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Title: Epithelial stem and progenitor cells of the upper airway

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Abstract

The upper airway acts as a conduit for the passage of air to the respiratory system and is implicated in several chronic diseases. Whilst the cell biology of the distal respiratory system has been described in great detail, less is known about the proximal upper airway. In this review, we describe the relevant anatomy of the upper airway and discuss the literature detailing the identification and roles of the progenitor cells of these regions.

Introduction

The upper airway, or respiratory tract, commences at the nasal cavity and ends at the bifurcation of the trachea, or carina, where the airway divides into the right and left bronchi. The upper airway is important in its function of conducting air to the distal respiratory system and acts as a barrier tissue in the face of external foreign particles including infectious agents, allergens and pollutants. The lining of respiratory epithelium is prone to dysfunction which can lead to multiple diseases causing major global morbidity, including chronic inflammatory diseases such as allergic rhinitis (AR), chronic rhinosinusitis (CRS), asthma and chronic pulmonary obstructive disease (COPD).

The respiratory epithelium is pseudostratified, consisting of multicilated cells, goblet cells and basal cells, the progenitor cells of the proximal airway. Basal cells are a self-renewing population of cells with the capacity to commit to further differentiation[1]. These stem, or progenitor, cells maintain normal organ homeostasis and are important in initiating repair following injury.

Stem cells exist across many epithelial tissues of the body and whilst very effective tissue-repair mechanisms exist, there are observed links between chronic tissue damage and inflammation, and carcinogenesis[2]. This begs the question of whether progenitor cells act as the cell of origin in tumor initiation and indeed in the lung, alveolar type II cells, the progenitor cell of the distal alveolar space, have been proposed as the cell of origin of lung adenocarcinoma cells[3, 4]. It is therefore imperative to gain a full understanding of the function and role of progenitor cells both in homeostasis and in disease.

This review will provide an overview into what is known about progenitor cells of the upper airway, specifically focussing on the nasal cavity, larynx and trachea. A summary is provided in Table 1.

Relevant Anatomy

The upper airway, or respiratory tract commences at the nose, which consists of two nasal cavities separated by the nasal septum. The lateral nasal wall houses three bony cushions, termed the superior, middle and inferior turbinates. Both the lateral nasal wall and nasal septum are lined by pseudostratified respiratory epithelium. Olfactory epithelium, which houses specialized cilia and olfactory receptor cells responsible for detecting odor molecules, lies above the superior turbinate.

The upper airway then continues posteriorly, or backwards, to the pharynx, a tube-like structure which is divided into the nasopharynx, oropharynx and laryngopharynx, or larynx. The larynx serves as a conduit for the passage of air to the airway below, and also provides vital function in allowing phonation and effective swallowing. The larynx is divided into the epiglottis, the glottis (where the vocal cords are situated) and the subglottis. The epithelial lining varies across the organ; the superior portion of the posterior glottis and anterior vocal cords are lined by keratinising stratified squamous epithelium whereas the rest of the organ is lined by pseudostratified ciliated columnar (respiratory) epithelium[5].

At the level of the cricoid cartilage, the subglottis then forms the trachea, or windpipe, which divides at the carina to the left and right main bronchi where the upper airway concludes.

Following this division in humans, the lower respiratory tract undergoes up to 23 further divisions from the main bronchi, down to the terminal bronchioles[6]. Thereafter, the respiratory bronchioles, along with the alveolar ducts, form the most distal respiratory airway zone with the function of gas exchange[7]. In mice, it is important to note that the terminal bronchioles transition directly into the alveolar space at the bronchoalveolar duct junction[8].

Basal cells – the progenitor cells of the trachea, bronchi and terminal bronchioles

When considering the progenitor cell population of the upper airway, the vast majority of characterization work has been performed thus far on the trachea, main bronchi and terminal bronchioles in both mice and human. We will therefore first discuss these anatomical regions.

Basal cells in the human extend from the upper airways to the terminal bronchioles, as shown in immunohistochemistry studies[9], whereas in mice basal cells are limited to the murine trachea[1]. In both species basal cells express the same commonly used markers, the transcription factor TP63 and CK5, a cytokeratin, and make up approximately 30% of the pseudostratified epithelium of the lung[1, 10]. Electron microscopy studies as well as immunohistochemistry of cytokeratin 14 (CK14) have also demonstrated two basal cell subpopulations in the normal human airway, with reduced CK14 expression and poorly developed hemidesmosomes and anchoring fibrils in the distal airways[11]. Other proposed basal cell markers include podoplanin (PDPN), nerve growth factor receptor (NGFR)[10] and integrin-alpha 6 (ITGA6), as well as KRT15, LGALS1, ITGB4, LAMA3, LAMB3, S100A2, NPPC, BCAM and DST[12].

The basal cells of the trachea have been most extensively studied in mice and humans and it is these studies that have determined their capacity to differentiate into goblet cells, characterized by MUC5AC expression and multicilated cells, characterised by ACT and FOXJ1 expression[13]; these cell types typically reside in the luminal compartment of the airway. Further basal cell characterisation studies are discussed below.

Characterisation of basal cells

In vitro techniques

First, it is important to discuss the various techniques that have been utilised to study the *in vitro* progenitor and differentiation capacity of basal cells from the trachea to the terminal bronchioles. Primary human bronchial epithelial cells (HBECs), sampled from either brushings or biopsies of the airway, can be successfully grown in 2D in optimised cell culture media[14] and rapidly expanded to clinically-relevant numbers for tissue engineering strategies[15]. Air-liquid interface (ALI) cultures enable differentiation of basal cells for functional studies whilst 3D culture of HBECs in an extracellular matrix promotes basal cell differentiation into the main differentiated luminal cell types of ciliated and secretory cells, in the proportions expected *in vivo*[15, 16]. Organoids provide a functional 3D unit in which to study both homeostasis and disease processes in response to injury *in vitro*; further advances have been made in the long-term culture of airway organoids, with Sachs *et al*[17] demonstrating the maintenance of pseudostratified human airway epithelium for over a year.

In vivo models

Studying homeostasis under normal conditions can be challenging due to basal cells' low background proliferative rate, however injury models have been utilised successfully, in combination with lineage tracing, to discover more about both lineage potential and cellular mechanisms of differentiation.

Rock *et al*[18] used a sulfur dioxide (SO_2) model of airway injury to demonstrate the importance of Notch signalling in driving terminal differentiation in repair and proposed a model in which differentiating basal cells divide asymmetrically to generate a multipotent population of suprabasal TP63- CK8+ (a cytokeratin) luminal cells, termed 'early progenitors'. They found that sustained Notch activation then promotes their luminal differentiation into ciliated and secretory cells. Pardo-Saganta *et al*[19] further investigated this heterogeneity of basal progenitor cells; a similar injury model was used to demonstrate discrete subpopulations of TP63+ basal cells that inherently express Notch pathway components following injury which are usually associated with luminal cells, c-myb+ and N2ICD+, corresponding to ciliated and secretory cell fate commitment respectively. These subsets of basal cells appear to have committed to either ciliated or secretory fate prior to

the formation of CK8+ luminal cells. Further evidence is provided by Watson *et al*[20] who used long-term lineage tracing and mathematical modelling to discover two distinct populations of CK5+ basal cells, multipotent basal progenitor cells and committed precursors, termed basal luminal progenitors or BLPs; the authors suggest those basal cell subpopulations found by Pardo-Saganta *et al*[19] correspond to the most differentiated BLPs.

Cytokeratin 14 (CK14) has also been used to demonstrate basal cell multipotentiality. CK14 is upregulated in a subpopulation of basal cells in murine trachea, and expression is widespread following injury. Using naphthalene injury, designed to specifically ablate secretory cells and recruit non-secretory cell progenitors[21], CK14-positive cells were shown to be both multipotent, possessing the ability to differentiate into basal, ciliated and secretory cells, as well as unipotent. Ghosh *et al*[22] used lineage tracing to further demonstrate that 'steady state' CK14+ cells were unipotent in homeostasis and multipotent following injury, and that in the context of injury, 'steady state' CK14+ cells were a direct progenitor for club and ciliated cells and that 'steady state' CK14-derived club cells were themselves progenitors for ciliated cells.

In the remaining murine airway beyond the trachea, club cells which express SCGB1A1 have been found to represent a population of self-renewing cells[23] as basal cells do not exist in these regions; these give rise to ciliated cells. Interestingly, Tata *et al*[24] demonstrated the plasticity of club cells, showing their ability to de-differentiate into basal cells using a doxycycline model of basal cell ablation and lineage tracing of SCGB1A1 cells.

Several important signalling pathways and their subsequent mechanistic effects on basal cell function have been uncovered in mice. Mori *et al*[25] unearthed the importance of the Jag 1 / 2 signalling pathways in controlling the pool of progenitor cells available for differentiation; they found that basal cells express Jag ligands in homeostasis and Notch 3 becomes selectively expressed in cells occupying a parabasal position (likely to be suprabasal cells), once the pool of p63+ basal cells are fully expanded, under the control of Jag 1 / 2 signalling from adjacent basal cells. These Notch 3+ progenitor cells then remain undifferentiated until Notch 1 and Notch 2 signalling drives secretory-multiciliated cell fate selection.

Single cell RNA-sequencing (scRNA-seq)

Single cell transcriptomic studies have demonstrated basal cell diversity throughout the murine trachea in homeostasis and the human airway in health, and provide an insight into cell lineage hierachies. Zhou *et al*[26] use scRNA-seq of murine trachea from the *Trp63*$^{CreERT2;Rosa26lox\text{-}STOP\text{-}lox\text{-}tdtomato}$ mouse line to find six distinct basal cell subpopulations with two spatially distinct major basal cell subpopulations. In humans, Goldfarbmuren *et al*[27] discover three basal cell subpopulations in tracheal epithelium, across smokers and non-smokers, based on differential gene expression signatures of proliferation, differentiation and squamous metaplastic response to injury, whereas in the distal airways, four distinct basal cell subpopulations were demonstrated by Carraro *et al*[28]. This is further shown by the functional heterogeneity of human CK5^{+}-basal cell-derived organoids as demonstrated by scRNA-seq[29], with two subtypes termed Basal 1 and Basal 2.

'Suprabasal cells', cells that are already committed to differentiation and characterised by low TP63 expression and higher expression of KRT19 and NOTCH3, have also been demonstrated[30]. Single-cell work has also demonstrated the expansion of specific basal cell subpopulations more distally in disease, with identification of a 'secretory primed basal' (SPB) cell subset in idiopathic pulmonary fibrosis[28] and demonstration of hypoxia and Notch signalling as possible drivers of a CK5+ basal-like state in human fibrotic AT2 cells[31].

Further single-cell RNA sequencing studies have been performed which also include the upper airway at the nose and are discussed below along with progenitor cells of the nose.

Effects of injury on basal cells

In homeostasis, it has been shown that the proximal airway epithelium in humans is maintained by an equipotent basal cell population, where the clonal dynamics are determined in a stochastic manner in *vivo*, in a 'neutral drift' model[32]. However, this does not preclude the possibility of a small subset of stem-like basal cells that become activated in injury, for instance in response to smoking exposure, which then expand in repair.

Indeed, this was further investigated by Yoshida and Gowers *et al*[33], who defined the genomic architecture of normal airway cells in children and never-smoking, smoking and ex-smoking adults, and discovered that the very earliest carcinogenic changes can be identified in "'normal-appearing" airway epithelium in patients, with and without lesions. They found considerable heterogeneity in mutational burden across the cells, discovering a population of proliferating basal cells in ex-smokers with near-normal mutational burden, which expand to repopulate the airway in response to injury. These cells also lacked the mutational signatures associated with tobacco exposure, more closely resembling signatures in cells of never- smokers. These findings underpin and provide a molecular basis for the epidemiological observation that ex-smokers have greater protection against lung cancer the longer they have stopped smoking and adds further weight to the known phenomenon of basal cell heterogeneity throughout the human airway.

In the content of injury, it is important to consider the role of airway progenitors in distal alveolar regeneration, as has been shown by the presence of lineage-negative epithelial progenitors (LNEPs)[34] or distal airway stem cells (DASCs)[35]. Vaughan *et al*[34] demonstrated the ability of LNEPs in the distal lung, reliant on Notch signalling, to activate a P63/CK5+ remodeling program after influenza or bleomycin injury, where cells then migrate and propagate to repair injured areas deplete of mature lineages. This finding was further validated by Zuo *et al*[35] who also showed that selective ablation of these DASCs prevents distal alveolar regeneration in the context of influenza injury.

Further, transplantation of exogenous basal cells into the airway epithelium following injury has been shown to have a durable response, with preserved self-renewal and differentiation capacity beyond two years following engraftment[36]; this has great potential in the field of cellular therapy for diseases such as cystic fibrosis and primary ciliary dyskinesia and demonstrates the translational possibilities once the resident epithelial stem cells of an organ are completely characterized.

Progenitor cells of the respiratory bronchioles – beyond mice and men

A key difference between the human and murine airway is that the respiratory bronchioles, along with the alveolar ducts, form the most distal respiratory airway zone in humans but in mice, the terminal bronchioles transition directly into the alveolar space at the bronchoalveolar duct junction. The respiratory bronchiole similarly does not exist in rats, hamsters, guinea pigs, gerbils or rabbits[8]. Ferrets and primates have been used as alternative animal models in studying the progenitor cells of the respiratory bronchioles[7, 37, 38] and offer promise in examining human homeostasis and modelling disease, particularly relevant for cell replacement or gene therapy work. Pigs have been found to also have comparable cellular lineages and composition to human airways[39, 40].

A recent study by Basil *et al*[7] has proposed a new population of progenitor cells of the respiratory bronchiole region, termed respiratory alveolar secretory (RAS) cells. Single cell RNA sequencing was employed to transcriptionally demonstrate the presence of these cells, between canonical secretory cells and alveolar type 2 cells (AT2) cells, based on expression of SCGB3A2, which was further corroborated using immunohistochemistry and RNAscope analysis of this region, in humans and in ferrets. Using in vitro organoid models, they also showed the ability of RAS cells to differentiate into AT2 cells through Notch and Wnt signalling. Similarly, Murthy *et al*[41] used spatial transcriptomics and single-cell analysis of the human distal airways, specifically the terminal and respiratory bronchioles (TRBs), to identify several new cell types, including TRB-specific alveolar type-0 (AT0 cells) and TRB secretory cells (TRB-SCs). In a non-human primate model of lung injury, AT2 cells were found to transiently acquire an AT0 state, termed a bipotent progenitor, from which AT1 cells and TRB-SCs could differentiate.

Progenitor cells of the nose

Basal cells, as the progenitor cells of the trachea, bronchi and terminal bronchioles, are the likely putative progenitor cells of the nasal respiratory epithelium. Indeed, the airway epithelium is typically considered as one entity rather than anatomically distinct compartments, and is mainly composed of basal cells, multicilated cells, goblet cells and club cells, as described[42]. Changes in gene expression profiling of the nasal epithelium has

been shown to be detectable in lung cancer patients, suggestive of a carcinogenic "field effect"[43] and supportive of the "one airway, one disease" stance[44].

There has, however, been limited scrutinization or characterization of basal cells of the nose in isolation thus far in the literature, in terms of stem cell function and differentiation capacity. One example is by Yu et al[45], who have isolated and cultured basal cells from nasal polyp tissue and control nasal mucosa, demonstrating reduced colony-forming efficiency and proliferation rate in the nasal polyp tissue compared to healthy controls. Progenitor cell function has not yet been compared between the nose and trachea or bronchi, however.

Nasal epithelial cells are often used as control samples for normal respiratory epithelium in human studies due to the inherent "easy access" of nasal brushings from patients and healthy volunteers[46]. Ruiz Garcia *et al*[47] used single-cell RNA sequencing to establish a single-cell atlas through the time course of airway differentiation *in vitro*, using human nasal inferior turbinate tissue and an air-liquid interface culture system. They propose that goblet cells are possible precursors of multicilated cells and identify several subpopulations of basal, suprabasal, club and multicilated cells, and extrapolate these findings to both human and murine airway epithelium *in vivo*. They do, however, acknowledge that this inference may be limited as the differences, if any, between the spatially distinct nasal, tracheal and bronchial compartments are yet to be determined. Further, Deprez *et al*[30] performed single-cell transcriptomics of the proximal to distal upper respiratory tract (nose to 12th division of airway), identifying region-specific subclusters of suprabasal, secretory and multicilated cells of the nose, characterised by differentially expressed genes that could only be found in the nasal samples.

While questions remain as to whether nasal epithelial phenotype and progenitor capability clearly mirrors the rest of the upper airway, Giovannini-Chami *et al*[48] question whether the different regions do follow similar pathophysiological processes in disease states along the proximal-to-distal axis. In the context of allergic respiratory disease, the authors use flow cytometric analysis and RNA sequencing of human samples to conclude that nasal epithelial cells are not good surrogates for bronchial epithelial cells in the specific evaluation of type 2 T-helper cell (Th) status. Whether this dichotomy can be extrapolated to other studies of

respiratory epithelial function in homeostasis and disease remains to be seen. Indeed, in the context of malignancy, cancers which affect the nasal cavity, often considered in conjunction with those of the paranasal sinuses as sinonasal cancers, are much rarer than lung cancers in terms of incidence, despite carcinogenic tobacco smoke exposure being a common risk factor; the incidence per year of lung cancers is typically 100 times higher than sinonasal cancers in the USA[49, 50]. This contrast may also suggest that caution should be taken when considering the upper airway as one continuous entity.

Basal cells of the nose have been shown to be of importance in the context of disease. Chronic rhinosinusitis (CRS) is a disease that ranges from rhinitis to severe nasal polyposis, and results from persistent activation of Type 2 immunity, with basal cell hyperplasia a hallmark of severe disease; Ordovas-Monatanes *et al*[51] examined this using Seq-Well for massively parallel single-cell RNA sequencing to profile primary human surgical CRS samples, reporting transcriptomes for human respiratory epithelial, immune and stromal cell types, enabling comparison with healthy tissue. In severe polyposis, they found a global reduction in cellular diversity characterized by basal cell hyperplasia and reduction in glandular cells, as well as an aberrant basal progenitor differentiation trajectory. They propose that a reduction in epithelial diversity, arising from functional shifts in basal cells secondary to formation of 'memories' of chronic exposure to an inflammatory Type 2 environment, underlies the barrier dysfunction observed in this context and contributes to this chronic inflammatory disease.

In the context of the SARS-Cov-2 pandemic, there is also heightened interest in the function of the nasal respiratory epithelium as the nose is viewed as the initial route of entry of the virus; indeed, in symptomatic patients, nasal swabs have yielded higher viral loads than throat swabs[52]. Sungnak *et al*[53] used single-cell RNA sequencing to discover that SARS-Cov-2 entry-associated genes, namely ACE2 and TMPRSS2, are enriched in nasal goblet and ciliated cells, a finding further corroborated by Ziegler *et al*[54, 55]. Nasal samples, along with matched tracheal, bronchial and blood samples, have also been utilised in single-cell multi-omic profiling to demonstrate the presence of a higher steady-state expression of interferon-responsive genes in the airway epithelium of pediatric patients compared to adults, with a more naïve systemic immune state; these findings provide a basis for the observed milder

clinical symptoms in children following SARS-Cov-2 infection[56]. Cultured epithelial cells from the nose also more robustly replicate SARS-Cov-2 compared to those from the large or small airways[57], further suggesting the existence of functional differences.

Progenitor cells of the larynx

The larynx is a 'patchwork' of stratified squamous and respiratory epithelium as detailed above and whilst the nature of progenitor cells of the respiratory epithelium of the nose is yet to be fully elucidated, even less is known about these cells in the larynx. Like the nose, it is probable that basal cells are the progenitor cells of the respiratory epithelium that lines the laryngeal surfaces, however little work has thus far been performed to characterise these. Further weight is added to this since embryologically, the larynx, trachea and esophagus are intrinsically linked via shared organogenic pathways within the foregut endoderm of the upper aerodigestive tract[58].

In developmental biology, the stratified squamous vocal fold epithelium of the mouse has been characterized, with evidence of the presence of p63-positive basal cells during postnatal stages (up to adulthood) with expression of CK5 and CK14 throughout all the layers of the vocal fold, as opposed to just the basal cell layer of the trachea, with persistent expression of CK8 expression in the luminal layer[59].

Long-term culture of primary epithelial cells is fundamental to investigating the cell biology of an organ. Mou *et al*[60] previously demonstrated the benefit of dual SMAD signalling inhibition for in vitro expansion of epithelial basal cells, using laryngeal cells, amongst other cell types. They show that expanded laryngeal basal cells formed stratified epithelia when differentiated in air-liquid interface (ALI) culture and suggest that prior cultures failed because of senescence due to unwanted differentiation. Dong *et al*[61] have shown the use of conditional reprogramming to establish a primary culture system of normal and laryngeal and hypopharyngeal squamous cell carcinoma (LHSCC), using a feeder layer of mitotically-inactivated 3T3-J2 mouse embryonic fibroblasts. However, it has been demonstrated that such a culture system tends to favour expansion of normal epithelial cells[62] and it must be

noted that the cancers in this system were not sequenced to prove their nature. It was also possible to manipulate these cells into 3D organoids, corroborating the findings of Driehuis *et al*[63] who investigated the feasibility of growing patient-derived organoids from samples of laryngeal cancer, amongst other head and neck squamous cell carcinoma samples (HNSCC). Furthermore, the LHSCC-derived organoids in Dong *et al*[61] were found to be positive for the basal cell markers CK5+ and P63+ in the outer layer, whilst keratinized cells were located on the inside of the organoids, suggestive of possible trans-differentiation to a squamous phenotype. *In vitro* culture of laryngeal respiratory airway basal cells appears to remain challenging.

Conclusions and future directions

Whilst much is known about the progenitor cells of the upper airway beyond the trachea, there is limited knowledge regarding the proliferation and differentiation capacity of the putative progenitor cells of the nose and larynx. Are there differences in phenotypic function between these subsites, the trachea and the rest of the conducting airways? Are these cells transcriptionally comparable? Are cells from these regions differentially susceptible to disease or cancer? Further investigation and scrutinization of the progenitor cells of these upper airway subsites is required to be able to answer these outstanding questions.

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Table 1. **Epithelial progenitor cells of the human upper airway**

Anatomical Location	Cell type	Human marker genes	Function in adult organ	References
Nose	Basal cells	KRT5, KRT14, TP63	Putative progenitor cell - self-renewal and differentiation capability not yet fully characterised	Ruiz Garcia et al 2019[1], Vieira Braga et al 2019[2]
	Suprabasal cells	KRT5, SERPINB4, KRT19, FABP5, TFCP2L1, S100A9, KRT16, KRT23	More committed to differentiation than basal cells	Deprez et al 2020[3], Ruiz Garcia et al[1]
Larynx	Basal cells	KRT5, P63	Putative progenitor cell - self-renewal and differentiation capability not yet fully characterised - distinction between stratified squamous and respiratory epithelium progenitor cells not yet characterised	Lungova et al 2015[4]
	Suprabasal cells	KRT8?	Not yet fully characterised	Lungova et al 2015[4]
Trachea/main bronchi/terminal bronchioles	Basal cells	KRT5, KRT14, KRT15, TP63, $DLK2_{high,}$, PDPN, NGFR, LGALS1, ITGA6, ITGB4, LAMA3, LAMB3, NPPC	Main airway progenitor cell – self-renewal and differentiation capability	Rock et al 2009[5], Hogan et al 2014[6], Nikolic et al 2018[7], Deprez et al 2020[3], Hewitt and Lloyd 2021[8], Vieira Braga et al 2019[2], Zaragosi et al 2020[9]
	Suprabasal cells	KRT5, KRT19, NOTCH3, $TP63_{low,}$ SERPINB4	More committed to differentiation than basal cells	Deprez et al 2020[3], Hewitt and Lloyd 2021[8]
Respiratory bronchioles	RAS cells	SCGB3A2, SCGB1A1	Differentiation into alveolar AT2 cells	Basil et al 2022[10]

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