# Modelling the lung in vitro

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# Chapter 7

Summary and General discussion

Chronic and acute lung diseases affect many people worldwide and are a major cause of death (World Health Organization 2018). In order to study the mechanisms and signalling pathways that underlie these diseases, advanced patient specific models are required. In addition to studying lung health and disease, these models are also important and useful for their application in high-throughput screening of potential new therapeutics. There has been an increased demand for more comprehensive in vitro models with better translatability to the *in vivo* situation. This increased demand also relates to the ethical considerations related to the use of animal models, as well as the fact that translation of results from animal models to humans is often problematic (Uhl and Warner 2015). As a result of this demand from both the scientific community and society, there is an urgent need for better human in vitro lung models, and a reduction in the use of animals for (respiratory) research purposes. This is furthermore highlighted by the notion that replacement of animal models is now high on the national (Transitie proefdiervrije innovatie 2020) and European (political) agenda (European Commission 2020-08-31).



In the last decades, much progress has been made in the development of in vitro models of the lung. Many research groups have transitioned to the use of primary cells instead of tumour cell lines or immortalized cell lines. Furthermore, culture techniques like air-liquid interface (ALI) and organoids have become widely accepted in the field of lung research. The in vitro models have also increased in complexity as illustrated by developments in the organ-on-chip field, despite the fact that this is still somewhat in its infancy. The field keeps developing and we understand that all these models have their own advantages and disadvantages. In this thesis, several new models and improvements in existing models are described, with the aim to contribute to the in vitro toolbox required for studying lung biology in health and disease.

In this thesis we have focussed on methods for obtaining patient-specific lung cells through different means. We used human induced pluripotent stem cells (hiPSC) to develop a differentiation protocol to obtain alveolar-like cells as well as endothelial cells from the same donor. We used hiPSC-derived alveolar-like cells to study the growth at the ALI and analysed wound repair (Chapter 2). We also developed a method to expand and establish cultures of primary cells derived from sources with limited amounts of cells (Chapter 3 and 4). We demonstrate that the few cells present in tracheal aspirates of preterm new-borns or the cells present in bronchoalveolar lavage (BAL)

fluid can be used to develop ALI cultures (Chapter 4). Furthermore, we explored the effects of the different substrates that the cells are cultured on. Regular culture plastic is much stiffer than the natural matrix in the lung, so possible substitutes need to be evaluated. We have studied the effects on cell differentiation and function of airway and alveolar cells when cultured on poly(trimethylene carbonate (PTMC) and Polydimethylsiloxane (PDMS) respectively (Chapter 3 and 7). Finally, as the lungs are at the forefront of defence against inhaled pollutants and pathogens, they are supported in their function by surrounding immune cells. We have therefore developed a co-culture of airway epithelial cells and polarized macrophages. This model allowed us to study the interaction and cross-talk between airway epithelial cells and macrophages, and the role of this cross-talk in host defence and epithelial repair (Chapter 5).

In the next paragraphs I will highlight and discuss the main findings of this thesis. The findings will be placed into perspective of the current field as well as their future perspectives.

## The use of human induced pluripotent stem cells in cellular models

The development of a model that can be used to analyse physiological relevant mechanisms or that can be used to test therapeutics is highly reliant on the cells and the sources used. In chapter 2 we used hiPSC-derived alveolar epithelial cells to model alveolar epithelial wound closure *in vitro* at the physiologically relevant air-liquid interface (ALI). We unexpectedly found that the canonical WNT activator CHIR99021 had a detrimental effect on the wound closure. We (as described in chapter 2) and other groups have found other instances were canonical WNT activation was beneficial for alveolar cell growth (Jacob et al. 2017). However, the unique setup in which the cells grown in 2D at the ALI might explain some of these differences. Additionally, while CHIR99021 is widely used as an effective canonical WNT activator it functions indirectly through the inhibition of GSK3β, which is part of the β-catenin destruction complex. It is known that CHIR99021 via inhibition of GSK3β can have additional effect on cellular signalling (An et al. 2010). Work is being done to compare the effects of CHIR99021 and recombinant WNT ligands on the induction of WNT. Unfortunately, this is still ongoing. Nevertheless, due to the relative instability of the recombinant WNT ligands CHIR99021 is predominantly used but the off target effects could explain some of the differences observed.

Furthermore, we found that EpCAM$^+$ selection of the cells alone was not enough to obtain a pure cell population. Other groups have used surface expression of carboxyl peptidase M (CPM) for selection or have used NKX-2.1 or pro-surfactant protein C (SFTPC) reporter lines for selection during, at the end of the differentiation protocol or with every consecutive passage (Jacob et al. 2017; Gotoh et al. 2014). The introduction of these reporters is highly useful for *in vitro* cultures but will remain an issue if these cells will eventually be used for the use in humans. The variation in efficiency of differentiation protocols and the accompanying heterogeneity of the obtained population highlights the largest hurdles that need to be overcome in hiPSC related research, both for modelling the alveoli *in vitro* and the eventual application in humans (D'Antonio-Chronowska et al. 2019; Kilpinen et al. 2017). For example, the direct single cell analysis of SFTPC-sorted hiPSC-derived alveolar cells revealed the presence of many different subpopulation (Hurley et al. 2020). This illustrates that even the current most leading protocols are not yet capable of overcoming this issue but also shows that the SFTPC$^+$ cell population might be very heterogeneous. Nevertheless, the application of single cell analysis following and during each step in the differentiation pathway will provide valuable insight in the different genes involved and cell subtypes traversed in generating the cell type of interest from the hiPSC state. If the homogeneity of the population is to be increased, we likely need to broaden the number of markers used in the validation of intermediate stages which are analysed and the methods used in the differentiation protocols.

The influence of epigenetic components in hiPSC-based models are currently understudied. For instance, it remains largely unclear what epigenetic changes occur when a cell changes from one type to another in a forced and relatively short timeframe, such as during hiPSC differentiation. Furthermore, it is unclear to which extent the residual epigenetic memory could influence cell behaviour after cellular reprogramming (Papp and Plath 2011; Takahashi and Yamanaka 2006; Brix, Zhou, and Luo 2015). *In vivo* organogenesis is a continuous and dynamic process that is driven by neighbouring tissue interaction. In contrast, most current methods of differentiation in vitro are directed and highly simplified compared the subtle changes that direct lung development in utero, and lack relevant interactions with the local (micro)environment. It was shown that the combined culture of anterior and posterior gut organoids without additional exogenous factors led to fusion of the organoids and further development of the organoid in the respective

anterior and posterior organs (Koike et al. 2019). This demonstrates that the differentiation and patterning of the structures is subtler and maybe more dependent on cell-cell interaction than expected previously. Furthermore, it was recently shown that differentiation and growth of hiPSC-derived alveolar cells benefited from intermittent withdrawal of triggers for WNT signalling (Hurley et al. 2020). The pulse wise activation of WNT signalling created a balance between proliferation and differentiation of the cells. This again is likely more representative of the *in vivo* situation where during development frequent fluctuations and gradients in concentrations of growth factors are expected (Christian 2012; Rosenbauer et al. 2020). A further aspect that is insufficiently integrated in many differentiation protocols is the influence of the extracellular matrix or signals that are downstream of the cell-matrix interaction. Some protocols already utilise 3D/organoid culture in Matrigel or substitutes thereof. This could for instance influence changes in geometry and polarization of the cells, which in turn affects differentiation and cell fate decisions (Kilian et al. 2010; Mosqueira et al. 2014; Warmflash et al. 2014), but is likely highly specific for different stages or organs (Goetzke et al. 2019). The modulation or supplementation of these Matrigel/hydrogel with extracellular matrix proteins can have beneficial effects on the growth and development as shown with pancreatic progenitors (Greggio et al. 2013). Furthermore, it is unclear how other mechanical queues could influence differentiation. For instance the interaction of the inner cell mass with the extraembryonic tissue and the uterus of the mother during or very shortly following gastrulation likely influences the polarization of the early embryo (Beddington and Robertson 1998).

Nevertheless, even with all these hurdles yet to be overcome, hiPSC are central in many research areas and more refined protocols are being developed continuously. The potential of generating an *in vitro* model in which all involved cells have the same genetic background would have major implications. We managed to generate $CD31^{+}CD144^{+}$ endothelial cells from the same hiPSC cell lines that we used to generate alveolar epithelial cells (AEC) 2-like. However, we did not manage to generate a stable co-culture due to the difficulties of fine-tuning a medium in which both cell types are comfortable.

## Co-culture of different cell types in *in vitro* lung models

As emphasized previously, the lungs consist of many different cells, which

all have to work in unison to maintain a healthy functioning organ. This interaction between cell types is often not reflected in *in vitro* models, which can be partly attributed to culture or cell availability issues. It is important to realize that our knowledge on the way different cell types contribute and interact during certain responses and mechanisms is still limited. Nevertheless, we know that many biological processes are guided by an interplay between different cell types, indicating the need for more complexity in *in vitro* models. Different studies have described methods of studying cell-cell interaction, such as precision cut lung slices (Akram et al. 2019; Liu et al. 2019), or *ex vivo* explants (Powley et al. 2020; Jager et al. 2014). However, using these methods it is not always clear what cells and in what numbers they are present. In chapter 5 we have therefore focussed on the development of a defined co-culture model of airway epithelial cells with monocyte-derived macrophages polarized using GM-CSF or M-CSF (M(GM-CSF) or M(M-CSF)). This model was used to study the contribution of macrophages to epithelial repair following a mechanically applied wound. We found that the macrophages and especially the M(GM-CSF) macrophages had a positive effect on the wound closure of the epithelial cells. It is already known that macrophages play a role in tissue repair and homeostasis, but these cells are often lacking in epithelial repair models (Wynn and Vannella 2016; Minutti et al. 2017). The process of wound repair can be studied using only the airway epithelial cells (Amatngalim et al. 2016), but the addition of macrophages provides another dimension and layer of complexity. *In vivo* even more cell types are involved and thus this model can be expanded by adding additional cell types. Individual cell types have been combined in previous studies with epithelial cultures that focussed on a specific process or mechanism of interest. For instance, the addition of neutrophils to the culture has been used to study migration through the epithelial barrier as a response to an inflammatory stimulus (Yonker et al. 2017). The co-culture of peripheral blood monocytes (PBMC) with epithelial cells was used to study the contribution of secreted compounds on immune cell differentiation (Luukkainen et al. 2018). The combination with fibroblasts has been described as a model to mimic airway remodelling or to study epithelial-mesenchymal cross-talk and stimulate epithelial growth (Ishikawa, Ishimori, and Ito 2017; Skibinski, Elborn, and Ennis 2007). Also, individual co-cultures with mesenchymal stem cells have been set up (Schmelzer et al. 2020). Even triple co-cultures have been attempted although not solely using primary cells (De Rudder et al. 2020; Blom et al. 2016; Alfaro-Moreno et al. 2008).

The effects of the indirect cross-talk between macrophages and epithelium that we describe in chapter 5 are based on a culture setup where the cells are cultured in different compartments. All effects observed are thus due to secreted compounds and we have not analysed cultures in which cell-cell contact was possible. Studies in which cell-cell contact between macrophages and epithelial cells was possible show differential effects between macrophage subsets on wound healing (Gindele et al. 2017), but also some changes in macrophage behaviour or polarization based on the cell-cell interaction (Ji et al. 2018; Bauer et al. 2015). However, when cell-cell contact is studied it is difficult to separate the interactions of different signalling pathways active in the various cell types, although techniques such as single cell or bulk RNA sequencing may be helpful in this respect. We show that the compound-based cross-talk between M(GM-CSF) and the epithelium results in an upregulation of the CAMP gene, that encodes hCAP18/LL-37, an antimicrobial peptide central in defence against pathogens and implicated in wound repair. We also demonstrate an upregulation in DEFB4B in the epithelium and the apical secretion of its gene product the antimicrobial peptide hBD-2. The effect of the co-culture on expression of these antimicrobial peptides make this model interesting to use for infection studies. Overall, macrophages have long been known for their role in both repair as well as host defence *in vivo*, and it will be interesting to see how macrophage-epithelial cell co-cultures will contribute to our understanding of the functioning of both epithelial cells and macrophages in a variety of processes.

## Organoid based expansion of limited cell populations

Both in chapter 3 and 4 organoid-based cultures were used to expand cells that do not proliferate well in 2D or samples that have low cell numbers. The use of organoids to grow airway epithelial cell cultures has been used in previous studies (Barkauskas et al. 2017; Benali et al. 1993; Tan et al. 2017). This culture method has increased in popularity since it was demonstrated in the Hubrecht institute that they could be cultured in a mesenchyme-free system (Sachs et al. 2019). Now, organoid cultures have become central in many fields of research. It was also shown that the organoids could be cultured over long periods of time (Sachs et al. 2019), experiencing less senescence compared to conventional 2D cultures, which eventually stop proliferating (Walters et al. 2013). Other groups have demonstrated that the use of dual SMAD inhibition together with Rho-kinase inhibition can be used to maintain the proliferative capacity of the airway basal cells (Mou et al.

2016). Although this method of expansion had a lower success rate when the starting population contained low numbers of cells (Lu et al. 2020). Our method of expansion using organoid cultures provides a promising and reliable method of obtaining sufficient cells. However, in contrast to studies that use organoid-based models (Dekkers et al. 2013), the lung is characterized by an air-exposed apical side. We have therefore focussed on expansion through organoids to ultimately establish air-exposed cultures. The possibility to expand cells from tracheal aspirates of preterm new-borns or bronchoalveolar lavage samples and subsequent establishment of *in vitro* cultures was previously not possible. The availability of these *in vitro* cultures of preterm new-borns to study, for instance, the development of Bronchopulmonary dysplasia (BPD) was an unmet need (Looi et al. 2019). It will now be possible to obtain samples from patients not or not yet suffering from lung diseases, building up biobanks before diseases arise. These can be diseases that manifest in childhood like asthma or BPD, but also later in life, like cancer or chronic obstructive pulmonary disease (COPD). This would enable side by side comparison of patient and control sample before onset of symptoms, thus allowing discovery of early biomarkers or risk factors. The clinical implications of this insight in cellular function before disease progression could be substantial.



We have demonstrated that epithelial cells present in human airway samples containing limited cell numbers of which most are luminal cells, can be expanded using organoids. Although the flexibility of the lung epithelium is suspected, de-differentiation of luminal to basal cells has so far only been demonstrated in animal models (Tata et al. 2013). This process has not been conclusively demonstrated in humans, although it is suspected. In our cultures, we cannot exclude the possibility that the few basal cells in our samples are responsible for the expansion. We demonstrate that the cultures generated from the samples containing primarily luminal cells do not respond to modulation of differentiation by IL-13 or Notch inhibition using DAPT as expected. An interesting possibility would be that the luminal cells in these cultures de-differentiate to a $TP63^{+}KRT5^{+}$ "basal" cell, but do not fully regain all functionality we classically ascribe to a basal cell. Another possibility could be that the very few basal cells initially in the culture have been exhausted to such an extent that their differentiation is impaired (Eenjes et al. 2018), but this remains unclear. We can also not exclude that other cells we did not consider to be able to form lung organoids do contribute to organoid formation, as was demonstrated in the intestine where not only

the stem cells are able to form gut organoids (Serra et al. 2019). Single cell analysis could potentially reveal underlaying non-genetic differences in these basal cells that could explain the different responses.

The expansion of the alveolar cells in organoids cultures that we describe in chapter 3, again emphasises the observed phenomena that cells cultured in a 3D matrix are more capable of maintaining phenotype and proliferative capacity, although it remains unclear why or what mechanisms are involved. We demonstrate that alveolar cells grown in a 3D matrix maintain their proliferative capacity and expression of type 2 alveolar epithelial cell markers such as HTII-280 and SFTPC over multiple weeks. The difficulty of expanding alveolar cells has led to many studies using either cell lines or poorly characterized commercial cells that lack essential features of the epithelial cells that line the alveolar lumen (Hassell et al. 2017; Akimoto et al. 2016). This method could provide a more accessible way of obtaining alveolar epithelial cell cultures. We have so far been unsuccessful in freezing pure alveolar cell populations, as single cells or organoids, but if the cells can be maintained in matrix they can be transported and used accordingly.

## Lung-on-chip

Lung-on-chip technology has emerged as a highly promising new area of study. It combines various different science disciplines and offers a range of possibilities for variation in parameters and adaption to the needs of the user. It can regulate the biomechanical forces that are sensed by tissue in health or disease, and will allow for the long-term study of cells, even allowing changing parameters during culture. Central parameters of the lung like shear stress (Galie et al. 2014), stretch (Huh 2015), concentration gradients (Wang et al. 2013), blood-epithelial barrier function (Booth and Kim 2012) or various other relevant physiological parameters can be integrated. It is only now when we are starting to expose various different cell types to these mechanical forces, that the impact of these forces on cellular function become more appreciated.

We cultured alveolar epithelial cells on PDMS S1 chips from Emulate to study the effect of the substrate and mechanical forces. We demonstrate that the alveolar cells seeded on chips are better capable of sustained HTII-280 expression compared to static ALI cultures on Transwell inserts with stiff PET membranes. When we expanded the alveolar cells as organoids, we observed that virtually all cells maintain HTII-280 expression at the

apical surface, which is in contrast to cells cultured on Transwell inserts. Our observations suggest that the alveolar cells on the chips maintained a balance between proliferation and differentiation. We observed that after 7 days there was a balance in cells expressing either AEC1 and AEC2 markers. Although various groups have analysed the growth of alveolar cells on chip, HTII-280 expression as a marker for AEC2 has not been used (Jain et al. 2018; Stucki et al. 2018). It remains unclear how long the cultures can be maintained and how long the culture will be stable as we stopped our experiments after 8 days on the chips. Furthermore, it is unclear through what mechanism the alveolar cells are able to maintain their phenotype on the chip. The control samples, in which there was a medium flow present, but that were not exposed to additional mechanical forces, also maintained HTII-280 expression. Whether this is due to the interaction with the different substrate or that the shear stress experienced by the medium has an effect is unclear.



When the cells were exposed to cyclic stretch, we observed changes in the orientation of the alveolar cells. The alveolar cells showed an elongated morphology and aligned perpendicular to the direction of the stretch. The effect observed is robust and is not present in airway cells exposed to stretch (unpublished observations) but it is unclear whether this is a process occurring *in vivo*. This observation is interesting, but also illustrates the gap in knowledge that needs to be overcome to utilize this technology to full effect.

Although the lung-on-chip technology is still in its infancy, it has the potential of making an impact and replacing many animal models (Reardon 2015). Although still expensive and not accessible to most laboratories, it has clear advantages over organoid cultures or static ALI cultures. The chips can be cultured submerged as well as at ALI whereas the organoids have their apical surface turned inwards. This makes it difficult to study physiological relevant exposures in organoid systems. The static Transwell culture systems do not allow for the application of physiologically relevant forces, however the apical surface of the culture is much better accessible. The choice of the model used will depend on the research question asked, but using one model will likely not be sufficient. More models should be used in parallel to corroborate and validate the results. However for this to happen, complex *in vitro* culture models need to become accessible and accepted.

## Validation and acceptance of biological models

A difficult, but central question that arises with every model, is whether the added layers of complexity are a better representation of the *in vivo* situation than other models, and whether this increased complexity is required to address the specific research question. New or improved models frequently can and are only compared to the previous model or the *in vivo* situation in its entirety. Will the addition of more cell types, different substrates or mechanical forces provide a better prediction of therapeutic success? This will highly depend on the mechanism and interactions of the compounds being researched, known or unknown. The effect will most likely be difficult to predict beforehand. There are examples of relatively simple and high-throughput models that provide a high translational value like the successful CFTR gut organoid swelling assay (Dekkers et al. 2013). Once an effective compound has been identified that could restore CFTR-function using this assay, it can be readily tested in patients with the same mutations (de Winter-de Groot et al. 2020; Berkers et al. 2019). More complex diseases that chronically progress and over time involve more and different cell types are more difficult to simulate and subsequently validate. It was shown that the application of stretch influenced the migration of tumour epithelial cells and influenced the subsequent effectiveness of tyrosine kinase inhibitors (Hassell et al. 2017). The complexity of the model and the added interdonor variation means that this can likely only be validated by strenuous comparison between patients, animal models and *in vitro* models.

As the *in vitro* models become more complex and comprehensive, they will have the capacity to substitute, replace or complement in part animal experiments. However, most of these models are used in academic settings, and predominantly in the labs that developed them. This also leads to the lack of standardization in the field as different labs have different interests, making it difficult to achieve some consensus. The distribution of many culture techniques - with some notable exemptions - is slow. This holds true even more for their acceptance by international regulatory authorities and industries. Only when extensive validation studies show that an *in vitro* model could replace (part) of animal testing, will it be possible to be considered by the regulatory authorities. Comprehensive testing and validation is paramount and should not be rushed but the search for *in vitro* replacements should also be intensified. In addition to increasing societal pressures that call for a decrease in animal testing, the improvements in culture models also make these transitions more feasible.

## Concluding remarks

Over the past decades, *in vitro* culture models have improved significantly and have become a vital tool in the field of lung biology. In this thesis we studied and developed different *in vitro* culture models and their roles in studying the lung. We have described different methods for obtaining cells, through differentiation of hiPSC (chapter 2), through isolation from lung tissue or using organoid-based expansion (chapter 3 and 4). We have studied the effects of the substrate the cells are cultured on (chapter 3 and 6), illustrating that the behaviour of the cells is strongly linked with its surroundings. We show impaired differentiation of airway epithelial cells on our PTMC membranes and a stable culture of alveolar cells on PDMS. This highlights the need for a strong cooperation between different academic fields to understand and integrate these findings in future *in vitro* models. We furthermore developed a co-culture setup of airway epithelial cells together with macrophages (chapter 5). We demonstrate that the addition of macrophages had a dominant effect on processes previously studies using only airway epithelial cells showing the importance of cross-talk between cell types.

Finally, the development of better more translatable *in vitro* models will need to be a cooperative effort of many fields. The combination of all topics described in this thesis could in the future lead to personalized *in vitro* models for therapeutically screening, as well as reduce the amount of required test animals. Although these advances are likely far in the future, the knowledge gained, the *in vitro* models studied and the methods developed could contribute to better protocols, screenings and clinically translatable results.

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