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Signaling Mechanisms that Control Ciliated Cell Differentiation

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UNIVERSITY OF MIAMI

SIGNALING MECHANISMS THAT CONTROL CILIATED CELL DIFFERENTIATION

By
Benjamin John Gerovac

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Doctor of Philosophy

SIGNALING MECHANISMS THAT CONTROL CILIATED CELL
DIFFERENTIATION

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An innate front-line defense mechanism termed mucociliary clearance (MCC) occurs in the airway epithelium and is critical for respiratory health. MCC is made possible by the coordinated efforts of goblet and multiciliated cells, two cell types that line the airway epithelium. Maintaining the relative populations of goblet and multiciliated cells is necessary for optimum MCC, however, decreases in the multiciliated cell population and dysfunctional MCC are seen in a variety of respiratory diseases. Despite the clinical relevance, there is still much to understand about the signaling pathways that control multiciliated cell fate.

Previous data has shown multiciliated cell differentiation of primary human airway epithelial cells occurred \textit{in vitro} if they were grown at an air:liquid interface (ALI), but multiciliated cell differentiation was inhibited if the cells were kept submerged in media. However, after two decades, the mechanism behind submersion dependent inhibition of multiciliated cell differentiation remained elusive. Published literature demonstrated submersion could create a low oxygen environment. Therefore, the hypotheses that submersion inhibits multiciliated cell differentiation by creating a low oxygen (hypoxic) environment and suppresses genes necessary for multiciliated cell differentiation were tested. The results showed both submersion and hypoxia repressed multicilin and FOXJ1 expression, genes necessary for multiciliated cell differentiation. It
also showed that inhibition was dependent upon the Notch signaling pathway, as pharmacological and neutralizing antibody inhibition of Notch signaling restored multiciliated cell differentiation. Additionally, activation of Notch signaling via expression of activated Notch intracellular domain (NICD) resulted in repression of multiciliated cell differentiation. Furthermore, Notch neutralizing antibody data indicated that Notch2 but not Notch1 was important for this repression. These results implicated submersion and hypoxia activated Notch signaling to prevent the differentiation of multiciliated cells by blocking the expression of genes required for multiciliated cell differentiation.

The finding that multiciliated cell differentiation does not require an ALI when Notch was inhibited lead to the discovery that it was possible to differentiate primary airway epithelial cells into multiciliated cells submerged in plastic wells. Moreover, transduction of airway epithelial cells with lentivirus containing the human FOXJ1 promoter driving the expression of a fluorescent protein was successful in detecting and quantifying multiciliated cell differentiation of airway epithelial cells differentiated in 96-wells. These data provided evidence that high-throughput approaches are possible to detect compounds and/or signaling pathways that regulate multiciliated cell differentiation.

Since submersion created a low oxygen environment, the influence of hypoxia inducible factor (HIF) signaling on multiciliated cell differentiation was tested. The data showed the expression of a lentiviral transduced constitutively active form of HIF-2α but not HIF-1α repressed multiciliated cell differentiation in airway epithelial cells. These
data suggested HIF signaling might be a cause of inhibition of multiciliated cell differentiation in low oxygen environments.

In addition to submersion via Notch signaling and/or HIF-2α signaling, the inflammation related cytokine, IL-13, has been shown to reduce multiciliated cell differentiation and is elevated in respiratory diseases such as asthma. Given Notch signaling inhibits multiciliated cell differentiation; the hypothesis was tested whether IL-13 was activating Notch to inhibit multiciliated cells. The results showed IL-13 repressed multiciliated cell differentiation through a Notch independent mechanism. Pharmacological inhibitor experiments showed IL-13 utilized the JAK/STAT but not MEK signaling pathway to repress multiciliated cell differentiation. Additionally, the data indicated IL-13, like Notch, repressed multiciliated cell differentiation by suppressing the expression of multicilin and FOXJ1.

The data in this thesis provide novel insights into the mechanisms that control human multiciliated cell differentiation. They show that low oxygen and/or chronically inflamed environments will hinder multiciliated cell differentiation through different mechanisms. They also reveal multicilin expression is regulated both by JAK/STAT and by Notch signaling pathways independently of each other. These insights have furthered our knowledge into the signals that control multiciliated cell fate and may lead to novel therapeutics to help those with respiratory disease. Additionally, these data demonstrate technical innovations for studying differentiating multiciliated cells, and show it is possible to use novel high-throughput assays that could one day reveal more aspects of multiciliated cell differentiation.
DEDICATION

I would like to dedicate this thesis to all my friends and family and dog that supported me throughout my time in graduate school.
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Table of Contents

List of Figures ................................................................. viii
List of Abbreviations ....................................................... x

Chapter

1 Introduction ..................................................................... 1
   Cell Types of the Lung .................................................... 1
   Mucociliary Clearance .................................................... 3
   Epithelial Remodeling in Respiratory Disease .................... 4
   Human Airway Progenitor Cells ....................................... 5
   Multiciliated Cell Differentiation in the Airway ................ 6
   Culture of Normal Human Bronchial Epithelial Cells .......... 7
   Oxygen Content and HIF Signaling .................................. 8
   Notch Signaling Pathway ................................................ 11
   Interleukin 13 and JAK/STAT Signaling ............................ 15
   Summary and Goals ....................................................... 22

2 Materials and Methods .................................................. 23
   Normal Human Bronchial Epithelial Cell Culture ................ 23
   Inhibitors ........................................................................ 24
   RNA purification and Quantitative RT-PCR (RT-qPCR) .......... 24
   Immunofluorescence and Confocal Microscopy .................. 25
   Protein Isolation and Western Blotting ............................. 26
Ciliary Beat Frequency

Cilia Length

Lentiviral Construction, Production, and NHBE Transduction

Statistical Analysis

3 SUBMERSION AND HYPOXIA INHIBIT MULTICILIATED CELL
DIFFERENTIATION VIA NOTCH SIGNALING

NHBE Multiciliated Cell Differentiation is Inhibited in an Apical-volume Dependent Manner

Submersion of NHBE Cells Creates a Hypoxic Environment

Submersion and Hypoxia Inhibit Multiciliated Cell Differentiation via a γ-secretase Dependent Pathway

MCIDAS is Suppressed in Submerged Culture and Promoted by DAPT

Ectopic expression of NICD in Hypoxic or Submerged Conditions with DAPT Inhibits Multiciliated Cell Differentiation

Inhibition of Notch2 but not Notch1 Promotes Multiciliated Cell Differentiation

Conclusions

4 MULTICILIATED CELL DIFFERENTIATION ON PLASTIC

Differentiation of Multiciliated Cells in 96-wells

Fluorescence Detection of Multiciliated Cell Differentiation in 96-wells

Conclusions

5 HIF SIGNALING AND MULTICILIATED CELL
DIFFERENTIATION
HIF-2α but not HIF-1α Inhibits Multiciliated Cell Differentiation .......... 55
Conclusions ................................................................................................. 58

6 THE INFLUENCE OF IL-13 ON MULTICILIATED CELL

Differential ................................................................................................. 59
IL-13 Inhibits Multiciliated Cell Differentiation Independent of Notch
Signaling ..................................................................................................... 59
IL-13 Inhibits MCIDAS and FOXJ1 Expression in the Presence of DAPT ... 61
Canonical Notch Regulated Transcriptional Repressors are not Promoted by
IL-13 ......................................................................................................... 61
IL-13 Depends on JAK Activity but not MEK Activity to Inhibit
Multiciliated Cell Differentiation ................................................................. 63
Conclusions ................................................................................................. 68

7 DISCUSSION ............................................................................................. 70

WORKS CITED .............................................................................................. 83

vii
LIST OF FIGURES

Figure 1.1 Branching generations and zones of the lung ........................................ 2
Figure 1.2 Mechanism of mucociliary clearance ................................................... 5
Figure 1.3 Processing the Notch Receptor .......................................................... 13

Figure 3.1 FOXJ1 expression and ciliated cell differentiation are reduced by
submersion in an apical volume dependent manner .............................................. 35
Figure 3.2 Submersion and hypoxia induce HIF-1α and HIF-2α proteins .............. 36
Figure 3.3 γ-secretase inhibition restores FOXJ1 expression and ciliated cell
differentiation in submerged and hypoxic conditions ........................................... 39
Figure 3.4 MCIDAS expression is inhibited by submersion in a γ-secretase
dependent manner ................................................................................................. 42
Figure 3.5 Expression of HA-NICD1 increases Notch signaling and inhibits FOXJ1
in DAPT treated submerged and hypoxic cells .................................................... 44
Figure 3.6 Ectopic expression of HA-NICD2 promotes Notch signaling and
represses FOXJ1 in submerged and hypoxic cells treated with DAPT .................... 45
Figure 3.7 Constitutive expression of NICD3 increases Notch signaling and
inhibits FOXJ1 in DAPT treated submerged and hypoxic cells ............................ 46
Figure 3.8 Inhibition of Notch2 but not Notch1 promotes multiciliated cell
differentiation in hypoxic and submerged condition .......................................... 48

Figure 4.1 DAPT enables FOXJ1 expression and ciliated cell differentiation of
NHBE cells in submerged culture on plastic in 96-well plates .............................. 51
Figure 4.2 Detection of multiciliated cell differentiation in 96-wells via lentiviral
transduction with pFC virus ............................................................................... 54
Figure 5.1 HIF-2α but not HIF-1α inhibits ciliogenesis independently of Notch signaling ................................................................. 57

Figure 6.1 Multiciliated cell differentiation in inhibited by IL-13 in the presence of DAPT ................................................................. 60

Figure 6.2 IL-13 inhibits the expression of MCIDAS and FOXJ1 ......................... 62

Figure 6.3 HES1, HES2, HEY1, and HEY2 expression is not induced by IL-13 ...... 63

Figure 6.4 JAK but not MEK activity is required for IL-13 dependent inhibition of multiciliated cell differentiation .............................................. 66

Figure 6.5 Inhibition of JAK activity increases MCIDAS and FOXJ1 in IL-13 treated cultures ............................................................... 67

Figure 6.6 JAK Inhibitor 1 and PD98059 inhibit STAT3 phosphorylation .............. 69

Figure 7.1 Model describing potential environmental cues and signaling pathways that control multiciliated cell differentiation ................................................. 80
LIST OF ABBREVIATIONS

ALDOC – Aldolase C, Fructose-Bisphosphate
ALI – Air:liquid interface
ANK – Ankyrin
ANOVA – Analysis of variance
ASM – Airway smooth muscle
B2M – Beta-2 microglobulin
BC – Basal Cell
BEGM – Bronchial epithelial growth media
bHLH – Basic helix-loop-helix
BSA- Bovine serum albumin
CBF – Ciliary beat frequency
cDNA – Complimentary DNA
CF – Cystic fibrosis
COPD – Chronic obstructive pulmonary disease
C-TAD – carboxyl-terminal transactivation domain
DAPT – N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DBZ – Dibenzazepine
DLL1 – Delta-like 1
DLL3 – Delta-like 3
DLL4 – Delta-like 4
DMSO – Dimethyl Sulfoxide
EF1 – elongation factor 1
EGF – Epidermal growth factor

ERK – Mitogen-Activated Protein Kinase

FERM – four-point-one, ezrin, radixin, moesin

FFT - Fast Fourier transform

FIH – Factor inhibiting HIF

FOXJ1 – Forkhead Box J1

Fringe – β1–3N-acetylglucosaminyl transferase

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

HA- Hemagglutinin

HD – Heterodomain

HES1 – Hes Family BHLH Transcription Factor 1

HES2 – Hes Family BHLH Transcription Factor 2

HEY1 – Hairy/enhancer-of-split related with YRPW motif 1

HEY2 – Hairy/enhancer-of-split related with YRPW motif 2

HIF-1α – Hypoxia inducible factor 1 alpha

HIF-1β – Hypoxia inducible factor 1 beta

HIF-2α – Hypoxia inducible factor 2 alpha

HIF-3α – Hypoxia inducible factor 3 alpha

HRP – Horseradish peroxidase

IL-13 – Interleukin 13

IL-13Rα1 – Interleukin 13 receptor, alpha 1

IL-13Rα2 – Interleukin 13 receptor, alpha 2

IL-4Rα – Interleukin 4 receptor, alpha
IRS-1 – Insulin receptor substrate 1
IRS-2 – Insulin receptor substrate 2
JAG1 – Jagged1
JAG2 – Jagged2
JAK1 – Janus Kinase 1
JAK2 – Janus Kinase 2
JAK3 – Janus Kinase 3
KIR – Kinase inhibitors region
LNR – LIN12 and Notch repeat
LTR – Long terminal repeat
MAML – Mastermind
Mb – Mindbomb
MCC – Mucociliary Clearance
MCIDAS – Multicilin
MCS – Multiplie cloning site
MEK – Mitogen-Activated Protein Kinase Kinase
NECD – Notch extracellular domain
Neu – Neuralized
NHBE – Normal human bronchial epithelial cells
NICD – Notch intracellular domain
Notch1 – Notch receptor 1
Notch2 – Notch receptor 2
Notch3 – Notch receptor 3
Notch4 – Notch receptor 4
NRR – Negative regulatory region
NRR1 – Notch1 negative regulatory region antibody
NRR2 – Notch2 negative regulatory region antibody
N-TAD – N-terminal transactivation domain
ODD – Oxygen-dependent degradation
OPA – Polyglutamine repeat-containing region
PAS – PER-ARNT-SIM
PBS – Phosphate buffered saline
PCL – Periciliary Layer
PEG – Polyethylene glycol
PEST – Proline, glutamic acid, serine, threonine-rich
PFA – Paraformaldehyde
PHD – Prolyl hydroxylase
Pofut1 – O-fucosyltransferase 1
PTP – Protein tyrosine phosphatases
PVDF – Polyvinylidene difluoride
qPCR – Quantitative PCR
RAM – Recombination binding protein-J associated molecule
RBPJK – Immunoglobulin kappa J region
RTE – Rat tracheal epithelial cells
Rumi – O-glucosyltransferase
s.d. – Standard deviation
SEM – Standard error of the mean
SH2 – Src homology-2
SHPs – SH2 containing phosphatases
SIN – Self-Inactivating
SOCS – Suppressors of cytokine signaling
STAT1 – Signal transducer and activator of transcription 1
STAT2 – Signal transducer and activator of transcription 2
STAT3 – Signal transducer and activator of transcription 3
STAT4 – Signal transducer and activator of transcription 4
STAT5 – Signal transducer and activator of transcription 5
STAT6 – Signal transducer and activator of transcription 6
TACE – Necrosis factor-α-converting enzyme
TAD – Transactivation domain
TBS-T – 0.1% Tris buffered saline-Tween 20
TM – Transmembrane
TRP63 – Transformation-related protein 63
TyK2 – Tyrosine kinase 2
VHL – von Hippel-Lindau tumor suppressor protein
Chapter 1: Introduction

Cell Types of the Lung

The lung can be divided into two zones, the conducting zone and respiratory zone (1). In these different zones, the epithelium is lined with a variety of cell types with unique characteristics and roles to play in maintaining lung function. The major cell types are basal, goblet, multiciliated, clara (also designated club), and alveolar type I and II cells. However, their relative populations vary depending on the respiratory zone location. The conducting zone is the most proximal to the esophagus and begins in the trachea followed by the bronchi and bronchioles and ends in the terminal bronchioles. The conducting zone ends and the respiratory zone begins with the respiratory bronchioles followed by alveolar ducts and ends in alveoli sacs (Figure 1.1).

Basal cells are thought to act as stem cells in the airways and act to restore other cell types during normal turnover and during injury. Basal cells are most abundant in the trachea and bronchi, representing roughly 30% of the cell population in these areas, but basal cell density tapers off as one enters the bronchioles eventually becoming undetectable in the respiratory zone (2, 3). Goblet cells are responsible for secretion of mucins into the airway lumen and are another cell type that is most abundant in the trachea and bronchi regions, representing approximately 4% of the cell population in regions of the conducting zone but eventually become undetectable as one enters the respiratory zone (4, 5). Multiciliated cells possess hair-like microtubule-based projections on their apical membranes and represent around 30% of the cell population throughout the trachea, bronchi, and bronchioles of the conducting airway. However, contrary to the two above cell types, they exist in the respiratory zone albeit as a lower percentage of
Figure 1.1. Branching generations and zones of the lung. The lung consists of 23 generations (denoted N) of branching tubes (denoted by orange lines). The initial 15 generations comprise the conducting zone where no gas exchange occurs and includes the trachea, bronchi, bronchioles and terminal bronchioles. Generations 16-23 are denoted the respiratory zone and contain the alveoli (denoted by black dots), which are the sites where gas exchange takes place. The respiratory zone includes the respiratory bronchioles, alveolar ducts and ends with the alveolar sacs.

the cell population (2). Clara cells are a secretory cell type thought to play roles in detoxification and immune regulation due to the expression of cytochrome P450 and clara cell specific protein. Clara cells are rarely detected in the trachea, bronchi, and bronchioles (0 – 0.4% of the cell population), but their numbers increase to around 10% of the cell population in the terminal bronchioles and upwards of 20% of the cell population in the respiratory bronchioles (6-8). Alveolar type I and type II cells are responsible for surfactant production and facilitating gas exchange. They are found exclusively in the alveoli of the respiratory zone (9, 10). Pulmonary neuroendocrine cells are thought to act as chemosensors of inhaled substances, and are found in the conducting and respiratory zones (11, 12). Pulmonary neuroendocrine cells are a very low abundance cell type that on average only contributes 0.04% to the cellular population (13). While
these cell types each have unique characteristics, they can work together in order to create mechanisms to benefit lung health and function such as in the case of mucociliary clearance.

**Mucociliary Clearance**

As we breathe, air travels to respiratory zone of the lung, where alveolar cells facilitate gas exchange between inhaled air and circulating blood. While gas exchange is necessary for survival, as it removes metabolic waste such as carbon dioxide and provides oxygen to cells in the body, it also presents a potential health risk because, along with oxygen, we also inhale potentially harmful airborne pathogens and toxins. To deal with this possible health hazard, our lungs possess an innate defense mechanism termed mucociliary clearance (MCC). MCC occurs in the trachea, bronchi, and proximal bronchioles of the airway where it removes dangerous inhaled substances from the lungs. MCC is accomplished by the coordinated efforts of goblet cells and multiciliated cells along with the periciliary layer (PCL) that resides directly above the epithelium. Goblet cells secrete large glycoproteins termed mucins, which when combined with salts and water form a viscoelastic mucus layer that floats atop the airway epithelium that ensnares and traps inhaled substances e.g. bacteria, smoke, etc. (14). Multiciliated cells then propel this mucus layer out of the lung by coordinated beating of their microtubule-based projections (cilia) in the PCL, which is a liquid layer responsible for both promoting mucus layer formation and preventing the mucus layer from collapsing the cilia and inhibiting beating (15). The propelling mucus layer out of the lung results in inhaled toxins and pathogens being removed from the lung where they can do the respiratory system no harm (16) (Figure 1.2). An example showing the importance of the PCL comes
from the respiratory disease cystic fibrosis (CF). In CF, the PCL is diminished due to a mutation in the cystic fibrosis transmembrane conductance regulator gene, which is chloride channel necessary for proper hydration of the PCL. The dehydration of the PCL leads to the collapse of the mucus layer onto the cilia and disruption of MCC (17). In addition to a PCL, MCC requires maintaining proper quantities of goblet and multiciliated cells to expel the mucus layer out of the lung and too few multiciliated cells or an increase in mucus secreting cells can cause MCC dysfunction. MCC dysfunction can lead to an elevated risk of bacterial infection (18) and/or development of mucus plugs, which block gas exchange and increase the chance of mortality for those with respiratory disease (19).

**Epithelial Remodeling in Respiratory Disease**

Respiratory diseases are prevalent in the United States with roughly 1 in 12 people afflicted with asthma (20), approximately 11.8 million people suffering with chronic obstructive pulmonary disease (COPD) (21), and an estimated 30,000 individuals with CF (22). A common characteristic of these respiratory diseases is remodeling of the airway epithelium. Studies have shown increases in the amount of mucus secreting cells along with decreases in the multiciliated cell population in individuals with these diseases (3, 23-26). This remodeling likely contributes to the impairment of MCC seen in patients with asthma and COPD (27, 28). Despite the immense clinical need to understand this epithelial remodeling, as it likely contributes to respiratory disease pathogenesis, there is still much that is unknown. Specifically, there is still much to be discovered about the environmental factors and signaling pathways involved in controlling the cell fate decisions of the basal stem cells that result in changes in epithelial cell populations.
**Figure 1.2.** Mechanism of mucociliary clearance. In this diagram the components of mucociliary clearance are displayed. The epithelium is composed of multiciliated cells, goblet cells, and basal cells. Goblet cells secrete mucins (dark red circles) that form a mucus layer (green) that resides above the epithelium that trap harmful inhaled substances (bright red). Multiciliated cells remove this mucus layer by beating of the cilia on the apical surface, which requires the periciliary layer (PCL) to keep the mucus layer from collapsing the cilia. The mucus and the trapped substances are then removed from the lungs.

**Human Airway Progenitor Cells**

In the adult lung, the airway epithelium is lined with basal cells (BCs), which are long-lived cells aptly named due to their proximity to the basal lamina located below the epithelium. BCs are distinguishable by their high expression of transformation-related protein 63 (TRP63), nerve growth factor receptor, and integrin α6 in both humans and mice (29-31). BCs encompass roughly 30% of the total cell population in the upper airways of humans where MCC occurs (2, 32). Moreover, studies have shown BCs are multipotent progenitor stem cells capable of repopulating both goblet and multiciliated cell populations lost due to normal turnover or during injury (33, 34). As BCs regenerate the airway epithelium, it has been proposed that changes in their microenvironment and/or genetic or epigenetic changes may alter BC cell fate decisions causing a
remodeling of the epithelium. Therefore, this proposal suggests that the alteration of BC fate decisions may be an underlying cause of the epithelial remodeling and MCC dysfunction seen in respiratory diseases such as COPD, CF, and asthma (3).

**Multiciliated Cell Differentiation in the Airway**

Multiciliated cells first appear in the human airway epithelium around 12 to 15 weeks of gestation during the pseudoglandular stage of human lung development and continue differentiation throughout development and after birth (35, 36). Differentiation of multiciliated cells is thought to involve five stages: commitment to a ciliated cell fate, replication of centrioles (centriologenesis), transportation of centrioles to the apical cell membrane, docking to the apical cell membrane after centrioles transition to basal bodies, which involves additional accessory structures such as basal feet and transition fibers being added to the centriole, and formation of the axoneme and elongation of the cilia (37-39). While there is still much to learn about the factors involved in each of these steps, previous studies have illuminated a few of these. Forkhead box J1 (FOXJ1; also designated HFH-4) is a transcription factor and member of the winged helix protein family, whose members are known to be important for differentiation of certain tissues including neural crest and islet cells (40, 41). Foxj1 expression was found to be limited to multiciliated cells in the airway epithelium in mice (42). In addition, multiciliated cells were absent in the airways of Foxj1-null mice, and this absence was thought to be caused by disorganized basal bodies that were not attached to the apical membrane in the epithelial cells (43). Gain-of-function experiments in Zebrafish found foxj1 was sufficient to promote expression of genes important for multiciliated cell differentiation and was sufficient to form ectopic motile biciliated cells in *Xenopus* (44, 45). These data
suggested FOXJ1 was necessary for multiciliated cell differentiation. However, the data also suggested FOXJ1 was only important for late stages of ciliogenesis and not early stages such as centriologenesis. More recently, multicilin (MCIDAS; previously designated IDAS (46)), a transcriptional regulator, was identified by microarray analysis to play an important role in multiciliated cell differentiation. Specifically, in mouse and *Xenopus* models, knockdown of MCIDAS or expression of a dominant negative MCIDAS inhibited multiciliated cell differentiation. In addition, expression of MCIDAS promoted transcription of genes necessary for multiciliated cell differentiation including foxj1, and MCIDAS was also shown to be sufficient to induce ectopic multiciliated cell differentiation (47). Importantly, the ability of MCIDAS to induce ectopic multiciliated cells demonstrated MCIDAS stimulated centriologenesis, which showed MCIDAS was sufficient to drive all four stages of multiciliated cell differentiation described above. Furthermore, while there have been other factors shown to be important for multiciliated cell differentiation such as myloblastosis proto-oncogene Myb (48) and another winged helix family member named regulatory factor X 3 (49), MCIDAS expression is the earliest known step in the multiciliated cell differentiation pathway.

**Culture of Normal Human Bronchial Epithelial Cells**

Normal human bronchial epithelial (NHBE) cell differentiation can be recapitulated and studied *in vitro* using air: liquid interface (ALI) culture techniques. This system may be suitable to study cell fate signaling mechanisms as cells grown using this system have been shown to have similar transcriptional profiles to *in vivo* airway epithelial cells (50, 51), and previous studies have used NHBE cells to investigate how Notch signaling and the cytokine interleukin 13 influence bronchial cell differentiation (52, 53). The culture of
NHBE cells at an ALI results in a pseudostratified epithelium containing goblet and multiciliated cells after three weeks. However, if NHBE cells are left submerged for this same time period, goblet cell differentiation still occurs, but multiciliated cell differentiation is strikingly inhibited (54). The mechanism of this inhibition is not well understood, but studies using rat tracheal epithelial (RTE) cells showed inhibition of multiciliated cell differentiation was dependent on the apical volume used to submerge the cells (55). However, inhibition of multiciliated cell differentiation by submersion is perplexing since multiciliated cells differentiate during human fetal development where the fetus is enclosed in the amniotic sac, which is filled with fluid (35). A possible clue as to the mechanism of submersion dependent repression of multiciliated cell differentiation is that during human fetal development there is an increase in amniotic fluid oxygen content at the same time multiciliated cell differentiation begins (56, 57). In addition, submersion has been shown to create a hypoxic environment due to the much lower diffusion of oxygen in liquid versus air (58). Therefore, a lack of oxygen may be inhibiting multiciliated cell differentiation of NHBE cells in submerged conditions.

**Oxygen Content and HIF Signaling**

Oxygen content can act as a determinant affecting the differentiation of stem cells including chondrocyte and neuronal differentiation (59, 60). Specifically, low oxygen (hypoxia) can inhibit differentiation of many different types of cells including pancreatic cells, adipocytes, and myoblasts (61-63). The mechanism by which hypoxia controls cell differentiation is not well understood.

A cellular response to hypoxia is modulation by HIF proteins. These are transcription factors whose abundance is regulated by oxygen levels. The HIF family consists of
hypoxia inducible factor 1α (HIF-1α), hypoxia inducible factor 2α (HIF-2α; also designated EPAS1), and hypoxia inducible factor 3α (HIF-3α) (64). HIF-1α is ubiquitously expressed while HIF-2α and HIF-3α are more tissue restricted, but all three HIFs expressed in the lung (65-67). HIF signaling also involves hypoxia inducible factor 1β (HIF-1β; also designated ARNT), which is a binding partner of HIF-α proteins and is involved in HIF target gene regulation (68). HIF-α proteins contain basic helix-loop-helix (bHLH), PER-ARNT-SIM (PAS), and oxygen-dependent degradation (ODD) regions. The bHLH region is necessary for HIF-α proteins to bind DNA. The bHLH is also involved in binding to HIF-1β in conjunction with the PAS region (69). The ODD region is responsible for the oxygen dependent regulation of HIF protein stability (70). HIF-1α, HIF-2α, and HIF-3α also contain a transactivation domain region at the carboxyl-terminus, and this region is partitioned into a N-terminus transactivation domain (NTAD) and a carboxyl-terminus transactivation domain (CTAD). Interestingly, HIF-1α and HIF-2α contain both the NTAD and CTAD while HIF-3α only contains the NTAD. Since the transactivation domain is important for the recruitment of co-factors that promote HIF target gene transcription such as CREB-binding protein (71), the lack of the CTAD in HIF-3α was thought to make HIF-3α act as a dominant-negative for HIF signaling. There is evidence for HIF-3α suppressing HIF signaling (72), however, HIF-3α can also increase HIF signaling (73). Therefore, the role of HIF-3α in HIF signaling is likely context specific.

In normoxic conditions, HIF-1α, HIF-2α, and HIF-3α proteins are rapidly degraded and HIF signaling suppressed. HIF-1β does not contain an ODD region so it is not regulated by oxygen content (64). This ODD dependent degradation is promoted by
hydroxylation of two specific proline residues in the ODD. Hydroxylation of the proline residues is carried out by prolyl hydroxylases (PHDs), and hydroxylation of only one of the proline residues is sufficient for protein degradation (74). PHDs require oxygen as a cofactor for hydroxylation. Therefore, in hypoxic conditions, PHDs do not hydroxylate HIF proline residues (75). Hydroxylated proline residues provide a platform for the von Hippel-Lindau tumor suppressor protein (VHL), which is an ubiquitin ligase that polyubiquitinates and targets HIF proteins for proteosomal degradation (76). HIF protein that is not hydroxylated binds to HIF-1β to form a heterodimer. This heterodimer translocates to the nucleus where it binds to hypoxia response elements in the promoters of target genes such as vascular endothelial growth factor and erythropoeitin and regulates their transcription (77, 78). Interestingly, the NTAD and CTAD of HIF-1α and HIF-2α bestow target gene specificity (79), and this has been proposed to explain despite HIF-1α and HIF-2α having similar sequences and structures, they can regulate different gene targets (80).

In addition to proline hydroxylation, HIF signaling is also negatively regulated by other mechanisms. For example, the asparaginyl hydroxylase factor inhibiting HIF (FIH) hydroxylates an asparagine residue in the CTAD of HIF proteins. This hydroxylation represses HIF signaling by disrupting recruitment of transcriptional co-activators (71). FIH also requires oxygen as a cofactor. Therefore, asparagine hydroxylation is decreased in hypoxic conditions (81). Additionally, the protein, acetyl transferase arrest-defective-1, has been shown to add an acetyl group (acetylate) a lysine residue in the ODD domain. This acetylation promotes the recruitment of VHL to HIF leading to its deterioration, and mutation of the lysine residue leads to an increase in the stability of HIF protein (82).
Intriguingly, histone deacetylase inhibitors were able to cause HIF protein degradation through association of HIF with heat shock protein 70 in a VHL and ubiquitin independent mechanism but still required the ODD domain (83), which shows a non-canonical pathway leading to HIF degradation. Finally, an alternatively spliced form of HIF-3α, named inhibitory PAS domain protein, inhibits HIF signaling by either binding up HIF-1β so HIF-1α, HIF-2α, and other isoforms of HIF-3α proteins cannot bind or by binding up HIF-α proteins and inhibiting their binding to DNA (84, 85).

Since oxygen content can control cell differentiation decisions and HIF signaling is oxygen dependent, it may be possible that HIF signaling is playing a role in controlling differentiation decisions for airway epithelial cells in low oxygen environments. Thus, HIF signaling may play a role in the suppression of multiciliated cell differentiation caused by submersion. Notch signaling may also play a role in the submersion dependent suppression as it has been shown to inhibit multiciliated cell differentiation and be potentiated in low oxygen environments (61, 86).

**Notch Signaling Pathway**

The Notch signaling pathway regulates cell differentiation decisions and tissue homeostasis (87-89). Notch signaling is an evolutionarily conserved pathway described in many species from fruit flies to sea urchins to humans (87, 90, 91). Notch signaling has been extensively studied and it is known Notch signaling occurs through a juxtracrine mechanism. There are four known Notch receptors (Notch1-4) and five known Notch ligands named jagged1 (JAG1), jagged2 (JAG2), delta-like 1 (DLL1), delta-like 3 (DLL3), and delta-like 4 (DLL4). All Notch receptors and ligands are all type I transmembrane proteins (92), and are expressed in human airway epithelial cells (93, 94).
The Notch receptors undergo multiple cleavage steps in order to produce the active form responsible for target gene regulation (Figure 1.3). Following translation, Notch receptors are cleaved in the Golgi by Furin (termed S1 cleavage) in the protein heterodomain (HD) region to produce a heterodimeric protein with the Notch extracellular domain (NECD) non-covalently bound to a peptide containing the transmembrane (TM) and Notch intracellular domains (NICD) (Figure 1.3). The NECD contains an epidermal growth factor (EGF)-like repeat region, which is essential for ligand binding. Binding of NECD to a Notch ligand results in a second cleavage (termed S2 cleavage) in the HD by necrosis factor-α-converting enzyme (TACE; also designated ADAM17) (95). The NECD also contains the LIN12 and Notch repeats (LNRs) region, which prevent ligand independent cleavage of Notch receptor by metalloproteases (96). Interestingly, the mechanism employed by the LNR domain to inhibit ligand independent S2 cleavage is due to steric hindrance by the LNR region that blocks TACE from reaching the S2 cleavage site. Notch neutralizing antibodies, which inhibit conformational changes in the LNR region following ligand binding, inhibited S2 cleavage. Thus, demonstrating steric hindrance of the S2 cleavage site in the LNR region is removed by conformational changes in the Notch receptor following ligand binding (95, 97). Following S2 cleavage, the TM-NICD product is cleaved by γ-secretase, a multi-unit protease complex that cleaves transmembrane proteins, in the TM domain (termed S3 cleavage) (98).
Figure 1.3. Processing of the Notch receptor. Notch proteins are synthesized as precursor forms that are cleaved by furin-like convertase (S1 cleavage) in the heterodomain (HD) to generate the mature receptor, which is composed of two subunits that are held together by non-covalent interactions. The extracellular domain (ECD) of the Notch protein is comprised of epidermal growth factor (EGF)-like repeats, three cysteine-rich LIN12 and Notch repeats (LNRs), followed by a carboxy-terminal hydrophobic region. The Notch intracellular domain (NICD) is composed of conserved protein domains: namely, the RBP-Jκ-associated module (RAM) domain, ankyrin (ANK) repeats, nuclear localization signals (NLSs) and the PEST domain. On binding to the Notch receptor, the ligand induces a conformational change, exposing the S2 cleavage site in the ECD to the metalloproteinase tumour necrosis factor-α-converting enzyme (TACE). Following S2 cleavage, Notch undergoes a third cleavage (S3) in the transmembrane (TM) region that is mediated by the presenilin-γ-secretase complex, which is composed of presenilin 1 (PSEN1), PSEN2, nicastrin (NCSTN), presenilin enhancer 2 (PEN2) and anterior pharynx-defective 1 (APH1). The S3 cleavage results in the release of the active NICD from the plasma membrane and the subsequent translocation into the nucleus. OPA, polyglutamine repeat-containing region. Modified and reprinted with permission from the corresponding author and from Macmillan Publishers Ltd: Nature Reviews Cancer Ranganathan et al. (92), copyright (2011).

S3 cleavage releases the NICD from the cell membrane where it translocates to the nucleus and interacts with Notch transcriptional complex components such as mastermind (MAML) and recombination signal binding protein for immunoglobulin kappa J region (RBPJK) to control expression of Notch target genes (89).
The NICD contains the recombination binding protein-J associated molecule (RAM), the ankyrin (ANK), transactivation, and the proline, glutamic acid, serine, threonine-rich (PEST) regions along with two nuclear localization signals (99) (See Figure 1.3). The ANK and RAM regions are responsible for interaction between the NICD and transcriptional cofactors such as MAML and RBJPK, which are essential cofactors of the Notch transcriptional complex, and deletion of ANK and RAM regions negatively regulates Notch signaling (100-102). The transactivation region is involved in recruitment of factors such as histone acetyl-transferases, which promote transcriptional activation (103). The PEST domain influences NICD stability, and it has been shown necessary for NICD ubiquitination and protein degradation (104, 105). This is carried out by Numb protein family members (106).

Notch signaling is also regulated by factors independent of the regulatory regions on the ligands and receptors. Mind bomb (Mb) and Neuralized (Neu) are ubiquitin ligases that interact with Notch ligands to target them for ubiquitination and internalization following receptor binding. Ligand internalization also internalizes the NECD domain of the Notch receptor and leads to the conformational changes resulting in S2 and S3 cleavage. Mutations in the Mb and Neu genes have been shown to negatively regulate Notch signaling in Zebrafish (107, 108). The addition of saccharides to the EGF-like domains in the NECD domain offer another level of Notch signaling regulation. Genes such as O-glucosyltransferase, β1–3N-acetylglucosaminyl transferase, and O-fucosyltransferase 1 (Pofut1), facilitate addition of sugars to the EGF-like domains, and all three have been shown to be indispensable for Notch signaling (109-111).
With regard to airway epithelial cell differentiation, transgenic mouse models have shown that Notch signaling is important to multiciliated cell differentiation. For example, Pofut1 and Rbpjk mutant mice showed inhibited Notch signaling and significant increases in the amount of multiciliated cells in their airways (109). In addition, transgenic constitutive activation of Notch signaling by overexpression of NICD1 in mouse airways led to significant decreases in the multiciliated cell population (86). Pharmacological studies have shown that the addition of γ-secretase inhibitors such as dibenzazepine, which reduce Notch signaling by suppressing S3 cleavage and the release of NICD to the nucleus, also promote multiciliated cell differentiation using mouse tracheal explants (86). These data agree with observations showing Notch signaling inhibits the expression of Foxj1, Mcidas, and multiciliated cell differentiation in animal models (47, 109). Overall, Notch signaling appears to be a negative regulator of multiciliated cell differentiation in airway epithelial cells.

**Interleukin 13 and JAK/STAT Signaling**

Similar to Notch signaling, interleukin 13 (IL-13) has been shown to inhibit multiciliated cell differentiation (112). IL-13 is a pleiotropic cytokine initially described in 1989 (113). IL-13 is a 132 amino acid protein produced by many adaptive and innate immune cell types including type 2 T helper cells, type 2 innate lymphoid cells (114), natural killer cells (115), mast and basophil cells (116), and eosinophils (117). IL-13 is also produced by non-immune cells such as airway epithelial cells (118). IL-13 is secreted from cells, and it can act in autocrine and paracrine fashions (119, 120). IL-13 binds to the IL-13 receptor, which is a heterodimer comprised of interleukin 4 receptor, alpha (IL-4Rα) and interleukin 13 receptor, alpha 1 (IL-13Rα1) proteins (121). IL-13Rα1
is a transmembrane protein that possesses an extracellular domain where secreted IL-13 binds and an intracellular domain that is essential for downstream signaling (122). IL-13Rα1 has weak affinity for IL-13 (K_d ~ 4 nM), but upon dimerization with IL-4Rα, the affinity increases over 100-fold (K_d ~ 30 pM) (123). IL-13 also binds to interleukin 13 receptor, alpha 2 (IL-13Rα2), which shares 37% protein sequence homology with IL-13Rα1 and binds IL-13 with greater affinity (K_d ~ 250 pM). However, IL-13Rα2 does not mediate IL-13 induced signaling despite the presence of IL-4Rα (124). It is thought this is because IL-13Rα2 lacks an extensive intracellular domain, which was reported to be essential for IL-13 to signal through IL-13Rα1 mentioned above. Because of this, IL-13Rα2 is thought to act as a decoy receptor for IL-13 and to negatively regulate IL-13 responses (125, 126). IL-4Rα is ubiquitously expressed (127, 128), and IL-13Rα1 and IL-13Rα2 have been found to have widespread expression including airway smooth muscle and airway epithelial cells (121, 125, 129), which likely explains the pleotropic nature of IL-13.

Binding of IL-13 to the IL-13 receptor triggers downstream signaling (130). The JAK/STAT pathway is the principal signaling mechanism for a variety of cytokines, including IL-13 (131). In the JAK/STAT pathway, there are four Janus Kinases (JAK1, JAK2, JAK3, Tyk2) and seven signal transducers and activators of transcription (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) family members (132). All of which are expressed in human airway epithelial cells (94).

JAK molecules contain 4 functional regions with the four-point-one, ezrin, radixin, moesin (FERM) region being the most N-terminal. The FERM region is important for regulating JAK activity by facilitating association of JAKs with receptors and auxiliary
proteins (133, 134). Mutations of tyrosine residues in the FERM region cause constitutive kinase activation despite the absence of cytokine simulation or can create proteins lacking kinase activity, depending on the mutation (133, 135). The region adjacent to the FERM is the Src homology-2 (SH2) region. This is an interesting region because, unlike canonical SH2 regions, it does not bind phosphorylated tyrosine residues (136). Another interesting observation is that the SH2 region has been reported to be necessary for binding of JAKs to certain receptors but dispensable for others (137). The pseudokinase region is next to the SH2 region, and while containing many of the conserved amino acids seen in canonical tyrosine kinase domains, the pseudokinase region lacks important residues necessary for catalytic activity. However, studies have shown it to play a pivotal role in negatively regulating JAK kinase activity (138, 139). The most carboxyl terminus region of the JAK protein is the tyrosine kinase region. The kinase activity occurs in this region as the name suggests. The region possesses dual, adjacent tyrosine residues whose phosphorylation regulates substrate binding and activation of JAKs (140-142). On a historical note, JAK kinases got their name “Janus kinase,” which refers to a roman god with two faces, because they possess two kinase domains (143).

STATs are transcription factors and contain six defined regions. These are, in order from the N-terminus to the C-terminus, the N-terminal region, the coiled-coiled region, the DNA binding region, the linker region, SH2 region, the transactivation region, and the . The N-terminal region is associated to a variety of STAT functions including dimerization, nuclear localization and deactivation, DNA binding, and receptor interactions (144-146). The coiled-coiled region interacts with important regulatory proteins and in receptor interactions and nuclear export (147, 148). The DNA binding is
important for binding STAT molecules to gamma activated sequence elements, short nucleotide sequences important for induction of transcription, in the promoters of target genes. In certain situations complexes of STATs and other molecules such as interferon regulatory factor 9 cause STATs to bind other DNA elements like the IFN-α/β-stimulated response element (149). The linker region acts as a spacer to maintain proper conformational arrangement between the DNA binding and SH2 regions (150). The SH2 region is important for three elements of STAT activity that are largely based on its ability bind phosphotyrosine residues, which is mediated by a conserved arginine amino acid. The first is recognition and recruitment of the STAT to specific phosphotyrosine motifs on a ligand bound cytokine receptor. The second is association with JAK molecules leading to phosphorylation of the STAT molecule at a specific tyrosine residue located downstream of the SH2 domain. The third is dimerization of STATs following tyrosine phosphorylation (150, 151). To note, phosphorylation of STAT tyrosine residues is not exclusively from JAKs. Other kinases such as SRC family tyrosine kinases members can also phosphorylate the same tyrosine residues (152). The transactivation region is responsible for transcriptional regulation of target genes by interaction with other transcription factors. Moreover, regulation of this region comes from phosphorylation of both tyrosine and serine residues and can be regulated by a variety of kinases including mitogen-activated protein kinases (132, 153).

In addition to the mechanisms mentioned above, JAK/STAT signaling is negatively regulated by multiple other mechanisms. SH2-containing phosphatases (SHPs), who are members of the protein tyrosine phosphatase (PTP) family, dephosphorylate tyrosine residues on proteins (154). SHPs dephosphorylate tyrosine residues through binding of
their SH2 domains to phosphorylated tyrosine residues followed by dephosphorylation of the tyrosine residue by their carboxyl-terminus phosphatase domain (155, 156). Specifically, SHPs directly dephosphorylate tyrosine residues on JAKs and cytokine receptors, which inhibits recruitment and phosphorylation of STAT molecules leading to inhibition of JAK/STAT signaling (157, 158). The protein inhibitor of activated STAT proteins represent another class of proteins that can negatively regulate JAK/STAT signaling. They inhibit JAK/STAT signaling by binding to STAT dimers and disrupting STAT dependent transcriptional regulation of target genes by inhibiting binding of STATs to DNA and by recruiting transcriptional repressors to STAT target gene promoters (159). Additionally, suppressors of cytokine signaling (SOCS) proteins are well-established inhibitors of JAK/STAT signaling. SOCS proteins contain an SH2 domain, like PTPs, along with a kinase inhibitors region (KIR) and a carboxyl-terminus SOCS box, which designates proteins for ubiquitination and proteosome degradation (159, 160). SOCS proteins are thought to be expressed at low levels in general conditions but rapidly induced in response to cytokines along with other stimuli such as certain pathogens and lipopolysaccharide binding protein (LPS) (161). SOCS proteins inhibit JAK/STAT signaling in a variety of ways. The first is by binding to tyrosine residues on activated receptors and thereby hindering binding of STAT molecules to receptors. The second is, by directly binding JAKs and inhibiting their kinase activity via their KIR. Lastly, SOCS proteins can target JAKs for degradation by their SOCS domain (159, 162-164).

With respect to IL-13 signaling, IL-13 receptor activation leads to the recruitment and phosphorylation/activation of JAK1, JAK2, and Tyk2 (156). Activation of JAKs leads to
the phosphorylation of tyrosine residues on IL-4Ra. This results in the recruitment of STAT6 to the receptor where it becomes phosphorylated by JAKs. The phosphorylation of STAT6 by JAKs causes dimerization of STAT6 molecules by their attraction of their SH2 domain to a phosphorylated tyrosine on another STAT. STAT6 dimers translocate to the nucleus where they bind to gamma activated sequence elements in DNA and regulate target gene expression (153). Once in the nucleus, STAT molecules are dephosphorylated/inactivated and exported from the nucleus for another potential round of activation (148, 150, 153, 156). Additionally, SOCS and SHPs negatively regulate IL-13 activation of JAK/STAT, which adds additional regulatory levels of IL-13 dependent JAK/STAT signaling (165, 166).

IL-13 receptor can also participate in other signaling pathways. For example, insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) are also recruited to the IL-13 receptor. Specifically, recruitment occurs following phosphorylation of tyrosine 497 on the IL-13 receptor, which results in phosphorylation and activation of the IRS proteins (167). Following activation, IRS-1 and IRS-2 have been observed to be involved in phosphoinositol 3 kinase signaling (167, 168). IL-13 has also been shown to use the mitogen activated protein kinase 13 signaling pathway to regulate mucin expression in airway epithelial cells (169), and pharmacological inhibition of the mitogen-activated protein kinase kinase abrogates IL-13 dependent goblet cell hyperplasia (170).

IL-13 has been associated with multiple human diseases including inflammatory diseases like atopic dermatitis (171, 172) and allergic rhinitis (173, 174). IL-13 has also been shown to fuel the pathogenesis of Hodgkin lymphoma (175). With respect to respiratory disease, IL-13 levels are elevated in respiratory diseases such as asthma (176).
and in virally infected lungs (177), and IL-13 has been shown to be a central mediator of asthma pathogenesis (178).

Phenotypically, both asthmatic lungs and virally infected lungs display airway hyperresponsiveness (179, 180), which is characterized by increased sensitivity for contraction of the bronchioles leading to a narrowing of the airways. Epithelial remodeling has been observed in asthmatic and virally infected lungs, which show increased numbers of goblet cells (181, 182) and decreased numbers of multiciliated cells (26, 183). Moreover, it has been shown that respiratory syncytial virus (RSV) infection of human basal cells in vitro results in an epithelium with goblet cell hyperplasia and less multiciliated cells (184). Given that IL-13 is elevated in individuals with asthma and by respiratory viral infection (176, 185) and that the IL-13 receptor is found on airway smooth muscle (ASM) and airway epithelial cells, as described above, it has been proposed that IL-13 could be responsible for these airway hyperresponsiveness and epithelial remodeling. Indeed in vivo studies in mice showed IL-13 induced airway hyperresponsiveness (178), and in vitro studies using human airway smooth muscle cells showed IL-13 enhanced contractility (186). Studies using normal primary human bronchial epithelial cells have shown IL-13 increases goblet cell differentiation and decreases multiciliated cell differentiation (31, 52, 86, 112, 187). Furthermore, IL-13 induced goblet cell hyperplasia is blocked human airway epithelial cells in vitro by the addition of pharmacological γ-secretase inhibitors, which inhibit Notch receptor S3 cleavage and Notch signaling (86). These results show a dependence of Notch in IL-13 induced goblet cell hyperplasia. Knockout mouse studies have also shown IL-13 increases mucus production and causes goblet cell hyperplasia using the JAK/STAT6 pathway.
pathway (86, 188). In contrast to the extensive studies investigating how IL-13 promotes goblet cell differentiation, little is understood as to how IL-13 inhibits multiciliated cell differentiation (187).

**Summary and Goals**

Dysfunction in mucociliary clearance (MCC) and epithelial remodeling, including decreases in multiciliated cells, is commonly seen in respiratory diseases such as COPD and asthma. Modulation of signaling pathways that influence basal/stem cell differentiation towards a non-multiciliated cell fate is the probable cause of this epithelial remodeling and MCC dysfunction. Therefore, understanding which signaling pathways are important in basal cell fate decisions may have great clinical importance and lead to novel therapies to restore MCC in the lungs of individuals with respiratory disease.

The goal of this research thesis is to determine the environmental cues and signaling pathways that influence human bronchial multiciliated cell fate decisions. Specifically, the mechanism(s) that control submersion dependent and IL-13 dependent inhibition of multiciliated cell differentiation will be investigated with the goal of understanding the molecular events in the multiciliated cell differentiation pathway that are being disrupted by these stimuli and the relevant signaling pathways utilized by submersion and IL-13 to inhibit multiciliated cell differentiation. Understanding how these factors are inhibiting multiciliated cell differentiation may increase our understanding of respiratory disease pathogenesis as IL-13 is elevated in respiratory diseases such as asthma, and submersion may be relevant to bronchial differentiation in low oxygen environments, which has been seen in the respiratory disease COPD (24, 67).
Chapter 2: Materials and Methods

Normal Human Bronchial Epithelial Cell Culture

Normal human bronchial epithelial (NHBE) cells were isolated from the epithelium of airways from human donor lungs by manual dissection followed by protease treatment to disassociate the cells from the basement membrane; as previously described (189). Lungs used were rejected for organ transplant. Lungs were provided by the Life Alliance Organ Recovery Agency of the University of Miami with institutional review board approval. NHBE cells were cultured as described previously (190) but will be briefly explained. Undifferentiated NHBE cells were expanded on 10 cm² collagen coated, tissue culture treated plates in bronchial epithelial cell growth media (BEGM) until reaching confluency. BEGM contains high amount of EGF (25 ng/ml), which promotes proliferation of NHBE cells. After the cells reach confluency, they were transferred and cultured on 1 cm² or 0.33 cm² Transwell® permeable membranes (3421 or 3460; Corning, Corning, NY) or 96-well plates (353219; BD Biosciences, San Jose, CA) coated with human collagen IV (C7521; Sigma, St. Louis, MO). Cells were plated at 150,000 or 50,000 cells for 1 cm² or 0.33 cm² membranes respectively and 100,000 for 96-wells. These cells were grown in air:liquid interface (ALI) media. For 96-well or 384-well florescence experiments, fluorescence was measured using a Synergy H1 Multi-Mode Plate Reader (BioTek, Winooski, VT). NHBE cells were either cultured at an ALI in air/normoxia (21% O₂), submerged under various apical volumes of media, or at an ALI in hypoxic conditions (0.5% O₂). Hypoxic conditions were established using the Coy oxygen control glove box (Coy Laboratory Products, Grass Lake, MI) using nitrogen gas to control the levels of oxygen. Dr. Claudia Rodrigues generously made the oxygen glove
box available. For IL-13 experiments, IL-13 (200-13; Peprotech, Rocky Hill, NJ) was dissolved in water with 0.1% BSA and various concentrations were used in experiments with NHBE cells. Passage 1 NHBE cells were used as higher passage cells can lose their differentiation capacity. Only NHBE cells obtained from the lungs of non-smokers were used. The number of individual donors used for experiments is stated in figure legends.

**Inhibitors**

To inhibit Notch signaling, N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester (DAPT) was used at a final concentration of 10 μM. Notch negative regulatory region (NRR) antibodies or IgG control were used at 20 μg/ml. Antibodies were generously provided by Dr. Chris Siebel’s lab at Genentech. To inhibit JAK/STAT signaling, JAK Inhibitor 1 (15146; Cayman Chemical, Ann Arbor, MI) was used at a final concentration of 9 μM. To inhibit MEK/ERK signaling, PD98059 (10006726; Cayman Chemical) was used at a final concentration of 50 μM. DAPT, JAK Inhibitor 1, and PD98059 were dissolved in 100% dimethyl sulfoxide (DMSO), and an equivalent amount of DMSO was used as a vehicle control.

**RNA purification and Quantitative RT-PCR (RT-qPCR)**

Total RNA was purified from NHBE cells using the E.Z.N.A HP Total RNA Isolation Kit (R6812-02; Omega Bio-Tek, Norcross, GA) adhering to the manufacturer’s instructions. RNA concentrations were determined using a Nanodrop 1000 (Thermo Scientific, Rockford, IL). Complimentary DNA (cDNA) was made using 0.5 μg total RNA with the iScript cDNA Synthesis Kit (170-8891; Bio-Rad, Hercules, CA). Real-time quantitative PCR (qPCR) was performed on a CFX96 iCycler (Bio-Rad) using 1/20th cDNA reaction volume. Taqman® probes were used for qPCR to detect *HES1*.
(Hs01114113_m1; Life Technologies), \textit{HES2} (Hs00219505_m1; Life Technologies), \textit{HEY1} (Hs0114113_m1; Life Technologies, Carlsbad, CA), \textit{HEY2} (Hs00232622_m1; Life Technologies), \textit{FOXJ1} (Hs00230964_m1; Life Technologies), \textit{MCIDAS} (Hs04234533_g1; Life Technologies), \textit{ALDOC} (Hs00193059_m1; Life Technologies), \textit{GAPDH} (Hs99999905_m1; Life Technologies), and \textit{B2M} (Hs99999907_m1; Life Technologies). All Taqman® qPCR reactions were performed in duplex with \textit{ALDOC}, \textit{FOXJ1}, \textit{HES1}, \textit{HES2}, \textit{HEY1}, \textit{HEY2}, or \textit{MCIDAS} being detected simultaneously with either \textit{GAPDH} or \textit{B2M}, which is described in the figure legends. Taqman® duplex qPCRs were done using SsoFast™ supermix (172-5321; Bio-Rad). \textit{MCIDAS} and \textit{GAPDH} were also detected by SYBR Green method using the iTaq Universal SYBR Green supermix (171-5120; Bio-Rad). The primers used were designed using Primer3 (NIH; Bethesda, MD) and are \textit{MCIDAS} forward (MCIDAS\_F) (CCAACAGAATGCTGGCACTG), \textit{MCIDAS} reverse (MCIDAS\_R) (CAACGCTCTCTGGTTCTGGT), \textit{GAPDH} forward (GAPDH\_F) (TGGTCTCCTCTGGTTCTGGT), and \textit{GAPDH} reverse (GAPDH\_R) (TGCTGTAGCCAAATTCGTTGT). The amounts of \textit{HEY1}, \textit{ALDOC}, \textit{HES1}, \textit{HES2}, \textit{HEY1}, \textit{HEY2}, \textit{FOXJ1} and \textit{MCIDAS} mRNAs relative to a reference housekeeping mRNA, either \textit{GAPDH} or \textit{B2M}, in different treatments, were calculated from the measured threshold cycles (Ct) using the equation: relative mRNA = 2^\text{(Ct reference mRNA – Ct gene of interest mRNA)}.

**Immunofluorescence and Confocal Microscopy**

NHBE cells were fixed on filters or in the wells of a 96-well plate using 4% paraformaldehyde (PFA) in 1X PBS for 20 minutes at room temperature followed by
washing with 1X PBS. NHBE cells were permeabilized with 1% Triton X-100 in 1X PBS for 15 minutes proceeded again by washing with 1X PBS. NHBE cells were blocked in 3% bovine serum albumin (BSA) in 1X PBS for 1 hour at room temperature. After blocking, primary antibodies anti-FOXJ1 (AF3619, 0.01 μg/ml; R&D Systems, Minneapolis, MN), anti-HA (2367, 1:500; Cell Signaling Technology, Danvers, MA), and/or anti-acetylated tubulin (T6793, 1:2000 dilution; Sigma) were added overnight at 4°C. After overnight incubation, unbound primary antibody was washed away with 1X PBS. After washing, species-specific Alexa Fluor® coupled secondary antibodies (A11055, A31571, A31572, and A21447, 1:500; Life Technologies) were added in 3% BSA for 1 hour at room temperature. Unbound secondary antibody was washed off with 1X PBS and nuclei were stained with Hoechst dye for 20 minutes at room temperature. Hoechst dye not bound to DNA was washed off with 1X PBS. Cells on Transwell® membranes were mounted onto slides with Fluoro-Gel and cells in 96-well plates were immersed in Fluoro-Gel (17985-10; Electron Microscopy Sciences, Hatfield, PA). Confocal images were taken on a DM6000 for filters and on a DMI6000 for 96-well plates with a SP5 confocal module (Leica, Wetzlar, Germany). Images were processed with Volocity version 6.1.1 software (Perkin-Elmer, Waltham, MA).

**Protein Isolation and Western Blotting**

Whole cell protein lysates were prepared from NHBE cells by lysing cells in whole cell lysis buffer (10 mM Tris pH 8.45, 1% SDS, 0.1 mM EDTA) supplemented with cOmplete Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail (04906837001 and 11697498001; Roche, Basal, Switzerland). Lysis buffer, 150 μl for 1 cm² or 90 μl for 0.33 cm² filters, was added directly to NHBE cells, and lysates were
sonicated using a Misonix W-225 sonicator. Sonicated samples were centrifuged using a refrigerated micro 18R centrifuge (VWR, Radnor, PA) at 14,100 x g at 4°C for 10 minutes to remove insoluble material. The supernatant was transferred to another tube and stored at -80°C. Protein concentrations were determined using a BCA assay kit (23225; Thermo Scientific).

After denaturing protein lysates by heating to 95°C for 5 minutes in protein loading buffer (60 mM Tris pH 6.8, 1.7% SDS, 10% glycerol, and 0.002% bromophenol blue) supplemented with 0.1M dithiothreitol (DTT), 30-50 μg were loaded onto 4-15% Mini-PROTEAN® TGX™ precast gels (456-1086; Bio-Rad) and separated by SDS-PAGE electrophoresis using 1X Tris/Tricine/SDS running buffer (161-0744; Bio-Rad). Proteins were transferred to a BioTrace™ polyvinylidene difluoride (PVDF) membrane (66543; PALL Life Sciences, Ann Arbor, MI) in 10 mM CAPS buffer (pH 11.0) at 320 mA for 90 minutes at 4°C. Following protein transfer, PVDF membranes were blocked with either 5% milk if probing for non-phosphorylated proteins or with 5% BSA if probing for phosphorylated proteins. Both blocking buffers were made by dissolving the milk or BSA in 0.1% Tris buffered saline-Tween 20 (TBS-T). After blocking PVDF membranes for at least 1 hour, they were probed for anti-NICD1 (4147, 1:1000 dilution; Cell Signaling Technology), anti-HIF-1α (610958; BD Biosciences), anti-HIF-2α (NB100-122; Novus Biologicals, Littleton, CO), or anti-phospho-Tyr641-STAT6 (9361; Cell Signaling Technology) overnight at 4°C. Primary antibodies were diluted in either 5% milk or 5% BSA solutions. After incubation, primary antibody solution was removed and blots were washed with TBS-T to remove unbound antibody. After washing, species-specific horseradish peroxidase (HRP) conjugated secondary antibodies (474-1056 and 474-1806;
KPL, Gaithersburg, MD) were added for 1 hour at room temperature. Unbound secondary antibody was removed by washing with TBS-T. Supersignal West Pico chemiluminescent HRP substrate (34087; Thermo Scientific) was added to the PVDF membranes and chemiluminescent signal was detected using a Chemidoc XRS+ system (Bio-Rad) with Quantity One software (Bio-Rad). After probing for the above proteins, β-actin was probed as a loading control using anti-β-actin (ab3820, 1:5000, Abcam, Cambridge, UK) for 1 hour at room temperature. The primary antibody solution was removed followed by washing with TBS-T and incubation with an HRP conjugated secondary antibody (KPL) for 1 hour. Chemiluminescent substrate was then added to PVDF membranes and detected on the Chemidoc XRS+ system.

**Ciliary Beat Frequency**

Ciliary beat frequency (CBF) was measured as previously described (191). Briefly, video of multiciliated NHBE cells was taken at room temperature using a charge-coupled device (CCD) camera (XC7500; Sony, Tokyo, Japan) connected to a Zeiss Axiovert 200M microscope with a 40X differential interference contrast (DIC) optics objective lens. Pixel intensity spectra from a fast Fourier transform (FFT) of multiciliated cells were recorded. CBF (Hz) was assessed from FFT data from four individual multiciliated cells from two different lungs. Data is represented as mean ± standard deviation (s.d.).

**Cilia Length**

Cilia length was determined by measuring the height of cilia from individual multiciliated NHBE cells using the z-axis from 40X confocal images of cultured on an ALI or submerged in 96-wells that were stained with acetylated α-tubulin. Measurements of NHBE cells were performed using Volocity version 6.1.1 software (Perkin-Elmer) on
images taken from two lungs from each condition. Cilia length was determined by averaging four randomly chosen multiciliated cells from each lung. Data is presented as mean ± s.d.

**Lentiviral Construction, Production, and NHBE Transduction**

Lentiviruses expressing HA tagged Notch Intracellular Domain 1, 2, and 3 (HA-NICD1-3), constitutively active HIF-1α (CA-HIF-1α) and constitutively active HIF-2α (CA-HIF-2α) were constructed using the pCDH-EF1-HA-MCS-FLAG-T2A-copGFP vector constructed by Dr. Nevis Fregien. This vector was based on the pCDH-MCS-T2A-copGFP (CD526A-1; System Biosciences, Mountain View, CA).

HA-NICD1, HA-NICD2, HA-NICD3 fragments were produced by PCR of human NICD1, human NICD2 or rat NICD3 sequence from pLU-CMV-NICD1-IRES-GFP, pLU-CMV-NICD2-IRES-GFP, or pLU-CMV-NICD3-IRES-GFP vectors (Gifts from Dr. Anthony Capobianco). To amplify NICD1, 5’-aagcggcgcctgaagggcctccggaatgc-3’ and 5’-aagcggcgcctgaagggcctccggaatgc-3’ were used. To amplify NICD2, 5’-aacgggtgcaaacaagctgaagcgtaagcatg-3’ and 5’-aacgggtgcaaacaagctgaagcgtaagcatg-3’ were used. To amplify NICD3, 5’-aacgggtgcaaacaagctgaagcgtaagcatg-3’ and 5’-aacgggtgcaaacaagctgaagcgtaagcatg-3’ primers were used. All primers contained AgeI and NotI restriction sites (shown in bold), which were used to insert them into the pCDH-EF1-HA-MCS-FLAG-T2A-copGFP vector. The PCR products were then restriction digested and inserted to produce pCDH-EF1-HA-NICDX-T2A-copGFP vectors (NICDX = NICD1, NICD2, or NICD3).

To construct CA-HIF-1α and CA-HIF-2α pro-lentiviral vectors, CA-HIF-1α and CA-HIF-2α sequences were PCR amplified from retroviral constructs purchased from
Addgene (Cambridge, MA). The HA-HIF1alpha P402A/P564A-pBabe-puro and HA-HIF2alpha-P405A/P531A-pBabe-puro constructs both came from William Kaelin (Addgene plasmids # 19005 and 19006) (192). Primer sequences 5’-ggatctagagcttacctggtacctacccc-3’ and 5’-gaagggcggttaacctgtaccaagctctgag-3’ were used to PCR amplify the HIF1α-P402A/P564A sequence, and 5’-cattacctagacccatgctacctacgagccc-3’ and 5’-gattatgctagctcctgggtcctgtccag-3’ primers were used to PCR amplify the HIF-2α-P405A/P531A sequence. For cloning of HIF-1α, XbaI and NotI were the restriction sites used (shown in bold). For cloning of HIF-2α, XbaI and BmtI sites were used (shown in bold). Following PCR fragments being digested with restriction enzymes; they were inserted to produce pCDH-EF1-HA-CA-HIF-Xα-T2A-copGFP vectors (CA-HIF-Xα = CA-HIF-1α or CA-HIF-2α). All pro-viral vectors had their sequences confirmed.

To produce lentiviruses, HEK293T cells were co-transfected with 10 μg pro-lentiviral vector and 3rd generation packaging plasmids 3.75 μg pRSV-REV, 7.5 μg pMDLg/RRE, and 4.5 μg pMD2.VSVG per 10 cm² dish. 3rd generation vectors and packaging plasmids were used because they offer the largest extent of biosafety in creating lentiviruses as there is no Tat, which is required for viral replication, and more recombination events to generate replication competent lentivirus (193). Furthermore, the pro-lentiviral vector contains self-inactivating (SIN) long terminal repeats (LTRs), which allow for control of the inserted sequence by the flanking inserted promoter without the influence of the viral promoter in the LTR (194). Transfection was carried out with the CalPhos™ mammalian transfection kit (631312; Clontech, Mountain View, CA, USA) according to the manufacturer’s protocol. HEK media (DMEM + 10% FBS + 1% Pen/Step) was changed
every 24 hours and lentivirus was collected at 48 and 72 hours after transfection. Virus-laden media was filtered through a 0.45 µm filter to remove any cellular debris, and 0.42 ml 40% polyethylene glycol (PEG) (9633P; Sigma) was added per ml media. PEG solution was incubated at 4°C for at least 48 hours to precipitate the virus. The virus was centrifuged for 30 minutes at 1500 x g at 4°C and the supernatant was aspirated off. The tube was then re-centrifuged again for 30 minutes at 1500 x g at 4°C to remove residual PEG. Virus pellets were suspended in BEGM media and aliquots were made and stored at -80°C. The viral concentration or viral titre was determined by p24 assay (NEK050001KT; Perkin-Elmer).

NHBE cells were transduced with lentiviruses by the spinoculation method, which entails mixing NHBE cells and lentivirus in suspension in BEGM media and then centrifuging for 2 hours at 1000 x g at room temperature. The resulting NHBE cell pellets were resuspended in the virus containing BEGM media before plating. For HA-NICD lentiviruses, NHBE cells were plated onto 10 cm² plates while for CA-HIF, NHBE cells were plated onto Transwell® membranes. The next day, the media was replaced with virus free BEGM media for HA-NICD cells or ALI media for CA-HIF cells, and these cells grew over the next few days with media being changed every 2-3 days. After 5 days, looking for copGFP expression in NHBE cells assessed lentivirus transduction. Following confirmation of copGFP positive (copGFP+) cells, HA-NICD transduced cells were either plated on 0.33 cm² Transwell® filters (3421, Corning) in ALI media or had their RNA purified using the E.Z.N.A total RNA kit (R6834-02; Omega Bio-Tek). HA-NICD transduced cells were cultured submerged (0.5 ml) or at an ALI in hypoxic (0.5% O₂) conditions + 10 µM DAPT for 3 weeks and then immunostained for HA and FOXJ1.
For CA-HIF-α transduced cells, upon confirmation of copGFP+ cells, cells were cultured at an ALI in air +/- 10 μM DAPT for 3 weeks and then immunostained for HA and FOXJ1 or RNA was purified and HIF target gene expression was analyzed.

**Statistical Analysis**

PRISM 5 software (GraphPad Software, San Diego, CA) was used for statistical analysis. Data presented as median +/- standard error of the mean (SEM) unless otherwise denoted. N represents the number of individual lungs used for the experiment. Analysis of two groups used an unpaired Student’s two-tailed t-test. For analysis of three or more groups, a one-way analysis of variance (ANOVA) was done with a Newman-Kuels, Dunnett, or Bonferroni multiple-comparisons post-test; depending on the goal of the experiment. A p-value less than 0.05 was considered significant.
Chapter 3: Submersion and Hypoxia Inhibit Multiciliated Cell Differentiation via Notch Signaling

NHBE Multiciliated Cell Differentiation is Inhibited in an Apical-volume Dependent Manner

Ostrowski et al. showed submersion inhibited multiciliated rat tracheal epithelial (RTE) cell differentiation in an apical-volume dependent manner (55), but it has never been investigated whether this is also true for human cells. Additionally, the effect of submersion on the expression of FOXJ1, a transcription factor necessary for multiciliated cell differentiation, has never been studied despite previously finding that knockout of FOXJ1 results in the lack of multiciliated cell differentiation (43). To answer if multiciliated cell differentiation was inhibited in NHBE cells in an apical-volume dependent manner and to assess the effect of submersion on FOXJ1 expression, NHBE cells were cultured under a variety of apical media volumes (0 ml, 0.125ml, 0.25 ml, or 0.5 ml) for three weeks. NHBE cells were either immunostained for FOXJ1 and acetylated-tubulin (cilia) or RNA was purified and FOXJ1 expression was analyzed. Qualitative analysis of confocal images suggested both FOXJ1 positive (FOXJ1+) and multiciliated cells were increased over air:liquid interface (ALI) control (0 ml) at 0.125 ml volume, were similar at 0.25 ml, and were drastically decreased at 0.5 ml (Figures 3.1A-H). Quantification of the immunofluorescence data (Figure 3.1I) showed a significant increase in FOXJ1+, multiciliated cells at 0.125 ml, no significant difference at 0.25 ml, and a significant decrease at 0.5 ml. Quantification of FOXJ1 mRNA (Figure 3.1J) showed expression patterns similar to what was observed by immunofluorescent data with the exception that FOXJ1 was decreased at 0.25 ml. While the increase in multiciliated cells was interesting, no further experiments investigating this were
performed. The influence of apical-volume on NHBE cell density was investigated by counting the number of nuclei per image field. This was done to ensure accurate percentages of ciliated cells in different conditions. The results showed (Figure 3.1K) there was no significant difference between cell densities between control (0 ml) and 0.125 ml or 0.25 ml, but there was a significant decrease (~33%) at 0.5 ml, however, the reason for this difference is unclear. These data indicate submersion inhibits multiciliated NHBE cell differentiation in an apical-volume dependent manner, and this inhibition may be due to repression of FOXJ1 expression.

Submersion of NHBE Cells Creates a Hypoxic Environment

The mechanism of inhibition of FOXJ1 expression and multiciliated cell differentiation by submersion was unclear. It was previously reported that oxygen concentration at the cellular level decreases as apical volume is increased (58, 195). Since hypoxia has been shown to inhibit differentiation of many cell types (61, 62), it was hypothesized that larger apical volumes may be inhibiting FOXJ1 expression and multiciliated cell differentiation by creating a hypoxic environment at the level of the cells.

Figure 3.1. FOXJ1 expression and ciliated cell differentiation are reduced by submersion in an apical volume dependent manner. (A-H) Representative extended focus confocal immunofluorescent images of NHBE cells cultured for 21 days with apical media volumes of: 0 ml (A, E), 0.125 ml (B, F), 0.25 ml (C, G), and 0.5 ml (D, H) stained for nuclei (Hoechst, blue) and cilia (acetylated α-tubulin, white; A-D) or FOXJ1 (green; E-H). (I) Percent FOXJ1 positive (white bars) and ciliated (black bars) cells at the apical media volumes, indicated on the X-axis, showing a significant increase at 0.125 ml and a significant decrease at 0.5 ml. (J) FOXJ1 mRNA levels after 21 days of differentiation at the apical media volumes, indicated on the X-axis, normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA, showing the amount of FOXJ1 mRNA increases at 0.125 ml and decreases significantly at higher apical volumes, which is similar to changes in FOXJ1+ cells. (K) Quantification of nuclear density after 21 days submerged with different volumes of media showing a significant reduction in cells in 0.5 ml. Scale Bar = 50 μm. One-way ANOVA; * p < 0.05. n=3. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac et al/2014/Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner/Am J Respir Cell Mol Biol/51/516-25.The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.
To test this, NHBE cells were cultured either at an ALI in hypoxia (0.5% O2), at an ALI in air (21% O2), or submerged under 0.5 ml apical volume, and the levels of HIF-1α and HIF-2α protein were measured by western blot. The data showed (Figures 3.2A and B) submersion increased both HIF-1α and HIF-2α protein levels compared to air controls, similar to hypoxia. These data suggest that submersion created a hypoxic environment.

**Figure 3.2.** Submersion and hypoxia induce HIF-1α and HIF-2α proteins. NHBE cells cultured 72 h at an ALI in air (21% O2) then switched to an ALI in hypoxia (0.5%) (Hyp.) or submerged (0.5 ml) (Sub.) for 5 hours. (A) Western blots of whole cell protein lysates with antibodies to HIF-1α, HIF-2α, and β-actin showing both HIF-1α and HIF-2α induction in submerged and hypoxic conditions compared to ALI (Air) control. (B) Densitometry quantification of the western blot data from 2 lungs. The data show increases in HIF proteins indicating submersion causes a hypoxic environment. β-Actin is used as a loading control. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac et al/2014/Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner/Am J Respir Cell Mol Biol/51/516-25. The *American Journal of Respiratory Cell and Molecular Biology* is an official journal of the American Thoracic Society.

**Submersion and Hypoxia Inhibit Multiciliated Cell Differentiation via a γ-secretase Dependent Pathway**

Since NHBE cells submerged under larger apical volumes were hypoxic, it suggested that hypoxia, and not submersion, was responsible for inhibiting FOXJ1 and multiciliated cell differentiation. To test this, NHBE were cultured at an ALI in 0.5% O2
for three weeks and ciliated cell differentiation was assessed by immunofluorescent staining. The results showed, similar to submerged cells (Figures 3.3A and 3.3B), hypoxia inhibited FOXJ1 expression and multiciliated cell differentiation (Figures 3.3E and F) compared to air control (Figures 3.3I and 3.3J). These data indicated the hypoxic environment created by submersion was responsible for suppression of FOXJ1 expression and multiciliated cell differentiation. However, the mechanism of this was not understood. In the literature, it has been reported that hypoxia-dependent inhibition of both neuronal and pancreatic cell differentiation requires Notch signaling (61, 62), and Notch signaling inhibits multiciliated cell differentiation (47, 86, 109). Therefore, it was hypothesized that submersion and hypoxia inhibited multiciliated cell differentiation by promoting Notch signaling. To test this, NHBE cells were cultured submerged or at an ALI in hypoxia with the γ-secretase inhibitor DAPT, which has been shown to inhibit Notch signaling (196). The results showed addition of DAPT restored FOXJ1 expression and multiciliated cell differentiation in both submersion (Figures 3.3B and 3.3C) and hypoxia (Figures 3.3G and 3.3H). This restoration was comparable to air levels (Figure 3.3K), which is consistent with the hypothesis that Notch signaling is mediating the reduction in multiciliated NHBE cells. In addition, to confirm DAPT inhibited Notch signaling, the levels of cleaved Notch Intracellular domain 1 (NICD1) protein was assessed in NHBE cells cultured at an ALI, at an ALI in hypoxia with or without DAPT, or submerged with or without DAPT. The results showed NICD1 was reduced in submerged and hypoxic conditions with DAPT, and the data showed neither hypoxia nor submersion induced NICD1 levels compared to ALI (Figure 3.3L). Furthermore, to ensure DAPT was inhibiting Notch target gene expression, the levels of Notch target
gene hairy/enhancer-of-split related with YRPW motif 1 (HEY1) were measured in submerged and hypoxic conditions with or without DAPT. The results revealed significant decreases of HEY1 in both conditions by DAPT, which indicated DAPT was inhibiting Notch target gene expression (Figure 3.3M). Together, these data imply that DAPT was inhibiting Notch signaling in the conditions tested.

MCIDAS Expression is Suppressed in Submerged Culture and Promoted by DAPT

MCIDAS is a transcriptional regulator that is necessary for multiciliated cell differentiation and suppressed by Notch signaling in Xenopus (47). Additionally, MCIDAS expression occurs before expression of FOXJ1 in the multiciliated cell differentiation pathway. Since FOXJ1 expression was inhibited by submersion, the next question was whether submersion also inhibited MCIDAS expression, and if so, could this be reversed by the addition of DAPT. To answer these questions, NHBE cells were cultured at an ALI in air, submerged (0.5 ml apical volume), or submerged with DAPT for sixteen days and expression of MCIDAS and FOXJ1 were measured at multiple time-points.

Figure 3.3. γ-secretase inhibition restores FOXJ1 expression and ciliated cell differentiation in submerged and hypoxic conditions. (A-J) Representative extended focus confocal immunofluorescent images of NHBE cells after 21 days cultured (A, B) submerged (0.5 ml), (C, D) submerged (0.5 ml) + 10 μM DAPT, (E, F) ALI in hypoxia (0.5% O2), (G, H) ALI in hypoxia (0.5% O2) + 10 μM DAPT, or (I, J) ALI in air and stained for nuclei (Hoechst, blue) and cilia (acetylated α-tubulin, white) or FOXJ1 (green). (K) Graph displaying quantification of the percent FOXJ1 positive (FOXJ1+) in each condition showing a significant decrease in submerged and hypoxic conditions compared to the air control and showing DAPT restores ciliogenesis to control ALI levels. Scale Bars = 50 μm. One-way ANOVA; * = p < 0.05. n=3. (L) Western blots of whole cell lysates (40 μg) from NHBE cells cultured 24 hours at an ALI in air, submerged (0.5 ml) +/- 10 μM DAPT (top panel) or 48 hours at an ALI in air, ALI in hypoxia (0.5% O2) +/- 10 μM DAPT (bottom panel) probed for activated-Notch1 (NICD1). The data show NICD1 levels do not increase over ALI (air) and DAPT decreases NICD1 protein levels in submersion and hypoxia. β-Actin is used as the loading control. (M) qRT-PCR quantification of Notch target gene, HEY1, mRNA from NHBE cells cultured for 21 days in submerged and ALI in hypoxia (0.5% O2) (Hyp.) +/- 10 μM DAPT. HEY1 mRNA is normalized to the housekeeping gene β2-microglobulin (B2M) and showing DAPT inhibits Notch signaling. Student’s t-test; * = p < 0.05. n=3. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac et al/2014/Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner/Am J Respir Cell Mol Biol/51/516-25. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.
The data (Figure 3.4A and 3.4B) demonstrated both *MCIDAS* and *FOXJ1* mRNA increased by seven days in ALI cultures and DAPT treated submerged cells. However, this increase was not seen in submerged conditions. This strongly suggested submersion inhibited MCIDAS and FOXJ1 expression required Notch signaling.

**Ectopic Expression of NICD in Hypoxic or Submerged Conditions with DAPT Inhibits Multiciliated Cell Differentiation**

DAPT is \(\gamma\)-secretase inhibitor and does not inhibit Notch signaling directly. Rather, it inhibits the proteolytic cleavage of the receptors to release NICD. This is problematic since \(\gamma\)-secretase cleaves other proteins that may influence cell differentiation e.g. E-cadherin (197, 198). Therefore, further experiments are necessary to determine whether it is Notch or another \(\gamma\)-secretase target that is responsible for the rescue of multiciliated cell differentiation in submerged and hypoxic conditions. To examine this, NHBE cells were transduced with a N-terminally HA-tagged NICD1 (HA-NICD1) lentivirus and cultured in submerged with DAPT and hypoxia with DAPT conditions. The DAPT will inhibit endogenous expression of NICD along with the other \(\gamma\)-secretase substrates while the ectopically expressed HA-NICD1 will be unaffected, as it does not need to be cleaved by \(\gamma\)-secretase because it is already expressed in the active form. If expression of HA-NICD1 were sufficient to inhibit FOXJ1 expression in the presence of DAPT, this would indicate that the cleavage Notch mediates the inhibition of multiciliated cell differentiation. However, if cells expressing HA-NICD1 express FOXJ1, this would suggest it is another \(\gamma\)-secretase substrate and not Notch that is responsible for the inhibition. To confirm expression of HA-NICD1 promoted Notch signaling in NHBE cells, the level of the Notch target gene HEY1 was measured by RT-qPCR. The data
(Figure 3.5A) show a significant increase in \textit{HEY1} compared to the pCDH-EF1-HA-MCS-FLAG-T2A-copGFP backbone vector with no insert (HA-Vector). This indicated HA-NICD1 stimulated Notch signaling in transduced NHBE cells. To determine the influence of HA-NICD1 on FOXJ1 expression, NHBE cells were cultured for 3 weeks submerged or at an ALI in hypoxia in the presence of DAPT, and cells were then fixed and stained for HA to identify HA-Vector and HA-NICD1 transduced cells and FOXJ1 to label multiciliated cells. Confocal images of HA-NICD1 (Figures 3.5G and 3.5I) show nuclear HA staining, which indicates HA-NICD1 was expressed and being translocated to the nucleus. This is in contrast to HA staining of HA-Vector (Figures 3.5F and 3.5H), which showed a diffuse cytoplasmic expression as expected because the HA-FLAG protein product does not possess any localization signals. FOXJ1+ nuclei were confirmed in all conditions tested (Figures 3.5A-D). However, in HA-NICD1 transduced cells there was a clear exclusion of FOXJ1 from HA positive (HA+) nuclei (Figures 3.5K and 3.5M). In contrast, there was prevalent FOXJ1+, HA+ cells in HA-Vector transduced cells (Figures 3.5J and 3.5L). To quantify the effect of HA-NICD1 on multiciliated cell differentiation, the percentage of FOXJ1+ (% FOXJ1+), HA+ were counted for both HA-NICD1 and HA-Vector and the data showed a significant decrease in the % FOXJ1+ cells in HA-NICD1 transduced cells compared to HA-Vector (Figure 3.5N and 3.5O). As a control for the extent of multiciliated cell differentiation in the different conditions, quantification of the % FOXJ1+ nuclei in non-transduced cells (HA negative; HA-), on the same filter as the transduced cells was measured. The data showed no
Figure 3.4. MCI expression is inhibited by submersion in a γ-secretase dependent manner. Expression of (A) MCI and (B) FOXJ1 mRNAs during NHBE differentiation at an ALI in air (21% O₂) (orange), submerged (0.5 ml) (blue), or submerged (0.5ml) + 10 μM DAPT (green). MCI and FOXJ1 mRNAs are normalized to the housekeeping gene β2-microglobulin (B2M). The graphs show expression of MCI and FOXJ1 mRNAs is inhibited by submersion but inhibition is overcome by adding DAPT. n=3. This experiment was performed by Monica Valenica. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac et al/2014/Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner/Am J Respir Cell Mol Biol/51/516-25.The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.
significant difference between non-transduced cells in HA-NICD1 and HA-Vector treated filters. This indicated multiciliated cell differentiation was occurring at similar levels and that is was the HA-NICD1 that was the cause of the decrease in multiciliated cell differentiation. Similar results were shown using HA-NICD2 (Figure 3.6) and HA-NICD3 (Figure 3.7) lentiviruses. These data suggested it was Notch and not another γ-secretase target that was inhibiting multiciliated cell differentiation. The data also show Notch1, Notch2, and Notch3 were sufficient for inhibition. This indicated the pathway(s) responsible for multiciliated cell repression could all be activated by multiple Notch receptors. These experiments corroborate a previous study in mice showing NICD1 suppresses multiciliated cell differentiation (86). However, the data show this hold true for humans and looks at NICD2 and NICD3.

**Inhibition of Notch2 but not Notch1 Promotes Multiciliated Cell Differentiation**

Ectopic expression experiments suggested NICD1, NICD2, or NICD3 were sufficient to inhibit ciliated cell differentiation. However, this experiment did not address how endogenous levels of these proteins affect multiciliated cell differentiation of NHBE cells. To investigate this question, antibodies against the Negative Regulatory Region (NRR) were used to prevent Notch signaling. NRR antibodies bind to the NRR region, comprised of the LNR and HD regions, on the Notch receptor and block ligand-dependent conformational changes. This inhibits the S2 cleavage and subsequent S3 cleavage to prevent NICD release from the cell membrane (95, 97). The major benefit of using these antibodies is they are specific for individual Notch receptors allowing inhibition of specific Notch receptor signaling. To determine the role of endogenous Notch1 and Notch2 on multiciliated cell differentiation in submerged and hypoxic
Figure 3.5. Expression of HA-NICD1 increases Notch signaling and inhibits FOXJ1 in DAPT treated submerged and hypoxic cells. (A) RT-qPCR of HEY1 mRNA from NHBE cells transduced with lentiviruses expressing either HA-tagged NICD1 (HA-NICD1) or vector control (HA-vector). HEY1 mRNA was normalized to the housekeeping gene β2-microglobulin mRNA. Student’s t-test; * = p < 0.05. n=3. (B-M) Representative extended focus confocal immunofluorescent images of NHBE cells transduced with HA-NICD1 or HA-vector lentiviruses. Cells were grown for 3 weeks in submerged + 10 μM DAPT or in hypoxia (0.5% O₂) + 10 μM DAPT and then stained for nuclei (Hoechst, blue), FOXJ1 (green) or HA (red). (B-E) Hoechst and FOXJ1 merged, (F-I) HA alone, and (J-M) HA and FOXJ1 merged. Quantification of percent FOXJ1 positive (FOXJ1+) HA-NICD1 or HA-vector transduced (HA+) and non-transduced (HA-) NHBE cells in submerged + DAPT (N) and hypoxia + DAPT (O) conditions. A minimum of 500 cells from 3 different lung donors was counted for each group. Scale bar = 50 μm. One-way ANOV with Bonferroni multiple comparison post-test; * p < 0.05. n=3. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac et al/2014/Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner/Am J Respir Cell Mol Biol/51/516-25. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.
Figure 3.6. Ectopic expression of HA-NICD2 promotes Notch signaling and represses FOXJ1 in submerged and hypoxic cells treated with DAPT. (A) RT-qPCR of HEY1 mRNA from NHBE cells transduced with lentiviruses expressing either HA-tagged NICD2 (HA-NICD2) or vector control (HA-vector). HEY1 mRNA was normalized to the housekeeping gene β2-microglobulin mRNA. Student’s t-test; * = p < 0.05. n=3. (B-M) Representative extended focus confocal immunofluorescent images of NHBE cells transduced with HA-NICD2 or HA-vector lentiviruses. Cells were grown for 3 weeks in submerged + 10μM DAPT or in hypoxia (0.5% O2) + 10μM DAPT and then stained for nuclei (Hoechst, blue), FOXJ1 (green) or HA (red). (B-E) Hoechst and FOXJ1 merged, (F-I) HA alone, and (J-M) HA and FOXJ1 merged. Quantification of percent FOXJ1 positive (FOXJ1+) HA-NICD2 or HA-vector transduced (HA+) and non-transduced (HA-) NHBE cells in submerged + DAPT (N) and hypoxia + DAPT (O) conditions. A minimum of 500 cells from 3 different lung donors was counted for each group. Scale bar = 50 μm. One-way ANOV with Bonferroni multiple comparison post-test; * p < 0.05. n=3.
Figure 3.7. Constitutive expression of NICD3 increases Notch signaling and inhibits FOXJ1 in DAPT treated submerged and hypoxic cells. (A) RT-qPCR of *HEY1* mRNA from NHBE cells transduced with lentiviruses expressing either HA-tagged NICD3 (HA-NICD3) or vector control (HA-vector) showing a significant increase of the Notch target gene *HEY1* in HA-NICD3 transduced cells compared to HA-vector control. *HEY1* mRNA was normalized to the housekeeping gene β2-microglobulin mRNA. Student’s t-test; * = p < 0.05. n=5. (B-M) Representative extended focus immunofluorescent images of NHBE cells transduced with either HA-NICD3 or HA-vector lentiviruses. Cells were grown for 3 weeks in submerged + 10μM DAPT or hypoxia (0.5% O₂) + 10μM DAPT, and then stained for nuclei (Hoechst, blue), FOXJ1 (green), or HA (red). (B-E) DAPI and FOXJ1 merged, (F-I) HA alone, and (J-M) HA and FOXJ1 merged. Quantification of percent FOXJ1 positive (FOXJ1+) HA-NICD3 or HA-vector transduced (HA+) and non-transduced (HA-) NHBE cells in submerged + DAPT (N) and hypoxia + DAPT (O) conditions showing significant reduction the number of FOXJ1+ cells in HA-NICD3 transduced cells compared to non-transduced cells and to HA-vector control transduced cells in DAPT treated submerged and hypoxic conditions. In fact, 0/2170 NICD3 HA+ cells counted in submerged and 0/484 NICD3 HA+ cells counted hypoxic conditions were FOXJ1+. A minimum of 150 cells was counted for each group. Scale bar = 50 μm. n = 4 for sub. + DAPT and n = 2 for hyp. + DAPT. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac et al/2014/Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner/Am J Respir Cell Mol Biol/51/516-25. The *American Journal of Respiratory Cell and Molecular Biology* is an official journal of the American Thoracic Society.
conditions, NHBE cells were cultured submerged (0.5 ml apical) or at an ALI in hypoxia (0.5% O2) with NRR1, NRR2, or IgG antibody control. NRR1 and NRR2 are anti-Notch1 and anti-Notch2 receptor antibodies; respectively. In addition, DAPT was used as a positive control for multiciliated cell differentiation in these conditions. After 3 weeks, fixing and staining NHBE cells for FOXJ1 and cilia assessed the extent of ciliated cell differentiation. The results showed NRR2 but not NRR1 caused significant promotion of multiciliated cell differentiation compared to the IgG control in hypoxia and submerged conditions (Figures 3.8A and 3.8B). In addition, combining NRR1 and NRR2 showed similar levels of multiciliated cell differentiation compared to NRR2 alone. These data suggested endogenous levels of Notch2 but not Notch1 inhibited multiciliated cell differentiation. Moreover, because NRR1 showed no effect on multiciliated cell differentiation, it could mean the antibody was not inhibiting NICD1 cleavage. To test this, the extent of NICD1 formation in NHBE cells treated with NRR1 or IgG control was measured by western blot. The data showed NRR1 inhibited NICD1 formation (Figure 3.8C). These data indicated inhibition of NICD1 did not inhibit multiciliated cell differentiation. Interestingly, while NRR2 increased multiciliated cell levels to the same extent as DAPT in hypoxia, there were significantly fewer in submerged conditions. The reason for this is not clear but may suggest different contributions of different Notch receptors in different culture conditions. For example, there could be a larger reliance on Notch3 in submerged cultured compared to hypoxia to suppress multiciliated cell differentiation and conversely less reliance on Notch2. NRR3 (anti-Notch3 receptor antibody) was never tested to determine this. Overall, it appears Notch2 but not Notch1 is important for repression of multiciliated cell differentiation.
Figure 3.8. Inhibition of Notch2 but not Notch1 promotes multiciliated cell differentiation in hypoxic and submerged condition. (A) Representative confocal images from NHBE cells cultured for 3 weeks submerged (A) or at an ALI in hypoxia (B) and immunostained for cilia (acetylated-tubulin, white), FOXJ1 (green), and nuclei (Hoeschst, blue). Quantification of the % FOXJ1+ cells is below each respective confocal image panel. Scale Bar = 50 μm. n=9. One-way ANOVA with Newman-Keuls multiple comparisons post-test. * p < 0.05, *** p < 0.001 (compared to IgG) and # p < 0.05 (NRR2 compared to DAPT). (C) Western blot of NHBE lysates treated submerged with IgG or NRR1 antibody for 48 hrs. Lysates were probed with anti-NICD1 antibody. β-Actin was used as the protein loading control. n=3.
Conclusions

In this chapter, inhibition of multiciliated cell differentiation in submerged culture was found to be dependent on the apical media volume on the NHBE cells. This led to the discovery that submersion causes NHBE cells to become hypoxic as assessed by an increase in HIF-1α and HIF-2α proteins. From there, it was shown hypoxia inhibited multiciliated cell differentiation in ALI cultures and this relied on γ-secretase activity as addition of DAPT restored multiciliated cell differentiation in both submerged and hypoxic conditions. Additionally, it was revealed submersion inhibited expression of both MCIDAS and FOXJ1, transcription factors essential for multiciliated cell differentiation (43). Moreover, this inhibition was in a γ-secretase dependent manner. Furthermore, expression of NICD1, NICD2, and NICD3 in DAPT treated submerged and hypoxic cultures showed NICD was sufficient to suppress multiciliated cell differentiation. However, experiments using NRR antibodies suggested that endogenous levels of Notch2 but not Notch1 play a role in the inhibition of multiciliated cell differentiation. Overall, these data illustrate the mechanism used by submersion to inhibit multiciliated cell differentiation. Specifically, submersion was shown to inhibit multiciliated cell differentiation by suppressing the expression of genes necessary for differentiation by the Notch signaling pathway. Some of these data were published in an article entitled Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner in the journal American Journal of Respiratory Cell and Molecular Biology (199).
Chapter 4: Multiciliated Cell Differentiation on Plastic

Differentiation of Multiciliated cells in 96-wells

Up to this point, differentiation of multiciliated cells was only possible when cells were cultured at an ALI on porous membranes. The discovery that multiciliated cell differentiation was possible in submerged culture by inhibiting Notch signaling suggests the possibility that multiciliated cell differentiation could be achievable using standard cell culture methods on plastic dishes. This would allow the potential to perform high-throughput experiments that would be difficult to do using Transwell® membranes. To test this, NHBE cells were cultured in a 96-well plate for three weeks submerged in ALI media with or without DAPT. The cells were then fixed and stained for cilia and FOXJ1. The media volume used to submerge NHBE cells in a 96-well was 150 μl, which is approximately equivalent in apical depth to 0.5 ml on a 1-cm² filter. After 21 days of culture without DAPT, no FOXJ1+ or multiciliated cells were detected, but FOXJ1+, multiciliated cells were detected in NHBE cells treated with DAPT (Figure 4.1). The cilia on these cells were motile with a ciliary beat frequency (CBF) of 10.18 ± 2.84 Hz, which is similar to previously published CBF reported for NHBE cells at an ALI, which was approximately 8 to 9.5 Hz (200). Furthermore, cilia length of multiciliated cells differentiated in 96-well plates was not significantly different to cilia on cells cultured at an ALI (8.82 ± 1.04 μm and 9.15 ± 1.56 μm, respectively). Therefore, differentiation of motile multiciliated cells requires neither an ALI nor porous membranes.

Fluorescence Detection of Multiciliated Cell Differentiation in 96-well Plates

Multiciliated NHBE cell differentiation is possible in 96-wells, but detection and quantification of these cells required staining them for multiciliated cell markers.
Figure 4.1. DAPT enables FOXJ1 expression and ciliated cell differentiation of NHBE cells in submerged culture on plastic in 96-well plates. Representative 3D opacity renderings of 40X confocal immunofluorescent images of NHBE cells cultured in wells of a 96-well plate submerged for 21 days (top) or submerged plus (+) 10 µM DAPT (middle) and a Z-stack of submerged + 10 µM DAPT is also shown (bottom). Cells were stained for cilia (acetylated α-tubulin, white), FOXJ1 (green), and nuclei (Hoechst, Blue). The percent FOXJ1 positive cells are indicate in upper right corner of the upper two panels. The 3D images were cropped and rotated along the Z-axis to view cells from above the apical surface. Scale bar = 20 µm. n=3. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac et al/2014/Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner/Am J Respir Cell Mol Biol/51/516-25. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.

While this is a good method to identify multiciliated cell differentiation in 96-wells, the this protocol is not practical for high throughput assays. In an attempt to improve detection of multiciliated cell differentiation for the development of high throughput screens, a fluorescence based method was developed to use lentivirus containing a 1-kilobase (Kb) fragment of the human FOXJ1 promoter driving the expression of the fluorescent protein, mCherry (pFOXJ1-mCherry (pFC)) (created by Dr. Nevis Fregien). Analysis of NHBE cells transduced with this lentivus showed mCherry expression only in differentiated multiciliated cells. Thus, it should be possible to detect multiciliated cell
differentiation by measuring mCherry fluorescence in living cells using a plate reader. To test this, NHBE transduced with the pFC lentivirus, were cultured submerged in 96-well plates with or without DAPT for three weeks and mCherry fluorescence was measured using a fluorescence plate reader after 21 days. Control included were media only and non-transduced NHBE cells to ascertain background fluorescent levels of media and NHBE cells in 96-wells. The results (Figure 4.2A) show significantly more fluorescence in wells containing pFC transduced, DAPT treated cells compared to pFC transduced cells without DAPT, DAPT treated non-transduced cells, and media only. These data suggested measuring FOXJ1 promoter driven mCherry fluorescent protein expression was an appropriate assay to measure ciliated cell differentiation. The fluorescence readings from technical triplicate wells from a single lung were used for quantification. However, the experiment was repeated in another lung with similar results. However, the data showed there was well-to-well consistency in fluorescence readings. To insure the fluorescence readings corresponded with multiciliated cell differentiation, after reading their fluorescence, the NHBE cells were fixed and stained for FOXJ1 and cilia. The images (Figures 4.2B-G) showed non-transduced DAPT treated cells contained FOXJ1+, multiciliated cells but they did not express detectable levels of mCherry. The images also showed vehicle control treated pFC transduced cells did not have multiciliated cells and contained no mCherry cells. Finally, the images showed DAPT treated cells transduced with pFC contained mCherry positive, multiciliated cells. Together, the immunofluorescent data corroborate the fluorescent data results and show that by transducing NHBE cells with pFC lentivirus, multiciliated cell differentiation can easily be assayed on a high throughput format. This data demonstrates the feasibility of high
throughput assays for multiciliated cell differentiation. Similar results were found with NHBE cells cultured on 384-wells (data not shown).

**Conclusions**

In this section, it was shown that NHBE cells could differentiate into multiciliated cells in plastic 96-well plates. Thus, showing multiciliated cell differentiation does not require NHBE cells to be cultured on porous membranes and opens up the possibilities of obtaining multiciliated cells on a variety of substrates. Moreover, it was also shown that multiciliated cell differentiation could be detected and quantified by transducing NHBE cells with a lentivirus containing the human FOXJ1 promoter driving the mCherry fluorescent protein. This discovery holds the potential to be able to easily detect and track multiciliated cell differentiation over time, as measuring fluorescence is performed using live cells that can be assayed multiple times over time. In addition, this could possibly be used as a high-throughput platform to find novel Notch inhibitors or to determine other signaling pathways that influence multiciliated cell differentiation. Figure 4.1 was published in the article entitled *Submersion and hypoxia inhibit ciliated cell differentiation in a notch-dependent manner* in the journal American Journal of Respiratory Cell and Molecular Biology (199).
Figure 4.2. Detection of multiciliated cell differentiation in 96-wells via lentiviral transduction with pFC virus. (A) Quantification of relative fluorescent units (RFUs) from technical triplicate wells of NHBE cells that were either not transduced or transduced with the FOXJ1 promoter driving mCherry lentivirus (pFC) cultured for 3 weeks submerged on a 96-well plate with DAPT or vehicle control (DMSO). An additional Media only control (only media in the well) was assessed to give background fluorescence of the media alone. One-Way ANOVA with Dunnett’s multiple comparisons post-test (Media Only well as the control). *** p < 0.001. n=3. (B-G) Representative confocal images of non-transduced or pFC transduced NHBE cells cultured in 96-wells for 21 days with vehicle control or DAPT. NHBE cells were stained for FOXJ1 (green), cilia (acetylated-tubulin, white), and nuclei (Hoeschst, blue). Non-transduced cells had FOXJ1+ cells but they did not express mCherry while pFC transduced cells had FOXJ1+ cells that expressed mCherry (white arrows). Scale bar = 50 μm. n=2.
Chapter 5: HIF Signaling and Multiciliated Cell Differentiation

HIF-2α but not HIF-1α Inhibits Multiciliated Cell Differentiation

Since submersion and hypoxia were shown to inhibit multiciliated cell differentiation (Figure 3.3) and HIF proteins are induced in NHBE cells under these conditions (Figure 3.2), it was possible HIF proteins play a role in influencing multiciliated cell differentiation. In addition, HIF signaling has been shown to influence the differentiation of multiple cell types (62, 201, 202). To assess the roles of HIF-1α and HIF-2α have on multiciliated cell differentiation, NHBE cells were transduced with lentiviruses expressing constitutively active HIF-1α (CA-HIF-1α), constitutively active HIF-2α (CA-HIF-2α) or HA only (lentiviral backbone with no insert) to differentiate the effects of HIF proteins from other effects induced by hypoxia such as metabolic differences. The CA-HIF-α proteins, HIF1α-P402A/P564A or HIF-2α-P405A/P531A, produce HIF proteins that have the proline residues in the oxygen-dependent degradation (ODD) region mutated to alanine. Thereby, preventing hydroxylation by prolyl hydroxylases (PHDs) and subsequent targeting for ubiquitination and degradation. Thus, these proteins are stable in normoxic conditions. Therefore, if HIF proteins are responsible for repressing ciliated cell differentiation in hypoxia, the constitutively active forms should inhibit ciliated cell differentiation in normoxia. To test this, CA-HIF1α or CA-HIF-2α transduced NHBE cells were cultured at an ALI in normoxia (21% O₂) for 3 weeks and immunostained for HA and FOXJ1 to detect transduced and FOXJ1 positive (FOXJ1+) cells. To measure HIF signaling, RNA was purified to assess HIF target gene expression. If HIF-1α or HIF-2α were sufficient to inhibit multiciliated cell differentiation, HA+, transduced cells would not be FOXJ1+. However, if HIF-1α or HIF-2α were not
sufficient, there would be no difference in the percentage of FOXJ1+ cells between transduced and non-transduced cells. Figure 5.1A shows CA-HIF-1α transduced NHBE cells significantly induced the expression of the HIF target gene aldolase C, fructose-bisphosphate (ALDOC) compared to NHBE cells transduced with the control virus. Thus, indicating CA-HIF-1α was promoting HIF signaling in normoxic cells. Immunofluorescent staining of CA-HIF-1α transduced cells cultured at an ALI showed an abundance of FOXJ1+ cells (Figure 5.1B) and showed CA-HIF-1α was expressed and translocated to the nucleus as detected by HA staining (Figure 5.1C). Many FOXJ1+ cells were also HA+. Quantification of the confocal images (Figure 5.1E) showed no significant difference the percentage of FOXJ1+ between NHBE cells transduced with CA-HIF-1α (HA+) and non-transduced cells (HA-). These data suggested HIF-1α does not inhibit multiciliated cell differentiation. Confocal images of immunofluorescent stained CA-HIF-2α transduced cells showed numerous FOXJ1+ cells (Figure 5.1F) and showed nuclear HA staining, as predicted (Figure 5.1G). However, there are few HA+, FOXJ1+ cells (Figure 5.1H). Quantification of confocal images showed significantly less FOXJ1+, HA+ cells compared to FOXJ1+, HA- cells (Figure 5.1I). These data indicated HIF-2α is sufficient to repress multiciliated cell differentiation in normoxic cells. Furthermore, CA-HIF-2α was also able to suppress multiciliated cell differentiation in the presence of DAPT (Figures 5.1J-M). These data suggest HIF-2α can block multiciliated cell differentiation despite Notch signaling being inhibited. This suggests HIF signaling can repress multiciliated cell differentiation independently of Notch signaling.
Figure 5.1. HIF-2α inhibits ciliogenesis independently of Notch signaling. A) qRT-PCR quantification of HIF target gene, ALDOC, mRNA levels in HA-HIF-1α DA or HA-vector (control) transduced cells. The data show a significant increase in ALDOC levels in the HA-HIF-1α DA cells, indicating an increase in HIF signaling. Beta-2 microglobulin was used as a loading control. B-E) Representative confocal images of NHBE cells transduced with HA-HIF-1α DA cultured at an ALI for 21 days and quantification of the % FOXJ1+ non-transduced (HA-) and transduced (HA+) showing no significant difference between HA- and HA+ cells. F-I) Representative confocal images of NHBE cells transduced with HA-HIF-2α DA cultured at an ALI for 21 days and quantification of the % FOXJ1+ non-transduced (HA-) and transduced (HA+) showing significantly less HA+ cells compared to HA-. J-M) Representative confocal images of NHBE cells transduced with HA-HIF-2α DA cultured at an ALI for 21 days + 10 μM DAPT and quantification of the % FOXJ1+ non-transduced (HA-) and transduced (HA+) showing significantly less HA+ cells compared to HA-. Scale bar = 50 μm. Student’s t-test; * = p < 0.05. n=3.
Conclusions

In this section, the role of HIF signaling in multiciliated cell differentiation was investigated. The data showed constitutively active HIF-2α but not constitutively HIF-1α was sufficient to inhibit multiciliated cell differentiation in NHBE cells grown at an ALI in normoxia. These data suggest that HIF-2α signaling may be a regulator of multiciliated cell differentiation in low oxygen environments. Additionally, the data demonstrated expression of CA-HIF-2α was able to repress multiciliated cell differentiation on ALI despite inhibition of Notch signaling. This indicates HIF-2α has the potential to inhibit FOXJ1 expression and multiciliated cell differentiation independently of Notch signaling, which may be significant as it would suggest that if HIF-2α signaling was potentiated enough, inhibiting Notch would no longer be sufficient to restore multiciliated cell differentiation in low oxygen environments.
Chapter 6: The Influence of IL-13 on Multiciliated Cell Differentiation

IL-13 Inhibits Multiciliated Cell Differentiation Independent of Notch Signaling

Interleukin 13 (IL-13) has been shown to inhibit multiciliated cell differentiation (31, 52, 112). In 2007, Gomperts et al. published work suggesting IL-13 inhibits multiciliated cell differentiation by binding to the FOXJ1 promoter and inhibiting transcription (187). However, the understanding of this process has not advanced. Guseh et al. showed γ-secretase inhibitors attenuated IL-13 driven goblet cell hyperplasia in NHBE cells (86), and data in this thesis showed Notch signaling inhibits multiciliated cell differentiation (Figure 3.5). Therefore, the role of Notch signaling in IL-13 induced repression of multiciliated cell differentiation was investigated by culturing NHBE cells submerged with the addition of DAPT, to inhibit Notch signaling, and various concentrations of IL-13 for three weeks. NHBE cells were then stained for FOXJ1 and cilia and immunofluorescence images were taken. If Notch signaling was required for IL-13 dependent inhibition, multiciliated cell differentiation would occur in the presence of IL-13 because DAPT inhibits Notch signaling. If Notch signaling was not required for IL-13 dependent inhibition, multiciliated cell differentiation would still be inhibited by IL-13 in the presence of DAPT, and would indicate IL-13 inhibits multiciliated cell differentiation independently of Notch signaling. Confocal immunofluorescent images (Figure 6.1A) showed DAPT induced multiciliated cell differentiation in submerged culture was inhibited by IL-13. Quantification of the % FOXJ1+ cells (Figure 6.1B) showed a dose-dependent inhibition of multiciiliated cell differentiation with significant inhibition occurring at IL-13 concentrations ≥ 1 ng/ml. To confirm DAPT was inhibiting γ-secretase and NICD formation in the presence of IL-13, NICD1 was measured by western
blot. The results showed DAPT inhibited NICD1 formation in the presence of IL-13 (Figure 6.1C). Moreover, the expression of the Notch target gene HEY1 was measured to confirm Notch target gene expression was still being inhibited by DAPT.

Figure 6.1. Multiciliated cell differentiation in inhibited by IL-13 in the presence of DAPT. NHBE cells cultured in submerged conditions for 3 weeks submerged, submerged with 10 μM DAPT, or submerged with 10 μM DAPT and various concentrations of IL-13. (A) Representative immunofluorescence confocal images of NHBE stained after 21 days. NHBE cells were stained for cilia (acetylated-tubulin, white), FOXJ1 (green), and nuclei (Hoeschst, blue). Scale Bar = 50 μm. (B) Quantification of the percent FOXJ1 positive cells (% FOXJ1+) in confocal images. n=5. (C) Western blot of whole cell protein lysates (40 μg) from NHBE cells treated for 48 hours with DMSO or 10 μM DAPT with and without 10 ng/ml IL-13 probed with anti-NICD1 antibody. n=3. (D) qRT-PCR quantification of HEY1 mRNA levels from NHBE cells treated for 21 days with DMSO or DAPT or DAPT with various concentrations of IL-13. HEY1 is normalized to the housekeeping gene β2-microglobulin (B2M). n=5. One-way ANOVA; Bonferroni multiple comparisons post-test. NS = not significant, * p < 0.05, ** p < 0.01, and *** p < 0.001. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac BJ and Fregien NL/2015/IL-13 inhibits multicilin expression and ciliogenesis via JAK/STAT independent of Notch cleavage/DOI: 10.1165/rcmb.2015-0227OC.The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.
The results showed DAPT dependent inhibition of HEY1 was unchanged by IL-13 at any concentration tested (Figure 6.1D). These data suggested DAPT was still inhibiting Notch signaling in the presence of IL-13, and indicated IL-13 inhibited multiciliated cell differentiation independent of Notch.

**IL-13 Inhibits MCIDAS and FOXJ1 Expression in the Presence of DAPT**

In addition to FOXJ1, multiciliated cell differentiation also requires multicilin (MCIDAS) expression, which occurs upstream of FOXJ1 (47). Since MCIDAS is required for multiciliated cell differentiation, the influence of IL-13 on the expression of MCIDAS was investigated. NHBE cells were cultured submerged with DAPT and various amounts of IL-13 for three weeks. After three weeks, RNA was purified and MCIDAS levels were measured by RT-qPCR. The results showed MCIDAS decreased with increasing concentrations of IL-13 with significant inhibition occurring at concentrations $\geq 1$ ng/ml IL-13 (Figure 6.2A). FOXJ1 levels were also found to be significantly inhibited at concentrations $\geq 1$ ng/ml IL-13 (Figure 6.2B), which is similar to immunofluorescent results in Figure 6.1 and consistent with data from a recent report (31). These data indicated, in the absence of Notch signaling, IL-13 suppresses MCIDAS expression leading to downstream inhibition of FOXJ1 and multiciliated cell differentiation. This is important because MCIDAS expression was previously shown to be repressed by Notch signaling (47). This suggests multiple signaling pathways negatively regulate MCIDAS expression.

**Canonical Notch Regulated Transcriptional Repressors are not Promoted by IL-13**

The HES and HEY family members are a group of transcriptional repressors that are induced by Notch signaling (92) and could be responsible for suppressing MCIDAS in
NHBE cells cultured in submerged conditions. Since IL-13 inhibits MCIDAS expression, it is plausible it could be doing so by inducing this family of repressors independently of Notch. Therefore, the effect of IL-13 on these genes was explored. To test this, NHBE cells were cultured submerged with DAPT with or without IL-13 for three weeks. RNA was then purified and expression of HES/HEY genes was evaluated by RT-qPCR. The results showed IL-13 did not significantly change HEY1 or Hes Family BHLH Transcription Factor 2 (HES2) (Figures 6.3B and 6.3C) gene expression. Additionally, IL-13 significantly reduced Hes Family BHLH Transcription Factor 1 (HES1) and hairy/enhancer-of-split related with YRPW motif 2 (HEY2) gene expression (Figures 6.3A and 6.3D). These data revealed IL-13 does not promote expression of these genes.

Figure 6.2. IL-13 inhibits the expression of MCIDAS and FOXJ1. Graphical depictions showing quantification of MCIDAS and FOXJ1 expression in NHBE cells differentiated for 21 days with 10 μM DAPT and the indicated concentrations of IL-13. (A) MCIDAS is normalized to the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH). n=3. (B) FOXJ1 is normalized to the housekeeping gene β2-microglobulin (B2M). One-way ANOVA; Dunnett post-test (0 ng/ml is the control). * p < 0.05, ** p < 0.01, *** P < 0.001. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac BJ and Fregien NL/2015/IL -13 inhibits multicilin expression and ciliogenesis via JAK/STAT independent of Notch cleavage/DOI: 10.1165/rcmb.2015-0227OC. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.
canonical Notch controlled transcriptional repressors and indicated IL-13 represses MCIDAS expression through another transcriptional repressor or other mechanism.

**Figure 6.3.** *HES1, HES2, HEY1,* and *HEY2* expression is not induced by IL-13. Quantification of mRNA levels in NHBE cells differentiated for 21 days submerged with 10 μM DAPT with or without 3 ng/ml IL-13 using RT-qPCR. (A) *HES1*, (B) *HES2*, (C) *HEY1*, and (D) *HEY2* were normalized to the housekeeping gene β2-microglobulin (B2M). n=3. Student’s t-test with Welch’s correction. NS = not significant, * p < 0.05. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac BJ and Fregien NL/2015/IL-13 inhibits multicilin e expression and ciliogenesis via JAK/STAT independent of Notch cleavage/DOI: 10.1165/rcmb.2015-0227OC. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.

**IL-13 Depends on JAK Activity but not MEK Activity to Inhibit Multiciliated Cell Differentiation**

IL-13 activates JAK/STAT6 signaling to cause goblet cell hyperplasia and increased mucin secretion in mice (87, 189). Additionally, IL-13 induced goblet cell hyperplasia has also been shown to be attenuated by pharmacological inhibition of mitogen activated...
protein kinase kinase (MEK) (170). IL-13 has also been shown to promote phosphorylation/activation of mitogen activated protein kinases (ERK1/2) (203). Since IL-13 does not appear to depend on Notch to inhibit multiciliated cell differentiation, other signaling pathways that could be responsible for this inhibition were investigated. To test alternative signaling pathways, NHBE cells were cultured submerged with or without DAPT with or without IL-13, with or without JAK Inhibitor 1, a pan-JAK inhibitor (204), or with or without PD98059, a MEK inhibitor, for three weeks. Multiciliated cell differentiation was assessed by immunofluorescent staining for cilia and FOXJ1 and by measuring MCIDAS and FOXJ1 expression by RT-qPCR. The ability of the pharmacological inhibitors to inhibit their respective pathways was assessed by measuring the phosphorylation of target proteins of JAK and MEK. For JAK, signal transducer and activator of transcription 6 (STAT6) was measured and for MEK, phosphorylation of ERK1/2 was measured by western blotting. The results showed both inhibitors attenuated phosphorylation of their respective target proteins to or below control (DAPT alone) levels in the presence of IL-13 (Figure 6.4A). Confocal immunofluorescent images of NHBE cells, stained for cilia and FOXJ1, showed DAPT induced multiciliated cell differentiation was inhibited by IL-13. However, addition of JAK Inhibitor 1 but not PD98059 permitted multiciliated cell differentiation in the presence of IL-13 (Figure 6.4B). Quantification of the % FOXJ1+ cells showed JAK Inhibitor 1 but not PD98059 significantly increased the amount of multiciliated cells compared to IL-13 (Figure 6.4C). Additionally, qRT-PCR showed JAK Inhibitor 1 significantly increased both **MCIDAS** and **FOXJ1** in IL-13 treated cultures while there was no significant increase by PD98059 (Figures 6.5A and 6.5B). These data indicate IL-
13 requires JAK but not MEK activity to inhibit MCIDAS expression and multiciliated cell differentiation. Neither JAK Inhibitor 1, PD98059, nor IL-13 induced multiciliated cell differentiation in submerged conditions without DAPT (Figure 6.4C). Interestingly, JAK Inhibitor 1 or PD98059 caused significant decreases in multiciliated cell differentiation in DAPT treated cultures and these effects were independent of IL-13 as they occurred without IL-13. The reason for this reduction was not clear, but Tadokoro et al. recently published that signal transducer and activator of transcription 3 (STAT3) phosphorylation promoted multiciliated cell differentiation (205). Thus, it was possible these inhibitors could be reducing multiciliated cell differentiation by inhibiting STAT3 phosphorylation. To test the effect of the inhibitors of STAT3 phosphorylation, NHBE cells were grown submerged in the presence of DAPT with or without JAK Inhibitor 1 or PD98059, and STAT3 phosphorylation was measured by western blotting. The data showed JAK Inhibitor 1 reduced STAT3 phosphorylation, on average, greater than 99% while PD98059 reduced levels 42% compared to DAPT alone (Figure 6.6).

Figure 6.4. JAK but not MEK activity is required for IL-13 dependent inhibition of multiciliated cell differentiation. (A) Western blot of whole cell lysates (30 μg) from submerged NHBE cells treated with 10 μM DAPT, DAPT with 3 ng/ml IL-13 with or without 9 μM JAK Inhibitor 1 (left panel) or 50 μM PD98059 (right panel), as indicated below, for 48 hours and probed with phosphorylated STAT6 (p-STAT6) or phosphorylated ERK1/2 (p-ERK1/2) with anti-p-STAT6 or anti-p-ERK1/2 antibody. β-actin was used as a loading control. n=3. (B) Representative immunofluorescence images of NHBE cells cultured for 3 weeks submerged in DMSO (vehicle control) with or without IL-13, DAPT with or without JAK Inhibitor 1 or PD98059 with or without DAPT with or without IL-13; as indicated at the top. Cells were stained for FOXJ1 (green), cilia (acetylated-tubulin, white), and nuclei (Hoeschst, blue). Scale Bar = 50 μm. (C) Quantification of percent FOXJ1 positive cells (% FOXJ1+). n≥3. One-way ANOVA; Bonferroni multiple comparison post-test. * p < 0.05, *** p < 0.001. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac BJ and Fregien NL/2015/IL-13 inhibits multicilin expression and ciliogenesis via JAK/STAT independent of Notch cleavage/DOI: 10.1165/rcmb.2015-0227OC. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.
Figure 6.5. Inhibition of JAK activity increases MCIDAS and FOXJ1 expression in IL-13 treated cultures. RT-qPCR quantification of MCIDAS and FOXJ1 mRNAs from NHBE cells differentiated for 21 days with 10 μM DAPT and 3 ng/ml IL-13 with or without 9 μM JAK Inhibitor 1. (A) MCIDAS is normalized to the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH). (B) FOXJ1 is normalized to the housekeeping gene β2-microglobulin (B2M). n=5. Unpaired Student’s t-test with Welch’s correction. * p < 0.05, ** p < 0.01. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac BJ and Fregien NL/2015/IL-13 inhibits multicilin expression and ciliogenesis via JAK/STAT independent of Notch cleavage/DOI: 10.1165/rcmb.2015-0227OC. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.

These results indicate that the pan JAK inhibitor, JAK Inhibitor 1, may be reducing the maximum level of multiciliated cell differentiation by inhibiting STAT3 phosphorylation and reducing induction of ciliated cell differentiation. PD98059 may also be inhibiting multiciliated cell differentiation by reducing the level of STAT3 phosphorylation; however, the reduction was much less severe than by JAK Inhibitor 1 inhibition. Interestingly, the significant decrease between submerged with with the addition of DAPT and PD98059 cultures treated with or without IL-13 showed IL-13 works through a different or additional mechanism than PD98059 utilizes to cause a more severe repression of multiciliated cell differentiation. Further studies into the mechanism behind the reduction in multiciliated cell differentiation caused by PD98059 and JAK Inhibitor 1 are areas of future study.
Conclusions

In this chapter the signaling pathway used in IL-13 dependent inhibition of multiciliated cell differentiation in NHBE cells was investigated. A novel cell culture system was used to ascertain if IL-13 could inhibit multiciliated cell differentiation despite Notch signaling being inhibited by DAPT. The data showed IL-13 inhibited MCIDAS and FOXJ1 expression and multiciliated cell differentiation in the absence of NICD formation and Notch target gene expression being reduced. This suggested IL-13 inhibited multiciliated cell differentiation using a Notch independent mechanism. The effect of IL-13 on canonical repressor Notch target genes was investigated as a possible mechanism for repression of MCDIAS. The results showed IL-13 either does not promote expression of HES/HEY transcriptional repressors or inhibited Notch target gene expression. Moreover, pharmacological inhibitor data showed IL-13 dependent inhibition of MCIDAS expression and multiciliated cell differentiation required JAK activity but not MEK activity. Furthermore, these data showed inhibition of JAK or MEK activity was sufficient to reduce the level of multiciliated cell differentiation independently of IL-13, possibly by inhibiting STAT3 phosphorylation. These data provide novel insights into IL-13 dependent inhibition of multiciliated cell inhibition and these data were published in an article entitled *IL-13 inhibit multicilin expression and ciliogenesis via JAK/STAT independently of Notch cleavage* in the journal American Journal of Respiratory Cell and Molecular Biology (207).
Figure 6.6. JAK inhibitor 1 and PD98059 inhibit STAT3 phosphorylation. Western blot of whole cell lysates (30 μg) from NHBE treated for 48 hrs submerged with 10 μM DAPT with or without 9 μM JAK Inhibitor 1 or 50 μM PD98059 that were probed with anti-phosphorylated STAT3 (pSTAT3) antibody. β-Actin was used as a loading control. Normalized relative amounts of pSTAT3 (DAPT alone is set to 1) are indicated above the corresponding bands. n=3. Reprinted with permission of the American Thoracic Society. Copyright © 2015 American Thoracic Society. Cite: Gerovac BJ and Fregien NL/2015/IL-13 inhibits multicilin expression and ciliogenesis via JAK/STAT independent of Notch cleavage/DOI: 10.1165/rcmb.2015-0227OC. The American Journal of Respiratory Cell and Molecular Biology is an official journal of the American Thoracic Society.
Chapter 7: Discussion

For over two decades, it had been known that submersion inhibited multiciliated NHBE cell differentiation; however, the mechanism had eluded researchers. The first clue regarding the mechanism of multiciliated cell differentiation inhibition by submersion came in 1995 when Ostrowski and Nettsheim showed inhibition of multiciliated rat tracheal epithelial (RTE) cells was dependent on the apical volume in which cells were grown under (55). In this thesis, the data found NHBE cells showed a similar response. However, RTE multiciliated cell differentiation inhibition occurred at a calculated apical depth of ≥ 1 mm, but NHBE cells did not show inhibition until a calculated apical depth of approximately 4 mm. In contrast, multiciliated cell differentiation was increased at about 1 mm in NHBE cells. The difference between calculated apical media depth and multiciliated cell differentiation could represent differences in the calculated apical depths as Transwell® filters with a smaller diameter (1 cm²) were used in these experiments than in the rat studies. This would lead to a roughly 1.8 times greater meniscus height compared to the larger diameter (4.5 cm²) filters. Despite these differences, there is a correlation between increased apical volume and greater inhibition of multiciliated cell differentiation in both species. This suggests there is a conserved submersion-dependent mechanism inhibiting multiciliated cell differentiation in rodents and humans. The data also revealed submersion inhibited multiciliated cell differentiation by inhibition of FOXJ1 expression, a transcription factor necessary for multiciliated cell differentiation (43), in an apical volume dependent manner. Although, the mechanism of how increased apical media volume caused
decreased multiciliated cell differentiation remained unclear. Interestingly, the data showed that a small amount (0.125 ml) of apical media increased multiciliated cell differentiation. The reason for this is unclear but may indicate an apical soluble factor that promotes multiciliated cell differentiation, and the small volume of apical liquid would facilitate diffusion to other cells to promote multiciliated cell differentiation. However, further studies are required to determine if this is true.

It has been published that the depth of the liquid in which cells are cultured under influences the availability of oxygen to the cells due to the low diffusion of oxygen in liquid compared to air; roughly 10,000 times less. The result is at greater liquid depths, oxygen is metabolized by the cells as soon as it diffuses to them for oxidative phosphorylation (58). The results in no excess oxygen available for other cellular processes. Thus, leading to the creation of a hypoxic environment (58). Based on this, it was hypothesized submersion could be creating a low oxygen environment at high apical volumes. This hypothesis was supported by showing NHBE cells induced both HIF-1α and HIF-2α proteins in submerged conditions compared to air. HIF-1α and HIF-2α proteins are markers for hypoxia as they are rapidly degraded in air/normoxic conditions via an oxygen dependent mechanism. Therefore, HIF proteins are stabilized in hypoxic conditions due to the lack of oxygen. Since both HIF-1α and HIF-2α proteins were increased in NHBE cells at a high apical volume, this suggested submersion was creating a low oxygen environment. Based on those data, hypoxia was tested to determine whether it also inhibited multiciliated cell differentiation. The results showed that, like submersion, hypoxia inhibited FOXJ1 expression and multiciliated cell differentiation and suggested submersion was inhibiting FOXJ1 expression and multiciliated cell
differentiation by creation of a hypoxic environment. Thus, the data implicated the mechanism underlying submersion dependent inhibition of multiciliated cell differentiation involved the creation of a hypoxic environment. While this is the first observation linking hypoxia to multiciliated cell differentiation, Polosukhin et al. showed hypoxia promotes goblet airway epithelial cell differentiation (24). Therefore, these observations support the idea that oxygen concentration can play a role in bronchial epithelial cell differentiation.

During embryonic development, oxygen concentration may play a role in lung development. Multiciliated cells are not observed until about the twelfth week of human gestation (35). This corresponds to a time during human fetal development when there is an increase of maternal blood flow to the intervillous region of the placenta. The increased blood flow causes an increase in the oxygen concentration from 40 mmHg to 80 mmHg with a resultant increase in the amount of oxygen delivered to the fetus (56, 57, 206). Hence, oxygen may play a pivotal role in inducing multiciliated cell differentiation during embryonic development with low oxygen concentrations preventing multiciliated cell differentiation and higher oxygen concentrations permitting differentiation. Moreover, oxygen may also play a role in multiciliated cell differentiation in respiratory disease e.g. COPD where the airway epithelial of patients have been found to be hypoxic, and a decrease in the multiciliated cell population has been reported (24, 25, 67). Thus, oxygen content could be a major player in regulation of multiciliated cell differentiation both during development and during respiratory disease in adults.

Hypoxia has been shown to potentiate Notch signaling (61). Furthermore, Notch signaling was also shown to affect the cell fate decisions of basal cells (BCs) towards a
secretory cell fate and away from a multiciliated cell fate in a variety of animal models (44, 86, 109). Therefore, NHBE cells were cultured in submerged or hypoxic conditions in the presence of the γ-secretase inhibitor DAPT, to inhibit Notch signaling, to ascertain whether Notch signaling was necessary for inhibition of multiciliated cell differentiation in these conditions. Results showed DAPT restored both FOXJ1 expression and multiciliated cell differentiation in submersion and hypoxia. Moreover, the data also showed multicilin (MCIDAS), a transcriptional regulator necessary for multiciliated cell differentiation (47), was suppressed by submersion and induced by DAPT. This suggested that low oxygen conditions repress both MCIDAS and FOXJ1 via Notch signaling, resulting in the inhibition of multiciliated cell differentiation. Additionally, the data showed ectopic expression of NICD1, NICD2, or NICD3 was sufficient to inhibit multiciliated cell differentiation in submerged or hypoxic conditions. This suggested any of the Notch1-3 receptors were capable of inhibiting multiciliated cell differentiation. However, experiments with Notch specific neutralizing antibodies showed endogenous Notch2 but not Notch1 played a role suppression of multiciliated cell differentiation. This finding is in line with other reports showing Notch2 as an inhibitor of multiciliated cell development in the lung (207, 208). These data suggested there is a common downstream target gene of Notch1, Notch2, and Notch3 that can repress MCIDAS and multiciliated cell differentiation, although, in NHBE cells Notch2 but not Notch1 activates this pathway. The discrepancy in the results between the ectopic NICD expression and endogenous Notch inhibition experiments may be due to differential expression of endogenous Notch receptor levels that are not a factor when expressing NICD ectopically driven by with the strong elongation factor 1 (EF1) promoter. In other words, expression
of NICD by EF1 may produce levels of NICD mRNA that are much higher than endogenous levels in the cell. However, the relative levels of Notch receptor mRNA were never determined and would need to be assessed to conclude if there was a difference. Support for this idea comes from published data showing endogenous Notch1 mRNA is expressed at roughly half the levels of Notch2 or Notch3 (93).

The exact mechanism of how hypoxia potentiates Notch signaling is still unclear. Western blots probed for NICD1 showed no differences in levels among ALI versus submersion or hypoxia. Although, the western blots were performed on the whole cell population, which means that if Notch receptor proteins decrease in some cells types and increase in others, the net results would appear to be no change. Indeed, it has been shown by Marcet et al. that cells destined to become multiciliated NHBE cells have lower Notch1 receptor protein levels (53). Additionally, Notch neutralizing antibody experiments would suggest measuring NICD1 may not be the best assay because inhibition of NICD1 showed no effect on multiciliated cell differentiation. Thus, it may be better to measure NICD2 and/or NICD3 levels. However, there are currently no commercially available antibodies that specifically recognize these proteins. If low oxygen does not potentiate Notch signaling by stabilization of NICD1 protein, there are alternative mechanisms that may exist. For example, hypoxia has been shown to increase Notch ligand expression, which could increase Notch signaling (209). In addition, enhancement of NICD activity could be achieved by hypoxia by reduction of asparaginyl hydroxylation of NICD, which is dependent on oxygen (210). Additionally, HIF-1α, which was shown in Figure 3.2 to be increased in NHBE cells cultured in hypoxic or
submerged cultures, has been shown to potentiate Notch signaling by binding to NICD (61).

The observation that hypoxia, which induces HIF signaling, inhibits multiciliated cell differentiation was the basis for further investigation into the potential roles of HIF proteins on multiciliated cell differentiation. The data showed constitutively active HIF-2α but not constitutively active HIF-1α inhibited multiciliated cell differentiation in ALI cultures. This suggested hypoxia could be inhibiting multiciliated cell differentiation in a HIF-2α dependent manner. However, these experiments were performed using a constitutively active protein being driving by the strong EF1 promoter, which could express high amounts of protein, and therefore, not reflect what would occur in the endogenous cell environment. Therefore, further studies are needed to look at the influence of endogenous HIF-2α on multiciliated cell differentiation. For example, knocking down HIF-2α levels via shRNA and analyzing multiciliated cell differentiation in hypoxia would be a way to assess this. Despite that, to my knowledge, this is the first study to report a potential role for HIF-2α affecting multiciliated cell differentiation. Additionally, the data showed constitutively active HIF-2α could inhibit multiciliated cell differentiation even in the presence of DAPT. This could mean there are alternative ways to inhibit multiciliated cell differentiation i.e. one Notch dependent and another HIF-2α dependent (possibly only when expressed at higher than endogenous levels as addition of DAPT is able to restore multiciliated cell differentiation in hypoxia (Figure 3.3)). Another explanation of the data may be high levels of HIF-2α are able to potentiate Notch signaling by enhancing the drastically reduced NICD levels caused by DAPT. Indeed, it has been reported in the literature that NICD directly interacts with HIF-2α.
Further investigation studying the involvement of HIF-2α in multiciliated cell differentiation is warranted and may reveal novel findings.

Many previous reports have shown IL-13 represses multiciliated cell differentiation (31, 52, 112, 187). However, despite the mechanism underlying IL-13 induced goblet cell hyperplasia and mucus secretion in the airways being well documented (86, 188), little was understood about the molecular mechanism involved in IL-13 dependent multiciliated cell inhibition. Since IL-13 dependent mucus cell hyperplasia can be abrogated by Notch inhibition (86), the effect of inhibiting Notch signaling on IL-13 dependent multiciliated cell inhibition was tested. The data showed inhibition of multiciliated cell differentiation was observed independently of Notch cleavage as repression occurred despite the presence of DAPT. This finding is similar to a recent publication showing IL-13 inhibited multiciliated cell differentiation of human bronchospheres in the presence of NRR2 antibody (31). Although, in those experiments, the possibility that IL-13 was inhibiting multiciliated cell differentiation using another Notch receptor was not addressed. Additionally, the use of DAPT, which will inhibit all Notch receptor cleavage, suggested IL-13 does not use any of the Notch receptors to stop multiciliated cell differentiation. These data revealed IL-13 drives mucus cell hyperplasia through a different pathway than it uses to repress multiciliated cell differentiation. In addition, the data showed IL-13 inhibited MCIDAS and FOXJ1 expression in the presence of DAPT. Since MCIDAS was previously described to be negatively regulated by Notch signaling (47, 199), these data imply MCIDAS acts as a molecular intersection for multiple signaling pathways, including Notch and IL-13, to affect multiciliated cell differentiation. Moreover, because Notch signaling represses MCIDAS expression, the
effect of IL-13 on Notch target repressor genes (HES1, HES2, HEY1, and HEY2) was evaluated because even though IL-13 does not require NICD, it could be upregulating the expression of canonical Notch target transcriptional repressors to inhibit MCIDAS expression. However, none of the four genes tested were elevated in the presence of IL-13. In fact, two showed IL-13 treatment significantly reduced mRNA levels. Subsequently, IL-13 has been reported to activate the JAK/STAT6 and MEK/ERK signaling cascades, which results in increased mucus production and bronchial goblet cells hyperplasia (86, 170, 188). With this in mind, the effect of these signaling pathways on IL-13 dependent multiciliated cell inhibition was investigated. The results revealed pharmacological inhibition of the JAK/STAT but not the MEK/ERK pathway promoted multiciliated cell differentiation in the presence of IL-13. These data showed IL-13 uses the JAK/STAT signaling pathway to promote goblet cell hyperplasia as well as to repress multiciliated cell differentiation. The exact mechanism of how Notch and IL-13 repress MCIDAS is still unknown and needs further study but these novel insights have furthered our understanding of how IL-13 inhibits multiciliated cell differentiation. In turn, these insights have shown the similarities and differences between the mechanisms employed by IL-13 to cause BCs to differentiate towards a goblet cell fate and not a multiciliated cell fate. The investigation into IL-13 also revealed JAK and MEK play a currently unknown roles in multiciliated cell differentiation as pharmacological inhibition of these pathways resulted in reduced levels of multiciliated cells in the absence of IL-13. Therefore, these data give insight into pathways that should be explored in more detail at a future time e.g. the role of ERK and different STATs in multiciliated cell differentiation.
Epithelial remodeling, including multiciliated cell loss, is observed in respiratory diseases. However, the factors that drive multiciliated cell loss in these diseases are not fully known. Previous data showed the airways of individuals with COPD and CF, where a decrease in multiciliated cells are observed (25, 212), were hypoxic (24, 67, 213). Data shown in this thesis demonstrated that hypoxia inhibits multiciliated cell differentiation via potentiating Notch signaling to inhibit genes necessary for multiciliated cell differentiation. Thus, the multiciliated cell loss observed in these respiratory diseases could be caused by the low oxygen conditions seen in diseased epithelium. Additionally, the data showed hypoxia suppressed multiciliated cell differentiation via Notch signaling. Therefore, Notch signaling inhibitors may be a potential therapy in treating epithelial remodeling in these lungs. Moreover, IL-13 is a cytokine that is elevated in respiratory disease such as asthma (176, 214) and during viral infection of the airways (185). Interestingly, multiciliated cells are decreased in asthmatics (26) and in virally infected airway epithelial cells from mice, bovine, and humans (182-184, 215). Taken together with observations in this thesis showing IL-13 suppresses multiciliated cell differentiation by activation of the JAK/STAT signaling pathway, these data suggest that specific JAK/STAT inhibitors may restore multiciliated cells in these individuals and may improve their lung function. This may give similar results as compounds that neutralize IL-13 signaling in a clinical trial setting (216). The results also showed how both Notch and IL-13 can inhibit multiciliated cell differentiation independent of each other. This could imply that modulation of multiple signaling pathways at once versus a single therapy may be necessary to restore multiciliated cell differentiation in an epithelium that is hypoxic and also chronically inflamed, which is another characteristic of COPD and
CF (217, 218). Figure 7.1 summarizes these data into a model showing multiple environmental cues e.g. inflammation and/or hypoxia can trigger the repression of multiciliated cells through different mechanisms. Therefore, the insights of this thesis add to our knowledge of potential causes of respiratory disease pathogenesis and may help give rise to therapeutics that could alleviate symptoms of disease in patients.

Until now, differentiation of multiciliated airway epithelial cells *in vitro* only occurred by culturing primary cells on an ALI. The discovery that NHBE cells will differentiate into multiciliated cells in the presence of the γ-secretase / Notch inhibitor, DAPT in plastic wells demonstrates that apical air exposure is not required for multiciliated cell differentiation and more traditional cell culture methods are adequate. These observations are a major technical advance and may open up the experimental possibilities for a variety of respiratory related fields. One such field is the study of cilia orientation and its role on fluid flow. This fluid flow is crucial for the physiology of many body systems and loss of the fluid flow can be a cause of disease (219). For example, loss of cilia driven fluid flow in the lung i.e. mucociliary clearance dysfunction can cause chronic sinusitis and bronchiectasis (220). Additionally, cilia driven fluid flow is necessary for the movement
Figure 7.1. Model describing potential environmental cues and signaling pathways that control multiciliated cell differentiation. The diagram shows multiciliated cell differentiation is controlled by regulation of MCIDAS and can be negatively regulated by both Notch and JAK/STAT signaling. It also describes the environmental conditions that activate these inhibitory pathways to suppress MCIDAS expression.

of cerebrospinal fluid (CSF), and without this flow, the CSF pressure will build leading to hydrocephalus (221). Cilia driven fluid flow is dependent on the orientation of the cilia and errors in cilia orientation disrupt this flow leading to disease (219). It has been shown in mouse ependymal cells in vivo that cilia orientation initially is random and then orientates based on the hydrodynamic force i.e. fluid flow already occurring in their environment (222). Therefore, with the ability now to generate multiciliated cells in submerged conditions, further studies into the mechanisms involved in this fluid flow dependent cilia reorientation can be studied. In addition to helping investigate current
fields of study, this technical advance may open up new or enhance established fields of study. 96-well and 384-well cell culture platforms are commonly considered “high throughput,” but to perform a high throughput assay requires the development of an easily measured and accurate screen for what you want to evaluate. Figure 4.2 showed multiciliated cell differentiation was easily and accurately measured in NHBE cells transduced with a lentivirus encoding the human FOXJ1 promoter driving the expression of the fluorescent protein, mCherry. Thus, these data showed an effective screen for multiciliated cell differentiation in NHBE cells in 96 and/or 384-wells can be created. Hence, we now have a high throughput assay for discovering compounds and factors that regulate FOXJ1 expression and multiciliated cell differentiation. In addition, this assay can also be used as a screen for novel Notch signaling inhibitors. If we extrapolate these results and say rather NHBE cells can be cultured on high throughput platforms and assays can be developed with them via lentiviral transduction, it opens the realm of possibilities for examining other biological relevant questions. For example, one could transduce NHBE cells with a lentivirus encoding the Muc5AC, which is a marker for airway epithelial goblet cells (223), promoter driving a fluorophore to develop an assay for studies involving the regulation of mucin expression and/or goblet cell hyperplasia. Furthermore, this system shows NHBE cells are a viable option for high throughput studies. This is likely advantageous from previous studies that used human cells lines in high throughput drug discovery studies in CF and respiratory viral studies (224, 225) because primary NHBE cells have been shown to emulate in vivo airway epithelial cells better than cell lines (50, 51). Additionally, in the past, NHBE cells would lose differentiation capacity, but a recent 2012 study by Suprynowicz et al. described a
technique where NHBE cells could be passaged for over 200 doublings and still retain their ability to differentiate into a pseudostratified epithelium containing goblet and multiciliated cells (226). Therefore, NHBE cells can be passaged to sufficient numbers for high throughput studies. Overall, the discovery that high throughput assays can be developed using primary NHBE cells opens up the future possibility that this system could be used to answer a multitude of biological questions in a range of research topics.
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