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PRIMARY CILIA MECHANOTRANSDUCTION AND MICROTUBULE STABILITY IN
MECHANICALLY STRETCHED LUNG ADENOCARCINOMA CELLS

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

by

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Table of Contents

Acknowledgements.....	ii
List of Figures.....	vii
Chapter	
1 INTRODUCTION	1
Mechanical Environment of the lung.....	3
Microtubules.....	3
Primary Cilia	4
Hedgehog signaling mechanism.....	6
SMO Inhibitors.....	8
TGF- β and Cilia.....	8
Taxol Resistance.....	10
Primary cilia and proliferation, adhesion and migration.....	10
2 MATERIALS AND METHODS.....	14
Cell Culture	14
Preparation of siRNA	14
Cell Transfections.....	15
Cell Stretch.....	16
Cell Proliferation and Survival.....	17
Scratch Wound Healing Assay.....	18
RNA isolation and Real-time quantitative PCR.....	18
Immunofluorescence	19
Smoothened Inhibitors Treatment.....	19

	TGF- β 1 Treatment.....	20
	Taxol Resistant cell lines.....	20
	Statistical Analysis	20
3	RESULTS	21
	Quantification of Primary Cilia in lung cancer cell lines	21
	H460 Migration-Primary cilia play a role	26
	Smoothened Inhibitors	28
	TGF- β 1 induced Epithelial to Mesenchymal Transition.....	30
	Taxol Resistant A549luc cell line	31
4	DISCUSSION.....	34
	Lung cancer cell lines have increased primary cilia on stretch.....	34
	Primary cilia aid in increased cell migration on stretch.....	37
	Smoothened Inhibitors reduce lung cancer cell proliferation and migration	38
	TGF- β 1 causes an increase of IFT88 gene expression and IFT88 inhibition causes partial reversal of TGF- β 1 induced EMT.....	39
	Taxol resistant cell line.....	41
5	CONCLUSION.....	43
6	FUTURE DIRECTIONS.....	45
	References.....	47
	SUPPLEMENTAL STUDIES.....	53
	Investigating the role of integrins in the mechanism of primary cilia.....	53
	Investigating the role of SMO gene in the Smoothened inhibitor mechanism	54

Quantifying primary cilia on TGF- β 1 Treatment.....	54
pINDUCER cell line.....	55
List of Primers for RT-qPCR genes.....	59

List of Figures

Figure 1: Basic structure of a primary cilium	5
Figure 2: Schematic of the Sonic Hedgehog signaling pathway	7
Figure 3: Cell survival is decreased with stretch and inhibition of IFT88.....	11
Figure 4: Inhibition of IFT88 decreases cell adhesion and migration	12
Figure 5: FlexCell™ tension plus system.....	17
Figure 6: Stretch induces primary cilia formation	22
Figure 7: Stretch increases and IFT88 inhibition abolishes primary cilia	23
Figure 8: A549luc cells in static and 15% stretch conditions for 2 days staining	23
Figure 9: H460 cells in static and 15% stretch conditions for 2 days.....	25
Figure 10: SKLU1 cells in static and 15% stretch conditions for 2 days	25
Figure 11: H838 cells in static and 15% stretch conditions for 2 days.....	26
Figure 12: H838-spag6 cells in static and 15% conditions for 2 days	26
Figure 13: Decrease in H460 migration of IFT88 inhibited samples under 15% stretch in 48 hrs.....	27
Figure 14: H460 migration scratch assay.....	27
Figure 15: Cyclopamine reduces cell proliferation.....	29
Figure 16: Jervine affects cell migration	30
Figure 17: TGF-β1 increases IFT88 expression	30
Figure 18: IFT88 inhibition causes reversal of TGF-β1 induced Vimentin up-regulation and E-Cadherin down-regulation.....	31
Figure 19: 12 nM Taxol resistant cells static and 15% stretch for 2 days	32
Figure 20: Taxol resistance increases cell proliferation	32

Figure 21: Epithelial and Mesechymal markers expression levels in Taxol Resistant Vs A549luc cells.....	33
Figure 22: Integrin western blots	53
Figure 23: SMO expression	54
Figure 24: Quantification of primary cilia with TGF- β 1 treatment.....	55
Figure 25: A549luc cells +/- TGF- β 1 for 2 days	55
Figure 26: Map of pINDUCER.....	56
Figure 27: Clone D1 Control and Dox group	57
Figure 28: IFT88 expression in clone D1.	58

Abstract

PRIMARY CILIA MECHANOTRANSDUCTION AND MICROTUBULE STABILITY IN MECHANICALLY STRETCHED LUNG ADENOCARCINOMA CELLS

By Monika Rassi Radhika, B.E.

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, 2015

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

Assistant Professor, Biomedical Engineering

The objective of this study is to investigate the role of microtubule based organelle, the primary cilia in lung adenocarcinoma by i) Quantifying the presence of primary cilia in several Non Small Cell Lung Cancer (NSCLC) cell lines in response to mechanical stimuli, ii) Attempting to determine the role of primary cilia in cell migration, iii) Investigating the effects of Paclitaxel(Taxol) resistance in lung cancer cells, iv) Analyzing the response of lung cancer cells to Smoothened Inhibitors and v) Determining the effects of Transforming Growth Factor Beta-1(TGF- β 1) induced Epithelial to Mesenchymal Transition(EMT) in lung cancer cells. To ascertain the effects of primary cilia in the hall marks of tumor progression, several experiments involved prohibition of primary cilia formation by silencing IFT88, the gene responsible using small interfering RNA. Three out of the five cell lines tested, showed increased expression of primary cilia under mechanical stretch. IFT88 inhibition of H460 cells decreased their migration rate to the injury site under stretch conditions. Smoothened (SMO) Inhibitors decreased proliferation and migration rates in human lung adenocarcinoma cell lines (A549luc) similar to the effects observed in IFT88 silenced cells. IFT88 silenced A549luc cells showed a partial reversal of TGF-beta1 induced up-regulation of a mesenchymal marker. These results indicate that primary cilia play a role in the progression and metastasis of lung cancer by aiding the adhesion, proliferation, migration and EMT of lung cancer cells.

CHAPTER 1 – INTRODUCTION

Lung cancer is the leading cancer killer in the United States. An estimated 158, 040 are expected to die from lung cancer in 2015 accounting for 27% of all cancer deaths. It is the most common cancer world wide with a survival rate of just 17.8% [1]. There are three main types of lung cancer: Non Small Cell Lung Cancer (NSCLC) , Small Cell Lung Cancer (SCLC) and Lung carcenoid tumor. NSCLC is the most common type accounting for 85% of lung cancers and is very difficult to treat. They are classified into three subtypes: Adenocarcinoma, Squamous cell and Large cell carcinoma are subtypes of NSCLC [2]. Adenocarcinoma is the more common type of NSCLC accounting for roughly 50% of NSCLC and usually develops in the peripheral portion of the lungs. They form recognizable glandular patterns. This type of NSCLC is slow growing and can remain undetected for a long period due to the absence of the typical symptoms of lung cancer such as chronic cough. This type is often found in non-smokers and is the most common type to afflict women. Adenocarcinoma is on the increase in recent years [3,4]. Squamous cell carcinoma usually starts in the tissue lining air passages in the lungs mostly located centrally in the bronchi. Smoking is known to be the major cause of this type of cancer. This type is very slow growing and can take years to become invasive. The incidence of this type has been on the decrease in recent years [3,5]. Large cell carcinoma occur in the outer edges of the lungs and tend to grow rapidly. These account for only 10% of NSLC. Since they develop in the outer regions of the lungs, they can cause fluid to develop between tissues and invade the chest wall causing pain [3,6]. The second category of lung cancer is small cell lung cancer also called oat cell cancer comprises 10-15% of lung cancers. This type mostly arises

within the lung and tends to spread very quickly. The last category lung carcinoid tumour comprises only less than 5% of lung cancers. Most of these tumors grow slowly and rarely spread [2]. Lung cancer cells are cultured from tumors of biopsy samples isolated from lung cancer patients. Although all major histological types of NSCLC could be cultured, assay conditions favored adenocarcinoma because of identified medium for their culture. After questions of tumor cells retaining their differentiated properties during cell culture arose, it was shown that true tumor cells retained their differentiated properties [7]. Hence cultured tumor cells accurately represent tumor cells in vivo with out the complex in vivo environment. In addition cell lines are capable of infinite replication and can be dispersed to laboratories worldwide allowing researchers to directly compare their results from identical studies [7]. A549 adenocarcinoma cell line was initiated by D.J. Giard in 1972 through explant culture of lung carcinomatous tissue from a 58 yr old Caucasian male. SKLU1 adenocarcinoma cell line was harvested from a 60 yr old Caucasian female. H460 cell line was derived by A.F. Gazdar in 1982 from a patient with large cell lung cancer. H838 cell line was established in 1984 from 59 yr old Caucasian male adenocarcinoma patient who was a smoker. In this study, A549luc cells were primarily used. These are a luciferase expressing cell line derived from A549 cells by stable transfection of the North American Firefly Luciferase gene to facilitate future in-vivo use in experimental tumor models. The H838-spag6 cell line used in this study was derived from H838 cell line incorporated with the Sperm Associated Antigen protein encoded by the spag 6 gene in humans obtained from Dr. Zhibin Zang.

The ability of cancer cells to metastasize and form tumors is partly dependent on the physical interactions and mechanical forces between cancer cells and their microenvironment. Interaction between cancer cells and the Extra Cellular Matrix (ECM) during migration, the elastic

deformations cancer cells undergo to penetrate cell-cell junctions during invasion, velocity and adhesion and shear forces cancer cells are subjected to in the vascular system to bind to tumor sites, and other factors in the microenvironment such as ECM thickness and pore size, all play a crucial role in the spread of cancer. A clearer understanding of the role of physical interactions and mechanical forces will provide new insights into cancer progression and new therapeutic approaches [41].

Mechanical Environment of the lung

The lung is subjected to several complex physical forces including breathing, blood flow and surface tension. The airways exhibit shear forces as air flows into the lungs and epithelial cells experience both tensile and compressive forces during inhalation and exhalation [8]. These mechanical forces also influence the cellular processes in the lung. Normal respiration is known to cause 5-40% change in surface area in the lung [10]. Mechanical forces play a critical role in lung tissue under pathological conditions. Lung cells are often subject to non-physiological stretching as a result of disease or injury [8]. Lung cancer mostly co-exists with other lung disorders such as bronchitis, emphysema, asthma, lung fibrosis, lung infections. These conditions cause significant changes to the structure of the lung and the extracellular matrix. Hence cancer cells experience more bilateral tensile stresses and shear strains than healthy lung cells [9].

Microtubules

Microtubules which are key components of the cytoskeleton are dynamic polymers of alpha/beta tubulins that form a well organized network of polarized tube filaments in eukaryotic cells. Regulation of microtubule dynamics is crucial for mitosis, cell migration, cell signaling and trafficking. Polymerization of microtubules occurs by a nucleation/elongation mechanism in

which the formation of a short microtubule nucleus is followed by elongation of the microtubule at its ends. The process of dynamic instability is one in which individual microtubules exist either in an elongation state or a rapidly shortening state with abrupt random transitions between these states. Cell proliferation and migration in cancer cells are controlled by microtubule dynamics. The critical role played by microtubules in cell division makes them a suitable target for the development of chemotherapeutic drugs against rapidly dividing cancer cells [11]. One type of post polymerization modification microtubules undergo is acetylation, the process is still not clearly understood but has been shown to increase microtubular stability. Acetylated α -tubulin is present in microtubules, that under depolymerizing conditions are more stable. Acetylation occurs after the assembly of tubulin and deacetylation occurs before their disassembly [42]. It has been demonstrated that cyclic stretch also causes this acetylation of microtubules which makes them more stable against mechanical forces [12]. Taxol which is a microtubule stabilizer is also known to cause acetylation of microtubules [42].

Primary cilia

Cilia are membrane bound microtubular projections emanating from the cell surface [13]. Primary cilia are immotile cilia and are found as solitary organelles. Research over the past decade has established that they transduce signaling information from the extra cellular matrix [13]. In normal cells, cilia are dynamically regulated during cell cycle progression present in G0 and G1 and G2 cells but invariably reabsorbed before mitotic entry to re-appear post cytokinesis, causing periodic resorption and reassembly of cilia. Defects in ciliary integrity cause a number of developmental disorders. The structure of a primary cilium is shown in Fig 1. The primary cilium consists of a scaffold comprised of microtubule triplets (9 triplets in a

hollow ring). The ciliary axoneme is anchored by the basal body near the cell surface. The axoneme consists of doublet microtubules [14].

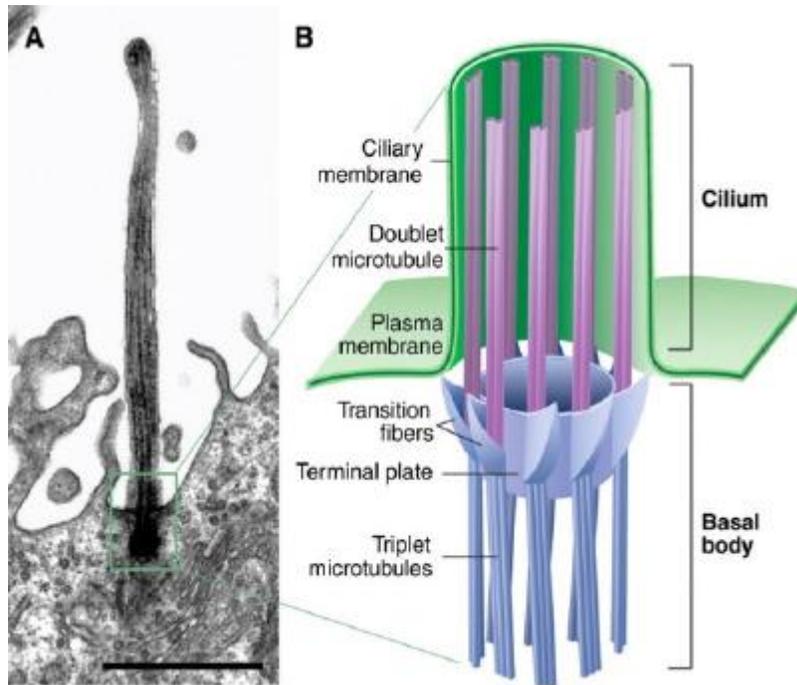


Fig 1. The structure of a primary cilium [43]

Intraflagellar transport (IFT) is the cellular process responsible for the formation and maintenance of cilia. The IFT protein complexes move along the axonemal doublet microtubules from the base to the tip of the organelle and returning. The rate of transport of building materials from the tip governs the length of the primary cilium. In mammalian cells, IFT88 is required for the formation of primary cilia. It also serves as a protein regulating G1-S transition in the cell cycle [14].

The ciliary axoneme is normally composed of polymers α and β tubulins. Primary cilia is comprised of α and/or γ tubulins which are found mostly only in specific microtubules such as centrosomes [16].

In 1997, mechanical properties of cultured renal epithelial cells were measured, and it was proposed that the primary cilia present in the cells were mechanically sensitive and that they could act as flow sensors. Further studies showed that MDCK cells respond to the bending of primary cilia by increasing intracellular calcium. Defects in primary cilia structure or function cause abnormalities like polycystic kidney disease. Kidney specific deletion of kif3a coding a subunit of kinesin protein results in loss of cilia and cyst formation in mice [15, 17]. Loss of IFT proteins resulted in the degeneration of the photoreceptor layer in zebra fish indicating that primary cilia serve as sensors to photoreceptors to sense their external environment [22].

Primary cilia have PC2 (TRP Ca²⁺ ion-gated channel) which detects fluctuations in intra-cellular calcium levels which can be caused when cells are subjected to stretch, localized on the surface [52].

These findings established the signal transduction properties of primary cilia and that primary cilia are a mechanism through which cells respond to their mechanical environment.

Primary cilia are known to be down regulated in renal carcinoma, breast and pancreatic cancer when compared to their surrounding normal tissue [18,19,20]. The presence and role of primary cilia in lung cancerous epithelial cells in comparison to their surrounding normal cells have not been studied to date. Manipulation of IFT88 will shed light on the role it plays on the signaling cascade of cancer cells. Stable transfection of IFT88 using small interfering RNA (siRNA) will render the cells unable to generate cilia [21]. In this study, double stranded sequences of siRNA of 21 base pair length replacing the initial part of the sequence of a targeted gene with complimentary forward and reverse primers was used.

Hedgehog signaling mechanism

Hedgehog signaling plays a critical role in the homeostasis of diverse adult tissues. Deregulated HH signaling has been implicated with several human cancers such as breast, pancreas, digestive tract, lung and kidney. Mammals express three HH family members Sonic hedgehog (SHH), Desert Hedgehog (DHH) and Indian hedgehog (IHH) [23]. Shh is the most broadly expressed gene. Fig 2 is a diagrammatic representation of the Shh pathway. Secreted Shh binds to the receptors Patched (Ptch) present in the cytoplasmic membrane of the receiving cell. Binding of Shh to Ptch releases the repression exerted by Ptch, on Smoothened (SMO) a G-protein coupled receptor essential for the transduction of hh signaling. Smoothened facilitates the interaction of several hh downstream effectors in primary cilia resulting in the activation of transcription factors Gli (Gli1, Gli2, Gli3) primarily Gli2A that transcribe into the nucleus [24]. The primary cilium is necessary for hh signaling in most cells. In the absence of HH ligand, Ptch1 localizes to the cilium and blocks hh signaling by preventing SMO entry. Gli3 undergoes processing to its repressor state Gli3R which dissociates from the SUFU gene and translocates into the nucleus [25].

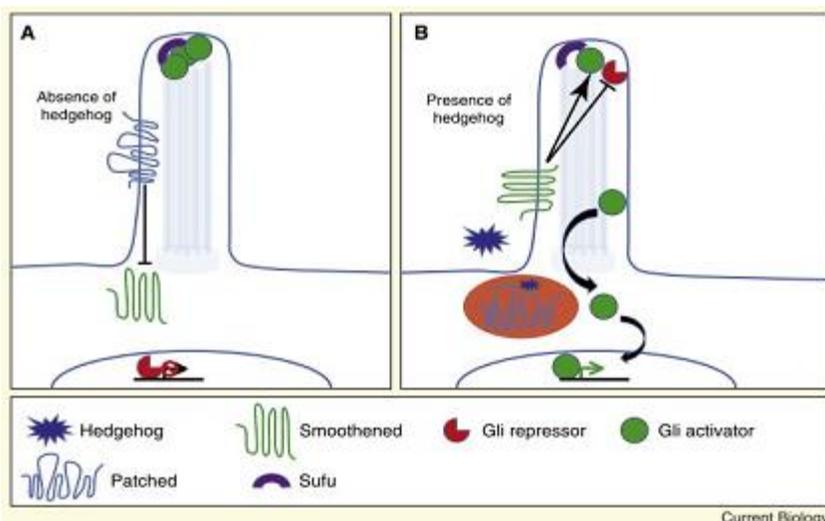


Fig 2. A schematic of the hh signaling pathway [26]

A study conducted on adult articular chondrocytes demonstrated for the first time that mechanical loading activates primary cilia mediated HH signaling in the cells but that this response is lost at high strains due to cilia disassembly [27]. Many NSCLC cell lines express Gli1 consistent with HH activation establishing the involvement of HH signaling pathway [28].

SMO Inhibitors

As SMO is known to be essential for transduction of HH signaling, various SMO antagonists have been used to demonstrate the HH signaling requirement of cancer cells. SMO antagonists at the right concentrations have been shown to inhibit the proliferation of HH producing cancer cells. However the specificity of the SMO antagonists has been under debate and a proper association between expression of the HH target gene GLI1 and sensitivity of a number of cancer cell lines to these antagonists has not been established [23]. One of the first SMO antagonists identified was the plant derived teratogen called Cyclopamine. Cyclopamine functions as an HH antagonist by binding and inactivating SMO function in the HH signaling pathway. It has been estimated that 25% of all human tumors require HH signaling to maintain tumor cell viability [28]. A549 adenocarcinoma cells when treated with Cyclopamine have shown a significant decrease in cell number especially at longer time points and cell survival especially at increasing doses of the inhibitor. The specificity of Cyclopamine was confirmed by SMO silencing which also affected the proliferation and viability of A549 and H520 cancer cells [24]. Jervine, another plant steroidal alkaloid similar to Cyclopamine also disrupts HH signaling by affecting SMO function similar to Cyclopamine. However, the complete mechanism of Cyclopamine and Jervine has not been fully resolved yet [29, 30].

TGF-Beta-1 and cilia

Epithelial to Mesenchymal Transition (EMT) plays a role in the early stages of metastasis of cancer cells. EMT is a process during which cells undergo a morphological switch from the epithelial polarized phenotype to a highly motile fibroblastic or mesenchymal phenotype. In the EMT process, epithelial cells lose their features, gain mesenchymal properties, and become motile and invasive. Transforming Growth Factor-Beta1 (TGF- β 1) is a known inducer of EMT. Tumor cells lose their ability to be growth arrested by TGF- β 1, undergoing EMT leading to increasing invasiveness and metastasis [33]. EMT phenotype has been associated with poor clinical outcome in several cancers especially NSCLC. It has been established that A549 cells undergo EMT phenotypic changes upon exposure to TGF- β 1. A549 cells treated with TGF- β 1 acquire EMT phenotype and show an increase in cell migration and invasion. TGF- β 1 treated A549 cells showed a dramatic increase in HH pathway ligand Shh both at the mRNA and protein levels where as parental A549 cells showed undetectable levels of Shh mRNA. A similar rise in Shh levels were seen in another NSCLC cell line H2030 when treated with TGF- β 1. These results suggest that TGF- β 1 induced EMT is mediated by transcriptional activation of Shh. It was found that knock-down of Shh significantly attenuated TGF- β 1 induced EMT [34]. Therefore Shh up regulation with TGF- β 1 is mechanistically linked with TGF- β 1 induced EMT in NSCLC. In addition, it was found that down-regulation or inhibition of Shh signaling causes an inhibition in cell migration, invasion and tumorigenic potential of TGF- β 1 treated A549 cells, implying that increase in Shh signaling in those cells could be responsible for their increase in migration and invasion. These results have been consistent with other NSCLC cell lines H1299 and H1650 subjected to the same conditions [34]. A study reported that TGF- β 1 signaling was found to be reduced in a fibroblast cell line with stunted primary cilia [35]. Clathrin dependent endocytosis (CDE) which is a regulator of TGF- β 1 signaling is found in the

pocket region of the primary cilium. These indicate that primary cilia regulate TGF- β 1 signaling and that the ciliary pocket is a compartment for CDE signal transduction. IFT88 was also shown to be regulated with regulation of CDE [35]. TGF- β 1 modulates the key components of HH pathway [36].

Taxol Resistance

Taxol is a valuable chemotherapeutic drug used for treatment of many types of cancer. Taxol's principle targets in cells are the spindle microtubules, it binds reversibly to tubulin along the surface of microtubules. One form of microtubule dynamics that is highly sensitive to Taxol is dynamic instability. Dynamic instability appears to be essential for progression through mitosis into anaphase. Taxol is an anti-mitotic drug that induces microtubule assembly and the formation of microtubule bundles [37,38]. Taxol's major effects on dynamic instability are to reduce the rate and extent of micro tubule shortening. Subtle suppression of microtubule dynamics by Taxol strongly inhibits the assembly and formation of the mitotic spindle, thereby preventing or slowing down cell cycle progression at the metaphase/anaphase checkpoint. Despite Taxol's success as an anti-tumor agent, cells usually become resistant to the drug over time thus severely limiting its long term effectiveness. Drug resistant cancer lines and human tumor tissues have shown to harbor tubulin gene mutations, alterations in total tubulin content, altered microtubule polymer levels and altered microtubule associated protein expressions. All of these modifications in tubulin could contribute to the resistance to Taxol [39]. The effects of subjecting Taxol resistant cells to mechanical stretch have not been studied so far.

Primary Cilia and Proliferation, Adhesion and Migration

Previous work on this project by Sagar Patel showed that primary cilia aid in the adhesion, proliferation, invasion and migration of A549 cells.

Loss of primary cilia negatively affects cell survival

We found that gene silencing of IFT88 negatively affected the cell's ability to survive (Figure 3). 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.

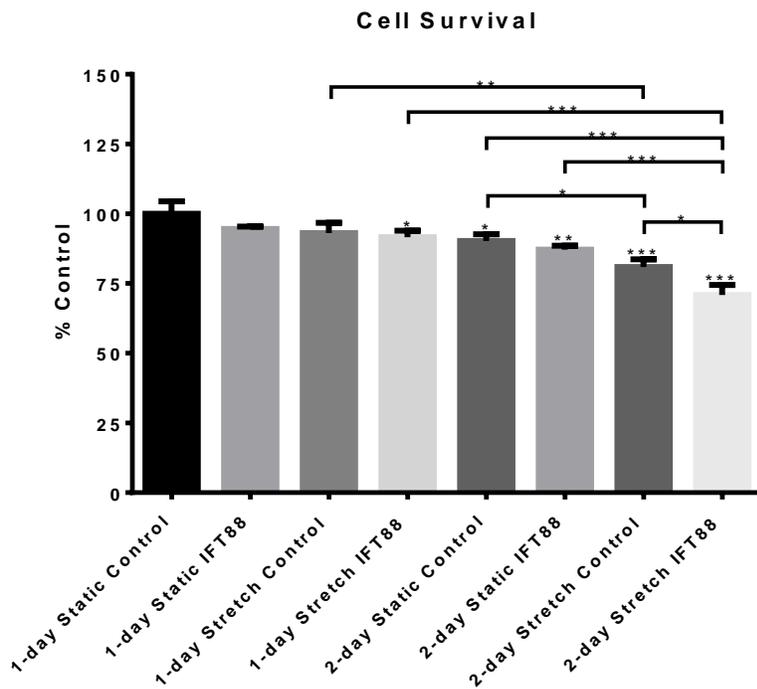


FIGURE 3.

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 4A) 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 result in a decrease in the migration rate of the cells. As shown in Figure 4B, cells without primary cilia showed significantly decreased migratory capabilities 48 hours after stretch.

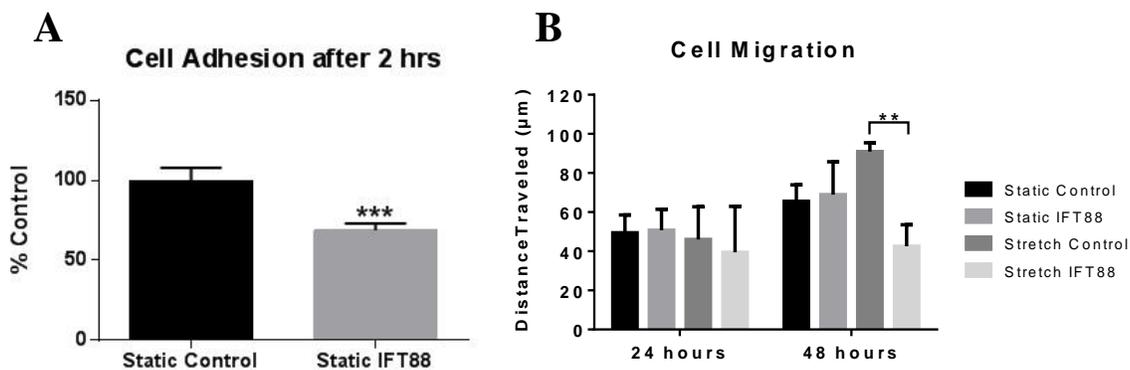


FIGURE 4.

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.

Previous work on this project has shown that primary cilia aid in cancer cell adhesion, proliferation and migration. We attempted to investigate the effects of lung cancer cells with/without primary cilia inhibition on the properties of tumor progression like proliferation, migration and EMT of those cells in response to stretch and other treatment. The following objectives will aid in the understanding of the significance of primary cilia in tumor progression in lung adenocarcinoma by quantifying their presence, analyzing their response to mechanical stretch and investigating their response to Smoothed inhibitors, chemotherapeutics and Epithelial to Mesenchymal Transition inducers.

Objectives

1. Quantify the presence of primary cilia in NSCLC cell lines
2. Investigate the role of mechanical stretch and IFT88 silencing in H460 cell line with a scratch wound healing migration assay
3. Observe the response of primary cilia in the cell proliferation and migration of A549 cells to Smoothed inhibitors
4. Observe the response of A549luc cells to EMT inducer TGF- β 1
5. Primary cilia quantification, cell proliferation and EMT of a Paclitaxel (Taxol) Resistant A549luc cell line in comparison to regular A549luc cells.
6. Supplemental studies

CHAPTER 2: MATERIALS AND METHODS

Cell Culture

Human lung adenocarcinoma cell line, A549(ATCC[®] CCL-185[™]), A549luc (ATCC[®] CCL-185[™]), SKLU1 (ATCC[®] HTB57[™]) (ATCC, Manassas, VA), H460, H838, H838-spag6 obtained from Dr. Zhibing Zang, Department of Obstetrics and Gynecology, VCU were used in this study. Unless otherwise noted, cells were cultured using Life Technology's RPMI 1640 medium (Invitrogen) 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 RPMI 1640 media supplemented with 10% FBS only. Custom siRNA sense and antisense oligonucleotides corresponding to the cDNA sequences of IFT88 and scrambled control were ordered from Dharmacon (Dharmacon, Lafayette, CO).

IFT88: CCGAAGCACUUAACACUUA | Control: GTGCGCTGCTGGTGCCAAC

Following manufacturer's protocol, 5x siRNA Buffer (Thermo Scientific, Lafayette, CO) was diluted to 1x concentration using sterile RNase-free water. A final concentration of 20 μM was used for siRNA transfections.

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, cells were suspended in 2 mL of RPMI-1640 media supplemented 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 RPMI 1640 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, RPMI-1640 media.

PEI Transfection

6-well amino treated BioFlex culture plates (Flexcell International Corp., Hillsborough, NC) were coated with Type I Collagen solution (Sigma-Aldrich, St. Louis, MO) (250 µg/mL) 2 hours prior to plating cells. A549LUC cells suspended in 2 mL of RPMI 1640 media supplemented with 10% FBS were used to plate 9×10^5 cells per well 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 Transfection Solution

PEI solution: Linear PEI (Polysciences, PA) 250 kDA was dissolved in ultrapure water at a concentration of 1mg/ml at room temperature at a pH of 3. The final pH was brought to 8. Prior to transfection, media from each of the wells was aspirated; cells were washed with serum free Hybridoma medium (Life Technologies, Grand Island, NY) and then suspended in 2 ml of Hybridoma per well. RNAi solutions +/- IFT88 (final concentration of 176 nM) were incubated with PEI solution and Hybridoma media for 10 mins. The mix was then added to the wells bringing the total volume to be 3.4 ml per well and incubated overnight. The next day, prior to stretch, the solutions were aspirated and the cells were suspended in RPMI 1640 media with 0.1% FBS, 1% PS.

Cell Stretch

Cells are plated in 6-well BioFlex culture plates and allowed to stretch at physiologically relevant stretch levels in the lung of 15% change in membrane surface area at 0.86 Hz for 48 hours. As shown in Figure 3, 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.

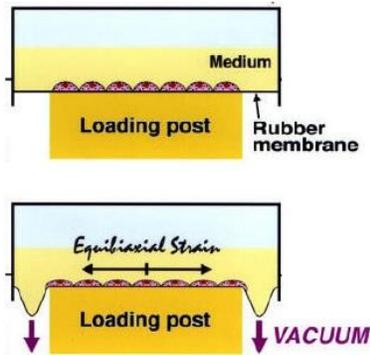


Figure 5: FlexCell™ tension plus system. www.flexcellint.com. Adenocarcinoma cell lines will be plated on collagen type I coated silicone membranes and cyclically stretched at 15% change in surface area.

Cell Proliferation

Cell proliferation and viability were measured using the MTT (Roche, Indianapolis, IN)

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 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

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.

RNA isolation and Real time quantitative PCR

RNA was collected and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). 600 μL 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. 30 μL 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 10 μL of nuclease-free DEPC -treated water. 4.2 μL of DEPC-treated water, 2 μL of 10x RT Buffer, 2 μL of 10x Random Primers, 0.8 μL of 25x dNTP (100 mM) and 1 μL of MultiScribe Reverse Transcriptase reagents were used to make 10 μL 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). qPCR was analyzed for $\Delta\Delta Cq$ values. GAPDH or 18s were used as controls.

Immunofluorescence

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

Primary antibodies:

Mouse Anti-acetylated tubulin: Sigma T7451 1:2000 (Sigma-Aldrich, St. Louis, MO).

Rabbit ARL13B 1:100 (Proteintech, Chicago, IL).

Secondary Antibodies:

Alexa Fluor 594 chicken anti-mouse IgG 1:800 (Life Technologies, Grand Island, NY).

FIT-C goat anti-rabbit IgG 1:200 (Proteintech, Chicago, IL).

Alexa Fluor 488 goat anti-mouse IgG 1:800 (Life Technologies, Grand Island, NY).

They were mounted on slides using Prolong gold anti-fade reagent with DAPI (Life Technologies, Grand Island, NY). Images were taken using scanning confocal microscopes (Zeiss LSM 700) at 40x magnification

Smoothed Inhibitor Treatment

Cells were treated with Cyclopamine hydrate - C4116 (Sigma-Aldrich, St. Louis, MO) of 5 μ M concentration or Jervine - J4145 (Sigma-Aldrich, St. Louis, MO) of 2.5 μ M concentration in

RPMI 1640 medium with 0.5% Dimethyl Sulfoxide, 0.5% Fetal Bovine Serum and 1% Penicillin Streptomycin.

TGF- β 1 Treatment

Cells were treated with Recombinant Human TGF- β 1 - 240-B-002 (R&D Systems, Minneapolis, MN) of 3ng/ml concentration in RPMI 1640 medium with 0.1% Fetal Bovine Serum and 1% Penicillin Streptomycin.

Taxol Resistant cell lines

A549luc cells were treated with increasing concentrations of Paclitaxel (Taxol) - T7191 (Sigma-Aldrich, St. Louis, MO) over a period of time. Starting at 1.5 nM, the concentrations were increased in multiples of two. 12 nM and 24 nM were the final concentrations and the cell lines developed resistance to these final concentrations of Taxol. 12 nM concentration cell line was used for experiments in this study.

Statistical Analysis

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

CHAPTER 3: RESULTS

Quantification of primary cilia in lung cancer cell lines

Lung cancer cell lines A549luc, SKLU1, H460, H838, H838-spag6 were used for this in-vitro immunofluorescence staining. The cells, under static and 15% cyclic stretch conditions for 2 days. Preliminary staining was done with DAPI and acetylated α -tubulin. For the cell lines that showed an increase of primary cilia on stretch, the experiment was repeated and cells were stained with DAPI, acetylated α -tubulin and ARL13B which is a protein that is localized in the cilia and plays a role in cilia formation and maintenance. This double staining is for confirmation of the structure to be a primary cilium and to differentiate cilia from α -acetylated tubulin. The presence of double stained acetylated α -tubulin and ARL13B structures were found to be increased in the stretch samples of A549luc and H460 cells when compared to their static controls. SKLU1, H838 and H838-spag6 cell lines did not show a significant difference in acetylated α -tubulin/acetylated α -tubulin and ARL13B between their static and stretch samples (Fig 6).

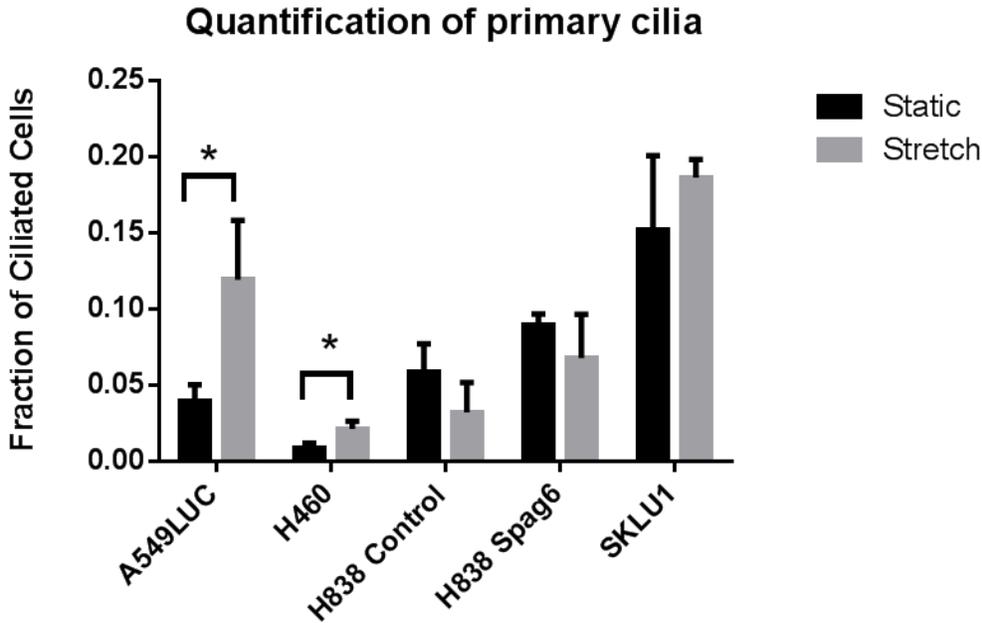


Fig 6. Stretch induces primary cilia formation. A549luc and H460 cells show a significant increase in primary cilia on stretch in comparison to the static controls in 2 days. Data is representative of mean +/- standard deviation, n= 3 per group. * p < 0.05 multiple t-tests using Holm-Sidak method.

Primary cilia inhibition on static and stretch conditions on A549luc cells

A549luc cells were stained for DAPI and acetylated α -tubulin and ARL13B. To inhibit primary cilia, IFT88 gene was silenced using siRNA. Controls and IFT88 inhibited samples were subjected to static or 15% cyclic stretch conditions for 2 days. Control samples on stretch showed a significant increase in primary cilia when compared to the static controls. IFT88 inhibited samples on stretch showed a significant decrease in primary cilia when compared to the stretch controls (Fig 7). The images show the staining for α -acetylated tubulin and ARL13B which confirms the presence of cilia under the four groups of static control, static IFT88, stretch control and stretch IFT88 (Fig 8A, 8B, 8C and 8D). Fig 8E is a zoomed image of a cluster of cells with α -acetylated tubulins and primary cilia.

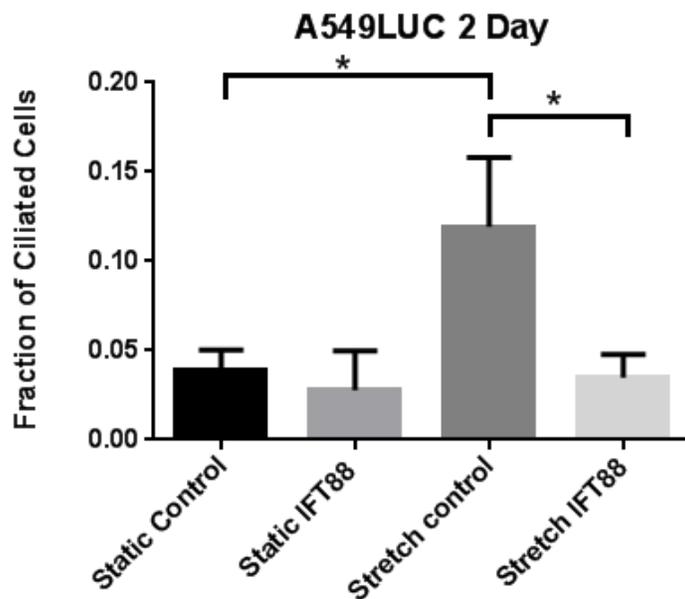
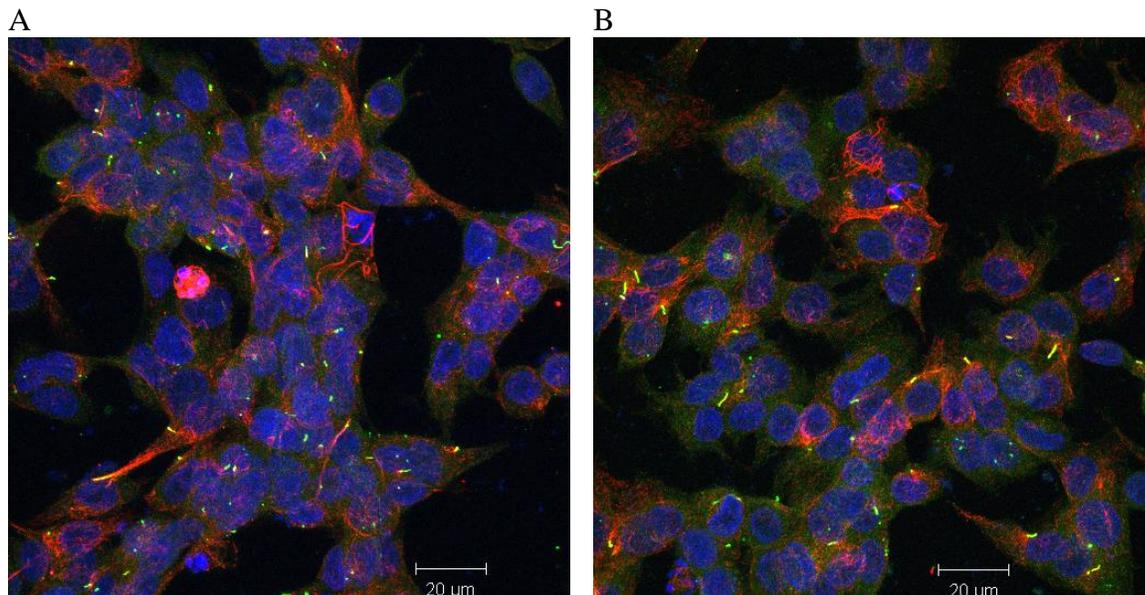


Fig 7. Stretch increases and IFT88 inhibition abolishes primary cilia. Control samples under stretch show a significant increase in primary cilia compared to the static controls where as IFT88 inhibited samples under stretch show a significant decrease in primary cilia compared to the stretch controls. Data is representative of mean \pm standard deviation, $n=3$ per group. * $p < 0.05$ One way Anova with multiple comparisons.



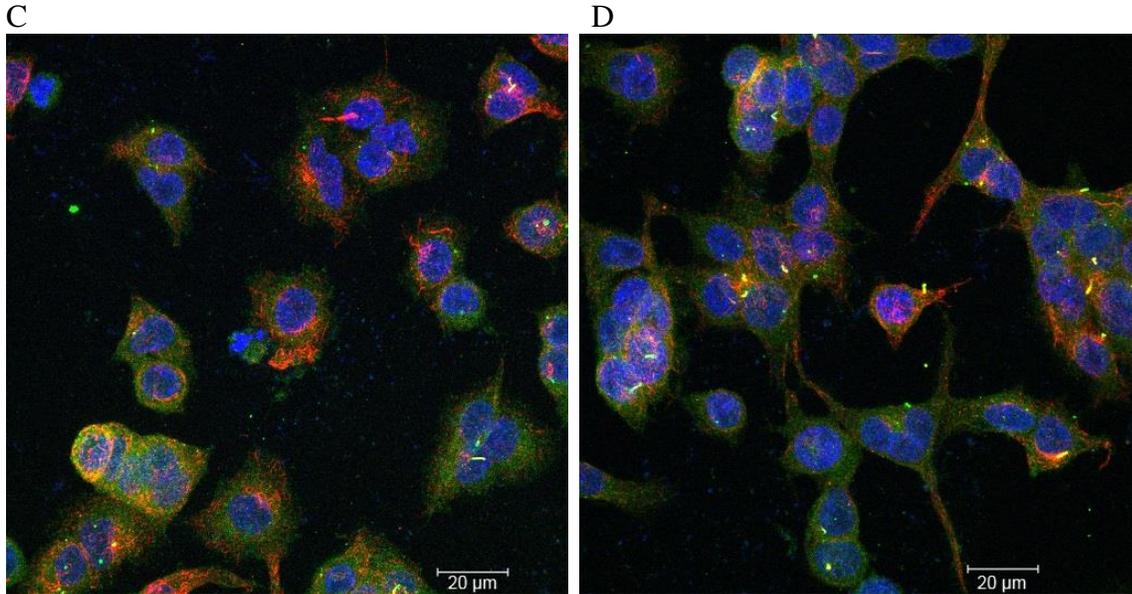


Fig 8. A549luc cells in static and 15% stretch conditions for 2 days were stained with DAPI(blue), α -acetylated tubulin(red) and ARL13B(green). A - Static Control, B- Stretch Control, C-Static IFT88, D- Stretch IFT88. A&B. Control samples under stretch show a significant increase in primary cilia than the static controls. B&D. IFT88 inhibited samples show a significant decrease in primary cilia than the stretch controls.

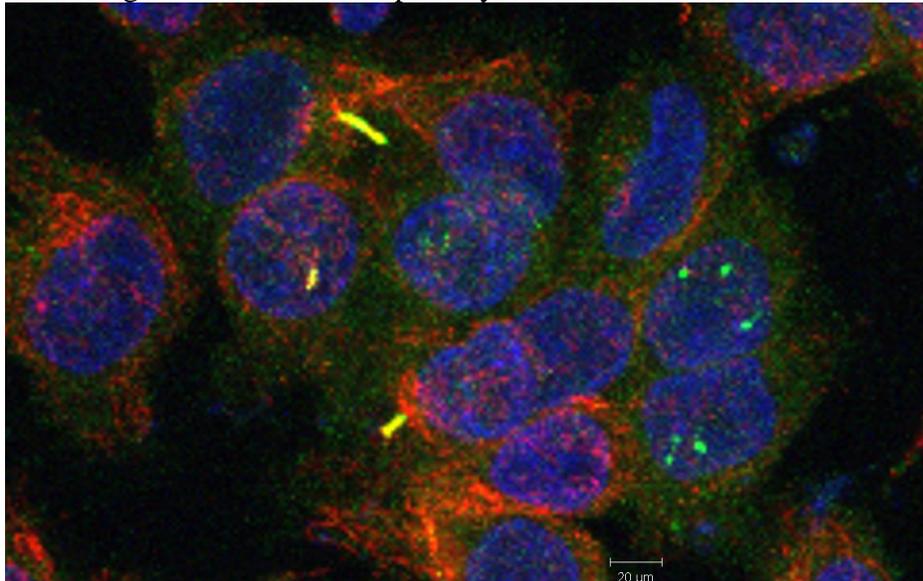


Fig 8E. Zoomed image of a cluster of A549luc cells stained with DAPI(blue), α -acetylated tubulin(red) and ARL13B(green). The double staining of α -acetylated tubulin and ARL13B confirms a structure to be a primary cilium. Typically, this double staining in these images would be yellow indicating the yellow structures to be primary cilia.

The images below show staining with DAPI and α -acetylated tubulin/ α -acetylated tubulin and ARL13B in H460, SKLU1, H838 and H838 spag6 cell lines

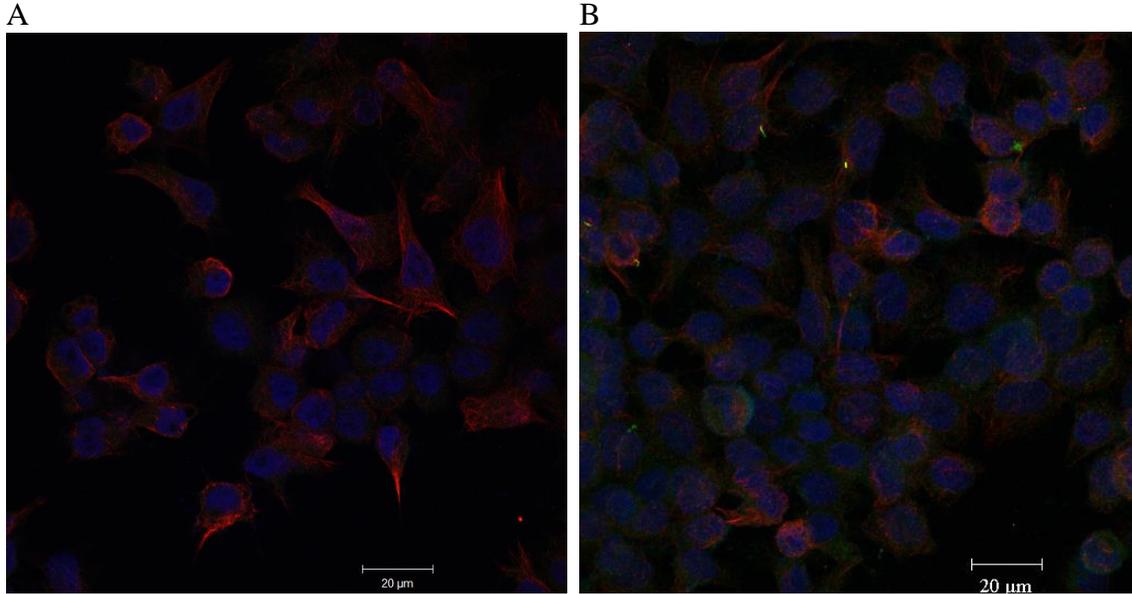


Fig 9. H460 cells under static and 15% cyclic stretch for 2 days stained with DAPI, α -acetylated tubulin and ARL13B. A- Static, B- Stretch. The stretch samples show a significant increase in primary cilia than the static controls.

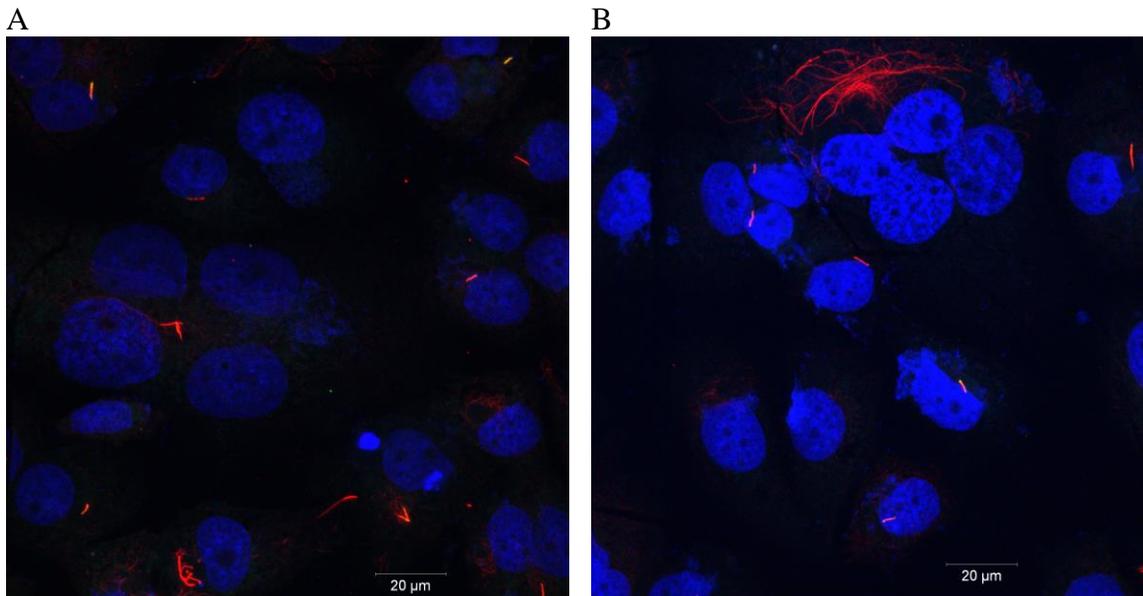


Fig 10. SKLU1 cells under static and 15% cyclic stretch for 2 days stained with DAPI, α -acetylated tubulin and ARL13B. A- Static, B- Stretch. The increase in primary cilia in the stretch samples was not significant compared to the static controls.

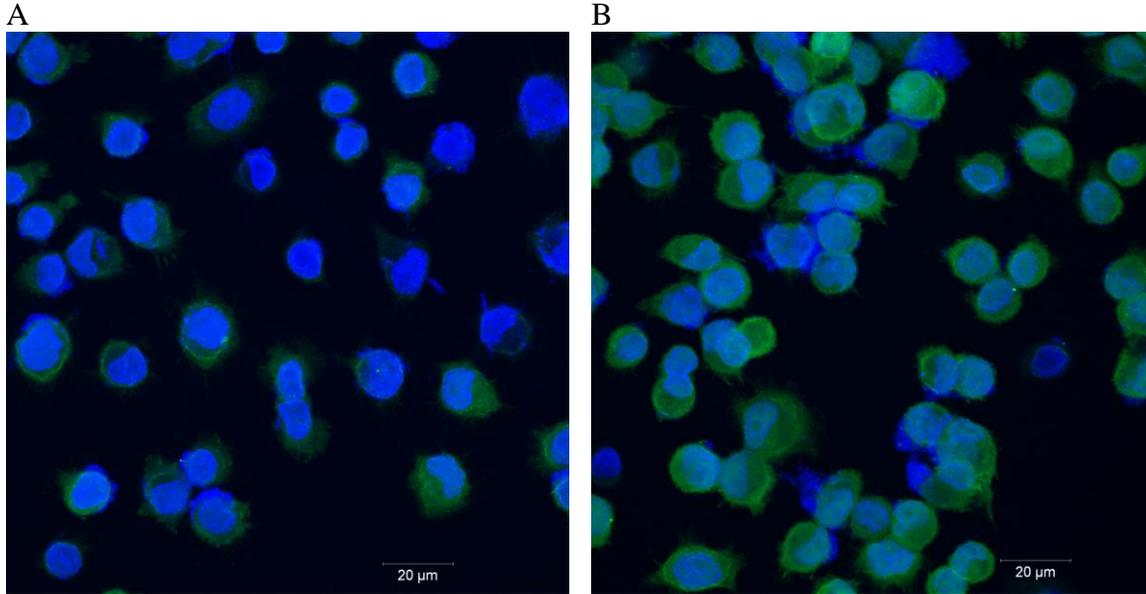


Fig 11. H838 cells under static and 15% cyclic stretch for 2 days stained with DAPI(blue) and α -acetylated tubulin(green). A- Static, B- Stretch. Stretch samples did not show an increase in primary cilia when compared to the static controls.

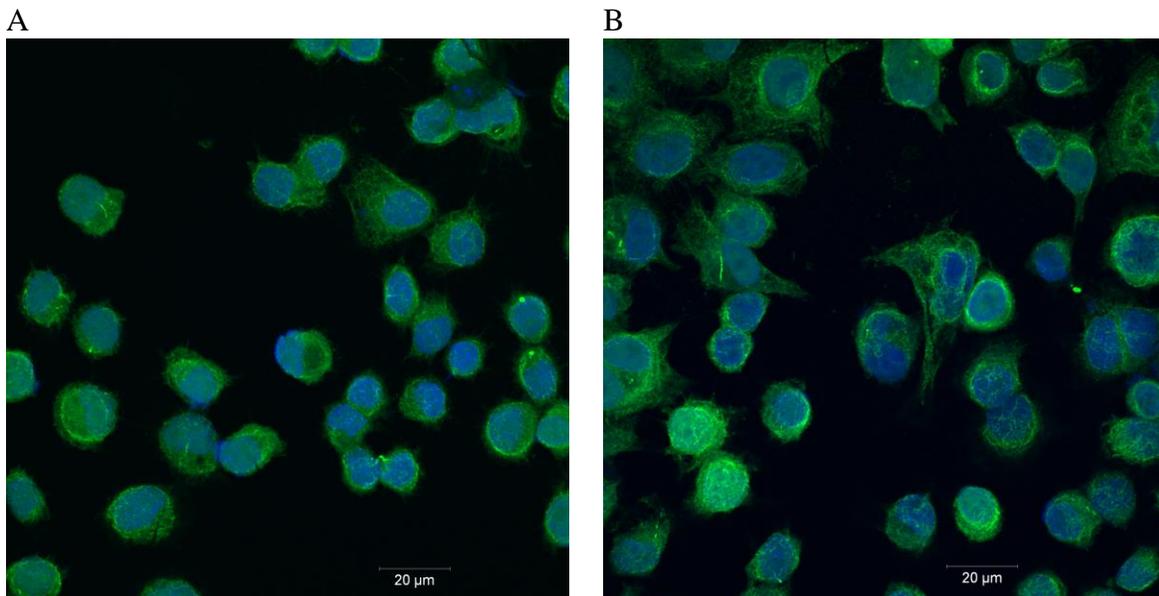


Fig 12. H838-spag6 cells under static and 15% cyclic stretch for 2 days were stained with DAPI(blue) and α -acetylated tubulin(green). A- Static, B- Stretch. Stretch samples did not show an increase in primary cilia compared to the static controls.

H460 migration - Primary cilia play a role

H460 cells on a scratch wound healing migration assay on static and 15% cyclic stretch for 24 and 48 hrs showed that the IFT88 inhibited samples on stretch showed a considerable decrease

in migration towards the injury site compared to the stretch controls (Fig 13). The images of the scratch assay for the IFT88 inhibited and control samples under static and stretch at 48 hrs are shown in Fig 14.

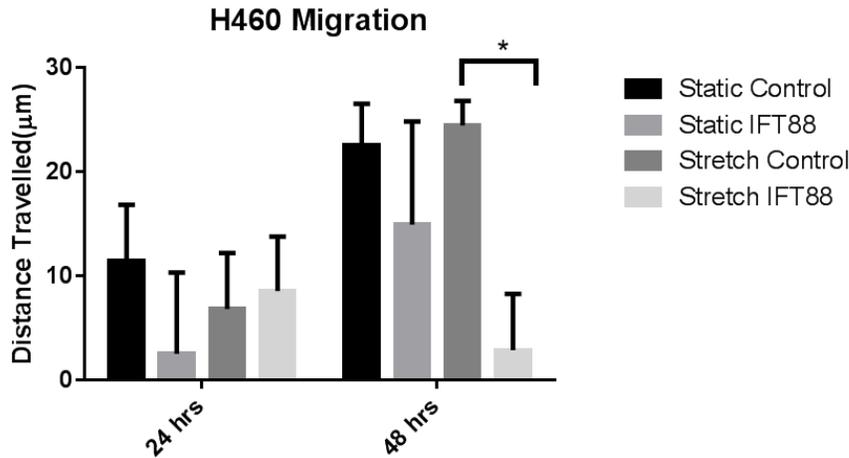
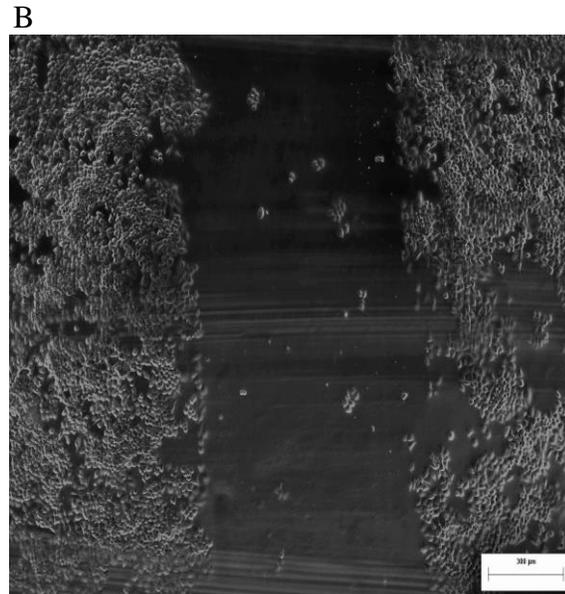
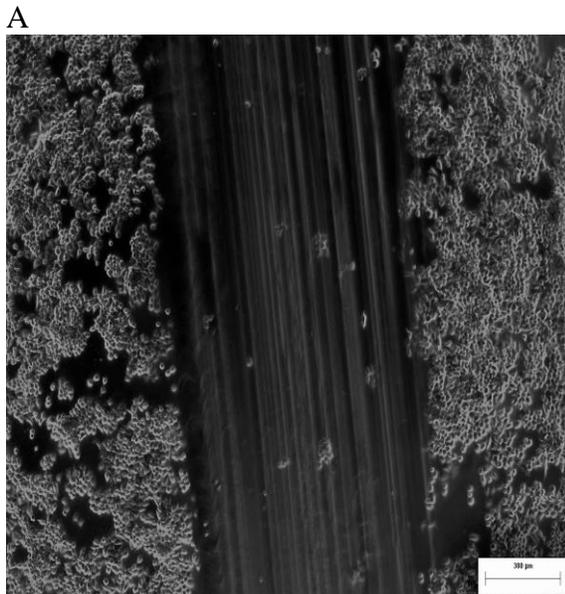


Fig 13. Decrease in migration of IFT88 inhibited samples under 15% stretch in 48 hrs. IFT88 inhibited samples showed a considerable decrease in migration compared to the control under stretch in 48 hrs. Data is representative of distance travelled +/- standard deviation, n= 3 per group. * p < 0.05 Two way Anova with multiple comparisons.



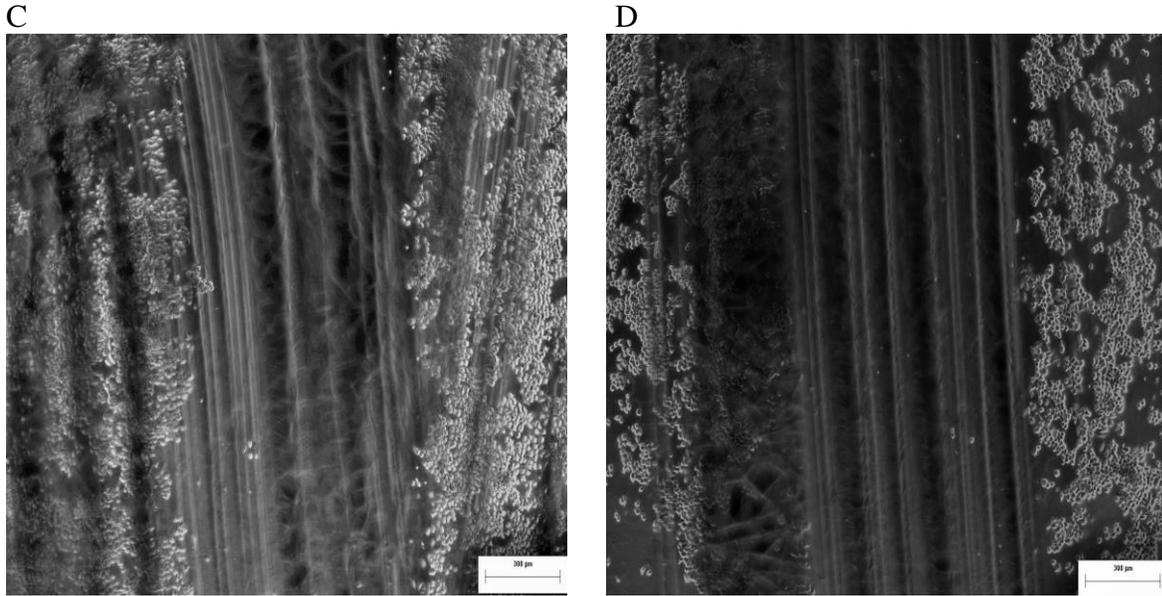


Fig 14. A- Static Control, B- Static IFT88, C- Stretch Control, D- Stretch IFT88 at 48 hrs. A&B. IFT88 inhibited samples migrate slower than controls in 48 hrs on stretch. B&D. The decrease in migration of IFT88 inhibited group compared to the controls is not significant in the static samples.

Smoothened Inhibitors

Cyclopamine reduces cell proliferation

A549luc cells were grown with/without inhibition of IFT88, with/without 5 μ M Cyclopamine treatment for 2 days. Cells with Cyclopamine and IFT88 inhibition, both showed a both showed a reduction in proliferation in an MTT assay (Fig 15)

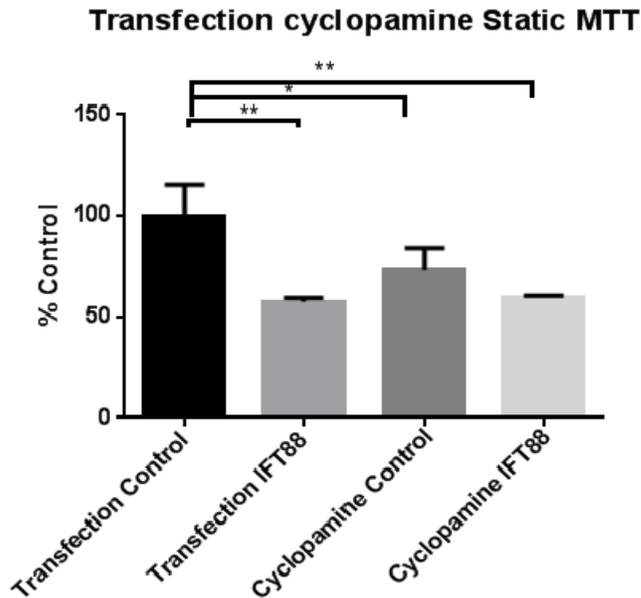


Fig 15. Cyclopamine reduces cell proliferation. Cell proliferation significantly decreased in IFT88 inhibited samples, 5 μ M of 2 day Cyclopamine treated samples and Cyclopamine treated IFT88 inhibited samples when compared to the control samples. Data is normalized to static control and representative of mean \pm standard deviation, n= 3 per group. * p < 0.05, ** p < 0.005 One-way ANOVA with multiple comparisons.

Jervine affects cell migration

A549 cells treated with 2.5 μ M Jervine on a scratch wound healing migration assay for 24 and 48 hrs showed a significant decrease in the migration of cells to the injury site in the Jervine treated samples compared to their controls at 48 hrs (Fig 16).

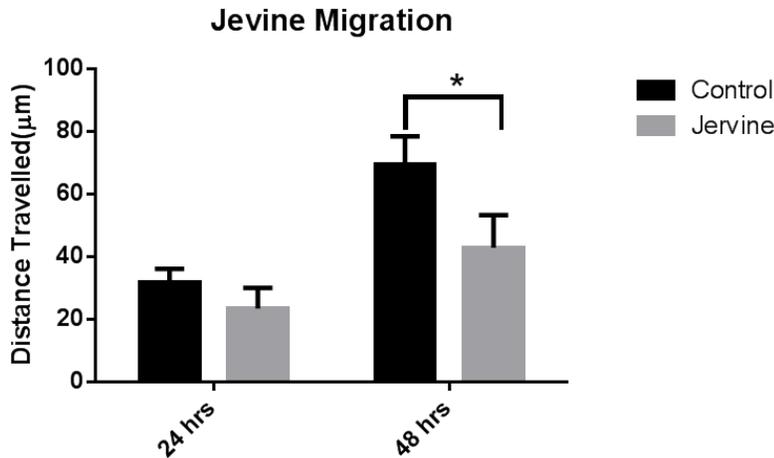


Fig 16. Jervine affects cell migration. Jervine treatment(2.5 µM) significantly decreases cell migration compared to the controls at 48 hrs. Data is representative of distance travelled +/- standard deviation, n= 3 per group. * p < 0.05 Two way Anova with multiple comparisons.

TGF-β1 induced Epithelial to Mesenchymal Transition
TGF-β1 causes an increase in IFT88 gene expression

A549luc cells with/without IFT88 inhibition were treated with 3 ng/ml of TGF-β1 for 2 days.

Control samples treated with TGF-β1 expressed a significant increase in IFT88 gene expression (Fig 17).

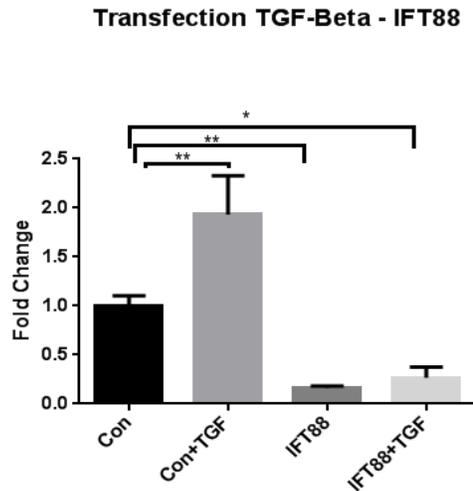


Fig 17. TGF-β1 increases IFT88 expression. Control samples treated with 3 ng/ml TGF-β1 for 2 days show a significant increase in IFT88 expression. Data is normalized to static control and representative of mean +/- standard deviation, n= 3 per group. * p < 0.05, ** p < 0.005 One-way ANOVA with multiple comparisons.

IFT88 inhibition causes partial reversal of TGF- β 1 induced up-regulation of Vimentin

A549luc cells with/without IFT88 inhibition were treated with 3 ng/ml of TGF- β 1 for 2 days.

The up-regulation of mesenchymal marker Vimentin caused by EMT inducer TGF- β 1 was partially reversed with a significant difference in the IFT88 inhibited samples. The down-regulation of epithelial marker E-Cadherin caused by TGF- β 1 was also reversed in the IFT88 inhibited samples but the difference was not significant (Fig 18A, 18B).

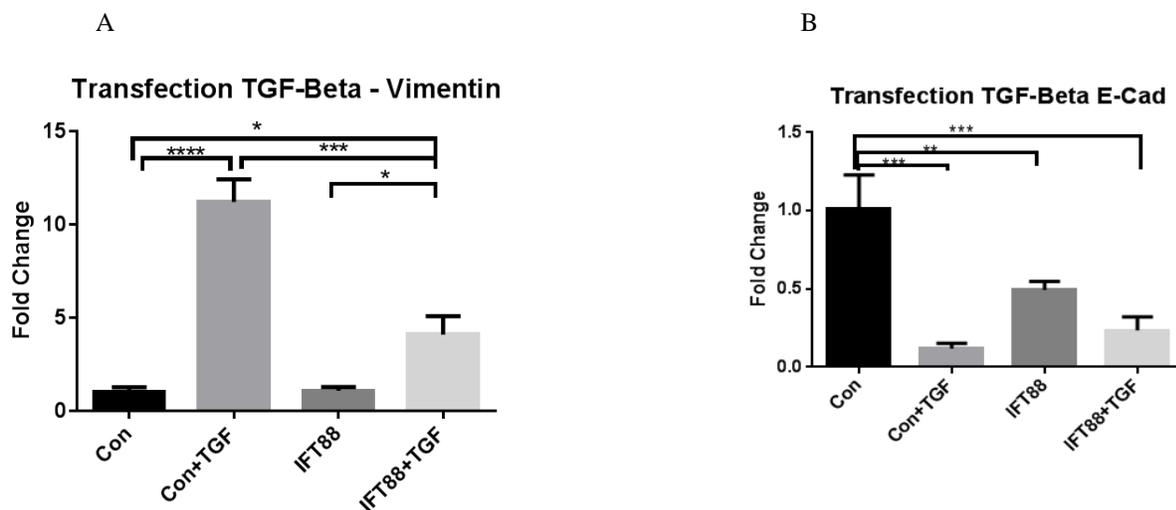


Fig 18. IFT88 inhibition causes reversal of TGF- β 1 induced Vimentin up-regulation and E-Cadherin down-regulation. A549luc cells were treated with 3 ng/ml TGF- β 1 with/without IFT88 inhibition for 2 days. IFT88 inhibited samples treated with TGF- β 1 show a significant decrease in Vimentin expression compared to the control treated with TGF- β 1. On the contrary, IFT88 inhibited samples with TGF- β 1 show a significant increase in E-Cadherin compared to the controls with TGF- β 1 but the difference was not significant. Data is normalized to static control and representative of mean \pm standard deviation, n= 3 per group. * p < 0.05, ** p < 0.005, *** p < 0.005 One-way ANOVA with multiple comparisons.

Taxol Resistant A549luc cells

Taxol resistant A549luc cells do not have primary cilia

A549luc cells resistant to 12nM concentration of Taxol was subjected to 2 day static and 15% cyclic stretch. They were stained for DAPI and α -acetylated tubulin. It was found that both static and stretch samples did not have primary cilia (Fig 19A, 19B)

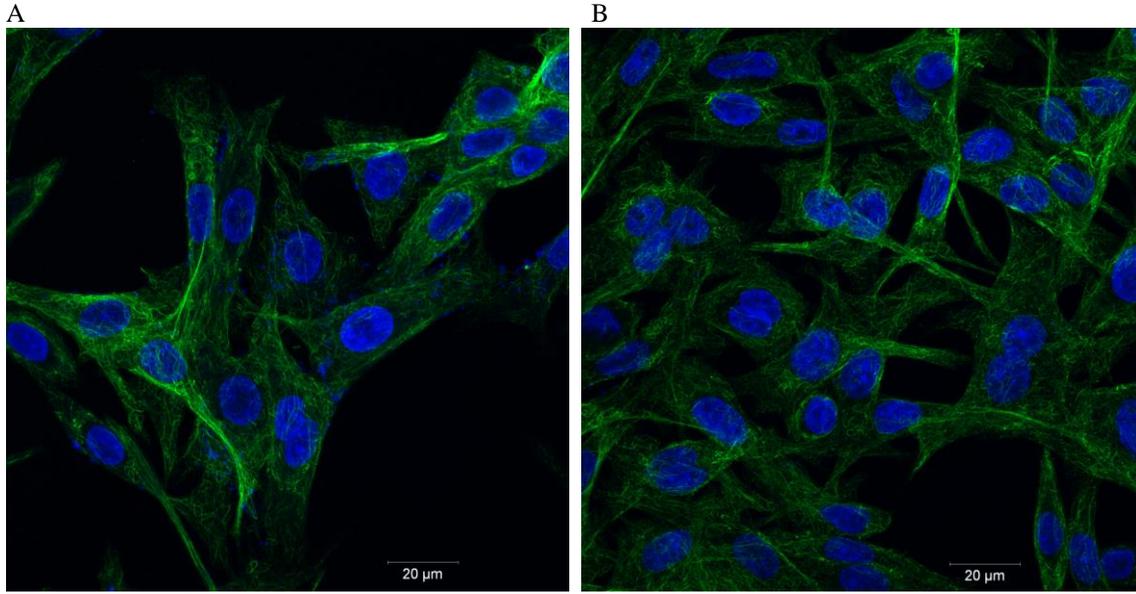


Fig 19. 12 nM Taxol resistant A549luc cells under static and 15% cyclic stretch for 2 days were stained with DAPI (blue) and α -acetylated tubulin (green). A- Static, B- Stretch. Primary Cilia was found to be absent in both static and stretch samples.

Taxol resistant cells proliferate more than regular cells

A549luc cells resistant to 12 nM concentration of Taxol and regular A549luc cells were under static and 15% cyclic stretch for 2 days. Taxol resistant cells have more proliferation than regular A549luc cells. Taxol resistant cells proliferated significantly higher than regular A549luc cells under static conditions (Fig 20).

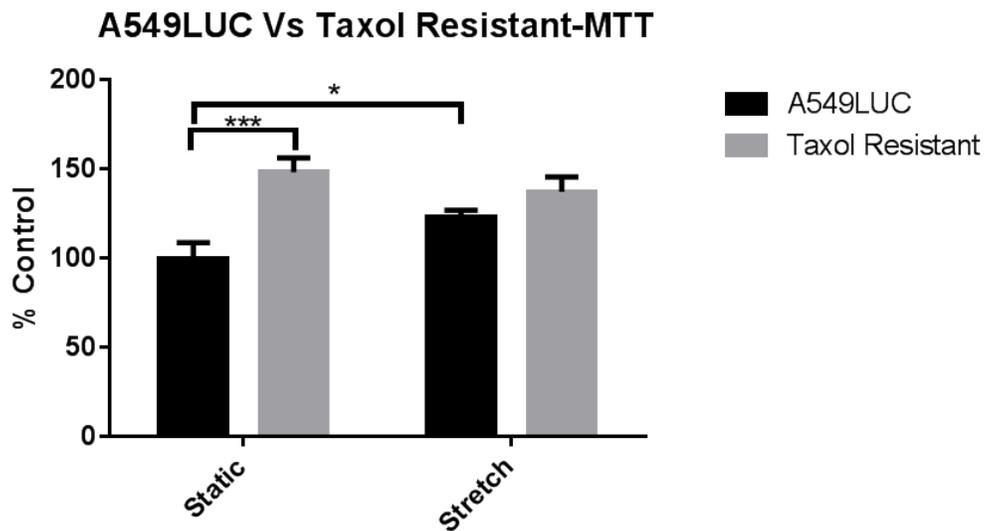
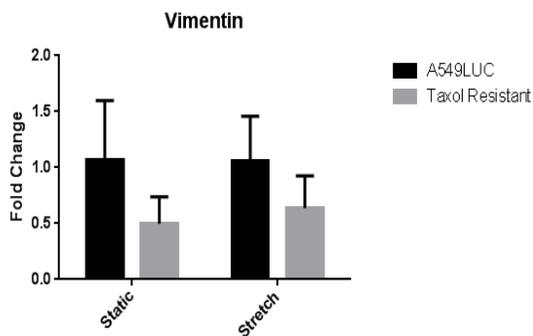


Fig 20. Taxol resistance increases cell proliferation. A549luc cells resistant to 12nM Taxol had significantly higher proliferation than regular A549luc cells under static conditions in 2 days. Data is normalized to static control and representative of mean +/- standard deviation, n= 3 per group. * p < 0.05, ** p < 0.005, *** p < 0.005 Two-way ANOVA with multiple comparisons.

Epithelial and Mesechymal markers expression levels in Taxol Resistant Vs A549luc cells.

12 nM Taxol resistant and regular A549luc cells under static and 15% cyclic stretch conditions for 2 days were analyzed for Vimentin and E-Cadherin expression levels. There was no significant difference between Vimentin or E-Cadherin expressions between Taxol resistant and regular A549luc cells in static or stretch conditions (Fig 21 A, 21 B).

A



B

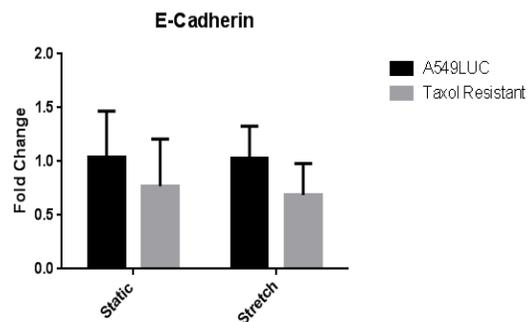


Fig 20. Vimentin and E-Cadherin expression levels of Taxol resistant and regular A549luc cells. 12 nM Taxol resistant and A549luc cells were under static and 15% cyclic stretch for 2 days. There was no significant difference between Vimentin or E-Cadherin expression levels between Taxol resistant and regular A549luc cells.

CHAPTER 4: DISCUSSION

Lung cancer is the leading cancer killer in the United States accounting for 27% of cancer deaths [1]. Adenocarcinoma occurring in the peripheral portion of the lungs is the most common type of lung cancer [3]. The lung is subjected to several complex physical forces during breathing and blood flow. Mechanical forces play a critical role in lung tissue under pathological conditions. Lung cells are often subject to non-physiological stretching as a result of disease or injury [8]. The ability of cancer cells to metastasize and form tumors is partly dependent on the physical interactions and mechanical forces between cancer cells and their microenvironment [41]. Primary cilia are known to transduce signal information from the ECM to the cells and act as mechanical sensors to the cells [13]. Primary cilia are known to be down regulated in renal carcinoma, breast and pancreatic cancer when compared to their surrounding normal tissue [18,19,20]. Primary cilia are found to be up regulated in human basal cell carcinomas and medulloblastomas. But their presence and role in these two cancer types is paradoxical as they can both mediate and suppress hedgehog dependent tumorigenesis in these cancer types by mediating or suppressing the activation of the hedgehog pathway [46,47]. Recent studies have shown that proliferating malignant cancer cells such as human epithelial HeLa adenocarcinoma cells and MG63 osteosarcoma cells robustly exhibit primary cilia [48]. The presence and role of primary cilia in lung cancerous cells in comparison to their surrounding normal cells have not been studied to date.

Lung cancer cell lines have increased primary cilia on stretch

We performed immunofluorescence staining on the lung cancer cell lines A549, H460, SKLU1, H838 and H838-spag6 under stretch and static conditions. A549 and H460 had increased expression of primary cilia in the stretch samples than the static. The increase in primary cilia in response to mechanical stretch was not significant in the SKLU1 cell line. The H838 and H838-spag6 cell lines did not show an increase in primary cilia on stretch. Primary cilia are up regulated in some type of cancers but down regulated in some others. Similarly, primary cilia are up regulated in some lung cancer cell lines but not in some others, the reasons for which are not clearly understood. Among the four well defined cell lines in our quantification study, the three that showed an up-regulation of primary cilia on stretch A549, H460 and SKLU1, express the KRAS mutation which is absent in H838 which is the cell line that did not show up-regulation of primary cilia on stretch [55]. The RAS genes encode a family of GTPs regulating cell growth, differentiation and apoptosis. Mutations in the RAS genes lead to the formation of RAS with impaired GTP activity leading to constitutive activation of RAS signaling. Mutations in the KRAS gene occur frequently in NSCLC particularly in adenocarcinoma [56]. Two of the cell lines used in our quantification, the H838 which did not show up-regulation of primary cilia on stretch and SKLU1 whose primary cilia up-regulation on stretch was not significant, are known to express the TP53 mutation [55]. The tumor suppressor gene TP53 is frequently mutated in human cancers. Inactivation of TP53 function or its attendant pathway is a common feature of human tumors that often co-relates with increased malignancy, poor patient survival and resistance to treatment. Abnormality of TP53 is common in NSCLC and plays an important role in tumorigenesis of lung epithelial cells [57]. Hence absence of KRAS and presence of TP53 mutations could be responsible for the contradictory primary cilia quantification results of the H838 cell line to A549 and H460. However, other

mutations such as Kelch like ECH associated protein1(KEPA1) which negatively regulates Nuclear Factor erythroid-2 related factor 2 (NRF2), common to A549, H460 and H838 and deficiency of LKB1 tumor suppressor in A549, H460 and H838 show that these two mutations are common to the two cell lines having increased primary cilia on stretch and the cell line which did not show an increase in primary cilia, indicating these two mutations have no bearing on the occurrence of primary cilia [49, 50]. A non-sense mutation involving G-T transversion was detected specific to H838 but no further evidence of this mutation having correlation with primary cilia occurrence could be found [49]. In addition to these well defined adenocarcinoma cell lines, we examined the H838 cell line with spag-6 over expression. Sperm associated antigen 6(spag-6) is a protein that is encoded by the spag6 gene. Spag6 is known to be up-regulated in lung and breast cancers. Sperm associated antigens are being studied to use in immunotherapy of cancer. mRNA expression of spag6 is shown to be elevated in tumors ranging from 10-70% [51]. Hence the study of the H838 spag-6 over expressing cell line would allow us to explore possibilities for a co-relation between spag6 gene and primary cilia. However, the H838-spag6 cell line showed similar results in the quantification of primary cilia to H838 cell line. Hence spag6 does not seem to influence the formation of primary cilia in H838 lung cancer cells. So to our knowledge, the KRAS and TP53 gene mutations are two possible factors that could play a role in the non-increase of primary cilia on stretch in the H838 cell line contradictory to the other cell lines tested in the study. Hence we attempted to address the possible factors that could mediate primary cilia response in A549luc cells which showed an increase in primary cilia on stretch as a supplemental study. Integrins are transmembrane receptors consisting of 18 α and 8 β sub units. Integrins are well known mediators in the attachment of cells to each other and to their surrounding extra cellular

matrix. They also mediate signals for the control of diverse cell functions, including survival, proliferation, differentiation, adhesion and migration. Low activation state of integrins on cancer cells is thought to account for the highly metastatic and motile behavior. Increased expression of $\alpha1\beta1$ and $\alpha2\beta1$ integrins correlates to metastatic ability in squamous cell carcinoma. Reduced $\alpha3\beta1$ expression correlates to increased aggressiveness in SCLC and NSCLC. $\beta1$ integrin mediates adhesion of SCLC to ECM proteins and prevents effectiveness of chemotherapy [31]. Hence integrins can be molecular targets for the diagnosis and treatment of cancer because of their contribution to cancer progression by mediating tumor cell proliferation, invasion, metastasis and development of resistance to chemotherapy. Even though many studies have tried to profile the integrin types in several types of lung cancer, there has been no report on comprehensive integrin expression profiles in lung cancer cells so far [31]. It has been shown that $\beta1$, $\alpha3$ and $\alpha5$ integrins are localized to the primary cilium of MDCK cells. $\beta1$ integrins in the primary cilium of MDCK cells potentiate fibronectin induced intracellular calcium signaling even though the calcium signaling is not mediated through $\beta1$ activation [32]. Hence integrins could influence the signal transduction of primary cilia in cancer. Therefore, we performed western blotting of A549 +/- IFT88 inhibition under static or stretch protein samples for 24 and 48 hrs for integrins. But the blots showed no difference in integrin expression levels between IFT88 inhibited and control samples for either of the time points (Fig 22).

Primary Cilia aid in increased cell migration on stretch

Primary cilia has been known to co-ordinate signaling pathways critical to cell migration during development and wound healing. They are known to compartmentalize Platelet Derived Growth Factor Receptor alpha (PDGFR α) and modulate Heat Shock Protein27 (Hsp27) which regulate cytoskeleton re-organization and organization required for directional migration [53, 54]. H460

stretch cells in our migration experiment would contain increased primary cilia due to stretch which would co-ordinate signaling for wound healing and directional migration and hence the cells migrated significantly more towards the injury site in the stretch group when compared to the static and IFT88 inhibited groups (Fig 13).

Smoothed Inhibitors reduce lung cancer cell proliferation and migration

Smoothed(SMO) is a G coupled protein receptor essential for hedgehog signaling(hh) [24]. SMO antagonists at the right concentrations have been shown to inhibit the proliferation of HH producing cancer cells [23]. One of the first SMO antagonists identified was the plant derived teratogen called Cyclopamine. Cyclopamine functions as an HH antagonist by binding and inactivating SMO function in the HH signaling pathway [28]. A549 adenocarcinoma cells when treated with Cyclopamine have shown a significant decrease in cell number [24]. Jervine, another plant steroidal alkaloid similar to Cyclopamine also disrupts HH signaling by affecting SMO function similar to Cyclopamine [29]. Supporting these facts, in this study A549luc cells +/- IFT88 inhibition and +/- Cyclopamine in an MTT assay showed a significant reduction in proliferation in both the Cyclopamine treated and IFT88 inhibited groups showing a similar effect on proliferation for Cyclopamine treatment and IFT88 silencing (Fig 15). However, these effects of Cyclopamine and IFT88 silencing were not additive in the Cyclopamine treated IFT88 inhibited group. In addition, A549 cells +/- Jervine showed a significant decrease in migration of the cells to the injury site of the Jervine treated samples at 48 hrs in a scratch wound healing assay implying Jervine significantly affects A549 cell invasion (Fig 16). Hence Cyclopamine decreases proliferation and migration in A549 and H460 cell lines. As Cyclopamine and IFT88 inhibition cause a similar decrease in proliferation, in order to determine the role of SMO in IFT88 inhibition, we analyzed the expression of mRNA levels of the SMO gene in A549luc

cells +/- IFT88 inhibition and +/- Cyclopamine as a supplemental study. But the groups did not show any considerable differences in SMO expression (Fig 23). Spann et al showed that the presence of primary cilia does not indicate their responsiveness to Smoothed activation or inhibition based on their mRNA levels [44]. Hence the action of SMO gene in IFT88 inhibition may be at the protein and not the RNA levels.

As SMO antagonists have been known to inhibit proliferation of hh producing cancer cells and SMO inhibitors such as Cyclopamine have been known to inhibit proliferation of lung adenocarcinoma cell lines such as A549 [23,24], SMO inhibitors can become effective drugs in treating lung cancer. Hh signaling plays a major role in the homeostasis of diverse tissues and have also been implicated in cancer signaling. Deregulated hh signaling has been implicated with several human cancers such as breast, pancreas, digestive tract, lung and kidney [23].

Primary cilia are mediators of the hh pathway and can both mediate and suppress hh dependent tumorigenesis [46, 47]. SMO is a protein essential for the transduction of hh signaling [24].

Understanding the mechanism of action of SMO antagonists will enable us to map out the hh signaling pathway better and in turn shed more light on the correlation between primary cilia and the hh signaling mediation.

TGF- β 1 causes an increase of IFT88 gene expression and IFT88 inhibition causes partial reversal of TGF- β 1 induced EMT

Transforming Growth Factor-Beta1 (TGF- β 1) is a known inducer of EMT. Tumor cells lose their ability to be growth arrested by TGF- β 1, undergoing EMT leading to increasing invasiveness and metastasis [33]. EMT phenotype has been associated with poor clinical outcome in several cancers especially NSCLC. It has been established that A549 cells undergo EMT phenotypic changes upon exposure to TGF- β 1. In this study, TGF- β 1 treated A549luc

cells exhibited an increase in IFT88 gene expression (Fig 16). To ascertain whether this increase in IFT88 expression causes an increase in primary cilia, we quantified the presence of primary cilia in control and TGF- β 1 treated samples through immunofluorescence as a supplementary study. However, the increase of primary cilia in the TGF- β 1 treated samples was not significant and did not explain the huge surge of IFT88 expression in those samples (Fig 24). To observe the effects of IFT88 inhibition to TGF- β 1 induced EMT, we analyzed the expression levels of mesenchymal marker Vimentin and epithelial marker E-Cadherin in TGF- β 1 treated A549luc cells +/- IFT88 inhibition. We found that there was a reversal of the TGF- β 1 induced up-regulation of Vimentin and down-regulation of E-Cadherin in the IFT88 inhibited samples (Fig 17). These results support previous findings from literature that primary cilia regulates TGF- β 1 signaling elaborated below.

TGF- β 1 treated A549 cells showed a dramatic increase in HH pathway ligand Shh both at the mRNA and protein levels where as parental A549 cells showed undetectable levels of Shh mRNA. A similar rise in Shh levels were seen in another NSCLC cell line H2030 when treated with TGF- β 1 [34]. These results suggest that TGF- β 1 induced EMT is mediated by transcriptional activation of Shh. Maitah et al found that knock-down of Shh significantly attenuated TGF- β 1 induced EMT [34]. Therefore Shh up regulation with TGF- β 1 is mechanistically linked with TGF- β 1 induced EMT in NSCLC. In addition, it was found that down-regulation or inhibition of Shh signaling causes an inhibition in cell migration, invasion and tumorigenic potential of TGF- β 1 treated A549 cells, implying that increase in Shh signaling in those cells could be responsible for their increase in migration and invasion. These results have been consistent with other NSCLS cell lines H1299 and H1650 subjected to the same conditions [34]. Cigna et al found that TGF- β 1 modulates the key components of HH pathway

[36]. A study reported that TGF- β 1 signaling was found to be reduced in a fibroblast cell line with stunted primary cilia [35]. Clement et al found that Clathrin dependent endocytosis (CDE) which is a regulator of TGF- β 1 signaling is found in the pocket region of the primary cilium. These indicate that primary cilia regulate TGF- β 1 signaling and that the ciliary pocket is a compartment for CDE signal transduction. IFT88 was also shown to be regulated with regulation of CDE [35]. Hence the reversal of TGF- β 1 induced Vimentin up regulation and E-Cadherin down regulation on IFT88 inhibition could be due to the absence of primary cilia to regulate the TGF- β 1 signaling.

Taxol resistant cell line

Despite Taxol's success as an anti-tumor agent, cells usually become resistant to the drug over time thus severely limiting its long term effectiveness. Taxol is an anti-mitotic drug that induces microtubule assembly by affecting dynamic instability of the microtubules and induces the formation of microtubule bundles [37,38]. On immunofluorescence staining, we found that primary cilia are absent in Taxol resistant cells both under static and stretch conditions. Taxol is known to cause acetylation of microtubules and act as a microtubule stabilizer [43]. Exit from the cell cycle and entry into quiescence is known to be the most permissible condition for the formation of primary cilium and it is therefore believed proliferating cells are unable to ciliate. Hence primary cilia assembly is thought to occur during cell-cycle exit [45]. Subtle suppression of microtubule dynamics by Taxol strongly inhibits the assembly and formation of the mitotic spindle, thereby preventing or slowing down cell cycle progression at the metaphase/anaphase checkpoint [39]. As primary cilia assembly occurs during cell cycle exit, the prevention of cell cycle progression by Taxol might be the reason for the absence of primary cilia in Taxol resistant cells. We observed the effects of Taxol resistant A549luc cells to proliferation under

static and stretch conditions. Taxol resistant A549luc cells proliferated more than regular cells and the difference was significant in static condition. Taxol resistance may cause increased microtubule dynamics [37]. This may be behind the increase in proliferation rates of those cells on static. Both Taxol and cyclic stretch cause acetylation of microtubules [12, 42]. Microtubule stabilization due to over acetylation by both these factors might be the reason behind the decrease in proliferation of Taxol resistant cells under stretch when compared to static. We attempted to ascertain the EMT properties of Taxol resistant cells by analyzing the expressions of Vimentin and E-Cadherin in these cells in comparison to regular A549luc cells. However, there was no significant difference in the expressions of Vimentin or E-Cadherin in Taxol resistant cells compared to regular cells. Previous work in our laboratory showed that A549 cells undergo EMT on 4 days of stretch. In our experiment, we have done only a 2 day time point for consistency as that is the time point used in all the experiments in this study. That could be the reason for the absence of any signs of EMT in both the regular and Taxol resistant group. The EMT could also have occurred at the protein level and not at the RNA level.

CHAPTER 5: CONCLUSIONS

Primary cilia play an important role in the hallmarks of tumor progression. Previous work on this project showed that human lung biopsies with cancer had more primary cilia than regular lung biopsies. This was further supported by previous work in our laboratory by specifically targeting IFT88 and inhibiting the formation of primary cilia using siRNA that primary cilia aid in the adhesion, proliferation and migration of A549 lung adenocarcinoma cells. In order to assess the significance of primary cilia in lung adenocarcinoma, quantification of primary cilia in NSCLC cell lines was performed. There was an increase of primary cilia in stretch conditions in three out of the five cell lines tested indicating that primary cilia must be essential for the propagation of most cell types in lung cancer and that those cancer cells respond to the cyclic stretch they were subjected to by forming more cilia on stretch than static conditions. The absence of gene mutation KRAS present in A549, H460 and SKLU1 and the presence of TP53 mutation absent in A549 and H460, in the H838 cell line could be responsible for H838 having no increase in primary cilia formation on stretch contradictory to A549, H460 and SKLU1. A549luc cells with IFT88 inhibition showed considerably decreased cilia in both stretch and static confirming successful siRNA silencing of IFT88. Supporting the cell line quantification results that lung cancer cells subjected to cyclic stretch respond by producing more cilia than in the static state, H460 cells showed a decrease in migration of cells to the injury site under stretch for the IFT88 inhibited samples indicating that primary cilia is essential for migration of cancer cells thus enabling the control stretch samples with increased primary cilia to migrate faster to the injury

site than the static and IFT88 inhibited groups. Smoothened Inhibitor treatment of A549 cells resulted in a decrease in proliferation and migration of those cells similar to the effects of IFT88 inhibition. This shows that Shh activation is necessary for the propagation of the cancer cells which was disrupted by SMO inhibition. Primary cilia is a major player in Shh mediation. Therefore figuring out the mechanism of SMO inhibitors would shed more light on the Shh signaling pathway and primary cilia mediation of the pathway. TGF- β 1 induced Epithelial to Mesenchymal Transition shows a reversal in IFT88 inhibited A549 cells confirming that primary cilia regulate TGF- β 1 induced EMT supporting the theory that primary cilia regulate TGF- β 1 signaling. Taxol is a popular chemotherapeutic drug whose effectiveness is affected by developing resistance to it over time. Taxol resistant A549luc cells show an absence of primary cilia implying that the microtubule stability and prevention of cell cycle progression induced by Taxol prevents the formation of primary cilia which is assembled mostly at the cell-cycle exit point. Increased tubulin acetylation caused by both Taxol and cyclic stretch may have resulted in increased microtubule stability in stretch samples resulting in static samples proliferating more than the stretch group. Hence the primary cilia play a critical role in progression and metastasis of lung cancer and in the Sonic Hedgehog pathway. Primary cilia can be potential targets for novel therapeutic approaches to cancer drugs and treatment.

CHAPTER 6: FUTURE DIRECTIONS

The quantification of primary cilia done on five cell lines in this study can be expanded to a larger panel of different types of lung cancer cell lines to assess the significance of primary cilia's role in the different types of lung cancer. We have obtained human lung tissue biopsies from TDAAC through IRB approval. Investigating the presence of primary cilia and Shh related proteins in those samples through immunofluorescence staining could give us more information on the potential of primary cilia as a therapeutic target for cancer treatment.

Immunofluorescence staining of SMO inhibitor treated lung cancer cells for α -acetylated tubulin and ARL13B would enable us to quantify the formation of primary cilia in SMO inhibitor treated and regular lung cancer cells. A significant increase or decrease of primary cilia in SMO inhibitor treated cells would give us further insights into the co-relation between SMO and primary cilia there by also providing insight into the primary cilia and Shh mediation mechanism. More studies on the role of SMO gene in IFT88 inhibition through western blotting analysis of protein samples can be done as the mRNA levels of SMO gene did not give any information on it.

The reversal of TGF- β 1 induced EMT gene expressions in IFT88 inhibited A549 cells can be further investigated by immunofluorescence staining of a mesenchymal or epithelial marker with staining for ARL13B which is specific to primary cilia. The expression levels of ARL13B which would show the presence or absence of primary cilia, relative to a mesenchymal or

epithelial marker would provide an idea into primary cilia's role in the transition of cells into mesenchymal phenotype.

The Taxol resistant cell line can be investigated for mRNA analysis of primary cilia associated gene expressions which could give insight into the signal transduction of the Taxol resistant cells in the absence of primary cilia. The Epithelial and Mesenchymal marker expressions for the Taxol resistant cells in comparison to the regular A549luc cells can be analyzed at the protein level through western blotting as EMT may have occurred at the protein level and not at the RNA level.

The IFT88 inhibition through siRNA performed in this study were for short time points of 2 days. In order to move forward with the effects of primary cilia abolishment on a long term basis, a stable cell line with a vector ON/OFF system which will facilitate ease of future use in in-vivo studies developed for us by the Molecular Biology Core facility, VCU should be subjected to more testing for use in an in-vivo mouse model. These pINDUCER plasmid clones tested in the supplementary study should be further tested for consistent knockdown of IFT88.

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Supplemental studies - Results

Investigating the role of integrins in the mechanism of primary cilia

Control and IFT88 inhibited protein samples of A549 cells on static and 15% stretch conditions for 1 and 2 days were analyzed for integrin expression levels through western blotting. There was no significant difference in integrin expressions between control and IFT88 inhibited samples in static or stretch conditions at 1 or 2 day time points (Fig 22).

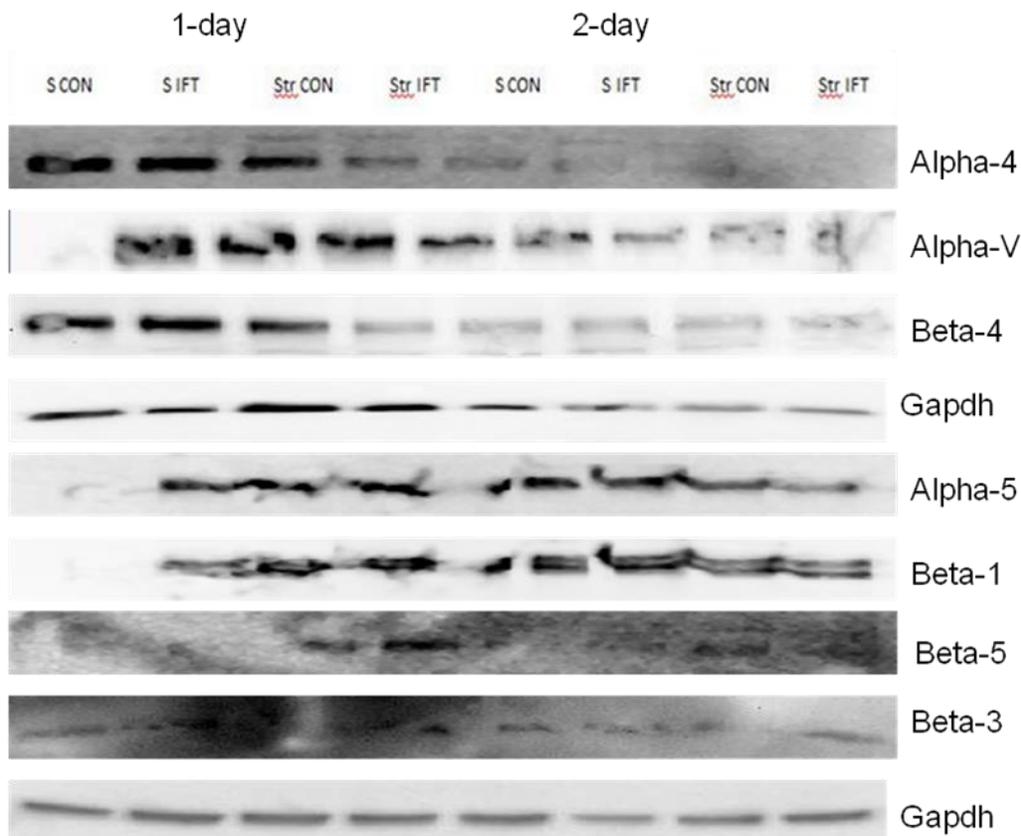


Fig 22. Integrin expressions of IFT88 inhibited and control samples of A549 cells. IFT88 inhibited and control protein samples of A549 cells at static and 15% cyclic stretch at 1 and 2 day time points did not show a difference in integrin expression levels.

Investigating the role of SMO gene in the mechanism of Smoothened inhibitors

A549luc cells with/without IFT88 inhibition, +/- 5 μ M Cyclopamine were analyzed for SMO gene expression levels through RT-qPCR. There was no difference in the expression levels of SMO between the groups (Fig 23).

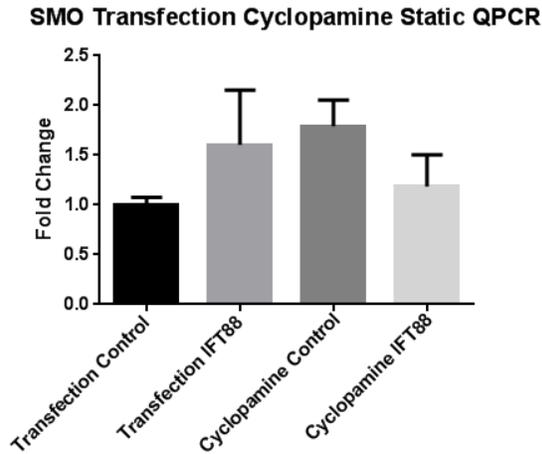


Fig 23 SMO expression. A549luc cells with/without IFT88 inhibition, +/- 5 μ M Cyclopamine were analyzed for SMO gene expression. There was found to be no significant difference between the groups. Data is representative of mean +/- standard deviation, n= 3 per group. One-way ANOVA with multiple comparisons.

Quantification of primary cilia on TGF- β 1 Treatment

A549luc cells were treated with/without 3 ng/ml TGF- β 1 for 2 days. They were stained with DAPI and α -acetylated tubulin. The increase of primary cilia in the TGF- β 1 treated samples compared to the controls was not significant (Fig 24). The images below show A549luc cells +/- TGF- β 1 stained with DAPI and α -acetylated tubulin (Fig 25A, 25B).

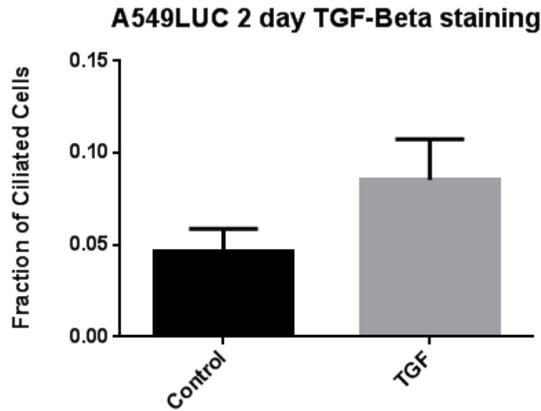


Fig 24. Quantification of primary cilia with TGF- β 1 treatment. A549luc cells +/- 3 ng/ml TGF- β 1 did not show a significant difference in the increase of primary cilia in the TGF- β 1 treated samples. Data is representative of mean +/- standard deviation, n= 3 per group. Mann-Whitney un-paired t-test.

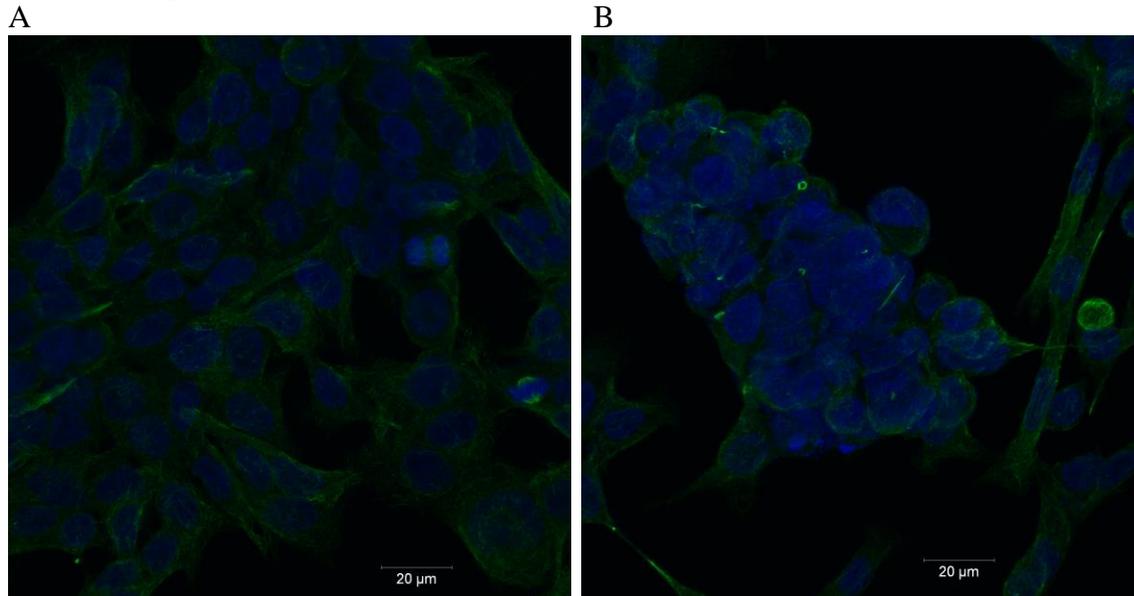


Fig 25 A549luc cells +/- TGF- β 1 for 2 days were stained with DAPI (blue) and α -acetylated tubulin(green). A- Control, B- TGF- β 1. The increase in primary cilia in the TGF- β 1 samples compared to their controls was not significant.

pINDUCER cell line

We wanted to develop and test a tailored pINDUCER A549LUC stable cell line having an IFT88 gene silencing lenti-viral vector incorporated inside operating using a Doxycycline (Dox) ON/Off system to facilitate future in-vivo applications. The objective was to perform RT-qPCR and fluorescence imaging to confirm the operation of the vector +/- Dox and choose the most

efficient clone based on fluorescence and RT-qPCR among the 37 provided by the Molecular Biology Core Facility, VCU for further studies.

The pINDUCER stable cell line is an inducible system that can provide temporal and reversible control of gene expression. The pINDUCER lentiviral system encodes rtTA3 and an interchangeable marker and a bicistronic transcript. Upon the addition of doxycycline (dox) transcription of the turboRFP-shRNA cassette is activated. Without dox, the cells with the lentiviral vector would fluoresce with eGFP [40]. In this plasmid, Pinducer11 was used with the plasmid located in backbone 6. A map of the pINDUCER backbone is shown in Fig 26.

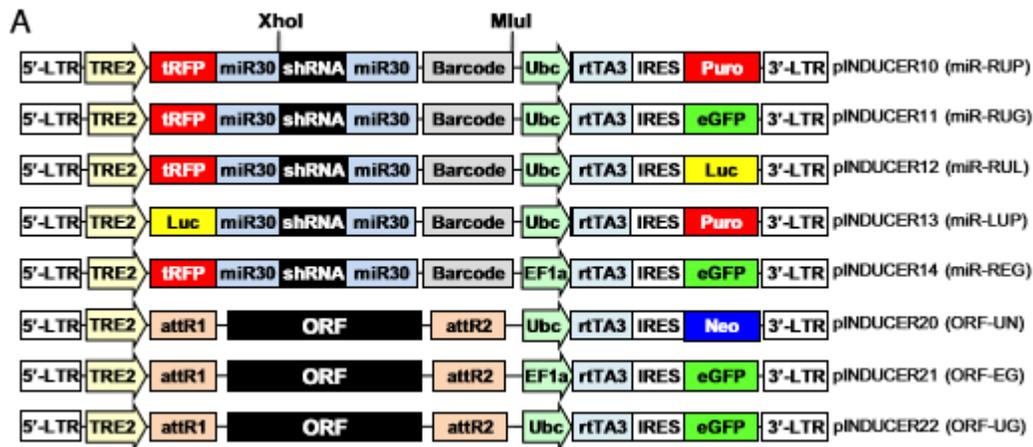


Fig 26. Map of pINDUCER [40]

Out of 37 clones, clone D1 was chosen based on fluorescence in FITC and TRITC channels and PCR results for suppression of IFT88 gene. 333 ng/ml of Dox is used to activate the shRNA and to turn the lentiviral vector 'ON'.

Fluorescence test of clone D1

Clone D1 +/- Dox was imaged in FIT-C and TRIT-C channels. The control group with out Dox glowed green in the FIT-C channel and was blank in the TRIT-C channel indicating the vector

being in the 'OFF' state. The Dox treated group glowed green in FIT-C and red in TRIT-C indicating the vector was turned 'ON' on Dox addition (Fig 26A, 26B, 26C, 26D).

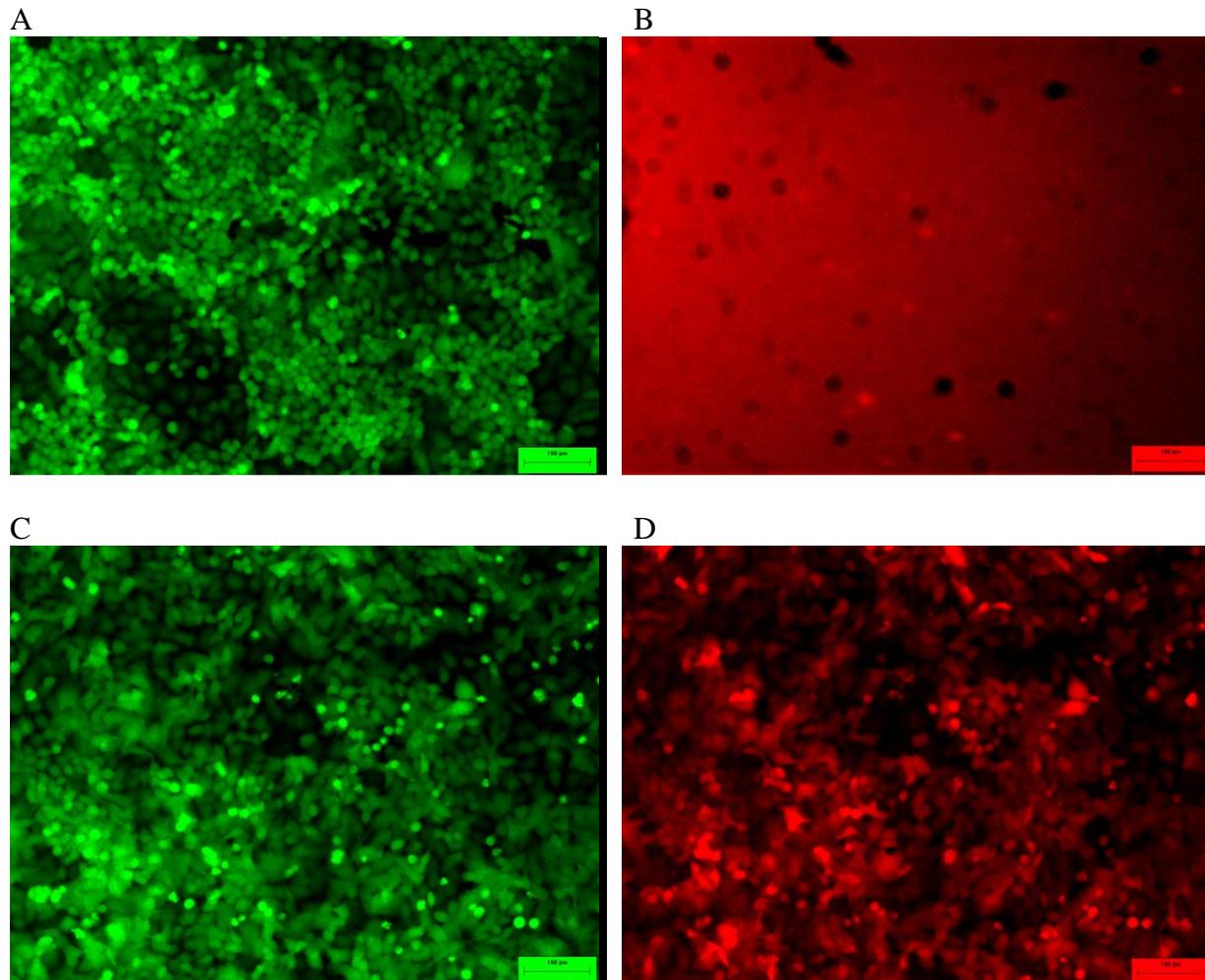


Fig 26. Clone D1. A- Control in FIT-C, B- Control in TRIT-C, C- Dox in FIT-C, Dox in TRIT-C

RT-qPCR confirmation of IFT88 inhibition.

Clone D1 +/- Dox was analyzed for IFT88 expression. The Dox treated samples had significantly lower expression of IFT88 compared to the control samples (Fig 27). However, this IFT88 inhibition was found to be in-consistent on multiple repetitions of the experiment and their RT-qPCRs.

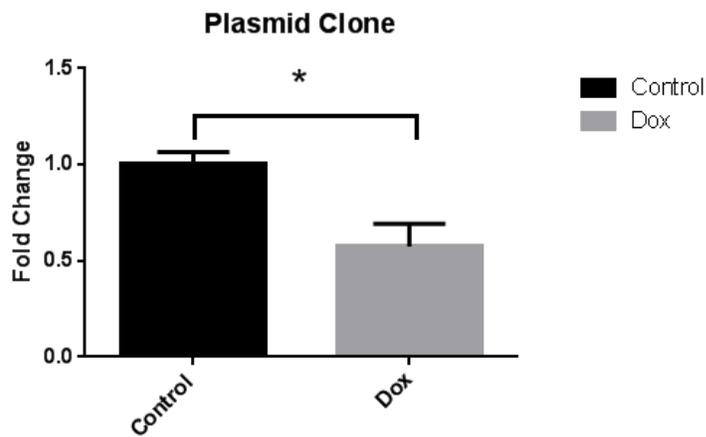


Fig 27. IFT88 expression in clone D1. A549luc pINDUCER cells +/- Dox show a significant decrease in IFT88 expression in the Dox group compared to the controls. Data is representative of mean +/- standard deviation, n= 3 per group. Un-paired t-test with Welch's correction.

List of Primers for genes used for RT-qPCR

IFT88

Forward: GACCGAGAAAAAATGAAGAAGGC

Reverse: CCTTTCACGTTCCATTTGCC

Smoothened (SMO)

Forward: GCACTCCTGACCGCTTCC

Reverse: CTCTTGGGGTTGTCTGTCCG

Vimentin

Forward: GCTAACCCAACGACAAAGCC

Reverse: CCTCTCTCTGAAGCATCTCCTC

E-Cadherin

Forward: CGGGAATGCAGTTGAGGATC

Reverse: AGGATGGTGTAAGCGATGGC

18s (Control)

Forward: GCAATTATCCCCATGAACG

Reverse: GGGACTTAATCAACGCAAGC

GAPDH (Control)

Forward: ACGTGTCAGTGGTGGACCT

Reverse: GTCCACCACCCTGTTGCTG

VITA

Monika Rassi Radhika was born on December 19, 1982 in Tamil Nadu, India. She graduated from SRM University, India with a Bachelor of Engineering degree in Electronics and Communication Engineering in 2004. During this time, she was a member of the IEEE. For her under graduate project, she developed a secure image encryption system using a micro-controller as the cryptographic tool. She worked as a Junior QA analyst for a year in 2006. Migrating to Canada on marriage, after a string of odd jobs in retail, she decided to pursue her long term dream of pursuing her Masters in Biotechnology/Bio-sciences in the US. Currently she is a graduate student in the Mechanobiology laboratory in the Department of Biomedical Engineering and mother of a 1 yr old.