Probing for the cellular identity of Bronchioalveolar Stem Cells (BASCs) derived from variant club cells post antibody-mediated Notch inhibition.

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by

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INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled “Probing for the cellular identity of Bronchioalveolar Stem Cells (BASCs) derived from variant Club cells after antibody-mediated Notch inhibition” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Sukanya Bhat at Institute for Stem Cell Science and Regenerative Medicine under the supervision of Dr. Arjun Guha, Associate Professor, Department of Mechanisms Regulating Barrier Tissue Homeostasis, InStem Bangalore, during the academic year 2024-2025.

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This thesis is dedicated to my parents and sister, whose love, encouragement, and belief in me have been my significant source of strength. I also dedicate this work to my supervisor, Dr. Arjun Guha, for his invaluable guidance and belief in me. Also, to the readers and all those who share my passion for regeneration—may this work inspire curiosity and discovery.

Declaration

I hereby declare that the matter embodied in the report entitled “Probing for the cellular identity of Bronchioalveolar Stem Cells (BASCs) derived from variant Club cells after antibody-mediated Notch inhibition” are the results of the work carried out by me at the Department of Mechanisms Regulating Barrier Tissue Homeostasis, Institute for Stem Cell Science and Regenerative Medicine, under the supervision of Dr. Arjun Guha, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Sukanya Bhat

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Abstract

Cell fate in adult tissues is tightly regulated by signaling mechanisms that maintain homeostasis and facilitate repair. In the lung, club cells act as facultative progenitors, self-renewing and giving rise to multiciliated cells. Under specific injury conditions, they can also contribute to alveolar regeneration. While Notch signaling maintains club cell identity, its inhibition typically drives transdifferentiation into ciliated cells.

However, a recent study from our lab identified a subset of variant Club cells that evade this fate switch and instead enter a dual-identity state, co-expressing the Club cell marker CC10/Scgb1a1 and the alveolar type 2 (AT2) marker SPC/Sftpc, resembling bronchioalveolar stem cells (BASCs). These cells are enriched at neuroepithelial bodies (NEBs) and bronchioalveolar duct junctions (BADJs), niches known for regenerative potential.

To characterize these BASCs derived from club cells derived from variant club cells, we took two approaches. First, the immunohistochemistry approach is to see if they express any other gene at the protein level of the club or alveolar type 2 cell. Second, I contributed to isolating these cells with my lab members, on which single-cell RNA sequencing analysis was done. Our findings show that while these cells express markers of both club and AT2 cells, they lack other definitive markers, suggesting their lineage-ambiguous nature. This intermediate state allows them to either revert to Club cells or transition into other epithelial lineages, highlighting their potential role in lung repair and regeneration and providing new insights into airway epithelial plasticity.

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I am deeply grateful to my supervisor, Dr. Arjun Guha, for giving me the opportunity to work on this exciting project for my master's thesis and also for allowing me to blend into different projects and learn so many new things. It has been a privilege to learn from him and be a part of his lab. I sincerely thank Professor Girish Ratnaparkhi for his valuable feedback during the mid-year review.

To all the members of the Guha lab, I am immensely thankful for the constant support during challenging times has helped me grow both as a researcher and as a person. Especially to Adi and Sai, the two people who have helped me throughout by teaching me skills from the genotyping PCRs to immunohistochemistry.

I extend my gratitude to IISER Pune for providing me with this incredible learning experience and the many opportunities that have enriched my academic journey. I am grateful to my friends, who stood by me through my highs and lows. I would like to thank my parents and sister, my pillars of strength; your encouragement and belief in me have meant everything. From taking care of even the smallest things to always believing in me, I will be forever grateful.

I'd like to take a moment and appreciate myself for making it this far, something that my younger self could never have imagined.

Contributions

Contributor name	Contributor role
AG	Conceptualization Ideas
AG SM PV SB	Methodology
SB PV	Software
SB	Validation
PV	Formal analysis
SB PV SM	Investigation
AG	Resources
SB PV	Data Curation
SB	Writing - original draft preparation
SB AG	Writing - review and editing
SB PV	Visualization
AG	Supervision
AG	Project administration
AG	Funding acquisition

AG- Dr. Arjun Guha, SB- Sukanya Bhat, VP-Vasam Prabantu, SM- Sai Manoj

1. Introduction

1.1 Cellular fate dynamics in tissue repair and regeneration

The capability of tissues to repair and regenerate is fundamental to maintaining homeostasis and recovering from injury. It is an essential process that enables organisms to recover from injury and daily wear and tear. Traditionally, cell fate has been viewed as largely deterministic, as depicted by the Waddington landscape, where cells follow predefined developmental trajectories and reach their terminally differentiated states in adulthood. However, emerging evidence from the past few decades has challenged this classical view by revealing that cell identity and fate are more flexible than once thought. Pioneering discoveries by Shinya Yamanaka demonstrated that even fully differentiated cells can be reprogrammed, overturning the long-held belief that cell fate is irreversible (Takahashi K et al., 2006; Takahashi K et al., 2007). This plasticity enables them to contribute to tissue repair and regeneration through dedifferentiation and transdifferentiation.

Dedifferentiation refers to the process where differentiated cells lose their mature characteristics, returning to a progenitor-like state with the ability to develop into a different cell type. In contrast, transdifferentiation occurs when a fully differentiated cell directly converts into another cell type, bypassing an intermediate progenitor state. These processes highlight the dynamic nature of cellular identity in adult tissues, challenging the traditional notion of irreversible cell fate commitment and expanding our understanding of tissue plasticity and regeneration.

During homeostasis and repair, the tissues rely on two critical cellular populations: professional stem cells, which possess an intrinsic capability of self-renewing and differentiation in dedicated regions of the tissues, and facultative stem cells, which are typically differentiated cells with specialised functions that can acquire regenerative potential in response to injury. Various tissues have facultative stem cells, such as the intestine, skin, and lungs (Merrell AJ et al., 2016). For example, tissues in the gut epithelium, skin with high cellular turnover, primarily depend on professional stem cells to sustain continuous regeneration and maintain homeostasis. In contrast, tissues with slower turnover rates, including the lung and liver epithelium, have a more dynamic strategy, utilizing both professional and facultative stem cells. In these tissues, facultative stem cells play a significant role in replenishing lost or damaged cells where and when required. This adaptive mechanism ensures effective tissue repair and long-

term organ function, again highlighting the plasticity of some of the differentiated cells in response to injury.

Following an injury, the lung demonstrates a remarkable capacity for regeneration and functional recovery. In the case of unilateral pneumectomy, stem cell populations increase, and the remaining lung adapts through compensatory growth to regain respiratory function. (Eisenhauer, P. et al., 2013). Furthermore, cell lineage tracing studies carried out in the murine lung have revealed extensive evidence of remarkable cellular plasticity during both homeostasis and injury-induced repair (Tata PR et al., 2013; Jain R et al., 2015; Lynch TJ et al., 2018; Tata A et al., 2018; Guha A et al., 2017; Ouadah Y et al., 2019). Consequently, the murine lung has become an essential model for studying the regulatory mechanisms that govern cell fate transitions. The lung's ability to regenerate depends on its diverse epithelial cell populations, each playing a distinct role in maintenance and repair.

1.2. The murine lung epithelium: structural organization and protective functions

The lung is a highly specialized branched organ that facilitates gas exchange. In addition to its respiratory function, the epithelial lining of the lung serves as a crucial barrier against inhaled pathogens, toxins, and pollutants, preserving pulmonary homeostasis. The airway epithelium in the adult lung is a heterogeneous structure, with varying cell populations spanning as we go from the proximal to distal region (Rawlins, E. L. et al., 2006; Franks, T. J. et al., 2008). The respiratory tract is broadly divided into three distinct regions: the trachea, the bronchioles, and the alveoli (Figure 1). The airway epithelium primarily consists of club cells, goblet cells, basal cells, ciliated cells, and rare neuroendocrine cells, whereas the alveolar epithelium is composed mainly of alveolar type1(AT1) and alveolar type2 (AT2) cells.

The maintenance and regeneration of these distinct epithelial regions are governed by resident stem cell populations. The basal cells serve as the principal stem cell population, in homeostasis and injury conditions in the trachea (Rock JR et al., 2009; Rock JR et al., 2011; Hong KU et al., 2004; Hong KU et al., 2004). The club cells function as facultative progenitors capable of self-renewing and differentiating into multiciliated cells for airway maintenance in the distal airway. Under certain conditions, they can also give rise to AT2 cells, aiding alveolar repair and regeneration. (Rawlins, E. L. et al., 2009; Rokicki, W. et al., 2016). In the alveoli, the AT2 cells are the bonafide stem cell population, contributing to tissue maintenance during homeostasis and repair

following injury. (Barkauskas CE et al., 2013; Desai TJ et al., 2014). Additionally, bronchioalveolar stem cells (BASCs), a multipotent stem cell population located at the bronchioalveolar duct junction (BADJ), play a crucial role in repairing both bronchiolar and alveolar epithelium in response to lung injury (Liu Q et al., 2019; Salwig I et al., 2019).

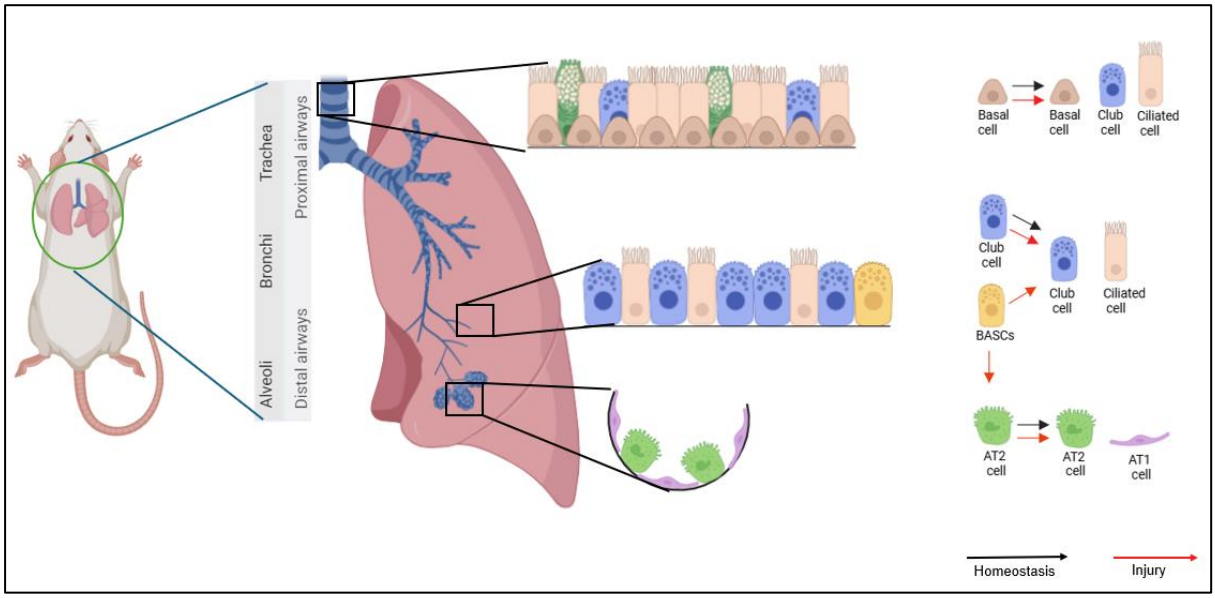

Figure1. Schematic of the murine respiratory system and the resident stem cells. The murine respiratory tract is divided into three distinct regions, the trachea, the bronchi and the alveoli. As we go along the proximal to distal axis of the airway epithelium, the composition of the epithelium varies. Each compartment is maintained by its own resident stem cells, as shown on the right side of the figure. In the trachea, the basal cells act as the stem cells. In the distal bronchi, club cells are the facultative stem cells, and in the alveoli, the AT2 cells are the stem cells. There is a junction between the terminal bronchi and airway epithelium known as the Bronchioalveolar duct junction (BADJ), where the Bronchiolaveolar stem cells reside. They do not contribute to the maintenance of the airway in homeostasis but get activated under injury condition to give rise to either club cell in the airway or alveolar type2 cell in the alveoli. (Generated in Biorender).

1.3. The human and mouse lung epithelium: similarities and differences

While the overall organization of the lung epithelium is conserved across species, significant differences between human and murine lungs must be accounted for when investigating lung regeneration and disease modeling. (Rao Tata P et al., 2017). Murine models are powerful tools for lung biology research due to their genetic tractability; however, key anatomical and cellular differences must be considered when applying findings to human physiology. In humans, cartilage rings provide structural support from the trachea to the bronchioles, whereas in mice, cartilage is limited to the trachea and main bronchi. In mice, basal cells are present from the trachea to the main

bronchi, whereas in humans, they extend throughout the airways, including distal regions. The human lung has a higher abundance of goblet cells in the proximal airways, facilitating mucus production, whereas Club cells are primarily limited to the small bronchioles. In contrary, the mouse lung contains a greater number of club cells, which are distributed from the trachea to the bronchi, while goblet cells are relatively scarce. In both human and mouse airway epithelium, rare neuroendocrine cells are found either as solitary cells or in innervated clusters known as neuroepithelial bodies (NEBs), which are surrounded by club cells. (Kuo CS et al., 2015 ; Noguchi M et al., 2015; Avadhanam KP et al., 1997). Besides the differences in cell composition and abundance, an important anatomical difference is the presence of distinct respiratory bronchioles at the point where the airways transition into the alveoli in the human lung, which are not there in the mouse lung.

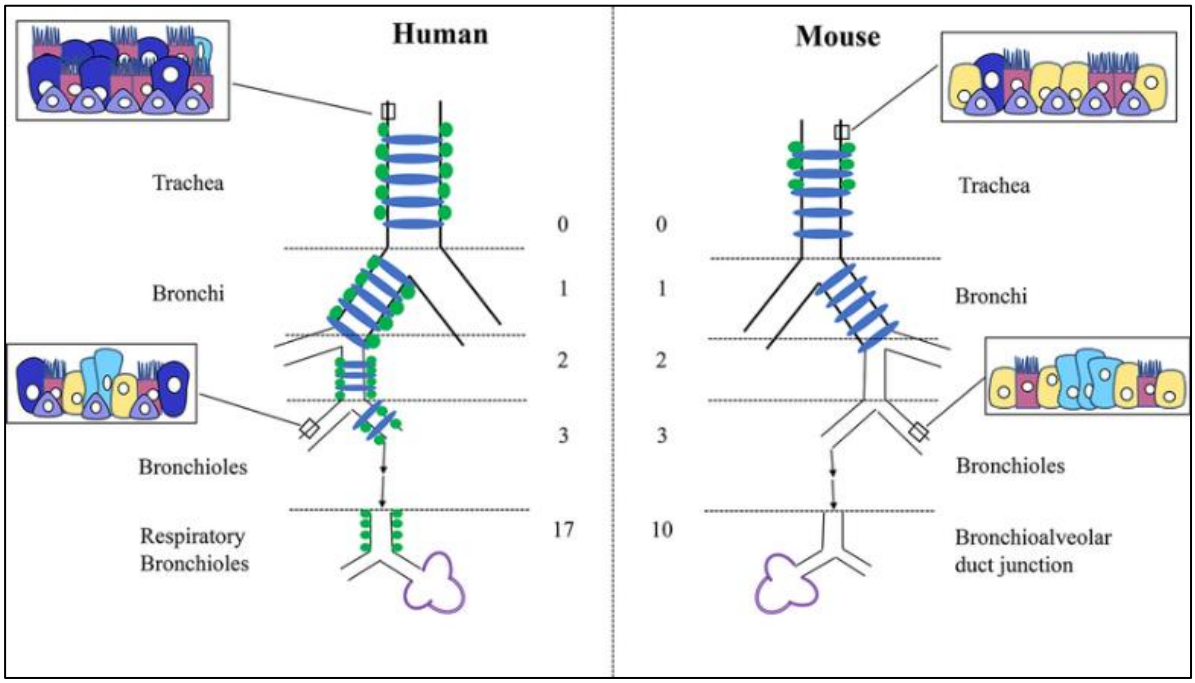

Figure2. Diagram depicting the variation between human and mouse lung structure and cellular composition. Cartilage rings (blue) are present from the trachea to the bronchioles in human lungs but are present only in the trachea and bronchi in mouse. Submucosal glands (green) are restricted to the trachea in mouse but are present into the smaller distal airways in humans. In mouse, only the trachea and main stem bronchi are lined with pseudostratified epithelium, whereas in humans, all the conducting airways are lined with pseudostratified epithelium. Basal cells (purple) exists till the bronchioles only in humans. The human lung consists of more goblet cells (dark blue) in the proximal epithelium, with Club cells (yellow) mostly restricted to the smaller airways. Conversely, the mouse lung contains more Club cells

(yellow) through the trachea and bronchi, with less goblet cells throughout.(Club cells = yellow, Ciliated cells = pink, Basal cells = purple, Goblet cells = dark blue, Neuroendocrine cells = light blue). (Adapted from Soula Danopoulos et al., 2019)(open-access article)

Given these structural and functional parallels, murine models remain indispensable for studying lung regeneration and disease pathogenesis. Their genetic accessibility has facilitated the identification of cellular origins of various lung pathologies, the mechanisms governing cell fate transitions, and the role of facultative progenitors in repair. The development of conditional knockout strategies, lineage-tracing approaches, and single-cell technologies in mice has significantly enhanced our understanding of stem cell dynamics and disease progression, paving the way for translational advancements in respiratory medicine. Studying the regenerative capacity of the small airways in the murine lung is particularly important, as these regions are highly vulnerable to human lung diseases.

1.4. Club cell: function and importance

In the mammalian airway, the predominant cell type is the secretory club cells. Club cells, in coordination with the multiciliated cells, play a vital role in mucociliary clearance, an important defense mechanism that removes inhaled pathogens and debris from the respiratory tract (Bustamante-Marin XM et al., 2017). These specialized epithelial cells secrete secretoglobins and other bioactive molecules that help regulate inflammatory responses (Chichester CH et al., 1991; Oesch F et al., 2019) and support xenobiotic metabolism (Tokita E et al.,2014; Snyder JC et al., 2010; Wiesner DL et al., 2020), contributing to airway homeostasis and protection. Club cells express various members of Secretoglobin family (Scgb1a1, Scgb3a2, and Scgb3a1), with distinct distribution patterns throughout the airway tree (Reynolds SD et al., 2002). Among these, Scgb1a1 is the most widely recognized marker for club cells, as it is also expressed in the distal airways of the human lung. They are typically quiescent under homeostasis, carrying out their functions, but in response to injury, they also contribute to airway maintenance by differentiating into multiciliated cells (Rawlins, E. L. et al., 2009). Furthermore, club cells may relocate in response to certain types of alveolar injury and generate alveolar type 1 and alveolar type 2 cells (Guha A et al., 2017; Zheng D et al., 2013).

1.5. Notch signaling actively maintains the club cell fate:

Notch signaling is one of the signals that is important during the mammalian lung development and is essential for specifying the club cell fate It is an evolutionary conserved, juxtacrine cell communication pathway that regulates cell fate determination among various cell process. In mammals, this pathway involves the interaction between transmembrane Notch receptors (Notch1, 2, 3 and 4) on cell that is receiving signal and transmembrane ligands (Jagged1 and 2, Delta-like 1,3 and 4) on the neighbouring cell that is sending the signal. This receptor-ligand interaction induces conformational changes, leading to the sequential proteolytic cleavages, with the final cleavage mediated by γ-secretase. This cleavage releases the Notch intracellular domain (NICD), which then translocates to the nucleus, where the NICD binds to the transcription factor RBPJk, and activates expression of Notch target genes (as depicted in Figure 3) (Kopan R, 2012; Bray SJ, 2006).

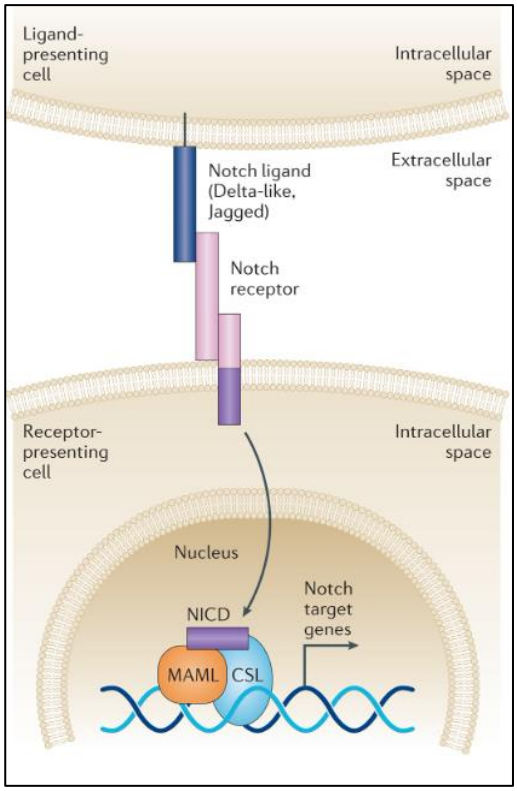

Figure3. Diagram explaining the Notch signaling pathway. The Notch signaling pathway involves the interaction between the Notch receptors and Notch ligands on neighbouring cells. This interaction induces conformational changes, leading to the proteolytic cleavage of Notch Intracellular Domain (NICD). The Notch intracellular domain (NICD) is released into the cytoplasm and translocates to the nucleus, where it binds to transcriptional co-activators and activates downstream target gene expression. (Adapted from Andersson, E.R. et al., 2014)

Recent studies indicate that in the adult lung, Notch signaling plays a crucial role in the regulation and maintenance of club cell fate. Genetic analysis has demonstrated that Notch signaling is essential for airway epithelial differentiation and club cell fate determination. In the absence of Notch signaling, progenitor cells fail to form club cells, resulting in numerous multiciliated cells (Tsao PN et al., 2009). Therefore, this highlights the crucial role of Notch activation during development in directing progenitor cells toward the club cell fate. It has also been shown that Notch2 is the major contributing receptor in the selection between club cell and ciliated cell fate (Morimoto M et al., 2012). Similarly, several studies have shown that Notch signaling is indispensable for maintaining the fate of club cells in the trachea (Pardo-Saganta A et al., 2015) and airway tree (Lafkas D et al., 2015). Together, these findings support the idea that Notch signaling is important not only for the initial lineage specification but also for the long-term maintenance of club cells in the adult lung. In the airway epithelium, it maintains the balance between secretory and ciliated cell populations by promoting secretory cell fate while suppressing ciliated cell differentiation.

The recent findings from our lab reveal that canonical Notch signaling actively maintains the fate of all the club cells in the airways and its inhibition leads to its transdifferentiation into multiciliated cells. However, a subset of club cells termed as variant club cells escaped this transdifferentiation and enter a lineage-ambiguous state resembling Bronchioalveolar Stem Cells (BASCs) that are capable to give rise to alveolar cells as well airway cells upon injury. These variant club cells appeared to be enriched at Neuroepithelial bodies (NEBs) and near the transitioning zone of airways to alveoli (Bronchioalveolar Duct Junctions (BADJs)). The preliminary analysis shows that the differential response of variant club cells to Notch inhibition is mediated by Wnt signaling (Lingamallu SM et al., 2024).

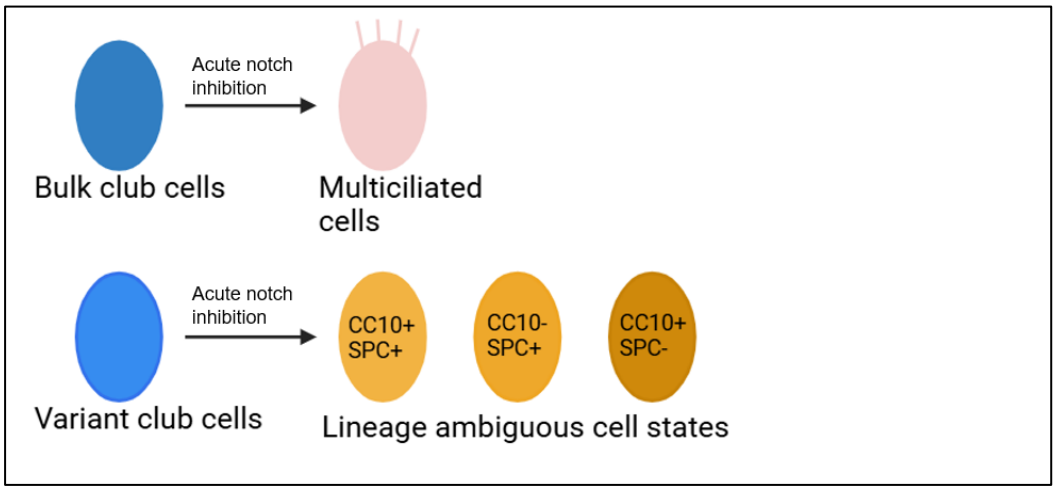

Figure4. Schematic showing the response of bulk club cells and variant club cells to acute Notch inhibition. While most of the club cells transdifferentiate into multiciliated cells, variant club cells resist the transdifferentiation and transition into lineage ambiguous cell stated, with around 80% of them being CC10+ SPC+. (Generated in biorender)

While Notch signaling is known to be essential for maintaining Club cell fate, the findings from our lab suggest that its inhibition triggers remarkable plasticity in a subset of Club cells (Lingamallu SM et al., 2024). However, the mechanisms underlying the differential response of variant Club cells remain unclear. Our research aims to dissect these regulatory mechanisms and their role in lung regeneration

1.6. Research Focus and the Broader Goal

Our lab's overarching objective is to understand how cellular fates are maintained across different regions of the lung and to identify the mechanisms that drive intercellular fate transitions. This includes both the extrinsic signals that direct cell fate and the intracellular processes that mediate these transitions.

1.7. Goal of the project:

Amongst the epithelial cell populations, club cells function as facultative stem cells in the smaller airways, with the ability to transition into multiple cell lineages under different conditions. My project specifically investigates one such cell fate transition; the transition of club cells to an $CC10^{+}$ SPC^{+} cell state (resembling BASCs), which can have implications for lung regeneration in future. Upon Notch inhibition, a subset of club cells resists transdifferentiation into multiciliated cells and instead enters a

lineage-ambiguous state. Understanding this intermediate state is key to deciphering the mechanisms that regulate cellular plasticity in the lung.

A key defining feature of this lineage-ambiguous population is the co-expression of CC10/Scgb1a1(club cell marker) and SPC(alveolar type 2 cell marker), which we are leveraging as a molecular handle to investigate this state. While $CC10^{+}$ SPC^{+} cells constitute a significant subset of lineage-ambiguous cells, they do not fully represent the entire population. Our next step is to identify additional markers that will allow for a more precise and comprehensive characterization of this transient state, either by expanding the known molecular profile of these cells or by capturing a broader spectrum of lineage-ambiguous populations. To better understand this dual-cell state, we have characterized their fate transition at the gene expression level via single cell RNA sequencing, with the broader goal of identifying the molecular insights to this process.

This project is significant as it addresses a fundamentally important question in regenerative biology: how a cell's fate is actively maintained, and how certain cells resist the transition to a different fate. The long-term benefit of the study may help create targeted therapies to improve lung repair from injury or disease by exploring how certain club cells can resist transdifferentiation and regain their regenerative capabilities. The perspective could extend to understanding how facultative stem cells behave across different tissues.

2. Materials and Methods

All experimental animals were maintained in a Specific Pathogen-Free (SPF) environment at the Animal Care and Resource Center (ACRC). C57BL/6J mice were administered either a control antibody (anti-gp120) or Notch1/2-blocking antibodies for durations of 3 or 7 days.

Notch antibody administration–

Animals received intraperitoneal injections of either a non-targeting IgG control (anti-gp120) or Anti-Notch1/Notch2 antibodies, diluted in PBS, at specific time points. The administered antibody dosages were as follows: non-targeting IgG (anti-gp120) at 40 mg/kg, Anti-Notch1 at 10 mg/kg, and Anti-Notch2 at 30 mg/kg. Animals were sacrificed at the designated time points as shown in the figures.

Tissue processing –

The animal was anaesthetized, followed by cervical dislocation. The lungs were then inflated with the mixture of Paraformaldehyde (1% (wt/vol)) and low-melting agarose (2%) in PBS and kept in the same fixative mixture for around 1hr at RT. The lobes were then separated, and the left lobe was used for doing thick section analysis and the remaining lobes were kept in the same fixative for overnight at 4°C and processed for paraffin embedding. After the overnight fixative, the tissues were given 3 PBS(1x) washes for 5 minutes each on the rocker. Then, the tissues were soaked in 0.85% NaCl for 1 hr likewise on the rocker. It was then transferred to 0.85% NaCl:100% ethanol for 1 hr on the rocker. The serial dilutions of ethanol of 70, 80, 90 and 100% was used for the remaining washes and it was kept overnight in 100% ethanol. The tissues were then given 3 xylene washes of 45 minutes each on the rocker. Then the tissues were immersed in 1:1 solution of paraffin and xylene for 2 hrs at 70°C. The tissues were then given 2 washes of paraffin for 1 hr each at 70°C with vacuum on.

Tissue embedding –

A hot plate was set to 65 °C during the second paraffin wash. Moulds were kept ready on the hot plate. After the second paraffin wash, fresh paraffin was poured into the moulds, and the tissue was transferred to the mould and oriented properly. After placing the ring on the mould, more paraffin was poured to fill the ring. The block was placed down on a cold surface to let it cool overnight at RT.

Tissue sectioning –

The slide warmer was set to 40 °C and a water bath to 45 °C with DEPC water. The blocks were kept on ice for 15-20 min before the sectioning to avoid folding and wrinkling of sections. The block was trimmed till enough representation of the tissue(airways) can be seen. 5 um sections were cut and taken on the slides, dried underneath the slides and around the sections with tissue paper, and kept on the slide warmer overnight for drying.

Immunostaining –

Deparaffinization and rehydration –

Slides were kept in the following solutions in coplin jars: Xylene two washes for 5 min each, then two 100% ethanol washes for 5 min each, one 90% ethanol wash for 2 min, one 70% ethanol for 2 min, one 50% ethanol for 2 min and then lastly, three Milli-Q water washes for 2 min each.

Antigen retrieval –

7 ml of Tris-based(100x) antigen retrieval buffer (pH-9) was added to 700 ml of Milli-Q water in a beaker. The beaker was covered with a lid and boiled at 100% power for 10 min. The slides were then placed in the slide holder and were quickly immersed into the boiling solution, covered with lid and reheated at 80% power for 15 min. After reheating, the beaker was kept to cool to room temperature without the (takes ~45 min-1 hr to cool).

Antibody staining –

The slides were washed three times with 1x PBS for 5 min each in coplin jars and then the sections were kept for permeabilization with 0.5% PBST (0.5% Triton X-100 in 1X PBS) for 30 min in a coplin jar. The slides were then given three mores washes of 1x PBS for 5 min each in coplin jars.

The slides were wiped underneath and around the sections with a tissue paper to remove the solution and surrounded the sections with a reagent blocker pen to make a hydrophobic boundary. Around 60-80 ul of blocking solution was added onto the sections and kept for incubation for 30 min -1 hr at RT. After which the blocking solution was discarded and 60-80 ul of the primary antibody solution was put on the section and kept for incubation in a hydration chamber overnight at 4^0C.

After the overnight primary antibody incubation, it was removed, and the slides were washed with 1x PBS for 5 min (3x) in coplin jars. Around 60-80 ul of the secondary antibody solution onto the sections and incubated in a hydration chamber at room temperature for 2 hours. The antibody solution was then removed, and the slides were washed with 1x PBS for 5 min (3x) in coplin jars. The slides were then kept for 3-4 hrs in DAPI. The slides were then dried with an aluminium foil kept on top to prevent bleaching and mounted with mowiol.

Immunofluorescence analysis –

Following antisera were used for the immunofluorescence: Goat anti-Uteroglobin (Merk, 1:1000 dilution), Rabbit anti-CGRP (Sigma, 1:1000 dilution), Rabbit anti-Sftpc (Seven Hills Bioreagents, 1:300 dilution), Mouse anti-Foxj1 (Bioscience, 1:200 dilution), Rabbit anti-ABCA3 (Seven Hills Bioreagents, 1:500 dilution), Goat anti-CGRP (Abcam, 1:500 dilution). Alexa 488/568/647-conjugated Donkey anti-mouse/rabbit/goat (Invitrogen, 1:300 dilution). The antibody-stained sections were imaged on Zeiss LSM-780 lasers and laser-scanning confocal microscopes. Multichannel images were acquired using confocal microscope and processed using Fiji (ImageJ). Nuclei were identified by the presence of DAPI and cells were identified as positive if the respective markers expression was seen.

Single cell RNA sequencing (scRNA seq) protocol –

Tissue digestion –

The animal was anaesthetized, followed by cervical dislocation. The lungs were exposed and perfused with 10 ml of cold PBS through the right ventricle. The lung was inflated with 2 ml of cold 5 U/ml dispase and the trachea was tied to prevent backflow. The lungs were dissected out and incubate on ice for 10 mins. The lobes were then separated, and the trachea and extraneous tissue were discarded. The lungs were minced finely using blades and the tissue was then transferred to 10 ml of digestion buffer in a 50 ml centrifuge tube. The tube was incubated horizontally at 37°C, 90 RPM for 1 hr. The enzymes were then neutralized with a 2-fold excess volume of neutralizing buffer (10% FBS in PBS + 20 U/ml Dnase1 and 2 mM EDTA).

Note: All steps from now on including incubations, washes and centrifugation should be performed at 4°C or in the cold room. Minimise temperature fluctuations as much as possible. Make sure to use temperature-controlled centrifuges and set them at 4°C beforehand.

RBC lysis using ACK buffer –

The sample was divided into two 15 mL falcons and spun down at 500 g for 5 min to collect the pellet. The two pellets were pooled and resuspended in 5 ml of RBC lysis buffer and incubated on ice for 3 min. The solution was passed through a 70 µm filter into a fresh 15 ml falcon tube, and the buffer was quenched with DMEM: F12K (1:1) complete media. Then again, spun down at 500 g for 5 min, resuspend the pellet in 500 µL of FACS buffer, and did cell count using trypan blue for live cell percentage.

Epithelial cell enrichment using MACS (Magnetic Activated Cell Sorting) –

Biotin-conjugated primary antibodies anti-CD45, anti-CD31, and anti-Ter119 were added at a concentration of 1 µL of the antibody for ≤ 5×10^6 cells and incubated at 4℃ for 25 min with gentle intermittent shaking (~ every 5-10 min). Note: Cells should remain in suspension for efficient antibody staining.

The primary antibody was then washed by diluting the cell suspension with 5 ml of FACS buffer and spun down at 500 g for 5 min. Then, resuspend the pellet in 500 µL of FACS buffer.

Surface staining for airway epithelial cells and inclusion of single-cell stains –

Anti-biotin microbeads at 2 µL per million cells were added and incubated at 4℃ for 20 min with gentle intermittent shaking (~ every 5-10 min). Note: Cells should remain in suspension for efficient antibody staining.

The secondary antibody (beads) was washed by diluting the cell suspension with 5 ml of FACS buffer and spun down at 500 g for 5 min. The pellet was resuspended in 500 µL of FACS buffer and that was gently added onto a previously equilibrated LS column. The elute was collected into a fresh 15 ml falcon tube. (The elute is the fraction of interest since it contains the epithelial cells which are not labelled). Once the sample went through; an additional 5 ml of FACS buffer was added to the column and collected the total elute. The flow through was spun down at 500 g for 5 min and resuspend the pellet in 100 µL of FACS buffer. The cells were counted using a haemocytometer and checked for live cell percentage using trypan blue.

Fluorescence Activated Cell Sorting (FACS) and generation of cell suspension–

FACS was set up using single-stained samples to set gates for live (DAPI-), epithelial (EpCAM+), and airway (CD24+) cells. DAPI was added to the double-stained sample, and cells were sorted for DAPI-, EpCAM+, CD24+ into BSA-coated tubes at 5°C. The cells were then pelleted, resuspended, and the concentration was adjusted to 500-700 cells/µl.

Library prep and sequencing was done using 10x Genomics Chromium Controller and their protocol was followed by NGS department.

GEM Generation and cDNA preparation–

Cell suspension was loaded into the 10x Genomics Chromium Controller with GEM reagents and generated Gel Bead-in-Emulsion (GEMs). These GEMs are then transferred to a PCR tube and reverse transcription is performed to convert mRNA to cDNA.

cDNA Amplification and Library Preparation –

The cDNA was amplified via PCR. The amplified cDNA was purified using beads. Sequencing library (fragmentation, adapter ligation, indexing) was constructed.

Quality Control and Sequencing –

The library quality was analyzed on an Agilent Bioanalyzer High Sensitivity chip for size and concentration and once passed the quality check, was sequenced.

Data analysis –

ScRNA seq data analysis –

We have used CellRanger (10X) to align reads with mouse genome and to count unique molecular identifiers. Doublets were identified and removed from individual datasets with DoubletFinder (v.2.0)49 and SoupX (v.1.5.0)50 was used to remove ambient background RNA from droplets of datasets. Seurat (v.5) was used for downstream analysis of individual datasets including normalization, scaling, find clusters, linear dimension reduction (Principal Component Analysis), UMAP. Differential gene expression analysis was done using FindMarkers. Additionally, marker genes were identified using FindAllMarkers.

The sequencing quality of all raw sequencing data in FASTQ format was evaluated using cellranger-7.2.0. Reads were mapped to the Mouse mm10 (GENCODE vM23/Ensembl98) genome assemblies by cell ranger with default settings and counting was performed. The reads were analysed using the Seurat package (Version 5.1.0) in R (Version 4.3.2). We obtained two independent datasets from separate experiments: (1) a gain-of-function and Notch-treated sample, and (2) a 7-day Notch-treated cell sample. To complement these experimentally derived samples, we leveraged a reference dataset of cells from publicly available datasets deposited in the Gene Expression Omnibus (GEO) database ([reference]). Cell-type annotations and corresponding experiment information were carefully curated and integrated into our analysis. The combination of these datasets enabled us to validate and extend our study in distinct experimental contexts.

To elucidate the gene expression profiles of Bronchioalveolar Stem Cells (BASCs), we employed a multi-step analytical pipeline integrating bioinformatic tools and statistical analysis. The initial step involved pre-processing the gene expression data to ensure comparability and accuracy. Specifically, we performed the following steps on all datasets:

1. Quality filtering: To ensure data quality, cells were filtered to remove doublets using SoupX and dblfinder, and single-cell experiment objects were generated.

2. Normalization: Normalization of gene expression data was performed to account for variations in sequencing depth and library size, enabling meaningful comparisons between cells.

3. Scaling: Normalized data were scaled to reduce gene-specific variability and emphasize differences in gene expression profiles between cells.

4. Dimensionality reduction and clustering: Uniform Manifold Approximation and Projection (UMAP) was applied to reduce high-dimensional gene expression data to a lower-

dimensional representation, facilitating visualization and clustering analysis. The Seurat package was utilized to identify distinct cell clusters based on gene expression profiles.

5. Highly variable genes were selected for principal-component analysis (PCA). Following PCA, significant principal components were used as input for nearest

neighbour based clustering and UMAP generation, both implemented in the Seurat package. Log2FoldChange expression values were computed using default methods.

Software and Versions

The following software and versions were used in this analysis:

R (version 4.3.2)

Seurat package (Version 5.1.0)

Table 1: 10x PBS buffer composition for single-cell experiment

10x PBS buffer composition	For 500 ml
KH_2PO_4	720 mg
NaCl	45000 mg
Na_2HPO_4	2105.56 mg

Table 2: RBC lysis buffer composition for single-cell experiment

RBC lysis buffer composition	For 100 ml
NH_4Cl	829 mg
$KHCO_3$	100 mg
EDTA(0.2M stock)	3.7 mg

Table 3: FACS buffer composition for single-cell experiment

FACS buffer composition	For 500 ml
FBS	10 ml
200mM EDTA	5 ml
100x penstrep	5 ml
10x PBS	50 ml
ddH_2O	430 ml

Table 4: Digestion buffer composition for single-cell experiment

Digestion buffer composition	For 10 ml
Trypsin EDTA (0.25%)	1 ml
Dispase (50 U/ml)	1 ml
CollegenaseD	10 mg
DNaseI (2000 U/ml)	100 µl
1x HBSS	7.87 ml

Table 5: HBSS buffer composition for single-cell experiment

10x HBSS buffer composition	For 500 ml
$CaCl_2$	700 mg
$MgCl_2.6H_2O$	500 mg
$MgSO_4.7H_2O$	500 mg
KCl	2000 mg
KH_2PO_4	300 mg
NaCl	40,000 mg
Na_2HPO_4 (anhyd)	240 mg
$NaHCO_3$	875 mg
D-glucose	5000 mg
HEPES	29,790 mg

3. Results and Discussion

3.1 Antibody-mediated Notch inhibition reveals multiple cell fate trajectories of airway club cells.

Under homeostasis condition, it has been shown that Notch signaling is necessary for the maintenance of the club cell fate in the airway epithelium. Inhibition of Notch signaling using antibody that prevents activation of the downstream cascade leads to the transdifferentiation of the majority of club cells into multiciliated cells. But, under these conditions, rare club cells, associated with NEBs and at terminal bronchioles, resist this transdifferentiation and express CC10 (a marker used to identify club cells).

The first step was to recapitulate these findings. To achieve this, the mice were administered with either non targeting IgG or Notch2/Notch1 antibody. The non targeting IgG that is gp120 is a viral antibody, it is being used as a negative control because it doesn't recognize any proteins in the mouse. Its only function is to mimic the presence of an antibody so that the effects of your anti-Notch antibody treatment can be specifically attributed to Notch inhibition. The anti-Notch antibody will bind to the Notch receptors, hindering the receptor-ligand interaction, and thereby inhibiting the Notch signaling pathway. The immunostainings were done on thin tissue sections of control treated with IgG antibody, 3 days and 7 days post-Notch inhibition. These sections were stained for CGRP (marker for NEBs), FOXJ1(marker for ciliated cells), CC10 (marker for club cells).

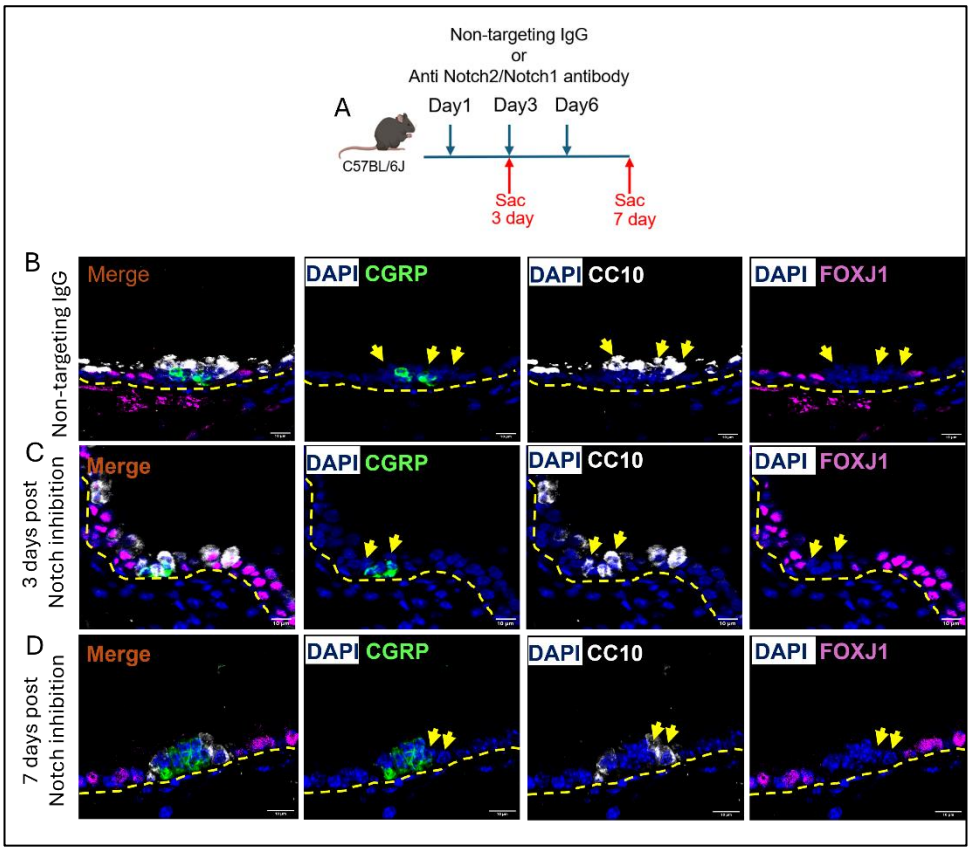

Figure5. Antibody-mediated inhibition of Notch changes the fate of airway Club cells to multiciliated cells except at NEBs. (A) Schematic of the treatment administered using either non-targeting IgG or anti-Notch2/Notch1 antibodies. (B) Thin section of a control lung, showing the distribution of ciliated cells (FOXJ1, magenta) and club cells (CC10, white) and NEBs (CGRP, green). (C) Thin section of a 3 days post Notch inhibition lung, showing the distribution of ciliated cells (FOXJ1, magenta) and club cells (CC10, white) and NEBs (CGRP, green). Note that the club cells transition to a double positive state ($CC10^{+}$, $FOXJ1^{+}$), post 3 days Notch inhibition, except at NEBs. (D) Thin section of a 3 day post Notch inhibition lung, showing the distribution of ciliated cells (FOXJ1, magenta) and club cells (CC10, white) and NEBs (CGRP,green). Club cells transdifferentiate into multiciliated cells post 7 days of Notch inhibition, except at NEBs. Yellow arrows point to the club cells associated with NEBs which resists transdifferentiation. Yellow dashed line indicates the airway epithelium. Scale bar- 10µm.

Immunostaining of thin sections from control lungs (Figure 5(B)) shows that majorly club cells are present, and they are also associated with NEBs, and some mutilated cells are present in the distal airways. After 3 days of antibody-mediated inhibition of Notch (Figure 5(C)), we can see that the club cells have gained FOXJ1. After 7 days

of antibody-mediated inhibition of Notch (Figure 5(C)), we can see that the club cells have transdifferentiated into multiciliated cells. Note that the club cells surrounding NEBs do not transdifferentiate and remain CC10^{+}. This was observed in the terminal bronchioles too. Also, we know that these are the club cells that transdifferentiate into multiciliated cells because lineage tracing experiments were done with *scgb1a1* cre labelled animals, and the multiciliated cells at 7 days post Notch inhibition are tdtomato positive.

Most of these cells that resist transdifferentiation are found at NEBs and terminal bronchioles. Next, to check whether these club cells that are resistant to transdifferentiation, express SPC, we stained the sections from 3 days and 7 days post Notch inhibition with SPC antibody.

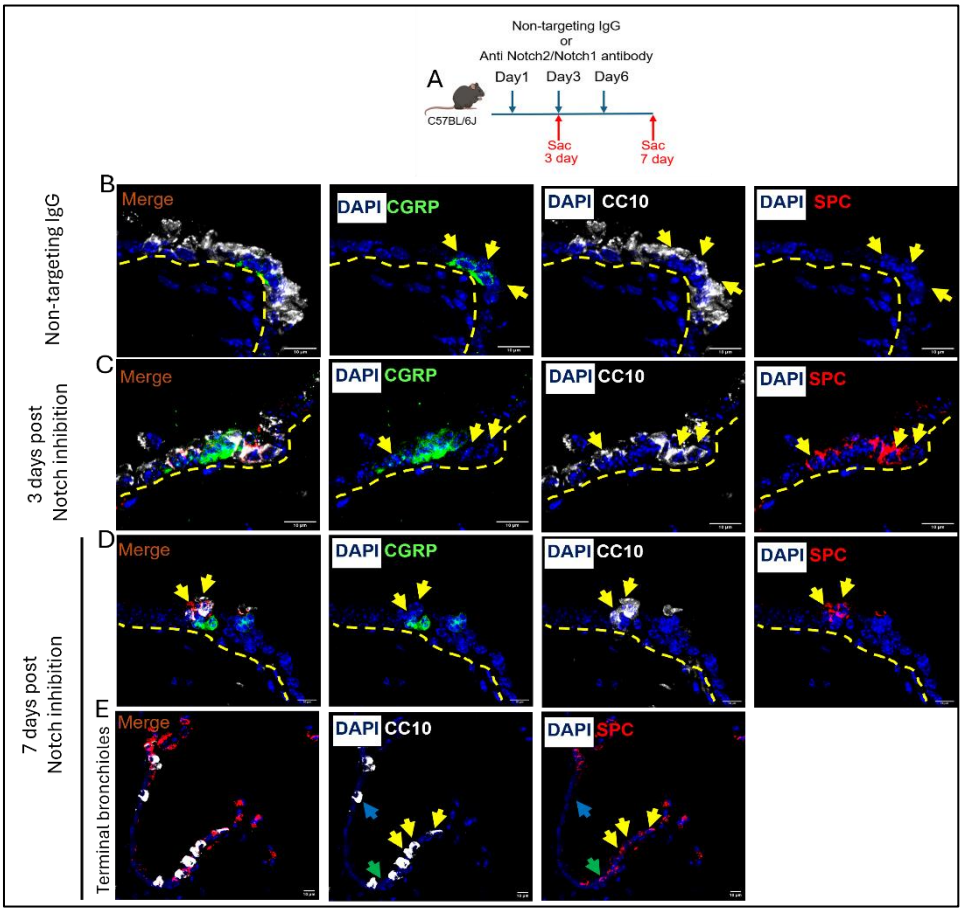

Figure6. The club cells surrounding NEBs that resist transdifferentiation resemble BASCs post Notch inhibition. (A) Schematic of the treatment administered using either non-targeting IgG or anti-Notch2/Notch1 antibodies. (B) Thin section of a control lung, showing the distribution of Alveolar type 2 cells (SPC, red) and club cells (CC10, white) and NEBs (CGRP, green). (C) Thin section of a 3 days post Notch inhibition lung, showing the distribution of Alveolar type 2 cells (SPC, red) and club cells (CC10, white) and NEBs (CGRP, green). Club cells associated with

NEBs become double positive state ($CC10^+$, SPC^+), post 3 days Notch inhibition. (D) Thin section of a 7 days post Notch inhibition lung, showing the distribution of alveolar type 2 cells (SPC, red) and club cells (CC10, white) and NEBs (CGRP, green). Club cells associated with NEBs co-express CC10 and SPC, post 7 days Notch inhibition. (E) Thin section of a 7 days post Notch inhibition lung, showing the distribution of alveolar type 2 cells (SPC, red) and club cells (CC10, white) in the terminal bronchioles. Club cells in the terminal bronchioles co-express CC10 and SPC, post 7 days Notch inhibition. Yellow dashed line indicates the airway epithelium. Yellow arrows point to the double positive CC10, SPC cells at 3- and 7-days post Notch inhibition. Green arrows point to the cell that is SPC^+ but $CC10^-$. Blue arrows point to the cell that are $CC10^+$ but SPC^-. Scale bar- 10µm.

Immunostaining of thin sections from control lungs (Figure 6(B)) shows that there is no SPC expression in the airway regions. We can see the appearance of CC10 and SPC double-positive cells at 3 days post Notch inhibition (Figure 6(C)), and these cells remain double positive after 7 days of Notch inhibition(Figure 6(D)). There are some cells post Notch inhibition that are $CC10^+$ SPC^- or $CC10^-$ SPC^+, but the majority of them are $CC10^+$ SPC^+. The double-positive CC10 and SPC resemble BASCs because of the co-expression of CC10 and SPC. These data align with the recent findings from the lab (Lingamallu SM et al., 2024).

3.2 Characterization of BASCs derived from variant club cells that escape transdifferentiation upon inhibition of Notch signaling.

Next, we wanted to characterize these BASCs derived from club cells to check what other markers these cells express. As they express one of the definitive markers for both club and alveolar type 2 cells, we wanted to check if we can find any other known markers for either of these cell types. To do that, we immunostained the thin sections from 3- and 7- days post Notch inhibition with SCGB3A2(another club cell marker) and ABCA3 (another alveolar type 2 cell marker).

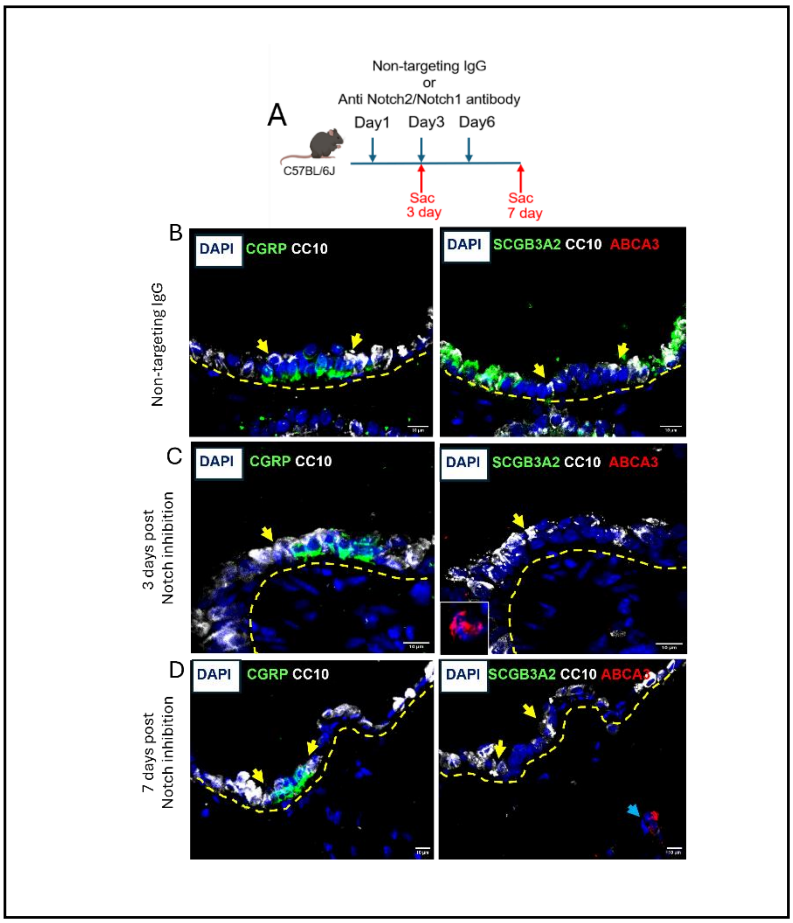

Figure7. The BASCs derived from variant club cells do not express SCGB3A2 and ABCA3 post Notch inhibition. (A) Schematic of the treatment administered using either non-targeting IgG or anti-Notch2/Notch1 antibodies. (B) Thin section of a control lung, showing the distribution of club cells(CGRP (green), CC10 (white) and CC10 (white), SCGB3A2(green), ABCA3(red) on serial tissue sections. (C) Thin section of a 3 days post Notch inhibition lung, showing the distribution of club cells(CGRP (green), CC10 (white) and CC10 (white), SCGB3A2(green), ABCA3(red) on serial tissue sections. The insert on the left side shows the AT2 cell(ABCA3 (red)) Post 3 days of Notch inhibition the club cells have lost their SCGB3A2 expression and do not express ABCA3. (D) Thin section of a 3 days post Notch inhibition lung, showing the distribution of club cells(CGRP (green), CC10 (white) and CC10 (white), SCGB3A2(green), ABCA3(red) on serial tissue sections. Note that the post 7 days of Notch inhibition the club cells do not express neither SCGB3A2 nor ABCA3. Yellow dashed line indicates the airway epithelium. Yellow arrows point to the club cells associated with NEBs. Blue arrows point to the AT2 cells. Scale bar- 10µm.

Immunostaining of thin serial sections from control lungs (Figure 7(B)) shows that almost all the club cells express CC10 and SCGB3A2 in the airway regions. The club cells associated with NEBs too. We see that the remaining club cells that resist transdifferentiation in the distal airways, though they express CC10, have lost their SCGB3A2 expression after 3 days of Notch inhibition(Figure 7(C)) and do not express SCGB3A2 post 7 days of Notch inhibition(Figure 7(D)). We also show that the remaining club cells that resist transdifferentiation though they expresss SPC, do not express ABCA3 at any time point in the airways (Figure 7(C,D)). Though these BASC resembling cells express one of the club cell or AT2 cell marker, they do not express the other definitive marker of either of these two cell types.

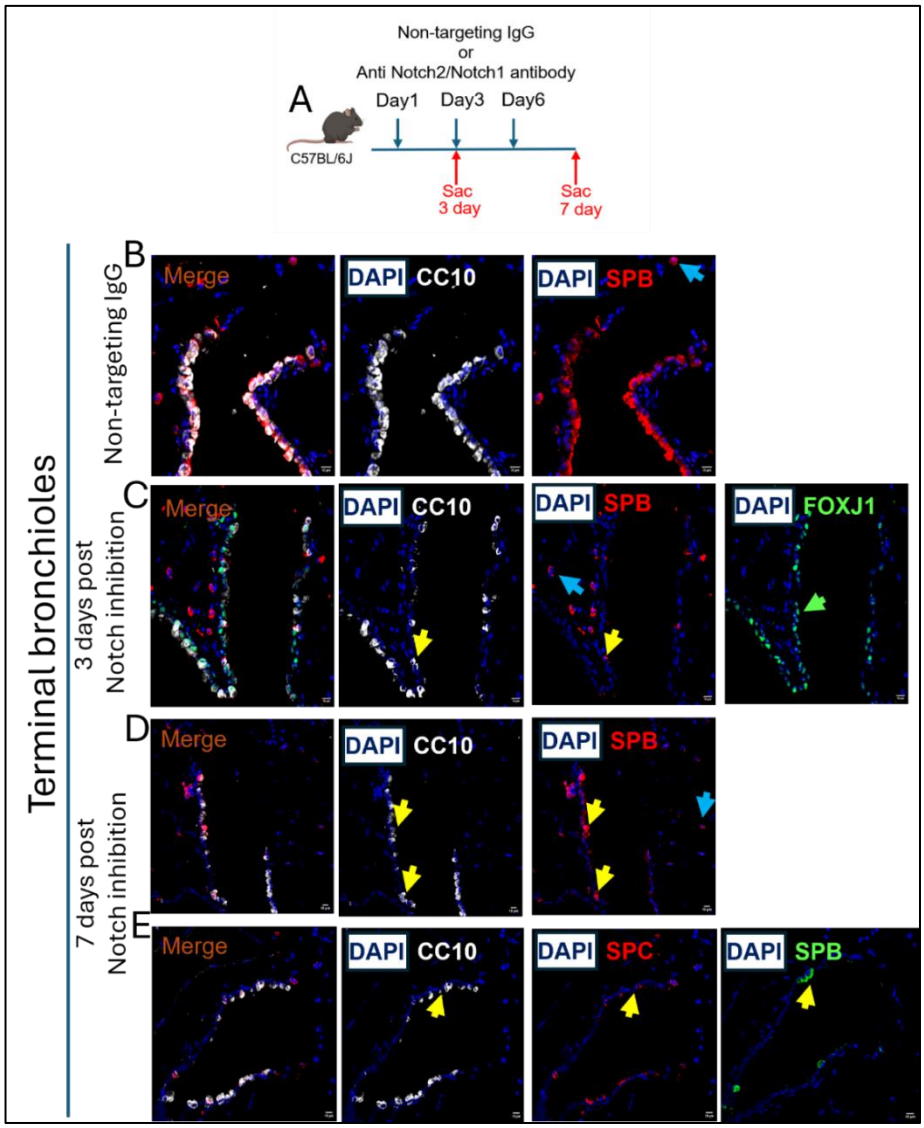

Figure8. Some of the BASCs derived from variant club cells express SFTPB(SPB). (A) Schematic of the treatment administered using either non-targeting IgG or anti-Notch2/Notch1 antibodies. (B) Thin section of a control lung, showing the

distribution of club cells (CC10 (white)) in the terminal bronchioles. (C) Thin section of a 3 day post Notch inhibition lung, showing the distribution of club cells (CC10 (white)) and multiciliated cell (FOXJ1 (green)) in the terminal bronchioles. Post 3 days of notch inhibition, many of the club cells that resist transdifferentiation into multiciliated cells express SPB. (D) Thin section of a 7 day post Notch inhibition lung, showing the distribution of club cells (CC10 (white)) in the terminal bronchioles. Post 7 days of notch inhibition, many of the club cells that resist transdifferentiation into multiciliated cells express SPB. (E) Thin section of a 7 day post Notch inhibition lung, showing the distribution of club cells (CC10 (white)) in the terminal bronchioles. Note that post 7 days of notch inhibition, some of the $CC10^{+}$ SPC^{+} cells express SPB. Yellow arrows point to the club cells that express SPB. Green arrow points to the muliticilated cells. Blue arrow points to the AT2 cell. Scale bar- 10µm.

We also tried the immunostaining of the sections post 3 and 7 days of Notch inhibition with SPB(marker for AT2 cells). There are club cells which express SPB post 3 and 7 day Notch ihhibition (Figure 7(C,D)). The multiiciliated cells do not express the SPB (Figure7(C)). Some of the $CC10^{+}$ SPC^{+} cells also express SPB (Figure7 (E)). So, But, as during homeostasis too, we can see the expression of SPB in most of the club cells, therefore, we cannot conclude SPB as the marker for $CC10^{+}SPC^{+}$ cells.

3.3 Single cell RNA sequencing data reveals that BASCs derived from club cells correlate with known BASCs better than other known differentiated cell populations.

As these $CC10^{+}$ SPC^{+} cells resemble BASCs, and there is evidence in the lab that these BASCs derived from club cells aid in alveolar repair. We then took a single cell RNA sequencing (scRNA seq) approach to address the following questions. What are these cells transcriptomically? What is their gene expression profile? How do they correlate with all other known cell types of lung airway epithelium? As these cells are phenotypically like BASCs are they similar to them at transcriptomic level too?

For this, we did a scRNA analysis of 3 days post Notch inhibition and 7 days post Notch inhibition and wnt activation (we get more number of these double positive cells in this condition (Lingamallu SM et al., 2024). We integrated the single cell RNA data from Liu et al., 2019 (for BASCs reference) with our data set for the analysis.

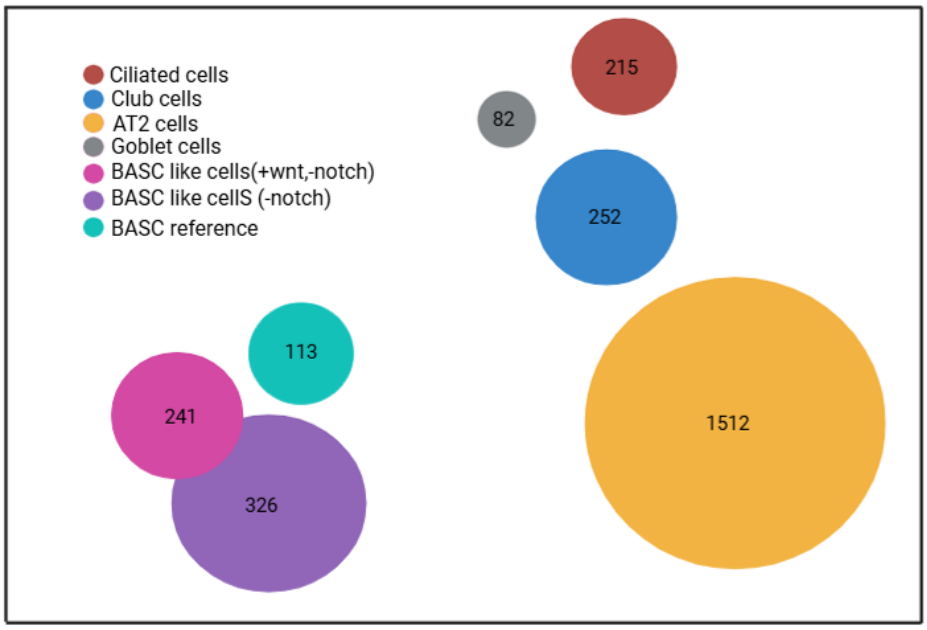

Figure9. Schematic of UMAP clustering of different cell populations from the single cell RNA sequencing analysis.

By the single cell data, we got clusters of cells as depicted in (Figure 9). The numbers inside the clusters represent the number of cells we got for the different cell types from the single cell data analysis. The unsupervised UMAP clustering shows that the double positive cells from the 3 days post Notch inhibition and 7 days post Notch inhibition and Wnt activation cluster closely as compared to the other cell types, indicating that the cells from these two conditions share similar gene expressions. Also, the cells from these two conditions together cluster close to the reference BASCs cell population(Lui et al.).

	AT2 Cells	BASC(Anti -notch)	BASCs(Liu. et al)	BASCs(anti -notch, wnt activated)	Ciliated cell	Club cell	Goblet cell
AT2 Cells	1	-0.17	-0.41	-0.08	-0.14	-0.07	-0.012
BASC(Anti -notch)	-0.17	1	0.24	0.74	-0.64	-0.71	-0.65
BASCs(Liu. et al)	-0.41	0.24	1	0.21	-0.48	-0.32	-0.32
BASCs(anti -notch, wnt activated)	-0.08	0.74	0.21	1	-0.68	-0.64	-0.69
Ciliated cell	-0.12	-0.64	-0.48	-0.68	1	0.54	-0.54
Club cell	-0.07	-0.71	-0.32	-0.64	0.54	1	-0.54
Goblet cell	-0.12	-0.65	-0.326	-0.69	0.54	0.54	1

Figure10. Table showing the correlation matrix of different cell types.

The correlation matrix (figure 10) shows the correlation value between different cell types. BASCs cell population shows a strong positive correlation with BASCs (Anti-Notch, Wnt activated), a correlation value of 0.74, suggesting a close relationship between these populations. Again, this emphasizes that the double positive cells formed in the Notch inhibited and Notch inhibited, Wnt activated conditions are similar.

BASCs from both Notch inhibited, and Notch inhibited plus Wnt activated condition have a moderate positive correlation with BASCs from Liu.et al., 2019 indicating that the BASCs derived from variant club cells resemble more closely to the BASCs than any other cell type. Though at the protein level BASCs derived from variant club cells express CC10 and SPC, but they correlate poorly with both the club cells as well as AT2 cells. The negative correlation of BASCs derived from variant club cells from both Notch inhibited and Notch inhibited plus Wnt activated conditions with all other cell types suggests that these double-positive cell populations do not show similar gene signature profiles with any of the known differentiated cell types in the lung epithelium.

4. Conclusion

Upon antibody-mediated Notch inhibition, most of the bulk club cells transdifferentiate into multiciliated cells, but a subset of club cells resists this transdifferentiation, termed as variant-club cells. These variant club cells transition into a dual identity cell state, which resembles BASCs. We used an immunostainings-based approach to look at whether BASCs derived from variant club cells express features of either club cells or alveolar type 2 cell or both. Also, we isolated cells from lungs that have been treated with the Notch antibodies to analyze the transcriptomics and characterize this cell population by using *cc10* and *spc* to identify this cell population. Our findings from both the approaches reveal that the BASCs derived from club cells adopt transient stable state and do not resemble any of the known differentiated cell types in the lung.

Notably, these BASCs derived from variant club cells can revert to the variant club cell state when Notch signaling is restored and proliferates to restore cellular balance in the airway. This suggests that specific lung niches harbor variant club cells capable of entering a stable cell state similar to BASCs, from which they can either return to their original identity or generate other epithelial cell types upon injury, contributing to lung regeneration.

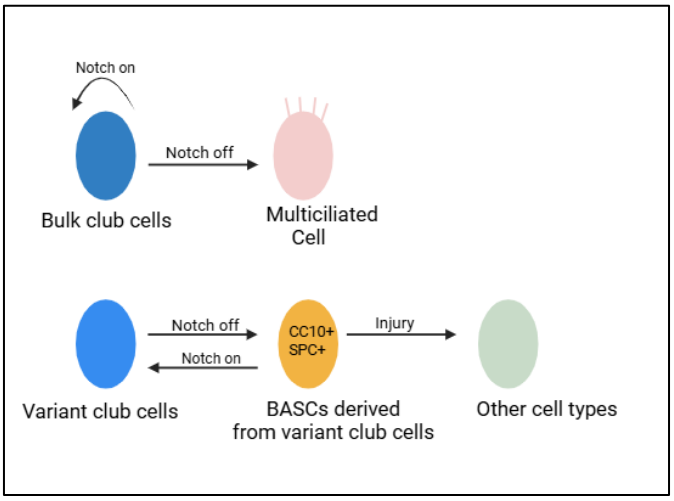

Figure11. Model figure

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