Modelling the lung in vitro

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Chapter 1

Introduction

Scope of this thesis

The lungs are a complex organ that can be sub-divided in regional sections that are defined by their function, structure and cellular composition. The respiratory compartment consists of the trachea, bronchi, bronchioles or small airways, alveoli or air sacs, and the supporting mesenchymal compartment of connective tissue, blood vessels and lymphoid tissue. The lungs consists of as many as 40 different resident cells types and more cell types are being discovered or redefined with the current advances in single cell RNA sequencing technology (Montoro et al. 2018; Plasschaert et al. 2018; Schiller et al. 2019). The function of the lungs is to facilitate the exchange of oxygen and carbon dioxide between the oxygen carriers in the blood and the air that we breathe. For the lungs to achieve this, the different compartments need to work together seamlessly. The conducting lower airways are composed of the trachea that divides into bronchi and bronchioles. The bronchioles lead to the alveoli that constitute most of the lung surface area and enable gas exchange with the circulating blood. The conducting airways are lined with highly specialized pseudostratified epithelial cells including ciliated cells, mucus-producing goblet cells and club cells, while the underlying basal cells act as progenitors (Crystal et al. 2008). The distal part of the lungs contain an average of 450 million pulmonary alveoli lined by thin, flat, non-dividing alveolar epithelial type 1 cells (AEC1) that form a semi-permeable barrier that selectively exchanges O_2 and CO_2. The AEC1 are accompanied by cuboidal alveolar epithelial type 2 cells (AEC2) that produce pulmonary surfactant proteins that reduce surface tension, and function as a progenitor for the alveolar epithelium (Barkauskas et al. 2013). The lung epithelium has the capacity to provide defence against pathogens and the toxins and pollutants in the inhaled air from the outside world (Hiemstra, McCray, and Bals 2015). They are supported in their role in host defence by numerous resident - and circulating immune cells (Byrne et al. 2015). Among all immune cells present, the alveolar macrophages are of special note as they reside permanently in the alveolar compartment to both protect the alveolar epithelium from external threats as well as controlling the inflammatory response to mitigate collateral damage (Guilliams et al. 2013; Garbi and Lambrecht 2017). These defences are vital as the lungs are continuously challenged by inhaled pathogens, noxious particles, and toxic gases present in inhaled air. These inhaled substances pose a health threat as both acute and repeated exposure can cause and/or contribute to a range of acute and chronic lung conditions that constitute a global health burden. All respiratory diseases combined, of which the most frequent are chronic

obstructive pulmonary disease (COPD), respiratory infections and lung cancer, are directly responsible for one in six deaths worldwide (World Health Organization 2018). Furthermore, chronic respiratory conditions affect more than one billion people worldwide (World Health Organization 2017).

The study of lung biology and pathology is therefore vital but also complicated when considering the high diversity in cell types, numbers, function and tissue morphology among the different lung regions and disease states. Animal models have been widely used to study the lungs, but ethical considerations, poor translatability of results obtained from animal experiments to human physiology and substantial differences in lung structure and function between humans and e.g. mice, have led to an increased demand for alternative human *in vitro* methods to study both lung biology and pathology. However, the aforementioned complexity and diversity in lung cell populations are important elements to consider when designing an *in vitro* model of the human lungs. This also indicates that at this point in time a single *in vitro* model to study the lung in its entirety is not feasible. Instead, the appropriate model will depend on the experimental questions one wishes to answer, with the goal of recapitulating the key morphological and functional features of (part of) the organ.

Modelling of the human lung

The study of lung disease pathogenesis and drug development, as well as inhalation toxicology, requires physiologically relevant models of human lung tissue. For many years, animal models have been central in many forms of research and still play a role in disease modelling and the study of lung development, as well as the development and testing of new drugs. However, it is becoming more clear that animal models are not always suitable to study human processes (Leist and Hartung 2013). While animals have provided seminal insight into lung physiology and pathophysiology, they are limited in recapitulating the development, structure, disease symptoms, and responses of the human respiratory system. Notably, cellular composition differs between mouse and human lungs. In the mouse airways, mucus-producing goblet cells are rare and secretory club cells are abundant, whereas the opposite applies to human airways (Rock, Randell, and Hogan 2010). Although animal models provide access to native tissues and are indeed used for modelling human diseases and assessing efficacy of therapeutics in

a number of tissues and organs, they are often poor predictors of clinical success due to species-species differences in mechanisms of action or toxicities of drugs (Uhl and Warner 2015). Lung specific studies equally suffer from these limitations, and over-reliance on animals alone to model complex human respiratory diseases such as asthma has contributed to the lack of new efficacious treatment despite huge research efforts, as animal models have a long record of failing to predict clinical efficacy of novel therapies in human (Mullane 2011). Furthermore, gene mutations can induce different, if any, phenotypes in mice compared to humans (Liao and Zhang 2008). Taken together, this summary of limitations demonstrates that animals alone cannot be relied upon and are often an imperfect model to provide answers for increasingly specialized questions regarding a range of human lung diseases and their drug treatment, necessitating the need for human-specific preclinical or patient specific *in vitro* models of the lung.

2D cultures and cell sources

The initial difficulty in modelling the lung or features of any organ *in vitro* lies in the challenge to identify and recapitulate the essential structural and functional elements of the human organ that govern both healthy and pathological responses. In the lung, as in various other organs, the epithelial cells lining the organ surfaces facilitate many functions of the lung. Since epithelial function and dysfunction is frequently associated with organ disease, epithelial cells are a topic of intense research. In trying to mimic the lung epithelial layer *in vitro*, scientists have predominantly relied on simpler surrogate models to gain insight into human physiology and pathophysiology. 2D monolayer cultures of cancer or immortalized cell lines still represents the "standard" and most common culture model and alternative to animal models. Frequently used examples are the cell line A549 that is derived from a lung carcinoma from a patient in 1972 and has an alveolar background which enables it to mimic some alveolar features (Lieber et al. 1976). Another frequently used cell line is the BEAS-2B cell line, which is derived from the bronchial epithelium obtained during autopsy of an individual without cancer and immortalized using replication-defective SV40/adenovirus transfection (Reddel et al. 1988). These cell lines are relatively cheap to maintain, remain stable in culture and can be expanded reliably, making them relatively easy to use in (high-throughput) experiments. However, these advantages are offset by their limited physiological relevance and clinical predictivity. Due to the derivation from tumours, or the immortalization by oncogenic

transformation, these cells do not recapitulate the differentiated cell types of the adult airway, resulting in an improper replication of tissue specific function, and also their barrier formation is limited. It often remains unclear to what extent the cell lines truly recapitulate the physiological situation, which restricts their experimental use. This limited physiological relevance results in improper prediction of *in vivo* tissue function and clinical predictivity of drug efficiency.

A function that is lacking in most cell line cultures and which is central to lung function is the exposure to air. The next level of complexity from 2D cultures and a more advanced representation of the epithelial compartment of the lung can be obtained by air-liquid interface cultures. Although this can be done with some cell lines, notably Calu-3 (Banga et al. 2012), these types of cultures are frequently established with primary cells. Primary cells are usually freshly isolated from tissue and used directly or after limited expansion. Notable drawbacks of the use of these cells are their limited lifespan, proliferative capacity and specific culture requirements. The primary tracheal and bronchial epithelial cells can be obtained from various sources. For instance, from macroscopically normal, tumour-free resected lung tissue from patients that are diagnosed with lung cancer, collected during lung volume reduction surgery for late stage COPD, derived from tissue that has become available in the context of lung transplantation, or from bronchial biopsies or brushings/scrapings that are obtained during bronchoscopy. The obtained epithelial cells can be expanded as basal cells and frozen until required (Amatngalim et al. 2016). Upon thawing and expansion, the basal cells can be seeded on Transwell inserts, with a porous membrane, that facilitate both air exposure and nutrient absorption from the bottom compartment. The exposure to air initiates differentiation of the basal cells and over the course of 2-3 weeks these will develop in a pseudostratified epithelial culture resembling the composition of the lining of the airways. These cultures have a similar gene expression signature as the human airway epithelium *in vivo* (Dvorak et al. 2011; Ross et al. 2007), recapitulate several key functional hallmarks of human airway epithelium, and have been successfully used in studying numerous biological processes in lung repair, function, host defence and pathophysiology (Gray et al. 1996; Crystal et al. 2008; Livraghi and Randell 2007; Malavia et al. 2009; Amatngalim et al. 2018). As mentioned above, the epithelial cells are often isolated from tissue derived from patients that had to undergone surgery

because of a disease, mostly lung cancer or COPD. The influence of these known and unknown lung diseases or other pathologies on the epithelial cell function *in vitro* is often unknown and can further increase the already existing donor variability. Alternative ways in which primary nasal, tracheal or bronchial epithelial cells can be obtained is via biopsies or brushes (Banerjee et al. 2009; Brewington et al. 2018). These methods do not rely on surgical resection and allow sampling of otherwise healthy individuals, although especially bronchoscopy required for collection of tracheal and bronchial epithelial cells is still invasive. The cells obtained through these methods are limited in number and require expansion for use in further experiments. This can be problematic as primary cells have a limited proliferative capacity and there is thus a risk of cellular senescence. Interestingly, another culture technique has shown that this exhaustion could potentially be overcome. The inhibition of TGF-β/BMP/SMAD signalling pathways was recently found to enable the long-term expansion of primary $TP63^{+}$ airway basal cells by inhibiting terminal differentiation and promoting self-renewal (Mou et al. 2016). However, it is at present insufficiently clear whether the cells pushed beyond their "normal" limits behave in a similar manner compared to the freshly obtained cells. Nevertheless, this technique could prove very useful in expanding limited cell populations for use in further experiments.

Human induced pluripotent stem cells

When human embryonic stem (ES) cells were first isolated (Thomson et al. 1998) there was a strong interest in using these cells in therapy. These cells could be obtained from the inner cell mass of a blastocyst and remained karyotypically stable in culture and could differentiate to different cell types of all three germ layers (Xu et al. 2002). Protocols were developed to direct this differentiation but the ethical considerations remained pressing. Then in 2006, Yamanaka and colleagues offered new opportunities when they first described a method for generating mouse induced pluripotent stem cells (Takahashi and Yamanaka 2006). The following year they reported successful generation of human induced pluripotent stem cells (hiPSC) (Takahashi et al. 2007). By forcefully expressing 4 specific transcription factors, now known as the Yamanaka factors, adult human somatic cells can be reprogrammed to a pluripotent state. From this pluripotent state, the cells can be directed through the embryonic development to the organ or tissue of interest. In addition, these cells can proliferate virtually indefinitely while maintaining genetic integrity. hiPSC retain the genomic makeup of the

donor and therefore hold great potential to build advanced patient specific *in vitro* models of human tissue and to further our understanding of lung physiology and disease. Although the genetic sequence is unaltered during reprogramming to hiPSC, the remaining epigenetic memory could influence cell behaviour (Takahashi and Yamanaka 2006; Papp and Plath 2011; Brix, Zhou, and Luo 2015; Nishizawa et al. 2016). Retaining epigenetic memory from the source cell could be interesting for studying patient specific features but can also interfere with reprogramming as it can lead to insufficient silencing of tissue specific modification (Vaskova et al. 2013; Horvath 2013). Nevertheless, these cells give interesting prospects for their use in regenerative medicine. As they retain the genetic makeup of the donor, upon transplantation there is no risk of graft versus host disease, and their use allows for the intriguing possibility to become one's own donor. As of yet it is not possible to generate a complete functioning organ, but transplantation of organ specific cells or progenitor cells is an enticing possibility. This approach has in fact been implemented in various case studies, notably in Japan. Initial data of implantation of retinal cells in an individual suffering from age-related macular degeneration has shown positive results (reviewed in (Cuevas, Parmar, and Sowden 2019; Oswald and Baranov 2018)). The retinal progenitor cells were engrafted in the eye and were able to slow or completely stop disease progression. As of yet no side effects have been reported, although the long-term effects remain unclear.

Initial studies first reported efficient induction into mesodermal and ectodermal lineages; however, maturation into the third endodermal germ layer remained limited (Kadzik and Morrisey 2012). The induction of posterior endoderm cell derivatives that give rise to organs such as the liver, intestine, and pancreas became possible in the early 2000s (Spence et al. 2011; D'Amour et al. 2006; Cai et al. 2007). In 2011, a key study elucidated a mechanism permitting stem cell differentiation into lung-specific endoderm precursor (Green et al. 2011).

Generation of endodermal cells from hiPSC *in vitro* is accomplished through directed differentiation, a process in which *in vivo* developmental stages are mimicked *in vitro* using controlled sequences of endogenous signalling factors (Murry and Keller 2008). hiPSC are first directed into definitive endoderm through activin A simulation, followed by anterior foregut endoderm (AFE) induction through dual inhibition of the SMAD pathway by inhibiting thebone morphogenic protein (BMP) and transforming growth factor (TGF-β)

signalling pathways (Green et al. 2011). Following AFE establishment, a ventral patterning step is needed mirroring the morphogenesis of the endoderm where the trachea and lung buds eventually emerge ventrally (Morrisey and Hogan 2010). AFE ventralization is largely achieved through WNT, BMP, retinoic acid and fibroblast growth factor (FGF) signalling (Lee et al. 2014; Green et al. 2011) yielding cells expressing transcription factor NKX2–1, the earliest marker for lung epithelial lineages (Kimura et al. 1996; Xu et al. 2016). From this overall lung progenitor cell, the differentiation can be directed to either a proximal or distal fate, to obtain airway and alveolar epithelial cells, respectively (Fig. 1).

hiPSC-derived airway epithelial cells

Progression in the differentiation of NKX2-1^{+} lung progenitor cells towards proximal airway epithelial cells advanced in the past few years. Differentiation of hiPSC into airway epithelial cells was achieved in spheroid cultures using FGF10, KGF, WNT agonist and Notch inhibition (Konishi et al. 2016). More recently, it was shown that activating WNT signalling during differentiation efficiently induced a distal fate, whereas suppressing WNT signalling following ventralization directed cells towards a proximal fate, and resulted in epithelial cells expressing the classic markers SCGB1A1/CC10 for club cells, MUC5AC (goblet cells), and p63 and Keratin 5 (basal cells) (McCauley et al. 2017). Although the timing, concentration and method of WNT activation likely plays an important role, since in another study it was shown that promoting WNT by inhibiting GSK-3β resulted in a mixed population (de Carvalho et al. 2019). To promote cilia development of the airway derived hiPSC, Notch inhibition was needed to produce motile multiciliated cells characterized by acetylated alpha-tubulin (α-Tub) staining (McCauley et al. 2017; Wong et al. 2012; Firth et al. 2014). Another protocol used 2D air-liquid interface cultures with FGF18 stimulation to generate a mature and polarized epithelial layer, in which motile cilia and mucus could be observed at the apical surface (Wong et al. 2015). It was shown that the scaffold and micro-environment of hiPSC-derived lung progenitors is a major determinant for achieving a mature airway epithelial phenotype, as shown using human hiPSC-derived lung organoids (Dye et al. 2015; Dye et al. 2016).

hiPSC-derived alveolar epithelial cells

Over the past years, notable progress has been made using NKX2-1$^+$ lung progenitors to generate human alveolar epithelial cells by establishing protocols to direct these progenitors towards a distal fate. The combination of glucocorticoids, growth factors, and cAMP effectors (dexamethasone, 8-br-cAMP, IBMX, and KGF/FGF7; collectively known as DCIK) (Gonzales et al. 2002; Wade et al. 2006) has been shown to induce alveolar maturation through activation of PKA and CDP-choline pathways which upregulate lamellar body surfactant production in AEC2 (Andreeva, Kutuzov, and Voyno-Yasenetskaya 2007) (Fig. 1). Recognized markers of the distal alveolar epithelium include Surfactant Protein C (SFTPC), Surfactant Protein B (SFTPB), HTII-280, ABCA3, and LAMP3/DC-LAMP for AEC2, while Podoplanin (PDPN), Caveolin (CAV1), and Aquaporin 5 (AQP5) primarily define AEC1 cells. Stimulation of 2D cultures of NKX2-1$^+$ differentiated distal progenitors with DCIK, FGF10 and WNT activators leads to expression of AEC1 and AEC2 markers, mature phenotypic characteristics of lamellar bodies, and functional surfactant uptake capability (Huang et al. 2015). AEC2 markers were found to be abundant in this protocol while AEC1 markers were minimally expressed (Chen et al. 2017).

Taken together, the advances in hiPSC differentiation protocols have made these approaches very interesting for the study of lung biology. The major drawback however is the lengthy and labour-intensive maintenance and

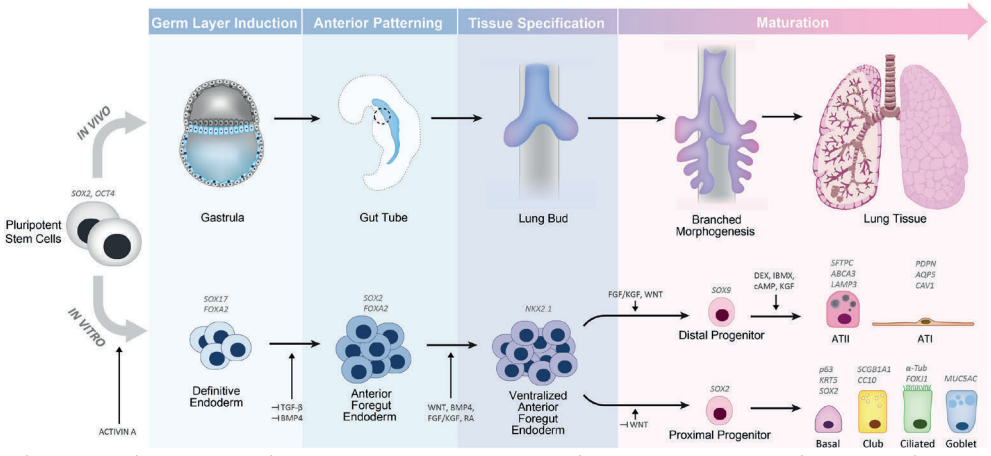

Fig. 1. Overview of the major stages of lung development in humans corresponding to the directed differentiation pathways of pluripotent stem cells towards a lung epithelial fate. The various intermediate steps of development with key signalling factors and common markers are indicated. Reproduced from Nawroth et al. (Nawroth et al. 2019).

differentiation timelines resulting in limited efficiency. Despite substantial advances in differentiation of lung stem cells, full maturation is still a challenge. For example, demonstrating SFTPC and SFTPB gene expression was initially sufficient to claim AEC2 cell differentiation. Furthermore, achieving an optimal balance between AEC1 and ACE2 is complicated. However, with the accelerated progress in lung PSC protocols, more strict metrics are now being considered to reach the ultimate goal of generating a supply of cells that recapitulates the same molecular, biochemical, phenotypical, and functional features found in the adult lung (Beers and Moodley 2017). Nevertheless, the capability of generating patient specific cell lines that can be directed to any cell types will be crucial in eventually developing a truly patient specific model.

The 3D culture of lung organoids

Whereas hiPSC have gained importance next to primary lung cells to obtain airway and alveolar cells, there have also been important developments in culture models. One culture model that has risen in prominence during the past couple of years is the culture of spheroids or organoids. An organoid can be described as a "miniature organ" suspended in a hydrogel which overcomes the 2D constraints and relies on the self-organisation of the cells to form 3D structures. Such a structure need consists of different organ specific cells and rely on self-organization and self-renewal. As this culture technique focusses on the progenitor/stem cells of the organ and because the culture conditions facilitate the selection of these cells, organoids can be passaged and propagated longer when compared to their 2D equivalents.

Airway organoids

The first airway organoids were described as early as 1993 (Benali et al. 1993). Since then, lung organoids derived from human lung tissues or derived from hiPSC have been successfully used to study lung development and disease (Barkauskas et al. 2013; Tata et al. 2013; Chen et al. 2017). These methods previously required supporting feeder cells to grow, but more recently the long-term mesenchymal cell-free expansion of airway organoids was demonstrated (Sachs et al. 2019). This allows for the reliable clonal expansion of airway cells and has opened new avenues of research.

Alveolar organoids

Growing organoids from alveolar tissue has proven to be more challenging, however the reward could potentially be much greater as the proliferating AEC2, that serves as a progenitor for the non-proliferating AEC1, rapidly loses its phenotype when plated on conventional culture plastic (Mao et al. 2015). However, when seeded in a 3D hydrogel the phenotype appears to be maintained for longer periods of time and the cells could be expanded and used for experiments (Glisinski et al. 2020; Barkauskas et al. 2017). To date, unfortunately, limited work has been done on primary human alveolar epithelial organoids and various studies describe the need for supporting mesenchymal cells. The ability to expand the AEC2 reliably under feeder-free conditions while maintaining phenotype could have major implications for the field. The 3D culture also has major implications in the hiPSC field, both in differentiation and in propagation. hiPSC-derived organoid cultures showed cell maturation of the alveolar epithelium (Jacob et al. 2017; Dye et al. 2015). Interestingly, 3D co-culture with human fetal lung fibroblasts and stimulation with DCIK resulted in expression of AEC2 specific markers and lamellar bodies formation, although functional maturity was not confirmed (Gotoh et al. 2014). Long-term expansion of hiPSC-derived alveolar epithelial cells, a challenge with conventional 2D distal lung cell cultures, was recently achieved using an organoid approach and a refined differentiation sequence after AFE induction (Yamamoto et al. 2017). This sequence differs from previous protocols by more closely mimicking the distal tip cell microenvironment through a preconditioning step of WNT activation, Notch inhibition, and FGF10 plus KGF supplementation. These cells were then matured using DCIK in organoids co-cultured with fibroblasts. Interestingly, co-cultures with fetal lung fibroblasts lines resulted in SFTPC-expressing cells (with substantial variability ranging from 2% to 51% $SFPTC^{+}$ cells), whereas incorporating a dermal fibroblast line showed no SFTPC induction. Upon passaging, the proportion of AEC2 cells in the culture increased to approximately 70%, and AEC1-like cells were also present.

Nevertheless, there are also limitations as reviewed by Fatehullah et al. (Fatehullah, Tan, and Barker 2016). Lung organoids consist solely of epithelial cells (with or without supporting feeder fibroblasts) and thus lack the native organ microenvironment and appropriate architecture that is facilitated by structural cells and is often essential in disease development, tissue growth and repair. A further drawback of organoid culture is that they are fluid-filled

with an inward turned lumen, making the apical side of the epithelial cells inaccessible. Analysis or stimulation via the physiological relevant side (e.g. using airborne substances) is impossible or at least impractical. Measuring transmembrane transporter activity via indirect methods in gut organoids have been optimized in the context of cystic fibrosis research (Dekkers et al. 2016). Evaluating the transport and metabolism of drugs across the cell layer, or quantifying the release of inflammatory mediators or newly formed virions, is often precluded by the difficulty of accessing and sampling luminal contents. Furthermore, as the organoids are suspended in a hydrogel matrix, it is unclear if all stimuli concentrations are equally distributed as their distribution may be hampered by slow diffusion through the matrix. In addition, as the organoids consist of only epithelial cells, they lack tissue-tissue interfaces, such as the interface between vascular endothelium and surrounding stroma and the accompanying immune cells, which are essential for the morphology and function of virtually all organs.

All the above discussed culture methods or techniques are frequently used in pulmonary research and have provided seminal insight in lung biology. However, all these methods lack basic biomechanical forces. The lung is a flexible dynamic organ that is in continuous motion. The next step in modelling the human lung is incorporating physiological fluidic and biomechanical cues, including flow of air or liquid, in an effort to mimic what cells experience *in vivo*.

Organ-on-Chip technology

Organ-on-Chip or Lung-on-Chip are another culture model that has rapidly evolved in the past decade. These platforms are micro-engineered culture systems that allow continuous perfusion of microchannels on which the cells can be exposed to the biomechanical forces discussed in the previous section. The "chip" can be constructed to focus on key aspects of the organ of interest. For lung physiology, air and liquid flow are important as well as cyclic stretch which represents breathing (Benam et al. 2016; Huh, Hamilton, and Ingber 2011; Huh et al. 2010). The lung-on-chip would provide more complexity than can be achieved using static air-liquid interface culture. This is important as the influence of the biomechanical forces on the lung and alveolar biology is incompletely understood and the lung-on-chip could play a critical role in elucidating these interactions (Waters, Roan, and Navajas 2012). The Lung-on-Chip has the potential to play a role in large scale screening of compounds

and therapeutics, although further development in both our understanding of the biology of cell behaviour in chips as well as a wider distribution and acceptance is needed to achieve this. This is however important, since for instance the mechanical forces, like stretch, of the chip on the cells influence cell function and can alter drug and compound responses (Hassell et al. 2017), and therefore testing on a Lung-on-Chip may better predict the *in vivo* situation. Another interesting possibility would be to mimic the mechanical consequences of disease progression like the remodelling of the epithelium that occurs in COPD or asthma (Grainge et al. 2011). A further consideration is the material used in the chip as the properties of the material influence different aspects. The materials need to be selected to provide structural support to the cells, but also needs to tolerate the mechanical forces, and support instrumentation for tissue maintenance and experimental readouts. Optimally, the material of the chip needs to be non-toxic, optically clear, affordable, non-degrading under cell culture conditions, flexible to allow stretching, and not altering the chemistry of the fluid environment. Furthermore, the properties of the substrate the cells are grown on, such as the stiffness, flexibility, curvature or adsorption, among others can influence different cellular differentiation and function (Riaz et al. 2016; Discher, Janmey, and Wang 2005). These parameters can influence migration (Zaman et al. 2006), proliferation (Ulrich, de Juan Pardo, and Kumar 2009), metabolism (Park et al. 2020) or differentiation (Engler et al. 2004) and likely much more cellular properties, that we are only just starting to uncover. The integration all these factors and considerations into a chip allows for many new lines of research which have previously been impossible.

At present the organ-on-chip technology is in its infancy despite major developments in the last decade. Further development is essential and requires research performed by multidisciplinary teams, including biologists and engineers. The fact that the technique is both novel and expensive, explains why only few studies have been performed using this technology, making it difficult to validate and assess the applications.

Scope of this thesis

The study of airway repair, homeostasis and function requires suitable models. Human *in vitro* culture models could provide a way to reduce the use of animal models and generate data that can be better translated to patients. In the studies described in this thesis, an effort is made to explore various improvements of different current human *in vitro* models. In **chapter 2**, the use of human induced pluripotent stem cells as a source for obtaining alveolar cells is described. The use of these cells as a cell source could provide a patient-specific model for *in vitro* screenings. The generation of alveolar type 2 (AEC2)-like cells using our protocol is discussed and these cells were subsequently cultured at the air-liquid interface (ALI) to create a model of alveolar wound healing at the ALI. In **chapter 3**, we describe a method to utilize organoids to expand human alveolar cells to obtain sufficient numbers to reliably establish alveolar epithelial cell cultures. We isolated AEC2 from resected lung tissue using HTII-280 coated magnetic beads, and expanded these using organoid culture. Once sufficient cells were obtained, we seeded the cells on a lung-on-chip system to study the effect of flow and mechanical stretch on the alveolar cells. In **chapter 4** we continue on the use of organoids as a method of expanding cell populations. The technique is applied to obtain airway epithelial from clinical samples with low epithelial cell numbers. broncho-alveolar lavage (BAL) fluid from adults and tracheal aspirates (TA) from preterm new-borns was used to expand airway epithelial cells in organoid culture, followed by establishment of ALI cultures to allow exposure to airborne substances which is not possible using organoid models. In these studies, expansion using organoid or conventional submerged 2D culture (from respectively BAL and TA, or bronchial tissue) was compared using the properties of ALI cultures as read-out. The proposed culture method could provide a tool to study the early factors involved in the development of lung diseases, including those occurring early in life such as bronchopulmonary dysplasia (BPD). In **chapter 5**, we increased the cellular complexity of our models by including the cross-talk between polarized macrophages and airway epithelial cells. In lung tissue, every process relies on the interplay between various cell types, which is often not reflected in *in vitro* models. We therefore developed a co-culture model to study the effect of macrophage-epithelial interactions on airway epithelial wound repair. In **chapter 6**, we focussed on the substrates on which lung epithelial cells can be grown. To this end, we evaluated the use of a bio-compatible polymer of poly(trimethylene carbonate) (PTMC) for generation of culture membranes. PTMC has the

theoretical advantage over competitors used in organ-chips, that it is not rigid, more flexible (and thus allows stretching) and may be less susceptible to non-specific adsorption. We thus aimed evaluated the use of PTMC membranes in our primary human airway epithelial culture model. Finally, in **chapter 7** the findings of the studies presented in this thesis are summarized and discussed in a wider context of the field together with possible future perspectives.

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