Recent Advances in Human Respiratory Epithelium Models for Drug Discovery

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Abstract

The respiratory epithelium is intimately associated with the pathophysiologies of highly infectious viral contagions and chronic illnesses such as chronic obstructive pulmonary disorder, presently the third leading cause of death worldwide with a projected economic burden of £1.7 trillion by 2030. Preclinical studies of respiratory physiology have almost exclusively utilised non-humanised animal models, alongside reductionistic cell line-based models, and primary epithelial cell models cultured at an air-liquid interface (ALI). Despite their utility, these model systems have been limited by their poor correlation to the human condition. This has undermined the ability to identify novel therapeutics, evidenced by a 15% chance of success for medicinal respiratory compounds entering clinical trials in 2018. Consequently, preclinical studies require new translational efficacy models to address the problem of respiratory drug attrition. This review describes the utility of the current *in vivo* (rodent), *ex vivo* (isolated perfused lungs and precision cut lung slices), two-dimensional *in vitro* cell-line (A549, BEAS-2B, Calu-3) and three-dimensional *in vitro* ALI (gold-standard and co-culture) and organoid respiratory epithelium models. The limitations to the application of these model systems in drug discovery research are discussed, in addition to perspectives of the future innovations required to facilitate the next generation of human-relevant respiratory models.

Keywords

Respiratory Epithelium, *In vitro*, *In vivo*, *Ex vivo*, 3D Cell Culture, Organoids, Air-liquid Interface.

Abbreviations

3Rs (Refinement, Reduction and Replacement), Absorption, Distribution, Metabolism, Excretion and Toxicology (ADMET), Air-liquid Interface (ALI), Arginine-Glycine-Aspartic acid-Alanine (RGDA), ATP-binding Cassette (ABC), Chronic Obstructive Pulmonary Disorder (COPD), Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9), Cystic Fibrosis (CF), Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), Drug Metabolism and Pharmacokinetic (DMPK), Epithelial-mesenchymal transition (EMT), Extra-cellular matrix (ECM), Forkhead Box protein J1 (FOX J1), Genome-wide Association Studies (GWAS), Hemagglutinin Type 1 and Neuraminidase Type 1 (H1N1), Human Bronchial Epithelial Cell (HBEC), Human Immunodeficiency Virus (HIV), Human Nasal Epithelial Cell (HNEC), Intercellular Adhesion Molecule 1 (ICAM-1), Interleukin (IL), Isolated Perfused Lungs (IPL), Knockout (KO), Matrix Metalloproteinase 1 (MMP-1), Middle East Respiratory Syndrome Coronavirus (MERS-CoV), Mucociliary Clearance (MCC), National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), Poly(ethylene glycol) (PEG), Precision Cut Lung Slices (PCLS), Quantitative Polymerase Chain Reaction (qPCR), Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), Thermally induced Phase-separation (TIPS), Three-dimensional (3D), Trans-epithelial Electrical Resistance (TEER), Transforming Growth Factor β (TGF-β), Tumor Necrosis factor-α (TNFα), Two-dimensional (2D), Tyrosine-Isoleucine-Glycine-Serine-Arginine (YIGSR), Zonula Occuldins (ZO).

1. Introduction

There exists a clear and present need to improve the human relevant tools at our disposal for mechanistic investigations of respiratory pathogenesis and therapeutic drug development. Current *in vivo* models almost exclusively utilise non-primate animals which have been indispensable for aiding advancements in the mechanistic understanding of pulmonary pathogenesis and therapeutic drug development (Bonniaud et al., 2018). However, despite their utility, these animal models have been limited by their poor correlation to the human condition. Organisations such as the NC3Rs (UK National Centre for the Replacement, Refinement and Reduction of Animals in Research) have accelerated efforts to move beyond the use of animals for scientific purposes. Recent advances in the multidisciplinary fields of complex 3D cell culture, biofabrication and microfluidics offer unique opportunities to address the problem of generating models which faithfully replicate the biological processes of human organs *in vivo*. This review highlights the current state-of-the-art of respiratory epithelium modelling, and describes some of the inefficiencies of the current respiratory translational models used in drug discovery and target validation (Hendrickx et al., 2018).

Preclinical drug development processes have been optimised with the aim of determining potential toxicities and efficacies of novel compounds to reduce the inherent risk of first-in-man studies. Whilst these processes vary according to the disease and target being studied, they utilise common frameworks. For example: Initial target discovery stages serve to establish potential therapeutic roles of enzymes and membrane-bound receptors for a known pathological hall mark of disease while assessing known mechanisms of toxicity (also selectivity, tractability etc). Next, tens of thousands to millions of novel or repurposed molecules housed in 'compound libraries' are screened for 'hits' against these identified targets ('hit identification'), usually via 2D cell-line based, high-throughput *in vitro* assays (Marx et al., 2016). More complex *in vitro* models are used during both the initial target discovery stages and subsequent target validation and lead optimisation studies, where promising early lead molecules are screened for their toxicity and efficacy and subsequently cut to candidate molecules. Finally, animal models are utilised during DMPK (drug metabolism and pharmacokinetic) and ADMET (absorption, distribution, metabolism, excretion and toxicology) studies to determine optimal dosages, potential side effects and drug-drug interactions. Only compounds delivering continual success to this stage are selected to progress into human clinical trials. However, the effectiveness of the current drug development process

has been undermined by its reliance upon the implementation of inadequate models that lack translatability to the human condition.

Despite earnest efforts, drug development has increasingly failed at phase II/III of human clinical trials, attributable to the lack of predictivity of human *in vivo* efficacy from the current *in vitro* models used in early stage studies, thus emphasising the need for better design and validation of models (Booth and Zemmel, 2004; Harrison, 2016). Novel therapeutic compounds entering clinical trials for respiratory disorders have shown a 7% and 15% chance of success from phase I and II trials respectively, and an overall success rate of less than 7% (Dowden and Munro, 2019). Though this figure is in the middle echelons of therapeutic-area dependent averages for novel compound success in clinical trials (3-16%) (Dowden and Munro, 2019), it still represents a huge drug attrition problem for the sector as a whole. Analysis into the causes of clinical failure across therapeutic areas showed between 2013-2015, 73% and 69% of all failures in phase II and III trials respectively, were due to insufficient efficacy and safety (48% and 25% in phase II and 55% and 14% in phase III respectively) (Harrison, 2016). Perhaps most worryingly, these figures remain largely unchanged as of 2016-2018, with 79% of overall failures due to safety and efficacy (with the remaining 21% citing operational, strategic and/or commercial reasons for failure) (Dowden and Munro, 2019).

Importantly, failures due to insufficient efficacy are almost twice as likely in phase II, and more than twice as likely in phase III, than failures due to toxicity (Harrison, 2016). This is directly attributable to the implementation of large-scale, standardised *in vitro* safety assays early in drug discovery. Here, the use of standardised safety assays via reductionist *in vitro* screening models, allow for efficient testing of molecules during hit-to-lead studies. This approach has been largely successful, and has contributed to a significant reduction in the number of failures due to toxicity in phase I and II trials (compared to efficacy failures (Dowden and Munro, 2019; Harrison, 2016)). However, this approach has yet failed to address the current drug attrition problem. Concerns pertaining to effectively determining efficacy have especially been undermined (Ledford, 2011).

Toxicity failures may be addressed in a number of ways, such as a greater use of humanized monoclonal antibodies relative to small molecules due to their reduced off-target toxicity (Paul et al., 2010). Efficacy failures have been attributed to the lack of control of bias in preclinical proof of concept studies, where removing such biases in preclinical assessments of efficacy may serve as an effective accelerator of clinical success (Lindner, 2007). Furthermore, a recent

analysis of 28 projects at AstraZeneca attributed 40% of project failures to insufficient target linkage to the disease and the availability, or lack thereof, of validated models (Cook et al., 2014). Therefore, addressing the unmet preclinical needs for efficacy testing with either more appropriate animal models (Kola and Landis, 2004), or increased use of complex *in vitro* models, may serve to significantly improve compound efficacy studies. Certainly, there exists a requirement for more predictive models in the target validation stages of drug development, practically in the form of organotypic *in vitro* human assays.

A wide array of functional *in vitro* models have been utilised for the study of the pathologies associated with the human respiratory system (Ball and Padalia, 2019; Fraser, 2005) (see FIG 1). Many of these systems utilise models of the respiratory epithelium, which serves to warm, moisten and remove harmful pathogens and particulates from inspired air. The most commonly utilised *in vitro* model of the respiratory airway is that of the tracheo-bronchial epithelium. Here, the extrapulmonary conducting airways are comprised of C-shaped hyaline cartilaginous rings (Kia'i and Bajaj, 2020), a collagenous submucosa (Fraser, 2005), and a pseudo-stratified ciliated epithelium supported by a fibroblast-laden lamina propria and basement membrane (Khan and Lynch, 2020). The tracheo-bronchial respiratory epithelium subsequently contains a number of specialised cellular phenotypes (see Table 1). Replicating each of these components in a model system, as well as the array of specialised cell phenotypes present, remains a challenge.

Bacterial and viral infections most frequently affect the upper respiratory tract in humans (Thomas and Bomar, 2020). The rhinovirus or common cold remains the most common of the viral infections, but others include the coronavirus, respiratory syncytial virus and the adenovirus. Inflammatory lung diseases also characteristically involve pathologies pertaining to the respiratory airway epithelia (Huang et al., 2011). These include chronic obstructive pulmonary disorder (COPD) which is currently the third leading cause of death worldwide, with estimates for a COPD-derived economic burden of £1.7 trillion by 2030 (Quaderi and Hurst, 2018).

Furthermore, asthma, the most prevalent respiratory disease in the world (GBD 2015 Chronic Respiratory Disease Collaborators, B et al., 2017), and the genetic, autosomal recessive disorder cystic fibrosis (CF), both develop pathologies that arise from a loss of respiratory epithelium function. The primary pathology of CF is characterised by the secretion of abnormally viscous mucus from goblet cells and serous mucus glands, which inhibits mucociliary clearance (MCC) and causes an increased risk of infection due to improper

clearance of the respiratory airways (Huang et al., 2011). Similarly, a loss of MCC caused by a reduced number of ciliated cells and goblet cell hyperplasia is an underlying physiology of bronchitis and COPD (Gohy et al., 2019). Loss of functions of the respiratory epithelium, such as a defective epithelial barrier derived from inhalation of cigarette smoke and environmental insults have been linked to the onset of COPD and asthma respectively (Gon and Hashimoto, 2018a; Xiao et al., 2011). These insults can damage the protein complexes present between various cells in the respiratory epithelia and cause a breakdown of paracellular transport mechanisms and a loss of efficient control of substance diffusion in/out of the subepithelial space (Brune et al., 2015) (see table 1). Ciliated epithelial cell dysregulation, squamous metaplasia and goblet cell hyperplasia are also associated with COPD (Gohy et al., 2019). Therefore, the use of models which effectively model these phenomena are vital for the effective development of novel therapeutics.

It is true that *all* models are reductionist in nature, and therefore will ultimately fail to fully recapitulate the complexity of a target organism. Therefore, it's imperative that we seek models that provide an 'economical description of the natural phenomena' while remaining alert to their underlying failings (Box, 1976). Current translational approaches lack the ability to provide the required understanding of disease mechanisms and signalling pathways that underpin respiratory pathogenesis. Existing *in vitro* models are complementary rather than alternative models to animal studies (albeit they can serve to reduce the number of animal studies required), with the sole use of multiple *in vitro* models remaining insufficient (Bonniaud et al., 2018). As a result, the transition from *in vitro* modelling and animal testing of novel compounds to first-in-man studies remains a "leap of faith" (Bonniaud et al., 2018).

The focus of this review is to highlight the current state-of-the-science of respiratory translational models used in drug discovery and target validation (Hendrickx et al., 2018). Here, the utility and limitations of *in vivo* and *ex vivo* modelling of the respiratory epithelium are described, with a focus on reviewing the current *in vitro* models of the tracheo-bronchial epithelium.

2. In vivo models

2.1 In vivo lung models

Traditionally, disease modelling of respiratory disorders in small animals has been the primary method of understanding the mechanisms and pathologies of a disorder in man. Schanker's seminal work in the development of pulmonary drug absorption and inhaled therapeutic drug deposition in *in vivo* respiratory models has remained a foundation for respiratory models (Burton and Schanker, 1974; Enna and Schanker, 1972a, 1972b; Mahato and Narang, 2010; Schanker and Burton, 1976). The development of this work with innovative dosing devices, and optimisation of drug delivery sites and anaesthetics have maintained their usability with small rodents and their relative importance in pharmacokinetic studies (reviewed here (Sakagami, 2006)).

Animal models are now most commonly used to bridge the gap between animal-to-man translation of ADMET testing (Tanner et al., 2019), drug dosage studies (Hu et al., 2019) and drug delivery studies (Cryan et al., 2007a). Models of this nature aim to improve the determination of a therapeutic human dosage for efficacious, first-in-man clinical studies, and to validate novel compounds via standardised end-point readouts (reviewed by Altamirano-Lagos et al., 2019a; Coraux et al., 2005; Gretebeck and Subbarao, 2015; Kips et al., 2003; Takayama, 2020a; Yuan et al., 2020). Particular advancements of current *in vivo* pulmonary models have targeted utilisation for translational pharmacokinetic studies (Trist, 2011). A recent multi-compartmental rat model consisting of plasma and a deep lung compartment was developed to predict human plasma profiles to known, soluble, bronchodilator compounds, and showed efficient cross-species translatability (to relevant dog and human data) of physicochemical, pharmacological, and pharmacokinetic properties (Hendrickx et al., 2018). The recent application of CRISPR/Cas9 genome editing technologies has served to increase the applicability of small rodent models for preclinical drug discovery (Zuberi and Lutz, 2016). Other efforts such as providing a murine milieu with a ‘human immune system’, also function to increase the predictive validity of small rodents as models of human disease (Allen et al., 2019).

The model compliance with genome editing technologies allows for its more effective use in drug efficacy studies. As a result, animal experimentation remains key to novel inhaled drug development and validation, as well as progressing our understanding of respiratory

pathogenesis and pathology (Cryan et al., 2007b). Small animal models of cystic fibrosis via conditional cystic fibrosis transmembrane conductance regulator ($CFTR^{-/-}$) knockout (KO) mice are still providing key insights into the functions of CFTR in neurons and T-cells (Keiser and Engelhardt, 2011). The utilisation of novel $CFTR^{-/-}$ pig and ferret models have also allowed for mechanistic studies of CF progression in the lung and pancreas which have not been previously identified in mice (Keiser and Engelhardt, 2011). Further still, efforts to model lung cancer (Janker et al., 2018; Kellar et al., 2015; Kwon and Berns, 2013), COPD (Ghorani et al., 2017; Tanner and Single, 2020; Vlahos and Bozinovski, 2014), respiratory syncytial virus (Altamirano-Lagos et al., 2019b; Bem et al., 2011), and uniquely human diseases such as asthma (Holmes et al., 2011) have also been developed and enhanced in recent years.

2.2 Limitations of *in vivo* lung models

A reliance on the use of non-humanised animal models remains a major issue in drug development (Ledford, 2011). The 2018 European respiratory society task force concluded that there remains no single animal model which captures all of the clinical features of asthma, COPD, pulmonary fibrosis or acute lung injury (Bonniaud et al., 2018). Therefore, despite the advances with *in vivo* models' abilities to reflect individual features of pulmonary disorders, they should be selected based on specific hypotheses and with any strong conclusions being drawn with their respective limitations in mind (Bonniaud et al., 2018). One may also argue that genetically homogeneous animals raised and experimented upon in a controlled, clean environment will inherently fail to create the translatability required to accurately model human diseases (G. Liu et al., 2019). Furthermore, rodent models may also be hindered by possible genetic discrepancies, i.e. the reserve-capacity hypothesis of transformed telomere length (Weinstein and Ciszek, 2002), which remain to be addressed.

The phylogenetic differences between small rodents and humans restrict their effectiveness as models of human pathology (Mestas and Hughes, 2004). The development of these models while utilising non-foetal tissue (or risk major ethical concerns for use in large-scale preclinical studies) is one of many developments that are still required. Currently, many animal models of respiratory pathologies fail to translate to the human condition. For instance, respiratory syncytial virus models are generated via the administration of large doses of the virus directly introduced into the lungs of small rodents, diametrically opposed to the small dose exposure to the virus over time seen in humans and thus drastically reducing their validity (Taylor, 2017).

The ability of rodents to produce a systemic response to a drug, e.g. an inhaled aerosol, has remained a defining reason in their use over *in vitro* models (Prytherch and Berube, 2014). However, structural differences in the relative size of the respiratory tract in rats and mice and their bronchiolar divisions i.e. the absence of a left lung lobe division, are all significant variations to human anatomy (Perinel et al., 2017). Functional differences in cell types have also been identified which are trickier, and perhaps impossible, to normalise. For example, it has been found in rodents that non-ciliated secretory cells act as a progenitor cell for the respiratory airways, whereas in humans it is the basal cell that plays this role (Bonniaud et al., 2018). Physiological variations such as the significantly greater rate of respiration at rest (80 breaths/min in rodents compared to 12-20 breaths/min in man), have also been shown to significantly alter the deposition of inhaled aerosols in the rodent lung (Perinel et al., 2017). The relatively high cost and strenuous amount of manual handling required for the use of *in vivo* models also reduces their utility in any preclinical high-throughput setting (see Table 3). Animal models will also continue to struggle to accurately mimic uniquely human diseases such as asthma (Barnes et al., 2015).

Non-human primates, such as baboons and macaque monkeys, have been utilised as models of the child respiratory tract. The increased resemblance of these species to humans in cellular expression, macroscopic and microstructure anatomy and functional genomics allow for more accurate and valid conclusions to be drawn from their experimental data (Tanner and Single, 2020). However, a number of key issues persist, such as the very high cost of study and physiological differences including an altered inspired-to-expired ratio during spontaneous breathing (Perinel et al., 2017). These factors highlight how current *in vivo* models may fail to predict toxicity in man (discussed in detail here (Van Norman, 2020)). A greater limitation to the use of non-human primate models, is one of fundamental ethical considerations, such as those outlined in the European directive passed in 2010 adopting the principles of the NC3Rs. The 3Rs (replacement, reduction and refinement) have evolved since their conception in 1953, with the NC3Rs now stating to accelerate the development of models and tools that address current scientific questions without the use of animals (Tannenbaum and Bennett, 2015). The implementation of the 3Rs has helped reduce the overall use of animals in research (7% year-over-year fall in 2018 (UK Home Office, 2018)), as well as to drive the development of alternative methodologies such as *in silico* and *in vitro* modelling.

3. Ex vivo models

3.1 Ex vivo lung models

The limitations of the current animal models and 3D cell culture systems to accurately mimic the respiratory epithelium have led to the use of human *ex vivo* tissue models, involving the culture of explanted human lung tissue. The resulting *ex vivo* tissue possesses the cellular composition of the native human lung, as well as all of the correct extracellular matrix components and complexity (G. Liu et al., 2019).

Isolated perfused lungs (IPL), derived from rejected lungs for transplantation, have proved valuable for testing of pulmonary drug absorption and novel therapeutic interventions for lung (Tronde et al., 2008) (reviewed by Briot et al., 2016; Chan et al., 2020; Costa and Andrade, 2016; Tane et al., 2017)**)**. The process of lung selection, preparation, perfusion, ventilation and oxygenation has been well described in Ross *et al* (2019). Their use in the validation of therapeutics such as keratinocyte growth factors have led to subsequent progression into clinical trials (McAuley et al., 2017; Perkins et al., 2014). Furthermore, a number of standardised endpoints for isolated perfused lung experimentation exist including alveolar fluid clearance, lung weight gain and pulmonary arterial pressure (Ross et al., 2019). This improves their feasibility for preclinical studies as validated, pre-defined endpoints are required to allow for comparability between compounds.

The use of animal IPLs for drug absorption studies remains more common place than the use of human IPLs due to a number of intrinsic limitations associated with the latter (discussed in the next section). Moreover, the use of non-human primates for IPL studies remains controversial (aptly discussed by Dahlmann and Sewald, 2017). Consequently, several advances with rodent (Eriksson et al., 2018), rabbit (Beck-Broichsitter et al., 2009) and porcine (Klassen et al., 2018; Mccormack et al., n.d.) *ex vivo* pulmonary models have been established. These recent developments include an *ex vivo* IPL rodent model which was proven to accurately predict rat *in vivo* plasma concentration-time profiles after inhalation of several compounds (Eriksson et al., 2020). The model hopes to improve the accuracy of drug performance predictions when compared to *in vitro* data, and to ultimately increase mechanistic understanding of pulmonary drug absorption (Eriksson et al., 2020). A porcine *ex vivo* model with controllable breathing parameters has also been recently developed consisting of a human plastinated head connected to an artificially ventilated *ex vivo* porcine pulmonary tract (Perinel et al., 2017). The authors showed physiologically relevant ventilation characteristics (i.e. tidal

volume, rate and homogeneity of ventilation) in aerosol deposition studies. Furthermore, comparative studies between *in vivo*, *ex vivo* and *in vitro* models of intratracheal inhalation (Sciuscio et al., 2019), pharmacokinetic modelling (Sakagami, 2006) and drug absorption (Bosquillon et al., 2017) have established *ex vivo* IPL systems to be as predictive as *in vivo* models (see Table 3). Porcine *ex vivo* lung perfusion models have also been used to successfully predict the outcome of organ transplantation (Sommer et al., 2019).

Precision cut lung slices (PCLS) have also emerged as a useful tool for studying the inflammatory response and pathogenesis in the respiratory system (G. Liu et al., 2019) (reviewed by Alsafadi et al., 2020; Henjakovic et al., 2008; Morin et al., 2013; Rosales Gerpe et al., 2018). This *ex vivo* tissue is derived from slices cut using a microtome (and therefore highly precise with less than 5% variability in the thickness of each slice) from selected healthy/diseased human donor lung tissue with minimum co-morbidities (G. Liu et al., 2019). This method markedly increases the number of tests obtainable from a single lobe of a human lung in comparison to an IPL set-up thus increasing its utility. The resultant models have recently been used to study asthma (Bourke et al., 2020), COPD (Dvornikov et al., 2019; Herbert et al., 2019; Maarsingh et al., 2019), pulmonary fibrosis (Cedilak et al., 2019b, 2019a; Yanagihara et al., 2020) and respiratory infections (Caid et al., 2019; Danov et al., 2019). Functional innovations of PCLS with siRNA knockdown in animals (Ruigrok, 2019), live-cell imaging (Akram et al., 2019), and ECM-mimicking biomaterials (Bailey et al., 2020) further enhances the applicability of PCLS methodology for pulmonary studies. Furthermore, animal-derived PCLS cultures have been shown to be compatible for high-throughput screening applications (Watson et al., 2016). The generally short lifespan of PCLS cultures was overcome with successful cryopreservation. Whilst he authors did show differences between fresh PCLS cultures and those having undergone a single freeze-thaw cycle (including higher glutathione levels in the latter), cryopreservation was concluded not to have any significant negative effect on the cultures. As a result, both mouse and rat PCLS cultures were utilised in a high-throughput toxicological screen (toxicity of zinc chloride at 6 varying concentrations in a 96 well plate format) (Watson et al., 2016). A continuation of this optimisation research will facilitate PCLS cultures to be used in high-throughput screening applications, though much further protocol development and validation is required at this stage.

Human lung tissue explants have also been utilised as a source of a number of primary human alveolar epithelial type II (Chu et al., 2020) and bronchial (Tane et al., 2017) epithelial cells.

Ex vivo mesenchymal fibroblasts obtained from primary human tissue were analysed via single-cell RNA sequencing to discover the expression of Sonic Hedgehog, a key secreted protein present in the hedgehog signalling pathway, to be preferentially expressed in the proximal respiratory airways (Ross et al., 2019). This data, in combination with genome-wide association studies (GWAS) mapping COPD loci to genes which alter the hedgehog pathway, have provided significant insight into the onset of respiratory disorders (Kheirallah et al., 2016). Tissue explants obtained from cancer patients have recently been optimised to study tumour pathophysiology, overcoming shortcomings with traditional explants regarding drug uptake, tissue stratification, and modelling of immune-responses to drug treatments (Powley et al., 2020). Similarly, *ex vivo* tissue explants have been used to study the pathophysiology of lung carcinomas (Karekla et al., 2017), asthma (Morin et al., 2005), viral infections (Thakker et al., 2019), environmental insults (KC et al., 2020) and COPD (Lea et al., 2019; Mathyssen et al., 2020).

3.2 Limitations of *ex vivo* lung models

Although human *ex vivo* IPL models are highly relevant, they are rarely used in preclinical studies due to the sporadic supply of healthy, normal donor tissue which inhibits proper experimental planning (Huang et al., 2011). Therefore, human IPL, PCLS and tissue explant models may remain unsuitable for most preclinical drug discovery applications. It is also important to distinguish between healthy tissue and one which has been surgically sliced and removed from its native environment, thus inherently reducing the comparability of *ex vivo* tissue models to native human tissue. Moreover, donor variability arising from the intrinsic heterogeneity of human lungs arising from sex, age and smoking history of the donor, extending in this context to include any trauma suffered to the individual, the cause of death and co-morbidities, has a significant effect on the quality and reliability of lung samples (Perinel et al., 2017). This variability limits comparability between studies. There is also an inability to utilise genome editing technologies to interrogate targets/proteins of interest due to ethical considerations for genome editing of human-derived cells and tissue (e.g. embryos and sperm cells), which would require genetically engineered humans from an early developmental stage before the organ is collected. Conversely, genome editing of primary human cells is well established, and has been effectively applied to an *in vitro* respiratory model (Rapiteanu et al., 2020).

Possibly the greatest challenge to the use of *ex vivo* human lung tissue culture in drug discovery is the rapid degradation of tissue slices or whole IPLs. Most IPL experiments are restricted from time of tissue preparation to fixation for analysis e.g. for histological staining, in 6-10 hours, which maybe prolonged to 48 hours with the use of bioreactors (Perinel et al., 2017). These time constraints, in combination with the sporadic supply of human tissue (Ross et al., 2019), therefore require laboratories to have flexible working hours for dedicated research staff, further increasing the costs associated with this relatively expensive method e.g. costs of organ transportation, dedicated experimental set-ups and support staff necessary for immediate analysis. Recent advances for PCLS cultures have increased their culture time (from 7-10 days) up to 21 days via the embedding of slices into extra-cellular matrix (ECM)-mimicking hydrogel (poly(ethylene glycol)-based hydrogels with RGDA and YIGSR ligand cohesions) support structures (Bailey et al., 2020). Further advances are required to standardise such protocols to overcome this critical limitation in coming years. The introduction of validated and standardised cryopreservation protocols for human ex vivo models will help improve their utility for target identification and validation research. A greater availability of genetic expression and/or single-cell RNA-sequencing data obtained from human lung tissue explants may also serve as an invaluable resource to improve the understanding of genetically driven mechanisms of human respiratory pathology.

4. Two-dimensional in vitro models

4.1 2D cell-line airway models

Two-dimensional (2D) monolayer culture systems are still heavily used in preclinical drug development for high-throughput toxicity screening of novel compounds due to their ease-of-use, availability and convenience e.g. rapid time to cell confluency (reviewed by Castellani et al., 2018; Faber and McCullough, 2018; Hiemstra et al., 2018; Lechanteur and das Neves, 2018; Nikolić et al., 2018). The relatively successful implementation of these reductionist models in standardised high-throughput safety screening has helped reduce novel therapeutic compound attrition due to insufficient safety in recent years (Dowden and Munro, 2019; Harrison, 2016; Lindner, 2007; Paul et al., 2010). Most 2D *in vitro* models of the tracheo-bronchial respiratory airways commonly utilise the adenocarcinomic human alveolar basal epithelial A549 cell line, the virally transformed BEAS-2B cell line derived from a non-cancerous human bronchial epithelium, and the bronchial adenocarcinoma-derived Calu-3 cell line. Such immortalised cell lines, either carcinoma-derived or virally-transformed, have many

desirable and promising characteristics for drug development studies (Prytherch and Berube, 2014). They are usually expanded and cultured on rigid 2D tissue-culture treated polystyrene surfaces and are commonly used for high-throughput *in vitro* screening assays utilising small compound libraries for lead identification (Langhans, 2018). These models therefore aim to predict non-specific, toxic and/or adverse reactions to novel compounds in humans and to determine the efficacy of novel targets and compounds in the respiratory airways.

The culture of cell lines is relatively simple, cost-effective, and allow studies into specific pathological processes. The relative low requirements for manual handling and manipulation also increases their utility in drug discovery and their applicability for automation. A549 cells have been recently utilised to determine the anti-inflammatory properties of novel plant-based compounds (Henz Ryen et al., 2020). The carcinomic nature of A549 cells have directed their use for compound efficacy testing of novel anti-cancer treatments (Pokrovsky et al., 2019). Recent studies have shown metformin-dependant upregulation of microRNA-7 can supress A549 cell growth via regulation of several signalling pathways (Dong et al., 2020). Furthermore, a potential anti-tumoral mechanism of action for the low-molecular weight heparin enoxaparin, was determined in A549 cells (Henz Ryen et al., 2020). The applicability of cancerous 2D cell lines to human physiology is inherently limited, yet such studies may be useful in narrowing the search for novel therapeutics.

The BEAS-2B cell line is able to maintain their ability to undergo squamous differentiation *in vitro* in response to foetal bovine serum or transforming growth factor-beta (Huang et al., 2011). However worryingly, new data has shown evidence discounting the well-established bronchial epithelial origins of BEAS-2B in favour of a mesenchymal-derived lineage (Han et al., 2020). Despite this, BEAS-2B cells were recently utilised as a normal human respiratory system to analyse the cytotoxic effects of electronic cigarette fluids (Hua et al., 2019). BEAS-2B cells have also been utilised to analyse the effect of cigarette smoke and particulates in the airways (Dugour et al., 2013), and for mechanistic and toxicological studies (Ong et al., 2013). Further still, both A549 and BEAS-2B cell lines were utilised in a recent proof-of-principle drug toxicity study, concluding that aerosolizable marine phytotoxins effectively downregulate the mTOR pathway without significant toxicity, providing support for the biogenic amine hypothesis (Van Acker et al., 2020).

Calu-3 cells were recently used as a reference cell line for *in vitro* toxicity studies of intranasal delivery of zonisamide for central nervous system diseases, and were effectively used to inform

subsequent *in vivo* testing (Gonçalves et al., 2020). This cancerous cell line has been heavily utilised to investigate the biological mechanisms (Hoffmann et al., 2020; Kong et al., 2020; Zecha et al., 2020) and efficacy of novel compounds (Felgenhauer et al., 2020; C. da Silva et al., 2020) or repurposed drugs (Felgenhauer et al., 2020; C. da Silva et al., 2020; C. S. B. da Silva et al., 2020; Yamamoto et al., 2020) for the treatment of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Calu-3 cells were also recently shown to functionally express the ABC transporter MDR1, which were shown as missing from healthy human bronchi and in the commercial 3D airway model EpiAirway™(Rotoli et al., 2020). Despite this, these cells have additionally been utilised as *in vitro* cigarette smoke exposure models to validate bioinformatic data in a study of ATP-binding cassette (ABC) transporters (Aguiar et al., 2019). Notably, Calu-3 have a high expression of CFTR and thus have been widely utilised to study CF (Gróf et al., 2020; Ramsey et al., 2020; Sultan et al., 2020; Yang et al., 2020).

4.2 Limitations of 2D airway models

The limitations of 2D models have become increasingly obvious in recent years, which has created a significant shift towards the use of 3D *in vitro* models. Importantly, 2D cellular models force cells to adapt to a rigid environment to survive, creating non-physiological changes in morphology, proliferation, functionality, metabolism and cytoskeletal organization that are not translatable to the cells native state (Sunyer et al., 2012; Wells, 2008).

Junctional complex proteins present between epithelial cells *in vivo* form an epithelial barrier which acts as an immunological and physical barrier to pathogenic particulates (Brune et al., 2015) (see Table 1). This epithelial barrier is primarily formed by zonula occuldins (ZO-1/2/3) and claudin proteins forming tight junctions, and the transmembrane protein E-cadherin forming adherens junctions between adjacent cells of the epithelium (Wittekindt, 2017). BEAS-2B cells fail to express characteristic airway mucins and tight junction formation, with the latter limiting their ability to form an effective epithelial barrier when compared to A549 cells (Prytherch and Berube, 2014). Calu-3 cells lack physiological features of the bronchial airways such as ZO-1 protein expression and consequently fail to differentiate to form tight junctions (Huang et al., 2011). They also lack functional motile cilia and fail to form a pseudostratified epithelium (Huang et al., 2011). Further still, BEAS-2B cells maintain their ability to form primary cilia, yet they do not form motile cilia which are characteristic of the native airways (Prytherch and Berube, 2014).

Moreover, the transformation processes of all immortalised cell lines create a dysregulation of signal transduction networks within the cells to varying degrees. Genetic changes lead to chromosomal mutations and often a loss of any inherent differentiation capabilities (Huang et al., 2011). In this way, respiratory cell lines cannot give rise to the differentiated cell phenotypes present in respiratory airways and their respective functional properties e.g. ZO-1/E-cadherin protein expression and subsequent barrier formation, motile cilia formation and subsequent cilia beating, mucociliary differentiation and subsequent mucus secretion. In comparison, 3D cultures provide deeper insights into cell–cell and cell–matrix interactions *in vitro* than 2D culture systems by more faithfully replicating *in vivo*-like spatial arrangements and cellular migration speeds, thus allowing for a greater translatability to the human condition (Pampaloni et al., 2007) (see Table 3). Such systems also allow for mechanical and chemical cues to be isolated and examined individually, increasing our ability to understand their role in pathophysiological states (East et al., 2010).

Therefore, the use of 2D cell lines for respiratory target validation research, is limited in number of significant ways. And with improvements in the automation of 3D cell cultures, as well as the significant translational improvements provided by 3D co-cultures (see discussion in later sections), it may be the right time to give sincere thought to moving towards exclusively using 3D models in novel therapeutic compound validation research (Watson et al., 2016).

5. Three dimensional in vitro models

5.1 3D air-liquid interface respiratory epithelium models

To overcome the issues related with 2D culture of cell-line based airway models, the modern gold-standard for human respiratory airway models consist of culturing primary human bronchial epithelial cells (HBECs) (also applied to human nasal epithelial cells (HNECs)) at an air-liquid interface (ALI) (see FIG. 2). HBECs, often obtained from bronchial brushings or via cadaveric donor tissue, are cultured to confluency on transwell inserts (characteristically in a 24-well plate format). Once confluent, medium is removed from the apical aspect of the transwell, with cells maintaining basal exposure to culture medium through a porous transwell membrane, thus forming an air-liquid interface (Awatade et al., 2018). The HBECs are subsequently cultured for 4-6 weeks. The *in vitro* ALI culture of HBECs functionally resembles the *in vivo* human airways (reviewed by BéruBé et al., 2010; Lacroix et al., 2018; Patel et al., 2012). Consequently, HBECs are able to form apical-basal cell polarity (Chen and Schoen, 2019), and differentiate into secretory goblet cells and ciliated epithelial cells while self-assembling into a pseudo-stratified columnar epithelium (Rayner et al., 2019).

The model therefore captures many of the relevant cellular phenotypes that are present in the lower respiratory airways *in vivo* (Ball and Padalia, 2019; Fraser, 2005; Khan and Lynch, 2020; Kia'i and Bajaj, 2020), and therefore remains the checkpoint measure for many novel drug candidates before entering clinical trials (Awatade et al., 2018). ALI models can be produced using explant human tissue or primary HBECs as a source of cells, with the former providing greater translatability (greater predictive validity) and the latter providing greater flexibility (genetic editing and long-term culture). HBEC ALI cultures exhibit clear epithelial barrier formations via the expression of the junctional complex proteins ZO-1 and E-cadherin, thus creating a significant advantage for their use over cell-line based models. 3D ALI models also provide certain advantages over animals models such as the significant lower costs of maintenance and setup, use of human tissue, and ease of handling (Chen and Schoen, 2019). In comparison to *ex vivo* human tissue models, 3D ALI models possess long-term feasibility, commercially available consumables (i.e. primary human cells), and well-established genome-editing protocols to investigate genetic targets of interest (Rapiteanu et al., 2020).

The improved translatability, in addition to the use of standardised end-points, enables functional utilisation of the 3D HBEC ALI model in preclinical target validation and lead optimisation research (Awatade et al., 2018). For example, the reproduction of a genetic lesion

(CDKN2A disruption) *in vitro* was shown to induce dysregulation in SOX2 production in the HBEC ALI model. This provided sufficient predictive validity to human bronchial dysplasia to enable preclinical screens of novel agents to treat squamous lung cancer (Porter et al., 2019). Additionally, basic research studies subjecting the 3D ALI epithelium to cigarette smoke were shown to increase matrix metalloproteinase 1 (MMP-1) release post-single exposure to cigarette smoke, accurately mimicking the response seen in the *in vivo* airways (Mathis et al., 2013). An overview of critical pathways involved with COPD and asthma pathologies generated from *in vitro* respiratory epithelium modelling are illustrated in figure 3 and 4 respectively.

The epithelial barrier, created *in vitro* via the expression of junctional complex proteins, namely E-cadherin and ZO-1 (Nawijn et al., 2011), has been implicated to play a role in COPD (Aghapour et al., 2018) (see FIG. 3), asthma (Gon and Hashimoto, 2018b; Xiao et al., 2011) (see FIG. 4) and other chronic respiratory disorders (Roche et al., 2020; Xiao et al., 2011). *In vitro* analysis using 3D ALI models has proven the importance of the epithelial barrier for cell proliferation, matter permeability, maintenance of apical-basal cell polarity and modulating the immune response to insult and injury of the epithelium (Nawijn et al., 2011). These numerous functions (and their dysfunction in several disorders) mark the epithelial barrier as a key modulator of the respiratory epithelium and therefore highly essential to model in order to effectively validate novel compounds for respiratory disorders. As a result, recent advances in the development of novel quantitative, high-throughput immunofluorescent imaging protocols have been utilised to qualitatively assess the formation of these junctional complex proteins (Buckley et al., 2018; Pell et al., 2021). Automated trans-epithelial electrical resistance (TEER) protocols have also been enhanced to enable reliable, real-time and high-throughput quantification of epithelial barrier integrity in ALI models (Srinivasan et al., 2015).

Mucociliary differentiation of basal progenitor cells into motile ciliated cell phenotypes is sufficiently captured in the 3D ALI model, which is lacking in 2D cell line-based models. As a result, methods for quantifying cilia have progressively evolved with manual counting of immunohistochemical/immunofluorescent preparations (Gohy et al., 2019; Tadokoro et al., 2014a), to automated analysis via single-cell image cytometry using relevant fluorescent markers (Rapiteanu et al., 2020). Functional readouts such as quantified cilia beat frequency via high-resolution video microscopy have also developed using open-source software

(CiliaFa) (Smith et al., 2012)). These readouts have been used to functionally analyse motile cilia (Gsell et al., 2020; Khelloufi et al., 2018; Yaghi and Dolovich, 2016a).

The loss of the ciliated cell phenotype is a central pathology associated with asthma, HIV infection (Strulovici-Barel et al., 2019), pathologic genetic mutation (Fassad et al., 2018), influenza virus (Wu et al., 2016), and a number of other chronic respiratory diseases. Exposure of human airway cells derived from diseased and healthy lung explant tissue to exogenous transforming growth factor β1 (TGF-β1) has been shown to recapitulate the reduced mucociliary differentiation and subsequent reduced number of ciliated cells in COPD airways (Gohy et al., 2019). Similarly, explant nasal tissue from CF patients has been used to successfully develop CF ALI models (Gianotti et al., 2018)·(Scholte et al., 2006). Consequently, the mechanistic pathways involved in ciliagenesis have been extensively studied using the 3D ALI respiratory model. The role of the Notch signalling pathway (Rock et al., 2011), interleukin (IL)-6 signalling (Tadokoro et al., 2014b) and FoxJ1 expression (Brekman et al., 2014a) have been identified in multiple studies as vital to normal ciliagenesis. CRISPR/Cas9 mediated depletion of FoxJ1 and subsequent multi-parametric analysis of HBEC ALI models, showed significant reduction of mucociliary differentiation into the ciliated cell phenotype without affecting barrier integrity (Rapiteanu et al., 2020). Efficacy testing of novel compounds, such as proprotein convertase inhibitors, acting via Notch-dependant mechanisms have been shown to promote ciliagenesis in the 3D ALI models (Lee et al., 2017).

The functional utility and flexibility of respiratory ALI models has enabled the *in vitro* study of multiple chronic, viral respiratory infections, including the pandemic H1N1 influenza virus generated by the H275Y genetic mutation. Here, genetic editing enabled the reverse-engineering of the H275Y mutation into human airway cells, while the ALI model provided an efficient platform to study the growth kinetics and tolerance of the wild-type and mutant strains of the H1N1 virus to the mutation (Brookes et al., 2011). Transmission electron imaging of the severe acute respiratory syndrome coronavirus (SARS-CoV, 2002) in HBEC ALI models, showed concentrated viral particles in between ciliated cells, as well as in the ciliated cell microenvironment. Further *in vitro* analysis determined SARS-CoV entry, replication and release all occurred exclusively in the ciliated cell phenotype of the respiratory epithelium (Sims et al., 2008).

Furthermore, efficacy testing of anti-viral compounds for the Middle East respiratory syndrome coronavirus (MERS-CoV) was conducted using human airway ALI models (alongside animal models) (Agostini et al., 2018). These studies were able to show sufficient efficacy of GS-441524 and its pro-drug GS-5734 (Remdesivir) against MERS-CoV (Agostini et al., 2018). More recently, HBEC ALI models were utilised effectively to isolate the SARS-CoV-2 virus. The point of entry of SARS-CoV-2 virus was isolated to ACE2 and its co-factor TMPRSS2-expressing respiratory cells using single-cell genomic analysis with HBEC ALI models (Lukassen et al., 2020).

5.2 Limitations of 3D air-liquid interface respiratory epithelium models

The finite number of passages for primary HBECs, and by extension for all primary human cell lines, before cells begin to lose functional *in vitro* characteristics (i.e. differentiation ability) have limited the utility of these ALI models, while raising costs associated with the sourcing of the cells. Recent optimisation research aiming to improve these models have shown HBECs to maintain their differentiation capabilities up to passage 6 (Rayner et al., 2019). However, further protocol validations are required before these practices are widely accepted. The 3-4 weeks of cell culture required until ALI cultures are fully differentiated increases the time required for validation studies. Also, ALI-media dependant variations to the quality of cultured airways have also been recently described (Leung et al., 2020). Validation of ALI media prior to experimentation increases labour, time and cost of experimentation, but also inhibits reliable comparisons to be drawn between studies. Furthermore, the rigid polyethylene terephthalate (PET) porous substrate of ALI transwell inserts adds additional physical stress to the cells and creates a mechanically mismatched interface which may negatively influence epithelium differentiation.

The extensive manual handling of transwells utilised in ALI models limits the scalability of the model for early drug discovery toxicology testing as well as for target validation studies. This is due to the introduction of human errors into the culture system such as variations in media levels which result in variations in the integrity of the epithelial layers. These issues are beginning to be addressed via the development of automated, software-driven, long-term, cultivation systems (e.g. CULTEX®). Here, transwells are placed into an incubator which regulates adjustments to media levels to minimise manual handling while increasing the consistency of the cultivation process, thus enabling scaling of the model system (Aufderheide et al., 2016). Scalability issues extend to methods of analysis, where recent advances such as

in-situ immunofluorescent imaging and automated *in vitro* TEER recordings (Pell et al., 2021) (discussed previously), are aiming to address. Provided further optimisation of these technologies is completed (to reduce cost and improve validation), it may enable the use of ALI models for high-throughput screening assays in early drug discovery. This would serve to greatly improve our ability to screen compounds using more translatable 3D models.

Human genetic heterogeneity can create donor-dependant responses in *in vitro* HBEC cultures, which hinders the reproducibility and consequently the reliability, of certain aspects of model-generated data. However, donor variability at this stage could be effectively utilised to reduce attrition in later stage clinical trials. To accomplish this, genomic and proteomic analysis of a large donor pool would be required to enable the identification of cellular and molecular mechanisms underlying donor-to-donor variability (Huang et al., 2011). The time and opportunity cost required to accomplish this could prove beneficial, as utilising the donor variation between disease and healthy population may allow for targeting of novel compounds for personalised medicines (Prytherch and Berube, 2014). The lack of representation of cell types in the native respiratory airways poses a major limitation to the translatability of the current gold-standard ALI model. The tracheo-bronchial respiratory epithelium, and nasal and alveolar epitheliums respectively, consist of several cellular phenotypes, including but not limited to, mesenchymal fibroblasts of the lamina propria, endothelial cells of the associated vasculature and chondrocytes of the hyaline cartilaginous housing of the airways (Khan and Lynch, 2020). These cellular phenotypes are crucial in the functioning of the airway epithelium *in vivo*. The mesenchymal fibroblasts of the respiratory airway have been shown to regulate *in vivo* inflammatory responses and repair (Fraser, 2005). Further still, they have been shown to produce cytokines and chemokines to various stimuli and act as local sentinel cells in response to inflammation (Evans et al., 1999). Respiratory fibroblasts also have a significant role for *in vitro* disease modelling of the respiratory epithelium. This has been shown in asthma where fibroblasts relay information to epithelial and inflammatory cells (Evans et al., 1999), and where altered fibroblast-epithelial interactions alter EMT in COPD patients (Nishioka et al., 2015). Roles of fibroblast-epithelial interactions in the respiratory epithelium have been reviewed for pulmonary fibrosis (2013), COPD (2014), fibrosis (Krieg et al., 2007), asthma (Halwani et al., 2010) and EMT (Knight et al., 2020) respectively. Epithelial-endothelial cell cross talk in response to injury has also been well documented in the literature. For instance, an epithelial-endothelial, microfluidic co-culture model showed the release of TNF-α by epithelial cells activated endothelial cells and induced expression of endothelial adhesion

molecules (E-selectin and ICAM-1) (Blume et al., 2017). Many endothelial-epithelial and fibroblast-epithelial dependant signalling pathways are therefore lacking in the current gold-standard HBEC ALI models.

5.3 3D co-culture air-liquid interface *in vitro* airway models

A functional human 3D ALI tracheo-bronchial model of cultured human fibroblasts and human tracheo-bronchial cells was effectively applied to the study of host-pathogen infection (Marrazzo et al., 2016). The model showed histological similarity to native tissue, expression of epithelial barrier, biomarker expressions including cytokeratin-5 (CK5) and CK14 and presence of Club cells in the upper epithelial layer. However, deeper comparison into the fibroblast-epithelial interactions of such models are required. Similarly, healthy HBECs derived from severe asthmatic patients were cultured in co-culture with monocyte-derived dendritic cells from *ex vivo* explant tissue. The model was applied to study the epithelial–dendritic unit and its modulation of CXC-chemokine ligand (CXCL)8, IL-33 and thymic stromal lymphopoietin (TSLP) in asthma (Gras et al., 2017). The study concluded that the bronchial epithelial phenotype was significantly involved in modulating the epithelial-dendritic response (Gras et al., 2017).

Co-culture models have also been generated aimed at recapitulating particular morphologies of the airway such as the lung branching morphogenesis during development. The fibroblast-epithelial crosstalk *in vitro* was created via a co-culture consisting of a fully-differentiated epithelium of HBECs cultured onto human foetal lung fibroblasts (IMR-90) seeded into a collagen matrix (Ishikawa et al., 2017). The presence of mesenchymal cell populations in this ALI respiratory epithelium model was able to recreate epithelial-mesenchymal transition (EMT) in the epithelium and ECM deposition in airway models induced by TGF-β1 (Ishikawa et al., 2017). Co-culture models of other areas of the respiratory airways and using cell lines as well as primary cells have also been developed. A co-culture of human macrophages and dendritic cells was used to model the airway epithelial barrier with A549 epithelial cells, and to model the alveolar type II epithelial barrier by replacing A549 cells for 16HBE14o-epithelial cells (Lehmann et al., 2011). Respiratory airway co-culture models for immunological studies have also been developed (Papazian et al., 2016). A human bone-marrow derived mesenchymal stem cell co-culture system with HBECs promoted a matured epithelial tissue analogue utilising a 3D-TIPS (thermally induced phase-separation) printed soft elastomer scaffold (Wu et al., 2020) (see FIG. 2). The substantial enhancement of mucin expression, ciliation, well-

683 constructed intercellular tight junctions and adherens junctions of HBECs in this co-culture
684 model demonstrates a more robust and biologically relevant tissue model for target validation
685 research (Wu et al., 2020).

686 Many of the models that have been described rely on protocols which limits their usefulness
687 e.g. time consuming to develop and culture, labour intensive and costly. As a result,
688 commercial *in vitro* lung models have been developed which are sold as differentiated cellular
689 consumables for research purposes. These commercially available *in vitro* ALI models
690 overcome traditional limitations of 3D ALI models, including reduced labour and model
691 developmental costs. As a result, there exist multiple major competitors in the *in vitro* lung
692 market (Mordor Intelligence, 2018) (see Table 2). The market is expected to grow in the
693 coming years, mainly from increased awareness of the 3Rs, and the subsequent move away
694 from non-humanised animal models, as well as increased funding and investments for the
695 research and development of advanced *in vitro* models (Mordor Intelligence, 2018).

696 Differentiated (ready-to-use) 3D human airway models, which can be acquired with nasal,
697 tracheal or bronchial epithelial cells, from healthy or diseased tissue, are readily commercially
698 available (Huang et al., 2011). Commercial monoculture models (MucilAir™, Epithelix) have
699 been used to characterise CBF in an *in vitro* nasal epithelium (Tratnjek et al., 2020) and study
700 drug interaction with native respiratory ABC transporters (Mercier et al., 2019). Furthermore,
701 toxicity studies involving possibly negative effects of polycyclic hydrocarbons have also been
702 investigated using this model (Cervena et al., 2019). Commercial co-culture models
703 incorporating fibroblasts in a 3D collagen matrix beneath a fully differentiated HBEC epithelial
704 layer have also been developed in an attempt to create a 'full-thickness' airway model.
705 Commercial co-culture models of this type (EpiAirway™ , MatTek) were reported to show
706 more characteristic expression of ABC transporters when compared to Calu-3 cells (Rotoli et
707 al., 2020), as well as a greater toxicology resistant phenotype when compared to cancerous
708 A549 (Zavala et al., 2016). These models have also been employed to study inhalation
709 toxicology (Jackson et al., 2018), and electronic cigarette smoke (Fields et al., 2017).
710 Significant differences between the response of a co-culture (EpiAirwayFT™) model in
711 modulating goblet cell hyperplasia when compared with traditional HBEC ALI models have
712 also been reported (Bolmarcich et al., 2018). Oral epithelial co-culture models have also been
713 developed (Schlage et al., 2014). An *in vitro* lung-on-a-chip model incorporating changes in
714 mechanical stress during respiration recently developed by AlveoliX™ was reported to

effectively model the air-blood barrier (Stucki et al., 2018). This model is also the first of its kind to be utilised in a physiological stretch-induced scratch-wound assay (Felder et al., 2019). Co-cultures with respiratory cells are envisioned for near-future developments of this innovative model.

5.4 Limitations of 3D co-culture air-liquid interface *in vitro* airway models

There remains a number of limitations with the current *in vitro* co-culture models of the respiratory epithelium (Duell et al., 2011). Co-cultures utilising cell lines (A549 etc.) do not show translatable phenotypes *in vitro* when compared to primary human cell culture (discussed previously). Their utilisation is therefore less desirable when compared to exclusive primary cell models. Also, sufficient nutrient requirements (glucose, serum, vitamins etc) for ALI co-cultures remains to be addressed**.** At the time of writing, no commercially available and dedicated fibroblast-epithelial or epithelial-endothelial media is available. Validation of in-house generated co-culture media further increases the time, cost and labour associated with the culture and maintenance of ALI models. ALI media-dependant variations in the quality of the cultures is therefore likely to be amplified in co-culture models (Leung et al., 2020). The use of organ-on-a-chip and associated microfluidic technologies shows potential to overcome these concerns by utilising multi-channel systems that allow for the inflow of several bespoke medias. However, these models often struggle to fully recapitulate the complex ECM and tissue architecture of native airways, thus limiting their ability to mimic lung cell-cell interactions *in vitro* (G. Liu et al., 2019). Additionally, the use of 3D printed soft porous scaffolds shows stronger integration between the epithelium layer and substrate compared to the standard PET transwell-based ALI model, which not only enhances the differentiation of HBECs but also improves sample collection and processing for post-culture characterisations (Wu et al., 2020).

Moreover, the absence of accurate multi-level analysis of some co-culture models inhibit their usefulness. It is therefore necessary to ensure optimal analysis methodology as well as model development to fully realise the benefits associated with the improved cell-cell interactions that are present in these models. The comparatively higher costs of 3D models can serve to limit their usage (Edmondson et al., 2014). However, these costs need to be weighed against their advantages, as commercial models come ready-to-use, often with significant prior validation studies. The physiological translatability of current co-cultures can also be further improved. Co-cultures of lung fibroblasts seeded on the basal aspect of the porous transwell membrane, with bronchial epithelial cells seeded in the apical layer are also commercially available (Mas

et al., 2016). These enable co-culture of cells in relatively close proximity, however, the relative distance between the cell types in some models remains much greater than the contact distance found *in vivo*, potentially limiting close proximity cell-cell interactions in the co-culture system, though it may still facilitate the activation of some native pathways via the release of signalling molecules. Several platforms are required to model a complete respiratory epithelium using pre-constructed co-culture *in vitro* lung models, e.g. modelling fibroblast, chondrocyte, immune and epithelial cell interactions within the respiratory epithelium. As a complete model is yet to be realised, procuring multiple models (where possible) remains highly inefficient for high-throughput applications. Therefore, the ability to create co-culture models with fully functional cell-cell interactions while maintaining the flexibility to modify the model to a specific disease or functional output, is still required.

5.5 3D organoid *in vitro* airway models

Airway organoid models have become increasingly important for functional respiratory drug discovery. Organoids can be defined as complex, self-assembling 3D clusters of organ-specific progenitor, stem or terminally-differentiated cell phenotypes. Organoids have been reviewed extensively, both generally (Clevers, 2016; Fang and Eglen, 2017) and specifically with regards to airway organoids (Y. Li et al., 2020). The significance of airway organoids for drug discovery applications cannot be understated. The potential ability for physiologically relevant and translatable models allowing for high-throughput, functional assays would significantly improve upon the current 2D *in vitro* models being utilised in early stage target identification and for target validation. The application of organoid models for personalised cancer therapeutics, e.g. for drug penetration studies, are particularly promising due to improved cell-cell interactions and heterogeneity of the *in vitro* cultured cell clusters. Briefly, organoids utilise adult or pluripotent stem cells (the former from patient biopsies), an ECM-based basement scaffold (commonly Matrigel) and media with the relevant growth factors. The cells are cultured for around 7 days in a multi-well plate format to produce a sufficient number of organoids for a given application. Specific protocols for the generation of airway organoids are freely available (Hild and Jaffe, 2016).

Lung organoid models have been shown to be capable of high-throughput drug candidate analysis, by seeding into 384-well plates with multiple endpoints including gene expression analysis and high-content immunofluorescent imaging. Recently, the identification of several SARS-CoV-2 entry inhibitors via high-throughput screens of FDA approved compounds (in

Matrigel-coated 384-well plates with several compounds) was reported using a human pluripotent stem cell-lung organoid (Han et al., 2021). The study of using lung organoids overcame limitations of traditional 2D cell lines, which failed to capture the physiologically-relevant processes of SARS-CoV-2 infection in humans, such as binding with ACE2 and TMPRSS2 (ACE2 expression and it's relation to SARS-CoV-2 infection, previously reviewed in detail in Zamorano Cuervo and Grandvaux, 2020). Multiple functional endpoints were also utilised including qPCR, immunofluorescent imaging, single-cell RNA sequencing and cell viability assays. Similar organoids derived from pluripotent cells have also been shown to allow for multiple endpoint measurements including functional cilia beating and expression marker analysis for ciliated, goblet and club cell phenotypes (Zhou et al., 2018a). Other human adult alveolar stem cell-derived organoids have also been applied to study the pathogenesis of SARS-CoV-2 (Salahudeen et al., 2020; Youk et al., 2020) and Influenza A virus (Zhou et al., 2018b).

Recently, there has been a growing body of evidence for the applicability and effectiveness of large libraries of airway cancer organoids created from human tumour resections, for the development of patient-specific cancer therapeutics. Normal and cancer human bronchial organoids from surgically resected patient lung samples were shown to histologically replicate the hallmarks of multiple subtypes of lung cancer (including squamous cell, adeno-, adenosquamous, large cell and small cell carcinomas), comprising up to 95% of lung cancer patients (Kim et al., 2019). This is a significant advantage over traditional 2D cell line cultures, which are also used for high-throughput screening, but do not exhibit the cellular heterogeneity required to accurately mimic human tumours. Over 80 lung cancer organoids (cultured in commercial Matrigel, CORNING) were shown to be applicable for a high-throughput drug screening application (4 compounds in 96-well plates) after reconstitution from cryopreservation in this study (Kim et al., 2019). Shi et al (2020) reported the development of large libraries of non-small cell lung carcinoma organoids (NSCLC), which were generated using patient-derived tissue (adeno- and small-cell adenocarcinoma). The study similarly bio-banked lung cancer organoids and showed full recapitulation of histological and functional outputs after reconstitution, and also utilised these organoids in a high-throughput drug screening platform (Matrigel-coated 384 well plate format) (Shi et al., 2020). The ability to cryopreserve airway organoids that maintain their complex tissue architecture after thawing significantly improves the utility and lifespan of these models. Sachs et al (2019) also developed NSCLC organoids which formed a pseudostratified epithelium, and which were

shown to regenerate their respective cancer phenotypes *in vitro*. These organoids were similarly utilised to generate an *in vitro* drug screening platform (8 compounds, 384 well plate coated with basement membrane extract (Trevigen)), but were also shown to form functional CFTR disease organoids, and to be susceptible to respiratory syncytial virus infection (Sachs et al., 2019). Finally, Li et al (2020) reported the generation of 12 adenocarcinoma organoid lines from patient tumour resections which were morphologically characterised with their parental tumours. The organoids were utilised for high-throughput screening applications (24 compounds, 348 well plate format) and successfully determined the dose-response for the 24 compounds tested, as well isolating and determining previously undefined clinical tumour prognostic biomarkers (Li et al., 2020a).

These recent efforts clearly demonstrate a markedly improved ability to utilise organoids for the study of the human respiratory epithelium (see Table 3). Though not without its limitations, the use of patient-derived tissue allows for the development of personalised targeted therapies, and therefore, should be supported as a useful tool for drug discovery.

5.6 Limitations of 3D organoid *in vitro* airway models

The traditional difficulties associated with the use of respiratory organoids in drug discovery include establishing validated, standardised culture protocols for creating uniform organoids of a defined shape and size. This issue is being addressed with recent publications of high-throughput organoid culture protocols (Boehnke et al., 2016). However, as few protocols, currently exist, difficulties for identifying the optimal culture protocol for a specific research goal still remain. Another traditional limitation of organoids is the generation of a large number of organoids for use in high-throughput screening, which are still challenging despite proven possibilities in the research laboratory, including the cryopreservation of organoids while maintaining complex tissue architecture (Kim et al., 2019; Sachs et al., 2019; Shi et al., 2020; Zhou et al., 2018a).

Furthermore, the dependency of the current culture protocols on the use of complex biological hydrogels (e.g. Matrigel) as the basement membrane remains a larger issue (Kim et al., 2019; Z. Li et al., 2020). The use of growth-factor reduced versions of these hydrogels will help reduce inherent biological variability (Shi et al., 2020). Therefore, the search for synthetic scaffold matrices that are able to support the growth of organoids remains an important avenue of current research.

It is also important to consider a balance of throughput and complexity for the organoids that are to be used for drug discovery applications, as the general appeal of organoids is their ability to provide complex 3D models applicable for high-throughput screening. For high-throughput target identification research, the amount of manual handling required and the complexity of culture protocols may also become limiting factors to the effectiveness of airway organoids. The application of CRISPR/Cas9 gene editing is yet to be fully realised in these models (Driehuis and Clevers, 2017). With regards to cancer organoids, a key missing factor in this culture is cancerous micro-environment present in vivo, which is not represented in the organoid modes, including associated effects of immune cell components and changes in the relevant tumour micro-vasculature (Kim et al., 2019; Paolicelli et al., 2019).

Finally, the longer time-scale required for generating organoids experiments increase their associative costs and risks, as for all 3D cell culture models. An ALI model requires 21-42 days of differentiation after initial cell expansion (~7 days) to form a fully-differentiated respiratory epithelium. In contrast, some organoid cultures can be formed in around 7-14 days, which reduces their relative costs (Hild and Jaffe, 2016). In comparison, human pluripotent stem cell-derived organoids can take from 50-85 days to fully differentiate (Miller et al., 2019). Therefore, the longer times and high costs should be taken into account for organoid models where the reprogramming of iPSCs or growth of human pluripotent stem cells are involved. Selecting between using organoids developed from either pluripotent or adult stem cells, should be decided based upon their relevant merits (discussed in Vaart and Clevers, 2021) and the proposed scientific application in drug discovery.

6. Concluding remarks and future outlook for modelling the respiratory epithelium

All the methodologies presently reviewed serve various functions aimed at studying respiratory physiology. The advancements in 3D cell culture technologies have vastly improved the ability to create functional *in vitro* models that recapitulate *in vivo* organ structures (Moroni et al., 2018). Furthermore, biofabrication methods such as 3D printing (Wu et al., 2020) and bioprinting (Ma et al., 2018; Ong et al., 2017; Vanderburgh et al., 2017; Zhang et al., 2019), electrospinning (Chen et al., 2018), tumoroid/organoid cell culture (Rossi et al., 2018; Takahashi, 2019; Xu et al., 2018), microfluidics (Cui and Wang, 2019; Mullard, 2018; Sun et al., 2019) and organ-on-chip technologies (Esch et al., 2015; Sontheimer-Phelps et al., 2019; Zhang et al., 2018), have already been successfully implemented to generate translational and predictive *in vitro* respiratory models. Bioprinting for instance, has been applied for *in vitro*

model fabrication aimed at high-throughput target selection, toxicity screening, and ADME testing (Peng et al., 2017). However, many issues remain to be resolved with these technologies, such as a limited capacity to generate large scale tissue constructs due to inadequate vascularisation (reducing nutrient supply, mass transport and waste removal (Hutmacher et al., 2015)). Therefore, a multi-disciplinary convergence of biofabrication methods is demanded. A successful integration of these innovative technologies is likely to be required prior to the next generation of predictive, functional, and biologically relevant *in vitro* human organ cultures being realised. Furthermore, recent developments in advanced functional biomaterials aiding biofabrication technologies are assisting to significantly advance this effort (Place et al., 2009). Other model systems such as complex respiratory organoid cultures have shown promise for the study of SARS-CoV-2 infection (Lamers et al., 2020; Alyssa J. Miller et al., 2019; Monteil et al., 2020; Takayama, 2020b; Varga et al., 2020). The use of patient-derived induced pluripotent cells (iPSCs) for respiratory modelling is yet to be fully realised (Calvert and Ryan (Firth), 2019). Specifically, with regards to the use of isogenic iPSC pairs which show impressive utility for their applicability in determining the efficacy of novel compounds, by using common statistical comparisons but with an impeccable experimental control.

Many *in vitro* models fail to cross the 'Valley of Death' (Coller and Califf, 2009), the term now used to describe the gap between the development of a novel 3Rs methodology and its subsequent implementation in industrial applications (Williams and Andrews, 2019). In many cases, this has been attributed to a lack of sufficient model validation, which in turn may have served to create a lack of confidence in these methodologies to create reproducible effects at a mid-to-high throughput. Regulatory hurdles, not discussed in the current review, will also need to be overcome before wide-scale implementation and acceptance of novel 3D cell culture methodologies are seen in preclinical research (Booth and Zemmel, 2004; Moffat et al., 2017; Pridgeon et al., 2018). Furthermore, enhanced collaboration between academia and industry, as well as increased funding for preclinical model development and the use of standardised end-point measurements and commercially available consumables (rather than bespoke instruments/tools) is required to accelerate this process. Ultimately, a well-funded, multi-disciplinary and perseverant approach to novel model development will serve to realise the ability to accurately mimic microscale human respiratory functions.

Figures, Figure Captions and Tables:

	Morphology / Structure	Function(s)	Genetic Markers	Signaling Pathways	Associated Pathologies
Respiratory Cellular Phenotypes					
Ciliated	Elongated columnar terminally differentiated cells (Bustamante-Marin and Ostrowski, 2017), 200-300 motile cilia (5-10μm long, 250nm thick, flagellar ciliary axoneme of 9 peripheral doublets and attached dynein arms with 2 central microtubules (Fliegauf et al., 2007)), and ~400 microvilli per cell (Beule, 2010)	Motile mucus clearance to pharynx to be swallowed or coughed (Bustamante-Marin and Ostrowski, 2017), extracellular fluid flow mechano-sensation (Fliegauf et al., 2007)	Acetylated α-tubulin (Schamberger et al., 2015; Xiaojun et al., 2016), DNAI1 (Zuo et al., 2018), CBE1 (Yoshisue et al., 2004), FoxJ1 (Brekman et al., 2014b, 2014a; Gomperts et al., 2004; Rapiteanu et al., 2020; Thomas et al., 2010) TGF-β1 (Gohy et al., 2019)	Notch (Rock et al., 2011), Il-6 signalling (Tadokoro et al., 2014b), STAT3 pathway (Johnson et al., 2018), STK11/MARK3/ERK1/2 (Chu et al., 2019), Hedgehog/Wnt (Anvarian et al., 2019; Christensen et al., 2007; Pala et al., 2017)	Asthma (Jevnikar et al., 2019; Vieira Braga et al., 2019a), HIV infection (Strulovici-Barel et al., 2019), genetic mutation (Fassad et al., 2018), influenza virus (Wu et al., 2016), squamous metaplasia and goblet cell hyperplasia associated COPD (Gohy et al., 2019), SARS-CoV entry, replication and release (Sims et al., 2008), PCD (Leigh et al., 2019), Kartagner's syndrome (Yaghi and Dolovich, 2016b), epithelial hypoplasia (Rock et al., 2010)
Goblet	Highly polarised, organelles situated at the top of the cell, containing high molecular weight mucin glycoproteins (Bustamante-Marin and Ostrowski, 2017)	Primary secretory cell phenotype of the respiratory epithelium (Fraser, 2005). produce and secrete mucus (comprising water (~95%, pH 5.5–6.5), and the large (0.5-20MDa), hydrated mucin glycoprotein (Bansil and Turner, 2006)	MUC5AC (Schamberger et al., 2015; Vieira Braga et al., 2019b) MUC5B (Fahy and Dickey, 2010), CEACAM5 (Vieira Braga et al., 2019b), S100A4 (Vieira Braga et al., 2019b), SARS-CoV-2 entry receptor ACE2 and viral entry-associated protease TMPRSS2 (Gengler et al., 2020) region/disease-dependant: KRT4, CD36, IDO1, NOS2, IL19, CSF3, CXCL10 (Vieira Braga et al., 2019b)	Muc5Ac expression: IL-13 activating Jak1/Stat6 (Fahy and Dickey, 2010), C3 receptor and β2-adrenergic–receptor signalling (Fahy and Dickey, 2010) Development: Sox2, Notch, E2f4, and Math (Fahy and Dickey, 2010) Mucin expression: Purinergic (P2Y2 purinergic receptors (P2Y2R)) (Fahy and Dickey, 2010)	Goblet cell hyperplasia causal role in bronchitis & COPD (Gohy et al., 2019), IPF(Whitsett, 2018), CF (Huang et al., 2011; Zoso et al., 2019), Mucous metaplasia in asthma and COPD (Rock et al., 2010)

Basal	Cuboidal cell population attached directly to the basement membrane (Kia'i and Bajaj, 2020), region-specific multipotent stem cells (Rock et al., 2010)	Key progenitor cell type in the tracheo-bronchial epithelium (Rock et al., 2009), (bronchioalveolar stem cells (Lee et al., 2014) and type 2 alveolar cells (Barkauskas et al., 2013) in bronchi and alveoli respectively) maintains homeostasis in the normal epithelium (Rock et al., 2010)	KRT5 and KRT14 (Rock et al., 2010; Schamberger et al., 2015; Zuo et al., 2018), TP63 and NPPC (Vieira Braga et al., 2019b), TRP63 (Rock et al., 2010)	Notch (Morimoto et al., 2010; Tsao et al., 2009), Jag2 (Whitsett, 2018), EGF and VEGF pathways (Xu et al., 2016), p63 (Sheikh et al., 2004), IL-27/CXCL10 via PI3K-Akt*(Cao et al., 2012), αvβ6 integrin activation via TGF-β1 signalling*(Branchet t and Lloyd, 2019)	IPF (Jonsdottir et al., 2015), MUC2 expression in CF and COPD (Whitsett, 2018), basal cell hyperplasia (Voynow et al., 2005) associated COPD and CF (Rock et al., 2010)
Club (*previously Clara*)	Non-ciliated, bronchiolar secretory cell, characteristic apical dome shape with microvilli (Fraser, 2005)	Endogenous, stem cell/progenitor population (Fraser, 2005; Rawlins et al., 2009) Secrete mucins post-bronchiolar injury, give rise to alveolar type 1 & 2 cells during repair of the alveolar epithelium (Zheng et al., 2017)	SCGB1A1 (also called CC10, CC16, uteroglobin) (Fraser, 2005; Q. Liu et al., 2019; Schamberger et al., 2015), Muc5b (Zhu et al., 2008), (C16orf89, CLDN22, GNPNAT1, UBL4A, CCDC68, EPSTI1, and OSBPL7) (Zuo et al., 2018)	IL-13/CCSP expression via an EGFR- and leukocyte-dependent pathways (Kim et al., 2002), IL-4 and/or IL-13 for allergen-induced mucus production (Kuperman et al., 2005)	Clara cells in pathologic states focus on pulmonary tumors (Singh and Katyal, 1997), squamous metaplasia in CF and COPD (Rock et al., 2010)
Functional Epithelium Phenomena					
Epithelial Barrier	Physical and immunological barrier to inhaled pathogens, composed namely of E-cadherin (lower-basally expressed), ZO-1 (apically expressed) (Nawijn et al., 2011)	Maintain apical-basal cell polarization, regulate EMT, cell proliferation, matter permeability, modulating the immune response to insult and injury *in vivo* (Nawijn et al., 2011), control paracellular transport pathways between adjacent cells (Wittekindt, 2017)	ZO-1 and claudin proteins (Wittekindt, 2017), Crumbs complex and partitioning defective complex (Fahy and Dickey, 2010)	TGF-β induced EMT (Shirakihara et al., 2011), MEK-Erk pathway (Shirakihara et al., 2011)	Asthma (Gon and Hashimoto, 2018b; Tsicopoulos et al., 2013; Xiao et al., 2011), COPD (Aghapour et al., 2018; Roche et al., 2020; Xiao et al., 2011)

Mucociliary Clearance (MCC) via Ciliary Beating	Planar motion of motile cilia, created by an ATP-consuming sliding filament mechanism (Beule, 2010), aided by cough clearance (Fahy and Dickey, 2010), average CBF of 10-20 Hz (Satir and Christensen, 2007), average MCC velocity 5.5mm/min (Klein et al., 2013)	Clearance of the 10–15 μm mucus layer coating ciliated, columnar epithelial cells of the respiratory mucosa (Beule, 2010)	*See ciliated and goblet cells*	Cholinergic, and adenosine-receptor agonists (Fahy and Dickey, 2010), β-adrenergic agonists and increased Ca^{2+}, cAMP and cGMP (via PKA and PKB)(Salathe, 2007; Sears et al., 2015) purinergic pathways (P2X and P2Y receptors) (Davis and Lazarowski, 2008), pH (Salathe, 2007), PKC inhibits ciliary beating (Salathe, 2007)	CCNO-deficiency dependant defective ciliary mother centriole generation and placement (Wallmeier et al., 2014), PCD (Bustamante-Marin and Ostrowski, 2017), COPD (Bhowmik et al., 2008; Yaghi and Dolovich, 2016b)

* in airway epithelial cells, + in small airways, ACE2 - Angiotensin-converting enzyme 2, cAMP – Cyclic adenosine monophosphate, CBE1 - Ciliated Bronchial Epithelium 1, CBF – Ciliary beat frequency, CC- Club Cell Protein, CCNO - Cyclin O, CCsp - Club-cell secretory protein, CD - Cluster of differentiation , CEACAM - Carcinoembryonic antigen-related adhesion molecule, CF – Cystic Fibrosis, cGMP - Cyclic guanosine monophosphate, COPD – Chronic obstructory pulmonary disorder, CSF3 - Colony Stimulating Factor 3, CXCL10 - CXC motif chemokine 10, EGF - Epidermal growth factor, EGFR - Epidermal growth factor receptor, EMT - Epithelial-mesenchymal transition, ERK - Extracellular signal-regulated kinase, FoxJ1 - Forkhead box protein J1, HIV - Human immunodeficiency virus, IDO1 - Indoleamine 2,3-dioxygenase, IL – Interleukin, IPF - Idiopathic pulmonary fibrosis, JAG2 - Jagged Canonical Notch Ligand 2, Jak1 – Janus Kinas 1, KRT – Keratin, MUC - Mucin, NOS2 - Nitric Oxide Synthase 2, NPPC - Natriuretic peptide type C, PCD – Primary cilia dyskinesia, PI3K-AKT - Phosphatidylinositol 3-kinase/protein kinase B, PKA/PKB/PKC – Protein kinase A/B/C, SARS-CoV - Severe acute respiratory syndrome coronavirus, Sox2 - Sex determining region Y-box 2, STAT3 - Signal transducer and activator of transcription 3, TGF-β – Transforming growth factor β, TMPRSS2 - Transmembrane protease, serine 2, TP63 - Tumor Protein P63, Trp63 - Transformation related protein 63, VEGF - Vascular endothelial growth factor, ZO-1 - Zonula occuldin 1.

Table 1 | Functional Cellular Phenotypes of the Respiratory Epithelium: Structure, function, genetic expression profiles and associated pathophysiology of ciliated, goblet, basal and club cell phenotypes, and of the respiratory epithelial barrier and functional mucociliary clearance of the tracheobronchial respiratory epithelium.

In Vitro Lung Model Providers[+]	Technology/Platforms	Products/Services
Epithelix	Provider of cells, 3D transwell-based ALI tissues models and testing services	MucilAir™ - 3D Human Airway Epithelia (Chioccioli et al., 2019) SmallAir™ - 3D Human Small Airway Epithelia (Huang et al., 2017) OncoCilAir™ - Human 3D Lung Cancer Model (Benainous et al., 2017)
MatTek	*In vitro* tissues, primary human cells and culture ware	EpiAirway™ - model of the tracheobronchial epithelium (Czekala et al., 2019) EpiAlveolar™ - co-culture model alveolar epithelial and pulmonary endothelial and fibroblasts (Guseh et al., 2009)
Emulate	Human Emulation System comprised of proprietary Chip-S1 'stretchable' Organ-Chips	Alveolus Lung-chip (gaseous exchange of the alveolus)(Jain et al., 2018) Airway Lung-chip (small airways)(Henry et al., 2017)
AlveoliX	Organ-on-a-chip technologies	AlveoliX Lung-on-a-chip tissue model (Stucki et al., 2018)
ATCC®	Cell lines and *In vitro* model system	ATTCC® CCL-185EMT™ Lung cancer model using ATCC A549 cell line (Thiery and Sleeman, 2006)
Mimetas	Microfluidic 3D cell culture plate - OrganoPlate®	OrganoTEER® - real-time epithelial barrier function OrganoPlate 2/3-lane® - multiple perfusion channel systems for co-cultures (Beaurivage et al., 2019; Petrosyan et al., 2019; Wevers et al., 2018) OrganoFlow® - rocker to maintain perfusion
TissUse	Multi-organ/Human-on-a-chip technology platform – Devices, Chips and Cells/accessories	Devices, Chips and Cells/Accessories Chip Design service using primary cells, iPSC stem cells etc (Ramme et al., 2019)
Insphero	Prequalified, assay-ready 3D models - 3D InSight™ Microtissue models	3D Liver, Islet & Tumour models (Boos et al., 2019) *3D InSight™ Services*
Cn Bio Innovations	Lab-on-a-chip microphysiological systems - PhysioMimix™ OOC benchtop device	Lung-on-a-chip Transwell® model - Multi-MPS 12 (T12) Consumable Plate (Edington et al., 2018)

Table 2 | Various commercial suppliers of *in vitro* lung models: Examples of current technology/platforms, products and services provided by commercial developers of *in vitro* respiratory models. This table is intended to provide examples only (Mordor Intelligence, 2018). [+] The commercial entities listed may provide additional services to those listed above and other commercial entities may provide products and services not included in this table.

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Model Type	Applications in Drug Discovery	Advantage	Disadvantage	References
***In vivo* models**	Pharmacokinetic studies ADME-Tox testing Drug dosage studies Drug delivery studies Disease modelling e.g. lung cancer, COPD, respiratory syncytial virus	Compatible with CRISPR/Cas9 genome editing e.g. CFTR knockout mice	Incompatible for HTS Physiological variations to human anatomy e.g. inspired-to-expired ratio during spontaneous breathing High cost	(Yuan et al., 2020)(Sakagami, 2006)(Tanner et al., 2019)(Hu et al., 2019) (Tanner and Single, 2020)(Altamirano-Lagos et al., 2019b)(Zuberi and Lutz, 2016)(Keiser and Engelhardt, 2011)(Perinel et al., 2017)
***Ex vivo* models**	Intratracheal inhalation Pharmacokinetic modelling Drug absorption Disease modelling e.g. COPD and pathophysiology of lung carcinomas, asthma High-throughput toxicological screening	Standardised endpoints for IPL models e.g. alveolar fluid clearance, lung weight gain and pulmonary arterial pressure. PCLS models are compatible with siRNA knockdown in animals Functional endpoints e.g. live-cell imaging PCLS models are compatible with ECM-mimicking biomaterials	Largely incompatible for HTS toxicity screening Sporadic supply of healthy Large Donor-to-Donor variability Expensive method e.g. costs of organ transportation, dedicated experimental set-ups and support staff necessary for immediate analysis	(Sciuscio et al., 2019)(Alsafadi et al., 2020)(Sakagami, 2006)(Bosquillon et al., 2017)(Mathyssen et al., 2020)(Karekla et al., 2017)(Morin et al., 2005)(Watson et al., 2016)(Ross et al., 2019)(Ruigrok, 2019)(Akram et al., 2019)(Bailey et al., 2020)(Huang et al., 2011)
2D **Cell-line** ***In vitro* models**	Compound efficacy testing Toxicity testing Drug toxicity Mechanistic studies Disease modelling e.g. CF	Compliant with HTS Small time delay to generate data Cost-effective Low level of manual handling and manipulation required increases applicability for automation	Limited translatability in morphology, proliferation, functionality, metabolism and cytoskeletal organization to the native state Failure to express physiologically-relevant features e.g. BEAS-2B fail to express mucins and tight junctions Calu-3 cultures lack ZO-1 expression, functional motile cilia and an *in vitro* pseudostratified epithelium	(Felgenhauer et al., 2020)(Nikolić et al., 2018)(Van Acker et al., 2020)(Ong et al., 2013)(Hoffmann et al., 2020)(Gróf et al., 2020)(Langhans, 2018)(Prytherch and Berube, 2014)(Huang et al., 2011)

3D **Air-Liquid Interface** ***In vitro* models**	Preclinical target validation and Lead optimisation research Preclinical compound efficacy screening of Disease modelling e.g. HIV infection, influenza virus, CF	Semi-automated endpoints e.g. TEER and immunofluorescent imaging Functional endpoints e.g. CBF Compatible with genome editing Compliant with use of patient-derived cells Automated, cultivation systems	Donor-to-donor variability Low compatibility for Tox screening Large time delay to generate data (3-6 weeks) Media dependant model variations Relative high cost compared to other in vitro methods Limited lifespan of HBECs	(Lacroix et al., 2018)(Awatade et al., 2018)(Porter et al., 2019)(Strulovici-Barel et al., 2019)(Wu et al., 2016)(Gianotti et al., 2018) (Pell et al., 2021)(Gsell et al., 2020)(Leung et al., 2020)(Rayner et al., 2019)(Aufderheide et al., 2016)(Rapiteanu et al., 2020)
3D **Organoid** ***In vitro* models**	High-throughput compound screening with patient-derived cells	Variable time delay to generate data e.g. 7-14 days for human adult stem cells and 22-50 days for human pluripotent stem cells Compatible with cryopreservation Availability of standardised and high-throughput culture protocols Compatible with multi-parametric analysis e.g. qPCR, IF imaging, scRNA-seq and metabolic assays	The application of CRISPR/Cas9 technology is yet to be fully realised in these models Dependent on use of Matrigel basement membrane, introducing small biological variability Missing tissue micro-environment, including immune component	(Kim et al., 2019)(Vaart and Clevers, 2021)(Nikolić and Rawlins, 2017) (Hild and Jaffe, 2016)(K Shi et al., 2020) (Boehnke et al., 2016)(Han et al., 2021)(Driehuis and Clevers, 2017).

Abbreviations: ADME-Tox - Absorption, Distribution, Metabolism, Excretion and Toxicity, ALI – Air-liquid interface, CBF – Ciliary beat frequency, CF – Cystic Fibrosis, HBECs – Human bronchial epithelial cells, hiPSCs – Human induced pluripotent stem cells, HIV - Human immunodeficiency virus**,** HTS – High-throughput screening, qPCR – Quantitative polymerase chain reaction, scRNA-seq - Single cell RNA-sequencing, TEER – Trans-epithelial electrical resistance.

Table 3 | Summary of the current respiratory epithelium models for use in drug discovery: The table presents a non-exhaustive comparison of the relative advantages and disadvantages for models of the respiratory tract, generated from the primary literature discussed in this review and referenced in the table.

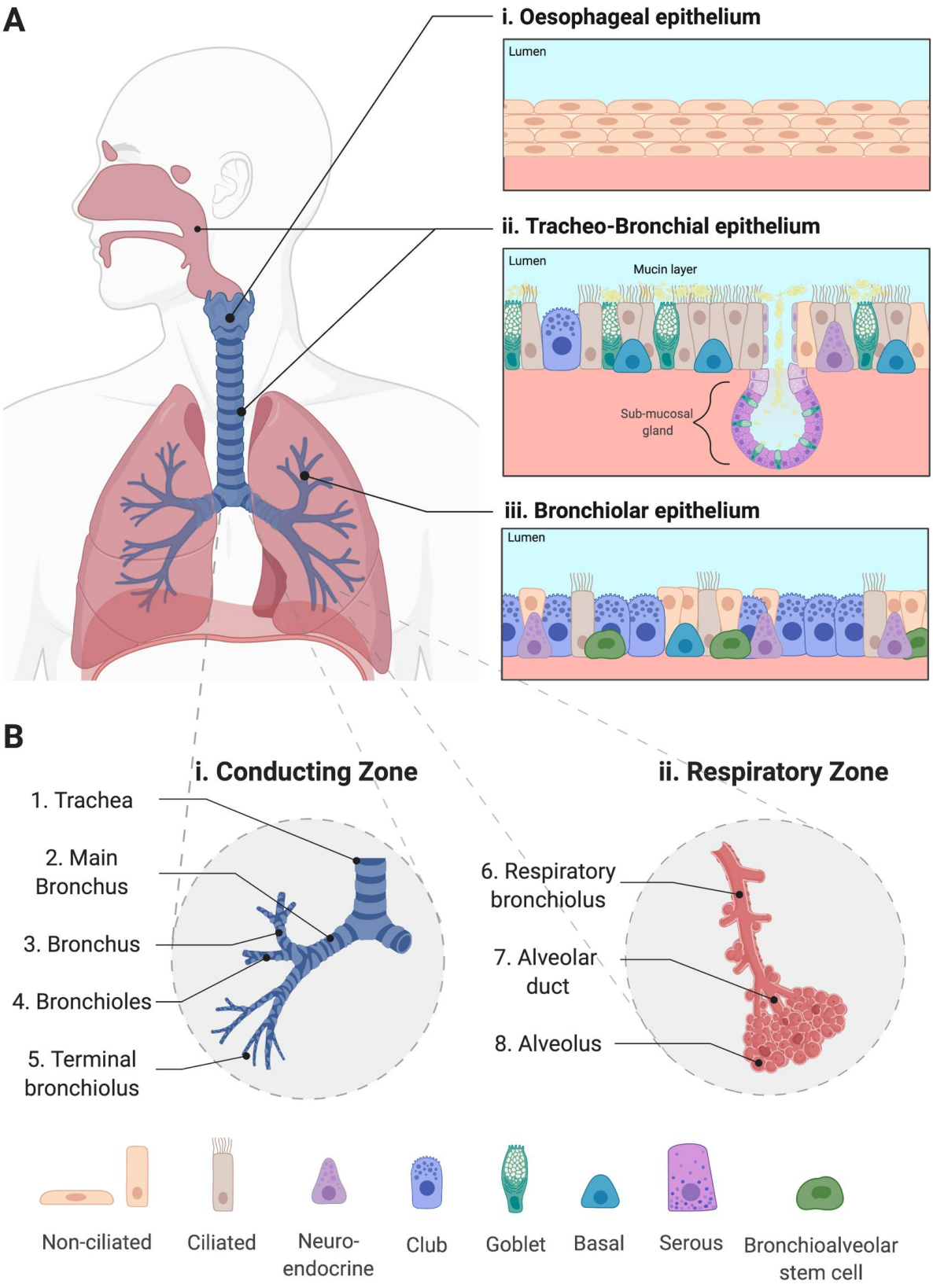


Figure 1 |. Illustration depicting gross human respiratory tract anatomy and relative cell densities present in various airway epithelia. (A) (i) The nonkeratinized stratified squamous

oesophageal epithelium serves a protective function, supported by the loose connective tissue of the laminar propria (not shown). **(ii)** The pseudo-stratified ciliated, columnar epithelium of the ***nasal*** and ***tracheal*** airways is made up of ciliated, goblet, basal, club cells, neuroendocrine and serous cells, with seromucous glands extending into the mucosal space. **(iii)** A simple cuboidal epithelium of the ***bronchiolar epithelium*** comprises of bronchoalveolar stem cells and club cells, the predominant cell type in the simple cuboidal epithelium of the bronchiolar epithelium. **(B) (i)** The ***conducting zone*** airways includes the nasal cavity, pharynx and the larynx which houses the organs of the epiglottis, the vocal cords or voice box and glottis. The larynx is continuous with the *trachea* which bifurcates into the lungs via the *main bronchi*, which divide serially into conducting, terminal (or membranous) and respiratory (alveoli-containing) *bronchioles*. **(ii)** The ***respiratory zone*** of the airways facilitates gaseous exchange and begins with *respiratory bronchiole*s. Each bronchiole gives rise to *alveolar ducts* and later to alveolar sacs containing *alveoli*.

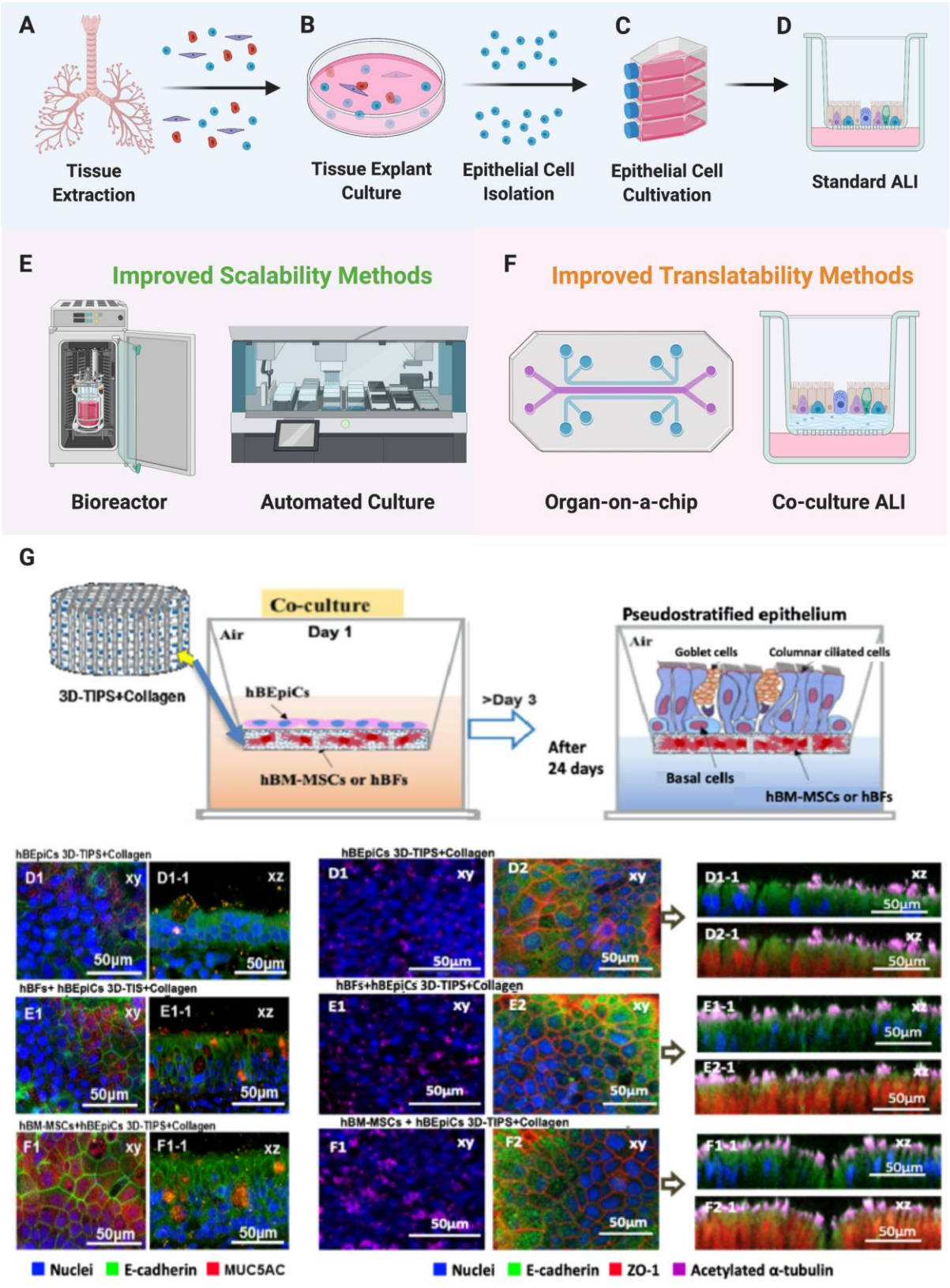
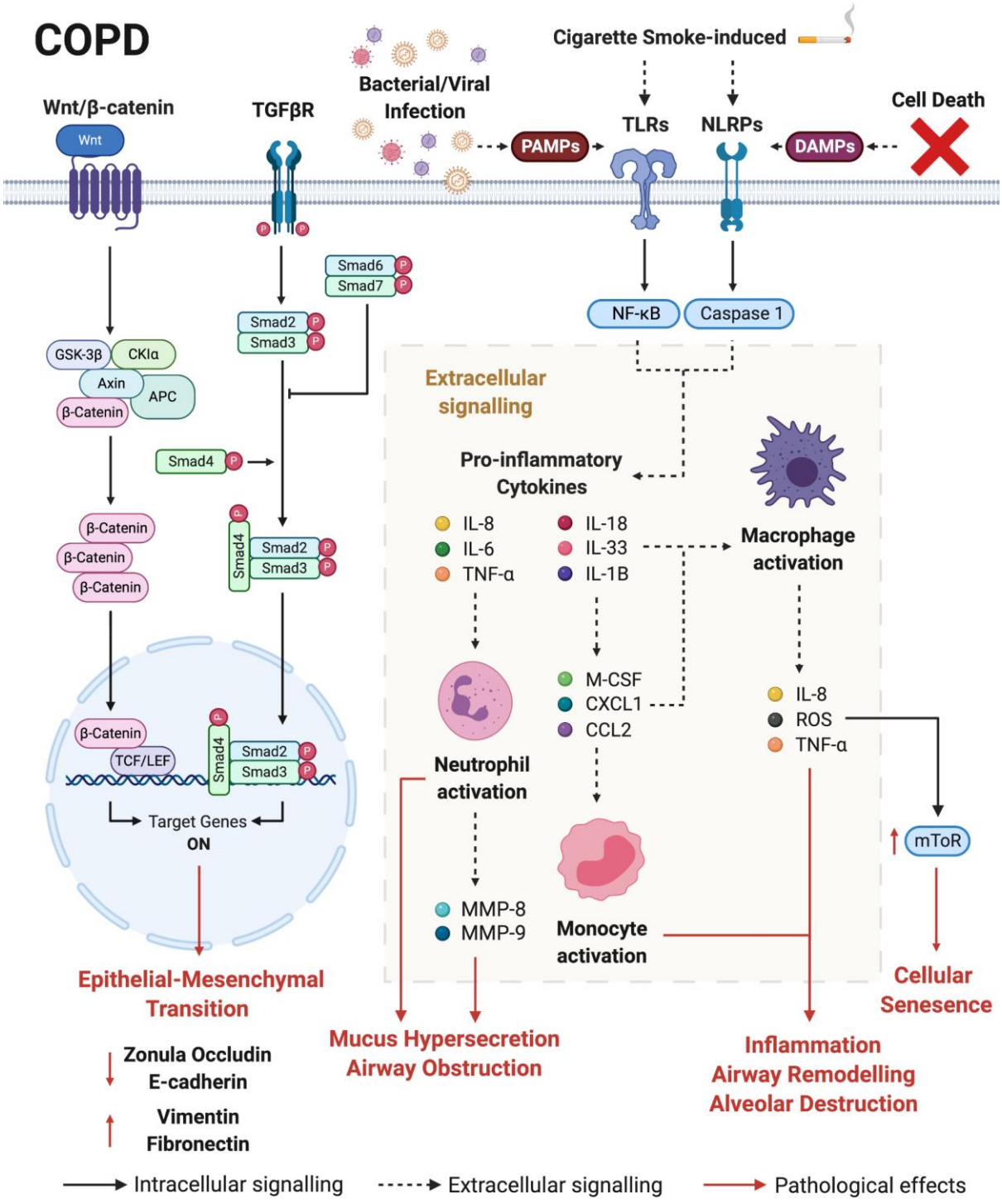


Figure 2 | Overview of current 3D Air-liquid interface (ALI) culture protocols. A generic protocol is described from which significant differences may exist between commercial vendors and current variations of respiratory air-liquid interface models. **(A)** Lung *tissue*

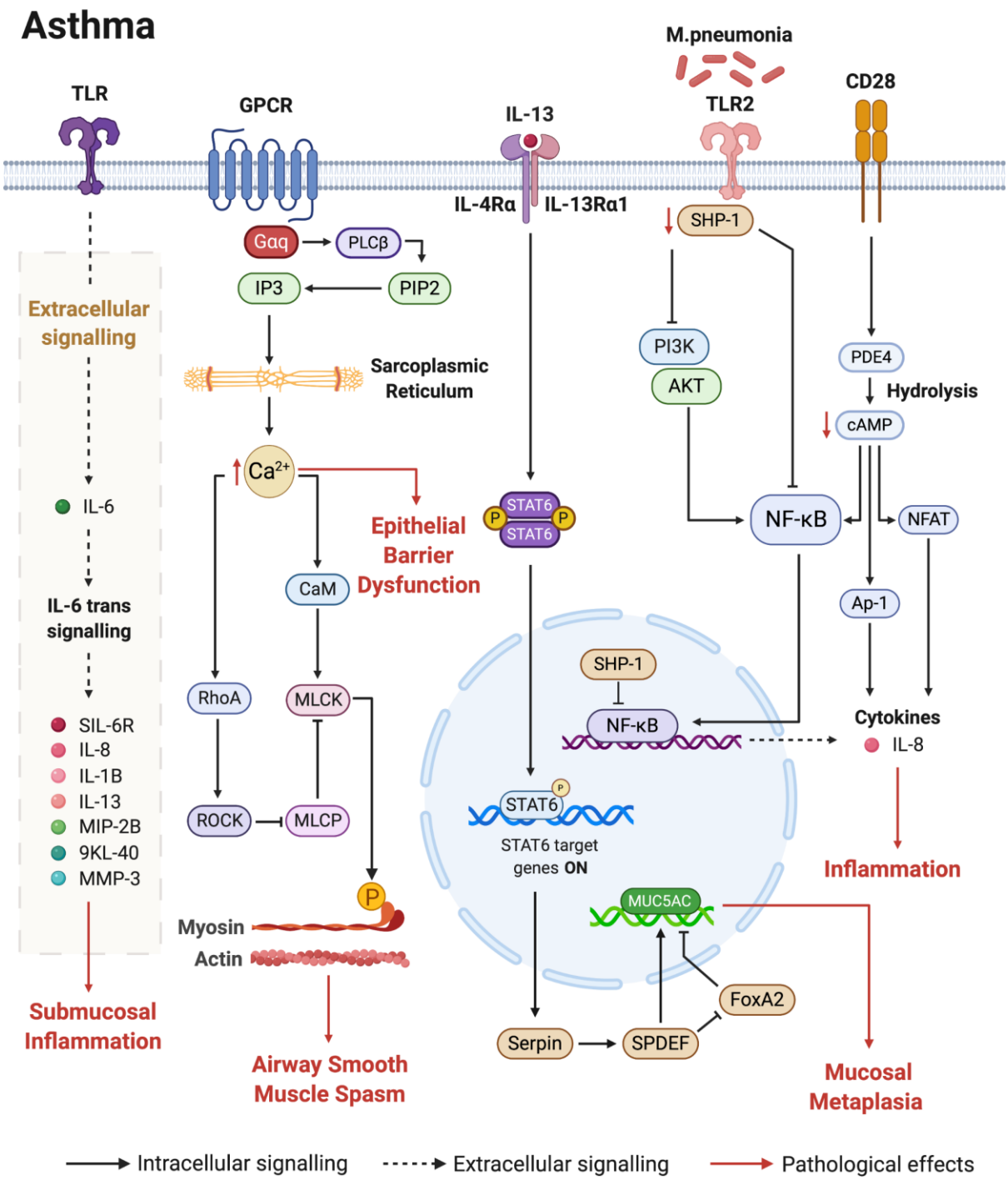
extraction is commonly acquired from post-mortem human cadavers. **(B)** Tissue *explant culture* using standardised protocols allows for bronchial/tracheal *epithelial cell isolation*. **(C)** Epithelial cell cultivation is often carried out to passage 1-2, with *epithelial cell expansion* at passage 2-4 and subsequent ALI differentiation between passage 1-6. **(D)** *Standard ALI* refers to the typical 3D ALI model widely adopted in preclinical studies. Advancements to increase scalability by reducing the manual labour involved, and translatability by introducing multiple native cell populations are shown in **(E)** and **(F)** respectively. **(G)** An example co-culture ALI model is shown, adapted with permission from Wu et al, 2020. Here, a human lung fibroblast (hBFs) and human bone-marrow derived stem cell (hBM-MSCs) co-culture with bronchial epithelial cells (hBEpiCs), utilising a 3D-TIPS (thermally induced phase-separation) printed soft elastomer scaffold, enabled enhanced epithelial barrier formation (increased ZO-1 and E-cadherin staining shown in red and green respectively) and ciliation (increases acetylated alpha-tubulin staining shown in purple) in matured epithelial tissue analogues.



Abbreviations: **APC** - Adenomatous polyposis coli, **CCL** - CC chemokine ligand, **CKIa** - Casein kinase Iα, **COPD** - Chronic obstructive pulmonary disease, **CXCL** - CXC chemokine ligand, **DAMPs** - Damage-associated molecular patterns, **ECM** – Extracellular matrix, **G-CSF** - Granulocyte colony-stimulating factor, **GSK-3b** - Glycogen synthase kinase, **IL** -Interleukin, **M-CSF** - Macrophage colony-stimulating factor, **MMP** - Matrix metalloproteinase, **mTOR** - Mechanistic target of rapamycin pathway, **NF-κB** - Nuclear factor-κB, **NLR** - Nucleotide-binding oligomerization domain like receptor, **NLRP** - Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing proteins, **PAMPs** - Pathogen-associated molecular patterns, **PRR** - Pattern recognition receptor, ROS - Reactive oxygen species, **TCF/LEF** - T-cell factor/lymphoid enhancer factor, **TGF-βR** - Transforming growth factor-β receptor, **TLRs** – Toll-like receptors, **TNF-α** - Tumor necrosis factor-α, **TSLP** - Thymic stromal lymphopoietin, **Wnt** - Wingless-related integration site.

Figure 3 | Illustration depicting the multifaceted signalling mechanisms of the respiratory epithelium in COPD. TGFβR activation leads to SMAD2/3-SMAD4 complex translocation into the nucleus, while Wnt receptor activation leads to β-catenin translocation into the nucleus

of epithelial cells. These pathways cooperatively induce transcriptional changes leading to EMT in bronchial epithelial. Activation of PRRs, namely NLRPs and TLRs in COPD leads to prolonged, inflammatory responses. PRRs are activated by DAMPs released after tissue injury, while TLRs interact with PAMPs. Their activation leads to the production of inflammatory cytokines IL-1β, IL-33, and IL-18, and subsequent increases in TNF-α, IL-6 and IL-8. Activation of TLRs directly produces IL-8 via the NF-κB pathway. IL-1β induces the release of M-CSF which potentiates chronic inflammatory disease via induction of monocytes. The proinflammatory cytokines IL-1β, IL-8 and IL-18 activate neutrophils and macrophages. Cigarette-smoke induced activation of TLRs leads to ROS production via macrophage activity, which produces a plethora of effects in bronchial epithelial cells including, but not limited to, remodelling of the ECM, cell apoptosis, altered mitochondrial respiration, causing both direct and indirect epithelial injury. Pathway data was adapted from the relevant literature and generated utilising *in vitro* respiratory models (Baarsma and Königshoff, 2017; Barnes et al., 2003; Chung and Adcock, 2008; De Rose et al., 2018; Eapen et al., 2019; Hikichi et al., 2019; Mortaz et al., 2011).



Abbreviations: **AKT** - Protein kinase B, **AP-1** - Activator protein 1, **Ca2+** - Calcium, **CaM** – Calmodulin, **cAMP** – Cyclic adenosine monophosphate, **CD** - Cluster of Differentiation, **GαQ** - Gq protein alpha subunit, **GPCR** - G protein–coupled receptor, **IL** – Interleukin, **IP3** - Inositol 1,4,5-Triphosphate, **KCl** - Potassium chloride, **MIP-2B** - Macrophage inflammatory protein-2-beta, **MLCK** - Myosin light chain kinase, **MLCP** - Myosin light chain phosphatase, **MMP** - Matrix metallopeptidase, **MUC5AC** – Mucin 5AC, **NFAT** - Nuclear factor of activated T cells, **PDE4** - Phosphodiesterase-4, **PI3K** - Phosphoinositide 3-kinase, **PIP2** - Phosphatidylinositol 4,5-bisphosphate, **PLCβ** - Phospholipase C beta, **RhoA** - Ras Homolog Family Member A, **ROCK** - Rho-associated coiled-coil protein kinase, **SHP-1** - Src homology 2 domain-containing protein tyrosine phosphatase 1, **SPDEF** - SAM pointed domain containing ETS transcription factor, **STAT6** - Signal transducer and activator of transcription 6, **TLR** - Toll-like receptor, **YKL-40** - Chitinase 3-like protein 1.

Figure 4 | Illustration depicting the core signalling mechanisms involved in the asthmatic respiratory epithelium. In a novel subset of asthmatic patients, TLR-mediated increase in epithelial IL-6 trans signalling leads to increased submucosal inflammation. Activation of Gαq,

subsequently activates PLCβ and enables IP3 binding to the sarcoplasmic reticulum to increase intracellular calcium. Calcium initiates myosin phosphorylation via CaM activation. Subsequently, MLCK phosphorylation and disinhibition of MLCP causes airway smooth muscle spasm in the asthmatic airway. Activation of the IL-13 receptor in airway mucous progenitors phosphorylates STAT6 which subsequently translocates into the nucleus to activate STAT6-gene promoters. The subsequent downstream processes include a SERPIN-mediated activation of the transcription factor SPDEF, activating mucosal cell differentiation directly or via the disinhibition of FOXA2, leading to bronchial airway mucosal metaplasia. M. pneumoniae-activated TLR2 signalling in asthmatic patients fails to recruit SHP-1, which normally inhibits NF-κB function, therefore increasing NF-κB activity alongside increased PI3K/Akt signalling and contributes to excessive IL-8 production. Similarly, CD28 receptor activation induces PDE4, which hydrolyses cAMP and increases AP-1, NFAT and NF-κB activity serving to increase proinflammatory cytokine production. Pathway data was adapted from the relevant literature and generated utilising *in vitro* respiratory models (Athari, 2019; Erle and Sheppard, 2014; Heijink et al., 2020; Jevnikar et al., 2019; Wang et al., 2012).

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