ABSTRACT

CASEY, ASHLEIGH. Elucidating the Role of Canonical Wnt/β-catenin Signaling in Primary Human Airway Basal Cell Proliferation and Mucociliary Differentiation Capacity *in vitro*. (Under the direction of Dr. Bala Rao).

The objective of this study is to better understand human airway epithelial basal cell biology by enhancing current knowledge surrounding the canonical Wnt signaling pathway in the airway niche, which has been previously implicated in proliferation and mucociliary differentiation. The strategy presented in this thesis is to develop a media formulation exploiting canonical Wnt activity to promote proliferation and maintain differentiation capacity of primary human small airway epithelial (SAEP) cells *in vitro*. This approach will require cells to undergo continuous self-renewal while retaining their functional ability to reconstitute the pseudostratified epithelium via multipotent differentiation. It was hypothesized that exposure to GSK3-inhibitors during the cell growth phase would enhance Wnt activity, thereby promoting proliferation of the p63+ basal cell population with an enhanced capacity to undergo differentiation into non-basal subtypes once exposed to ALI culture. The first aim was to develop a culture platform, primarily through the development of a control media formulation to serve as a baseline for assessing Wnt activity through the addition of GSK3-inhibitors, changes in basal media, and serum reduction. After finalizing the control formulation, the impact of Wnt on basal cell proliferation was evaluated using nuclear counting methods to calculate total cell yield, along with immunocytochemistry to quantify the proportion of proliferating basal cells. Differentiation capacity was assessed using air-liquid interface (ALI) cultures and subsequent immunofluorescent imaging to distinguish expression levels of the non-basal subtypes. Overall, SAEP cells expanded in the presence of Wnt agonists expressed the highest transcription levels of downstream Wnt targets, demonstrated nuclear stabilization of β-catenin, generated a greater yield of p63+ basal cells in planar culture, and effectively reconstituted the pseudostratified epithelium in ALI.

Elucidating the Role of Canonical Wnt/β-Catenin Signaling in Primary Human Airway Basal Cell Proliferation and Mucociliary Differentiation Capacity *in vitro*

by
Ashleigh Casey

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Biomanufacturing

Raleigh, North Carolina

2021

APPROVED BY:

_____	_____
Dr. Bala Rao Committee Chair	Dr. Laila Roudsari External Member
_____	_____
Dr. Scott Randell	Dr. Roger Ilagan External Member
_____	
Dr. Scott Magness	

BIOGRAPHY

Ashleigh Casey received her Bachelor of Science degree in Microbiology from North Carolina State University in 2016. As an undergrad, she held internships at Novozymes in the Microbiology QC group and Grifols in the Bioanalytics R&D department. After graduating, she was hired as a Research Technician in the Regenerative Medicine Lab at United Therapeutics, where she now works as a Research Associate II in the Cell Biology R&D group. To further her career development and gain industry knowledge, Ashleigh enrolled part-time in the Biomanufacturing graduate program at NCSU's Biotechnology Education Center (BTEC) with a concentration in the upstream track. Despite an upstream concentration, her thesis research secured her interest in understanding the mechanisms underlying airway basal cell function.

ACKNOWLEDGEMENTS

First and foremost, I want to give sincere thanks to Laila for her constant support and mentorship throughout this effort, which has undoubtedly shaped me into a better, more thoughtful researcher.

I also want to thank my committee members Dr. Roger Ilagan, Dr. Scott Randell, Dr. Scott Magness, and Dr. Bala Rao for their time, guidance, and advice, which has steered me in the right direction.

Finally, I would like to acknowledge my husband, Ben, for his continued support, even though he has expressly stated that he does not plan to read this thesis (in his own words: “I love you, but don’t ask me to read this”).

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CHAPTER 1

Background

The lung is a functionally intricate organ which, at its most proximal region, acts as a first line defense against airborne pathogens and irritants (Haas et al., 2019; Schmid et al., 2017; Rock, Randell, & Hogan, 2010). This defense is facilitated by the resident multipotent progenitor population, or airway basal cells, which respond during natural turnover or following injury by replenishing the highly specialized cell populations that comprise the pseudostratified epithelium (Rock, Randell, & Hogan, 2010; Hackett et al., 2011; Walters et al., 2013). These airway basal cells are controlled through a balance of signaling pathways that regulate proliferation, self-renewal, and lineage commitment into non-basal subtypes. However, disruption of this balance can impede the ability for progenitor cells to maintain homeostasis of the lung epithelium, and therefore contribute to a multitude of epithelial abnormalities, including basal- and goblet-cell hyperplasia (the failure to differentiate), squamous- and goblet-cell metaplasia (abnormal differentiation), dysplasia (or neoplastic growth), and progression to malignant transformation (Rock, Randell, & Hogan, 2010; Schmid et al., 2017). The signaling mechanisms facilitating differentiation and proliferation of the human airway basal cell population during tissue repair have yet to be completely defined, leading to many unanswered questions surrounding the etiology of chronic lung disease (Rock, Randell, & Hogan, 2010; Huang et al., 2017). Many studies have implicated Wnt/β-catenin signaling in regeneration and remodeling of the airway epithelia, with particular importance placed on β-catenin activity in regulating basal cell fate (Haas et al., 2019; Aros et al., 2020; Huang et al., 2017; Kim et al., 2013; Hu et al., 2019; Schmid et al., 2017). However, these studies often yield conflicting results and the underlying mechanisms of the Wnt/β-catenin pathway remain elusive, particularly in the context of the adult human airway epithelia. Therefore, a better understanding of Wnt/β-catenin signaling could allow for tighter control over airway basal cell self-renewal and differentiation processes *in vitro* (Huang et al., 2017). This is of critical importance, as the ability to recapitulate a functional model of the human airway epithelia relies on the efficient and consistent generation of a highly functional progenitor population.

Despite the ability for epithelial basal cells to undergo continuous self-renewal during their lifespan *in vivo*, these same cells expand for a limited number of passages *in vitro* before succumbing to growth arrest (Walters et al., 2013; Zhang et al., 2018). This growth arrest greatly limits researchers' ability to obtain sufficient quantities of basal cells that maintain their original phenotype and function and is therefore a major constraint for being able to utilize airway basal cells for their regenerative potential. Encouraging progress has been made in overcoming these limitations, such as the use of feeder cells and Rho kinase inhibitor (Y-27632) to generate "conditionally reprogrammed" epithelial cells (CRC) with the ability to undergo continuous self-renewal in culture (Liu et al., 2017; Suprynowicz et al., 2012; Gentzsch et al., 2017; Bove et al., 2014). Often, these methods involve regulation of several major signaling pathways, primarily TGF-β, Wnt, and Notch (Zhang et al., 2018; Mou et al., 2016). Although highly effective, the use of feeder cells and manipulation of multiple pathways can complicate biological interpretations (i.e. cell signaling events), lead to functional abnormalities, and create additional regulatory hurdles for manufacturing cell therapy products (Zhang et al., 2018).

1.1 Specialized Cell Types of the Airway Epithelium

Like progenitor cells of other adult tissues and organs, airway epithelial basal cells (KRT5+, p63+) in the adult lung are a subset of undifferentiated, multipotent progenitor cells that exhibit the capacity to self-renew, proliferate, and give rise to various specialized cell types that comprise the pseudostratified airway epithelium (Levardon et al., 2018). The majority of the normal human airway is lined by a pseudostratified epithelium of ciliated cells, secretory cells, goblet cells, ionocytes, and basal cells located directly on the basement membrane (Rock, Randell, & Hogan, 2010; Walters et al., 2013). The proportional distribution of each cell type is dependent upon the position along the proximal-distal axis, with the basal cell population being especially variable, ranging anywhere between 6-30% of the total epithelial population depending on positioning (Rock, Randell, & Hogan, 2010).

1.1.1 Comparison of Human & Mouse Airway Structure

The canonical Wnt/β-catenin pathway is highly conserved among mammalian species, which has enabled the use of animal models to elucidate crucial discoveries regarding airway cell function and airway disease pathology (Rock, Randell, & Hogan, 2010). The pseudostratified epithelium of the genetically tractable mouse trachea, in particular, has served as a well-developed model

for these discoveries (Rock & Hogan, 2011). Functionally, basal cells found in the mouse trachea and human airway are similar in that they function as epithelial progenitors with the ability to self-renew and generate luminal daughters (Rock & Hogan, 2011). Despite similarities, there are some inherent differences in cellular distribution and general architecture between the mouse and human airways (**Fig. 1**). In humans, basal cells are present throughout the airways, even in the smaller, more distal bronchioles with diameters less than 0.5 mm. By contrast, basal cells in the mouse lung are confined to the trachea, mainstem bronchi, and the most proximal regions of the intralobar airways (Rock & Hogan, 2011).

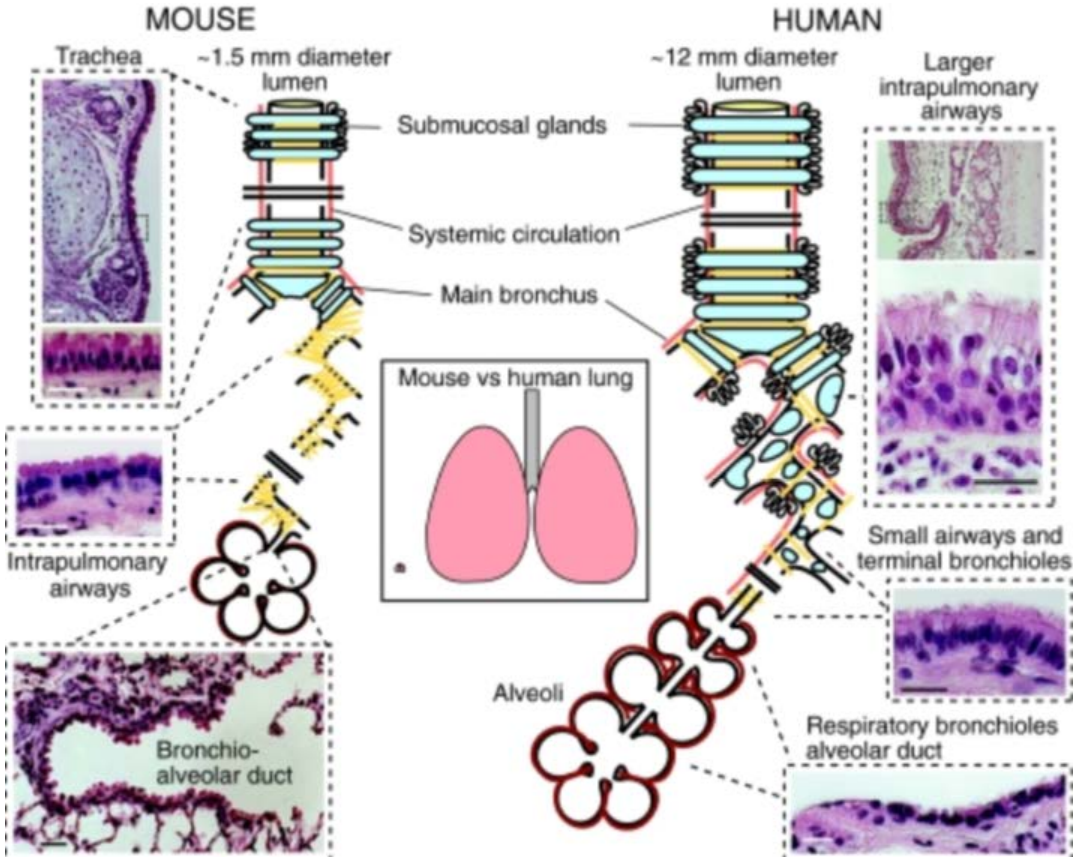

Figure 1: Comparison of lung structure and epithelial organization between human and mouse lungs. Figure copied from "Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling", by Rock, JR, Randell SH, and Hogan BL, 2010, *Dis Model Mech.* 3(9-10): p. 545-556

1.2 Overview of Canonical Wnt/β-catenin Signaling Pathway

The highly complex Canonical Wnt/β-catenin signaling pathway has long been implicated in controlling various biological processes via autocrine and paracrine activities in different organ systems, particularly related to cell fate decisions, spatial tissue patterning during development, and tissue repair in response to injury (Hu et al., 2019; Mou et al., 2016; Malsin et al., 2019). Wnts are glycoproteins secreted by various cell types within a local niche, primarily macrophages (Malsin et al., 2019) and distinct alveolar Lgr5+ and bronchiolar Lgr6+ mesenchymal populations (Kim et al., 2013; Lee et al., 2017). Wnt signaling can activate gene expression either through a β-catenin-dependent (canonical) or, β-catenin-independent (non-canonical) signaling pathway depending on the presence or absence of additional co-receptors. In the lung, canonical Wnt signaling has been known to play an important role in bronchiolar and alveolar biology (Aros et al., 2020) as one of the key reciprocal signals involved in branching morphogenesis (Rock & Hogan, 2011). In humans, 19 Wnt proteins have been identified (Malsin et al., 2019), each of which are thought to have different roles in relation to the regulation of stem cell function. In reference to the inherent biochemical differences among the Wnt proteins, Roel Nusse, a leader in the field of Wnt signaling asserts that “not all Wnt proteins are created equal” (Nusse, 2012). Historically, it has been understood that the extent to which β-catenin is activated during a Wnt response is dictated by the particular Wnt ligand being expressed. For example, Wnt3a and Wnt1 are thought to induce β-catenin activation in the canonical pathway, whereas others, including Wnt5a, Wnt11, and Wnt4, are thought to act antagonistically and/or independently of β-catenin (Malsin et al., 2019). More specifically, there is evidence to suggest that within the airway niche, Wnt7a and Wnt4 are the most important for regulating proliferation and self-renewal of airway basal cells (Schmid et al., 2017), whereas Wnt3a and Wnt5a have been associated with facilitating differentiation into the ciliated lineage (Aros et al., 2020). More recently, it has been shown that basal cells in the human airway primarily express Wnt7a whereas basal cells in the mouse airway also express Wnt3a, Wnt5b, and Wnt9a (Schmid et al., 2017).

1.2.1 Canonical Wnt Signaling Mechanism

After Wnt proteins are secreted from the niche-specific cell source, activation of canonical Wnt signaling is contingent upon the successful binding of Wnt proteins to frizzled transmembrane receptors and LRP5/6 co-receptors. Once activation of canonical Wnt signaling is initiated,

dishevelled (Dvl/Dsh) is recruited intracellularly to prevent phosphorylation of β-catenin at serine/threonine residues (41, 37, and 33) (Huelskin et al., 2000) by glycogen synthase kinase (GSK) 3, a component of the β-catenin degradation complex (APC/Axin/GSK). Inhibition of GSK3 activity allows for the stabilization of non-phosphorylated (active) β-catenin in the cytosol, where it accumulates before undergoing subsequent translocation into the nucleus. Once in the nucleus, β-catenin heterodimerizes with DNA binding proteins (LEF1 and TCF1) to transactivate Wnt target genes including *AXIN2*, *CCND1*, *CMYC*, and *MMP7*.

1.2.2 Modulation of Wnt Signaling

Over time, multiple strategies have been developed for studying Wnt proteins and their activity. One common method is the use of soluble Wnt proteins, such as Wnt3a, isolated from conditioned media of cells known to overexpress Wnt. Although this method has been used extensively, the conditioned media often contains contaminating proteins that may impede interpretations of Wnt-specific biological activity (Willert & Nusse, 2012). Alternatively, small molecules have proven to be a highly effective, tunable approach for modulating differentiation and cellular functions, and have been successfully utilized to improve the ability for stem cells to undergo a more efficient and directed differentiation process (Huang et al., 2017; Naujok et al., 2014). Many studies have demonstrated the utility of small molecules for modulating the canonical Wnt pathway. The most widely used class of small molecule agonists of Wnt are GSK3-inhibitors (Willert & Nusse, 2012), which act to inhibit GSK3 activity to prevent β-catenin degradation, thereby allowing β-catenin to undergo nuclear translocation and transactivate transcription of downstream Wnt target genes. However, GSK3 serves as a major signaling hub, playing a key role in integrating various upstream and downstream signaling pathways (Willert & Nusse, 2012; Ngkelo et al., 2015). Therefore, its inhibition could potentially alter the activity of other signaling pathways in addition to Wnt, including Hedgehog signaling and the insulin signaling pathway (Willert & Nusse, 2012). For this reason, the specific compound used for research studies must be carefully selected, and the potential impact on other major signaling pathways should be considered. It is also important to consider the concentration to be used, as small molecules often differ in activity and can induce side effects like cytotoxicity or dysregulation of other vital pathways. In previous comparative studies evaluating the effectiveness of various GSK3 inhibitors, it was determined that CHIR99021 led to the strongest induction of Wnt/β-catenin activity while also demonstrating the lowest cytotoxicity compared to

SB216763, BIO, TWS119, and CHIR98014 (Huang et al., 2017; Naujok et al., 2014). Therefore, due to its known effectiveness and minimal side effects when used at an appropriate concentration (typically around 3 μM), CHIR99021 was the primary Wnt agonist used in this thesis to determine whether enhanced Wnt signaling (via GSK3 inhibition) could be utilized for controlled regulation of long-term basal cell self-renewal and differentiation.

Table 1: Small molecule agonists and inhibitors of Wnt/B-catenin signaling.

Component	Wnt-related Function	Mechanism
CHIR99021	Agonist	Inhibits GSK3α and GSK3β
BIO	Agonist	Inhibits GSK3β and
SB216763	Agonist	Inhibits GSK3α and GSK3β
TWS119	Agonist	Inhibits GSK3β
CHIR98014	Agonist	Inhibits GSK3α and GSK3β
XAV939	Inhibitor	Inhibits TNKS activity
IWR-1-endo	Inhibitor	Stabilizes AXIN2

1.3 Purpose of Research

The intent of this research was to inform the development of a media formulation to culture and expand human airway basal stem cells while preserving their stem cell properties and capacity for subsequent mucociliary differentiation. The long-term culture of these progenitor cells has proved challenging due to spontaneous differentiation and short-term self-renewal capacity *in vitro*, especially at later passages (> passage 2). This outcome highlights the importance of understanding of key signals required to overcome these current limitations by fostering a culture environment in which basal cells can maintain physiological function and long-term proliferation so that these cells that may be used to enhance the current understanding of lung disease etiology, cell function, and epithelial repair mechanisms.

Despite the great progress that has been made in overcoming these challenges, like development of the CRC method and dual-SMAD inhibition (Zhang et al. 2018; Mou et al., 2016), relatively little is known about airway basal cell biology and the underlying signaling mechanisms that regulate their behavior (Rock & Hogan, 2009). In particular, the use of GSK3-inhibition to specifically regulate adult human airway basal cell growth and function remains poorly defined, thus leaving an open area for investigation. Therefore, this thesis was focused on elucidating the role of the Wnt/β-catenin pathway in primary human airway basal epithelial cells *in vitro*. For this thesis, the following aims were proposed: 1) Establish a culture platform to serve as a baseline for assessing Wnt activity. A simplified control media formulation will be established to eliminate interference of extraneous cell signaling, allowing for the isolation of Wnt-derived cell behavior. 2) Test the hypothesis that the self-renewal of human airway epithelial basal cells in vitro is regulated by canonical Wnt signaling. Proliferation of the p63+ basal population will be assessed after exposure to Wnt agonists and inhibitors. 3) Test the hypothesis that canonical Wnt signaling is required for efficient differentiation into specialized non-basal cell types. After treatment with various Wnt agonists in culture, differentiation capacity into ciliated and secretory subtypes will be assessed in air-liquid interface culture. The studies proposed in Aims 1 and 2 will establish the relationship between canonical Wnt activity and the proliferative capacity of $p63^{+}$ airway basal cell population. Through testing the impact of Wnt signaling on the fundamental stem cell property of self-renewal, the culture system and media formulation developed through these experiments will be utilized to address the third and final aim. Aim 3 will provide information regarding the role of canonical Wnt signaling in the regulation of airway epithelial cell differentiation capacity. Together, these studies will enhance the contextualized understanding of canonical Wnt activity in the human airway niche and offer useful research tools for recapitulating a biologically relevant human airway epithelium *in vitro*.

CHAPTER 2

Materials & Methods

2.1 Cell Sourcing

The research presented in this paper was conducted on primary human small airway epithelial (SAEP) cells. The cells were purchased from the American Type Culture Collection (ATCC) at passage 0 (one expansion after initial isolation). Although the full detailed isolation procedure remains unclear due to its proprietary nature, it is known that the cells are isolated from a portion of airway below the trachea and main bronchus.

2.2 Primary Airway Basal Cell Culture

This section outlines the materials and methods used to culture the SAEP cells for developing a base media formulation and generating cell samples used for subsequent experiments to isolate the role of Wnt signaling.

2.2.1 Description of Cell Culture Procedure

SAEP cells obtained from ATCC at passage 0 were grown in collagen 1-coated (rat tail, Corning) T75-flasks (Corning Biocoat). After thaw, the cells were seeded at a density between 3000-5000 cells/cm^2 and incubated at a regulated temperature of 37°C and 5% CO_2. Cell culture medium was exchanged on Day 3 of culture. Harvest occurred on Day 5-7 of culture when the cells had reached ~60-80% confluence. At harvest, the flasks were rinsed with DPBS followed by detachment via pre-warmed trypsin-EDTA (0.25%) (Gibco). After the cells had detached from the flask surface, quench solution of DPBS + 5% Fetal Bovine Serum (FBS; Gibco) was added to the flasks and the cell suspension was collected in a sterile 15 mL conical tube. To concentrate the cells into a pellet, the cell suspension was centrifuged at 300 x g for 5 minutes. Supernatant was aspirated and the cells were suspended in DMEM/F-12 or Advanced DMEM-F12 prior to counting on the Nexcelom Cellometer using ViaStain™ Acridine Orange/Propidium Iodide (AO/PI) staining solution (Nexcelom).

2.3 Analytical Methods

Various analytical methods were used to compare and assess the impact of media components on Wnt activity, basal cell proliferation, and differentiation capacity. Wnt activity was assessed through qRT-PCR by measuring transcription levels of downstream Wnt target genes, along with

western blot to confirm nuclear stabilization of β-catenin. Changes in cell number were determined via nuclei quantification after growth in planar culture, whereas specific changes in basal cell proliferation were assessed via fluorescent staining. Air Liquid Interface (ALI) culture was the method used for analyzing the ability of the cells to undergo mucociliary differentiation to form a pseudostratified epithelium.

2.3.1 Western Blot

Cell lysate was prepared by adding 500 µL Pierce™ RIPA buffer to the cell pellet (~1e6 cells) for 15 minutes on ice. The lysate was clarified via centrifugation (10,000 x g for 5 minutes). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo). After adding 1X and 10X reducing agents, the samples were heated for 10 minutes at 70°C. Protein samples were loaded at 20 µg into Bolt™ 8% Bis-Tris Plus (Invitrogen) mini gels using the Thermo mini tank system with NuPAGE MOPS SDS Running Buffer (20X). Anti-β-actin was used for loading control. After loading the samples and 5µL of Novex™ Sharp Pre-stained protein standard, the gels were run for one hour at a constant voltage of 165V. The gels were then transferred onto membranes using the Invitrogen iBlot 2 membrane transfer system**.** The membranes were blocked with 5% non-fat milk in DPBS + 0.05% Tween-20 (DPBS-T) for one hour at room temperature while rocking. Primary antibody dilutions were prepared in 1% non-fat milk in DPBS-T buffer and the membranes were incubated overnight at 4°C while rocking. The following day, the membranes were washed for five minutes with DPBS-T (x3) to remove unbound/excess primary antibody and incubated with a secondary horseradish peroxidase-labeled antibody (1:5000). The membranes were washed an additional 3 times before applying Pierce ECL substrate to detect chemiluminescent signal using the Syngene G:BOX.

Table 2: Antibodies used for western blot analysis.

Antibody	Source	Catalog #	Clone	MW (kDa)	Dilution
β-actin	Abcam	ab8226	mAbcam8226	~42	1:2000
β-catenin	Cell Signaling Technology	8480	D10A8	~85	1:1000
p63-α	Cell Signaling Technology	13109	D2K8X	~75	1:1000
TCF1/TCF7	Cell Signaling Technology	2203	C63D9	~48,50	1:1000

2.3.1.1 Nuclear & Cytoplasmic Extraction

Nuclear and cytoplasmic protein fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo) to assess β-catenin stabilization and localization via western blot. After treatment with various media formulations, cell pellets (1 x 10^6 cells) were collected via trypsinization and centrifugation. On ice, the first two reagents (CER I and CER II) were added to the cell pellet to initiate cell membrane disruption and subsequent release of cytoplasmic contents. After microcentrifugation (~16,000 x g for 5 minutes) the supernatant (cytoplasmic proteins) was transferred to a separate, clean tube and the intact nuclei were recovered through microcentrifugation (~16,000 x g for 5 minutes). The third reagent (NER) was added to extract the nuclear proteins, which were recovered after another round of microcentrifugation (~16,000 x g for 5 minutes).

2.3.2 Quantitative RT-PCR

Quantitative real-time PCR was performed to determine the mRNA expression of known downstream Wnt target genes in the epithelial compartment. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and quantified with a NanoDrop 1000. cDNA was synthesized from total RNA using random hexamer primers per manufacturer's instructions targeting 20-100 ng per reaction. mRNA of interest was quantified by quantitative RT-PCR using a BioRad iCycler and the following TaqMan gene expression probes: *AXIN2* (Hs00610344_m1), *TCF7*

(Hs01556515_m1)**,** and *MMP7* (Hs01042796_m1). Fold change in expression was normalized against the PPIA housekeeping gene and calculated using the $\Delta\Delta C_t$ method.

2.3.3 Immunocytochemistry (ICC)

Cell cultures in 24-well plates were rinsed with 1mL DPBS/well and fixed with 4% PFA for 15 minutes at room temperature. After fixing, the cells were permeabilized with 0.2% Triton-X buffer for 7 minutes at room temperature and blocked for 1.5 hours in ICC blocking buffer (0.1% Triton-X + 2% goat serum + 5% BSA). Primary antibodies were diluted in blocking buffer and 1mL was added to each well and set to incubate overnight at 4℃. The following day, the primary antibodies were removed, and each well was rinsed with 1mL DPBS (x2). The secondary antibody dilutions were prepared 1:500 in ICC blocking buffer and 1mL was added to each well. The samples were incubated in the dark at room temperature for 1 hour before washing with 1mL DPBS (x2) and counterstaining cell nuclei with DAPI (prepared 1:1000 in DPBS) for an addition hour in the dark at room temperature. Fluorescence imaging was performed on the Leica DMI 6000SD inverted fluorescence microscope at 20X magnification.

2.3.4 Celígo Cell Quantification

Cell nuclei were visualized using a Hoechst staining solution prepared at a concentration of 1 µg/mL in DPBS. After ensuring the solution was thoroughly mixed, 1 mL was added to each well of the 24-well plate. Incubation took place in the dark for 10 minutes at room temperature before running a whole-well cell quantification assay on the Celígo ® (Nexcelom).

2.3.5 Air Liquid Interface (ALI) Culture

The ALI assay is an effective culture method used to study the capacity of human airway basal cells to differentiate into non-basal subtypes. To initiate the culture, undifferentiated SAEP cells were seeded onto a polyester membrane (0.40 µM pore size, 0.33 cm^2) of Costar transwell inserts (Corning) at a density of ~600,000 cells/cm^2. The cells were cultured for an additional two days in their respective growth medium to allow for monolayer formation on the transwell membrane. Once the cells reached confluence, the media was removed from the upper chamber to expose the apical surface to air and establish the ALI (referred to as ALI ‘‘day 1’’). The cells were then grown at a regulated temperature of 37℃, 5% CO_2, and the culture medium was changed every other day for a total of 14 days until the cultures were fixed with 4% paraformaldehyde for 30 minutes. Following fixation, the membranes were processed for whole-

membrane analysis via immunocytochemistry with an anti-CC10 and anti-acetylated-tubulin antibody to assess differentiation into secretory or ciliated cells, respectively. The cells were permeabilized with 0.2% triton X-100 for 7 minutes at room temperature, then blocked in 0.1% tritonX-100 + 5% BSA + 2% normal goat serum for 1.5 hours at room temperature. The samples were stained for the presence of ciliated and secretory cells using mouse IgG2b acetylated-tubulin (1:100) and rabbit polyclonal CC10 (1:200) primary antibodies incubated overnight at 4°C. APC-conjugated goat anti-rabbit H+L and AF488-conjugated goat anti-mouse H+L were the secondary antibodies used. The sections were counterstained with DAPI to identify cell nuclei. Upon completion of staining, a scalpel was used to cut the membrane from the transwell insert, which was then placed cell-side-down on a drop of DPBS in a chamber slide. Immunofluorescence microscopy was performed using a Leica DMI 6000SD inverted fluorescence microscope.

2.4 Analysis Software

2.4.1 Statistical Analysis

GraphPad Prism Software was used to conduct statistical analysis. Results were compared by one-way ANOVA followed by parametric or non-parametric analysis for comparison of individual groups if a significant difference was found. In some instances, t-tests were used to compare groups, as indicated in the figure description. Comparisons with p-value of <0.05 were accepted as statistically significant.

2.4.2 Image Quantification

ImageJ (FIJI) software (downloaded from https://imagej.nih.gov/ij/) was used for removing fluorescent background from images, preparing multi-channel overlays, and cell quantification for which a macro was developed. In brief, the .tiff images were first converted to a16-bit format before being changed into binary images. The “fill holes” and “watershed” tools were then used to better define cell boundaries, thereby preventing one cell from being quantified as multiple and/or cell clusters from being quantified as a single cell. The final step was to run “3D object counter” analysis to obtain cell counts.

CHAPTER 3

Development of Culture System (Aim 1)

3.1 Purpose of Experiments

This chapter details the development of a simplified control media formulation to serve as the baseline for experiments. Rather than using a commercially available airway epithelial growth medium as the control for this study, a simplified control media was developed to contain as few components as possible to minimize extraneous influences on other major signaling pathways. Unlike commercially available growth media, the intent of this formulation was not to maximize growth, but rather to support cell survival and promote enough proliferation of the SAEP cells to achieve sufficient cell yield for performing downstream assays. The purpose of avoiding maximal cell growth in the control was to ensure that the role of Wnt on basal cell proliferation could be easily evaluated after the addition of Wnt agonists despite changes in base media, serum concentration, or the addition of other growth factors.

3.2 Control Media Formulation

3.2.1 Selection of Basal Medium

DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) with GlutaMAX^{TM} is a widely used basal medium used to support growth of various mammalian cell types including MDCK, glial cells, fibroblasts, and bronchial epithelial cells (Brockman-Schneider et al., 2008; Gillette et al., 2013; Ulrich et al., 2004). It is a commercially available 1:1 mixture of DMEM and Ham's F-12, combining DMEM's high concentrations of glucose, amino acids, and vitamins with F-12's wide variety of components (Gibco). The addition of GlutaMAX ^{TM} supplement enhances culture stability by minimizing toxic ammonia build-up, thereby improving cell growth and viability. However, since this formulation does not contain any proteins, lipids, or growth factors, the media by itself is often not capable of promoting sufficient cell growth, in which case additional supplementation is required. LHC (Gibco) is another commonly used serum-free base media designed for bronchial epithelial cell cultures and has a lower $CaCl_2$ concentration than DMEM/F12. However, extracellular Ca^{2+} has been shown to influence nuclear trafficking of β-catenin and subsequent Wnt target gene expression (Nusse & Clevers, 2017). To ensure that the effects of low extracellular Ca^{2+} would not overshadow the activity of Wnt agonists, as was

suspected during the development of EpiX medium (Zhang et al., 2018), DMEM/F12 was selected as the base medium for the control.

3.2.2 Determining Optimal Serum Concentration

Since DMEM/F12 does not contain any lipids, proteins, or growth factors, the manufacturer recommends supplementation with fetal bovine serum (FBS) to promote cell growth. Therefore, a serum titration study was performed to determine the optimal concentration of FBS for the media formulation. For this experiment, three concentrations of FBS (Gibco) were tested: 2%, 5%, and 10%. The SAEP cells were seeded at a density of ~3000 cells/cm2 in T75 flasks (collagen 1coated), one flask per condition. Despite being fed with freshly prepared media on Day 4 of culture, it appeared that the cells were failing to proliferate in all conditions and were beginning to detach from the flask. The cultures were carried out for an addition 3 days (7 days total) before the cells were collected via trypsinization despite being <5% confluent (**Fig. 2A**). After collection, live cell nuclei were quantified using the Nexcelom Cellometer (**Fig. 2B**). A very slight increase in growth was seen with the addition of 5% and 10% FBS, although the total live cell yield from these conditions was less than 250 cells/cm^2 which is insufficient for performing any downstream assays. Since 5% and 10% serum were remarkably similar in outcome, either concentration would have been a suitable choice for the media formulation. However, in both cases, supplementation with additional components would need to be tested to further promote growth. To minimize any potential influence that serum may have on cell signaling pathways, 5% FBS was chosen as the concentration for the control media moving forward.

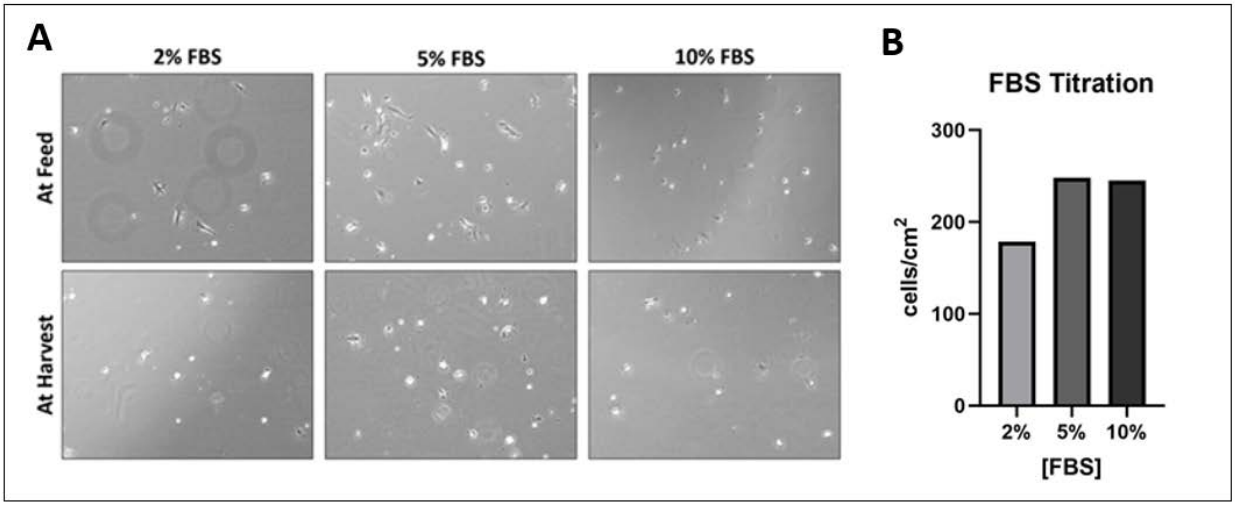

Figure 2: Serum titration study to determine optimal concentration for control media. SAEP cells were cultured in T75 flasks (n=1) for 7 days in media containing different concentrations of FBS: 2%, 5%, 10%. After 7 days of culture, the cells were <5% confluent (2A). Cell quantification (2B) shows comparable growth between conditions, with 5% and 10% FBS resulting in a slightly higher cell yield compared to 2% FBS.

3.2.3 Development of Serum-Free Formulation

The presence of serum in cell culture media is known to affect proliferation and differentiation capacity of stem cells. Before moving forward with isolating the impact of Wnt activity on airway basal cells, it was important to confirm that the 5% FBS in the control media was not interfering with signal transduction. To test this, SAEP cells were cultured in media containing two different concentrations of FBS (2% and 5%) with and without CHIR99021. As predicted, the SAEP cells grown in serum-containing media without CHIR99021 demonstrated the lowest expression levels of three main Wnt target genes *MMP7*, *TCF7*, and *AXIN2* **(Fig. 3)** compared to the control and CHIR99021-only conditions. The highest gene expression was seen in the CHIR99021-only condition, with the most notable difference in *AXIN2*, which showed a fold change of ~50. These results suggest that FBS abrogates Wnt activity in culture, even at low concentrations, and therefore should be reduced or removed altogether from the control media to maximize Wnt activity.

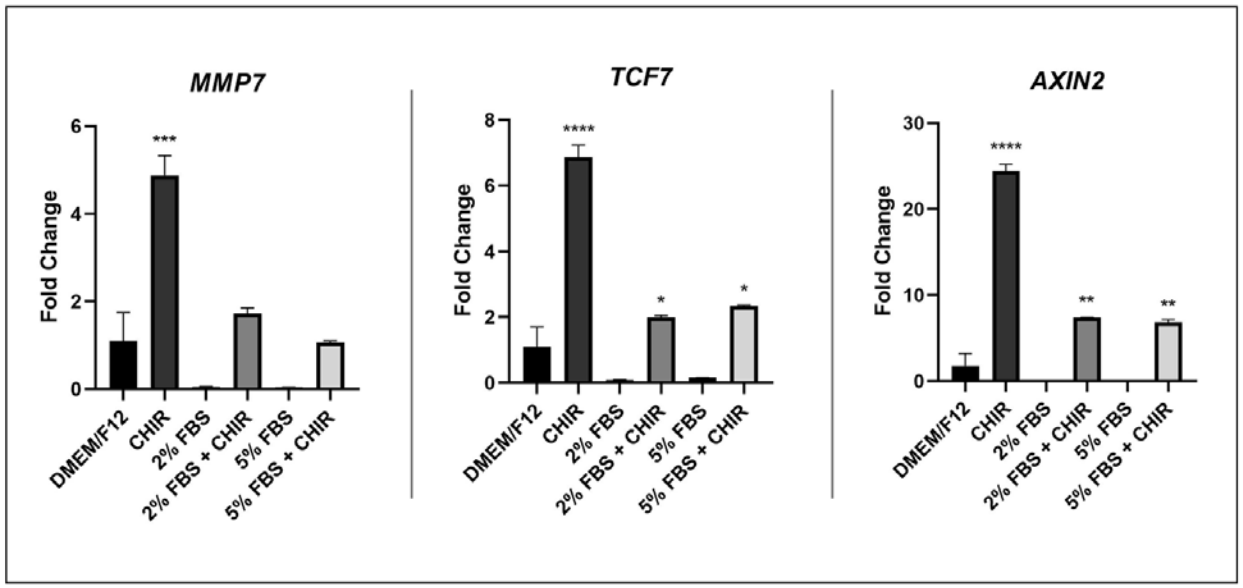

Figure 3: Impact of FBS on transcription of downstream Wnt target genes. qRT-PCR was performed to assess the impact of FBS (2% and 5%) on the expression of downstream Wnt target genes: *MMP7, TCF7, AXIN2* with and without the addition of CHIR99021. The data is representative of n=2 technical replicates per condition. Fold change for *MMP7, TCF7*, and *AXIN2* expression was significantly higher in the CHIR99021-only conditions compared to the DMEM/F-12 control. A significant increase in fold change was also observed for *TCF7* and *AXIN2* for all conditions containing CHIR99021, with and without FBS (2% and 5%), although the increase was less than CHIR99021 alone. Statistical significance was determined via one-way ANOVA, multiple comparisons, a=0.05 (**** $p < 0.0001$; *** $p = 0.0001$; ** $p < 0.001$; * $p < 0.05$).

A commercially available serum-free alternative (Knockout Serum Replacement, KSR) was tested as a replacement for FBS alongside an alternate base medium, Advanced DMEM/F12. Advanced DMEM/F12 (Gibco) is a widely used basal medium which, unlike classic DMEM/F12, has been formulated specifically to allow for reduced serum supplementation by 50-90% without altering mammalian cell growth rate or morphology (Thermo). The following ingredients are present in Advanced DMEM/F12 to allow for serum reduction: ethanolamine, glutathione, ascorbic acid, insulin, transferrin, AlbuMAX® II, along with the trace elements sodium selenite, ammonium metavanadate, cupric sulfate, and manganous chloride (Thermo). In accordance with the manufacturer's recommendation, Advanced DMEM/F-12 was supplemented with GlutaMAX™ to maximize performance. Although B-27™ and N2 supplements are typically used for supporting survival and maintenance of neuronal progenitor cells and iPSCs, recent studies have demonstrated their utility as additives in Advanced DMEM/F12 media to

generate lung organoids (Kaushik et al., 2018; Miller et al., 2019). Therefore, the Advanced DMEM/F12 conditions were tested with and without the addition of B-27™ and N2.

3.2.3.1 Knockout Serum Replacement

KSR is a serum-free formulation designed to directly replace FBS in feeder-based stem cell cultures. Based on the cell quantification data obtained via Celígo, FBS and KSR were comparable in terms of cell growth at 2% and 5% concentrations, with and without the addition of CHIR99021 **(fig. 4).** Overall, this data demonstrates that KSR can serve as a direct substitute for FBS in serum-free media formulations, despite the absence of a feeder layer. However, for the control media, KSR did not offer a clear advantage as a replacement for FBS in terms of enhancing proliferation.

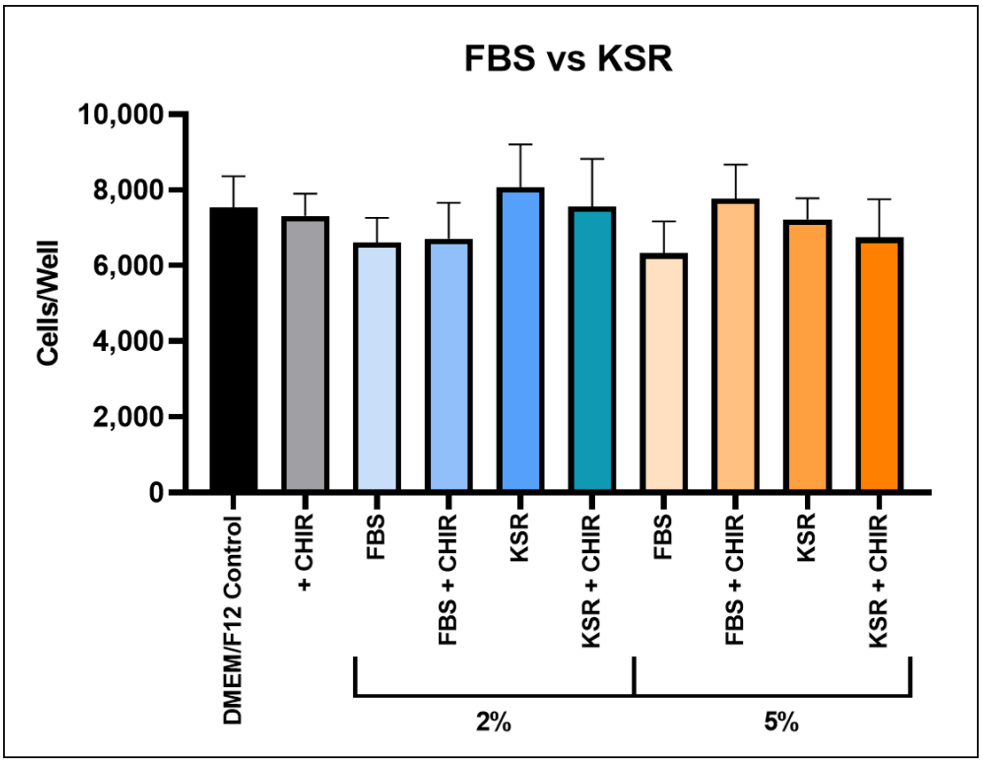

Figure 4: Celígo cell quantification evaluating serum reduction in control media. Cells were seeded in 24-well plates (n=6) and cultured for 5 days to assess the impact of FBS and KSR (± CHIR99021) on cell growth. Cell nuclei were stained with Hoescht and quantified via Celigo. FBS and KSR demonstrated comparable growth at both concentrations tested (2% and 5%) with and without CHIR99021 (3 µM). DMEM/F12 + rhEGF (25 ng/mL) was used as the control. No significant differences in cells/well (24-well plate) were observed between the conditions (one-way ANOVA, multiple comparisons, a=0.05).

3.2.3.2 Advanced DMEM/F-12

DMEM/F12 and Advanced DMEM/F12 controls demonstrated comparable growth. However, based on the cell quantification data obtained from the Celígo assay, the addition of B-27™ and N2 to the Advanced DMEM/F12 media led to a statistically significant increase in cell growth compared to the Advanced DMEM/F12 control and regular DMEM/F12 control. The total cell yield was further increased with the addition of CHIR99021 (3uM) to the Advanced DMEM/F12 media containing B-27™ and N2. Among the conditions in Advanced DMEM/F12, the combination of B-27™ + N2 + CHIR99021 demonstrated the highest expression for the three downstream Wnt target genes: *AXIN2, TCF7,* and *MMP7* **(Fig. 5).** This formulation also produced the highest cell yield **(Fig. 6),** providing a sufficient number of cells for performing downstream assays. Overall, these findings supported the decision to replace DMEM/F12 + 5% FBS with Advanced DMEM/F12 + B-27™ + N2 as the final control media formulation (complete formulation outlined in **Table 3** below).

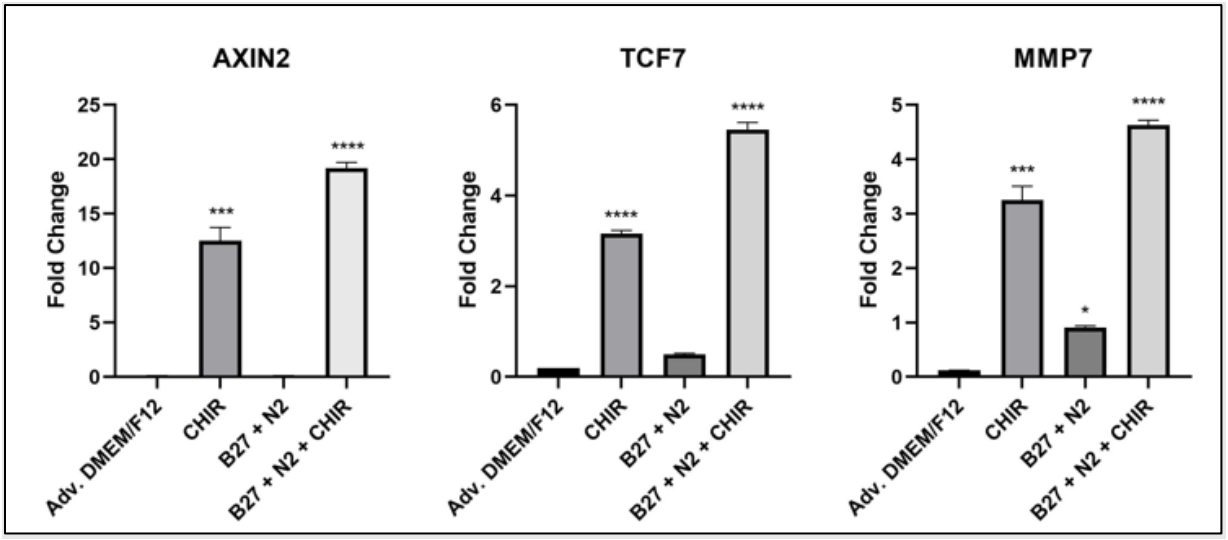

Figure 5: Assessment of dowstream Wnt target genes. qRT-PCR was performed to assess the impact of DMEM/F12 (± CHIR99021) and Advanced DMEM/F12 (± CHIR99021, ± B-27 + N2) on the expression of Wnt target genes: *MMP7, TCF7, AXIN2.* The data is representative of n=2 biological replicates per condition. (**** $p < 0.0001$; *** $p = 0.0002$; ** $p < 0.001$; * $p < 0.05$).

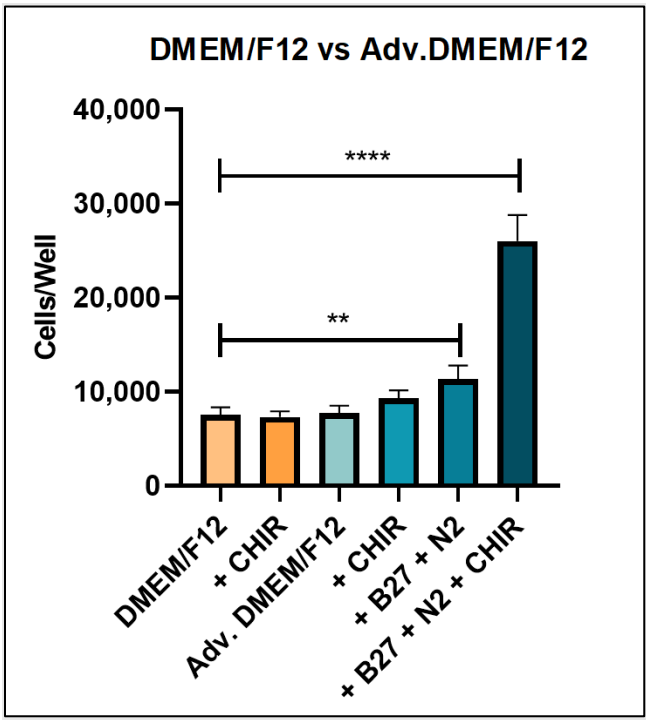

Figure 6: Celígo cell quantification evaluating alternate basal medium. Cells were seeded in 24-well plates (n=6) and cultured for 5 days to compare cell growth in DMEM/F12 (± CHIR99021) and Advanced DMEM/F12 (± CHIR99021, ± B-27 + N2). Cell nuclei were stained with Hoescht and quantified via Celigo. The conditions containing B-27 and N2 demonstrated significantly higher cell yield compared to the standard DMEM/F12 control. Statistical significance was determined via one-way ANOVA, multiple comparisons, a=0.05. (**** $p < 0.0001$; ** $p = 0.0065$).

Table 3: Serum-free Control Media Formulation

Component	Concentration	Mfr./Catalog #
Advanced DMEM/F-12	N/A	Gibco/12634010
GlutaMAX™ (100X)	1X	Gibco/35050079
B-27™ (50X)	1X	Gibco/17504044
N-2 (100X)	1X	Gibco/17502048

3.3 Confirming Wnt Activation

Before proceeding with the current formulation for additional testing, it was crucial to determine whether Wnt signaling was being activated in the basal cells after being treated with CHIR99021. Successful activation of Wnt was confirmed by assessing transcription levels of downstream target genes and β-catenin stabilization, as nuclear localization of β-catenin is highly dependent on the successful inhibition of GSK3.

3.3.1 CHIR99021 Titration

To determine the optimal concentration of CHIR99021 required for enhancing Wnt activity without cytotoxicity, a titration study was performed in which SAEPs were treated with 1 µM, 3 µM, and 5 µM CHIR99021. After 5 days of culture in media containing the various concentrations of CHIR99021, the cells were harvested for further analysis via qRT-PCR to compare mRNA levels of two key downstream Wnt targets: *AXIN2* and *TCF7*. The results showed that CHIR99021 treatment, specifically at 3 µM and 5 µM, lead to a significant increase *(one-way ANOVA, $p<0.05$)* in the mRNA levels of both *AXIN2* and *TCF7* compared to the DMSO control and 1 µM condition (**Fig**.**7**). Despite the large fold change compared to control seen with the 5 µM treatment, literature suggests that overactivation of Wnt can result in neoplastic cell behavior and subsequent malignant transformation (Aros et al., 2020). Therefore, 3 µM was selected as the concentration of CHIR99021 to be used in the media moving forward, as it still led to a significant increase in expression of downstream Wnt target genes.

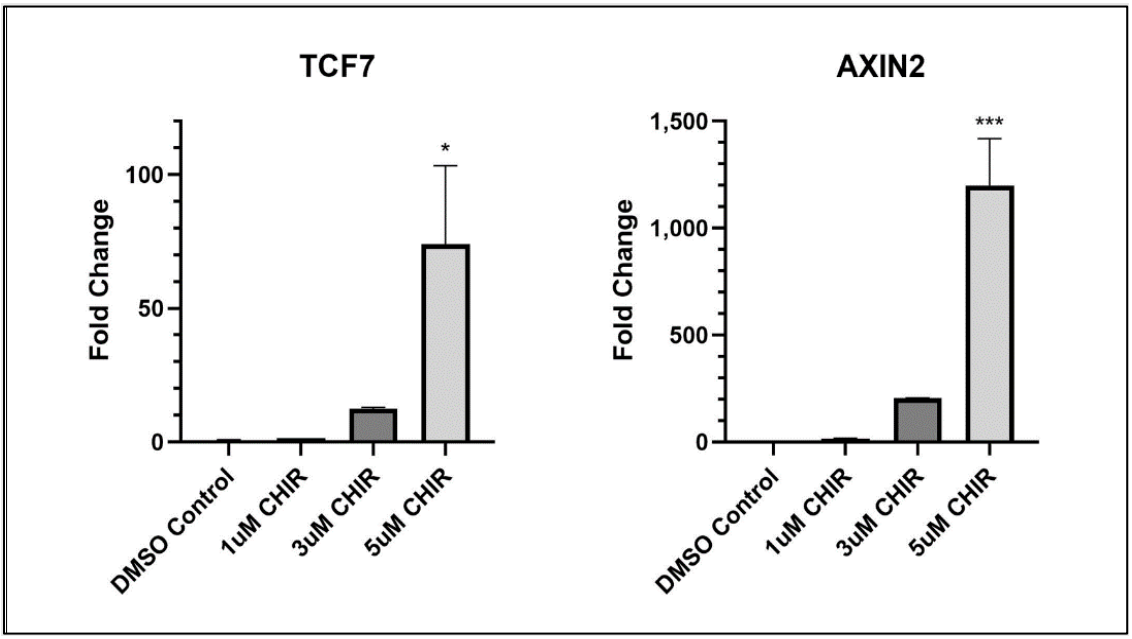

Figure 7: Dose-dependent transcription of downstream Wnt target genes. A significant increase was observed in the mRNA levels of both *AXIN2* and *TCF7* in the 5 µM CHIR99021 condition compared to the DMSO control. An increase in fold change was also seen for the 3 µM condition, but this increase was not significant compared to the control. Statistical significance was determined via one-way ANOVA, multiple comparisons, a=0.05. (*** $p = 0.0009$; * $p < 0.05$).

3.3.2 β-catenin Stabilization

If CHIR99021 is effective in promoting Wnt activity through the successful inhibition of GSK3β, it would be expected to see an increase in nuclear localization of β-catenin rather than stabilization within the cytosol. Immunoreactivity against p63 was used as a marker of airway basal cells and co-stained with non-phosphorylated (active) β-catenin to assess nuclear colocalization via ICC. Western blot analysis for β-catenin was also performed to assess differences in protein level between the cytoplasmic and nuclear fractions. As expected, the ICC results showed an increase in overall β-catenin expression in the CHIR99021-treated cells compared to the control throughout the course of the study (Day 2, Day 4, Day 7, Day 9; see **Fig. 8**). A potential difference in β-catenin localization was also observed between the CHIR99021-treated cells, which appeared to have increased nuclear localization compared to the control in which the low level of β-catenin remained localized within the cytoplasm. The addition of B-27™ and N2 to the media did not greatly alter the expression level compared to the media-only control, although it appeared to work synergistically with CHIR99021 to enhance β-catenin

expression as seen in the B-27™ + N2 + CHIR99021 condition, particularly on Day 9 of culture. In support of the ICC results, western blot analysis also showed the highest β-catenin expression in CHIR99021-treated cells, most notably in the nuclear fraction (**Fig. 9**). As expected, β-catenin expression was detected at a low level in cells treated with XAV939, the DMSO control, and B-27™ +N2 conditions. Additionally, TCF1/TCF7 expression was only present in the CHIR99021-treated cells and was undetectable in both the nuclear and cytoplasmic fraction of the XAV939, DMSO control, and B-27™ + N2 conditions. Together, these results suggest successful activation of Wnt signaling in SAEPs treated with CHIR99021 at 3 µM, enhanced by the presence of B-27™ + N2.

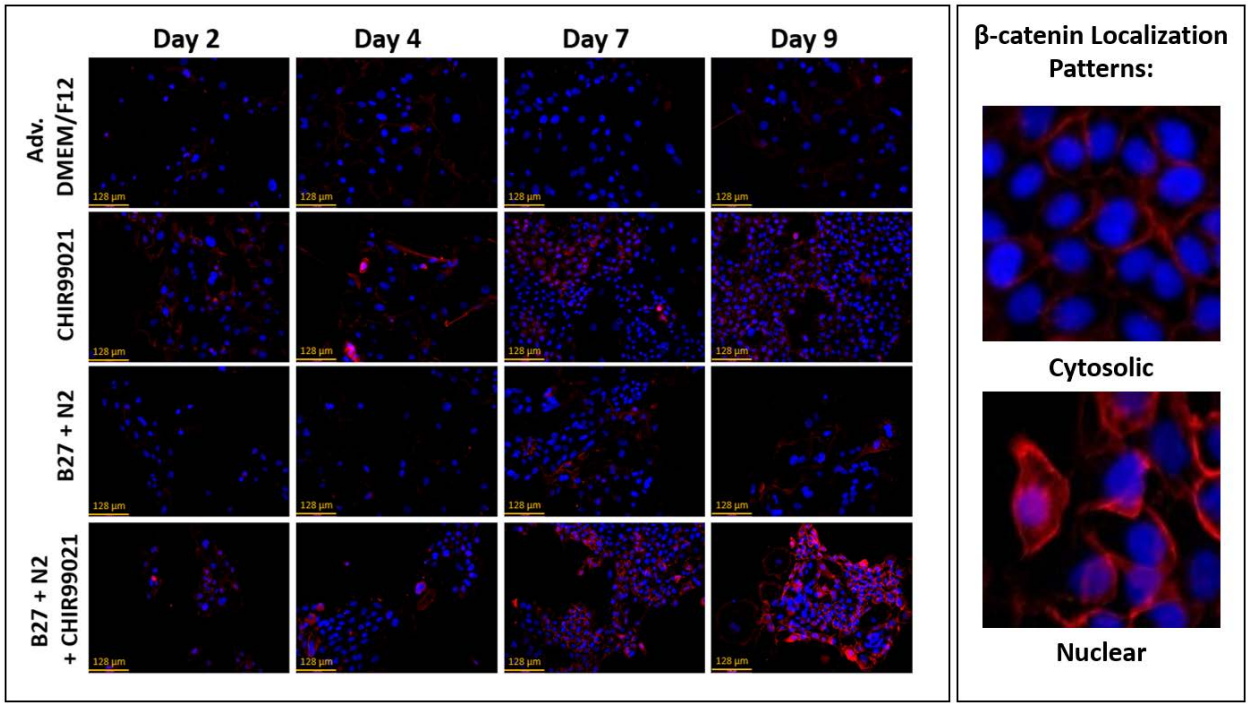

Figure 8: Time course of non-phosphorylated (active) β-catenin expression level and stabilization. β-catenin (red) is highly expressed in the cells treated with Wnt agonist CHIR99021, particularly on days 7 and 9 of culture. The strongest expression is seen in the cell junctions of tightly packed cell clusters formed in the +CHIR99021 conditions, although high nuclear expression is also apparent. As expected, there is little to no expression observed in the controls throughout the 9-day culture period (Adv. DMEM/F12 and B-27 + N2), and in the cases where it is expressed at low levels (i.e., Day 7 and 9 for B-27 + N2) no nuclear expression is observed. Scale bar = 128 µm.

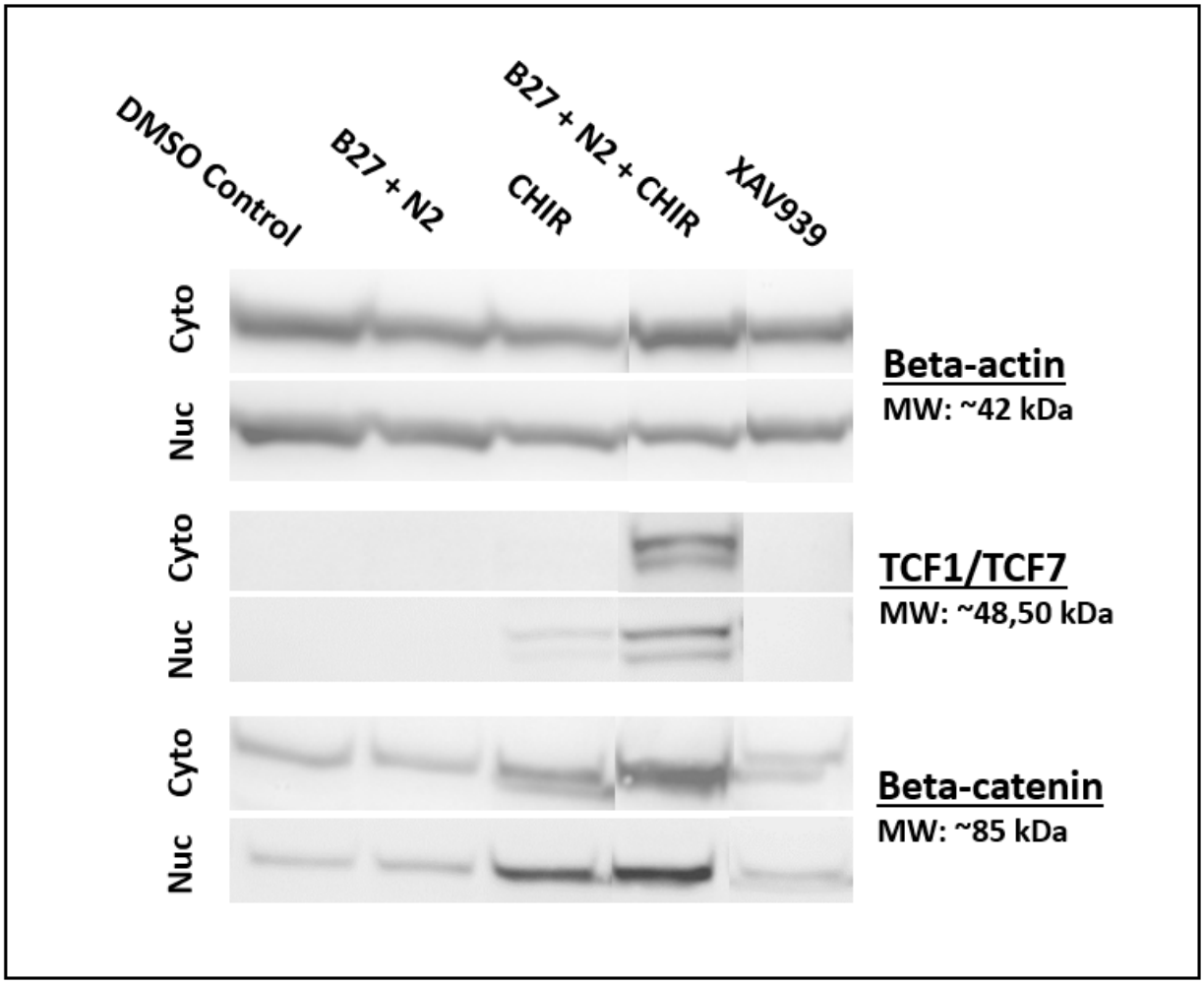

Figure 9: Comparative assessment of Wnt-associated protein expression in nuclear and cytoplasmic extracts of SAEPs. β-catenin is highly expressed in the +CHIR99021 conditions, particularly in the nuclear fraction, indicating successful transduction of the canonical Wnt pathway. TCF1/7 is only detectable in the +CHIR99021 samples, with increased nuclear and cytoplasmic expression in the presence of B-27™ + N2. As expected, TCF1/7 is undetectable in the control conditions and after exposure to Wnt inhibitor XAV939. β-actin of the same blots is shown for normalization of protein loading for the cytoplasmic fraction. Blot images were spliced between CHIR and XAV939 samples to omit data from an unrelated study.

CHAPTER 4

Assessing the Role of Wnt on Basal Cell Proliferation (Aim 2)

4.1 Purpose of Experiments

The experiments discussed in this chapter were aimed to elucidate the impact of canonical Wnt on SAEP cell proliferation through treatment with various Wnt agonists and inhibitors. The ways in which CHIR99021 treatment impacts overall cell yield was investigated, followed by experiments aimed specifically at monitoring changes in the Tumor Protein (p63) positive population. This population was of particular interest, as p63 is a well-established marker of normal epithelial basal cells involved in the regulation of self-renewal vs differentiation of mucociliary cells (Haas et al., 2019) and is highly expressed within basal cells of adult proximal airways (Hashimoto et al., 2012). Among the literature, there is a general consensus that Wnt/β-catenin is responsible for the upregulation of p63 in basal stem cells, thus maintaining self-renewal capacity and preventing early differentiation into non-basal cell types (Aros et al., 2020; Haas et al., 2019; Schmid et al., 2017). Since these findings are typically from studies using non-human (mouse, zebra fish, and/or *Xenopus*) airway epithelia, it was important to determine whether p63 is upregulated in the same manner in primary human airway epithelial basal cells *in vitro* by evaluating differences in expression and proliferation after activation of the Wnt pathway.

4.2 Impact of Wnt/β-catenin on SAEP Yield

To evaluate the impact of Wnt signaling on SAEP proliferation, cells were expanded in 24-well plates for a total of 9 days with and without CHIR99021 (3 µM) in the media. Samples were fixed with 4% PFA at four timepoints (Day 2, Day 4, Day 7, Day 9) and cell nuclei were quantified using the 24-well Celígo cell counting assay. The results showed that CHIR99021 treatment led to an increase in total cell yield, especially when combined with B-27™ + N2 **(Fig. 10**). On Day 2, both conditions containing B-27™ + N2 demonstrated a significantly higher cell yield compared to the Advanced DMEM/F12 control and CHIR99021 condition. One potential reason for this observed outcome is that B-27™ + N2 may be acting to enhance initial attachment and/or survival of Wnt-responsive basal cells after plating, which is plausible considering the known utility of B-27™ supplementation for enhancing survival of neuronal stem cells in culture, as mentioned in Chapter 3. Follow-up experiments were performed with

similar growth outcomes, showing a consistent increase in total SAEP cell yield after exposure to CHIR99021, particularly when added in combination with B-27™ + N2 (data not shown).

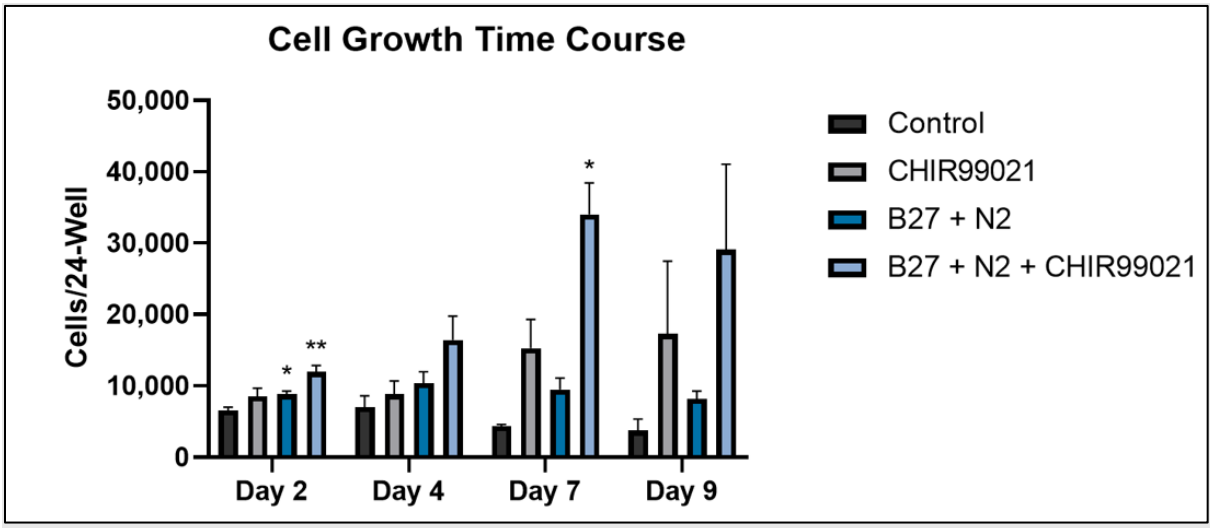

Figure 10: SAEP Cell Growth Time Course Study. **S**AEPs (passage 1) were expanded with and without the addition of 3 µM CHIR99021 to the media formulations, and 24-well samples were collected on Days 2, 4, 7, and 9 of culture (n=4 per condition). On Day 2 of culture, a significant difference in cell yield was observed in the B-27™ + N2 conditions (± CHIR99021) compared to the Advanced DMEM/F12 control. On Day 7, the only significance was seen for the B-27™ + N2 + CHIR99021 condition. Significance was determined by two-way ANOVA compared to the control for each time point *(Tukey's multiple comparison test, a = 0.05, ** p = 0.0100; * p < 0.05).*

4.3 Impact of Wnt/β-catenin on p63+ Basal Cell Proliferation

After determining that CHIR99021 treatment led to an overall improvement in SAEP cell yield, the next step was to assess impacts on the p63+ subpopulation. To achieve this, ICC was performed on samples obtained from a short-term time course study (4 hours, 24 hours, and 48 hours) using proliferation marker ki67 to evaluate the proliferation of the p63+ basal subset in response to CHIR99021 treatment. Based on the ICC images, the addition of CHIR99021 alone appeared to increase proliferation of the p63+ population compared to the control, as there was a noticeable increase in the dual expression of p63 and ki67. This response was even greater when CHIR99021 was added to the control media containing B-27™ and N2. As shown in **Fig. 11**, treatment with CHIR99021 lead to a significantly higher proportion of proliferating basal cells beginning 24 hours post-seed, which was maintained throughout the 48-hour culture period.

To evaluate maintenance of the p63 phenotype over extended culture time, a 7-day time course study was performed. Similar to the 48-hour study, SAEP cells were seeded into 24-well plates and expanded in various media formulations (with and without CHIR99021) until time of collection: Day 2, Day 4, and Day 7 **(Fig. 12).** The cells were then co-stained with ki67 and p63 for ICC analysis, which qualitatively appears to demonstrate an increase in the overall proportion of p63+ cells, along with an increase in the proportion of cells expressing dual positivity for ki67 and p63. The temporal changes of expression observed in both time course studies suggest that CHIR99021 treatment increases the proliferation of p63+ basal cells.

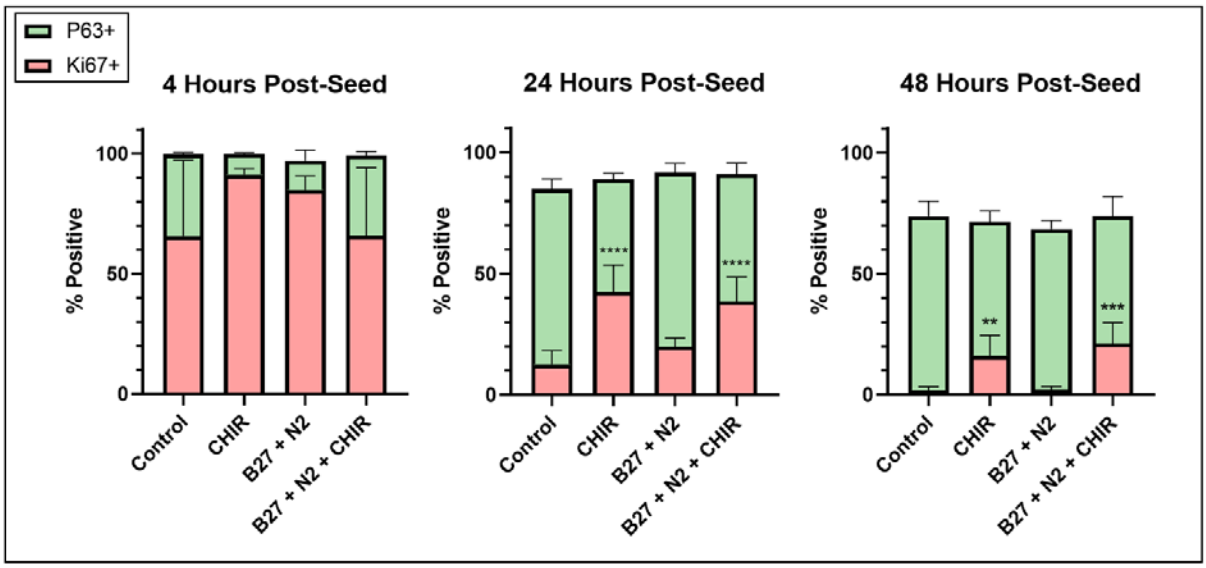

Figure 11: Assessment of proliferative p63+ basal population over short-term 48-hour time course. The proportion of dual-positive p63/ki67 basal cells was assessed at three culture timepoints: 4 hours, 24 hours, and 48 hours. Cell quantification was performed using ICC images (20X) using ImageJ (FIJI) software. Significance was determined by one-way ANOVA compared to the control for each time point *(Tukey's multiple comparison test, a = 0.05, **** $p < 0.0001$; *** $p = 0.0004$; ** $p = 0.0073$).*

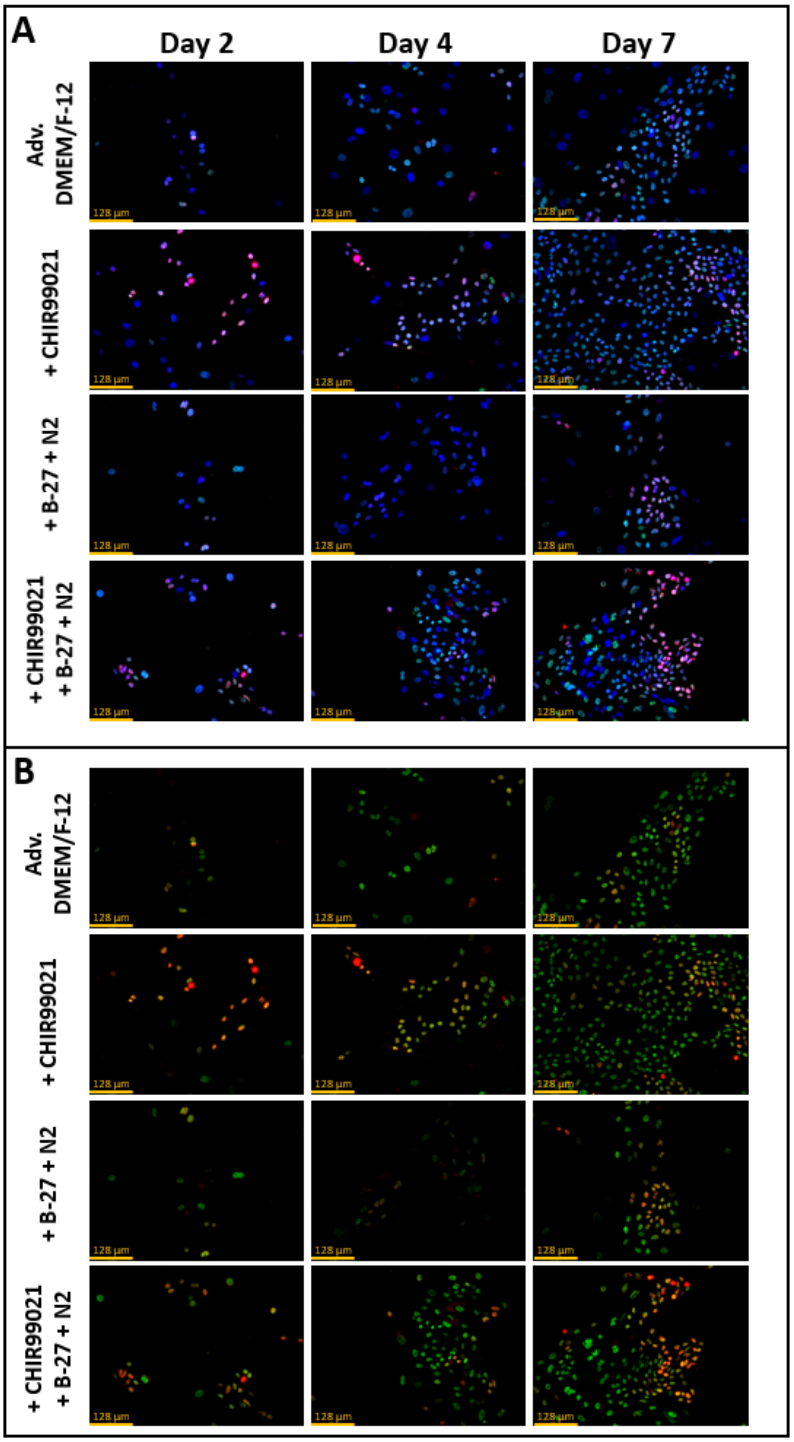

Figure 12: Visual assessment of proliferative p63+ basal population over 7-day time course. Fluorescent overlays were generated using ImageJ (FIJI) software to aid in the visual assessment of the dual-positive p63/ki67 basal cell population. Changes in population proportion were assessed at three time points: Day 2, Day 4, and Day 7. Figure B is a copy of Figure A without the DAPI overlay to allow for easier visualization of the dual positive p63/ki67 population. (A: blue = DAPI, red = ki67, green = p63). Scale bar = 128 µm.

An additional study was performed to assess protein-level transcription of p63 in both the nuclear and cytoplasmic fractions of cells exposed to CHIR99021 during a 5-day culture compared to cells exposed to Wnt inhibitor, XAV939. Western blot analysis (**Fig. 13**) showed a clear increase in p63 expression in SAEPs treated with CHIR99021, specifically within the nuclear cell fraction. As expected, there was an absence of p63 expression in both the nuclear and cytoplasmic fractions of the cells exposed to XAV939 during expansion.

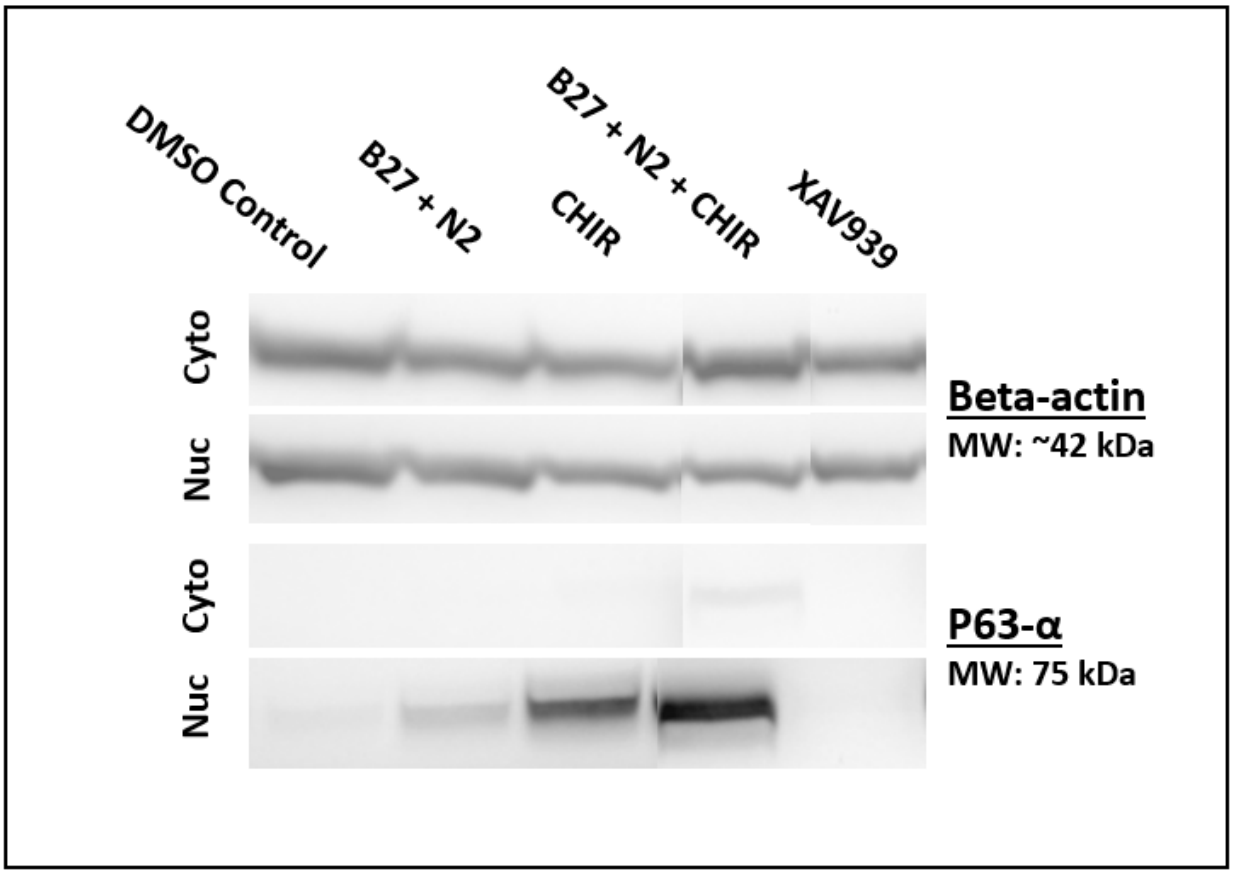

Figure 13: Comparative assessment of p63-α basal marker expression in nuclear and cytoplasmic extracts. In the +CHIR99021 conditions, p63-α was highly expressed, indicating Wnt-induced upregulation of p63-α at the protein level. Expression was slightly detectable in the nuclear fraction of the DMSO control and B27™ + N2 conditions but appeared to be completely absent from both the nuclear and cytoplasmic fractions of the XAV939-treated cells. β-actin of the same blots is shown for normalization of protein loading for the cytoplasmic fraction. Blot images were spliced between CHIR and XAV939 samples to omit data from an unrelated study.

Overall, the results from these experiments support existing evidence that CHIR99021 treatment enhances proliferation of p63+ cells (Aros et al., 2020; Haas et al., 2019), suggesting that it likely plays a role in regulating self-renewal of basal cells, which is essential for basal cell repair in response to injury in the adult human airway.

4.4 Testing Alternative Wnt Agonists

To build confidence in the conclusion that Wnt activity may play a role in regulating basal cell proliferation, the effect of CHIR99021 on the activation of Wnt/β-catenin pathway and p63+ cell proliferation was compared against other known Wnt agonists, including human recombinant Wnt3a and TWS119. Numerous studies have implicated the family of RSpondin (RSpo) proteins in amplification and modulation of the canonical Wnt pathway, with RSpo1 being of particular importance (Kim et al., 2008; Nusse, 2012). Although RSpo proteins by themselves are not known to have intrinsic Wnt signaling activity, there is strong evidence to suggest that the RSpo/Lgr signaling axis serves to elevate Wnt signaling or "uncover" endogenous Wnt signaling activities (Willert & Nusse, 2012). Therefore, RSpo1 was added in combination with each agonist to determine whether Wnt activity could be further enhanced and thereby elicit a stronger cell response. After five days of continuous exposure to the various Wnt agonists (± RSpo1), the cells were co-stained with ki67 and p63 to compare the effectiveness of each treatment on proliferation and phenotypic maintenance **(Fig. 14**). Of the agonists tested, CHIR99021 seemed to have the greatest impact on proliferation. Based on the ICC results, the addition of RSpo1 to the media did not appear to impact, or enhance, the activity of the Wnt agonists as hypothesized.

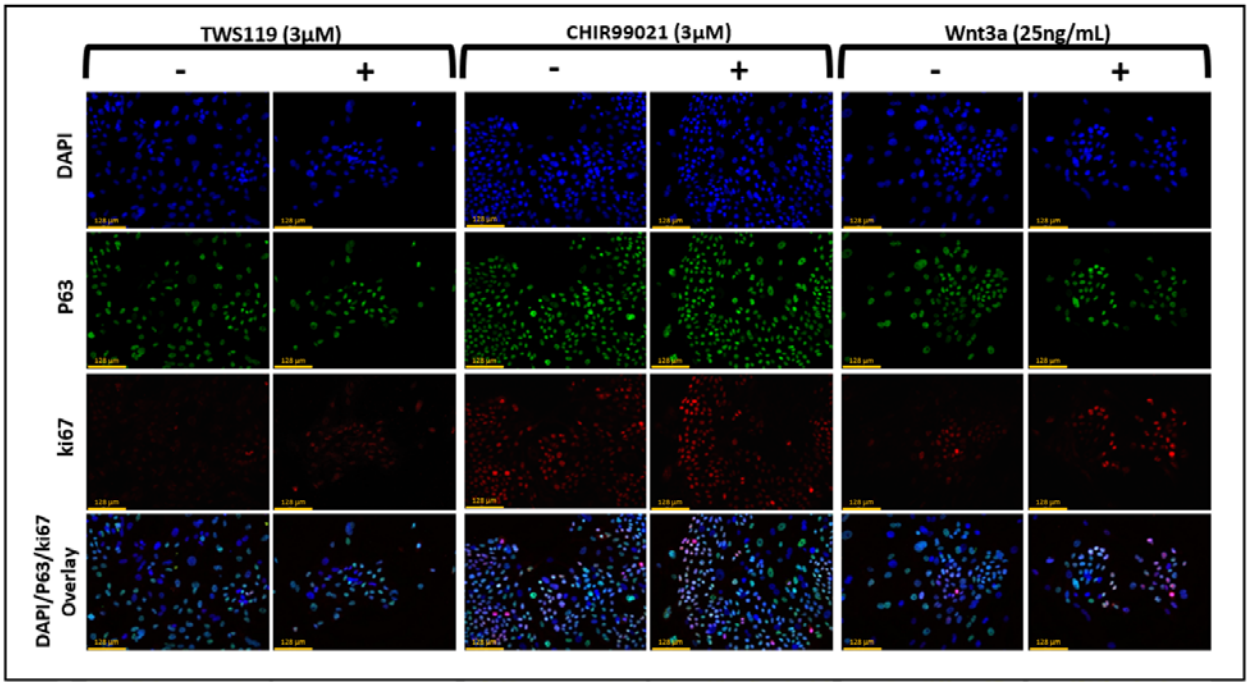

Figure 14: Impact of Wnt Agonists on p63+ basal cell proliferation. The proportion of proliferative p63+ basal cells was observed after treatment with TWS119 (3 µM), CHIR99021 (3 µM), and Wnt3a (25 ng/mL) without (-) and with (+) the addition of RSpo1 (25 ng/mL) (blue = DAPI, red = ki67, green = p63). Scale bar = 128 µm.

CHAPTER 5

Assessing the Role of Wnt on Multipotent Differentiation Capacity (Aim 3)

5.1 Purpose of Experiments

In this chapter, experiments were performed to evaluate the impact of Wnt signaling on SAEP differentiation capacity. There are often contradictions found among studies surrounding the role of canonical Wnt signaling, particularly in the context of differentiation (Haas et al., 2019; Schmid et al., 2017). Multiple studies have demonstrated the importance of Wnt/β-catenin signaling for normal ciliogenesis in vertebrate systems, as it has been shown to co-regulate expression of *FOXJ1*, the master transcription factor for motile cilia (Aros et al., 2020; Haas et al., 2019). However, Schmid et al. (2017) reported the opposite effect, showing that enhanced Wnt/B-catenin signaling caused a decrease in the number of ciliated cells in ALI culture, whereas exposure to Wnt inhibitor Dickkopf-1 (Dkk1) lead to an increase in ciliated cells (Schmid et al., 2017). According to Haas et al. (2019), a reduction or loss of p63 expression results in basal cell differentiation, thus maintenance of the p63 phenotype is required for the cells to continue the self-renewal process. Therefore, for this thesis, it was hypothesized that SAEP cells expanded in the presence of CHIR99021 would more efficiently differentiate into the specialized non-basal cell types that comprise the functional airway epithelia, as previous experiments provide evidence that CHIR99021 treatment may lead to enhanced p63 expression in culture.

5.2 Mucociliary Differentiation in ALI Culture

ALI culture is an effective model of airway epithelial repair and was used to assess changes in differentiation capacity of SAEP cells after being exposed to Wnt agonists during culture. Prior to ALI culture, SAEP cells were expanded in T75 flasks for five days in media containing either XAV939, RSpo1, CHIR99021 (± RSpo1), TWS119 (± RSpo1), or Wnt3a (± RSpo1). At harvest, the cells were seeded onto transwell inserts (n=2) and cultured for a total of 16 days: 2 days of expansion and 14 days of ALI. After 14 days in ALI culture, each well was visually observed to assess ciliary movement, and the cells were fixed with 4% PFA for further analysis of the distinct non-basal populations. Identity and absence of differentiated cells at the apical surface was confirmed via ICC imaging using antibodies against CC10 and acetylated-Tubulin proteins to identify secretory and ciliated subtypes, respectively (**Fig. 15**). As expected, there was an

absence of differentiation in the control (Adv. DMEM/F12) and negative control (XAV939) conditions. By contrast, the cells treated with CHIR99021, RSpo1, and Wnt3a all displayed a well-differentiated population of ciliated and secretory cells, whereas the TWS119 treatment resulted in poor differentiation of both cell types. Although green ac-tubulin staining is evident in the TWS119-only condition, it does not show specific localization to cilia (see **Fig. 15** below for an example of localized vs. non-localized ac-tubulin staining).

Interestingly, when CHIR99021 was added in combination with RSpo1, secretory differentiation was maintained whereas ciliary differentiation appeared to decrease. Although the reason for this decrease is unknown, it is possible that the combination of RSpo1 and CHIR99021 lead to overexpression of Wnt and therefore disrupted normal ciliogenesis. If true, this would support the findings reported by Schmid et al. (2017; mentioned above) that enhanced Wnt/β-catenin activity decreases the abundance of ciliated cells in ALI culture. Surprisingly, the opposite was observed in the TWS119/RSpo1 condition, in which the addition of RSpo1 appeared to enhance ciliary and secretory cell differentiation. Future experiments will need to be performed to determine whether this differentiation capacity can be maintained over passage after treatment with Wnt agonist(s).

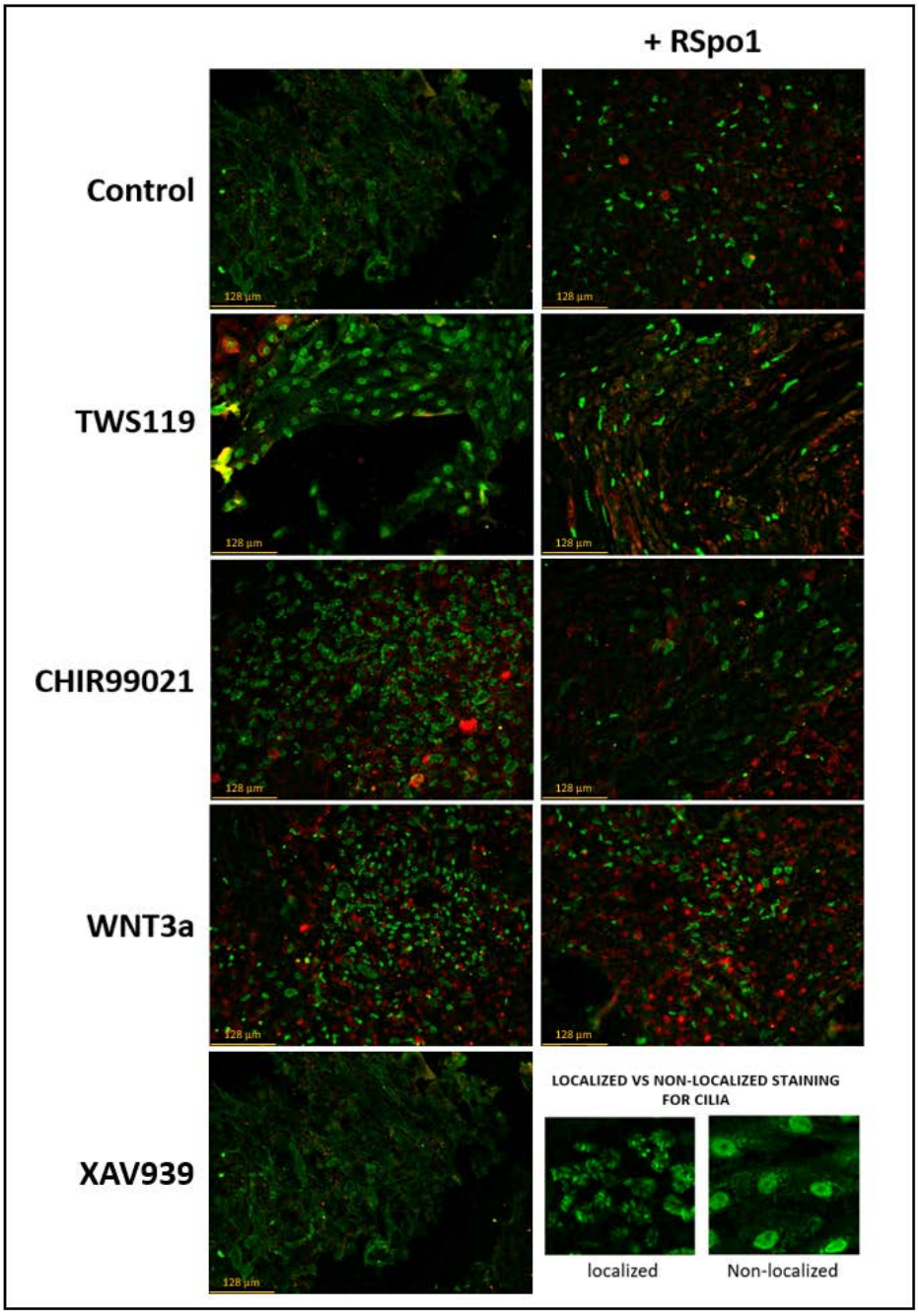

Figure 15: Impact of Wnt Agonists on basal cell differentiation. Differentiation into ciliated (ac-Tub+, green) and Club (CC10+, red) cell types was assessed after 14 days in ALI using fluorescence microscopy. Images were taken at 20X magnification. The cells were expanded in media containing TWS119 (3 µM), CHIR99021 (3 µM), or Wnt3a (25 ng/mL) without (-) and with (+) the addition of RSpo1 (25 ng/mL) for 5 days prior to ALI culture. Cells were also cultured in media containing XAV939 (3 µM) as a negative control. Once seeded into transwell inserts (n=2), the cells were expanded for an additional day in their respective growth medias. Pneumacult ALI media was replaced in the bottom chamber every other day for 14 days until the wells were fixed with 4% PFA and stained for fluorescence microscopy. Scale bar = 128 µm.

CHAPTER 6

Conclusions & Future Work

6.1 Conclusions

Overall, the data presented in this thesis highlights some potential ways in which canonical Wnt/β-catenin signaling may be involved in the proliferation and multipotent differentiation capacity of airway basal cells. After exposure to CHIR99021 in culture, SAEP cells demonstrated high transcription levels of downstream Wnt target genes (*AXIN2, TCF7, MMP7*) and increased nuclear localization of β-catenin compared to the control. Additionally, the CHIR99021 treatment appeared to increase the proportion of basal cells expressing both ki67 and P63 in planar culture, and resulted in effective mucociliary differentiation into secretory and ciliated cell types in ALI culture. This apparent increase in ki67+ basal cells and successful mucociliary differentiation in ALI was also observed after treatment with Wnt3 and TWS119, but notably absent in the negative control treated with Wnt inhibitor XAV939. Therefore, there is evidence to suggest that canonical Wnt/β-catenin signaling may be involved in basal cell proliferation within the airway epithelium. This information regarding Wnt-induced basal cell behavior could potentially be utilized for future studies aimed to exploit canonical Wnt activity to better maintain 'stemness' of airway basal cells, with the ultimate goal of long-term expansion *in vitro* while maintaining the functional capacity to differentiate into the specialized cell types of the airway epithelium.

6.2 Future Work

Because of the inherent complexity of the canonical Wnt pathway, there are many areas of potential investigation to further enhance the current understanding of Wnt signaling in human airway basal cells.

6.2.1 Investigating Basal Signatures

To build on the current understanding of how Wnt activity impacts human airway epithelial cell behavior, it may be beneficial to further elucidate the Wnt-reactive basal subpopulation(s) through investigating unique basal signatures pre- and post-exposure to Wnt agonists. Recently published single cell sequencing data by Carraro et al. (2020) and others provides great insight into phenotypic expression of cells predestined, or primed, for specific non-basal lineages. In

other words, within the general basal cell population, there are distinct subpopulations including Multipotent Basal (MPB), Secretory Primed Basal (SPB), and Proliferating Basal (PB), all with distinct marker expression (Carraro et al., 2020). This information could serve as a useful tool for determining the distinct basal signatures and the most common lineage fate of the Wnt-reactive basal population.

6.2.2 Identifying Cell Source of Wnt in Airway Niche

Many Wnt-producing and Wnt-responsive cell populations have been identified within the lung. A study performed by Aros et al. (2020) demonstrated that CK5+ airway basal stem cells secrete Wnt ligand, Nabhan et al. (2018) has reported a Wnt-producing fibroblast in the AT2 cell niche, and Lee et al. (2017) has identified and characterized the Wnt-producing Lgr5+ and Wnt-responsive Lgr6+ mesenchymal populations. Through identification of Wnt-source cells in the human lung, secreted Wnt protein(s) can be directly isolated from spent culture media and potentially serve as a biologically relevant replacement for small molecule agonists.

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