2013

THE MECHANOSENSING OF PRIMARY CILIA IN TUMOR PROGRESSION OF LUNG ADENOCARCINOMA

Sagar Patel
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd

Part of the Biomedical Engineering and Bioengineering Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/3071

This Thesis is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
THE MECHANOTRANSDUCTION OF PRIMARY CILIA IN
TUMOR PROGRESSION OF LUNG ADENOCARCINOMA

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Engineering at Virginia Commonwealth University

by

SAGAR SUNIL PATEL
Bachelor of Science, Virginia Commonwealth University, 2009

Director: REBECCA L. HEISE, PH. D.
Assistant Professor, Biomedical Engineering

Virginia Commonwealth University
Richmond, Virginia
May, 2013
Acknowledgments

First and foremost, I would like to offer my sincere gratitude to Dr. Rebecca L. Heise. I cannot begin to describe how incredible it has been to work for you. You have been a great mentor teaching me everything I needed to know and provided unlimited knowledge and insights for my project over the past two years. Thank you very much for being this patient with me throughout the years. It has been and will remain some of the most unforgettable experiences in my life. Hopefully I can and will continue to try to be as great of a scientist as you are.

I would also like to extend my special thanks to Dr. Hu Yang and Dr. Ramesh Natarajan for being a part of my committee. Both of you have been exceptional mentors to me throughout the whole project. I have learned some important concepts such as quantitative PCR, targeted gene therapy, drug delivery and therapeutics that I would not have been able to understand without your help.

I would like to thank all of my lab colleagues, Monika Radhika, Robert Pouliot, Joe Herbert, Kevin Lessard, Dilawar Khokhar, David Schweer, Mia Warlick, Puja Patel, Meher Malik, Shane Diller, Arjun Venkatasubramanian, Dustin Jones, Ananya Mishra, and Paul Howell. All of you have been amazing to work with. Thank you for all of your help and support and making the lab such a fun place to be.

I would also like to recognize several facilities for all of their help. Human tissues, patient consents, clinical data (or other appropriate service) were provided by the VCU Tissue and Data Acquisition and Analysis Core (TDAAC) Facility, supported, in part, with the funding from NIH-NCI Cancer Center Core Support Grant P30 CA016059, as well as through the Dept.
of Pathology, School of Medicine, and Massey Cancer Center of Virginia Commonwealth University. Microscopy was performed at the VCU Department of Anatomy and Neurobiology Microscopy Facility, supported, in part, with funding from NIH-NINDS Center core grant (5P30NS047463). Microarray gene analysis was performed at the MicroArraying Core in the Nucleic Acids Research Facilities. Services and products for cell cycle analysis in support of this research project were generated by the VCU Massey Cancer Center Flow Cytometry Shared Resource, supported, in part, with funding from NIH-NCI Cancer Center Support Grant P30 CA016059. Funding for this project was provided by the Jeffress Memorial Trust and American Cancer Society Institutional Research Grant.

I would like to thank all of my friends for their support. You guys were very understanding and were always there during my times of need. Lastly, this would not have been possible without the undying love and support from my family. I cannot thank them enough and the hard work invested in this project is my dedication to them. Thank you and I love you all.
# Table of Contents

Acknowledgements .......................................................................................................................... ii

List of Figures ..................................................................................................................................... vii

Chapter

1 INTRODUCTION ......................................................................................................................... 1

   Mechanical Environment of the Lung ............................................................................................ 3

   Background of Primary Cilia ........................................................................................................ 4

   Mechanosensory behavior of primary cilia .................................................................................. 6

   Kidney epithelial cells and primary cilia ....................................................................................... 7

   Signal Transduction by Primary Cilia ........................................................................................... 9

   Hedgehog signaling mechanism ................................................................................................. 10

   Role of IntraFlagellar Transport in Primary Cilia ...................................................................... 11

   Primary cilia and cancer ............................................................................................................... 13

   Objectives .................................................................................................................................... 14

2 MATERIALS AND METHODS .................................................................................................... 16

   Cell Culture ................................................................................................................................ 16

   Preparation of siRNA ................................................................................................................... 16

   Cell Transfections ....................................................................................................................... 17
3 RESULTS .............................................................. 26

Cancerous tissue show presence of primary cilia ........................................ 26
Regulation of primary cilia affects the phases of cell cycle .......................... 30
Loss of primary cilia negatively affects cell survival ................................... 32
Primary cilia aid in cell adhesion and migration ........................................ 33
Sonic Hedgehog signaling pathway plays a role in cell mechanotransduction
via primary cilia ...................................................................................... 35

MicroArray Gene Analysis ........................................................................ 40

4 DISCUSSION .............................................................................. 51

Cancerous tissue show presence of primary cilia ........................................ 51
Regulation of primary cilia affects the phases of cell cycle ........................................ 52
Loss of primary cilia negatively affects cell survival ................................................. 54
Primary cilia aid in cell adhesion and migration .................................................. 55
Sonic Hedgehog signaling pathway plays a role in cell mechanotransduction
via primary cilia ........................................................................................................ 58
MicroArray Gene Analysis ..................................................................................... 61

5 CONCLUSION .............................................................................................................. 66

6 FUTURE DIRECTIONS ............................................................................................... 68

References ................................................................................................................. 70

SUPPLEMENTAL INFORMATION .................................................................................. 83

Results ....................................................................................................................... 83
Discussion .................................................................................................................. 87
List of Primers for Hedgehog signaling related genes ......................................... 88

Appendices ................................................................................................................ 89

A Flow Cytometry Analysis data files ................................................................. 89
List of Figures

Figure 1: Basic structure of primary cilia ................................................................. 5

Figure 2: Mechanism in primary cilia in renal epithelial cells................................. 7

Figure 3: An overall signaling mechanism in Sonic Hedgehog pathway .................. 10

Figure 4: Role of IFT88 in Hedgehog signaling mechanism and some other cellular functions... 12

Figure 5: FlexCell™ tension plus system ................................................................. 19

Figure 6: Lung cancerous tissue has more primary cilia........................................... 26

Figure 7: Successful knockdown of IFT88 gene abolishes the formation of primary cilia ....... 27

Figure 8: Stretch increases primary cilia in 1-day samples....................................... 28

Figure 9: Stretch in 2-day samples induces an increase in primary cilia ..................... 29

Figure 10: Inhibition of primary cilia promotes cell cycle progression after 24 hours ........ 30

Figure 11: Cell cycle progression start to return to base levels after 48 hours ............. 31

Figure 12: Cell survival is decreased with stretch and with inhibition of IFT88 ............. 32

Figure 13: Inhibition of IFT88 decreases cell adhesion and migration ....................... 33

Figure 14: Cell migration increases in SKLU1 control samples after 48 hours static condition... 34

Figure 15: Successful knockdown of IFT88 gene abolishes the formation of primary cilia for 3-days ................................................................. 35

Figure 16: Sonic hedgehog gene is down-regulated in the absence of primary cilia ....... 36
Figure 17: Regulation of Ptch and Smo genes under stretch conditions ........................................ 37

Figure 18: Up-regulation of Gli2 expression for 1-day and 2-day samples ................................... 39

Figure 19: The number of probe sets from the three pairwise comparisons ............................. 42

Figure 20: GeneArray for static control vs. static IFT88 ............................................................... 43

Figure 21: GeneArray for static control vs. stretch control ........................................................... 44

Figure 22: GeneArray for static control vs. stretch IFT88 ............................................................. 45

Figure 23: GeneArray for stretch control vs. stretch IFT88 ........................................................... 46

Figure 24: GeneArray for stretch IFT88 vs. static IFT88 .............................................................. 47

Figure 25: Gene expression increased in stretch control compared with static control

  where further stretch control is also higher than stretch IFT88 .............................................. 48

Figure 26: Gene expression decreased in stretch control compared with static control

  where further stretch control is also lower than stretch IFT88 ........................................... 49

Figure 27: Heatmap of the genes that are regulated under stretch control samples compared to static

  control samples, which are also regulated in rest of the comparisons ................................. 50

Figure 28: Cells without primary cilia proliferated significantly less from 2 – 4 days ............... 71

Figure 29: Migration is significantly affected with the treatment of ammonium sulfate ............ 72

Figure 30: Gene expressions of hedgehog pathway related genes after 48 hours ................. 74

Figure 31: Cell cycle analysis for 1-day static control samples ..................................................... 78

Figure 32: Cell cycle analysis for 1-day static IFT88 samples ...................................................... 79

Figure 33: Cell cycle analysis for 1-day stretch control samples ............................................... 80
Figure 34: Cell cycle analysis for 1-day stretch IFT88 samples .................................................... 81

Figure 35: Cell cycle analysis for 2-day static control samples ..................................................... 82

Figure 36: Cell cycle analysis for 2-day static IFT88 samples ...................................................... 83

Figure 37: Cell cycle analysis for 2-day stretch control samples ................................................... 84

Figure 38: Cell cycle analysis for 2-day stretch IFT88 samples .................................................... 85
Abstract

THE MECHANOTRANSDUCTION OF PRIMARY CILIA IN TUMOR PROGRESSION OF LUNG ADENOCARCINOMA

By Sagar Sunil Patel, B.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Biomedical Engineering at Virginia Commonwealth University.

Virginia Commonwealth University, 2013

Major Director: Rebecca L. Heise, Ph.D.

Assistant Professor, Biomedical Engineering
The objective of this study was to investigate primary cilia and their mechanotransduction role in lung adenocarcinoma tumor progression. The main focus investigated the effect of primary cilia on cell cycle progression, survival, adhesion and migration analysis of these cells and the role of sonic hedgehog signaling pathway in mechanotransduction. Human Non-Small Cell Lung Cancer (NSCLC) adenocarcinoma biopsies contain more primary cilia than non-tumor lung sections. To observe the effects of primary cilia presence in lung cancer cells \textit{in-vitro}, formation of primary cilia is inhibited using small interfering RNA. A549 cells with intact primary cilia observe less cell cycle progression than cells deficient in primary cilia under static and cyclic stretch conditions. Primary cilia cause higher cell survival and adhesion. Increase in cell adhesion also increases the migration and wound closure rates in control samples compared to samples treated with inhibition of IFT88, thereby increasing the metastasis of these cells. Several downstream regulatory genes in sonic hedgehog signaling pathway observe significantly decreased gene expressions in primary cilia deficient cells, thus indicating inefficient mechanotransduction. Therefore, cancer cells need primary cilia to survive, adhere and migrate and continue tumor progression.
CHAPTER 1: INTRODUCTION

Lung cancer accounts for over 159,000 deaths annually [1]. According to the American Cancer Society, in 2013, 228,190 new cases of lung cancer are expected in the US only, with 71% mortality rate in these cases [1]. Lung cancer is divided into two different types: Small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Small cell lung cancer accounts for 10-15% of all lung cancer. They are observed on the bronchi towards the center of the chest cavity and are very metastatic from the beginning stages of this cancer [1,2]. Due to their small size, excision of the tumor through surgery is almost never the only type of treatment that is given. Chemotherapy is the best type of treatment for this cancer. Since NSCLC accounts for the remaining 85-90%, it is the type of lung cancer that requires the most attention [1]. NSCLC is classified as any malignant lung tumor that lacks a small-cell component [3]. NSCLC are extremely resistant to chemotherapy and hence, very difficult to treat [4]. It is classified into three main categories: squamous cell carcinoma, large-cell carcinomas and adenocarcinoma. Squamous cell lung carcinoma is a type of malignant tumor that forms within the lining of the lungs’ bronchi. This carcinoma type accounts for 25-30% of all the non-small lung cancer cells [5]. Even though lungs are the main locations for this cancer, it is also known to metastasize to other important organs of the body, including bones, liver and brain [5]. Their unique ability of keratinization and forming squamous pearls sets them apart compared to the other cancer cell types. Smoking is the primary risk for contracting this type of cancer. Other causes may also include age, family history and secondhand smoking [5]. The growth activity for squamous lung carcinoma is very small and will generally take years before they are deemed harmful and invasive.
Large-cell carcinomas are undifferentiated tumor cells that lack the specific characteristics of a small cancer cells and non-small cancer cells [3]. Unlike squamous cell carcinoma, they do not keratinize into pearl-like appearance. They also lack glandular differentiation, a unique characteristic of the lung adenocarcinomas [3]. They take place in the periphery of the lungs and account for only 5% of the lung cancers [6]. Adenocarcinomas are the most common type of NSCLC. They occur in the periphery of the lungs and account for over 40% of all lung cancers [6]. In the past few years due to a decrease in smoking, there are more cases of adenocarcinomas compared to any other types of non-small cancer cells in Western countries [6]. Alveolar, bronchial, or bronchiolar epithelial cells are the primary contributors to the development of these malignant epithelial tumors [6].

For in-vitro studies, immortalized cell lines are created from lung cancer tissue that has been removed from a patient and cultured under in-vitro conditions. Lung tissues can be extracted from different patients to create their own cell lines. Under in-vitro conditions, these cells can be cultured indefinitely without affecting its physical and functional nature. A549 cell line was developed by D.J. Giard in 1972 after removing and harvesting tissue from a 58 year Caucasian male while SK-LU-1 cells were harvested from a 60 year old Caucasian female [7,8]. As adherent cells, they attach to the culture flasks and grow in monolayer. Upon reaching confluency, these cells can also grow over one another and create multilayer tumors. Over the last few decades, A549 and SK-LU-1 cells have been main targets of several studies and are considered two of the most widely used in-vitro cell lines.

Understanding cell cycle, survival, adhesion and migration in these cells will help understand tumor progression. Cell cycle consists of the different stages in cell division, starting from the quiescent stages, G₀, G₁ phases, synthesis of DNA, S phase, which eventually leads to
cell mitosis, M phase. It can be used to understand the cancer cell growth and survival. Cancer cells have increased functionality of adhesion and migration. Adhesion involves attachment of cancer cells to other cells and the extracellular matrix while migration refers to their ability to metastasize and spread to other parts of the body. In order to better understand the functions and effects of these factors on cancer growth, it is vital to understand the physiological and mechanical environment in the lungs.

**Mechanical Environment of the Lung**

Normal lung epithelial cells constantly undergo bilateral tensile stresses and shear strains during normal respiration [9,10]. During inhalation, they experience tensile stresses due to the expansion in the chest cavity while they undergo compressive forces during exhalation. In vitro studies have shown that cells experience up to 30% change in surface area during normal respiration [11]. Stretch greater than 37%, change in cellular surface area greater than total lung capacity, was found to be harmful and decrease cell viability [12,13]. Due to a loss in normal lung function, patients suffering from lung cancer also experience complex mechanical environments from other pre-existing lung diseases such as chronic bronchitis, emphysema, primary lung fibrosis and other tumor associated changes [14]. These conditions significantly alter the structure of the lung cells, its phenotype and the extracellular matrix surrounding them [15,16]. Therefore, lung cancer cells undergo higher mechanical forces compared to normal cells [11]. With pre-existing diseases, cancerous cells can experience up to 30% stretch without injuring them [11]. Almost every normal cell in the body possess immotile, solitary structures known as primary cilia [17]. Until recently, their role as sensory organelles and mechanotransducers had been fairly unknown. New studies have now established the role of primary cilia as sensory organelles in several different types of cells in the body including
fibroblasts, chondrocytes, vascular smooth muscle cells and renal epithelial cells [17–26]. Photoreceptors and the olfactory cells have also been found as modified versions of primary cilia [27–29]. Moreover, primary cilia are considered important centers for various mechanotransduction signaling pathways in normal and cancerous cells [18,19,22,30–34].

**Background of Primary Cilia**

Primary cilia were first discovered and cited by KW Zimmerman in 1898 [35]. Unlike motile cilia, primary cilia are antenna-like structures that protrude from almost every cell of the human body [17]. The structure of the cilia is the key reason for its immotility. Within the structure, axoneme is the most important element and is primarily responsible for its structural integrity [20]. Axoneme within each motile cilium consists of nine doublet microtubules around its periphery and two doublet microtubules towards the center (9 + 2 arrangement). The central pairs are primarily responsible to provide motility for the cilia [36]. However, primary cilia lack these central pairs of microtubules (9 + 0 arrangement). Each axoneme is connected to a basal body, which is located at the base of axoneme and is made up of many triplet microtubules. Microtubules consist of at least three types of tubulin: α-, β-, and γ-tubulins [37]. α and β-tubulins combine to form dimers that are important for structural integrity of microtubules whereas γ-tubulin are more specialized and found only in certain types of microtubules such as centrosomes [38,39]. Most of the studies
have showed the presence of α and/or γ-tubulins is necessary for the formation of primary cilia [14,17,18,21,25,28,29,31,38–55]. Figure 1 represents an accurate structure of the primary cilia [20]. When first observed in 1898, they were thought to be functional in one of three possible different ways. First, they were assumed to have become vestigial organs and lost all capabilities of proper functionality [26]. Primary cilia are present in abundance during the deactivation of mitosis while their number decreases during cell division and proliferation [25,56]. Therefore, they played a vital role in controlling the mitotic stages of all the cells [56]. Since the basal bodies of primary cilia are constructed from the same material of microstructures as the centrioles, the formation of cilia was temporarily deactivated in order to create the mitotic spindle in 3T3 cells [56]. It was shown that these cilia were critical sensory organelles for earlier forms of eukaryotes and the modern day visual and auditory systems consists of modified, more enhanced cilia [17]. Henceforth, these assumptions can also be applied to the third hypothesis: primary cilia are very important sensory organelles and administer the signal transductions between all the cells and the extracellular matrix [17,20].

**Mechanosensory behavior of primary cilia**

There are two main advantages of primary cilia. They can be used as sensory organelles to detect the physiological and mechanical change in the external environment of the cell (28). They also serve as signaling centers between the cells and the extracellular matrix (16). Primary cilia are one way through which the cells can sense the change in the mechanical environment [17]. In chondrocytes, Mcglashan et. al. showed the presence of extracellular matrix receptors and the associated integrins located on the primary cilia. These enable the primary cilia to detect the mechanical and physiochemical changes in the extracellular matrix and regulate the cell-ECM interactions [18]. The integrins, polycystin1 (PC1), polycystin2 (PC2), α3 and β1, were
also found on primary cilia in vascular smooth muscle cells in mice and were shown to increase migration of these cells compared to the cells with the inhibition of β1 integrin [19]. They play an important role in detecting the mechanical change in the external environment of the cells as well as serve as signaling centers between cells and extracellular matrix [18,20]. Ciliary dysfunction was also found to be a leading cause of liver cystogenesis [57]. They can regulate pressure, touch and vibration [23,24]. Photoreceptors and olfactory cells use modified primary cilia to detect changes in the external environment [27–29]. Deformation of intraflagellar transport IFT57 protein, which is required to maintain cilia structure and regulate ciliogenesis, led to the degeneration of photoreceptors in zebrafish [21,27]. Similar changes in the genetic makeup of these personalized primary cilia have led to anosmia [28]. The function of the primary cilia is most understood through its key role in the kidney epithelium [17]. In kidneys, proteins detect the change in fluid flow within the kidney tubule and initiate intracellular calcium signal. This led to human polycystic kidney disease (PKD). The ion-gated channels responsible to detect fluid flow are located on the primary cilia [22,31].

**Kidney epithelial cells and primary cilia**

Primary cilia originates from the epithelial cells around the nephrons and afloat in the lumen of the ducts [20]. As shown in Figure 2, Transient Receptor Potential (TRP) ion channels, which are located on the primary cilia, act as mechanosensors to detect the change in fluid flow within the tubule. PC1 and PC2 are localized on the surface of the primary cilia [18]. PC1 is a large integral protein whereas PC2 is a TRP Ca\(^{2+}\) ion-gated channel [58]. These proteins on the primary cilia combine with P100 and STAT6 to form a complex that has an ability to detect change in volumetric flow within the tubule and control the proliferation, differentiation and/or
apoptosis of kidney epithelium cells. P100 and STAT6 are transcription factors that are involved with the regulation of the kidney epithelia cells [43].

Under normal kidney function, flow of urine in the tubule lumen is recorded by PC1 and activates the signaling pathways within the cilia, causing it to bend. This increases the intracellular concentration of calcium ions entering through the TRP channels of PC2 and deactivates the regulated intracellular proteolysis (RIP) of PC1 [31]. The inbound calcium ions ensure that the transcription factors stay bound with PC1 and prevent them from their translocation to the cell nucleus, where they can signal the proliferation of the cells [31]. In the event of kidney failure or a mutation in either of these proteins, the motility of the primary cilium is compromised. PC1 is no longer able to detect the changes in fluid flow and limits the influx of calcium ions through the PC2 channel. This activates RIP, which in turn cleaves PC1. P100 and STAT6 migrate to the nuclear membrane and activate unregulated proliferation of the epithelium cells. These cells clump together to form cysts, a condition also known as Polycystic Kidney Disease [20]. A general summary is represented in Figure 2.

Besides the kidneys, the role of primary cilia has also been investigated in several other organs and cells. In human bronchial smooth muscle cells (HBSMCs), Wu et. al. demonstrated the dependence of these cells on primary cilia in order to maintain, remodel and repair. They
have been shown to promote wound healing by promoting directional cell migration and chemotaxis in fibroblasts [44,52]. Intraflagellar proteins that make up the structure of primary cilia are necessary for the development of chondrocytes in bone growth-plates [47]. Integrins on the primary cilia are needed to communicate with the ECM and regulate cell proliferation and migration through mechanochemical sensing[33]. Primary cilia are found to be precursors to the development of motile cilia in human tracheal epithelial cells [34]. Primary cilia have not been identified in normal and cancerous alveolar epithelial cells.

**Signal Transduction by Primary Cilia**

Primary cilia are also very important in facilitating the signal transduction pathways. They are primary locations for PDGFα receptors (PDGFRα), which are necessary for downstream signaling of the PDGFRαα and Mek1/2-Erk1/2 pathway. In the absence of primary cilia in Tg737ompk mutant mice fibroblasts, PDGFRαα signaling is significantly down-regulated, which hinders normal regulation of tissues such as proliferation and apoptosis [43,52,59]. Simons et. al. presented that inversin is a necessary protein to activate the non-canonical Wnt pathway by inhibiting the expression of dishevelled [32]. Inversin and dishevelled proteins are colocalized on the primary cilia and play a competitively inhibition role against each other. The role of primary cilia is best described largely due to its role in the mechanotransduction of Hedgehog signaling mechanism [18,30,36,51,54,60–62].

**Hedgehog signaling mechanism**

Hedgehog and its associated components belong to a family of lipoproteins that signal and control their expressions. Hedgehog pathway “regulate(s) tissue patterning, cell proliferation, and many other biological processes” [63] and can lead to serious birth defects and
cancer. There are three types of homologues, desert hedgehog (DHh), indian hedgehog (IHh) and sonic hedgehog (SHh), of which SHh has been mostly widely studied.

The sonic hedgehog pathway requires the help of two main transmembrane proteins, Patched (Ptch) and Smoothened (Smo), and several transcription factors. During the absence of the Hedgehog (Hh) ligand (Figure 3A), Ptch is absorbed by cellular organelles and prevents the inactivation of the Smo protein. This in turn activates a cascade of events on the primary cilia

![Figure 3: An overall signaling mechanism in Sonic Hedgehog pathway [30].](image)

that involves the activation of Gli transcription factors that are located on the surface of the primary cilia. Each phase of the Gli pathway has its own set of regulation that will eventually lead to the transcription of the DNA with or without the expression of Hh gene. In this case, inactivation of the Smo proteins causes the binding of Sufu proteins to the Gli activators and releasing of Gli repressors. The repressor factors travel to the nucleus and initiate transcription without the expression of SHh. The presence of SHh prevents the deactivation of Smo by Ptch
and ensures that Smo travels along the primary cilia and activate the Gli activator and inhibit Gli repressor factors (Figure 3B). Similar to the repressors, these activated factors travel down the cilia and to the nucleus, where it will bind to the DNA and activate the expression of the gene Hh during its transcription [36]. This allows the hedgehog gene to be successfully expressed during the transcription of the DNA. Figure 3 shows a summary of the sonic hedgehog signaling pathway. Therefore, the role of Hedgehog signaling pathway is dependent upon the formation of primary cilia.

**Role of IntraFlagellar Transport in Primary cilia**

Intraflagellar transport (IFT88) is one of the most important multi-protein complexes that make up the base of the primary cilia. Along with a motor unit, Kif3a, IFT88 is responsible for the formation of primary cilia as well as maintenance of several cellular functions. They have been shown to be necessary for ciliary and flagellar assembly, and prevent mutation in Tg737 gene and PKD in Chlamydomonas [64]. They are also important in assembly and maintenance of vertebrate photoreceptors [55]. Inhibition of IFT88 decreases primary cilia formation and has been shown to regulate several cellular functions including cell cycle and migration [39,44,47]. Hedgehog signaling pathway in primary cilia has been studied in several *in-vitro* and *in-vivo* studies and is found to be regulated through IFT88 [36,43,54,60–62,65–68]. To this day, the role of IFT88 in primary cilia has not been investigated in lung adenocarcinoma cells. Regulation of IFT88 helps understand how cellular functions and signaling pathways are affected in the presence or absence of primary cilia. This is been shown through the use of small interfering ribonucleic acid (siRNA). They are double-stranded sequences that are 20-25 base pairs in length and involve replacing the beginning section of the nucleotide sequence of a targeted gene with complementary sequence using forward and reverse primers. They have been used successfully
Figure 4: Hedgehog signaling mechanism and some cellular functions involves the presence of IFT88.

**Primary cilia and cancer**

Primary cilia have been shown to be downregulated in clear cell renal cell carcinoma, breast cancers, and pancreatic cancer compared to their adjacent normal tissue [46,70,71]. The role of primary cilia in cancer has been connected with the activation of sonic hedgehog (Shh) [72]. In basal cell carcinomas and medulloblastomas, the primary cilia can enhance or suppress tumorigenesis dependent upon Shh activation [65,66]. In human bronchial smooth muscle cells, primary cilia have been shown to regulate the signaling between the cells and the extracellular matrix via EGFR, α2, α5, and β1 integrins [33]. However, the presence and the role of primary cilia have not been studied in normal and cancerous lung epithelial cells.
Understanding the role primary cilia will play towards cell cycle, survival, adhesion and migration can significantly help understand the progression in tumor growth. Research has indicated that primary cilia are shown to regulate cell division cycle by inhibiting the G\textsubscript{1} – S transition [39,42,73–75]. Thus, it inhibited cell division and proliferation. Presence of primary cilia has also been used to test cell’s ability to survive [51,76]. Decreasing the cell-cell adhesion, cell-ECM adhesion and cell migration can reduce the metastasis of cancer to other organs of the body [68]. Mutant Kif3A, PC1 and PC2, which are some of the components of primary cilia, show decrease and ineffective adhesions of FAK to the focal adhesion and regulate ECM protein-protein bindings, thereby limiting cell adhesion [47,68]. This would disrupt their migration rate towards a site of injury or away from the primary cancerous organ [48,77–79].
The primary objectives discussed below will help understand the importance of primary cilia towards tumor progression in lung adenocarcinoma cells. It will also help define the response of lung cancer cells on cell ciliogenesis to mechanical stimuli and its effect on cell division cycle, proliferation, adhesion and migration.

Objectives:

1. Identify the presence of primary cilia in human lung adenocarcinoma biopsies

2. Develop an *in-vitro* model for the lung adenocarcinoma cell lines, A549 and SK-LU-1, under static and high-stretch (15% change in cells’ surface areas) conditions for 1-day and 2-day timepoints.

3. Use small interfering RNA (siRNA) to specifically target and silence the gene expression of IFT88, in order to inhibit the formation of primary cilia.
   
   a. Confirm the down-regulation of the genetic expression of IFT88 gene, thus confirming the deformation of primary cilia.

   b. Analyze high-quality confocal microscope images to validate successful knockdown of primary cilia in samples treated with siRNA IFT88 compared to the control samples under static and high-stretch conditions.

   c. Analyze the difference observed in IFT88-silenced samples compared to the control samples for the following categories:

      i. Cell division cycle

      ii. Cell survival
iii. Adhesion

iv. Migration

d. To identify the role of primary cilia in the mechanotransduction of Sonic Hedgehog signaling pathway

i. RT-qPCR analysis of SHh-pathway related genes for the treated samples versus control samples.

e. Microarray gene analysis will highlight the effect of inhibition of primary cilia formation on the gene expressions involved in most signaling pathway mechanisms.

Supplemental Study: Abolish primary cilia using ammonium sulfate.

a. Compare cell proliferation rate, migration and gene expressions for samples +/- primary cilia under static and stretch conditions.
CHAPTER 2: MATERIALS AND METHODS

Cell Culture

Human lung adenocarcinoma cell line, A549 (ATCC® CCL-185™) and SKLU1 (ATCC® HTB57™) (ATCC, Manassas, VA), were used to perform the experiments. Unless otherwise noted, cells were cultured using Dulbecco’s modified Eagle / F-12 medium (HyClone, Logan, UT) with 10% Fetal Bovine Serum (HyClone, Logan, UT) and 1% Penicillin/Streptomycin (Mediatech Inc., Manassas, VA). Using T-75 flasks, cells were grown until 70-80% confluence, trypsinized and passaged. They were incubated at 37 °C, 95% air and 5% CO2. Prior to plating the cells for the experiments, each 6-well BioFlex plate is coated with 1 mL of Collagen Type I solution for 2 hours at biological conditions.

Preparation of siRNA

T-75 flasks that are 70-80% confluent were used to prepare the cells for siRNA experimentation. Cells are plated using DME/F-12 media supplemented with 10% FBS only. Custom siRNA sense and antisense oligonucleotides corresponding to the cDNA sequences of IFT88 [42] and scrambled control [41] were ordered from Dharmacon (Dharmacon, Lafayette, CO).

IFT88: CCGAAGCACUUAACACUUA | Control: GTGCCTGCTGGTGCCAAC

Following manufacturer’s protocol, 5x siRNA Buffer (Thermo Scientific, Lafayette, CO) was diluted to 1x concentration using sterile RNase-free water to anneal the oligonucleotides. An optimized transfection protocol followed by the laboratory of Charles Chalfant Ph.D., was used to determine a final concentration of 20 μM for siRNA transfections on A549 and SK-LU-1 adenocarcinoma cell lines. The protocol was further optimized using 40 μM concentration
resulting in almost similar transfection efficiency but lower cell survival compared to the lower concentration. Therefore, 20 μM concentration was the best concentration for siRNA transfections of both cell lines.

**Cell Transfections**

6-well untreated BioFlex culture plates (Flexcell International Corp., Hillsborough, NC) were coated with 1 mL of Type I Collagen solution (Sigma-Aldrich, St. Louis, MO) (250 μg/mL) at 37 °C, 95% air and 5% CO2 for 2 hours prior to plating cells. After aspirating the collagen solution, A549, SK-LU-1 cells were suspended in 2 mL of DME/F-12 media supplemented only with 10% FBS and incubated at biological conditions overnight. The cells were treated with siRNA +/- inhibition of expression of IFT88 gene. siRNA without IFT88 were treated with scrambled gene sequence and were used as controls.

**Preparation of Transfections solutions**

DharmaFECT solution: For each well, 4 μL of DharmaFECT 1 reagent (Dharmacon, Lafayette, CO) (1 mg/mL) was diluted with 11 μL of 1x OPTI-MEM (without Phenol Red) and incubated at room temperature for 10 minutes. A total volume of 15 μL DharmaFECT solution is used per well.

RNAi solutions: 10 μL of 20 μM control and IFT88 siRNA were diluted in 175 μL of 1x OPTI-MEM individually. For each siRNA, 15 μL of DharmaFECT solutions were added, gently mixed and incubated at room temperature for 20 minutes.

Prior to treating the cells with the siRNA solutions, old DME/F-12 media from each of the wells was aspirated; cells are washed with 1x Dulbecco’s Phosphate Buffered Saline (Life
Technologies, Grand Island, NY) and 1x OPTI-MEM (no supplements added). 800 μL of 1x OPTI-MEM is added in each of the wells along with the siRNA solutions +/- IFT88 are added to each corresponding wells and incubated at biological conditions for 4 hours (final concentration of 200 nM). 500 μL of 1x OPTI-MEM supplemented with 30% Fetal Bovine Serum only is added to each well and further incubated for 4 hours at biological conditions. At the end of the incubation, solutions are aspirated, cells are washed with 1x D-PBS and incubated overnight with 0.1% FBS, 1% PS, DME/F-12 media.

Cell Stretch

A549 cells are plated in 6-well BioFlex culture plates and allowed to stretch at 15% change in membrane surface area at 0.86 Hz for 24 and 48 hours. As shown in Figure 5, stretch was performed using the 25 mm loading station on the FlexCell Tension Plus system (Flexcell International Corporation, Hillsborough, NC). The system uses vacuum pressure to apply equiaxial stresses to the cells cultured on silicone membrane wells. After the timepoint, plates are removed from the system to analyze different aspects of the project.

Figure 5: FlexCell™ tension plus system. www.flexcellint.com. Adenocarcinoma cell lines will be plated on collagen type I coated silicone membranes and cyclically stretched at 15% change in surface area.
**Cell Proliferation and Survival**

Cell proliferation and viability were measured using the MTT (Roche, Indianapolis, IN) and AlamarBlue cell viability assay (Invitrogen, Grand Island, NY).

**MTT** - Cleaving of a particular tetrazolium salt, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and its conversion to formazan was used to analyze the number of viable cells in the well. Initially, MTT was introduced in each of the well through the addition of labeling reagent and incubated at physiological conditions for 4 hrs. Solubilization solution is added to the wells and incubated overnight to solubilize formazan salt. The absorbance was measured at 550 and 650 nm using the BioTek microplate spectrophotometer (Winooski, VT). For each treatment in order to eliminate any extra background interference, the values for 650 nm were subtracted from the values at 550 nm. Per treatment, the average of these values is compared to the averages of the rest of the treatments. Data is normalized by comparing the values for each treatment to the static control of that timepoint. N = 3 per group and significance is represented by * p < 0.05, ** p < 0.01, *** p < 0.005

**AlamarBlue** - The assay assessed cell’s capability to reduce resazurin into resorufin to analyze the viability in the wells. AlamarBlue contains an active ingredient, Resazurin, which is a non-toxic, non-fluorescing compound that is cell permeable and blue in color. Once resazurin enters the cells, it is reduced into resorufin, a highly fluorescent red compound. Once alamarblue is added to the plates that contain cells, they are incubated at 37 °C, 5% CO₂ for 15, 30, 45 and 60 minutes. The absorbance was measured at 570 and 600 nm using the BioTek microplate spectrophotometer. Background interference is eliminated.
by finding the ratio of 600nm / 570nm for each of the samples. Per treatment, the average of these values is compared to the averages of the rest of the treatments. Data is normalized by comparing the values for each treatment to the static control of that timepoint. Two-way ANOVA with Tukey post-hoc analysis with N = 3 per group and significance is represented by * p < 0.05, ** p < 0.01, *** p < 0.005.

**Scratch Wound Healing Assay**

Cells were grown to near confluence in flexcell plates while a scratch wound was made vertically towards the center of the well using a 200 μL pipet tip. To use the same reference area, a line was drawn under the well horizontally using a marker pen. Plates were either exposed to static or stretch conditions for a period of 48 hours while the wound was imaged every 24 hours to calculate the change in wound closure at each timepoint. Images for each well are taken above and below the marker pen line on the scratch wound site. The migration of the cells towards the site of injury was measured by analyzing the distance traveled by the cells over a span of 48 hours. Images were taken with an Olympus IX71 inverted research microscope while the distances were calculated using QCapture Pro 6.0 image and analysis software.

**Cell Adhesion**

Cells are transfected using scrambled control and siRNA IFT88 on 24-well BioFlex plate prior to experimentation. Each well of a hard-bottom 24-well plate was coated using 400 μL collagen type I solution for 2 hours and blocked with 400 μL of 10 mg/mL bovine serum albumin (Fisher Scientific, Pittsburgh, PA) for 30 minutes prior to plating cells. Transfected cells were counted, plated on the hard-bottom 24-well plate, incubated for 2 hours at 37°C, 5% CO₂ and allowed to attach to the bottom of the plate wells. They were fixed with 5% Glutaldehyde (Fisher
Scientific, Pittsburgh, PA) for 5 minutes and washed with PBS. They were again fixed for 20 minutes with 5% Glutaldehyde and washed three times with de-ionized water before getting stained with 400 μL of 0.1% Crystal Violet (Amresco, Solon, OH). They were washed three times with de-ionized water and lysed with 400 μL of 10% acetic acid after 5 minutes. The absorbance of the stained cells was measured at 570 nm using BioTek Epoch microplate spectrophotometer and Gen5 data analysis software.

**RNA isolation and Real time quantitative PCR**

RNA was collected and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). 600μL of Buffer RLT was used to lyse and collect the RNA. Equal amount of 70% ethanol was also added and vortexed. The solution was loaded into a RNeasy mini spin column where RNA was collected and purified using Buffer RW1 and Buffer RPE solutions. 30μL of Rnase-free water was used to dilute and collect the RNA from the spin column. Reverse transcription was performed on the RNA to convert to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For each sample, 1.2 μg of RNA was diluted with 10μL of nuclease-free DEPC -treated water. 4.2μL of DEPC-treated water, 2μL of 10x RT Buffer, 2μL of 10x Random Primers, 0.8μL of 25x dNTP (100 mM) and 1μL of MultiScribe Reverse Transcriptase reagents were used to make 10μL of 2x RT master mix solution. Once added to the RNA, a thermal cycler was used to convert it to cDNA. The thermal cycler conditions involve incubation at 25 °C for 10 minutes, 37 °C for 120 minutes and 85 °C for 5 minutes. Real time quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Primers were obtained from Life Technologies (Grand Island, NY) and their sequences are shown in Supplemental section. qPCR was analyzed for ΔΔCq values where Static Control samples were used to determine the relative
gene expression for the rest of the treated samples. The gene GAPDH was used as an internal control.

**Immunofluorescence**

Cells were fixed using 4% Paraformaldehyde solution, permeabilized with 0.2% Triton x-100 and blocked with 5% bovine serum albumin.

Primary antibodies were incubated at room temperature for 3 hours.
Mouse anti-acetylated tubulin: Sigma T7451 1:500 (Sigma-Aldrich, St. Louis, MO).
Rabbit PolyAb Anti-IFT88 1:50 (Proteintech, Chicago, IL).

Secondary Antibodies:
Goat anti-mouse Alexa Fluor 488 IgG 1:800 (Life Technologies, Grand Island, NY).
Goat anti-rabbit Rhodamine-conjugated IgG 1:100 (Proteintech, Chicago, IL).

They were mounted on slides using Prolong gold anti-fade reagent with DAPI (Life Technologies, Grand Island, NY). Z-stack images were taken using scanning confocal microscopes (Leica TCS-SP2 AOBS and Zeiss LSM 700) at 40x and 63x magnification and analyzed using Leica LCS and Zen 2011 image analysis softwares.

**Flow Cytometry**

Cells (1x10^6 cells/well) were plated, transfected on 6-well BioFlex plates and stretched for 24 and 48 hours with DME/F-12 media supplemented with only 1% Penicillin/Streptomycin to mimic starvation conditions. They were trypsinized, neutralized with appropriate media and aspirated. A staining solution with 0.1% TritonX-100, 2mg DNase-free RNase and 200 µg of 1 mg/mL propidium iodide was used to stain the samples before being analyzed by BD FACSCANTO II™ and acquired by FACSDIVA software. 10,000 cells from each 1-day and 2-
day post-transfected samples were analyzed for 3 main phases of cell cycle: G1, S and G2 phases. Statistical analysis was performed using ModFit LT analysis software. Data was presented as percentage of cells +/- standard deviation in each phase. Comparisons were made using two-way ANOVA tests with Tukey post-hoc analysis where p-value < 0.05 was considered significant.

**Microarray Gene Analysis**

Cells (3x10^5 cells/well) were plated, transfected on 6-well BioFlex plates and kept under static and stretch conditions for 24 hours. Rna was collected for each treatment (N=3) and purified using RNeasy Mini Kit. Reverse transcription was performed on the RNA to convert to cDNA using the High Capacity cDNA Reverse Transcription Kit. Real time quantitative PCR was performed using Power SYBR Green PCR Master Mix to confirm inhibition of IFT88 gene expression. Microarray gene analysis was performed to observe gene expression changes on 26,000 genes using Affymetrix Human Genome U133A 2.0 GeneChip®. Data is filtered under different conditions for seven significant comparisons. They are acquired using Bioconductor analysis software and R statistical programming software. The gene expressions in all four treatment samples are compared in pairs for significance differences between them. A list of different comparisons is defined below:

1. Static Control vs. Static IFT88
2. Static Control vs. Stretch Control
3. Static Control vs. Stretch IFT88
4. Stretch Control vs. Stretch IFT88
5. Stretch IFT88 vs. Static IFT88

6. Increase in Stretch Control compared to Static Control, where further Stretch IFT88 is decreased compared to Stretch Control

7. Decrease in Stretch Control compared to Static Control, where further Stretch IFT88 is increased compared to Stretch Control.

Each of these data sets was uploaded onto the Ingenuity Interactive Pathway Analysis software to analyze significant differences in the genetic expressions between the two group sets. The genes that are regulated in the static control versus stretch control comparison are analyzed for their expressions in first 5 comparisons. Heatmap is created using their p-value expressions in the GeneCluster and TreeView software created by the EisenLab. Genes are hierarchically clustered according to their relative distance from rest of the genes using a complete linkage clustering with an uncentered correlation similarity.

Statistical Analysis

Unless mentioned otherwise, GraphPad Prism was used for statistical analysis of the data. Data are presented as mean +/- standard deviation. One-way and Two-way ANOVA tests with Tukey pot-hoc comparisons were performed where appropriate. * p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.005 were considered significant.
CHAPTER 3: RESULTS

_Cancerous tissue show presence of primary cilia_

Initially, we checked whether the lung adenocarcinoma lung biopsies exhibited an increase in primary cilia when compared to normal surrounding lung tissue. Human lung biopsies were stained with DAPI and acetylated $\alpha$-tubulin. Acetylated $\alpha$-tubulin was found to be more in abundance in the cancerous tissue (Fig. 6A). When the same settings were applied to observe similar structures in the normal surrounding lung tissue, only faint $\alpha$-tubulin staining was found (Fig. 6B). These preliminary data suggested that there is an increase in the amount of $\alpha$-tubulin present in cancerous cells.

**FIGURE 6.** Lung cancerous tissue has more primary cilia. Normal and cancerous tissue biopsies were acquired from lung cancer patients. Samples were stained with DAPI (blue) and anti-tubulin (green). A. Cancerous tissue contained more primary cilia (arrows) as observed by the high intensity of $\alpha$-tubulin around cell surface. B. Primary cilia were not observed in normal surrounding tissue acquired from the same patient. Biopsies were obtained through Virginia commonwealth university tissue and data acquisition and analysis core (TDAAC).

To support our initial results, we conducted an _in-vitro_ immunofluorescence staining on A549, lung epithelial adenocarcinoma cell line, for DAPI and $\alpha$-tubulin. In order to inhibit the formation of primary cilia, the IFT88 gene was silenced using the small interfering ribonucleic
acid (siRNA). Figure 7 below represents a successful inhibition of IFT88 mRNA levels in the samples treated with siRNA compared to the samples treated with scrambled control. All samples underwent a 15% cyclic stretch in surface area for 24 and 48 hours to better mimic the dynamic conditions of the lungs. Similar results were found for 24 hours and 48 hours (Figure 8-9) post-stretch samples. Samples treated for the inhibition of IFT88 (Fig. 8B, 8D, 9B, 9D) exhibited decreased expression of α-tubulin (green), which shows a decrease in the presence of primary cilia compared to the control samples (Fig. 8A, 8C, 9A, 9C).

**FIGURE 7:**
**Successful knockdown of IFT88 gene abolishes the formation of primary cilia.** For static and stretch conditions, samples treated with siRNA IFT88 show down-regulation of IFT88 genetic expression for 1-day and 2-day samples. Data is normalized to static control for each timepoint and representative of mean +/- standard deviation, n=3 per group. * p<0.05, ** p<0.01, *** p<0.005. Two-way ANOVA with Tukey post-hoc test.
1-day Samples: A) Static Control, B) Static IFT88, C) Stretch Control, D) Stretch IFT88

FIGURE 8.
Stretch increases primary cilia in 1-day samples. Lung adenocarcinoma cells (A549) were stained with DAPI (blue) and α-acetylated tubulin (green) after stretched for 24 hours. Darker stained regions of α-tubulin are noted primary cilia. A. Static control samples show presence of few primary cilia around the periphery of cell nuclei. B. Inhibition of IFT88 successfully decreased primary cilia formation. C. Stretch increased primary cilia formation as shown in the control samples. D. Inhibition of IFT88 decreased the formation of primary cilia under stretch condition.
2-day Samples: A) Static Control, B) Static IFT88, C) Stretch Control, D) Stretch IFT88

FIGURE 9.
Stretch in 2-day sample induces an increase in primary cilia. Lung adenocarcinoma cells (A549) were stained with DAPI (blue) and α-acetylated tubulin (green) after stretched for 48 hours. Darker stained regions of α-tubulin are noted primary cilia. A. Static control samples show presence of few primary cilia around the periphery of cell nuclei. B. Inhibition of IFT88 successfully decreased primary cilia formation. C. Stretch increased primary cilia formation as shown in the control samples. D. Inhibition of IFT88 decreased the formation of primary cilia under stretch condition.
Regulation of primary cilia affects the phases in cell cycle

To identify the role of primary cilia on the stages of cell cycle, flow cytometry was performed. As shown in figure 10, we observed that the static and stretch IFT88-silenced samples progressed farther in the cell cycle compared to the static or stretch controls after 24 hours. Under static and stretch conditions, IFT88 inhibited samples were significantly lower in the G₁ phase while they were significantly higher in the S phase compared to control samples. Stretch IFT88 samples were significantly higher in the G₂ phase compared to the stretch controls, thus showing that stretch plays a role in regulating cell cycle.

FIGURE 10.
Inhibition of primary cilia promotes cell cycle progression after 24 hours. Static and stretch IFT88-treated samples were significantly lower in G₁ phase and significantly higher in S phase compared to the static and stretch control. Stretch IFT88 samples were also found significantly higher in the G₂ phase compared to the stretch controls after 24 hours under 15% stretch conditions. Data is representative of percentage of cells +/- standard deviation, n= 3 per group. * p < 0.05, ** p < 0.01, *** p < 0.005 Two-way ANOVA with Tukey post-hoc test.
Cell cycle progression observed in IFT88-silenced samples is returned back to basic levels after 48 hours under stretch conditions (Figure 11). Under static conditions, treated samples still exhibit significant down-regulation during the G₁ phase while they are up-regulated during the G₂ phase. An increase in cell cycle progression usually concludes an increase in cell proliferation. However, the role of cell cycle progression towards cells’ survival has yet to be examined.

**FIGURE 11.**
Cell cycle progression start to return to base levels after 48 hours. Samples with inhibition of IFT88 have progressed farther in the cell cycle under static condition. These samples under stretch returned back to the control levels faster than the static samples. Although stretch control samples are significantly lower than the static controls in G₁ phase, there was no significance found in the other phases. Data is representative of percentage of cells +/- standard deviation, n= 3 per group. * p < 0.05, ** p < 0.01, *** p < 0.005 Two-way ANOVA with Tukey post-hoc test.
**Loss of primary cilia negatively affects cell survival**

We found that gene silencing of IFT88 negatively affected the cell’s ability to survive (Figure 12). The knockdown of IFT88 significantly decreased cell survival under stretch at 1-day. The difference is better represented with the 2-day samples where static and stretch treated samples were significantly lower than the control samples. With the exceptions of the 1-day static IFT88 and 1-day stretch control samples, all of the samples are also significantly decreased compared to the 1-day static control. Both of the stretch samples at 2-days are also significantly lower than their respective 1-day stretch samples.

*FIGURE 12. Cell survival is decreased with stretch and with inhibition of IFT88.* 1-day static and stretch samples shows that cell survival significantly decreased for IFT88 treated and stretch samples when compared to 1-day static control. These results are further supported by the 2-day samples where significant decreases are also found for IFT88 treated and stretch control samples when compared to the 1-day static control. Data is normalized to static control and representative of mean +/- standard deviation, n= 3 per group. * p < 0.05, ** p < 0.01, *** p < 0.005 two-way ANOVA with Tukey post-hoc test.
Primary cilia aid in cell adhesion and migration

Decrease in cell survival lowers the number of cells that can potentially adhere to other cells or extracellular matrix. Under static conditions, IFT88 silenced samples (Figure 13A) were found 30% less likely to attach to the collagen coated BioFlex well membrane compared to the control samples after 2 hours suggesting a decrease in cellular function to adhere. The adhesion rate directly determines the rate at which the cells are able to enter the bloodstream or lymphatic system and can potentially become metastatic. A decrease in the number of cells that attach can

**FIGURE 13.**
Inhibition of IFT88 decreases cell adhesion and migration. A. In the presence of primary cilia, cells attach 30% better (left) than cells without primary cilia (right). Data is normalized to static control and representative of mean +/- standard deviation, n= 5 per group. *** p < 0.005 unpaired two-tail t-test.

B. Cells with primary cilia traveled almost twice as far as IFT88-treated samples after 48 hours stretch. Data is normalized to static control and representative of mean +/- standard deviation, n=3 per group. ** p < 0.01 Two-way ANOVA with Tukey post-hoc test. Control sample showing cells migrated closer towards the scratch wound injury (bottom left) compared to the cells that are treated with siRNA IFT88 (bottom right).
result in a decrease in the migration rate of the cells. As shown in Figure 13B, cells without primary cilia showed significantly decreased migratory capabilities 48 hours after stretch. The images visually show the difference in migration experienced by samples in the presence of primary cilia (bottom left) compared to the treated samples with no primary cilia (bottom right).

Migration was also performed on another lung adenocarcinoma cell line, SK-LU-1. Under static condition, scrambled control samples significantly migrated closer towards the site of injury than samples inhibited of primary cilia after incubation at biological conditions for 48 hours (Figure 14). Once the role of primary cilia is confirmed in these critical aspects of tumor progression mentioned above, it was important to find out the signaling mechanism through which cilia are able to conduct the mechanotransduction within these cells.

**Figure 14.**

**Increase in cell migration in SK-LU-1 control samples after 48 hours static condition.** Control samples observed higher migration rate after 48 hours compared to IFT88-silenced samples. Data is normalized to static control and representative of percent gap closure ± standard deviation, n= 3 per group. * p < 0.05 unpaired two-tail t-test for each timepoint.
**Sonic Hedgehog signaling pathway plays a role in cell mechanotransduction via primary cilia**

Since several studies had linked primary cilia with the sonic hedgehog pathway [18,30,36,51,54,60–62], we tested the genetic expressions of several genes associated with this pathway. To ensure if primary cilia play a significant role in the signaling pathway in lung adenocarcinoma cells, successful down-regulation in the IFT88 mRNA expression in the treated samples under static and stretch conditions for 1-day, 2-day and 3-day samples are shown below (Figure 15). In this case, 3-day samples are presented to confirm consistent inhibition of gene expression.

![IFT88 gene expression](image)

*FIGURE 15.*
**Successful knockdown of IFT88 gene abolishes the formation of primary cilia for 3-days.** For static and stretch conditions, samples treated with siRNA IFT88 show down-regulation of IFT88 genetic expression for 1-day, 2-day and 3-day samples. Data is normalized to static control for each timepoint and representative of mean +/- standard deviation, n=3 for most groups. * p<0.05, ** p < 0.01, *** p<0.005. Two-way ANOVA with Tukey post-hoc test.

The effects of siRNA IFT88 were observed on the following Sonic Hedgehog related genes: Sonic Hedgehog (SHh), Patched (Ptch), Smoothened (Smo) (Figure 16-17). In each graph, static IFT88, stretch control and stretch IFT88 are compared and normalized to Static Control. Figure 16 shows that after 24 hours timepoint, the gene expressions are significantly down-regulated for sonic hedgehog under stretch conditions. Static IFT88 is also down-
regulated for sonic hedgehog. After 48 hours, sonic hedgehog expression show that the Static IFT88 and Stretch IFT88 samples have returned back to static control level while Stretch Control is significantly up-regulated.

Figure 17 shows that the gene expressions are significantly down-regulated for patched and smoothened under stretch conditions after 24 hours timepoint. Static IFT88 remains unchanged for patched and smoothened. After 48 hours, the 2-day samples show that patched and smoothened gene levels have also recovered back to base levels compared to their 1-day samples indicating a swift recovery response.

Figure 16.
Sonic Hedgehog gene is down-regulated in the absence of primary cilia. At 24 hours, both samples of SHh are significantly down-regulated under stretch. Static IFT88 is also down-regulated for SHh. After 48 hours, stretch control is the only that is up-regulated for SHh gene. Data is normalized to static control for each timepoint and representative of mean +/- standard deviation, n= 3 per group. * p < 0.05, ** p < 0.01, *** p < 0.005 two-way ANOVA with Tukey post-hoc test.
Figure 17.
**Regulation of Ptch and Smo genes under stretch conditions.** At 24 hours, both samples of Ptch and Smo are significantly down-regulated under stretch. After 48 hours, stretch IFT88 is down-regulated for Smo. Control and treated samples for Ptch return back to base levels after 48 hours. Data is normalized to static control for each timepoint and representative of mean +/- standard deviation, n= 3 per group. * p < 0.05, ** p < 0.01, *** p < 0.005 two-way ANOVA with Tukey post-hoc test.

Gli1 and Gli2 are two of the main transcription factors in the hedgehog signaling pathway. Their gene expressions will also help understand the signaling mechanism. No significant changes are found for Gli1 expression for 1-day and 2-day timepoints (Figure 16). For Gli2 gene, stretch IFT88 samples are up-regulated when compared to both, static control and stretch control samples, 24 hours after stretch. Significant up-regulation is also found for IFT88-
silenced samples for static and stretch after 48 hours. Stretch control samples have returned back to base levels.

**Figure 18.**

*Up-regulation of Gli2 for 1-day and 2-day samples.* No significance is observed for Gli1 samples for both days. For Gli2, Stretch IFT88 is up-regulated for 1-day and 2-days compared to static control. It is also up-regulated when compared to stretch control at 1-day while static IFT88 samples are only up-regulated after 48 hours. Data is normalized to static control for each timepoint and representative of mean +/- standard deviation, n= 3 per group. * p < 0.05, ** p < 0.01, *** p < 0.005 two-way ANOVA with Tukey post-hoc test.
**MicroArray Gene Analysis**

To understand how the inhibition of primary cilia affected the gene expressions for all of the genes, we conduct a microarray gene analysis on the 1-day samples. Comparing the genetic expressions of all the treated samples to static control allow us to visualize how different genes are up-regulated and down-regulated throughout all of the signaling pathways (Table 1, Figure 19). Once the data is collected, seven different comparisons were made between two group sets as described in the methods sections.

The bar charts show a list of all of the categories of genes that observed significant differences in their molecular and cellular functions by the different 1-day treatments. Throughout the seven comparisons, genes associated with cell cycle and cell survival are affected the most. In order to observe the overall effects of the treatments on all of the aspects, which include the physiological functions, molecular functions as well as diseases and disorders, heat maps are incorporated. Genes that were up-regulated or down-regulated the most are represented with the darker colors (Orange for up-regulation and Blue for down-regulation). Besides the categories of molecular and cellular functions that are most affected, cancer and tissue morphology are found to be affected the most. Figures 20 – 26 shows the bars and heat maps for each different statistical group set. Genes, which are regulated in control samples under stretch as compared to static, are analyzed to observe the difference in expression levels in each of the first 5 statistical comparisons. A heatmap showing the significant regulation of these genes is represented in Figure 27. Divisive hierarchical clustering is used to classify the genes according to their relative distances from each other. This clustering combines a group of genes that are closest to one another into a cluster. It is combined with the next closest gene cluster to create a branch. Similarly, genes that are farther are clustered with the existing branch according
to the relative distance to create bigger branches. The hierarchy lastly combines the cluster of genes that are farthest with the existing branches in order to create a tree [80,81]. A complete linkage clustering is applied between two closest clusters by calculating the distance between the genes that are farthest apart [81,82]. Pearson correlation coefficient can be used to predict and determine the similarity between two clusters of genes by creating a linear comparison [80]. This is also known as centered correlation. Figure 27 shows the genes that are significantly regulated in the comparison of static control versus stretch control samples. These genes only are further observed for their expression in the remaining four pairwise comparisons. Since the gene expressions in one comparison are focused more than the remaining comparisons, it is extremely difficult to calculate the overall similarity using the Pearson correlation coefficient. Therefore, an uncentered correlation was applied to observe the differences between the clusters of genes that are being regulated.
Table 1. Results from the linear contrasts comparing each group to the Static: Control group.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Number of probe sets having FDR&lt;0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static: Control versus Static:IFT88</td>
<td>3103</td>
</tr>
<tr>
<td>Static: Control versus Stretch: Control</td>
<td>78</td>
</tr>
<tr>
<td>Static: Control versus Stretch:IFT88</td>
<td>4015</td>
</tr>
</tbody>
</table>

Figure 19.
The number of probe sets from the three pairwise comparisons. Each group compared to Static: Control. Probe sets were considered significant if the FDR<0.01.
Figure 20.
GeneArray for static control vs. static IFT88.
Bar chart showing genes important in molecular and cellular functions (Left).
Heat map exhibiting the important pathways that are significantly regulated between the two subsets (Right).
Figure 21.
GeneArray for static control vs. stretch control.
Bar chart showing genes important in molecular and cellular functions (Left), Heat map exhibiting the important pathways that are significantly regulated between the two subsets (Right)
Figure 22.
GeneArray for static control vs. stretch IFT88.
Bar chart showing genes important in molecular and cellular functions (Left),
Heat map exhibiting the important pathways that are significantly regulated between the two subsets (Right).
Figure 23.
GeneArray for stretch control vs. stretch IFT88.
Bar chart showing genes important in molecular and cellular functions (Left),
Heat map exhibiting the important pathways that are significantly regulated between the two subsets (Right).
Figure 24.
GeneArray for stretch IFT88 vs. static IFT88
Bar chart showing genes important in molecular and cellular functions (Left),
Heat map exhibiting the important pathways that are significantly regulated between the two subsets (Right).
Figure 25.
Gene expression increased in Stretch Control compared with Static Control where further Stretch Control is also higher than Stretch IFT88.
Bar chart showing genes important in molecular and cellular functions (Left),
Heat map exhibiting the important pathways that are significantly regulated between the two subsets (Right).
Figure 26. Gene expression decreased in Stretch Control compared with Static Control where further Stretch Control is also lower than Stretch IFT88. Bar chart showing genes important in molecular and cellular functions (Left), Heat map exhibiting the important pathways that are significantly regulated between the two subsets (Right).
Figure 27. Heatmap of the genes regulated under stretch control condition compared to static control samples which are also regulated in rest of the comparisons.

Genes are clustered using complete linkage clustering with an uncentered similarity correlation. Gene upregulation and downregulation are expressed in red and green respectively, where lighter shades represent higher significant p-values. Genes that are not observed or significantly regulated in the corresponding comparisons are represented by grey shades. GeneCluster andTreeView softwares created by EisenLab were used to analyze and create the heatmap.
CHAPTER 4: DISCUSSION

Lung cancer comprises of 27% of all cancer-related mortality and is one of the most lethal cancers.[1]. Specifically adenocarcinoma, which occurs towards the periphery of the lungs, is the most common type of lung cancer and contribute over 40% of all lung cancers [6]. Normal lung epithelial cells continuously undergo tensile stresses and shear strains [9]. Along with these stresses, lung cancer cells experience higher stresses due to pre-existing lung diseases such as chronic bronchitis, emphysema, primary lung fibrosis and other tumor associated changes [11,14]. The extracellular mechanical environment of the cells has a great impact on cellular activity and plays a crucial role towards tumor formation and metastasis [18,20]. It has been shown that one way cells can detect the changes and initiate mechanotransduction is by forming primary cilia [17–34]. Cancerous cells of some tissues have been shown to be deficient in primary cilia [46,70,71]. Hence, the mechanosensory and the cellular signaling functions are carried out through other mediums. However, the relationship between cancer tumorigenesis and primary cilia in the lung has yet to be significantly examined.

Cancerous tissue show presence of primary cilia

In order to learn more on primary cilia in the lungs, we performed immuno-fluorescence on normal and cancerous human lung tissue biopsies. We found primary cilia are not present in normal lung epithelial cells (Figure 6B). However, they are highly expressed in lung adenocarcinoma cells (Figure 6A). The importance of primary cilia has not been studied in lung cancer cells. This initial observation helped us further investigate and understand the role of primary cilia in lung adenocarcinoma cell lines. IFT88 has been shown to be responsible for the formation of cilia and flagella in Chlamydomonas [53]. To specifically target primary cilia on
the cells, siRNA was used to inhibit the gene expression of IFT88. Figure 7 shows the decreased expression fold change observed in samples treated with siRNA IFT88 at 1 and 2-day timepoints.

Normal cells in the lungs constantly undergo mechanical stresses and strain. Due to the loss of proper structure, shape and function, cancerous lung cells undergo much higher mechanical forces compared to normal cells [11,14–16]. To better mimic the physical environment experienced by the cells in the lungs, a bioreactor was used to induce biaxial stresses on the cells under biological conditions. Cells were treated with a scrambled control and siRNA IFT88, inhibiting the expression of IFT88, under static and stretch biological conditions for 24 and 48 hours. We found that the scrambled control samples for both timepoints had significantly higher number of primary cilia than the treated samples (Figure 8-9). This suggests that the IFT88-inhibited samples lead to the depletion of primary cilia.

**Regulation of primary cilia affects cell cycle**

To further understand if primary cilia aid cancerous growth, we investigated cell cycle. Cell cycle analysis summarizes the progression of cell division for each sample treatment. Studies have linked this progression to be dependent on the formation of primary cilia [73]. Centrosomes consist of centromeres that make the basal bodies of primary cilia [74]. During the resting phases of the cells, G₀ and G₁, basal bodies aid in formation of primary cilia. During mitosis, the cilia are resorbed and the basal bodies localize to the spindle poles and are used for cell division [42]. More specifically, IFT88 has also been shown to regulate cell cycle. A decrease in the expression of IFT88 is related to the resorption of cilia and cell cycle progression. The transition from G1-S phases is also severely hindered with the expression of IFT88 [39].
Cells without primary cilia have mutated function to respond to external stimuli that control cell proliferation [75]. Cell cycle analysis for 1-day and 2-day samples showed similar results (Figure 10-11). IFT88 silenced cells were found more in S and G2 phases compared to the control samples. However, applying bilateral mechanical stretch has also been shown to affect cell cycle. In fetal lung fibroblasts, mechanical stretch inhibited cell cycle progression and activated cell apoptosis through the activation of caspase-3 [83]. Similarly in rat alveolar type II cells, cyclic stretch was found to induce cell apoptosis and secretion by applying 22% stretch in surface area of the cells, thereby significantly decreasing cell proliferation and survival [84]. Stretch-induced inhibition of cell proliferation in rat smooth muscle cells was found to be dependent upon the inhibition of growth factors, such as platelet derived growth factor and thrombin. These growth factors inhibited the transition between the G1 and S phases, thus decreasing the proliferation rate [85]. McAdams et al. [11] confirmed that a stretch of 16% in cells’ surface areas resulted in minimal effect on the proliferation rate of the cells. In order to limit the negative effects of stretch on the cell stresses and survival, 15% change in cell surface area was chosen for the described experiments. Since several studies have shown the effect of primary cilia in cell cycle in many different types of cells, it was important to learn how cell cycle is affected in lung adenocarcinoma cells in the presence and absence of primary cilia. Our initial experiments found results similar to those in the previous studies. The transition between G1 and S phases was inhibited due to the presence of primary cilia. Under stretch conditions, cells in IFT88 silenced samples progressed further into S and G2 phases compared to the control at 1-day timepoint. Cells in static IFT88-treated samples also progressed into S phase more than static control (Figure 10). After 2 days, only static IFT88 samples were found to be higher in G2 phase compared to the static control samples. No progression is observed in the stretch IFT88-
treated samples (Figure 11). This can be related to starvation with no serum. Samples were treated with media without any serum supplements to mimic extreme starvation conditions. Since serum starvation has been shown to decrease cell survival and induce cell death, it might be necessary for the cells to retain or form primary cilia under such conditions for survival [10, 51, 71, 86]. Therefore, the next step was to investigate the effects of cilia on cell survival in these cells.

**Loss of primary cilia negatively affects cell survival**

From our preliminary observations of cell cycle analysis, using no serum might have played a small role in affecting cell cycle. In order to understand how primary cilia regulate cell survival as accurate as possible, it is vital to limit other sources that might potentially affect the result. Media was supplemented with 0.1% fetal bovine serum for cell sustainability and limit cell death. Cell proliferation and survival have been studied in primary cilia. Song et. al. showed that mice deficient in Kif3a, a component of the Kinesin-II motor complex that helps IFT proteins form and maintain primary cilia, experienced loss of primary cilia. This resulted in decreased proliferation of growth-plate cells and development of post-natal dwarfism [47]. Contrary to what has been shown about the dependence of cell growth on cell cycle progression, primary cilia promote cell survival.

Inhibition of Spag17 led to dysfunction of motile cilia and low survival rate in neonatal mice [76]. Astrocytes in the central nervous system that readily forms primary cilia were treated with siRNA IFT20 [51]. Samples were also treated with serum-free media to promote cell survival. Cell survival was significantly compromised through the inhibition of Hedgehog signaling [51]. Our cell survival experiments were conducted using low levels of serum (0.1%
FBS) to mimic similar conditions and test cell survival. We found that cell survival was dependent on the duration of the stretch and inhibition of IFT88 expression. All samples at 2-day timepoint had significantly lower cell survival when compared to 1-day static control sample. In each timepoint, control samples experienced significantly higher cell survival rates compared to the IFT88-knockdown samples (Figure 12), concluding that primary cilia, specifically IFT88, is an essential regulator in cell survival. Decreased survival rate lowers the amount of cells that can potentially attach to ECM or other cells and eventually metastasize to other parts of the body.

**Primary cilia are necessary for cell adhesion and migration**

Several studies have shown that primary cilia are linked with cell adhesion. In chondrocytes, McGlashan et. al. showed the presence of extracellular matrix receptors and the associated integrins located on the primary cilia. They enable the primary cilia to detect the mechanical and physiochemical changes in the extracellular matrix and regulate the cell-ECM interactions [18]. Primary cilia were absent in mice with mutant Kif3A, which resulted in improper adhesion of FAK to the focal adhesion [47]. Integrins such as polycystins genes, PC1, PC2, α2, α3 and β1, have also been shown to play an important role in chondrocytes, vascular smooth muscle cells as well as regulation polycystic kidney disease [18,58]. PC1 aids in mechanosensing as well as cell adhesion with the extracellular matrix and adjacent PC1 molecules [87]. It activates the calcium channel PC2, thereby inducing cell signaling [68]. They are both members of focal adhesion complex family and regulate several ECM protein-protein interactions [68]. Photoreceptors and cholangiocytes develop glycoprotein-dependent cilia-cilia contacts that sense the extracellular environment and transduce proper signaling [88]. In our experiment, cells were transfected on 24-well BioFlex plates 1 day prior to analysis. As directed
in the methods section, cell adhesion assay using 0.1% crystal violet was performed after these cells were re-plated on hard-bottom 24-well plate, incubated under static biological conditions for 2 hours and allowed to adhere to the bottom of the wells. The knockdown of IFT88 significantly reduced the cell’s efficiency to adhere to the collagen and BSA coated hard-bottom wells under static conditions compared to the scrambled samples (Figure 13A).

Schneider et. al. concluded that in the presence of platelet-derived growth factor αα (PDGFαα), mice embryonic fibroblasts oriented its primary cilia towards the site of wound injury and underwent an increase in migration of the cells [44,59]. Fibroblasts with an inhibition of IFT88 showcased little or no cilia and migrated significantly less [44]. The receptors (PDGFRαα) are localized on the primary cilia in fibroblasts and are responsible to activate signaling pathway to mediate cell cycle control and directional cell migration [48,79]. Similarly in mice aortic endothelial cells and interneuronal cells, primary cilia were found to control formation of focal adhesion, and directional migration [77,78]. Wu et. al. demonstrated that the integrins located on the primary cilia are needed to communicate with the ECM and regulate cell proliferation and migration through mechanochemical sensing in order to maintain, remodel and repair in human bronchial smooth muscle cells (HBSMCs) [33]. Our experiments measured the distance traveled by the control and the IFT88 silenced samples towards the site of injury (Figure 13B). After 48 hours, the stretch controls with the presence of primary cilia traveled significantly farther towards the injury compared to the primary cilia abolished samples. The image (bottom left) represents the distance travelled by the cells in the control samples was much higher than the distance by primary cilia deficient treated samples. We also conducted migration on a different lung adenocarcinoma cell line, SK-LU-1. The migration analysis is shown in Figure 14. Under static conditions, cell without primary cilia exhibited lower closure rate of
wound injury than the control samples after 48 hours. These results further helped support our observations through the A549 cell lines. Investigating the signaling mechanism through which these primary cilia regulated the above functions of the cells became the next important priority.

*Cell signaling occurs on primary cilia via Sonic hedgehog pathway-related genes*

In basal cell carcinomas and medulloblastomas, tumorigenesis can be activated or inhibited through the sonic hedgehog pathway depending on the type of initiated response. By increasing the activation of smoothened protein, tumor formation diminished. Contrastingly, tumorigenesis was also activated due to an increased expression of Gli2 transcription factor in the pathway [65,66]. The role of primary cilia has been shown to be primarily dependent on the activation of Hedgehog signaling pathway [72]. An in-vivo study showed the correlation between genes in Hedgehog signaling pathway and primary cilia in several phenotypic and morphologic mutant mouse embryos [54]. More specifically, membrane proteins essential for the primary cilia such as intraflagellar transports (IFT88 and IFT172) and Kif3a were more significantly affected due to the changes in the expression of hedgehog related genes [60]. This pathway serves as a mechanotransduction center between cells [63]. Two genes within the hedgehog signaling pathway, Patched (Ptch1) and Gli1, were significantly down regulated in post-natal IFT88-deleted growth plate chondrocytes compared to the controls [67]. Intraflagellar proteins IFT88 and IFT172 play a significant role in regulating the sonic hedgehog signaling pathway [67].

Hedgehog signaling pathways are also significantly affected with the simulation of stretch conditions. Indian hedgehog molecule (Ihh) was necessary in mechanotransduction and promotes proliferation in 17-day-old embryonic chick chondrocytes. The enhanced chondrocyte
proliferation was in part due to a brief increase in the mRNA levels of Ihh observed after 2-day stretch conditions [89]. The effect of sonic hedgehog signaling molecules were also investigated. Unlike the observations made by Wu et. al, Morrow et. al. concluded that the exposure of 10% cyclic strain on the rats vascular smooth muscle cells significantly decreased the mRNA expressions of Shh, Ptch, Smo and Gli after 24 hours [90]. This played an important role in diminished cell growth and increased apoptosis.

Figures 15-18 show the gene expressions for Sonic Hedgehog related genes. In each graph, the data was compared and analyzed to static control samples. To make sure that the primary cilia is involved in Hedgehog signaling pathway, it was essential to observe expressions for some of the genes involved in the pathway in the absence of cilia. If these gene expressions are significantly altered, primary cilia might be responsible in the regulation of the signaling pathway. Specifically inhibiting the expression of IFT88 inhibited the formation of primary cilia. Figure 15 shows successful inhibition of IFT88 gene under static and stretch conditions for 1-day and 2-day timepoints. Figures 8-9 shows that there are more cells that show the presence of primary cilia under stretch control conditions for both days compared to the static control samples. This suggests that the stretch control samples would observe higher expression for IFT88. However, the mRNA level of IFT88 does not show a significant increase in stretch control. We believe that this effect might not be only dependent on stretch conditions. The mRNA levels only conclude the genetic expression of IFT88 and are not completely indicative of presence of primary cilia. To further investigate, western blotting will be needed to observe the difference in protein expression levels between the static and stretch samples. The effects of siRNA IFT88 were similarly observed on the following Sonic Hedgehog related genes: Sonic Hedgehog (SHh) (Figure 16), Patched (Ptch) and Smoothened (Smo) (Figure 17), and
transcriptional factors: Gli1, Gli2 (Figure 18). After 24 hours timepoint, the gene expressions are significantly down-regulated for sonic hedgehog, patched and smoothened under stretch conditions. Our results validated the results collected by Wu et. al. and morrow et. al. The mRNA levels of SHh, Ptch and Smo were dependent on the stretch conditions. Static IFT88 is also down-regulated for sonic hedgehog, while it remains unchanged for patched and smoothened. No significant changes are found for the 1-day Gli1 transcription factor. However, Stretch IFT88 is significantly up-regulated when compared to both, Static Control and Stretch Control samples 24 hours post-stretch in Gli2 transcription factor.

After 48 hours, the static IFT88 and stretch IFT88 samples of sonic hedgehog expression have already returned back to normal levels. Similar to the results by Wu et. al., stretch control is significantly up-regulated 2-days post-stretch [89]. Compared to their 1-day samples, patched and smoothened gene levels have also recovered back to base levels indicating a swift recovery response. Although no significance was determined, Gli1 indicate an increasing trend in the IFT88 silenced samples suggesting a slower genetic expression response. Gli2 is also significantly up-regulated under static and stretch conditions in the knockdown samples, thus continuing their up-regulation from 1-day timepoint.

Our results validated the data found in several other studies. The hedgehog signaling pathway is significantly affected by the inhibition of primary cilia. Down-regulation of IFT88, the genes and the transcription factors involved in the hedgehog pathway concludes that cells are unable to form primary cilia as well as create the subsequent proteins necessary for proper mechanotransduction within the cells. Since hedgehog pathway is significantly affected by the inhibition of primary cilia, primary cilia might also play a role in other mechanotransduction pathways.
**Microarray Gene Analysis**

It became important to conduct a microarray gene analysis to understand how the different types of treatments affected the gene expressions associated with different signaling pathways that take place within the cells. Foldbjerg et al. concluded that silica (SiO$_2$) nanoparticles supplemented with bovine serum albumin did not only increase the oxidative stress but also up-regulated genes involved in lipid metabolism and biosynthesis in six different mammalian (normal and carcinoma) cell lines including A549 [91]. Genes in cell transcription, cell junction, and ECM-receptor interactions were also found to be down-regulated in these treatments [91]. These down-regulated genes were also investigated in a study by Price et al. in which matrigels were used to form three dimensional cell cultures. They are very rich in extracellular matrix components and have been shown to play a role in gene regulation and cell phenotype alterations [92]. Price et al. presented that they are important to regulate the microRNA expression and ECM-associated cell growth in five human cancer cell lines [92]. A gene array revealed that matrigels significantly regulated ECM genes associated with cell stress responses, survival, adhesion, migration, and metastasis. Anti-cancer drugs treatments on A549 revealed that FDXR, a gene involved in p53-mediated cell apoptosis, was significantly up-regulated in six of the drugs [82]. Inhibition of cell cycle progression, decrease in cell growth and migration were also found to be regulated [82]. Microarray analysis performed on main populations and side populations in four human lung cancer cell lines found that eight of the ten signaling pathways that observed the most significant changes in gene expression were associated with cell cycle, adhesion and survival [93]. In our experiments, microarray gene analysis was performed not only to validate the importance of the above mentioned signaling pathways, but also investigate important gene regulations that might also be involved in other
signaling pathways. The analysis recorded the expression of every gene for the three treatments and compared them to the expression in static control samples. Seven different comparisons were made to find a list of genes that are significantly regulated between two individual treatments. Table 1 shows the number of gene probe sets that were significantly regulated when each of the three treatments were compared to static control group pairwise. Figure 19 presents a Venn diagram that compares the number of probes sets that are shared between each of the three pairwise comparisons. As observed, stretch control samples had the lowest number while the stretch IFT88 samples had the highest number of genes that were significantly different compared to static controls.

Heat maps are the best tools to observe the overall effects of the treatments on the gene expressions of all aspects of cells, which range from the microscopic molecular and cellular functioning of the cells to the macroscopic cancers and diseases. Categories of genes that are up-regulated or down-regulated the most are represented with the darker colors (Orange for up-regulation and Blue for down-regulation). Throughout all of the comparisons, we found that cancer, tissue morphology, cell cycle, and cell survival are few of the categories in which genes are most significantly affected.

When the gene expressions collected from static IFT88 and stretch IFT88 treated samples were individually compared to the gene expressions observed in static controls samples, most genes associated with cancer and tissue morphology exhibited an up-regulation (Figure 20, 22). The cancer cells in the lungs have primary cilia whereas the normal epithelial lung cells do not. Unlike the lungs, the normal epithelial cells in the rest of the body show a presence of primary cilia while the corresponding cancer cells lack them. Cell cycle is also up-regulated while cell survival shows mixed results under stretch treated samples. Static treated samples resulted in
mixed findings for cell cycle and cell survival compared to static control samples. Stretch control samples are significantly down-regulated for cell survival and cancer associated genes while the genes in tissue morphology provided mixed results compared to static control (Figure 21).

Cancer, tissue morphology, and cell cycle are all up-regulated when stretch treated samples are compared to stretch control (Figure 23). Cell survival is found to be negatively regulated. Comparing the static treated samples to stretch treated samples, cancer, cell cycle, and tissue morphology are significantly up-regulated while cell cycle provided mixed results (Figure 24). In the remaining two comparisons, no significant changes are found in the four categories (Figure 25 – 26).

The bar charts show a list of all of the categories of genes that observed significant difference in their molecular and cellular functions by the different treatments. These are helpful to understand the genes that are involved in different signaling pathways that are significantly affected. Validating the observations made by previous studies as discussed above, genes associated with cell cycle and survival are affected the most and other pathways such as cellular development and cell proliferation are also greatly affected. Specifically, significantly regulated genes in the static control versus stretch control comparison were used to create a heatmap (Figure 27). It also compared the relative expressions for the same genes in the remaining four main comparisons, static control – static IFT88, static control – stretch IFT88, stretch control – stretch IFT88, and stretch IFT88 – static IFT88. Comparing static control – stretch control and static control – stretch IFT88, two important genes of interest were observed. Dual specificity protein phosphatase 10 (DUSP10) is up-regulated with increase in stretch and presence of primary cilia. However, in the presence of stretch condition, the gene expression was down-regulated with an inhibition in IFT88. Therefore, we can conclude that primary cilia might
play an important medium through which the mechanotransduction of DUSP10 signaling pathway takes place. Shi et. al. showed that DUSP10 regulated JNK and p38K MAPK signaling to decrease the muscle stem cell proliferation as well as increase muscular cell differentiation in mice. This improved the muscular regenerative myogenesis [94]. A similar study conducted by Campbell et. al showed DUSP10 as an important regulator in p38K signaling. DUSP10 is up-regulated after partial hepatectomy and inhibits the expression of p38K, which significantly increases the tissue regeneration in the liver [95]. Insulin-like growth factor-binding protein 3 (IGFBP3) was also regulated in our study. In the absence of primary cilia, IGFBP3 was up-regulated suggesting that its inverse relationship on the presence of primary cilia. Oh et. al. have shown that it significantly decreases migration and invasion in two NSCLC cell lines including A549. This further decreased the metastasis, improving the survival of human lung fibroblasts [96]. Similarly, IGFBP3 induces cell apoptosis by arresting G_1 – S transition and inhibiting cell cycle progression in 789-O, A549 and MCF-7 cell lines [97]. Several other genes are also related to signaling mechanisms in cell apoptosis, which can be further investigated to understand their specific functions.
Primary cilia are necessary for the hallmarks of tumor progression. Preliminary data suggest that human lung cancer biopsies show an increase in primary cilia compared to the normal surrounding tissue. This is further confirmed by specifically targeting IFT88 and inhibiting the formation of primary cilia using siRNA. Several 1-day and 2-day staining show that samples treated with siRNA IFT88 has significantly less primary cilia under static and 15% stretch conditions, therefore confirming that the gene IFT88 is successfully silenced and inhibited formation of primary cilia. The effects of the knockdown are compared to the controls under static and stretch conditions for several different factors of tumor progression for 24 and 48 hours.

Primary cilia are responsible for the arrest of G1 – S transition and severely inhibit cell cycle progression. In their absence, the cell survival rate is also significantly decreased in siRNA IFT88 samples for 24 and 48 hours timepoints. Studies have shown that absence of primary cilia results in ineffective cell adhesion and migration. Our study confirmed lower adhesion rate in treated samples under static conditions compared to the control samples after 2 hours. Similarly, cell migration is also negatively affected by the absence of cilia. Treated samples traveled almost 50 μm less than the control samples after 48 hours under stretch. These prove that cells with no primary cilia are less metastatic and have lower chances to spreading to other parts of the body.

Genetic expressions for some of the genes that play a role in Sonic Hedgehog pathway were analyzed after 24 and 48 hours. After 24 hours, SHh, Smo and Ptch are all down-regulated, whereas after 48 hours post-stretch, recover back to normal levels. Gli2 levels were significantly
up-regulated in the treated samples after 24 and 48 hours. These results suggest that Sonic Hedgehog pathway is necessary for the hallmarks of tumor progression. Microarray analysis also supported the above results. Genes that play important roles in cancer, tissue morphology, cell cycle, cell survival and several other important signaling pathways are greatly affected as a result of inhibition of primary cilia. Formation and maintenance of primary cilia are two of the top priorities and their inhibition can significantly reduce tumor progression in lung adenocarcinoma cells.
CHAPTER 6: FUTURE DIRECTIONS

Observing the presence of primary cilia in more human lung cancer biopsies will allow us to visualize if primary cilia are only present in lung cancer cells. We have recently received an IRB approval which will enable us to acquire higher number of human lung tissue biopsies. Confirming primary cilia presence in these biopsies, it will be important to create more in-vitro models to test for tumor progression in other lung adenocarcinoma cell lines. In-vivo models will also be able to provide more results and will be more closely associated with the functionality and the mechanical environment of living lungs. This project focused mainly on the gene silencing effects of siRNA in lung adenocarcinoma cells. It is also imperative to investigate that the siRNA treatment does not have any negative effects on the normal lung epithelial cells. The specificity of siRNA treatment to only cancer epithelial cells will ensure a great possible drug delivery and therapeutic target in the lungs. 1-day and 2-day are considered short-term timepoints. In order to find the long-term effects of siRNA, longer timepoints should be incorporated as a part of the study.

The gene array data acquired through the samples treated at 1-day will help learn about the thousands of genes that are affected by the different treatments and span across several hundred signaling pathways through the cells. It is a great resource for future studies in order to observe the gene regulation and target several other signaling pathways of interest. One specific pathway to observe would be cell apoptosis. Cells without primary cilia had low survival rates, thus proving the involvement of cell apoptosis. Caspase9 is a specific gene that is very important in cell apoptosis signaling pathway and could be potentially a great target to understand the role of cilia in cell death. Some other pathways including cellular development and tissue morphology will be very important in understanding the epithelial-to-mesenchymal transition
(EMT) in lung adenocarcinoma cells. More extensive study will be needed to understand the importance of primary cilia in the mechanotransduction of DUSP10 and IGFBP3 signaling pathways and their effect on cell proliferation, differentiation and survival. Lastly, understanding and using the best possible medium to deliver drugs to specific genes, drug therapeutics will not only allow researchers to clinically inhibit tumor progression, but also to successfully treat and cure cancer.
REFERENCES


69. Gradilone S a, Radtke BN, Bogert PS, Huang BQ, Gajdos GB, Larusso NF. HDAC6 Inhibition Restores Ciliary Expression and Decreases Tumor Growth. Cancer research. 2013 Mar 27;


SUPPLEMENTAL INFORMATION

RESULTS

Preliminary studies included treatment of ammonium sulfate as means to eliminate the formation of primary cilia. Samples with treated with 30 mM ammonium sulfate diluted into 0.1% FBS, 1% P/S DME/F-12 medium while the control samples did not get treated with ammonium sulfate. The asterisks above the graph denote the significance compared to the 1-day static control samples. Cells treated with ammonium sulfate proliferated significantly less compared to the 1-day static control. At 2-days, stretch controls are also found to be lower than static control. After 3-day and 4-day, static and stretch samples treated with ammonium sulfate were significantly lower than the static and stretch control samples for that timepoint (Figure 28).
Figure 28.
Cells without primary cilia proliferated significantly less from 2 – 4 days. Control samples proliferated more than samples with ammonium sulfate treatment. Static and stretch treated samples were significantly lower from 2 – 4 days compared to 1-day static control as well each individual timepoint.

To check whether the cells with primary cilia are proliferating towards the site of wound injury, migration assay is performed. Migration can also explain cell’s ability to metastasize. Cells with cilia have higher migratory rate than the treated samples. Ammonium sulfate decreased migration significantly after 1 day in static condition. They were even more significant after 2-days where static and stretch samples with ammonium sulfate travelled much less than the static and stretch control samples (Figure 29).
Figure 29. **Migration is significantly affected with the treatment of Ammonium Sulfate.** Treated samples under 1-day static condition migrated significantly less than static controls. 2-day post-stretch showed that static and stretch treated samples travelled much less towards the site of injury than the 2-day static and stretch controls. Data is normalized to static control and representative of mean of distance travelled +/- standard deviation, n=3 per group. *p < 0.05 and **p < 0.01, Two-way ANOVA with Tukey post-hoc test.

The role of primary cilia was confirmed through the proliferation and the migration rate of lung cancer cells. To investigate the signaling mechanism, the gene expressions for some of the genes in the Hedgehog mechanotransduction pathway were performed for samples after 48 hours (Figure 30). Down-regulation of IFT88 gene is shown to ensure successful knockdown of primary cilia in the ammonium sulfate treated samples as compared to the control samples. The gene expressions for SHh, Ptch and the transcription factor Gli1 are significantly decreased in the treated sample under stretch condition. Static treated samples were significantly decreased in Ptch and Gli1 genes. Stretch controls were found to be significantly up-regulated than the static controls for Smo gene.
Figure 30
Gene expressions of Hedgehog related genes after 48 hours. Successful knockdown of IFT88 gene in the treated samples showed significant down-regulation of Ptch and Gli1 genes under static and stretch conditions. The stretch treated samples also found decreased expression of Shh while stretch control was up-regulated for Smo compared to their respective static controls. Data is normalized to static control for each timepoint and representative of mean ±/− standard deviation, n= 3 per group. * p < 0.05, ** p < 0.01, *** p < 0.005 two-way ANOVA with Tukey post-hoc test.
DISCUSSION

Prior to the actual experimentation on primary cilia using siRNA, ammonium sulfate was used to test and collect preliminary results. Ammonium sulfate has been widely used for precipitation of proteins using hydrophobic interaction chromatography [98,99]. They have been shown as great dehydrating reagents enabling protein crystallization and precipitation [100]. They function as slightly acidic salts which cleave water molecules from proteins and crystallizing them [101]. Since the extracellular matrix and the surface of the cells consists of several important proteins including the primary cilia, addition of ammonium sulfate as a treatment leads to degradation of these proteins [98]. The proliferation rate of samples treated with 30 mM ammonium sulfate under static and stretch conditions upto 4 days conclude that these cells proliferate significantly less than the untreated control samples. Similarly, the 2-day migration rate and the gene expressions for sonic hedgehog signaling pathway are also significantly deregulated for the ammonium sulfate treated samples. These results do not necessarily emphasize the importance of primary cilia in the above hallmarks of cancer since ammonium sulfate is a very non-specific treatment for abolishing primary cilia. Therefore, it was used as only a preliminary step for the experiments using the gene-specific inhibition of IFT88, thus ensure the role in primary cilia in tumor progression.
List of Primers for Hedgehog signaling related genes

IFT88
Forward: GACCGAGAAAAAATGAAGAAGGC
Reverse: CCTTTTCACGTTCCATTTGCC

Sonic Hedgehog (SHh)
Forward: GCTTCGACTGGGTGTACTACG
Reverse: GCCACCGAGTTCTCTGCT

Patched (Ptch)
Forward: CCTCGAGACCAACGTGGAG
Reverse: GGGGTCTGTATCATGAGTTGAGG

Smoothened (Smo)
Forward: GCACTCCTGACCGCTTCC
Reverse: CTCTTGGGGTTGTCTGTCCG

Gli1
Forward: CGAGAAGCCACACAAAGTGC
Reverse: CACATGTATGGCTTCTCACCC

Gli2
Forward: CCTCCATCAATGCCACG
Reverse: GTTGACGGTGCTGCTGAC

18s (Control)
Forward: GCAATTATTCCCCATGAACG
Reverse: GGGACTTAATCAACGCAAGC

GAPDH (Control)
Forward: ACGTGTCAGTGGTGGAACCT
Reverse: GTCCACCACCCTGTTGCTG
APPENDIX A

Flow Cytometry Analysis data files

This data was collected using BD FACSCANTO II™, acquired by FACSDIVA software and analyzed using ModFit LT analysis software.
Figure 31. Cell cycle analysis for 1-day static control samples. Percent of number of cells observed in each of the phases. Data is analyzed using ModFit LT analysis software where $G_2/G_1 = 1.92$. 
Figure 32.  
Cell cycle analysis for 1-day static IFT88 samples.  Percent of number of cells observed in each of the phases. Data is analyzed using ModFit LT analysis software where $G_2/G_1 = 1.92$. 

82
Figure 33. Cell cycle analysis for 1-day stretch control samples. Percent of number of cells observed in each of the phases. Data is analyzed using ModFit LT analysis software where $G_2/G_1 = 1.92$.
Figure 34. Cell cycle analysis for 1-day stretch IFT88 samples. Percent of number of cells observed in each of the phases. Data is analyzed using ModFit LT analysis software where G2/G1 = 1.92
Figure 35. Cell cycle analysis for 2-day static control samples for 1-day. Percent of number of cells observed in each of the phases. Data is analyzed using ModFit LT analysis software where $G_2/G_1 = 1.92$
Figure 36.
Cell cycle analysis for 2-day static IFT88 samples. Percent of number of cells observed in each of the phases. Data is analyzed using ModFit LT analysis software where G2/G1 = 1.92.
Figure 37. 
Cell cycle analysis for 2-day stretch control samples. Percent of number of cells observed in each of the phases. Data is analyzed using ModFit LT analysis software where G2/G1 = 1.92
Figure 38. Cell cycle analysis for 2-day stretch IFT88 samples. Percent of number of cells observed in each of the phases. Data is analyzed using ModFit LT analysis software where $G_2/G_1 = 1.92$.
Sagar S. Patel was born on November 27, 1986 in Gujarat, India. In 2001, he moved to Springfield, Virginia at the age of fourteen. He graduated West Springfield High School in 2005. He graduated from Virginia Commonwealth University in 2009 with a Bachelor of Science degree in Biomedical Engineering with minors in Physics, Chemistry and Mathematical Sciences. During his time, he was the chair of philanthropy committee and community service for Sigma Beta Rho Fraternity, Inc. and was an active member in Biomedical Engineering Society and Indian Student Association. For his senior design project, he worked with a fellow partner to design and develop an artificial cost-effective simulation system that performed basic motions of the elbow joint. During his senior year, he also started working as a research assistant in the Department of Anatomy and Neurobiology, where he observed the effects of Traumatic Brain Injury on the brain activities of in-vivo rat models. He was named an author in a publication *Mechanisms of Dendritic spine Remodeling in a Rat Model of Traumatic Brain Injury* submitted in the Journal of NeuroTrauma in 2011 for his contribution in the project. Currently, he is a Master of Science candidate and works as a graduate research assistant in the Pulmonary Mechanobiology Laboratory in the Department of Biomedical Engineering.