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Targeting basal cell-mediated epithelial repair: preclinical evidence on their role in treating chronic obstructive pulmonary disease (COPD)

By

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Contents

Abstract

Chronic obstructive pulmonary disease (COPD) is a chronic and progressive lung disease that is characterized by chronic inflammation and airflow obstruction due to airway remodeling, leading to a decline in lung function. COPD is the fourth most common cause of death and affects the daily lives of millions of patients globally. There are currently no curative treatments, meaning novel treatments are greatly desired. When lungs are injured, various progenitor cells, including basal cells, proliferate to restore the epithelium layer. However, upon persistent injury, basal cells become susceptible to genomic changes and lose their ability to regenerate the epithelium. This literature review therefore aimed to evaluate the therapeutic potential of stimulating basal cell functionality in COPD via Hedgehog and Notch signaling pathways to induce epithelial repair. Taken together, evaluated studies showed inhibition or downregulation of Hhip, Ptch1 and Notch 2 receptors (via Hhip, Ptch1 or Notch 2 antagonists) and upregulation or activation of the SHH ligand (via a SHH agonist) in HH and Notch signaling pathways may be therapeutically beneficial as both lead to increased basal stem/progenitor cells and a balanced differentiation into secretory and multiciliated cells, thereby inducing epithelial repair at the earliest stages of COPD progression. These therapeutic strategies should be evaluated using a combination of cigarette smoke induced and elastase induced mice models, lung organoids (of basal stem/progenitor cells) and clinical studies.

Introduction

Chronic obstructive pulmonary disease (COPD) is a currently incurable lung disease that is characterized by chronic inflammation and obstructions of airflow due to airway remodeling. This leads to an accelerated lung function decline.[1,2,3,4] Given enough time, COPD can develop into respiratory failure, often requiring lung transplantation.[3] COPD is not reversible and affects gas exchange. Several phenotypes exist for this disease, from which chronic bronchitis (airway inflammation and excessive mucus hypersecretion) and emphysema (alveolar destruction) are the most common (**Figure 1** [5]).[1] In some cases, COPD patients may develop both phenotypes.[6]

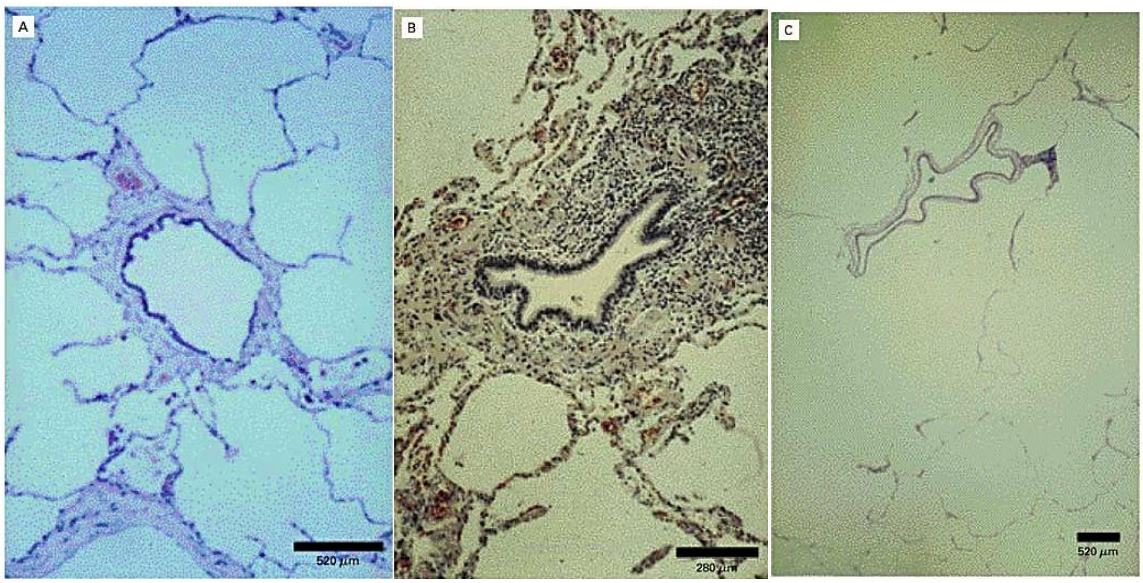

Figure 1. Histological features of COPD upon Hematoxylin and eosin (H&E) staining. A: healthy airways; B: chronic obstructive bronchiolitis with accompanying airway thickening and infiltration with immune cells; C: emphysema with alveolar wall destruction and airway collapse.[7]

Several studies indicate that COPD is the fourth most common cause of death (41.9 deaths per 100,000 individuals), affecting the daily lives of approximately 174 million people globally in 2015.[1,2,8,9] In 2017, COPD remained the most prevalent form of chronic respiratory disease worldwide. This chronic respiratory disease prevalence was estimated to be 55.1% among men and 54.8% among women globally ([Bill F, Foundation, 2020]).[8] Terzikhan et al. (Rotterdam Study) estimated a COPD prevalence of 4.7% and an overall incidence rate of approximately 9/1000 person-years in the elderly population (aged 45 and older) in the Netherlands. According to this study, COPD is more prevalent in males than females.[7,10] The prevalence has been shown

to increase with age (age group 55 – 60 years) and therefore, the disease progression and its financial impact are predicted to increase in the aging population.[10]

Risk factors for COPD include long-term exposure to tobacco smoke (approximately 20% of the cases [10]).[11] Tobacco smoking is one of the leading causes of COPD.[11] Other risk factors include inhalation of combustion products from fossil fuel, particulate matter from air pollution (household and environmental), respiratory infections, asthma with airway remodeling, genetic susceptibility (e.g., hereditary deficiency of alpha-1 antitrypsin [12]), alterations in MUC_5AC – and MUC_5B expression [13], and abnormal lung development.[1,2,3,9,10] The COPD pathogenesis is unclear. However, it has been suggested to be related to inflammation, oxidative stress, excessive protease production (causing an imbalance in protease/anti-protease) and decreased immunity.[3] These factors lead to lung airway remodeling and airflow obstruction. [1,3]

This airflow obstruction and related symptoms are diagnosed by performing a lung function test (spirometry). Using the acquired FEV_1/FVC ratio (**Figure 2**), diagnosis for respiratory diseases (independent of lung size) can be done. The FEV_1/FVC ratio describes the proportion of the vital capacity of an individual, that is exhaled in the first second of forced expiration. A reduction in this ratio indicates possible airflow obstruction.[14] Based on the severity of lung function impairment and exacerbation encountered in this test, the patient is categorized according to the GOLD Guidelines.[1,5,15,16] This information determines the treatment choice given by the General Practitioner or Chest Physician (**Figure 3**).[1]

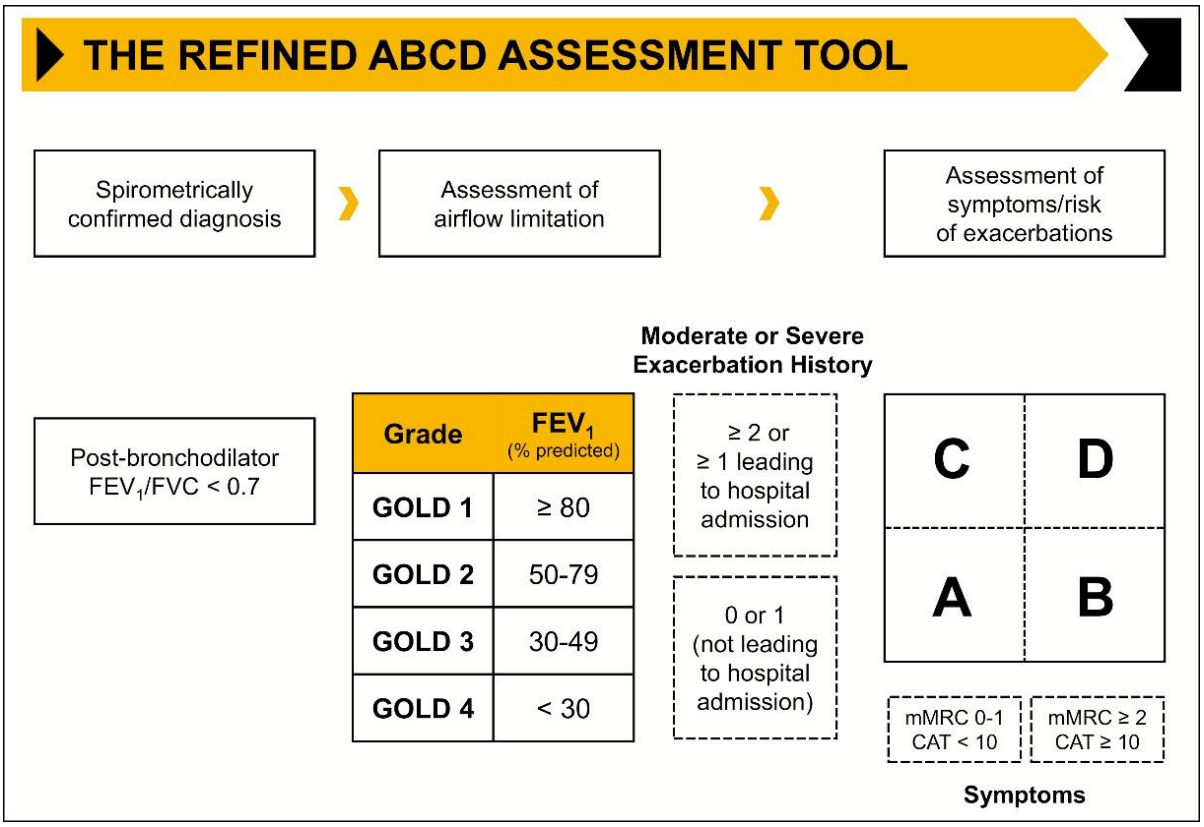

Figure 2. **Overview of COPD severity and categorization**. GOLD 1: mild lung function problems; GOLD 2, GOLD 3 or GOLD 4: severe; FEV_1: volume of exhaled air in the first second. This is used as index measure for airway obstruction; FVC: forced vital capacity. This is the amount of air forcibly exhaled from the lungs after taking a deep breath; FEV_1/FVC: proportion of the vital capacity of an individual, that is exhaled in the first second of forced expiration.[1,14,15]

Currently available COPD therapies (medication, oxygen treatment and rehabilitation therapy[3]) deal with this airflow obstruction and aim to relieve symptoms (e.g., dyspnea, cough and/or sputum production), to slow the decline in lung function, or to prevent exacerbations.[1,3,5,16] These treatments include the use of long acting muscarinic receptor antagonists (LAMA), long-acting β2-agonist (LABA), inhaled corticosteroid (ICS), bronchodilators (β2 receptor agonist and anticholinergics), Phosphodiesterase (PDEi) and Azithromycin (**Figure 3** [5]).[1,5] The severity of COPD is a leading factor in making treatment choices. However these therapies do not prevent or reverse disease progression.[3]

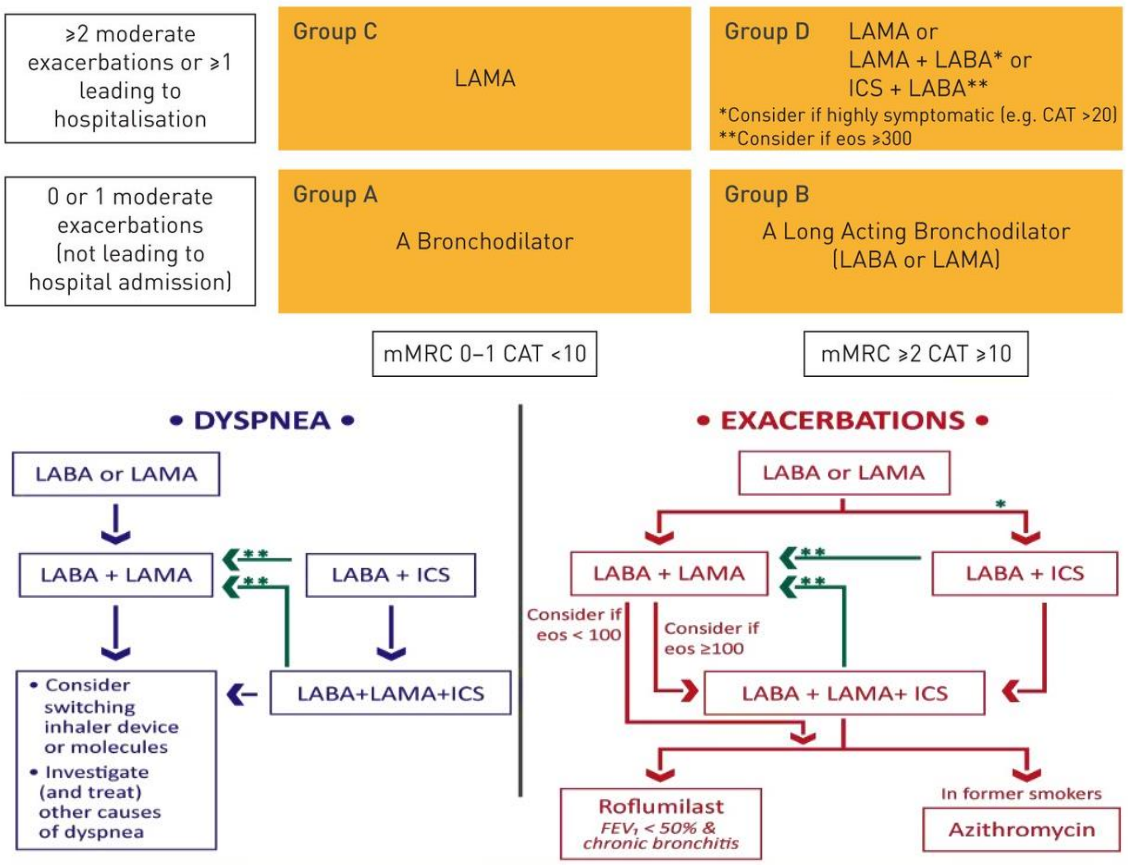

Figure 3. Schematic overview of the initial and follow-up treatments of COPD. Category A patients have a mild form of the disease. Category B includes patients with severe lung function impairment with little exacerbation. Category C indicates impaired lung function and high risk for exacerbation. Category D indicates severe symptoms and high exacerbation. LAMA: long-acting muscarinic antagonist; LABA: Long-acting β_2-agonist; ICS: inhaled corticosteroid. [5]

COPD progression involves major changes in the airway architecture with characteristic changes in epithelial cell populations.[17] Dysregulation in epithelial repair is one of the characteristics of both phenotypes of COPD.[2] Understanding epithelial remodeling and epithelial repair are thus important to develop innovative therapeutics.[2] When lungs are injured, various progenitor cells, including ciliated cells, goblet cells, club cells and basal cells, proliferate to restore the epithelium layer. Basal cells are typically found in the larger airways and function as progenitors of ciliated and secretory cells. Upon persistent injury, basal cells become susceptible to genomic changes and lose their ability to regenerate the epithelium. Thereby leading to the major changes in the airway (characterizing COPD) and accelerated lung function loss. [17]

Stimulating basal cell functionality may be a useful therapeutic approach to normalize epithelial repair in COPD. The aim of this review was therefore to evaluate the therapeutic potential of stimulating basal cell functionality in COPD from studies conducted in this research area. Thereby providing recommendations for the associated challenges. In this review, background information on normal respiratory tract epithelium, COPD pathophysiology and basal cell mediated epithelial repair will be discussed first. Thereafter, signaling pathways and beneficial therapeutic targets for basal cell-mediated epithelial repair (from the analyzed publications) will be evaluated and discussed. Finally, recommendations for the challenges and future basal-cell-mediated-epithelial-repair therapy will be proposed.

COPD Pathophysiology

The development of COPD is a complex process, which involves many cellular and molecular mechanisms.[3] To understand the complexity and pathophysiology of this disease, it is essential to be familiar with normal wound-healing upon injury (e.g., because of exposure to xenobiotics, toxic gasses, dust particles, and tobacco smoking). [11,18] The four overlapping phases of wound-healing include the coagulation phase, inflammation phase, fibroblast recruitment/proliferation phase, and remodeling phase (**Figure 4**[19]).[11] Following lung injury, epithelial cells release inflammatory cytokines to initiate an antifibrinolytic coagulation cascade. This cascade triggers platelet activation to enable blood clot formation. Afterwards, leukocytes (e.g., neutrophils, macrophages, and T cells) infiltrate the affected area, secrete profibrotic cytokines, and remove dead cells and invading pathogens. Thereafter, bone-marrow derived fibrocytes and resident fibroblasts become activated and differentiate into myofibroblasts to produce extracellular matrix (ECM) proteins. Fibroblasts and myofibroblasts may also come from other sources, such as by epithelial-mesenchymal transition (EMT).[19] Finally, activated myofibroblasts promote wound contraction and repair. Epithelial and endothelial cells then cover freshly produced ECM. Afterwards the remaining myofibroblasts are instructed to undergo apoptosis or to become senescent.[11,19] These epithelial repair processes are severely altered in the lungs of COPD patients favoring excessive ECM and mucus production and airflow obstruction.[18]

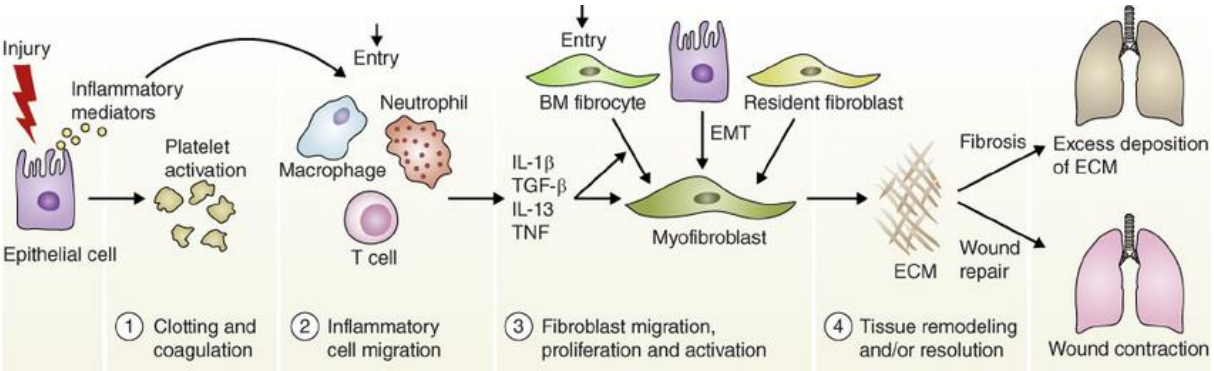

Figure 4. Schematic overview of normal lung epithelial repair (lung wound healing). [19]

In COPD, there is persistent inflammation in the lungs, airway hyperresponsiveness (towards inhaled particles), mucus hypersecretion and impaired ciliary clearance. This leads to airway and lung tissue remodeling (chronic bronchitis), (small) airway fibrosis and destruction of alveoli (emphysema) causing the airways to become narrow and the lumen to become smaller (**Figure 5**).[1,11] The airways are not held open by alveolar attachments anymore leading to enlargement of the airspaces due to the disrupted elastic fibers. These changes in the lungs are

caused by smoking and other irritants which are major factors in COPD pathogenesis.[20] Inhalation of smoke, for example, activate epithelial cells and macrophages to release chemotactic factors which attract inflammatory cells and cytotoxic T cells to the lungs. Thereafter, activated macrophages, epithelial cells and inflammatory cells release proteases such as matrix metalloproteinase 9 (MMP9) causing elastin destruction, emphysema and release of neutrophil-derived elastase leading to mucus hypersecretion. In the small airways, fibrosis is caused by stimulation of fibroblast proliferation due to transforming growth factor-β (TGF-β) (released by epithelial cells and macrophages).[1,6,20] In some cases, COPD patients may develop both phenotypes. However, one of these phenotypes is usually predominant.[6] Dysregulation of epithelial repair is one of the characteristics of both phenotypes. In order to target this dysregulated epithelial repair in COPD, it is crucial to discuss the structural integrity and function of the airway and alveolar epithelium.[11]

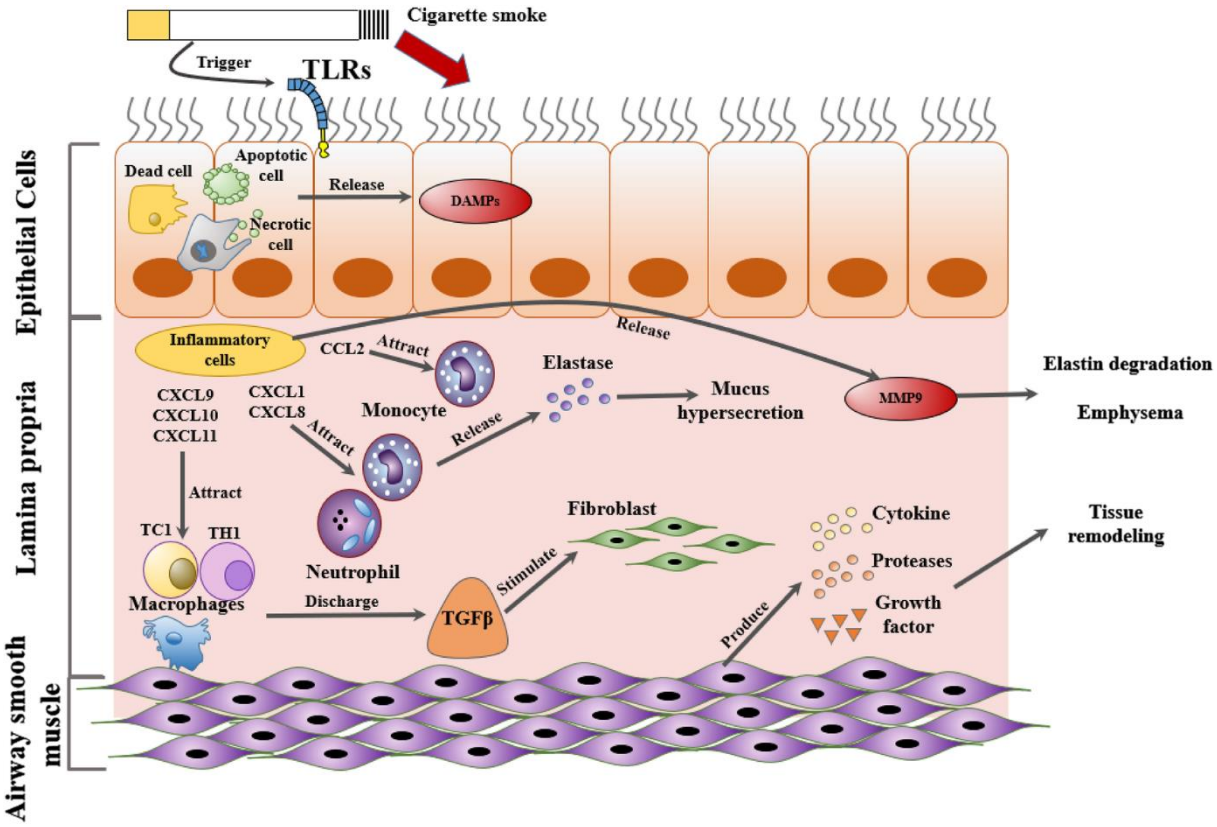

Figure 5. Schematic overview of the COPD pathophysiology.[21]

Regulating respiratory epithelial repair

The human respiratory tract is lined with a cell layer comprising a large variation of cells. The composition of (small) airway epithelium includes ciliated cells (for mucociliary transport), mucus producing goblet cells, non-ciliated club cells (secretory proteins), neuroendocrine cells and basal cells (**Figure 6**[24]).[11,22] The latter one has stem cell properties and acts as progenitor for ciliated cells and goblet cells.[11,24] When moving toward the smaller airways (lower area of the respiratory tract), the number of ciliated cells and goblet cells decrease, whereas the amount of club cells increase.[1] On the contrary, the alveolar epithelium consists of cuboidal alveolar epithelial type II cells (AEC2) and alveolar epithelial type I cells (AEC1). AEC1 are flat cells that make up for 95% of the alveolar surface and facilitate gas exchange with neighboring pulmonary microcirculation.[1,22] AEC2 produces surfactant and functions as progenitor cells for AEC1. This surfactant is critical for homeostasis as it lowers surface tension, ensures lung expansion upon inhalation, and prevents alveolar collapse upon exhalation.[1,22] The airway epithelium is the first line of defense against toxins, pathogens and other unwanted substances which enter the airways through inhaled air. However, it maintains a selective permeable barrier which is formed by epithelial junctions (epithelial cells connected by intercellular junctional complexes).[11,22] These epithelial junctions allow epithelial repolarization by separating the basal from the apical membrane. This may thus be crucial for basal epithelial cell differentiation into mucociliary epithelium.[11] This barrier function can be interrupted by toxins (e.g., tobacco smoke) leading to the release of inflammatory signals that contribute to onset of disease.[23]

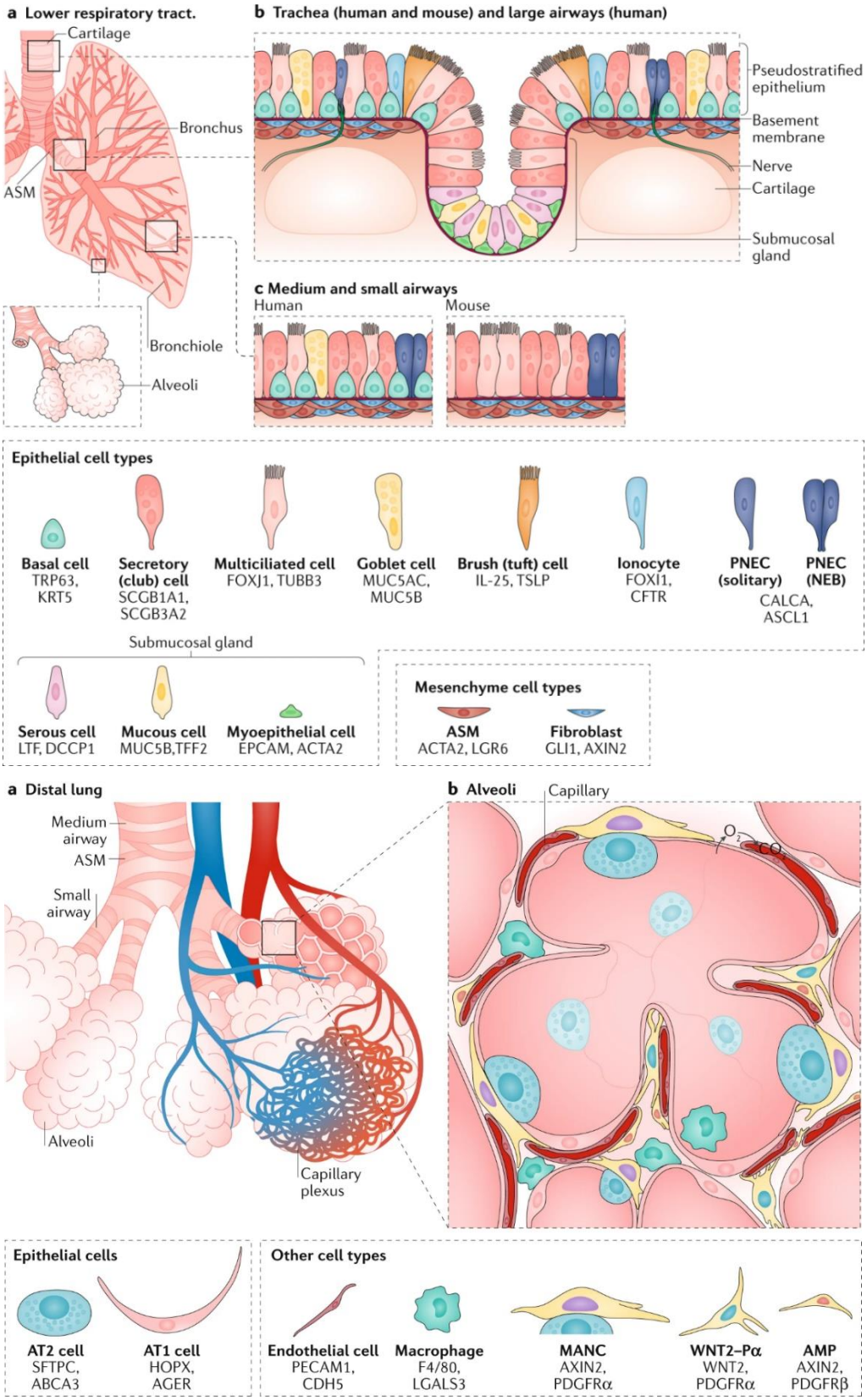

Figure 6. Overview of the airway – and alveolar epithelial cell heterogeneity. A: cellular composition of the airways; B: cellular composition of the distal lung (alveoli).[24]

Dysregulation in epithelial repair is one of the characteristics of both aforementioned phenotypes of COPD.[2] Understanding epithelial remodeling and regulation of epithelial repair are thus important to develop innovative therapeutics. Upon lung injury, progenitor cells including ciliated cells, goblet cells, club cells and basal cells proliferate to generate epithelial repair. In humans, basal cells are typically found in the larger airways (and throughout most of the airway tree) and thus may have a crucial role in maintenance and repair of the airway epithelium.[17,24] Basal cells, progenitor cells for the airway epithelia, are able to repopulate the pseudostratified epithelium during homeostasis and after epithelial injury.[24] These cells constantly self-renew and generate multiciliated and secretory cells even during homeostasis when the turnover of airway epithelium is low. [24] However it does so at a lower rate. In response to epithelial injury, these basal cells can increase the number of both secretory and multiciliated cells.[24] Recently, the difference in stem cell abilities of basal (progenitor) cells (under homeostatic conditions versus regeneration after acute injury) was demonstrated by the combination of lineage-tracing and single-cell RNA sequencing.[24] During homeostasis, basal (progenitor) cells differentiate into secretory cells and in a subsequent trans-differentiation step, this causes an increase in multiciliated lineage. After acute injury, these cells can differentiate directly into secretory – or multiciliated lineages.[24] Epithelial repair and the flexibility of basal cell proliferation upon tissue injury are facilitated by various signaling pathways including the Hedgehog (HH) and Notch pathways.[24]

Hedgehog – and Notch signaling pathways in the lungs

Several studies have shown that Hedgehog and Notch signaling are aberrant in COPD as these pathways are crucial for proliferation of epithelial cells and regulation of airway epithelial repair and maintenance.[25,26,27] Hedgehog (HH) signaling is crucial during lung development, epithelial repair and regeneration of the epithelium after damage (**Figure 7**). HH signaling involves canonical and non-canonical HH pathways. Ligands in these pathways include Sonic Hedgehog (SHH), Desert Hedgehog (DHH) and Indian hedgehog (IHH). In canonical HH signaling, binding of these ligands to their receptors (transmembrane protein receptor, Ptch) releases G-protein-coupled receptor smoothened (Smo) and activates (and translocates[25]) Zinc-finger DNA-binding proteins (Gli) family transcription factor. This causes activation of the HH pathway.[2,28] Non-canonical HH signaling includes three activation modes, namely: ligand-dependent/Smo-independent activation, ligand-dependent/Gli-independent activation, and Gli-only activation.[2] When hedgehog ligands are not bound to their receptors, Ptch1 inhibits the HH signaling through the Smo (negative feedback loop). However this Ptch1 dissociates from the Smo upon hedgehog ligand binding. The latter results in downstream activation of the GLI family transcription factors.[28] Recently, it was confirmed that this pathway is dysregulated in COPD pathogenesis and that hedgehog interacting protein (Hhip) and pathway patched homolog 1 (Ptch1) are important regulators of COPD.[2,28] In COPD, Ptch1 is upregulated and causes airway cell proliferation, goblet cell hyperplasia, elevated mucus production, airway epithelial thickening and airflow limitation.[6] Thereby leading to mucus-producing phenotypes in COPD patients.[28] Hedgehog signaling maintains a balance between epithelial cell proliferation and mesenchymal quiescence during homeostasis and regeneration in COPD. Upon injury, this balance is disturbed leading to perturbations in the mesenchyme and epithelial regeneration. Activation of HH signaling, during epithelial injury, impairs lung mesenchyme expansion, whereas its inactivation prevents the restoration of quiescence while resolving the injury.[6,24]

Another pathway which plays an important role in lung development is the Notch signaling pathway. Notch signaling is a cell-cell signaling pathway which consists of four (4) homologous receptors (Notch1/2/3/4) and five (5) canonical ligands (Delta1/3/4 and Jagged1/2).[29,30] In mammals, these Notch receptors and ligands mediate communication between neighboring cells to decide cell fate during organ development. This is done through RBPJ (or CSL) transcriptional effector.[26,29] The Notch signaling pathway functions based on three transmission modes, namely (1) lateral inhibition, (2) induction, and (3) lineage decisions.[30] In the first mode, lateral inhibition, Notch-active and Notch-negative cells are delineated by stochastically appearing ligand-expressing cells in the uniform progenitor population. This is done upon activation of Notch signaling in adjacent cells and inactivation of ligand expression by the ligand-expressing cells. In the second mode, induction, ligand-expressing cells and receptor-expressing cells are derived from different cell lineages and Notch signaling is only activated in receptor-expressing cell lineage. The last mode involves lineage decisions which are primarily dependent on Notch regulators such as Numb (a negative regulator of Notch signaling that antagonizes receptors). This leads to the downregulation of Notch signaling in the Numb-positive daughter cells and upregulation of Notch signaling in the Notch-negative daughter cells. Thereby, generating Notch-active and Notch-negative daughter cells and dynamic fate decisions (e.g., asymmetric division of stem/progenitor cells).[30] Notch signaling also maintains a balance between multiciliated – and secretory cell differentiation and promotes the maturation of mesenchymal fibroblasts (crucial for alveoli formation) during the final stages of lung development.[6,24] Activation of Notch leads to mucus cell metaplasia and an increase in mucus producing secretory cells. Whereas loss of Notch leads to an increased amount of multiciliated cells, as the trans-differentiation of secretory cells into multiciliated cells is not inhibited anymore (**Figure 7**).[24] In COPD, this pathway is downregulated (Notch3 and DLL1[30]) leading to secretory cell differentiation, goblet cell metaplasia in chronic bronchitis.[6]

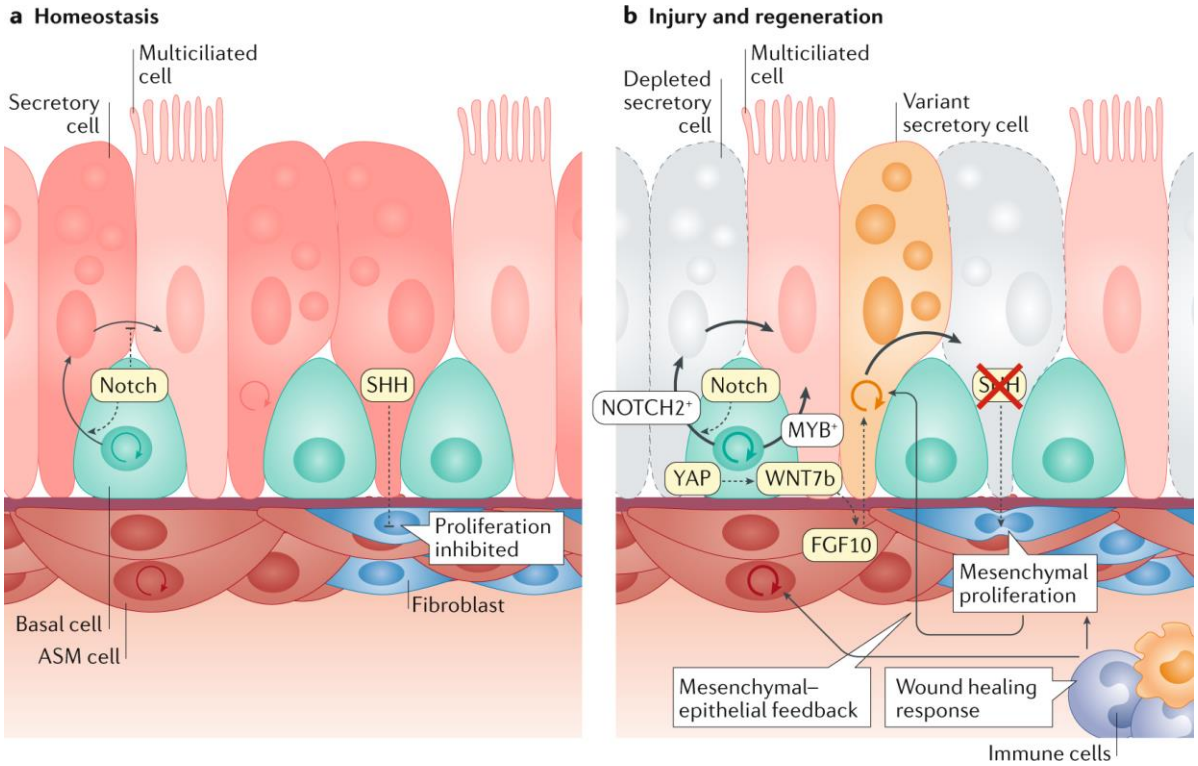

Figure 7. Overview of the epithelial cell proliferation and epithelial repair during homeostasis and after injury.[24]

The Hedgehog – and Notch pathways are crucial for epithelial cell proliferation and airway epithelium repair and maintenance (**Figure 7**). In COPD both of these pathways are perturbed leading to increased mucus production and airway remodeling. Other changes include basal cell hyperplasia, squamous metaplasia, alterations of cilia, loss of secretory cells and loss of the epithelial junction barrier.[31] These changes could be resolved by targeting basal cell-mediated epithelial repair using the Hedgehog – and Notch signaling pathways. This, because airway basal cells, stem/progenitor cells of the airway epithelium, contribute to tissue maintenance and epithelial – repair and regeneration upon injury.[30] They are crucial for maintaining the airway epithelial structure and do so by self-renewing, differentiating into ciliated and secretory cells, and establishing interactions with mesenchymal cells.[31] The flexibility of basal cell differentiating potential upon tissue injury is caused by the heterogeneity in this cell population with cells expressing either Notch2 (to generate the secretory lineage) or transcriptional activator, MYB (to generate the multiciliated lineage).[24] Hedgehog (i.e., SHH), produced at homeostasis in the adult airway epithelium, maintains adjacent mesenchymal quiescence. Deletion of this SHH led to the loss of mesenchymal quiescence (mostly fibroblasts) giving rise to a feedback mechanism (on the epithelium) which increased the epithelial proliferation.[24]

Basal cell-mediated epithelial repair: evaluation of published studies

Basal cells, keratin 5-positive cuboidal cells lining the basement membrane, represent approximately 34% of the tracheal cell population and gradually decrease in the lower areas of the tracheobronchial tree (**Figure 8**[32]).[17] Human and mouse airways contain subsets of $K5^+$ basal cells with different differentiation properties, namely unipotent basal cells (generating basal cells), bipotent cells (generating basal and secretory cells) and multipotent basal cells (generating basal, secretory and ciliated cells) [33]. Well-known airway basal cell markers include keratins KRT5 and KRT17 (intermediate filament constituents crucial for cytoskeleton assembly); integrins ITGA6, ITGB1 and ITGB4 (regulate interactions with ECM components of the basement membrane); and transcription factors TP63 and basonuclin (crucial for basal cell renewal and maintenance).[31] The basal cell population comprises stem/progenitor cells that can contribute to epithelial differentiation and repair by self-renewing, replenishing themselves, proliferating and differentiating to give intermediate cells (also called "parabasal cells" and "indetermined cells"). Subsequently, these intermediate cells differentiate into ciliated cells and secretory cells (multipotentiality)[33].[17] Another function of basal cells is to influence and respond to surrounding cells by respectively secreting polypeptides (e.g., bone morphogenetic proteins 1 and 2, transforming growth factors 1 and 2, fibroblast growth factors 2 and 11 and Notch ligand Jagged) and via expressing a variety of receptors (for e.g., transforming growth factor, epidermal growth factor, and tumor necrosis factors).[17] These cells, thus, play a crucial role in the airway homeostasis by functioning as stem/progenitor cells and by influencing surrounding cells via secretion of aforementioned growth factors. [17] However in COPD, these airway basal cells contribute to disease pathogenesis by abnormal proliferation/differentiation processes (stem/progenitor fatigue), basal cell hyperplasia, mucus cell hyperplasia, ciliary dysfunction, generating proinflammatory microenvironment and perturbing epithelial-mesenchymal interactions leading to a deranged epithelial architecture, decreased epithelial barrier integrity and airway remodeling.[17]

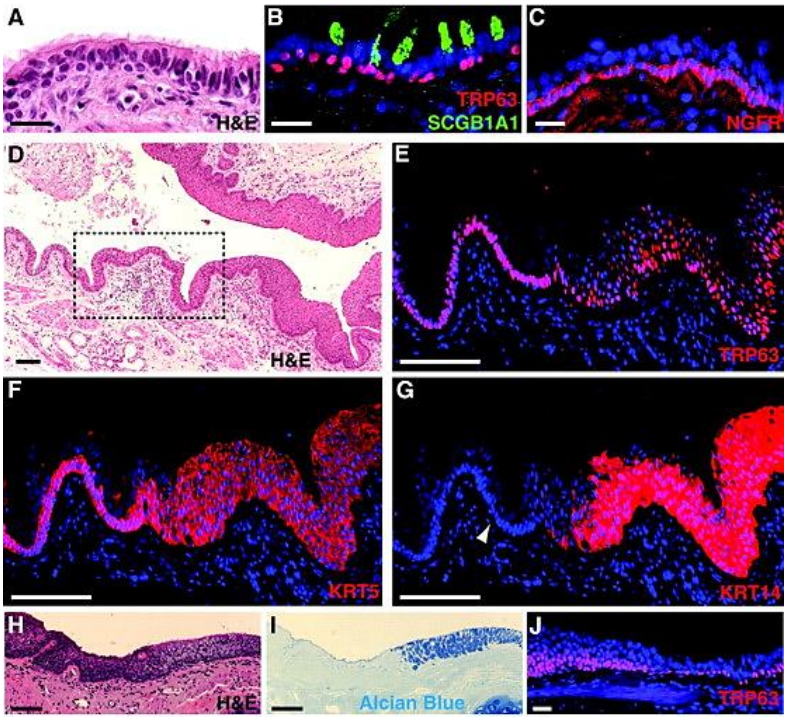

Figure 8. Histologic features of basal cells in normal (small) – and COPD airways. A-C: sections (approximate diameter of 2 mm) of airways of a non-smoker, acquired after lobectomy. A: Hematoxylin and eosin (H&E) stained sections of basal cells of the normal pseudostratified airway epithelium. B-C: section of basal cells identified by expression of TRP63 (B) and NGFR (C) in adjacent sections. D: Airways of a smoker with COPD (H&E stained) showing transition from a normal epithelium into squamous metaplasia (box). E-G: high magnification of D (adjacent regions of the box in D) with expansion of TRP63+ (red in E), KRT5+ (red in F) and KRT14+ (red in G) cells in the squamous metaplasia regions. G: monoclonal antibody staining of rare basal cells in normal airways against KRT14 (indicated with an arrow head). H-J: regions of mucus hyperplasia and squamous metaplasia in airways of smokers with COPD upon H&E (H), Alcian Blue (I) and anti- TRP63 (red in J) staining.[32]

Ghosh et al.[33], examined the role of basal progenitor cells in COPD and non-COPD airways. To do so, these authors conducted a cross-sectional study in a patient population of ≥ 40 years, undergoing baseline bronchoscopy, with a smoking history of ≥10 pack-year. These subjects were divided into two clinical studies ("Biomarkers") to study the progenitor count (primary outcome from clone-forming basal cells in biopsies), progenitor self-renewal, multipotentiality (air-liquid interface (ALI) culture, immunostaining for basal cells ($K5^+$), secretory cells ($Muc5b^+$), and ciliated cells ($ATC1^+$)) and lung function measurements (i.e., lung volume, airflow, and carbon dioxide diffusion) (secondary outcomes). The normal epithelium is composed of cell types which are differentiated from clone-forming (or sphere-forming) basal

progenitors during *in vitro* culture. However in COPD, basal progenitors generated epithelium with a 1.5-fold increase in basal cells, 2-fold increase in secretory cells and 88% reduction in ciliated cells. Thereby indicating basal cell "exhaustion" and reduced capacity of basal progenitor cells to differentiate into a normal mucociliary epithelium. Time-lapse imaging showed that cilia were lost upon epithelium generation by COPD progenitors.[33] The loss of multipotent basal progenitors and basal cells with restricted differentiation abilities could explain hyperplasia of basal cells.[33] The overall conclusion of this study was that airway basal progenitor cells are exhausted in COPD, thereby creating a "COPD-like" epithelium and a reduced differentiation into a normal mucociliary epithelium. Thus, treating this exhaustion of airway basal progenitor cells in COPD to proliferate and differentiate into a normal mucociliary epithelium, may be therapeutically beneficial.[33]

Hedgehog signaling

More recently, Ancel et al., examined basal progenitor differentiation processes in COPD by studying the HH pathway, its activating ligand (SHH), main receptor Ptch1, main co-receptor Hhip, and main transcription factor Gli2 (**Table 1**).[25] Ancel et al., evaluated this HH pathway in COPD and non-COPD subjects using endobronchial samples, characterized cell populations and localizations using immunostaining, identified SHH proteins (in bronchoalveolar lavage (BAL) and lung tissues) using ELISA and RNA sequencing and validated this with and external independent cohort. Results of this study showed that *in vitro* inhibition of the HH pathway caused a deficit in SHH signaling and COPD-like epithelial remodeling with an increase in basal cells, a decrease in Gli2-positive cell nuclei in basal cells, and a drastic decrease in ciliated cells (**Figures 9** and **10**). Thereby implying an impairment in differentiation processes of airway basal progenitor cells.[25,28] These authors also found that Ptch1 and Gli2 are able to transduce SHH signaling and that Gli2 present in basal and ciliated cells may imply the existence of autocrine and paracrine signaling in the epithelium. Another finding was that Hhip, a SHH receptor, inhibited the HH pathway. Genetic alterations in Hhip were associated with COPD and emphysema and influenced protein function (instead of protein expression).[25] Altogether this study indicated that HH pathway is defect (and inactivated), due to the loss of its canonical ligand, SHH, which resulted in COPD-like epithelial remodeling with an increase in basal cells and secretory cells and a decrease in ciliated cells. Investigations of molecular mechanisms involved in inactivation of this HH pathway may be therapeutically beneficial.[25]

These alterations of HH pathway were also observed in the study conducted by Belgacemi et al. and Tam et al..[2,28] Belgacemi et al., investigated the participation of (altered) HH pathway (Gli1, Gli2, Gli3, Smo, and Ptch1) in airway epithelial cell differentiation (cell fate) *in vitro* and in COPD and non-COPD subjects (patients undergoing lung resection for cancer and patients who underwent routine fiberoptic bronchoscopy with bronchial brushings under local anesthesia). Thereby focusing on cell proliferation, reduction in ciliated cells, mucus production and epithelial barrier integrity. Experiments using ALI medium and immunostaining showed that SHH was found in supernatants from airway epithelial cell culture and total protein extracts from human bronchi therewith demonstrating that its production is mediated by airway basal epithelial cells. To inhibit the canonical and non-canonical signaling and to avoid off-target effects, an antibody was used against SHH (AB5E1 treatment in the *in vitro* experiments). These results showed that upon addition of ALI switch to AB5E1, basal cell proliferation and ciliated cells decreased. Nuclear localization of Gli also decreased upon this antibody-directed treatment throughout differentiation, whereas it was still detected in progenitor and ciliated cells. This showed that Gli2 is crucial for airway epithelial cell differentiation and basal cell fate and that HH pathway inhibition mainly influences progenitors. This was concluded after the reduction of mRNA and protein levels in the first differentiation steps.[2] These authors demonstrated that activation of HH pathway is crucial for generating a fully functional epithelium and that inhibition of this pathway led to a reduced basal cell proliferation and a reduced amount of ciliated cells, partially due to Gli2. Gli2, Smo and Ptch1 were mainly found in progenitors, which makes them potential markers of airway remodeling in COPD.[2] The presence of Gli2 in nuclei could be used to study HH activation in homeostasis and COPD. Gli2 was found to be a novel important regulator for basal cell fate to indicate the importance of progenitor cell homeostasis and localization of Gli2 in the airways may be important to develop therapeutics which oppose airway remodeling and improve respiratory functions.[2]

Tam et al., investigated the role of Ptch1 in airways of COPD patients using lung tissues and bronchial epithelial cells from COPD and non-COPD subjects, human epithelial cell lines and Ptch1+/- mice models.[28] The results from this study showed an increased amount of airway epithelial-specific Ptch1 protein expression in COPD subjects and an impaired wound closure and mucus expression upon Ptch1 knockdown. Thereby suggesting an upregulation of Ptch1 protein and mucus expression in airways of COPD subjects (the chronic bronchitis phenotype of COPD) accompanied by airway epithelial thickening and airflow limitations.[28] These perturbations were studied using an *in vitro* model, wherein Tam et al., showed a reduction in

cell proliferation, goblet cell hyperplasia, and therefore a reduction in mucus hypersecretion (upon Ptch1 attenuation). These results also corresponded *in vivo* with the HDM-exposed (house dust mites) Ptch1+/- mice model, thereby indicating a therapeutic potential of Ptch1 attenuation for treating COPD patients.[28]

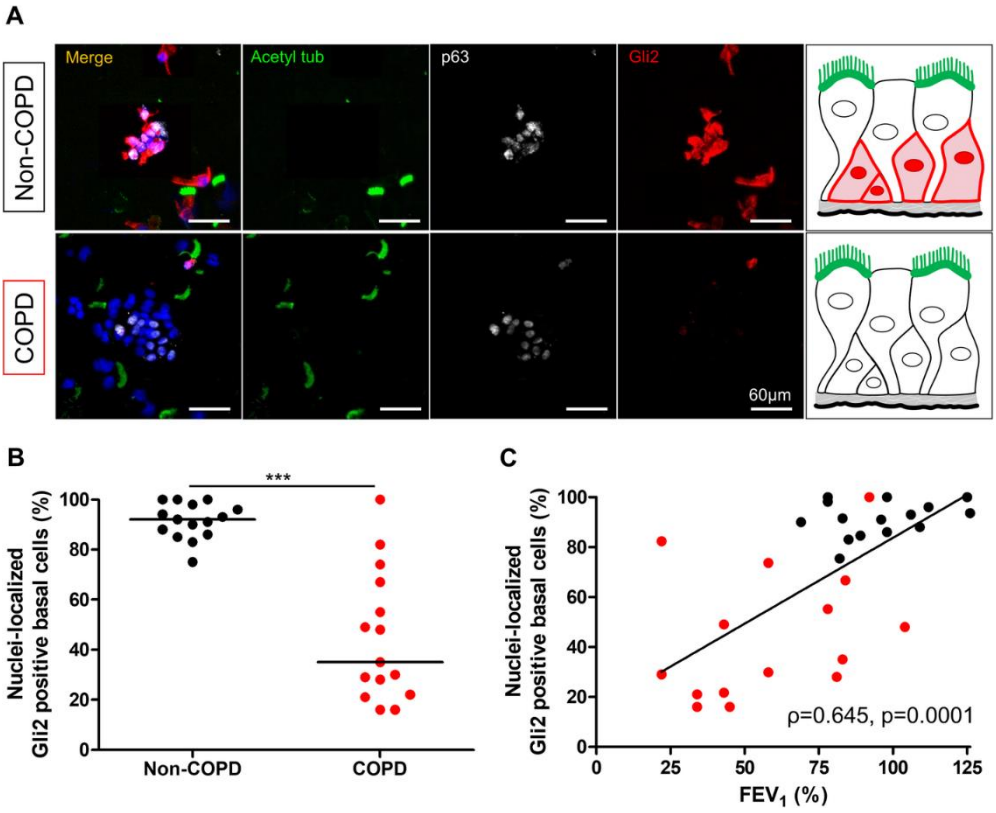

Figure 9. **Histological features and dot plots of decreased Gli2 expression in the nuclei of airway basal progenitor cells from COPD patients.** A: microscopic representation of a bronchial brushing stained for cilia (green, acetylated tubulin), Gli2 (red, GLI2), basal cells (white, P63) and cell nuclei (blue, DAPI) in COPD and non-COPD subjects. B: dot plot with median indicating the percentage of Gli2-positive basal cell nuclei in non-COPD (black circles) and COPD (red circles) subjects (n = 15 for both); $p < 0.0001$. C: Linear regression of B according to FEV1 (% predicted).[25]

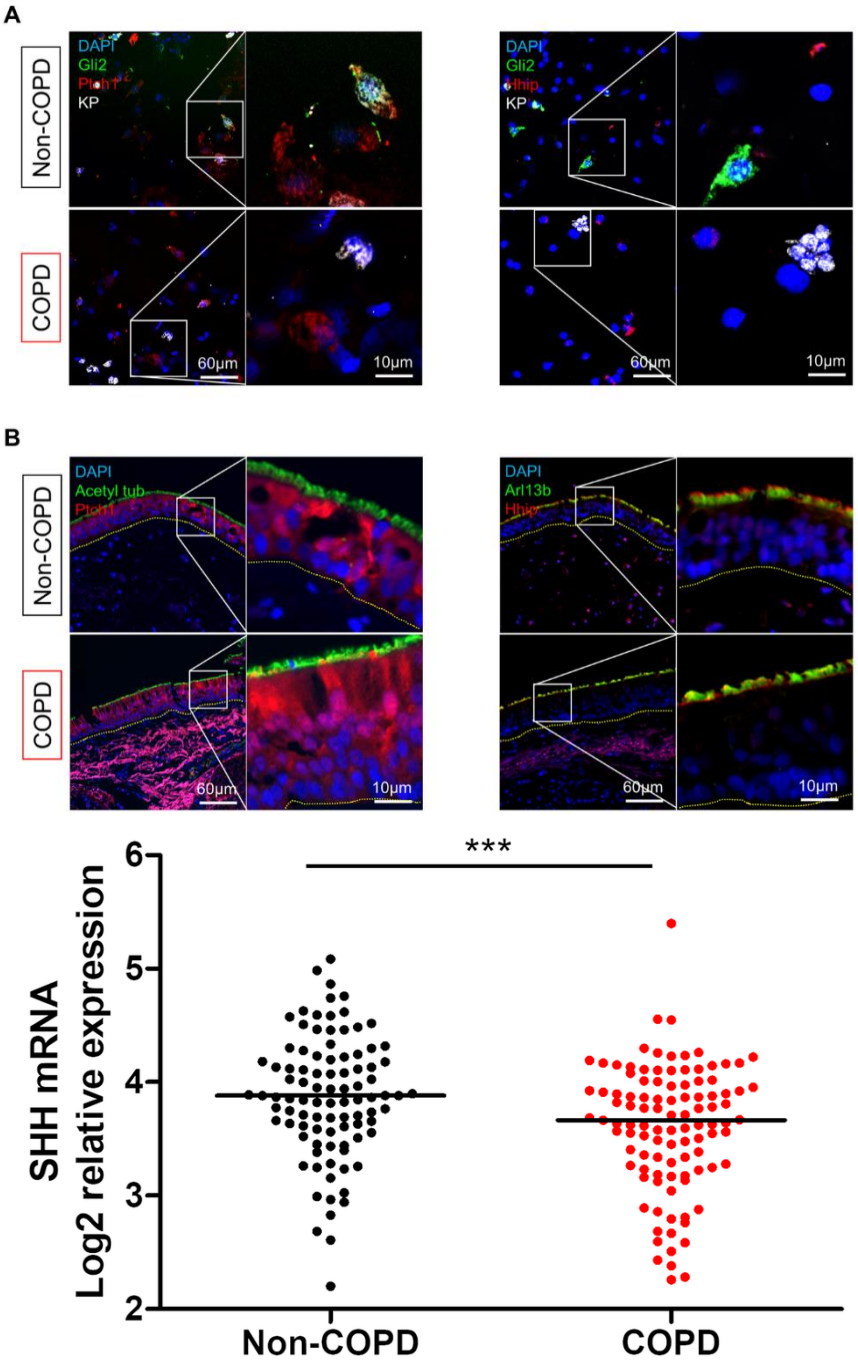

Figure 10. Histological features and dot plot of decreased SHH activating ligand in the bronchi from COPD patients. A: microscopic representation of a bronchial brushing stained for basal cells (white, pancytokeratine, KP), Gli2 (green), Ptch1 (red, left panel), Hhip (red, right panel) and cell nuclei (blue, DAPI). B: microscopic representation of a bronchial brushing stained for cilia (white, pancytokeratine, KP), Gli2 (green), Ptch1 (red, left panel), Hhip (red, right panel) and cell nuclei (blue, DAPI). Dot plot with median indicates decreased SHH transcript levels in COPD patients (red circles; n = 145); normalized expression of log2 transformed SHH expression in non-COPD patients (black circles; n = 91); p <0.0001.[25]

Notch signaling

The other pathway, crucial for regulating basal progenitor cell behavior, is Notch signaling (**Figure 11; Table 1**). Rock et al., studied the mouse tracheobronchial epithelium (as a genetically traceable model of human airways) to show that Notch signaling regulates basal stem/progenitor cell behavior in mouse and human airways. This role of Notch in human airway basal cell differentiation was shown by studying primary human bronchial epithelial cells.[11] During epithelial repair, Notch signaling increases upon generation of undifferentiated daughter cells by basal progenitor cells.[11] These authors showed that sustained activation of Notch signaling promotes differentiation of basal cells into secretory cells. Airway epithelial stem/progenitor cells show relatively little proliferation and differentiation, therewith indicating that Notch signaling is active under stable conditions (and does not increase). Therefore, the increasing activity of Notch signaling (during enhanced cell turnover conditions *in vivo*) during epithelial repair upon basal cell differentiation to undifferentiated, luminal daughters, was shown using this mouse model. With this model, Rock et al., also showed that most of the luminal cells died, whereas the basal cells were left behind throughout this tracheobronchial region. Basal cells remain adjacent to the basal membrane, thereby causing an efficient epithelial repair process and continuous expression of basal cell markers (e.g., TRP63, KRT5).[11] In order to investigate if Notch signaling is sufficient to promote basal cell differentiation into luminal daughter cells *in vivo*, the authors activated this pathway in basal cells using a ROSA^{NOTCH} mouse line and afterwards tested this with the addition of gamma secretase inhibitor (DBZ) and vehicle (DMSO).[11] This also corresponded with the aforementioned results, indicating that basal cell differentiation into secretory lineages depends on the activation of Notch signaling. However its self-renewal and proliferation is not dependent on Notch signaling. Basal cells are found throughout the tracheobronchial tree of the human lungs and these cells in COPD (KRT5+ and KRT14+ basal cell populations) expand in regions with squamous metaplasia which could be the result of a decreased level of Notch signaling (**Figure 12**).[11] These findings were confirmed by Kiyokawa et al., who showed that Notch signaling (i.e., Notch3, DLL1, HES5 and HEY1/2 genes) is inhibited in COPD in comparison to non smokers (using microarray analysis). Persistent activation of the Notch pathway by e.g., tobacco smoke favored the differentiation of basal cells into secretory cells by blocking the ciliated cell fate (by e.g., Notch2)[24,30]. Thereby causing mucosal hyperplasia in COPD. These perturbations were attenuated using Notch2 inhibitor, indicating a therapeutic potential of this pathway for treating COPD patients.[30]

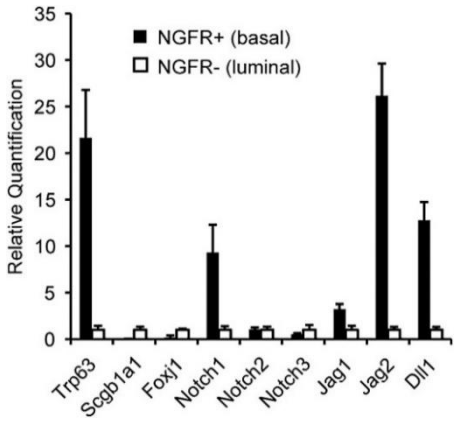

Figure 11. Overview of the Notch signaling pathway genes expressed in basal cells (black bars) and luminal cells (open bars) acquired from qRT-PCR analysis. The y-axis shows the relative quantification. Based on triplicate samples and three independent experiments, the error bars show a confidence interval of 95%.[11]

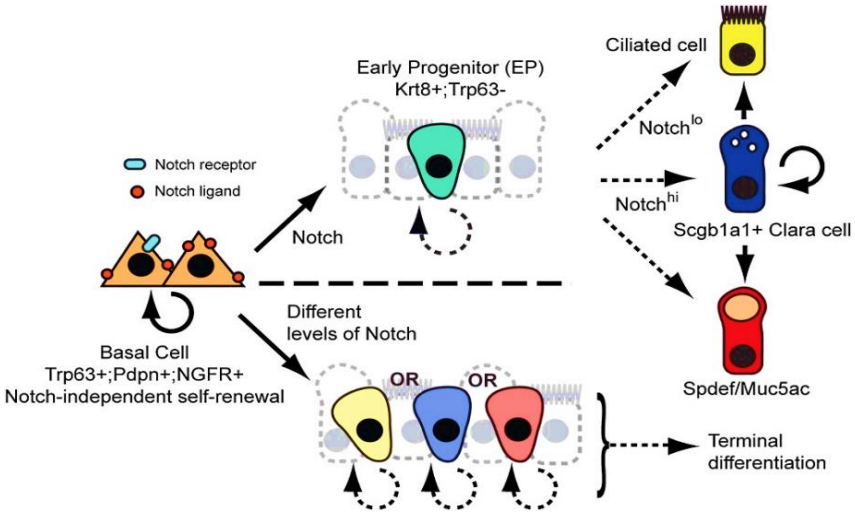

Figure 12. Schematic overview of the role of Notch signaling in basal cells. Notch signaling is only involved in luminal differentiation of basal cells and do not contribute to self-renewal of these cells.[11]

Collectively, these studies show that the HH signaling pathway, its inverse correlation with mesenchymal proliferation during repair and regeneration processes and Notch signaling pathway are dysregulated in COPD (**Table 1**).[34] These changes, among others, impact basal cell proliferation/differentiation processes and epithelial repair and regeneration. In COPD, Ptch1 is upregulated (in moderate and severe COPD stages; shown using *in vitro* and *in vivo* models by Tam et al.[2,28]), whereas SHH, Hhip, Gli2, Gli1+, and Smo (shown using bleomycin and naphthalene injury mice models[34]) are downregulated.[6,25,28] Notch signaling in COPD is also inhibited leading to downregulation of Notch3, DLL1, HES5 and HEY1/2 and upregulation of Notch 2.[6,30]

Table 1. Dysregulated signaling pathways with their respective targets and study models in COPD

Signaling pathway	Target	Consequences	Species	Model
Airway basal progenitor cells	Airway basal progenitor cell "fatigue"	**Ghosh et al., 2018[33]:** ⁘ Airway basal progenitor cell differentiation capacity ↓ → regeneration of epithelium ↓ ⁘ Basal cells 1.5-fold ↑ → Secretory cells 2-fold ↑ → Ciliated cells 88% ↓ [33] ⁘ Notch ↓ → Differentiation of basal cells and parabasal (p63+) cells ↓ → ↑ multiciliated cells and ↓ secretory cells[33]	Mice Human [33]	ALI [33]
HH	Ptch1 ↑ SHH ↓ Hhip ↓ Gli2 ↓ Gli1+ ↓ Smo ↓	**Tam et al., 2019[28]; Ancel et al., 2020[25]:** **Normal**: ⁘ HH ligand binding → Ptch1 dissociation from Smo → Smo release ↑ and activation of Gli-family transcription factors [28] **COPD**: ⁘ No HH ligand binding → Ptch1 inhibits signaling through Smo [28] → Smo release ↓ → ↓ HH pathway (also shown by Peng et al., 2015[34]) ⁘ Ptch1 ↑ → secretory cells ↑ → mucus production ↑ → mucus hypersecretion → chronic bronchitis phenotype → airway epithelial thickening → airway remodeling → airflow limitation [28] ⁘ Hhip mRNA and protein ↓ → indirect activation of HH as Hhip is a antagonist for SHH, IHH and DHH [25,28] ⁘ Fibroblast from COPD smokers SHH > fibroblasts from COPD non-smokers [28] ⁘ SHH ↓ → inactivated HH → Gli2 nuclear translocation ↓ (transcription activator ↓) → impairment in basal progenitor cells → basal cells ↑, Gli2-positive cell nuclei in basal cells ↓ and ciliated cells ↓ → COPD-like epithelial remodeling [25]	Mice Human [28]	⁘ Bleomycin[34] ⁘ napthalene injury[34] ⁘ *in vitro* [25,28] ⁘ *in vivo* [28] ⁘ ALI [28]
Notch	Notch 3 ↓ DLL1 ↓ HES5 ↓ HEY1/2 ↓ Notch 2 ↑ NICD1/HEY2+ ↑	**Hadzic et al., 2020; Rock et al., 2011; Zepp et al., 2019; Kiyokawa et al., 2020, Ghosh et al., 2018:** ⁘ **Enhanced cell turn over conditions** : Notch ↑ → basal cell differentiation → secretory lineages ↑ [11] ; ↓ multiciilated cells and mucus metaplasia ⁘ Notch ↓ → lead secretory cell differentiation → ↑ multiciilated cells and ↓ secretory [24]→ goblet cell metaplasia [6] → cells; basal cells (KRT5+ and KRT14+) expand in squamous metaplasia regions [11] ⁘ **COPD**: Notch ↑↑↑ → NICD1/HEY2+ ↑ (in epithelial cells of COPD patients notably in mucosal hyperplasia areas) → basal cell differentiation to secretory lineages ↑ → secretory cell differentiation to multiciliated lineages ↓[30]	Mice [6,11,24] Human [11,24,30]	⁘ injury/ repair model of mouse tracheobronchial epithelium to study Notch *in vivo* [11] ⁘ Primary human airway bronchial epithelial cells [11]

HH = Hedgehog; Ptch1 = Patched homolog 1; SHH = Sonic Hedgehog; Hhip = Hedgehog interacting protein; Gli2 = transcription factor; Smo = G-protein-coupled receptor smoothened; DLL1 = Delta-like ligand 1; HES5 = hairy and enhancer of split 5; HEY1 = Hairy/enhancer-of-split related with YRPW motif 1; HEY2 = Hairy/enhancer-of-split related with YRPW motif 2 (downstream effectors of Notch); NICD1 = Notch1 intracellular domain[30]; reduction in consequences is shown with arrows pointing downwards (↓), whereas increase is shown with arrows pointing upwards (↑)[18].

Challenges and recommendations

Evaluating the therapeutic potential of stimulating basal cell functionality in COPD, via HH and Notch signaling pathways, showed that under normal conditions these pathways are activated leading to an increase in airway basal progenitor cells and its differentiation into secretory and multiciliated cells. Hence, inducing epithelial repair and maintaining a balance between the mesenchymal quiescence and epithelial cell proliferation.[6,24] However in COPD, the HH pathway is inactivated, whereas the Notch pathway is persistently activated leading to an increased amount of basal and secretory cells and a decreased amount of multiciliated cells. This, then leads to increased mucus production, COPD-like airway remodeling and airflow limitation. Crucial receptors and ligands involved in COPD pathogenesis include Hhip receptor, Ptch1 receptor, SHH ligand (HH) and Notch 2 (Notch) receptor (**Table 1**).

Aforementioned studies tested the attenuation of Ptch1, upregulation of SHH, expression of Hhip and inhibition of Notch 2 in mice models (*in vivo* and *in vitro*) and human samples (*in vitro*) to induce basal cell-mediated epithelial repair and regeneration in COPD. In order to do so, these authors conducted target validation wherein several limitations were encountered. Ghosh et al. conducted a non-prospectively designed cross-sectional study which limited the possibility to test underlying hypotheses such as examination of the relationship between progenitor count and lung function over time. Another limitation was that the subjects were white males, therefore restricting generalizability to other populations.[33] As COPD is a heterogenous and complex disease single animal models cannot reproduce this pathogenesis. Therefore, different animal models that mimic different features of the COPD stages were used by Hadzic et al., and Tam et al..[6,28] The mice model used by Tam et al. did not cause mucus expression and goblet metaplasia in the distal lung compared to air-exposed controls (upon chronic smoke exposure up to 6 months). Therefore, different (ovalbumin or house dust mite (HDM)) models were used to initiate goblet metaplasia.[28] Tam et al. also conducted a cross-sectional study, which made it difficult to draw strong conclusions on the relationship between Ptch1 and mucus expression in COPD patients.[28] Downregulation of Ptch1 could dysregulate mucus expression in COPD, but other factors such as oxidative stress and local inflammation could also have contributed to activation of HH signaling.[28] Other epithelial-specific Ptch1 mRNA and protein expression studies showed positive correlation of Ptch1 with FEV1/FVC in whole lung tissues from COPD and non-COPD patients, whereas in this study epithelial-specific Ptch1 expression were quantified (which led to paradoxical findings).[28]

Therefore it is recommended to upregulate the HH pathway and downregulate the Notch pathway to induce basal cell-mediated epithelial repair and epithelial regeneration in COPD.[28,35] This could be done using antagonists for Hhip, Ptch1 or Notch 2 receptors or agonists for SHH.[2,25,28,30] Antagonist for Hhip or agonist for SHH could lead to an increase in SHH ligand which on its turn will cause dissociation of Ptch1 from Smo, release of Smo and translocation of Gli2 transcription factor. This will activate the HH pathway leading to an increase in basal progenitor cells, its differentiation into secretory cells and trans-differentiation of secretory cells into multiciliated cells. SHH activates the HH pathway by binding and inhibiting Ptch1, thereby initiating the downstream signaling. SHH does so by antagonizing the Ptch1 receptor and binding and inhibiting this with only its short palmitoylated N-terminal fragment. The rest of the SHH ligand also binds and internalizes this receptor through a binding site, but does not contribute to activation of HH signaling.[36] Antagonist for the Notch 2 receptor could downregulate the persistent activation of Notch and therefore basal progenitor cells will be able to differentiate into secretory cells and multiciliated cells using the Notch2 expressed in this cell population.[24,30]

Small-molecule inhibitors for Notch (Notch/ γ-secretase inhibitors[37]) and Hedgehog (e.g., Vismodegib targeting basal cell carcinoma[38]) are also therapeutically beneficial to target basal cells in COPD. These novel therapeutic approaches should be tested in COPD patients. These inhibitors could be administered locally via inhalation using e.g., dry powder inhalers, metered-dose inhalers, or nebulizers to minimize side effects in other parts of the human body, to increase the efficacy and to get a high dose at the site of action (small molecules up to 20 kDa for a feasible lung deposition).[39]

These agonists and antagonists and its role in basal cell-mediated epithelial repair can be evaluated using *in vitro* and *in vivo* cigarette smoke (CS) and elastase induced injury/repair mouse models and human tracheobronchial epithelial cell cultures (prospectively designed study) as these are low of costs, easy to use and give reproducible results.[40] Mouse models (tracheobronchial epithelium with resident basal progenitor cells[11]) are recommended as they show genetic and biological similarities with humans, thus, allowing e.g., gene manipulation.[40] In the cigarette smoke induced models, mice (whole body or only via the nose for a certain time period) could be exposed to CS in a smoking equipment in order to demonstrate the chronic bronchitis phenotype in COPD, upregulation of Ptch1, the effect of Ptch1 antagonists (SHH) on these receptors and Notch2 antagonists/inhibitors. In the elastase induced models, intratracheal instillation could be used to introduce elastase into the trachea of mice to reproduce

the emphysema phenotype, and demonstrate the effect of Hhip antagonists.[40] Elastase mice models are technically easy to initiate COPD by a single tracheal instillation of elastolytic enzymes (e.g., porcine pancreatic elastase (PPE), papain and human neutrophilic elastase) and to control disease severity using enzyme adjustments.[40] Although both models are advantageous and could be used for studying COPD in mice, these also have limitations. These limitations are e.g., that there are no standardized protocols yet for animal exposure (a limitation of CS-induced COPD *in vivo*), therefore the source of smoke (commercial or research cigarettes), components of CS, delivery (entire body or via the nose), and the dose of CS are important factors which should be kept in mind.[40] A disadvantage of the elastase model is that various pathophysiological mechanisms determine elastase function in COPD (emphysema) and bring up a lot of clinical events.[40] Therefore, these therapeutics could also be tested using *in vitro* pharmacokinetic models (for e.g., drug dosing, ADME, bioavailability, delivery route) and lung organoids from airway epithelial stem/progenitor cells (or induced pluripotent stem cells, iSPC[22]) using high-throughput screening, air-liquid interface (ALI) systems, immunofluorescence microscopy and gene editing technology (CRISPR/Cas9). These derived lung organoids, similar to *in vivo*, are able to differentiate and self-organize, however it does not yet cover all the complex mechanisms and interactions in the lung. Therefore, it can be used in combination with *in vivo* animal/human models to give reproducible results and to better understand cell behavior, molecule production (by mesenchymal cells from stem cell niche or adjacent epithelial cells), and the role of ECM and physical forces in their regulation processes in human to provide curative treatment for COPD.[22,41]

Conclusions

This literature study evaluated the therapeutic potential of stimulating basal cell functionality in COPD, based on published studies, and provided recommendations for identified challenges. This review discussed the normal respiratory tract epithelium, COPD pathophysiology, and the role of basal cells, Hedgehog and Notch signaling pathways and beneficial therapeutic targets for basal cell-mediated epithelial repair. It has been shown these basal progenitor cells could contribute to lung epithelial repair by self-renewing, differentiating into ciliated and secretory cells, and establishing interactions with mesenchymal cells.[31] They could do so using signaling pathways such as HH signaling, that maintains a balance between epithelial cell proliferation and mesenchymal quiescence during homeostasis and regeneration in COPD, and Notch, that control lung development when reactivated upon lung regeneration and regulate basal cell differentiation into the secretory lineage.[6,24] In COPD, SHH and Notch are downregulated causing upregulation of Ptch1 and Notch2 and downregulation of Hhip, Gli2, Gli1+, Smo, Notch3, DLL1, HES5 and HEY1/2.[6,25,28,30,34] Evaluating basal cell-mediated epithelial repair using either Hhip, Notch2 or Ptch1 antagonists or SHH agonist could be beneficial to develop innovative therapeutics which target epithelial remodeling and its dysregulated repair at the earliest stages of COPD progression. These therapeutic strategies could be evaluated using a combination of CS induced and elastase induced mice models (*in vivo*), lung organoids (of basal stem/progenitor cells) and human models (*in vivo* and *in vitro*).[31,42]

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