# A novel subset of airway basal cells: Identification of a stem/progenitor cell population in the human respiratory system

By

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CERTIFICATE OF APPROVAL

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# List of Abbreviations

| | |
|---|---|
| AEC1 | Alveolar epithelial cell 1 |
| AEC2 | Alveolar epithelial cell 2 |
| AQP5 | Aquaporin 5 |
| ATI | Alveolar type I |
| ATII | Alveolar type II |
| ALI | Air liquid interface |
| ASMC | Airway smooth muscle cell |
| BEGM | Bronchial epithelial growth media |
| BMP | Bone morphogenetic protein |
| CCSP | Club cell secretory protein |
| CGRP | Calcitonin gene-related peptide |
| CRC | Conditionally reprogrammed cell |
| CYP2F2 | Cytochrome P450, family 2, subfamily f, polypeptide 2 |
| DLL | Delta like ligand |
| EGF | Epidermal growth factor |
| EpCam | Epithelial cell adhesion molecule |
| FACS | Fluorescence activated cell sorting |
| FBS | Fetal bovine serum |
| FGF | Fibroblast growth factor |
| GFP | Green fluorescent protein |
| GRP | Gastrin release peptide |

| | |
|---|---|
| HAT | Hypoxanthine-aminopterin-thymidine |
| hBECs | Human bronchial epithelial cells |
| HH | Hedgehog |
| HOPX | Homeodomain only protein |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IHH | Indian hedgehog |
| iPSC | Induced pluripotent stem cell |
| ITGA6 | Integrin alpha 6 |
| ITGB4 | Integrin beta 4 |
| KRT5 | Cytokeratin 5 |
| KRT8 | Cytokeratin 8 |
| KRT14 | Cytokeratin 15 |
| KRT18 | Cytokeratin 18 |
| mAb | Monoclonal antibody |
| MMLV | Moloney murine leukemia virus |
| MUC5AC | Mucin 5AC |
| MUC5B | Mucin 5B |
| NCAM | Neural cell adhesion molecule |
| NGFR | Nerve growth factor receptor |
| NICD | Notch intracellular domain |
| OCT | Optimal cutting temperature |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PDPN | Podoplanin |
| PE | Phycoerythrin |
| PECAM | Platelet endothelial cell adhesion molecule |
| PTFE | Polytetrafluoroethylene |
| qPCR | Quantitative polymerase chain reaction |
| RA | Retinoic acid |
| RPMI | Roswell Park Memorial Institute |
| SCGB1A1 | Secretoglobin Family 1A Member 1 |
| SCGB3A1 | Secretoglobin Family 3A Member 1 |
| SCGB3A2 | Secretoglobin Family 3A Member 2 |
| SFTPC | Surfactant protein C |
| SHH | Sonic hedgehog |
| SPA | Surfactant protein A |
| SPA | Surfactant protein B |
| SPDEF | SAM pointed domain containing ETS transcription factor |
| TGFβ | Transforming growth factor beta |
| TP63 | Tumor repressor protein 63 |
| TUBA4A | Tubulin alpha 4A |
| TUBB4A | Tubulin beta 4A |
| WNT | Wingless/integrated |

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## Abstract

The basal cell is the reported resident stem cell population in the human proximal airway and is responsible for maintaining the respiratory epithelium during homeostasis and in response to injury. However, we still do not fully understand the stem cell capabilities of this epithelial cell population. Basal cells have been shown to be capable of self-renewal and differentiation to generate the ciliated, secretory, and neuroendocrine cell lineages in the airway. Whether every basal cell has the inherent capacity to function as a stem cell however is still unknown. The hypothesis that only a fraction of the total basal cell population can act as stem cells suggests that there is functional heterogeneity within the population. Determining if there is basal cell heterogeneity will require the ability to identify and isolate distinct subsets of the overall population for evaluation of their functional properties.

The goal of this thesis dissertation is to address the question of whether there is heterogeneity within the human airway basal cell population and how we can answer the question experimentally. I will start by reviewing the physiology and cellular composition of the human respiratory system to highlight its complexity as it has two distinct regions, each with its own distinct morphology and cell types that dictate function. From there I will discuss the efforts of the Philip Streeter lab to generate and characterize novel monoclonal antibodies as molecular tools for the identification and isolation of cell subsets. During the course of my thesis research, we developed and experimentally validated a panel of monoclonal antibodies that allow us to study lung stem cell biology. After that I will discuss the historical development and current

repertoire of *in vitro* assays that allow us to evaluate cell populations for stem cell function. Organoid assay systems are a useful tool for studying stem cells, especially in humans since *in vivo* assays are not available. Organoid assays are capable of assessing the stem cell properties of self-renewal and potency, or ability to differentiate. I adapted a bronchosphere assay to be able to assess stem cell function in uncultured, primary human airway epithelial cells. I will then discuss how the culmination of the monoclonal antibody characterization studies and the development of the bronchosphere assay allows me to identify a subset of airway basal cells that proves there is functional heterogeneity within the airway basal cell population. My novel HLO1-6H5 antibody identifies a subset of basal cells that is enriched for stem/progenitor cells. I will also discuss the results of additional preliminary studies that will provide an opportunity to advance the study of human airway stem cells and the molecular pathways that regulate their function. I will conclude my dissertation with a discussion of future research and translational opportunities for the HLO1-6H5 antibody and the novel cell subset that it identifies.

# Chapter 1. Introduction

## 1.1. The human respiratory system: anatomy, physiology, and development

### 1.1.1. Anatomy and physiology of the respiratory system

The human respiratory system is comprised of the airways, which conduct air between the external environment and the lungs where gas exchange occurs (Figure 1.1). The lungs comprise the largest mass of the respiratory system and reside on either side of the heart. The right lung has three distinct lobes while the left lung has two, which is dictated by the branching pattern of the airway tree during development.

The respiratory system is divided into two functionally distinct regions, conducting and respiratory (Figure 1.1). The conducting region is further subdivided into the upper region, containing the nasal cavities and pharynx, and the lower region that consists of: the larynx, trachea, bronchi, and bronchioles[1,2]. The lower region of the conducting airway is characterized by the continuous branching and narrowing of airways to form the airway tree. Branching starts with the bifurcation of the trachea into the two stem bronchi and stops at the terminal bronchioles. The number of secondary bronchi that branch from the two stem bronchi results in the right lung possessing three lobes and the left lung having two lobes. Each lung has a pulmonary artery that branches in a pattern that aligns with the airway tree (Figure 1.1)[2]. The termination of the conducting region is at the distally-located terminal bronchioles, which branch into the respiratory bronchioles that mark the beginning of the respiratory region. The respiratory bronchioles transition into alveolar ducts and then terminate at the alveolar

sacs, which comprise most of the lung space. The function of each region is determined by its specific morphology and cellular composition.

The trachea and bronchi are hollow tubes structurally supported by hyaline cartilage and smooth muscle to keep the airways open and allow for the flow and filtering of air between the environment and the gas-exchange region of the lung [1,2]. The airways of the conducting region are lined with pseudostratified respiratory epithelium. This epithelium is comprised primarily of ciliated columnar cells, secretory columnar goblet cells, cuboidal basal cells, and to a lesser extent sensory brush cells and neuroendocrine cells (Figure 1.2)[3,4]. The ciliated and goblet cells work together to perform the airway's mucociliary function to filter out contaminants or foreign particles carried in from the external environment during air intake[1,2]. For mucociliary action, the goblet cells produce mucus to cover the airway surface and trap foreign particles, then the ciliated cells move the mucus upwards for expulsion from the body. Submucosal glands further supplement the secretions of goblet cells to cover the airway surface with mucus[2]. The basal cells are the reported stem cell population of the conducting airways in humans, but there is a question of whether this population is heterogeneous with only a subpopulation of basal cells that functions as stem cells, which will be addressed at greater depth later in this chapter. Identifying the most enriched population of airway stem cells is important to translational research efforts that aim to regenerate and repair lung tissue.

The cellular composition of the respiratory epithelium changes between the trachea and the terminal bronchioles, reflecting the switch in function from conducting

air to gas-exchange. In the bronchioles, ciliated cell and goblet cell frequencies decrease, secretory club cells emerge, and the epithelium transitions from pseudostratified to a simplified columnar morphology (Figure 1.2). Other structural changes that occur when descending the airway tree include the loss of supporting hyaline cartilage and submucosal glands in the bronchioles. The terminal bronchioles branch into respiratory bronchioles, marking the transition from the conducting region into the respiratory region.

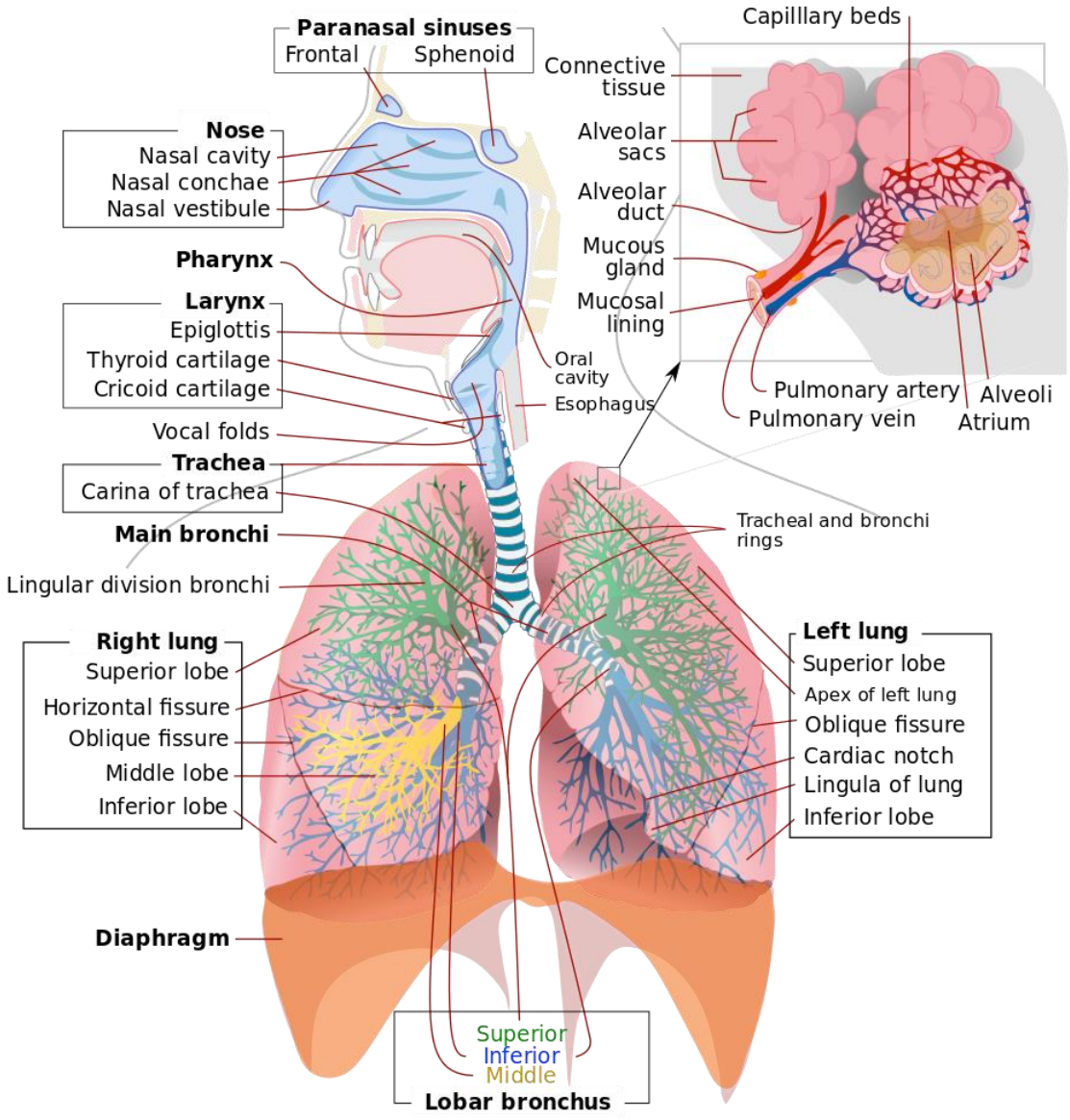


**Figure 1.1. Human respiratory system anatomy.**

The human respiratory system starts at the oral/nasal cavities in the head and descends into the chest where the lungs reside. The right lung has 3 lobes and the left has 2 lobes. Descending the branching airway tree eventually leads to the alveolar regions, where gas exchange occurs in coordination with the capillary beds of the pulmonary vasculature. *From Public Domain.*

The respiratory bronchioles possess a simple cuboidal epithelium comprised primarily of secretory club cells and the rare ciliated, neuroendocrine, or brush cell[1]. The various secretions of the club cells function as a surfactant to protect the local epithelium from surface tension and as a defense against foreign microbes and substances[1,2,5]. The bronchioles then terminally branch into the alveolar ducts, which connect to the small grape-like sacs known as alveoli. The ducts and alveoli are lined with simple squamous epithelium and are surrounded by elastic and reticular fibers to permit the epithelial stretching that is characteristic of respiration[1,2]. The epithelial lining of the alveoli is composed of squamous alveolar type I (ATI) cells interspersed with cuboidal alveolar type II (ATII) cells located at the junctions between alveolar sacs (Figure 1.2). The concurrent branching of the lung vasculature that begins in the conducting region becomes a capillary bed in the respiratory region. This network of capillaries surrounds the thin-walled alveoli for gas exchange across the ATI cells (Figure 1.1). The ATII cells produce surfactant to alleviate the surface tension in the alveoli. This complex system of air conduction and gas-exchange originates from the foregut endoderm during embryonic develpment[1,6,7].

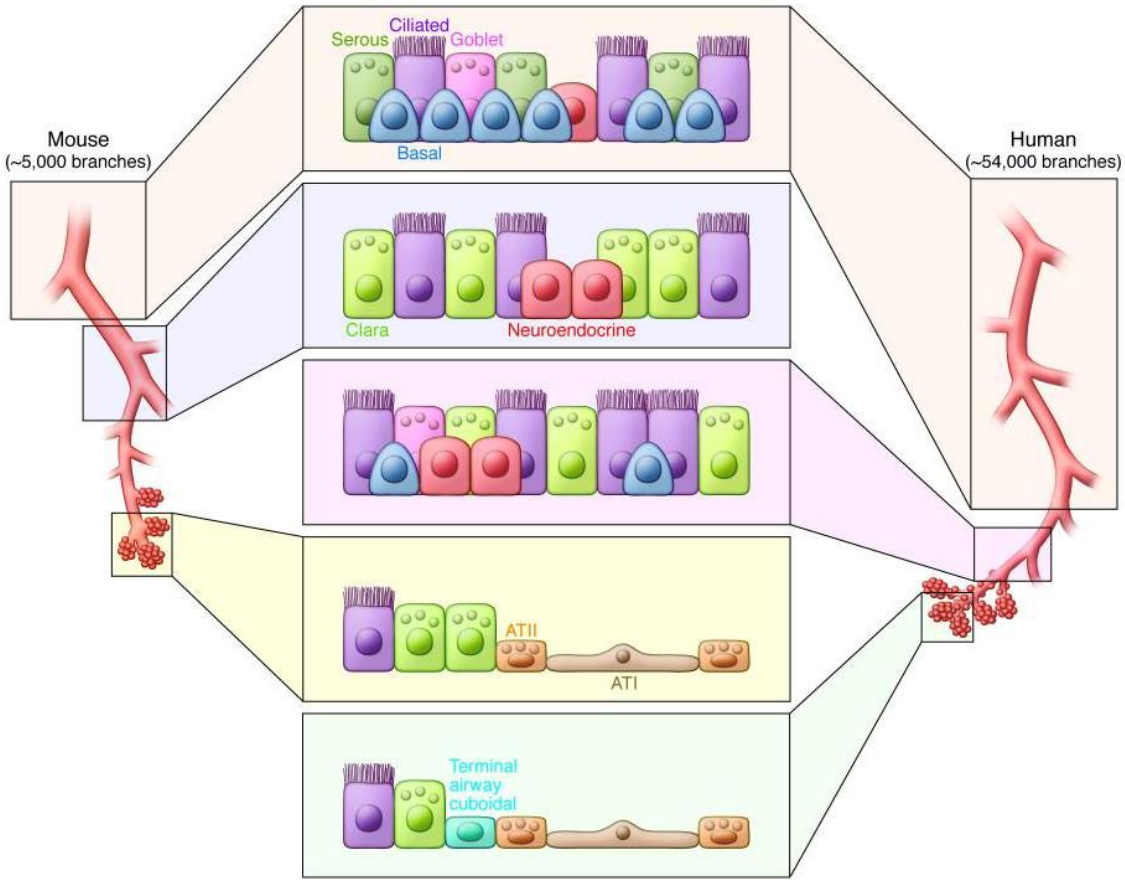


**Figure 1.2**. **Epithelial cell types of the mammalian respiratory system.**
The human and mouse airways are lined with epithelium, with variations in cellular composition and organization between the two species. Focusing on the human respiratory system, the trachea and bronchi are lined with a pseudostratified respiratory epithelium containing ciliated, basal, and secretory cells (goblet and serous). Descending down the airway tree to the more distal airways leads to a shift from pseudostratified to simple columnar or cuboidal epithelium. Cell composition changes as goblet cells are replaced with secretory Clara (club) cells and ciliated cell frequency decreases. In the alveolar region, the epithelium flattens due to the squamous alveolar type I (ATI) cells with the occasional cuboidal alveolar type II (ATII) cell present to provide secretory functions. *From Rackley et al. 2012 with permission*[8].

### 1.1.2. Embryonic development of the respiratory system

The development of the human lung begins around 3-4 weeks post-conception and progresses through five overlapping stages: embryonic, pseudoglandular,

canalicular, saccular, and alveolar[6,8] (Figure 1.3). At the onset of the embryonic phase, two buds form from the ventral anterior foregut endoderm and begin branching to create the early precursors of the left and right lungs[6–8]. Meanwhile, the trachea forms as a branching event from the primary foregut tube[8]. Concurrently, the early pulmonary vasculature initiates from the mesoderm surrounding the developing airway tree [6,9,10].

Around week 5, the pseudoglandular phase initiates as the lung buds continue growing due to ongoing branching of the airways. Differentiation begins to take place as cellular specification leads to the formation of smooth muscle, cartilage, and vasculature from the mesoderm, and early formation of epithelium and glands from the endoderm[6]. The blood and lymphatic vasculature systems of the lung undergoes branching morphogenesis to align with the developing airway tree. Specific airway epithelial cell types begin to emerge in the form of ciliated and neuroendocrine cells as proximal-distal patterning of the lung takes place[8]. The completed formation of the airway tree marks the end of the pseudoglandular phase[6,8].

During the canalicular phase, the final branching events take place to form the alveolar region. The terminal airway tubes widen to occupy the empty spaces of the lungs and form the early basis of the alveolar region[6]. The widening of the terminal airway tubes facilitates contact between the future alveoli and the developing capillary beds of the pulmonary vasculature system. Meanwhile previously formed airways grow in diameter. Further cellular differentiation occurs as the first secretory cell emerges, the club cell[8].

The initiation of the saccular phase is highlighted by the termination of branching processes and the enlargement and thinning of the first alveolar sacs[6]. The capillary beds that have formed from the mesoderm also begin to surround the growing alveoli[6]. With the development of the alveolar sacs, cellular differentiation into the ATI and ATII cell types initiates[6,8]. ATII cell emergence is confirmed by the detection of pulmonary surfactant in the alveolar region[11]. The surfactant protects the alveolar epithelium from surface tension that arises while the sacs grow and stretch.

Finally, the alveolar phase begins at birth, at which time the primitive alveolar sacs mature and septation occurs to generate distinct alveoli that contribute to the increased surface area needed for gas exchange[6]. Coinciding with the formation of mature alveoli, the capillary matrix also matures and fully surrounds the alveoli[6]. As for cellular differentiation, the final cell types of the proximal or airway region of the respiratory system emerge as secretory goblet cells and basal cells arise, thus completing the proximal-distal patterning of the lung[8].

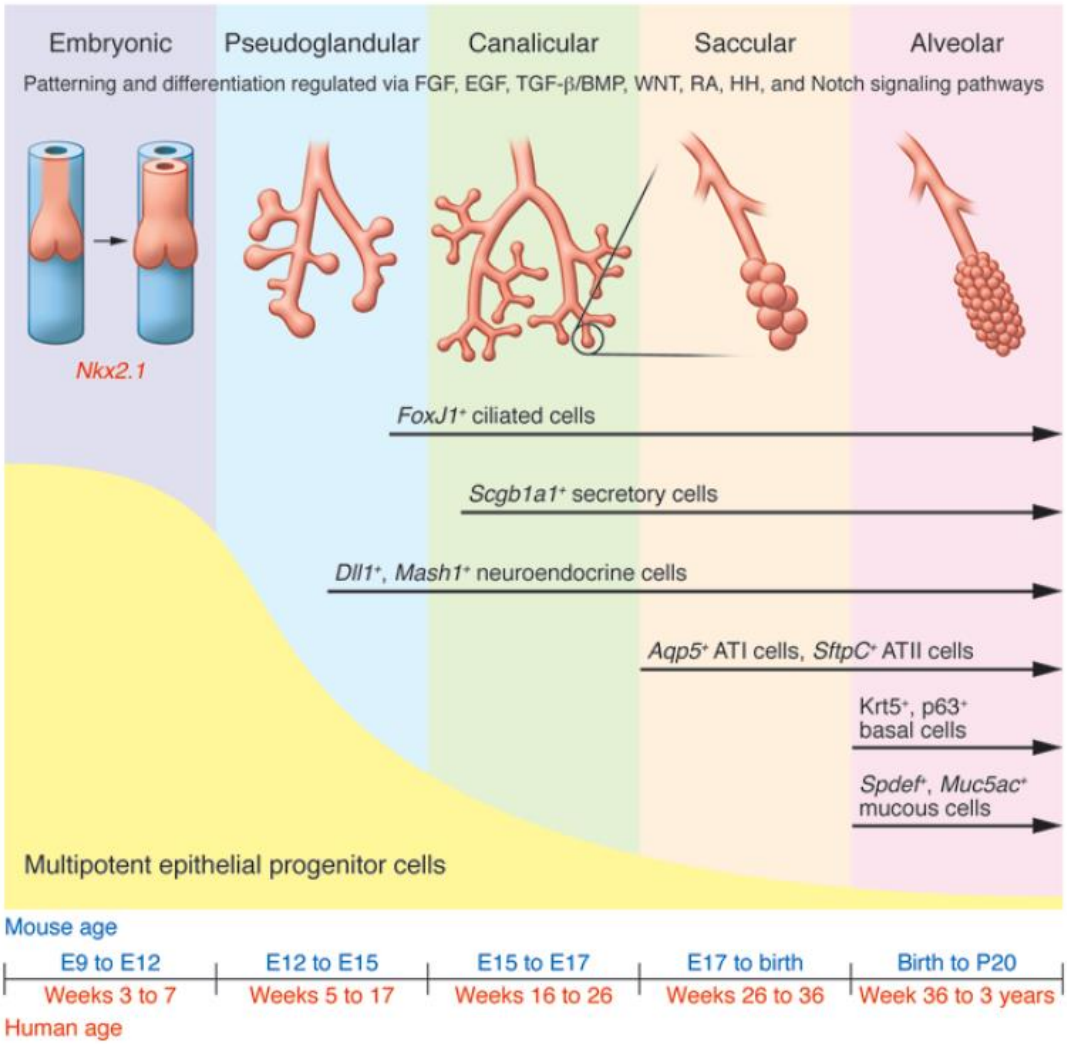


**Figure 1.3. The five stages of embryonic development of the respiratory system.**

Development of the respiratory system epithelium initiates from the ventral foregut endoderm around embryonic week 3 and proceeds according to five overlapping stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar. The embryonic, pseudoglandular, and canalicular stages are highlighted by the continuous branching and growth of the airway tree. The final stages, saccular and alveolar, are highlighted by the formation and maturation of the alveolar sacs. Throughout the process, proximal-distal patterning of the airways takes place via cellular differentiation. FGF = fibroblast growth factor, EGF = epidermal growth factor, TGFβ = transforming growth factor beta, BMP = bone morphogenetic protein, WNT = wingless/ integrated, RA = retinoic acid, HH = hedgehog, SCGB = secretoglobin, DLL = delta like ligand, AQP = aquaporin, SFTPC = surfactant protein C, SPDEF = SAM pointed domain containing ETS transcription factor, KRT = keratin, MUC = mucin. *From Rackley et al. 2012 with permission*[8].

### 1.1.3. Regulation of respiratory system development

Molecular regulators of human lung development are largely unknown, but some developmental transcription factors and signaling pathways have been identified using mouse models. The retinoic acid pathway regulates initiation of the first lung buds from the endoderm during the embryonic phase in both mouse and human. In mouse retinoic acid receptor knockout models, the lung buds do not form[12]. In human embryonic stem cells, retinoic acid is necessary to induce differentiation of endodermal cells to respiratory cell lineages, as indicated by the expression of the Nkx homeobox 1 (Nkx2.1) transcription factor[13].

The first lung bud cells in mice are characterized by expression of Nkx2.1, which serves as a lung lineage marker during development and in adulthood[6,8,10]. Nkx2.1 is also detected in human fetal lung at embryonic week 11 and in the ATII, basal, and club cell lineages of the adult lung[14]. The upregulation of Nkx2.1 is likely a result of multiple signaling pathways originating from the developing lung mesenchyme that surrounds the growing epithelium. The molecular pathways that regulate Nkx2.1, based on mouse models, are fibroblast growth factor (FGF), wingless/integrated (WNT), retinoic acid, transforming growth factor beta (TGFβ), Notch, and bone morphogenetic protein (BMP), which are commonly associated with almost all developmental processes[6,8,10,15].

Branching morphogenesis during development is a highly regulated process. A proposed model of mesenchymal/epithelial interaction in airway branching, based on data from mice, involves Wnt and FGF10 signaling in the distal mesenchyme to drive

BMP4 expression and the associated growth and elongation of the distal tips during the pseudoglandular stage[6,7,12,15,16] (Figure 1.4). In this model, WNT and FGF10 are negatively regulated by TGFβ, but the retinoic acid pathway inhibits the repressive effects of TGFβ[12]. FGF10 signaling also plays a key role in proximal-distal patterning of developing airways by controlling the interplay of SOX2 and SOX9 in epithelial progenitors. The mesenchymal FGF10 induces SOX9 expression and suppresses SOX2 in distal epithelial progenitors; meanwhile proximal epithelial progenitors increase SOX2 expression and lose SOX9 expression as they become more distant from the FGF10 source[16]. The mouse model shows that this segregation of SOX2 and SOX9 epithelial progenitors occurs in the first phase of airway branching, the pseudoglandular stage. Recent data in humans however show some differences in the molecular regulation of airway branching from the mouse model. *In vitro* cultures of human fetal lung explants show that during the pseudoglandular phase, the distal tips of growing branches possess double-positive SOX2+/SOX9+ progenitor cells, which segregate into SOX2+ proximal progenitors and SOX9+ distal progenitors upon entering the canalicular stage[17,18]. A comparison of transcriptome data for human tip cells versus mouse tip cells shows a 96% overlap in gene expression, but there are some clear differences in activated signaling pathways. For example, BMP2, BMP7, and IHH (Indian hedgehog) are enriched in human tip cells while BMP4 and Sonic hedgehog (SHH) are enriched in mouse[18].

Several of the aforementioned pathways have been manipulated *in vitro* to promote and/or maintain growth of primary human lung epithelium or to regulate differentiation of embryonic stem cells towards lung lineages[6,8,10,19–22]. The *in vitro*

growth of airway epithelial organoids utilizes growth factors from development and branching morphogenesis such as retinoic acid, FGF10, and R-spondin (WNT pathway) [18,23]. Blocking the Notch pathway *in vitro* disrupts differentiation of stem/progenitor cell types such as human basal cells and embryonic stem cells into the airway ciliated lineage and suppresses differentiation into the secretory lineages[20,24–26].

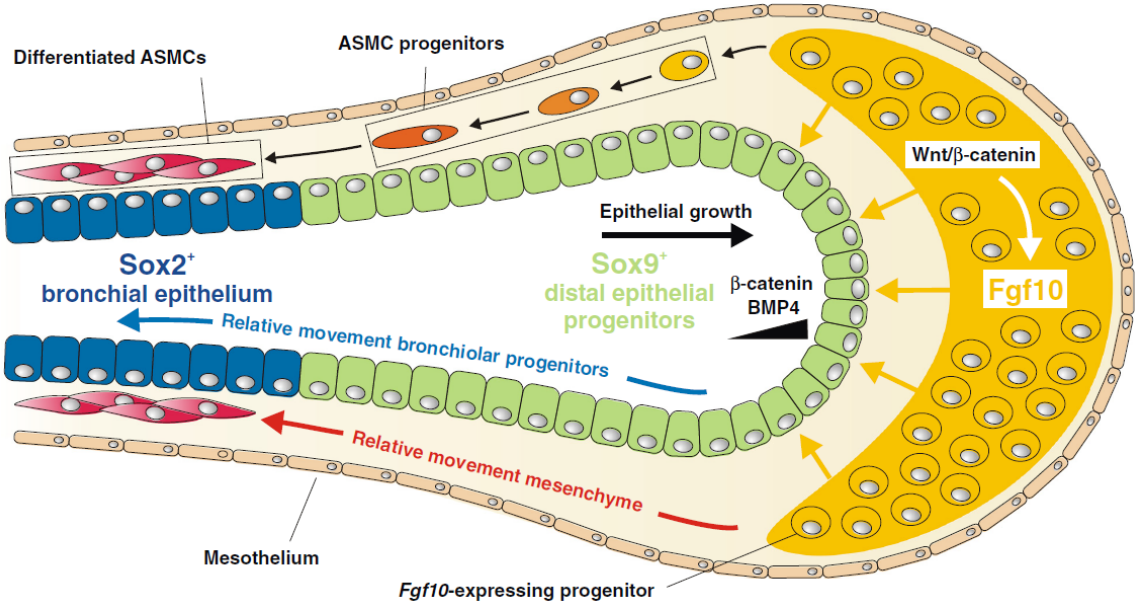


**Figure 1.4. Mouse model of airway branch elongation.**
Airway distal tip elongation is driven by the molecular signals between the early epithelial cells and the surrounding mesenchyme. WNT regulates signaling of FGF10 from the distal tip mesenchyme to the nearby distal epithelial progenitor cells (SOX9+), which grow towards the source of FGF10. As epithelial cells become more distant from FGF10, SOX2 expression increases, leading to proximal-distal patterning of the airway branch. *From Volckaert et al. 2014 with permission*[16].

An understanding of lung development, morphology, and function is critical to identifying what specific cell types are responsible for maintenance and repair of this

organ system. Some developmental lung progenitor cells and their associated markers persist into adulthood after the lung develops, providing a basis for identifying resident adult stem cells. Lung researchers have identified specific epithelial cell populations within the various regions of the lung that function as stem cells to drive localized maintenance and repair[7,19,27,28]. These adult stem cells are critical for maintaining homeostatic function of the respiratory system. Several stem cell populations and models have been hypothesized for the adult human respiratory system.

## 1.2. Epithelial stem cell populations of the respiratory system

### 1.2.1. Stem cells

Stem cells are defined as cells that have the ability to self-renew, thereby maintaining their own population, and the ability to differentiate into other specialized cell types. Potency is the ability of a cell to differentiate into other cell types that contribute to the overall function of the tissue in which they reside. For example, bipotent stem cells can differentiate into two other cell types and totipotent stem cells can differentiate into any of the cells found in the body. There are also progenitor cells, which are a step down from stem cells in that they are capable of differentiation, but they have already initiated the process and are therefore committed to become a specific cell type. Progenitor cells also have limited self-renewal capabilities compared to stem cells. Stem cells are further categorized as embryonic or adult stem cells.

### 1.2.2. Embryonic stem cells

Embryonic stem cells are pluripotent and therefore are able to differentiate into cells from any of the three germ layers of the body. As the name suggests, embryonic stem cells are derived from embryos during development. These cells do not persist into adulthood.

### 1.2.3. Adult stem cells

Adult stem cells have limited potency as they are restricted to differentiating into only certain types of cells, typically the ones that are found in the organ in which the stem cells reside. Unlike embryonic stem cells, adult stem cells persist long after development ends. Each organ has its own population of resident adult stem cells. In the respiratory system, there are multiple reported epithelial stem cell populations that correspond with the specific region of the lung in which they are located (Table 1.1).

### 1.2.4. Reported epithelial stem cells in the respiratory region

Over the last decade, pulmonary researchers have been able to identify multiple candidate epithelial stem cell populations using murine models, some of which have also been verified in the human lung (Table 1.1). The most highly reported and well-studied stem cell population in the both the human and mouse respiratory system is the alveolar type II (ATII) cell in the respiratory region of the lung[29–31]. ATII cells self-renew and can differentiate into the alveolar type I (ATI) cells that comprise the majority of the epithelium in the alveolar space.

Another reported stem cell population in the alveolar space of both humans and mice is a rare subset of the ATII population that expresses the integrin dimer α6 (ITGA6) and β4 (ITGB4)[32,33]. The studies isolated cells using an ATII specific protocol, and then FACS-purified subsets using ITGA6B4. The ITGA6B4 subset of ATII cells has low expression of the keratin 5 (KRT5) basal cell marker, the club cell marker CCSP (club cell secretory protein), and the SFTPC (surfactant protein C) marker for ATII cells[32]. The low expression of SFTPC raises the debate of whether this rare subset is actually ATII cells. In humans, this subset is able to grow and form colonies *in vitro* that contain a mix of basal cells (KRT5+), secretory club cells (CCSP+), and secretory goblet cells (mucin 5AC+)[32].

Recently in early 2018, another subset of ATII cells was identified, called alveolar epithelial progenitor (AEP) cells in both mouse and human alveolar tissue[34]. The human and mouse AEP cells express the ATII cell marker SFTPC and a novel cell surface marker TM4SF1 (transmembrane 4 Superfamily Member 1) that had been identified by RNAseq. Isolated human AEP cells were able to proliferate and differentiate to form colonies *in vitro*.

Mouse cancer models show another stem cell population in the respiratory region known as broncho-alveolar stem cells (BASC) that reside at the junction of the bronchiolar airways and alveolar space[35]. These cells form colonies *in vitro* that contained a mix of secretory club cells, ATI, and ATII cells[36]. However, there are no data to support the presence of BASCs in the human respiratory system.

Another recently reported stem cell in the respiratory region is the lineage-negative epithelial progenitor (LNEP), first identified in the bronchioles of a severe

mouse injury model by molecular phenotyping[37,38]. A phenotypically identical population of LNEP cells has been reported in human airway tissue afflicted with fibrosis, but the functional properties of these human cells is unknown[21,38].

As the aforementioned studies show, there are multiple stem cell populations within the respiratory region that have been verified in human tissue. However, research efforts into identifying and characterizing stem cell populations of the conducting airway region are lacking, especially in human tissue. Lung researchers now recognize that there is a need to focus on investigating the conducting airways and on developing new tools to facilitate such studies.

**Table 1.1. Identified lung stem/progenitor cell populations in adult mouse models.**

| Stem and progenitor populations | Marker genes | Proliferative | Differentiation repertoire | Cell-lineage labeling |
|---|---|---|---|---|
| **Proposed multipotent cells** | | | | |
| Alveolar Itga6$^{+}$Itgb4$^{+}$Sftpc$^{-}$ | *Itga6, Itgb4* | Yes | Self, AEC1, AEC2, club, ciliated | Excluded Sftpc+ cells (using Sftpc–CreERT2) |
| BASCs | *Sftpc, Scgb1a1* | Yes | Self, club, AEC2 | Scgb1a1-CreERT2 (not BASC specific) |
| Basal cell | *Trp63, Krt5, Krt14, Ngfr, Pdpn* | Yes | Self, club, ciliated, AEC1, AEC2 | Krt14-CreERT2 after injury |
| **Airway progenitors** | | | | |
| Basal cell | *Trp63, Krt5, Krt14, Ngfr, Pdpn* | Yes | Self, ciliated, basal | Krt5-CreERT2, Krt14-CreERT2 |
| Club cell | *Scgb1a1, Cyp2f2* | Yes | Self, ciliated, basal | Scgb1a1-CreERT2 |
| Variant club cell | *Scgb1a1, Cyp2f2* | Yes | Self, club, ciliated | Scgb1a1-CreERT2, Upk3a–CreERT2 |
| **Alveolar progenitors** | | | | |
| AEC2 | *Sftpc* | Yes | Self, AEC1 | Sftpc-CreERT2a |
| **Nonprogenitor lineages** | | | | |
| AEC1 | *Aqp5, Pdpn, Hopx* | No | NK | Aqp5-Crea |
| Ciliated epithelium | *Foxj1, Tubb4a* | No | NK | Foxj1-CreERT2 |

*Adapted from Kotton et al. 2014 with permission*[19].

### 1.2.5. Reported epithelial stem cells in the conducting airway region

Adult human airway epithelium is a slow turnover organ compared to other tissues, but there is still an estimated turnover of 30-50 days[39,40]. Resident airway stem cells maintain the epithelium by replacing the mature ciliated and secretory cells that gradually turnover. Several candidate stem cell populations have been identified in the epithelium of the airways (Table 1.1, Figure 1.2). The most highly reported airway stem

cell population is the basal cell population of the respiratory epithelium. Basal cells are cuboidal in shape and reside on the basement membrane, unexposed to the airway lumen through which air travels from the external environment (Figure 1.2). In both the mouse and human airways, the basal cell is capable of self-renewal and differentiation into the ciliated and secretory cell lineages that line the airway lumen[20,26,41–44].

Another proposed epithelial stem cell population in the airways is the secretory club cell population (Figure 1.2). Club cells become more prominent when descending the airway tree into the bronchioles. Mouse models show that the club cell is capable of self-renewal and differentiation to generate ciliated cells[45,46]. In naphthalene injury models, there is a damage-resistant subset of the club cell population called the variant club cell, which expresses one of the club cell markers, secretoglobin family 1a member 1 (SCGB1A1), but not the other, cytochrome P450 family 2 subfamily f polypeptide 2 (CYP2F2). Despite the evidence of stem cell properties in the mouse club cell population, human club cells are not functionally verified as a stem cell population for the distal airway epithelium.

A recently proposed stem cell population in the airways is the SOX2+ progenitor cell, which does not express basal cell marker cytokeratin 5 (KRT5) or club cell marker SCGB1A1[47]. As previously mentioned, progenitor cells are similar to stem cells in that they are capable of differentiation and self-renewal, but at a lesser extent when compared to stem cells. This progenitor cell population is found in a mouse influenza-injury model, in which KRT5+ stem cells emerged to repair the damaged lung epithelium. These KRT5+ cells arise from a common progenitor cell identified as SOX2+/

KRT5-/ SCGB1A1-[47]. The KRT5+ cells did not arise from lineage-traced SCGB1A1+ club cells, HOPX+ (homeodomain only protein) ATI cells, or SFTPC+ ATII cells[47]. Thus, indicating that the ATI, ATII, and club cells did not dedifferentiate from their original phenotype to produce the KRT5+ cells. However, there is no data to support the presence of this new stem/progenitor cell population in human respiratory epithelium.

Overall, there are several reported airway epithelial stem cell populations in mouse injury models, but aside from the basal cell population, the rest are not verified as stem cells in the human respiratory system (Table 1.1). Despite our understanding that the human basal cell population functions as an airway stem cell pool, there is still a question of whether every basal cell can act as a stem cell.

## 1.3. Basal cells as the stem cells of conducting airway

### 1.3.1. Reported stem cell function in human basal cells

The basal cell population represents ~6-30% of the total cells in the respiratory epithelium, with the highest percentages existing at the most proximal airways and the lowest in the distal airways[48,49]. As previously mentioned in Chapter 1.2, the basal cells are the reported adult stem cell for the human airway epithelium. *In vitro* studies of the human airway basal cell population shows that basal cells exhibit the stem cell properties of self-renewal and potency[26,44,50–52]. Within the human basal cell population, there is a subset of mitotically-active cells identified by expression of KRT14 (cytokeratin 14)[6,7,19,50,53]. The presence of a KRT14+ subset within the overall airway basal cell population provides evidence that there is heterogeneity within the population both

phenotypically and functionally. This notion of heterogeneity raises the question of whether every basal cell is a stem cell or if there is a subset of stem cells within the larger population.

### 1.3.2. Heterogeneity in the airway basal cell population

The question of whether all basal cells are functionally identical stem cells remains unanswered in the human airways. However, mouse studies over the last five years are beginning to answer that question. A study by Pardo-Saganta et al. proposes that there are two subtypes of mouse basal cells based on commitment to either the ciliated or secretory lineages, identified by an associated intracellular marker, C-MYB (transcriptional activator Myb) and N2ICD (Notch2 intracellular domain) respectively[54]. However, in their model they postulate that the two basal cell subtypes derive from an earlier (or parent) basal cell phenotype that does not express any markers of lineage commitment (Figure 1.5A). Therefore, their model depicts the proposed lineage-committed basal cell subtypes as more of a precursor cell phenotype that has differentiated away from the original basal cell phenotype, rather than actual subsets of the undifferentiated basal cell population. The precursor cell phenotype is an intermediate state between the undifferentiated basal cell population and the differentiated luminal cells. Their observation has not yet been verified in human airway basal cells. The question of whether the original basal cell population, that is negative

for lineage markers, is heterogeneous still needs to be addressed in humans.

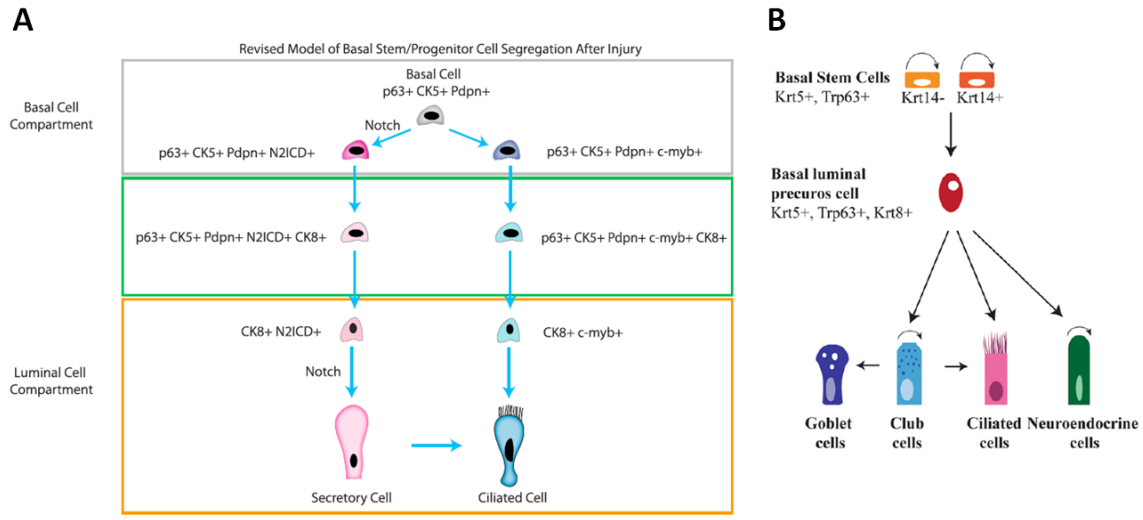


**Figure 1.5. Proposed models of basal cell subsets in mice.**

**A)** In injury models, basal cells segregate into two functionally distinct, lineage-committed subsets that are phenotypically identified by N2ICD or C-MYB expression; **B)** In homeostatic lung models, the basal stem cell subset asymmetrically divides to produce a subset of non-dividing basal luminal precursor cells that are capable of differentiation; *From Pardo-Saganta et al. 2015 with permission (Panel A)*[54] *and from Schilder et al. 2016 with permission (panel B)*[27]

Another study identifies two types of mouse basal cells in the homeostatic airway using clonal dynamics and mathematical modeling. The two subsets are identified as basal stem cells (BSCs) and basal luminal progenitor cells (BLPCs). In the study, long term lineage traces show that the subset of basal stem cells can divide asymmetrically to self-renew the basal stem cell population and to generate the subset of basal luminal progenitor cells (Figure 1.5B)[55]. Meanwhile, the BLPCs do not divide and instead differentiate into luminal lineages. Both the BSC and BLPC subsets are morphologically identical and are KRT5+/TP63+. However, the BLPCs are KRT8+ (cytokeratin 8), making them phenotypically distinct from the BSCs. KRT8 is a marker of

luminal cells in the airway epithelium. The study's proposed model is similar to the study by Pardo-Saganta et al., as it establishes the existence of a precursor cell phenotype that has differentiated away from the original basal cell phenotype, but is not yet a differentiated luminal cell[54]. BSCs and BLPCs have not yet been observed in human airway basal cells, leaving the question of basal cell heterogeneity in humans still unanswered. Addressing the question of heterogeneity in the human airway basal cell population is important in the effort to identify a subset of basal cells that functions as the reparative stem cell of the respiratory epithelium.

One method of determining whether a cell population is heterogeneous is by identifying distinct subsets phenotypically. Each type of epithelial cell in the human airway is identified by distinguishing protein markers (Table 1.2). As previously shown with the club cell population (SCGB1A1+/CYP2F2+) and its subset of variant club cells (SCGB1A1+/ CYP2F2-), differences in marker expression can be indicative of population heterogeneity. The basal cell population is defined by its expression of markers cytokeratin 5 (KRT5) and tumor repressor protein 63 (TP63)[44,56,57]. Basal cells can be further subdivided phenotypically by expression of an intracellular marker, cytokeratin 14 (KRT14)[42,44]. In both mouse and human basal cells, KRT14 is shown to identify a mitotically active subset of KRT5+ basal cells[42,50]. The KRT14 marker, along with the C-MYB and N2ICD markers, do appear to identify either subsets of basal cells or subsets of differentiating basal cells, but all three markers are intracellular proteins. These three markers cannot be used to isolate viable, phenotypically pure subsets of basal cells from primary human tissue. Efforts however are being made to identify cell surface markers

through RNAseq (of bulk populations or single cells) or flow cytometric screening with CD marker panels that will facilitate isolation of basal cells, with the goal of isolating subsets of the basal cell population[28,58,59].

**Table 1.2. Canonical markers for identifying cells in the mammalian respiratory system.**

| Human cell population | Markers* |
|---|---|
| Basal cell | KRT5, (KRT14), (NGFR), TP63, PDPN‡ |
| Ciliated cell | Acetylated tubulin, β3-tubulin, FOXJ1 |
| Secretory club cell | (PLUNC), SCGB1A1, (SCGB3A1), (SCGB3A2) |
| Secretory goblet cell | MUC5AC, MUC5B, SPDEF |
| Type 1 alveolar epithelial cell | AQP5‡, HOPX, HTI-56‡, PDPN‡, RAGE |
| Type 2 alveolar epithelial cells | ABCA3, HTII-280, LAMP3, LPCAT1‡, pro-SFTPC‡, SPA‡, SPB‡ |
| Distal tip | SOX9‡, SOX2‡, MYCN, GATA6, ETV5, HMGA1, HMGA2, HNF1B, ID2, CPM, CD47, (pro-SFTPC) |
| Stalk bronchiolar progenitors | SOX2 |
| Embryonic alveolar progenitors | Co-expression of HTII-280, HOPX, PDPN |
| Neuroendocrine cells | ASCL1, CGRP, chromogranin A, GRP, NCAM, substance P |
| Vascular endothelium | PECAM1, VECAD‡ |
| Lymphatic endothelium | PECAM1, PDPN‡, VECAD‡ |

*Markers in brackets are found in a subset of this cell type.
‡Markers expressed in more than one cell type.

*Adapted from Nikolic et al. 2018 with permission*[6].

### 1.3.3. Isolating human basal stem cells via cell surface markers

In 2009, Rock et al. identified two cell surface markers that are currently used to identify and isolate human and mouse airway basal cells, NGFR (nerve growth factor receptor) and ITGA6 (integrin alpha 6)[44,48]. RNAseq of lineage traced mouse airway basal cells (KRT5-CreER$^{T2}$) shows NGFR and ITGA6 as cell surface markers that are enriched in basal cells[44]. In their study, harvested mouse and human airway epithelial cells were cultured overnight to selectively expand the TP63+ basal cell population. After

expansion, NGFR and ITGA6 were used together to purify airway basal cells by FACS (fluorescence assisted cell sorting). In an *in vitro* sphere formation assay, the NGFR+/ITGA6+ basal cells demonstrate the stem cell properties of self-renewal and differentiation as they are able to generate the luminal ciliated cell lineage found in respiratory epithelium. The NGFR and ITGA6 markers provide lung researchers with the tools to begin investigating the human basal cell population and its stem cell properties. The next step, is to identify additional novel cell surface markers that can isolate a viable stem cell subset from within the human basal cell population[7,19,27].

# Chapter 2. Experimental strategies to identify, isolate, and analyze cell subsets in the human airway*

## 2.1. Introduction

The conducting region of the human respiratory system is lined with a pseudostratified respiratory epithelium which consists of ciliated cells, secretory goblet and club cells, neuroendocrine cells, and basal cells[7,19]. The columnar ciliated and secretory cells function in coordination to perform mucociliary clearance of foreign particles that are trapped in the airway[1,2]. Meanwhile the basal cells have been reported to be the stem cell population of the human airway, capable of both self-renewal and differentiation into the secretory and ciliated cell lineages [26,48,57,60–62]. This stem cell property makes the basal cell population the focus of lung regeneration and engineering studies. In addition to basal cells, other airway cell subsets have been reported in mice to exhibit stem cell properties: SCGB1A1+ club cells and SOX2+ airway epithelial cells[47,63]. Studying these various potential airway stem cell populations is difficult due to the dearth of molecular tools to target cell-surface markers for identification and isolation of specific cell subsets. Hence the need for developing new tool to study the various cell types of the human respiratory airways, with specific interest towards finding a basal stem cell population[27,64,65].

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Researchers of other human organ systems such as the pancreas, liver, and gall bladder faced a similar barrier to their study efforts as their counterparts in the lung regeneration field, a need for new molecular tools to target and/or isolate specific cell types and new subpopulations[66–70]. In the lung research field, very few cell-surface markers for human airway stem cells have been identified in the past decade. There has been success in developing new markers for the isolation of basal cells by targeting NGFR and ITGA6 expression, but there is still a lack of tools for targeting other epithelial cell types in human airway[44].

The novel monoclonal antibodies (mAbs) described here were developed against human tissue for the targeting of the major epithelial cell types of the airway: ciliated, secretory, and basal. These monoclonal antibodies allow for the identification of specific epithelial cell types in airway tissue sections and the isolation of enriched populations of viable cells from dispersed human bronchial epithelial cells (hBECs). Co-staining of these novel antibodies with canonical markers for specific airway cell types reveal the different cell lineages that these antibodies target. Flow cytometric analyses confirm that these antibodies target cell-surface antigens, enabling the isolation of viable cell subsets for downstream transcriptional analyses to verify the specific cell type that these antibodies target. Overall, we have generated a panel of novel monoclonal antibodies for the selective targeting of specific cell lineages that are physiologically relevant, as indicated by their reactivity with cells in primary human airway.

## 2.2. Materials and Methods

### 2.2.1. Human airway tissue specimens

Normal human airway epithelial cells and fresh, frozen lung tissue in OCT (optimal cutting temperature) compound were sourced from Marisco Lung Institute's CF (cystic fibrosis) Center Tissue Procurement and Cell Culture Core at the University of North Carolina in Chapel Hill. The Core staff dissected trachea and bronchi away from connective tissue and lymph nodes from cadaveric lungs. Dissected airways for isolating viable epithelial cells were cut into 1x2cm segments for overnight enzymatic digestion with 1% Protease XIV with 0.01% DNAse and then manually scraped with a scalpel to yield dispersed viable human bronchial epithelial cells (hBECs)[23,71]. Cells were suspended in F12 media and shipped overnight at 4°C to OHSU.

Freshly dissected airways designated for making tissue blocks were cut into segments and embedded into OCT compound for freezing. Airway tissue blocks were sent overnight on dry ice to OHSU.

### 2.2.2. Antibody Production

Animal husbandry and immunizations were performed under an OHSU Institutional Animal Care and Use Committee approved protocol. Mice were immunized according to one of two strategies: 1) three intraperitoneal injections with a target preparation of cells or 2) a single injection with a preparation of negative variant cells followed by cyclophosphamide depletion of B cells for subtractive immunization, then followed by two injections with the target cell preparation. The following combinations

of immunogen preparations were used: hBECs cultured in basal epithelial growth media (BEGM) (hBECs provided by Jeffrey Whitsett Lab at Cincinnati Children's Hospital Medical Center); subtractive immunization with hBECs cultured over six passages followed by positive immunizations with hBECs cultured for a single passage (hBECs provided by Scott Randell Lab at University of North Carolina in Chapel Hill); or lung organoids grown from primary hBECs according to the protocol published by Clevers et al[23,72–74].

Mice were sacrificed four days after the final immunization to harvest splenocytes. The splenocytes were fused with SP2/0 Ag14 myeloma cells and grown in methylcellulose-containing HAT (hypoxanthine-aminopterin-thymidine) medium to select for hybridoma clones[66,70]. Over 4,000 hybridoma clones were selected and isolated for culture expansion. The monoclonal antibody-containing media of each clone was collected for screening by immunohistochemistry on acetone-fixed tissue sections from fresh, frozen human airway and by flow cytometric analyses on hBECs. Clones of particular interest were further expanded in culture to generate additional monoclonal antibody (mAb) and then cryopreserved in DMEM with 10% FBS and 10% DMSO. Antibody isotyping was done with either a mouse immunoglobulin isotyping ELISA (enzyme-linked immunosorbent assay) kit (BD Biosciences) or by flow cytometry using isotype-specific fluorescent goat anti-mouse secondary antibodies (Jackson ImmunoResearch).

### 2.2.3. Fluorescent Immunohistochemistry

Cryosections (5-7µm) from fresh, frozen human airway were prepared on a Reichert 2800 Frigocut cryostat (Reichert Scientific Instruments), fixed in acetone for 5 minutes at -20°C, air-dried at room temperature, and then stored under dry conditions at -80°C for up to 6 months. Cut sections were blocked with 5% fetal bovine serum (FBS) and 5% normal goat serum, then stained with desired mAb, followed by 3 PBS washes. Bound primary mAb was detected with Cy3-conjugated goat anti-mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch) in PBS with 5% FBS. Sections were then washed 3 times, with the second wash containing Hoescht 33342 (Invitrogen) nuclear dye as a counterstain, and then mounted with Fluormount G solution (Southern Biotech). Stained sections were analyzed with a Zeiss Axioskop 2 plus microscope (Carl Zeiss).

Commercially available airway-specific epithelial cell markers were used for colocalization studies. Tissue sections were incubated with candidate cell-type specific primary mAbs and with either: polyclonal rabbit anti-human Keratin 5 (BioLegend), rabbit anti-human acetylated α-tubulin (Cell Signaling Technology), or rabbit anti-human Mucin 5B (Seven Hills). Detection of the rabbit anti-human primary antibodies was achieved with Dylight488-conjugated goat anti-rabbit IgG (H+L) secondary antibody (ThermoScientific).

### 2.2.4. Flow Cytometry

Dispersed hBECs were stained with candidate cell-type specific primary monoclonal antibodies for 30 min. at 4°C and then washed twice with cold staining buffer (RPMI media with 5% FBS). After washing, bound primary antibody was detected using PE-conjugated (phycoerythrin) goat anti-mouse IgM (immunoglobulin M) secondary antibody (Jackson ImmunoResearch) or A488-conjugated goat anti-mouse IgG (immunoglobulin G) secondary antibody (Jackson ImmunoResearch). Cells were then washed twice with cold staining buffer and then incubated with viability dye propidium iodide. Fluorescently-stained cells were analyzed using a LSRII Flow Cytometer (BD Biosciences) or sorted on an Influx Cell Sorter with a 140-micron nozzle (BD Biosciences).

### 2.2.5. RNA isolation and qPCR

Immunostained primary hBECs were FACS-isolated on an Influx Cell Sorter with a 140-micron nozzle (BD Biosciences) to collect antibody-negative and -positive fractions after doublet and dead cell exclusion. Cells were sorted directly into TriReagent (Molecular Research Center). RNA was phenol chloroform extracted and DNase treated for removal of genomic DNA (Invitrogen). First-strand cDNA synthesis utilized Moloney murine leukemia virus (MMLV) reverse transcriptase and random hexamer primers (Invitrogen). RNA was prepared with the Sybr-based Roche Faststart Essential DNA Master Mix and measured with the Roche Lightcycler 96 platform. Quantitative polymerase chain reaction (qPCR) reactions were performed with 45 cycles of 10 seconds at 95°C, 10 seconds at 60°C, and 15 seconds at 72°C. Gene expression levels

were reported as Cq values normalized by subtracting the mean Cq of the *GAPDH* housekeeping gene. The primers were for the following genes: *Tp63, Foxj1, Scgb1a1, and Muc5AC*. Primer sequences are listed in Table 2.1.

## 2.3. Results

### 2.3.1. Immunohistochemical and flow cytometric screening of monoclonal antibodies

Mouse anti-human monoclonal antibodies were generated by fusing mouse splenocytes with myeloma cells to create immortalized hybridoma clones. Multiple immunization strategies were used in generation of the hybridomas. The HBEC3 hybridomas were derived from immunizations with hBECs cultured on collagen-coated plastic under growth media conditions[23,71]. HBEC4 hybridomas were derived following subtractive immunization with human airway epithelial cells grown over six passages, followed by immunization with airway cells grown over only a single passage. HLO1 hybridomas were generated following immunizations with intact lung organoids grown from primary hBECs[72,73]. Additional hybridomas, the DHIC series, were developed following immunization with human endocrine cells. Monoclonal antibodies from over 4,000 hybridoma clones spanning at least seven distinct immunization strategies were screened on acetone-fixed human airway tissue sections and/or on dispersed single-cell suspensions of hBECs.

Flow cytometric analyses of monoclonal antibodies on single-cell suspensions of uncultured primary hBECs showed that some of the antibodies targeted cell-surface antigens (Figure 2.1). The frequency of staining varied across donors, but each selected

mAb consistently stained a subset of cells across multiple biological replicates of primary hBECs, ranging from very rare populations that were less than 1% of the bulk hBECs population, HBEC4-3B5, to more abundant subsets that comprised ~60% of total hBECs like HLO1-6H5 (Table 2.2, Figure 2.1D).

Immunohistochemical staining of human airway tissue demonstrated varying patterns of epithelial cell targeting. Some of the more interesting mAbs stained distinct cell types such as basal or luminal cells, while others stained subsets of these two populations (Table 2.2). The HBEC3-1F3 and HLO1-6H5 antibodies showed basal cell or basal cell subset staining patterns (Figure 2.2A). The HLO1-6A6 and DHIC3-5H10 antibodies showed labeling of a subset of luminal cells, specifically a pattern that matches secretory cells (Figure 2.3A). The HBEC4-3B5 antibody showed staining for a subset of luminal cells, specifically a ciliated cell pattern (Figure 2.4A). The cell types that the monoclonal antibodies target were verified by co-labeling with commercially available antibodies that identify specific cell types in human airway epithelium.

### 2.3.2. Immunohistochemical colocalizations confirm targeting of distinct airway epithelial cell types

After initial screening by flow cytometry and immunohistochemistry (IHC), five monoclonal antibodies were selected for further characterization based on consistent staining intensity, patterns, and frequency across multiple biological replicates (Table 2.2). The panel of five selected antibodies demonstrated selective targeting of specific epithelial cell types in human airway tissue sections. In the initial immunohistochemical

screens, antibodies HBEC3-1F3 and HLO1-6H5 targeted only cells that resided along the basement membrane and away from the luminal surface of respiratory epithelium. Meanwhile, antibodies HLO1-6A6, DHIC3-5H10, and HBEC4-3B5 targeted luminal cells of the respiratory epithelium. Colocalization of the five mAbs with the following list of established markers of airway epithelial cell types was performed: the basal cell marker cytokeratin 5 (KRT5), the ciliated cell marker acetylated alpha-tubulin (TUBA4A), and the secretory cell marker mucin 5B (MUC5B)[7,19,64,65]. These colocalization studies identified the target cell type for each antibody. Both HBEC3-1F3 and HLO1-6H5 appeared to label a subset of KRT5+ cells as not every KRT5+ cell was stained by the mAbs (Figure 2.2A). This pattern was more obvious with HBEC3-1F3 as it labeled fewer cells in the epithelium than HLO1-6H5. HLO1-6H5 appeared to target KRT5+ cells that were attached to the basement membrane but did not target KRT5+ cells that were located suprabasally or luminally (Figure 2.2A). HLO1-6H5 and HBEC3-1F3 did not target luminal cells that expressed MUC5B or TUBA4A, indicating that neither mAb identified ciliated or secretory cells (Figure 2.2B-C).

HLO1-6A6 and DHIC3-5H10 targeted luminal cells that expressed MUC5B, indicating a selectivity for secretory cells (Figure 2.3A). However, DHIC3-5H10 occasionally colocalized with a subset of TUBA4A+ cells, suggesting that it might not differentiate between the two luminal cell lineages (Figure 2.3B). Neither mAb targeted KRT5+ basal cells (Figure 2.3C). HBEC4-3B5 also targeted luminal cells but appeared to selectively colocalize with TUBA4A+ cells more than MUC5B+ cells, as there were

multiple cells that were HBEC4-3B5+/MUC5B- (Figure 2.4A-B). HBEC4-3B5 did not target KRT5+ basal cells (Figure 2.4C).

### 2.3.3. Transcriptional analyses of FACS-isolated cell subsets

The identified panel of monoclonal antibodies were used to sort viable, phenotypically distinct cell subsets from dispersed primary human airway epithelium (Figure 2.1). Transcriptional analysis by qPCR with a canonical gene that identified each airway epithelial cell type provided additional data to verify the target cell type of each of the five novel mAbs established in the colocalization studies. The genes of interest in human airway epithelium are tumor repressor protein (*Tp63*) for identifying basal cells, the *Foxj1* transcription factor for ciliated cells, *Scgb1a1* for secretory club cells, and *Muc5AC* for secretory goblet cells[7,19,64,65]. Antibody-positive and -negative subsets were collected for four of the mAbs of interest, with two biological replicates for each mAb.

The transcriptional profile of the proposed basal cell-targeting antibodies HBEC3-1F3 and HLO1-6H5 supported the immunohistochemical data that these two mAbs specifically target basal cells in the airway epithelium. The antibody-positive subset of each mAb show an average fold change >1 for *Tp63*, indicating enrichment for basal cells, while expression of the ciliated (*Foxj1*) and secretory genes (*Scgb1a1* and *Muc5AC*) was <1, indicating a depletion for all of the luminal cell types of the airway (Figure 2.5A). HLO1-6H5+ cells showed higher upregulation for the basal cell gene *Tp63* than HBEC3-1F3+ cells with an average fold change >2.5. Overall the qPCR data and the

immunohistochemistry data together support the categorization of HBEC3-1F3 and HLO1-6H5 as antibodies that target basal cells.

DHIC3-5H10 and HLO1-6A6 were tentatively identified as antibodies that selectively target secretory cells based on the IHC colocalization data, but there was some evidence that the mAbs also targets the ciliated cell lineage. The IHC and qPCR data do agree that DHIC3-5H10 does not target basal cells (average *Tp63* fold change <0.5). However, the transcriptional profile of DHIC3-5H10+ hBECs does not clarify which luminal cell type it specifically identifies. The mAb positive subset of cells shows enrichment for the secretory goblet and club cell lineages with an average fold change >11 for both *Scgb1a1* and *Muc5AC*, but it also showed >5 average fold enrichment for *Foxj1*, indicating targeting of ciliated cells (Figure 2.5B). Although the most highly upregulated gene in DHIC3-5H10+ cells was for the club cell gene *Scgb1a1*, at a >30 average fold increase versus the mAb negative subset, the data still does not conclusively support categorizing DHIC3-5H10 as an antibody that strictly targets secretory cells. The IHC and qPCR data however does suggest that DHIC3-5H10 is able to selectively enrich for secretory cells over ciliated cells. HLO1-6A6 was not analyzed by qPCR due to low sample availability, preventing FACS-isolation of cell subsets for transcriptional analyses.

HBEC4-3B5 was tentatively identified as an antibody to selectively target ciliated cells based on the IHC colocalization data, but there was evidence that the mAb also targets the secretory cell lineage. Similar to DHIC3-5H10, the IHC and qPCR data both show that HBEC4-3B5 does not target basal cells (*Tp63* fold change <0.5) (Figure 2.5C).

But the qPCR data does not support labelling HBEC4-3B5 as a ciliated cell-specific antibody, due to the upregulation of both secretory genes; *Scgb1a1* was >23 fold upregulated and *Muc5AC* was >16 fold upregulated. The qPCR data does not support the IHC data to conclusively establish that HBEC4-3B5 is specific for ciliated cells.

## 2.4. Discussion

We have generated a panel of novel monoclonal antibodies for primary human airway epithelium. These antibodies can be used to visualize different epithelial cell types in respiratory tissue and identify different cell types in suspensions of uncultured primary hBECs. This panel of mAbs targets cell surface antigens on airway cells, which provides the added benefit of allowing for isolation of viable, phenotypically distinct populations of cells for downstream applications such as culture expansion or functional assays. Targeting cell surface antigens is critical for the study of viable, specific cell types from human organs as we are unable to lineage trace or genetically label target cells for study as can be done in rodent models. Antibodies towards cell surface markers provide us with the tools to investigate the biology of the human lung in greater depth.

The data for our novel panel of mAbs comes from uncultured primary human airway tissue. The rationale for assessment of primary tissue was to identify antibodies that exhibit utility towards the broad diversity of cell phenotypes present in the physiological organ. Culturing cells can result in biased selection towards specific cell types and/or induction of phenotypic changes in cells that may not be representative of primary tissue. By restricting our studies to uncultured primary tissue, we can identify

and develop physiologically relevant monoclonal antibodies as molecular tools for applications such as the identification of phenotypically distinct subsets of cell or specific cell targeting *in vivo*.

The luminal cell-reactive antibodies described here do not appear to clearly distinguish between the two luminal cell lineages as they did not exclusively identify a single cell type based on the qPCR and IHC data. However, the IHC data suggests that each of the luminal cell-targeting mAbs has some selectivity for either the secretory or ciliated cell lineages, but the purity of viable cell subsets isolated with the luminal cell-reactive mAbs may suffer due to a lack of specificity. However, that does not mean that these mAbs are not useful. Enrichment for certain cell types is still possible if the correct combination of markers is used. Instead of using them to purify specific cell types, these antibodies can be used in combinatorial panels to deplete certain cell types from a population. Basal cells are a population of interest to stem cell researchers and having these luminal cell antibodies would be useful for depletion of ciliated and secretory cells.

The IHC and qPCR data for HBEC3-1F3 and HLO1-6H5 support that they are basal cell-specific antibodies. The staining pattern of these two antibodies on airway tissue suggest there is heterogeneity in the human airway basal cell population. The basal cell-specific HBEC3-1F3 and HLO1-6H5 monoclonal antibodies do not recognize all KRT5+ cells, the canonical marker of basal cells (Figure 2.2A). This suggests that within the basal cell population, there is some phenotypic heterogeneity. The question of whether there is heterogeneity in the basal cell population is of great interest to the lung stem

cell research community[7,19,54,64]. The basal cell population is reported to contain stem cells for the respiratory epithelium that lines human proximal airway. Although numerous studies can verify that the basal cell population contains stem cells, we still do not know if every basal cell in human tissue can function as a stem cell[48,52,61,62]. Some progress has been made in studying basal cell heterogeneity in mouse models[54,55]. However, these observations may not be applicable to human airway stem cells as there are clear differences in cellular composition between human and mouse airways, most notably the absence of basal cells in the distal airway of the mouse. In order to advance our knowledge of the basal cell compartment, new molecular tools are needed. With our novel basal cell markers, we can FACS-isolate basal cell subsets to determine if there are any functional differences between basal cell subsets. These mAbs will allow us to address the question of heterogeneity in basal cells, the reported stem cell population of the human proximal airway.

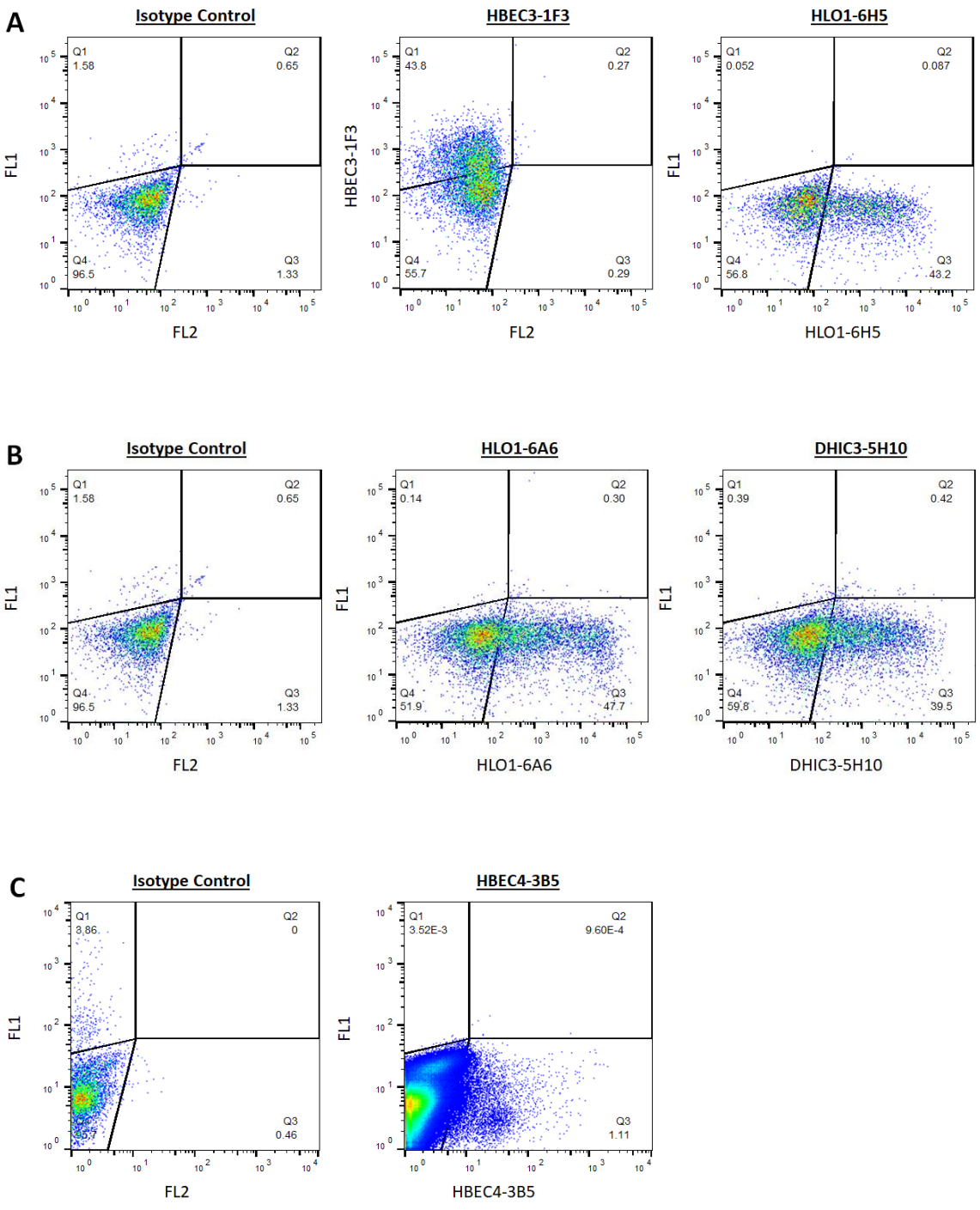
## 2.5. Figures and Tables

**Table 2.1. List of primer sequences for qPCR.**

| Gene | Associated cell lineage | 5' Primer sequence | 3' Primer sequence |
|---|---|---|---|
| *Tp63* | Basal cells | CCACAGTACACGAACCTGGG | CCGTTCTGAATCTGCTGGTCC |
| *Foxj1* | Ciliated cells | CAACTTCTGCTACTTCCGCC | CGAGGCACTTTGATGAAGC |
| *Scgb1a1* | Secretory club cell | ATGAAACTCGCTGTCACCCT | GTTTCGATGACACGCTGAAA |
| *Muc5AC* | Secretory goblet cell | GCACCAACGACAGGAAGGATGAG | CACGTTCCAGAGCCGGACAT |
| *GAPDH* | Housekeeping gene | AATGAAGGGGTCATTGATGG | AAGGTGAAGGTCGGAGTCAA |

**Table 2.2. Monoclonal antibodies reactive to antigens in primary human bronchial epithelial cells (hBECs).**

| Antibody | Isotype (mouse) | Target cell type by immunofluorescence with phenotypic markers in human airway | Avg. frequency of positive cells in primary hBECs |
|---|---|---|---|
| **HBEC3-1F3** | IgG | Basal Cells | 46% |
| **HLO1-6H5** | IgM | Basal Cells | 58% |
| **HLO1-6A6** | IgM | Secretory Cells | 30% |
| **DHIC3-5H10** | IgM | Secretory Cells | 22% |
| **HBEC4-3B5** | IgM | Ciliated Cells | <2% |


A
Isotype Control
Q1 1.58
Q2 0.65
Q4 96.5
Q3 1.33
FL1
FL2
HBEC3-1F3
Q1 43.8
Q2 0.27
Q4 55.7
Q3 0.29
HBEC3-1F3
FL2
HLO1-6H5
Q1 0.052
Q2 0.087
Q4 56.8
Q3 43.2
FL1
HLO1-6H5
B
Isotype Control
Q1 1.58
Q2 0.65
Q4 96.5
Q3 1.33
FL1
FL2
HLO1-6A6
Q1 0.14
Q2 0.30
Q4 51.9
Q3 47.7
FL1
HLO1-6A6
DHIC3-5H10
Q1 0.39
Q2 0.42
Q4 59.8
Q3 39.5
FL1
DHIC3-5H10
C
Isotype Control
Q1 3.86
Q2 0
Q3 0.46
FL1
FL2
HBEC4-3B5
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Q2 9.60E-4
Q3 1.11
FL1
HBEC4-3B5

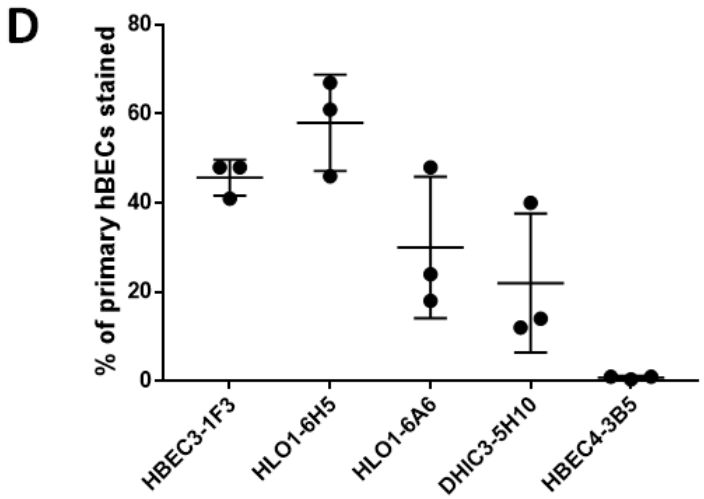
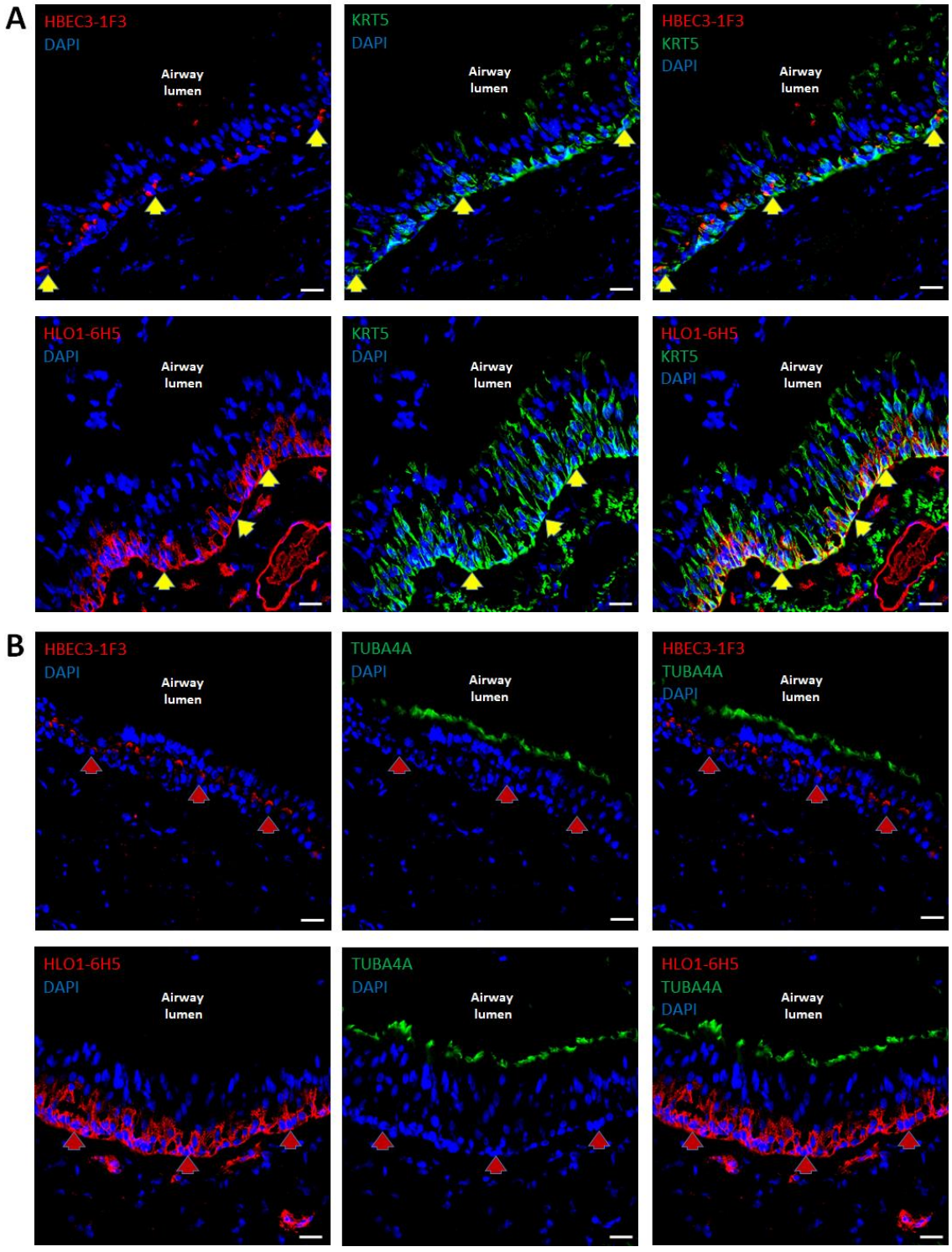



**Figure 2.1. Novel monoclonal antibodies target cell surface antigens for cell sorting.** Representative flow cytometric analyses of novel marker expression in primary dispersed human bronchial epithelial cells for antibodies that recognize cell surface antigens specific to airway **A)** basal cells; **B)** secretory cells; and **C)** ciliated cells. **D)** The frequency of positive cells for each antibody across 3 biological replicates is represented in a box and whisker plot. Data presented as mean (+/- S.D.).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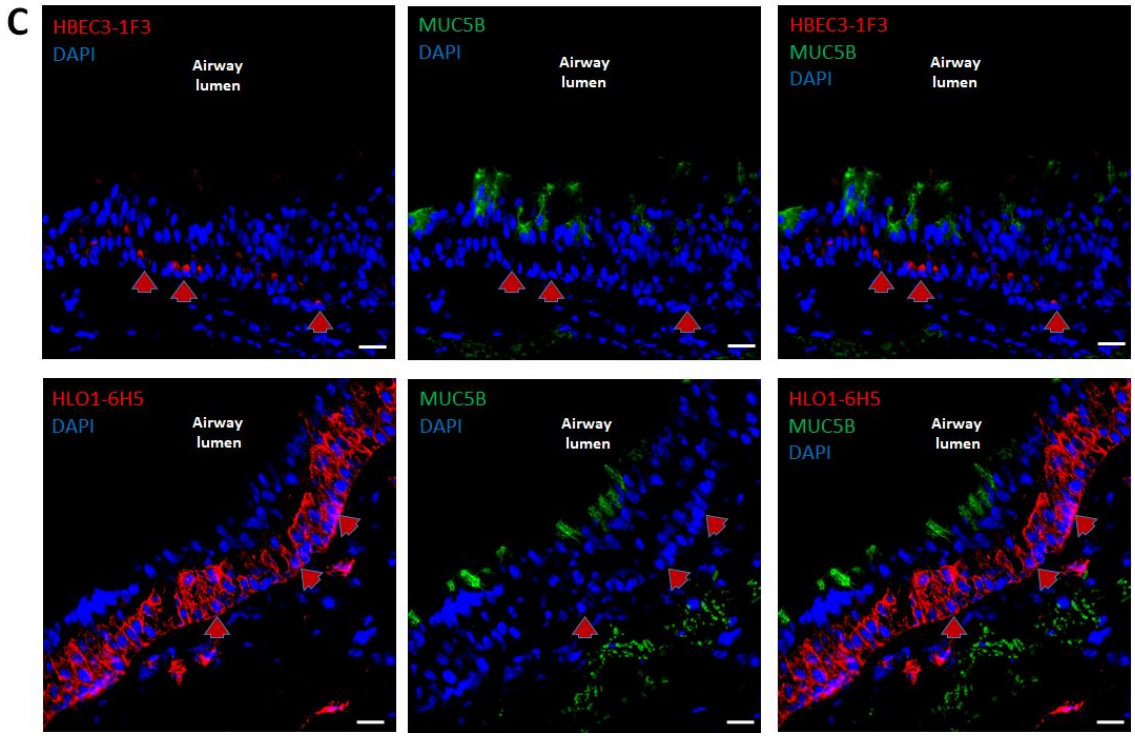
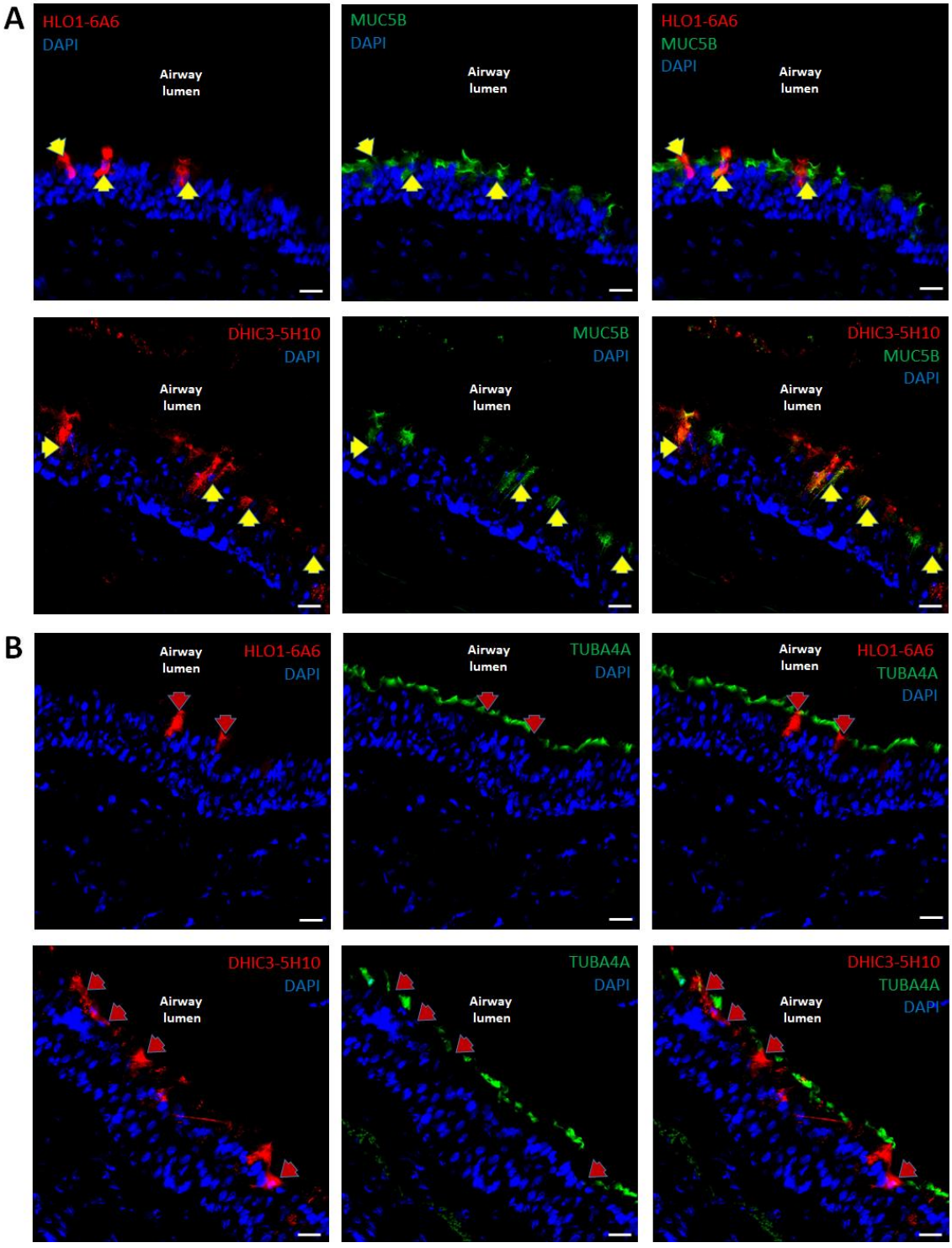



**Figure 2.2. Novel markers towards airway basal cells.**

Novel markers HBEC3-1F3 and HLO1-6H5 that target airway basal cells in primary human airway were co-stained with **A)** the canonical basal cell marker cytokeratin 5 (KRT5) with yellow arrows indicating cells that co-express the novel marker and KRT5; **B)** the canonical ciliated cell marker acetylated-alpha tubulin (TUBA4A) with red arrows indicating cells that express only the novel marker and not TUBA4A; **C)** the canonical secretory goblet cell marker mucin 5B (MUC5B) with red arrows indicating cells that express only the novel marker and not MUC5B. Scale bar: 50 µm.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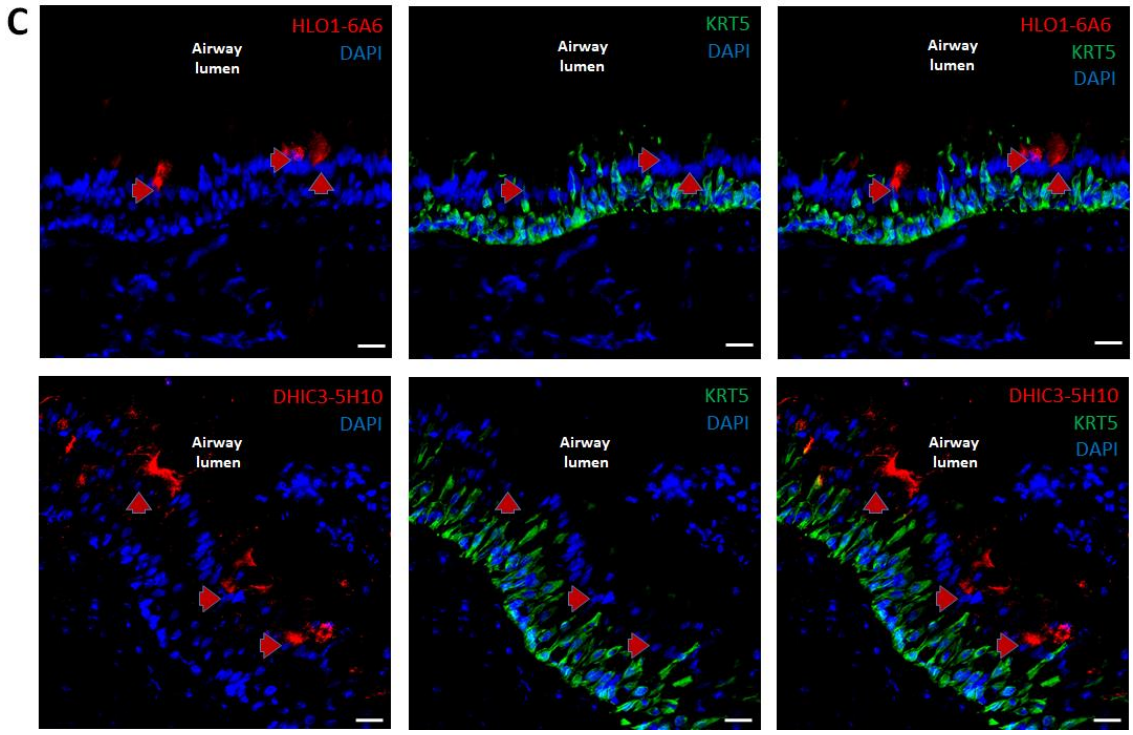



**Figure 2.3. Novel markers towards airway secretory cells.**

Novel markers HLO1-6A6 and DHIC3-5H10 that target airway secretory cells in primary human airway were co-stained with **A)** the canonical secretory goblet cell marker mucin 5B (MUC5B) with yellow arrows indicating cells that co-express the novel marker and MUC5B; **B)** the canonical ciliated cell marker acetylated-alpha tubulin (TUBA4A) with red arrows indicating cells that express only the novel marker and not TUBA4A; **C)** with the canonical basal cell marker cytokeratin 5 (KRT5) with red arrows indicating cells that express only the novel marker and not KRT5. Scale bar: 50 µm.

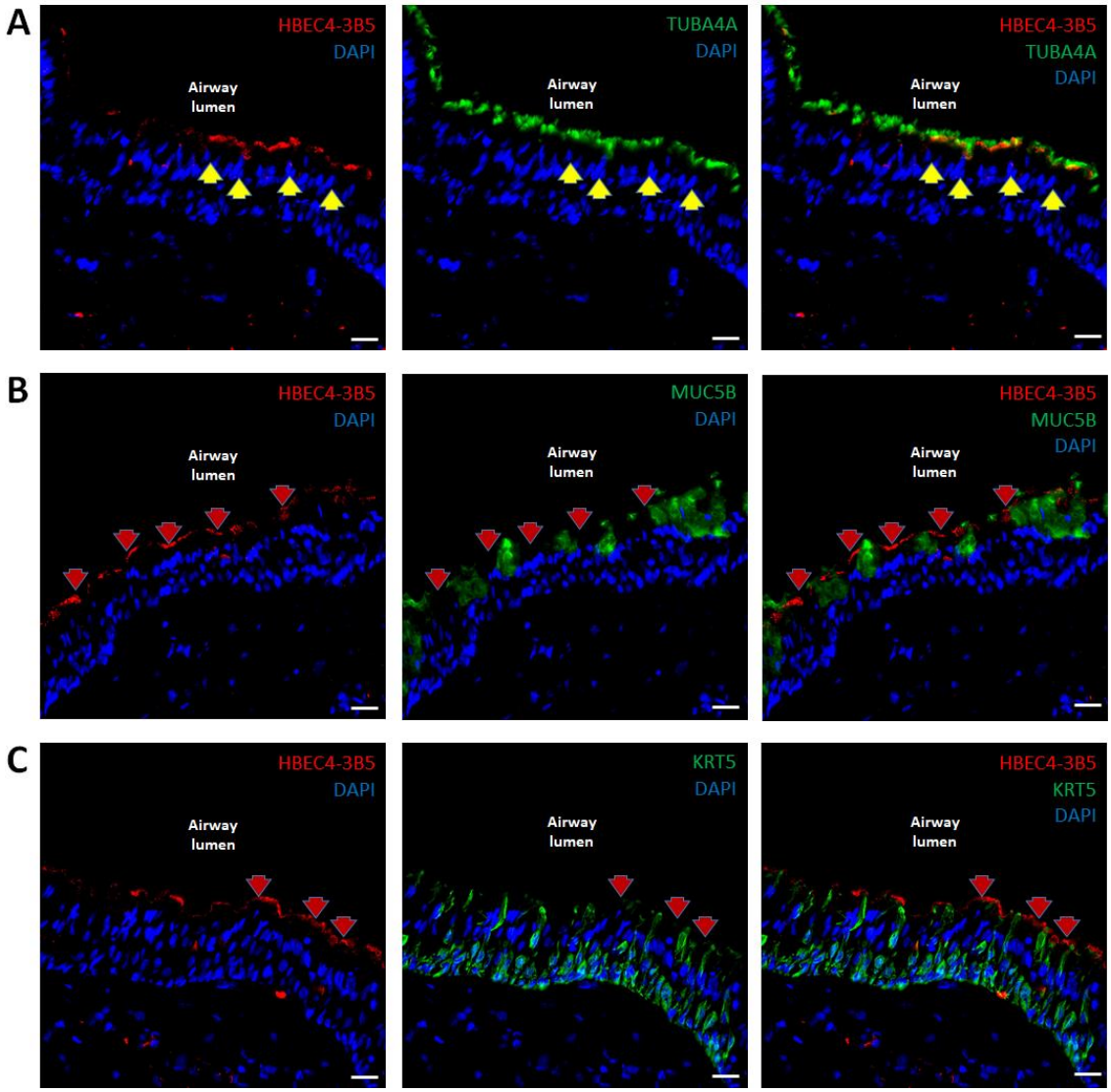
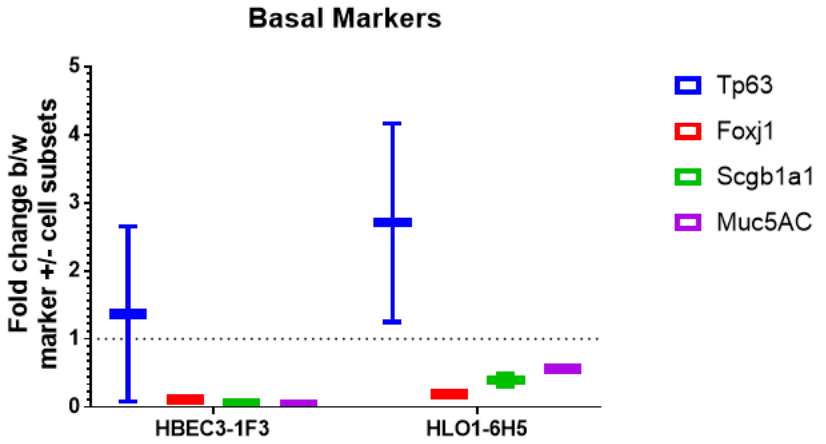



**Figure 2.4. Novel marker towards airway ciliated cells.**

A novel marker HBEC4-3B5 that targets airway ciliated cells in primary human airway was co-stained with **A)** the canonical ciliated cell marker acetylated-alpha tubulin (TUBA4A) with yellow arrows indicating cells that co-express the novel marker and TUBA4A; **B)** the canonical secretory goblet cell marker mucin 5B (MUC5B) with red arrows indicating cells that express only the novel marker and not MUC5B; **C)** with the canonical basal cell marker cytokeratin 5 (KRT5) with red arrows indicating cells that express only the novel marker and not KRT5. Scale bar: 50 µm.

A


Basal Markers
Fold change b/w marker +/- cell subsets
0
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4
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HBEC3-1F3
HLO1-6H5
Tp63
Foxj1
Scgb1a1
Muc5AC

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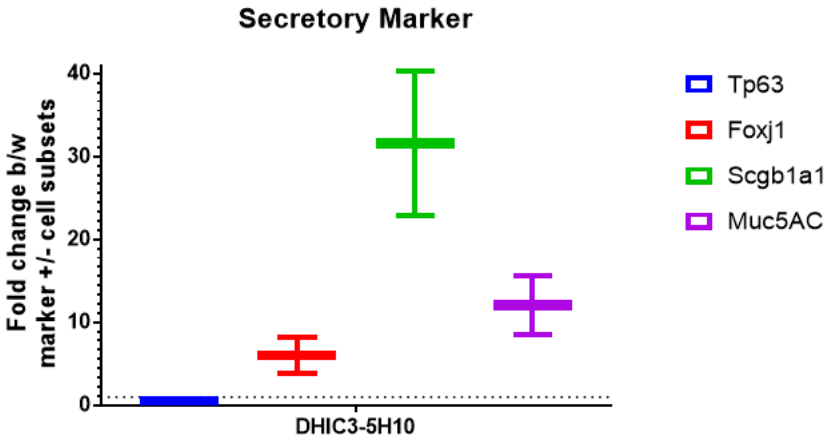
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Tp63
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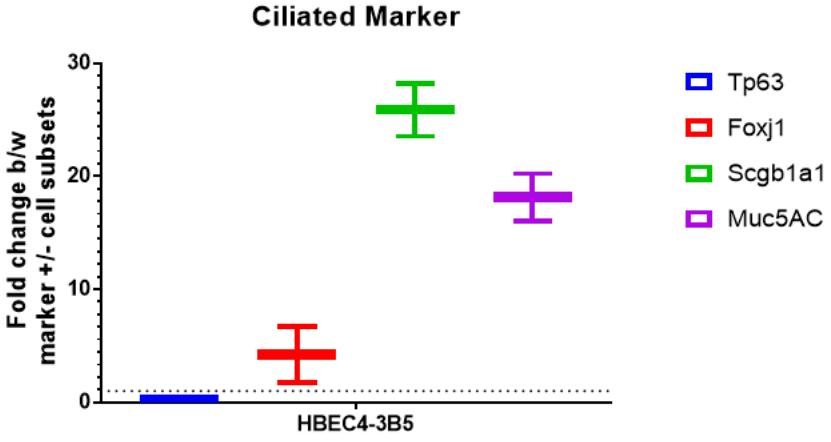
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Scgb1a1
Muc5AC

**Figure 2.5. Gene expression analysis of FACS-isolated primary human bronchial epithelial cells (hBECs) to verify specific airway cell type targeting by novel antibodies.**

Expression analysis of genes specific to basal cells (*Tp63*), ciliated cells (*Foxj1*), secretory club cells (*Scgb1a1*), and secretory goblet cells (*Muc5AC*) was done on FACS-isolated subsets of antibody positive and negative cells. Box and whisker plots of linear ratios of the aforementioned genes to compare antibody positive and negative cell subsets that were identified by immunohistochemistry as **A)** basal cell-specific markers based on colocalization with KRT5; **B)** secretory cell-specific markers based on colocalization with MUC5B; or **C)** ciliated cell-specific markers based on colocalization with TUBA4A. Relative mRNA expression of FACS-isolated marker positive and negative cell subsets was calculated based on qPCR ΔCq values, which are the differences between the Cq of the gene of interest and the Cq of housekeeping gene GAPDH. Each mAb was assessed on two biological replicates of primary hBECs. Data presented as mean (+/- S.D.).

# Chapter 3. *In vitro* assays and models to assess stem/progenitor cell function of human airway epithelial cells

The majority of identified adult stem cell populations identified in the mammalian respiratory system mentioned in Chapter 1 originate from mouse models. The reason for this is due to the reductionist approach that is necessary for identifying if a cell or cell population is the resident stem cell of an organ. The definition of a stem cell is a cell that can: 1) self-renew by proliferation and 2) differentiate into other cell types, also known as potency. There are also progenitor cells that are similar to stem cells but are limited in their differentiation potential as they are already committed to specific lineages and have limited self-renewal capabilities. It would be next to impossible to identify adult stem cells from an organ or mixed cell population without purification or specific labelling of individual cells or specific cell types. Mouse models allow for lineage tracing to track the proliferation and differentiation of individual cells or cell types during homeostasis or tissue repair *in vivo*. Such *in vivo* studies are not possible with humans; therefore *in vitro* assays are the method of choice for assessing stem cell properties in human cell populations.

## 3.1. *In vitro* assays for stemness in human organs and tissue systems

One of the earliest *in vitro* assays to identify stem cells is the 3-dimensional (3D) colony formation assay developed in 1956 by Puck and Marcus to study the effects of

radiation on HeLa cells from human cervical cancer[75]. Since then, the colony forming cell assay has been used for *in vitro* assessment of stem/progenitor cells for their ability to proliferate and differentiate, particularly in studies of hematopoietic stem/progenitor cells[76]. The design of colony forming assays is to isolate individual cells that can proliferate to form a multi-cellular colony in a culture matrix, thereby demonstrating the property of self-renewal. The assay also assesses the property of differentiation by determining if there are any cells in the colony that are phenotypically or morphologically distinct from the original input cell, thereby demonstrating the property of potency. Aside from the hematopoietic field, this colony formation assay is also used to identify potential stem cell populations in homeostatic or diseased human tissues like the hair follicles, prostate, and breast[76–81]. The next evolution of the 3D colony formation assay are the organoid/spheroid assays.

Organoids are structures containing multiple cell types that organize to mimic the functionality of the organ from which the input stem/progenitor cell is derived. Cells that can generate these complex structures are considered stem/progenitor cells because they must be capable of proliferation and differentiation to generate the diverse cell types of the organoid, thereby demonstrate the stem cell properties of self-renewal and potency. Organoid assays consist of an extracellular matrix gel to embed the cells within, along with a surrounding growth media. The media contains organ-specific growth factors to mimic the physiological environment and to promote cell proliferation and differentiation. The earliest applications of organoids from human tissue came from Mina Bissell's studies of organoid formation from healthy and

cancerous breast tissue and from Chung Lee's studies of human prostate cells[82,83]. Some of the most noteworthy breakthroughs in the development of human organoid systems came from Yoshiki Sasai and his team's creation of brain organoids in 2008 and Hans Clevers and his team's generation of intestinal organoids in 2011[84–86]. Organoid assays are now applied across many human tissue systems that span all three germ layers[85,87–89]. Some of these organoids are called spheroids based on their morphology, e.g. mammospheres from mammary gland, neurospheres from neural stem cells, and alveolospheres from alveolar lung[90–92]. Much like the 3D colony formation assays that preceded them, organoid assays are a functional assessment of whether a cell is a stem/progenitor cell.

## 3.2. *In vitro* assays for stemness in the human respiratory system

The earliest *in vitro* assay of stemness for human lung tissue is the air-liquid interface (ALI) model. Developed in 1999 by Scott Randell's team to study mechanisms behind airway mucus secretion, the ALI culture system places human bronchial epithelial cells (hBECs) derived from airway tissue onto permeable Transwell® supports that are placed inside tissue culture wells[93]. Media is initially added into the support to submerge the hBECs and into the well surrounding the support. Once the seeded cells have proliferated to form a monolayer, the media inside the support is removed to expose the apical surface of the cells to air. The air exposure promotes differentiation of the cells to form a pseudostratified mucociliary epithelium containing secretory cells, ciliated cells, and basal cells, mimicking the physiology of the human airway

epithelium[20,23,24]. These cultures have great utility in measuring the effects of disease or manipulated molecular pathways on respiratory epithelium morphology[20,21,25,94]. The limitation of the ALI model is that it requires an input of bulk cells from the airway, making it difficult to determine which cell type(s) are proliferating and/or differentiating to generate the mucociliary epithelium. To address this limitation, an organoid assay is needed that can assess the formation of airway epithelium from individual cells or from a specific cell type.

Bronchospheres are an organoid assay specific to cells of the airway epithelium. Pioneered by Rock et al. in 2009, the bronchosphere assay embeds a single cell suspension of hBECs into MatriGel® extracellular matrix, which is then placed into porous supports and culture wells for apical exposure to air, similar to the ALI monolayer cultures[44]. If the seeded single cells are stem or progenitor cells in nature, they will proliferate, differentiate, and organize in to organoids/spheroids called bronchospheres. Rock et al.'s 2009 study shows FACS-isolated NGFR+/ITGA6+ basal cells form bronchospheres that contain ciliated and basal cells, demonstrating that basal cells are stem cells as they can self-renew and differentiate[48]. Wu et al.'s study shows that culture-expanded, unsorted hBECs form bronchospheres that contain basal cells and two types of secretory cells[52]. As evident by the aforementioned studies, the cellular composition of bronchospheres can vary depending on the input cell and growth conditions. For example, the EGF concentration is higher in the Wu et al. study, while the Rock et al. study uses a higher concentration of MatriGel® matrix in their

bronchosphere assay, which could explain the differences in cellular composition of their respective bronchopsheres[44,52].

In addition to measuring stem cell properties, the bronchosphere assay is also a model system for mechanistic studies into molecular regulators of proliferation and differentiation. Danahay et al.'s study with human basal cells shows spheroids to contain basal cells, ciliated cells, and goblet cells, but the goblet cell frequency increases with cytokine IL17A treatment and the ciliated cell frequency increases with inhibition of Notch2[61]. The use of IL17A in Danahay's study mimics the cytokine secretions produced by helper T-cells in severe asthma. Lange et al.'s study shows that bulk hBECs can form bronchospheres consisting of basal, ciliated, and goblet cells, but activation of the Hippo/Yap signaling pathway causes spheroids to increase in diameter and contain a lower frequency of ciliated and secretory cell lineages compared to an untreated control[94]. The bronchosphere assay is a versatile platform for identifying signaling pathways or conditions that regulate airway stem/progenitor cell function, which may be representative of what happens physiologically.

## 3.3. Clonogenicity of the bronchosphere assay for measuring self-renewal[†]

One of the primary assumptions of the bronchosphere assay is that the spheroids are clonal, meaning that each colony arises from a single input cell. In Rock et al.'s study, the clonogenicity of their bronchosphere assay is validated using a mixture of

[†] This work is in preparation for publication at the time of this writing. First author: Chris Cheng; Additional authors: Yong-Ping Zhong, Claire Turina, Maria Grompe, Anusha Sridharan, Scott Randell, Jeffrey Whitsett, Philip Streeter.

GFP- and RFP-labelled mouse airway cells as the input cells[44]. Their results show that their bronchosphere assay is clonogenic as every colony that forms is a uniform color, either red or green, and never mixed, which would be indicative of a colony arising from >1 input cell[44].

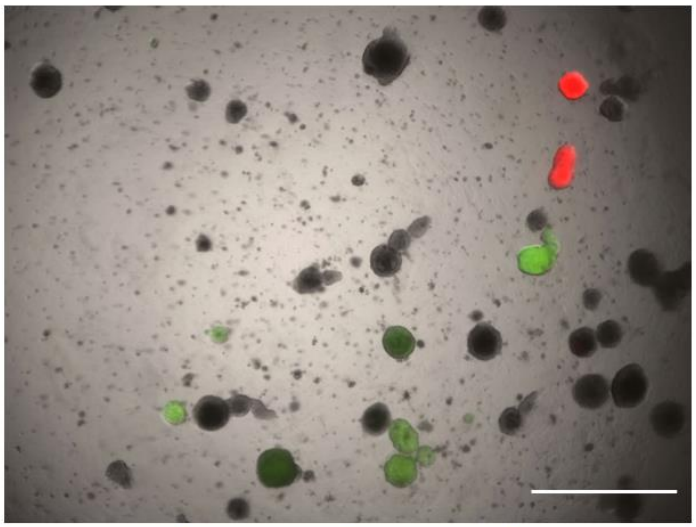
The stem cell property of self-renewal stipulates that a stem cell must be able to regenerate itself and maintain the resident population. This attribute is closely associated with clonogenicity in organoid and spheroid assays. If the colonies form as a result of cell migration and aggregation of multiple input cells, then the assay would be unable to accurately detect a stem cell. One method to get around this is to seed single cells into assay conditions, but this is highly inefficient if the stem cell frequency of the input cell population is unknown. The response to that of course is to seed more cells in a way that ensures that the cells do not aggregate. The use of extracellular matrices has helped in that regard. For uncultured primary airway cells, the bronchosphere assay requires input of multiple cells to assay for stem cell function. Therefore, much like Rock et al., the clonogenicity of my bronchosphere assay protocol requires validation[44].

Using a mixture of GFP- and RFP-labelled hBECs as input cells, I generated bronchospheres under standard incubator conditions of 37°C and 5% $CO_2$. The input hBECs were GFP- or dsRed-labelled by lentiviral transduction at low efficiency to reduce the chance of colonies forming from two cells that were labelled with the same fluorescent protein[‡]. After 2 weeks of culture, the colonies that formed were counted

[‡]Lentivirus for GFP and dsRed labelling was generously provided by Amita Tiyaboonchai of Markus Grompe's Lab at OHSU.

and assessed for green fluorescent protein (GFP) and dsRed expression (Figure 3.1). The bronchospheres that formed were either homogenously GFP-expressing, dsRed-expressing, or not fluorescently labelled at all (Figure 3.1A). There were no bronchospheres of mixed GFP and dsRed expression, which would have been indicative of a spheroid that formed from more than one cell; therefore, the bronchospheres were clonally derived (Figure 3.1B). The bronchosphere assay must be clonal in order to demonstrate that the spheroids arise from a proliferating, self-renewing cell, which is a key requirement for being a stem cell.

A



B

| Replicate | Red Colonies | Green Colonies | Red & Green Colonies |
|---|---|---|---|
| 1 | 14 | 22 | 0 |
| 2 | 8 | 13 | 0 |
| 3 | 8 | 23 | 0 |

**Figure 3.1. Bronchospheres are clonogenic and derived from single cells.**

**A)** Representative image of bronchospheres grown for 14 days from unsorted hBECs transduced for GFP- or dsRed-expression. Scale bar: 2000 µm; **B)** Spheroids that formed are either GFP-labelled, dsRed-labelled, or unlabeled. There were no colonies that were a mixture of GFP-and dsRed-labelling.

## 3.4. Assessing cellular differentiation in the bronchosphere assay[§]

Stem cells must be capable of differentiation to demonstrate the property of potency. Organoid/spheroid assays are readouts of potency as the colonies are multicellular structures comprised of diverse cell types that arise from a single parent cell or cell type[85,87–89]. As previously mentioned, numerous studies with bronchospheres show that the spheroid can contain basal cells, ciliated cells, and/or secretory cells depending on the input cell type and growth conditions. My bronchosphere assay is also able to detect cellular differentiation.

The bronchosphere assay allows for the assessment of the stem cell property of potency by evaluating the expression of airway lineage markers. Bronchospheres were assessed at day 42, when lumen formation was evident in almost all colonies, mimicking airway morphology. Immunocytochemistry of acetone-fixed bronchosphere sections showed that the colonies expressed markers of all three major cell types found in the airway respiratory epithelium: basal cells, ciliated cells, and goblet cells (Figure 3.2)[1,2]. All the bronchospheres contained basal cells as evidenced by positive expression of cytokeratins 5 and 14, KRT5 and KRT14 respectively (Figure 3.2A). The expression of KRT14 in my bronchospheres indicated that the constituent basal cells were a mitotically active subset[7,19]. Luminal cell lineages were evident in most of the spheroids that had formed lumens, as indicated by the expression of TUBA4A (acetylated alpha tubulin),

[§] This work is in preparation for publication at the time of this writing. First author: Chris Cheng; Additional authors: Yong-Ping Zhong, Claire Turina, Maria Grompe, Anusha Sridharan, Scott Randell, Jeffrey Whitsett, Philip Streeter.

indicating ciliated cells, and MUC5B (mucin 5B), indicating secretory goblet cells (Figure 3.2C-D). In summary, the bronchosphere assay is an effective readout of the stem cell properties of self-renewal and potency in primary hBECs.

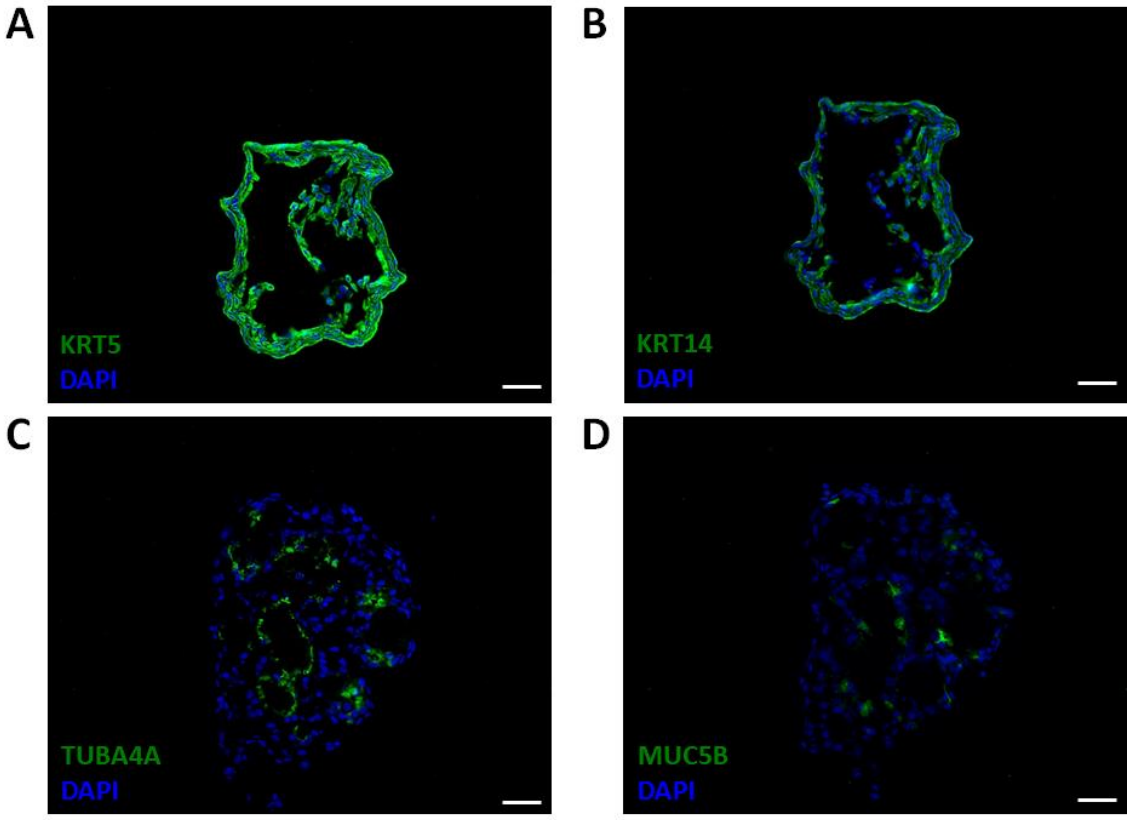



**Figure 3.2. Bronchospheres contain basal cells, ciliated cells, and secretory cells.** Bronchospheres grown for 42 days from unsorted hBECs form lumens in the interior of the spheroid and contain **A)** basal cells that are KRT5+ (cytokeratin 5), **B)** mitotically active basal cell subset (KRT14+), **C)** ciliated cells that are TUBA4A+ (acetylated alpha tubulin), and **D)** secretory goblet cells that are MUC5B+ (mucin 5B). Scale bar: 50 µm.

## 3.5. Primary airway stem cells require support cells in the bronchosphere assay[**]

Published applications of the bronchosphere assay show that various inputs of hBECs can yield spheroid formation. However, many of those studies use culture-expanded hBECs. The original bronchosphere assay from Rock et al.'s 2009 study uses hBECs that are cultured overnight as a monolayer on collagen-coated plastic prior to input into the bronchosphere assay[44]. An identical overnight culture protocol is used in Lange et al.'s study[94]. In Wu et al.'s and Danahay et al.'s respective studies, the input hBECs are cultured-expanded on plastic as a monolayer for a minimum of one passage prior to bronchosphere conditions[52,61]. Additionally, a protocol published in *Current Protocols in Stem Cell Biology* describing the use of human airway basal cells to establish bronchosphere cultures specifies the expansion of input basal cells by 2D monolayer culture as an initial step[95]. One group does distinguish itself from the rest in that it successfully grow bronchospheres from NGFR+ basal cells sorted from uncultured primary hBECs[62]. Their spheres however did not display strong evidence of differentiation and also the frequency of colony formation was dependent on the high amount of hyperplasia in the donor tissue, resulting in highly variable rates of bronchosphere formation. In summary, most of the currently published studies or protocols use cultured hBECs from primary human airway for consistent results.

[**] This work is in preparation for publication at the time of this writing. First author: Chris Cheng; Additional authors: Yong-Ping Zhong, Claire Turina, Maria Grompe, Anusha Sridharan, Scott Randell, Jeffrey Whitsett, Philip Streeter.

My work focuses on the identification of human airway stem cells that are the most physiologically relevant, and therefore monolayer culture-expanded cells are not assessed in my bronchosphere assays. I use the bronchosphere assay to assess stem cell function in subsets directly isolated from uncultured primary human airway. Through the course of my work, I have found that formation of bronchospheres from primary hBECs require the addition of support cells (Figure 3.3). The MRC5 stromal cell line is derived from human fetal lung and has been used in human bronchosphere and alveolosphere culture assays in order to increase colony forming efficiency[41,92,96]. The inclusion of MRC5 cells is not required for bronchosphere formation with hBECs that are culture-expanded[44,52,61,94]. I have found that without the presence of MRC5 cells, I do not see any bronchospheres form from primary cells, thereby preventing me from assessing stem cell function (n=6) (Figure 3.3A). Including MRC5 cells in my protocol recovers colony forming capabilities and allows me to assess the stemness of primary airway cells in the bronchosphere assay (Figure 3.3A). The MRC5 cells also seem to reduce variability in terms of my ability to observe bronchosphere formation as I consistently get colonies under these conditions. In summary, I have developed a modified bronchosphere assay that can consistently evaluate stemness in primary airway epithelial cell populations.

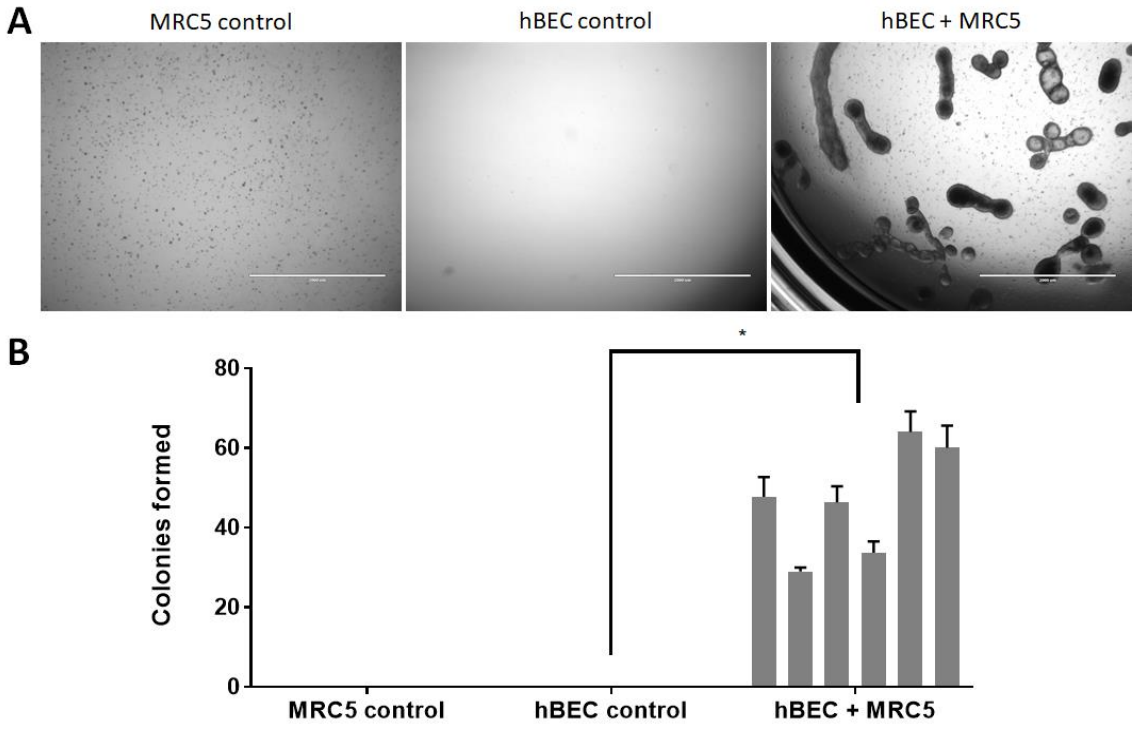



**Figure 3.3. Primary hBECs require MRC5 stromal cells to form bronchospheres.**

**A)** Bronchospheres grown for 14 days from unsorted hBECs co-cultured with MRC5 human fetal lung fibroblasts. Scale bars, 2000µm; **B)** Bar graph of number of bronchospheres formed by MRC5 fibroblasts, hBECs, and hBECs co-cultured with MRC5 fibroblasts across 6 donors, 3 replicates per donor. Data presented as mean (+/- S.D.). Paired t-test performed *$p<0.05$.

## 3.6. Discussion

Despite the limited number of models/methods for assessing stem cell functionality in human tissue, the development of organoid culture provides a suitable assay for the functional properties of self-renewal and potency. The organoid assay is versatile in that it can be modified to mimic the physiological environment or niche in which the target cell type resides. Identifying a suitable *in vitro* model for assessing stem cell function in uncultured primary airway cells was a challenge, as most investigators

prefer to culture-expand their primary tissue prior to analysis[24,51,52,94]. The rationale for culture expansion is that it allows for a larger amount of starting material for study, as uncultured primary lung tissue for research is scarce. The bronchosphere was an ideal assay and model system for studying stem cells of the respiratory system as it was specifically designed for that purpose. However, I still had to optimize the assay to meet my research needs.

The currently available bronchosphere protocols do not require stromal cell co-culture to generate colonies from culture-expanded hBECs. My data shows that without this stromal cell line however, the bronchosphere assay is not suitable for assessing un-cultured primary hBECs as colonies do not form. Although one study showed the ability to generate bronchospheres from uncultured hBECs without stromal cells by using hyperplastic airway tissue, the goal of my study was to identify stem cells from healthy airway tissue. Hence, I had to optimize the assay protocol with MRC5 stromal cells to enable the analysis of uncultured epithelial cells from healthy human airway.

For my study of airway stem cells, the inclusion of an air-liquid interface and stromal support cells provides the necessary conditions to assess stem cell function of uncultured primary cells. The limited availability of uncultured primary tissue did not provide many opportunities for assay optimization. However, insights from publications and other pulmonary researchers facilitated the process of identifying fibroblasts as a means of optimizing the assay for primary stem cell assessment[97,98]. Combined with the novel monoclonal antibodies from Chapter 2, I can isolate viable cell subsets from human airway epithelium and assess them for stem cell function.

# Chapter 4. HLO1-6H5 identifies bipotent clonogenic basal cells in primary human airway [††]

## 4.1. Introduction

The human respiratory system can be broken down into two regions based on function, the gas-exchange alveolar space and the conducting airways consisting of the trachea, bronchi, and bronchioles. The basal cell population maintains the pseudostratified respiratory epithelium that lines the conducting airway. The basal cell has been reported to be the stem cell population of the human airway, capable of both self-renewal and differentiation into the secretory and ciliated cell lineages[20,24,44,57,60]. This stem cell property makes the basal cell population the focus of lung regeneration and tissue engineering studies. However, recent studies have shown that the basal cell compartment is heterogeneous and that there are subpopulations of basal cells with different stem cell capabilities based on lineage commitment[54,55]. Studying these various potential airway stem cell subsets has been difficult due to the dearth of molecular tools to target cell-surface markers for identifying and isolating viable, phenotypically distinct cell subsets. Hence the need to develop new tools to subdivide the airway basal cell population[27,64,65].

The airway basal cell is defined as a cuboidal cell that resides along the basement membrane, unexposed to the airway lumen, and expresses cytokeratin 5 and TP63[7,19,64].

[††] This work is in preparation for publication at the time of this writing. First author: Chris Cheng; Additional authors: Yong-Ping Zhong, Claire Turina, Maria Grompe, Anusha Sridharan, Scott Randell, Jeffrey Whitsett, Philip Streeter.

Rock et al. discovered a pair of cell surface markers, NGFR and ITGA6, that were enriched in basal cells in both mouse and human airway epithelium[44]. These two markers have been used together and individually to identify and isolate bulk basal cells from primary or cultured airway tissue[26,41,61,63]. Further subdivision of the basal cell population to address questions of population heterogeneity however requires identification of new markers[7,19]. Several studies have identified subsets of basal cells based on intracellular markers and lineage tracing in mouse models[54,55]. Although these studies have identified phenotypically and functionally distinct subsets of basal cells, such findings have yet to be replicated in human airway. Also, it is impossible to use these markers for isolating viable subsets of basal cells because they are intracellular proteins. Targeting markers on the surface of basal cells remedies this issue.

Our lab has identified a novel subset of human basal cells using a monoclonal antibody that targets a cell surface antigen. The monoclonal antibody, HLO1-6H5, specifically identifies a subset of cytokeratin 5+ basal cells in primary human airway epithelium. The HLO1-6H5 antibody was compared to established cell surface markers to confirm that it identified a novel subset of the airway basal cell population[44]. HLO1-6H5 subsets from conducting airway epithelium have been isolated by FACS and assessed for functional differences, specifically in the stem cell properties of self-renewal/proliferation and differentiation in an *in vitro* clonogenic assay. My analyses have shown that the HLO1-6H5+ basal cell subset is highly enriched for a bipotent stem/progenitor cell population in primary human airway epithelium.

## 4.2. Methods

### 4.2.1. Human airway epithelial cell isolation

As mentioned previously in Chapter 2, donor airways deemed unacceptable for transplantation were acquired by the University of North Carolina's Cystic Fibrosis Center Tissue Procurement and Tissue Culture Core under IRB-approved protocols. Tracheal-bronchial segments of airway were disassociated with 0.1% protease XIV (Sigma) with 0.001% DNase (Sigma) then manually scraped with a scalpel to yield dispersed viable human bronchial epithelial cells (hBECs)[71]. Isolated airway epithelial cells were resuspended in F12 media and shipped overnight at 4°C to OHSU for flow cytometric and functional analyses.

### 4.2.2. Fluorescent Immunohistochemistry/Immunocytochemistry

As mentioned previously in Chapter 2, fresh human lung tissue from healthy donors was frozen in OCT (Optimal Cutting Temperature) compound blocks at the Marisco Lung Institute's Cystic Fibrosis Center Tissue Procurement and Cell Culture Core at the University of North Carolina in Chapel Hill. The Core staff dissected trachea and bronchi away from connective tissue and lymph nodes of cadaveric lungs. Freshly dissected airways were cut into segments and embedded into OCT compound and frozen. Airway tissue blocks were sent overnight on dry ice to OHSU.

Airway tissue sections (5-7µm) were cut using a Reichert 2800 Frigocut cryostat (Reichert Scientific Instruments). Tissue sections were fixed in acetone for 5 min at -20°C, air-dried at room temperature, and then stored under dry conditions at -80°C for

up to 6 months. Tissue sections were blocked with 5% fetal bovine serum (FBS) and 5% normal goat serum in a saponin-based permeabilization buffer (eBioscience), then incubated with primary antibodies for 30 min, followed by three PBS washes. The primary antibodies were detected with appropriate fluorescent secondary antibodies for 30min. followed by three PBS washes, with the second wash containing Hoescht 33342 nuclear dye as a counterstain (ThermoFisher), then mounted with Fluormount G (Southern Biotech). The staining buffer was 1x permeabilization buffer with 5% FBS. Stained sections were analyzed with a Zeiss Axioskop 2 plus microscope (Carl Zeiss).

For colocalization analyses, tissue sections were stained with HLO1-6H5 antibody and one of the following primaries: polyclonal rabbit anti-human keratin 5 (BioLegend), rabbit anti-human keratin 14 (ThermoFisher), rabbit anti-human acetylated α-tubulin (Cell Signaling Technology), rabbit anti-human mucin 5B (Santa Cruz) or rat anti-human keratin 8/18 (DSHB #TROMA-I-C). Secondaries are as follows: Cy3-conjugated goat anti-mouse IgG (Heavy & Light chains; EMD Millipore) and DyLight488-conjugated goat anti-rabbit IgG (Heavy & Light chains; ThermoFisher).

### 4.2.3. Flow cytometric analyses/FACS (Fluorescence-activated cell sorting)

Enzyme/mechanically dispersed hBECs were stained with various antibodies for flow cytometric analyses and fluorescence-activated cell sorting. Staining was conducted in staining buffer consisting of RPMI media (Gibco) with 5% FBS. Cells were incubated with primary antibodies for 30min. at 4°C, washed twice with cold staining buffer, stained with appropriate fluorescent secondary antibody 30min. at 4°C, washed

twice with cold staining buffer, and then stained for viability with 2ug/mL propidium iodide. The primary antibody used were: HLO1-6H5 antibody in hydridoma conditioned media, mouse anti-human NGFR (EMD Millipore), and rat anti-human ITGA6 (BioLegend). The secondary antibodies used were: PE-conjugated goat anti-mouse IgM secondary F(ab') $_2$ fragment (Jackson ImmunoResearch), A488-conjugated goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch), APC-conjugated donkey anti-rat secondary F(ab')$_2$ fragment (Jackson ImmunoResearch), DyLight488-conjugated goat anti-rabbit IgG (Heavy & Light chains) (ThermoFisher).

Intracellular flow cytometry followed the above protocol with the following steps added: staining with e450 Fixable Viability dye (eBioscience) for 30 min at 4°C prior to staining with the primary cell-surface antibodies and associated secondaries, then fixation in IC Fixation Buffer (4% paraformaldehyde) (eBioscience) for 30 min at 4°C after the primary cell-surface antibodies and secondaries stains, permeabilization with eBioscience Permeabilization Buffer (eBioscience) for 30 min at 4°C, staining with intracellular markers rabbit anti-human keratin 5 (BioLegend), rabbit anti-human keratin 14 (ThermoFisher) for 30 min at 4°C, two washes with the Permeabilization buffer, and then DyLight488-conjugated goat anti-rabbit IgG (Heavy & Light chains; ThermoFisher) secondaries. The propidium iodide was omitted for e450 Fixable Viability dye staining.

Fluorescently-stained cells were analyzed with an analytical flow cytometer (BD LSRII; BD Biosciences) or sorted with a BD Influx Cell Sorter (BD Biosciences) with a 140-micron nozzle after live/dead exclusion and doublet discrimination.

### 4.2.4. Bronchosphere assay

FACS-isolated cells for bronchosphere assay were directly sorted into 24 well plates containing Transwell® inserts (Sigma) pre-coated with 1:1 mixture of MatriGel (Corning) and air liquid interface media (ALI)[23,71]. Transwell® inserts are 12mm in diameter with a 0.4uM PTFE membrane that is pre-coated with 100% MatriGel prior to addition of the 1:1 MatriGel/ALI media mixture. 24,000 MRC5 human fetal lung stromal cells were added to the MatriGel/ALI media mixture to provide a support function. The ALI media formulation was reported by Fulcher et al[23,71]. Cultures were grown in standard 5% CO2, 37°C incubator conditions for 2 weeks, at which time the number of colonies was counted.

At 6 weeks, growing bronchospheres were harvested by dissolving MatriGel with Corning Cell Recovery Solution for 1 hour at 4°C. Released bronchospheres were washed with PBS, concentrated by centrifugation, embedded in OCT, and stored at -80°C. Bronchospheres embedded in OCT were cryosectioned (7µm), fixed in acetone for 5 minutes at -20°C, air-dried at room temperature, and then stored under dry conditions at -80°C for up to 6 months.

### 4.2.5. Conditional reprogramming cell culture

Embryonic mouse 3T3-j2 fibroblast feeder cells were irradiated to 22gy for mitotic arrest and then frozen in aliquots for use. Three million irradiated mouse 3T3-j2 feeders were seeded 2-24 hours in advance of human cell seeding. hBECs and feeders were cultured at a 30:1 hBEC to 3T3 ratio in the presence of growth media containing

5µmol/L Rho kinase inhibitor Y-27632 and 10ng/mL human EGF (Sigma)[74]. Cultures were grown at 37°C and 5% $CO_2$ in a humidified incubator for one week until harvest, at which point the hBECs are ~90% confluent and most of the mouse 3T3 feeder cells had detached from the plate surface. This method is known as CRC (conditional reprogramming cell) culture. Harvested passage 1 hBECs were stained for flow cytometry according to the previously listed protocol.

#### 4.2.6. Organoid culture of human bronchial epithelial cells

Isolated hBECs were grown under the organoid method developed by the Hans Clevers Laboratory for formation of pancreatic and liver organoids[73,84]. Primary hBECs were embedded in >95% Matrigel and submerged in growth media containing ALK5 inhibitor SB431542 and supplemented with WNT-agonist R-spondin, Noggin, and FGF-10[72,73]. Intact hBEC-derived organoids were harvested after 7 days of culturing, when lumen formation was evident, and used as the immunogen for antibody production.

## 4.3. Results

### 4.3.1. Generation and identification of a novel monoclonal antibody for human airway basal cells

As mentioned in Chapter 2, novel mouse anti-human monoclonal antibodies were generated by fusing mouse splenocytes with myeloma cells to create antibody producing hybridoma clones. The mouse splenocytes were produced using a strategy of immunizing mice with intact lung organoids grown from primary hBECs[67,72,73,84].

Hybridomas were sub-cloned and screened by immunohistochemistry of acetone-fixed human airway tissue sections and flow cytometric analysis of single-cell suspensions of uncultured primary hBECs. The isotype of each monoclonal antibody (mAb) was determined during screening. Immunohistochemical staining of airway tissue showed the HLO1-6H5 antibody targeted only cells in the basal compartment of the respiratory epithelium (Figure 4.1). Flow cytometric analyses of the HLO1-6H5 antibody indicated that it targeted epithelial cell subsets by cell-surface antigens.

### 4.3.2. The novel HLO1-6H5 monoclonal antibody targets a subset of basal cells in human proximal airway basal cells

The pseudostratified respiratory epithelium of human proximal airway is composed of columnar ciliated and secretory cells that are apically exposed to the lumen and cuboidal basal cells that reside along the basement membrane away from the lumen. The HLO1-6H5 antibody targeted an antigen expressed only on basal cells, not on ciliated or secretory cell in human airway epithelium (Figure 4.1A). The only cells that expressed the HLO1-6H5 target marker were located close to the basement membrane and were not exposed to the airway lumen. Basal cells are also known to express cytokeratin 5 (KRT5), with a mitotically active basal cell subset also expressing cytokeratin 14 (KRT14). The HLO1-6H5 target marker colocalized with a large subset of KRT5+ cells in airway tissue and targeted the entire subset of mitotically active basal cells that are KRT14+ (Figure 4.1A). HLO1-6H5+ cells did not express the luminal differentiation markers cytokeratin 8 (KRT8), the ciliated cell marker acetylated-alpha

tubulin (TUBA4A), or the secretory goblet cell marker Mucin 5B (MUC5B) (Figure 4.1B). In all of the tissue sections, the HLO1-6H5 antibody also identified a subset of cells in the submucosa, below the respiratory epithelium. In data not shown here, co-staining of airway tissue sections with HLO1-6H5 and anti-CD31 (endothelial cell marker) primary antibodies showed that HLO1-6H5 also identifies endothelial cells. HLO1-6H5 targeting of endothelial cells did not impact flow cytometric analyses or FACS of uncultured primary cells, as the hBEC harvesting protocol for primary airway did not isolate endothelial cells.

In an *in vitro* model of the human proximal airway, the HLO1-6H5 mAb also stained basal cells. As mentioned in Chapter 3, bronchospheres are 3-dimensional, multicellular structures that form from airway stem cells. Bronchospheres contain multiple types of airway cells, but KRT5+ basal cells comprise the majority of the cells within the structure (Figure 4.1C). Immunostaining of bronchosphere sections with HLO1-6H5 showed that the mAb identified a subset of KRT5+ basal cells located on the exterior layers of the bronchosphere wall. This was evidence that the HLO1-6H5 mAb targeted a subset of basal cells.

The HLO1-6H5 pattern of targeting airway basal cells was maintained in uncultured hBECs from proximal airway. Flow cytometric analyses of uncultured primary hBECs showed that the HLO1-6H5 antibody co-expressed on a subset of KRT5+ cells (Figure 4-1D). Flow cytometry revealed uncultured primary hBECs are composed almost entirely of cells that are KRT5+, indicating that most of the hBECs were basal cells. The high KRT5 expression was confirmed and quantified by flow cytometry on primary

hBECs from three independent adult donors. Flow cytometry showed that the HLO1-6H5+ subset was part of a larger subset of Krt5+ cells as >55% of total Krt5+ cells were HLO1-6H5+ across three different donors (Figure 4-1D). The HLO1-6H5+ subset also contained a subset of Krt14+ cells, with a frequency that varies from donor to donor, ranging from 34-40% (Figure 4-1D). Overall the immunohistochemistry and flow cytometry data indicated that the HLO1-6H5 antibody labels a discrete subset of KRT5+ basal cells, which was even more apparent in uncultured primary hBECs.

### 4.3.3. An *in vitro* assay to functionally measure stem/progenitor cell properties in human airway epithelial cells

As previously detailed in Chapter 3, the bronchosphere assay established by Rock et al. is a functional measurement of input cell clonogenicity for the stem cell properties of self-renewal and proliferation[44]. Bronchosphere colonies are derived from single cells and therefore they asses the stem cell property of self-renewal. The assay also allows for the assessment of the stem cell property of potency by evaluating bronchospheres for expression of airway lineage markers. In summary, the bronchosphere assay is an effective readout for evaluating the stem cell capabilities of any input cell type of subset, like the ones identified by the HLO1-6H5 mAb.

### 4.3.4. Stem cells of the human airway express the HLO1-6H5 target marker

The HLO1-6H5 antibody was screened by flow cytometry on freshly dispersed viable human bronchial epithelial cells isolated from tracheal-bronchial segments to

determine cell-surface reactivity as mentioned in Chapter 2. The antibody labeled 25-65% of total hBECs across multiple donors, demonstrating that the novel marker is a cell surface antigen (n=9). Primary hBEC subsets were FACS-isolated with HLO1-6H5 into bronchosphere assay wells and grown under standard incubator conditions (Figure 4.2A). After two weeks of culture, bronchospheres were evident in the assay, especially in the HLO1-6H5+ subset (Figure 4.2B). Bronchospheres were counted to determine the colony forming efficiency (CFE) of the two HLO1-6H5 sorted subsets. The HLO1-6H5 marker positively enriched for clonogenic cells in the bronchosphere assay as there was a five-fold difference in CFE between the antibody-positive and -negative subsets that was statistically significant (p=0.0067) (Figure 4.2B). Immunocytochemistry of bronchospheres grown for 42 days showed that the HLO1-6H5+ basal cells were able to differentiate and generate colonies that contained basal cells (KRT5 and KRT14), secretory goblet cells (MUC5B), and ciliated cells (TUBA4A) (Figure 4.2C). The pattern of HLO1-6H5 enrichment for clonogenic cells held up over five biological replicates. Next, I had to compare the HLO1-6H5 target marker to other published basal cell makers to verify that my antibody targeted a novel cell-surface marker.

### 4.3.5. The HLO1-6H5 target marker subdivides ITGA6+ basal cells and is distinct from NGFR+ basal cells

Rock et al. discovered two cell-surface markers for basal cells: nerve growth factor receptor (NGFR) and integrin alpha-6 (ITGA6)[44]. I created two marker panels of HLO1-6H5 with either NGFR or ITGA6, and then FACS-isolated novel subsets from

uncultured primary hBECs using the marker combinations. When I analyzed the hBECs with HLO1-6H5 and ITGA6, I saw that ITGA6 stained over half the cells (Figure 4.3A). HLO1-6H5 also stained over 50% of the cells, but the pattern of staining differed from ITGA6. HLO1-6H5 was able to subdivide ITGA6+ cells into two subsets: HLO1-6H5+Low/ITGA6+ and HLO1-6H5+Hi/ITGA6+. The two subsets and the double negative population were FACS-purified into the bronchosphere assay and showed the HLO1-6H5+Hi/ITGA6+ to be highly enriched for clonogenic cells. Sorts with this marker panel over three biological replicates showed that enrichment for clonogenic cells in the HLO1-6H5+Hi/ITGA6+ subset over the HLO1-6H5+Low/ITGA6+ and the HLO1-6H5-/ITGA6- subsets is statistically significant (Figure 4.3B)

I next looked at cell subsets identified by a marker panel of HLO1-6H5 and NGFR. Flow cytometry revealed that these two markers identify two phenotypically distinct basal cell populations in uncultured primary hBECs (Figure 4.3C). There was no evidence of a HLO1-6H5+/NGFR+ cells (n=3). The phenotypically distinct basal cells were then assessed for functional differences via the clonogenic bronchosphere assay. After 14 days, colonies were counted to determine the colony forming efficiency of each sorted hBEC subset (Figure 4.3D). The bronchosphere assay for HLO1-6H5/NGFR subsets shows the HLO1-6H5+/NGFR- subset to be highly enriched for clonogenic cells with a CFE >3.3% across three donors. Meanwhile the HLO1-6H5-/NGFR+ subset was severely depleted of clonogenic cells with a CFE <0.01%, and in most wells, no colonies formed. Although both the HLO1-6H5 and NGFR antibodies target basal cells, there is clearly a difference in the stem cell capabilities of these distinct basal cell subsets from

uncultured hBECs. In data not shown here, flow cytometry revealed that NGFR and ITGA6 are differentially expressed in uncultured primary hBECs, with no clear evidence of an ITGA6+/NGFR+ population, similar to the expression pattern of HLO1-6H5 and NGFR in these cells. In summary, the HLO1-6H5+ basal cell subset is distinct from NGFR+ basal cells in terms of stem cell enrichment. Meanwhile, the ITGA6 marker can be optimized by pairing with HLO1-6H5 in a marker panel to obtain an enriched population of basal stem cells.

### 4.3.6. Expression of cell surface markers that identify basal cells increase after *in vitro* passage

Primary hBECs were cultured expanded on irradiated 3T3-j2 feeder cells in the presence of Rho kinase inhibitor and then harvested at 80-90% confluence. This method, known as conditional reprogrammed cell (CRC) culture, was developed for the *ex vivo* expansion and maintenance of healthy and cancerous primary human epithelial cells[74]. Culturing hBECs for 1 passage increases EpCam expression to >99%, suggesting that there was a selective expansion of epithelial cells in this system. The flow cytometric analyses of cultured passage 1 hBECs showed increased levels of NGFR and ITGA6 as all cultured hBECs in this system appeared to express both markers at frequencies exceeding 95% of the total hBEC population (Figure 4.4). HLO1-6H5 expression however fluctuated across three donor matched passage 0 and passage 1 hBECs, and still labelled a subset of cultured hBECs. Of the four markers analyzed

however, only NGFR showed a statistically significant change in expression between uncultured and passage 1 hBECs.

## 4.4. Discussion

The basal epithelial cell compartment has long been established as a major source of adult stem/progenitor cells for maintenance of the airway respiratory epithelium. The novel HLO1-6H5 monoclonal antibody allowed me to explore the heterogeneity within the basal cell compartment by identifying a novel subset of keratin 5+ basal cells (Figure 4.1). The intracellular markers cytokeratin 5 and 14 are definitive markers of basal cells and a mitotically active basal cell subset respectively. The HLO1-6H5+ subset in respiratory epithelium is predominantly comprised of basal cells as indicated by the co-expression of KRT5 (Figure 4.1A). There is the occasional rare KRT5+/HLO1-6H5- basal cell in tissue sections but overall HLO1-6H5 appears to co-stain all KRT5+ cells. This does not hold true however in uncultured hBECs. Intracellular flow cytometry with KRT5 showed that HLO1-6H5 targeted a subset of basal cells (Figure 4.1D). The reason for this discrepancy can be explained by the nature of tissue processing techniques. Dispersing viable cells from tissue requires harsh reagents and enzymes. Such conditions are not conducive to retaining all the cell types that are present in the physiological organ. Despite the staining pattern from tissue sections, HLO1-6H5 does identify a subset of basal cells in both uncultured hBECs and in bronchospheres.

It is likely that the HLO1-6H5+ subset represents an earlier stage basal cell that is not yet lineage committed, which the bronchosphere morphology seems to suggest (Figure 4.1C). Sectioned bronchospheres tend to have at minimum two layers of cells forming the wall of the structure. The majority of cells in bronchospheres are KRT5 and KRT14+, suggesting a mitotically active basal cell phenotype. However, only a subset of the bronchosphere cells are HLO1-6H5+/KRT5+, and these cells comprised the outer layer of the structure. This structural localization of HLO-6H5+ cells in bronchospheres mimics the physiological morphology of respiratory epithelium, in which the basal stem cells reside along the basement membrane, furthest from the airway lumen. This evidence points to the HLO1-6H5+ population to be a subset of basal epithelial cells that are not yet lineage committed.

A limitation of using intracellular markers to identify and target specific cell types or cell subsets is that they cannot be used to FACS-isolate viable cells for functional assays. This obstacle can be overcome in mouse models through lineage tracing techniques, but it cannot be overcome in human studies. The use of cell surface markers overcomes this obstacle in human tissue studies. The HLO1-6H5 antibody identifies a cell surface antigen on primary hBECs (Figure 4.2A). Currently the most well-established surface markers for isolating basal cells are NGFR and ITGA6[20,44,62]. The HLO1-6H5 target marker is distinct from NGFR as each marker identifies a discrete basal cell population (Figure 4.3C). This distinction is further magnified in the bronchosphere assay as all the clonogenic cells are found in the HLO1-6H5+ basal cell subset, not in the NGFR+ basal cells (Figure 4.3D). The HLO1-6H5 target marker is co-expressed with ITGA6 but allows

for the subdividing of ITGA6+ cells by high and low expression of HLO1-6H5 (Figure 4.3A). These two subsets, HLO1-6H5+Hi/ITGA6+ and HLO1-6H5+Low/ITGA6+, are functionally distinct as well. The HLO1-6H5+Hi subset is the most enriched for clonogenic cells compared to the HLO1-6H5+Low subset, further distinguishing the HLO1-6H5+ subsets from ITGA6+ subsets. Other publications have found the NGFR and ITGA6 markers to be suitable for isolating basal cells that can form colonies, but this study is different from those in the aforementioned publications[44,52,61].

The hBECs used in this study are primary and have never been cultured prior to FACS. Other studies however, have frequently followed the standard practice of culturing airway epithelium overnight on collagen-coated plastic prior to FACS purification or by inducing a stem-like state via culturing on irradiated feeder cells. The culture-expanded hBECs are functionally different from primary hBECs, because they do not require stromal cell co-culture to form bronchospheres[44,94]. This observation suggests that cultured hBECs are either being induced towards a stem cell phenotype or the culture conditions selectively expand stem cells. NGFR and ITGA6's optimal utility is for isolating basal cells from cultured airway epithelial cells on collagen-coated plastic. Meanwhile the HLO1-6H5 antibody is optimized for isolating a clonogenic basal cell subset from uncultured primary hBECs. However, the NGFR and ITGA6 markers would be ineffective for isolating basal cells from hBECs cultured under CRC growth conditions as both markers are expressed in almost 100% of the cells (Figure 4.4). The HLO1-6H5 target marker may be useful for this culture system as it only targeted a subset of the cultured cells, but the functional relevance of this would have to be tested. The utility of

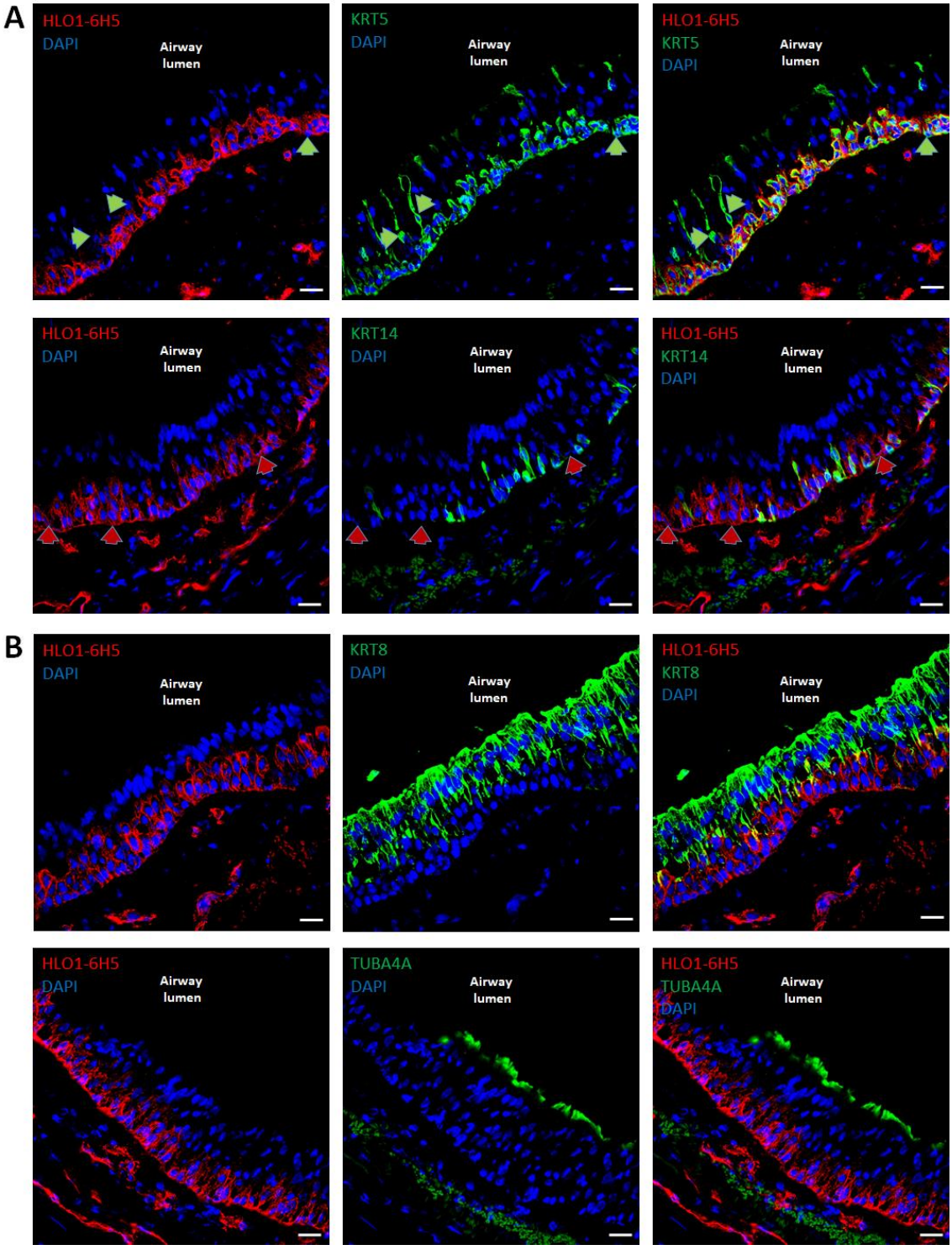
the HLO1-6H5 antibody is this study has primarily focusing on stem cell subsets *ex vivo*, but there is a possible application for the antibody in some *in vitro* contexts such as the CRC (conditionally reprogrammed cell) growth conditions. In summary I have shown that the HLO1-6H5 antibody is optimized for identifying and isolating a novel basal cell subset that is highly enriched for bipotent stem/progenitor cells from primary human airway epithelium. Based on the data presented, I propose a model of basal cell heterogeneity in which there are three subsets of basal cells: HLO1-6H5-/ITGA6-/NGFR+, HLO1-6H5+Low/ITGA6+/NGFR-, and HLO1-6H5+Hi/ITGA6+/NGFR-, of which the HLO1-6H5+Hi basal cell subset is enriched for self-renewing, bipotent stem/progenitor cells (Figure 4.5).

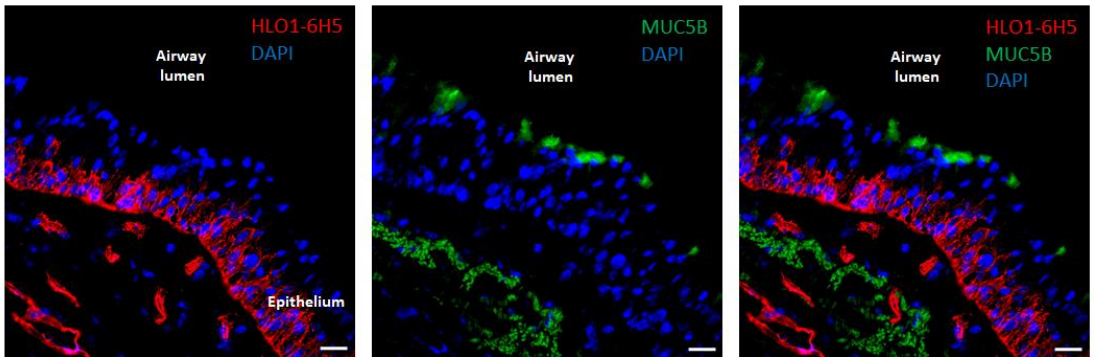
In addition to basal cells, other airway cell subsets have been reported to exhibit stem cell properties in mice: rare Scgb1a1+ club cells by Tata et al. and rare Sox2+ airway epithelial cells by Stripp et al[47,63]. These putative stem cell populations are distinguished by intracellular (SOX2) markers or extracellular/secreted markers (SCGB1A1), which makes them difficult to study outside of mouse models. However, studies in mice have shown these rare stem cell subsets to be able to repair airway epithelium under specific injury models, which raises the question of whether different epithelial injuries activate different stem cell populations for repair. It would be interesting to study and compare these stem cells subsets to the HLO1-6H5+ basal stem cell subset in regenerating human airway epithelial tissue sections.

The potential utility for the HLO1-6H5 target cell surface marker extends into both research and translational applications. Lung stem cell researchers have been

focused on subdividing the basal cell population into subsets that are phenotypically, functionally, and transcriptionally distinct. This focus on basal cell heterogeneity addresses questions regarding the specific cells that are responsible for airway homeostasis and epithelial repair. This monoclonal antibody addresses that need by isolating a novel subset of KRT5+ human basal cells. This subset is also a potential target for *ex vivo* expansion of donor tissue for not just research purposes, but potentially for translational applications. The HLO1-6H5+ basal cell subset could be relevant as an *in vivo* target or diagnostic marker for respiratory disease therapies such as cystic fibrosis, where correction of the CFTR protein in a basal stem/progenitor population would be advantageous due to its potential to repopulate the airway epithelium. Lung engineering and/or cell-based therapeutics development would also benefit from the identification of a subset that is highly enriched for stem/progenitor cells.

## 4.5. Figures and Tables

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Epithelium
MUC5B
DAPI
Airway lumen
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DAPI
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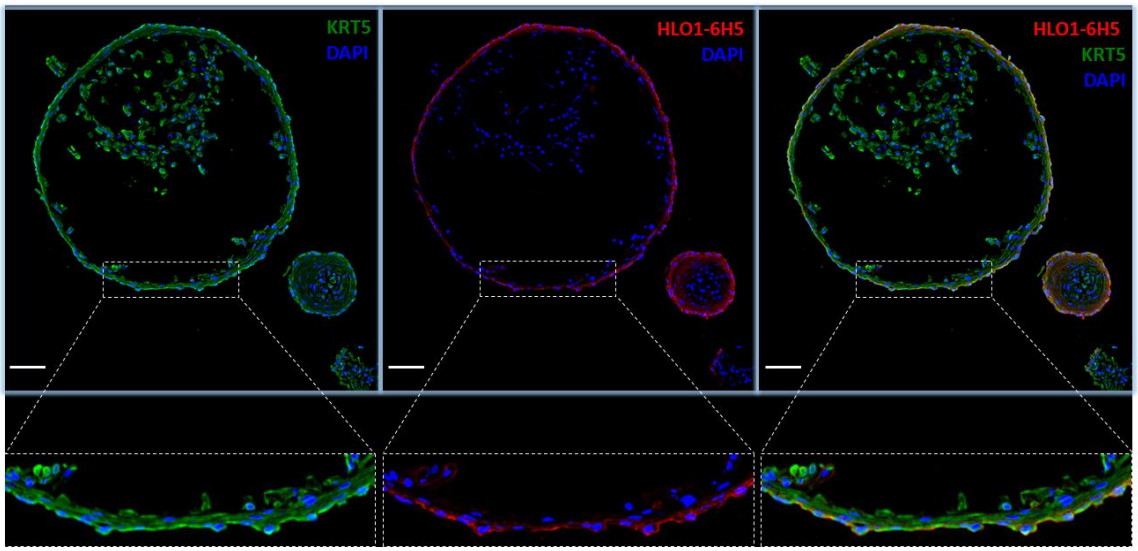
C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KRT5
DAPI

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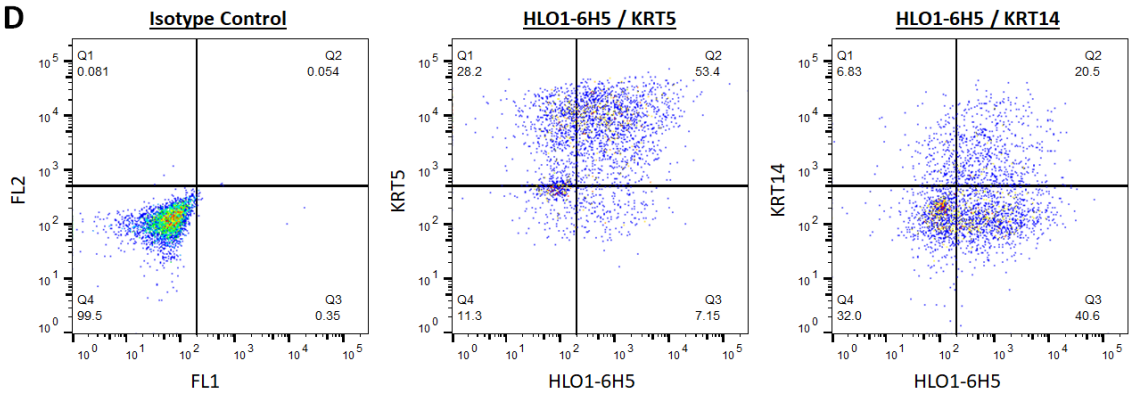
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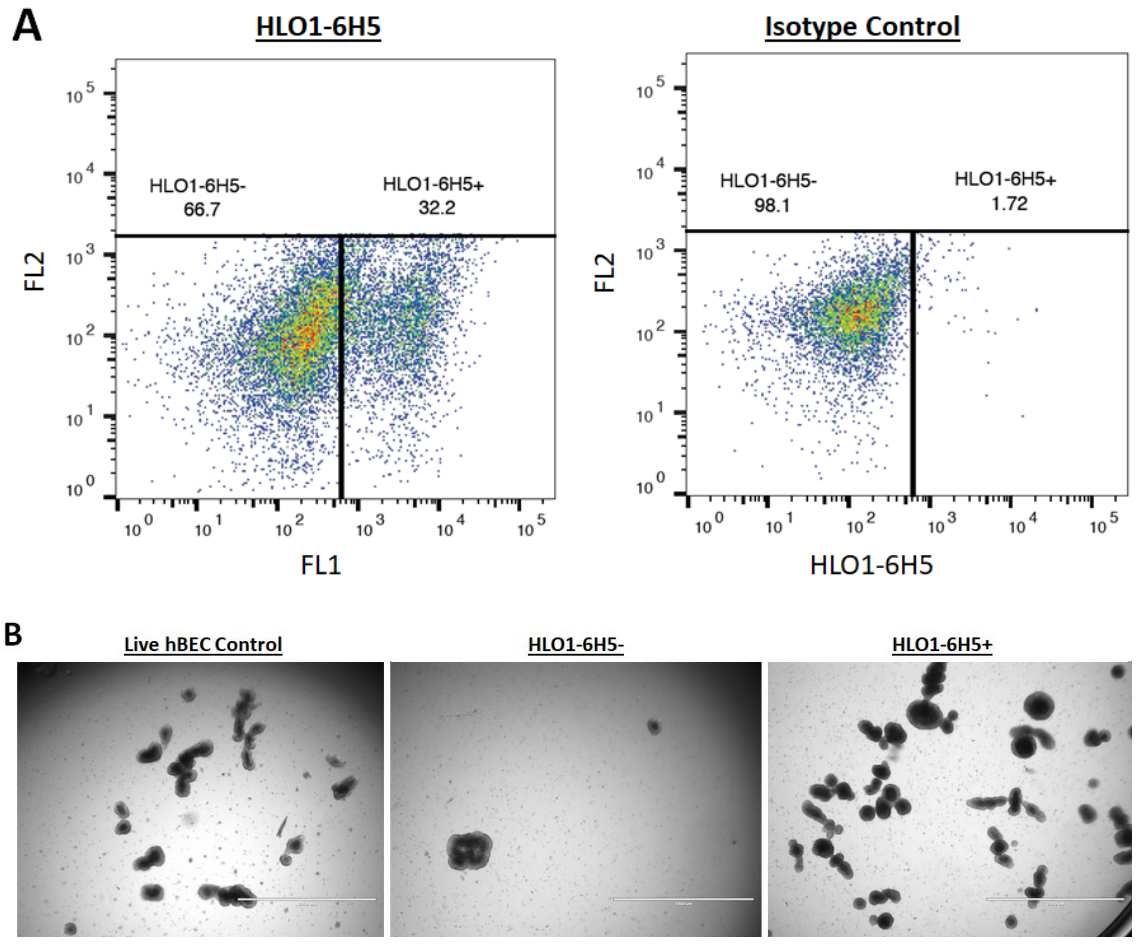
Q2 0.054
Q4 99.5
Q3 0.35
FL2
FL1
HLO1-6H5 / KRT5
Q1 28.2
Q2 53.4
Q4 11.3
Q3 7.15
KRT5
HLO1-6H5
HLO1-6H5 / KRT14
Q1 6.83
Q2 20.5
Q4 32.0
Q3 40.6
KRT14
HLO1-6H5

**Figure 4.1. HLO1-6H5 identifies a subset of human basal cells.**

Representative immunohistochemical co-staining of human proximal airway with the HLO1-6H5 mAb in combination with canonical markers that identify specific airway epithelial cell types such as **A)** basal cells (KRT5) and mitotically active basal cells (KRT14). Green arrows indicate basal cells that are KRT5+/HLO1-6H5-. Red arrows indicate HLO1-6H5+ cells along the basal laminae that are KRT14-. HLO1-6H5 were also co-stained with markers for **B)** luminal columnar cells (KRT8) such as ciliated cells (TUBA4A), and secretory cells (Muc5B). (n=3) Scale bar: 20µm. **C)** Day 28 bronchospheres derived from hBECs are also stained for basal cells (KRT5) and HLO1-6H5+ basal cells. Scale bar: 200µm **D)** Representative flow cytometry plots of HLO1-6H5 co-expression with KRT5 or KRT14 in uncultured primary hBECs. (n=3).



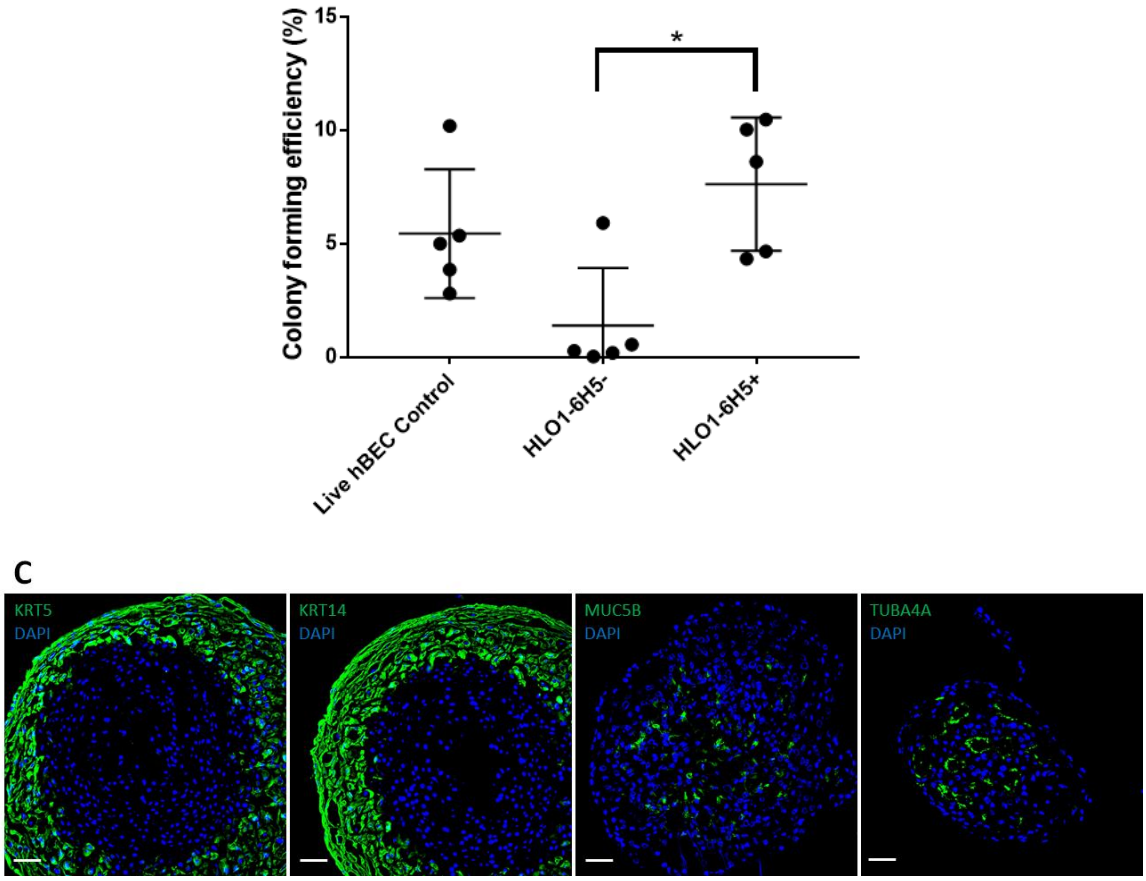
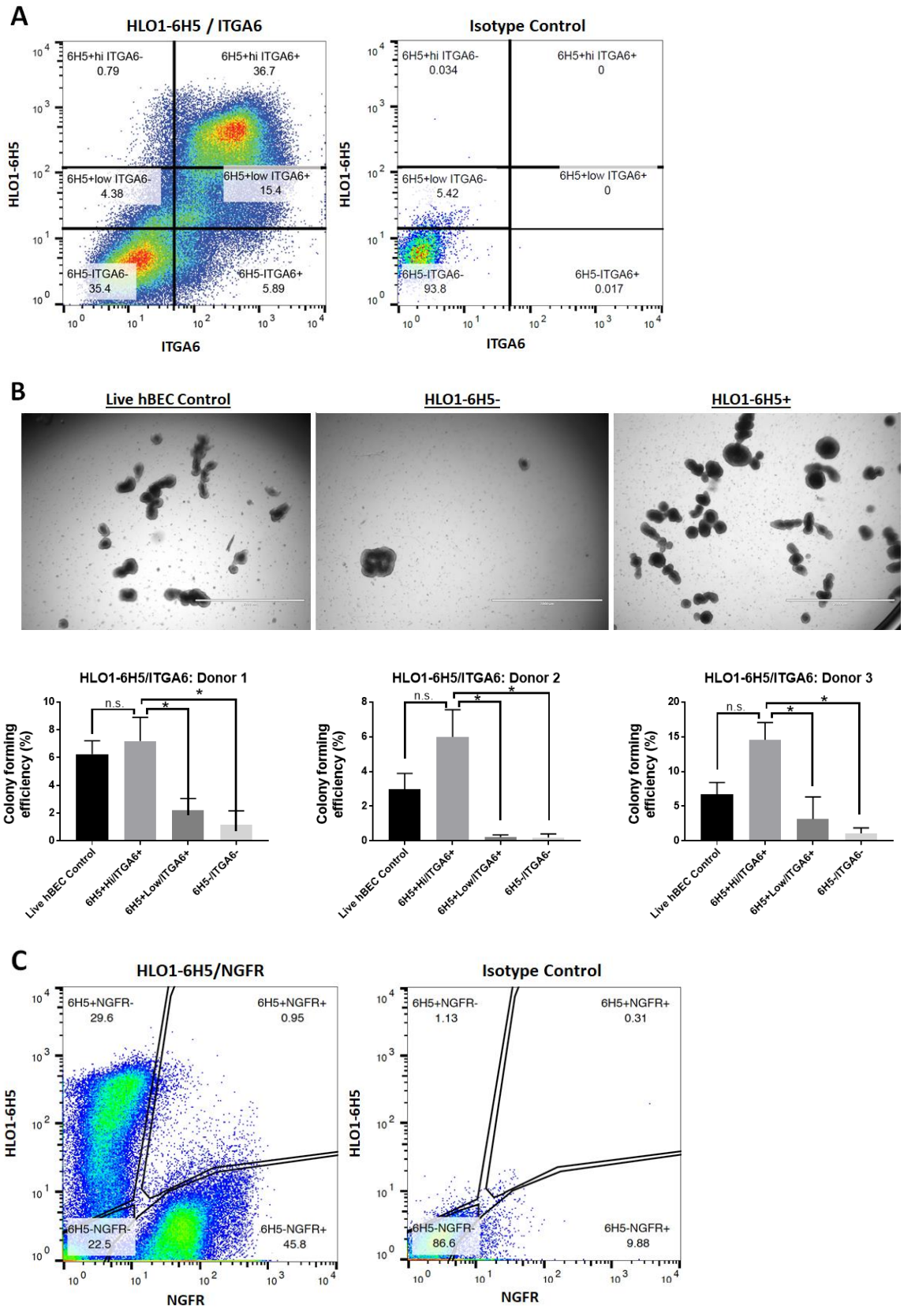



**Figure 4.2. FACS-isolated HLO1-6H5+ basal cells are functionally enriched for a bipotent stem/progenitor cell population.**

**A)** Representative data from FACS-isolation of HLO1-6H5 subsets for assessment of clonogenicity and potency in the bronchosphere assay (n=5); **B)** After 14 days of culture, colonies were counted to determine which HLO1-6H5 subset was enriched for clonogenic cells. Scatter plot shows the number of colonies formed as a percentage relative to the number of input cells (colony forming efficiency). Paired t-test performed, *$p<0.05$. Scale bar: 2000µm. **C)** Bronchospheres derived from HLO1-6H5+basal cells were sectioned and analyzed by immunocytochemistry to determine if the cellular composition of the colonies included basal cells (KRT5), mitotically active basal cells (KRT14), ciliated cells (TUBA4A), and/or secretory cells (MUC5B). Scale bar: 50µm.


A
HLO1-6H5 / ITGA6
6H5+hi ITGA6-
0.79
6H5+hi ITGA6+
36.7
6H5+low ITGA6-
4.38
6H5+low ITGA6+
15.4
6H5-ITGA6-
35.4
6H5-ITGA6+
5.89
HLO1-6H5
ITGA6
Isotype Control
6H5+hi ITGA6-
0.034
6H5+hi ITGA6+
0
6H5+low ITGA6-
5.42
6H5+low ITGA6+
0
6H5-ITGA6-
93.8
6H5-ITGA6+
0.017
HLO1-6H5
ITGA6
B
Live hBEC Control
HLO1-6H5-
HLO1-6H5+
HLO1-6H5/ITGA6: Donor 1
HLO1-6H5/ITGA6: Donor 2
HLO1-6H5/ITGA6: Donor 3
Colony forming efficiency (%)
n.s.
*
Live hBEC Control
6H5+Hi/ITGA6+
6H5+Low/ITGA6+
6H5-/ITGA6-
C
HLO1-6H5/NGFR
6H5+NGFR-
29.6
6H5+NGFR+
0.95
6H5-NGFR-
22.5
6H5-NGFR+
45.8
HLO1-6H5
NGFR
Isotype Control
6H5+NGFR-
1.13
6H5+NGFR+
0.31
6H5-NGFR-
86.6
6H5-NGFR+
9.88
HLO1-6H5
NGFR

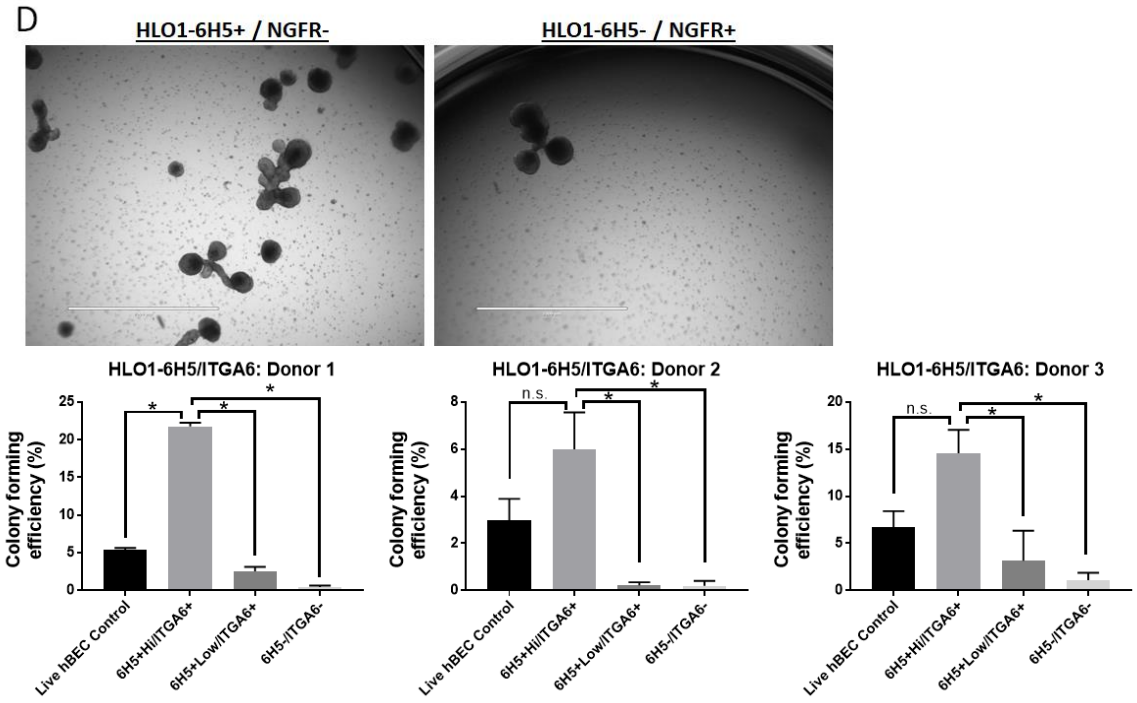



**Figure 4.3. HLO1-6H5+ basal cells are a subset of ITGA6+ airway epithelial cells and are distinct from NGFR+ basal cells in uncultured primary hBECs.**

**A)** Representative flow cytometry plots of HLO1-6H5 combined with ITGA6 to FACS-isolate cell subsets for assessment of clonogenicity in the bronchosphere assay. (n=3) **B)** At day 14, colonies were imaged and counted to determine which sorted subsets were enriched for clonogenic cells. Graphs of colony forming efficiency of each HLO1-6H5/ITGA6 cell subset across three biological replicates. **C)** Representative flow cytometry plots of HLO1-6H5 combined with NGFR to FACS-isolate cell subsets for assessment of clonogenicity in the bronchosphere assay. (n=3) **D)** At day 14, colonies were imaged and counted to determine which sorted subsets were enriched for clonogenic cells. Graphs of colony forming efficiency of each HLO1-6H5/NGFR cell subset across three biological replicates. Paired t-test performed for each biological replicate, *$p<0.05$. n.s. = not statistically significant. Data presented as mean (+/- S.D.). Scale bar: 2000µm.

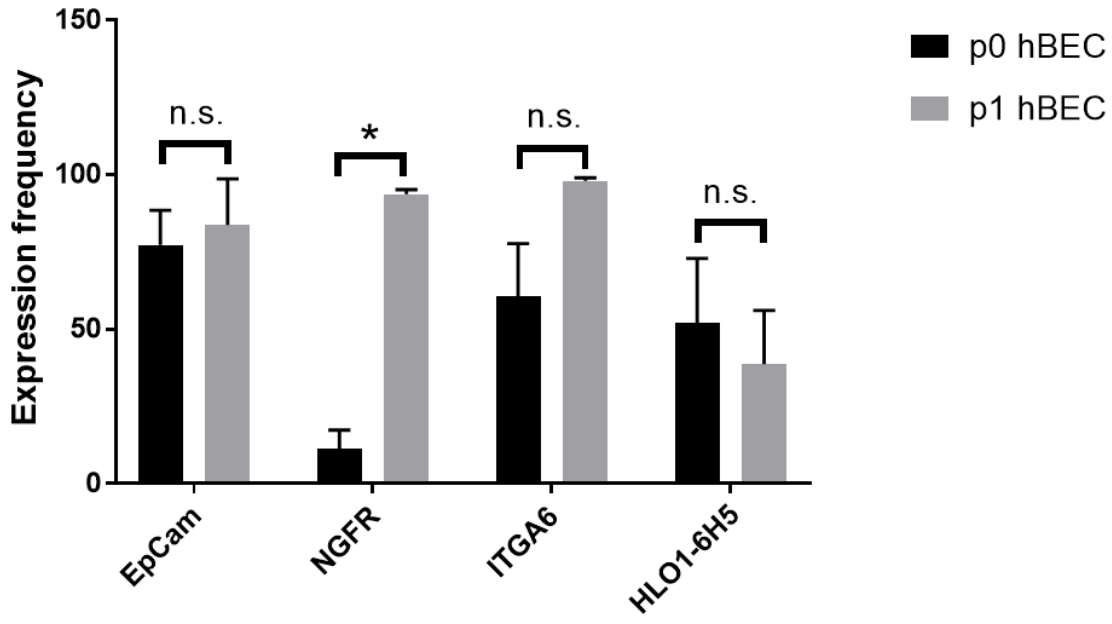



**Figure 4.4. Expression of cell surface markers increases from uncultured primary hBECs to passage 1 hBECs grown under conditional reprogramming culture conditions.**

Bar graph comparing expression frequency of the HLO1-6H5, NGFR, ITGA6, and EpCam cell surface markers in donor matched passage 0 and passage 1 hBECs grown on irradiated mouse 3T3-j2 embryonic fibroblast feeder cells. (n=3) Paired t-test, *$p<0.05$. n.s. = not statistically significant. Data presented as mean (+/- S.D.).

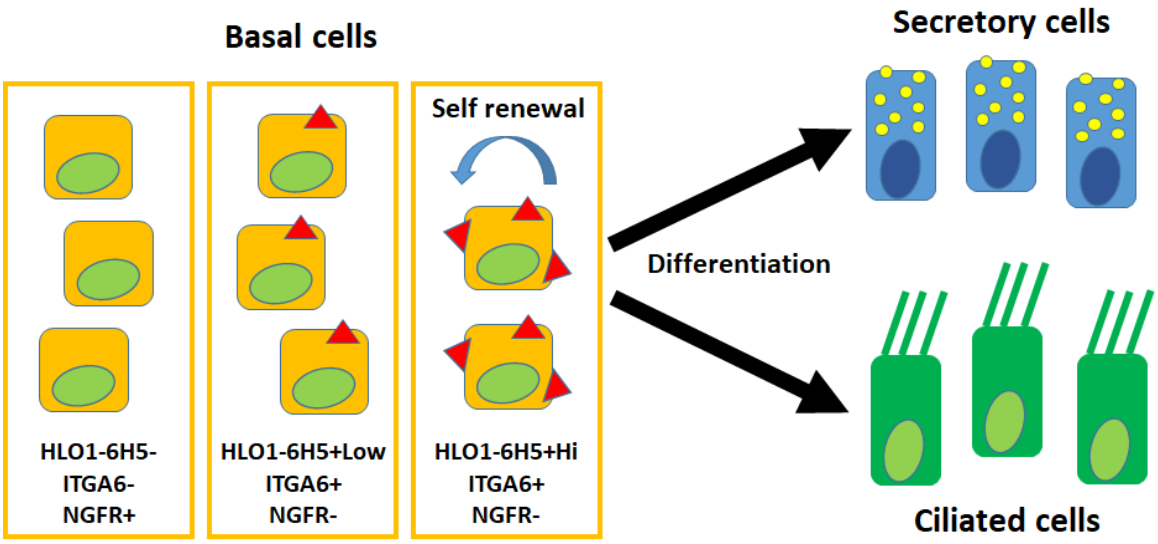



**Figure 4.5. A subset of airway basal cells enriched for bipotent stem/progenitor cells that express the target marker of the HLO1-6H5 antibody.**

The model proposed by this study is that of a novel subset within the human basal cell population of the proximal airway identified by expression of the target antigen of the HLO1-6H5 antibody. The HLO1-6H5+ basal cell subset demonstrates that the basal cell population possesses phenotypic heterogeneity. The enrichment of stem/progenitor cells in HLO1-6H5+Hi basal cells is indicative of functional heterogeneity in the physiological basal cell population. The basal cell subset identified by high expression of the HLO1-6H5 target antigen, ITGA6 expression, and no NGFR expression is capable of self-renewal and differentiation into ciliated cells and secretory cells. Meanwhile the HLO1-6H5+Low/ITGA6+/NGFR- and HLO1-6H5-/ITGA6-/NGFR+ subsets of basal cells are depleted of bipotent stem/progenitor cells.

# Chapter 5. Looking forward, opportunities to address more airway stem cell questions[‡‡]

Throughout the course of my work, additional biological questions arose as I progressed in developing the bronchosphere assay as a model of stemness in uncultured hBECs and in characterizing the HLO1-6H5+ subset of basal stem cells. I made initial efforts to address some of these questions, and preliminary data was generated. Three key questions that I attempted to address were: What is the molecular pathway underlying *in vitro* stem cell activation in the bronchosphere assay? What is the target antigen of the HLO1-6H5 antibody and is it functionally significant? Are there HLO1-6H5+ cell subsets in other human organ systems?

## 5.1. The Notch pathway regulates bronchosphere morphology *in vitro*

As mentioned in Chapter 3, I modified the bronchosphere assay from published protocols by including MRC5 stromal cells, because it was necessary in order to assess stem cell function in primary airway tissue[44,51,52,94]. This discovery raised the question: What were the MRC5 stromal cells' contribution to promoting stem cell function in lung epithelial cells? I sought to identify the underlying molecular mechanism of MRC5-mediated stem cell activation in primary airway cells in the bronchosphere assay.

[‡‡] This work is unpublished. The cystic fibrosis work was performed by our collaborator Anusha Sridharan and Jeffrey Whitsett.

The first investigation aimed to determine whether the interaction was mediated by paracrine or juxtracrine signaling between the MRC5 support cells and the hBECs. Paracrine signaling is mediated through secreted factors, while juxtacrine signaling requires cell-cell contact. MRC5-conditioned media was generated and added to the bronchosphere assay, but it failed to recapitulate the proliferative effects that MRC5 co-culture conditions had on stem cells. Additional attempts used contact inhibition, whereby the MRC5 cells were seeded outside of the Transwell® insert and away from the hBECs; these conditions also failed to promote stem cells to proliferate and form colonies. The conditioned media and contact inhibition experiments suggested that the underlying mechanism of interaction between the MRC5 stromal cells and hBECs was by cell-cell contact.

One of the most well-characterized juxtacrine signaling pathways is the Notch pathway. The Notch pathway initiates from an interaction between a cell's Notch receptors and the Notch ligands on a neighboring cell. In mammals, canonical Notch signaling involves one of four transmembrane receptors (Notch1, 2, 3, or 4) binding one of five ligands from the Jagged (JAG1 or 2) family or Delta-like (DLL1, 3, or 4) family[99–104]. Upon binding, there is proteolytic cleavage of the receptor to release its intracellular domain (NICD), which translocates into the nucleus to complex with the Recombination Signal Binding Protein For Immunoglobulin Kappa J (RBPJ) and Mastermind Like Transcriptional Coactivator 1 (MAML1) proteins for transcriptional activation[100–102]. There is also non-canonical Notch signaling, which is Notch signaling that deviates from canonical Notch ligands or the downstream transcriptional complex of

NICD/RBPJ/MAML1[101]. The Notch pathway is a known regulator of embryonic and adult stem cells in the formation and/or maintenance of organs such as skin, blood, intestine, bone, muscle, liver, and lungs[99,102]. In some epithelial organs, the Notch pathway regulates stem cell proliferation. The most noteworthy example is in mammalian intestine, where LGR5+ stem cells require Notch to proliferate for tissue maintenance and repair[101,102,105]. Evidence for a similar role for Notch in human airway basal cells is shown in a study by Hegab et al. that shows constitutive activation of Notch signaling increases human basal cell proliferation in monolayer cultures[62]. Based on the aforementioned findings for a potential role of Notch signaling in basal cell proliferation, I hypothesized that the Notch signaling pathway regulates the mechanism of stromal cell activation of airway stem cells towards proliferation.

Uncultured bulk hBECs were seeded into the bronchosphere assay according to the Chapter 4 Materials & Methods. The cultures were treated with 1μM of the γ-secretase inhibitor dibenzazepine (DBZ)[24]. DBZ inhibits the Notch pathway by preventing the proteolytic cleavage of the Notch receptor after ligand binding, preventing the formation of the NICD that translocates to the nucleus for transcriptional activation[106,107]. After 21 days of culture, it was apparent that Notch inhibition induced a morphological change in the bronchospheres as there was a dramatic increase in the number of elongated, branching colonies in the cultures (Figure 5.1A). These branching colonies differed from the spherical colonies seen in previous chapters (Chapters 3 and 4) as they are elongated in shape with at least a single branch emerging from the structure of the colony. Three colony morphologies were defined and counted: spherical

colonies, branching colonies that had at least 3 elongated branches, and complexes that were neither spherical or contained elongated branches. I compared the relative frequency of each morphology under 1μM DBZ-treatment (Notch-inhibited) and no treatment (Notch-permissive, DMSO vehicle control) and saw that the frequency of spherical colonies decreased by over half. Donor 1 spherical colony frequency decreased from 55% to 14% between the no treatment control and the DBZ-treatment, meanwhile donor 2 decreased from 79% to 36% (Figure 5.1B). Conversely, the frequency of branching colonies increased by at least 7-fold as donor 1 branching colony frequency increased from 4% to 33% and donor 2 increased from 4% to 29% when comparing control Notch-permissive to Notch-inhibited conditions respectively. The formation of complex colonies also increased under Notch-inhibited conditions as donor 1 increased from 41% to 53% and donor 2 increased from 17% to 35%. Overall, the inhibition of Notch shifted the proportion of bronchosphere colonies formed towards non-spherical morphologies, with the greatest shift being an increase in branching colony morphology and a decrease in spherical colonies.

HLO1-6H5+ basal cells were also assessed in the bronchosphere assay with 1μM DBZ-treatment to determine if the cells would respond in a similar manner as bulk hBECs. HLO1-6H5+ basal cells were FACS-isolated into the bronchosphere assay according to the Chapter 4 protocol. After 14 days of culture under treatment with 1μM DBZ-treatment or no treatment, it was apparent that Notch inhibition had the same effect on colony morphology that was seen in unsorted bulk hBECs as there was an increase in the number of elongated, branching colonies in the cultures (Figure 5.1C). I

again compared the relative frequency of each morphology under DBZ-treated and no treatment control and saw similar relative frequencies to what was observed in bronchospheres grown from bulk hBECs. Donor 1 sphere morphology frequency decreased from 43% to 26% between no treatment and DBZ-treatment, meanwhile donor 2 decreased from 59% to 51% (Figure 5.1D). Similar to the changes observed in branching morphology frequency in bulk hBEC colonies, the frequency of branching colonies derived from HLO1-6H5+ basal cells increased from zero to 16% in donor 1 and zero to 8% in donor 2 from Notch-permissive to Notch-inhibited conditions respectively. The formation of complexes did not change drastically from Notch-permissive to Notch-inhibited conditions as donor 1 went from 57% to 58% and donor 2 slightly decreased from 41% to 40%, which did not match the trend seen in uncultured bulk hBECs. In summary, Notch inhibition increases the frequency of branching colonies that emerge from primary hBECs in the bronchosphere assay and decreases the frequency of spherical colonies.

Regarding my hypothesis that Notch signaling is the underlying mechanism behind fibroblasts increasing airway stem cell proliferation, I did not see an increase in stem cell activation towards proliferation, as measured by colony forming efficiency in the bronchosphere assay. Counts of colonies across all three of the previously mentioned morphologies shows that Notch inhibition does not increase the number of colonies that emerge from primary hBECs and that there is not a statistically significant difference in colony count across four biological replicates (Figure 5.2). Therefore, the data does not support the initial hypothesis that Notch signaling regulate the

mechanism of lung stromal cells inducing airway stem cells out of quiescence and towards proliferation.

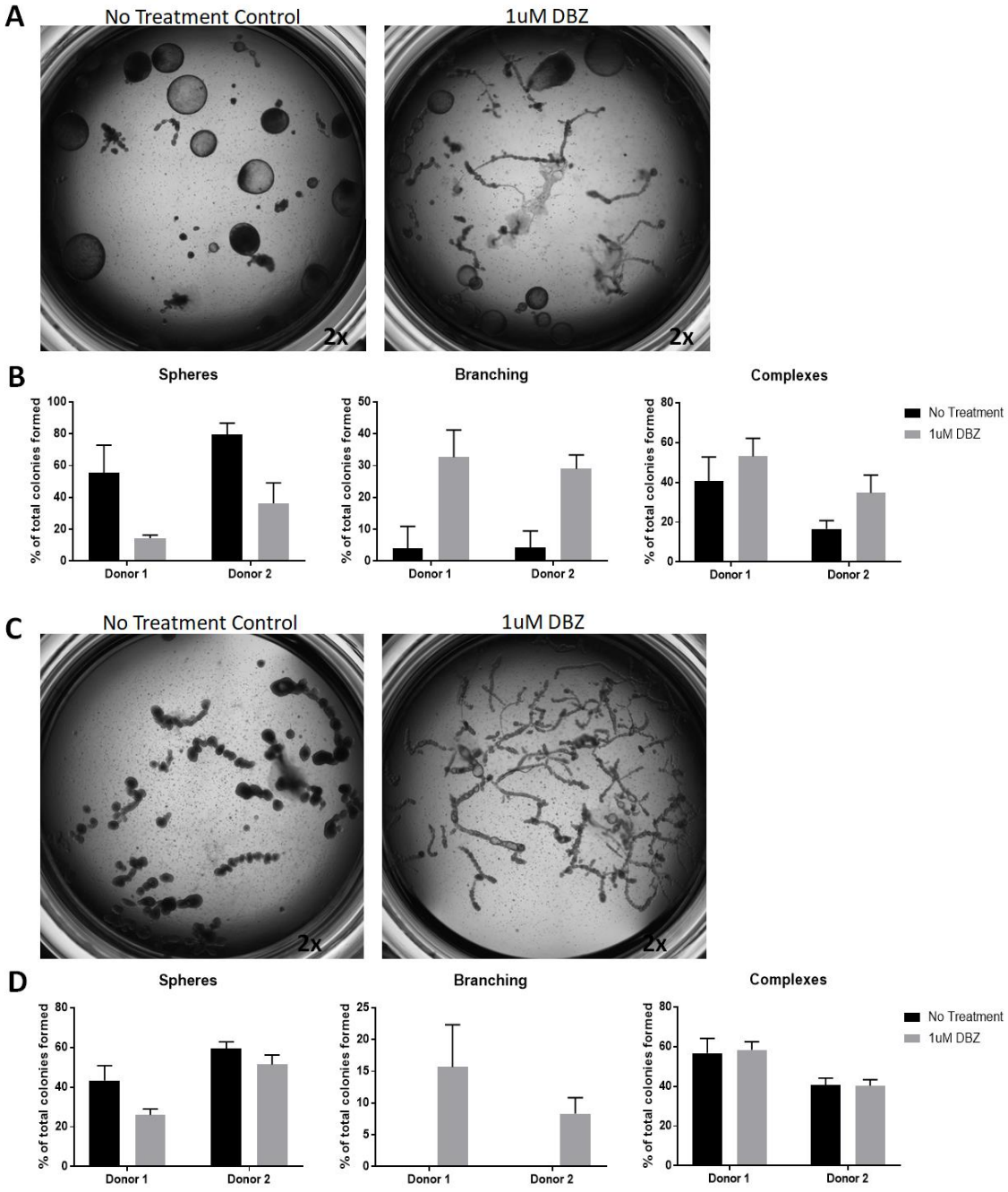


**Figure 5.1. Bronchospheres exhibit branching morphology under Notch-inhibition.**
**A)** Representative images of bronchospheres grown for 21 days from 1200 unsorted hBECs in co-culture with MRC5 support cells. Cultures are treated for 21 days with 1μM DBZ (dibenzazepine) to inhibit the Notch pathway or with no treatment (DMSO vehicle) control; **B)** Bar graphs comparing the number of bronchospheres categorized into three distinct morphologies under Notch permissive and inhibited growth conditions. The three bronchosphere morphologies are sphere shaped, branching (at least 3 branches), and complex (neither spherical or branching). Data from 2 donors, n=3 per donor. **C)** Representative images of bronchospheres grown for 14 days from 1200 FACS-isolated HLO1-6H5+ hBECs in co-culture with MRC5 support cells. Cultures are treated for 14 days with 1μM DBZ to inhibit the Notch pathway or with no treatment (DMSO vehicle) control; **D)** Bar graphs comparing the number of bronchospheres categorized into three distinct morphologies under Notch permissive and inhibited growth conditions. Data from 2 donors, n=3 per donor. Data presented as mean (+/- S.D.).

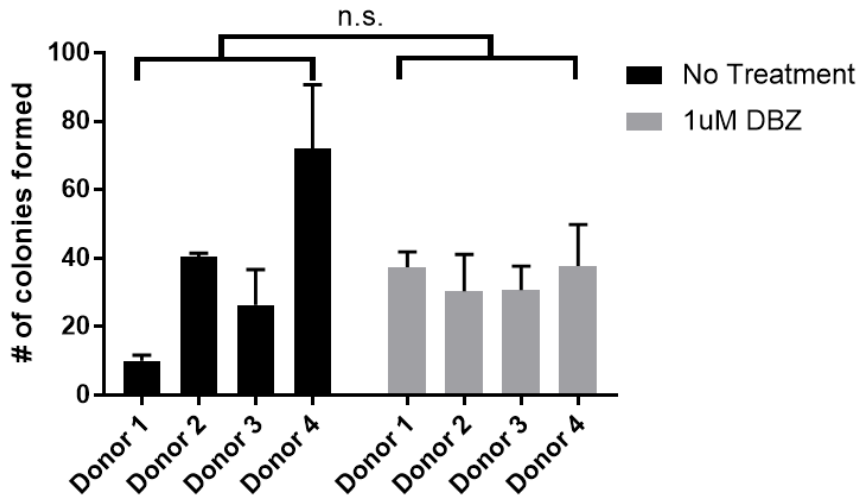



**Figure 5.2. Notch-inhibition does not increase bronchosphere formation frequency.**
Bar graph of number of bronchospheres formed by seeding 1200 primary hBECs in co-culture with MRC5 fibroblasts under 1μM DBZ to inhibit the Notch pathway or with no treatment (DMSO vehicle) control across 4 donors, n=3 per donor. Data presented as mean (+/- S.D.). Paired t-test performed, n.s. not significant.

Published studies have shown that epithelial cells from adult human airway preferentially differentiated towards ciliated cells and not towards the secretory lineage under Notch inhibition[24,60,61]. I evaluated the effects of Notch inhibition on airway basal cell differentiation in the bronchosphere assay. Colonies derived from FACS-isolated HLO1-6H5+ basal cells were grown for 28 days under Notch inhibition (1μM DBZ) or with no treatment (DMSO vehicle control), harvested as intact bronchospheres, embedded into OCT compound, sectioned, and then acetone-fixed as detailed in the Chapter 4 Materials and Methods. Bronchospheres formed under Notch-permissive or -inhibited conditions contained basal cells as indicated by KRT5 expression; these basal cells were also mitotically active as indicated by KRT14 expression (Figure 5.3A). However, there was a difference in secretory cell emergence in bronchospheres from the DBZ-treated growth conditions versus the no treatment control. Bronchospheres showed reduced secretory cell emergence under DBZ-treatment, and overall there was a decrease in the number of colonies that contained MUC5B+ secretory goblet cells (Figure 5.3). Counting of MUC5B-expressing colonies showed that Notch inhibition resulted in a greater than 75% reduction in frequency of colonies that contained goblet cells, as donor 1 decreased from 44% to 9% and donor 2 decreased from 28% to 0% (Figure 5.3B). The loss of goblet cell differentiation in the bronchospheres suggests a requirement for Notch pathway activation for basal cell differentiation to occur. The absence of ciliated cells in bronchospheres under both growth conditions is indicative of the timepoint at which these colonies were harvested, day 28. As shown in Chapter 2, ciliated cell differentiation is evident in bronchospheres harvested at a later timepoint, day 42.

Overall the data indicated that the MRC5 stromal cells do not use the Notch pathway to regulate airway stem cell activation towards proliferation. However, the stromal cells do use Notch signaling to regulate differentiation into the secretory lineage and branching morphogenesis.

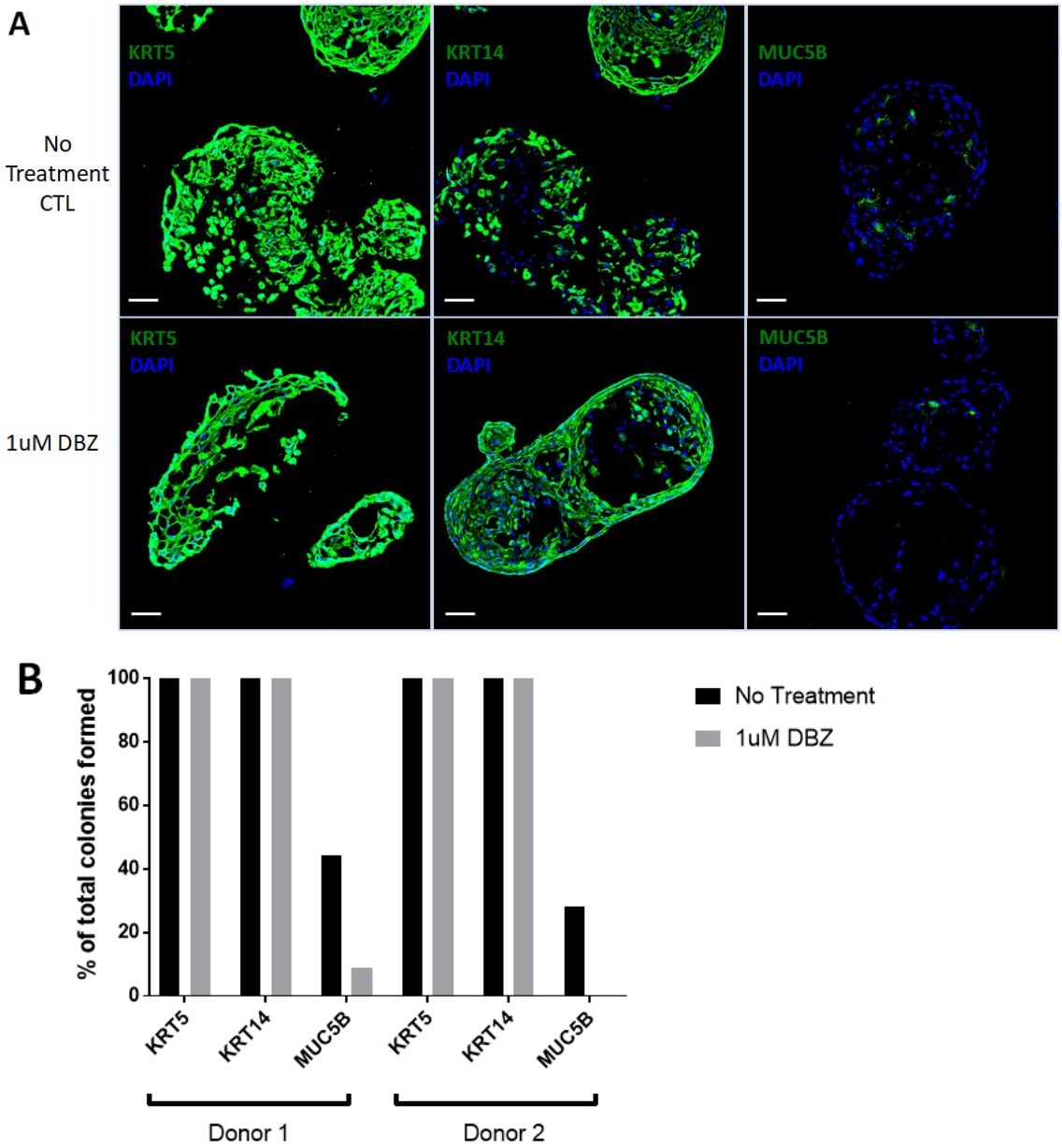


**Figure 5.3. Loss of secretory cell differentiation in basal-cell derived bronchospheres under Notch-inhibition.**

**A)** Representative images of acetone-fixed bronchosphere sections. Bronchospheres were grown for 28 days from HLO1-6H5+ basal cells with MRC5 support cells. Cultures were treated for 28 days with 1μM DBZ (dibenzazepine) to inhibit the Notch pathway or with no treatment (DMSO vehicle) control. Scale bar: 50 μm.; **B)** Bar graphs comparing the number of bronchospheres that have secretory goblet cells (MUC5B) from 2 donors.

The Notch pathway is a known regulator of airway basal cell function and in lung development, as previously mentioned in Chapter 1[6–8,10,19]. Although a recent study by Hegab et al. shows that the Notch pathway positively regulates basal cell proliferation, my data does not validate this hypothesis[62]. However, my data does support the hypothesis that differentiation of human airway basal cells towards the ciliated or secretory lineages is controlled through the Notch pathway. Multiple studies show that human airway basal cells and pluripotent stem cells can be biased towards differentiating into ciliated cells over secretory cells under conditions of Notch inhibition, or conversely biased towards the secretory lineages under constitutive Notch activation[20,24,60,61,108–110]. There is however some debate as to which specific Notch receptors are most important in cell fate determination for human basal cell differentiation. The study by Danahay et al. points to the Notch2 receptor as the key regulator of differentiation to the secretory lineage, as Notch2 blockade decreased expression of secretory genes and increased expression of ciliated cell genes[61]. Their study also shows Notch3 blockade decreases secretory cell gene expression, but to a lesser degree than Notch2 blockade, but does not increase ciliated cell gene expression

like Notch2 blockade does[61]. Conversely, Gomi et al.'s study shows that constitutive activation of Notch1 and Notch3 individually could increase ciliated cell differentiation and reduce secretory cell differentiation[60]. Meanwhile constitutive activation of Notch2 in their study shows no impact on basal cell differentiation towards one lineage over another, counter to the findings of Danahay et al. However, both Danahay et al. and Gomi et al. agree that Notch4 does not regulate basal cell differentiation.

The role of Notch in branching morphogenesis has been observed in models of drosophila and mouse airway development. Drosophila studies show that during development, Notch signaling is necessary in order to prevent unwanted branching of the tracheal tube[111]. Branching of the tracheal tube is driven by cells that have a tip progenitor phenotype which allows them to grows outward to form new airway branches[111]. As the airway branches, the tip progenitor cells use the Notch pathway to restrict cells in the airway stalk from adopting a tip cell phenotype, thereby blocking initiation of new airway branches[111]. Tsao et al. observed a similar role for Notch in an *in vitro* culture model of embryonic mouse lung in which Notch inhibition results in a higher frequency of airway branching compared to an untreated control[112].

Airway branching morphogenesis has also been observed in human bronchosphere models. Franzdóttir et al.'s study shows branching morphogenesis in their bronchospheres with an established hBEC line under co-culture conditions with human umbilical vein endothelial cells (HUVECs)[113]. In their study, they identify a paracrine mechanism of interaction between the HUVECs and hBECs by blocking the two cell types from direct contact with each other. This paracrine mechanism induces

branching morphology in the hBEC-derived bronchospheres, much like my data with the Notch inhibition. A more comparable experimental model to mine comes from the Shay Lab at University of Texas Southwestern Medical Center in Dallas, Texas. In two of their studies, they use a bronchosphere assay in which the hBECs are seeded on top of a MatriGel® matrix that contains IMR90, a human fetal lung fibroblast line. In their studies, they see formation of branching bronchospheres, but a key difference in their data from mine is that their method of seeding the hBECs allows them to aggregate and form colonies, meanwhile my colonies are clonally-derived. Overall, there are several methods of recapitulating the branching morphology seen in bronchospheres, but I have yet to identify an exact molecular mechanism to explain this phenomenon.

The role of Notch signaling in *in vitro* branching morphogenesis provides an opportunity to better understand a molecular mechanism underlying a key process in respiratory system development. Studies of lung development in human tissue models is restricted to *in vitro* models, making it difficult to mimic and recapitulate the physiological conditions and timing of processes such as airway branching, proximal distal patterning, and lineage commitment. My observations of Notch in the bronchosphere assay provides preliminary data that can facilitate investigations of what cellular processes and downstream molecular pathways are responsible for airway branching. It would be particularly interesting to see which of the four Notch receptors are responsible for this branching morphology by testing separate inhibitors of each receptor. My work also provides a model system for future studies into studying branching morphogenesis of the human respiratory system.

## 5.2. Identification of the HLO1-6H5 antibody's target antigen

The HLO1-6H5 monoclonal antibody targets a cell surface antigen on airway stem cells as previously mentioned in Chapters 2 and 4. The identity of this antigen is unknown, but its utility in isolating a subset of human airway basal cells that is enriched for stem/progenitor cells is clearly established by the data in Chapter 4. A key benefit to knowing the antigen of the HLO1-6H5 antibody is to determine if the identified protein and associated gene have a functional role in regulating stem cell properties. Understanding if the target antigen impacts stem cell quiescence, self-renewal, proliferation, or differentiation would provide an opportunity for mechanistic studies. Such investigations could involve knocking in or knocking out the HLO1-6H5 target antigen to determine if stem cell properties can be induced or abrogated. Another benefit to identifying the antigen is for additional experimental verification of the purity of stem cell/progenitor cell subsets that are FACS-isolated using HLO1-6H5. Knowing the protein and associated gene would allow us to verify the purity of the cell subsets through transcriptional analyses and by molecular methods if commercial reagents are available. Attempts were made to identify the HLO1-6H5 antigen by two different protocols.

The first method for antigen identification utilized version 5.1 of the human open reading frame library hORFeome that was cloned into a lentiviral vector[114]. The library was transduced into the C6 rat glioma cell line[67]. These cells were screened for expression of the HLO1-6H5 target antigen by flow cytometry, and the library came up

negative for the HLO1-6H5 target marker. This outcome was not unexpected as the hORFeome libraries do not fully represent the entirety of the human genome. At best, the hORFeome libraries captures only 63% of the total open reading frames known[114]. Had this approach worked and the library contained the open reading frame that corresponds to the HLO1-6H5 antigen, the next phase would have been to FACS-purify and culture-expand HLO1-6H5+ C6 cells. Once a sufficient population of cells had been collected for DNA recovery, I would have PCR amplified the open reading frame insert, Sanger sequenced the amplicon, and then identify the protein coding gene bioinformatically. For instances such as the HLO1-6H5 antibody, in which the target antigen is not represented in the hORFeome library, a proteomics approach is needed.

The second attempt at identifying the HLO1-6H5 antigen entailed immunoprecipitation of the protein for analysis by mass spectrometry. I identified multiple primary hBEC lines that I had propagated in the lab along with the HEK293 cell line as sources of the target protein antigen. The HUH7 human hepatocyte cell line was identified as a negative control cell line. An irrelevant IgM isotype antibody control was identified in HIC3-2D12, a novel marker developed by our lab as an endocrine cell marker for the pancreas[68,115,116]. To ensure that the target cell lysate contained the HLO1-6H5 antigen, I spotted the lysate onto nitrocellulose membranes and was able to detect the antigen with the HLO1-6H5 antibody. Once I had confirmed that the target antigen was solubilized in the cell lysate, I proceeded to immunoprecipitation.

My immunoprecipitation protocol was developed specifically for the HLO1-6H5 antibody, as there are very few published protocols for immunoprecipitation with an

IgM antibody like HLO1-6H5. IgM antibodies are distinct from the more common IgG antibodies in terms of structure. IgG exist as monomeric units with two binding sites, while an IgM is actually a large pentamer of five identical monomeric units to give it ten binding sites[117]. I was concerned with the possibility of IgM degradation during the immunoprecipitation, as it is a larger structure comprised of more constituent parts than an IgG antibody. Degradation of the IgM antibody could potentially contaminate the final purified product and increase background noise in the downstream mass spectrometry analysis. To avoid potential contamination by degraded IgM antibody, I crosslinked the monoclonal antibodies to anti-IgM beads and eluted the target antigen from the beads.

The mass spectrometry analyses were unsuccessful in identifying an enriched protein from the immunoprecipitations. Each attempt yielded a long list of candidate proteins, despite attempts to analytically filter the results. Three candidate proteins were selected based on the following criteria: absent in the negative cell line lysate, absent in pulldowns with the irrelevant antibody, reported cell surface expression in the Uniprot database, and relatively high abundance in the target cell lysate. The three candidate proteins were SLC4A7, SLC12A6, and LRRC8A.

To determine if any of the three candidate proteins were the antigen of HLO1-6H5, I designed sgRNA (single guide RNA) strands with the Crispor program to perform Crispr-Cas9 knockout of each candidate protein coding gene[118]. Assessment of target protein knockout was performed by flow cytometry, which showed that none of the target genes corresponded with the HLO1-6H5 antigen.

Future attempts at identifying the target antigen would benefit from optimizing the protocols established here. It is possible that the HLO1-6H5 antigen is not a protein, but instead the carbohydrate group of a glycoprotein. Treatment of viable HLO1-6H5+ cells with any variety of glycosidase will enzymatically remove glycans from a protein to experimentally determine if the antibody is targeting a carbohydrate. Identifying the target antigen has numerous benefits in terms of increasing our understanding of HLO1-6H5+ stem cells. Knowing the protein coding gene for the antibody would allow for future studies into the physiological context of the marker and associated cell subset as we would be able to examine them *in vivo* in the mouse respiratory system. Moving towards an *in vivo* model would allow for lineage trace studies to see how HLO1-6H5+ stem cells are regulated at homeostasis and during regeneration. Knowing the antigen's identity also allows for studies in the functional significance of the target protein and whether it plays a role in stem cell behavior. Additionally, knowing the antigen may allow for investigations into other tissue systems to determine if the HLO1-6H5 target marker plays a role in their resident stem cell population.

## 5.3. HLO1-6H5 cell subsets in other organ/tissue systems

The work presented in this thesis dissertation has focused on the HLO1-6H5 marker in the human airway epithelium, but this antibody's utility may not be restricted to the respiratory system. As evidenced in Chapter 5.2, the HLO1-6H5 antigen is also expressed on human embryonic kidney cells (HEK293). This evidence already indicates that the HLO1-6H5 target marker may be expressed in other organ systems. Additional

preliminary data also shows that the HLO1-6H5 target marker is expressed in human large intestine, pancreas, and stomach (Figure 5.4). In the large intestine, HLO1-6H5 expression is localized primarily to the middle and the base of the crypts, with the latter being the reported niche for intestinal stem cells[119,120§§]. In the pancreas, HLO1-6H5 is specific to the exocrine acinar cells and not the endocrine cells within the islet. In the stomach, the HLO1-6H5 antibody appears to label a secreted molecule originating from the gastric glands and covering the surface of the stomach lining. The added utility of the HLO1-6H5 antibody in other tissue systems provides research opportunities to potentially study other stem/progenitor cell subsets in contexts outside of the adult respiratory system.

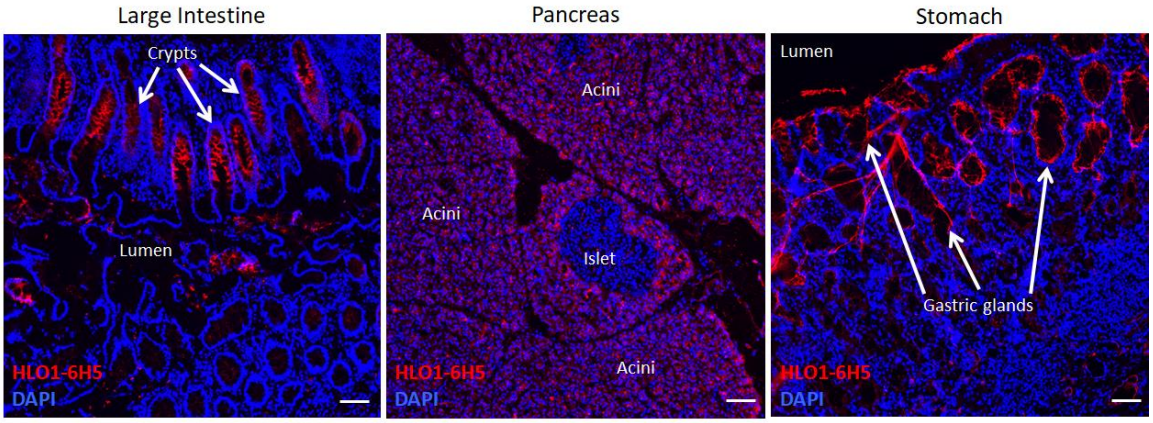



**Figure 5.4. The HLO1-6H5 target marker is expressed in multiple human organs.**
Acetone-fixed sections of human large intestine, pancreas, and stomach stained with HLO1-6H5 antibody. Scale bar: 50 µm.

§§ Intestinal tissue was generously provided by Nick Smith of Melissa Wong's Lab at OHSU.

## 5.4. Discussion

The HLO1-6H5 antibody has been shown to identify a subset of airway stem/progenitor cells, but there are still more questions that need to be answered about this novel subset. Identifying the HLO1-6H5 antigen is one endeavor that can increase understanding of airway stem cells as it will allow us to determine if there is a functional reason that stem cells express this marker. Transcriptional profiling by RNAseq of the HLO1-6H5 cell subsets would be an indirect method of antigen identification as the transcripts for the HLO1-6H5 target protein should be enriched in the positive subset. The RNAseq data could also yield insights into lineage specification and potential mechanisms of airway stem cell activation. One useful application of RNAseq technology would be to interrogate bronchosphere cells under Notch inhibition to identify downstream molecular targets that are suppressed or activated during branching morphogenesis. My data shows that the bronchosphere model may be useful in the study of the developmental processes of airway branching *in vitro*.

*In vitro* models of stem cells such as the bronchosphere assay are conducive to not only identifying stem cell populations, but also for evaluating some of the molecular mechanisms that regulate their function. These models provide a scalable and manageable system to observe how airway stem cells respond to activation or suppression of molecular pathways that may be informative of developmental or tissue regeneration processes. Bronchosphere models are already being used to evaluate Notch blockade as a therapeutic strategies to treat goblet cell metaplasia in some lung disease[61]. These *in vitro* models are scalable and amenable to manipulation, making

them ideal for continued use to evaluate stem/progenitor cell populations like the HLO-6H5+ subset in the respiratory system, and potentially in other human epithelial tissues like the intestine.

# Chapter 6. Conclusions and future applications of HLO1-6H5+ basal stem/progenitor cells and the HLO1-6H5 antibody

## 6.1. Conclusions

In this thesis dissertation, I have provided a detailed account of current approaches used for studying adult stem cells in the human respiratory system. Stem cell studies in humans are limited to *in vitro* assays, but the advancement of organoid technologies over the last decade has provided researchers with a more physiologically relevant tool to identify and characterize these cells. In Chapter 3, I provided a brief history on the development and application of organoid methodologies across multiple organ systems, starting with the early history of CFC assays. Currently, there are organoid models for almost every organ system and they can be generated from embryonic stem cells, induced pluripotent stem cells (iPSCs), and from harvested adult stem cells[85]. In the respiratory system, there are several different organoid systems in use as the complexity and diversity inherent to the various regions of the lung require exact culture conditions in order to recapitulate airway or alveolar morphologies. My work has focused on the stem cells of the proximal airways. There is a need to identify and characterize cell types in the airways, as there is a larger diversity of epithelial cells in that region compared to the alveolar space. The alveolar lung has two epithelial cell types, ATII and ATI cells. The ATII cells are a well characterized adult stem cell population for the alveolar region[29,31,92,121]. In the airway however, there are still

questions regarding whether the basal cells are the only stem cells of the region and whether all basal cells are stem cells. To address these questions, the bronchosphere assay was developed to evaluate airway cells for stem cell function[44,95]. I incorporated this assay into my thesis work to help identify stem/progenitor cell populations in the airway. Unfortunately, the assay was not originally designed for uncultured primary hBECs. Researchers that used the bronchosphere assay would culture-expand their cells at minimum overnight[44,52,94]. In order to use this assay, I had to modify it to meet my research goals by incorporating stromal cell co-culturing into the protocol. The addition of stromal cells to the assay is not a novel idea as other labs have used it to increase colony formation frequency[97,98]. But no other lab has reported the drastic effects that I have seen when incorporating stromal cells into the bronchosphere assay for assessment of stem cell function in uncultured hBECs. Primary uncultured hBECs require the presence of stromal cells in order to exhibit any stem cell function *in vitro*. This observation is important for the reason that identifying stem cells from uncultured primary tissue provides a more accurate representation of which cell or cell types in physiological tissue have the ability to self-renew and differentiate. Culturing cells can introduce artifacts into an analysis as the cells will change in response to the culture conditions and it's also possible that some cell types could be selected against and lost from the culture. I focused my thesis on identifying stem cells from uncultured primary cells and on developing a method to isolate them for functional analysis. My modified bronchosphere assay is the result of that effort.

In Chapter 3, the optimization of the bronchosphere assay revealed that stromal cells are necessary to promote stem cell proliferation in uncultured primary airway cells. This observation indicates that the mesenchyme plays a role in regulating the epithelial stem cells of the airway. One potential mechanism of mesenchymal/epithelial interactions is through FGF10 signaling from the mesenchyme to drive epithelial stem cell proliferation and differentiation[16,122]. The model of branching morphogenesis shown in Chapter 1 (Figure 1.4) shows that in the developing airway tree, the mesenchyme uses FGF10 signaling to drive BMP signaling and expansion of nearby epithelial cells for growth of the airway tube[16]. Mouse airway injury models have shown that FGF10 from mesenchymal cells promote airway stem cell proliferation for epithelial repair[123]. *In vitro* studies of mouse airway cells have also shown that FGF10-expressing stromal cells promote proliferation of resident epithelial stem cells[124,125]. In Chapter 5, I hypothesized that the mechanism of stromal cell promotion of airway stem cell proliferation was mediated by the Notch pathway. The preliminary data did not validate this hypothesis, but did confirm that the Notch pathway does regulate basal stem cell differentiation towards the ciliated or secretory cell lineages, as has been reported by other investigators[24,60,110]. Notch signaling is reported to originate with the epithelial basal cell population and not from the mesenchyme[112,126]. However, the role of the mesenchyme in Notch signaling cannot be ruled out as mesenchymal cells must promote a Notch-permissive niche in order for basal stem cells to differentiate. My observations that stromal cells are required for uncultured airway cells to proliferate and that the Notch

pathway regulates basal cell differentiation provides evidence that mesenchymal cells play a role in the regulation of basal stem cell function.

Knowing that I had an assay system that could evaluate cell populations for stemness, I also looked to identify a method of isolating novel cell phenotypes. Chapter 2 detailed my efforts in characterizing novel monoclonal antibodies developed in our lab in the hope of finding a tool that could target airway stem cells. Over the course of my thesis project, I have helped screen over 4000 antibody-producing hybridomas that were developed against human lung cells. Throughout the process I discovered several antibodies that were useful for targeting specific cell types and subsets in airway epithelium, but I identified only one mAb that demonstrated functional significance, HLO1-6H5.

In Chapter 4 of my thesis dissertation, I provide data that shows HLO1-6H5+ cells are a subset of human airway basal cells. Demonstrating that these cells are a basal cell subset is critical as the lung stem cell field at the time is still trying to address the question of whether the basal cells are heterogeneous in their ability to function as stem cells. Some studies identify KRT14, C-MYB, and N2ICD as intracellular markers that could define a subpopulation of basal cells[42,126]. However, it is impossible to use intracellular markers to purify a viable subset of basal cells. The HLO1-6H5 mAb addresses this issue because it targets a cell surface antigen on some basal cells. The mAb allowed me to FACS-purify subsets of basal cells from uncultured primary hBECs and evaluate their stem cell properties in the bronchosphere assay. The work in this thesis dissertation shows that HLO1-6H5+ basal cells are enriched for bipotent

stem/progenitor cells. The HLO1-6H5 target marker is distinct from the NGFR and ITGA6 markers that are currently employed to isolate basal cells from of culture-expanded epithelial cells[44,51,127]. I have been able to use the HLO1-6H5 mAb to show that there is functional heterogeneity within uncultured primary human basal cells. My HLO1-6H5 antibody also confirms that there is phenotypic heterogeneity, in agreement with the published intracellular markers found in mice and humans[42,126].

## 6.2. Future applications for the HLO1-6H5 monoclonal antibody and HLO1-6H5+ cells

Once the data on the HLO1-6H5 mAb is made available to the general research community, there will be interest in using this molecular tool in other scientific endeavors and possibly for the development or optimization of therapeutic strategies to repair tissue damage. Since my mAb can target and isolate viable cells, it would be useful for tissue engineering and reprogramming. Using antibodies for targeted delivery of therapeutic compounds to specific cell types is of great interest in the cancer research field[128]. It would also be useful for developing targeted therapies for airway disease. In the context of airway remodeling, having a tool like HLO1-6H5 that can target stem cells may help induce regenerative processes *in vivo*. Alternatively, the mAb can be used to isolate HLO1-6H5+ basal stem cells from primary tissue for *ex vivo* expansion or cellular engineering. Cystic fibrosis is a respiratory disease that researchers are currently developing both gene therapy approaches to correct the mutated gene and cell-based therapies to transplant healthy cells for repair of the tissue[129]. The mAb could also be

used in cultured cells as my data showed that HLO1-6H5 targets only a subset of culture-expanded cells. The antibody might be useful in purifying the most proliferative cells for prolonged cultures.

As mentioned in Chapter 5, there are HLO1-6H5+ cells in other human organs as well. Evaluating whether these cell subsets are stem/progenitor cells as well could increase our understanding of the biology of the HLO1-6H5+ subset. If the subset functions as a stem cell population in multiple organs, it suggests the possibility that the antigen may have a functional role. Identifying the antigen is a logical next step for the HLO1-6H5 mAb, as it would allow for *in vivo* characterization of the marker in mouse models to give a more physiological context to the cell subset. There is a lot of opportunity to increase our understanding of HLO1-6H5+ stem cells and their role in the respiratory system.

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