows for biobanking cells in limited supply. Culture vessels are usually coated with extracellular matrix proteins, such as collagen, gelatin, or fibronectin, to aid cell attachment and differentiation. To date, a number of culture media and methods have been developed for establishing ALI airway models (Gray *et al.* 1996; Fulcher *et al.* 2005; Liu *et al.* 2007; Prytherch *et al.* 2011; Cao *et al.* 2018). Although the medium formulations vary, retinoic acid (RA) is a common and essential ingredient in all recipes for promoting mucociliary differentiation of NHBE cells. The extent of differentiation, proportions of the different cell populations, and the overall morphology depend on the culture methods as well as the passage number of the NHBE cells. Variability in these factors is expected to influence the results of toxicity assessments. Commercial vendors may supply immature cultures as a measure of cost-saving. Under such circumstances, end-users can either request the vendors deliver well-differentiated cultures, which may increase the unit cost of the cultures, or maintain immature cultures in their own laboratories until they are fully differentiated. No matter which option end-users prefer, it should be emphasized to researchers using ALI airway models that only fully differentiated cultures should be employed for experimentation to ensure the validity and reproducibility of the studies. With NHBE cells and expansion and differentiation media becoming commercially available, it is now possible to reproducibly

produce ALI cultures in batches of 100 s in-house using easy-to-follow protocols (Cao *et al.* 2018; Rayner *et al.* 2019). Generating relatively large numbers of ALI tissue models allows the quantitative measurement of toxicity endpoints using a range of test article concentrations and with sufficient replicates to detect differences between exposure conditions.

Commercial Models While the methods for production of ALI airway cultures are well established in the literature and cell sources and required materials are readily available, production of well-differentiated ALI airway cultures is a time-consuming endeavor and can also be technically challenging for some laboratories. This situation has led to the establishment of several ready-to-use ALI airway models as commercial products. The first commercially available ALI airway models (EpiAirway™ and EpiAirway-FT™) were introduced by MatTek Corporation (Ashland, MA). Additional commercial ALI models (MucilAir™, SmallAir™, and OncoCilAir™) are available from Epithelix Sarl (Plan-les-Ouates, Switzerland). These products are shipped as viable tissues that are ready-to-use for experimentation following a brief recovery period or additional culturing to allow further differentiation in the end-user laboratory.

"Home-Made" Models Making airway ALI cultures in-house offers flexibility in designing and carrying out experiments and "home-made" models can be made at lower cost than purchasing commercial models. PneumaCult™ Expansion and Differentiation medium kits developed by STEMCELL Technologies (Vancouver, Canada) are one of the commercial ALI culture methods that are widely used for generating "home-made" ALI airway models. The recently developed PneumaCult™-Ex Plus medium kit supports expansion of primary NHBE cells for up to 6 passages without significantly losing their differentiation potential (Rayner *et al.* 2019). CnT Airway Proliferation and Differentiation Medium kits developed by CELLnTEC (Bern, Switzerland) and BronchiaLife™ Epithelial Airway Medium Complete Kit coupled with HBTEC Air-Liquid Interface Differentiation Medium (LifeLine Cell Technologies, Frederick, MD) represent other commercial method for establishing "home-made" ALI airway cultures. The components of these media are chemically defined and free of materials of animal or human origins. A range of progenitor cell-targeted growth factors and co-factors as well as trace elements, protective antioxidants, and vitamins are supplemented in the basal proliferation medium to enhance the longevity of the primary large airway epithelial cells. ALI airway cultures generated using these Medium kits have been widely utilized in various areas of respiratory research (Tanaka *et al.* 2018; Hess *et al.* 2019; Xia *et al.* 2020).

To make airway ALI cultures, NHBE cells first are grown in expansion medium on collagen-coated plastic culture dishes until they reach approximately 80% confluence. Cells are gently detached from the culture dish with low concentrations of trypsin-EDTA solution (e.g., 0.025%). Expanded cells are then seeded onto culture inserts at a density of 1.2×10^5 cells/cm^2 and allowed to further proliferate on the inserts with expansion medium added to both the apical and basolateral compartments. When they attain complete confluence, the cells are "air-lifted" by removing the medium in the apical compartment and adding differentiation medium to the basolateral compartment. This leaves the apical side of the cultures exposed to air and the basolateral side in contact with the differentiation medium. As discussed above, such culture conditions simulate the native environment and are essential for driving the differentiation of the NHBE cells into the mucociliary phenotypes found in the airway. Achieving fully differentiated ALI cultures takes approximately 4 wk of feeding every 2 or 3 d with fresh differentiation medium. A set of quality standards (e.g., cilia motility, mucus production, and histological examination) is necessary to ensure the quality of the cell cultures and the validity of in vitro findings using these cultures.

Exposure Systems Used with ALI Airway Cultures

Before specialized exposure systems became commercially available, testing of airborne substances often was conducted by applying dissolved test articles directly onto the lung cells. Such exposures not only fail to capture what happens in vivo, but also may introduce irrelevant chemical interactions, limiting the translational value of any findings (Aufderheide 2005; Limbach *et al.* 2005; Gminski *et al.* 2010; Raemy *et al.* 2012; Lenz *et al.* 2013; Loret *et al.* 2016; Upadhyay and Palmberg 2018). Dwivedi and colleagues demonstrated that solubilization of chemicals in culture medium blunted the effects of three pulmonary irritants (i.e., acrolein, crotonaldehyde, and hexanal), making direct comparison between the vapor- and aqueous-phase exposures difficult (Dwivedi *et al.* 2018). Furthermore, interpretation of studies on chemicals known to either react with components of culture medium (Coyle *et al.* 2018) or partition into the non-bioavailable compartments of the medium, such as lipids and proteins (Gülden and Seibert 2003), must consider the effects of these factors on chemical bioavailability. The latter scenario applies particularly to lipophilic agents, such as aryl hydrocarbon receptor agonists (Lee *et al.* 2011; Boei *et al.* 2017). Finally, agglomeration in culture medium is a major problem in testing NPs. Conceivably, these confounding factors could limit the value of in vitro toxicity assessments.

Tarkington and colleagues first described exposing cultures directly to test atmospheres at the air interface to simulate in vivo inhalation exposures (Tarkington *et al.* 1994). Ideally, design of such exposure systems meets the following

criteria: (1) cultivation of lung cell models under ALI conditions, (2) direct contact between airborne test articles and cells, and (3) homogeneous deposition of the test article across the culture surface. Commercial exposure systems have been developed based on these criteria (Aufderheide and Mohr 1999). Direct exposure of the semi-dry apical side of the airway epithelium to test articles in aerosol, vapor, or gas form not only reproduces the proximal airway exposure experienced by humans, but also facilitates more realistic cell-chemical interactions and faster uptake kinetics of the test articles than achieved by treatment of submerged cultures (Lenz *et al.* 2013; Loret *et al.* 2016). It is recommended to wash the apical side of the ALI airway cultures with PBS to remove excess mucus secreted by the goblet cells. This is an important practice to ensure homogenous delivery of test articles throughout the culture surface (Jackson *et al.* 2018). Dosimetry measurement under such conditions can be made by chemical analysis or using quartz crystal microbalances (QCMs) (Lenz *et al.* 2014; Wang *et al.* 2019a). However, QCM measurement must be vigorously validated to decouple mass deposition from the influence of viscoelasticity of test articles on vibration of the crystals (Parlak *et al.* 2013; Wang *et al.* 2019a).

A variety of exposure systems have been developed for direct exposure of lung cells to test articles in their physiologically relevant forms (Aufderheide and Mohr 1999; Bitterle *et al.* 2006; Lenz *et al.* 2013; Thorne and Adamson 2013; Lacroix *et al.* 2018; Tsoutsoulopoulos *et al.* 2019; Wang *et al.* 2019a; Wang *et al.* 2019b). These exposure systems range from those designed and used by individual research laboratories (Müller *et al.* 2011; Gualerzi *et al.* 2012; Amatngalim *et al.* 2015; Ji *et al.* 2017; Dwivedi *et al.* 2018) to widely available systems that are commercially marketed (Aufderheide and Mohr 1999; Okuwa *et al.* 2010; Li *et al.* 2012; Tsoutsoulopoulos *et al.* 2019). Gases, NPs, aerosols, particles, and complex aerosols (e.g., CS and diesel exhaust) have been tested using specialized systems that expose the apical surface of ALI airway cultures (Aufderheide and Mohr 2004; Aufderheide and Gressmann 2007; Okuwa *et al.* 2010; Lenz *et al.* 2013; Nara *et al.* 2013; Lenz *et al.* 2014; Wang *et al.* 2019a; Wang *et al.* 2019b). In the following sections, we will discuss the adaptability and applicability of select commercial exposure systems manufactured by Vitrocell® (Vitrocell® Systems GmbH, Waldkirch, Germany) and CULTEX® (CULTEX Laboratories GmbH, Hannover, Germany), two major equipment manufacturers in the field of in vitro inhalation toxicology.

Vitrocell® Systems A variety of airborne substances, such as NPs, gases, and complex mixtures (e.g., CS and e-cigarette [e-cig] aerosols) have been tested using exposure systems designed and manufactured by Vitrocell® (Lenz *et al.* 2014; Neilson *et al.* 2015; Polk *et al.* 2016; Ding *et al.* 2017; Fields *et al.* 2017; Wang *et al.* 2019a). These Vitrocell® exposure systems are discussed in this section.

a Liquid Aerosol Exposure Systems

The Cloud System was developed for single exposures of liquid aerosols under ALI conditions. It consists of a nebulizer (Aeroneb® Pro, Aerogen, Galway, Ireland), a removable exposure chamber, a base cultivation module with one position having a QCM for real-time mass deposition measurement, and a heating unit for maintaining a temperature of 37°C during exposure (Fig. 3*b*). During aerosol exposure, a dense cloud of droplets is produced by aerosolization of chemical solutions/suspensions through the piezoelectrically driven vibrating membrane in the nebulizer. Droplets form a uniform aerosol cloud inside the exposure chamber and uniformly deposit on the apical side of the cells housed in the base module over a period of 5 minutes. The overall deposition efficiency and dose rate of test articles is high, making it amenable for drug testing. The Cloud System has been used for the efficacy testing of pulmonary drugs and the safety evaluation of NPs and airborne particles (Lenz *et al.* 2014; Chortarea *et al.* 2017; Röhm *et al.* 2017; Wang *et al.* 2019a). Reproducible, spatially uniform deposition of test aerosols in each position of the exposure module was confirmed by fluorometric and chemistry analysis (Lenz *et al.* 2014; Röhm *et al.* 2017; Wang *et al.*, 2019a). Furthermore, aerosolized vehicles (e.g., phosphate-buffered saline) by themselves do not affect cell viability, CBF, or IL-8 induction in lung models, indicating that operation of the Cloud System does not induce undesirable stress to the cells (Lenz *et al.* 2014; Wang *et al.*, 2019a). Altogether, these observations support its application for exposing cells to liquid aerosols generated from non-volatile agents, NPs, and therapeutics. Compared with the more complex aerosol generation systems, the Cloud System is lower in cost, smaller in dimensions, and easier to maintain. However, this simple setup may not be optimal for simulating environmental or occupational exposures where subjects are exposed to airborne toxicants for hours on a daily basis. A continuous aerosol generation system, such as the BioAerosol Nebulizing Generator, equipped with a reservoir for solutions and suspensions, in combination with an in vitro exposure system, such as the 24/48 exposure module, could overcome this limitation. Test articles are continuously delivered to the nebulizer at a predefined speed by a peristaltic pump, allowing precise adjustment of aerosol concentrations without altering the airflow rate. Aerosols can be further diluted to lower concentrations before entering the exposure module. This setup has greatly broadened the range of test substances as well as dosing regimens for toxicity assessment. Manche and colleagues evaluated the genotoxicity of 3 alcohols that are commonly used for prevention of nosocomial infections (Manche *et al.* 2018). Mucoepidermal lung carcinoma NCI-H292 cells grown under ALI conditions were exposed intermittently to up to 85% (w/w) alcohol aerosols for a total

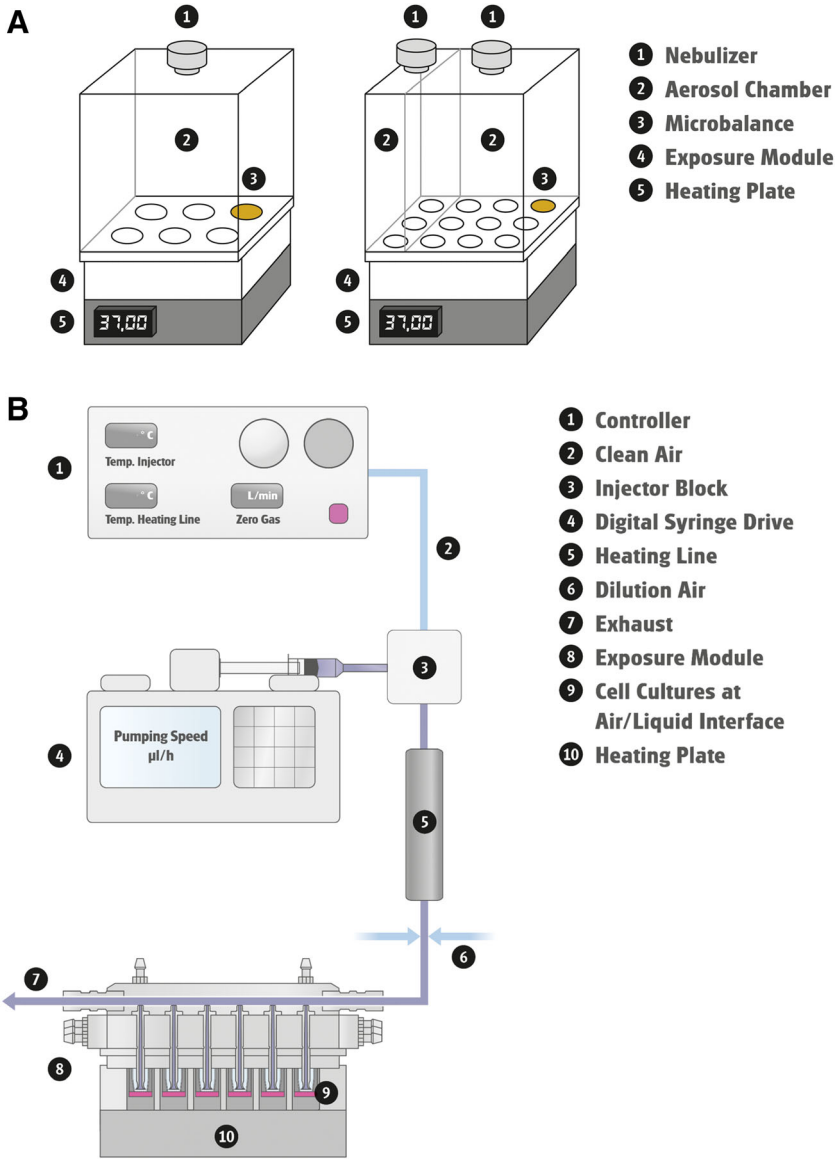

Figure 3. Representative in vitro exposure system from Vitrocell®. (A) Vitrocell® cloud system. Configurations with single or double aerosol chambers and various insert sizes are available. The setup with double chambers (on the right) allows conducting vehicle exposures simultaneously with chemical exposures. (B) Vitrocell® Spiking System.

of 60 minutes. Responses in both in vitro micronucleus and comet assays were negative at all concentrations tested.

b Vapor Generation and Exposure Systems

The Vitrocell® Spiking System is designed for exposing ALI airway cultures to gases, vapors, and gases generated from volatile and semi-volatile substances. This system consists of two components, a vapor generation system and a cell exposure module. The vapor generation system has a controller for adjusting the flow of clean air and system temperature and a digital syringe drive for injecting test articles at a predefined speed (Fig. 3*B*). Volatiles or semi-volatiles are vaporized by heating to their boiling points at the injection site. Vapors then are delivered through a heated line to the cell exposure module, where secondary dilution of the test articles takes place by mixing incoming vapors perpendicularly with clean (dilution) air. Concentrations of the test articles can be further adjusted by changing the rate of secondary dilution air flow. The cell exposure module houses the lung models, supplies culture medium to basolateral side of the ALI cultures, and maintains temperature and humidity during the treatment. The test articles are drawn from the dilution system into the exposure module and through a trumpet system by a steady vacuum. Most Vitrocell® exposure modules employ the trumpet technology for active diffusion and deposition of vaporized and aerosolized test articles onto the cells. Performance of the 24/48 exposure module, which exposes up to 48 culture inserts simultaneously, was characterized for CS delivery (Majeed *et al.* 2014). Results from multiple measurements, including photometry, in-line QCM, and chemistry analysis, demonstrated uniform distribution of CS vapors and particles,

with an observed coefficient of variation of 12.2% between inserts.

Recently, Oldham and colleagues conducted an in-depth evaluation of the 24/48 exposure module on the deposition efficiency and uniformity of monodisperse solid fluorescent particles with various mass median aerodynamic diameters (MMAD) and compared the experimental measurements with computational fluid dynamics (CFD)–predicted values (Oldham *et al.* 2020). Deposition of these particles across the surface of culture inserts was visualized using fluorescence microscopy. In general, deposition efficiency was highly dependent upon MMAD and in good agreement with CFD prediction. However, uniformity of deposition within individual insert exceeded ± 45% of the mean for particles with smaller MMAD. The heterogeneity of particle deposition within the culture inserts revealed by this study stressed the importance of determining the appropriate number of culture replicates based on both aerosol size and the sensitivity of the in vitro endpoints when using this exposure system for particle toxicty testing.

For particles and aerosols, QCMs can be incorporated into the exposure module for real-time monitoring of the delivered doses (Adamson *et al.* 2013). Although QCMs are not applicable for a system intended for gas exposures, in-line Fourier transform infrared (FTIR) spectroscopy may be used to provide dose measurements in real time. By using the Spiking System, cells can be efficiently and consistently exposed at the ALI to ppm or ppb concentrations of vapors and gases under conditions simulating respiratory exposures in vivo.

The vaporization efficiency of volatile and semi-volatile test substances depends on their chemical and physical properties, such as boiling point, stability, and purity. Theoretically, the Spiking System can vaporize chemicals with boiling points of up to 400°C. Some volatiles and semi-volatiles, however, become degraded or polymerize at temperatures even below their boiling points. When working with these types of chemicals, the highest temperature, at which these undesirable side reactions do not occur, should be used to guide the choice of a heating temperature. However, vaporization efficiency will decrease at lower temperatures, as will the maximal achievable concentration. The range of in vitro test concentrations, therefore, may be limited, which is a major disadvantage for testing thermally unstable chemicals or chemicals with tendency to polymerize at elevated temperatures. The vaporization capacity of the Spiking System also can be limiting. When the injection volume per unit time exceeds the system capacity, droplets will form at the tip of the injection syringe, which will interfere with vapor generation, resulting in large variations in delivered concentrations. Droplet formation is highly likely to occur with chemicals in aqueous solutions, such as formaldehyde.

To ensure consistent generation of vapors and gases, in-line real-time dose monitoring systems, such as gas chromatography-mass spectrometry or FTIR, are highly recommended. Alternatively, test articles can be collected into impingers connected at the end of the vapor generation system and analyzed chemically. However, such methods examine only the average vapor concentrations over a predefined period of time and, therefore, may overlook short-term variations in vapor concentrations. To ensure exposure of cells to consistent concentrations of vapors throughout the study, especially when conducting repeated exposures, it is recommended that the vapor generation system be validated on a daily basis.

Very few studies have been conducted using the Spiking System and most of them have not been published in peer-reviewed journals. However, the Spiking System has been used to evaluate the respiratory toxicity of vapors generated from styrene, formaldehyde, and acrolein (Wang *et al.* 2019b; Brandwein *et al.* 2020; Ren *et al.* 2020). Wang and colleagues repeatedly exposed human ALI cultures to styrene vapors to simulate inhalation exposure in humans. Styrene vapors were collected into impingers containing methanol and the concentration measured by analytical chemistry before conducting each cell exposure. System validation demonstrated a vapor generation efficiency with variations of less than 10% (Wang *et al.* 2019b). The 3M Strategic Toxicology Laboratory used the Spiking System to expose cells to acrolein vapors at the ALI. Comparison with findings made under the submerged culture conditions revealed that acrolein vapor induced dose-dependent toxicity at much lower concentrations (Brandwein *et al.* 2020). More testing should be conducted in order to comprehensively evaluate the performance and capability of the Spiking System and develop standard methods for system validation and cell exposure.

c Cigarette Smoking Robots

Early in vitro assessments of CS were conducted by exposing submerged lung cells to cigarette smoke extract (CSE) or condensate (CSC) diluted in physiological buffer or culture medium (Thaikoottathil *et al.* 2009; Schamberger *et al.* 2015). Given the complex composition of CS, such exposure methods fail to simulate the exposure experienced by smokers and may influence the outcomes of in vitro studies (St-Laurent *et al.* 2009). To overcome these limitations, whole smoke exposure systems have been developed for conducting cell exposures. Commercial smoking robots, such as Vitrocell® VC1 and VC10 (Adamson *et al.* 2017; Keyser *et al.* 2019) and Borgwaldt RM20S (Phillips *et al.* 2005; Azzopardi *et al.* 2015), have been used for exposing cells to freshly generated CS aerosols under ALI conditions and using standard

or customized smoke-generating regimens. The Vitrocell® Smoke Exposure Systems allow for consistent and reproducible generation and delivery of CS or e-cig aerosols to ALI cultures (Adamson *et al.* 2014; Behrsing *et al.* 2018). In general, these systems consist of two main components, i.e., a smoking machine for generating CS or e-cig aerosols and an exposure chamber for diluting and delivering the aerosols to the cells. VC1 is a manual smoking machine. Its design is based on the VC10 Smoking Robot technology. The scope of application of both VC1 and VC10 ranges from in vitro experiments to chemical analysis (Adamson *et al.* 2013; Thorne *et al.* 2013).

Extensive characterization of the Vitrocell® Smoke Exposure System demonstrated consistent particulate deposition, vapor phase marker CO delivery, and biological responses (Thorne *et al.* 2013). In a recent study, nicotine was validated as a CS dosimetry marker for the VC1 smoking robot (Behrsing *et al.* 2018). In another study, deposition of CS particles generated by the VC10 smoking robot was measured using QCM and results compared across 6 independent laboratories. Little or no difference in particle deposition between positions within the exposure module was observed, demonstrating reproducible performance of the VC10 smoking robots. To adapt the conventional smoking robots for e-cig research, Vitrocell® VapeStarters are used for activating various e-cig products and, therefore, have greatly expanded the range of tobacco products that can be tested with the systems. Aerosol deposited mass per puff as well as nicotine and carbonyl concentrations are commonly used for e-cig dosimetry measurement (Adamson *et al.* 2017; Iskandar *et al*, 2019; Bishop *et al.* 2019).

CULTEX® Systems First-generation CULTEX® exposure modules exposed cells to test chemicals at the ALI using specially designed inlet nozzles positioned in close proximity to the cells. These exposure modules were used for the in vitro assessment of particles, mineral fibers, wood dust, volatile compounds, CS, and therapeutics under ALI conditions (Ritter *et al.* 2001; Aufderheide *et al.* 2003; Aufderheide and Mohr 2004; Aufderheide and Gressmann 2007; Deschl *et al.* 2011). Characterization of the CULTEX® modules was carried out by validating gas delivery in the exposure module and assessing particulate deposition using fluorescence spectrophotometry (Ritter *et al.* 2001; Aufderheide *et al.* 2003; Deschl *et al.* 2011). Reproducible and dose-dependent particle deposition was demonstrated in these studies.

Unlike the linear design of the Vitrocell® Exposure Modules and first-generation CULTEX systems, newer CULTEX® Exposure Systems have adopted a radial flow system (RFS) (Aufderheide *et al.* 2011). Radial arrangement of the cell cultures mitigates against formation of a test article concentration gradient. The test atmosphere is introduced into the exposure module through a central inlet, allowing uniform delivery, distribution, and deposition of the test article within the device (Aufderheide *et al.* 2013). CFD was also used to determine particle number and mass distribution of the test articles in this study. CULTEX® RFS Exposure Systems have been used for toxicity assessment of CS, NPs, and gases (Aufderheide *et al.* 2011; Aufderheide *et al.* 2013; Rach *et al.* 2014; Steinritz *et al.* 2013; Tsoutsoulopoulos *et al.* 2019). A modified version of the exposure module was used for analyzing the mutagenic potency of airborne substances in the AMES assay (Aufderheide *et al.*, 2011). In a recent study, the acute toxicity of a set of 20 pre-selected test articles was assessed using the CULTEX® RFS and compared with in vivo reference data (Tsoutsoulopoulos *et al.* 2019). This study demonstrates a high level of concordance, specificity, and sensitivity as well as good intra/inter-laboratory reproducibility for the in vitro findings. CULTEX® offers a diverse range of exposure systems for in vitro toxicology research, including the CULTEX® RFS, CULTEX® RFS Compact, and CULTEX® LongTermCultivation-Continuous (LTC-C), all based on the radial flow design.

Applications of ALI Cultures

Owing to their physiological similarity to in vivo airway epithelium, primary cell–based ALI tissue cultures have been employed as in vitro models for elucidating the mechanisms underlying respiratory diseases, including acute epithelial injury, fibrosis, COPD, asthma, and cancer, as well as for studying pathogen-host interactions. In recent years, these models have been increasingly adapted for in vitro respiratory toxicity testing. Early studies relied on the direct application of the test article onto the apical side of the cultures to mimic inhalation exposure or addition to the basolateral medium to simulate systemic exposure. Although such exposures are easy to conduct, the physiological relevance of treating the air interface of ALI cultures with liquid solutions has been questioned. The development of aerosol/vapor generation and delivery systems has greatly expanded the range of test articles to include nanomaterials, volatile chemicals, and non-volatile chemicals, as well as CS aerosols and e-cig vapors, with exposures simulating in vivo inhalation exposures under an in vitro setting. Key applications of ALI cultures for respiratory research (summarized in Table 1) will be highlighted in the following sections.

In Vitro Toxicity Testing for Inhaled Agents

a Nanoparticles

Engineered nanomaterials (ENMs) are used in a wide range of applications. As the production and usage of

Table 1. Summary of applications using primary cell–based ALI airway tissue models

Areas of application	Description	References
Pulmonary drug testing	Studying deposition and absorption of inhalable drugs	Acosta *et al.* 2016; Reus *et al.* 2014
	Studying active and passive drug transport	Lin *et al.* 2007; Madlova *et al.* 2009
	Evaluate efficacy of antiviral and antibiotic drug candidates	Brockman-Schneider *et al.* 2014; Mata *et al.* 2012; Outlaw *et al.* 2019; Palermo *et al.* 2009; Tanabe *et al.* 2011; Triana-Baltzer *et al.* 2010; Zimmermann *et al.* 2009
Pathogen-host interaction	Respiratory viral infection, e.g., influenza virus, rhinovirus, respiratory syncytial virus, and SARS-CoV-2 coronavirus	Matrosovich *et al.* 2004; Zhang *et al.* 2005a; Jakiela *et al.* 2008; Zhang *et al.* 2002; Dijkman *et al.* 2009; Kindler *et al.* 2013; Zhu *et al.* 2020; Pyrc *et al.* 2010; Banach *et al.* 2009
	Respiratory bacterial infection, e.g., *Bacillus anthracis*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa*	Powell *et al.* 2015; Gasperini *et al.* 2017; Guevara *et al.* 2016; Raffel *et al.* 2013; Schwab *et al.* 2002; Balder *et al.* 2009; Prince *et al.* 2018; Reuschl *et al.* 2017; Matsuyama *et al.* 2018; Zhang *et al.* 2005b; Zulianello *et al.* 2006; Soong *et al.* 2011; Verkaik *et al.* 2014
Toxicity testing for inhaled chemical agents	Nanoparticles (NPs)	
	Evaluating biological and toxicological effects of inhaled NPs	Tilly *et al.* 2020; Kooter *et al.* 2017; Geiser *et al.* 2017
	Assessing pathological effects of NPs on normal and diseased airway and relate the data to existing epidemiological studies	Sacks *et al.* 2011; Kooter *et al.* 2019; Ji *et al.* 2019; Geiser *et al.* 2017; Beyeler *et al.* 2018
	Complex mixtures	
	Testing toxicity and biological effects of inhaled complex mixtures, e.g., e-cig, gasoline/diesel exhaust, and wood-burning stove particulates	Adamson *et al.* 2013; Adamson *et al.* 2014; Thorne *et al.* 2013; Behrsing *et al.* 2018; Corbett *et al.* 2019; Moses *et al.* 2017; Antherieu *et al.* 2017; Fields *et al.* 2017; Scheffler *et al.* 2015; Zarcone *et al.* 2016; Zarcone *et al.* 2017; Zarcone *et al.* 2018; Künzi *et al.* 2015; Vaughan *et al.* 2019; Hawley and Volckens, 2013; Shaykhiev *et al.* 2011; Shaykhiev and Crystal, 2014; Forteza *et al.* 2012; Iskandar *et al.* 2015; Ishikawa and Ito, 2017; Aufderheide *et al.* 2017; Amatngalim *et al.* 2018; Mertens *et al.* 2017; Schamberger *et al.* 2015
	Studying genome-wide association of ontological responses to complex mixture exposure	Corbett *et al.* 2019; Moses *et al.* 2017
	Single chemicals	
	Testing acute and sub-acute toxicity of inhaled single chemicals	Seagrave *et al.* 2010; Lantz *et al.* 2001; Tollstadius *et al.* 2019; Wang *et al.* 2019a
	Evaluating mechanisms of toxicity induced by single chemical exposure	Gwinn *et al.* 2017; Foster *et al.* 2017; McGraw *et al.* 2020; Kelly *et al.* 2014; Foster *et al.* 2017; McGraw *et al.* 2020 Brass *et al.* 2017; Kelly *et al.* 2019

ENMs surge, risks of exposure to NPs also increase, especially considering that aerosolization of NPs is unavoidable during manufacturing processes (Cassee *et al.* 2011; Lu *et al.* 2012; Zhu *et al.* 2017; Evangelista *et al.* 2019). Respiratory exposure to NPs is anticipated during the production, usage, and disposal of ENMs, which have demonstrated their cytotoxicity, genotoxicity, fibrogenicity, and carcinogenic potential in both in vivo and in vitro models (Shvedova *et al.* 2008; Li *et al.* 2010; Wang *et al.* 2011). However, such risks have not been well characterized due to many challenges, including the impracticality of assessing the toxicities of the vast number of ENMs in animals. With the rapid development in nanotechnology resulting in an expected increase in human respiratory exposure, cost- and time-effective methods are urgently needed to expedite the toxicity evaluation of ENM aerosols (Durantie *et al.* 2017; Clippinger *et al.* 2018).

Traditional in vitro approaches using submerged cell cultures have been widely used as a primary choice for toxicity screening of inhaled NPs. However, it is increasingly recognized that the characteristics of NPs under submerged conditions deviate from that of the inhaled "dry" NPs to which humans are exposed in vivo. For example, interaction of NPs with culture medium components could affect their hydrodynamic dimeter, agglomeration, morphology, and

surface modification; these factors will affect the settling rate and efficiency of NPs, which directly impact their delivered doses in vitro (DeLoid *et al.* 2015; Moore *et al.* 2015; Roszak *et al.* 2016). Some metal oxide NPs can be solubilized in culture medium; solubilization of NPs, however, is known to artificially alter their biological activities under submerged exposure conditions (Utembe *et al.* 2015; Kornberg *et al.* 2017). Exposing NP aerosols at the air interface could circumvent many of these issues and, overall, better represents how pulmonary NP exposures occur in vivo. Several studies have compared cellular responses between submerged and air-interface conditions (Raemy *et al.* 2012; Tilly *et al.* 2020). The deposited doses per surface area were first calculated for the respective studies to ensure that the comparison was made at equivalent doses. For submerged cultures, deposited doses can be estimated based on the administered concentrations, particle characteristics, and particokinetics (Deloid *et al.* 2014). For NP aerosol exposure, surface doses can be directly measured using QCM or atomic absorption spectroscopy (Raemy *et al.* 2012; Tilly *et al.* 2020). Differences were noted in dose- and time-response patterns and lowest-observed-adverse-effect levels. Primary cell–based ALI airway cultures have been increasingly used for NP toxicity evaluation (Kooter *et al.* 2017). The physiological relevance of the primary cell–based ALI cultures, along with their air interface, provides several advantages for nanotoxicology research, including the following: (1) minimal NP agglomeration, (2) avoidance of hydrolytic reactions or surface modifications of NPs by culture medium, (3) controlled and measurable delivered doses, and (4) assessment of airway tissue responses to test NPs. Geiser and colleagues conducted toxicity evaluations of silver (Ag) and carbon (C) NP aerosols in ALI cultures using a portable nano aerosol exposure system (Geiser *et al.* 2017). A single, short-time exposure to these aerosols induced moderate cytotoxicity and pro-inflammatory responses in a dose-response manner. In another study, Kooter and colleagues compared the toxicity of pristine (nCuO) and carboxylated ($nCuO^{COOH}$) NP aerosols in ALI cultures from asthmatic and healthy donors (Kooter *et al.* 2019). Asthma enhanced the sensitivity of the airway models to these NPs, possibly due to a combination of the hyperreactive respiratory tract and inefficient MCC in these cultures. The effect of donor disease state on tissue vulnerability to external stimuli was further explored in a study on aerosolized carbon nanoparticles (CNPs). ALI cultures mimicking normal and chronic bronchitis (CB) mucosa were exposed to CNP aerosols using the XposeALI® system (Ji *et al.* 2019). In general, responses involving inflammation, oxidative stress, tissue repair, and cell type–specific marker gene expression were more pronounced in the ALI airway cultures simulating CB. These in vitro findings are consistent with epidemiological studies where an association was established between the susceptibility of CB subjects and exposure to particulate matter (Sacks *et al.* 2011). The concordance between the in vitro and in vivo observations supports the value of employing ALI airway tissue models for elucidating mechanisms underlying the vulnerability of predisposed individuals to NP exposures. The reliability of this approach was demonstrated in studies that explored the toxicity of multi-walled carbon nanotubes (MWCNTs) (Beyeler *et al.* 2018). Airway ALI cultures derived from healthy and COPD donors were exposed acutely for 24 h to concentrations of MWCNTs that were set based on the permissible exposure limit of 1 $\mu g/m^3$ recommended by the National Institute for Occupational Safety and Health (NIOSH). Acute treatment at low doses of MWCNTs caused minimal changes in epithelial integrity, cell death, and inflammatory responses, while the concurrent positive control Dörentruper Quartz, which is known to induce pro-inflammatory cytokine expression, upregulated expression of a range of inflammatory and oxidative stress genes, regardless of the disease status. As MWCNTs pose hazards to workers at manufacturing facilities, long-term in vitro studies using repeated exposure regimens at human-relevant doses will be necessary to appropriately assess their potential risks. Such exposure regimens are only possible in cell models that have relatively long life spans, like ALI airway cultures. Taken together, these studies highlight the applicability and unique advantages of such in vitro approaches for comprehensive assessments of the health hazards posed by NPs.

b CS and e-Cig Aerosols

CS is a complex mixture of combustion products in the form of particulate matter, vapors, and aerosols (Rodgman and Perfetti, 2013), many of which are known carcinogens (Hecht 2012) and pulmonary irritants (Thorne *et al.* 2015; Gonzalez-Suarez *et al.* 2016). COPD, one of the respiratory diseases that are highly associated with CS exposure, is characterized in the airway epithelium by basal cell hyperplasia, squamous cell formation, loss of barrier function, decreased cilia biogenesis and function, and goblet cell hyperplasia (Shaykhiev and Crystal, 2014). These adverse tissue responses, in addition to key early molecular initiating events, have been reproduced using primary cell–based ALI airway cultures. Acute exposure to CS caused ontological, cytological, and genome-wide changes reflective of the in vivo pathogenesis associated with CS (Shaykhiev *et al.* 2011; Forteza *et al.* 2012). For example, CS-induced changes in the expression of xenobiotic metabolism gene in co-cultures of differentiated ALI cultures with fibroblasts are well-correlated with those observed in bronchial scrapings from smokers (Iskandar *et al.* 2015).

The in vitro endpoints measured in ALI cultures under different exposure protocols can be compared to the pathology observed in human airway epithelium to associate the impact of exposure metrics on CS toxicity. While several smoking regimens are accepted as the standards, the

Canadian Intense (CI) regimen, which often generates higher levels of reactive aldehydes and nicotine (Pazo *et al.* 2016), caused a more rapid induction in cytotoxicity compared to the International Organization for Standardization (ISO) 3308 regimen, although the maximal responses produced by the two protocols were similar (Bishop *et al.* 2019). Aufderheide and colleagues examined the effects of repeated CS exposures on metaplasia in ALI airway mono- and co-cultures with lung fibroblasts (Aufderheide *et al.* 2017). A total of 13 CS exposures induced the formation of non-hyperplastic cytokeratin 13–positive areas, accompanied by reduced mucus secretion, in both mono- and co-cultures. Studies examining the effects of repeated CS exposures on differentiation of the ALI cultures have also be conducted. Findings from these studies support the use of these cultures to model bronchial re-epithelialization during tissue repair as well as normal epithelial maintenance (Ishikawa and Ito, 2017). Differences in the toxicity of CS generated from cigarettes with different product designs can be characterized using the ALI systems. Although acute exposures are often used for these studies, the relatively longer life span of ALI airway cultures makes them amenable to repeated, subacute treatments (i.e., treatments that last up to several months), which can provide useful information on CS toxicity.

Apart from assessing the toxicity of CS by itself, studies have been conducted to understand the association between the host-pathogen interaction and pulmonary injuries in smokers. CS suppresses host defenses in the lungs of smokers (Herr *et al.* 2009). Sub-acute exposures of ALI cultures to CS decrease the expression of respiratory host defense proteins as a result of aberrant epithelial differentiation (Ishikawa and Ito, 2017), leading to a deficiency in the transepithelial transport of IgA (Amatngalim *et al.* 2018). Mertens and colleagues co-exposed ALI airway cultures to CS and IL-13 and investigated the effects of CS on pathogenesis of asthma (Mertens *et al.* 2017). The gene expression signature of IL-13 was irreversibly modulated by CS, demonstrating the importance of the interaction between the Th2 genes and CS in the development of asthma. These studies provided mechanistic evidence on the vulnerability of smokers to respiratory pathogens.

Electronic cigarettes (e-cigs) have been promoted as a safer alternative to conventional cigarettes; however, e-cig aerosols contain chemicals associated with pulmonary injury, including reactive aldehydes, particulate matter, and volatile organic compounds (Kosmider *et al.* 2014). Despite rapid growth in the use of e-cigs, their long-term health effects remain largely unknown. The recent outbreak of acute respiratory injury among e-cig users in the USA highlights the urgency of developing methodologies for assessing the potential risks of e-cigs and other novel tobacco products and has stimulated research on the toxicodynamics of e-cig aerosols (Perrine *et al.* 2019). Genome-wide association studies have correlated ontological responses in e-cig-exposed ALI cultures to those in bronchial scrapings from e-cig users (Moses *et al.* 2017; Corbett *et al.* 2019). The concordance between the in vitro and in vivo data suggests the suitability of employing ALI airway cultures for evaluating the pathological changes induced by inhaled substances, including CS and e-cig aerosols.

With regard to e-cig dosimetry, Shen and colleagues demonstrated that nicotine content was a leading predictor of e-cig aerosol cytotoxicity, as aerosols from nicotine-containing e-cig products were more potent than their nicotine-free analogues in ALI cultures (Shen *et al.* 2016). Relatively easy to quantitate, nicotine has been suggested as a dosimetry marker for in vitro e-cig aerosol exposures (Adamson *et al.* 2017; Bishop *et al.* 2019).

To date, most investigations on e-cig aerosol exposures have employed acute exposures (Scheffler *et al.* 2015; Moses *et al.* 2017; Antherieu *et al.* 2017; Fields *et al.* 2017). With the experience gained from repeated CS exposures, it should be possible to use airway ALI cultures to conduct sub-acute exposure studies with e-cig aerosols. Although the in vitro toxicity assessment of e-cig aerosols is still in its infancy, the primary cell–based ALI airway tissue model system has proven to be a relevant platform for screening alternative tobacco products as well as elucidating the mechanisms underpinning e-cig aerosol toxicity to human bronchial epithelium.

c Chemicals

Primary cell–based ALI culture systems have been used increasingly for chemical toxicity testing, particularly for testing irritants and oxidizers. Testing, however, has been impeded due to the difficulty in developing standardized methods for generating and delivering chemical aerosols and vapors, which is complicated by the vast differences in the physiochemical characteristics of test articles with potential health concerns. Gases and vapors readily reach pulmonary tissues by respiration. There are many examples where ALI cultures have been exposed to volatile agents using relatively crude exposure method that involve ad hoc approaches devised in-house, such as the vapor cup (Seagrave *et al.* 2010; Gwinn *et al.* 2017). A limitation of this approach, however, is that it relies on the theoretical estimation of a nominal vapor exposure concentration based on a head space calculation using the chemical partition coefficient and other factors (Kelly *et al.* 2014). True control of the exposure concentrations, ideally by performing real-time monitoring, can be technically and logistically challenging. Nevertheless, the simplicity of the vapor cup exposure method has a major advantage of eliminating the need for the complex vapor generation, monitoring, and exposure systems necessary for a

continuous flow-through in vitro inhalation exposure system. A simple procedure of direct application of chemical solutions or suspensions has also been shown as a quick and useful screening method for acute respiratory toxicants and irritants. After pipetting the suspension or solution of chemical test agent directly onto the apical tissue surface, the tissue insert is capped to prevent evaporation of vehicle or volatile components. Tissue viability is determined following a 3-h incubation period. This method correlates well with Global Harmonized System (GSH) and EPA acute inhalation toxicity categories determined by in vivo rat inhalation exposures studies and allows for relatively fast screening of large numbers of chemicals (Jackson *et al.* 2018).

Human ALI airway models have been extensively used to evaluate potential mechanisms of airway toxicity and fibrosis (obliterative bronchiolitis) induced by occupational exposure to artificial butter flavoring chemicals, such as 2,3-butanedione (diacetyl [DA]), including DA-induced basal cell effects (Gwinn *et al.* 2017; Foster *et al.* 2017; McGraw *et al.* 2020) and involvement of EGFR signaling pathway (Kelly *et al.* 2014; Foster *et al.* 2017; McGraw *et al.* 2020). Exposure of ALI airway mono- or co-cultures with donor-matched fibroblasts to DA vapors using vapor cups caused airway epithelial injury with histopathological evidence of basal/suprabasal cell spongiosis (Gwinn *et al.* 2017) as well as hyperphosphorylation and cross-linking of basal cell–specific keratins (Foster *et al.* 2017). In another study, McGraw and colleagues demonstrated increased ubiquitination of keratin 5 and decreased expression of p63, both of which are basal cell markers, and proteasome activity was shown to be involved in basal cell repair of injury induced by DA vapors (McGraw *et al.* 2020). Collectively, these findings suggest that basal cells are a major target of DA toxicity. Analyses of the secreted proteasome in ALI airway cultures exposed to DA vapors revealed secretion of pro-inflammatory/pro-fibrotic cytokines and chemokines, including the EGFR ligands, TGFα, and amphiregulin (AREG), as well as IL-1α, IL-6, and IL-8 (which is EGFR-dependent) (Kelly *et al.* 2014; Brass *et al.* 2017; Gwinn *et al.* 2017; Kelly *et al.* 2019). These observations suggest an important role for EGFR signaling in DA toxicity.

Exposure to non-volatile agents also presents difficulties, considering the technical feasibility of agent delivery, dose quantitation, reactivity/partition of test agents within the delivery system, and the physiological relevance of the exposure modality. A balance between practicality and relevance must be struck to produce responses relevant to humans and with minimal artifacts. The development of aerosol exposure modules, such as the Vitrocell® Cloud System and similar setups, has significantly eased toxicodynamic studies of aerosolized non-volatiles (Lantz *et al.* 2001; Tollstadius *et al.* 2019), particularly for reproducibly generating aerosols over extended periods of time. Wang and colleagues repeatedly exposed ALI airway cultures to dihydroxyacetone (DHA), an active constituent of the sunless tanning products, once a week for up to 5 weeks (Wang *et al.* 2019a). While DHA induced dose-dependent reduction in mucin secretion and CBF, these effects were reversed upon cessation of the treatment, suggesting that DHA has only transient effects on pulmonary clearance and host defense mechanisms.

The adverse effects of gasoline and DE emissions also have been investigated in ALI airway tissue models (Künzi *et al.* 2015; Zarcone *et al.* 2018). DE was found to be cytotoxic in ALI cultures from healthy donors and increase intercellular permeability as well as expression of markers for inflammation and oxidative stress (Zarcone *et al.* 2016). A follow-up study using ALI airway cultures having underlying co-morbidities, i.e., cultures made from donors with COPD and cultures infected with *Haemophilus influenzae*, revealed that COPD and *Haemophilus* infection did not modulate the tissue responses to DE emissions (Zarcone *et al.* 2017). These results are not surprising as pulmonary changes observed in vivo rely on endogenous cell types (Zhao *et al.* 2009) that are not present in differentiated in vitro ALI airway cultures.

With the increasing use of biodiesel to offset fossil fuel consumption, the toxicity of biodiesel blends has become a health concern. Vaughan and colleagues assessed the cytotoxicity and inflammatory responses in ALI airway cultures to emissions from biodiesel blended with and without the fuel additive triacetin (Vaughan *et al.* 2019). Although combustion of biodiesel blends generated less particulate matter than diesel alone, their combustion products caused significantly greater cytotoxicity and secretion of IL-8, particularly with blends containing 10% triacetin. These findings are congruent with an in vivo study that reported increased inflammation and oxidative stress responses in mice exposed to biodiesel emissions (Yanamala *et al.* 2013).

In another study, the cytotoxicity and inflammatory responses induced by gasoline engine emissions were examined in ALI cultures using the CULTEX® exposure system equipped with an electrostatic precipitator (Vaughan *et al.* 2019). This study showed that gasoline engine emissions produced a dose-dependent induction of cytotoxicity, oxidative stress, and inflammatory responses. A customized electrostatic aerosol exposure system (Volckens *et al.* 2009) has been used to characterize exposures to indoor wood smoke in a manner simulating smoke exposure during cooking (Hawley and Volckens 2013). Increased generation of particulate matter 10 (PM_{10}: inhaled particles with diameters of 10 μm or less) was generally correlated with modulation of markers for

inflammation and oxidative stress, suggesting that traditional wood-burning stoves may lead to chronic diseases, a result that is in accordance with epidemiological findings (Po *et al.* 2011). Taken together, studies conducted in ALI airway cultures have generated valuable in vitro toxicity profiles of vehicle exhaust and environmental emissions that could be valuable for making in vitro-in vivo comparisons. While some studies have included ALI airway models made from sensitive populations, e.g., COPD, more research is required to examine the accuracy of these models to recapitulate toxicity responses among high-risk populations.

Pulmonary Drug Testing The large surface area of human lungs is characterized by a unique air-epithelium interface that is well-suited for drug absorption and uptake. Drug administration by inhalation is a non-invasive route that can bypass the first-pass metabolism by the liver, overcome poor absorption of drugs by the gastrointestinal tract, and reduce systemic side effects (Patton and Byron 2007). Pulmonary drug delivery also provides an excellent solution for delivering locally acting drugs to treat respiratory diseases, such as asthma, cystic fibrosis, COPD, and CB (Laube 2014). Novel aerosol delivery devices have enabled the local deposition of accurately controlled concentrations of aerosolized medications in the lung (Chandel *et al.* 2019). So far, there are more than 20 active pharmacological ingredients (APIs) that have been formulated as inhaler products (Labiris and Dolovich 2003; Stein and Thiel 2017). The clinical efficacy of inhaled drugs depends on the effectiveness of aerosol generation as well as factors affecting their bioavailability, such as their regional deposition, absorption, and clearance (Olsson *et al.* 2011).

After deposition on the luminal surface of the respiratory epithelium, aerosolized drugs first need to overcome removal by mucociliary escalators (Haghi *et al.* 2014). Residual drugs then are transported into the cell via either passive or active mechanisms, depending on their molecular size, lipid solubility, and site of absorption (Olsson *et al.* 2011). Ninety percent of small molecular weight (150–200 Da) hydrophobic drugs are rapidly absorbed by passive diffusion (Patton *et al.* 2004). In vitro ALI airway models have been employed for assessing the deposition and absorption of inhalable drugs across the epithelium as well as for distinguishing between passive diffusion and active transport (Reus *et al.* 2014; Acosta *et al.* 2016). Lin and colleagues reported that the transepithelial permeability of a panel of anti-allergy drugs, including fexofenadine-HCl, albuterol, albuterol sulfate, dexamethasone, budesonide, and triamcinolone acetonide, was linearly correlated with their lipophilicity (Lin *et al.* 2007). Among these drugs, fexofenadine-HCl was transported symmetrically between the apical and basolateral sides of the ALI cultures. This observation is consistent with findings in human airways (Lechapt-Zalcman *et al.* 1997; Thiebaut *et al.* 1987) and may reflect either a relatively low expression of P-gp transporters in the ALI cultures or saturation of this drug transporter by high concentrations of fexofenadine-HCl.

Because of the importance of P-gp transporters for drug disposition, the effect of selective inhibitors, such as GF120918A, was evaluated using digoxin as a model substrate (Madlova *et al.* 2009). In this study, the P_{app} and efflux values were calculated for digoxin transport in the ALI cultures. GF120918A significantly increased the efflux ratio of digoxin transport only in ALI cultures that had been cultured for 21 days or longer. These results suggest that P-gp activity may depend on the differentiation status of the cultures, emphasizing the importance of using fully differentiated primary cell–based ALI cultures for drug transport research. In another study, Hoffmann and colleagues evaluated the absorption of over 30 chemicals and established threshold values for their permeability coefficient in ALI airway cultures (Hoffmann *et al.* 2018). Results from this work demonstrated that these cultures are an effective barrier model for studying the absorption kinetics of different classes of chemicals and generating useful information for toxicokinetic modeling and chemical risk assessment.

Human ALI airway cultures have been increasingly used for testing antiviral and antibiotic drug candidates (Zimmermann *et al.* 2009; Triana-Baltzer *et al.* 2010; Mata *et al.* 2012). Antimicrobial agents of different classes, such as macrolides, quinolones, and β-lactams, have been used widely in treating respiratory tract infections. Macrolides (e.g., azithromycin [AZM] and clarithromycin) and fluoroquinolones (e.g., maxifloxacin [MXF]) exert anti-inflammatory effects by attenuating cytokine release both in vitro and in vivo (Rubin and Henke 2004; Shalit *et al.* 2006; Tauber and Nau 2008). Zimmermann and colleagues further examined whether or not such immunomodulatory effects could be reproduced in airway epithelial cells (Zimmermann *et al.* 2009). ALI cultures were treated with cefuroxime (CXM), AZM, levofloxacin (LVX), and MXF at therapeutic concentrations, followed by treatment with TNF-α to simulate pro-inflammatory conditions. AZM and MXF were more effective than CXM and LVX in inhibiting the release of TNF-α-induced cytokines, such as GM-CSF and IL-8. The MXF finding is consistent with previous observations, suggesting that it may attenuate inflammation in bronchial epithelial cells.

In addition to their anti-microbial and anti-inflammatory activities, macrolides also mitigate mucus hypersecretion, a common feature seen in many respiratory diseases, such as asthma and COPD (Rubin *et al.* 1997; Kaneko *et al.* 2003; López-Boado and Rubin 2008; Ou *et al.* 2008). For instance, clarithromycin was found to inhibit IL-13-induced mucus hypersecretion and goblet cell hyperplasia in ALI cultures by inactivating the NF-κB signaling pathway (Tanabe *et al.* 2011). IL-13 is a pro-fibrotic cytokine secreted by T helper type 2 (Th2) cells that is associated with allergic responses in

humans (Gour and Wills-Karp 2015). These findings indicate that the ALI airway model may also be a useful tool for screening drugs to treat secretory hyperresponsive diseases, such as asthma.

Pathogen-Host Interaction Animal models are important tools for studying infectious diseases of the respiratory system. However, the animal model should be selected carefully due to differences in anatomy, physiology, respiration rates, and inhalation pharmacokinetics between species (Davies and Morris 1993; Harkema *et al.* 2006; Corley *et al.* 2012; Swearengen 2018). Furthermore, elucidating the mechanisms underlying microbial infections using animal models has always been challenging. In addition, the high cost and ethical concerns about conducting animal research contribute to the increasing popularity of using in vitro pulmonary cell models for conducting studies on respiratory pathogens. Among the existing in vitro lung models, physiologically relevant human ALI airway cultures provide a natural infection target for a variety of human respiratory pathogens, and thus are a valuable culture system for mechanistic studies on pathogen-host interactions.

***a*. Virus-Host Interaction** The interplay between host cells and viral factors in the pathogenesis of several common human respiratory viruses, including influenza virus (Matrosovich *et al.* 2004; Zhang *et al.* 2005a), rhinovirus (Jakiela *et al.* 2008), and respiratory syncytial virus (Zhang *et al.* 2002), has been investigated in ALI airway cultures. Utilization of ALI cultures derived from individuals with respiratory diseases, such as asthma (Hackett *et al.* 2011; Bai *et al.* 2015) or COPD (Osei *et al.* 2016; Osei and Hackett 2020), also allows the investigation of the role of pathogens in disease exacerbation. Human ALI airway cultures also have been employed for identifying novel antiviral drug candidates (Palermo *et al.* 2009; Mata *et al.* 2012; Brockman-Schneider *et al.* 2014; Outlaw *et al.* 2019). Triana-Baltzer and colleagues initially evaluated the pharmacodynamics and antiviral activity of DAS181 in ALI cultures (Triana-Baltzer *et al.* 2010). DAS181 is a sialidase fusion protein that effectively inhibits the entry of influenza virus by cleaving sialic acid receptors from airway epithelium. Approximately 80% of sialic acid receptors could be removed by DAS181 within 15 minutes, with the effects persisting for at least 2 days. Findings from this study also provided useful information on the treatment regimen and dose selection for DAS181, which is currently under clinical trials as Fludase® (Davidson 2018).

ALI airway cultures have been used to study the propagation kinetics and cytopathic effects of novel human viruses targeting the respiratory system; these are measurements that are difficult to make using traditional approaches (Banach *et al.* 2009; Dijkman *et al.* 2009; Pyrc *et al.* 2010; Kindler *et al.* 2013; Zhu *et al.* 2020). Recently, Zhu and colleagues used ALI airway cultures to characterize the cytopathic effects of a novel betacoronavirus associated with the COVID-19 outbreak (Zhu *et al.* 2020). Bronchoalveolar lavage fluid from patients with pneumonia was directly inoculated onto the apical surface of the ALI cultures and genome sequencing of isolated virus was conducted. Functional assays also revealed significant reduction in cilia beating and morphological changes were observed as early as 4 days after inoculation.

***b*. Bacteria-Host Interaction** Bacterial infections of the respiratory system normally begin with the adherence and colonization of pathogens on the apical surface of the airway epithelium, followed by internalization into host cells or bacterial invasion to reach the subepithelial space (Hasan *et al.* 2018). Polarized ALI airway cultures are a powerful tool for studying the infection of a range of human respiratory pathogens, such as *Bacillus anthracis* (Powell *et al.* 2015), *Bordetella pertussis* (Gasperini *et al.* 2017), *Haemophilus influenzae* (Raffel *et al.* 2013), *Burkholderia cepacia* (Schwab *et al.* 2002), *Moraxella catarrhalis* (Balder *et al.* 2009), *Mycoplasma pneumoniae* (Prince *et al.* 2018), *Mycobacterium tuberculosis* (Reuschl *et al.* 2017), *Mycobacteroides abscessus* (Matsuyama *et al.* 2018), *Pseudomonas aeruginosa* (Zulianello *et al.* 2006), *Staphylococcus aureus* (Soong *et al.* 2011), and *Streptococcus pneumoniae* (Verkaik *et al.* 2014).

Opportunistic bacteria, such as *P. aeruginosa*, invade airway epithelium with compromised TJs (Plotkowski *et al.* 1999). Zulianello and colleagues exposed ALI cultures to strains of *P. aeruginosa* expressing different virulence factors (Zulianello *et al.* 2006). The strains that secrete rhamnolipids most efficiently compromised barrier integrity and increased the paracellular permeability of the ALI airway cultures. Work by Zhang and colleagues identified Toll-like receptor 5 (TLR5) on the apical surface of the ALI cultures and defined its key role in mediating epithelial responses to *P. aeruginosa* (Zhang *et al*. 2005b). Considering the importance of TLRs in recognizing pathogens and activating the innate immunity of airway epithelium (Hopkins and Sriskandan 2005), findings from this study provide a better understanding of the mechanisms underlying bacterial invasion and the responses provoked in airway epithelial cells.

The multiple cell types that make up the ALI cultures have been used to study the cell type–specific pathogenic effects of respiratory pathogens like *Haemophilus influenzae* and *Bordetella pertussis*, which primarily adhere to non-ciliated cells (Ketterer *et al.* 1999) and ciliated cells (Tuomanen and Hendley 1983; Wilson *et al.* 1991; Soane *et al.* 2000), respectively. *B. pertussis* is the major pathogen responsible for whooping cough or pertussis in humans (Mallory and Hornor 1912). It is known to cause ciliostasis and disrupt MCC in airway epithelium. However, the molecular mechanism underlying its adherence and colonization has not been thoroughly investigated due to the lack of suitable in vitro

models. Guevara and colleagues developed a quantitative adherence assay in ALI airway cultures and identified multiple mutations in the fimbrial adhesin subunits that may contribute to *B. pertussis* adherence, confirming the essential role of FimD adhesion in this process (Guevara *et al.* 2016).

Conclusions

Efforts are currently underway to develop alternatives for in vivo inhalation toxicity testing by the development of in vitro airway/lung approaches (e.g., ALI airway models) consistent with the 3Rs principles of replacement, reduction, and refinement (Russell and Burch 1959). Conducting in vivo inhalation toxicity studies using whole-body or nose-only exposure systems is expensive and time-consuming and typically requires a large number of animals. The goal of using alternative methods, like human in vitro ALI airway cultures, ultimately is to replace inhalation toxicity testing in animals with in vitro approaches.

Before in vitro approaches can ever replace in vivo inhalation studies, however, ALI culture models must be fully validated to optimally reproduce the airway/lung biology of native tissue. Validation also should include assessing the reproducibility of the endpoints that can be measured with ALI cultures across different batches of both commercial and "home-made" models as well as the transferability of results between independent testing laboratories. One important element for validating any new assay for making regulatory decisions is determining its performance relative to an accepted standard (see, for instance, OECD, 2005). One problem with validating performance of ALI airway assays is that these models and endpoints are mainly developed using human tissue, while most reference data from an accepted validated test have been generated with rodents (e.g., studies conforming with OECD Test Guidelines 412 and 413). Although it is clearly the case that a human-based system will be more valuable for assessing human health risk than a rodent system, in this case, it may be necessary to develop rodent ALI airway data as an approach to bridge data between rodent and human. Ultimately, validated in vitro assays must be accepted by both regulatory agencies (e.g., US EPA, US FDA, Health Canada, NIEHS) and the industry as an alternative or replacement to in vivo testing. Acceptance often involves consensus-forming mediated by groups such as the Organisation for Economic Cooperation and Development (OECD) and The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).

Although ALI airway cultures have not yet been incorporated into regulatory guidelines, international workshops have been organized to standardize approaches for assessing airborne substances in these cultures (Clippinger *et al.* 2018; Lacroix *et al.* 2018). Efforts are ongoing to develop a set of criteria for standardizing every aspect of the studies utilizing ALI airway cultures, including culture preparation, assay performance, and exposure procedures. For instance, Institute for In Vitro Sciences (Bethesda, MD) has coordinated a multi-institutional validation study to develop standards for CBF measurement in ALI airway cultures. At this time, ALI airway culture models are not ready to fully replace inhalation studies in animals but can be effectively used to complement in vivo studies as a tool (1) to perform initial screening for chemical- or drug-induced toxicity in order to prioritize compounds for further (and more extensive) testing in vivo and/or (2) for mechanistic and follow-up evaluations. Such applications of ALI airway models can potentially refine how in vivo testing is conducted and reduce both the number of animals used for inhalation toxicity testing and the time for conducting the studies.

The use of standard in vivo models to predict adverse chemical- or drug-induced airway effects in humans can be complicated by species-specific differences in the respiratory tract between rodents and humans. Although these species differences produce challenges for validating in vitro airway models, viewed differently, a major advantage of ALI cultures is that the tissues can be of human origin and thus are reflective of human airway/lung biology. ALI cultures are composed of the critical functioning cell types present within the airway epithelium and, therefore, can be used effectively for evaluating human-relevant toxic effects and mechanisms involved in airway responses to chemical or drug exposures. In addition, ALI cultures can be used in conjunction with physiologically relevant exposure systems for vapors/gases, aerosols, or particles, although further optimization of high-throughput approaches is needed for efficient screening of large numbers of compounds.

Resident cells within ALI airway epithelial models can secrete pro- and anti-inflammatory cytokines; however, these cultures lack mesenchymal cells as well immune cells, such as neutrophils, monocytes/macrophages, and lymphocytes that are recruited from the circulation. These cells perform a critical role in regulating inflammation and the immune responses induced within the airways by external stimuli. Improved ALI culture models, therefore, are needed to incorporate these additional cell types as well as endothelial and mesenchymal cells to more realistically reproduce inflammatory responses. Full-thickness (FT) ALI models have been developed to include a thin basement membrane-like lining with a subepithelial collagen-rich matrix that contains donor-matched fibroblasts. Mechanical forces exerted within the airway/lung can also be engineered into ALI cultures. Microphysiological systems, such as lung-on-a-chip, have integrated flow and/or mechanical forces (e.g., stretching to reflect the breathing motion) into in vitro airway models. Additional work is needed to determine how well these in vitro airway models can be created to best recapitulate

airway fibrosis, remodeling, and repair from repeated injury with relevance to chemical/drug-induced sub-acute/chronic toxicity. It should be noted that one of the features of the basic ALI human airway model is that they remain structurally and functionally useful for months in culture, which lends them to conducting sub-acute dosing protocols, similar to those conducted in animal models and that occur with human exposures. Making a more complex model, however, may affect the stability of airway models, reducing their useable life span in culture.

Most testing with human ALI cultures has been conducted with culture models generated from single donors, with little consideration given to the age, sex, race, and health condition of the donor. There are data indicating that pre-existing diseases of the donors, such as asthma (Hackett *et al.* 2011; Bai *et al.* 2015) and COPD (Osei *et al.* 2016; Osei and Hackett 2020), can affect the biology of airway models in a way that can be used to assess the effects of inhalation exposures on susceptible populations. However, effects of lifestyle factors, including use of tobacco or electronic cigarettes and occupational or environmental chemical exposures, on airway epithelium may possibly be reversible when airway cells are removed from the context of the in vivo lung environment and subjected to long-term in vitro culturing in the absence of the initiating stimulus. For instance, while some gene expression changes caused by tobacco smoke are reported to be irreversible, many other changes are reversible following smoking cessation (Beane *et al.* 2007). Nevertheless, certain aspects of in vivo disease states can be recapitulated in vitro by supplementing the missing factors in cell culture. One example is to recapitulate goblet cell hyperplasia in in vitro bronchial models by exposing the cultures to cigarette smoke or by adding T cell–derived cytokines, such as IL-13, or other exogenous stimuli (Bolmarcich *et al.* 2018).

Thus, in order to use data from ALI airway cultures in a scientifically rigorous manner and understand variability among results, the effects of the inherent demographic differences among healthy, non-smoking donors on the responses measured in ALI cultures must be understood. One potential solution for minimizing variability among donors is developing models derived from a single induced pluripotent stem cell line that could be used for standardized testing. On the other hand, the study of cultures derived from a variety of healthy and diseased individuals (Hackett *et al.* 2011, Bai *et al.* 2015) will enable a more comprehensive understanding of the responses of both healthy and diseased human populations to environmental agents and therapeutics.

Conducting inhalation toxicology studies in vitro, under conditions simulating human inhalation exposures, is technically challenging. Although improvement and standardization are still needed, the combination of ALI airway tissue models and progress in developing exposure systems offer unique opportunities to advance in vitro respiratory toxicology and the human risk assessment for a broad range of airborne substances as well as chemicals that indirectly impact lung health.

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Compliance with Ethical Standards

Disclaimer The opinions expressed in this review are those of the authors and do not necessarily represent the official positions of the Food and Drug Administration, the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, or the National Toxicology Program positions or policies. Mention of any company or product does not constitute endorsement by the FDA, NIOSH/CDC, or NTP.

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