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**The Roles of Primary Cilia in
Cardiovascular Diseases and Cancer**

A Dissertation by

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Chapman University

Irvine, CA

School of Pharmacy

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Pharmaceutical Sciences

August 2020

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The Roles of Primary Cilia in Cardiovascular Diseases and Cancer

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- Alquraini A, **Jamal M**, Zhang L, Schmidt T, Jay GD, Elsaid KA. The autocrine role of proteoglycan-4 (PRG4) in modulating osteoarthritic synoviocyte proliferation and expression of matrix degrading enzymes. *Arthritis Research & Therapy*. 2017;19:89.
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- **Jamal, M. H.**, Nunes, A. C. F., Vaziri, N. D., Ramchandran, R., Bacallao, R. L., Nauli, A. M., & Nauli, S. M. (2020). Rapamycin treatment correlates changes in primary cilia expression with cell cycle regulation in epithelial cells. *Biochemical Pharmacology*, 114056. <https://doi.org/10.1016/j.bcp.2020.114056>

ABSTRACT

The Roles of Primary Cilia in Cardiovascular Diseases and cancer

by Maha Hassan Jamal

Primary cilia are sensory organelles present in most mammalian cell types and regulate cell cycle and signaling pathways. Biochemical and molecular dysfunctions of primary cilia are associated with a wide range of diseases, including cancer, ciliopathies polycystic kidney disease (PKD), liver disorders, mental retardation, and obesity to cardiovascular diseases. Dysfunction in endothelial cilia contributes to aberrant fluid-sensing and results in vascular disorders, such as hypertension, aneurysm, and atherosclerosis. In this dissertation, the most recent outcomes on the roles of endothelial primary cilia within vascular biology have been summarized. Moreover, we evaluate the correlation between cilia formation or length and cell cycle or division using PKD and cancer epithelia. The results show that these cells were associated with abnormal ploidy and were highly proliferative compared with normal kidney epithelia (NK). Importantly, the cancer epithelial cells show a reduction in the presence and/or length of primary cilia. Restoration of the expression and length of primary cilia in these cells using rapamycin were inversely correlated with cell proliferation. Our data suggest that primary cilia may serve as a novel target in cardiovascular disorder and cancer.

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LIST OF ABBREVIATIONS

Abbreviation Meaning

ADPKD	autosomal dominant polycystic kidney disease
BCA	bicinchoninic acid assay
BMP	Bone Morphogenic Protein
BrdU	bromodeoxyuridine
Cep164	centrosomal protein 164
DR	Dopamine Receptor
PKB	protein kinase B
PKC	calcium-dependent protein kinase
CaM	Calmodulin
eNOS	endothelial nitric oxide synthase
IFT	Intraflagellar Transport
FBS	Fetal Bovine Serum
FITC	fluorescein isothiocyanate
NK	Normal Kidney
NO	Nitric oxide
PBS	Phosphate-Buffered Saline
PC1	Polycystin-1

PC2	Polycystin-2
PDGFR α	platelet-derived growth factor receptor- α
PKD	Polycystic Kidney Disease
RIP	regulated intramembrane proteolysis
STAT	signal transducer and activator of transcription
TGF- β	Transforming Growth Factor- β

Chapter 1: Primary Cilia Preview

1.1. Introduction:

Cilia are sensory organelles expressed on the surface of most of the non-haematological cells [1]. The structure of the cilium is divided into 3 parts: the basal body (BB), the axoneme, and the transition zone (TZ) [2,3]. Cilia are classified into motile and non-motile (primary) cilia. Motile cilia have (9+2) microtubule arrangement while primary or non-motile cilia lack the central pair of microtubules, with a (9+0) microtubule arrangement [4]. Primary cilia act as antennae that transmit extracellular signals into intracellular biochemical responses that regulate proliferation, differentiation, and migration [5,6].

1.2. Primary Cilia Structure:

The cilium is a membrane bound structure that is composed of microtubule bundles (ciliary axoneme) originating from the basal body. The axonemal structure contains microtubules that are made of α and β tubulins that are post translationally acetylated to support the cilia structure [7].

Intra-flagellar transport (IFT) is an essential protein complex required for growth and maintenance of both motile and non-motile cilia. Kinesin 2 motor proteins transport the cargo proteins in the anterograde direction to the tip of the cilium while cytoplasmic dynein 2 motor proteins transport cargo proteins in the retrograde direction towards the basal body [8].

1.3. Ciliogenesis and Cell Cycle Regulation:

Ciliogenesis or the timing of cilium formation is controlled by the phases of cell cycle [9]. Formation of primary cilia typically begins at the G1/G0 phase of the cell cycle when the mother centriole in the centrosome acts as a basal body to start cilia formation [10]. As cells re-enter the cell cycle, the cilium and the basal body disassembled releasing the centrioles to work as the organizing center for the mitotic spindles during cell division [11]. As the cells enter the quiescence phase (G0), the mother centriole forms the basal body and the primary cilium is re-assembled [12]. So, cilium acts as a brake for cell cycle progression by holding the basal body and can stop abnormal cell growth by restricting cell cycle [2].

1.4. Primary Cilia as Mechanosensors:

Primary cilia are physical–chemical sensors that respond to many stimuli, such as flow stress [4]. Physical stimulation, such as fluid flow, causes primary cilia deflection to increase intracellular calcium, decrease intracellular cAMP, and stimulate downstream mechanotransduction signaling cascades that regulate cell proliferation [2]. Defects in proteins, receptors or ion channels, that localize to the primary cilium, basal body, and/ or centrosomes, such as the case in polycystic kidney disease, result in defective flow sensing leading to uncontrolled cell proliferation and cyst formation [13–15].

1.5. Signaling Pathways:

Cilia have been implicated in signaling pathways including the Hedgehog (Hh), Wnt and Platelets-Derived Growth Factor (PDGF) pathways [1,14].

Hedgehog (Hh) Signaling Pathway:

Hedgehog (Hh) is one of the essential signaling pathways that is involved in development as well as in homeostasis and regeneration. In the absence of Hh ligand, the Patched (PTCH1) receptor binds and inhibits the activity of the seven transmembrane-domain protein Smoothed (SMO), a central activator of the pathway. On the other hand, binding of Hh ligand to Ptch1 relieves its inhibition of Smo, thus smo is allowed to stimulate downstream Gli activators and inhibit the formation of Gli repressors [16]. Hedgehog deregulation was observed in variety of cancer types [17] such as basal cell carcinoma [18], breast cancer [19], liver cancer [20], and colon cancer [21]. Previous studies have shown that the primary cilium has a crucial role in regulating Hh signaling [22]. Wong S. et al showed that primary cilia can either induce or suppress tumor formation depending on the nature of the oncogenic initiating factor [23].

Wnt Signaling Pathway:

Wnt signaling pathway regulates the balance between cellular differentiation, polarity controls and proliferation to regulate tissue homeostasis. In the absence of canonical Wnt-signaling, the “destruction complex”, which is composed of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3- β (GSK3- β) targets β -catenin to the proteasome for degradation, inhibiting translocation of β -catenin into the nucleus to activate gene expression. Binding of Wnt ligands to a membrane-bound Frizzled (Fzd) receptor, which then binds lipoprotein receptor related protein 5 and 6 (LRP5/6), allows the recruitment of Axin with LRP5/6; thus the Axin/APC/GSK3- β “destruction complex” can no longer degrade β -catenin. Then β -catenin can freely enter the nucleus, with the help of AHI1, to interact with transcription factors to activate Wnt gene expression. The Wnt signal is transduced through Disheveled (Dvl), which is recruited

to the membrane and binds Axin upon stimulation. The presence of primary cilium controls the level of expression of Wnt target genes by (1) controlled degradation of Dvl by the proteins: inversin (INVS) and nephrolithiasis (NPHP) that are localized in the primary cilium and (2) sequestering AHI1 at the cilium so it becomes unable to help β -catenin to translocate into the nucleus [2,9].

In this thesis, the first part reviews the crucial role of primary cilia in cardiovascular disease while the second part assesses the relationship among Wnt signaling pathway, cell proliferation and primary cilia.

Hypothesis:

Cilia length is associated with cancer progression, and specific pathways in cilia associated with cancer cell cycle can be modulated.

Aims:

1. To karyotype and validate the cancer cells.
2. To characterize the expression of the primary cilia and assess the cell proliferation and Wnt signaling pathway in cancer cells.
3. To restore primary cilia expression in cancer cells using Sirolimus (Rapamycin) and then:
 - a) Characterize the expression of the primary cilia.
 - b) Evaluate the association between re-expression of primary cilia and cancer cell proliferation.
 - c) Investigate the correlation between primary cilia and Wnt signaling pathways after restoring primary cilia expression in cancer cells.

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Chapter 2: The Roles of Primary Cilia in Cardiovascular Diseases

Pala R, Jamal M, Alshammari Q, Nauli SM. The Roles of Primary Cilia in Cardiovascular Diseases. *Cells*. 2018;7(12):233.

2.1. Abstract

Primary cilia are microtubule-based organelles found in most mammalian cell types. Cilia act as sensory organelles that transmit extracellular clues into intracellular signals for molecular and cellular responses. Biochemical and molecular defects in primary cilia are associated with a wide range of diseases, termed ciliopathies, with phenotypes ranging from polycystic kidney disease, liver disorders, mental retardation, and obesity to cardiovascular diseases. Primary cilia in vascular endothelia protrude into the lumen of blood vessels and function as molecular switches for calcium (Ca^{2+}) and nitric oxide (NO) signaling. As mechanosensory organelles, endothelial cilia are involved in blood flow sensing. Dysfunction in endothelial cilia contributes to aberrant fluid-sensing and thus results in vascular disorders, including hypertension, aneurysm, and atherosclerosis. This review focuses on the most recent findings on the roles of endothelial primary cilia within vascular biology and alludes to the possibility of primary cilium as a therapeutic target for cardiovascular disorders.

2.2. Introduction

Cilia have been studied for their motile functions of lung epithelium, sperm cells, as well as in other organisms (such as algae) [1]. These motile cilia, in most cases, have 9 + 2 microtubule structural arrangement. Researchers have also gained interest in studying non-motile cilia [2]. Non-motile cilia also known as primary cilia have a 9 + 0 microtubule structural arrangement. Defects in primary cilia could cause various life-threatening diseases in humans, such as neural tube defects, which result in numerous abnormalities of the brain and spinal cord in patients diagnosed with Meckel syndrome, for example [3]. Several studies have shown that primary cilia are recognized as mechanical and chemical sensory organelles which serve as antennae to transmit extracellular to intracellular signaling mechanisms. Because primary cilia act as sensory organelles by which cells sense and transduce extracellular signals [4], any defects in primary cilia function could potentially cause several diseases which are collectively known as ‘ciliopathies’ (Table 1). The list of human ciliopathies has increased in recent years [5,6]. Mutations in approximately 50 genes have revealed to alter ciliary assembly or function, and as many as 1000 different ciliary proteins are still with undetermined functions and required further investigation. Hence, abnormal ciliary proteins can be associated with a single organ dysfunction to systemic multiple organ complications depending on the type of cells affected [7].

Table 0.1: Ciliary Function and Disease Relevance.

Function	Disease Relevance	Reference
Nodal flow sensing	Situs inversus; Situs ambiguous; Situs isomerism	[14,15]

Function	Disease Relevance	Reference
Mechano-sensing	Kidney, Liver, and Pancreas Diseases	[16,17,18]
Shear stress sensing	Hypertension; Atherosclerosis; Aneurysm formation	[10,11,12,13]
Osmolarity sensing	Respiratory diseases; Infertility	[19,20]
Gravitational sensing	Osteoporosis; Chondroporosis	[21,22,23]
Olfaction sensing	Anosmia; Hyposmia	[24,25]
Light sensing	Retinitis pigmentosa; Blindness	[26,27,28]
Chemo-sensing	Nephrocystin; Diabetes; Obesity	[29,30,31]
Neurotransmitter sensing	Impaired brain plasticity	[32]
Developmental regulatory sensing	Developmental defects; Cancer	[33,34,35]
Pressure sensing	Bone maintenance, development	[22,36,37]

Cilia dysfunction has been implicated in polycystic kidney disease (PKD), obesity, nephronophthisis, mental retardation, Bardet-Biedl syndrome, oral facial syndrome, vascular diseases and others [7]. Specifically, impaired primary cilia on endothelial cells have important clinical consequences and are associated with many vascular diseases [8,9]. Although it has been over a century since primary cilia have been visualized, the study of their mechano- and chemosensory roles remains relatively a new field of study. Additionally, studies on endothelial primary cilia function of the vascular system and primary cilia as a therapeutic target for vascular diseases are still limited. In this review, the roles of primary cilia will be discussed with emphasis on the cardiovascular diseases [10,11,12,13]. Hence, it is important to have a clear understanding about ciliary structure and functional ciliary proteins to investigate how cilia dysfunction can contribute to vascular disorders of hypertension, aneurysm and atherosclerosis.

2.3 Cilia Structure

A cilium is considered as a cellular organelle, which is primarily composed of a membrane, soluble compartment, axoneme, basal body, and ciliary tip [38]. A cilium extends from a basal body complex, which is mainly composed of two centrioles. One of the centrioles is known as the mother centriole, to which the ciliary axoneme is ingrained beneath the cell membrane. The cilium structure contains the microtubular portions of cytoskeletal core unit called the axoneme (Figure 1). The axonemal structure contains nine peripheral doublet microtubules, which are made of alpha- and beta-tubulins and are post-translationally acetylated to support the long cilia structure [39,40,41]. The non-motile axoneme structure lacks the central pair of microtubules (9 + 0) [42,43]. In the blastocyst nodal cilia, the axoneme lacks the central pair of microtubules (9 + 0) but shows motility. This exception requires both dynein arms for motility [44]. Of note is that the lack of radial spokes induces rotational motion instead of beating motion, suggesting that the

absence of radial spokes allows nodal cilia to rotate unidirectionally but, as a trade-off, renders them ultrastructurally fragile [44]. While in most cases of motile cilia, the axoneme contains nine peripheral doublet microtubules and a central pair of microtubules (9 + 2) [45]. There is a connection between the microtubular portion of the cytoskeleton and the ciliary axoneme, and the disruption of cytoplasmic microtubules or actin filaments, which could affect microtubules assembly resulting in the loss of ciliary structural integrity and mechanosensory function [46,47,48]. On the other hand, protein entering and exiting through cilium is controlled by a proteomic barrier at the ciliary base that encompasses a transition zone which separates cytosol from the cilia [49,50,51,52]. The ciliary membrane is connected with the plasma membrane but possesses a lipid bilayer composition that differs from the plasma membrane compositions [53]. The periciliary membrane, also known as the transition-membrane, connects ciliary and plasma membrane to form the ciliary pocket [54,55]. In addition to its fundamental structural role, the basal body connected to the transition-membrane is thought to regulate protein entry and exit from the ciliary compartment. Furthermore, the mechanoreceptors, protein transporters, sensory proteins and ligand-gated ion channels are involved in signal transduction enclosed within the ciliary membrane. The axoneme allows the intraflagellar signaling and intraflagellar transport (IFT) activities along with ciliary shaft using the soluble compartment, also called cilioplasm. Primary cilium lacking ribosomes is incapable of producing its own proteins required for the elongation and continuous turnover of axoneme necessary for self-safeguarding. Moreover, each part of this cilia structure is crucial to support various signaling molecules. Some of the more established cilia-dependent signaling pathways are already described [38].

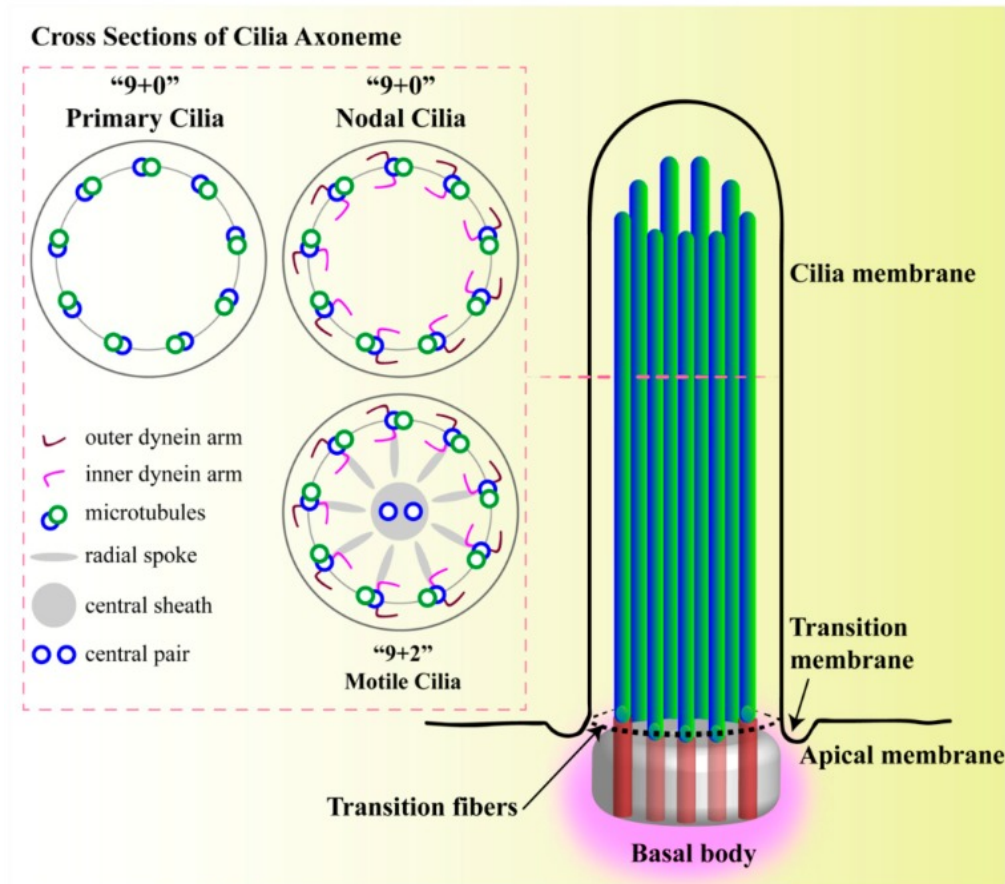


Figure 2.1 Structure of primary cilium

A cilium is a membrane-bound structure and composed of multiple central pairs of microtubules (axoneme) running from the basal body. A basal body is a microtubule-based structure composed of mother and daughter centrioles. The ciliary membrane and axoneme contributes to the upper part of the cilium. The ciliary membrane is continuous with the cell membrane, but they have their own proteins, ion channels, and/or receptors. The ciliary skeleton may have 9 + 0 or 9 + 2 axoneme compositions. Most 9 + 0 cilia lack inner and outer dynein arms, radial spokes, and central sheath and are commonly referred as non-motile primary cilia. Some 9 + 0 cilia lack the central microtubule only and are motile. Between the cell membrane and cilium, there is a transition-membrane at the junction of the basal body acting as a barrier for molecules to enter or exit from the primary cilium.

2.4 Primary Cilia as a Blood Flow Sensor

Flow sensing by the cilia permit cells to sense blood flow along the blood vessels, urine flow through kidneys, bile acid in the liver, pancreatic secretions in the duodenum, nodal flow in

Hansen's node (the site which determines the patterns the anterior-posterior axis of the embryo during gastrulation), interstitial fluid flow within the bone canaliculi, and potentially other systems/organs [56]. The function of the vascular system depends on the mechanical fluid flow signaling from the blood flow. Several studies have also reported that the presence of primary cilia in major circulatory systems including endocardia [13,57], arteries [58,59], veins [60], corneal endothelium [61,62], and smooth muscle cells of both arterial and airway endothelia [63,64]. The continuous contraction and relaxation of smooth muscle cells produce changes in the blood vessels diameter, which is important for normal blood flow [65,66,67]. Increase in vascular stiffness is a major cause of hypertension, which leads to complications including ventricular hypertrophy, vascular aneurysm and atherosclerosis [68,69,70,71,72]. These changes suggest that smooth muscle cells or neuronal regulations are important in regulating the vascular tone in addition to the mechanical fluid-flow within the blood vessel. The regulation of circulatory function is acquired by neuronal regulators through central and/or peripheral neurons [73,74,75].

The mechanical fluid-flow provides local regulation or autoregulation within a blood vessel. For example, autoregulation is required to achieve immediate blood flow control in specified area of the tissue. Autoregulation is independent of the neighboring tissues and has little to no effect on the surrounding tissues [76,77]. In an isolated blood vessel, the sudden increase of transmural blood pressure causes a reduced vessel diameter [78,79,80], whereas high flow stress increases vessel diameter [67,80,81,82]. As such, the lining of the inner surface of vascular blood vessels are endothelial cells with primary cilia protrusions, which can sense changes in blood velocity and pressure and convert these mechanical signals into changes of vascular smooth muscle tone [83,84]. In a biophysical perspective, fluid-shear stress refers to the partial or frictional force of blood flow as it brushes against the vascular endothelia [85]. This frictional force is not stable

because blood flow changes with each heart muscle contraction, resulting in pulsatile patterns of blood flow [85]. As a result, blood flow through a vessel creates different types of forces such as stretch, compression, cyclic strain, pressure and shear stress. While these forces may be practically impossible to differentiate *in vivo*, they can be independently studied in *in vitro* and *ex vivo* studies [86].

Our earlier studies show that the primary endothelial cilia act as a mechanosensor in *in vitro* (mouse aortic endothelial cells), *ex vivo* (isolated mouse arteries, blood vessels from human patients) and *in vivo* (mouse models) [11,12,58]. Ciliary length is also positively correlated with mechanosensory action. Blood vessels with relatively a low fluid force have longer cilia while blood vessels with a high fluid force are devoid of cilia or have very short cilia. In addition, the changes in fluid dynamics affect endothelial cilia distribution and depend on fluid-flow intensity with longer cilia present in lower fluid-flow areas [13,87]. This is because of the inability of primary cilia to stand against high levels of fluid flow, which results in ciliary disassembly and loss of intraflagellar transport which is necessary for ciliary reassembly [88]. Subsequently, the mechanosensing function of cilia in high fluid flow areas could be replaced by other mechanisms like glycocalyx to sense higher shear forces [89].

Primary cilia have a critical role in sensing the extracellular stimuli, such as odorant or chemical (chemosensory) and movement (mechanosensory). These stimulations are then translated into intracellular signals. As a mechanosensor, a primary cilium can sense the fluid-flow in multiple cell types including renal epithelial and vascular endothelial cells [12,18,90]. Polycystin-1 (PC1) and polycystin-2 (PC2) form a mechanosensory complex in the primary cilia. It is recently shown that the PC1 and PC2 form a complex and are assembled in a stoichiometry of 3 PC2 for every PC1 molecule [91]. The PC1 and PC2 complex detects the bending of the cilia by the fluid flow

leading to an increase in Ca^{2+} influx and an inhibition of the regulated intramembrane proteolysis (RIP) of PC1 by keeping the signal transducer and activator of transcription (STAT) factor 6 and its coactivator P100 in a complex bound to PC1 tail [92,93]. This is how primary cilia is thought to promote proliferation and differentiation through fluid-shear stress. On the other hand, the absence or lack of flow as well as loss or dysfunction of cilia, PC1, or PC2 decrease Ca^{2+} influx and activate RIP that allows STAT6 and P100 to translocate to the nucleus and stimulate transcription resulting in uncontrolled cell proliferation and cyst formation [94,95]. In particular, PC1 and PC2 are widely expressed across the vasculature, and they are hypothesized to play a major role in the development, maintenance, and function of the myoelastic arteries [96,97,98]. These observations indicate a direct pathogenic role for both PC1 and PC2 in the vascular complications of hypertension, aneurysm and/or atherosclerosis.

2.5 Role of Primary Cilia in Heart Development

Nodal cilia probably have the earliest cilia function during embryonic development. During gastrulation period, both motile (nodal) and non-motile cilia at the embryonic node play an important role in regulating signaling cascades required for the formation of left-right asymmetry, a process which regulates the early stages of cardiogenesis and connection to the blood vessels [15,99,100,101,102]. Fluid flow plays an important role in trabeculation, cardiac cell proliferation, and formation of conduction system, in addition to changes in fluid-shear forces, which lead to cardiac diseases. Cilia in cardiomyocytes have a series of receptors, which take part in regulating cellular signaling mechanisms required for the continuous differentiation, morphogenesis and development of the heart [103,104,105,106]. Independent studies have established the important role of heart cilia in cardiac development. Defects in cilia structure or function lead to severe

inherited cardiac diseases. Also, defects in cilia result in a variety of heart developmental defects such as arterial and ventricular septum defects [107,108], abnormal looping, and remodeling of the heart tube into a multi-chambered organ [109,110,111,112,113,114] or myocardial wall disorganization [115]. Moreover, mice with a mutation in cilia structural gene *ift88*, *kif3a* or *kif3b* are characterized by severe heart phenotypes including hypoplasia of the endocardial cushions, a reduction in ventricular trabeculation, and an increase in volume of pericardial space including defective cardiac looping [102]. A variety of signaling pathways are involved directly or indirectly in heart development. For example, Hedgehog (Hh) signaling coordinated by primary cilia in a variety of cells controls tissue patterning and promotes the activation of different transcriptional factors involved in different cellular signaling mechanisms during homeostasis in vertebrates [116,117]. As a result, defects in primary cilia Hh signaling leads to severe cardiac disorders including congenital heart diseases [118]. Another example of a signaling pathway which plays an important role in cardiac morphogenesis is the superfamily of Transforming Growth Factor β /Bone Morphogenic Protein (TGF β /BMP). TGF β /BMP signaling network is involved in a wide range of cellular mechanisms and processes and is therefore fundamentally vital during tissue homeostasis and morphogenesis [119]. Recent studies show that primary cilia can regulate the canonical TGF β signaling network through the activation of transcription factors Smad2/3 at the ciliary pocket [104]. Furthermore, the TGF β ligand, TGF- β 1, stimulates the differentiation of stem cells into cardiomyocytes and that *Ift88/Tg737* (*Tg737^{orpk}*) mouse embryonic fibroblasts are characterized by decreased TGF β activity associated with reduced clathrin-dependent endocytosis activity at the ciliary base, suggesting that cardiac primary cilia play a direct role in regulating TGF β signaling during cardiomyogenesis. Recent findings further show that platelet-derived growth factor receptor- α (PDGFR α) localizes to primary cilia in

mutant mouse heart, indicating that a portion of the PDGF signaling pathway is associated with cardiac primary cilia during cardiac morphogenesis and development [105]. The localization of PDGFR α causes downregulation of Hh signaling in primary cilia and causes diminished ventricular wall thickness and ventricular septal defect [105]. Further, mice studies show that mutated or the absence of PDGFR α , consequences arise in prenatal mortality such as heart defects including weakened myocardium, thinned septa and valve, outflow tract, and aortic branch malformations [120,121,122]. Taken together, the PDGF signaling system might be specifically coordinated by cardiac primary cilia, potentially acting as signaling hubs facilitating the cross-talk between different signaling networks in order to coordinate cardiogenesis.

2.6 Role of Primary Cilia in Biochemical Signaling and Hypertension

As mechanosensory organelles, primary cilia depend on various receptors expressed on the ciliary membrane. Vascular endothelial cells lining the blood vessel wall are in continuous contact with blood flow forces. Activation of primary cilia by blood flow leads to the activation of PC1 and PC2 resulting in an intracellular Ca²⁺ signaling network involving calmodulin (CaM), calcium-dependent protein kinase (PKC), serine-threonine kinase/protein kinase B (Akt/PKB) and endothelial nitric oxide synthase (eNOS). Such biochemical reaction generates nitric oxide (NO) leading to vasodilation (Figure 2). There are two major proposed mechanisms for primary cilia detection of blood flow forces [85]. The first suggests that ciliary bending occurs upon exposure to blood flow-pressure force, which triggers cytoskeletal distortion. The second suggests that cilia bending triggers activation of PC1 mechanosensory protein and PC2 cation Ca²⁺ channels. It is proposed that the increase in intracellular Ca²⁺ is caused by an increase in intraciliary Ca²⁺ [123], whereas another study has suggested that Ca²⁺ could be rallied in both directions between the cilia and the cytoplasm [124]. While differences in the intraciliary Ca²⁺ can be due to the sensitivity of

the cilia-specific Ca^{2+} probes [125], both studies show a consensus that mechanosensing function of cilium involves cytosolic Ca^{2+} signaling as shown independently by other laboratories [17,126,127]. Thus, it is fair to assess that primary cilia are Ca^{2+} -responsive mechanosensors that can trigger a diverse biochemical signaling.

Regardless, the cytosolic Ca^{2+} forms complexes with CaM, and the Ca^{2+} -CaM complex has been shown to indirectly activate eNOS through activation of the AKT/PKB signaling which activates AMPK, a known stimulator of eNOS [128]. Inhibition of Ca^{2+} -dependent PKC, Akt/PKB, or CaM activity downstream of Ca^{2+} signaling have no effect on the flow induced intracellular Ca^{2+} increase, although there is a loss of NO synthesis [11]. This indicates that the Ca^{2+} signaling is upstream of the biochemical reaction in producing NO. Though eNOS triggering is principally a Ca^{2+} -dependent process, some studies have suggested a Ca^{2+} -independent pathway in NO biosynthesis is also possible. This Ca^{2+} -independent pathway depends on the heat shock protein 90 (HSP90) [129,130]. HSP90 is a molecular chaperone, but it may also act as a signal transduction agent concomitant with eNOS in several systems, including the cardiovascular system. HSP90 also localizes to primary cilia [131]. Although its activation can increase eNOS action in presence of Ca^{2+} -CaM [129,132,133], it is unclear if cytosolic HSP90 is involved in this signaling pathway.

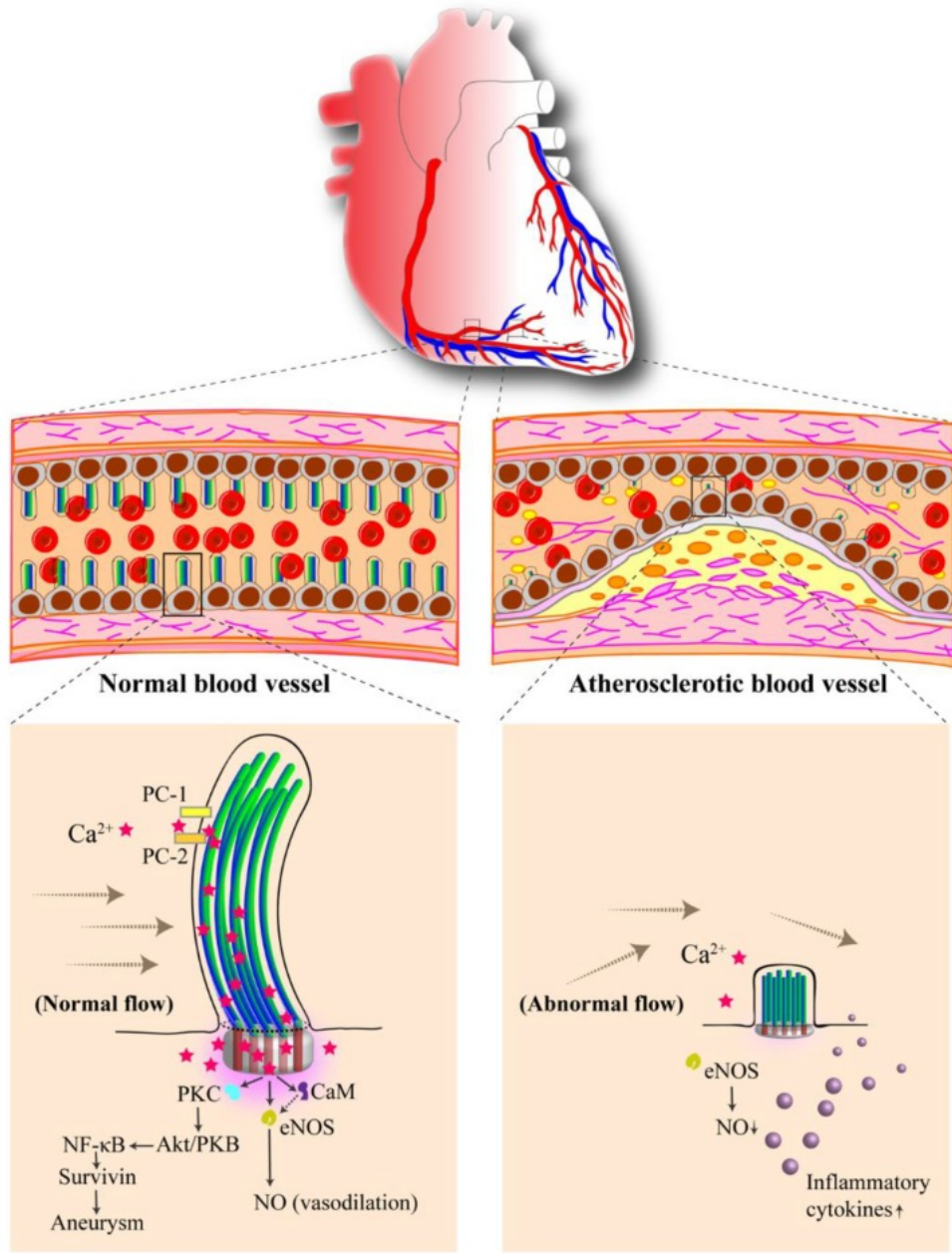


Figure 2.2 Vascular endothelial cilia sense the blood flow along the blood vessel. Primary cilia are structural compartments that house many mechanosensory proteins. Ciliary bending occurs upon blood-flow stimulation, and polycystin-1 (PC1) activates polycystin-2 (PC2), resulting in calcium (Ca^{2+}) influx. This generates a cascade of various protein activation and ultimately leads to endothelial nitric oxide synthase (eNOS) activation, producing vasodilator nitric oxide (NO). Calmodulin (CaM), calcium-dependent protein kinase (PKC) and serine-threonine kinase/protein kinase B (Akt/PKB) are involved in maintaining a healthy vascular structure. Abnormality in primary cilia has been proposed to promote vascular atherosclerotic formation.

Dopamine signaling is considered to be an important signaling mechanism in the nervous, immune, cardiovascular, and renal systems [134]. Dopamine is an endogenous catecholamine hormone that is mainly produced in the brain and adrenal gland and is also biosynthesized in renal proximal tubules [135,136,137]. Dopamine, an endogenous hormone in the sympathetic nervous system, is known to be intricately involved in the regulation of hypertension. For example, abnormalities in dopamine signaling can contribute to high blood pressure in humans. The five G-protein-coupled dopamine receptors (DR) are categorized into D1-like (DR1 and DR5) and D2-like (DR2, DR3, and DR4) families. Several *in vitro* and *in vivo* experiments confirm the presence of Dopamine 1-like receptors, DR1 and DR5, on primary cilia [59,138,139,140,141]. Studies have identified DR5 receptors in cultured mouse vascular endothelial cilia and mouse arteries *in vivo*. The DR modulates cilia mechanosensory function by altering fluid flow sensitivity. Rat studies also show that dis-integrin and metalloproteinase with thrombospondin motifs 16 (*Adams16*) play a crucial role in blood pressure control. Further, interruption of the *Adams16* gene results in longer vascular endothelial primary cilia and significantly lower systolic blood pressure [58]. To date, there are no drugs available that specifically target DR in the cilia, but studies using agents selective for DR1-like receptor subtypes have shown vasodilatory outcomes in peripheral arteries. Activation of DR5 using dopamine increases ciliary length while inhibition of DR5 leads to the loss of ciliary sensory (chemo and mechano) activity [59]. These results are confirmed by challenging endothelial ciliary knockout cells, *Pkd1*^{-/-} and no or short cilia *Tg737^{orpk/orpk}* with dopamine under static conditions, resulted in a considerably less Ca²⁺ influx than wild-type endothelial cells. As Ca²⁺ fluxes in these cells are often concomitant with activation of eNOS, the results may indicate a potential reestablishment of the missing vasodilatory reactions caused by a failed ciliary generation of NO

biosynthesis. Likewise, there are DR within blood vessels in human, and activation of DR triggers a vasodilatory action [142].

Cilia dysfunction causes abnormal Ca^{2+} signaling and kidney disorders such as autosomal dominant polycystic kidney disease (ADPKD), which is a genetic disease caused by a mutation in ciliary PC1 or PC2 [6]. Cardiovascular malformations including high blood pressure and left ventricular hypertrophy notably contribute to mortality in ADPKD patients. A recent clinical review involving 1877 ADPKD patients shows that the use of antihypertensive medications in ADPKD patients have been increased from 32% in 1991 to 62% in 2008 [143]. This has important clinical consequences as another study has found that border-line hypertension in ADPKD patients show a better response with a dopamine precursor relatively to the angiotensin-converting enzyme inhibitor [144]. When individuals are perfused with 0.25–0.5 $\mu\text{g}/\text{kg}/\text{min}$ of dopamine, the results indicated an upward trend in flow-mediated dilation in ADPKD patients and reported a statistically significant decrease in hypertension [145]. It is currently studied to better understand if the dopamine-induced vasodilation is a cilia-dependent process [146]. A more recent study, however, seems to support the idea of cilia involvement in hypertension [147]. The study shows that cilia function is impaired in endothelial cells from patients with pulmonary arterial hypertension due to the inflammation, and cilia length plays an important role in response to inflammatory signaling, such as pro-inflammatory cytokines and/or anti-inflammatory interleukins. The results show that the pro-inflammatory cytokines help in increase cilia length and is PKA/PKC-dependent, whereas anti-inflammatory interleukins induce a reverse effect on cilia length. It is therefore postulated that the length of endothelial cilia is associated with endothelial function and pulmonary arterial pressure.

2.7 Role of Primary Cilia in Vascular Aneurysm

An aneurysm is a formation of an abnormal swelling in a weak area of a blood vessel that can rupture, leading to bleeding and possibly to death. The most common arteries that can be affected by aneurysm are cerebral arteries and aortic artery. Aneurysm formation and rupture are considered one of the major complications associated with ADPKD, in which PC1 is required for structural integrity of blood vessel [148]. Thus, PC1 and PC2 functions are required in blood vessels [97,98,149], and, any abnormalities in either protein leads to aneurysm formation [150]. Of note: In ADPKD patients, the aneurysm can occur in different arteries such as the aorta, splenic, coronary, and cerebral arteries [151,152,153,154].

Within the arteries, primary cilia play an important role in the structure and the function of endothelial cells [12,60]. Therefore, the absence or dysfunction of primary cilia can induce aneurysm formation and progression during vascular injuries [10,155]. Vascular aneurysms are associated with tissue remodeling due to unusual proliferation of the endothelial cell layers through the hemodynamic fluctuations in fluid-shear forces [156]. Endothelial cilia are required for shear stress-induced Ca^{2+} influx and NO signaling [11], and eNOS deficiency is the hallmark of endothelial dysfunction and associated with cardiovascular complications including aneurysm, indicating the protective role of eNOS [157]. Primary cilia regulate endothelial actin organization and focal adhesion assembly that can affect directional migration and cell permeability through hsp27 and Notch/foxc1b signaling [158,159]. It is therefore thought that the mechano-sensation of primary cilia is essential in promoting proper vascular development.

Previously, we showed that the similarity of the pathogenesis between cyst formation and aneurysm associated with PKD in mice models (*PdgfβCre:Survivin^{flox/flox}*, *PdgfβCre:Pkd1^{flox/flox}* and *PdgfβCre:Tg737^{flox/flox}*). Dysfunction of the primary cilia induces an abnormal survivin expression that results in irregular cytokinesis leading to cell polyploidy, multi-mitotic spindle formation and aberrant cell division orientation. This abnormality in symmetrical cell division and cell ploidy leads to the extension of tissue architecture, developing cysts in the kidney and aneurysm in the vasculature [10]. PKC and Akt are downstream signaling messengers of primary cilia, and they regulate survivin expression following primary cilia activation. Akt is downstream of PKC and can regulate Nuclear Factor- κ B, which regulates the expression of survivin. All in all, the inability of primary endothelial cilia to respond to fluid flow can contribute to the vascular aneurysm.

2.8 Role of Primary Cilia in Atherosclerosis

Atherosclerosis plaques mainly develop in the arterial system with bifurcations, branch points, or the inner curvature of arched arteries. Atherosclerosis plaques are often observed at sites with low and oscillating fluid-flow within the embryonic cardiovascular system [13,57]. Plaques happen most frequently in areas of great curvature and branch points in addition to low fluid forces or non-unidirectional flow [160,161]. Like cilia which are present only at the regions of inner curvature of the artery arch [13], atherosclerotic plaques do not happen homogenously along the circulatory system. A recent report confirms that removing endothelial cilia from the vascular branch points causes abnormal fluid-flow responses that contribute to the atherosclerosis [162]. Moreover, exposure of endothelial cells to oscillatory fluid-flow results in the disengagement of eNOS, which promotes reactive oxygen species (ROS) formation rather than NO, leading to atherosclerosis

plaque growth [163]. There is an upregulation of inflammatory gene expression in areas with disturbed blood flow, and this further promotes plaque formation and hyperlipidemia [164,165].

The role of primary cilia in the development of atherosclerosis has been revealed in the apolipoprotein-E-deficient mouse model (*ApoE*^{-/-}) with a high fat and cholesterol diet [162]. Increasing numbers of the endothelial primary cilia existed in atherogenesis areas under hyperlipidemia-induced lesion formation. *Tek-Cre*•*Ift88*^{C/-}•*ApoE*^{-/-}, in which endothelial *Ift88* was specifically ablated, displayed a significantly greater increase in plaque formation compared to that established by their wildtype littermates. The lack of endothelial cilia in vascular branches result in significant upregulation lymphocyte markers, macrophage marker genes, along with proinflammatory cytokines [162]. Atherosclerosis lesions increase in the mice who lack endothelial cilia by 59% in females, and 67% in males as compared to the control mice. This is measured by counting atherosclerotic lesioned surface area. Furthermore, lacking endothelial cilia enhances inflammatory gene expression and a decrease in endothelial nitric oxide synthase activity. Hence, it is proposed that vascular endothelial cilia play an important role in control of atherosclerosis.

2.9 Role of Primary Cilia in Cell Proliferation

Not only do primary cilia provide a sensory signaling hub, they also play an important role in cell proliferation. Ciliogenesis begins at the G1/G0 phase of the cell cycle, and resorption or disassembly of cilia starts after the cell cycle re-entry. Primary cilia formation is influenced by the coordination of assembly/disassembly equilibrium, IFT system, and membrane trafficking [166]. Specifically, ciliogenesis involves multiple steps and is correlated with cell division. First, the centrosome travels to the cell surface and the basal body is formed by the mother centriole to

nucleate ciliary axoneme at the G1/G0 phase of the cell cycle. This step which involves membrane docking is regulated by the distal appendage proteins, such as centrosomal protein 164 (Cep164). On the other hand, CP110, Odf1, and trichoplein are negative regulators of ciliogenesis targeting ciliary extension. Second, elongation of the cilium and maintenance of ciliary length occur. This process is negatively regulated by Ndel until mature primary cilium is formed. Third, upon cell cycle entry, ciliary resorption occurs followed by axoneme shortening. Ciliary disassembly is controlled by Aurora A-HDAC6, Nek2-Kif24, and Plk1-Kif2A pathways. Fourth, the basal body is released from cilia; thus, centrioles (centrosome) become free to act as microtubule organizing center (MTOC) or spindle poles during mitosis [166,167,168].

In tumors, cilia are not present on most proliferative cells suggesting that although cilia are not directly required during cell proliferation, they do play a key role in the entry and exit of mitosis [169,170,171]. PC1 has been shown to mediate JAK/STAT pathway [172]. Ciliary PC1 is able to activate STAT3; when the cytoplasmic tail of PC1 is cleaved in response to fluid-flow, it can coactivate STAT-1, 3, and 6 as well as JAK2 [92]. The PC1 tail triggers several cytokines and growth factor signaling, amplifying the cellular response and potentially leading to an increase in L-arginine thus arresting cell proliferation.

Although the reason of the absence of cilia in cancer cells is not exactly known, this phenomenon is arguably not surprising given that the presence of cilia is a cell-cycle-dependent process [173]. Thus, cilia are not expected to be present in highly proliferative cells. However, what complicates the discussion is that primary cilia have also been reported in cancers, including in medulloblastoma [34,174], basal cell [33] and gastrointestinal stroma cells [175]. A recent study suggests a possibility of an enzymatic effect in cancer cells [176]. It is shown that posttranslational modification of ciliary tubulin is affected and resulted in less robust formation of primary cilia.

Lacking proper posttranslational modification in ciliary exoneme may therefore increase a risk factor for cancer development [176].

2.10 Conclusion and Perspectives

Both primary cilia structure and sensory functions are essential for normal tissue homeostasis and function. The *in vitro* and *ex vivo* fluid-flow studies have greatly advanced our knowledge of the chemo- and mechano-sensory function of primary cilia in cardiovascular systems. More studies are warranted towards clinical intervention for hypertension, aneurysm and atherosclerosis. Unfortunately, there are no pharmacological agents available that selectively target primary cilia. While this review mostly represents a small portion of possible connections between primary cilia and cardiovascular disorders, we may need a large-scale screening study to include potential pharmacological agents in order to understand whether or not targeting sensory functions of primary cilia would result in better cardiovascular outcomes.

Primary cilia are ubiquitously present in many organ systems, including the cardiovascular system. Emerging data suggest that cilium dysfunction is a primary cause in many cardiac and vascular disorders. Over the past years, researchers have provided tremendous advances in understanding of the basic cellular and molecular functions of primary cilia. Despite the fact that more research is needed, we should also extend ourselves by integrating the basic science knowledge into clinical considerations and perspectives. Otherwise, we are not able to see the forest because we are too focused on the trees.

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Chapter 3: Rapamycin treatment correlates changes in primary cilia expression with cell cycle regulation in epithelial cells

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Author Contribution:

MHJ conceived the idea, performed the majority of the work and prepared the manuscript. ACFN and NDV provided advice and technical assistance on cancer cells; RLB on NK and PKD cells. RR helped in editing and finalizing the manuscript. AMN assisted in data and statistical analyses. SMN conceived the idea, confirmed data analysis and finalized the manuscript. All authors read and approved the final draft manuscript.

3.1. Abstract

Primary cilia are sensory organelles that regulate cell cycle and signaling pathways. In addition to its association with cancer, dysfunction of primary cilia is responsible for the pathogenesis of polycystic kidney disease (PKD) and other ciliopathies. Because the association between cilia formation or length and cell cycle or division is poorly understood, we here evaluated their correlation in this study. Using Spectral Karyotyping (SKY) technique, we showed that PKD and

the cancer/tumorigenic epithelial cells PC3, DU145, and NL20-TA were associated with abnormal ploidy. We also showed that PKD and the cancer epithelia were highly proliferative. Importantly, the cancer epithelial cells had a reduction in the presence and/or length of primary cilia relative to the normal kidney (NK) cells. We then used rapamycin to restore the expression and length of primary cilia in these cells. Our subsequent analyses indicated that both the presence and length of primary cilia were inversely correlated with cell proliferation. Collectively, our data suggest that restoring the presence and/or length of primary cilia may serve as a novel approach to inhibit cancer cell proliferation.

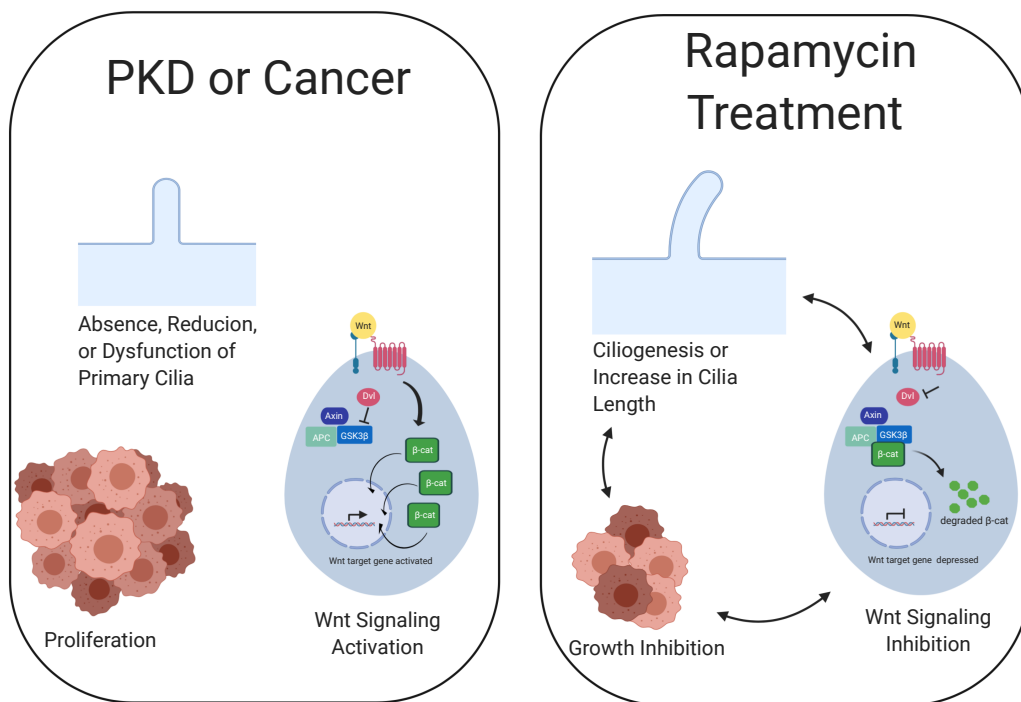


Figure 3.1 Graphical Abstract

3.2. Introduction

Most of the non-hematological cells in humans display sensory primary cilia, which are expressed on the cell surface [1]. Primary cilia act as antennae that transmit extracellular signals into intracellular biochemical responses. Primary cilia regulate cell signaling and key cellular processes, such as proliferation, differentiation, and migration [2-5]. Genetic mutations that disrupt the function of primary cilia can therefore result in a diverse set of diseases called ciliopathies. These disorders involve not only rare congenital syndromes like Joubert syndrome, Bardet-Biedl syndrome, and Meckel syndrome, but also more common diseases such as polycystic kidney disease (PKD) [6-10]. Furthermore, cancer has been proposed as a ciliopathy [11]. The most essential role of cilia in cancer pathogenesis is presumably its regulation on cell cycle and malignancy-related signaling pathways [12-14].

The structure of the cilium can be divided into 3 parts: the basal body, the axoneme, and the transition zone. The timing of cilium formation or ciliogenesis is controlled by the phases of cell cycle [15, 16]. Formation of primary cilia typically begins at the G_1/G_0 phase of the cell cycle when the mother centriole in the centrosome acts as a basal body to start cilia formation [17, 18]. As cells re-enter the cell cycle, the cilium and the basal body disassembled releasing the centrioles to work as the organizing center for the mitotic spindles during cell division [19, 20]. As the cells enter the quiescence or resting phase, the mother centriole forms the basal body and the primary cilium re-assembled. According to this finding [18, 21], primary cilia develop only in quiescent or differentiated cells; therefore, as the proliferation index increases, the number of ciliated cells decrease [22]. Thus, cilium has been hypothesized to regulate the cell cycle and is thought to halt abnormal cell growth by restricting cell cycle [16].

Previous studies report reduction or loss of primary cilia in a variety of cancer types, such as pancreatic cancer, renal cell carcinoma, breast cancer, and cholangiocarcinoma [7, 9, 23, 24]. Loss of the primary cilia in cancer cells may induce cell proliferation and may also participate in abnormal cellular signaling associated with cancer or its formation. Jenks et. al. recently report that enhanced ciliogenesis can facilitate resistance to a number of kinase inhibitors [25]. They show that both acquired and *de novo* resistant cancer cells show an increase in cilia number, and length. Based on the collective evidence and observations, we thus hypothesize that cilia length is associated with cancer progression, and specific pathways in cilia associated with cancer cell cycle can be modulated.

The major ciliary signaling pathways include the Hedgehog [14], Wnt [26] and Platelet-Derived Growth Factor [27]. In particular, Wnt signaling pathway modulates the balance between cellular differentiation, polarity controls and proliferation to regulate tissue homeostasis [1, 28]. The presence of primary cilium controls the expression levels of Wnt target genes by regulating the degradation of Disheveled (Dvl), a protein that is recruited to the membrane and binds axin to prevent β -catenin degradation. Specifically, inversin and nephrolithiasis-3 localized in the primary cilium are involved in the regulation of Dvl level [26, 29]. In addition, sequestering ciliary protein AHI1 to the cilium has been shown to prevent β -catenin to translocate into the nucleus [30].

In this study, we characterized the presence and the length of primary cilia in human cancer cells. We also examined the correlation between primary cilia expression and Wnt signaling pathway. We showed that primary cilia presence and length are reduced in cancer. Moreover, we demonstrated that this loss of primary cilia is associated with an increase in the baseline β -catenin level as a measure of Wnt signaling. Because recent studies have shown that cilia length in

vascular endothelia and renal epithelia of normal and cancer tissues can be regulated pharmacologically [31, 32], we further aimed to restore primary cilia expression in cancer cells using sirolimus (or rapamycin). Our goal was to understand the relationship among Wnt signaling pathway, cell proliferation and primary cilia.

3.3. Materials and Methods

Cell Lines and Culture Conditions

Only human epithelial cells were used in our studies. Both normal kidney (NK) and *PKD2* cells with abnormal cilia function (PKD) have been previously characterized [33, 34]. NK has fully functional primary cilia, while PKD is a well-known model for dysfunctional cilia; thus, we used them as controls in our study. Human prostate cancer cells PC3 (ATCC CRL-1435) [35], DU145 (ATCC HTB-81) [36] and bronchial tumorigenic epithelial cells NL20-TA or NL (ATCC CRL-2504) [37] were obtained from the American Type Culture Collection (ATCC, Manassas, VA). We used these epithelial cells to obtain independent correlation between hyperproliferation and cilia length or cilia formation in the presence or absence of rapamycin (AK Scientific, Union City, CA) treatment. Thus, the presence studies were to examine if there was a correlation in the changes between hyperproliferation and cilia length or cilia formation using these human epithelial cell lines. Cells were supplied with epithelia growth medium (PromoCell, Heidelberg, Germany) supplemented with 15% fetal bovine serum (FBS; Seradignm, Radnor, PA), and were maintained in 5% CO₂ at 37°C under humidified culture conditions. In the experiments that cell confluence was required to induce cilia formation, the cultured cells were incubated with media containing 2% FBS and 0, 1 or 10 μM of rapamycin for 1, 3, and 8 days [31, 32, 38]. For the 8-days treatment, the media and rapamycin were replaced with the fresh preparation on the fourth day. Both

concentrations and durations of rapamycin treatment had also been used in previous studies [31, 32].

Spectral Karyotyping (HiSKY)

We have previously described this methodology in detail [39]. Briefly, after the cells were grown to 60-70% confluent, 0.05 µg/ml of colcemid solution (Adipogen, San Diego, CA) was added to the cells and incubated for 48 hours. After harvesting the cells, they were incubated with a hypotonic solution (0.56% KCl) followed by a fixing solution (methanol/acetic acid). KCl, methanol and acetic were purchased from Fisher Scientific (Fair Lawn, NJ). The chromosomes were next spread on a slide and hybridized with a cocktail of human fluorescence-labeled probes specific for individual chromosomes (Applied Spectral Imaging, Carlsbad, CA). Data were analyzed with the HiSKY Spectral Imaging system from Applied Spectral Imaging.

Immunofluorescent Staining

While cilia may lose some of their structural integrity upon fixation, certain fixation techniques can preserve the substructure of primary cilia and ciliary proteins [40, 41]. Selecting a proper fixation method depends on which ciliary proteins are of interest to the investigators. Generally, proteins that are localized along the axoneme are best preserved with paraformaldehyde fixation. Since axoneme is a microtubule-rich structure that forms the core of primary cilia, antibodies against acetylated- α -tubulin can be used to detect axoneme. Paraformaldehyde fixation (10-min incubation at room temperature) provides a replicable result, maintains an intact microtubule cytoskeleton, and preserves the cytoskeletal labeling. The same technique was therefore used in our study to maintain consistency with what was already established in the cilia field [40, 41].

Briefly, cells were seeded onto coverslips placed in six-well plates. After the cells have reached the required confluency (60-70%), they were cultured for the various time points in maintenance medium with or without rapamycin. The cells on the coverslips were then subjected to a 10 minute-fixation using 4% paraformaldehyde (EMS, Hartfield, PA) and 2% sucrose (Fischer Scientific, Fair Lawn, NJ) in phosphate-buffered saline (PBS; Corning, Manassas, VA). After a PBS wash, the cells were permeabilized using 1% TritonX (Fischer Scientific, Fair Lawn, NJ) in PBS. Acetylated- α -tubulin antibody (1:10,000 dilution, Sigma Aldrich, St. Louis, MO; catalog# T6793) was added to the primary cilia and incubated overnight at 4°C followed by a 1-hour incubation at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (1:1000 dilution, Vector Labs Burlingame, CA; catalog# F1-2000; lot# ZE0803). Actin filaments were stained by incubating the cells for 1 hour at room temperature with Texas Red-conjugated phalloidin (1:400 dilution, Invitrogen, Carlsbad, CA; catalog# T7471, lot# 23749W). Nuclei were stained with mounting media containing Dapi blue fluorescence (Vector Labs Burlingame, CA). The images of the primary cilia were captured by a fluorescence microscope, and their presence and length were analyzed by NIS-Elements software.

Cell Growth

To quantify their growth rate of cells, cells were counted every day for 5 days. On day 0, 3×10^5 cells were seeded and plated on 10 cm culture dishes supplied with growth medium. The evenly distributed cells in each dish were then counted every 24 hours until they were 100% confluent.

Cell Cycle and Proliferation Analyses

After harvesting the cells using trypsin (Corning, Manassas, VA), the cells were fixed using 95% ethanol and incubated at -20°C overnight. In some experiment, cells were first labeled with 10

μ M bromodeoxyuridine/fluorodeoxyuridine (BrdU; Acros Organics, Pittsburg, PA; catalog# AC228595000) for 1 hour at 37°C and 5% CO₂. For DNA denaturing, the cells were incubated with 2N HCl for 30 minutes at room temperature followed by neutralization with 0.1 M sodium borate for 30 minutes at room temperature. Afterwards, the cells were incubated with Alexa 488 conjugated BrdU antibody (Invitrogen, Carlsbad, CA; catalog# A21305, lot# 571730) for 1 hour at room temperature in the dark for BrdU experiments. In other experiments, the cells were stained with propidium iodide (PI) for 1 hour at room temperature in the dark. Cells were then analyzed with flow cytometry BDFacsverse.

Western Blot Analysis

Cells were lysed using lysis buffer (Thermoscientific, Rockford, IL) supplemented with protease inhibitor cocktail (Complete, Mannheim, Germany). The concentrations of protein were determined by using micro bicinchoninic acid assay (BCA) (Thermoscientific, Rockford, IL). A 30 μ g of protein was prepared using 2x Laemmli sample buffer (BioRad, Hercules, CA) and loaded on a 10% SDS-polyacrylamide gel. The gel was run for 1-2 hours at 120V. After transferring the protein from the gel to the membrane (BioRad, Hercules, CA), the membrane was blocked with 5% non-fat dry milk (Lab Scientific, Livingston, NJ) for 2 hours at room temperature. The membrane was then incubated overnight at 4°C with primary antibodies specific for Gli1 Anti-Gli1 antibody (Abcam, Burlingame, CA; catalog# ab49314) [42], smo (LSBio, Seattle, WA; catalog# LS-A2666-50) [43], β -catenin (Abcam, Burlingame, CA; catalog# ab6302, lot# GR3314727-5) [44], phospho-mTOR (Ser2448) (Cell Signaling Technologies, Danver, MA; catalog #2976, clone# 49F9) [45], phospho-mTOR (Ser2481) (Cell Signaling Technologies, Danver, MA; catalog #2974) [45], phospho-p70 S6 Kinase (Thr389) (Cell Signaling Technologies,

Danver, MA; catalog# 9234) [46] and β -actin (Cell Biolabs, San Diego, CA; catalog# AKR-002). Afterwards, the membrane was incubated with secondary anti-rabbit (catalog# 7074) or anti-mouse (catalog# 7076) HRP-linked antibody (Cell Signaling Technologies, Danver, MA) for 1 hour at room temperature. The signals were analyzed by an imager (BioRad, Hercules, CA) after the membrane was subjected to SuperSignal West Pico PLUS Chemiluminescence Substrate (Thermoscientific, Rockford, IL; catalog# 34580, lot# UH290793).

β -catenin immunofluorescence

To differentiate nucleus and cytoplasmic β -catenin, cells were plated onto coverslips placed in six-well plates and allowed to grow to 50-70% confluency before treatments. After cells were treated for the different time points (0, 1, 3 and 8 days) in maintenance medium with or without rapamycin, cells were fixed using 4% paraformaldehyde and 2% sucrose in PBS for 10 minutes. For the 8-days treatment, the media and rapamycin were replaced with the fresh preparation on the fourth day. Cells then were washed with PBS and permeabilized using 1% TritonX in PBS. Next, cells were incubated with anti- β -catenin primary antibody (1:2000) at 4°C overnight followed by a 1-hour incubation at room temperature with FITC-conjugated anti-rabbit IgG secondary antibody (1:1000 dilution, Vector Labs Burlingame, CA; catalog# F1-1000, lot# ZC0202). Nuclei were stained with mounting media containing Dapi blue fluorescence. Images were acquired using NIS-Elements software. For analysis, all images were viewed and randomly captured at 100 \times magnification. For quantification, cells were counted from three different microscopic fields. A region of interest (ROI) was randomly selected in the nucleus and cytoplasm. The ratio of FITC fluorescence in nucleus versus cytoplasm was determined by measuring the FITC fluorescence of the ROI in the nucleus and cytoplasm.

Data and Statistical Analysis

We used NIS-Elements software (version 4.3), Microsoft Excel (version 16.32), and GraphPad Prism (version 8.3) to analyze the presence and length of primary cilia. Whenever feasible, the data was confirmed to be normally distributed prior to the subsequent analyses. Otherwise, the data were transformed logarithmically. The slope of the growth curve was measured by fitting the curve into the sigmoidal-fitted graph. For Western blot analysis, band intensity was captured with Bio-Rad imager (Model no. Universal Hood III. Serial no. 731BR02716. version 5.1), quantified with the NIH Fiji ImageJ (version 2.0), and analyzed with GraphPad. For all studies, a minimum of three independent experiments were performed. The exact number of independent experiments for each study is indicated in the scattered bar graphs or in the figure legends.

The correlation analyses were performed by using Pearson correlation coefficient test. Multiple variable analyses were further performed by using multiple-linear regression test. The Pearson correlation was studied before and after rapamycin treatment with 1-dimensional (before vs. after of one variable) or 2-dimensional (before vs. after of two variables) analysis. To clarify the correlation analyses, scattered plots (before vs. after) were provided to show the strength and weakness of Pearson correlation coefficient. For the 2-dimensional analysis, the correlation for the variables (slope) was first identified before (or after) treatment followed by the correlation before vs. after analysis in the corresponding scattered plot.

All data were reported as mean±standard error of mean (SEM). A *p* value of <0.05 was considered statistically significant. Statistical analysis comparing multiple groups was performed by using ANOVA test followed by Tukey's post-test or Dunnet post-test. Significant differences relative to the control baseline within each group are indicated in asterisk (*). Differences with normal

kidney (NK) epithelia are indicated in a hash sign (#). The level of significant difference (p value) is indicated in each graph and figure legends.

3.4. Results

PKD and Cancer Cells were Characterized by Abnormal Ploidy.

Because genomic instability has been associated with dysfunction of primary cilia [33, 47-49], we studied chromosome numbers in PKD and cancer cells using SKY technique to authenticate our cells. Karyotyping analyses revealed that NK had a normal chromosomal composition (Fig. 2.2A). In contrast, PKD had abnormal ploidy (77,XX) (Fig. 2.2B). PC3 karyotyping analysis also showed abnormal ploidy (104,XY) (Fig. 2.2C). The abnormal polyploidy was also apparent in DU145 (72,XY) (Fig. 2.2D). Karyotyping analysis of NL showed an abnormal increase in the number of chromosomes (109,XX) (Fig. 2.2E). Overall, karyotype analysis of individual cells confirmed that the abnormal ploidy was associated with PKD and cancer cells (Fig. 2.2F). The chromosomal spread for each representative image is shown (Fig. 2.3). A more specific ploidy of each chromosome of each cell type is also presented (Table 2.1).

Primary Cilia Expression was Decreased in Cancer Cells.

To characterize the expression of primary cilia in different cell types, the presence of cilia was determined by immunofluorescence using antibody against acetylated- β -tubulin (**Fig. 2.4A**). Actin filaments was stained with phalloidin and nuclei were stained with DAPI to simply identify individual cells. The representative images show that primary cilia were expressed in NK, PKD, and PC3, but they were absent in both DU145 and NL (**Fig. 2.4B**). Even though cilia were present

in PC3, the percentages of the primary cilia were significantly lower than those observed in NK. However, there was no significant difference in cilia length among NK, PKD, and PC3. The distribution of cilia lengths in each cell type was tabulated (**Fig. 2.4C**).

PKD and Cancer Epithelia were Highly Proliferative.

Since primary cilia play an important role in cell cycle regulation [16], we assessed the proliferation profile of each cell type. The growth rates of the five cell types over a period of five days were examined by counting the cell number every day (**Fig. 2.5A**). The growth rates were significantly higher in PKD and cancer cells than NK cells. We also assessed the proliferative index by examining the DNA contents because some of the cancer cells continued to divide even after they have become confluent. The relative percentage of cells in each phase (G_1 and G_2/M) was quantified and analyzed with flow cytometry. The post-analysis graphs (**Fig. 2.5B**) and pre-analysis histogram (**Fig. 2.6**) from flow cytometry studies are presented. In confluent condition, the percentage of the cells in the G_2/M phase was significantly higher in PC3 and DU145 than NK. This effect was associated with lower percentage of PC3 and DU145 cells in the G_1 phase. In non-confluent condition, there was a significant increase in the accumulation of the cells in the G_2/M phase and a significant decrease in the accumulation of the cells in the G_1 phase in PKD, PC3, DU145, and NL cells compared to NK. Our data indicated that compared to control normal NK cells, both PKD and cancer epithelia had higher proliferative rate.

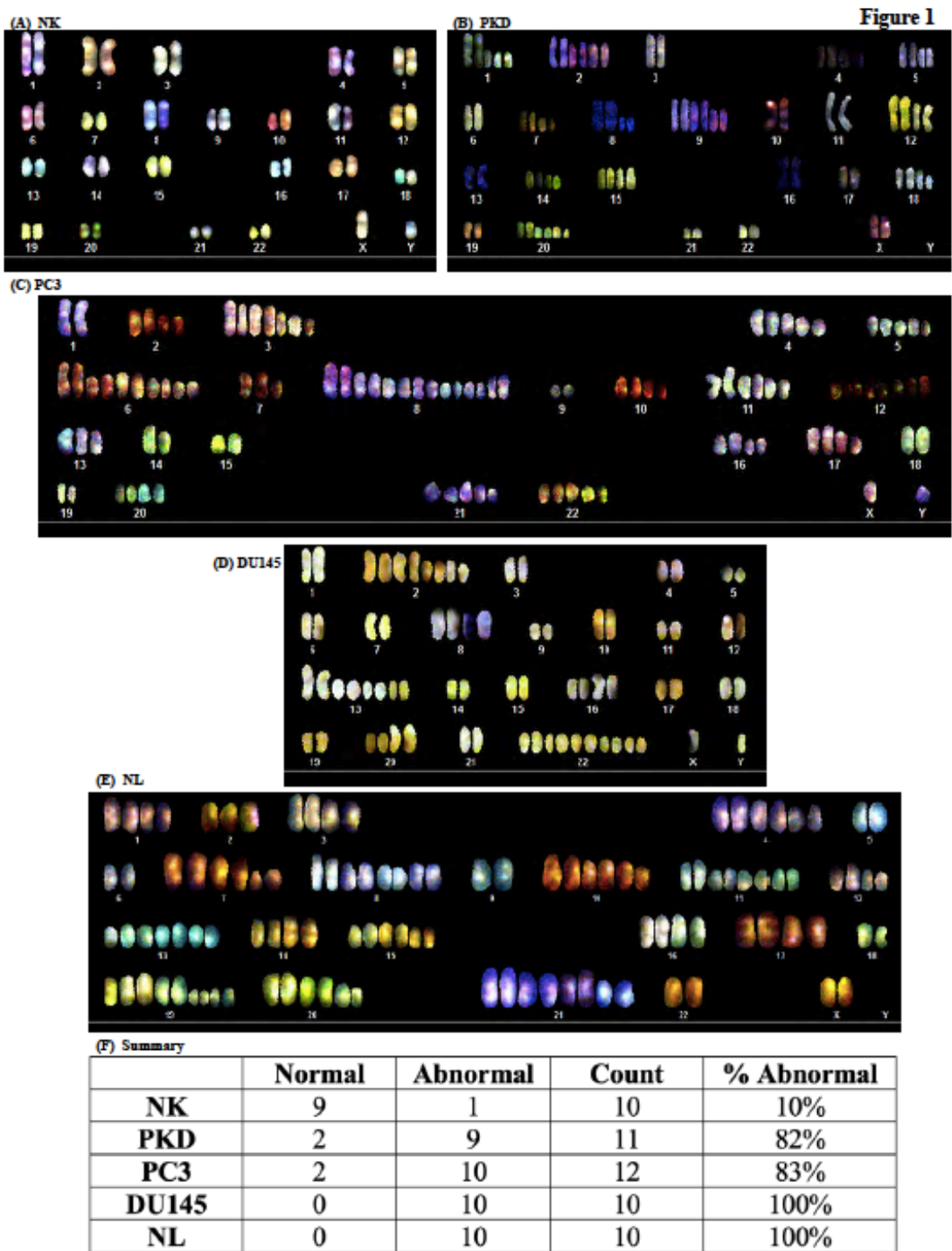


Figure 3.2 Karyotyping analyses of human epithelial cells.

Spectral karyotyping shows somatic chromosomes (1 to 22) with a pair of sex chromosomes (XY). Representative images show epithelium from (A) normal kidney (NK) with normal chromosome number (46, XY), (B) PKD (77,XX), (C) PC3 prostate cancer (104,XY), (D) DU145 (72,XY), and (E) NL (109,XX). (F) Summary of overall karyotype analysis of individual cells confirmed the abnormal ploidy associated with PKD and cancer cells. N=10-12 for each cell type.

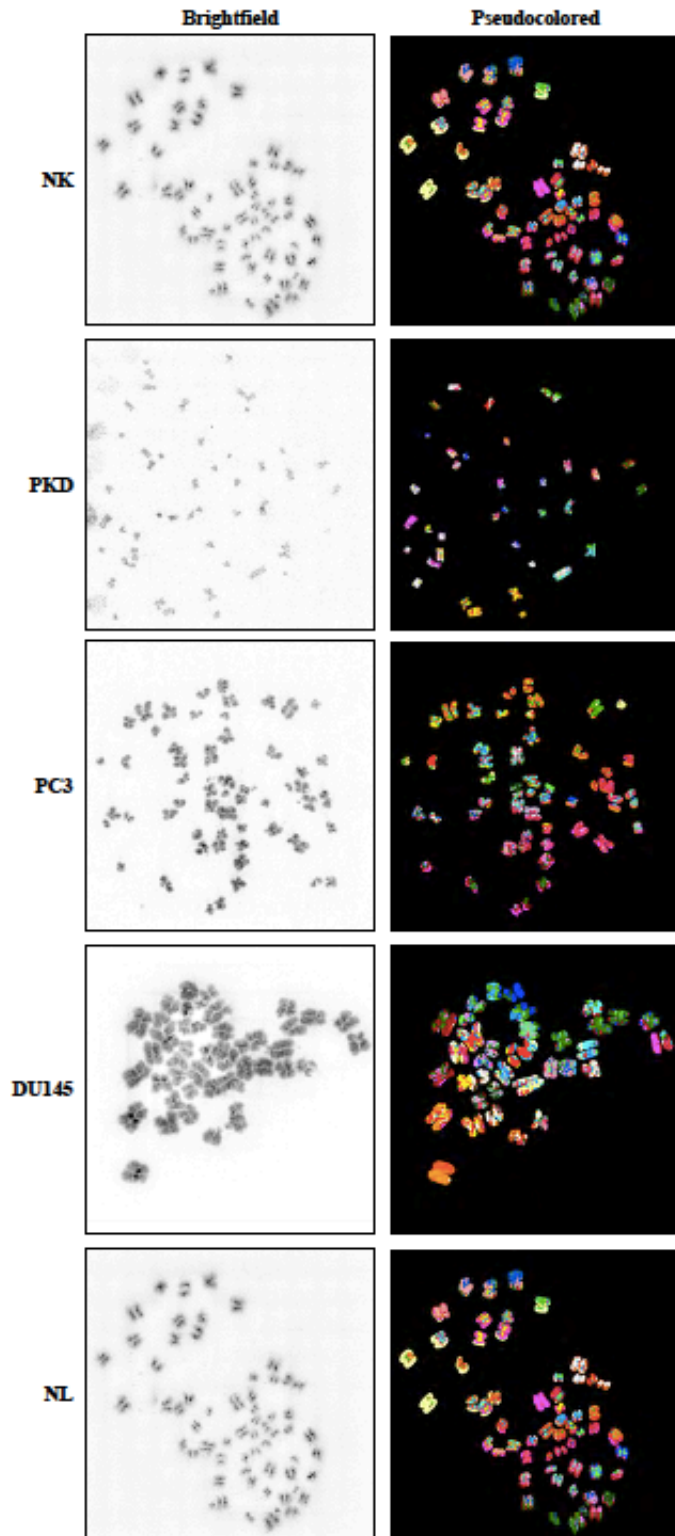


Figure 0.3 Representative images of metaphase spread.

Shown here are images in brightfield (on the left) and pseudocolored (on the right) of NK, PKD, PC3, DU145, and NL.

Table 0.1 Chromosomal abnormality (frequency) in epithelia

Chromosome number	NK	PKD	PC3	DU145	NL
1	Normal	Polyploidy / Aneuploidy (9/11)	Normal	Normal	Polyploidy (10/10)
2	Normal	Polyploidy / Aneuploidy (9/11)	Polyploidy / Aneuploidy (10/12)	Polyploidy (10/10)	Polyploidy / Aneuploidy (10/10)
3	Normal	Normal	Polyploidy / Aneuploidy (10/12)	Normal	Polyploidy (10/10)
4	Normal	Polyploidy / Aneuploidy (9/11)	Polyploidy / Aneuploidy (10/12)	Normal	Polyploidy (10/10)
5	Normal	Polyploidy (9/11)	Polyploidy / Aneuploidy (10/12)	Normal	Normal
6	Normal	Normal	Polyploidy / Aneuploidy (10/12)	Normal	Normal
7	Aneuploidy (1/10)	Polyploidy / Aneuploidy (9/11)	Polyploidy / Aneuploidy (10/12)	Normal	Polyploidy (10/10)
8	Normal	Polyploidy (9/11)	Polyploidy / Aneuploidy (10/12)	Polyploidy (10/10)	Polyploidy (10/10)
9	Aneuploidy (1/10)	Polyploidy / Aneuploidy (9/11)	Normal	Normal	Normal
10	Polyploidy (1/10)	Normal	Polyploidy / Aneuploidy (10/12)	Normal	Polyploidy (10/10)
11	Normal	Normal	Polyploidy / Aneuploidy (10/12)	Normal	Polyploidy (10/10)
12	Normal	Polyploidy / Aneuploidy (9/11)	Polyploidy / Aneuploidy (10/12)	Normal	Polyploidy (10/10)
13	Normal	Normal	Polyploidy / Aneuploidy (10/12)	Polyploidy (10/10)	Polyploidy / Aneuploidy (10/10)
14	Normal	Polyploidy / Aneuploidy (9/11)	Normal	Normal	Polyploidy (10/10)
15	Normal	Polyploidy / Aneuploidy (9/11)	Normal	Normal	Polyploidy (10/10)
16	Normal	Normal	Polyploidy (10/12)	Polyploidy (10/10)	Polyploidy (10/10)
17	Polyploidy (1/10)	Normal	Polyploidy / Aneuploidy (10/12)	Normal	Polyploidy (10/10)
18	Normal	Polyploidy / Aneuploidy (9/11)	Normal	Normal	Normal
19	Normal	Normal	Normal	Normal	Polyploidy / Aneuploidy (10/10)
20	Normal	Polyploidy / Aneuploidy (9/11)	Polyploidy / Aneuploidy (10/12)	Polyploidy (10/10)	Polyploidy / Aneuploidy (10/10)

21	Aneuploidy (1/10)	Normal	Polyploidy / Aneuploidy (10/12)	Normal	Polyploidy (10/10)
22	Normal	Normal	Polyploidy / Aneuploidy (10/12)	Polyploidy (10/10)	Polyploidy / Aneuploidy (10/10)
X	Normal	Normal	Normal	Normal	Normal
Y	Normal		Normal	Normal	

Note: NK, normal kidney epithelia; PKD, polycystic kidney epithelia; PC3, prostate cancer epithelia;

DU145, prostate cancer epithelia; NL, cancer lung epithelia

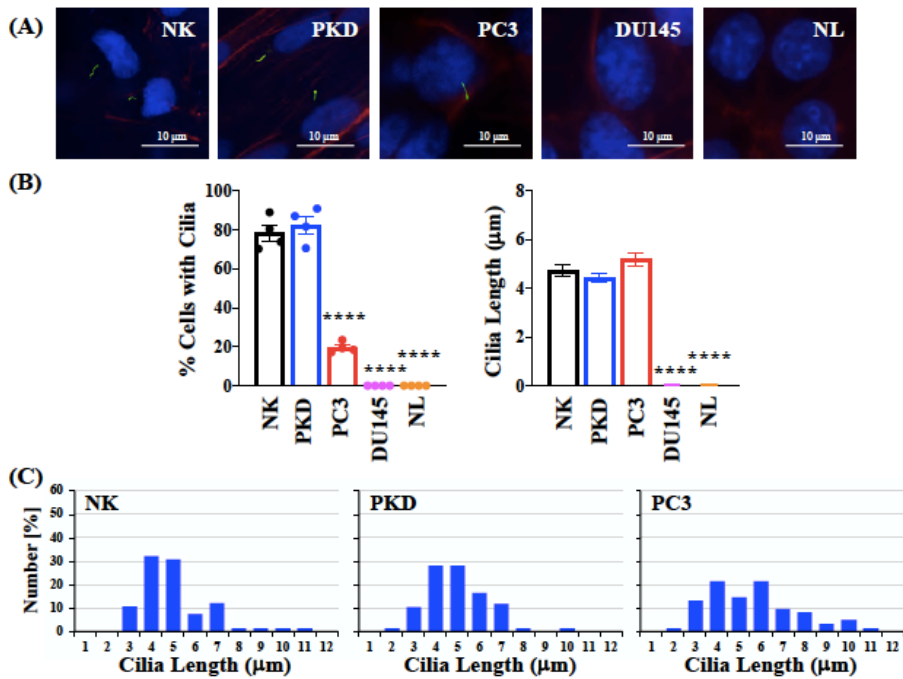


Figure 4.4 Evaluation of primary cilia expression and length in epithelial cells.

(A) Representative images of primary cilia in human epithelial cells. Primary cilia were identified by immunofluorescence using antibody against acetylated α -tubulin (green); actin filaments using texas red-conjugated phalloidin (red); and nuclei using DAPI (blue). (B) The percent of cells with cilia and the average cilia length of each distribution of cilia in each cell type. (C) Histograms depict the distribution of cilia lengths in each cell type. Values are represented as mean \pm SEM. ****, $p < 0.0001$ compared with the control (NK) cells. N=4 independent experiments.

Rapamycin Partially Restored Primary Cilium Expression in Cancer Cells.

Because primary cilium regulates cell cycle progression and can stop abnormal cell growth by restricting cell cycle [16, 22], restoration of the primary cilium in cancer cells may reduce cell proliferation. Rapamycin has been previously shown to increase cilia formation and length [31, 32]. Therefore, we treated the cells with different concentrations of rapamycin at different time points (1, 3, and 8 days). Rapamycin did not induce ciliogenesis on day-1 and day-3 (data not shown), while 1 μ M and 10 μ M of rapamycin treatment on day-8 appeared to restore cilia formation in PC3, DU145, and NL (**Fig. 2.7A**). Treatment of the cells with 1 μ M and 10 μ M of rapamycin significantly increased the expression of primary cilia in PC3, DU145, and NL compared to the control cells (**Fig. 2.7B**). The cilia length was significantly increased with 1 or 10 μ M of rapamycin treatment compared to the control cells in NK, PKD, DU145, and NL but not in PC3. Cilia lengths of each cell type were tabulated (**Fig. 2.7C**).

Rapamycin Treatment Inhibited Cell Proliferation.

Treatment with 10 μ M of rapamycin caused a significant increase in primary cilia expression in the cancer epithelial cells. A concentration of 10 μ M was therefore selected for the rest of our experiments. Before and after the cells were treated with 10 μ M of rapamycin for 1, 3, and 8 days, cell proliferation (defined as the percentage of cells with an increase DNA synthesis) was assessed and analyzed by flow cytometry (**Fig. 2.8**). In all cell lines, rapamycin treatment at different time points significantly increased the percentages of the cells in G₁ phase (**Fig. 2.8A**). Conversely, the

percentages of the cells in G₂/M phase were significantly decreased by rapamycin treatment (Fig. 2.8B). We also validated the cell proliferation data using an independent BrdU staining method by determining the incorporation of the thymine analogs into newly synthesized DNA (Fig. 2.9). We found that rapamycin treatment for 8 days significantly reduced the percentage of BrdU-positive cells compared to untreated control cells (Fig. 2.10).

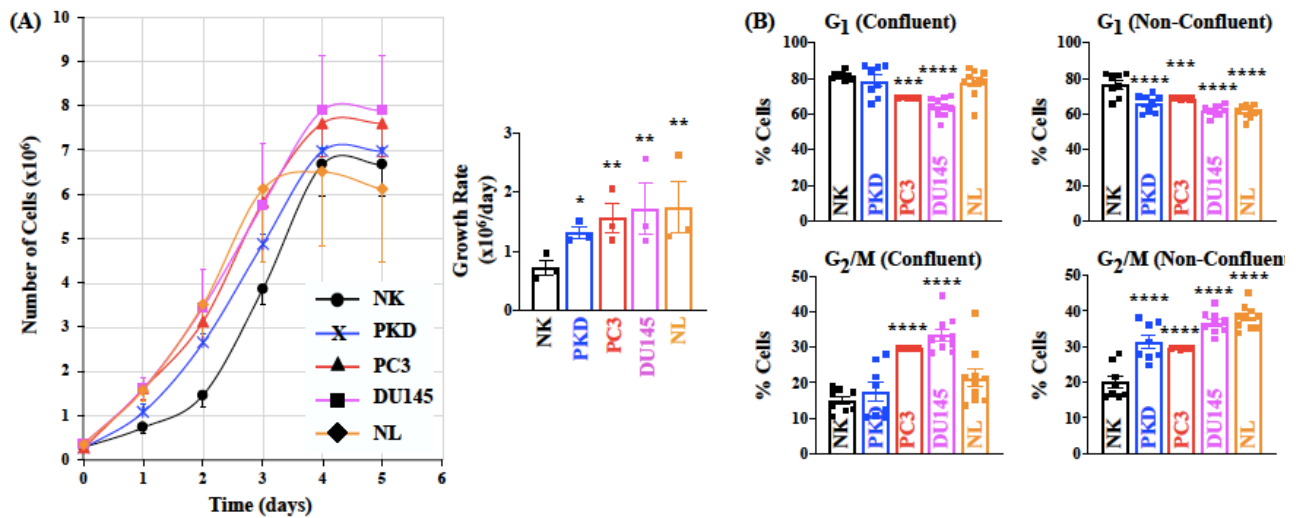


Figure 3.5 PKD and Cancer Epithelia were Highly Proliferative.

(A) The growth rates of the five cell types over a period of five days were examined by counting the cell number in each of the five days. (B) Quantitation of cell cycle phases in selected cells using propidium iodide. The relative percentages of cells in G₁ and G₂/M under confluent condition or non-confluent condition are shown on this graph. Values are represented as mean±SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$ compared with the control NK. N=3 for cell growth; N=8 for cell cycle analysis.

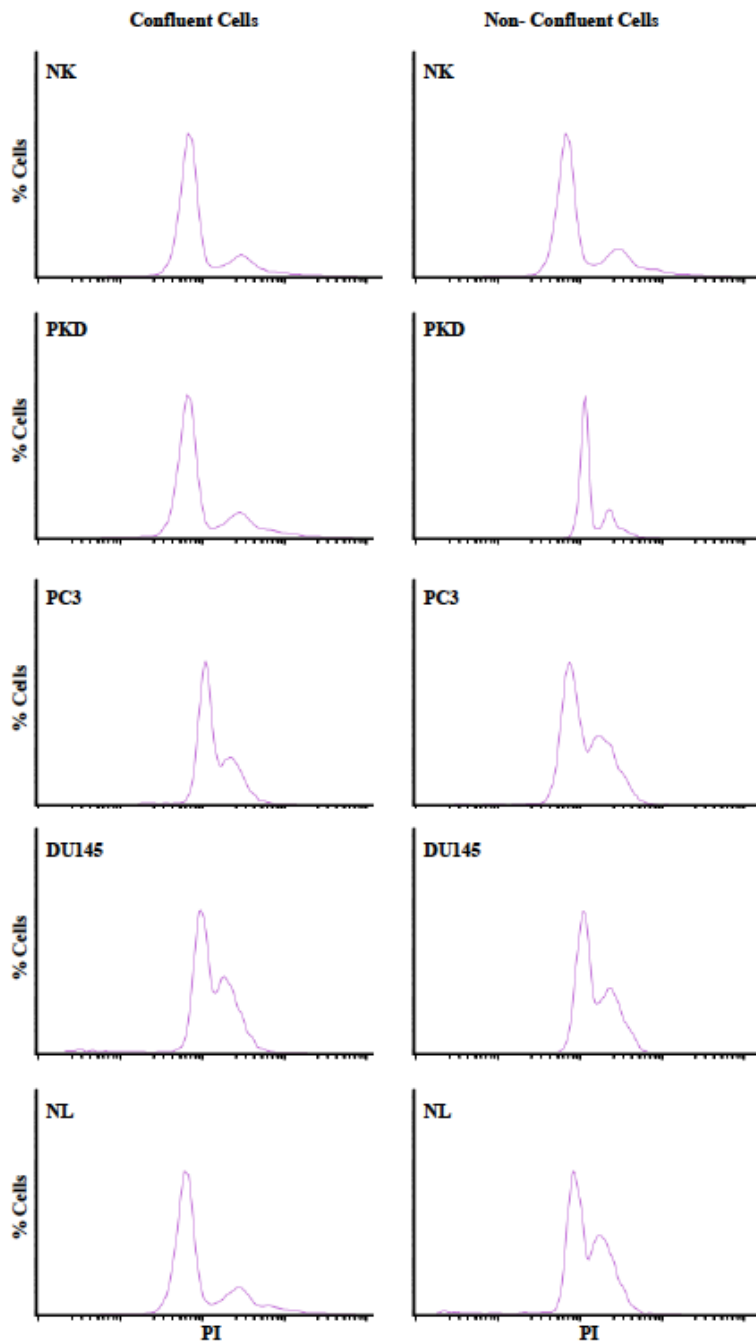


Figure 3.6 Quantitation of G₁ and G₂/M phases.

Representative graphs show the percentages of cells with varying intensity of PI (propidium iodide) staining of NK, PKD, PC3, DU145, and NL under confluent and non-confluent conditions.

Effects of Rapamycin Treatment on Cell Proliferation, Cilia Expression, and Cilia Length.

Pearson's correlation coefficient was used to measure the strength of the association between control and rapamycin treatment on the changes in cell proliferation, cilia expression or cilia length (**Fig. 2.11**). The summary graphs before and after rapamycin treatment (**Fig. 2.11A**) were analyzed and derived using Pearson's correlation, in which linear regression graphs were used to show the strength of the correlation (**Fig. 2.11B**). The rapamycin treatment was inversely correlated with the percent of cells in G₂/M phase ($r=0.730$, $p=0.162$) but was positively correlated with the percent of cells with cilia ($r=0.986$, $p=0.002$) and cilia length ($r=0.869$, $p=0.056$).

We subsequently analyzed the associations among cell proliferation, cilia expression and cilia length (**Fig. 2.12**). The summary graphs between each association (**Fig. 2.12A**) were analyzed and derived using Pearson's correlation, in which linear regression graphs were used to show the strength of the correlation (**Fig. 2.12B**). The percent of cells with cilia was inversely correlated with the percent of cells in G₂/M phase ($r=0.843$, $p=0.028$). Cilia length was also inversely correlated with the percent of cells in G₂/M phase ($r=0.964$, $p=0.003$). As expected, cilia length was positively correlated with the percent of cells with cilia ($r=0.515$, $p=0.172$). These results indicated that rapamycin treatment was associated with increased cilia expression/length and decreased cell proliferation.

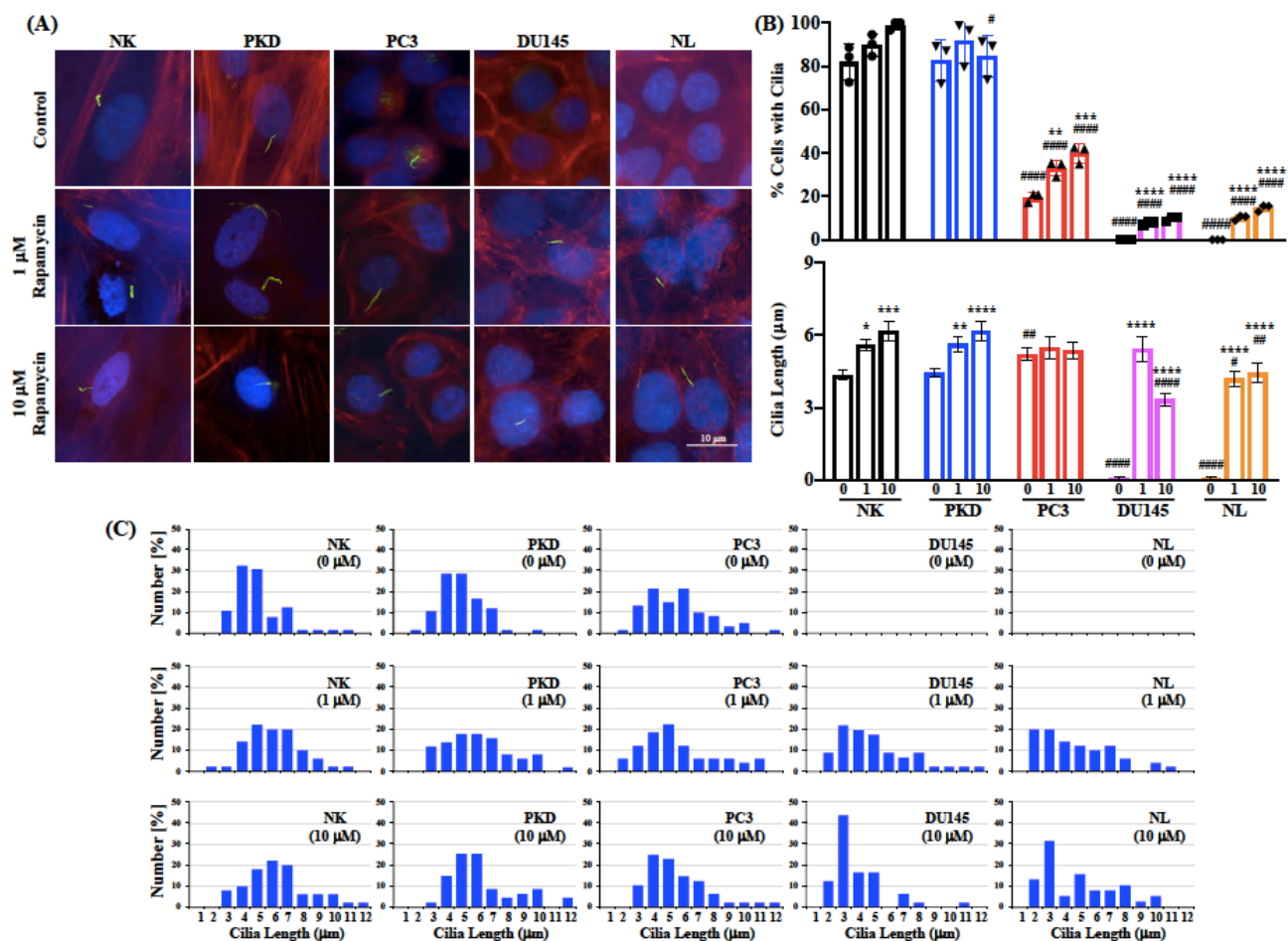


Figure 3.7 The effect of rapamycin treatment on ciliogenesis.

(A) The representative images that show primary cilia expression after treatment with 0, 1 or 10 μM of rapamycin for 8 days in NK, PKD, PC3, DU145, and NL. Primary cilia were identified by immunofluorescence using antibody against acetylated α -tubulin (green); actin filaments using texas red-conjugated phalloidin (red); and nuclei using DAPI (blue). (B) The percentages of cells with cilia and the average cilia length after treatment with 0, 1, or 10 μM of rapamycin for 8 days in NK, PKD, PC3, DU145, and NL. (C) Histograms show the distribution of cilia length after rapamycin treatment (0, 1, or 10 μM). Values are represented as mean \pm SEM. *, $p < .05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$ compared to control baseline of corresponding group. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; and ####, $p < 0.0001$ compared to normal kidney (NK) epithelia. $N=3$ independent experiments with a total of at least 150 cilia measurements. (NOTE: technically the ANOVA test results should be reported first, i.e., their p values. Only if their p values are significant, then the post-test analysis need to be performed. As of now, the ANOVA p values are not reported.)

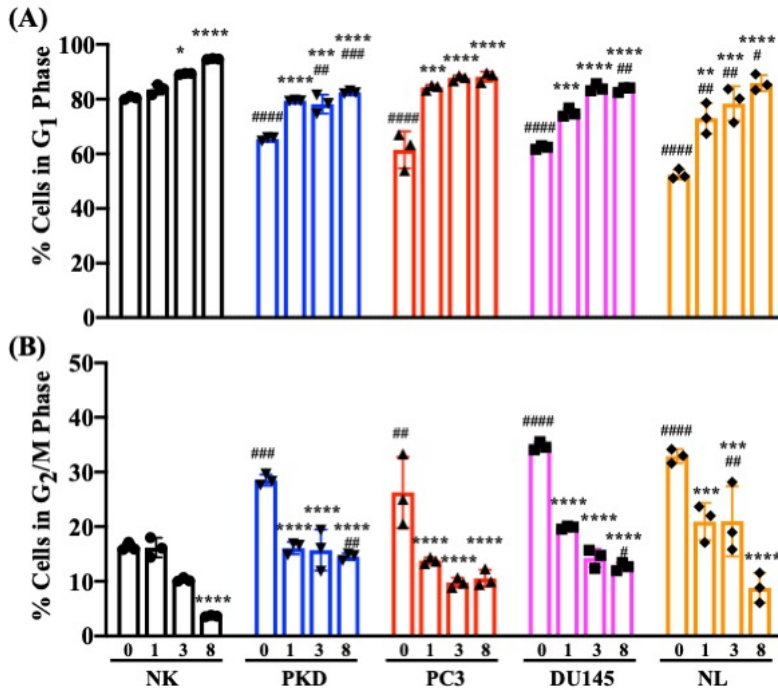


Figure 3.8 Inhibition of cell proliferation by rapamycin using propidium iodide.

Quantitation of cell cycle phases using propidium iodide. The relative percentages of cells in (A) G₁ and (B) G₂/M before and after treatment with 10 μ M of rapamycin for 1, 3, and 8 days in NK, PKD, PC3, DU145, and NL. Values are represented as mean \pm SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$ compared to control baseline of corresponding group. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; and ####, $p < 0.0001$ compared to control NK. N=3 independent experiments.

Rapamycin Treatment in Wnt/ β -catenin Signaling Pathway.

The level of hedgehog as well as Wnt/ β -catenin signaling molecules were compared among different cell types using Western blot analyses (data not shown). The expression levels of β -catenin, the hallmark indicator of the canonical Wnt signaling pathway, were higher in PKD, PC3, DU145, and NL compared to NK. However, there seemed to be no difference in the expression levels of Gli1 and smoothened (smo) among the different cell types.

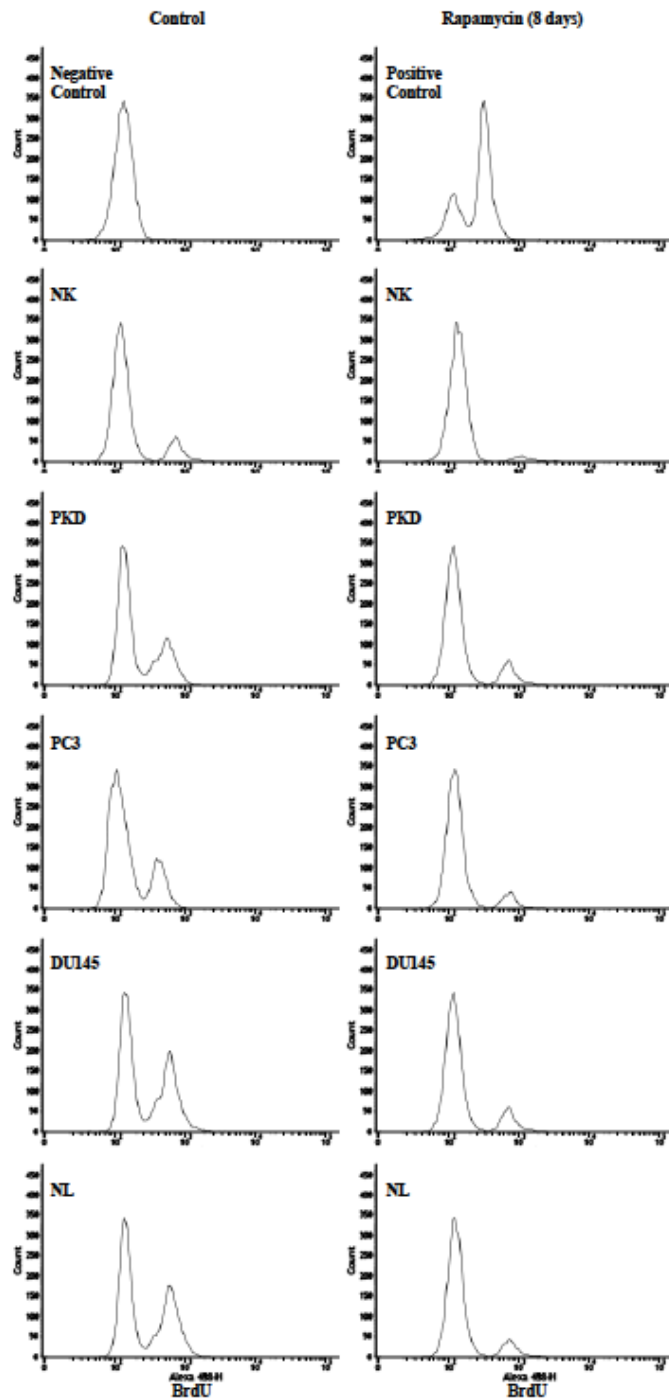


Figure 3.9 Analysis of BrdU incorporation.

Representative graphs show the numbers of cells (count) with varying incorporation (intensity) of BrdU staining in NK, PKD, PC3, DU145, and NL before and after treatment with 10 μ M rapamycin for 8 days.

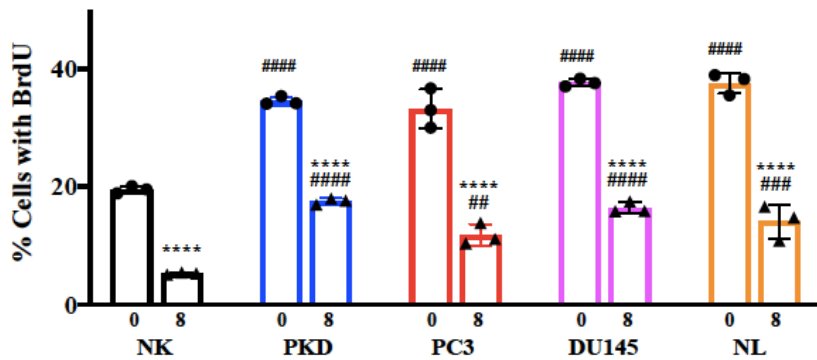


Figure 3.10 Inhibition of cell proliferation by rapamycin using BrdU.

The relative percentages of cells with BrdU before and after treatment with 10 μ M rapamycin for 8 days in NK, PKD, PC3, DU145, and NL. Values are represented as mean \pm SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$ compared to control baseline of corresponding group. #, $p<0.05$; ##, $p<0.01$; ###, $p<0.001$; and ####, $p<0.0001$ compared to control NK. N=3

We subsequently evaluated the effects of 10 μ M of rapamycin treatment for 1, 3, and 8 days on β -catenin expression level (**Fig. 2.13A**). On day 1 and 3 of rapamycin treatment, the expression level of β -catenin significantly increased in NK, PKD, and DU145 compared to their untreated cells. The expression level of β -catenin significantly decreased in NK, PKD, and PC3 compared to their untreated cells on day 8. Because rapamycin is a potent inhibitor of mTOR (mammalian target of rapamycin), the effects of rapamycin on the phosphorylation of mTOR at Ser2448 and its downstream target p70 S6-Kinase (S6K) were measured using Western blot. The phosphorylation of mTOR at Ser2448 was significantly reduced on day 1, 3, and 8 after rapamycin treatment

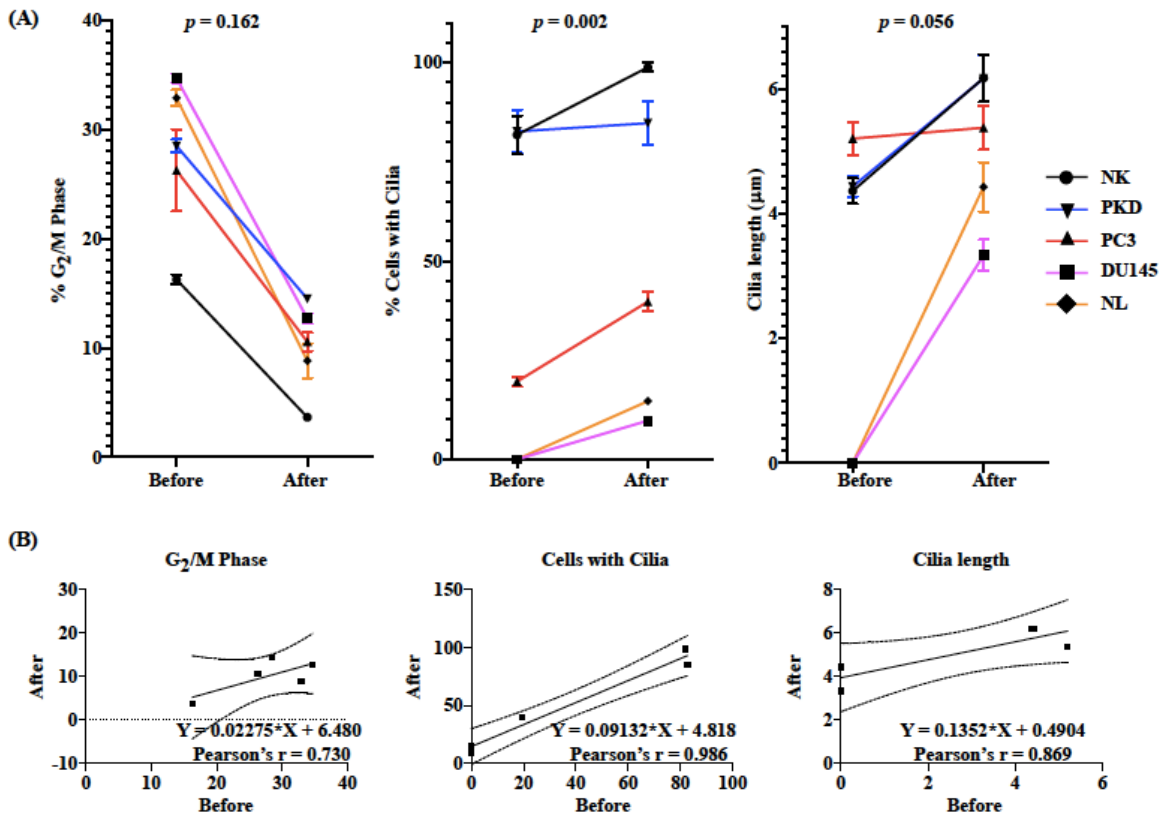


Figure 3.11 One-Dimensional Correlation Analysis.

(A) Pearson correlation was used to evaluate the association before and after rapamycin treatment on the changes in the percentage of cells in G₂/M phase, percentage of cells with cilia, and cilia length. The p-value (*p*) represents the significance of the correlation coefficient. (B) The results of Pearson linear regression analysis are shown in scattered plots. The scattered plots show changes in each variable before and after rapamycin treatment. Pearson correlation coefficient (*r*) shows the regression line and the upper and lower 95% confidence limits.

compared to the corresponding non-treated NK, PKD, PC3 and DU145. In NL, rapamycin did not change the phosphorylation of mTOR at Ser2448 on day 1 and 3; however, on day 8 rapamycin significantly increased mTOR phosphorylation. The phosphorylation of S6K was significantly reduced at the following days: 8 days after rapamycin treatment in NK; 1, 3, and 8 days after rapamycin treatment in PKD and PC3 cells; 1 and 3 days after rapamycin treatment in DU145 cells; and 3 days after rapamycin treatment in NL. On the other hand, the phosphorylation of S6K

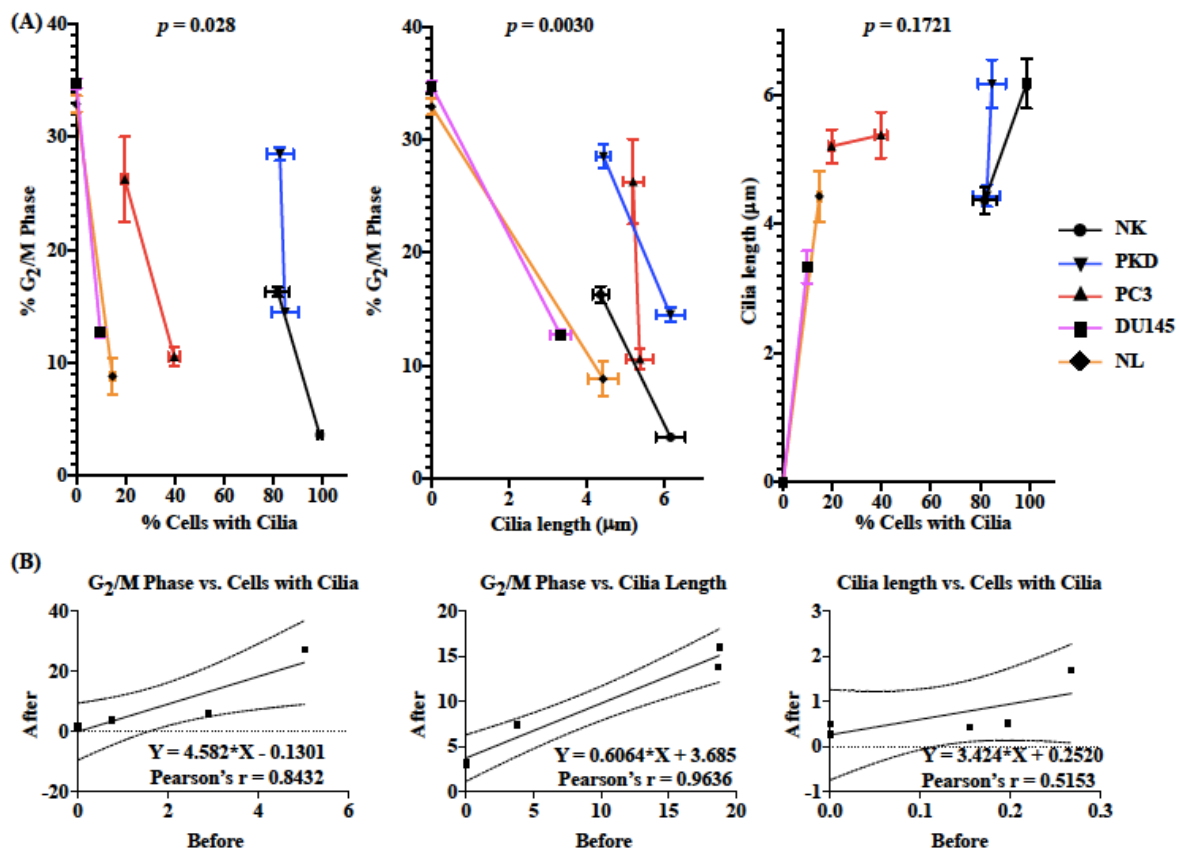


Figure 3.12 Two-Dimension Correlation analysis data

(A) Pearson correlation was used to evaluate the correlations of the changes in cilia expression vs. cell proliferation, cilia length vs. cell proliferation, and cilia expression vs. cilia length. The p-value (p) represents the significance of the correlation coefficient. (B) The results of Pearson linear regression analysis are shown in scattered plots. The scattered plots show changes in two variables before and after rapamycin treatment. Pearson correlation coefficient (r) shows the regression line and the upper and lower 95% confidence limits.

was significantly elevated on day 8 of rapamycin treatment compared to untreated NL. Because rapamycin did not inhibit the phosphorylation of mTOR at Ser2448 in NL, the effect of rapamycin on the phosphorylation of mTOR at another major site (Ser2481) was examined (**Fig. 2.13B**). The

phosphorylation of mTOR Ser2481 was significantly reduced on day 1, 3, and 8 after rapamycin treatment compared to the expression in the absence of rapamycin in NL.

Immunofluorescence analysis was performed to determine the translocation of β -catenin into the nucleus (**Fig. 2.13C**). In NK and PKD, 10 μ M rapamycin treatment for 1 day significantly increased the translocation of β -catenin into the nucleus while treatment for 8 days significantly decreased the β -catenin nuclear translocation compared to untreated control. In PC3 and DU145, treating the cells with rapamycin for 3 days significantly increased the β -catenin nuclear translocation and significantly decreased nuclear β -catenin after 8 days of treatment. The nuclear β -catenin was significantly reduced by rapamycin treatment in NL.

Original Western blot images prior to cropping are presented to show the effects of rapamycin on β -catenin, S6K, mTOR phosphorylation at S2448 (**Fig. 2.14A**) and S2481 (**Fig. 2.14B**).

Representative images are also shown to determine cytosolic and nuclear β -catenin (**Fig. 2.15**).

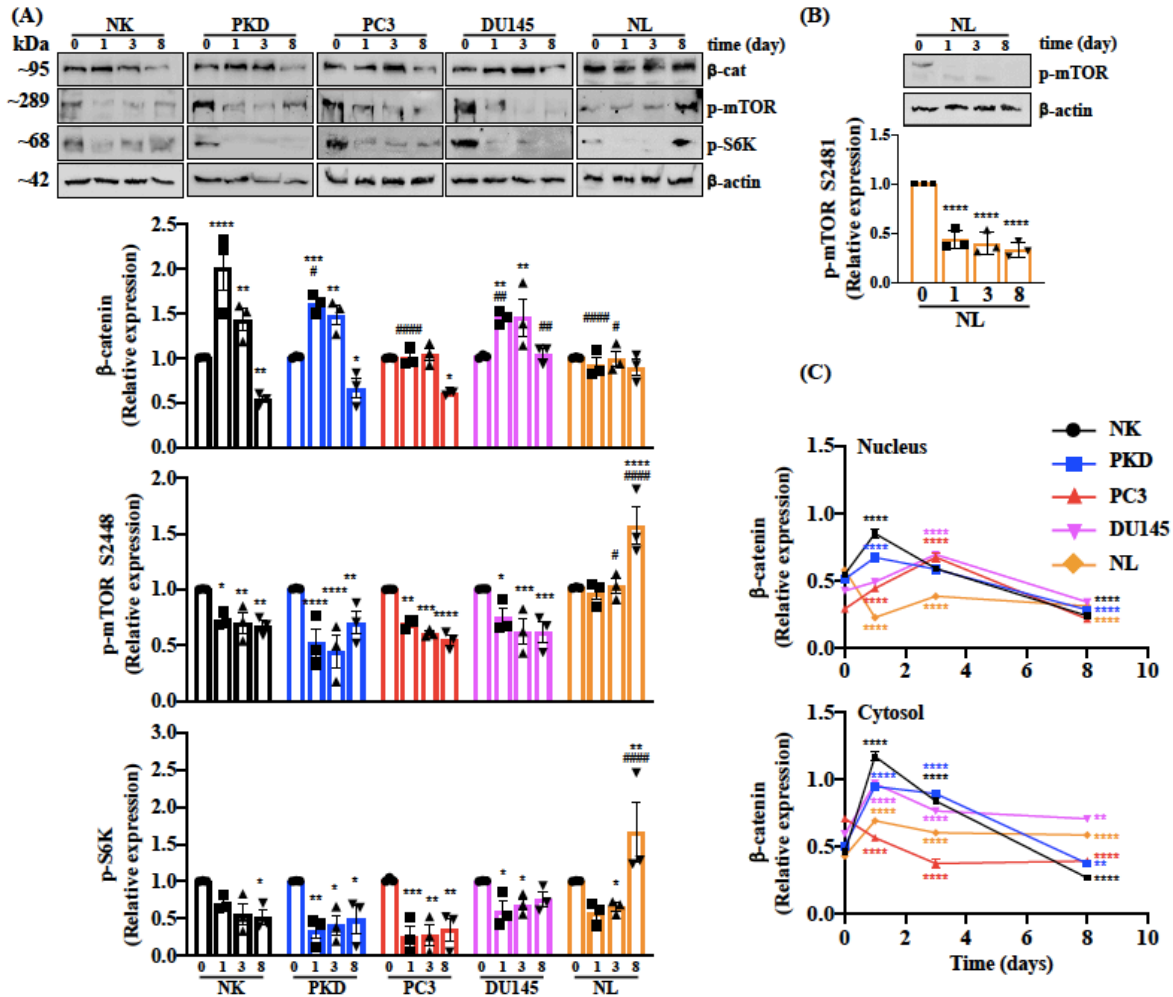


Figure 3.13 Effects of Rapamycin on Signaling Molecules.

(A) The protein expressions of β -catenin, p-mTOR (Ser2448), p-S6k, and β -actin were analyzed before and after treatment with 10 μ M of rapamycin for 1, 3, and 8 days in NK, PKD, PC3, DU145, and NL. (B) The protein expressions of p-mTOR (Ser2481) was separately analyzed in NL. Relative expression levels are expressed as the density ratio relative to β -actin. (C) Quantifications of nuclear and cytosolic accumulation of β -catenin were measured before and after treatment with 10 μ M of rapamycin for 1, 3, and 8 days in NK, PKD, PC3, DU145, and NL. Values are represented as mean \pm SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$ compared to control baseline of corresponding group. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; and ####, $p < 0.0001$ compared to control NK. N=3 independent experiments.

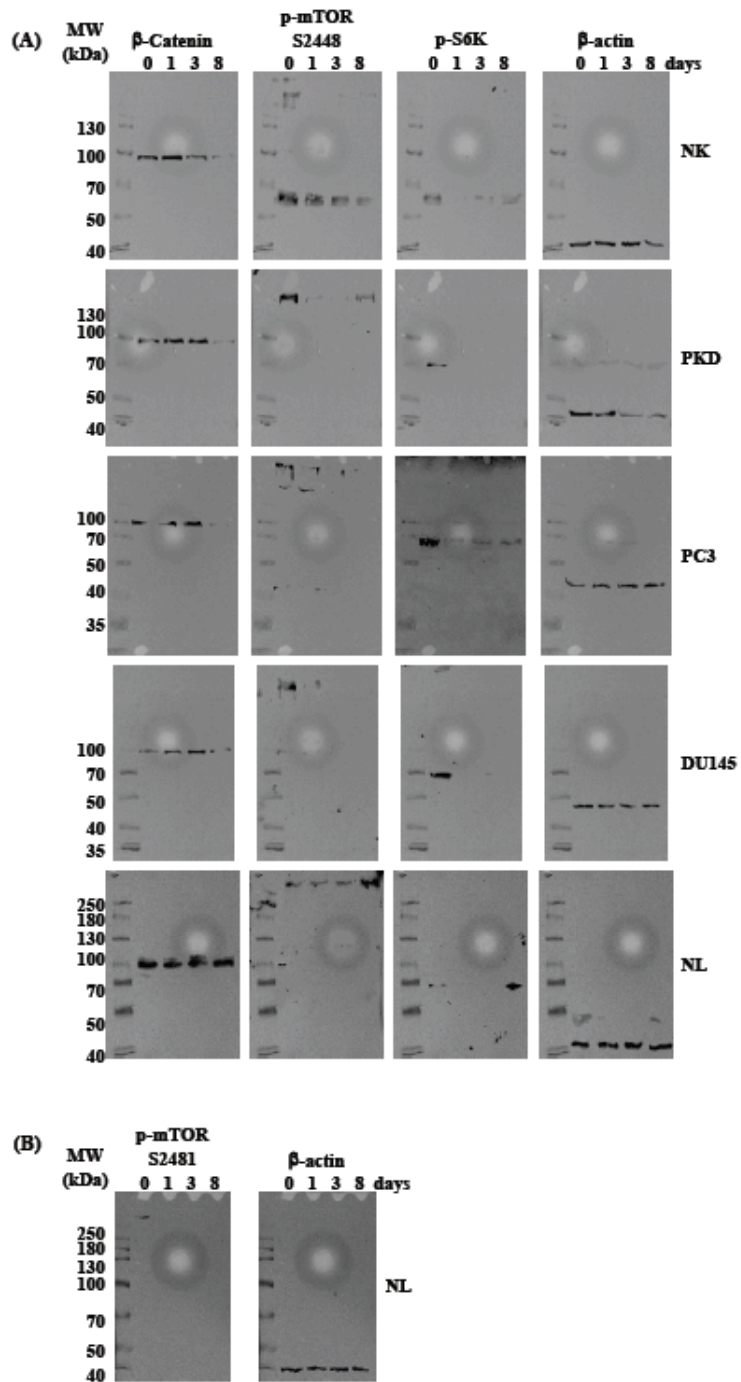


Figure 3.14 Representative Western blot images.

(A) Original, uncropped immunoblots of β -catenin, p-mTOR (Ser2448), p-S6k, and β -actin are shown before and after treatment with 10 μ M of rapamycin for 1, 3, and 8 days in NK, PKD, PC3, DU145, and NL. (B) Original blots of p-mTOR (Ser2481) and β -actin are shown before and after treatment with 10 μ M of rapamycin for 1, 3, and 8 days in NL. The molecular weight (MWs) of the proteins are shown on the left of each corresponding blot.

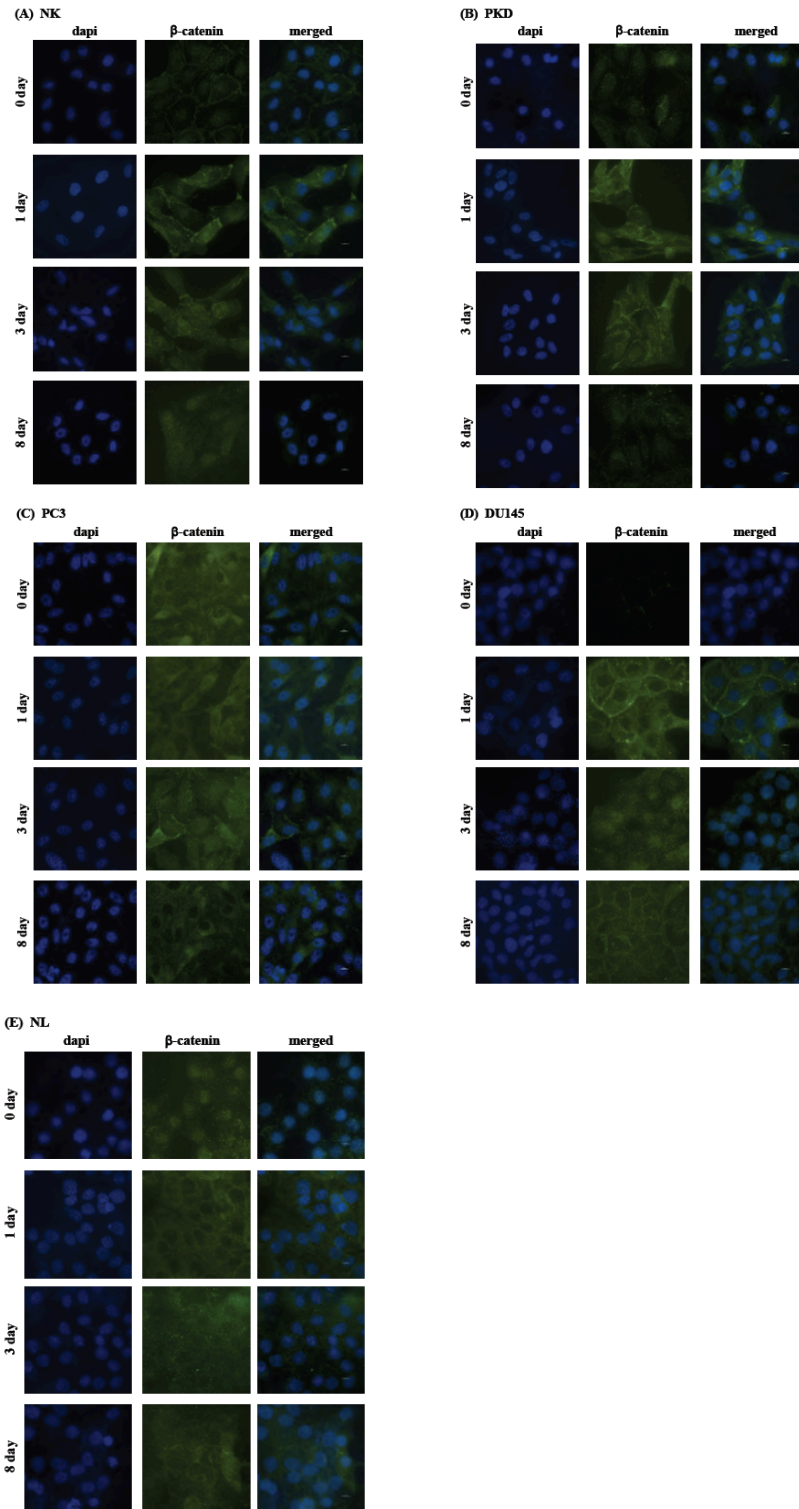


Figure 3.15 Representative immunofluorescent images of β -catenin.

β -catenin translocation was assessed before and after treatment with 10 μ M of rapamycin for 1, 3, and 8 days in NK, PKD, PC3, DU145, and NL.

3.5. Discussion

Based on the emerging concept that cancer is associated with loss of primary cilia [7, 9, 23, 24], we postulate that restoration of primary cilia formation may attenuate cancer proliferation. In order to restore ciliogenesis in cancer cells, we treated the cells with rapamycin because rapamycin has been previously shown to increase cilia formation and length [31, 32]. We indeed found that rapamycin restored cilia formation and attenuated cell proliferation. Furthermore, our analyses suggest that ciliogenesis and antiproliferative effects by rapamycin treatment are highly correlated with one another.

Dysfunction of primary cilia has been associated with genomic instability [33, 47-49]. Cancer cells are also known to have genomic instability [50, 51]. Thus, we speculate that primary cilia may be involved in cancer pathogenesis. Abnormal ploidy formation was indeed observed in PKD and cancer cells. Moreover, we found that dysfunction or loss of primary cilia is associated with increased proliferation rate.

DU145 prostate cancer and NL bronchial tumorigenic cells did not express primary cilia while PC3 prostate cancer cells expressed low level of primary cilia. Our results are consistent with the previous studies that show the absence of primary cilia in PC3 and DU145 prostate cancer [52]. Our studies also agree with the previous report showing that prostate cancer tissues have a reduction in the percentage of ciliated cells [53]. After excluding cilia length of 1 μ m or less, we did not find any significant difference in cilia length among PC3, PKD, and NK. In contrast, a previous study show that there are more primary cilia in lung adenocarcinoma as well as in other cancers, such as adenocarcinoma of the colon, follicular lymphoma, and pancreatic adenocarcinoma [54]. Another study shows that ciliogenesis has a role in promoting cancer drug

resistance [25]. Even in the same cancer type, primary cilia can have an opposing role in tumorigenesis depending on the oncogenic initiating event [14, 55], suggesting the complexity of the roles of cilia in cancer.

We found that PKD, PC3, DU145, and NL are more proliferative than NK. We observed that even after becoming confluent, a condition of growth arrest, PC3 and DU145 cells are still significantly more proliferative than NK cells. The main physiological difference between immortalized cells and cancer cells is the loss of cell-cell contact inhibition in cancer cells (Fig. 4B). Cancer cells continue to proliferate even after they have become confluent. E-cadherin adhesive junctions are thought to play an important role in mediating contact inhibition through homophilic interactions of E-cadherin molecules between the two neighboring cells [56-58]. Previous studies have shown that over-expression of cadherins can antagonize β -catenin signaling by binding and sequestering it from the nuclear signaling [59, 60]. In cancer cells, loss of E-cadherin expression can contribute to upregulation of β -catenin signaling pathway [61]. It has been reported that overexpression of β -catenin in epithelial cells promotes cell proliferation [62]. Compared to immortalized non-tumorigenic cells, the genes involved in cell proliferation and cell cycle are significantly deregulated in tumorigenic cells [63]. Cyclin inhibitors and negative regulators of cell proliferation are progressively downregulated during tumorigenesis.

The mammalian target of rapamycin (mTOR) signaling pathway is an essential regulator of cell proliferation and metabolism processes, which are directly controlled by the mTORC1 pathway, such as protein, lipid and nucleotide synthesis, energy metabolism, and autophagy. Dysregulation of the mTOR pathway is involved in several diseases including cancer, diabetes, obesity, neurological diseases, and genetic disorders [64, 65]. Activation of mTORC1 stimulates glycolysis and lipid biosynthesis [66] and positively regulates glutamine metabolism [67].

Recently it is found that mTORC1 is also has an important role in aging and age-related diseases [68]. Rapamycin is a selective inhibitor of mTORC1 and a potent inhibitor of S6K1 activation (the downstream target of mTOR) [69]. It is found that rapamycin treatment improves insulin sensitivity by preventing a S6K-mediated feedback loop [70]. Moreover, rapamycin treatment prevents the differentiation of human adipocyte [71] and protects against high-fat-diet-induced obesity [72]. Rapamycin also has a role in extending the lifespan and preventing the onset of many age-related diseases [73, 74]. This information signifies a broad spectrum of rapamycin in cellular signaling and cell processes. Within the context of our work on cilia and cell proliferation, our studies do not differentiate cause-and-effect between cilia and cell proliferation. We thus use rapamycin only as a pharmacological tool to examine the correlation between the changes in the cilia and cell proliferation.

Rapamycin is an mTOR inhibitor and one of the most potent inducers of cilia formation. Rapamycin shows a statistically significant increase (up to 6-fold) in the percentage of cells with cilia compared to vehicle-treated cells [32]. Moreover, it has been shown that rapamycin increases primary cilia length and function in renal epithelia and vascular endothelia [31]. Consistent with these previous studies, our work demonstrates that rapamycin treatment for 8 days partially restores primary cilium expression in DU145 and NL cancer cells and significantly increases cilia length in NK, PKD, DU145, and NL. We found that 10 μ M of rapamycin increased the cilia length more than 1 μ M of rapamycin treatment. Cell cycle before and after 1, 3, and 8 days of rapamycin treatment was evaluated to determine if restoration of primary cilia was associated with attenuation of cell proliferation. Our results show that rapamycin inhibited cell proliferation significantly after 1, 3, and 8 days of treatment compared to the untreated cells. Our analyses also indicated that there was a significant correlation between the percent of cells with cilia and cell proliferation.

Consistent with our finding, Khan et al. have previously shown that rapamycin exerts its antiproliferative effect in cancer cells at least in part through its ability to restore primary cilium formation [32].

In unstimulated cells, β -catenin protein exists very little in cytoplasmic or nuclear fractions due to rapid degradation of β -catenin in the cytoplasm by the destruction complex that composed of the adenomatous polyposis coli protein, GSK-3 β , and Axin/Conductin. However, in the presence of a Wnt signal, a Frizzled family receptor and the downstream component Dvl are activated. Dvl in turn leads to the inactivation of GSK-3 β , resulting in the accumulation of cytoplasmic β -catenin. High levels of β -catenin in the cytosol result in its translocation into the nucleus and activation of expression of Wnt-responsive genes. The presence of primary cilium controls the levels of expression of Wnt target genes by regulating the degradation of Disheveled (Dvl) [30]. Wnt signaling activation was observed in many cancers and may contribute to the cancer progression [75-78].

We showed a higher level of β -catenin expression, which suggests the utilization of canonical Wnt signaling pathway in PKD, PC3, DU145, and NL. Similar to the previous studies [79-81], our data indicated that dysfunction or loss of primary cilia was associated with the activation of Wnt signaling pathways. However, other studies show a low activation of Wnt signaling pathway in prostate cancer [53, 82]. Due to the concept that the presence of primary cilium controls the levels of expression of Wnt target genes [26, 29], we evaluated the effect of primary cilia restoration on Wnt/ β -catenin signaling pathway by measuring the total protein expression of β -catenin as well as β -catenin nuclear translocation. We found that on day 1 and 3 of rapamycin treatment, the expression level of β -catenin was significantly increased in NK, PKD, and DU145 compared to

the untreated cells and the nuclear translocation of β -catenin increased significantly in NK, PKD, PC3 and DU145 compared to the untreated cells. This effect is consistent with the previous study that shows that mTORC1 activation suppressed Wnt/ β -catenin signaling and that rapamycin could activate Wnt/ β -catenin signaling pathway [83, 84]. However, the expression level of β -catenin significantly decreased in NK, PKD, and PC3 compared to untreated cells after 8 days of rapamycin treatment. Likewise, the β -catenin nuclear translocation significantly decreased in NK, PKD, PC3, and DU145 compared to untreated cells after 8 days of rapamycin treatment. This reduction in β -catenin level is presumably due to the presence or increase length of primary cilia. Generally, there is a trend of an initial increase followed by a decrease of β -catenin level as well as the translocation of β -catenin into the nucleus with rapamycin treatment. Moreover, we confirm the effect of rapamycin on inhibiting the phosphorylation of mTOR and its downstream target p70 S6 Kinase (S6K). In NL, rapamycin neither changes the level of β -catenin expression nor reduces the phosphorylation of both mTOR at Ser2448 and S6K. However, rapamycin significantly inhibits the phosphorylation of mTOR at Ser2481 in NL. The inhibition of mTOR phosphorylation at a different site (Ser2481) may trigger the compensatory increase in the phosphorylation of mTOR at Ser2448 and S6K on day 8 treatment. In addition to the different phosphorylation site of mTOR, the accumulation of β -catenin in the cytosol instead of nucleus in NL may be the reason that NL behaves differently from other cells.

3.6. Conclusion

In summary, we showed that rapamycin increased the expression and/or length of primary cilia. Both the presence and length of primary cilia were correlated significantly with cell proliferation. Our study supports the idea that the antiproliferative effects of rapamycin are correlated with ciliogenesis.

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Conclusion

Most of the non-hematological cells express sensory organelles on their surface called primary cilia. Primary cilia transmit extracellular clues into intracellular signals for molecular and cellular responses that regulate proliferation, differentiation, and migration. Defects in primary cilia could result in various life-threatening diseases including cardiovascular diseases and cancer.

In this review, we explained the ciliary structure and functional ciliary proteins to understand how cilia dysfunction can be associated with vascular disorders such as hypertension, aneurysm and atherosclerosis. We also demonstrate the possible connections between primary cilia and cardiovascular disorders. Future studies that involve pharmacological agents are needed in order to understand whether or not targeting sensory functions of primary cilia would improve cardiovascular outcomes.

In our study, we evaluated the association between cilia formation or length and cell cycle or division. Using Spectral Karyotyping (SKY) technique, we showed that PKD and the cancer/tumorigenic epithelial cells were associated with abnormal ploidy. We also showed that PKD and the cancer epithelia were highly proliferative. Our findings indicate that the cancer epithelial cells had a reduction in the presence and/or length of primary cilia compared with the normal kidney (NK) cells. After restoring the expression and length of primary cilia in these cells using rapamycin, the following analyses implied that both the presence and length of primary cilia were inversely correlated with cell proliferation. Collectively, our data suggest that restoring the presence and/or length of primary cilia may serve as a novel approach to inhibit cancer cell proliferation.