Airway Progenitor Cell Development and Function

Mimicking *in vivo* behavior *in vitro*

Evelien Eenjes

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Mimicking *in vivo* behavior *in vitro*

Luchtweg voorloper cel ontwikkeling en functie
Nabootsen van *in vivo* gedrag *in vitro*

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Chapter 1.1

Introduction

Scope of this thesis

The lung is composed of a highly branched airway structure, which humidifies and warms the air before entering the alveolar compartment. In the alveoli, a thin layer of epithelium is in close proximity with the capillary endothelium, allowing for an efficient exchange of oxygen and carbon dioxide. During development proliferation and differentiation of progenitor cells generates the lung architecture, and in the adult lung a proper function of progenitor cells is needed to regenerate after injury. Malfunctioning of progenitors during development results in various congenital lung disorders, like Congenital Diaphragmatic Hernia (CDH) and Congenital Pulmonary Adenomatoid Malformation (CPAM). In addition, many premature newborns experience continuous insults on the lung caused by artificial ventilation and supplemental oxygen, which requires a highly controlled mechanism of airway repair. Malfunctioning of airway progenitors during regeneration can result in reduction of respiratory function or (chronic) airway diseases. It is hypothesized that pathways that are active during development are re-activated upon damage, or at least in part. Understanding basic mechanisms of progenitor cell behavior during development, and regeneration can help to gain a better understanding about the underlying cause of lung diseases, especially those occurring in prenatal development or in the immediate postnatal period of life.

LUNG DEVELOPMENT

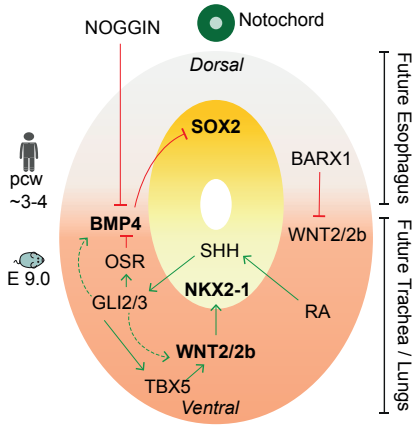
After fertilization, many cell fate decisions are made to evolve from a one-cell embryo to a full-grown organism with optimal lung function at birth. Each cell fate decision is tightly regulated and closely monitored, from the segregation into ectoderm, mesoderm and endoderm, till the late commitment towards lung specific cell types. The trachea, airway, and alveolar epithelial cells of the lung, develop from the endodermal lineage. Simultaneously with lung endodermal development, the lung mesoderm develops and generates various cell lineages such as vascular cells, smooth muscle cells, pericytes and cartilage precursors. The lung mesoderm and endoderm promote each other's growth and differentiation during all stages of development [1, 2]. Here, we focus on the development of lung epithelium out of endodermal progenitors.

Origin and specification of the lung

Specification of lung and esophagus starts from the anterior foregut endoderm. A dorsalventral (D-V) patterning of the foregut, separates dorsal, Sry-related HMG box 2 positive (SOX2+) esophagus progenitors from ventral, NK2 Homeobox 1 positive (NKX2-1+) lung progenitors [3, 4] (for details see Fig. 1). Multiple reciprocal signaling cues between mesoderm and endoderm that contribute to a proper localization of *Nkx2*-1 expression in the ventral foregut endoderm have been identified (Fig. 1) [2, 5, 6]. Two main events are important, the initiation of *Nkx2-1* expression and repression of *Sox2*. First, *Nkx2-1* expression is induced

by both canonical WNT2 and WNT2b ligands from the ventral mesoderm and FGF2 secretion from adjacent developing cardiac mesoderm [7-9]. Second, the repression of *Sox2* in the ventral foregut endoderm is due to the expression of *Bmp4* from the ventral mesoderm [10]. NOGGIN is secreted by cells residing in the notochord on the dorsal side, suppressing BMP signaling in de dorsal mesoderm and allowing *Sox*2 expression [11, 12]. SOX2, in its turn, is able to repress *Nkx2-1* expression, thereby restricting its expression to the ventral foregut endoderm [10] (Fig. 1).

The expression of *Sox2* and *Nkx2-1* demarcating the D-V boundary of the foregut endoderm is important in separating the trachea from the esophagus. Mouse models with expression levels of *Sox2* below a 15% threshold or lacking *Nkx2*-1, both resulted in separation defects, resembling the human congenital condition called tracheoesophageal fistula (TEF), where the airway is connected with the stomach and/or esophageal atresia (EA), a short and blunted esophagus [3, 4, 11]. Multiple factors that contribute to trachea and esophagus D-V patterning have been identified using genetic mouse models resembling a TEF/EA phenotype, or with genetic screens of human infants born with EA/TEF (Fig. 1, Table 1) [5, 11]. Although genetic analyses of human EA/TEF patients and animal models revealed genes associated with EA/TEF, the cellular mechanisms causing the separation defect are poorly understood.



Mouse gene	Function	Phenotype	Reference
Nkx2-1$^{-/-}$	Homeodomain containing transcription factor	TEF	[3]
Sox2$^{GFP/COND}$	HMG-box transcription factor	EA/TEF	[4]
BMP4^{COND}	secreted bone-morphogenetic protein signaling ligand	TA	[82]
Barx1$^{-/-}$	Homeodomain transcription factor	EA/TEF	[83]
Noggin$^{-/-}$	BMP inhibitor	EA/TEF	[11, 12]
Gli2/3	Shh zinc-finger transcription factors	EA/TEF/ TA / Pulmonary agenesis	[84]
Shh	Secreted hedgehoc family ligand	EA/TEF	[85, 86]
Human gene	**Mutation**	**Phenotype**	**Reference**
NOGGIN	Point mutation	EA/TEF	[87]
SOX2	HMG-box transcription factor	EA +/- TEF	[88]

Fig. 1. Proteins involved in lung specification. *During lung specification, Nkx2-1 expression is restricted to the ventral side and Sox2 to the dorsal side of the foregut endoderm. Retinoic acid (RA)-signaling activates RA receptors in the surrounding mesoderm driving cells to secrete Sonic Hedgehog (SHH) (HH ligand) in the ventral foregut mesoderm. SHH-responsive cells subsequently trigger activation of GLI2 and GLI3 transcription factors in the ventral mesoderm, which stimulate expression of WNT2/2b and BMP4 [89]. Odd-skipped related zinc finger transcriptional repressor, OSR, and SHH signaling target TBX5 are important modulators of WNT2/2b and BMP4 signaling [90, 91]. The transcription factor, BARX1, is expressed in the dorsal mesenchyme thereby repressing WNT signaling [83].*

Table 1. Mutations in genes associated with defects in tracheoesophageal development in mouse and human. *The contribution of these genes listed have shown to play a role in the specification of lung progenitors and their involvement is indicated in figure 1. TEF = tracheoesophagel fistula, EA = esophageal atresia.*

Trachea and primary lung bud formation

After specification of lung progenitors, the single common foregut tube begins to compartmentalize [1]. A timed and localized expression of retinoic acid (RA) induces mesenchymal expression of FGF10, which activates NKX2-1+ lung progenitor cells by binding to its receptor FGFR2B and subsequently induces lung bud formation [13-15] (Fig. 2). At the same time of lung bud formation, the trachea, separates from the esophagus proximal of the lung buds [11, 16]. Of note, FGF10 knock out mice show normal formation of the trachea, while the lung buds do not form [17, 18], suggesting that a distinct mechanism of FGF10 signaling is involved in formation and separation of the trachea from the esophagus.

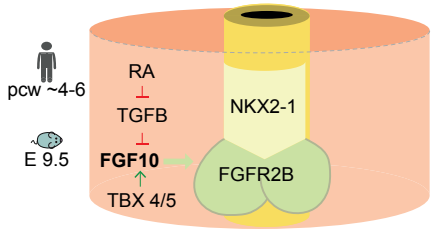


Fig. 2. Primary lung bud formation *FGF10 from the ventral mesoderm is essential in lung bud formation, and is regulated by RA and Transforming Growth Factor-β (TGF-β) signaling. TBX transcription factors present in the foregut mesoderm have shown to be essential in regulating FGF10 expression as well [92, 93]. E = embryonic day, pcw = post-conceptional week*

Branching Morphogenesis

A complex tree-like structure of airways is formed in the pseudoglandular stage, with a repetitive pattern of formation of new buds, bifurcation and outgrowth of buds [19]. During branching of the airways SOX9+, Inhibitors of DNA binding 2+ progenitor cells (ID2+) reside at the branching distal tips. These tip progenitors, are multipotent and give rise to the SOX2+ progenitor cells which will form the airway epithelium [20-22]. In contrast to the mouse branching airways, in human lung the tip progenitors express both SOX9 and SOX2 (Fig. 4A) [23, 24].

Maintaining a proximal-distal patterning during lung development is crucial for a proper branching of the airways. We previously illustrated formation of cystic airway structures in a mouse model where *Sox2* expression was induced in the distal tip progenitor cells [20]. During the last decades, the use of transgenic mouse models contributed highly to the identification of multiple epithelial-mesenchymal signaling pathways important for maintaining a proximal-distal patterning and coordinating initiation and outgrowth of lung buds (for further references [1, 25] and the legend of Fig. 3). As described, FGF10 is a major player in primary bud formation, and FGF10 continues to be present in the mesenchyme surrounding the outgrowing buds during branching morphogenesis [17, 26]. The localized source of FGF10 within the “tipmicroenvironment” regulates multiple factors to control expansion of the bud by inducing proliferation while suppressing S*ox2* expression to prevent differentiation (Fig. 3) [27-30]. When the lung bud grows, cells become displaced from the FGF10 source and differentiate to SOX2+ airway progenitor cells. FGF10 plays a central role in the branching morphogenesis of mouse lungs, however, FGF10 is not essential for branching of human fetal lungs *in vitro* [31]. Further studies are needed

to elucidate the differences and similarities in molecular mechanisms controlling mouse and human proximal-distal patterning. This will help to reveal the mechanisms of abnormal development, which give rise to a variety of life threatening congenital anomalies, such as CDH, CPAM and EA/TEF. Defects or interruptions in these signaling and gene expression pathways, like *Sox2* overexpression, have shown to lead to branching defects resembling the congenital lung disease, CPAM [20]. Despite, the strong correlation between SOX2 and the formation of airway cysts, it is not known how SOX2 exerts its activity.

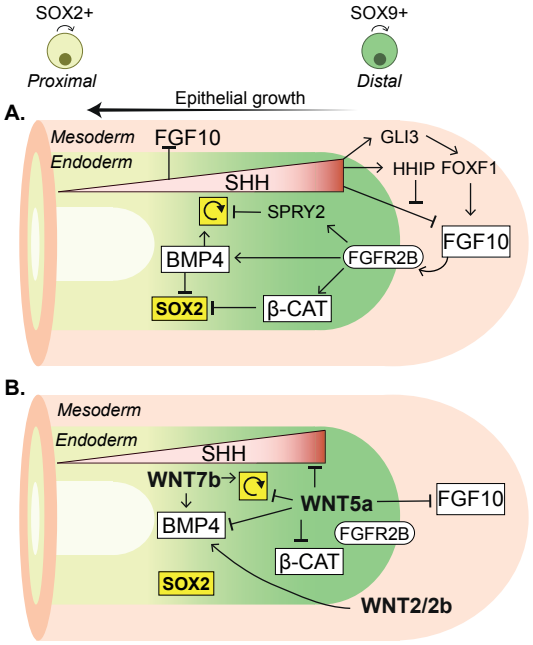


Fig. 3. Lung bud outgrowth *(A) Several reciprocal interactions between mesoderm and endoderm regulate the expansion of the distal tip through proliferation and suppression of Sox2 expression. SHH is expressed in a gradient with the highest expression in the distal bud. SHH inhibits mesenchymal FGF10 expression just proximal of the distal bud. At high concentrations, SHH induces expression of HH inhibitory protein (HHIP) in the distal mesenchyme to allow for FGF10 expression via regulation of GLI3 and FOXF1 [25]. Proliferation of progenitor cells is positively regulated via BMP4 induction or inhibited via its antagonist SPRY2 [29, 94-96]. Sox2 expression is inhibited via Wnt-β-Catenin and BMP4 signaling [28, 30]. (B) Knock-out mouse models of WNT ligands demonstrated defects in lung development; WNT2/2b(canonical) [97] in distal mesenchyme, WNT5a (non-canonical) [98-100] and WNT7b (canonical) in distal epithelium [101], each suggested to be involved in the regulation of BMP4, β-Catenin, SHH signaling or cell proliferation. ⟳ = proliferation.*

Development of proximal airway and distal alveolar lineages

During branching morphogenesis, SOX2+ progenitor cells proliferate but also start to differentiate to proximal airway cell lineages (Fig. 2B). SOX2 remains in airway epithelium after progenitor cells differentiate, and deletion of SOX2 during development shows a severe reduction in basal, ciliated and secretory cells [21].

The first evidence of differentiation is the appearance of a few basal cells (Transformationrelated protein 63, TRP63+) at E9.5 in the trachea and in proximal regions of the lung bud in mice. Lineage tracing studies using *Trp63-CreERT*, shows that basal cells at <E9.5 are able to give rise to both airway and alveolar epithelial cells (Fig. 4B). From E10.5 onward, basal cells are only progenitors for the cells in the pseudostratified epithelium of the extrapulmonary airways (trachea and main bronchi) [32] (Fig. 4B). Vice versa, lineage tracing of tip progenitor cells using *Sox9-Cre* or *Id2-Cre* induced at <E9.5, shows that tip progenitor cells give rise to airway epithelial cells both in the extra- and intra-pulmonary airways, and lineage tracing of SOX9+ or ID2+ progenitors from E11.5 shows that tip progenitor cells only give rise

to the intrapulmonary airways [22, 32]. Thus, during lung specification and lung bud formation (E8.5-E9.5), two complementary lineages are defined early in trachea/ lung development, both contributing to the epithelial cells of the respiratory tract.

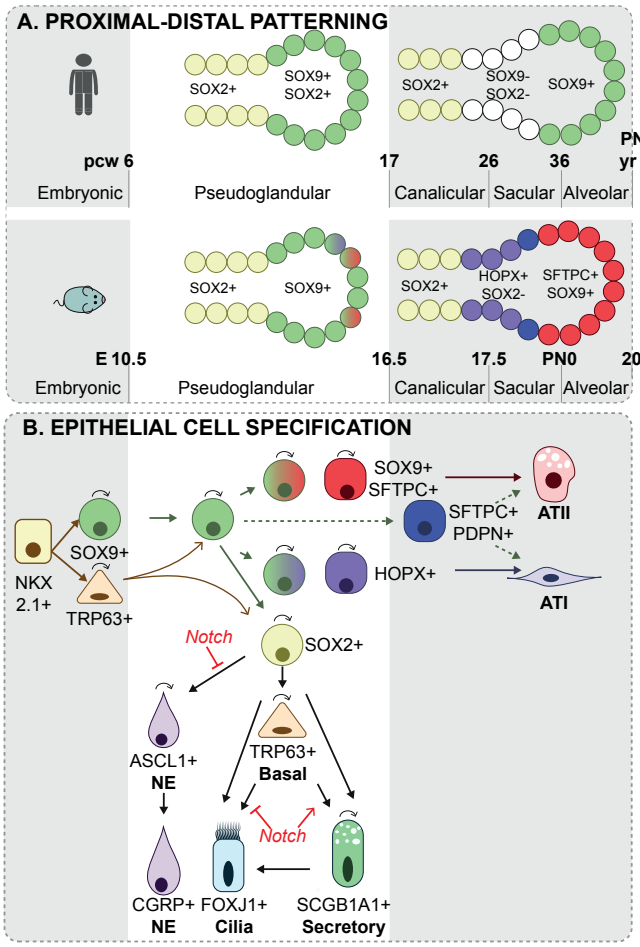


Fig. 4. Endodermal lineage specification during lung development. *(A) A proximal-distal patterning of the lung bud regionalizes the airway epithelium during branching. In human, distal bud progenitor cells express SOX9 and SOX2, while in mice these cells only express Sox9. After the pseudoglandular stage, SOX9+ progenitors are present in the tip of the distal bud, SOX2- SOX9- just proximal of the distal bud and SOX2+ progenitors in the proximal airways. In mice, the patterning of the distal bud is further specified by the expression of Sftpc and Hopx. (B) During the growth of the primary lung buds a, SOX9+ progenitor cells give rise to the SOX2+ airway progenitor and a few basal cells are present. Some SOX9+ progenitors start to specify to alveolar type (AT) I or ATII cells around E13.5. SOX2+ progenitors differentiate to neuroendocrine cells (NE) and basal cells. The basal cells that develop at this stage, can self-renew and differentiate to ciliated and* secretory cells in the extrapulmonary airways. SOX2+ progenitor cells also further differentiate to secretory and ciliated cells. Secretory cells can self-renew and give rise to ciliated cells [63]. Notch signaling inhibits or stimulates differention at different stages of lung development. During the canalicular and saccular stage, numerous alveolar sacs develop that are the precursors of the alveoli. SFTPC+SOX9+, SFTPC+PDPN+ and HOPX+ progenitor cells further differentiate into ATI or ATII cells. At the end of embryonic lung development, the alveolar sacs are subdivided by the formation of secondary septae and the ATI cells become closely associated with the endothelial cells [25]. ↷ cell division, E = embryonic day, pcw = post-conceptional week, PN = post-natal.

At E13.5, as the bronchial tree is expanding, SOX2+ progenitor cells give rise to Neuroendocrine (NE) cells and non-NE cells (Fig. 2B). Precursor of NE cells, are first scattered throughout the proximal airway epithelium and subsequently migrate to form NE clusters, which are mostly located at the bifurcations of airways [33, 34]. Notch activity controls the choice between NE and non-NE cell fate [35, 36]. Inhibition of Notch signaling results in an increase in NE cells, but also in an increase in ciliated cells at the expense of secretory cells. This shows that Notch signaling balances the differentiation between secretory and ciliated cells at later stages in development (after E15.5) (Fig. 4B) [37-39]. NE cell hyperplasia is associated with

CDH, but the underlying cause and whether this contributes to the onset or specific pathology related to CDH is not yet investigated [40]. Previously, it was shown that overexpression of *Sox2* during lung development resulted in increased basal cell numbers, but also to an increase in NE cells. However, the underlying molecular mechanisms that guide the SOX2+ airway progenitors to differentiate to basal or NE cells is not yet understood [20].

Mature alveoli exist of cuboidal surfactant producing alveolar type 2 cells (ATII) and flattened alveolar type I (ATI) cells. The first specification of SOX9+ tip progenitors to either ATI or ATII cells is observed at E13.5 [41] (Fig. 4A, B). From E15.5 onward, SOX9+ progenitors are still involved in branching of distal tips, but cells in the recently branched epithelium do not express Sox2, as they do early in development, but rather express the ATI marker, *Hopx* (Fig. 4A) [41, 42]. In addition, bipotent progenitor cells expressing both ATII and ATI markers, can be found in the distal bud but they show only minor contribution to the alveolar compartment during development [41, 43, 44] (Fig 4B). In human lung development, tip progenitors loose *SOX2* expression and remain only SOX9+ in the canalicular and saccular stage (Fig. 4A). However, tip progenitor cells already start to express both markers of ATI and ATII cells 5 weeks prior to the canalicular stage and in co-expression with SOX2 [23]. The functional significance of *SOX2* expression in human tip progenitor cells during the pseudoglandular stage is currently unknown.

LINEAGE DIVERSIFICATION AND CELL PLASTICITY UPON AIRWAY REGENERATION

As a result of lung development, the airway epithelium is aligned with a wide range of cell types (Fig. 5). During steady state, the airway epithelium is a low turnover tissue, but upon severe damage, quiescent progenitor cells can regenerate the airway epithelium. An imbalance in homeostatic turnover or regeneration, can cause severe lung diseases like, Bronchopulmonary Dysplasia (BPD), Chronic Obstructive Pulmonary Disease (COPD), asthma or lung cancers. Lineage tracing studies in mice have demonstrated that within the airway epithelium, most adult epithelial cells retain plasticity to de- or transdifferentiate under stress or damage conditions. Ciliated cells are an exception, they have no potential to proliferate or differentiate after injury [37]. Here, a focus is made on the main adult airway cell types that are known to contribute to repair after injury. A more extensive description of lung regeneration and *in vitro* models to study adult airway epithelium is given in our published review (chapter 1.2;[45]).

Basal cells

The basal cell is one of the most studied cell types of the lung regarding regeneration. In the mouse, basal cells are mainly located in the extrapulmonary airway epithelium.

In the human lung, basal cells are present in the trachea to the smallest airways and are only absent in the terminal bronchioles just proximal of the alveoli (Fig. 5) [46]. *In vitro* cultures using isolated mouse and human basal cells have shown that these cells can self-renew and are multipotent, meaning that they can differentiate to secretory and ciliated cells [47].

In both, mouse and human, basal cells are characterized by the expression of *Trp63*, and *Trp63* knock-out mice lack basal cells [48-50]. Besides *Trp63* expression, all basal cells also express *Cytokeratin 5* (*Krt5)*, while a subpopulation of basal cells express *Cytokeratin 14* (*Krt14)*, which greatly expands upon injury [51, 52]. In human airway epithelium, *KRT14* also shows a more restricted expression pattern than *KRT5*, but increases in regions of squamous metaplasia in COPD patients [46]. The functional difference between KRT14+ and KRT14- basal cells is not yet explored. Furthermore, basal cells are thought to be the source of lung squamous cell carcinoma through increased expression of both *SOX2* and *TRP63* [53, 54]. The regulation of basal cell maintenance, proliferation and differentiation in relation to SOX2 is poorly understood.

A very small population of *Trp63* expressing cells reside in the mouse intrapulmonary airways, which substantially increases upon severe lung injury. Lineage tracing showed that these cells contributed to both alveolar and airway lineages, showing the high potential of distal TRP63+ cell population to regenerate lung epithelium [32, 55, 56]. Although, a similar population of basal cells was identified in human terminal bronchioles, its expansion or differentiation potential and contribution to airway regeneration is still uncertain [55].

Submucosal glands

Submucosal glands (SMGs) are specialized secretory glands with a grape like structure embedded within the connective tissue, just underneath the proximal tracheal epithelium of the mouse and the cartilaginous airways of the human [57] (Fig. 5). The submucosal glands can be subdivided in ducts and acini. The ducts contain a similar cellular composition as the surface epithelium of the airways. The acini contain basally located myoepithelial cells expressing *Krt14*, *Krt5* and smooth muscle actin 2 (*Acta2*), and luminal cells secreting mucous and fluids rich in antimicrobial enzymes [58, 59]. Upon injury, basal myoepithelial cells migrate to the surface epithelium of the trachea and aid in repopulating the airway due to proliferation and differentiation to basal, ciliated and secretory cells [60, 61]. In pigs, similar to human, SMGs are present throughout the cartilaginous airways and exposure to chlorine gas showed that SMG derived cells contributed to the repair of the airway [61].

Secretory cells

Secretory (Club) cells produce mucins and microbial peptides to capture inhaled

substances, which are propelled out of the lung through cilia movement. Different subsets of secretory cells in mouse and human airways are identified by the secretion of different members of secretoglobins; SCGB1A1, SCGB3A1 or SCGB3A2 [62] (Fig. 5). Lineage tracing studies, using secretory cell marker SCGB1A1, showed that besides the protective function, secretory cells have the potency to self-renew, differentiate to ciliated cells, and de- differentiate to basal cells [63, 64].

Naphthalene-induced injury is a common used mouse model to study airway regeneration [65]. Secretory cells are most vulnerable to naphthalene exposure due to their expression of *Cytrochrome P450* enzyme (*Cyp2f2*), which converts naphthalene to a cytotoxic product [66]. A subset of secretory cells, the variant club cells, was identified because they lack *Cyp2f2* expression, and survive naphthalene exposure [67, 68]. The variant club cell is located closely to neuroendocrine cell clusters, and expresses *Uroplakin3a* (*UPK3a*) [69] in addition to *Scgb1a1* (Fig. 5). A similar localization of UPK3a+ secretory cells near neuroendocrine cells was observed in human lung sections, suggesting a similar progenitor cell population is present [69].

Neuroendocrine cells

The neuroendocrine (NE) cells form a rare subpopulation in the airway epithelium and act as chemosensory cells, communicating with the nervous system and influencing smooth muscle tone as well as regulating immune responses [70-72]. NE cells also have the ability to contribute to airway epithelial repair after naphthalene induced injury [73, 74]. As mentioned, hyperplasia of NE cells has been implicated in a number of lung diseases, some of which are pediatric lung diseases, like BPD and CDH [40, 75]. Furthermore, NE cell markers are found in small cell lung cancer (SCLC) [76], and *in vivo* studies in mouse showed the NE cells are the origin for SCLC development [73, 74]. How and why NE cells associate with such a wide range of lung diseases is unknown and therefore an interesting airway population to study.

Bronchioalveolar stem cells

In the zone where bronchiole transition to the alveoli, there are epithelial cells which carry both the secretory cell marker SCGB1A1 and ATII marker SFTPC [77] (Fig. 5). These, so called Broncho-Alveolar Stem Cells (BASCs), show self-renewal potential and are able to differentiate to bronchiolar and alveolar cell types *in vitro* [77-79]. A novel dual-lineage tracing approach, showed that SFTPC+ SCGB1A1+ cells contribute to bronchiolar and alveolar epithelium after naphthalene-induced airway injury or bleomycin-induced alveolar injury, respectively [80, 81]. BASCs are relatively stable in normal lung homeostasis, showing that BASCs are only activated upon injury [80, 81]. In addition, lineage tracing studies using *Scgb1a1-Cre* showed

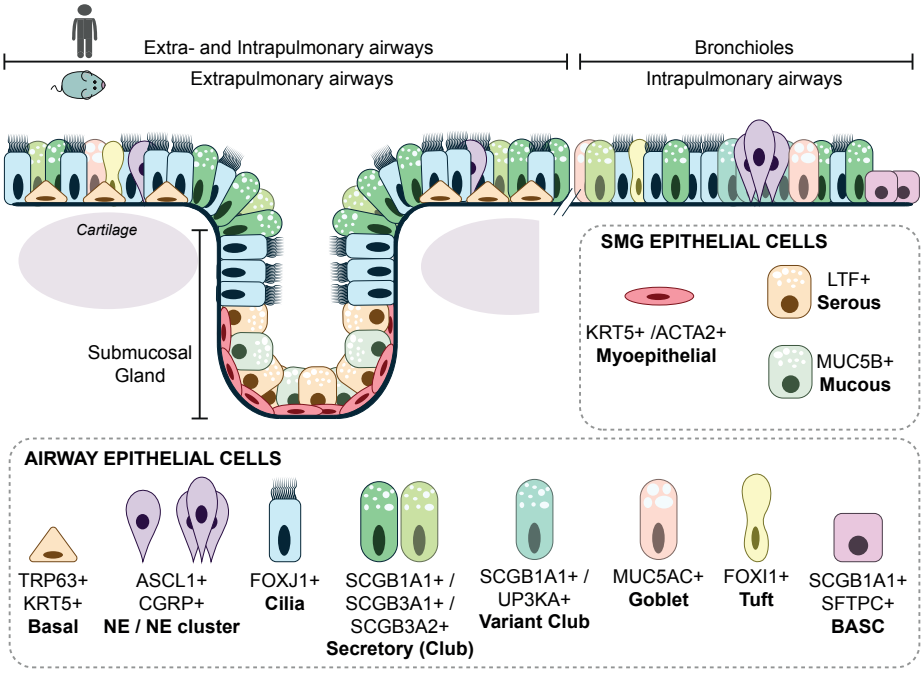


Fig. 5. Cellular composition of the airways. *The extrapulmonary epithelium (trachea and main bronchi) of the mouse consists of a pseudostratified epithelium containing basal cells, while in the human lung, basal cells are only absent in the bronchioles proximal of the alveoli. Submucosal glands are in mouse and human only present in cartilaginous airways. The airway epithelium consists of many different epithelial cells, the main epithelial cells are described within the text. A rare epithelial cell type is the ionocyte. They appear to be the main source of cystic fibrosis transmembrane conductance regulator (CFTR) activity, thereby regulating mucous production [102]. Goblet cells are nearly absent in mouse airway epithelium, but are more frequently found in human airway epithelium and together with other secretory cells are responsible for mucous production.*

that; SCGB1A1+ cells did not contribute to alveolar repair after hyperoxic aveolar injury [63], suggesting that the contribution of SCGB1A1+ cells to alveolar repair depends on the type and possibly severity of injury.

In conclusion, airway epithelial cells have the ability to regenerate the airway epithelium. The contribution to this by different cell types can be assessed by the use of lineage tracing tools and different injury models. However, the identification of progenitor lineages is much easier and faster than understanding the underlying mechanisms that regulate the contribution of each cell type to regeneration. Furthermore, most of the airway epithelial cell plasticity is observed in mouse models, translating these findings to either the quiescent human airway epithelium or the misregulation of cellular plasticity upon disease is a considerable challenge. Importantly, the proliferation of *in vitro* lung development models, such as lung organoids, air-liquid interphase cultures and lung-on-a-chip model, are likely to increase our understanding of human airway plasticity in development, homeostasis and disease.

SCOPE OF THIS THESIS

During the development and regeneration of the airways, a proper maintenance, expansion and differentiation of progenitor cells is needed for proper lung formation and correct repair. Prior studies, highlighted the importance of SOX2 in airway progenitor function. In this thesis, the role of SOX2 in murine and human airway development and regeneration is investiged. As part of this, *in vitro* airway models are developed to translate our findings to human airway functions. In **chapter 2**, a focus is on the development of the expansion of basal cells *in vitro* to reduce the number of mice needed for air-liquid interface (ALI) cultures. In this manner, basal cell differentiation can be studied *in vitro* from mouse models that require intensive breeding strategies to obtain sufficient numbers of mice and cells. **Chapter 3** describes the development of a human *in vitro* airway model using available lung material. This will allow translation of findings obtained by *in vitro* mouse studies or *in vivo* mouse models to human airway progenitor functions in health and diseases. The clinical samples used contain low numbers of airway epithelial cells and therefore the possibility is assessed of expanding these cells as organoids to study the differentiation of basal cells on subsequent ALI culture. ALI cultures are air-exposed and therefore an interesting model for air exposure studies, like cigarette smoke. Furthermore, with the use of tracheal aspirates of neonates, a human airway *in vitro* model is developed to study airway epithelial function of premature newborns that have the potential to develop bronchopulmonary dysplasia (BPD). **Chapter 4** elaborates on SOX2+ airway epithelial cells that specifically express SOX21 during development and regeneration. SOX2 and SOX21 function together in maintenance, proliferation and differentiation of stem cells during neuronal development, as well as in embryonic stem cells. Therefore, differentiation during lung development and repair after naphthalene injury is studied in mouse models deficient in SOX2 or SOX21. Additionally, the expression of SOX21 is studied in human airways by using fetal lung organoids and ALI cultures of adult bronchial epithelial cells. As published previously, increased *Sox2* expression during lung development results in neuroendocrine (NE) cell hyperplasia, which is often observed in airway diseases. **Chapter 5** focusses on the potential role of SOX2 and SOX21 in the initiation, differentiation, migration and clustering of NE cells. Finally, **chapter 6** dicusses the findings made in this thesis, future perspectives and contributions to the field.

Taken together, this thesis aims to increase the understanding of airway progenitor cell behavior during development and regeneration in both human and mouse by developing novel *in vitro* models. In understanding basic mechanisms of progenitor cell behavior, we hope to identify therapeutic targets when airway progenitor behavior goes awry resulting in disease.

REFERENCES

1. Cardoso, W.V. and J. Lu, Regulation of early lung morphogenesis: questions, facts and controversies. Development, 2006. 133(9): p. 1611-24.
2. Swarr, D.T. and E.E. Morrisey, Lung endoderm morphogenesis: gasping for form and function. Annu Rev Cell Dev Biol, 2015. 31: p. 553-73.
3. Minoo, P., et al., Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(-/-) mouse embryos. Dev Biol, 1999. 209(1): p. 60-71.
4. Que, J., et al., Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. Development, 2007. 134(13): p. 2521-31.
5. Billmyre, K.K., M. Hutson, and J. Klingensmith, One shall become two: Separation of the esophagus and trachea from the common foregut tube. Dev Dyn, 2015. 244(3): p. 277-88.
6. Morrisey, E.E. and A.K. Rustgi, The Lung and Esophagus: Developmental and Regenerative Overlap. Trends Cell Biol, 2018. 28(9): p. 738-748.
7. Goss, A.M., et al., Wnt2/2b and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. Dev Cell, 2009. 17(2): p. 290-8.
8. Harris-Johnson, K.S., et al., beta-Catenin promotes respiratory progenitor identity in mouse foregut. Proc Natl Acad Sci U S A, 2009. 106(38): p. 16287-92.
9. Serls, A.E., et al., Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. Development, 2005. 132(1): p. 35-47.
10. Domyan, E.T., et al., Signaling through BMP receptors promotes respiratory identity in the foregut via repression of Sox2. Development, 2011. 138(5): p. 971-81.
11. Que, J., et al., Morphogenesis of the trachea and esophagus: current players and new roles for noggin and Bmps. Differentiation, 2006. 74(7): p. 422-37.
12. Li, Y., et al., Aberrant Bmp signaling and notochord delamination in the pathogenesis of esophageal atresia. Dev Dyn, 2007. 236(3): p. 746-54.
13. Desai, T.J., et al., Retinoic acid selectively regulates Fgf10 expression and maintains cell identity in the prospective lung field of the developing foregut. Dev Biol, 2004. 273(2): p. 402-15.
14. Malpel, S., C. Mendelsohn, and W.V. Cardoso, Regulation of retinoic acid signaling during lung morphogenesis. Development, 2000. 127(14): p. 3057-67.
15. Chen, F., et al., Inhibition of Tgf beta signaling by endogenous retinoic acid is essential for primary lung bud induction. Development, 2007. 134(16): p. 2969-79.
16. Kishimoto, K., et al., Synchronized mesenchymal cell polarization and differentiation shape the formation of the murine trachea and esophagus. Nat Commun, 2018. 9(1): p. 2816.
17. Bellusci, S., et al., Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development, 1997. 124(23): p. 4867-78.
18. Sekine, K., et al., Fgf10 is essential for limb and lung formation. Nat Genet, 1999. 21(1): p. 138-41.
19. Metzger, R.J., et al., The branching programme of mouse lung development. Nature, 2008. 453(7196): p. 745-50.
20. Gontan, C., et al., Sox2 is important for two crucial processes in lung development: branching morphogenesis and epithelial cell differentiation. Dev Biol, 2008. 317(1): p. 296-309.
21. Que, J., et al., Multiple roles for Sox2 in the developing and adult mouse trachea. Development, 2009. 136(11): p. 1899-907.
22. Rawlins, E.L., et al., The Id2+ distal tip lung epithelium contains individual multipotent embryonic progenitor cells. Development, 2009. 136(22): p. 3741-5.
23. Nikolic, M.Z., et al., Human embryonic lung epithelial tips are multipotent progenitors that can be expanded in vitro as long-term self-renewing organoids. Elife, 2017. 6.
24. Danopoulos, S., et al., Human lung branching morphogenesis is orchestrated by the spatiotemporal distribution of ACTA2, SOX2, and SOX9. Am J Physiol Lung Cell Mol Physiol, 2018. 314(1): p. L144-L149.
25. Morrisey, E.E. and B.L. Hogan, Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell, 2010. 18(1): p. 8-23.
26. Yuan, T., et al., Fgf10 Signaling in Lung Development, Homeostasis, Disease, and Repair After Injury. Front Genet, 2018. 9: p. 418.
27. Shu, W., et al., Wnt/beta-catenin signaling acts upstream of N-myc, BMP4, and FGF signaling to regulate proximal-distal patterning in the lung. Dev Biol, 2005. 283(1): p. 226-39.
28. Volckaert, T., et al., Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. Development, 2013. 140(18): p. 3731-42.
29. Hyatt, B.A., X. Shangguan, and J.M. Shannon, FGF-10 induces SP-C and Bmp4 and regulates proximal-distal patterning in

embryonic tracheal epithelium. Am J Physiol Lung Cell Mol Physiol, 2004. 287(6): p. L1116-26.
30. Wang, Y., et al., Development and regeneration of Sox2+ endoderm progenitors are regulated by a Hdac1/2-Bmp4/Rb1 regulatory pathway. Dev Cell, 2013. 24(4): p. 345-58.
31. Danopoulos, S., J. Shiosaki, and D. Al Alam, FGF Signaling in Lung Development and Disease: Human Versus Mouse. Front Genet, 2019. 10: p. 170.
32. Yang, Y., et al., Spatial-Temporal Lineage Restrictions of Embryonic p63(+) Progenitors Establish Distinct Stem Cell Pools in Adult Airways. Dev Cell, 2018. 44(6): p. 752-761 e4.
33. Kuo, C.S. and M.A. Krasnow, Formation of a Neurosensory Organ by Epithelial Cell Slithering. Cell, 2015. 163(2): p. 394-405.
34. Noguchi, M., K. Sumiyama, and M. Morimoto, Directed Migration of Pulmonary Neuroendocrine Cells toward Airway Branches Organizes the Stereotypic Location of Neuroepithelial Bodies. Cell Rep, 2015. 13(12): p. 2679-86.
35. Ito, T., et al., Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium. Development, 2000. 127(18): p. 3913-21.
36. Jia, S., H. Wildner, and C. Birchmeier, Insm1 controls the differentiation of pulmonary neuroendocrine cells by repressing Hes1. Dev Biol, 2015. 408(1): p. 90-8.
37. Rawlins, E.L., et al., Lung development and repair: contribution of the ciliated lineage. Proc Natl Acad Sci U S A, 2007. 104(2): p. 410-7.
38. Tsao, P.N., et al., Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. Development, 2009. 136(13): p. 2297-307.
39. Morimoto, M., et al., Different assemblies of Notch receptors coordinate the distribution of the major bronchial Clara, ciliated and neuroendocrine cells. Development, 2012. 139(23): p. 4365-73.
40. Ijsselstijn, H., et al., Abnormal expression of pulmonary bombesin-like peptide immunostaining cells in infants with congenital diaphragmatic hernia. Pediatr Res, 1997. 42(5): p. 715-20.
41. Frank, D.B., et al., Early lineage specification defines alveolar epithelial ontogeny in the murine lung. Proc Natl Acad Sci U S A, 2019. 116(10): p. 4362-4371.
42. Alanis, D.M., et al., Two nested developmental waves demarcate a compartment boundary in the mouse lung. Nat Commun, 2014. 5: p. 3923.
43. Treutlein, B., et al., Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. Nature, 2014. 509(7500): p. 371-5.
44. Desai, T.J., D.G. Brownfield, and M.A. Krasnow, Alveolar progenitor and stem cells in lung development, renewal and cancer. Nature, 2014. 507(7491): p. 190-4.
45. Schilders, K.A., et al., Regeneration of the lung: Lung stem cells and the development of lung mimicking devices. Respir Res, 2016. 17: p. 44.
46. Rock, J.R., S.H. Randell, and B.L. Hogan, Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. Dis Model Mech, 2010. 3(9-10): p. 545-56.
47. Rock, J.R., et al., Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc Natl Acad Sci U S A, 2009. 106(31): p. 12771-5.
48. Daniely, Y., et al., Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium. Am J Physiol Cell Physiol, 2004. 287(1): p. C171-81.
49. Yang, A., et al., p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature, 1999. 398(6729): p. 714-8.
50. Mills, A.A., et al., p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature, 1999. 398(6729): p. 708-13.
51. Hong, K.U., et al., Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. Am J Pathol, 2004. 164(2): p. 577-88.
52. Hong, K.U., et al., In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. Am J Physiol Lung Cell Mol Physiol, 2004. 286(4): p. L643-9.
53. Ferone, G., et al., SOX2 Is the Determining Oncogenic Switch in Promoting Lung Squamous Cell Carcinoma from Different Cells of Origin. Cancer Cell, 2016. 30(4): p. 519-532.
54. Bass, A.J., et al., SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. Nat Genet, 2009. 41(11): p. 1238-42.
55. Vaughan, A.E., et al., Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury. Nature, 2015. 517(7536): p. 621-5.
56. Zuo, W., et al., p63(+)Krt5(+) distal airway stem cells are essential for lung regeneration. Nature, 2015. 517(7536): p. 616-20.
57. Tata, P.R. and J. Rajagopal, Plasticity in

the lung: making and breaking cell identity. Development, 2017. 144(5): p. 755-766.
58. Lynch, T.J. and J.F. Engelhardt, Progenitor cells in proximal airway epithelial development and regeneration. J Cell Biochem, 2014. 115(10): p. 1637-45.
59. 59. Hegab, A.E., et al., Novel stem/progenitor cell population from murine tracheal submucosal gland ducts with multipotent regenerative potential. Stem Cells, 2011. 29(8): p. 1283-93.
60. Lynch, T.J., et al., Submucosal Gland Myoepithelial Cells Are Reserve Stem Cells That Can Regenerate Mouse Tracheal Epithelium. Cell Stem Cell, 2018. 22(5): p. 779.
61. Tata, A., et al., Myoepithelial Cells of Submucosal Glands Can Function as Reserve Stem Cells to Regenerate Airways after Injury. Cell Stem Cell, 2018. 22(5): p. 668-683 e6.
62. Reynolds, S.D., et al., Secretoglobins SCGB3A1 and SCGB3A2 define secretory cell subsets in mouse and human airways. Am J Respir Crit Care Med, 2002. 166(11): p. 1498-509.
63. Rawlins, E.L., et al., The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. Cell Stem Cell, 2009. 4(6): p. 525-34.
64. Tata, P.R., et al., Dedifferentiation of committed epithelial cells into stem cells in vivo. Nature, 2013. 503(7475): p. 218-23.
65. Van Winkle, L.S., et al., Cellular response in naphthalene-induced Clara cell injury and bronchiolar epithelial repair in mice. Am J Physiol, 1995. 269(6 Pt 1): p. L800-18.
66. Plopper, C.G., et al., Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. I. Histopathologic comparison of the respiratory tract of mice, rats and hamsters after parenteral administration of naphthalene. J Pharmacol Exp Ther, 1992. 261(1): p. 353-63.
67. Hong, K.U., et al., Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. Am J Respir Cell Mol Biol, 2001. 24(6): p. 671-81.
68. Reynolds, S.D., et al., Conditional clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. Am J Physiol Lung Cell Mol Physiol, 2000. 278(6): p. L1256-63.
69. Guha, A., et al., Uroplakin 3a(+) Cells Are a Distinctive Population of Epithelial Progenitors that Contribute to Airway Maintenance and Post-injury Repair. Cell Rep, 2017. 19(2): p. 246-254.
70. Garg, A., et al., Consider the lung as a sensory organ: A tip from pulmonary neuroendocrine cells. Curr Top Dev Biol, 2019. 132: p. 67-89.
71. Sui, P., et al., Pulmonary neuroendocrine cells amplify allergic asthma responses. Science, 2018. 360(6393).
72. Branchfield, K., et al., Pulmonary neuroendocrine cells function as airway sensors to control lung immune response. Science, 2016. 351(6274): p. 707-10.
73. Song, H., et al., Functional characterization of pulmonary neuroendocrine cells in lung development, injury, and tumorigenesis. Proc Natl Acad Sci U S A, 2012. 109(43): p. 17531-6.
74. Ouadah, Y., et al., Rare Pulmonary Neuroendocrine Cells Are Stem Cells Regulated by Rb, p53, and Notch. Cell, 2019. 179(2): p. 403-416 e23.
75. Cutz, E., H. Yeger, and J. Pan, Pulmonary neuroendocrine cell system in pediatric lung disease-recent advances. Pediatr Dev Pathol, 2007. 10(6): p. 419-35.
76. van Meerbeeck, J.P., D.A. Fennell, and D.K. De Ruysscher, Small-cell lung cancer. Lancet, 2011. 378(9804): p. 1741-55.
77. Kim, C.F., et al., Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell, 2005. 121(6): p. 823-35.
78. 78. Lee, J.H., et al., Lung stem cell differentiation in mice directed by endothelial cells via a BMP4-NFATc1-thrombospondin-1 axis. Cell, 2014. 156(3): p. 440-55.
79. Lee, J.H., et al., Anatomically and Functionally Distinct Lung Mesenchymal Populations Marked by Lgr5 and Lgr6. Cell, 2017. 170(6): p. 1149-1163 e12.
80. Salwig, I., et al., Bronchioalveolar stem cells are a main source for regeneration of distal lung epithelia in vivo. EMBO J, 2019. 38(12).
81. Liu, Q., et al., Lung regeneration by multipotent stem cells residing at the bronchioalveolar-duct junction. Nat Genet, 2019. 51(4): p. 728-738.
82. Li, Y., et al., Bmp4 is required for tracheal formation: a novel mouse model for tracheal agenesis. Dev Biol, 2008. 322(1): p. 145-55.
83. Woo, J., et al., Barx1-mediated inhibition of Wnt signaling in the mouse thoracic foregut controls tracheo-esophageal septation and epithelial differentiation. PLoS One, 2011. 6(7): p. e22493.
84. Motoyama, J., et al., Essential function of Gli2 and Gli3 in the formation of lung, trachea and oesophagus. Nat Genet, 1998. 20(1): p. 54-7.
85. Litingtung, Y., et al., Sonic hedgehog is essential to foregut development. Nat Genet, 1998. 20(1): p. 58-61.
86. Pepicelli, C.V., P.M. Lewis, and A.P. McMahon,

Sonic hedgehog regulates branching morphogenesis in the mammalian lung. curr Biol, 1998. 8(19): p. 1083-6.
87. Murphy, A.J., et al., Mutational analysis of NOG in esophageal atresia and tracheoesophageal fistula patients. Pediatr Surg Int, 2012. 28(4): p. 335-40.
88. Williamson, K.A., et al., Mutations in SOX2 cause anophthalmia-esophageal-genital (AEG) syndrome. Hum Mol Genet, 2006. 15(9): p. 1413-22.
89. Rankin, S.A., et al., A Retinoic Acid-Hedgehog Cascade Coordinates Mesoderm-Inducing Signals and Endoderm Competence during Lung Specification. Cell Rep, 2016. 16(1): p. 66-78.
90. Han, L., et al., Osr1 functions downstream of Hedgehog pathway to regulate foregut development. Dev Biol, 2017. 427(1): p. 72-83.
91. Steimle, J.D., et al., Evolutionarily conserved Tbx5-Wnt2/2b pathway orchestrates cardiopulmonary development. Proc Natl Acad Sci U S A, 2018. 115(45): p. E10615-E10624.
92. Sakiyama, J., A. Yamagishi, and A. Kuroiwa, Tbx4-Fgf10 system controls lung bud formation during chicken embryonic development. Development, 2003. 130(7): p. 1225-34.
93. Arora, R., R.J. Metzger, and V.E. Papaioannou, Multiple roles and interactions of Tbx4 and Tbx5 in development of the respiratory system. PLoS Genet, 2012. 8(8): p. e1002866.
94. Weaver, M., N.R. Dunn, and B.L. Hogan, Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. Development, 2000. 127(12): p. 2695-704.
95. Eblaghie, M.C., et al., Evidence that autocrine signaling through Bmpr1a regulates the proliferation, survival and morphogenetic behavior of distal lung epithelial cells. Dev Biol, 2006. 291(1): p. 67-82.
96. Mailleux, A.A., et al., Evidence that SPROUTY2 functions as an inhibitor of mouse embryonic lung growth and morphogenesis. Mech Dev, 2001. 102(1-2): p. 81-94.
97. Hrycaj, S.M., et al., Hox5 Genes Regulate the Wnt2/2b-Bmp4-Signaling Axis during Lung Development. Cell Rep, 2015. 12(6): p. 903-12.
98. Li, C., et al., Wnt5a participates in distal lung morphogenesis. Dev Biol, 2002. 248(1): p. 68-81.
99. Li, C., et al., Wnt5a regulates Shh and Fgf10 signaling during lung development. Dev Biol, 2005. 287(1): p. 86-97.
100. Volckaert, T. and S.P. De Langhe, Wnt and FGF mediated epithelial-mesenchymal crosstalk during lung development. Dev Dyn, 2015. 244(3): p. 342-66.
101. Rajagopal, J., et al., Wnt7b stimulates embryonic lung growth by coordinately increasing the replication of epithelium and mesenchyme. Development, 2008. 135(9): p. 1625-34.
102. Montoro, D.T., et al., A revised airway epithelial hierarchy includes CFTR-expressing ionocytes. Nature, 2018. 560(7718): p. 319-324.

Chapter 1.2

Regeneration of the lung: Lung stem cells and the development of lung mimicking devices

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ABSTRACT

Inspired by the increasing burden of lung associated diseases in society and an growing demand to accommodate patients, great efforts by the scientific community produce an increasing stream of data that are focused on delineating the basic principles of lung development and growth, as well as understanding the biomechanical properties to build artificial lung devices. In addition, the continuing efforts to better define the disease origin, progression and pathology by basic scientists and clinicians contributes to insights in the basic principles of lung biology. However, the use of different model systems, experimental approaches and readout systems may generate somewhat conflicting or contradictory results. In an effort to summarize the latest developments in the lung epithelial stem cell biology, we provide an overview of the current status of the field. We first describe the different stem cells, or progenitor cells, residing in the homeostatic lung. Next, we focus on the plasticity of the different cell types upon several injury-induced activation or repair models, and highlight the regenerative capacity of lung cells. Lastly, we summarize the generation of lung mimics, such as air-liquid interface cultures, organoids and lung on a chip, that are required to test emerging hypotheses. Moreover, the increasing collaboration between distinct specializations will contribute to the eventual development of an artificial lung device capable of assisting reduced lung function and capacity in human patients.

BACKGROUND

Although the lung has a low rate of cellular turnover during homeostasis, it has a remarkable ability to regenerate cells after injury [1]. Disruption of this regeneration potential is the cause of several lung diseases. Therefore, understanding the underlying mechanisms of the regenerative capacity of the lung offers potential in identifying novel therapeutic targets. Much can be learned from studies on lung development as processes involved in the differentiation of cell lineages during development are recapitulated during repair [2]. Recent advances in the identification of new cell markers, the analysis of cell fate by in vivo lineage tracing experiments, the use of embryonic and induced pluripotent stem cells, and improvements in organoid cultures have increased the knowledge about the presence of potential stem cells in the lung [3-6]. The goal of this review is to survey the latest developments in endogenous lung regeneration and bioengineering of lung models for therapeutic applications in the future. We will first provide an overview of the latest insights in lung progenitor cells and their potential to differentiate into lung epithelial cells, which is of interest for the in vivo regeneration of lung tissue. Next, we will discuss the plasticity of the different epithelial cells in the lung and their potential to contribute to epithelial regeneration. Finally, we will highlight the possible clinical applications of this knowledge, focusing on different populations of stem cells, lung mimics and tissue engineering.

POTENTIAL EPITHELIAL STEM CELLS OF THE LUNG

Different subsets of epithelial cells and potential stem cell niches have been identified in the lung. The airways of the human lung are lined by a pseudostratified epithelium made up of basal cells, secretory cells (Scgb1a1+ club cells and goblet cells), ciliated cells and neuroendocrine cells (Fig. 1A). The trachea of the mouse, a frequently used model in research, has a similar architecture as the human airways. In human airways, basal cells decrease in frequency from the large to the distal airways [7]. The airways of the mouse and the respiratory smallest bronchioles of the human lung are covered by a cuboidal epithelium. This epithelium lacks basal cells and contains ciliated cells, secretory cells and neuroendocrine cells that are usually clustered in neuroendocrine bodies (NEBs) (Fig. 2A) [8]. The alveoli of both human and mouse are composed of two functional distinct cell types, flat and extended alveolar type I (AT-I) cells to allow gas exchange and cuboidal alveolar type II (AT-II) cells for surfactant protein production and secretion (Fig. 2A) [2, 9]. New emerging technologies, such as single cell RNA-sequencing and proteomic analysis, revealed molecular signatures that hint at different subpopulations of type I and type II cells. It remains to be seen whether such signatures reflect functionally different cell types, or that it represents similar cells at physiologically or metabolically different phases. However interesting, this is not the focus of this review, and therefore we only refer to the current literature [10-12].

Basal-like stem cells: the stem cells of the epithelium

Basal cells are being characterized by the expression of Trp63, Ngfr, Podoplanin (Pdpn, also known as T1α), GSIβ4 lectin and Cytokeratin5 (Krt5). They have the capacity to self-renew and to form secretory and ciliated cells (Fig. 1B) [13-15]. Notch signaling plays a major role in determining the differentiation of basal cells to either the secretory lineage or the ciliated lineage [15-17]. A small subset of the basal cells (<20%) expresses Krt14 under homeostatic

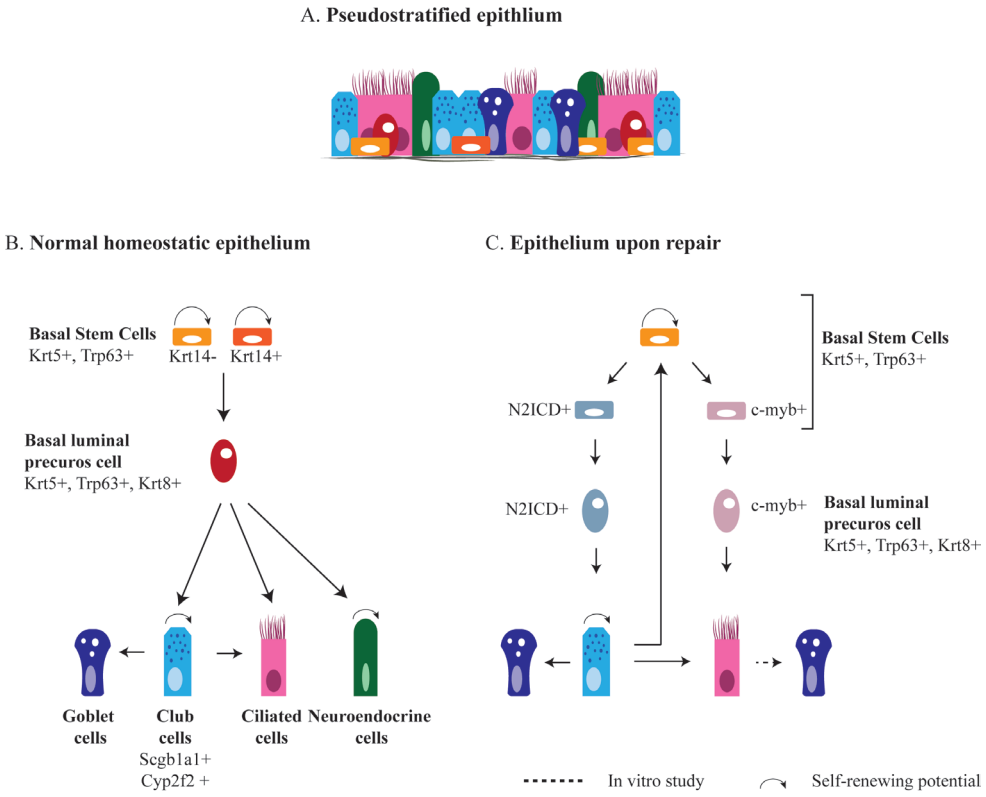

Fig. 1. Regeneration of pseudostratified airway epithelium of the lung. *(A) The airways are lined by a pseudostratified epithelium consisting of secretory cells (goblet and club cells), ciliated cells, neuroendocrine cells and basal cells. Goblet cells are abundant in the human epithelium, but are rare in mice. (B) The relationship between the different epithelial cells during normal homeostasis. The basal cells are progenitor cells of the pseudostratified epithelium which are heterogeneous for the expression of Krt14. The basal cell becomes a Krt8 positive luminal precursor cell before further differentiation. A basal cell differentiate into secretory cells and neuroendocrine cells under homeostatic conditions. Neuroendocrine cells are also capable to self-renew [168]. Scgb1a1+ secretory cells are a self-renewing population and can give rise to ciliated cells. In homeostatic epithelium, there is a very low turnover of cells. It is likely that the dividing secretory cell population is sufficient to regenerate ciliated cells in homeostatic condition. However, their generation from basal cells is not excluded. Upon allergen exposure, secretory cells are the main source of goblet cells [169], but it is unknown whether basal cells can directly differentiate into goblet cells. (C) Upon depletion of luminal cells by SO2exposure, basal cells proliferate and subdivide into two populations, N2ICD and c-myb positive, respectively, differentiating into secretory and ciliated cells. After the loss of basal cells, secretory cells (de)differentiate into functional progenitor basal stem cells. In a normal pseudostratified epithelium, only a few scattered goblet cells are present. Increases in goblet cells are observed upon immune stimuli and in diseases like COPD. Lineage tracing studies show that goblet cells can arise from Scgb1a1+ secretory cells and recently a trans-differentiation of Foxj1+ ciliated cells to goblet cells was observed upon smoke exposure in culture.*

conditions. These cells are thought to be a self-renewing population involved in maintenance of the Krt5+ basal cell population. This proportion is highly increased and becomes multipotent after naphthalene-induced depletion of secretory cells [18, 19]. Lineage tracing studies show that Krt14+ cells can directly regenerate secretory and ciliated cells [18, 20]. Recently, two distinct populations of basal cells were identified in the adult lung using long-term lineage tracing experiments and single-cell gene expression profiling: basal stem cells (BSCs) and basal luminal precursor cells (BLPCs). Both cell types are Krt5+ and Trp63+ with rare detection of Krt14, indicating that Krt14 is not a robust marker for stem cell identity [21]. However, the rapid up-regulation of Krt14 post-injury suggests that Krt14 may be an important marker to identify activated stem cells in the regenerating epithelium. Within homeostatic conditions, BSCs divide via asymmetric division to produce one new BSC and one BLPC, which can further differentiate into a neuro-endocrine and secretory cell (Fig. 1B). The BLPCs have a low or negligible rate of self-amplification, lack any overt signs of differentiation, and are distinct from BSCs by their expression of Krt8 [21]. This model is consistent with a previous observation in human basal cells addressing the potential of individual basal cells to self-renew and differentiate [22]. Additionally, the emergence of a Krt5+/Krt8+ parabasal cell population, which have comparable characteristics as the previously described BLPCs, was shown to be controlled by active Notch3 signaling [16]. Notch3-/- mice showed an increase in basal cells and parabasal cells, but not in multiciliated and secretory cells, suggesting that Notch3 is involved in restricting the expansion of the basal and parabasal population [16]. Interestingly, binding of the transcription factor Grainyhead-like 2 (Grhl2) to the promotor region of Notch3 was observed, suggesting a role for Grhl2 in the transcription of Notch3 [23]. BSC-specific ablation of Grhl2 showed only a decrease in the number of ciliated cells, but no other changes in the morphology of the epithelium [24]. Whether Grhl2 is important in the Notch3 dependent regulation of the BSC and parabasal cell population still has to be explored. Krt8+/Krt5+ double positive cells were previously identified in mice as a marker for progenitor cells upon regeneration following injury induced by reactive oxygen species and sulfur dioxide (SO2) [15, 25]. Interestingly, using the SO2 injury model, it was observed that Trp63+ basal cell populations segregate in subpopulations prior to the formation of the Krt8+ progenitor cell. These dividing Trp63+ basal stem cell populations are either N2ICD+ (the active Notch2 intracellular domain) cells that differentiate into mature secretory cells, or c-myb+ cells that differentiate into ciliated cells (Fig. 1C) [26]. This specific segregation of progenitor cells was not found in homeostatic epithelium, which indicates that post-injury mechanisms may lead to different subsets of progenitor cells compared to the homeostatic epithelium [26]. A new study shows Trp73 as a regulator of ciliated cell differentiation, which expression was observed in terminally differentiated ciliated cells as well as in Trp63+ basal cells. This indicates a direct transition from basal cell to ciliated cell as well as a

segregation of epithelial cell fate at the basal cell level [27]. The role for Trp73 in response to damage and the trigger that is responsible for a Trp73+ basal cell to initiate ciliated cell differentiation is not yet studied . This would be essential in understanding the role of Trp73 in the Trp63+ basal cell population.

Clusters of Trp63+/Krt5+ cells, called distal alveolar stem cells (DASCs), are present in the distal airways after H1N1 influenza virus infection and have the capacity to replace injured alveolar cells (Fig. 2B) [28, 29]. Despite sharing similarity in markers, the tracheal basal stem cells (TBSCs) and DASCs show different fates in culture and *in vivo* transplantation. The TBSCs give rise to more proximal epithelium both in culture and *in vivo*, while the DASCs can form alveolar spheres *in vitro* and give rise to alveolar cells and secretory cells *in vivo* [29]. Krt5 lineage tracing studies concluded that these cells were not present before infection and were generated as a response to injury [29]. In addition to this finding, Vaughan and colleagues proposed a lineage negative epithelial precursor (LNEP) cell expressing Trp63+ and Krt5+ that helps to regenerate the alveoli after bleomycin injury. Transcriptional profiling of these cells indicate a very heterogeneous population suggesting that different cell types are present in the Trp63+/Krt5+ population [30]. Moreover, active Notch signaling was required to activate Trp63+/Krt5+ expression in LNEPs and active Notch prevents the further differentiation into AT-II cells [31]. This suggests that the hyperactive Notch signaling observed in lung diseases possibly contributes to failure of regeneration. In conclusion, basal cells can function as tissue-specific stem cells of the airway epithelium, but the heterogeneity in the population of basal cells is not yet completely understood. Since the identification of different subsets of basal cells is studied using lineage-tracing studies in mice, validation of these subsets of basal cells in human lung is of importance. Differences in progenitor populations are found in homeostatic epithelium compared to damaged epithelium. This suggests that in response to injury, molecular mechanisms are triggered that lead to the appearance of different subsets of epithelial progenitor cells, perhaps derived from one general homeostatic basal cell. Currently, signaling pathways are being identified that influence the expansion of basal cells and differentiation into specific cell types, but the precise underlying molecular mechanisms still need to be identified (Table 1). Furthermore, it is increasingly recognized that basal cells not only contribute to tissue repair, but are also a target for respiratory pathogens and contribute to host defense against infection [32]. Further studies, including those aimed at identifying subsets of basal cells that display these properties, are needed to better understand the link between this immune basal cell response and repair of the epithelium.

Other epithelial progenitor cells

Basal cells are not the only identified multipotent cells in the lung (Table 2). Variant

Cell type	Subtypes	Differentiation potential	Signaling Cues
(Tracheal) Basal Stem Cells	Trp63, Krt5, Krt14$^{+/-}$	Self-Renewal	Notch [25], Hippo signaling [33]
	Trp63, Krt5, Krt8	Basal Luminal	Notch 3 signaling [16], Grhl2[24]
		Precursor Cell	
		Neuroendocrine	Notch1[34, 35] and Hes1[36]
	Trp63, Krt5, N2ICD	Club	Notch^{high} signaling [15], Notch1 [37], Notch2 [34]
	Trp63, Krt5, c-Myb / Trp73	Ciliated	Notch^{low} signaling [15], Notch 1 and 2 [34]
Distal Alveolar Stem Cells [a]	Trp63, Krt5	Self-Renewal	Increased Notch activity, Notch1 [31]
		AT-II	Inhibition of Notch [31]
		Club	

[a]Only observed after H1N1 influenza virus infection or bleomycin induced injury, AT-II=Alveolar Type II Cells

Table 1 *Overview Basal-like Stem Cell Populations*

club cells, a subset of secretory cells that are positive for Secretoglobin family 1a member 1 (Scgb1a1) and negative for Cyp2f2, have been shown to self-renew and to differentiate into Cyp2f2+ secretory cells after naphthalene injury [3, 38, 39]. Interestingly, another subset of Scgb1a1+ cells co-expressing the AT-II marker Surfactant protein C (Sftpc) was shown to differentiate into bronchiolar and alveolar lineages in vitro. These cells were called broncho-alveolar stem cells (BASCs) and are located at the broncho-alveolar duct junction (BADJ) (Fig. 2B) [40]. However, conflicting results are reported based on lineage tracing of Scgb1a1+ cells after lung injury. Scgb1a1+ cells differentiate into alveolar epithelial cells after influenza and bleomycin-induced injury, but not after hyperoxia-induced alveolar injury [39, 41]. This contradiction could result from different subsets of cells being labeled by the Scgb1a1-driven Cre driver, or from the activation of different pathways by hyperoxia and bleomycin. Cell-specific lineage tracing tools are required to give more clarity about the potential of BASCs and the variant club cells. Different alveolar progenitors and associating markers have been identified in response to lung injury and are summarized in Fig. 2B. AT-II cells expressing Sftpc are capable of self-renewal and a small fraction of mature type II cells can differentiate into AT-I cells in homeostasis and after injury [42, 43]. Besides the progenitor potential of AT-II cells, another progenitor subpopulation for alveolar epithelial cells has been identified. These cells co-express α6 and α4 integrins, but lack expression of Scgb1a1 or Sftpc. They respond to lung injury and can differentiate into AT-II cells and club cells. These cells reside in the alveoli as well as in the BADJ and their differentiation potential in vivo is most likely restricted by their niches [44]. Furthermore, a distinct population of Sca1+/Sftpc+ AT-II cells appeared at the onset of repair after infection of the lung by Pseudomonas aeruginosa intratracheal

instillation [45, 46]. Most of these cells were negative for α4 integrin, Trp63 and Scgb1a1, separating them from respectively other distal progenitor cells and BASCs [28, 40, 44, 46]. Lineage tracing experiments showed that Sca1+ AT-II cells may arise from Sftpc+/Scgb1a1- cell and further differentiate into AT-I cell (Fig. 2B). This conversion of Sca1+ AT-II cells to AT-I cells depends on an active Wnt/β-catenin pathway [47]. Taken together, several populations are being marked as progenitor cells and the activity of subsets of progenitor populations seems to depend on their niches and kind of epithelial damage. The current challenge is to elucidate whether the different progenitor cells are indeed different cells, or if these cells are variants of a single precursor cell that are induced by different damaging agents. Single-cell RNA sequencing of the developing distal lung epithelium has helped in defining more precisely the different types of (progenitor) cells in the distal region of the developing lung [12]. A similar approach during regeneration of the proximal and distal lung epithelium might provide additional clues on the heterogeneity of epithelial cells upon repair.

Cell type	Marker genes	Differentiation potential	Hallmarks
Variant Club Cells	Scgb1a1$^+$, Cyp2f2$^-$	Club, Ciliated	Located near NEBs
		Ciliated	Survive Naphthalene injury
Broncho-Alveolar Stem Cells	Scgb1a1$^+$, Sftpc$^+$	AT-II, Ciliated	Wnt signaling induces proliferation BASC [48]
			Located at BADJ
Itgα6$^+$, Itgβ4$^+$Alveolar progenitor	Scgb1a1$^-$, Sftpc$^-$, Itgα6$^+$, Itgβ4$^+$	AT-II, Club	Located at BADJ and Alveolar wall
AT-II	Sftpc$^+$, LysM	Sca1$^+$, Sftpc$^+$ AT-I progenitor cell	EGF induced proliferation [42]
		AT-I	Wnt dependent conversion to AT-I [46]

NEBs=Neuroendocrine Bodies, BADJ=Broncho-Alveolar Duct Junction, AT-I/II = Alveolar-Type I/II cells

Table 2 *Other Potential Epithelial Stem cells*

PLASTICITY OF THE LUNG

Further complexity and challenges in lung regeneration are generated by the plasticity of differentiated cells (Table 3). Independent studies have pointed at the potential of Scgb1a1+ secretory cells to dedifferentiate into Trp63+/Krt5+ basal cells upon depletion of the basal cell lineage or after damage of the lung epithelium [14, 49]. These dedifferentiated basal cells have the full capacity to redifferentiate into ciliated or secretory cells (Fig. 1C). The Hippo pathway and its down-stream effector Yap are required for the dedifferentiation of secretory cells [33]. Moreover, Yap has been shown to regulate stem cell proliferation and differentiation during normal epithelial homeostasis and regeneration upon injury in the adult lung [33, 50]. Further research showed that the nuclear-cytoplasmic distribution of Yap is important in the differentiation of adult lung epithelium and during development [16, 51]. Thus,

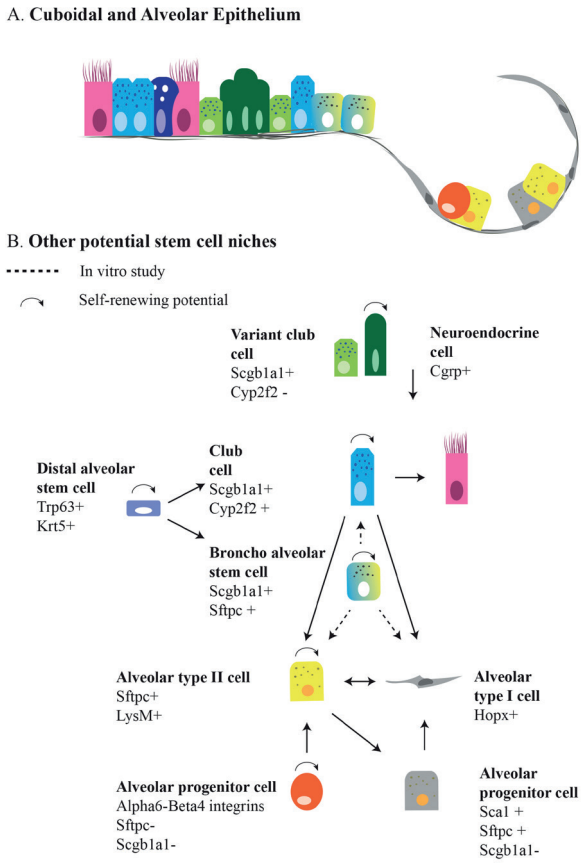

Fig. 2. Regeneration of distal and alveolar airway epithelium after injury. *(A) The small airways lack basal cells and consist of cuboidal epithelium, containing secretory and ciliated cells, as well as clusters of neuroendocrine cells. The cuboidal epithelium passes into a broncho-alveolar duct junction which is the niche of broncho-alveolar stem cells. The alveolar epithelium consists of alveolar type I, type II cells and alveolar progenitor cells. (B) Variant club cells (Cyp2f2-) are a variant of secretory cells that survive naphtalene injury and give rise to cyp2f2+ club cells. Lineage tracing of Cgrp+ cells showed that after depletion of club cells by naphtalene injury neuroendocrine cells contribute to the regeneration of these cells. At the broncho-alveolar duct junction, broncho-alveolar stem cells were isolated and shown to differentiate into bronchiolar and alveolar lineages in culture (dashed lines). Scgb1a1+ cells have the potential to form alveolar type I and type II cells after bleomycin injury, but not after hyperoxia-induced injury (dashed line). AT-II cells can self-renew and differentiate to AT-I cells. After pneumonectomy, a contribution of AT-I cells to regenerate AT-II cells was observed. An alveolar progenitor cell expressing α6-β4 integrins can regenerate AT-II cells after injury. Yet another cell type was identified expressing Sca1+ arising from AT-II cells and regenerating AT-I cells. Distal alveolar stem cells appear after severe injury and give rise to secretory and alveolar cells.*

Hippo signaling may be important in stimulating regeneration of the pseudostratified epithelium by controlling basal stem cell differentiation as well as luminal cell plasticity.

Differentiation of Foxj1+ ciliated cells to mucus-producing goblet cells was observed in human primary bronchial epithelial cell culture after exposure to IL-13, an important mediator in asthma [52]. Interestingly, this plasticity was not confirmed by a Foxj1+ lineage tracing study in mice using an ovalbumin-induced injury model [53]. Either the difference of damage to the epithelium, smoke versus ovalbumin, or the use of different species could account for the different outcomes.

Previous lineage tracing studies using lysozyme M as marker for mature AT-II cells already demonstrated that AT-II cells can differentiate into AT-I cells [42]. More recently, a plasticity AT-I cells after pneumonectomy has been shown. To regenerate the alveoli, Hopx+ AT-I cells proliferate and differentiate into Sftpc+ AT-II cells (Fig. 2B) [54]. The formation of AT-II cells from Hopx+ AT-I cells in organoid culture seems to be modulated by TGF-β signaling [54]. These results suggest a bi-directional transition between the two types of mature alveolar cells. However,

after pneumonectomy the contribution of AT-I cells to regenerate AT-II cells is small (~10%). Vice versa, approximately 16% of regenerated AT-I cells are derived from Sftpc+ AT-II cells, indicating that other cell sources also contribute to re-alveolarization [54]. Thus, strategies for regeneration of lung epithelium in disease, includes targeting of progenitor cell populations and activating the plasticity or fate of differentiated lung cells. Signaling cues to induce endogenous lung regeneration are starting to be identified and might be targets for disease therapies in the future. In line with initiating differentiation through signaling, it has been demonstrated that conversion of a specific cell type can be induced by changing the expression of a single protein. Ectopic expression of Sox2 in AT-II cells changed its alveolar cell type to a more proximal cell fate expressing Scgb1a1 and Trp63, even though the cells remained in the niche for distal cells [55]. A similar approach was used to show the plasticity of AT-I cells, where overexpression of Sox2 was sufficient to reprogram AT-I cells towards a proximal airway cell fate with expression of Trp63 [56]. The differentiation potential and plasticity of the lung epithelial cells as described in the above sections are illustrated in Figs. 1 and 2 to show the complexity of the cells involved in regenerating the lung epithelium.

Cell type	**Marker genes**	**Differentiation potential**	**Signaling Cues**
Club cells	Scgb1a1^{+}, Cyp2f2^{+}	Basal	Hippo pathway [33]
		Ciliated	*Unknown*
		Goblet	IL-13 exposure [57]
AT-I	Hopx	AT-II	Modulated by TGF-β signaling [54]
			Proximal cell fate by overexpressing Sox2 [56]
AT-II cells	Sftpc^{+}, LysM	Proximal cells	Ectopic Sox2 expression [55]
Ciliated cells	TubIVa, Foxj1	Goblet	IL-13 exposure[52]

AT-I /AT-II = Alveolar type-I/II Cell

Table 3 *Plasticity Of Differentiated Cells*

REGENERATIVE MEDICINE

Drugs to induce lung regeneration

Different signaling pathways are involved in either maintaining a quiescent homeostatic or inducing a proliferating regenerating epithelium [3]. Signaling consists of cross-talk and feedback loops between epithelial cells but also between epithelial and mesenchymal cells. Such interplay between mesenchymal and epithelial cells is for example important in Hedgehog (Hh) signaling. In the adult lung, Hh signaling balances between stimulating proliferation and quiescence. In the homeostatic lung Hh signaling is active to maintain quiescence, however upon injury Hh signaling is inhibited to stimulate epithelial proliferation [58]. A shift in the balance can lead to failure of repair but can also play a role in promoting tumorigenesis [58, 59].

Several pathways involved in lung development and regeneration are relevant in lung disease, and drugs that either inhibit or induce these pathways could have a beneficial effect for patients. Recently, it was shown that deletion of Notch3 leads to an expansion of basal cells, a hallmark of smokers and individuals with chronic obstructive pulmonary disease (COPD) [16, 60, 61]. Interestingly, Notch3 down-regulation was observed in smokers and in COPD lung, making it a potential target for controlling the balance between basal and luminal cells [16, 62] Candidate pathways for targeting in COPD include Hedgehog signaling, Notch signaling, the retinoic acid pathway and the transforming growth factor-β (TGF-β) pathway [63]. The TGF-β pathway, as well as bone morphogenetic proteins (BMPs), growth differentiation factors and activins are also linked to asthma and these pathways could be potential drug targeting candidates [64]. In COPD there is mucus hypersecretion, and there are several ongoing studies that examine the effect of already marketed drugs on the production and secretion of mucus in COPD models [65]. Recently, it was shown that interference of Notch signaling with specific antibodies against the ligands Jag1 and Jag2 results in an increase in ciliated cells at the expense of club cells [66]. Moreover, jagged inhibition also reversed goblet cell hyperplasia, which could potentially be important in COPD patients to reduce the mucus production and to increase clearance by the ciliated cells. Fibroblast growth factors (FGFs) also play a role in regeneration of several tissues including the lung [67]. FGF1 and FGF2 are thought to play a role in the protection of epithelial stem cells and lung maintenance, and are linked to pulmonary hypertension. FGF1 is also thought to play a role in idiopathic pulmonary fibrosis. FGF7 and FGF10 are involved in lung regeneration and several different injury models show that these FGFs are important for repair of the lung. Several recombinant FGFs (FGF1, FGF2) and truncated forms of FGFs (FGF7, FGF10) are already used in clinical applications, like angiogenic therapies, coronary heart disease and treatment of ulcers [67]. Although these therapies are not yet available for lung diseases, there may be some future perspectives, either in inducing or inhibiting pathways involved in disease or by activation of endogenous lung progenitor cells.

STEM CELLS

Stem cells are functionally characterized by their undifferentiated state and their properties of self-renewal and pluripotency to become specialized cells. Because of these characteristics, they are appealing to be used for the regeneration of damaged tissue. A distinction can be made between embryonic stem cells (ESCs) and adult stem cells. ESCs are derived from the inner cell mass of the blastocyst and these cells have the ability to differentiate into ectodermal, mesodermal and endodermal cell types. Human ESCs could be useful to study early embryonic development, for cell replacement therapy, to study disease pathways, and for drug discovery, although ethical and therapeutic issues hamper the use of these cells.

Besides ESCs, several types of adult stem cells throughout the human body exist, like hematopoietic stem cells, intestinal stem cells, mammary stem cells, olfactory stem cells, mesenchymal stem cells, endothelial stem cells, and neural stem cells. Adult stem cells are also capable of self-renewal and may differentiate into several cell types, but their differentiation potential is more restricted [68-70]. As indicated, there is accumulating evidence that differentiated cells show more plasticity than previously thought. Moreover, the number of different progenitor cells in the lung is higher than previously expected, depending on the type of injury or disease.

Induced pluripotent stem cells

In 2006, the group of Yamanaka introduced a method to generate cells with properties similar to ESCs [71]. These so-called induced pluripotent stem cells (iPSCs) are somatic cells that are reprogrammed into a multipotent stem cell-like stage using only four different factors: Oct4, Sox2, cMyc and Klf4 (Yamanaka factors) [70]. Culturing these cells under distinct conditions induces several specialized cell types. iPSCs can be used for numerous applications, like disease modeling, regenerative medicine, drug discovery, and toxicity studies [70, 72]. The lung is a very complex organ that consists of many different specialized cell types, which makes it challenging to generate human airway and alveolar epithelial cells from iPSCs. First, definitive endoderm should be derived from human iPSCs (hiPSCs), followed by generation of anterior foregut endoderm [73]. From this anterior endoderm, lung endoderm can be derived, which can subsequently be guided towards bronchial progenitor cells (Sox2+) or alveolar progenitor cells (Sox9+), and finally towards bronchial or alveolar epithelial faith [70]. Several studies have shown the differentiation of ESCs and iPSCs into AT-II cells [74-79]. Other groups have shown the differentiation of iPSCs into multiciliated cells [80], mature airway epithelium expressing functional CFTR protein [81], multipotent lung and airway progenitors [82], purified lung and thyroid progenitors [83], purified distal lung alveolar epithelium [84], lung and airway epithelial cells [85], and lung and airway progenitor cells [86]. An overview of these differentiation protocols is given by Ghaedi and co-workers, although optimization is clearly required before these cells may be used in clinical applications [70].

iPSCs may be used for the generation of patient-specific disease models and (large scale) drug screening, as shown for example with cells derived from patients suffering from cystic fibrosis [87]. A more clinical use of iPSCs in lung disease therapy is not yet approved and more knowledge is necessary before this will be applicable[88].

Mesenchymal stem cells

Mesenchymal stem/stromal cells (MSCs) are adult stem cells that have the potential to differentiate into cells derived from the mesoderm lineage. MSCs were first derived from bone marrow, but many other sources are reported, including umbilical

cord blood, placenta, skin, liver and brain [89, 90]. MSCs refer to a heterogeneous population of cells, making it difficult to isolate them. Therefore, MSCs are defined by a number of criteria based on the expression of specific cell surface antigens and their functionality. Cells should express CD75, CD90 and CD105, but not CD34, CD45, HLA-DR, CD11b, CD19 and CD14. MSCs should be capable to differentiate into chondrocytes, osteoblasts and adipocytes, and should adhere to plastic for stable cell culture [68, 89-91]. Recent studies have shown that MSCs may differentiate in other cell types, including lung cells, although this is still controversial [92, 93]. It has been reported that MSCs can also be isolated from the lung. Martin et al. reported the isolation of MSCs from tracheal aspirates of neonates and from adult broncho-alveolar lavage [94]. More recently, Gong and co-workers isolated lung resident MSCs and showed that these cells have the potential to differentiate into AT-II cells [95]. MSCs derived from other sources than the lung can also be differentiated into alveolar epithelium. These alveolar cells were generated from MSCs derived from human umbilical cord blood by culturing them in lung-specific differentiation media [93].

There are many completed and ongoing clinical trials using MSCs for applications in the nervous system, heart, liver and kidney. In lung disease, therapies with MSCs could be useful in bronchopulmonary dysplasia (BPD), COPD, acute respiratory distress syndrome and idiopathic pulmonary fibrosis [68, 89, 91, 96, 97]. However, given the low percentage of engraftment of the instilled MSCs as demonstrated in animal models, it is very likely that the beneficial effects of MSC therapy are not due to the differentiation potential of MSCs itself, but rather due to paracrine and immunomodulatory effects [89, 98-100].

Endothelial progenitor cells

There are two different subsets of endothelial progenitor cells (EPCs), proangiogenic hematopoietic cells and endothelial colony-forming cells (ECFCs) [101]. Proangiogenic hematopoietic cells are derived from the bone marrow and are involved in vascular repair. It is thought that these cells circulate to injury sites and there facilitate formation of new vessels using paracrine mechanisms, but lack direct vessel-forming ability. ECFCs are rare circulating blood cells that have the potential to generate cells that express genes from the endothelial lineage. They also have the potential to form blood vessels *in vivo* [101]. There is increasing evidence that EPCs are involved in several lung diseases, including COPD, BPD and pulmonary hypertension. Several lung injury animal models have shown (partial) reversal of the induced phenotype by systemic administration of EPCs, including improvement of pulmonary function and repair of the alveolar and vascular structure of the lung [102-104]. These therapeutic effects could be caused by structural conditions of the cells, by paracrine effects or by a combination of

both [88]. The interaction between the pulmonary vasculature and the airways is important for proper growth and regeneration of the lung (reviewed in [105]). This was recently supported by the identification of endothelial derived angiocrine signals promoting alveolar regeneration after pneumonectomy [106, 107]. The interactions between the vasculature and epithelial cells upon repair are still elusive, but the identification of signaling molecules, like stromal cell-derived factor-1 (SDF-1), may be important for potential therapies. Systemic administration of EPCs has shown to be beneficial in patients with primary pulmonary hypertension [108, 109]. Several pre-clinical and clinical trials are ongoing to test the potential of using EPCs in lung disease therapies [88].

Besides the stem cells mentioned in this section, there are also endogenous lung progenitor cells that were discussed in previous sections. All these different stem/progenitor cells are potentially targets for therapeutic strategies. While MSCs and EPCs could be effective because of their paracrine effects, iPSCs could be useful in the development of lung mimics and tissue engineering. Pathways involved in differentiation of lung progenitor cells to other cell types and plasticity of these cells, could be induced or inhibited by medication to induce lung regeneration.

LUNG MIMICS

Most studies on cell biology and tissue regulation are based on 2D cell-culture models. Although these models are valuable to answer specific scientific questions, it is clear that these models have limitations and fail to reconstitute the *in vivo* cellular microenvironment. Therefore, 3D cell-culture models were developed, which mimic a more realistic tissue- and organ-specific micro-architecture, although some aspects, including tissue-tissue interfaces and a mechanically active microenvironment are still missing. However, these models are very useful in patient-specific disease models, drug-screening and as a source of cells for transplantation [110].

Air-liquid interface cultures

Air-liquid interface (ALI) cultures mimic a more realistic lung environment and make it possible for airway epithelial cells to proliferate and differentiate *in vitro*. Whitcutt et al. were the first to demonstrate mucociliary differentiation using ALI cultures [111]. Culturing human airway epithelial cells from patients, makes it possible to conduct patient-specific research and drug-screening, for example in cystic fibrosis and asthma [112, 113]. ALI cultures were also used to model the effects of smoke exposure on epithelial cells, which could be used to gain more insight in mechanisms involved in the pathogenesis of COPD [114, 115]. In 2015, a new computer-controlled ALI culture system was introduced in order to generate more stable and comparable cultures, which may be useful for large-scale toxicology studies [116].

Organoids

The concept of stem cell-derived organoids has already been discovered in the 1950's [117]. Organoid models use the pluri- or multi-potent properties of stem cells to differentiate into specialized cell types and to self-organize into a 3D structure with organ- or tissue-specific morphogenetic and histological properties [118-120]. Overviews of tissues and diseases modeled with organoids have been topics of recent reviews [119-121]. These tissues include intestinal buds, liver bud derivatives and retinal derivatives. In the intestine, single Lgr5+ stem cells can be isolated and grow into intestinal organoids [122]. Generation of lung organoids from one single stem cell have not been reported yet, but several studies have reported the generation of lung organoids derived from human pluripotent stem cells (hPSCs), primary respiratory cells and cell lines (reviewed in [123]). These organoids include trachea/bronchospheres [15, 124-126], bronchiolar organoids [127], bronchioalveolar organoids [126, 127], alveolospheres [43, 126, 127], branching structures [128-130], alveolar spheroids [131] and multi-lineage organoids [132]. In 2015, Dye et al. established a protocol to successfully generate lung organoids derived from hPSCs (embryonic and induced). hPSCs were first differentiated into anterior foregut spheroids, using ActivinA, BMP and TGF-β inhibitors. This anterior foregut endoderm was subsequently induced into a lung lineage by modulating FGF and Hedgehog signaling. In this way, the foregut spheroids gave rise to lung organoids. These organoids possess both proximal airway-like structures and immature alveolar airway-like structures and are globally similar to fetal human lung. These human lung organoids can be used to study lung development and regeneration [133]. Previously, tracheospheres were used to show the capacity of basal cells to self-renew and the potential to form secretory and ciliated cells [13]. Jain et al. used organoid cultures to show the potential of Hopx+ AT-I cells to form AT-II cells [54]. Furthermore, application of the Clustered Regularly Interspaced Short Palindromic Repeats/Crispr associated protein (CRISPR/Cas) system in organoid culture might be a method to identify important players in epithelial cell differentiation. Recently, this approach was used to identify the role of transcription factor Grhl2 in the differentiation of ciliated cells [24]. In the future, the loss or gain of function by manipulation of genes in culture, will lead to more insight in potential stem cell populations in the lung. Organoids are very useful to answer specific questions about lung development and regeneration, but so far they are not exposed to air, resulting in incomplete differentiation of adult airway cells. Furthermore, it does not allow to expose organoids to air pollutants such as toxic gasses and micro- or nanoparticles, making it impossible to use them to study the effects of air pollutants on the airway epithelium.

Lung-on-a-Chip

Organs-on-chips refer to bioengineered devices that mimic tissue properties and functions in a well-controlled environment [118]. Additionally, there are also (acellular)

lung-mimicking microfluidic devices not specifically to study lung biology, but as respiratory assist devices or oxygenators [134]. Over the past decade, several micro-engineered organ models have been developed to study liver, kidney, intestine, and heart, among others [135]. The first lung-on-a-chip was introduced by Huh and co-workers, which mimicked the vascular-alveolar structure by using lung epithelial cells exposed to air on one side and pulmonary vascular endothelial cells exposed to flowing culture medium on the other side of a permeable synthetic membrane (Fig. 3, [136]). This model incorporated a microfluidics system and applied mechanical stress, and as such was capable of mimicking gas exchange. However, it also has some limitations, since it uses a flat 2D membrane, cell lines instead of primary cells, and lacks interstitial fibroblasts and alveolar macrophages [136-138]. In 2015, Stucki and colleagues reported a lung-on-a-chip with an integrated, bio-inspired respiration mechanism. This model used primary human pulmonary alveolar epithelial cells, which were co-cultured with endothelial cells and exposed to a 3D cyclic mechanical strain to mimic respiration [139]. The group of Blume developed a 3D model, consisting of an air- liquid interface culture of human primary airway epithelial cells in a microfluidic culture system. This system had a continuous exchange of fluids and mediators, thereby simulating the interstitial flow in the lung [140]. The power of using the lung-on-a-chip approach includes the possibility of connecting multiple devices, thereby creating a more realistic lung mimic by integrating microfluidics, stretch, curvature and primary cells. In addition to air-liquid-interfaces and mimicking stretch during in- and exhalation, the microfluidic approach allows to apply pressure and shear flow profiles both in alveoli and attached blood capillaries. Compartmentalized microfluidic systems make bioartificial/-engineered lung tissues also amenable for higher-throughput screening of the influence/impact of concentrations and mixtures of soluble factors in the blood/medium compartment, and of gases and particles in the air compartment.

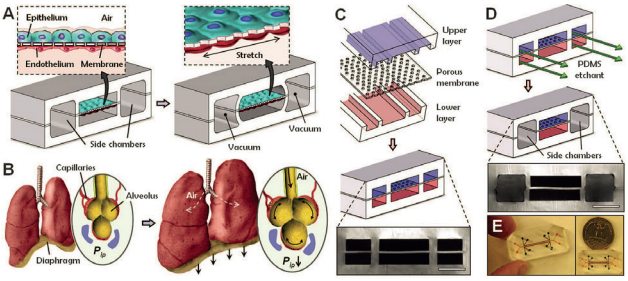

Fig. 3. Example of a human breathing lung-on-a-chip microdevice. *Lung-on-a-chip microfluidic device with compartmentalized microchannels to mimic a breathing lung (From Huh et al., "Reconstituting organ-level lung functions on a chip", Science 2010; 328:1662-8. Reprinted with permission from the AAAS [106]).See original reference for detailed description of the figure. In brief, (A) indicates the creation of mechanical breathing movements causing mechanical stretch of the membrane, (B) shows the physiology of the normal breathing human lung, (C) and (D) show the assembly and etching of the microdevice, and (E) visualizes the actual size of the device.*

TISSUE ENGINEERING

Although the above described systems are rapidly evolving, a huge hurdle is the generation of whole tissues and organs. There are three important demands to

successfully create tissues and organs: the source of cells, the type of scaffold, and the composition of the extracellular matrix (ECM). An appropriate mixture of cells should be used for the recapitulation of cell-cell interactions [141]. Appropriate scaffolds are necessary to obtain a 3D structure and can be either synthetic or biological, and biodegradable or non-biodegradable. In addition to the template three-dimensional structure, there is mechanical support and tissue instruction by engineered mechanical (e.g., through material or geometry-related matrix elasticity or stiffness), geometrical/topographical (e.g. through surface roughness or designed micro- or nanotextures) or (bio)chemical cues (e.g. RGD-adhesion moieties). An advantage of biodegradable scaffolds is that these are absorbed by the body. However, in the case of synthetic biodegradable scaffolds this may result in acidic degradation products causing inflammation in the surrounding tissue, e.g. when aliphatic polyesters like polylactic acid are used [142]. Compared to synthetic scaffolds, biological scaffolds are more similar to the tissue or organ that they should substitute, although biological scaffolds may lack sufficient mechanical properties [143]. Different types of biological scaffolds can be used like collagen, Matrigel® and decellularized organs [143]. Decellularization of organs has to be done in a proper way to as much as possible preserve all components of the extracellular space/ extracellular matrix components and their instructive properties. Several chemical, physical and enzymatical methods have been described to achieve this [68]. After decellularization, a process that does affect the extracellular matrix, the scaffold can be recellularized. Cells from different sources, as previously described, can be used for this purpose: embryonic, fetal or adult stem cells, autologous cells from the patient or iPSCs [68]. It is also possible to use allogeneic cells, e.g. in the case of transplantation of islets of Langerhans. There is also need for cells that are involved in vascularization and innervation, and cells with supportive, structural and barrier functions. Using autologous cells would be ideal to prevent rejection of the tissue-engineered organ in the patient, but could cause difficulties in the case of genetic or metabolic disorders [141]. Successful generation of tissue-engineered autologous bladders [144] and bio-engineered skin substitutes [145] have been reported as well as successful 3D bioprinting of several tissues and organs including multilayered skin, vascular grafts, heart tissue and tracheal splints [141, 146]. The structure and composition of the ECM should resemble that of embryonic organogenesis. It has been demonstrated that ECM signals are important to form pulmonary tissue structures in vitro [147]. Other signals, like cell-cell interactions, are also of importance to mimic the micro-environment of the organ [118, 148].

Tracheal bioengineering

In patients with a tracheal defect of 50% of total length in adults or 30% in children, artificial tracheal grafting is required [149]. Several approaches for tracheal epithelial differentiation have been tested, including co-culturing of tracheal epithelial cells with

fibroblasts or adipose-derived stem cells [150-152] and cell sheet engineering with tracheal epithelial cells [153, 154]. In spite of the controversies and success rate, Macchiarini et al. were the first group that transplanted a tissue-engineered airway [155]. The group of Steinke produced a bioartificial airway tissue using autologous primary cells to re-endothelialize and reseed a biological vascularized scaffold. After transplantation they observed complete airway healing and no evidence of tissue dedifferentiation [156]. Park and co-workers showed that human turbinated mesenchymal stromal cells cultured as intact sheets were able to differentiate into tracheal epithelium. These sheets were transplanted onto artificial grafts and tested in a rabbit model. After one month, regeneration of functional tracheal epithelium was observed [149]. Still, considerable problems are observed using tracheal grafts including failure to integrate and the formation of cartilaginous tissue [4, 68].

Vascular bioengineering

Interactions between epithelium, mesenchyme and endothelium are necessary for proper lung development and regeneration. Blood vessels secrete angiocrine factors that are involved in these processes including KLEIP, HIF-2α, VEGF, BMP-4, FGF, MMP14, EET and TSP-1. Angiogenesis, the process where vessels are formed from a pre-existing network, is important for adult vascular homeostasis, regeneration and adaption. Angiocrine signaling is necessary for this process [105]. The important role of the vasculature is also recognized in tissue engineering. Ren et al. attempted to generate transplantable rat lung grafts by seeding epithelial and endothelial cells into the airway and vascular compartments of a decellularized lung scaffold from the rat. The major problem was poor vascular performance, causing incomplete endothelial coverage of the scaffold vessels. They optimized their protocol by co-seeding endothelial and perivascular cells which resulted in an endothelial coverage of 75% [157]. Even during decellularization of lung scaffolds, vascularization is important to preserve the integrity of the scaffold [158]. Orlova and co-workers showed that it is possible to generate endothelial cells and pericytes from human PSCs. This could provide a source of patient-specific vascular cells used in vascular bioengineering[159].

Whole lung bioengineering

Bioengineering of the whole lung is more complex than tracheal bioengineering due to the complexity of the lung. Lungs that are not suitable for transplantation can be decellularized and the scaffold can subsequently be used for seeding cells to regenerate the lung. It is still unknown which cell source is most suitable to repopulate the decellularized lung: MSCs, lung resident cells or a combination of both. Recently, it was shown that lung epithelial stem cells require co-culture with stromal cells to proliferate and differentiate. Fibroblasts have shown the highest efficiency in this support, and also the tissue origin of these cells gives

varying patterns of support. Also, the use of FGFs and LIF-, ALK5- and ROCK-inhibitors activates proliferation and differentiation of quiescent lung stem cells [126]. Several methods were developed to decellularize lungs of rats, pigs, non-human primates and humans and to subsequently recellularize these scaffolds [160-166]. An overview of the currently available respiratory tract models, including the used cell sources and scaffolds, is reviewed by Nichols et al. [167].

CONCLUDING REMARKS

Knowledge about potential stem cells in the lung has markedly increased through various recent developments. One of the challenges will be to merge all the data from different species and obtained with various techniques into a simplified model of lung stem cells and their role in the normal and diseased lung. Furthermore, a comprehensive view of all the (un)differentiated cells is still missing, because our repertoire of cell specific markers is still inadequate to identify the various cell types. One concern is that the use of different markers in individual studies might lead to the misconception that several subpopulations of progenitor cells exist, whereas there may possibly be only a few. In the future, the increase of cell-specific markers combined with single-cell lineage tracing should improve the definition of different (stem) cell populations in the lung. Additionally, a universal and unambiguous biological read out system to test the quality and purity of lung stem cells is also unavailable. So far, different systems, such as ALI cultures, organoids and explants, are successfully employed to fill this gap, but this makes it cumbersome to compare the various studies. Together with ESCs, iPSCs, MSCs and EPCs, local lung stem or progenitor cells could be used for diverse clinical applications in the field of regenerative medicine. Current approaches to direct differentiation of stem cells, like iPSCs and MSCs, do generate lung-specific cells, but the specific lineages and the percentages of differentiated cells vary substantially. Therefore, optimization, improvement and expansion of the existing protocols is mandatory before clinical applications are possible. The manipulation of stem cells, like iPSCs, is required and useful for the development of lung mimics, for tissue engineering and for the generation of complete lung tissue. For tissue engineering applications, current scaffolds need to be improved or alternative suitable scaffolds need to be developed, which can be of synthetic and/or biological origin, and should contain appropriate ECM signals. Alternatively to bio-engineered lungs, specific pathways involved in differentiation of lung progenitor cells and plasticity of these cells may be targeted by novel compounds to induce their contribution to lung regeneration. Collectively, significant progress will be made through the interaction between very distinct scientific disciplines, such as developmental biology, biomedical engineering, and physics. These new and rapid developments in lung repair and regeneration offers a promising perspective for future patients with irreversible lung injury.

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AUTHORS' CONTRIBUTIONS

KAAS, EE, and RJR designed the concept and organized the review. KAAS, EE, SvR, AAP, DS, RT, PSH and RJR critically evaluated and improved the manuscript with significant additions.

REFERENCES

1. Beers MF, Morrisey EE: The three R's of lung health and disease: repair, remodeling, and regeneration. Journal of Clinical Investigation 2011, 121:2065-2073.
2. Volckaert T, De Langhe S: Lung epithelial stem cells and their niches: Fgf10 takes center stage. Fibrogenesis Tissue Repair 2014, 7:8.
3. Kotton DN, Morrisey EE: Lung regeneration: mechanisms, applications and emerging stem cell populations. Nature Medicine 2014, 20:822-832.
4. Hogan BLM, Barkauskas CE, Chapman HA, Epstein JA, Jain R, Hsia CCW, Niklason L, Calle E, Le A, Randell SH, et al: Repair and Regeneration of the Respiratory System: Complexity, Plasticity, and Mechanisms of Lung Stem Cell Function. Cell Stem Cell 2014, 15:123-138.
5. Lin C, Yao E, Chuang PT: A conserved MST1/2-YAP axis mediates Hippo signaling during lung growth. Dev Biol 2015, 403:101-113.
6. Rock JR, Hogan BLM: Epithelial Progenitor Cells in Lung Development, Maintenance, Repair, and Disease. Annual Review of Cell and Developmental Biology, Vol 27 2011, 27:493-512.
7. Boers JE, Ambergen AW, Thunnissen FB: Number and proliferation of basal and parabasal cells in normal human airway epithelium. Am J Respir Crit Care Med 1998, 157:2000-2006.
8. Rock JR, Randell SH, Hogan BL: Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. Dis Model Mech 2010, 3:545-556.
9. Morrisey EE, Hogan BL: Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell 2010, 18:8-23.
10. Guo M, Wang H, Potter SS, Whitsett JA, Xu Y: SINCERA: A Pipeline for Single-Cell RNA-Seq Profiling Analysis. PLoS Comput Biol 2015, 11:e1004575.
11. Du Y, Guo M, Whitsett JA, Xu Y: 'LungGENS': a web-based tool for mapping single-cell gene expression in the developing lung. Thorax 2015, 70:1092-1094.
12. Treutlein B, Brownfield DG, Wu AR, Neff NF, Mantalas GL, Espinoza FH, Desai TJ, Krasnow MA, Quake SR: Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. Nature 2014, 509:371-375.
13. Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, Hogan BLM: Basal cells as stem cells of the mouse trachea and human airway epithelium. Proceedings of the National Academy of Sciences of the United States of America 2009, 106:12771-12775.
14. Tata PR, Mou H, Pardo-Saganta A, Zhao R, Prabhu M, Law BM, Vinarsky V, Cho JL, Breton S, Sahay A, et al: Dedifferentiation of committed epithelial cells into stem cells in vivo. Nature 2013, 503:218-223.
15. Rock JR, Gao X, Xue Y, Randell SH, Kong YY, Hogan BL: Notch-dependent differentiation of adult airway basal stem cells. Cell Stem Cell 2011, 8:639-648.
16. Mori M, Mahoney JE, Stupnikov MR, Paez-Cortez JR, Szymaniak AD, Varelas X, Herrick DB, Schwob J, Zhang H, Cardoso WV: Notch3-Jagged signaling controls the Development 2015, 142:258-267.
17. Tsao PN, Vasconcelos M, Izvolsky KI, Qian J, Lu JN, Cardoso WV: Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. Development 2009, 136:2297-2307.
18. Ghosh M, Helm KM, Smith RW, Giordanengo MS, Li B, Shen H, Reynolds SD: A single cell functions as a tissue-specific stem cell and the in vitro niche-forming cell. Am J Respir Cell Mol Biol 2011, 45:459-469.
19. Cole BB, Smith RW, Jenkins KM, Graham BB, Reynolds PR, Reynolds SD: Tracheal Basal cells: a facultative progenitor cell pool. Am J Pathol 2010, 177:362-376.

20. Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR: In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. Am J Physiol Lung Cell Mol Physiol 2004, 286:L643-649.
21. Watson JK, Rulands S, Wilkinson AC, Wuidart A, Ousset M, Van Keymeulen A, Gottgens B, Blanpain C, Simons BD, Rawlins EL: Clonal Dynamics Reveal Two Distinct Populations of Basal Cells in Slow-Turnover Airway Epithelium. Cell Rep 2015.
22. Teixeira VH, Nadarajan P, Graham TA, Pipinikas CP, Brown JM, Falzon M, Nye E, Poulsom R, Lawrence D, Wright NA, et al: Stochastic homeostasis in human airway epithelium is achieved by neutral competition of basal cell progenitors. Elife 2013, 2:e00966.
23. Gao X, Vockley CM, Pauli F, Newberry KM, Xue Y, Randell SH, Reddy TE, Hogan BL: Evidence for multiple roles for grainyhead-like 2 in the establishment and maintenance of human mucociliary airway epithelium. [corrected]. Proc Natl Acad Sci U S A 2013, 110:9356-9361.
24. Gao X, Bali AS, Randell SH, Hogan BL: GRHL2 coordinates regeneration of a polarized mucociliary epithelium from basal stem cells. J Cell Biol 2015.
25. Paul MK, Bisht B, Darmawan DO, Chiou R, Ha VL, Wallace WD, Chon AT, Hegab AE, Grogan T, Elashoff DA, et al: Dynamic changes in intracellular ROS levels regulate airway basal stem cell homeostasis through Nrf2-dependent Notch signaling. Cell Stem Cell 2014, 15:199-214.
26. Pardo-Saganta A, Law BM, Tata PR, Villoria J, Saez B, Mou H, Zhao R, Rajagopal J: Injury induces direct lineage segregation of functionally distinct airway Basal stem/progenitor cell subpopulations. Cell Stem Cell 2015, 16:184-197.
27. Marshall CB, Mays DJ, Beeler JS, Rosenbluth JM, Boyd KL, Santos Guasch GL, Shaver TM, Tang LJ, Liu Q, Shyr Y, et al: p73 Is Required for Multiciliogenesis and Regulates the Foxj1-Associated Gene Network. Cell Rep 2016.
28. Kumar PA, Hu Y, Yamamoto Y, Hoe NB, Wei TS, Mu D, Sun Y, Joo LS, Dagher R, Zielonka EM, et al: Distal airway stem cells yield alveoli in vitro and during lung regeneration following H1N1 influenza infection. Cell 2011, 147:525-538.
29. Zuo W, Zhang T, Wu DZ, Guan SP, Liew AA, Yamamoto Y, Wang X, Lim SJ, Vincent M, Lessard M, et al: p63Krt5 distal airway stem cells are essential for lung regeneration. Nature 2014.
30. Rawlins EL: Stem cells: Emergency back-up for lung repair. Nature 2015, 517:556-557.
31. Vaughan AE, Brumwell AN, Xi Y, Gotts JE, Brownfield DG, Treutlein B, Tan K, Tan V, Liu FC, Looney MR, et al: Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury. Nature 2015, 517:621-625.
32. Shaykhiev R: Multitasking basal cells: combining stem cell and innate immune duties. Eur Respir J 2015, 46:894-897.
33. Zhao R, Fallon TR, Saladi SV, Pardo-Saganta A, Villoria J, Mou H, Vinarsky V, Gonzalez-Celeiro M, Nunna N, Hariri LP, et al: Yap tunes airway epithelial size and architecture by regulating the identity, maintenance, and self-renewal of stem cells. Dev Cell 2014, 30:151-165.
34. Morimoto M, Nishinakamura R, Saga Y, Kopan R: Different assemblies of Notch receptors coordinate the distribution of the major bronchial Clara, ciliated and neuroendocrine cells. Development 2012, 139:4365-4373.
35. Shan L, Aster JC, Sklar J, Sunday ME: Notch-1 regulates pulmonary neuroendocrine cell differentiation in cell lines and in transgenic mice. Am J Physiol Lung Cell Mol Physiol 2007, 292:L500-509.
36. Ito T, Udaka N, Yazawa T, Okudela K, Hayashi H, Sudo T, Guillemot F, Kageyama R, Kitamura H: Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium. Development 2000, 127:3913-3921.
37. Xing Y, Li A, Borok Z, Li C, Minoo P: NOTCH1 is required for regeneration of Clara cells during repair of airway injury. Stem Cells 2012, 30:946-955.
38. Reynolds SD, Hong KU, Giangreco A, Mango GW, Guron C, Morimoto Y, Stripp BR: Conditional clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. Am J Physiol Lung Cell Mol Physiol 2000, 278:L1256-1263.
39. Rawlins EL, Okubo T, Xue Y, Brass DM, Auten RL, Hasegawa H, Wang F, Hogan BL: The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. Cell Stem Cell 2009, 4:525-534.
40. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T: Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 2005, 121:823-835.
41. Zheng D, Limmon GV, Yin L, Leung NH, Yu H, Chow VT, Chen J: Regeneration of alveolar type I and II cells from Scgb1a1-expressing cells following severe pulmonary damage induced by bleomycin and influenza. PLoS One 2012, 7:e48451.

42. Desai TJ, Brownfield DG, Krasnow MA: Alveolar progenitor and stem cells in lung development, renewal and cancer. Nature 2014, 507:190-194.
43. Barkauskas CE, Cronce MJ, Rackley CR, Bowie EJ, Keene DR, Stripp BR, Randell SH, Noble PW, Hogan BL: Type 2 alveolar cells are stem cells in adult lung. J Clin Invest 2013, 123:3025-3036.
44. Chapman HA, Li X, Alexander JP, Brumwell A, Lorizio W, Tan K, Sonnenberg A, Wei Y, Vu TH: Integrin alpha6beta4 identifies an adult distal lung epithelial population with regenerative potential in mice. J Clin Invest 2011, 121:2855-2862.
45. Liu Y, Sadikot RT, Adami GR, Kalinichenko VV, Pendyala S, Natarajan V, Zhao YY, Malik AB: FoxM1 mediates the progenitor function of type II epithelial cells in repairing alveolar injury induced by Pseudomonas aeruginosa. J Exp Med 2011, 208:1473-1484.
46. Liu Y, Kumar VS, Zhang W, Rehman J, Malik AB: Activation of type II cells into regenerative stem cell antigen-1(+) cells during alveolar repair. Am J Respir Cell Mol Biol 2015, 53:113--124.
47. Liu Y, Kumar VS, Zhang W, Rehman J, Malik AB: Activation of type II cells into regenerative stem cell antigen-1(+) cells during alveolar repair. Am J Respir Cell Mol Biol 2015, 53.
48. Zhang Y, Goss AM, Cohen ED, Kadzik R, Lepore JJ, Muthukumaraswamy K, Yang J, DeMayo FJ, Whitsett JA, Parmacek MS, Morrisey EE: A Gata6-Wnt pathway required for epithelial stem cell development and airway regeneration. Nat Genet 2008, 40:862-870.
49. Zheng D, Yin L, Chen J: Evidence for Scgb1a1(+) cells in the generation of p63(+) cells in the damaged lung parenchyma. Am J Respir Cell Mol Biol 2014, 50:595-604.
50. Lange AW, Sridharan A, Xu Y, Stripp BR, Perl AK, Whitsett JA: Hippo/Yap signaling controls epithelial progenitor cell proliferation and differentiation in the embryonic and adult lung. Journal of Molecular Cell Biology 2015, 7:35-47.
51. Mahoney JE, Mori M, Szymaniak AD, Varelas X, Cardoso WV: The hippo pathway effector Yap controls patterning and differentiation of airway epithelial progenitors. Dev Cell 2014, 30:137-150.
52. Turner J, Roger J, Fitau J, Combe D, Giddings J, Heeke GV, Jones CE: Goblet cells are derived from a FOXJ1-expressing progenitor in a human airway epithelium. Am J Respir Cell Mol Biol 2011, 44:276-284.
53. Pardo-Saganta A, Law BM, Gonzalez-Celeiro M, Vinarsky V, Rajagopal J: Ciliated cells of pseudostratified airway epithelium do not become mucous cells after ovalbumin challenge. Am J Respir Cell Mol Biol 2013, 48:364-373.
54. Jain R, Barkauskas CE, Takeda N, Bowie EJ, Aghajanian H, Wang Q, Padmanabhan A, Manderfield LJ, Gupta M, Li D, et al: Plasticity of Hopx(+) type I alveolar cells to regenerate type II cells in the lung. Nat Commun 2015, 6:6727.
55. Kapere Ochieng J, Schilders K, Kool H, Buscop-van Kempen M, Boerema-De Munck A, Grosveld F, Wijnen R, Tibboel D, Rottier RJ: Differentiated Type II Pneumocytes Can Be Reprogrammed by Ectopic Sox2 Expression. PLoS One 2014, 9:e107248.
56. Yang J, Hernandez BJ, Martinez Alanis D, Narvaez Del Pilar O, Vila-Ellis L, Akiyama H, Evans SE, Ostrin EJ, Chen J: The development and plasticity of alveolar type 1 cells. Development 2016, 143:54-65.
57. Park KS, Korfhagen TR, Bruno MD, Kitzmiller JA, Wan H, Wert SE, Khurana Hershey GK, Chen G, Whitsett JA: SPDEF regulates goblet cell hyperplasia in the airway epithelium. J Clin Invest 2007, 117:978-988.
58. Peng T, Frank DB, Kadzik RS, Morley MP, Rathi KS, Wang T, Zhou S, Cheng L, Lu MM, Morrisey EE: Hedgehog actively maintains adult lung quiescence and regulates repair and regeneration. Nature 2015, 526:578-582.
59. Kugler MC, Joyner AL, Loomis CA, Munger JS: Sonic hedgehog signaling in the lung. From development to disease. Am J Respir Cell Mol Biol 2015, 52:1-13.
60. Khuri FR, Lee JS, Lippman SM, Lee JJ, Kalapurakal S, Yu R, Ro JY, Morice RC, Hong WK, Hittelman WN: Modulation of proliferating cell nuclear antigen in the bronchial epithelium of smokers. Cancer Epidemiol Biomarkers Prev 2001, 10:311-318.
61. Snitow ME, Li SR, Morley MP, Rathi K, Lu MM, Kadzik RS, Stewart KM, Morrisey EE: Ezh2 represses the basal cell lineage during lung endoderm development. Development 2015, 142:108-117.
62. Tilley AE, Harvey BG, Heguy A, Hackett NR, Wang R, O'Connor TP, Crystal RG: Down-regulation of the notch pathway in human airway epithelium in association with smoking and chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2009, 179:457-466.
63. Shi W, Chen F, Cardoso WV: Mechanisms of lung development: contribution to adult lung disease and relevance to chronic obstructive pulmonary disease. Proc Am Thorac Soc 2009, 6:558-563.
64. Verhamme FM, Bracke KR, Joos GF, Brusselle GG: Transforming Growth Factor-beta

Superfamily in Obstructive Lung Diseases. American Journal of Respiratory Cell and Molecular Biology 2015, 52:653-662.
65. Martin C, Frija-Masson J, Burgel PR: Targeting mucus hypersecretion: new therapeutic opportunities for COPD? Drugs 2014, 74:1073-1089.
66. Lafkas D, Shelton A, Chiu C, de Leon Boenig G, Chen Y, Stawicki SS, Siltanen C, Reichelt M, Zhou M, Wu X, et al: Therapeutic antibodies reveal Notch control of transdifferentiation in the adult lung. Nature 2015, 528:127-131.
67. El Agha E, Kosanovic D, Schermuly RT, Bellusci S: Role of fibroblast growth factors in organ regeneration and repair. Semin Cell Dev Biol 2015.
68. Stoltz JF, de Isla N, Li YP, Bensoussan D, Zhang L, Huselstein C, Chen Y, Decot V, Magdalou J, Li N, et al: Stem Cells and Regenerative Medicine: Myth or Reality of the 21th Century. Stem Cells International 2015.
69. Quan Y, Wang D: Clinical potentials of human pluripotent stem cells in lung diseases. Clin Transl Med 2014, 3:15.
70. Ghaedi M, Niklason LE, Williams J: Development of Lung Epithelium from Induced Pluripotent Stem Cells. Curr Transplant Rep 2015, 2:81-89.
71. Takahashi K, Yamanaka S: Induction of pluripotent stem cells from mouse embryonic nd adult fibroblast cultures by defined factors. Cell 2006, 126:663-676.
72. Singh VK, Kumar N, Kalsan M, Saini A, Chandra R: Mechanism of Induction: Induced Pluripotent Stem Cells (iPSCs). J Stem Cells 2015, 10:43-62.
73. Green MD, Chen A, Nostro MC, d'Souza SL, Schaniel C, Lemischka IR, Gouon-Evans V, Keller G, Snoeck HW: Generation of anterior foregut endoderm from human embryonic and induced pluripotent stem cells. Nature Biotechnology 2011, 29:267-U153.
74. Ali NN, Edgar AJ, Samadikuchaksaraei A, Timson CM, Romanska HM, Polak JM, Bishop AE: Derivation of type II alveolar epithelial cells from murine embryonic stem cells. Tissue Eng 2002, 8:541-550.
75. Van Haute L, De Block G, Liebaers I, Sermon K, De Rycke M: Generation of lung epithelial-like tissue from human embryonic stem cells. Respir Res 2009, 10:105.
76. Banerjee ER, Laflamme MA, Papayannopoulou T, Kahn M, Murry CE, Henderson WR, Jr.: Human embryonic stem cells differentiated to lung lineage-specific cells ameliorate pulmonary fibrosis in a xenograft transplant mouse model. PLoS One 2012, 7:e33165.
77. Samadikuchaksaraei A, Cohen S, Isaac K, Rippon HJ, Polak JM, Bielby RC, Bishop AE: Derivation of distal airway epithelium from human embryonic stem cells. Tissue Eng 2006, 12:867-875.
78. Wang D, Haviland DL, Burns AR, Zsigmond E, Wetsel RA: A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2007, 104:4449-4454.
79. Rippon HJ, Polak JM, Qin M, Bishop AE: Derivation of distal lung epithelial progenitors from murine embryonic stem cells using a novel three-step differentiation protocol. Stem Cells 2006, 24:1389-1398.
80. Firth AL, Dargitz CT, Qualls SJ, Menon T, Wright R, Singer O, Gage FH, Khanna A, Verma IM: Generation of multiciliated cells in functional airway epithelia from human induced pluripotent stem cells. Proc Natl Acad Sci U S A 2014, 111:E1723-1730.
81. Wong AP, Bear CE, Chin S, Pasceri P, Thompson TO, Huan LJ, Ratjen F, Ellis J, Rossant J: Directed differentiation of human pluripotent stem cells into mature airway epithelia expressing functional CFTR protein. Nat Biotechnol 2012, 30:876-882.
82. Mou H, Zhao R, Sherwood R, Ahfeldt T, Lapey A, Wain J, Sicilian L, Izvolsky K, Musunuru K, Cowan C, Rajagopal J: Generation of multipotent lung and airway progenitors from mouse ESCs and patient-specific cystic fibrosis iPSCs. Cell Stem Cell 2012, 10:385-397.
83. Longmire TA, Ikonomou L, Hawkins F, Christodoulou C, Cao Y, Jean JC, Kwok LW, Mou H, Rajagopal J, Shen SS, et al: Efficient derivation of purified lung and thyroid progenitors from embryonic stem cells. Cell Stem Cell 2012, 10:398-411.
84. Ghaedi M, Calle EA, Mendez JJ, Gard AL, Balestrini J, Booth A, Bove PF, Gui L, White ES, Niklason LE: Human iPS cell-derived alveolar epithelium repopulates lung extracellular matrix. J Clin Invest 2013, 123:4950-4962.
85. Huang SX, Islam MN, O'Neill J, Hu Z, Yang YG, Chen YW, Mumau M, Green MD, Vunjak-Novakovic G, Bhattacharya J, Snoeck HW: Efficient generation of lung and airway epithelial cells from human pluripotent stem cells. Nat Biotechnol 2014, 32:84-91.
86. Huang SXL, Green MD, de Carvalho AT, Mumau M, Chen YW, D'Souza SL, Snoeck HW: The in vitro generation of lung and airway progenitor cells from human pluripotent stem cells. Nature Protocols 2015, 10.
87. Martin U: Pluripotent stem cells for disease modeling and drug screening: new perspectives for treatment of cystic fibrosis? Mol Cell Pediatr 2015, 2:15.
88. Weiss DJ: Concise review: current status of stem cells and regenerative medicine in lung

biology and diseases. Stem Cells 2014, 32:16-25.
89. D'souza N, Rossignoli F, Golinelli G, Grisendi G, Spano C, Candini O, Osturu S, Catani F, Paolucci P, Horwitz EM, Dominici M: Mesenchymal stem/stromal cells as a delivery platform in cell and gene therapies. Bmc Medicine 2015, 13.
90. Neofytou E, Deuse T, Beygui RE, Schrepfer S: Mesenchymal stromal cell therapy: different sources exhibit different immunobiological properties. Transplantation 2015, 99:1113-1118.
91. Mobius MA, Thebaud B: Stem Cells and Their Mediators - Next Generation Therapy for Bronchopulmonary Dysplasia. Front Med (Lausanne) 2015, 2:50.
92. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E: Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006, 8:315-317.
93. Sueblinvong V, Loi R, Eisenhauer PL, Bernstein IM, Suratt BT, Spees JL, Weiss DJ: Derivation of lung epithelium from human cord blood-derived mesenchymal stem cells. Am J Respir Crit Care Med 2008, 177:701-711.
94. Martin J, Helm K, Ruegg P, Varella-Garcia M, Burnham E, Majka S: Adult lung side population cells have mesenchymal stem cell potential. Cytotherapy 2008, 10:140-151.
95. Gong X, Sun Z, Cui D, Xu X, Zhu H, Wang L, Qian W, Han X: Isolation and characterization of lung resident mesenchymal stem cells capable of differentiating into alveolar epithelial type II cells. Cell Biol Int 2012.
96. Stabler CT, Lecht S, Lazarovici P, Lelkes PI: Mesenchymal stem cells for therapeutic applications in pulmonary medicine. British Medical Bulletin 2015, 115:45-56.
97. Weiss DJ, Casaburi R, Flannery R, LeRoux-Williams M, Tashkin DP: A Placebo-Controlled, Randomized Trial of Mesenchymal Stem Cells in COPD. Chest 2013, 143:1590-1598.
98. Petrella F, Rizzo S, Borri A, Casiraghi M, Spaggiari L: Current Perspectives in Mesenchymal Stromal Cell Therapies for Airway Tissue Defects. Stem Cells International 2015.
99. Rosen C, Shezen E, Aronovich A, Klionsky YZ, Yaakov Y, Assayag M, Biton IE, Tal O, Shakhar G, Ben-Hur H, et al: Preconditioning allows engraftment of mouse and human embryonic lung cells, enabling lung repair in mice. Nat Med 2015, 21:869-879.
100. Li Q, Wang Y, Deng Z: Pre-conditioned mesenchymal stem cells: a better way for cell-based therapy. Stem Cell Res Ther 2013, 4:63.
101. Weiss DJ, Chambers D, Giangreco A, Keating A, Kotton D, Lelkes PI, Wagner DE, Prockop DJ, Cells ATSSoS, Cell T: An official American Thoracic Society workshop report: stem cells and cell therapies in lung biology and diseases. Ann Am Thorac Soc 2015, 12:S79-97.
102. Zhao YD, Courtman DW, Deng Y, Kugathasan L, Zhang Q, Stewart DJ: Rescue of monocrotaline-induced pulmonary arterial hypertension using bone marrow-derived endothelial-like progenitor cells: efficacy of combined cell and eNOS gene therapy in established disease. Circ Res 2005, 96:442-450.
103. Lam CF, Roan JN, Lee CH, Chang PJ, Huang CC, Liu YC, Jiang MJ, Tsai YC: Transplantation of endothelial progenitor cells improves pulmonary endothelial function and gas exchange in rabbits with endotoxin-induced acute lung injury. Anesth Analg 2011, 112:620-627.
104. Balasubramaniam V, Ryan SL, Seedorf GJ, Roth EV, Heumann TR, Yoder MC, Ingram DA, Hogan CJ, Markham NE, Abman SH: Bone marrow-derived angiogenic cells restore lung alveolar and vascular structure after neonatal hyperoxia in infant mice. Am J Physiol Lung Cell Mol Physiol 2010, 298:L315-323.
105. Woik N, Kroll J: Regulation of lung development and regeneration by the vascular system. Cell Mol Life Sci 2015, 72:2709-2718.
106. Ding BS, Nolan DJ, Guo P, Babazadeh AO, Cao Z, Rosenwaks Z, Crystal RG, Simons M, Sato TN, Worgall S, et al: Endothelial-derived angiocrine signals induce and sustain regenerative lung alveolarization. Cell 2011, 147:539-553.
107. Rafii S, Cao Z, Lis R, Siempos, II, Chavez D, Shido K, Rabbany SY, Ding BS: Platelet-derived SDF-1 primes the pulmonary capillary vascular niche to drive lung alveolar regeneration. Nat Cell Biol 2015, 17:123-136.
108. Wang XX, Zhang FR, Shang YP, Zhu JH, Xie XD, Tao QM, Zhu JH, Chen JZ: Transplantation of autologous endothelial progenitor cells may be beneficial in patients with idiopathic pulmonary arterial hypertension: a pilot randomized controlled trial. J Am Coll Cardiol 2007, 49:1566-1571.
109. Zhu JH, Wang XX, Zhang FR, Shang YP, Tao QM, Zhu JH, Chen JZ: Safety and efficacy of autologous endothelial progenitor cells transplantation in children with idiopathic pulmonary arterial hypertension: open-label pilot study. Pediatr Transplant 2008, 12:650-655.
110. Huh D, Hamilton GA, Ingber DE: From 3D cell culture to organs-on-chips. Trends in Cell

Biology 2011, 21:745-754.
111. Whitcutt MJ, Adler KB, Wu R: A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. In Vitro Cell Dev Biol 1988, 24:420-428.
112. Randell SH, Fulcher ML, O'Neal W, Olsen JC: Primary epithelial cell models for cystic fibrosis research. Methods Mol Biol 2011, 742:285-310.
113. Wark PA, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, Holgate ST, Davies DE: Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. J Exp Med 2005, 201:937-947.
114. Herr C, Beisswenger C, Hess C, Kandler K, Suttorp N, Welte T, Schroeder JM, Vogelmeier C, Bals R, Grp CS: Suppression of pulmonary innate host defence in smokers. Thorax 2009, 64:144-149.
115. Amatngalim GD, van Wijck Y, de Mooij-Eijk Y, Verhoosel RM, Harder J, Lekkerkerker AN, Janssen RA, Hiemstra PS: Basal cells contribute to innate immunity of the airway epithelium through production of the antimicrobial protein RNase 7. J Immunol 2015, 194:3340-3350.
116. Aufderheide M, Forster C, Beschay M, Branscheid D, Emura M: A new computer-controlled air-liquid interface cultivation system for the generation of differentiated cell cultures of the airway epithelium. Exp Toxicol Pathol 2015.
117. Ranga A, Gjorevski N, Lutolf MP: Drug discovery through stem cell-based organoid models. Advanced Drug Delivery Reviews 2014, 69:19-28.
118. Gjorevski N, Ranga A, Lutolf MP: Bioengineering approaches to guide stem cell-based organogenesis. Development 2014, 141:1794-1804.
119. Huch M, Koo BK: Modeling mouse and human development using organoid cultures. Development 2015, 142:3113-3125.
120. Yin X, Mead BE, Safaee H, Langer R, Karp JM, Levy O: Engineering Stem Cell Organoids. Cell Stem Cell 2016, 18:25-38.
121. Willyard C: The boom in mini stomachs, brains, breasts, kidneys and more. Nature 2015, 523:520-522.
122. Sato T, Clevers H: Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. Science 2013, 340:1190-1194.
123. Nadkarni RR, Abed S, Draper JS: Organoids as a model system for studying human lung development and disease. Biochem Biophys Res Commun 2015.
124. Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, Hogan BL: Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc Natl Acad Sci U S A 2009, 106:12771-12775.
125. Danahay H, Pessotti AD, Coote J, Montgomery BE, Xia D, Wilson A, Yang H, Wang Z, Bevan L, Thomas C, et al: Notch2 is required for inflammatory cytokine-driven goblet cell metaplasia in the lung. Cell Rep 2015, 10:239-252.
126. Hegab AE, Arai D, Gao J, Kuroda A, Yasuda H, Ishii M, Naoki K, Soejima K, Betsuyaku T: Mimicking the niche of lung epithelial stem cells and characterization of several effectors of their in vitro behavior. Stem Cell Res 2015, 15:109-121.
127. Lee JH, Bhang DH, Beede A, Huang TL, Stripp BR, Bloch KD, Wagers AJ, Tseng YH, Ryeom S, Kim CF: Lung stem cell differentiation in mice directed by endothelial cells via a BMP4-NFATc1-thrombospondin-1 axis. Cell 2014, 156:440-455.
128. Delgado O, Kaisani AA, Spinola M, Xie XJ, Batten KG, Minna JD, Wright WE, Shay JW: Multipotent capacity of immortalized human bronchial epithelial cells. PLoS One 2011, 6:e22023.
129. Franzdottir SR, Axelsson IT, Arason AJ, Baldursson O, Gudjonsson T, Magnusson MK: Airway branching morphogenesis in three dimensional culture. Respir Res 2010, 11:162.
130. Kaisani A, Delgado O, Fasciani G, Kim SB, Wright WE, Minna JD, Shay JW: Branching morphogenesis of immortalized human bronchial epithelial cells in three-dimensional culture. Differentiation 2014, 87:119-126.
131. Gotoh S, Ito I, Nagasaki T, Yamamoto Y, Konishi S, Korogi Y, Matsumoto H, Muro S, Hirai T, Funato M, et al: Generation of alveolar epithelial spheroids via isolated progenitor cells from human pluripotent stem cells. Stem Cell Reports 2014, 3:394-403.
132. Dye BR, Hill DR, Ferguson MA, Tsai YH, Nagy MS, Dyal R, Wells JM, Mayhew CN, Nattiv R, Klein OD, et al: In vitro generation of human pluripotent stem cell derived lung organoids. Elife 2015, 4.
133. Dye BR, Hill DR, Ferguson MAH, Tsai YH, Nagy MS, Dyal R, Wells JM, Mayhew CN, Nattiv R, Klein OD, et al: In vitro generation of human pluripotent stem cell derived lung organoids. Elife 2015, 4.
134. Kniazeva T, Hsiao JC, Charest JL, Borenstein JT: A microfluidic respiratory assist device with high gas permeance for artificial lung applications. Biomed Microdevices 2011, 13:315-323.
135. Bhatia SN, Ingber DE: Microfluidic organs-on-

chips. Nat Biotechnol 2014, 32:760-772.
136. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE: Reconstituting Organ-Level Lung Functions on a Chip. Science 2010, 328:1662-1668.
137. Huh D, Leslie DC, Matthews BD, Fraser JP, Jurek S, Hamilton GA, Thorneloe KS, McAlexander MA, Ingber DE: A Human Disease Model of Drug Toxicity-Induced Pulmonary Edema in a Lung-on-a-Chip Microdevice. Science Translational Medicine 2012, 4.
138. Fukumoto J, Kolliputi N: Human lung on a chip: innovative approach for understanding disease processes and effective drug testing. Front Pharmacol 2012, 3:205.
139. Stucki AO, Stucki JD, Hall SRR, Felder M, Mermoud Y, Schmid RA, Geiser T, Guenat OT: A lung-on-a-chip array with an integrated bio-inspired respiration mechanism. Lab on a Chip 2015, 15:1302-1310.
140. Blume C, Reale R, Held M, Millar TM, Collins JE, Davies DE, Morgan H, Swindle EJ: Temporal Monitoring of Differentiated Human Airway Epithelial Cells Using Microfluidics. Plos One 2015, 10.
141. Murphy SV, Atala A: 3D bioprinting of tissues and organs. Nature Biotechnology 2014, 32:773-785.
142. Patel M, Fisher JP: Biomaterial scaffolds in pediatric tissue engineering. Pediatr Res 2008, 63:497-501.
143. Li Q, Uygun BE, Geerts S, Ozer S, Scalf M, Gilpin SE, Ott HC, Yarmush ML, Smith LM, Welham NV, Frey BL: Proteomic Analysis of Naturally-Sourced Biological Scaffolds. Biomaterials 2015.
144. Atala A, Bauer SB, Soker S, Yoo JJ, Retik AB: Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet 2006, 367:1241-1246.
145. Pham C, Greenwood J, Cleland H, Woodruff P, Maddern G: Bioengineered skin substitutes for the management of burns: a systematic review. Burns 2007, 33:946-957.
146. Zopf DA, Hollister SJ, Nelson ME, Ohye RG, Green GE: Bioresorbable airway splint created with a three-dimensional printer. N Engl J Med 2013, 368:2043-2045.
147. Mondrinos MJ, Jones PL, Finck CM, Lelkes PI: Engineering De Novo Assembly of Fetal Pulmonary Organoids. Tissue Engineering Part A 2014, 20:2892-2907.
148. Ranga A, Lutolf MP: High-throughput approaches for the analysis of extrinsic regulators of stem cell fate. Curr Opin Cell Biol 2012, 24:236-244.
149. Park JH, Park JY, Nam IC, Hwang SH, Kim CS, Jung JW, Jang J, Lee H, Choi Y, Park SH, et al: Human turbinate mesenchymal stromal cell sheets with bellows graft for rapid tracheal epithelial regeneration. Acta Biomaterialia 2015, 25:56-64.
150. Kobayashi K, Nomoto Y, Suzuki T, Tada Y, Miyake M, Hazama A, Kanemaru S, Nakamura T, Omori K: Effect of fibroblasts on tracheal epithelial regeneration in vitro. Tissue Eng 2006, 12:2619-2628.
151. Kobayashi K, Suzuki T, Nomoto Y, Tada Y, Miyake M, Hazama A, Wada I, Nakamura T, Omori K: A tissue-engineered trachea derived from a framed collagen scaffold, gingival fibroblasts and adipose-derived stem cells. Biomaterials 2010, 31:4855-4863.
152. Okano W, Nomoto Y, Wada I, Kobayashi K, Miyake M, Nakamura T, Omori K: Bioengineered trachea with fibroblasts in a rabbit model. Ann Otol Rhinol Laryngol 2009, 118:796-804.
153. Yang J, Yamato M, Shimizu T, Sekine H, Ohashi K, Kanzaki M, Ohki T, Nishida K, Okano T: Reconstruction of functional tissues with cell sheet engineering. Biomaterials 2007, 28:5033-5043.
154. Kanzaki M, Yamato M, Hatakeyama H, Kohno C, Yang J, Umemoto T, Kikuchi A, Okano T, Onuki T: Tissue engineered epithelial cell sheets for the creation of a bioartificial trachea. Tissue Eng 2006, 12:1275-1283.
155. Macchiarini P, Jungebluth P, Go T, Asnaghi MA, Rees LE, Cogan TA, Dodson A, Martorell J, Bellini S, Parnigotto PP, et al: Clinical transplantation of a tissue-engineered airway. Lancet 2008, 372:2023-2030.
156. Steinke M, Dally I, Friedel G, Walles H, Walles T: Host-Integration of a Tissue-Engineered Airway Patch: Two-Year Follow-Up in a Single Patient. Tissue Engineering Part A 2015, 21:573-579.
157. Aad G, Abbott B, Abdallah J, Abdel Khalek S, Abdinov O, Aben R, Abi B, Abolins M, AbouZeid OS, Abramowicz H, et al: Search for Higgs boson pair production in the gammagammabb[over] final state using pp collision data at sqrt[s]=8 TeV from the ATLAS detector. Phys Rev Lett 2015, 114:081802.
158. da Palma RK, Campillo N, Uriarte JJ, Oliveira LVF, Navajas D, Farre R: Pressure- and flow-controlled media perfusion differently modify vascular mechanics in lung decellularization. Journal of the Mechanical Behavior of Biomedical Materials 2015, 49:69-79.
159. Orlova VV, van den Hil FE, Petrus-Reurer S, Drabsch Y, Ten Dijke P, Mummery CL: Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. Nat Protoc 2014, 9:1514-1531.

160. Ott HC, Clippinger B, Conrad C, Schuetz C, Pomerantseva I, Ikonomou L, Kotton D, Vacanti JP: Regeneration and orthotopic transplantation of a bioartificial lung. Nat Med 2010, 16:927-933.
161. Petersen TH, Calle EA, Colehour MB, Niklason LE: Bioreactor for the long-term culture of lung tissue. Cell Transplant 2011, 20:1117-1126.
162. Price AP, England KA, Matson AM, Blazar BR, Panoskaltsis-Mortari A: Development of a decellularized lung bioreactor system for bioengineering the lung: the matrix reloaded. Tissue Eng Part A 2010, 16:2581-2591.
163. Wallis JM, Borg ZD, Daly AB, Deng B, Ballif BA, Allen GB, Jaworski DM, Weiss DJ: Comparative assessment of detergent-based protocols for mouse lung de-cellularization and re-cellularization. Tissue Eng Part C Methods 2012, 18:420-432.
164. Booth AJ, Hadley R, Cornett AM, Dreffs AA, Matthes SA, Tsui JL, Weiss K, Horowitz JC, Fiore VF, Barker TH, et al: Acellular normal and fibrotic human lung matrices as a culture system for in vitro investigation. Am J Respir Crit Care Med 2012, 186:866-876.
165. Nichols JE, Niles J, Riddle M, Vargas G, Schilagard T, Ma L, Edward K, La Francesca S, Sakamoto J, Vega S, et al: Production and assessment of decellularized pig and human lung scaffolds. Tissue Eng Part A 2013, 19:2045-2062.
166. Wagner DE, Bonenfant NR, Sokocevic D, DeSarno MJ, Borg ZD, Parsons CS, Brooks EM, Platz JJ, Khalpey ZI, Hoganson DM, et al: Three-dimensional scaffolds of acellular human and porcine lungs for high throughput studies of lung disease and regeneration. Biomaterials 2014, 35:2664-2679.
167. Nichols JE, Niles JA, Vega SP, Argueta LB, Eastaway A, Cortiella J: Modeling the lung: Design and development of tissue engineered macro- and micro-physiologic lung models for research use. Experimental Biology and Medicine 2014, 239:1135-1169.
168. Song H, Yao E, Lin C, Gacayan R, Chen MH, Chuang PT: Functional characterization of pulmonary neuroendocrine cells in lung development, injury, and tumorigenesis. Proc Natl Acad Sci U S A 2012, 109:17531-17536.
169. Chen G, Korfhagen TR, Xu Y, Kitzmiller J, Wert SE, Maeda Y, Gregorieff A, Clevers H, Whitsett JA: SPDEF is required for mouse pulmonary goblet cell differentiation and regulates a network of genes associated with mucus production. J Clin Invest 2009, 119:2914-2924.

Chapter 2

A novel method for expansion and differentiation of mouse tracheal epithelial cells in culture

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ABSTRACT

Air-liquid interface (ALI) cultures of mouse tracheal epithelial cells (MTEC) are a well-established model to study airway epithelial cells, but current methods require large numbers of animals which is unwanted in view of the 3R principle and introduces variation. Moreover, stringent breeding schemes are frequently needed to generate sufficient numbers of genetically modified animals. Current protocols do not incorporate expansion of MTEC, and therefore we developed a protocol to expand MTEC while maintaining their differentiation capacity. MTEC were isolated and expanded using the ROCK inhibitor Y-27632 in presence or absence of the γ-secretase inhibitor DAPT, a Notch pathway inhibitor. Whereas MTEC proliferated without DAPT, growth rate and cell morphology improved in presence of DAPT. ALI-induced differentiation of expanded MTEC resulted in an altered capacity of basal cells to differentiate into ciliated cells, whereas IL-13-induced goblet cell differentiation remained unaffected. Ciliated cell differentiation improved by prolonging the ALI differentiation or by adding DAPT, suggesting that basal cells retain their ability to differentiate. This technique using expansion of MTEC and subsequent ALI differentiation drastically reduces animal numbers and costs for *in vitro* experiments, and will reduce biological variation. Additionally, we provide novel insights in the dynamics of basal cell populations *in vitro*.

INTRODUCTION

Airway epithelial cells play a pivotal role in protecting the lung by acting both as a mechanical and an immunological barrier. The epithelial cells of the upper respiratory tract form a pseudostratified epithelial layer consisting of several epithelial cell types that constitute an efficient host defense system that employs a variety of mechanisms, including its ability to clear inhaled particles and pathogens from the lung using mucociliary clearance. Efficient mucociliary clearance depends on a proper balance between basal, ciliated and secretory cells. The relative distribution of these cell types in the epithelial layer varies depending on the anatomic location within the conducting airways. Altered composition of these epithelial cell types has been implied in several chronic lung diseases, including asthma and chronic obstructive pulmonary disorder (COPD) [1-3].

Air-liquid interface (ALI) cultures of human primary airway epithelial cells (AEC) are a well-established *in vitro* model to investigate the role of airway epithelial cells in chronic lung diseases [4,5]. Primary AEC are isolated from bronchial biopsies, brushes or resected lung tissue, and can either be cultured directly onto transwell inserts or the cells can first be expanded *in vitro* for subsequent experimental use. AEC freshly isolated from lung tissue consist of multiple cell types, but during *in vitro* culture under submerged conditions the main population that will expand is the basal cells, the epithelial progenitor population [6,7]. Following *in vitro* expansion, primary AEC can be cultured on transwell inserts to establish ALI cultures. To this end, once the cultures have reached full confluence the apical medium is removed to induce an ALI that allows AEC to differentiate into a pseudostratified epithelial layer containing basal, ciliated and secretory cells [8]. Culturing primary airway epithelial cells at ALI provides a platform to investigate not only fully differentiated epithelial layers, but also the mechanisms of differentiation following airway epithelium damage and the dynamic processes of repair after injury [5]. Importantly, ALI cultures allow us to study the effect of airborne exposures on airway epithelial cells, e.g. whole cigarette smoke exposure [9].

In addition to primary human AEC, various research groups are using cultures of mouse tracheal epithelial cells (MTEC) [7]. These offer the opportunity to closely link *in vitro* and *in vivo* experiments, and make use of the large variety of transgenic mouse lines available. However, it is difficult to maintain MTEC in a proliferative state after isolation, and therefore MTEC are cultured directly onto transwell inserts without prior *in vitro* expansion. As a result, large animal numbers are needed to obtain adequate cell numbers for *in vitro* experiments. Therefore, novel methods are required to subculture MTEC in order to achieve a drastic reduction in animal numbers needed for experiments.

Expanding the progenitor cell population is essential to subculture MTEC. Basal epithelial cells are considered as the progenitor cell type for the maintenance of a

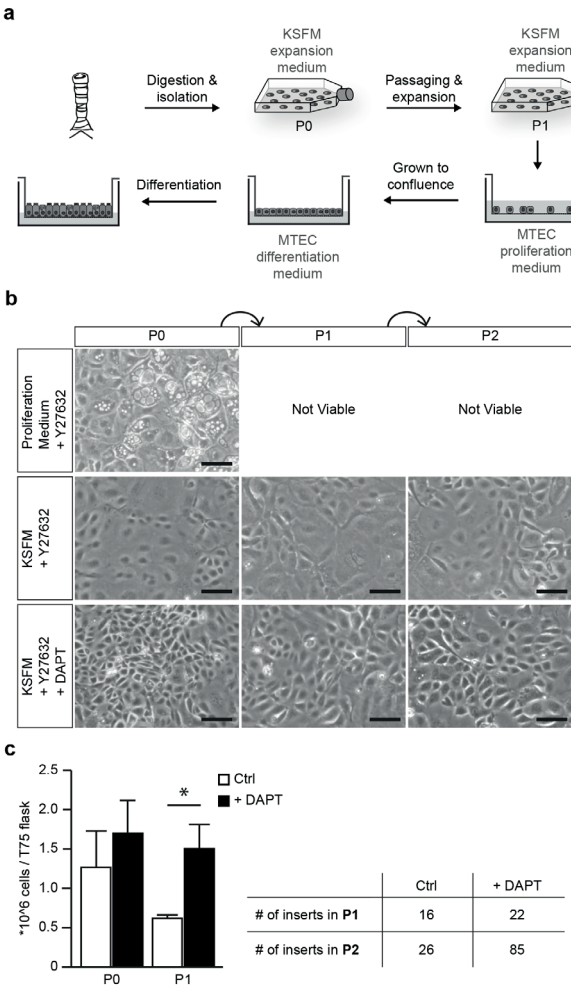
pseudostratified airway epithelium of the upper respiratory tract [6]. The mechanisms that control progenitor cell renewal and differentiation to maintain the airway epithelium are still being uncovered, mostly owing to the complex cell-cell interactions and subsequent signaling involved in the decision making towards a specific cell fate. Notch signaling has been implied in the regulation of basal cell self-renewal and differentiation towards the specialized cell types of the epithelial layer. Importantly, inhibition of Notch signaling has been shown to allow expansion of the basal cell population [10-12].

To investigate the possibility of expanding MTEC while retaining the ability to differentiate, we have developed an alternative culture method that will lead to a drastic reduction in animal numbers needed for *in vitro* experiments. Moreover, subculturing MTEC would allow for increased numbers of *in vitro* experiments without using additional difficult-to-breed transgenic mice. To this end, we have used a combination of Notch signaling inhibition together with adaptation of existing cell culture methods to explore the possibility of subculturing MTEC and subsequent ALI differentiation. Additionally, we also investigated the effect of passaging MTEC on the basal cell type population as these cells are essential for subsequent differentiation into a pseudostratified epithelial layer consisting of multiple epithelial cell types.

RESULTS

Serum free medium and the inhibition of Notch signaling enables basal cell expansion

Although the current methods of ALI culture represent the pseudostratified airway epithelium *in vitro*, a limitation of this technique is the relatively low number of cells obtained from a trachea. We first validated the existing culturing method of MTEC whereby isolated MTEC are cultured directly onto transwell inserts (Fig. S1). This culture method allowed us to plate approximately 4 to 5 transwell inserts (50,000 cells per insert) using two mice (±100,000 cells per trachea). Improving the expansion of MTEC will allow for multiple experiments to be performed with fewer mice. Ideally, following isolation from the trachea, cells can be expanded in culture for at least two passages to generate sufficient cells for multiple experiments (Fig. 1a). In previous studies, MTEC are isolated and plated on inserts in proliferation medium and upon reaching confluency, cultured at ALI in differentiation medium (Table S1) [13-14]. Following expansion in proliferation medium, MTEC showed a limited proliferation capacity, with the presence of swollen cells with enlarged vacuoles (Fig. 1b). We were unable to successfully passage MTEC grown in proliferation medium. Because we considered the possibility that proliferation medium may be deficient in factors preventing senescence, we switched to another medium. Keratinocyte Serum Free medium supplemented with epithelial growth factor (EGF), Bovine pituitary extract (BPE) and isoproterenol (KSFM) allows for expansion of human airway epithelial



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# of inserts in **P1**	16	22
# of inserts in **P2**	26	85

Fig. 1. Expanding MTEC in KSFM expansion medium with Notch signaling inhibitor. *(a) MTEC are expanded to passage 1 in KSFM expansion medium followed by passaging and culturing on transwell inserts. MTEC are grown to full confluence in MTEC proliferation medium, followed by air-liquid interface differentiation in MTEC differentiation medium. (b) Serial expansion of MTEC in different medium conditions. Epithelial cells are cultured in proliferation medium with ROCK inhibitor (Y27632), in KSFM medium with ROCK inhibitor or KSFM medium with ROCK inhibitor (Y27632) and γ-secretase inhibitor (DAPT). Phase-contrast images of the MTEC at various passages showing the morphology of the cells. Scale bar, 100μm. (c) The graph represents the number of cells obtained after passaging when cultured in KSFM with ROCK inhibitor alone or in the presence of DAPT (mean ± SEM). * $p < 0.05$ by unpaired t-test (n=5). The table shows the number of 12-well inserts that can be obtained after each passage.*

cells *in vitro* [15,16]. Additionally, adding a selective inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK), Y-27632, to the culture medium has been shown to increase proliferation of airway epithelial cells [17]. Growing MTEC in KSFM with Y-27632 indeed resulted in improved cellular expansion, survival and morphology (Fig. 1b). However, despite improved cell morphology and survival, the expansion rate was very slow and still a large number of enlarged cells were present. Notch signaling has been shown to play a pivotal role in preserving the basal cell population and in subsequent differentiation into specialized cell types. Furthermore, inhibition of Notch signaling has been shown to lead to an increased number of basal cells in mouse ALI cultures [10-12,18,19]. To evaluate whether inhibition of Notch signaling promotes the expansion of MTEC after isolation, we added DAPT, a γ-secretase inhibitor and indirect Notch signaling inhibitor, to the KSFM medium containing Y-27632. Growing MTEC in the presence of DAPT and Y-27632 in KSFM medium resulted in a marked increase in proliferation rate with a slightly better

morphology compared to cells grown in absence of DAPT; these differences were already apparent after 5 days of culture of P0 cells, but even more after passaging whereby an increased number of transwell inserts could be obtained when DAPT was present during expansion (Fig. 1c). KSFM medium containing Y-27632 and DAPT will be further referred to as KSFM expansion medium.

Notably, expanding MTEC from two mice in KSFM expansion medium resulted in 42.5 million cells, sufficient for 85 transwell inserts when plated at a density of 8 x 10^4 cells/cm^2, which is in stark contrast to the 200,000 cells required for 4-5 transwell inserts that are obtained if MTEC were isolated and cultured directly onto the transwell inserts. Also, no contaminating fibroblasts were observed during MTEC expansion. Taken together, we conclude that MTEC expansion is more efficient in the presence of the Notch signaling inhibitor DAPT than without.

Subcultured MTEC retain the ability to differentiate and develop a pseudostratified epithelial layer

Differentiation into a pseudostratified epithelial layer is essential when using MTEC cultures to mimic *in vivo* airway epithelium. To evaluate whether MTEC that were expanded in KSFM expansion medium retain the ability to form a pseudostratified epithelial layer *in vitro*, MTEC were isolated and cultured submerged in KSFM expansion medium. Subsequently, MTEC were cultured on inserts in MTEC proliferation medium until confluent, followed by differentiation at ALI in differentiation medium (Fig. 2a). Polarization of the pseudostratified epithelial layer in an apical and basal side was evaluated using keratin 8 (KRT8) and keratin 5 (KRT5), which mark the luminal and basal cell layer respectively. Cellular differentiation was analyzed by staining for specialized cell types, including ciliated cells using forkhead box 1 (FOXJ1) and beta-tubulin IV (TUBB4B), club cells using secretoglobin, family 1A, member 1 (SCGB1A1) and basal cells using transformation-related protein 63 (TRP63). Additionally, zona occludens 1 (ZO-1)-specific staining indicated epithelial tight junction formation, an important feature of epithelial cells to maintain a tight barrier function. After 8 days of differentiation at ALI we observed stratification of the epithelial cell layer, indicated by KRT5 expression in the basal cell layer and KRT8 expression in the luminal cell layer, similar to ALI-MTEC cultures of P0 (Fig. 2b and S1). Although most of the cells were single positive for either KRT5 or KRT8, some cells were KRT5/KRT8 double positive, indicating the presence of basal luminal precursors which are cells transitioning from a basal to a luminal cell phenotype [20]. Expanded MTEC developed tight junctions when cultured at ALI (Fig. 2b). Additionally, ciliated and secretory cells were detected after 8 days of differentiation at ALI (Fig. 2b). Epithelial polarization of expanded MTEC cultured at ALI was similar to the *in vivo* tracheal epithelium with TRP63 positive basal cells located at the basolateral side and the FOXJ1 positive ciliated cells at the apical side (Fig. 2b XZ plane, Fig. S1).

Taken together, these data show that expanded MTEC retain the ability to develop a pseudostratified epithelial layer, mimicking the *in vivo* pseudostratified epithelium.

We next compared the epithelial differentiation potential between P0, P1 and P2 MTEC by assessing ciliated cells and barrier development after 8 days ALI differentiation. Whereas the P2 ALI-MTEC were able to develop ciliated cells, the percentage of FOXJ1 positive cells was lower compared to passages P0 and P1 after 8 days of ALI differentiation (Figs 2d,e and S2). Barrier function was assessed using trans-epithelial resistance measurement, whereby the reduction in FOXJ1 positive

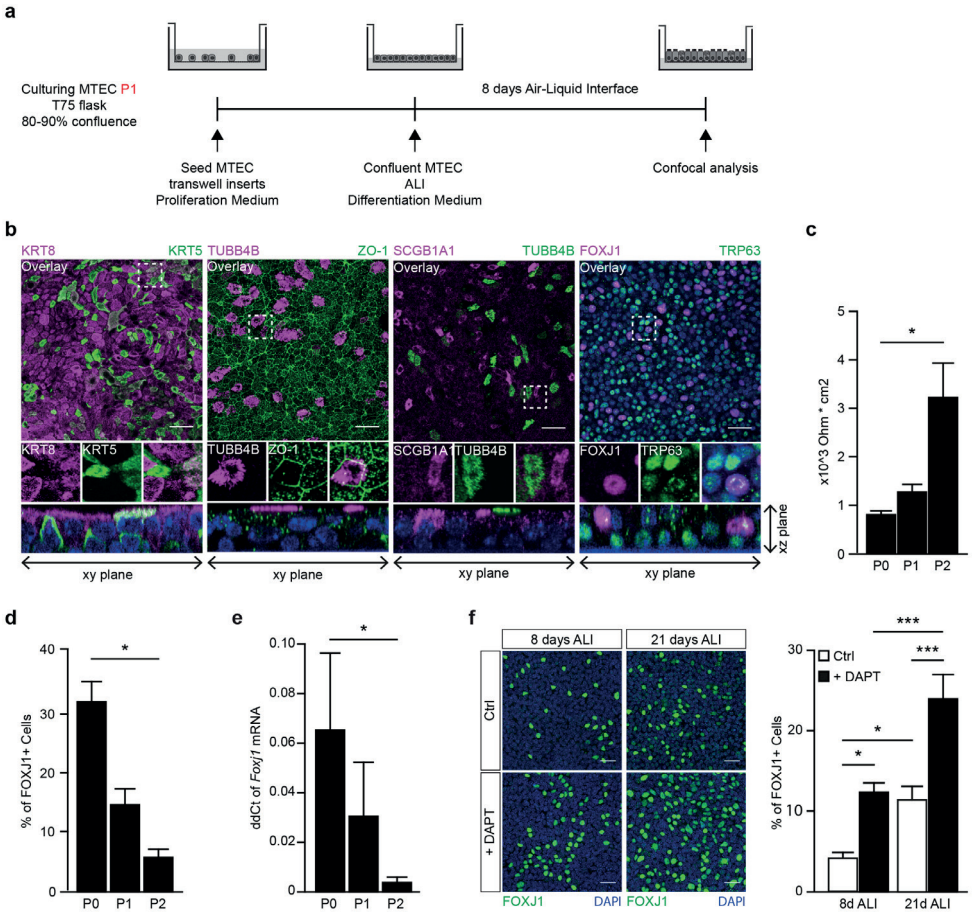

Fig. 2. Expansion of MTEC in vitro leads to decreased ciliary and club cell differentiation. *(a) Schematic representation of culture protocol. (b) Co-staining of different epithelial markers on MTEC passage 2 after 8 days of air liquid interface (ALI) culture. From left to right, inserts were stained with basal cell marker KRT5 and luminal marker KRT8, cilia cell TUBB4B with tight junction protein ZO-1, secretory club cell marker SCGB1A1 with cilia marker TUBB4B and the last panel shows TRP63 positive basal cells with ciliated cell marker FOXJ1. Nuclei are stained with DAPI (blue). Scale bar, 30 µm. (c) Resistance measurements of MTEC after different passages and 8 days of ALI (mean ± SEM). * p < 0.05 by one-way ANOVA (n=3). (d) Quantification of the percentage of FOXJ1 positive ciliated cells after 8 days of ALI comparing MTEC in passage (P) 0, 1 and 2 (mean ± SEM). * p < 0.05 by one-way ANOVA (n=3). (e) qRT-PCR analysis of Foxj1 in MTEC in passage (P) 0, 1 and 2 (mean± SEM). * p < 0.05 by one-way ANOVA (n=4). (f) Representative images and quantification of ciliated cells (FOXJ1) after 8 days or 21 days ALI and with or without the presence of DAPT during differentiation (mean ± SEM). * p < 0.05, *** P < 0.001 by two-way ANOVA (n=6). Scale bar, 30 µm.*

cells was accompanied by a significant increase in barrier development between P0 and P2 ALI MTEC (Fig. 2c-e). In line with our immunofluorescence imaging, we observed a significant decrease in *Foxj1* mRNA expression between P0 and P2 ALI MTEC (Fig. 2e). In addition to a decrease in FOXJ1 positive cells, we also observed a decrease in club cells as indicated by decrease in SCGB1A1 and SCGB3A2 positive cells between P0 and P2 ALI MTEC as detected by immunofluorescence staining and mRNA expression (Fig. S2c and d). Next we investigated whether the passaged MTEC could be treated in such a way that they develop ciliated cells in P2 ALI MTEC. Previously, it was shown that the presence of DAPT during differentiation increases the development of ciliated cells [11,21]. To this end, we differentiated expanded MTEC in the presence of DAPT during ALI. Aside from the expected decrease of club cells [18], we found an increased number of FOXJ1 positive ciliated cells compared to MTEC that were differentiated at ALI without DAPT Moreover, increasing the duration of ALI differentiation also increased the number of FOXJ1 positive cells (Fig. 2f). Overall, expanded MTEC cultured at ALI retain their ability to develop a pseudostratified epithelial layer. The differentiation of expanded MTEC appeared to be altered as indicated by a decrease in ciliated cells compared to P0. However, addition of DAPT during differentiation and increasing ALI duration increases the number of ciliated cells comparable to the numbers as observed in P0.

MTEC differentiation can be modulated using IL-13 to induce goblet cell metaplasia

Expanded MTEC cultured at ALI retained the ability to develop a pseudostratified epithelial layer. Next, we evaluated whether MTEC differentiation could be modulated using IL-13, which is important in the development of goblet cell metaplasia in mouse models of allergic airway inflammation [22,23]. We stained trachea of mice that were sensitized and exposed to house dust mite (HDM) allergen to induce allergic airways inflammation for the presence of goblet cells, and found these to be increased compared to placebo-treated mice (Fig. 3a). Previously, IL-13 has been shown to induce goblet cell metaplasia *in vitro* using ALI cultures of airway epithelial cells [15,24-26]. We exposed non-expanded (P0) and expanded (P1, P2) MTEC with DAPT to IL-13 during ALI culture and compared IL-13-induced MUC5AC expression patterns. Goblet cells are scarce in MTEC after 8 days of differentiation (Fig. 3c). However, we found that IL-13-exposed ALI MTEC cultures displayed increased numbers of goblet cells and there was no difference in the increase in goblet cells between P0 and P2 (Figs 3c and S3). Furthermore, IL-13 exposure resulted in a reduced number of ciliated cells in both ALI cultures with freshly isolated MTEC or with expanded MTEC (Fig. 3e). Finally, IL-13 decreases epithelial barrier function *in vitro* [27,28], which is in line with our results showing a similar reduction in barrier function following IL-13 exposure in expanded MTEC and isolated MTEC cultured directly onto inserts (Fig. 3d). Taken together our results indicate that expanding

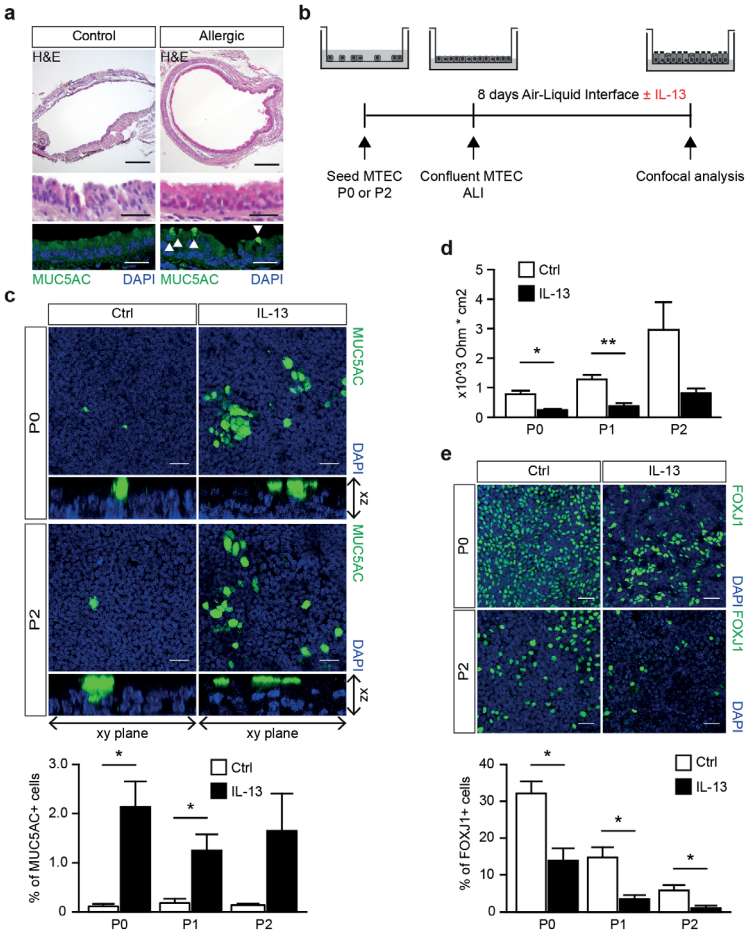

Fig. 3. IL-13 exposure during MTEC differentiation induces goblet cells. *(a) Hematoxylin and eosin on tracheal sections of control or house dust mite (HDM) treated mice. Immunofluorescence staining with Mucin 5AC (MUC5AC) shows the presence of goblet cells. MUC5AC expressing cells are indicated by the white arrows. Scale bar, 200 µm and 30 µm. (b) A schematic representation of the culture protocol. (c) Immunofluorescence staining of MUC5AC of MTEC passage 0 and 2 after 8 days of ALI culture with or without IL-13. Scale bar, 30 µM. Graph shows the percentage of MUC5AC expressing goblet cells in culture (mean ± SEM). * p < 0.05 by Student two-tailed t-test (n=3). (d) Resistance measurements after 8 days of ALI culture with or without IL-13 (5 ng/ml) treatment (mean ± SEM). * p < 0.05 by Student two-tailed t-test (n=3). Scale bar: 30 µm. (e) Immunofluorescence staining of FOXJ1 positive ciliated cells in a passage 0 (P0) and 2 (P2) after 8 days of ALI culture with or without IL-13 (5 ng/ml). Scale bar, 30 µm. Graph show the percentage of FOXJ1 expressing ciliated cells in culture (mean ± SEM). * p < 0.05 by Student two-tailed t-test (n=3).*

MTEC *in vitro* does not affect their ability to induce a physiologic response to IL-13 exposure, suggesting that this model could be an attractive alternative to evaluate new therapeutic compounds and their effects on airway epithelial cells *in vitro*.

Intrinsic changes of the basal cell population result in decreased differentiation potential

Expanding MTEC before ALI differentiation resulted in reduced numbers of ciliated cells, suggesting an altered differentiation potential of the basal cell population.

The basal cell population consists of various subtypes with different functionality that can be identified by the presence of different basal cell markers [5-7]. To evaluate whether the altered differentiation potential could be explained by a change in basal cell composition at baseline, we first examined the TRP63 positive basal cell population at day 0 of ALI differentiation for P0, P1 and P2 (Fig. 4a). No significant differences were detected in the percentage of TRP63 positive basal cells between P0, P1 and P2 (Fig. 4b). This indicates that a difference in the number of TRP63 positive basal cells is not the cause of an altered differentiation potential, suggesting that the identity of the TRP63 positive basal cell is changed. We therefore next evaluated the expression patterns of other previously published basal cell markers. KRT8 and KRT5 were already used to show stratification of the MTEC cultured at ALI (Fig. 2). As indicated, KRT5+KRT8 double positive cells mark a population of basal luminal precursor cells. Another marker used to distinguish basal cells is P75-nerve growth factor receptor (NGFR), which is enriched in murine tracheal basal cells [6]. We observed no overt differences in KRT5, KRT8 and NGFR positive basal cells in P2 compared with P0 (Figs 4c,d,e and S4).

A subpopulation of keratin 14 (KRT14) positive basal cells is reported to be present in a small subset of basal cells, but is highly increased after injury and serves as a progenitor for ciliated and secretory cells [29,30]. We investigated the presence of KRT14 positive basal cells in P0 and P2 MTEC on inserts at day 0 of ALI. The KRT14 positive basal cell fraction showed a marked increase in P2 compared to P0 (Fig. 4e and f). Overall, our data suggests that Notch signaling is involved in the maintenance of the basal cell population and that expanding MTEC during Notch signaling inhibition increases the KRT14 positive basal cell fraction without affecting the other investigated basal cell populations.

DISCUSSION

Using a combination of Notch signaling and Rho-associated kinase (ROCK) inhibition and specialized media, we were able to subculture MTEC while preserving the mucociliary differentiation potential when cultured at ALI. This protocol provides a much more efficient way to culture MTEC, increasing the number of experiments that can be performed with two mouse tracheas, with approximately 42.5 million cells after passaging, which is in stark contrast to 200 000 cells if MTEC are plated on transwell inserts directly after isolation. Our culture method will contribute to a reduction in the number of animals used, in line with the 3R principle, and also has the potential to decrease technical and biological variation between experiments. Furthermore, a reduction in the number of animals needed is relevant when MTEC are isolated from difficult-to-breed mouse strains. To increase the efficient use of mouse trachea for MTEC culture, we have used KSFM medium to prevent outgrowthof fibroblasts (a common problem in MTEC cultures) supplemented with the ROCK

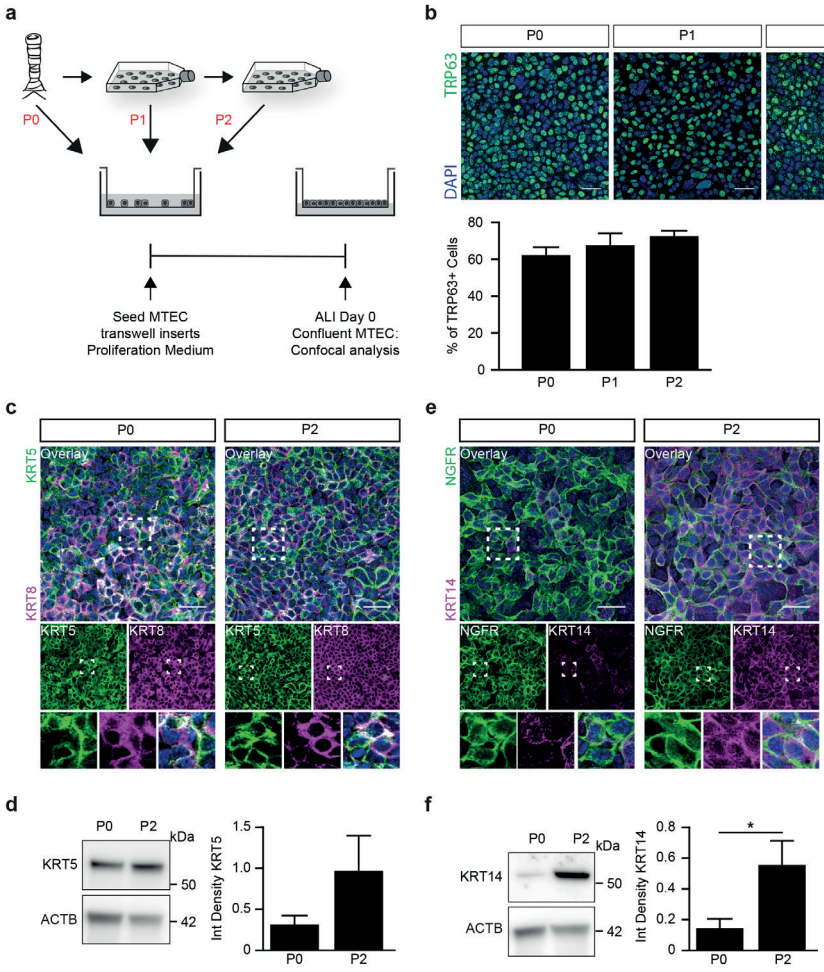

Fig. 4. The population of TRP63 positive basal cells change during expansion. *(a) Schematic representation of culture protocol. (b) Representative images of the number of TRP63 positive basal cells at ALI day 0. Scale bar, 30 µM. Graph shows the percentage of TRP63 positive basal cells at day 0 of ALI culture in a passage 0 (P0), passage 1 (P1) and passage 2 (P2) (mean ± SEM). One-way ANOVA (n=3). (c) Immunofluorescence of basal cell marker KRT5, and luminal cell maker KRT8 at day 0 of ALI. The overlay picture and the one-channel pictures are shown. The boxes indicate the area of which an enlarged image is presented. Scale bar: 30 µm. (d) Western blot analysis of KRT5 at day 0 of ALI in a passage (P) 0 and 2. Beta-actin (ACTB) is used as loading control. * p < 0.05 by Student two-tailed t-test (n=7) (e) Immunofluorescence of basal cell marker, P75-nerve growth factor (NGFR) and basal cell maker keratin14 (KRT14) at day 0 of ALI. Scale bar: 30 µm. (f) Western blot analysis of KRT14 at day 0 of ALI in a passage (P) 0 and 2. Beta-actin (ACTB) is used as loading control. * p < 0.05 by Student two-tailed t-test (n=9)*

inhibitor Y-27632 and the γ-secretase inhibitor and indirect Notch signaling inhibitor DAPT. Additionally, our results indicate that expanding MTEC *in vitro* results in altered stemness of the basal cell population which is accompanied by a shift of a KRT14 negative basal cell population observed at P0 towards a KRT14 positive basal cell population at P2. Further investigation is required to evaluate contribution of the KRT14 positive basal cell population towards the altered stemness of the basal cell fraction.

MTEC have previously been shown to have the ability to grow in submerged culture. However, the ability of the MTEC to differentiate at ALI was not explored [31]. More recently, SMAD signaling inhibition was shown to promote airway basal stem cell expansion *in vitro* from multiple species with subsequent ALI differentiation [32]. Although this study contributed significantly to the *in vitro* research field, the authors did not elaborate on cell culture techniques and growth media used. We used the SMAD signaling inhibitors described by Mou and colleagues in combination with KSFM medium, but observed that MTEC could not be expanded under these growth conditions (results not shown). In the present study, we have used KSFM to expand MTEC *in vitro* prior to culturing on transwell inserts. KSFM has previously been used successfully to expand primary human bronchial epithelial cells [15,16]. In addition, KSFM has the advantage of preventing outgrowth of fibroblasts, which is likely attributed to the low calcium levels, thus impairing fibroblast migration and proliferation [33-35]. An important advantage of the use of KSFM is therefore that it circumvents the need of culturing the isolated cell suspension for 4 to 5 hours on culture dishes to remove fibroblasts by adherence. This is important, since maintaining epithelial cells for several hours in suspension prevents them from adhering to a matrix, which may result in anoikis [36]. Our results indicate that MTEC do proliferate in KSFM, but the morphology, survival and expansion rates drastically improved when a ROCK inhibitor in combination with a Notch signaling inhibitor was used. Following expansion of MTEC in KSFM, we allowed MTEC to grow and differentiate on transwell inserts as previously described [14,17,37].

Expanding the progenitor basal cell population is essential for subculturing MTEC *in vitro*. ROCK inhibition is frequently used in embryonic stem cell cultures, induced pluripotent stem cells and some tissue-specific stem cell populations. More specifically, the ROCK inhibitor Y27632 has previously been shown to improve proliferation rates of human and mouse tracheal epithelial cells without affecting subsequent ALI differentiation [17]. Feeder layers or conditional medium from feeder layers has proven effective in expanding airway epithelial cells [38]. However, maintaining feeder layers adds significantly to the workload and exact mechanisms through which the feeder layers improve proliferation remain unknown. Therefore, using a defined culture medium without the need of feeder layers is preferred and is likely to result in increased consistence of the culture system.

A mechanism that has been suggested to underlie the ability of ROCK inhibitors to improve cell proliferation is the inhibition of downstream Notch signaling [39,40]. Whereas Y27632 improved MTEC proliferation rates in our experiments, cell morphology and survival suggested the presence of cell contact-induced impairment of cell proliferation. Cell-cell communication and related inhibition of proliferation has previously been attributed to Notch signaling [41]. Notch signaling has previously been shown to be involved in basal epithelial cell proliferation and differentiation

both *in vitro* and *in vivo* [10,11,18,21,41,41]. We therefore used DAPT, a γ-secretase inhibitor and indirect Notch signaling inhibitor, in combination with Y27632 in KSFM to expand MTEC *in vitro*. Our data shows that inhibiting Notch signaling is an effective way to expand the basal cell population.

Differentiation of the basal cell population into a pseudostratified epithelial layer containing secretory and ciliated cells is an important feature of the airway epithelium. We have shown that MTEC expanded to passage 2 retain the ability to differentiate into a pseudostratified epithelial layer. To this end, we used KRT5 and KRT8 staining to discriminate between basal and luminal precursor populations. Some cells show the presence of both KRT5 and KRT8 which may mark basal cells differentiating towards a luminal precursor cell [11,20]. Furthermore, the pseudostratified epithelium of the trachea contains various cell types including basal, ciliated and secretory cells. Using FOXJ1 and TUBB4B staining for ciliated cells and SCGB1A1 and SCGB3A2 staining for secretory cells in addition to qRT-PCR, we showed the presence of ciliated and club cells in expanded MTEC. However, the expanded MTEC had reduced numbers of ciliated and secretory cells present after 8 days of ALI, despite the unchanged KRT8 positive luminal fraction in P2 compared to P0. This suggests that the cells may require more time or an additional trigger for efficient end-stage differentiation. This hypothesis is strengthened by the observed increase in ciliated cells by extending the duration of ALI. This was further increased by adding the Notch inhibitor DAPT during ALI, which is in line with previous studies showing that inhibition of Notch signaling may drive epithelial cells towards ciliated cells [21,43]. Collectively, these findings indicate that after expansion in culture, basal cells retain the ability to develop a pseudostratified epithelial layer following ALI differentiation, but with an altered differentiation capacity, likely resulting from altered stemness of the basal cell population. Additional triggers generated by extended culture and Notch inhibition, are required to achieve more efficient mucociliary differentiation.

The ability to adapt to a changing environment to various stimuli is an important feature of airway epithelial cells. Furthermore, appropriate *in vitro* epithelial culture models should recapitulate the response to disease-specific stimuli observed in patients and/or animal models of disease. To this end, we used IL-13 exposure and assessment of subsequent goblet cell development of expanded MTEC cultured at ALI to mimic the *in vivo* allergic airways inflammation. IL-13 is a T helper 2 cytokine known to induce goblet cell metaplasia *in vivo* and *in vitro* [22,23,25,44,45]. IL-13 exposure during differentiation induced MUC5AC positive goblet cells in both expanded MTEC and in freshly isolated MTEC that were cultured directly onto inserts.

The presence of IL-13 during differentiation at the ALI resulted in an increased number of goblet cells, likely resulting from basal cells differentiating towards goblet cells rather than ciliated cells. Alternatively, the increased number of goblet cells may

be resulting from IL-13 induced trans-differentiation of ciliated cells towards goblet cells, resulting in fewer ciliated cells [26,45,46]. Moreover, IL-13 induced a reduction in barrier function in both expanded MTEC and isolated MTEC that had been cultured directly onto transwell inserts, which is in line with previous publications showing that IL-13 reduces barrier function *in vitro* [27,28]. Overall, expanded MTEC showed a robust response following IL-13 exposure including decreased barrier function and the development of goblet cells, indicating that expanding MTEC *in vitro* using a Notch signaling inhibitor does not affect their ability to respond to IL-13. The observation that goblet cell differentiation induced by IL-13 exposure is similar in P0 and P2, suggests that the basal cells retain the ability to differentiate into goblet cells.

Basal cells are the main progenitor cells of the conducting airways. They have the proficiency to self-renew and differentiate into various cell types found in a pseudostratified epithelial layer including ciliated and secretory cells. Various cellular markers have been described to distinguish basal cells in the airway epithelium. Furthermore, basal cells can be separated in various subsets depending on the combination of cellular markers present [5,7]. KRT5, TRP63 and NGFR are commonly used markers to delineate basal cell populations. In contrast, only a small fraction of basal cells is positive for KRT14 at baseline. Our results indicated that expansion significantly induced a KRT14 positive basal cell population in P2 compared to P0, whereas the other investigated basal cell populations remained unaltered between passages. Together these data suggest that expanding basal cells *in vitro* can alter the composition of the basal cell population, which may explain why we see a reduced number of ciliated cells in expanded MTEC compared to isolated MTEC cultured directly onto inserts. Whether this alteration of the basal cell population results from the KRT14 negative basal cell population turning KRT14 positive or that the KRT14 positive basal cell population has an increased capacity to expand *in vitro* compared to the KRT14 negative basal cell population remains to be further investigated. However, KRT14 positive basal cells are still able to differentiate in ciliated cells and goblet cells when exposed to additional triggers, respectively DAPT and IL-13, in a similar way as KRT14 negative basal cells. More research is needed to investigate the role of KRT14 positive basal cells and their ability to differentiate.

In conclusion, we developed a clearly defined culture method that allows ALI differentiation of MTEC into a pseudostratified epithelial layer following *in vitro* expansion. Expanding MTEC *in vitro* did result in decreased differentiation of basal cells to ciliated cells, likely resulting in part from an altered composition of the basal cell population as indicated by an increase in KRT14 positive basal cells. Despite the altered basal cell population, the Th2 cytokine IL-13 was still able to redirect epithelial differentiation towards a phenotype observed in allergic airways inflammation. Furthermore, this culture method may be useful to study repair following injury combined with *in vitro* lineage tracing if MTEC from transgenic animals are used.

Also, this new culture method will contribute to a reduction in animals needed for experimentation, in line with the principles of the 3R's (Replacement, Reduction and Refinement).

METHODS

Culture media and supplements

A detailed overview of all culture media and supplements can be found in supplemental table 1. "Ham´s F12" is defined as Ham´s F12 (Gibco, Bleiswijk, The Netherlands) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza, Verviers, Belgium). "MTEC basic" medium is defined as DMEM/F12 (Gibco) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 0.03% (w/v) $NaHCO_3$ (Gibco). "KSFM expansion medium" is defined as KSFM (Gibco) supplemented with 1% penicillin-streptomycin, 1 µM isoproterenol (Sigma-Aldrich, St. Louis, MO, USA), 0.03 mg/ml bovine pituitary extract (Gibco), 25 ng/ml murine epidermal growth factor (Peprotech, Rocky Hill, NJ, USA) and 10 µM Y-27632 hydrochloride (Cayman Chemical, Ann Arbor, MI, USA). "MTEC proliferation medium" is defined as MTEC basic supplemented with 5% (v/v) Fetal Bovine Serum (FBS), 1.5 mM L-glutamine (Lonza), 1x (v/v) Insulin-Transferrin-Selenium (Gibco), 0.1 µg/ml cholera toxin (Sigma-Aldrich), 25 ng/ml murine epidermal growth factor, 0.03 mg/ml bovine pituitary extract, 0.05 µM retinoic acid (Sigma-Aldrich) and 10 µM Y-27632 hydrochloride. "MTEC differentiation medium" is defined as MTEC basic supplemented with 0.1% (w/v) bovine serum albumin (BSA) (Gibco), 1.5 mM L-glutamine, 1% (v/v) Insulin-Transferrin-Selenium, 25 ng/ml cholera toxin, 5 ng/ml murine epidermal growth factor, 0.03 mg/ml bovine pituitary extract and 0.05 µM retinoic acid. Retinoic acid and Y-27632 hydrochloride stocks were prepared and stored at -80 °C and -20 °C respectively, and were supplemented fresh for cell culture usage and used within the same day. Culture media containing retinoic acid were protected from light.

Mouse tracheal epithelial cells (MTEC) isolation and culture

All animal experimental protocols were approved by the animal welfare committee of the veterinary authorities of the Leiden University Medical Center or the Erasmus Medical Center. Wild-type C57Bl/6 mice were used for isolating murine tracheas. MTEC were isolated as described previously [14]. After isolation, MTEC were resuspended in MTEC basic containing 10% FBS for direct culture on transwell inserts, or in KSFM for further passage and expansion of MTEC.

In experiments in which MTEC were grown on transwell inserts directly after isolation without passaging of the cells in KSFM expansion medium, the cells were first deprived of fibroblasts by incubating the cells on Primaria plates (Corning Costar, Cambridge, MA, USA) for 5 h at 37°C with 5% CO_2. Non-adherent cells were collected and centrifuged at 390xg for 10 min at room temperature. Cells were resuspended in MTEC proliferation medium, plated at 8 x 10^4 cells/cm^2 on transwell inserts (0.4 µm

pore size; Corning Costar) and cultured submerged until confluent at 37°C with 5% CO_2. MTEC proliferation medium was refreshed every two to three days.
For MTEC subculture experiments, trachea-derived cells were collected in KSFM expansion medium and plated in T75 flasks at 5 x 10^3 cells/cm^2. The adherence step using Primaria plates to remove fibroblasts was omitted when using KSFM, since this medium is formulated by the provider to prevent fibroblast growth. KSFM expansion medium was supplemented with DAPT (Sigma-Aldrich), a Notch signaling inhibitor, according to the experimental design described in the results section. At 80 – 90% confluence, MTEC were passaged using 0.25% (w/v) trypsin (Gibco) and 2.7 mM EDTA (Sigma-Aldrich) in Cell Dissociation Solution Non-enzymatic 1x (Sigma-Aldrich). MTEC were plated at 5 x 10^3 cells/cm^2 in KSFM. Passage 1 or passage 2 MTEC were used for further experiments. For culturing onto transwell inserts, cells were dissociated with Cell Dissociation Solution (Sigma-Aldrich) supplemented with 0.25% trypsin and 2.7 mM EDTA at 37°C for 15 min, centrifuged at 390 g for 10 min at room temperature and resuspended in MTEC proliferation medium. MTEC were plated on transwell inserts at 8 x 10^4 cells/cm^2 and cultured in MTEC proliferation medium until confluent at 37°C with 5% CO_2. MTEC proliferation medium was refreshed every two to three days. A schematic overview of the expansion method followed is given in figure 1.
After MTEC cultured on transwell inserts had grown to full confluence, apical medium was removed to achieve air-liquid interface (ALI) culture conditions. After the start of culture at ALI, the apical surface was washed with warm PBS and the basal medium was refreshed using MTEC differentiation medium every two to three days.
For experimental exposures, ALI-MTEC were exposed to either 5 ng/ml murine IL-13 (Peprotech) or 5 μM DAPT, added in the basolateral chamber of the transwell insert for the indicated duration.

RNA extraction, cDNA synthesis and Quantitative RT-PCR analysis

Cells were collected in PBS from the insert using cell-scrapers on ice. After centrifugation at 4 °C, 5000rpm for 5 min, the cell pellet was lysed using TRIzol™ reagent (Life Technologies, Rockville, MD, USA). RNA extraction was performed according to the TRIzol™ Reagent protocol. RNA concentrations were measured using Nanodrop (Thermofisher Scientific). First strand cDNA synthesis was synthesized using MLV Reverse transcriptase (Sigma, M1302) with Oligo(dT) primers (self-designed: 23xT + 1A, 23xT + 1C and 23xT + 1G). Quantitative RT-PCR analysis was performed with 0.5 μl of cDNA per reaction, Platinum Taq polymerase (Invitrogen, 18038042) and SybrGreen (Sigma, S9430). The primer combinations for the qRT-PCR are listed in supplemental table 3. Relative gene expression was calculated using the ΔΔCT method relative to GAPDH control.

Allergic airway inflammation in mice

To induce allergic airway inflammation in mice, 8 to 12 weeks old female C57BL/6 mice

were sensitized by intranasal instillation of 1 µg *Dermatophagoides pteronyssinus* extract (house dust mite extract (HDM)) in 50 µl PBS, followed by 5 daily intranasal challenges with 10 µg HDM in 50 µl PBS or PBS starting at 7 days after sensitization. Mice were sacrificed 2 days after the last challenge. Trachea were removed and fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) and stained for confocal analysis as described below.

Immunofluorescence of ALI-MTEC culture

MTEC on inserts were rinsed with PBS followed by fixation with 4% (w/v) paraformaldehyde (PFA) for 10 min at room temperature. After fixation cells were washed 3 times with PBS/0.2% (v/v) Triton-X100 (Sigma-Aldrich) to remove residual mucus from the apical surface. Non-specific binding sites were blocked with 5% (v/v) donkey serum (EMD Millipore, Billerica, MA, USA), 1% (w/v) BSA and 0.2% (v/v) Triton-X100 in PBS for 30 min at room temperature, followed by incubation with primary antibodies in blocking buffer at 4°C overnight (Supplemental table 2). The next day, MTEC were washed three times with PBS/0.02% Triton X-100 and incubated for 2 h at room temperature with Alexa Fluor labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) in blocking buffer (Supplemental table 2). After three washing steps with PBS/0.02% (v/v) Triton-X100 and one washing step with PBS, the cells were mounted with Vectashield hardset containing DAPI (Vector Laboratories, Burlingame, CA, USA). Images were acquired using a Leica SP5 confocal microscope. Cell counting was performed using ImageJ and 10-15 areas from each insert were analyzed for each replicate.

Immunofluorescence of tracheal sections

Mouse tracheas were fixed with 4% PFA overnight at 4°C, washed with PBS and embedded in paraffin for sectioning. Tracheas were sectioned (5 µm) and deparaffinized, rehydrated and subjected to antigen retrieval in Tris-EDTA (pH 9.0) at 600W for 15 min. After blocking with PBS plus 5% donkey serum and 0.05% Tween-20, sections were incubated with primary antibodies in blocking buffer overnight at 4°C (Supplemental table 2). The next day, sections were washed with PBS/0.05% Tween (3x, 5 min). Alexa Fluor labeled secondary antibodies were used in a 1:500 dilution in blocking buffer and sections were incubated for 2 h at room temperature. After incubation, sections were washed (3x, 5 min) with PBS/0.05% Tween-20 followed by one final wash with PBS only. Sections were mounted with Vectashield hardset containing DAPI (Vector Laboratories) and confocal images were obtained using a Leica SP5 confocal microscope.

Trans-epithelial electrical resistance (TEER)

TEER values were measured with an STX2 electrode (World Precision Instruments, Berlin, Germany) connected to a Millicell ERS voltohmmeter (World Precision Instruments). Prior to measurement, 700 µl prewarmed PBS was added apically to the

ALI-MTEC and incubated at room temperature for 10 min. Resistance measurements were corrected for the surface of the transwell inserts and expressed as Ohm * cm^2.

Western blot analysis

Cells were washed twice with PBS, scraped of the insert in PBS and pelleted. Cells were lysed in Carin lysis buffer containing 20mM Tris-HCl pH 8.0, 137 mM NaCl, 10 mM EDTA, 10% glycerol and 1% NP-40 and incubated on ice for 15 min. Complete protease inhibitors (Roche) was freshly added each time to the lysis buffer. Samples were centrifuged for 5 min at 18,711 g in an Eppendorf5417R at 4°C. Pellets were discarded and supernatant was used for western blot analysis. Protein concentrations were determined by the Pierce® BCA Protein Assay Kit (Thermos scientific) and equal concentrations of protein were eluted in 4x SDS sample buffer and 50mM 1,4-dithiothreitol (DTT, sigma). Samples were boiled and loaded on a 12% SDS-polyacrylamide gel and blotted onto a PVDF membrane (Immobilon®-P transfer membrane, Millipore). The blots were blocked for 1h in PBS containing 0.05% Tween-20 and 3% BSA at room temperature, and probed overnight with primary antibodies at 4°C. Next day, membranes were washed three times with PBS containing 0.05% Tween-20 and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO) at a dilution of 1:10,000. Signal was detected with Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare). Blots were developed using the Amersham Typhoon imaging system (GE Healthcare).

Statistical analysis

Data are represented as means with standard error of mean of measurements. Statistical differences between samples were assessed with a one way or two way analysis of variance (ANOVA) or unpaired t-test. Differences at P-values below 0.05 are considered significant (* $p<0.05$). All statistical analyses were performed using Graphpad PRISM version 5.02.

Data availability statement

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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AUTHOR CONTRIBUTION STATEMENT

E.E and T.M preformed the experiments, wrote the main manuscript and prepared the figures. M.J.B preformed cell culture experiments. Y.W obtained material from the house dust treated mice. R.J.R and P.S.H contributed to the experimental set-up, data interpretation and wrote the main manuscript. All authors reviewed the manuscript.

REFERENCES

1. Gao, W. et al. Bronchial epithelial cells: The key effector cells in the pathogenesis of chronic obstructive pulmonary disease? Respirology. 20, 722-729 (2015).
2. Loxham, M., Davies, D. E. & Blume, C. Epithelial function and dysfunction in asthma. Clin Exp Allergy. 44, 1299-1313 (2014).
3. Hiemstra, P. S., McCray, P. B., Jr. & Bals, R. The innate immune function of airway epithelial cells in inflammatory lung disease. Eur Respir J. 45, 1150-1162 (2015).
4. Nichols, J. E. et al. Modeling the lung: Design and development of tissue engineered macro- and micro-physiologic lung models for research use. Exp Biol Med (Maywood). 239, 1135-1169 (2014).
5. Schilders, K. A. et al. Regeneration of the lung: Lung stem cells and the development of lung mimicking devices. Respir Res. 17, 44 (2016)
6. Rock, J. R. et al. Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc Natl Acad Sci U S A. 106, 12771-12775 (2009).
7. Rock, J. R., Randell, S. H. & Hogan, B. L. Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. Dis Model Mech. 3, 545-556 (2010).
8. Pezzulo, A. A. et al. The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. Am J Physiol Lung Cell Mol Physiol. 300, L25-31 (2011).
9. Amatngalim, G. D. et al. Cigarette Smoke Modulates Repair and Innate Immunity following Injury to Airway Epithelial Cells. PLoS One. 11, e0166255 (2016).
10. Rock, J. R. et al. Notch-dependent differentiation of adult airway basal stem cells. Cell Stem Cell. 8, 639-648 (2011).
11. Mori, M. et al. Notch3-Jagged signaling controls the pool of undifferentiated airway progenitors. Development. 142, 258-267 (2015).
12. Pardo-Saganta, A. et al. Injury induces direct lineage segregation of functionally distinct airway basal stem/progenitor cell subpopulations. Cell Stem Cell. 16, 184-197 (2015).
13. You, Y. & Brody, S. L. Culture and differentiation of mouse tracheal epithelial cells. Methods Mol Biol. 945, 123-143 (2013).
14. Lam, H. C., Choi, A. M. & Ryter, S. W. Isolation of mouse respiratory epithelial cells and exposure to experimental cigarette smoke at air liquid interface. J Vis Exp. (2011).
15. Mertens, T. C., Hiemstra, P. S. & Taube, C. Azithromycin differentially affects the IL-13-induced expression profile in human bronchial epithelial cells. Pulm Pharmacol Ther. 39, 14-20 (2016).
16. van Wetering, S. et al. Regulation of SLPI and elafin release from bronchial epithelial cells by neutrophil defensins. Am J Physiol Lung Cell Mol Physiol. 278, L51-58 (2000).
17. Horani, A., Nath, A., Wasserman, M. G., Huang, T. & Brody, S. L. Rho-associated protein kinase inhibition enhances airway epithelial Basal-cell proliferation and lentivirus transduction. Am J Respir Cell Mol Biol. 49, 341-347 (2013).
18. Lafkas, D. et al. Therapeutic antibodies reveal Notch control of transdifferentiation in the adult lung. Nature. 528, 127-131 (2015).
19. Hunter, G. L. et al. Coordinated control of Notch/Delta signalling and cell cycle progression drives lateral inhibition-mediated tissue patterning. Development. 143, 2305-2310 (2016).
20. Watson, J. K. et al. Clonal Dynamics Reveal Two Distinct Populations of Basal Cells in Slow-Turnover Airway Epithelium. Cell Rep. 12, 90-101 (2015).
21. Gerovac, B. J. et al. Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner. Am J Respir Cell Mol Biol. 51, 516-525 (2014).
22. Kuperman, D. A. et al. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med. 8, 885-889 (2002).
23. Taube, C. et al. The role of IL-13 in established allergic airway disease. J Immunol. 169, 6482-6489 (2002).
24. Alevy, Y. G. et al. IL-13-induced airway mucus production is attenuated by MAPK13 inhibition. J Clin Invest. 122, 4555-4568 (2012).
25. Fujisawa, T. et al. Involvement of the p38 MAPK pathway in IL-13-induced mucous cell

metaplasia in mouse tracheal epithelial cells. Respirology. 13, 191-202 (2008).
26. Danahay, H. et al. Notch2 is required for inflammatory cytokine-driven goblet cell metaplasia in the lung. Cell Rep. 10, 239-252 (2015).
27. Ahdieh, M., Vandenbos, T. & Youakim, A. Lung epithelial barrier function and wound healing are decreased by IL-4 and IL-13 and enhanced by IFN-gamma. Am J Physiol Cell Physiol. 281, C2029-2038 (2001).
28. Saatian, B. et al. Interleukin-4 and interleukin-13 cause barrier dysfunction in human airway epithelial cells. Tissue Barriers. 1, e24333 (2013).
29. Hong, K. U., Reynolds, S. D., Watkins, S., Fuchs, E. & Stripp, B. R. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. Am J Pathol. 164, 577-588 (2004).
30. Hong, K. U., Reynolds, S. D., Watkins, S., Fuchs, E. & Stripp, B. R. In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. Am J Physiol Lung Cell Mol Physiol. 286, L643-649 (2004).
31. Brockman-Schneider, R. A., Amineva, S. P., Bulat, M. V. & Gern, J. E. Serial culture of murine primary airway epithelial cells and ex vivo replication of human rhinoviruses. J Immunol Methods. 339, 264-269 (2008).
32. Mou, H. et al. Dual SMAD Signaling Inhibition Enables Long-Term Expansion of Diverse Epithelial Basal Cells. Cell Stem Cell. 19, 217-231 (2016).
33. Balk, S. D. Calcium as a regulator of the proliferation of normal, but not of transformed, chicken fibroblasts in a plasma-containing medium. Proc Natl Acad Sci U S A. 68, 271-275 (1971).
34. Gail, M. H., Boone, C. W. & Thompson, C. S. A calcium requirement for fibroblast motility and prolifertion. Exp Cell Res. 79, 386-390 (1973).
35. Dulbecco, R. & Elkington, J. Induction of growth in resting fibroblastic cell cultures by Ca++. Proc Natl Acad Sci U S A. 72, 1584-1588 (1975).
36. Gilmore, A. P. Anoikis. Cell Death Differ. 12 Suppl 2, 1473-1477 (2005).
37. You, Y., Richer, E. J., Huang, T. & Brody, S. L. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. Am J Physiol Lung Cell Mol Physiol. 283, L1315-1321 (2002).
38. Butler, C. R. et al. Rapid Expansion of Human Epithelial Stem Cells Suitable for Airway Tissue Engineering. Am J Respir Crit Care Med. 194, 156-168 (2016).
39. Yugawa, T. et al. Noncanonical NOTCH signaling limits self-renewal of human epithelial and induced pluripotent stem cells through ROCK activation. Mol Cell Biol. 33, 4434-4447 (2013).
40. Venkatesh, D. et al. RhoA-mediated signaling in Notch-induced senescence-like growth arrest and endothelial barrier dysfunction. Arterioscler Thromb Vasc Biol. 31, 876-882 (2011).
41. Lai, E. C. Notch signaling: control of cell communication and cell fate. Development. 131, 965-973 (2004).
42. Kent, S. et al. DeltaNp63alpha promotes cellular quiescence via induction and activation of Notch3. Cell Cycle. 10, 3111-3118 (2011).
43. Tsao, P. N. et al. Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. Development. 136, 2297-2307 (2009).
44. Kuperman, D., Schofield, B., Wills-Karp, M. & Grusby, M. J. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. J Exp Med. 187, 939-948 (1998).
45. Tyner, J. W. et al. Blocking airway mucous cell metaplasia by inhibiting EGFR antiapoptosis and IL-13 transdifferentiation signals. J Clin Invest. 116, 309-321 (2006).
46. Gomperts, B. N., Kim, L. J., Flaherty, S. A. & Hackett, B. P. IL-13 regulates cilia loss and foxj1 expression in human airway epithelium. Am J Respir Cell Mol Biol. 37, 339-346 (2007).

SUPPLEMENTARY METHODS

Suppl. Table 1: *Overview of culture media and supplements*

Medium	Component	Provider (catalogue number)	Final concentration
KSFM expansion medium	KSFM	Gibco (17005034)	
	Penicillin Streptomycin	Lonza (DE17-602e)	100 U/ml 100 µg/ml
	Murine EGF	Peprotech (315-09)	0.025 µg/ml
	Bovine Pituitary Extract	Gibco (13028014)	0.03 mg/ml
	Isoproterenol	Sigma (I-6504)	1 µM
	Y-27632 (add fresh)	Cayman Chemical (10005583)	10 µM
	DAPT (add fresh)	Sigma (D5942)	5 µM
MTEC Basic (*)	DMEM/F12	Gibco (1133032)	
	Penicillin Streptomycin	Lonza (DE17-602e)	100 U/ml 100 µg/ml
	$NaHCO_3$	Gibco (25080094)	0.03% (w/v)
MTEC proliferation medium	MTEC basic	(*)	
	L-Glutamine	Gibco (25030081)	1.5 mM
	Fetal Calf Serum	HyClone (SH30071.03)	5%
	ITS-G	Gibco (41400045)	1x
	Cholera Toxin	Sigma (C8052-0.5mg)	0.1 µg/ml
	Murine EGF	Peprotech (315-09)	0.025 µg/ml
	Bovine Pituitary Extract	Gibco (13028014)	0.03 mg/ml
	Y-27632 (add fresh)	Cayman Chemical (10005583)	10 µM
	Retinoic Acid	Sigma (R2625-50mg)	0.05 µM
MTEC differentiation medium	MTEC basic	(*)	
	L-Glutamine	Gibco (25030081)	1.5 mM
	Bovine Serum Albumin	Gibco (15260037)	0.1% (w/v)
	ITS-G	Gibco (41400-045)	1x
	Cholera Toxin	Sigma (C8052-0.5mg)	0.025 µg/ml
	Murine EGF	Peprotech (315-09)	0.005 µg/ml
	Bovine Pituitary Extract	Gibco (13028-014)	0.03 mg/ml
	Retinoic Acid	Sigma (R2625-50mg)	0.05 µM

Suppl. Table 2: *Antibody overview*

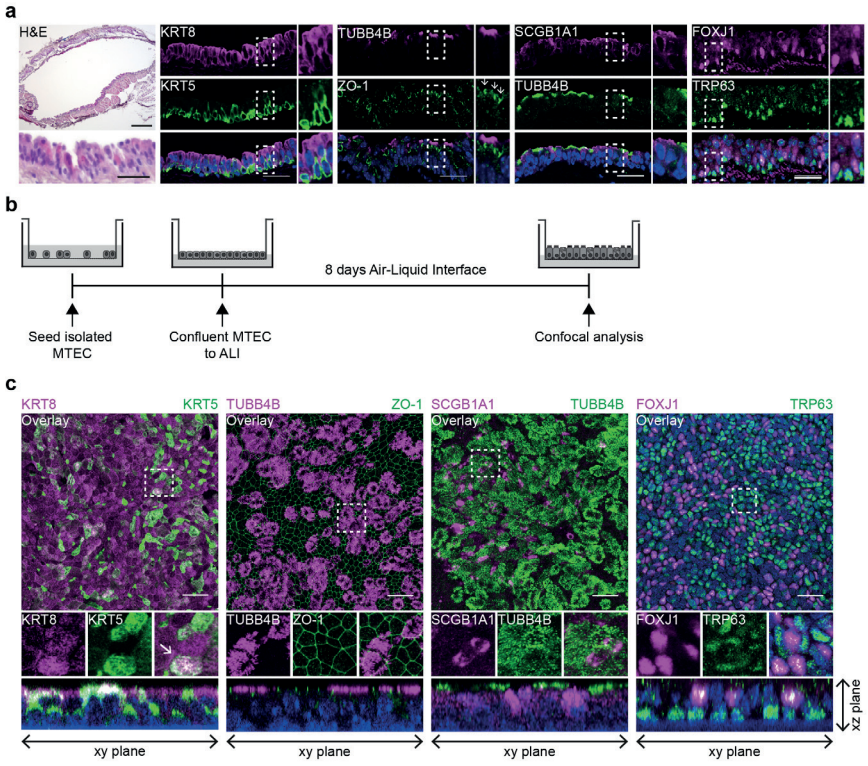
Primary Antibody	Provider (Catalogue number)	Dilution
Beta-Tubulin IV	BioGenex, MU178-UC, Mouse monoclonal	1:100
SCGB1A1 (CCSP)	From Dr. Barry Stripp, Goat polyclonal	1:5000
SCGB1A1 (CCSP, Uteroglobin)	Abcam, ab40873, Rabbit polyclonal	1:200
UGRP1/SCGB3A2	R&D Systems, af3465, Goat polyclonal	1:500
FOXJ1	eBioscience, 14-9965, Mouse monoclonal	1:300
KRT5	Biologend, Poly19055, Rabbit polyclonal	1:500
KRT8	DSHB, TROMA-I, Rat monoclonal	1:100
MUC5AC	Abcam, ab3649, Mouse monoclonal	1:500
TRP63	Santa Cruz, sc-8343, Rabbit polyclonal	1:50
Zona Occludens 1	Invitrogen, 61-7300, Rabbit polyclonal	1:100
KRT14	EMD Millipore, CBL197, Mouse monoclonal	1:100
NGFR	Promega, Anti-Human P75 pAb, Rabbit polyclonal	1:200
B-ACTIN	Cell Signaling, 4967, Rabbit polyclonal	1:1000
Secondary Antibody		
Alexa Fluor ®488, 594 Donkey anti Goat IgG	Jackson ImmunoResearch (111301), (112406)	1:500
Alexa Fluor ®488, 594 Donkey anti Mouse IgG	Jackson ImmunoResearch (112976), (110989)	1:500
Alexa Fluor ®488, 594 Donkey anti Rabbit IgG	Jackson ImmunoResearch (113254), (113078)	1:500
Alexa Fluor ®594 Donkey anti Rat IgG	Jackson ImmunoResearch (127426)	1:500
Anti-mouse HRP conjugated	DakoCytomation (P 0447)	1:10,000
Anti-rabbit HRP conjugated	DakoCytomation (P 0448)	1:10,000

Suppl. Table 3: *Primer overview*

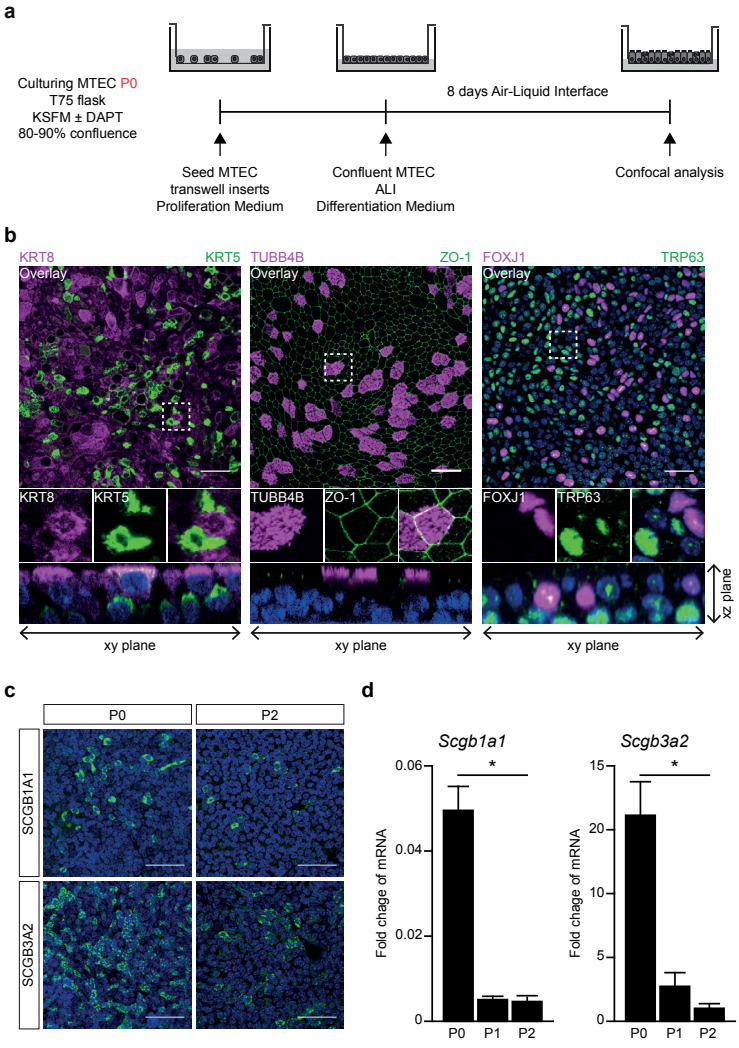
Primer	Forward primer	Reverse primer
Foxj1	5'-CAGACCCCACCTGGCAGAATTC-3'	5'-AAAGGCAGGGTGGATGTGGACT-3'
Scgb1a1	5'-GCAGCTCAGCTTCTTCGGACA-3'	5'-TCCTGGTCTCTTGTGGGAGGG -3'
Scgb3a2	5'-GTGGTTATTCTGCCACTGCCCTT-3'	5'-TCGTCCACACACTTCTTCAGTCC-3'
Trp63 (ΔNP63)[1]	5'-GGAAAACAATGCCCAGACTC-3'	5'-GATGGAGAGAGGGCATCAAA-3'
Krt5	5'-TACCAGACCAAGTATGAGGAG-3'	5'-TGGATCATTCGGTTCATCTCAG-3'
Krt8	5'-CTCATCAAGAAGGATGTGGAC-3'	5'-GAGGAAGTTGATCTCGTCGG-3'
Krt14	5'-CGGCAAGAGTGAGATTTCTG-3'	5'-AGGGATGCTTTCATGCTGAG-3'
Ngfr	5'-CGCTGACAACCTCATTCCTG-3'	5'-GCTGTTCCATCTCTTGAAAGC-3'
Gapdh	5'-CCTGCCAAGTATGATGACAT-3'	5'-GTCCTCAGTGTAGCCCAAG-3'

1. Zhao et al. Yap tunes airway epithelial size and architecture by regulating the identity, maintenance, and self-renewal of stem cells. Dev Cell 2014

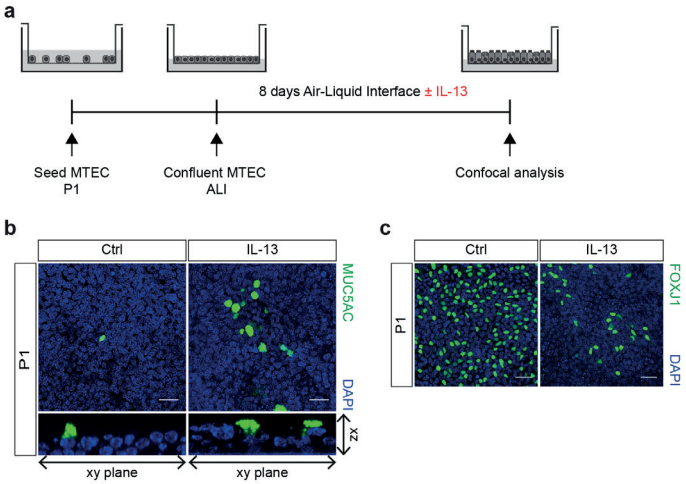
SUPPLEMENTARY FIGURES



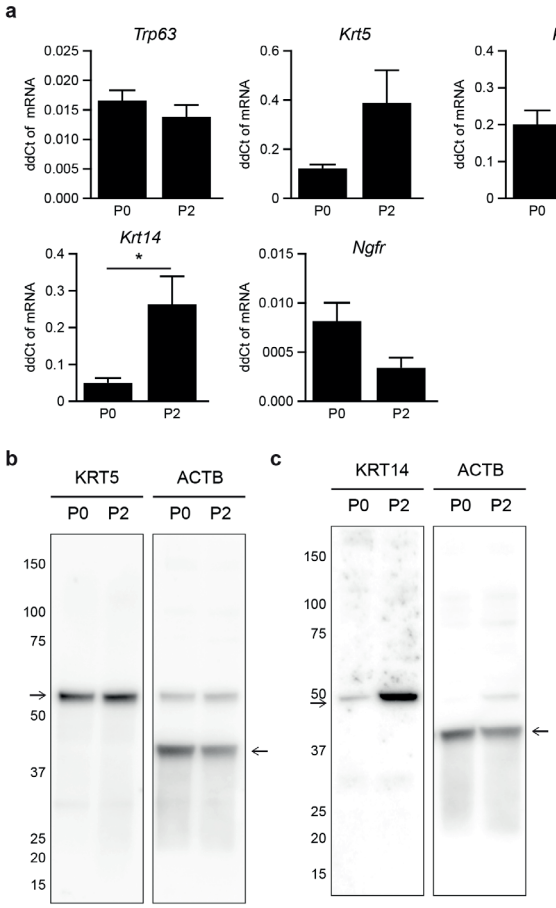
Suppl. Fig. 1. MTEC ALI cultures recapitulate the in vivo pseudostratified airway epithelium. *(a) Hematoxylin and eosin staining of a mouse tracheal sections and immunofluorescence co-staining on tracheal sections with basal cell marker KRT5 and luminal cell marker KRT8, cilia marker TUBB4B with tight junction protein ZO-1 (indicated by the arrows), secretory club cell marker SCGB1A1 with cilia marker TUBB4B and the last panel shows TRP63 positive basal cells with ciliated cell marker FOXJ1. Nuclei are stained with DAPI (blue). Scale bar, 200 μm and 30 μm. (b) Schematic representation of ALI culture. (c) Co-staining of the different epithelial markers on MTEC ALI culture. The arrow marks a basal luminal precursor cell that is both positive for KRT5 and KRT8. Orthogonal view shows a stratified epithelium. Nuclei are stained with DAPI (blue). Scale bar, 30 μm.*



Suppl. Fig. 2. Representative images of P1 on inserts. *(a) Schematic representation of ALI culture. (b) Co-staining of different epithelial markers on MTEC passage 1 after 8 days of Air Liquid Interface (ALI) culture. From left to right, inserts were stained with KRT5 for the basal cell layer and differentiation marker KRT8, Ciliated cell marker FOXJ1 with tight junction protein ZO-1, secretory cell marker SCGB1A1 with cilia marker TUBB4B and the last panel shows TRP63 positive basal cells with ciliated cells. Nuclei are stained with DAPI (blue). Scale bar, 30 µm. (c) Staining of SCGB1A1 an SCGB3A2 expressing secretory cells in MTEC passage (P) 0 and 2. Scale bar, 50 µm. (d) qRT-PCR of Scgb1a1 and Scgb3a2 mRNA in MTEC P0 and P2 at 8 days of ALI (mean± SEM). * p < 0.05 by one-way ANOVA (n=4).*



Suppl. Fig.3. IL-13 treatment stimulates the formation of goblet cells in vitro. *(a) Schematic representation of culture protocol. (b) Staining of Mucin 5AC (MUC5AC) expressing goblet cells in MTEC (Passage 1, P1) after 8 days of ALI with or without IL-13 (5 ng/ml). Nuclei are stained with DAPI (blue). Scale bar: 30 µM. (c) Staining of FOXJ1 positive ciliated cells after 8 days of ALI with or without IL-13 (5 ng/ml) in P1. Nuclei are stained with DAPI (blue). Scale bar: 30 µm.*



Suppl. Fig. 4. Western blot of Krt14 expression on ALI day 0 in P0 and P2 *(a) Expression of Trp63, Krt5, Krt8, Krt14 and Ngfr mRNA in MTEC Passage (P) 0 and 2 at day 0 of Air Liquid Interfase (ALI) ALI normalized to Gapdh (mean± SEM). * p < 0.05 by unpaired t-test (n=4). (b) Full length blots of the cropped blots of fig. 4d. Beta-Actin (ACTB) is used ad loading control and labeled on the same blot as KRT5. (c) Full length blots of the cropped blots of fig. 4f. ACTB is used ad loading control and labeled on the same blot as KRT14.*

Chapter 6

General Discussion

Pediatric lung diseases, such as congenital diaphragmatic hernia (CDH) and congenital pulmonary airway malformations (CPAM), often result from aberrant regulation of lung progenitor cells in their maintenance, proliferation or differentiation fate during development. Upon birth and in later life, it is equally important that residing progenitor cells are correctly regulated for homeostasis and repair after injury. For example, premature newborns require respiratory support to sufficiently oxygenate the blood. But due to the exposure of high oxygen by mechanical ventilation, the premature lungs are damaged and children can develop a clinical entity called Bronchopulmonary Dysplasia (BPD). This shows that proper repair by progenitor cells is important. It is thought that progenitor cells often activate identical processes during regeneration as those required for development. With this in mind and described in this thesis, we developed mouse and human airway *in vitro* models to study progenitor cell behavior. Furthermore we studied responses of different airway progenitor populations and the regulation of the transcription factors SOX2 and SOX21 in maintenance, proliferation and differentiation of airway progenitor cells. In this chapter we will discuss the potential mechanisms that contribute to SOX2+SOX21+ airway progenitor cell behavior, and the *in vitro* models that are being used to study airway progenitor cell function. Moreover we will reflect on the potential consequences of our findings in future clinical treatment strategies.

SOX2 AND SOX21 COORDINATE CELL FATE-DETERMINING TRANSCRIPTIONAL EVENTS

Intrinsic cellular changes occur as SOX2+ airway progenitor cells differentiate to airway specific cell types. Complete loss of SOX2 in airway epithelium results in a loss of basal cells, a decrease in cilia and secretory cells, during development and during regeneration [1]. In addition to the importance of SOX2 in airway progenitor differentiation, SOX2 is critical for pluripotency in embryonic stem cells and development of many other organs, e.g. brain, intestine and esophagus [2-4]. SOX2 is co-expressed with SOX21 in only some progenitor cells, e.g. in airway (**chapter 4**), inner ear, neuron, and embryonic stem cells [5-8]. Due to the widespread role of SOX2, its function is subject of multiple studies, but the interaction of SOX2 with SOX21 on progenitor cell behavior has mostly been ignored. In **chapter 4,** we showed that SOX2 and SOX21 have opposite effects on airway progenitor cell differentiation: SOX2 stimulates and SOX21 inhibits differentiation. We proposed that they regulate a balance in maintenance and turnover of airway progenitor cells. In **chapter 5** we show that deficiency in SOX2 and SOX21 have opposite phenotypes in clustering neuroendocrine (NE) cells during development. Thus, it seems that SOX2 and SOX21 stimulate and repress sets of genes in an opposite manner to influence airway development and regeneration.

The level of expression of SOX2 and SOX21 determines progenitor cell behavior

Common among stem-, progenitor-, and pluripotent- cells is their sensitivity to changes in SOX2 dosage [9, 10]. A notable difference among these tissues is the effect of SOX2 expression levels on either proliferation or differentiation. Neural progenitor cells exit the cell cycle upon *Sox2* deletion [11], while trachea epithelium exhibit altered differentiation programs without changes in cell proliferation (**chapter 4**). The high dosage depency of the regulatory function of SOX2 was nicely illustrated in neuronal progenitor cells. At high levels, SOX2 occupied low affinity binding sites to repress *Ccnd1* expression, while at low levels SOX2 was also found at high-affinity binding sites to activate *Ccnd1* [11]. In **chapter 4**, we showed that *in vitro* SOX2 and SOX21 levels increase during human and mouse basal cell differentiation, suggesting a dosage dependent regulatory function for both proteins. Like SOX2 transcriptional targets of SOX21 might also be dosage dependent, and may interfere with SOX2-induced genes, due to its conserved DNA binding domain and potential binding to SOX2 [8, 9] (Fig. 1).

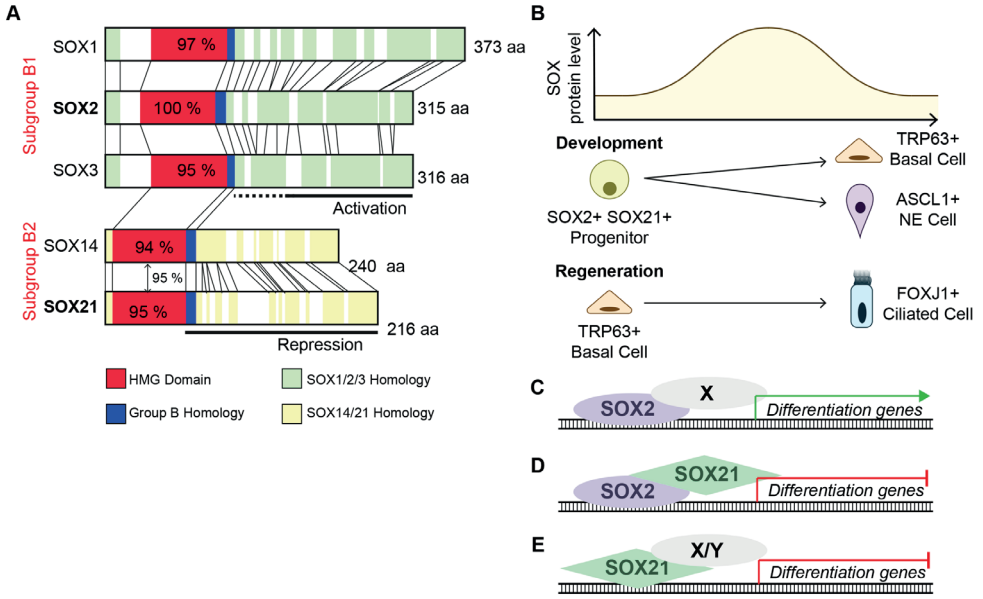


Fig. 1. *(A) Representative SOX proteins of group B1 and B2 are shown. Group B of the SOX protein family is subdivided into subgroup B1(activators) and B2 (repressors). Similarity score of the HMG domain amino acid (aa) sequence is relative to SOX2. Between subgroups B1 and B2, the similarities is found in 'Group B homology' besides the HMG domain [76]. (B) During the differentiation of airway progenitor cells and basal cells, SOX protein levels can fluctuate and competition can cause either transcription or repression of genes involved in differentiation. This figure illustrates a few examples on how this can influence gene transcription in relation to SOX2 and SOX21. (C) SOX2 can induce genes involved in differentiation, like TRP63 (chapter 4), but requires the binding of additional co-factors for active transcription. (D) SOX21 can interfere with this transcription to form a complex with SOX2, so additional factors cannot bind. This can either occur bound to the DNA (shown) or not bound to the DNA (not shown). (E) Another possibility is that, upon increasing levels of SOX21, SOX21 competes with SOX2 DNA binding by either binding to other additional cofactors (Y) or even by competing with similar co-factors (X) that bind SOX2.*

The importance of SOX2 dosage is further shown by a defect in the separation of trachea and esophagus early in development at decreased levels of SOX2 [4]. Upon separation *Sox2*, but also *Sox21* was increased 7 fold in the esophagus when compared to the trachea, suggesting that an early mechanism of SOX2 and SOX21 regulation of genes might be important in a correct separation [12]. However *Sox21*$^{-/-}$ mice do not show a defect in separation of the esophagus and trachea. Furthermore, *Sox21*$^{-/-}$ mice show an increased differentiation of airway progenitor cells, but does not cause any respiratory distress. Thus SOX21 may fine-tune SOX2 function, and be important in maintaining a progenitor state, but probably does not regulate sets of genes that are essential for the development of the esophagus, trachea or airways. Because *Sox21*$^{-/-}$ mice show increased differentiation, it would be interesting to pursue the effect of SOX21 on ageing of progenitor cells, as a higher turnover of progenitor populations might result in respiratory dysfunction later in life through depletion of the progenitor pool and thus homeostasis. Generating a conditional knock-out (KO) mouse of *Sox21* in airway epithelium would provide a model to study the effect of SOX21 on ageing of the lung specifically. In **chapter 4**, *Sox21*$^{+/-}$ mice did not show increased regeneration of airway epithelium compared to wild-type. An airway specific conditional Sox21 KO would provide further opportunities to study the effect of complete loss of SOX21 on airway specific injury, which is currently not possible due to the fragility of the *Sox21*$^{-/-}$ mouse.

Cofactors of SOX2 and SOX21 determine their regulatory function

How SOX2 and SOX21 regulate cell fate in individual cells depends further on the identity of its co-factors. SOX proteins alone do not elicit their action, but require binding to other transcription factors that provide specificity [9, 13, 14]. A number of putative SOX2 binding partners were identified in E18.5 lung using a mouse model containing a biotinylated SOX2 [15]. It would be interesting to identify co-factors that have the potential to bind SOX2 and SOX21, and thereby regulating transcription or repression of genes by forming a complex with either one. This might explain some of the contradicting phenotypes of increased or decreased differentiation upon loss of SOX2 or SOX21 respectively (**chapter 4 and 5**). Several hypotheses may explain this opposite effect, for example; (1) SOX2 and SOX21 can interact with each other, preventing additional binding of SOX2 or SOX21 to other co-factors, or (2) they have similar DNA binding motifs and bind similar co-factors due to their homology,, which would in a competition in both binding to the DNA and/or to their co-factors (Fig. 1B-E). As described previously, binding of SOX2 or SOX21 to DNA is dosage dependent. Similarly, the binding to their co-factors or each other might also be dosage dependent. Visualizing the interaction between SOX2 and SOX21 could be studied by the formation of a fluorescent complex when two non-fluorescent fragments are brought together [16]. The possibility to insert such fluorescent fragments into the SOX2 and SOX21 gene would provide the potential visualization

of the SOX2-SOX21 interaction within individual cells during development by live cell imaging and tissue clearing, or during adult progenitor differentiation in either air-liquid interface (ALI) or airway organoid cultures.

Determining co-factors of SOX2 and SOX21, is important to understand the mechanisms underlying changes in progenitor function resulting in airway malformations or disturbed differentiation. An example of an identified binding partner of SOX2 is the chromatin remodeling helicase CHD7. While SOX2 is associated with AEG (Anaphtalmia-EsophagealGenital)-syndrome, CHD7 is linked to CHARGE-syndrome (Coloboma, Heart malformations, Atresia of the choanae, Retarded growth, Genital anomalies and Ear anomalies). Clinical variation between these two syndromes frequently causes a failed diagnosis of these patients. The interaction between SOX2 and CHD7 provides a molecular explanation of the phenotypic gradient displayed by AEG and CHARGE patients and provides a connection between these at first sight genetic unrelated diseases [13]. SOX2 and SOX21 are often co-expressed in various progenitor cells and the identification of their common co-factors might also provide a link between now seemingly unrelated diseases, as seen in AEG and CHARGE syndrome. Identifying downstream targets of SOX2 and SOX21 and determining their co-factors is likely to provide a link between the underlying mechanisms causing congenital lung anomalies.

Both, squamous cell carcinoma (SQSC) originating from basal cells, and small cell lung cancer (SCLC) originating from NE cells, have been shown to have increased levels of SOX2 and/or SOX21 [17,19]. SOX proteins themselves are not druggable, hence identifying their targets would potentially help to develop new therapeutic strategies. Furthermore, interaction partners identified in SQSC, have been shown to be interaction partners of SOX2 in basal cells of healthy tissue [20]. The identification of co-factors of SOX2 and SOX21 with mass-spectrometry is difficult in low cell numbers or for specific cell types, therefore the use of cell lines originating from SCLC or SQSC with high expression levels of SOX2 and SOX21 may help to identify interacting partners in the normal development, homeostasis or differentiation of basal and NE cells.

In conclusion, identification of co-factors or DNA binding sites of SOX2 and SOX21, in a cell type specific manner and upon different dosages of SOX2 or SOX21 is a challenging perspective for future research. Recent methodology of single-cell RNA sequence provides a cell specific signature which could be related to expression levels of *Sox2* and *Sox21* [21]. In **chapter 4**, we showed that SOX2 and SOX21 levels differ in subsets of cells using previously published single cell RNA sequencing of human primary bronchial epithelial cells. Combining RNA sequencing, with ATAC-sequencing would provide a way to identify regulatory regions that direct transcription of cell-type specific genes [22]. Doing these experiments in airway specific cells depleted of SOX2, SOX21 or both, using either transgenic mice or siRNA mediated

depletion, would provide insight in the effect of loss of SOX2 and/or SOX21 on specific gene sets. In addition, with novel Chromatin Immunoprecipitation (CHIP) methodologies, less input material is needed which provides opportunities to study targets of SOX2 and SOX21 on possible FACS sorted specific cells. In future, a single-cell CHIP strategy might even be possible [23].

EXTRINSIC SIGNALING CUES COORDINATE LINEAGE DECISIONS AND THE MICROENVIRONMENT OF PROGENITOR CELLS

Cells undergo various differentiation, trans-differentiation and de-differentiation programs during development and regeneration, often regulated by the local tissue microenvironment. During development, different progenitors are present along the branching airways from the proximal to distal end and a subset of the adult progenitor cells remain in the epithelium at specific locations; submucosal glands, tracheal epithelium and neuroendocrine cells. Distinct niche components, including mesenchymal cells, neighboring epithelial cells, nerves, immune cells and extracellular matrix, can contribute to progenitor cell behavior, during development and from quiescence airway epithelium to active regeneration (Fig. 2).

Mesenchymal-epithelial signaling define the extrapulmonary airways

SOX21+ airway epithelium localizes with the presence of basal cells during development and in adult airway epithelium (**chapter 4**). Distal mesenchymal FGF10 signaling inhibits differentiation of SOX9+ tip progenitor cells early in lung development, but once epithelial cells are committed to the SOX2+ airway epithelium, FGF10 can also induce basal cell differentiation [24]. SOX21 might identify this proximal epithelial region where basal cell differentiation is induced and where underlying FGF10 signaling is present. In adult tracheal epithelium, basal cells maintain themselves and are recruited upon injury by mesenchymal expression of FGF10 (Fig. 2A). The secretion of FGF10 is regulated by epithelial activation of the Hippo pathway effector, YAP, leading to the expression and secretion of *Wnt7b* [25-27] (Fig 1A). YAP expression is essential for the maintenance of TRP63+ basal cells and loss of YAP results in increased differentiation to ciliated cells [26]. YAP regulates SOX2 expression in the transition from distal to proximal epithelium early in lung development [28]. Vice versa, SOX2 was shown to regulate transcription of YAP in cancer cells and osteo-adipo lineages [29, 30]. As SOX2 and SOX21 provides a balance between maintenance and differentiation of basal cells, it could well be that SOX2 and SOX21 are involved in transcription or silencing of YAP in basal cell differentiation and thereby influencing mesenchymal FGF10 signaling. Mutations in the *FGF10* gene are associated to an airway branch variation with high odds on the development of Chronic Obstructive Pulmonary Disease (COPD) [31]. Studying FGF and HIPPO signaling upon deletion of SOX2 and/or SOX21 would provide a potential link between SOX2, SOX21 and FGF10 signaling, which could

reveal potential novel ways of targeting COPD.

Notch signaling in airway epithelium guides differentiation

During development, we observed a heterogeneous expression of especially SOX21 within the airway epithelium (**chapter 4** and **5**). Notch signaling, is known for being expressed in a salt-and-pepper pattern, where receptor (Notch1 to Notch4) and ligand (Jag1, Jag2, Dll1, Dll3 and Dll4) are expressed from neighboring cells and influence cell fate decisions during organ development [32]. Inhibition of the Notch signaling pathway, increases the differentiation to ciliated and NE cells at the expense of secretory cells [33] (Fig. 2B). Jag2 shows a similar expression pattern as SOX21: starting proximal around E12.5 and from E13.5 onward it overlaps with the SOX2+ SOX21+ extrapulmonary airways, while Jag1 is only expressed in the SOX2+ intrapulmonary airways [34]. Within the inner ear and neurogenesis, SOX2 and SOX21 are linked to Notch activity by either regulating transcription of Jag1 and Notch responsive gene *Hes5* [5, 6, 13, 35]. SOX2 and SOX21 might regulate transcription of multiple Notch factors, like Jag1 and Jag2, in extrapulmonary airways, as well as *Hes1* in the development of Neuroendocrine (NE) and non-NE cell fate. Furthermore, the increase in NE precursor cells upon loss of SOX21, is similar to the phenotype observed by a loss of Dll ligands (**chapter 4** and **5**) [1, 34]. This would suggest that both SOX2 and SOX21 do not only target a single Notch pathway, but may actually regulate multiple Notch and non-Notch signaling pathways dependent on airway specific cell type, which can be a subject for future studies. Investigating the expression of SOX2 and SOX21 upon complete inhibition of the Notch pathway (*Rbpj* or *Pofut1* KO) or upon specific deletion of ligands or receptors can further establish whether SOX2 or SOX21 are up- or down-stream of Notch signaling. A complex role for Notch signaling, as both tumor suppressor or pro-tumorigenic, is defined in SCLC [36]. As, SOX2 and SOX21 are upregulated in a subset of SCLC, a potential link between Notch function and development of SCLC in relation with SOX2 or SOX21 might provide novel biomarkers to stratify the observed heterogeneity in SCLC.

Neuronal innervation influences progenitor cell differentiation

During the formation of the respiratory tract, an associated neural network is formed [37-39]. At the end of lung development, neurons innervate airway smooth muscle cells to control smooth muscle tone and trigger reflexes such as cough, but also innervate the SOX2+SOX21+ airway epithelium (Fig. 2C and 3A) , submucosal glands [40, 41] and NE cells (**chapter 5**, Fig. 2C). In **chapter 5**, we showed that the first histological sign of innervation of NE cells occurs around E16.5 and corresponds with a dip in SOX2 and SOX21 protein levels. During development of the salivary gland, it was shown that maintaining *Sox2* expression is dependent on innervation [42]. Such a role between *Sox2* expression and neuronal innervation has not yet

been explored in the airway epithelium.

Defective innervation of respiratory epithelium has been associated with the development of EA/TEF [43], as well as to branching defects in a CDH animal model [44]. Furthermore, disrupting neuronal innervation by laser ablation in explant cultures, resulted in a failure of local budding, suggesting involvement of neuronal innervation in the formation of the airway tree [45]. In the salivary gland, innervation

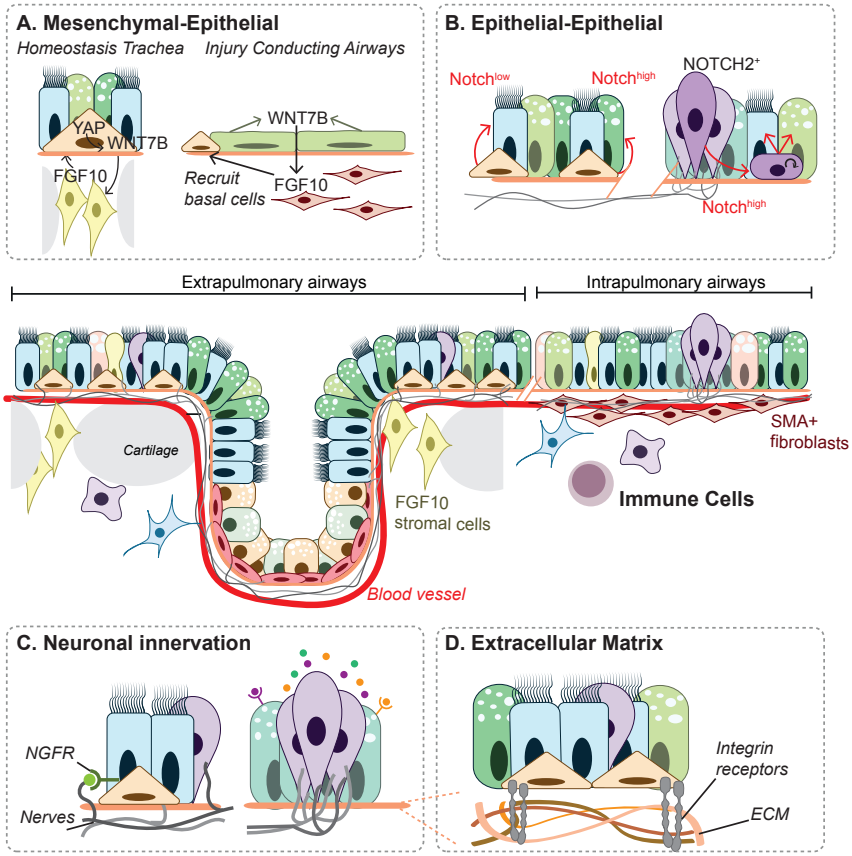


Fig. 2. *Around SOX2+SOX21+ airway epithelium, trachea, submucosal glands and neuroendocrine cells, different cellular (mesenchymal-, epithelial-, neuronal- and immune- cells) and non-cellular components (extracellular matrix) contribute to the behavior of progenitor cells. (A) HIPPO-WNT-FGF reciprocal signaling between basal and underlying stromal cells, contribute to the maintenance of basal cells in the tracheal epithelium. Upon injury, damaged epithelium secrete WNT7B and FGF10 is secreted from Smooth muscle actin positive (SMA+) fibroblasts, which recruit basal cells to regenerate the epithelium. (B) Notch signaling guides differentiation of basal cells to secretory (Notch^{high}) or ciliated (Notch^{low}) cells. A subset of neuroendocrine (NE) cells in a NE cluster in Notch2^{+}, and is suggested to function as a stem cell. Upon injury, the NE dedifferentiate to a transit amplifying cells under control of high Notch signaling. This transit amplifying cell subsequently differentiates to ciliated or secretory cells, probably also through receiving a high or low notch signal [73]. (C) The tracheal epithelium and neuroendocrine cells are innervated by neurons. Stimulation of NE can lead to the secretion of factors that might act on corresponding receptors present on the airway epithelium or can increase immune infiltrates [48, 74, 75]. Basal cells contain the P75-neuronal growth factor receptor (NGFR), which, potentially is stimulated by secretion of NGF by neuronal cells. However, this pathways has not yet been studied in the airway epithelium (D) Extracellular matrix (ECM) proteins are underlying the airway epithelial cells. Airway epithelial cells interact with ECM protein through integrin receptors, upon damage these interactions might change, which can influence progenitor cell behavior.*

has shown to be essential in maintaining the undifferentiated state of progenitor cells during development and regeneration [46, 47]. In future, explant culture with neuronal ablation or adding inhibitors of neuronal secretion could be used to specifically study the effect of innervation on progenitor cell differentiation during development and regeneration.

Immune cells increase complexity to regulation of airway progenitor behavior

In the lung, infiltration of immune cells correlates with lung diseases. It is known that basal cells and NE cells, both innervated, can have a function in the immune response and recruitment of immune cells [48-50]. In bronchopulmonary dysplasia (BPD), immune infiltration and NE cell hyperplasia is observed, but an interaction between neuronal innervation, immune response and onset of disease have has not yet been explored.

The infiltration of immune cells is a hallmark of tissue under stress, but they also have been thought as components that drive stem cell behavior within homeostatic conditions [51]. Macrophages have been shown to support stem cell survival in the intestine and regulatory T cells are found near the stem cell niche of hair follicles and promote regeneration [52, 53]. We (**chapter 2** and **3**) have shown that upon exposure of the inflammatory cytokine IL-13, basal cells differentiate to goblet cells at the cost of ciliated cells. However, the interaction between immune cells and airway progenitor cell differentiation under homeostatic conditions have yet to be explored.

The complexity of different cell types and their interaction between immune cells and other cells during lung development was recently studied by single cell sequencing of complete mouse lung tissues at various time points. Subsequently, the study examined ligand-receptor pairs between cell types in a spatial-temporal manner to map cellular interactions during development [54]. Most, interactions were found within the immune compartment and sporadic interactions were found between the immune and non-immune compartment, but these might include key signaling pathways for tissue development and homeostasis [54]. In future, immune cell-epithelial cell interactions during development, but also homeostasis, need to be confirmed and immunodeficient mouse models could help to identify the contribution of immune cells to progenitor cell fate decisions in the future.

The role of the extracellular matrix in the airway progenitor niche

Extracellular matrix (ECM) components can play a key part in determining cell fate during development and regeneration (Fig. 2D). The lung ECM continuously remodels during development [55], but the response of progenitor cells during development to ECM has not been studied well in the developing lung. The difference in dorsal-to-ventral localization of SOX2+SOX21+ airway epithelium early in lung development (**chapter 4**), could be due to difference in ECM composition,

in addition to differences in the mesenchymal population. Such a key role for cell-ECM interactions was demonstrated in the developing pancreas, where progenitors that encounter a laminin- or collagen-enriched environment results in endocrine differentiation, while progenitors exposed to a fibronectin- or vitronectin- enriched ECM maintain their progenitor state [56].

Several chronic respiratory diseases, including idiopathic pulmonary fibrosis, asthma, and chronic obstructive pulmonary disease (COPD), are associated with aberrant or excessive composition of ECM proteins. Besides immune infiltration, BPD also shows decreased vascularization, simplification of the alveolar compartment and a discomposed ECM [55, 57]. A key role was demonstrated for TGF-β in the remodeling of the ECM in a BPD animal model, and basal cells exposed to TGF-β can be a source of aberrant ECM production [58, 59]. Furthermore, production of matrix metalloproteinases by basal cells to remodel the ECM was shown to be essential for complete epithelial regeneration *in vivo* and differentiation towards the ciliated cell fate [60]. Studying the interaction between ECM components and progenitor cell behavior can help define therapeutic targets, but can also improve *in vitro* airway models to guide differentiation or to exactly mimic the physiological conditions found *in vivo*.

IN VITRO AIRWAY MODELLING TO STUDY AIRWAY PROGENITOR BEHAVIOR IN RELATION TO (CONGENITAL) AIRWAY DISEASES

Novel *in vitro* models provide an opportunity to study the different components contributing to progenitor behavior and can further bridge the knowledge gap between the findings made in animal models and human airway epithelium. Additionally, airway models resembling the *in vivo* environment freshly derived from patient material may further provide patient-specific cultures, which can be used for personal drug screening or sensitivity or identification of biomarkers. The risk of developing BPD in individual premature newborns which received oxygen supplementation and mechanical ventilation is difficult to predict. Comparing tracheal aspirates, either directly or after establishing airway-specific cultures, of premature newborns that develop BPD with those that do not develop BPD, will be especially be relevant for identification of biomarkers. The cultures would also be beneficial in providing the opportunity to gain mechanistic insight. For example, immune regulatory functions of basal cells in air-liquid interface cultures could be studied and compared between children that develop BPD and children that do not.

Studying lung development *in vitro*

The development of specific culture conditions for organoids, opens opportunities in studying human airway development and congenital lung diseases [61]. In **chapter 4**, we used fetal lung tip organoids, to compare the expression pattern of SOX21 in mouse to human lung development. In addition, specific airway organoid medium,

made it possible to culture airway organoids using primary tissue of CPAM patients (Fig. 3B). These primary cultures, make it possible to study CPAM versus healthy airway epithelial cells, as well as studying the effect of the mesenchymal populations on these cells. In future, combining healthy or CPAM epithelial cells with healthy or CPAM mesenchymal cells offers an opportunity to separate the epithelial intrinsic from the mesenchymal contribution to organoid formation. Despite these developments, human primary tissue is scarce and often difficult to genetically modify, therefore necissitating alternatives to study human lung development.

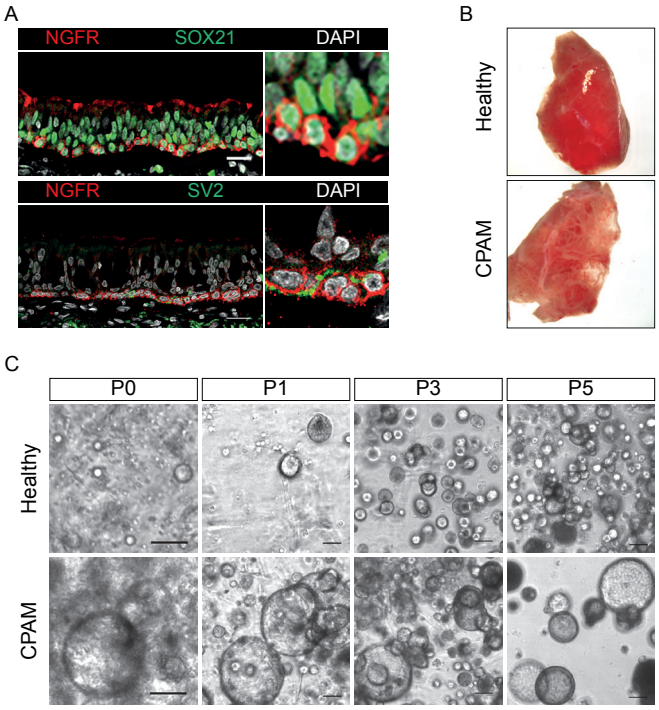


Fig. 3. *(A) The upper pictures show NGFR+ basal cells (red) in SOX21+ (green) human airway epithelium. In the lower figure, Synaptic Vesicle 2 (SV2) shows neuronal innervation of human airway epithelium. Scalebar = 50 µm. (B,C) Brightfield images from airway organoids obtained from lung resection material of a CPAM patient. Healthy lung material was obtained from healthy resection material, and CPAM was obtained from cystic lung tissue. P = passage number. Scale bar = 100 µm.*

Differentiation of human pluripotent stem cells into specific airway epithelial cells, like basal or neuroendocrine cells [62, 63], shows the potential to study every step of differentiation separately and further explore the role of SOX2 and SOX21 at each step. Using organoid cultures, it is possible to mimic different parts of the human lung, like the airway, alveolar compartment or even lung bud organoids [64, 65]. Furthermore, the lung bud organoids, which not only resemble the proximal-distal pattern of human branching morphogenesis, also contained the lung mesodermal lineage [66]. Despite some limitations, e.g. lack of vascular cells, this culture system provides a tool to study human lung development, epithelial-mesenchymal interactions and opens opportunities to study lung progenitor behavior with the use of genetically modified (human) pluripotent stem cells.

Although these cultures show the potential of studying airway development, they lack neighboring compartments during lung specification. A recent experiment, where a

posterior foregut spheroid was transferred adjacent to an anterior foregut spheroid, showed that without adding exogenous factors, the spheroids fused and start developing markers of biliary duct, pancreas and liver [67]. This specification of different organs by itself, would be interesting to study in respect to the separation of esophagus and trachea early in development, and how it goes awry in children born with EA/TEF.

Studying adult airway progenitors *in vitro*

With the use of *in vivo* lineage tracing studies, injury models and *in vitro* culturing, it became apparent that the airway epithelium contains a remarkable cellular plasticity. Dedifferentiation of mouse secretory cells to basal cells, with subsequent differentiation into full functional airway epithelium after injury was observed *in vivo* and *in vitro* [68]. We suggested in **chapter 3** that luminal cells obtained from either bronchoalveolar lavage (BAL) or tracheal aspirate (TA), also dedifferentiate *in vitro* to form airway organoids. However, we observed that basal cells obtained from the BAL and TA are less potent than basal cells obtained from primary bronchial tissue directly. Several questions remain: (1) is there indeed a dedifferentiation of luminal cells and (2) are dedifferentiated basal cells different from basal cells directly obtained from primary tissue? With the use of intestinal organoids, it was observed that both sorted LGR5+ (intestinal stem cells) and LGR5- cells can form intestinal organoids [69]. A similar separation of lung basal and non-basal cells in future would provide an opportunity to study dedifferentiation of luminal cells *in vitro*. This would allow a direct comparison between basal cells and dedifferentiated basal cells from the same tissue with respect to their gene expression patterns, epigenetic modifications, and response to simulations, like smoke exposure, Notch inhibition and IL-13 exposure.

By studying human airway diseases *in vitro*, we mostly depend on the availability of human primary tissue. With the use of organoid (3D) and culture plastic (2D) expansion, we show that air-exposed cultures can be derived from several sources, but that cultures differ in their response not only with respect of their source but also to their expansion method (**chapter 3**). Furthermore, it is important to realize that culturing basal cells may induce phenotypic changes, as is observed by an enrichment in KRT14+ basal cells in **chapter 2**. As for the study of human diseases, patient specific characteristics are of importance, and the effects of culturing cells should not be ignored. However, a recent study shows that strong intrinsic characteristics of basal cells, are being retained upon culture [70]. We also observed growth differences of primary cells obtained from cystic structures or healthy tissue of CPAM patients in culture (Fig. 3B), suggesting that some patient-specific characteristics are maintained. A challenge for future research will be to identify specific airway progenitor functions mimicking the *in vivo* situation rather than researching potential *in vitro* artefacts.

Due to the generation of airway specific culture media, the interaction between airway epithelial cells and other cell types is often ignored as for example, feeder cells are no longer needed. As the interaction between different cell types play a fundamental role in airway epithelium homeostasis and regeneration (see above), it would be important to include these compartments in future airway models [71, 72].

CONCLUDING REMARKS

Within this thesis, we elaborated on the understanding of airway progenitor behavior within airway development and regeneration. We identified, that changes in expression levels of SOX2 and SOX21 can influence the balance in progenitor maintenance and differentiation. Understanding the molecular mechanisms up- and down- stream of these two SOX transcription factors, might especially be of importance in the development of chronic airway or congenital lung diseases. The influence of SOX2 and SOX21 in progenitor function described in **chapter 4** and their upregulation in specific types of lung cancers, opens opportunities to further relate their function to tumor formation and progression and the potential findings of new therapeutic targets. We provided an *in vitro* model to study the airway epithelial compartment in relation to BPD for the first time through the development of human airway disease specific cultures, such as the tracheal aspirate from premature newborns (**chapter 3**). This can further contribute to the identification of biomarkers or understanding of disease progression. Furthermore, in **chapter 5**, we showed preliminary data of the contribution of SOX2 and SOX21 in the development of NE cells and using these models in future might help to understand NE cell function, for example, in the onset of CDH.

Finally, understanding airway progenitor functions and their niche *in vivo* will be essential for mimicking the human lung *in vitro*. In the future, regenerative medicine by either cell transplantation, or creating transplantable organs *in vitro*, might provide therapy for end-stage lung disease. Either synthetic (3D-printed) scaffolds or de-cellularised ECM scaffolds, combined with re-cellularisation with autologous progenitor cells, may lead to fully functional transplantable organs in the future. Although, such approaches might be far away in the future for lung diseases, the identification of specified SOX21+ SOX2+ airway regions, as well as the generation of novel *in vitro* airway models will contribute to the improvement of current protocols in the generation of lung specific cells types for clinical applications.

REFERENCES

1. Que, J., et al., Multiple roles for Sox2 in the developing and adult mouse trachea. Development, 2009. 136(11): p. 1899-907.
2. Arnold, K., et al., Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. Cell Stem Cell, 2011. 9(4): p. 317-29.
3. Mandalos, N.P. and E. Remboutsika, Sox2: To crest or not to crest? Semin Cell Dev Biol, 2017. 63: p. 43-49.
4. Que, J., et al., Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. Development, 2007. 134(13): p. 2521-31.
5. Freeman, S.D. and N. Daudet, Artificial induction of Sox21 regulates sensory cell formation in the embryonic chicken inner ear. PLoS One, 2012. 7(10): p. e46387.
6. Matsuda, S., et al., Sox21 promotes hippocampal adult neurogenesis via the transcriptional repression of the Hes5 gene. J Neurosci, 2012. 32(36): p. 12543-57.
7. Kuzmichev, A.N., et al., Sox2 acts through Sox21 to regulate transcription in pluripotent and differentiated cells. Curr Biol, 2012. 22(18): p. 1705-10.
8. Mallanna, S.K., et al., Proteomic analysis of Sox2-associated proteins during early stages of mouse embryonic stem cell differentiation identifies Sox21 as a novel regulator of stem cell fate. Stem Cells, 2010. 28(10): p. 1715-27.
9. Kamachi, Y. and H. Kondoh, Sox proteins: regulators of cell fate specification and differentiation. Development, 2013. 140(20): p. 4129-44.
10. Sarkar, A. and K. Hochedlinger, The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. Cell Stem Cell, 2013. 12(1): p. 15-30.
11. Hagey, D.W. and J. Muhr, Sox2 acts in a dose-dependent fashion to regulate proliferation of cortical progenitors. Cell Rep, 2014. 9(5): p. 1908-1920.
12. Kim, E., et al., Isl1 Regulation of Nkx2.1 in the Early Foregut Epithelium Is Required for Trachea-Esophageal Separation and Lung Lobation. Dev Cell, 2019. 51(6): p. 675-683 e4.
13. Engelen, E., et al., Sox2 cooperates with Chd7 to regulate genes that are mutated in human syndromes. Nat Genet, 2011. 43(6): p. 607-11.
14. Gontan, C., et al., Exportin 4 mediates a novel nuclear import pathway for Sox family transcription factors. J Cell Biol, 2009. 185(1): p. 27-34.
15. Schilders, K., et al., Generation of a biotinylatable Sox2 mouse model to identify Sox2 complexes in vivo. Transgenic Res, 2018. 27(1): p. 75-85.
16. Wolff, H., et al., Live-cell assay for simultaneous monitoring of expression and interaction of proteins. Biotechniques, 2006. 41(6): p. 688, 690, 692.
17. Ferone, G., et al., SOX2 Is the Determining Oncogenic Switch in Promoting Lung Squamous Cell Carcinoma from Different Cells of Origin. Cancer Cell, 2016. 30(4): p. 519-532.
18. Gure, A.O., et al., Serological identification of embryonic neural proteins as highly immunogenic tumor antigens in small cell lung cancer. Proc Natl Acad Sci U S A, 2000. 97(8): p. 4198-203.
19. Titulaer, M.J., et al., SOX antibodies in small-cell lung cancer and Lambert-Eaton myasthenic syndrome: frequency and relation with survival. J Clin Oncol, 2009. 27(26): p. 4260-7.
20. Kim, B.R., et al., Identification of the SOX2 Interactome by BioID Reveals EP300 as a Mediator of SOX2-dependent Squamous Differentiation and Lung Squamous Cell Carcinoma Growth. Mol Cell Proteomics, 2017. 16(10): p. 1864-1888.
21. Plasschaert, L.W., et al., A single-cell atlas of the airway epithelium reveals the CFTRrich pulmonary ionocyte. Nature, 2018. 560(7718): p. 377-381.
22. Ackermann, A.M., et al., Integration of ATAC-seq and RNA-seq identifies human alpha cell and beta cell signature genes. Mol Metab, 2016. 5(3): p. 233-244.
23. Dahl, J.A. and G.D. Gilfillan, How low can you go? Pushing the limits of low-input ChIPseq. Brief Funct Genomics, 2018. 17(2): p. 89-95.
24. Volckaert, T., et al., Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors. Development, 2013. 140(18): p. 3731-42.
25. Volckaert, T., et al., Fgf10-Hippo Epithelial-Mesenchymal Crosstalk Maintains and Recruits Lung Basal Stem Cells. Dev Cell, 2017. 43(1): p. 48-59 e5.
26. Zhao, R., et al., Yap tunes airway epithelialsize and architecture by regulating the identity, maintenance, and self-renewal of stem cells. Dev Cell, 2014. 30(2): p. 151-65.
27. Volckaert, T., et al., Parabronchial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. J Clin Invest, 2011. 121(11): p. 4409-19.
28. Mahoney, J.E., et al., The hippo pathway effector Yap controls patterning and differentiation of airway epithelial progenitors. Dev Cell, 2014. 30(2): p. 137-50.
29. Basu-Roy, U., et al., Sox2 antagonizes the Hippo pathway to maintain stemness in cancer

cells. Nat Commun, 2015. 6: p. 6411.
30. Seo, E., et al., SOX2 regulates YAP1 to maintain stemness and determine cell fate in the osteo-adipo lineage. Cell Rep, 2013. 3(6): p. 2075-87.
31. Smith, B.M., et al., Human airway branch variation and chronic obstructive pulmonary disease. Proc Natl Acad Sci U S A, 2018. 115(5): p. E974-E981.
32. Kiyokawa, H. and M. Morimoto, Notch signaling in the mammalian respiratory system, specifically the trachea and lungs, in development, homeostasis, regeneration, and disease. Dev Growth Differ, 2019.
33. Morimoto, M., et al., Different assemblies of Notch receptors coordinate the distribution of the major bronchial Clara, ciliated and neuroendocrine cells. Development, 2012. 139(23): p. 4365-73.
34. Stupnikov, M.R., et al., Jagged and Delta ligands control distinct events during airway progenitor cell differentiation. Elife, 2019. 8.
35. Pan, W., et al., Ectopic expression of activated notch or SOX2 reveals similar and unique roles in the development of the sensory cell progenitors in the mammalian inner ear. J Neurosci, 2013. 33(41): p. 16146-57.
36. Lim, J.S., et al., Intratumoural heterogeneity generated by Notch signalling promotes small-cell lung cancer. Nature, 2017. 545(7654): p. 360-364.
37. Aven, L. and X. Ai, Mechanisms of respiratory innervation during embryonic development. Organogenesis, 2013. 9(3): p. 194-8.
38. Tollet, J., A.W. Everett, and M.P. Sparrow, Development of neural tissue and airway smooth muscle in fetal mouse lung explants: a role for glial-derived neurotrophic factor in lung innervation. Am J Respir Cell Mol Biol, 2002. 26(4): p. 420-9.
39. Tollet, J., A.W. Everett, and M.P. Sparrow, Spatial and temporal distribution of nerves, ganglia, and smooth muscle during the early pseudoglandular stage of fetal mouse lung development. Dev Dyn, 2001. 221(1): p. 48-60.
40. Whitsett, J.A., Airway Epithelial Differentiation and Mucociliary Clearance. Ann Am Thorac Soc, 2018. 15(Suppl 3): p. S143-S148.
41. Carraro, G. and B.R. Stripp, Roles for Myoepithelial Cells in the Formation and Maintenance of Submucosal Glands. Am J Respir Cell Mol Biol, 2017. 56(6): p. 685686.
42. Emmerson, E., et al., SOX2 regulates acinar cell development in the salivary gland. Elife, 2017. 6.
43. Liu, X.M., et al., Abnormal development of lung innervation in experimental esophageal atresia. Eur J Pediatr Surg, 2012. 22(1): p. 67-73.
44. Rhodes, J., et al., Defective parasympathetic innervation is associated with airway branching abnormalities in experimental CDH. Am J Physiol Lung Cell Mol Physiol, 2015. 309(2): p. L168-74.
45. Bower, D.V., et al., Airway branching has conserved needs for local parasympathetic innervation but not neurotransmission. BMC Biol, 2014. 12: p. 92.
46. Knox, S.M., et al., Parasympathetic stimulation improves epithelial organ regeneration. Nat Commun, 2013. 4: p. 1494.
47. Knox, S.M., et al., Parasympathetic innervation maintains epithelial progenitor cells during salivary organogenesis. Science, 2010. 329(5999): p. 1645-7.
48. Branchfield, K., et al., Pulmonary neuroendocrine cells function as airway sensors to control lung immune response. Science, 2016. 351(6274): p. 707-10.
49. Whitsett, J.A. and T. Alenghat, Respiratory epithelial cells orchestrate pulmonary innate immunity. Nat Immunol, 2015. 16(1): p. 27-35.
50. Amatngalim, G.D., et al., Basal cells contribute to innate immunity of the airway epithelium through production of the antimicrobial protein RNase 7. J Immunol, 2015. 194(7): p. 3340-50.
51. Naik, S., et al., Two to Tango: Dialog between Immunity and Stem Cells in Health and Disease. Cell, 2018. 175(4): p. 908-920.
52. Ali, N., et al., Regulatory T Cells in Skin Facilitate Epithelial Stem Cell Differentiation. Cell, 2017. 169(6): p. 1119-1129 e11.
53. Sehgal, A., et al., The role of CSF1R-dependent macrophages in control of the intestinal stem-cell niche. Nat Commun, 2018. 9(1): p. 1272.
54. Cohen, M., et al., Lung Single-Cell Signaling Interaction Map Reveals Basophil Role in Macrophage Imprinting. Cell, 2018. 175(4): p. 1031-1044 e18.
55. Mizikova, I. and R.E. Morty, The Extracellular Matrix in Bronchopulmonary Dysplasia: Target and Source. Front Med (Lausanne), 2015. 2: p. 91.
56. Mamidi, A., et al., Mechanosignalling via integrins directs fate decisions of pancreatic progenitors. Nature, 2018. 564(7734): p. 114-118.
57. Alvira, C.M. and R.E. Morty, Can We Understand the Pathobiology of Bronchopulmonary Dysplasia? J Pediatr, 2017. 190: p. 27-37.
58. Nakanishi, H., et al., TGF-beta-neutralizing antibodies improve pulmonary alveologenesis and vasculogenesis in the injured newborn lung. Am J Physiol Lung Cell Mol Physiol, 2007. 293(1): p. L151-61.

59. van der Velden, J.L., et al., TGF-beta1-induced deposition of provisional extracellular matrix by tracheal basal cells promotes epithelial-to-mesenchymal transition in a c-Jun NH2-terminal kinase-1-dependent manner. Am J Physiol Lung Cell Mol Physiol, 2018. 314(6): p. L984-L997.
60. Gharib, S.A., et al., Matrix metalloproteinase-7 coordinates airway epithelial injury response and differentiation of ciliated cells. Am J Respir Cell Mol Biol, 2013. 48(3): p. 390-6.
61. Sachs, N., et al., Long-term expanding human airway organoids for disease modeling. EMBO J, 2019. 38(4).
62. Drick, N., et al., Generation of a NKX2.1 - p63 double transgenic knock-in reporter cell line from human induced pluripotent stem cells (MHHi006-A-4). Stem Cell Res, 2019. 42: p. 101659.
63. Chen, H.J., et al., Generation of pulmonary neuroendocrine cells and SCLC-like tumors from human embryonic stem cells. J Exp Med, 2019. 216(3): p. 674-687.
64. Dye, B.R., et al., In vitro generation of human pluripotent stem cell derived lung organoids. Elife, 2015. 4.
65. Yamamoto, Y., et al., Long-term expansion of alveolar stem cells derived from human iPS cells in organoids. Nat Methods, 2017. 14(11): p. 1097-1106.
66. Chen, Y.W., et al., A three-dimensional model of human lung development and disease from pluripotent stem cells. Nat Cell Biol, 2017. 19(5): p. 542-549.
67. Koike, H., et al., Modelling human hepato-biliary-pancreatic organogenesis from the foregut-midgut boundary. Nature, 2019. 574(7776): p. 112-116.
68. Tata, P.R., et al., Dedifferentiation of committed epithelial cells into stem cells in vivo. Nature, 2013. 503(7475): p. 218-23.
69. Serra, D., et al., Self-organization and symmetry breaking in intestinal organoid development. Nature, 2019. 569(7754): p. 66-72.
70. Ordovas-Montanes, J., et al., Allergic inflammatory memory in human respiratory epithelial progenitor cells. Nature, 2018. 560(7720): p. 649-654.
71. Takebe, T., B. Zhang, and M. Radisic, Synergistic Engineering: Organoids Meet Organs-on-a-Chip. Cell Stem Cell, 2017. 21(3): p. 297-300.
72. Park, S.E., A. Georgescu, and D. Huh, Organoids-on-a-chip. Science, 2019. 364(6444): p. 960-965.
73. Ouadah, Y., et al., Rare Pulmonary Neuroendocrine Cells Are Stem Cells Regulated by Rb, p53, and Notch. Cell, 2019. 179(2): p. 403-416 e23.
74. Barrios, J., et al., Pulmonary Neuroendocrine Cells Secrete gamma-Aminobutyric Acid to Induce Goblet Cell Hyperplasia in Primate Models. Am J Respir Cell Mol Biol, 2019. 60(6): p. 687-694.
75. Gu, X., et al., Chemosensory functions for pulmonary neuroendocrine cells. Am J Respir Cell Mol Biol, 2014. 50(3): p. 637-46.
76. Uchikawa, M., Y. Kamachi, and H. Kondoh, Two distinct subgroups of Group B Sox genes for transcriptional activators and repressors: their expression during embryonic organogenesis of the chicken. Mech Dev, 1999. 84(1-2): p. 103-20.

Appendix

Summary / Samenvatting

Curriculum Vitae

List of Publications

PhD Portfolio

Acknowledgements / Dankwoord

SUMMARY

Congenital lung diseases, like congenital diaphragmatic hernia and congenital pulmonary airway malformations, are often associated with an aberrant differentiation of endodermal progenitor cells. In addition, upon birth and later in life, proper airway progenitor function is important for maintaining airway epithelium homeostasis and preventing lung diseases. Both intrinsic and extrinsic mechanisms control progenitor maintenance, either by transcriptional regulation or by extracellular signals from the progenitor niche. Within these mechanisms, an overlap in airway progenitor function can be found during airway development, homeostasis and adult airway regeneration. This thesis describes the development and usage of *in vitro* and *in vivo* models to study the transcription factors SOX2 and SOX21 in airway progenitor function during development and regeneration.

In **chapter 2**, culture conditions are defined to expand mouse tracheal epithelial cells (MTECs), for the establishment of air-liquid interface (ALI) culture. In this way, a method was established to study the differentiation of basal cells to secretory- and ciliated cells with a limited number of mice. Despite the possibility to expand MTECs, a decreased differentiation of basal cells to ciliated cells was observed. This decreased differentiation potential was related to a shift in basal cell population, from a KRT14 negative population without expansion, to a KRT14 positive population with expansion. The decreased differentiation in basal cells to ciliated cells could be improved by prolonging the ALI-induced differentiation or by the inhibition of Notch signaling, suggesting that basal cells did retain their ability to differentiate.

In **chapter 3**, the possibility to use organoids for the expansion of a low number of human airway epithelial cells (AECs) obtained from available clinical sources was investigated. These organoids, were subsequently cultured on ALI system to develop a more relevant air-exposed model to study airway epithelial function. First, it was shown that the expansion method of AECs from bronchial tissue (BT), either as organoid- or the conventional 2D method, does not change ALI-induced differentiation. Next, ALI-induced differentiation, cigarette smoke induced ER stress and response to IL-13 and Notch inhibition, on organoid expanded AECs obtained from different sources; tracheal aspirate (TA), bronchoalveolar lavage (BAL) and BT was compared. From all sources, well-differentiated ALI cultures, containing basal, ciliated and secretory cells, could be established. However, BAL and TA derived ALI cultures showed to be less responsive to IL-13 and Notch inhibition, compared to BT derived ALI cultures. It was suggested that, when the source of AECs is absent in basal cells, like the TA and BAL but not BT, luminal cells de-differentiate to basal cells in organoid culture but do not regain all basal cell functionality. The potential to use this method to culture and expand limited number of cells present in TA of neonates, provides for the first time a humanized model to study the potential involvement of AECs in the development of bronchopulmonary dysplasia.

Chapter 4 describes that high levels of SOX2 during airway development coincides with expression of SOX21 in the trachea and main bronchi. This newly defined SOX2+ and SOX21+ region, overlaps with the differentiation of progenitor cells to basal- and neuroendocrine (NE) cells (**chapter 5**). By the use of mouse models deficient in either SOX2 or SOX21, it was shown that SOX2 stimulates and SOX21 suppresses airway progenitor differentiation during both development and regeneration of the extrapulmonary airways. In addition, SOX21 influenced SOX2 induced differentiation, by antagonizing SOX2 on different promotors involved in differentiation. The use of human fetal lung organoids and human adult airway ALI culture showed that SOX2 and SOX21 expression is conserved in human airway development.

In **chapter 5**, the function of SOX2 and SOX21 in a small subpopulation of airway epithelial cells, the NE cell was explored. Here, a distinct role for both SOX2 and SOX21 was observed at different stages of NE development. SOX21 suppresses differentiation of SOX2+ airway progenitors to NE cells, while deficiency in SOX2 only causes a delay in maturation of NE cells during development. Both, SOX2 and SOX21, were involved in the clustering of NE cells and show opposite phenotypes; deficiency of SOX2 resulted in more single solitary NE cells, while deficiency of SOX21 resulted in a higher percentage of NE cells present in clusters. This preliminary work is the start of understanding the basic mechanisms of how NE cell development is regulated and might contribute to the understanding of NE hyperplasia, which is associated with several lung disease.

In **chapter 6**, the main results and their contribution to the field of airway progenitor cell function, (congenital) airway diseases and *in vitro* culture models are discussed.

In **conclusion**, this thesis presents **novel *in vitro* models** to study airway epithelial function and shows that slight changes in expression pattern of **SOX2** and **SOX21** can disturb airway development and regeneration.

SAMENVATTING

De oorzaak van congenitale longaandoeningen, zoals Congenitale Hernia Diafragmatica en Congenitale pulmonale luchtwegafwijkingen, is vaak geassocieerd met een defect in de differentiatie van long voorlopercellen. Ook wanneer de longen volgroeid zijn, is het belangrijk dat luchtweg voorlopercellen goed functioneren om een homeostase van het luchtwegepitheel te behouden en aandoeningen aan de luchtwegen te voorkomen. Het behouden van de voorlopercel of het differentiëren naar andere luchtweg specifieke cellen, wordt gecontroleerd door intracellulaire veranderingen en extracellulaire signalering, die aanwezig is in de niche van de voorlopercellen. Er is een overlap in de mechanismes van luchtweg voorlopercel functie tijdens ontwikkeling van de luchtwegen en homeostase en regeneratie van het luchtwegepitheel. In dit proefschrift zijn *in vitro* en *in vivo* modellen gebruikt om de functie van transcriptiefactoren SOX2 en SOX21 te bestuderen in luchtweg voorlopercellen tijdens de ontwikkeling en regeneratie.

In **hoofdstuk 2**, zijn kweekcondities gegenereerd om muis tracheaal epitheelcellen (MTEC) te expanderen en vervolgens te laten differentiëren op een lucht geëxposeerde (airliquid interfase (ALI)) kweek. Hierdoor, kon met een minimaal aantal muizen, de differentiatie van basaal cellen naar secretoire- en gecillieerde-cellen worden bestudeerd. Het exanderen van MTEC, ging samen met een verminderde differentiatie naar gecilieerde cellen en een verrijking in een subpopulatie van basaal cellen, die positief zijn voor Keratine 14 (KRT14+). Deze verminderde differentiatie naar gecilieerde-cellen kon verbeterd worden door de tijd van de ALI-kweek te verlengen of door een remmer van de Notch signalering toe te voegen. Dit suggereert dat ondanks de verrijkingen van KRT14+ basaal cellen, ze de potentie behouden om te differentiëren.

In **hoofdstuk 3**, werd de mogelijkheid onderzocht om organoïden te gebruiken om luchtweg epitheelcellen (LEC) te expanderen. Deze organoïden, werden vervolgens gekweekt op een ALI-systeem om een relevanter lucht geëxposeerd model te creëren. Eerst is er aangetoond dat de expansiemethode van LEC verkregen van bronchiaal weefsel (BW), de conventionele 2D methode of door het gebruiken van organoïden, niet de differentiatie op de ALI kweek beïnvloedt. Vervolgens, werden LEC verkregen van tracheaal aspiraten (TA), bronchie-alveolaire lavage (BAL) en BW, in organoïds geëxpandeerd en op ALI-kweek gezet. De ALI-geïnduceerde differentiatie, ER stress geïnduceerd door sigaretten rook en respons op IL-13 en Notch inhibitie, werd vergeleken tussen deze kweken. De ALI-kweek van alle bronnen bevatte een gelijke hoeveelheid aan basaal-, gecilieerde- en secretoire-cellen. Desondanks, reageerde de BAL en TA opgezette ALI-kweken verminderd op blootstelling aan IL-13 of Notch inhibitie, dit in vergelijking met de BW opgezette ALI-kweken. Dit effect wordt mogelijk gecreëerd doordat TA en BAL maar niet BW, origineel geen basaal cellen bevatten en dat eerst luminale-cellen moeten de-differentiëren naar basaal cellen in

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de organoïde kweek. Deze ge-de-differentieerde basaal cellen verkrijgen vervolgens niet alle basaal voorlopercel potentie. In dit hoofdstuk, zijn wij de eerste die een model laten zien om LEC te bestuderen uit TA afkomstig van prematuur geboren kinderen. Dit is vooral van belang om te onderzoeken wat de rol van LECs zijn in de ontwikkeling van bronchopulmonaire dysplasie.

In **hoofdstuk 4** werd laten zien dat hoge niveaus van SOX2 expressie tijdens de ontwikkeling van de luchtwegen samengaat met de expressie van SOX21 in de trachea en de hoofd bronchi. Deze nieuw gedefinieerde SOX2+ SOX21+ regio gaat samen met de differentiatie van voorlopercellen naar basaal- en neuro-endocrine (NE)- cellen (**hoofdstuk 5**). Met het gebruik van muismodellen, die minder SOX2 of SOX21 tot expressie brengen, werd laten zien dat SOX2 belangrijk is in het stimuleren van differentiatie terwijl SOX21 de differentiatie van luchtweg voorlopercellen remt. SOX21 beïnvloedt de SOX2 geïnduceerde differentiatie door SOX2 functie te inhiberen bij het activeren van verschillende promotoren die betrokken zijn bij de differentiatie van voorlopercellen. Door humane foetale organoïden en humane volwassen luchtweg ALI kweken te gebruiken, is er aangetoond dat de expressie van SOX2 en SOX21 geconserveerd is in humaan luchtwegepitheel.

In **hoofdstuk 5** werd de functie van SOX2 en SOX21 verder bestudeerd in een kleine subpopulatie van luchtweg epitheelcellen, de NE-cellen. Verschillende SOX2 en SOX21 functies in de verschillende stadia van NE-cel ontwikkeling is in dit hoofdstuk beschreven. SOX21 remt de differentiatie van SOX2+ luchtweg voorlopercellen naar NE cellen, terwijl SOX2 vooral belangrijk is in de maturatie van NE cellen. Beide, SOX2 en SOX21, spelen een rol in het clusteren van NE-cellen; minder SOX2 liet meer enkele NE-cellen in de luchtweg zien, terwijl minder SOX21 leidde tot meer NE-cellen in clusters. Het preliminaire werk gepresenteerd in dit hoofdstuk is een start in het begrijpen van de basale mechanismes betrokken bij NE-cel ontwikkeling en kan bijdragen aan het begrijpen van NE cel hyperplasie, die aanwezig is in een verschillend aantal (congenitale) longaandoeningen.

In **hoofdstuk 6**, zijn de hoofdresultaten en hun bijdrage in het veld van luchtweg voorlopercellen, (congenitale) longaandoeningen en *in vitro* kweekmodellen bediscussieerd.

Tot slot, dit proefschrift presenteert **nieuwe *in vitro* modellen** om luchtweg epitheelfunctie te onderzoeken en laat zien dat kleine veranderingen in expressieniveaus van **SOX2** en **SOX21** kan leiden tot een afwijkende luchtweg ontwikkeling en luchtweg regeneratie.

CURRICULUM VITAE

Personal Information

Name:	Evelien
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Date of Birth:	July 20th, 1988
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Work Experience

2014 – 2019	**PhD student**, Dept. of Pediatric Surgery, Cell Biology, Sophia Children's hospital, Erasmus MC Promotor: Prof. Dr. D. Tibboel, Co-Promotor: Dr. R.J. Rottier
2012 – 2013	**Research assistant**, Dept. of Pathology and Cell Biology, Columbia University, New York City Supervisor: Dr. A. Yamamoto

Education

2010 – 2012	University of Groningen, **Master Biomedical Sciences** (Completed, degree: Master of Science) Research project 1 "Oligodendrocyte response to various stress" University of Groningen Supervisors: Dr. J. Vinet and Prof H.W.G.M. Boddeke Research project 2 "Modulating polyglutamine aggregation: A differential role for HSPB7 and ALFY", Columbia University, New York City Supervisors: Dr. A. Yamamoto (Columbia University) and Prof. H. H. Kampinga (University of Groningen)
2007 – 2010	University of Groningen, **Bachelor Biomedical Sciences** (Completed, degree: Bachelor of Science) Minor: Biomedical engineering

LIST OF PUBLICATIONS

Fox LM, Kim K, Johnson CW, Chen S, Croce KR, Victor MB, **Eenjes E**, Bosco JR, Randolph LK, Dragatsis I, Dragich JM, Yoo AS, Yamamoto A. Huntington's Disease Pathogenesis Is Modified In Vivo by Alfy/Wdfy3 and Selective Macroautophagy. *Neuron Published: 30 Dec 2019*

Eenjes E, Yang-Klingler YJ, Yamamoto A. Monitoring aggregate clearance and formation in cell-based assays. *Methods Mol Biol. 2019;1873:157-169*

Eenjes E*, Mertens TCJ*, Buscop-van Kempen MJ, van Wijck Y, Taube C, Rottier RJ, Hiemstra PS. A novel method for expansion and differentiation of mouse tracheal epithelial cells in culture. *Sci Rep. 2018 May 9;8(1):7349.*

Schilders K, **Eenjes E**, Edel G, de Munck AB, van Kempen MB, Demmers J, Wijnen R, Tibboel D, Rottier RJ. Generation of a biotinylatable Sox2 mouse model to identify Sox2 complexes in vivo. *Transgenic Res. 2018 Feb;27(1):75-85.*

Dragich JM, Kuwajima T, Hirose-Ikeda M, Yoon MS, **Eenjes E**, Bosco JR, Fox LM, Lystad AH, Oo TF, Yarygina O, Mita T, Waguri S, Ichimura Y, Komatsu M, Simonsen A, Burke RE, Mason CA, Yamamoto A. Autophagy linked FYVE (Alfy/WDFY3) is required for establishing neuronal connectivity in the mammalian brain. *Elife. 2016 Sep 20;5*

Schilders KA*, **Eenjes E***, van Riet S, Poot AA, Stamatialis D, Truckenmüller R, Hiemstra PS, Rottier RJ. Regeneration of the lung: Lung stem cells and the development of lung mimicking devices. *Respir Res. 2016 Apr 23;17:44.*

Eenjes E, Dragich JM, Kampinga HH, Yamamoto A. Distinguishing aggregate formation and aggregate clearance using cell-based assays. *J Cell Sci 2016 Mar 15; 129(6):1260-70*

PHD PORTFOLIO

Courses		
Biochemistry and Biophysics	2014	3
Genetics	2014	3
Laboratory animal sciences (Article 9)	2015	3
Scientific Integrity	2015	0,3
Microscopic image analysis: from theory to practice	2015	0,8
Ensemble Gene Browsing course	2015	0,6
English Biomedical Writing and Communications	2017	3
Biostatistical methods I: Basic Principles Part A	2017	2
Practical Linux course	2017	0,4
Safely working in Laboratory	2018	0,3
Seminar and workshops		
Monday morning weekly work discussions Cell Biology	2014-2019	2
MGC PhD workshop, Maastricht	2015	1
PhD meeting Cell Biology Department	2016-2018	1
Weekly Lab meeting	2014-2019	2
Bi-weekly Journal club	2014-2019	1
MGC PhD workshop, Leuven	2017	1
Monthly ACE-PH meeting	2017-2018	1
(Inter)national conferences		
NRS meeting: Animal models in science, Utrecht	2013	1
Longdagen, Utrecht	2015	1
NRS meeting: Young Inverstigator Symposium (presentation) Amsterdam	2016	1
Organoid meeting, Cambridge, UK	2017	2
GRC lung development, injury and Repair(poster), New London, USA	2017	2
European Pediatric Surgeons' Association (presentation), Belgrade, Serbia	2019	2
MGC Sypmposia (presentation), Rotterdam	2019	2
Themeday (presentation), Rotterdam	2019	1
Teaching		
Supervision Master student Molecular Medicine		5
Supervision Master student Molecular Medicine		5
Supervision Bachelor Student Practical Laboratory School		4
Supervision Bachelor Student Practical Laboratory School		4
Supervision Bachelor Student Practical Laboratory School		4
External Committee member thesis defence Hogeschool Rotterdam		1
Total		**60,4**