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Regulation of fluid-shear stress by mechanosensory primary cilia

Shakila Abdul-Majeed
The University of Toledo

A Dissertation

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by

Shakila Abdul-Majeed

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Medicinal & Biological Chemistry

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Dr. Surya Nauli, Major Advisor

_____

Dr. Paul Erhardt, Committee Member

_____

Dr. Zi-Jian Xie, Committee Member

_____

Dr. William Messer, Committee Member

_____

Dr. Anthony Quinn, Committee Member

_____

Dr. Patricia Komuniecki, Dean,

College of Graduate Studies

The University of Toledo

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An Abstract of

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The primary cilium is an important sensory organelle present in most mammalian cells. The cilium is involved in regulating various essential cellular processes and by virtue of its structure and location; the most important function of the primary cilium is to act as a sensor. The cilium senses the conditions in the extra cellular matrix and transduces the message to the cell interior resulting in changes in gene expression and protein synthesis. Dysfunctional cilia result in a variety of diseases, collectively called as "ciliopathies".

Our current studies have a two-fold aim. First, we aim to show that pharmacological agents modulate cilia length. To prove this, we examined intracellular molecules that regulate cilia length and/or cilia function *in vitro* and *ex vivo*. For the first time, we show that intracellular cAMP and cAMP-dependent protein kinase (PKA) regulate both cilia

length and function in vascular endothelial cells. Although calcium-dependent protein kinase (PKC) modulates cilia length, it does not play a significant role in cilia function. Cilia length regulation also involves mitogen-activated protein kinase (MAPK), protein phosphatase-1 (PP-1) and cofilin. Furthermore, cofilin regulates cilia length through actin rearrangement. Overall, our study suggests that the molecular interactions between cilia function and length can be independent of one another. We propose that cilia length and function are regulated by distinct, yet complex intertwined signaling pathways.

Our second aim is to show that dysfunctional dopamine/dopamine receptors are related to hypertension observed in polycystic kidney disease (PKD). PKD is characterized by cardiovascular irregularities, including hypertension. Dopamine, a circulating hormone, is implicated in essential hypertension in humans and animal models. Vascular endothelial primary cilia are known to function as mechano-sensory organelles. Though both primary cilia and dopamine receptors play important roles in vascular hypertension, their relationship has never been explored. We show for the first time that mouse vascular endothelia exhibit dopamine receptor-type 5 (DR5), which co-localizes to primary cilia in cultured cells and mouse arteries *in vivo*. DR5 activation increases cilia length in arteries and endothelial cells through cofilin and actin polymerization. DR5-activation also restores cilia function in the mutant cells. In addition, silencing DR5 completely abolishes mechano-ciliary function in WT cells. We find that DR5 plays very important roles in ciliary length and function. Furthermore, the chemosensory function of cilia can alter the mechanosensory function through changes in sensitivity to fluid-shear stress. We propose that activated ciliary DR5 has a functional mechanosensory role in endothelial cells.

Dedication

Dedicated to Almighty God

and

His last Messenger, Prophet Mohammad (peace be upon him)

Teachings of Prophet Muhammad (PBUH) which have inspired me to seek knowledge:

"Acquire knowledge from cradle to grave" (Mishkat)

"Acquiring knowledge is mandatory for ALL Muslims" (Ibn Majah, Mukaddamia section, Hadith-220)

To the loving memory of my Mother, who always encouraged me to do better than my best.

Acknowledgements

I sincerely thank my advisor, Dr. Surya M. Nauli, for giving me the opportunity to work with him towards my degree. I also thank my committee members, Drs. Erhardt, Xie, Quinn and Messer, who have encouraged, corrected and helped me to perfect my work and make it better science than I could on my own.

I sincerely appreciate the help of all my lab mates, Maki, Blair, Wissam, Shao, Brian, Jin and Ashraf, who have constantly supported me and helped me all the time. I also wish to thank NIH for their support in funding this work.

I would like to thank my late Mother, and my Father, who have always encouraged me to achieve my goals, irrespective of age.

Lastly, this work would not have been possible without the constant support of my beloved family, my husband, Abdul-Majeed, and my children, Ahmed, Emily, Ayesha and Zainab. Thank you my Dearests, for having put up with all I've dished out to you these past four years. Love you All!!!

Table of Content

List of Abbreviations

MTOC	Microtubule organizing center
IFT	Intraflagellar transport
ERK	Extracellular-signal related kinases
MEK	Mitogen-activated protein kinase kinase
MAPK	Mitogen-activated protein kinase
ORPK	Oak Ridge polycystic kidney
PC	Polycystin
PKD	Polycystic kidney disease
ADPKD	Autosomal dominant polycystic kidney disease
GFR	Glomerular filtrations rate

Chapter 1

Introduction

1.1. Cilia

Cilia are well-conserved, microtubule-based organelles which extend from the apical surface of most eukaryotic cells. Cilia can be categorized into multiple "9+2" (motile) or single "9+0" (primary/sensory) cilia, based on the geometry and composition of the axoneme (Bisgrove and Yost, 2006), with the exception of nodal cilia, which are 9+0 but motile (Fig. 1.1). The main artifact of the cilium, motile or primary, is the axoneme, which extends into the extra cellular matrix from the apical surface of the cell. The axoneme is a structure of 9 peripheral microtubule doublet rods arranged around a central core. It extends from the 9-triplet microtubules of basal body/ mother centriole of the cell, a microtubule-organizing center (MTOC). The triplet microtubules of the basal body and the microtubule doublets of the ciliary axoneme contain high levels of the more stable, acetylated and detyrosinated α-tubulin (Poole et al, 1997). The motile cilium is equipped with an additional microtubule pair in the center of the 9-doublets and is linked to dynein arms that are involved in the cilium's motility (Bisgrove et al, 2006, Dawe et al, 2007, Christensen et al, 2007, Pedersen et al, 2008,

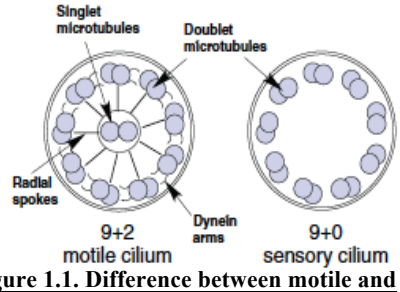

Figure 1.1. Difference between motile and primary/sensory cilia. Motile cilia express an additional pair of microtubule singlet in the center of the 9-doublet microtubules, absent in primary cilia.

Berbari et al, 2009). Motile cilia are present in large numbers on the epithelial cells of trachea, ependymal cells of the brain etc.

Primary cilia are present in every organ of the human body. For a long time, the primary cilia were considered vestigial organelles without specific functions. However, we now know that cilia are involved in most cellular processes (Fig. 1.2). Primary cilia are highly adapted to serve as specialized sensory organelles and are equipped with several

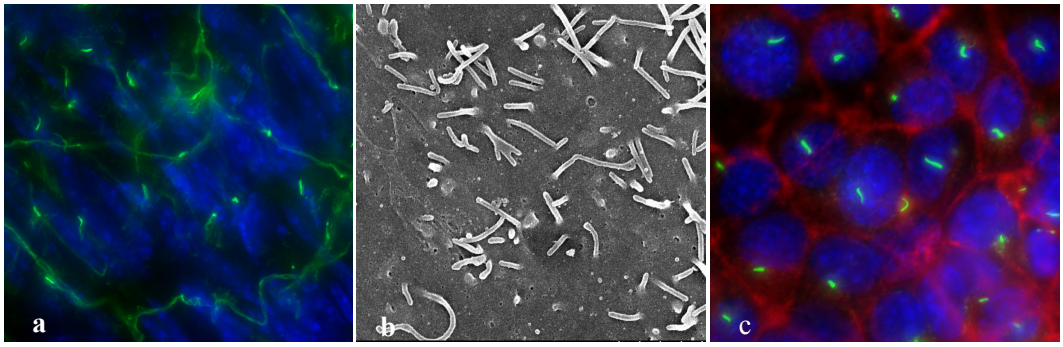

Figure 1.2. Immunofluorescence (IF) and scanning electron (SEM) microscopic images of primary cilia. a. IF image of primary cilia in femoral artery of wild type mouse. **b.** SEM image of primary cilia in *Tg737*$^{orpk/orpk}$ cells. **c.** IF image of primary cilia in wild type mouse aortic cells. (for IF images: **cilia**; **nucleus**; **actin**). Current work

specialized proteins, collectively known as ciliary proteins, to enable the cilium perform as a sensory organelle. Primary cilium, the hallmark of mammalian cells, is found in most cells except in hepatocytes, nucleated blood cells, adipocytes and cells that have motile cilia (Wheatley et al, 1996, Michaud and Yoder, 2006). The presence of different kinds of cilia types indicates that cilia have different functions.

Due to its ubiquitous nature and its role in several critical functions, any abnormalities in the primary cilia and/or the specialized ciliary proteins, results in severe defects, known as ciliopathies. Hence understanding this organelle becomes extremely important.

1.2. Structure of primary cilium

A typical cilium has an axoneme made of 9 outer-doublet microtubules rods, and protrudes into the extracellular matrix. The primary cilium arises from the mother centriole or the basal body, which provides a template for the nucleation of the microtubule axoneme. The major structure of the primary cilium is the axoneme, which is

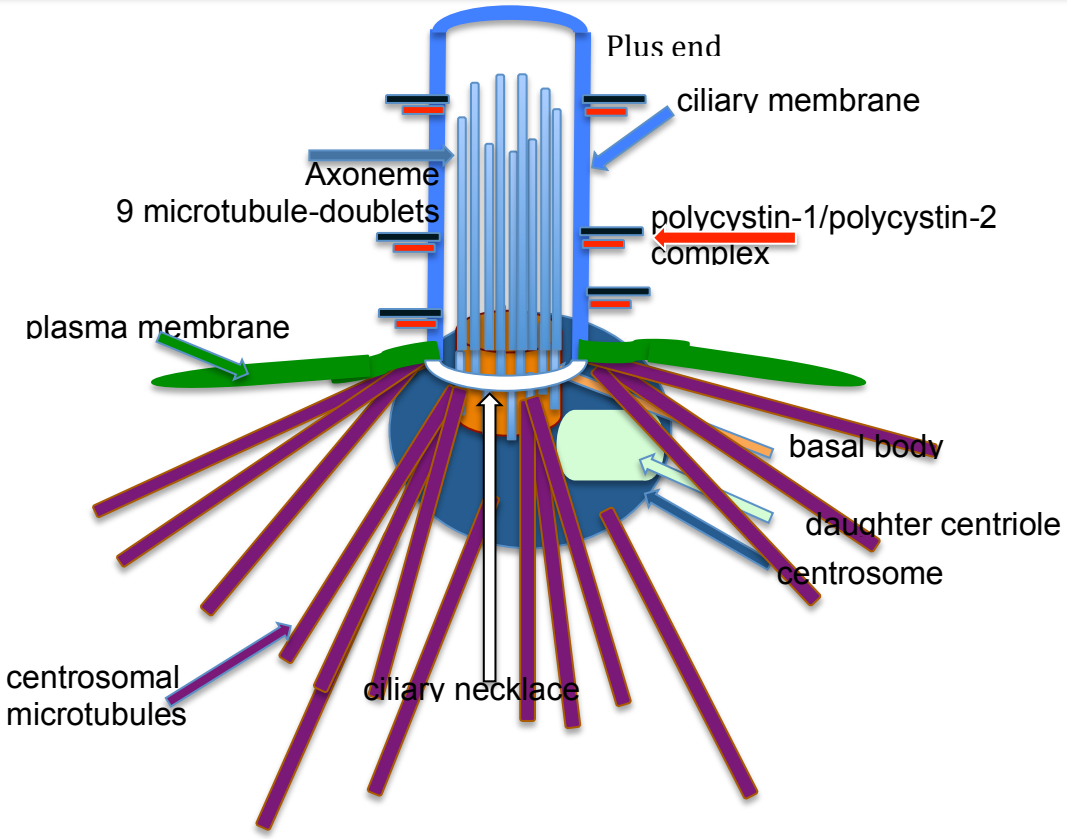

Figure 1.3. Structure of primary cilium. The axoneme, the main characteristic of the cilium, is made of 9-microtubule doublets and emerges from the 9-triplet microtubule basal body. The ciliary necklace forms a transition zone and controls movement of proteins from the cell body to the ciliary body. The cilium is connected to the cytoskeletal components via the centrosomal microtubules.

composed of 9-doublets of microtubules and sheathed with a lipid bilayer, the ciliary membrane (Fig. 1.3). Though continuous with the plasma membrane, the ciliary membrane is different from plasma membrane and houses distinct subset of receptors and specialized proteins, which are involved in signaling. The basal body also enables the

cilium to be connected to the network of the cellular microtubules. The ciliary membrane is firmly attached to the distal end of the centriole/basal body and the distal end of the basal body is linked to the cell membrane via a specialized transition zone, known as the ciliary necklace. The ciliary necklace acts like the nuclear membrane and controls the proteins entering or exiting the ciliary axoneme. IFT motor proteins are localized to the ciliary necklace, implying that the necklace region could be the site for coupling protein cargoes to the transport machinery (Satir & Christensen, 2007).

1.3. Formation of primary cilium

The ciliary microtubules are template from the same centrioles that are involved in organizing the mitotic spindle. Since the basal body is primarily a centriole, disassembly of the primary cilium is a prerequisite for the cell to enter into the cell cycle. Thus, the primary cilium is closely coupled to the cell cycle and actively regulates cell cycle and cell proliferation. Disassembly of the primary cilium liberates the basal body (mother centriole). Primary cilia are therefore post-mitotic structures. They are abundant during the G0/G1 phase and are also present during the S phase of the cell cycle, before the centrioles get involved in the formation (Fig. 1.4) of the mitotic spindle (Pan et al, 2007, Dawe et al, 2007, et al, 2009).

The primary cilium is formed as a solitary organelle, emerges from the distal centriole/ basal body during interphase when the centriole moves towards the plasma membrane and Golgi vesicles attach to the distal end of the mother centriole leading to the formation of the axoneme. With the accumulation of accessory structures, the centriole extends to become the basal body. As more vesicles fuse, a ciliary base is formed which then

shrouds the elongating axoneme (Pederson et al, 2008, Veland et al, 2009).

The processes of cilia assembly and disassembly are highly conserved processes. The cilia grow by the addition of axonemal subunits to the distal end of the growing cilia. However, the ciliary compartment does not house any protein syntheses units, and all proteins, including the subunits, are imported to the cilia by a specialized transportation

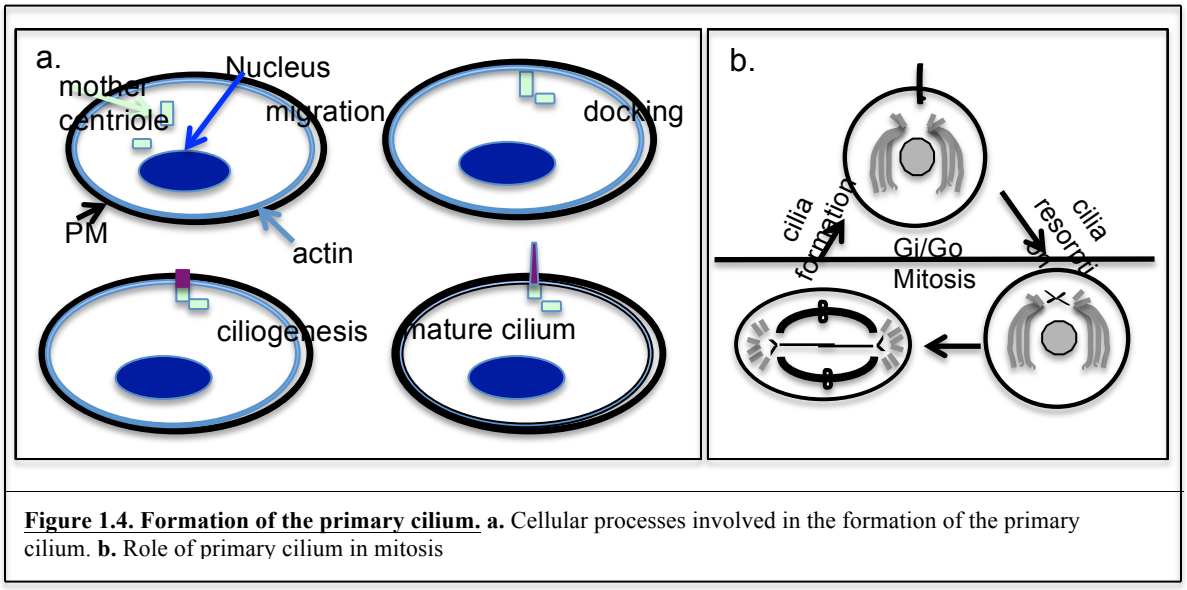

Figure 1.4. Formation of the primary cilium. a. Cellular processes involved in the formation of the primary cilium. **b.** Role of primary cilium in mitosis

system referred to as "intra flagellar transport (IFT)". In addition to IFT, actin dynamics and actin restructuring is also thought to play an important role in ciliogenesis. Actin polymerization inhibiting agents such as cytochalasin D and latrunculin have been found to induce ciliogenesis.

1.4. Intraflagellar Transport (IFT)

IFT, like the cilia, is a highly conserved, microtubule-based motility process, in which the IFT particles move bi-directionally between the basal body and the distal ciliary tip just underneath the ciliary membrane. The axonemal microtubules are oriented in parallel with their plus ends at the distal tip and their minus ends at the basal body. The

anterograde IFT particles (Fig. 1.5), driven by heterotrimeric kinesin-II motors, carries cargo into the ciliary body; while the retrograde IFT particles, driven by cytoplasmic

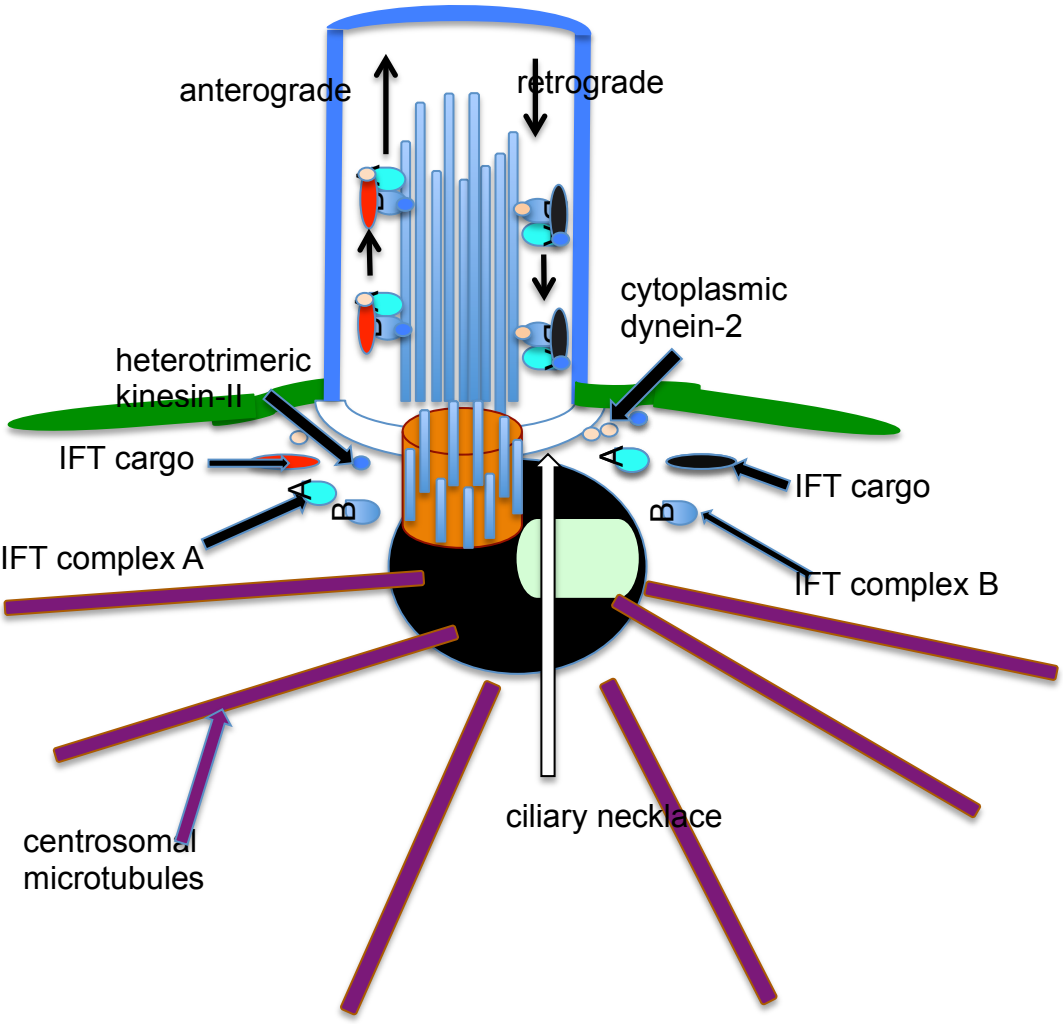

Figure 1.5. Intraflagellar transport (IFT). IFT, a microtubule-based transport mechanism, is a highly conserved mechanism involved in ferrying proteins to and from the ciliary axoneme to the cell body.

dynein 2 motors, recycles the breakdown proteins and IFT particles back to the cell body. The IFT motors, IFT particles and the cargo, all accumulate around the basal body and associate with the transition fibers/ciliary necklace, which functions as a loading dock. After assembling the cargo and the IFT particles, the IFT motors translocate to the axoneme to be transported by heterotrimeric kinesin-II motors on the axonemal

microtubules tracks. At the ciliary tip, the cargo gets unloaded and the proteins to be transported out of the cilium reassemble onto cytoplasmic dynein-2 motors (Rosenbaum and Whitman, 2002, Scholey, 2003). Though not much is known about the mechanisms involved in regulating IFT, Raf/MEK/ERK pathway is thought to phosphorylate and activate the motor proteins. IFT plays a crucial role in ciliogenesis and dysfunctional IFT mechanism result in loss of cilia, causing many genetic diseases.

1.5. Functions of primary cilium

The primary cilium extends into the extracellular space thereby vastly enhancing the area of contact between the cell interior and the extracellular environment. The shape and the location of the primary cilium bestow a unique ability to the primary cilia to act as a sensory organelle, capable of detecting and transmitting mechanical and chemical information from the extra cellular environment to the cell interior, resulting in changes in gene expression and protein synthesis. The cilia, therefore requires a large number of specialized proteins to function as a sensor and transducer of signals.

A cilium, like the nucleus, mitochondria, Golgi apparatus etc., can also be viewed as a separate entity within a cell (Singla and Reiter, 2006, Kolb and Nauli, 2008). In healthy tissues, the primary cilium is involved in many signal transduction pathways and acts as a photo- (Nishimura et al, 2004, Moore et al, 2006, Ghosh et al, 2009), chemo- (Winkelbauer et al 2005, Hearn et al, 2005, Davenport et al, 2007), osmo- (Andrade et al, 2005), gravitational- (McGlashan et al, 2007, Malone et al, 2007, Moorman and Shorr, 2008), and mechano-sensor (Nauli et al, 2003, Cano et al, 2006, Masyuk et al, 2006). Therefore, primary cilium can regulate many vital cellular processes such as cell

development, proliferation, differentiation, and other physiological responses (Satir and Christensen, 2007, AbouAlaiwi et al, 2009, Gerdes et al, 2009). Hence, a high density of specialized proteins such as signal modules, receptors, ion channels, kinases, phosphatases and secondary messengers are found in the ciliary compartment.

1.6. Primary cilium as a mechanosensor

The discovery that impaired functions of the primary cilium in *ift88TGN737Rpw* mouse (also known as ORPK mouse) causes cyst formation in the kidneys revealed the significance of the primary cilium. Subsequent research by various groups established the

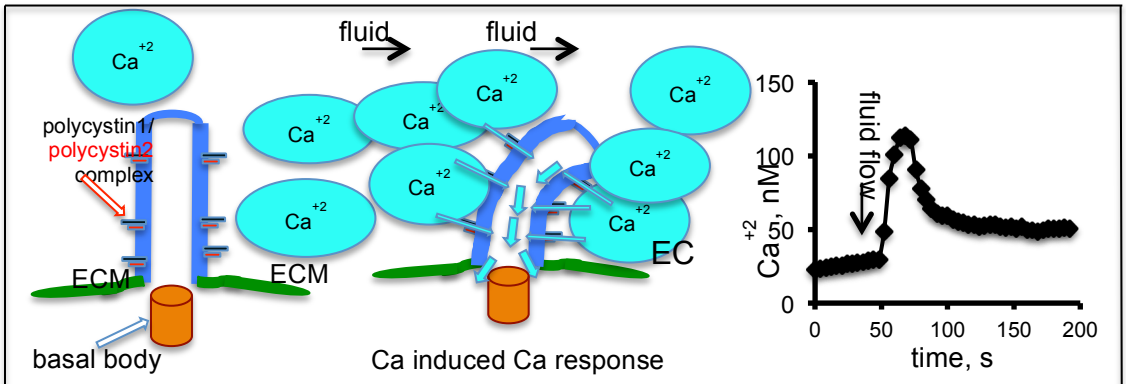

Figure 1.6. Primary cilium is a mecahnosensor. In absence of fluid flow, the cilium stands upright and bends in the presence of fluid flow. PC1-PC2 forms a complex and localize to the primary cilium. The bending of the cilium in presence of fluid flow activates PC1, a mechanosensor. Activated PC1 then activates PC2, a Ca-ion channel. Small amounts of calcium from the extra cellular matrix seep into the cell via PC2 in the ciliary membrane, resulting in a large flux of intracellular calcium by Ca-induced-calcium response. A typical graph resulting from the increase in intracellular calcium in presence of fluid flow is shown on the far right.

function of the primary cilium as a mechanosensor in renal epithelial cells (Moyer et al, 1994, Schrick et al, 1995, Pazour et al, 2000, Berbari et al, 2009, Veland et al, 2009). Thus, one of the most important functions of the primary cilia is detection of fluid flow in organs such as the kidneys, liver, pancreas etc. Primary cilia, expressed on the epithelial cells of these organs, sense fluid flow and transduce these signals into intra cellular calcium signaling response. Flow sensing is completely abolished in deciliated cells and

in cells with dysfunctional polycystin-1 (PC1) and polycystin-2 (PC2), two important ciliary proteins (Praetorius & Spring, 2002, Nauli et al, 2003, Nauli & Zhou, 2004, Nauli et al, 2006). PC-1 and PC-2 form a complex and localize to the ciliary membrane. PC1, encoded by the gene *PKD1*, is a mechanosensor, with a voluminous extracellular N-terminus, which acts as a GPCR. Polycystin-2, encoded by the gene *PKD2*, and belongs to the transient receptor potential (TRP) family and functions as a calcium channel. PC2 has for a long time been predicted to regulate cytosolic calcium (Gonzalez-Perrett et al, 2001, Vassilev et al, 2001), including modulating intra-organellar calcium release (Koulen et al, 2002) and extracellular calcium influx (Hanaoka et al, 2000). The cilium bends in the presence of fluid flow, thereby activating PC1, the mechanosensor (Fig. 1.6). PC1 then activates PC2, a calcium ion channel, which facilitates the flow of small amounts of calcium ions from the extra cellular matrix into the cell body via the cilium. This small flow of calcium ions creates a large influx of intracellular calcium. The calcium influx stimulates growth factors and hormonal releases, resulting in various cellular processes, including cell proliferation and regulation (Meijer et al, 2008).

1.7. Ciliopathies

Due to their ubiquitous nature and their role in various essential cellular processes, in addition to their sensory capabilities, any defect/impairment in the structure of the primary cilia results in a broad range of phenotypes (Badano et al, 2006, Veland et al, 2009). A large number of proteins contribute to regulating the sensory functions of the cilium, and are localized to the cilium and/or the basal body. These “ciliary” proteins

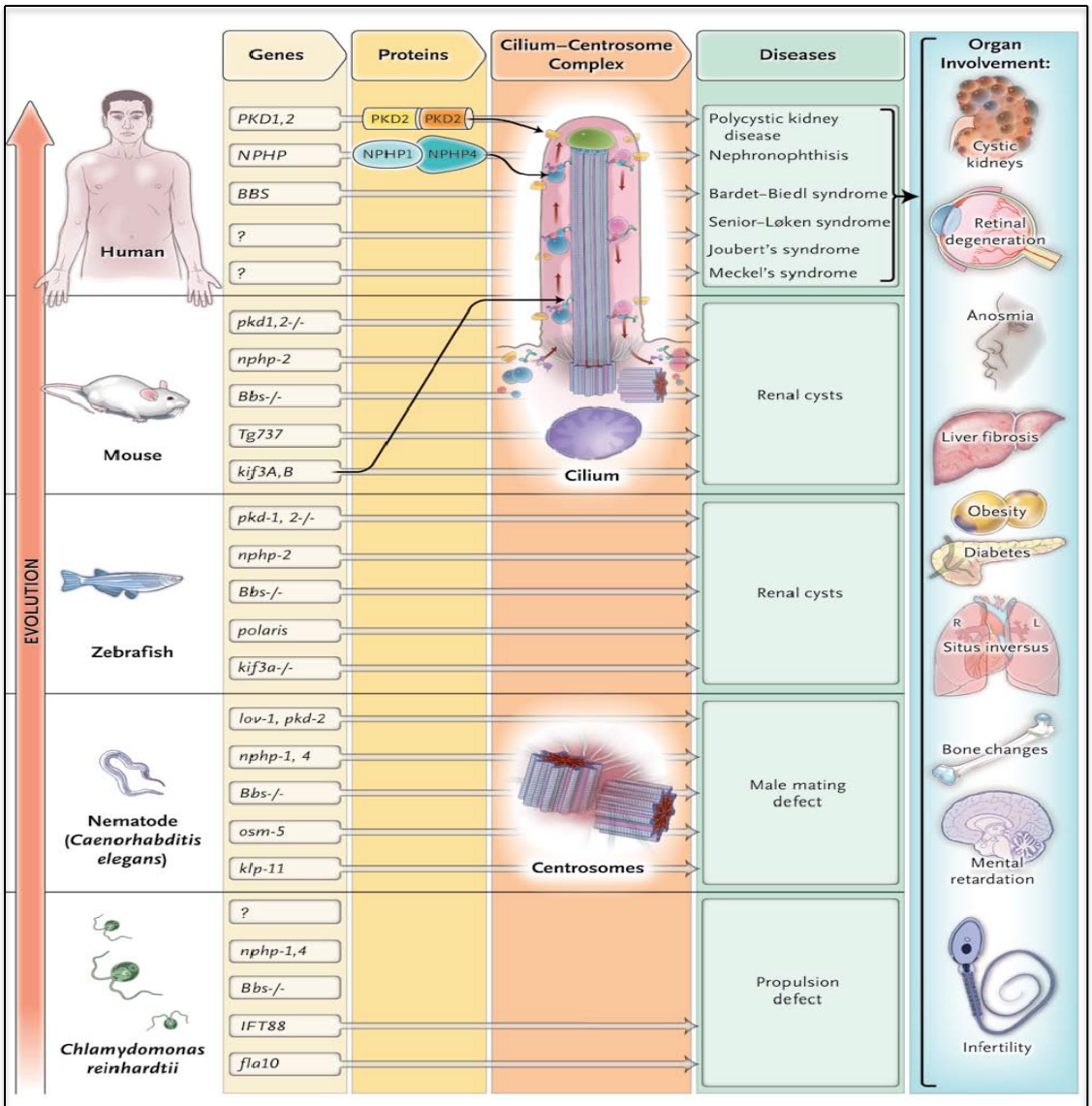

Figure 1.7. Ciliopathies. Various diseases have been linked to cilia and/or ciliary proteins in humans and animal models. Ref. Hildebrandt, F., Benzing, T. and Katsanis, N. Ciliopathies. *The New Engl J of Med*. 2011. 346(16):1533- 1543.

include polycystins (Pazour et al, 2002, Yoder et al, 2002), fibrocystin (Ward et al, 2003), nephrocystins (Otto et al, 2003, Mollet et al, 2005, Winkelbauer et al, 2005, Fliegauf et al, 2006), proteins that regulate Wnt signaling, hedgehog signaling etc. (Toftgård, R., 2009, Singla & Reiter, 2009, Wong et al, 2009, Han et al 2009, Hildebrandt et al, 2011, Winyard & Jenkins, 2011). The connection between dysfunctional primary cilia and diseases first came to light when dysfunctional *lov-1* gene, homologous to polycystin-1 in

humans, was linked to impaired mating in male *C. Elegans* (Barr and Sternberg, 1999). Since then, dysfunction of various proteins localized to the cilium or the basal body have been linked to diseases in humans and animal models, and are collectively known as "ciliopathies".

Recent discoveries in genetics and molecular fields, have transformed ciliopathy to a new class of disease in the past few years, with the unifying theme of dysfunctional proteins localized to the cilia and/or basal body. A table of these diseases, compiled in the New England Journal of Medicine in May 2011, is shown figure 1.7. One of the ciliopathy in which research is concentrated in our group is: autosomal dominant polycystic kidney disease (ADPKD)

1.8. Autosomal Dominant Polycystic Kidney Disease (PKD)

Polycystin-1 and polycystin-2 co-localize to the primary cilium of diverse cells. Vertebrate cells lacking PC1 and/or PC2 do not exhibit abnormal cilia. However, renal epithelial cells that exhibit dysfunctional PC1 and/or PC2, are incapable of sensing fluid flow resulting in autosomal dominant polycystic kidney disease (ADPKD). ADPKD is also found in animal models with abnormal ciliary structure, as those with mutated IFT genes, *ift88TGN737Rpw* mouse (aka Oak Ridge Polycystic kidney (orpk) mouse). ADPKD cystic cells are insensitive to

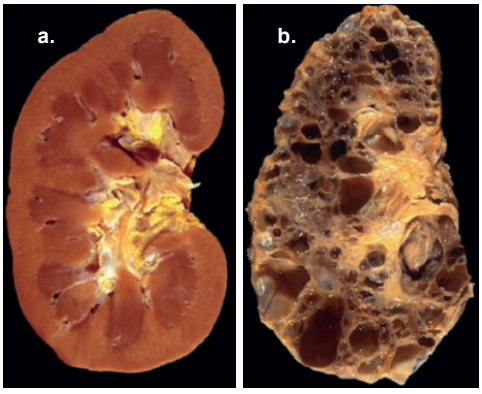

Figure 1.8. Manifestation of cysts in kidneys. **a.** Normal kidney. **b.** Fluid filled cysts in kidneys of ADPKD. Ref. Simons, M. & Mlodzik, M. *Annu* Rev *Genet.* 2008. 42:517-540.

fluid-flow calcium signaling and also show reduced levels of endoplasmic calcium store. Calcium release from intracellular stores gradually decreases with haplo insufficiency, over-expression or absence of polycystin2 (Torres, 2005, Harris and Torres, 2009). Decrease in the expression of polycystins below a critical threshold results in increased rates of proliferation and apoptosis, loss of planar polarity, expression of secretory phenotype and remodeling of the extra cellular matrix (Edelstein et al, 2008)

ADPKD is characterized by the presence of fluid-filled cysts in the kidneys (Fig. 1.8) finally resulting in end stage renal failure. Along with cystic manifestation, ADPKD patients and animal models also exhibit non-cystic phenotype, including hypertension, left ventricular hypertrophy, abnormal arterial remodeling, intra cranial aneurysm etc. Autopsy results of ADPKD patients show that more than 80% patients die of cardiovascular reasons than end stage renal failure (al-Nimri et al, 2003, al-Bhalal et al, 2005, Rahman et al, 2009, Chapman et al, 2010).

1.9. Hypertension in ADPKD

Hypertension occurs in 50-70% of ADPKD patients and starts often before renal functions are impaired. Most ADPKD patients with hypertension tend to have larger kidneys than normotensive ADPKD patients. Growth of cysts can lead to hypertension through several mechanisms such as displacement and narrowing of arterioles resulting in hypoperfusion and ischemia, increased release of endothelin into stretched or narrowed arterioles around expanding cysts, increase in afferent nerve activity from kidneys resulting in an increased sympathetic nerve activity. Several factors contribute to hypertension in ADPKD patients and animal models (Chapman et al, 2010).

Hypertensive ADPKD patients tend to exhibit high levels of intracellular sodium concentrations and a disturbed blood pressure and natriuresis relationship. Sodium balance is maintained at a cost of higher blood pressure required for excretion of same amount of sodium compared with normotensive patients (Schmid et al, 1990, Gabow, P.A., 1993, Wang & Strandgaard, 1997, Torres & Harris, 2007). Although hypertension is controlled using ACE and RAAS inhibitors in ADPKD patients, studies have shown that borderline hypertensive patients tend to excrete higher levels of dopamine and the amino acid, DOPA (3,4-dihydroxyphenylalanine- a precursor of endogenous dopamine) at all levels of sodium intake. This could imply that increased formation of dopamine could act as a compensatory mechanism to help maintain blood pressure and sodium balance in these patients (Barendregt et al, 1995).

PC1 and PC2 are also expressed in vascular endothelial and smooth muscle cells. Enhanced vascular smooth muscle contractility and impaired endothelial dependent vasorelaxation observed in ADPKD patients could imply the disruption of the polycystin function in the vasculature. In addition, ADPKD patients with normal glomerular filtration rates (GFR) exhibit reduced rates of endothelial vasodilation and constitutive nitric oxide synthase activity in their subcutaneous resistance vessels, along with impaired flow-induced vasodilation of the brachial artery. Finally, atherosclerosis occurs early in normotensive ADPKD patients due to reduced coronary flow velocity and increased carotid intima-media thickness (Clausen et al, 2006, Turgut et al, 2007, Borresen et al 2007, Nauli et al 2008, Turkmen et al, 2008).

1.10. Factors influencing ADPKD

Many pathways that couple cell surface receptors such as GPCRs, TRKs and integrins etc., are activated in ADPKD epithelial cells. Polycystins are generally localized to specialized structures that sense the extra cellular environment, such as primary cilia, focal adhesions and adherens complexes. In addition, they regulate calcium homoeostasis

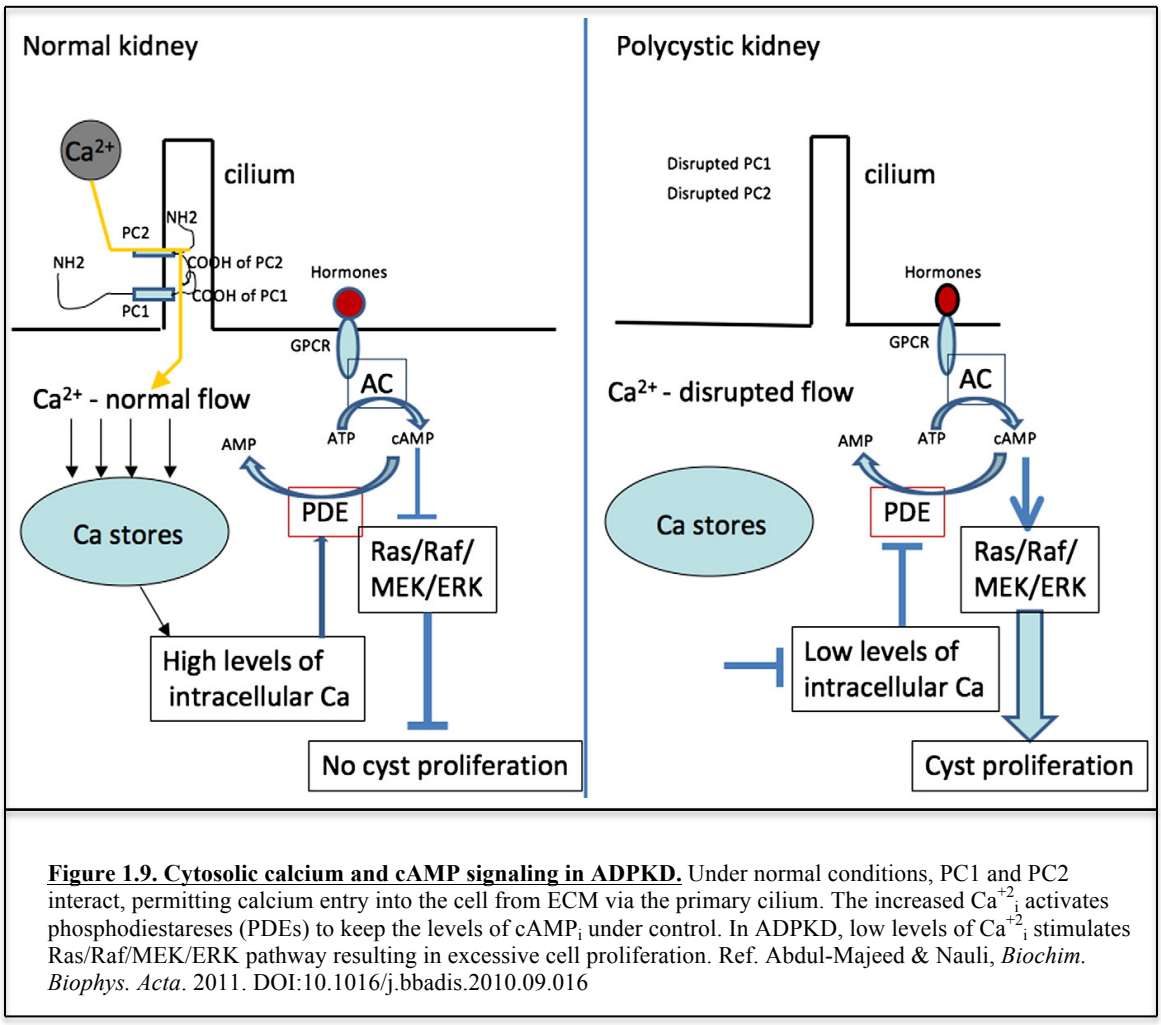

Figure 1.9. Cytosolic calcium and cAMP signaling in ADPKD. Under normal conditions, PC1 and PC2 interact, permitting calcium entry into the cell from ECM via the primary cilium. The increased $Ca^{+2}{}_i$ activates phosphodiestareses (PDEs) to keep the levels of $cAMP_i$ under control. In ADPKD, low levels of $Ca^{+2}{}_i$ stimulates Ras/Raf/MEK/ERK pathway resulting in excessive cell proliferation. Ref. Abdul-Majeed & Nauli, *Biochim. Biophys. Acta*. 2011. DOI:10.1016/j.bbadis.2010.09.016

and regulate tubular and vascular development in organs such as kidneys, liver, brain and pancreas (Grantham. J.D., 2008). Dysfunctional polycystins in ADPKD could lead to a reduced clearance of intracellular cAMP in renal epithelial cells (Cowley, B.D., 2008). Recent studies have indicated that ADPKD animal models exhibit increased levels of

cyclic adenosine monophosphate (cAMP) and expression of cAMP-dependent genes in their kidney, liver, vascular smooth muscles and choroid plexus. Altered cAMP catabolism could render cystic epithelia susceptible to upregulation of cAMP signaling, thereby contributing to disease progression in ADPKD (Harris &Torres, 2009, Wang et al. 2010). These two second messengers, calcium and cAMP, are intimately interconnected in ADPKD. PC1 and PC2 regulate intracellular calcium homeostasis; calcium regulates cAMP metabolism by stimulating calcium-inhibited phosphodiesterases (PDEs) or inhibiting calcium- dependent PDEs (Fig. 1.9).

cAMP plays an important role in effecting hormonal activation of various intracellular pathways. Though cAMP has been used as an anti-proliferative agent for many years, cAMP has been known to stimulate cell proliferation by activating mitogen activated protein kinase (MAPK). While under normal conditions, cAMP inhibits mitogen-activated protein kinase (MAPK) and cell proliferation, in ADPKD conditions or calcium-deprived conditions, cAMP stimulates MAPK and cell proliferation, thereby exasperating the cyst formation in ADPKD. The abnormal proliferative responses of cAMP directly co-relates to low levels of intracellular Ca^{+2} levels, as this situation can be reproduced in wild type (WT) cells by limiting $[Ca^{+2}]_i$. Conversely, treating cyst-derived cells with calcium ionophores or calcium activators improves this abnormal response to cAMP (Yamaguchi et al, 1997, 2000, 2003, 2004, 2006, Nagao et al, 2008, Abdul-Majeed & Nauli, 2011, doi:10.1016/j.bbadis.2010.09.016).

Thus, ciliary function and structure are important and necessary of maintain the architecture of kidney disease. The mechanosensory ability of the primary cilium is essential for maintaining intracellular calcium signaling. This research tries to explore the

mechanisms involved in regulating cilia length and cilia function as determined by change in intracellular calcium levels in the presence of fluid-flow shear stress. In addition, an effort has been made to try to explain the possible connection between hypertension and dopamine/dopamine receptors within ADPKD.

1.11. Hypotheses

Our main hypotheses include:

A. Cilia length can be manipulated by pharmacological agents
B. Cilia length and function are not co-related.
C. Dysfunctional dopamine/dopamine receptors are involved in hypertension observed in polycystic kidney disease.

We have designed experiments to study the following corollary hypotheses:

1. Cilia length can be manipulated using pharmacological agents.
2. These agents activate protein kinase A and C, which in turn activates MAPK.
3. Actin restructuring induces ciliogenesis.
4. Raf/MEK/ERK pathway activates actin-binding protein (ABP)-cofilin, thereby inducing ciliogenesis.
5. Cilia length and cilia function may or may not be regulated by the same molecular interactions.
6. Increase in cilia length need not necessarily translate into an increased cilia function, as determined by the increase in intracellular calcium in presence of fluid-flow shear stress.

7. It is known that polycystic kidney disease, caused by dysfunctional cilia/ciliary protein, is characterized by hypertension, in addition to formation of fluid dilled cysts in kidneys.
8. We also know that irregularities in endogenous dopamine and/or dopamine receptor also cause hypertension.
9. Therefore, if dopamine receptor is localized to the primary cilium in endothelial cells, dopamine receptor and hypertension could be inter-related within PKD.

Chapter 2

Mechanisms regulating cilia growth and cilia function in endothelial cells

Shakila Abdul-Majeed[1], Bryan C. Moloney[2], Surya M. Nauli [1,2,3, †]

[1]Department of Medicinal & Biological Chemistry, [2]Department of Medicine,

[3]Department of Pharmacology, University of Toledo, Health Science Campus, HEB 274,

3000 Arlington Avenue, MS 1015, Toledo, OH 43614, USA.

†Address correspondence and reprint requests to

Surya M.Nauli
College of Pharmacy
University of Toledo, Health Science Campus, HEB 274, 3000 Arlington Avenue,
MS 1015, Toledo, OH 43614, USA.
Tel: (419) 383 1921
Fax: (419) 383 xxxx
Email: surya.nauli@utoledo.edu

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2.1. Abstract

The primary cilium is an important sensory organelle present in most mammalian cells. Our current studies aim at examining intracellular molecules that regulate cilia length and/or cilia function in vitro and ex vivo. For the first time, we show that intracellular cAMP and cAMP-dependent protein kinase (PKA) regulate both cilia length and function in vascular endothelial cells. Although calcium-dependent protein kinase (PKC) modulates cilia length, it does not play a significant role in cilia function. Cilia length regulation also involves mitogen-activated protein kinase (MAPK), protein phosphatase-1 (PP-1), and cofilin. Furthermore, cofilin regulates cilia length through actin rearrangement. Overall, our study suggests that the molecular interactions between cilia function and length can be independent of one another. Although PKA regulates both cilia length and function, changes in cilia length by MAPK, PP-1, or cofilin do not have a direct correlation to changes in cilia function. We propose that cilia length and function are regulated by distinct, yet complex intertwined signaling pathways.

2.2. Introduction

Primary cilia are solitary organelles that extend from the basal body of the apical surface into the extra cellular matrix of most eukaryotic cells. Their unique position and structure bestow an added advantage of vastly increasing the area of contact with the cell exterior [1]. A primary cilium houses a large number of proteins. It has been suggested that a cilium has five distinct domains [2]. One of these domains includes the soluble compartment, which is also called the matrix compartment or cilioplasm. The cilioplasm is composed of fluid material to support various signaling proteins.

Cilia length and cilia function play a major role in maintaining a healthy cellular state. Studies on primary cilia in the renal epithelial system show that the length of the cilia is highly regulated, with the cilia being longer in larger lumen and shorter in smaller lumen [3]. Many studies have also found that cilia length can be regulated under different physiological conditions [4, 5]. Since the length of the cilia is very important in many organs involving fluid flow, such as the kidneys, pancreas, liver and many others, it is important to determine the mechanisms involved in cilia length and function. Most important, the relationship between cilia length and function remains unknown.

In the current study, we have used pharmacological agents to modify the level of intracellular cAMP and activity of various protein kinases and phosphatase. We identify molecular pathways that regulate cilia length. Interestingly, this regulation may also involve actin rearrangement. Our functional assay on cilia further indicates that mechanosensory cilia function may not always coincide with changes in the cilia length. Overall, our studies offer more detailed insight into regulation of cilia structure and function.

2.3. Materials and methods

Animal and cell culture

The Institutional Biosafety Committee of The University of Toledo approved the use of endothelial cells and other biohazard reagents. The University of Toledo Animal Care and Use Committee approved the use of animal tissues. In our studies, we used vascular endothelial cells that were previously described and characterized for various surface and intracellular markers [6, 7].

Cilia analysis and measurement

To measure cilia length, cells or femoral arteries were observed with fluorescence and scanning electron microscopes. For fluorescence analysis, cells and tissue were fixed with 4% paraformaldehyde in 2% sucrose solution for 10 min at room temperature. Acetylated α-tubulin was used as a ciliary marker and to measure cilia length. Antibodies used included acetylated α-tubulin (antimouse, Sigma clone 6-11B-1; 1:10,000 dilution), FITC (anti-mouse, 1:500, Vector Laboratory), Texas Red®-X phalloidin (anti-mouse, 1:100, Invitrogen) and DAPI (Vector Laboratory). The cover slip was then mounted on the microscope slide with mounting media containing DAPI. Cilia images were observed with an inverted Nikon Ti-U microscope and analyzed three dimensionally with Metamorph 7.0.

For the scanning electron micrograph, cells or femoral arteries were fixed with 2.5% paraformaldehyde/glutaraldehyde in sodium cacodylate buffer for 1 h at room temperature. In case of femoral artery, after fixing and drying the piece for 24 h, we made very fine cross sections of the artery (~1 mm) as such that the lumen would always be exposed for analysis. The samples were chemically dried using an initial 2-h incubation in 50% HMDS-ethyl alcohol mixture, followed by two half-hour incubations in 100% HMDS. Micrographs were obtained and analyzed using a Hitachi HD-2300 scanning electron microscope.

cAMP measurement

The measurement of cAMP was carried out according to the cAMP EIA kit with

acetylation step (Cayman Chemicals). In every measurement, a standard curve of cAMP was generated with a typical correlation coefficient of 0.992 ± 0.001. The standards and samples were read at a wavelength of 410 nm in duplicates and triplicates, respectively. The total cAMP was normalized with total protein content determined by a standard Bradford assay.

Cytosolic calcium measurement

For cilia function analysis, we used a similar protocol and setup as previously described [6, 7]. Briefly, cells were loaded with 5 μM Fura2-AM (Invitrogen) for 30 min at 39°C. Basal calcium was first determined for about a minute. Fluid flow at optimal shear stress was used to monitor changes in cytosolic calcium every 4 s. Changes in cytosolic calcium were monitored and recorded using a Nikon TE2000 microscope and Metafluor software. At the end of the experiment, the minimum fluorescence was determined by treating the cells with 2 mM EGTA and 10 μM ionomycin. After achieving the minimum signal, the maximum fluorescence was obtained by treating the cells with excess calcium (10 mM). All fluorescence measurements were corrected for auto-fluorescence.

Protein analysis

Measurement of protein was performed using a standard Western-blot analysis. Each well was loaded with 150 μg of protein. The membranes were initially blocked for 2 h with 5% milk in TBS containing 1% Tween. All the antibodies were diluted in 1% milk in TBST solution. The following antibodies were used in our analysis: anti-phosphoERK (anti-rabbit, Cell Signaling, 1:1,000 dilution, overnight at 4°C), p-cofilin (anti-rabbit,

Cell Signaling, 1:100, overnight at 4°C), anti-ERK (anti-rabbit, Cell Signaling, 1:1,000 dilution, overnight at 4° C), and anti-α tubulin (anti-rabbit, Abcam, 1:5,000 dilution, overnight at 4°C).

Pharmacological treatment

The following pharmacological agents and their concentrations were used on cells and tissues: cAMP analog (8pCPT-cAMP; 10 μmol/l), PKA activator (forskolin; 10 μmol/l), PKC activator (phorbol myristate acetate; 0.5 μmol/l), PKC inhibitor (bisindolylmaleimide XI hydrochloride; 0.5 μmol/l), and MAPK inhibitor (PD98059, 10 μmol/l). These concentrations were titrated to have optimal effects in our system. High-grade-quality pharmacological agents were selected and purchased from Sigma.

Agents were added after the cells were differentiated at 39°C to avoid any potential effect on cell growth. For femoral arteries, isolated tissues were briefly cleaned and rinsed with phosphate buffer containing glucose and calcium. Fresh buffer (2 ml) and the drug were added, and the samples were incubated at 39°C for the desired durations. Drugs were incubated with the samples for 4 or 16 h. The data for 4 h are not shown because of the inconsistency due to a non-optimal condition. Extreme caution was taken when adding the drugs. Drugs were diluted to different concentrations so that the exact same volume of the drug solution was added to the samples in an effort to maintain identical volume.

Statistical analysis

To examine effects of cAMP on cilia function, we determined the biological function by studying a typical logarithmic scale dose–response curve ($\log_e(\mathbf{x})$) [8, 9]. Likewise, we

used the same mathematical function for the cAMP measurement, which was also suggested by the manufacturer. The following equation was used to obtain the cAMP standard curve: $\mathbf{y} = \log_e (\mathbf{x}) + \mathbf{c}$; where **y** is $\log_{10}$ of signal intensity, **x** represents cAMP concentration, and **c** denotes proportional coefficient. Unless otherwise indicated, analysis was done after 16 h of treatment with pharmacological agents. All quantifiable experimental values are expressed as mean ± SEM, and values of $\mathbf{p<}0.05$ are considered significant. All comparisons between two groups were performed with Student's **t** test. Comparisons of three or more groups were done using ANOVA, followed with Tukey's post test. Data analysis was performed using SigmaPlot software version 11.

2.4. Results

Cilia length is regulated by various protein kinases in mouse endothelial cells and tissues

Previous studies have shown that intracellular cAMP regulates cilia length in cultured mono-layered epithelial cells [10, 11]. Our present studies also indicate that cAMP can increase cilia length in mouse endothelia of mono-layered cells in vitro and femoral arteries ex vivo (Fig. 2.1). We next utilized various pharmacological agents to examine the roles of other kinases. Activating cAMP-dependent protein kinase (PKA) or calcium-dependent protein kinase (PKC) is sufficient to significantly increase cilia length in cultured and femoral endothelial cells. On the other hand, inhibiting PKC or mitogen-activated protein kinase (MAPK) significantly decreases cilia length. Our data indicate that endothelial cilia can be modulated by cAMP, PKA, PKC, and MAPK. This modulation is observed in both cultured cells and vascular tissues.

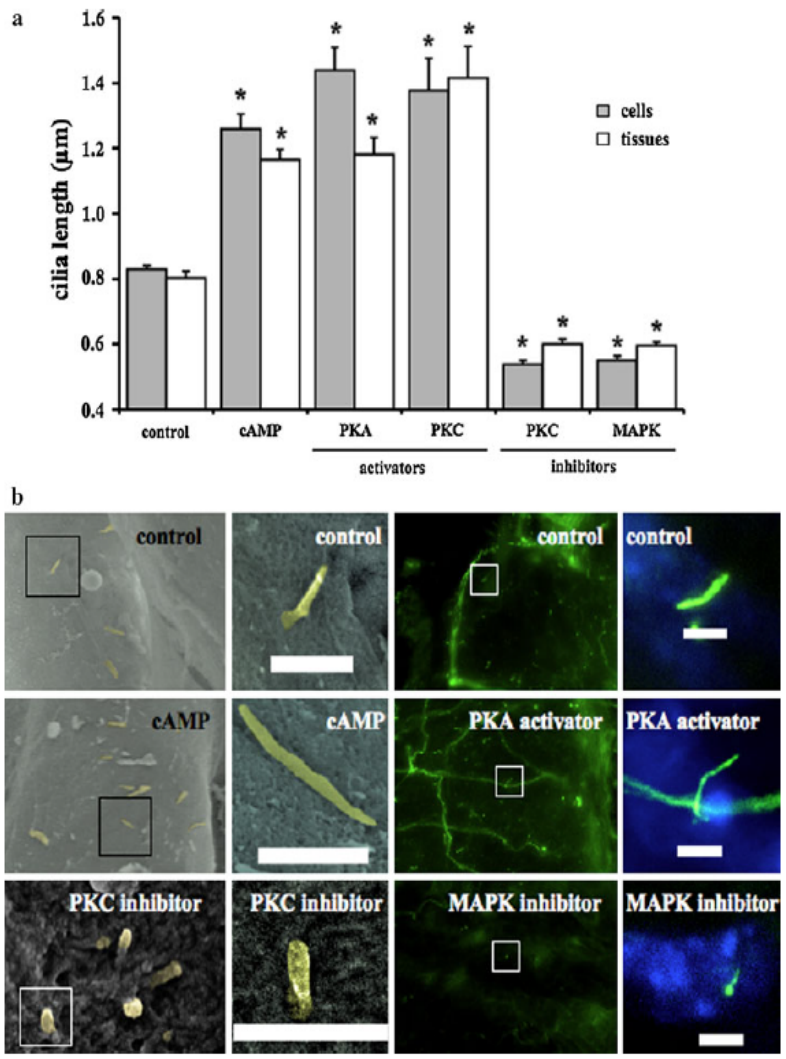

Figure 2.1. Cilia length can be modulated with pharmacological agents. **a**. Length of primary cilia in mono-layered cells and femoral arteries was measured before (control) and after treatment with 8-pCPT-cAMP (cAMP, 10 μmol/L), forskolin (PKA activator, 10 μmol/L), PMA (PKC activator, 0.5 μmol/L), bisindolylmaleimide (PKC inhibitor, 0.5 μmol/L), PD98059 (MAPK inhibitor, 10 μmol/L) for 16h. The cAMP and activators significantly increased cilia length, while the inhibitors significantly decrease cilia length. **b.** Representative scanning and immunofluorescence images from femoral arteries are shown. Acetyated-α-tubulin was used to identify primary cilia. Boxes indicate a greater magnification from the field of view. *Bar* 1μm. n=3 independent experiments; over 120 cilia were measured in cultured cells, and about 50 cilia were measured in femoral arteries. $*p<0.05$ compared to control group.

Cilia length is regulated by ERK and cofilin

To further understand the mechanism involved in cilia length regulation, we first examined the effects of the kinases' activities on intracellular cAMP level (Fig. 2.2a). PKA activation significantly increases intracellular cAMP level, indicating a possible synergistic pathway of cAMP and PKA in regulating cilia length. On the other hand, PKC activation does not increase cAMP level, although it increases cilia

length. In addition, although PKC or MAPK inhibition decreases cilia length, neither has a significant effect on cAMP level compared to control. This suggests that PKC/MAPK is downstream or has a separate pathway from cAMP.

We next investigated this possibility by measuring ERK phosphorylation, as an

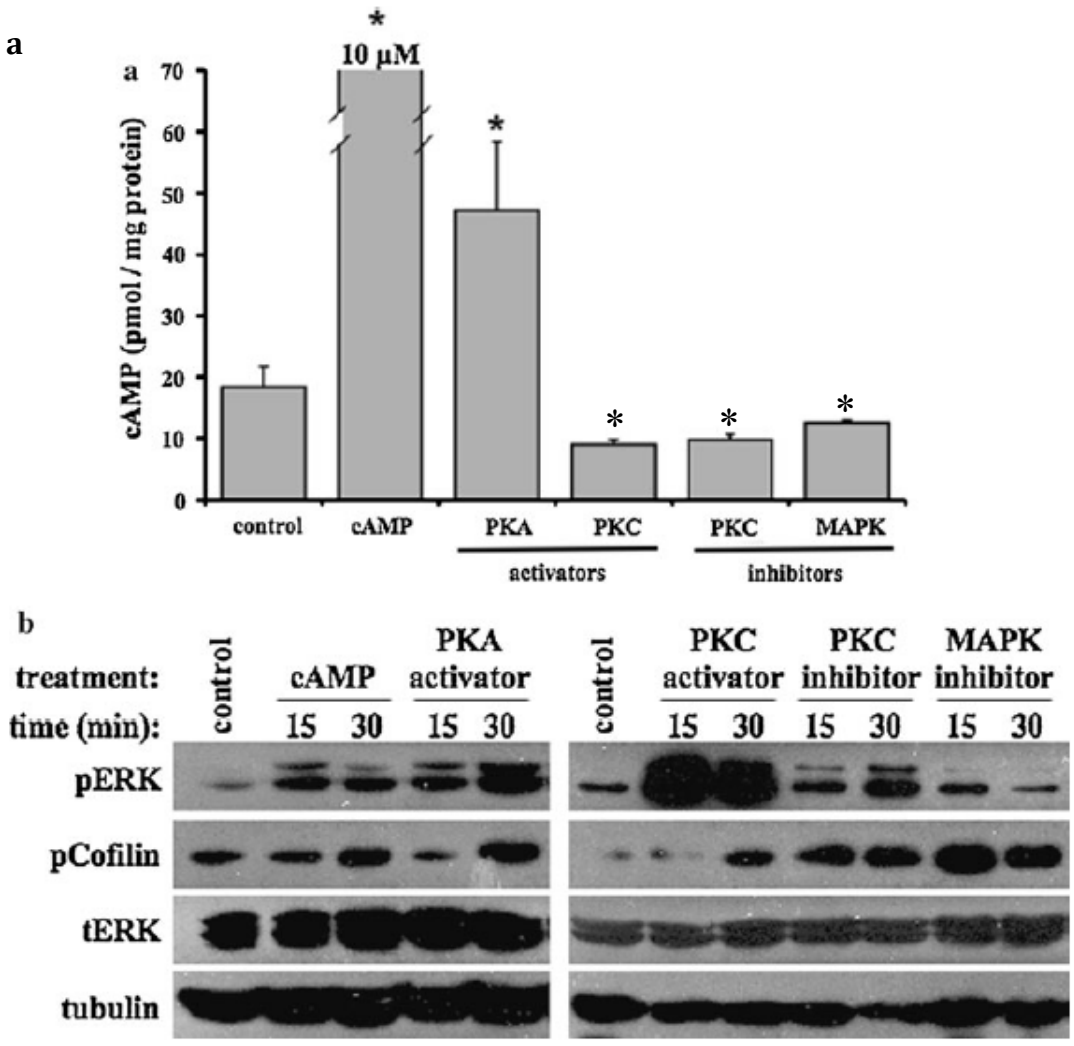

Fig. 2.2. Ciliary length modulation involves a combination of cAMP, pERK, and cofilin activation. **a**. Intracellular cAMP was measured in untreated cells (control) and cells treated with 8-pCPT-cAMP (cAMP), forskolin (PKA activator), PMA (PKC activator), bisindolylmaleimide (PKC inhibitor), or PD98059 (MAPK inhibitor). **b.** Western blots depicting phosphorylated ERK (pERK), phospho-cofilin (pCofilin), total ERK (tERK), and α-tubulin (tubulin) are shown in cells with corresponding treatments for 15 or 30 min. α-tubulin was used as a loading control. n = 3 independent experiments for cAMP measurement and two for Western blots. *$p<0.05$ compared to control group

indicator of MAPK activity. Because activation of MAPK can also promote cofilin dephosphorylation or activation [12,13], we also examined cofilin phosphorylation level (Fig. 2.2b). Our data show that ERK phosphorylation is consistently and substantially increased by cAMP, PKA activation, or PKC activation at 15 and 30 min. Compared to control, PKC or MAPK inhibition has a minimal effect on ERK phosphorylation.

Most interesting is that ERK phosphorylation within 15 min is inversely correlated with cofilin phosphorylation. In other words, ERK phosphorylation promotes activation of cofilin.

Cilia length is regulated by actin rearrangement and protein phosphatase-1

Cofilin is an actin-binding protein that regulates assembly and disassembly of cytoskeletal actin filament rearrangement [12, 13]. Thus, activation or dephosphorylation of cofilin induces actin reorganization. To examine this possibility in our endothelial cells, we analyzed actin cytoskeleton in the absence or presence of cAMP analog, PKA activator, PKC activator, PKC inhibitor or MAPK inhibitor (Fig. 2.3). As predicted, a normal distribution of actin stress fiber is consistently restructured to cortical filamentous actin in the presence of cAMP analog, PKA activator, or PKC activator. On the other hand, cells treated with PKC or MAPK inhibitors exhibit similar actin stress fibers as those observed in the control. Protein phosphatase-1 (PP-1) can dephosphorylate and thereby activate cofilin [14]. It is therefore expected that inhibition of PP-1 activity would block

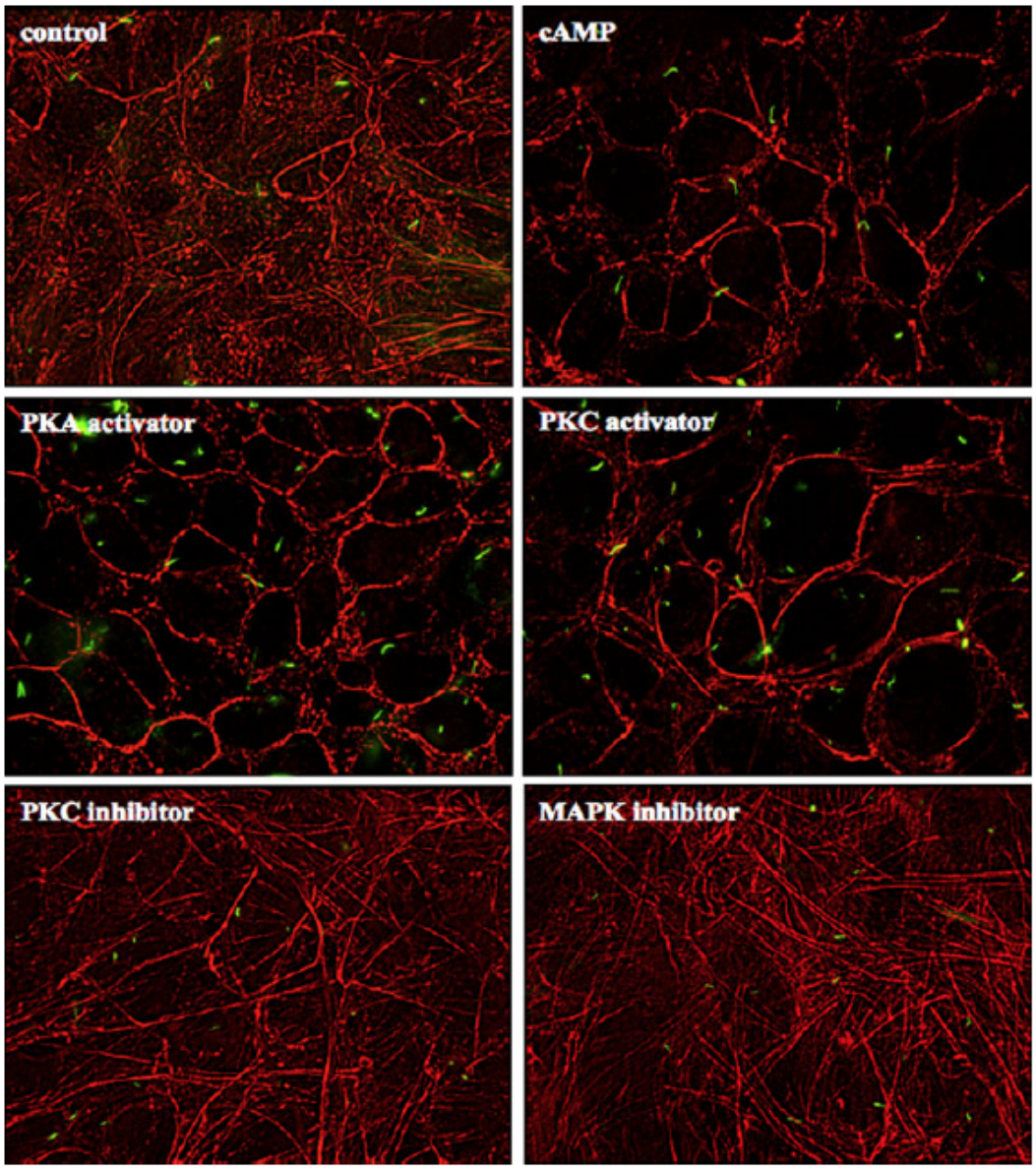

Fig. 2.3 Ciliary length modulation coincides with actin rearrangement. Representative images of primary cilia (green) and cytoskeletal actin filament (red) are shown. Actin filament was examined with phalloidin in untreated cells (control) and cells treated with 8-pCPTcAMP (cAMP), forskolin (PKA activator), PMA (PKC activator), bisindolylmaleimide (PKC inhibitor), or PD98059 (MAPK inhibitor). Filamentous actin stress fibers are reorganized to form cortical actin in cells treated with cAMP, PKA activator, or PKC activator. On the other hand, cells treated with PKC inhibitor or MAPK inhibitor did not show apparent difference in actin stress fibers when compared to control. n = 3 independent experiments

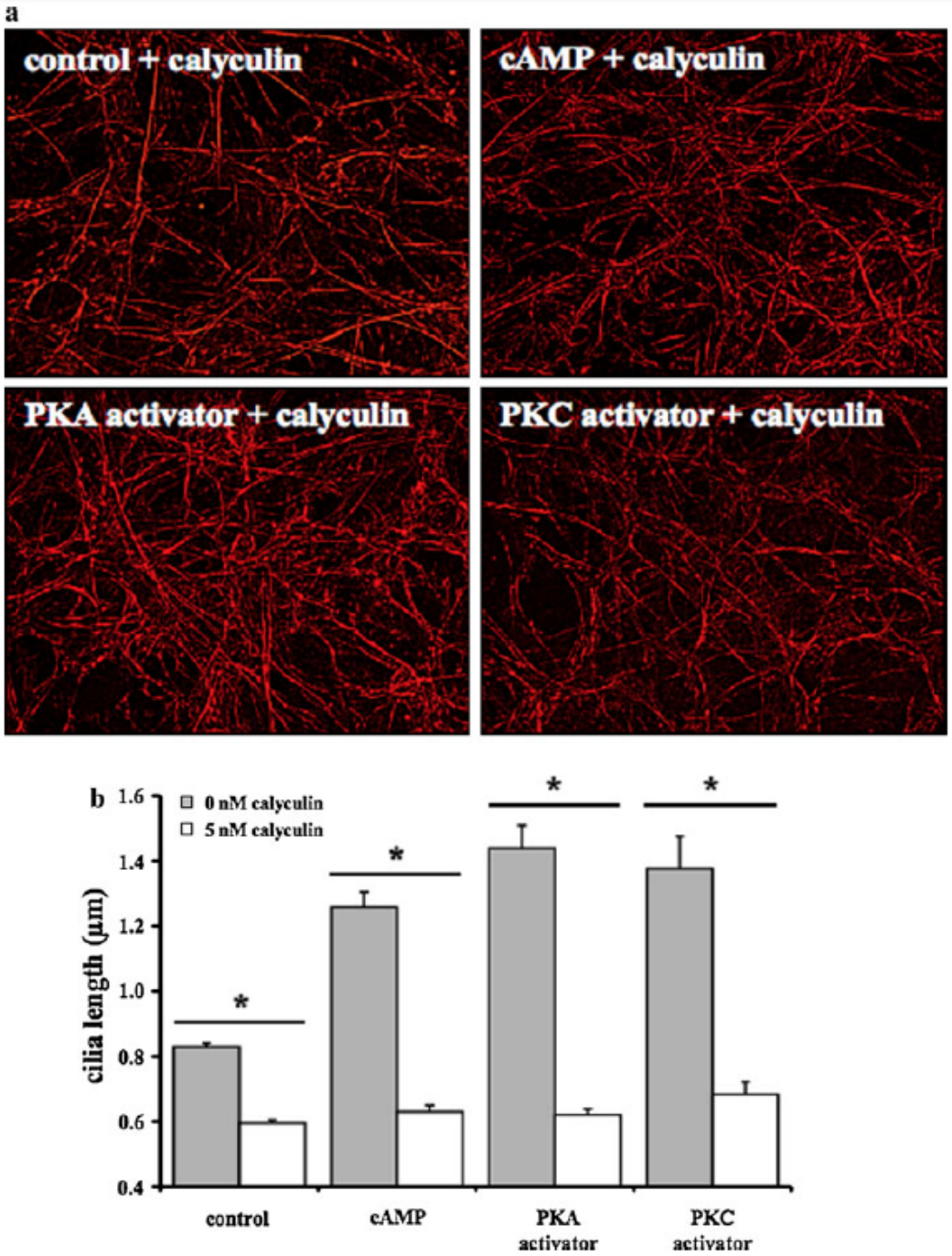

Fig. 4. Inhibition of actin rearrangement is sufficient to shorten ciliary length. **a.** Cytoskeletal actin filament was analyzed in cells before and after treatment with calyculin, a protein phosphatase-1 inhibitor. Regardless of the absence or presence of cAMP analog, PKA activator, or PKC activator, filamentous actin stress fibers are always observed in cells treated with calyculin. **b.** Calyculin-induced actin stress fiber maintenance is accompanied by a shortening in cilia length. n = 3 independent experiments. *p<0.05

cAMP-, PKA- or PKC-induced filamentous actin rearrangement (Fig. 2.4a). This further suggests that cofilin works downstream to PKA and MAPK. We hypothesize

that if cofilin is involved in regulating cilia length, inhibition of PP-1 will result in a decrease in cilia length. Supporting our hypothesis, co-incubation of PP-1 inhibitor with cAMP analog, PKA activator or PKC activator significantly decreases cilia length (Fig. 2.4b). Most surprising is that inhibition of PP-1 basal activity is sufficient to shorten cilia length, as indicated in the control group. We propose that PP-1 and cofilin-induced actin rearrangement play an important role in cilia length maintenance.

Cilia function is regulated by cAMP and PKA

One of the many roles of primary cilia is to function as a mechanical sensor [15, 16]. To understand if cilia function is also regulated by cAMP, PKA, PKC, and/or MAPK, we performed mechanical fluid-shear experiments to examine cilia function (Fig. 2.5 a). To enable us to assay relative functions of cilia, we calculated the amount of total cytosolic calcium increase in response to fluid-shear stress. Total changes in cytosolic calcium are determined by the area under the calcium-time curve (area under the curve). Compared to the control group, only cells treated with cAMP analog or PKA activator show a significant increase in cilia function (Fig. 2.5b). PKC and MAPK do not play a significant role in cilia function. When the area under the curve is correlated to intracellular cAMP concentration, a good correlation with R^2 of 0.81 is observed (Fig. 2.5c). For the first time, we show that cilia length and function are not regulated in the same precise manner.

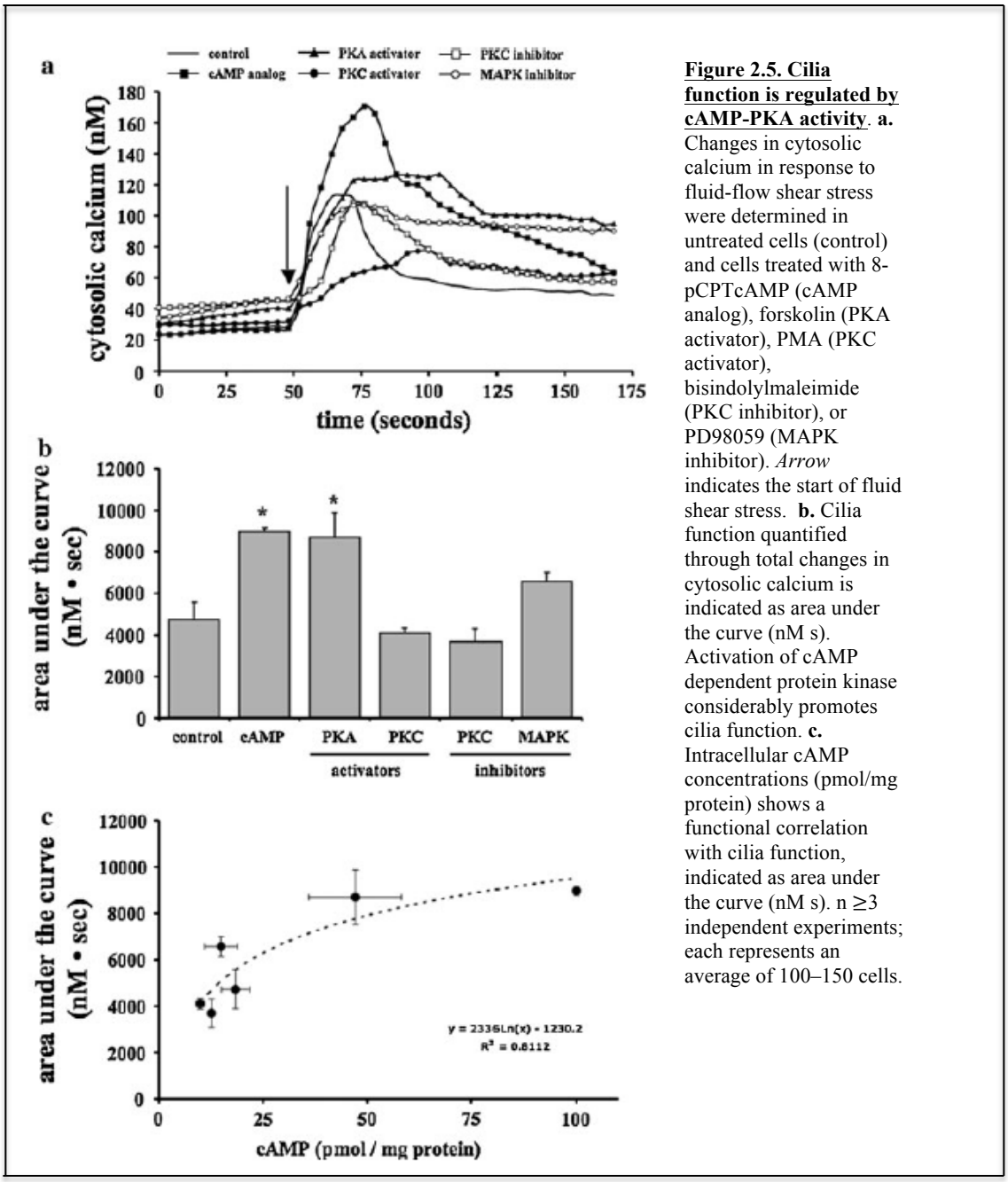

Figure 2.5. Cilia function is regulated by cAMP-PKA activity. **a.** Changes in cytosolic calcium in response to fluid-flow shear stress were determined in untreated cells (control) and cells treated with 8-pCPTcAMP (cAMP analog), forskolin (PKA activator), PMA (PKC activator), bisindolylmaleimide (PKC inhibitor), or PD98059 (MAPK inhibitor). *Arrow* indicates the start of fluid shear stress. **b.** Cilia function quantified through total changes in cytosolic calcium is indicated as area under the curve (nM s). Activation of cAMP dependent protein kinase considerably promotes cilia function. **c.** Intracellular cAMP concentrations (pmol/mg protein) shows a functional correlation with cilia function, indicated as area under the curve (nM s). n ≥3 independent experiments; each represents an average of 100–150 cells.

2.5. Discussion

For the first time, we show that endothelial cilia can be regulated through intracellular cAMP, cAMP-dependent protein kinase (PKA), calcium-dependent protein kinase (PKC), and mitogen-activated protein kinase (MAPK) in mono-layered cells in vitro and femoral arteries ex vivo. We further show that protein

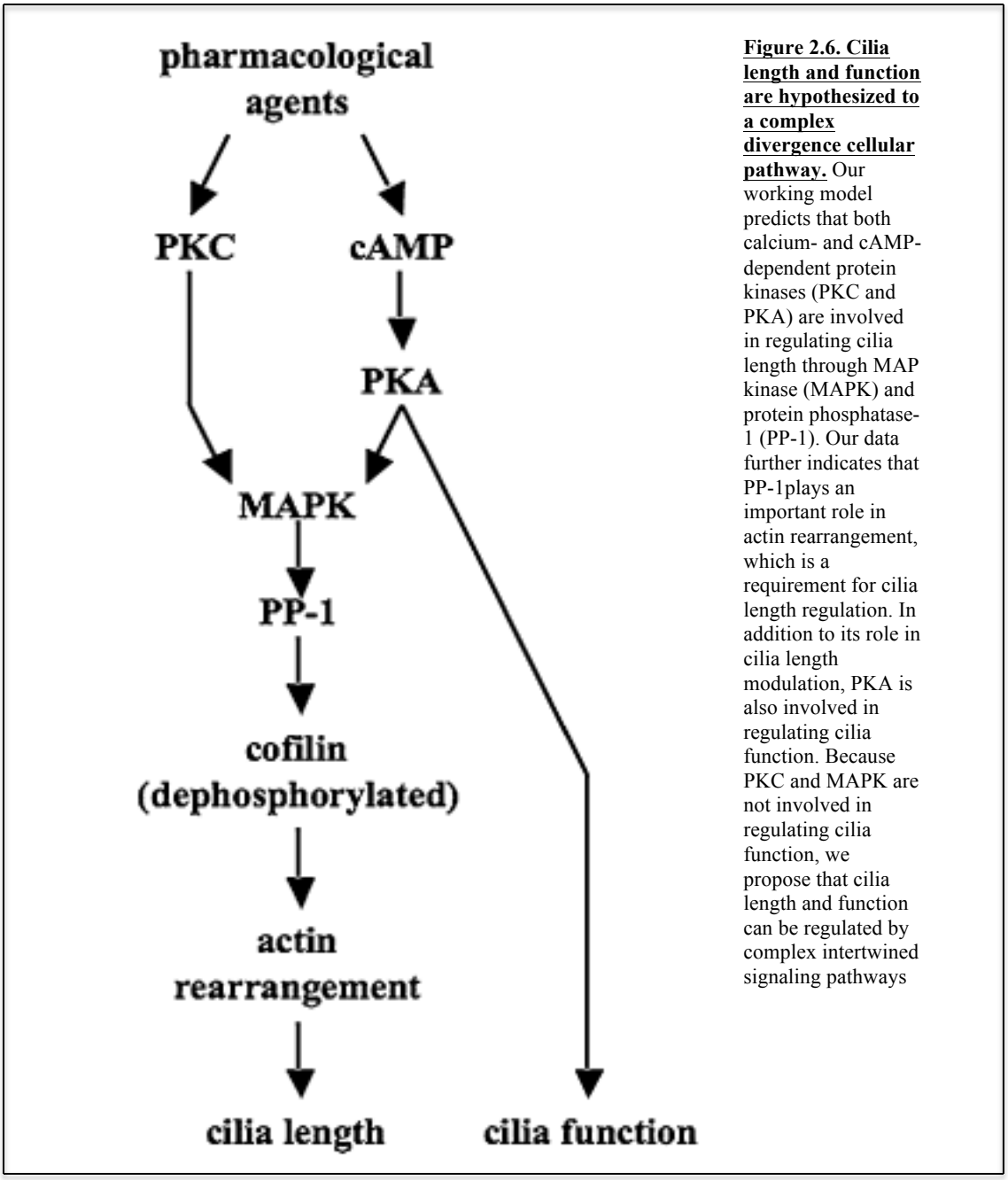

Figure 2.6. Cilia length and function are hypothesized to a complex divergence cellular pathway. Our working model predicts that both calcium- and cAMP-dependent protein kinases (PKC and PKA) are involved in regulating cilia length through MAP kinase (MAPK) and protein phosphatase-1 (PP-1). Our data further indicates that PP-1plays an important role in actin rearrangement, which is a requirement for cilia length regulation. In addition to its role in cilia length modulation, PKA is also involved in regulating cilia function. Because PKC and MAPK are not involved in regulating cilia function, we propose that cilia length and function can be regulated by complex intertwined signaling pathways

phosphatase-1 (PP-1), cofilin and actin rearrangement are also involved in regulating cilia length. Interestingly, only intracellular cAMP and PKA are involved in regulating both cilia length and function. We thus propose that cilia length and function can be regulated by distinct, yet complex intertwined signaling pathways (Fig. 2.6).

Primary cilium functions as a sensory organelle and houses a large number of specialized proteins. These specialized proteins are involved in various sensing of fluid shear stress, chemical, photon, gravity, and many others, which function may be dependent or independent from extracellular calcium influx [2,17]. Most importantly, these sensory proteins are not synthesized within the ciliary body. Intraflagellar transport (IFT) has been known to be responsible for ciliary function and growth, by carrying these sensory proteins and ciliary building blocks from the cell body to the ciliary body. Thus, the structural length and functional maintenance of primary cilium depends on proper functionality of IFT or IFT-associated molecules [18,19].

To further determine the mechanisms involved in ciliary growth and function, we used various well-recognized pharmacological agents in mouse aortic endothelial cells that have been previously generated and characterized [6, 7]. Our data suggest that cilia length can be increased by cAMP levels and PKA activation followed by MAPK activation. Interestingly, MAPK is involved in regulating IFT. MAPK is involved in phosphorylation of motor proteins and/or linker proteins associated with IFT [20]. In addition, MAPK has been proposed to play a similar role in both flagellar biogenesis and the sensory abilities of flagella in trypanosomatids and C. elegans [21, 22]. This opens the possibility that MAPK could regulate both the length of the primary cilium and its ability to function as a sensory organelle. Thus, inhibition of MAPK is expected to result in inefficient IFT mechanism resulting in decreased cilia length, as observed in our experiments.

Our study further shows that PKC activation can also induce MAPK activity,

thereby increasing cilia length. In addition, MAPK can induce PP-1 activity [23], which in turn activates cofilin [14]. Activation of cofilin is accompanied by filamentous actin rearrangement. The involvement of PP-1 and its substrate cofilin on actin rearrangement is further reinforced by the fact that inhibiting activity of PP-1 not only reverses the effects of MAPK but also sufficiently decreases the cilia length at the basal level. Consistent with this finding, modifying the stability of actin cytoskeleton has been implicated in cilia growth [24, 25].

Worth discussing is that activation of PKC tends to decrease cAMP, yet PKC activation increases cilia length. We propose that PKC-induced cilia length increase does not require cAMP/PKA-signaling pathway. However, it has been reported that PKC can also interact and activate various phosphodiesterases (PDEs). PDEs are known to hydrolyze cAMP, resulting in a low level of intracellular cAMP [26]. We thus believe that PKC/PDE/cAMP is a secondary pathway, while PKC/MAPK/PP-1 is the primary signaling pathway for cilia length regulation. Supporting our idea, PKC has been shown to directly activate MAPK activity [27], and MAPK is known to activate PP-1 [23].

PP-1 is a versatile phospho-Ser/Thr phosphatase involved in various eukaryotic cellular functions. Furthermore, PP-1 has been implicated in actin rearrangement in non-muscle cells [28]. At least in the endothelial cells, our data suggest the involvement of cofilin as the actin-related or actin-binding protein (ARP/ABP) involved in actin rearrangement. Of note is that cofilin is one of many PP-1 substrates [14]. Once activated by PP-1, cofilin is readily recruited to the leading edge of the actin filament, resulting in re-organization of the filament [29]. Whether

other ARPs/ABPs would be involved in regulating cilia length remains to be a possibility in other systems, such as in renal epithelial cells.

In addition to IFT, inhibition of actin polymerization is thought to have a major role in ciliogenesis. Actin dynamics, for example, plays an important role in ciliogenesis [24, 25]. In addition, a thick plank of actin at the apical surface of cells is required for docking of the basal body and the subsequent formation of the ciliary axoneme [30]. Thus, any activation or inhibition of actin polymerization could affect the microtubule-based cilium [31], which is in agreement with our current study. It has been reported that activation of the Raf/MEK/ERK pathway is accompanied by actin cytoskeletal reorganization, and cells with ciliary dysfunction exhibit altered actin-spindle organization resulting in substantially centrosomal over amplification and polyploidy [32]. Cofilin is an essential regulator of actin dynamics, participating in reorganization actin cytoskeletal structure among other cellular processes. Cofilin activity is also regulated by Ras/MEK/ERK pathway via PP-1. Ras/MEK/ERK can phosphorylate and activate PP-1, which in turn dephosphorylates and activates cofilin [14, 23]. Thus, treatment of our endothelial cells with MAPK inhibitor, PD98059, and PP-1 inhibitor, calyculin, would result in similar consequences, i.e., loss of actin rearrangement and subsequent decrease cilia length.

The primary cilium is structurally composed of acetylated and detyrosinated microtubules. Thus, activation or inhibition of any cell cytoskeletal proteins can affect the function of microtubule-based cilium [31]. Though our data suggest that MAPK/PP-1/cofilin may play an important role in cilia length, cilia function does not seem to be regulated by this mechanism. PKC activation, which promotes an

increase in cilia length, also does not seem to play any role in cilia function. Our functional studies indicate that activation of cAMP/PKA pathway can promote an increase in cilia function, in addition to cilia length. Overall, our study concludes that the molecular interactions between cilia function and length can be independent of one another. Furthermore, increasing cilia length does not necessarily translate into an increase in cilia function, and vice versa.

2.6. Acknowledgements

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Chapter 3

Dopamine Receptor Type 5 in the Primary Cilia Has Dual Chemo- and Mechano-Sensory Roles

Shakila Abdul-Majeed[1], Surya M. Nauli [1,2 †]

[1]Department of Medicinal & Biological Chemistry, [2]Department of Pharmacology,

University of Toledo, Health Science Campus, HEB 274, 3000 Arlington Avenue,

MS 1015, Toledo, OH 43614, USA.

†Address correspondence and reprint requests to

Surya M.Nauli
College of Pharmacy
University of Toledo, Health Science Campus, HEB 274, 3000 Arlington Avenue,
MS 1015, Toledo, OH 43614, USA.
Tel: (419) 383 1921
Fax: (419) 383 xxxx
Email: surya.nauli@utoledo.edu

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3.1. Abstract

Polycystic kidney disease is characterized by cardiovascular irregularities, including hypertension. Dopamine, a circulating hormone, is implicated in essential hypertension in humans and animal models. Vascular endothelial primary cilia are known to function as mechano-sensory organelles. Although both primary cilia and dopamine receptors play important roles in vascular hypertension, their relationship has never been explored. To determine the roles of the dopaminergic system and mechano-sensory cilia, we studied the effects of dopamine on ciliary length and function in wild-type and mechano-insensitive polycystic mutant cells ($Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$). We show for the first time that mouse vascular endothelia exhibit dopamine receptor-type 5 (DR5), which co-localizes to primary cilia in cultured cells and mouse arteries in vivo. DR5 activation increases cilia length in arteries and endothelial cells through cofilin and actin polymerization. DR5 activation also restores cilia function in the mutant cells. In addition, silencing DR5 completely abolishes mechano-ciliary function in WT cells. We found that DR5 plays very important roles in ciliary length and function. Furthermore, the chemo-sensory function of cilia can alter the mechano-sensory function through changes in sensitivity to fluid-shear stress. We propose that activated ciliary DR5 has a functional mechano-sensory role in endothelial cells.

3.2. Introduction

Primary cilium is a small hair-like projection present on the apical membrane of most cells. By virtue of its shape and location, the primary cilium is able to act as an

antenna, sensing and transmitting information from the extracellular matrix to the cell interior. To assist with its unique sensory roles, a high density of specialized proteins, such as receptors, ion channels, kinases, phosphatases, secondary messengers, and other signaling modules, is localized in the ciliary membrane, cilioplasm, or at the ciliary base[1]. These proteins enable the primary cilia to act as chemo-sensors and mechano-sensors. Dysfunctional cilia have been associated with a large number of diseases, such as polycystic kidney disease (PKD) and various other diseases, which have been collectively referred to as "ciliopathies." Improper structure and function of the primary cilia have been reported in patients experiencing PKD[2-4]. In addition to renal cyst formation, PKD is also characterized by noncystic manifestations, such as hypertension, left ventricular hypertrophy, cardiac valve abnormalities, intracranial aneurysms, and abdominal wall hernias, among others[5,6]. Furthermore, hypertension in PKD has been associated with abnormal mechanosensory cilia function and structure[7,8.]

Dopamine is an endogenous neuronal hormone, known to produce a wide range of cardiovascular and renal effects. Various subtypes of dopamine receptors are known to be present in different parts of the cardiovascular system[9]. Hence, dopamine is known to regulate systemic blood pressure, renal hemodynamics, and electrolyte balance. In humans, activation of dopamine receptors within the blood vessels can cause vasodilation[10]. Most importantly, circulating dopamine mediates vasodilation through both endothelium dependent (60%) and endothelium-independent (40%) mechanisms[11].

Within the vascular endothelial cells, dopamine receptors type 1 (DR1) and 5 (DR5)

are involved in endothelium-dependent relaxation (Ohlstein et al, 1984). Because of this, any abnormality in dopamine metabolism and/or receptor function has been implicated in essential hypertension in humans[13-15] and animal models (Nauli et al, 2003, Abdul-Majeed & Nauli, 2010,[16–18].

Despite the fact that cilia and dopamine play critical roles in hypertension, their relationship has not been explored. All we know from the clinical study is that PKD patients with borderline hypertension are better managed with DOPA (a dopamine precursor) than with angiotensin-converting enzyme inhibitors[19]. Our current studies show for the first time that the dopaminergic system regulates sensory cilia structure and function. Activation of the ciliary dopamine receptor increases cilia length. To examine the relationship between dopamine and cilia within PKD, we further used mechano insensitive $Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$ endothelial cells, derived previously from Pkd mouse models[7,8]. We show that ciliary dopamine activation can restore mechano-sensory cilia function in response to fluid-shear stress. We propose that localization of the dopamine receptor to cilia plays important chemo-sensory and mechano-sensory roles in vascular endothelial cells.

3.3. Materials and Methods

Animal and cell culture

In our studies, we used vascular endothelial cells that were previously generated and characterized for various surface and intracellular markers[1,2]. Cells were grown on a glass surface that had been coated with rat type I collagen and sterilized under UV light. Cells were grown to a confluent monolayer in Dulbeccos's Modified Eagle Medium with 2% fetal bovine serum at 39 ºC and a constant 5% CO2 and 95% O2

mixture for at least 2 to 3 days before the experiments.

Qualitative PCR analysis

Total RNA was isolated from mouse endothelial cells, heart and brain tissues using Qiagen RNeasy Midi kit. The cDNA was synthesized using Invitrogen SuperScript one step RT-PCR technique. The annealing temperature was at 60 ºC for 30 cycles in all cases. The primers for different types of dopamine receptors (DR) were designed based on the accession numbers from NCB database as follows: **DR1** (NM_010076; 5'-AAG ATG CCG AGG ATG ACA AC-3' and 5'-CCC TCT CCA AAG CTG AGA TG-3'), **DR2** (NM_010077; 5'- TGC CAT TGT TCT TGG TGT GT-3' and 5'-GTG AAG GCG CTG TAG AGG AC-3'), **DR3** (NM_007877; 5'-CCC TCA GCA GTC TTC CTG TC-3' and 5'-AGT CCT CTC CAC TTG GCT CA-3'), **DR4** (NM_007878; 5'-CGT CTC TGT GAC ACG CTC AT-3' and 5'-AAG GAG CAG ACG GAC GAG TA-3'), and **DR5** (NM_013503; 5'-ACC AAG ACA CGG TCT TCC AC-3' and 5'-CCT CCT CCT CAC AGT CAA GC-3')

Cilia analysis and measurement

Primary cilia were observed with fluorescence and scanning electron microscopes. For fluorescence microscopy, cells or femoral arteries were first fixed with 4% paraformaldehyde in 2% sucrose solution for 10 minutes at room temperature. Dopamine receptor-type 5 (EMD/MercSciences; 1:2500 dilution, 72 hours at 4 °C) and type 3 (Calbiochem; 1:5,000 dilution, 72 hours at 4 °C)-specific antibodies were used. Acetylated-**α**- tubulin (Sigma clone 6-11B-1; 1:10,000 dilution) was used both

as a cilia marker and to measure cilia length. The cover slip was then mounted on the microscope slide with mounting media containing dapi. When femoral artery was used, a segment of about 2 mm was cut open. The open lumen containing endothelia was then covered with microscope cover slip. Images were observed in an inverted Nikon Ti-U microscope and analyzed with Metamorph 7.0. All image analyses were performed by capturing series of Z-stack and compiled for a more accurate measurement.

For scanning electron micrograph, cells or femoral arteries were fixed with 2.5% paraformaldehyde / glutaraldehyde in sodium cacodylate buffer for one hour at room temperature. Samples were post-fixed with 1% aqueous osmium tetroxide solution and dehydrated using graded ethyl alcohol solutions. In case of femoral artery, after fixing and drying the piece for 24 hours, we made very fine cross-sections of the artery (~ 1mm) as such that the lumen would always be exposed for analysis. The samples were chemically dried using an initial 2-hour incubation in 50% HMDS-ethyl alcohol mixture, followed by two half-hour incubations in 100% HMDS. Micrographs were obtained and analyzed using Hitachi HD-2300 scanning electron microscope.

siRNA transfection

To examine cellular function of dopamine, various siRNAs were designed to knock down dopamine receptor type 5. Dividing cells were transfected with lipofectamine (Invitrogen), scramble siRNA, **siRNA1** (5'-AUC AUG UGG ACA UAG GCA GCA GCG A-3'), **siRNA2** (5'-AUG ACC AGC AAU GCC ACG AAG AGG U-3'), or

siRNA3 (5'-CAC ACU AGG ACG UUG CCG AGC AAG G-5'). All siRNAs were conjugated with GFP to monitor transfection efficiency, and 24 nM was used with transfection efficiency of about 95%.

Cytosolic calcium measurement

Endothelial cells were incubated with 5 μmol/L Fura2-AM for 30 minutes at 39 ºC. Basal calcium was equilibrated for about a minute. Agonist at optimal concentration or flow at optimal shear stress was used to monitor changes in cytosolic calcium as previously described[1,2]. Paired fluorescence images of Fura2 at excitation wavelengths of 340 (calcium-bound indicator) and 380 nm (calcium-free indicator) were monitored and recorded at every 4 seconds using Nikon TE2000 microscope and analyzed using Metafluor software. At the end of each experiment, a minimum fluorescence was determined by treating the cells with 2 mM EGTA and 10 μmol/L ionomycin. After achieving the minimum signal, the maximum fluorescence was obtained by treating the cells with excess calcium (10 mM) to calculate intracellular free calcium. All fluorescence measurements were corrected for auto-fluorescence.

Protein detection

Protein concentration was first measured using BSA kit (Pierce) with the linear regression of the standards of 0.999. A total protein of 50 or 150 mg was analyzed with a standard Western blot. The following antibodies and dilutions were used in our analyses: phospho-cofilin (Cell Signaling, 1:100), anti-cofilin (Cell Signaling, 1:250), dopamine receptor type5 (Calbiochem, 1:200), α-tubulin (Abcam, 1:5,000),

actin (Sigma, 1:500) and GAPDH (Cell Signaling, 1:1,500). Expression levels were quantified using NIH's ImageJ software.

Pharmacological treatment

Pharmacological agents include dopamine, dopamine receptor-3 specific antagonist (**S**)-Nafadotride tartrate, dopamine receptor-1/5 agonist (**R**)-(+)-SKF-38393 hydrochloride, and dopamine receptor-2/3 agonist (+)- bromocriptine methanesulfonate salt; all were purchased from Sigma. Pharmacological agents were added after the cells were differentiated at 39 ºC to avoid any potential effect on cell growth. Isolated femoral arteries were briefly cleaned and rinsed with PBS containing calcium. Fresh 2 mL media and the drug were added, and the samples were incubated at 39 ºC for the desired durations. In some cases, the samples were first pre-treated with an antagonist for 15 minutes before being re-challenged with an agonist for 4 or 16 hours. Extreme caution was taken when adding the drugs; drugs were pre-diluted to different concentrations so that the exact same volume of the drug solution was added to the samples, in an effort to maintain identical volume.

Statistical Analysis

All quantifiable experimental values are expressed as mean±SEM, and values of $\mathbf{p}<0.05$ were considered significant. All comparisons between two groups were performed with student's t-test with 2 samples assuming unequal variance. Comparisons of three or more groups were done using ANOVA, followed by Tukey's posttest. Data analysis was performed using Sigma Plot software version

11.

3.4. Results

DR5 Localizes to and Regulates Length of Primary Cilia

We show for the first time that DR5 is localized to the primary cilia of cultured

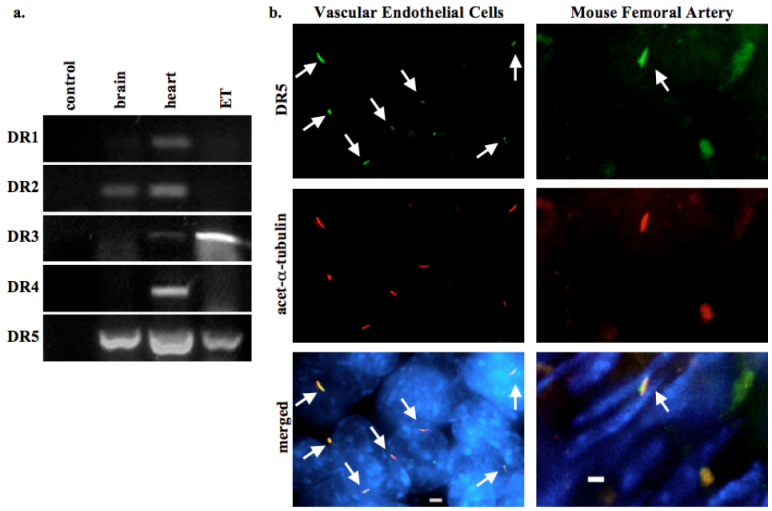

Figure S1. Dopamine receptor is expressed in endothelial cells. a. RNA message of dopamine receptor (DR) was analyzed from negative control (no mRNA) and positive controls (mRNA from mouse brain or heart). Wild-type vascular endothelial cells (ET) show the presence of DR3 and DR5 receptors. **b.** Immunolocalization study using specific antibody to dopamine receptor 5 (DR5) confirms ciliary expression (green) in monolayer endothelial cells (in vitro) and in femoral artery (in vivo). Acetylated-**α**-tubulin was used as a ciliary marker (red), and merged images are also shown. Arrows indicate the presence of cilia. N=3 independent experiments. Bar = 1μm.

endothelial cells and the femoral artery in vivo. Using well-characterized mouse endothelial cells, expressions of DR3 and DR5 are detected at the transcript level (Figure S1a, available in the online Data Supplement). Subcellular localization of these receptor subtypes was studied in 3D using DR3- and DR5-specific antibodies (Figure 3.1A). DR5 is localized to primary cilia of wild-type and $Pkd1^{-/-}$ endothelial cells. DR5 is also ocalized in short, stubby cilia of $Tg737^{orpk/orpk}$ cells. DR5 cilia localization was observed widely in a monolayer of endothelial cells and also in endothelia of the femoral artery in vivo (Figure S1b). No specific localization of

DR3 was observed in the cilia (data not shown).

Dopamine treatment for 4 or 16 hours increases cilia length in a dose-dependent manner (Figure 3.1B). Concentration of dopamine to induce maximal increase in

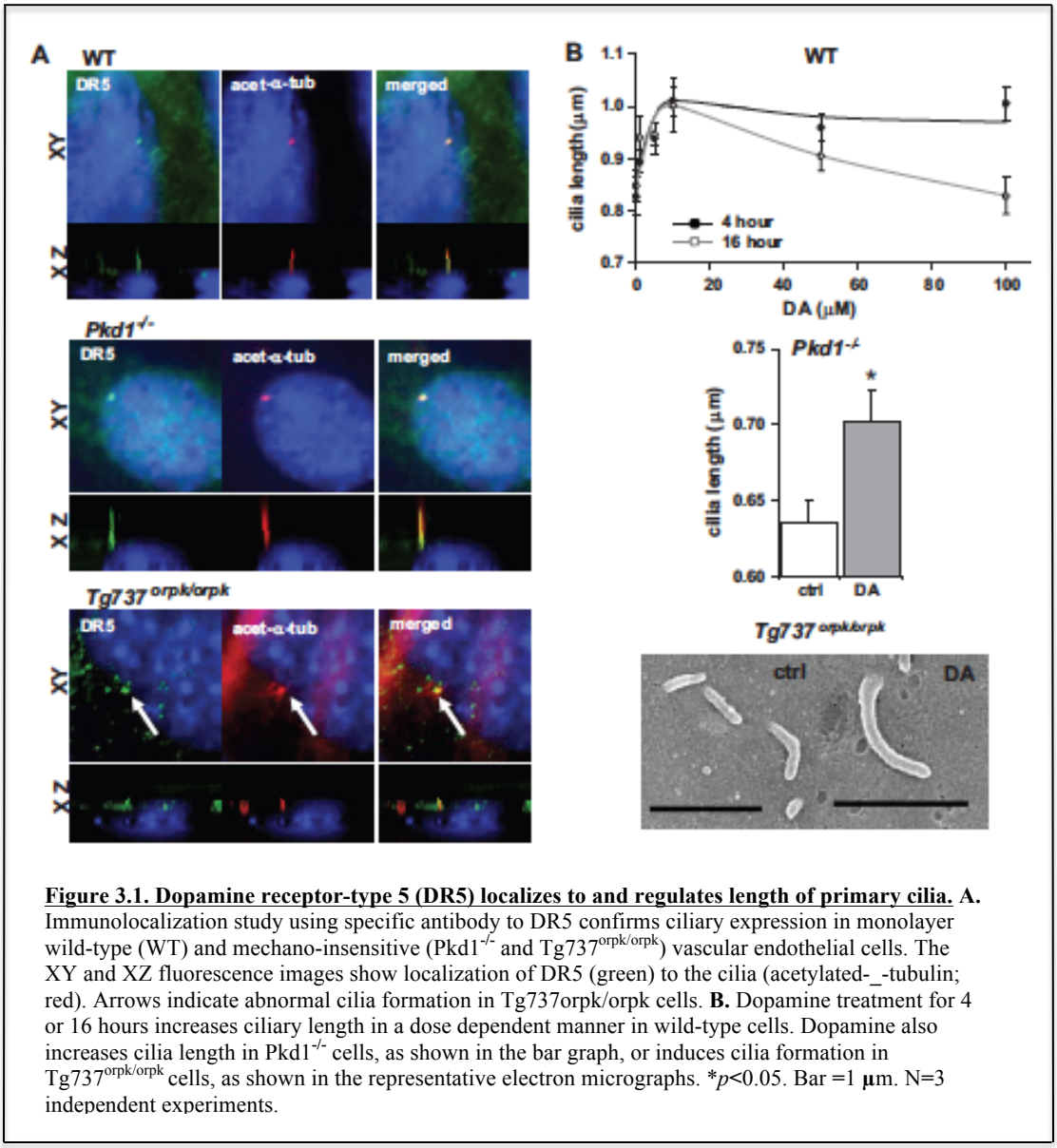

Figure 3.1. Dopamine receptor-type 5 (DR5) localizes to and regulates length of primary cilia. A. Immunolocalization study using specific antibody to DR5 confirms ciliary expression in monolayer wild-type (WT) and mechano-insensitive ($Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$) vascular endothelial cells. The XY and XZ fluorescence images show localization of DR5 (green) to the cilia (acetylated-_-tubulin; red). Arrows indicate abnormal cilia formation in Tg737orpk/orpk cells. **B.** Dopamine treatment for 4 or 16 hours increases ciliary length in a dose dependent manner in wild-type cells. Dopamine also increases cilia length in $Pkd1^{-/-}$ cells, as shown in the bar graph, or induces cilia formation in $Tg737^{orpk/orpk}$ cells, as shown in the representative electron micrographs. *p<0.05. Bar =1 μm. N=3 independent experiments.

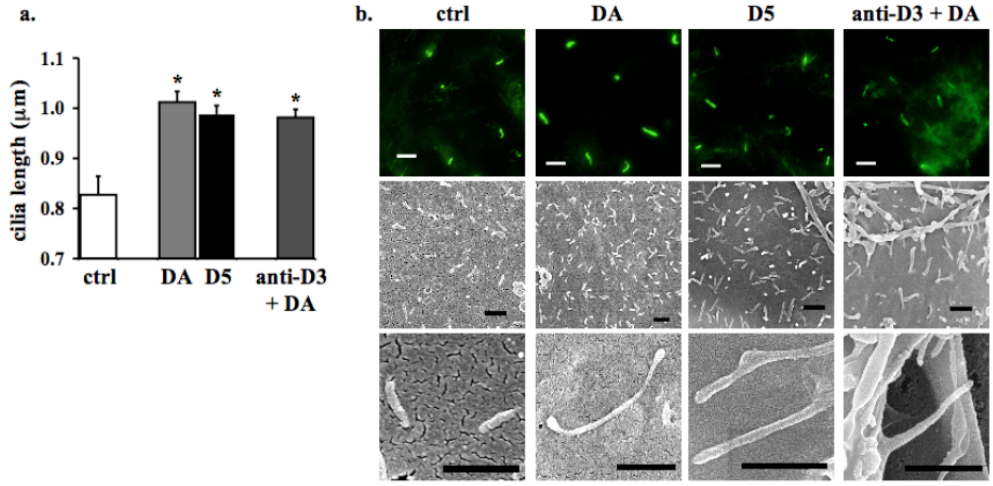

Figure S2. Dopamine increases ciliary length in cultured endothelial cells. a. Effects of dopamine (10 **μ**mol/L; 4 hours), partially selective DR5 agonist (SKF-38393 at 10 **μ**mol/L; 4 hours), and DR3 selective antagonist nafadotride tartrate (15 minutes at 10 **μ**mol/L, followed with 10 **μ**mol/L dopamine for 4 hours) indicate involvement of DR5-, but not DR3-induced cilia length increase. **b.** Representatives of ciliary length are shown in cells treated with vehicle as control (ctrl), dopamine (DA), SKF- 38393 (D5), and nafadotride tartrate and dopamine (anti-D3 + DA). Upper panels show cilia in endothelial cells, as observed with fluorescence microscopy. Lower panels represent electron micrographs at various magnifications. N>3 independent experiments; each with over 120 measurements. ***p**<0.05. Bar = 1μm.

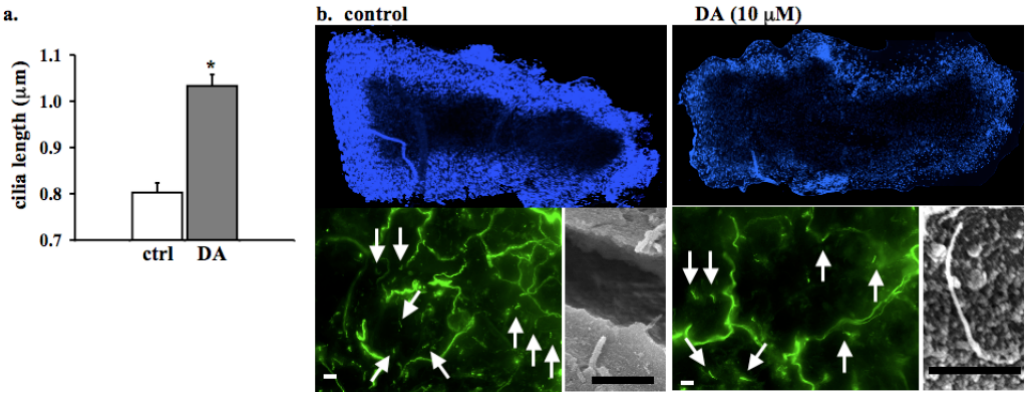

Figure S3. Dopamine increases ciliary length in endothelia of femoral artery. a. Isolated femoral arteries from adult mice were incubated with 10 **μ**mol/L dopamine for 16 hours. Dopamine significantly increased length of the cilia in vascular endothelia ex-vivo. b. Cilia length was studied with fluorescence and electron micrographs. Control (untreated) or dopamine-treated (10 **μ**mol/L) arteries shown in blue fluorescence represent the structural layout of a piece of femoral artery. Acetylated-**α**-tubulin is used to identify cilia length (green), and representative images were selected randomly. N=3 independent experiments; each with over 120 measurements. Arrows indicate the presence of cilia. ∗p<0.05. Bar = 1μm.

cilia length is optimal at10 **μ**mol/L for both 4 and 16 hours. Activation of DR5 is

sufficient to increase cilia length (Figure S2a). To further confirm that DR3 activation does not play a role in cilia length regulation, we used the DR3 inhibitor in the presence of dopamine. Observation with immunofluorescence and electron microscopy techniques shows that DR5 activation, either with dopamine or a DR5-specific agonist, increases in cilia length (Figure S2b). To further verify this finding, we isolated and treated mouse femoral arteries with either vehicle or 10 μmol/L of dopamine for 16 hours (Figure S3a). As expected, dopamine increases cilia length ex vivo comparable to that of cultured cells. Because the femoral artery contains smooth muscle cells, which also have primary cilia[20,21], the artery was laid down in such a way that only the first layer of cells from the intima was observed through both immunofluorescence and electron microscopy techniques (Figure S3b).

To understand the functional relevance of ciliary DR5 in PKD, we examined DR5 activation in $Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$ endothelial cells (Figure 3.1B). Interestingly, cilia length is also increased significantly in $Pkd1^{-/-}$ cells treated with dopamine. Because of their small and stubby cilia, we were not able to accurately determine the cilia length measurement in $Tg737^{orpk/orpk}$ cells. However, it is surprising that the length of cilia in $Tg737^{orpk/orpk}$ cells tends to be longer or occasionally restored, as seen in wild-type cells. In all of the genotypes, receptor activation with dopamine does not show an apparent subcellular redistribution of DR5 (data not shown).

Dopamine Increases Cilia Length Through Cellular Actin Differentiation via Cofilin Dephosphorylation

Inhibition of actin polymerization has been shown to play an important role in ciliogenesis[22–24]. Furthermore, the dephosphorylated or activated form of cofilin has

been shown to inhibit actin polymerization[25,26]. To examine this possibility in our system, we measured phosphorylated cofilin before and after treatment with dopamine for 15 and 60 minutes (Figure 3.2A). Supporting our idea, a significant

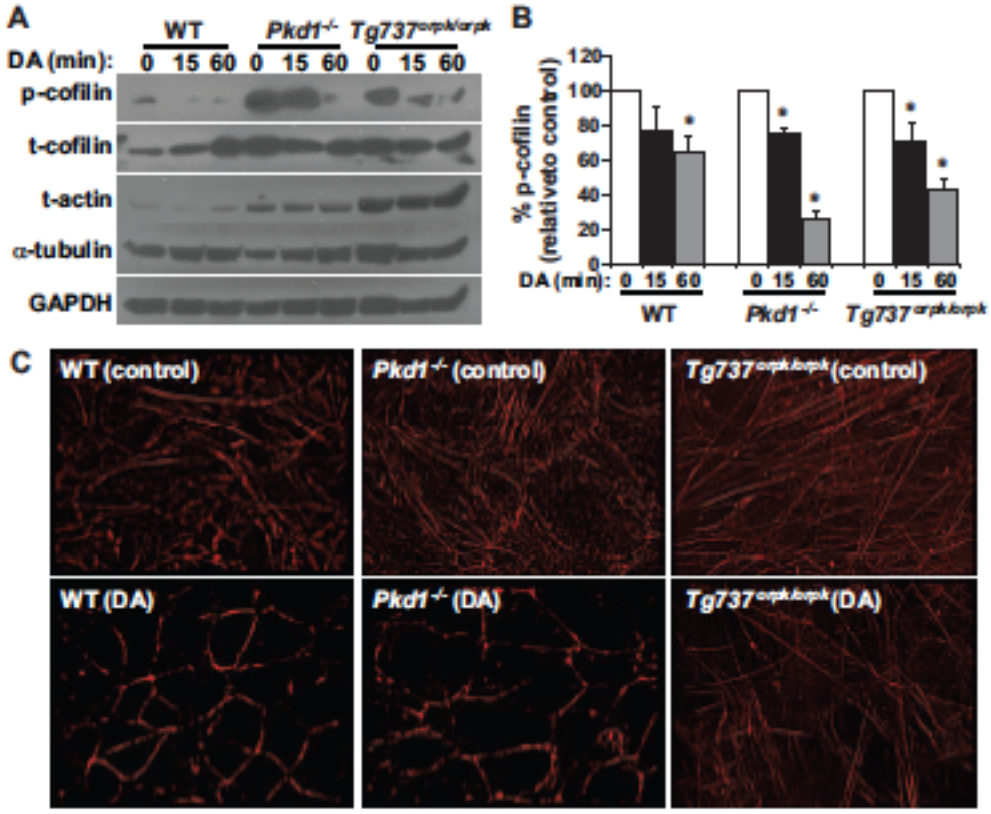

Figure 3.2. Dopamine increases cilia length through cellular actin differentiation via cofilin dephosphorylation. **A**.Wild-type (WT), Pkd1$^{-/-}$ and Tg737$^{orpk/orpk}$ cells were analyzed for the ratio of phosphorylated cofilin (p-cofilin) to total cofilin (t-cofilin) before and after 10 μmol/L of dopamine (DA) treatment for 15 or 60 minutes. Both α-tubulin and GAPDH were used as loading controls, whereas total actin (t-actin) consistently expresses at a higher level in the mutant cells. **B**. Dopamine significantly decreases levels of phosphorylated cofilin in wild-type, Pkd1$^{-/-}$ and Tg737$^{orpk/orpk}$ cells. **C**. Dopamine induces cellular differentiation, as evidenced from cytoskeletal actin rearrangement. Stress actin fibers and cortical actin formation are, respectively, presented before (control) and after 60 minutes of dopamine treatment. N=3 independent experiments. *P<0.05.

decrease of phosphorylated cofilin is observed in dopamine-treated cells (Figure 3.2B). Throughout our Western blot analyses, we also consistently observed the expression level of total actin to be greater in Pkd1$^{-/-}$ and Tg737$^{orpk/orpk}$ than in wild-type cells. Please note that we denoted the total actin as globular actin and

filamentous actin (F- actin) because we reduced and monomerized F-actin during our sample preparation. Thus, we next analyzed F-actin only to further understand

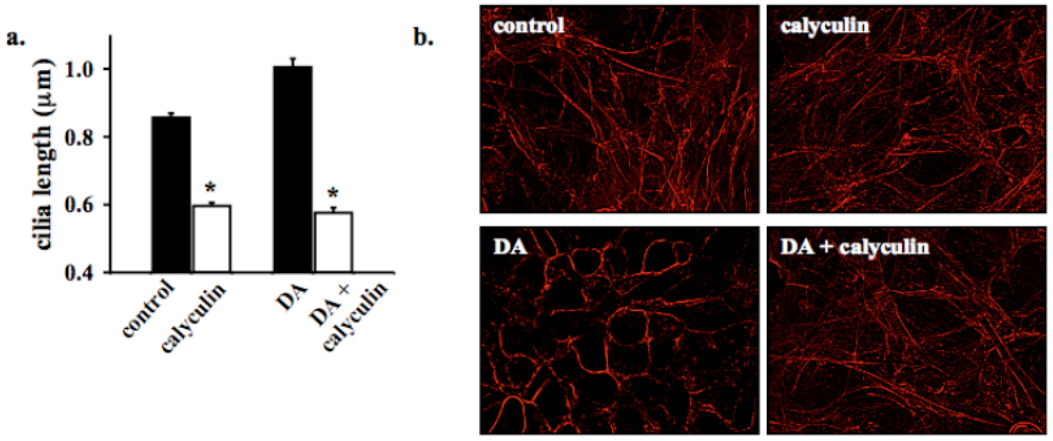

Figure S4. Protein phopatase-1 plays an important role in dopamine-induced cilia length and actin rearrangement. a. Protein phosphatase-1 (PP1) dephosphorylates and thereby activates cofilin. Calyculin, a protein phosphatase-1 inhibitor (5 nmol/L, 16 hours), significantly decreases in cilia length. Calyculin also inhibits dopamine (DA)-induced cilia length increase substantially. **b.** Dopamine-induced actin rearrangement is blocked by calyculin. This indicates that calyculin is downstream to dopamine, which regulates cilia length in wild-type vascular endothelial cells. N>3 independent experiments; each represents an average of 100-150 cells

the effects of dopamine in actin polymerization (Figure 3.2C). To our surprise, dopamine induces actin rearrangement in all of the cell types. Although the effect on Tg737$^{orpk/orpk}$ cells is not as substantial, dopamine induces redistribution of stress actin fibers to cortical actin. This actin redistribution has been associated with shear-induced cellular differentiation[27], a characteristic of mechanical-induced cilia activation[28]. To further confirm the roles of cofilin in regulating cilia length, we used calyculin to increase the basal phosphorylation level of cofilin by blocking protein phosphatase 1. Thus, calyculin would be constitutively inactivated. Blocking cofilin sufficiently and significantly decreases cilia length in the presence or absence of dopamine (Figure S4a). When the F-actin was analyzed, the association between actin rearrangement and cilia length was further confirmed (Figure S4b). We found

that calyculin could block dopamine-induced actin rearrangement.

Activation of Ciliary Dopamine Receptor Partially Restores Mechanosensory Function of Endothelial Cilia in Pkd1$^{-/-}$ and Tg737$^{orpk/orpk}$ Cells

Because primary cilia have been proposed to be chemosensory organelles[29,30] and to further verify the functional specificity of DR5 in the cilia, we challenged wild-type endothelial cells with dopamine, DR5-specific, and DR3-specific agonists (Figure S5a). Our data show that DR3 activation has no functional implication, at least in cytosolic calcium increases. Most important is that the agonist-induced cytosolic calcium studies validate the involvement of DR5 in cilia function. We also challenged Pkd1$^{-/-}$ and Tg737$^{orpk/orpk}$ cells with dopamine (Figure S5b). As in wild-type cells, the chemosensory role of the dopamine receptor in the cilia was verified in these mutant cells. Because of shorter cilia and, thus, lower DR5 expression level to cilia, a much smaller increase in cytosolic calcium was observed in Tg737$^{orpk/orpk}$

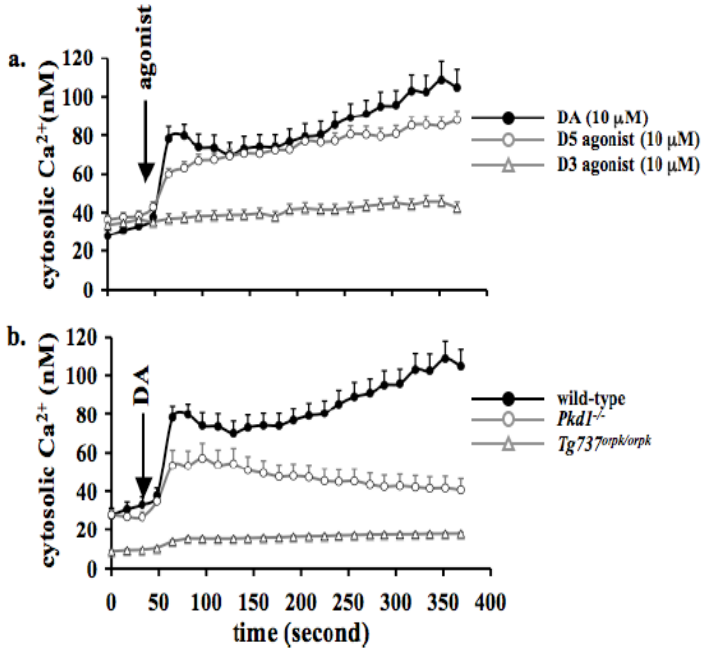

Figure S5. Chemosensory function of DR5 depends on proper cilia structure.
a. DR5-specific response was verified through independent studies with dopamine (10 µmol/L), partially selective DR5 agonist (SKF-38393, 10 µmol/L), and partially selective DR3 agonist (Bromocriptine, 10 µmol/L) in wild-type vascular endothelial cells. **b.** Dopamine-induced intracellular calcium increases in wildtype, Pkd1$^{-/-}$, but less in Tg737$^{orpk/orpk}$ cells reinforces sensory function of dopamine receptor in sensory primary cilia. N=5-9 independent experiments; each represents an average of 100-150 cells. *p<0.05.

cells.

Vascular endothelial cilia have also been proposed to function as mechanosensory organelles[31,32]. To examine the correlation between cilia length and function, we

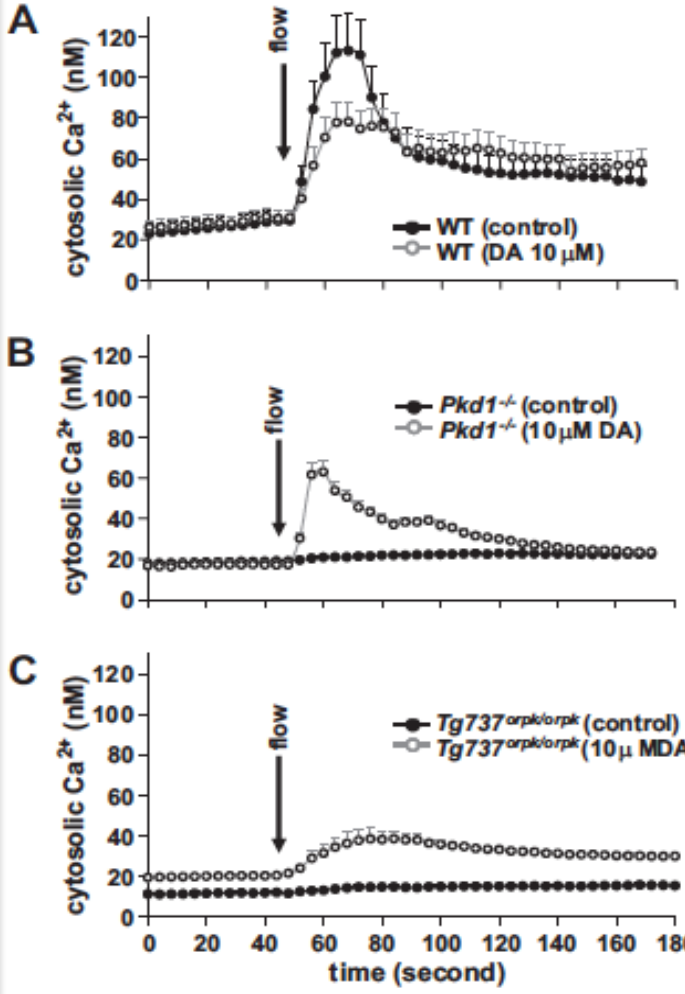

Figure 3.3. Activation of ciliary dopamine receptor partially restores mechano-sensory function of endothelial cilia in Pkd1$^{-/-}$ and Tg737$^{orpk/orpk}$ cells. A. Mechanosensory function of cilia is determined by studying changes in intracellular calcium in response to fluid-flow shear stress. Untreated wild-type (WT) cells and cells treated with dopamine (DA) for 16 hours do not exhibit significant changes in cytosolic calcium in response to fluid-flow shear stress. **B.** Pkd1$^{-/-}$ cells are incapable of responding to fluid-flow shear stress, and, hence, an increase in intracellular calcium is not observed in Pkd1$^{-/-}$in response to fluid flow. However, after treatment with dopamine for 16 hours, Pkd1$^{-/-}$ cells regain their ability to sense fluid flow, with a significant increase in intracellular calcium. **C.** Tg737$^{orpk/orpk}$ cells, which have short or stubby cilia, are incapable of sensing fluid flow shear stress. After dopamine treatment, these cells exhibit a small but significant rescue of the ciliary function. N=5 to 9 independent experiments; each represents an average of 100 to 150 cells.

performed fluid-shear stress experiments to analyze cilia function in wild-type cells treated with vehicle or 10 μmol/L of dopamine for 16 hours (Figure 3.3A). We found that the averaged areas under the curve in the presence and absence of dopamine were not significantly different. Thus, whereas dopamine significantly increases cilia length in wild-type endothelial cells, its effect on cilia function is minimal. Because dopamine also increases cilia length in Pkd1$^{-/-}$ cells, as well as

Tg737$^{orpk/orpk}$ cells, we next examined whether dopamine could have an effect on the mechanosensory function of cilia. Unexpectedly, the mechanosensory role in Pkd1$^{-/-}$ cells was restored in the presence of dopamine (Figure 3.3B). More surprisingly, the mechanosensory role in Tg737$^{orpk/orpk}$ cells was also restored in the presence of dopamine (Figure 3.3C).

Ciliary DR5 Is a Mechanosensory Receptor

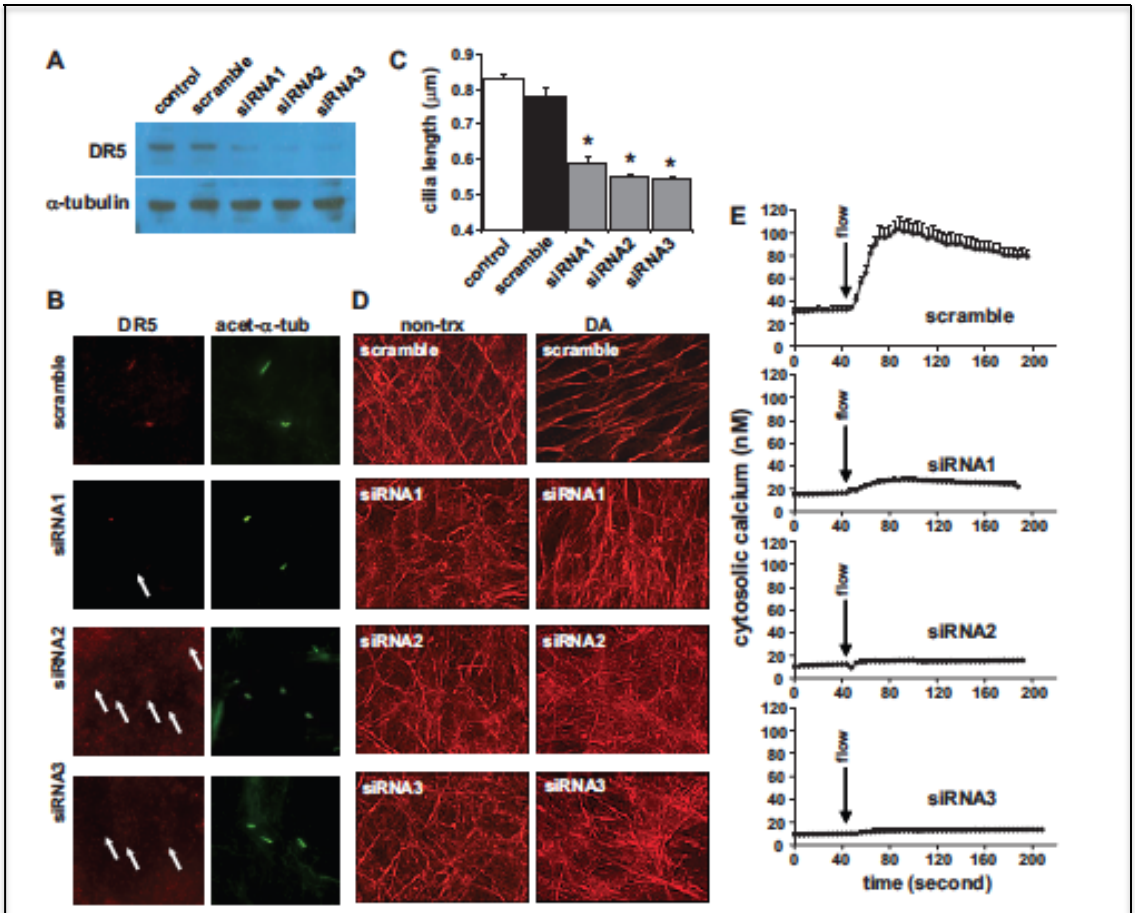

Figure 3.4. Ciliary dopamine receptor type 5 (DR5) is a mechano-sensory receptor. A. The presence of DR5 was analyzed by Western blot in wild-type vascular endothelial cells transfected with lipofectamine only (control), scramble RNA, small-interfering (si)RNA1, siRNA2, or siRNA3. α-tubulin was used as a loading control. **B.** The presence of ciliary dopamine receptor was verified in transfected cells, and acetylated α-tubulin was used as a ciliary marker. White arrows indicate the absence of dopamine receptor in the cilia. **C.** Cilia length decreases in siRNA-transfected cells compared with nontransfected (control) or scramble siRNA-transfected cells. *P<0.05. **D.** Cytoskeletal actin filament was analyzed in the transfected cells before treatment (nontrx) and after 16-hour dopamine treatment (DA). **E.** Mechano-sensory function of cilia was analyzed in the transfected cells. N=3 independent experiments; each represents an average of 100 to 150 cells.

To further confirm DR5 as a mechanosensory receptor, we transfected wild-type endothelial cells with various green fluorescent protein–tagged siRNAs, thereby knocking down DR5 level in the cells. Protein analysis shows substantially repressed DR5 expressions in siRNA-treated cells compared with untreated cells or transfected

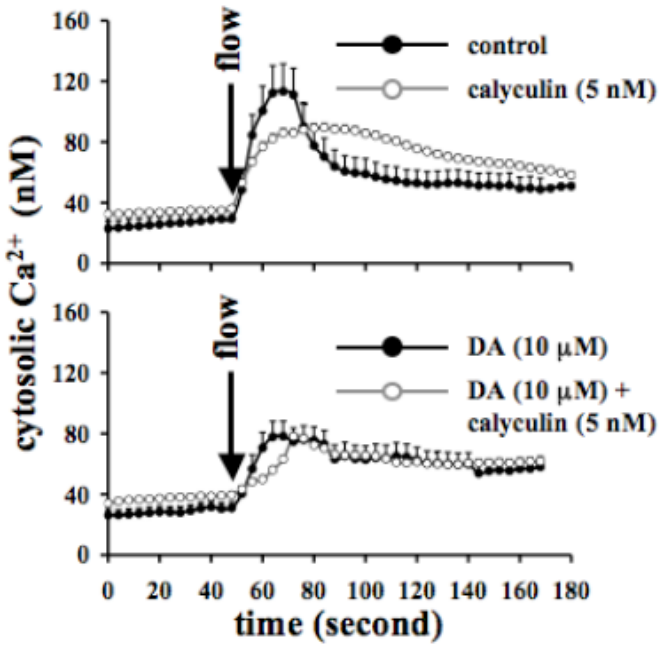

Figure S6. Protein phopatase-1 plays a minor role in cilia function. Though cilia length decreases in the presence of calyculin, mechanosensory cilia function is not compromised in the presence or absence of dopamine (DA). Area under the curve for calyculin-treated cells remains unchanged. N>3 independent experiments; each represents an average of 100-150 cells.

cells with scramble siRNA (Figure 3.4A). As expected, no DR5 was detected in the cilia of DR5-specific siRNA-treated cells (Figure 3.4B). Furthermore, DR5 knockdown cells have significantly shorter cilia, confirming the role of dopamine in regulating cilia length (Figure 3.4C). Consistent with our data showing that dopamine regulates cilia length through actin distribution, dopamine induced actin distribution is abolished in DR5 knockdown cells have knockdown cells (Figure 3.4D). To examine mechanosensory function of cilia, we next challenged the cells with fluid-shear stress (Figure 3.4E). Cells treated with siRNA became insensitive to

fluid flow, supporting the role of DR5 as a mechanosensory protein in vascular endothelial cells. Unlike the regulatory system of cilia length, however, blocking cofilin through the protein phosphatase 1 antagonist does not have any substantial effect on mechano- sensory cilia function (Figure S6). This indicates that regulatory pathways of cilia length and function may be differentially regulated.

3.5. Discussion

For the first time, we show that DR5 is localized to primary cilia in wild-type cells, as well as in ciliary abnormal $Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$ endothelial cells. We further provide evidence supporting the ciliary roles of DR5 as a chemo- and mechano-sensor. In addition, we show that endothelial cells can alter their sensitivity to fluid-shear stress through chemosensory function of cilia. The regulation of cilia structure involves dephosphorylated cofilin, which controls cellular cytoskeleton actin filament rearrangement. This cellular actin differentiation is required for extension of cilia length, including in $Tg737^{orpk/orpk}$ cells. Most interesting is that activation of ciliary DR5 would promote and restore cilia function, especially in $Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$ endothelial cells. We propose that ciliary DR5 within the vascular endothelia can provide a substantial implication in cilia related diseases, such as hypertension and PKD.

In the present study, we find that DR5 is specifically localized to vascular endothelial cilia in cultures and arteries in vivo. Dopamine through activation of DR5 increases cilia length in a concentration-dependent manner within 4 hours in cultures and mouse femoral arteries ex vivo. We did not perform in vivo study in

mice, because the dose-response study in vivo is still largely a challenge because of a drop in blood pressure caused by dopamine. More specifically, dopamine induces vasodilation in vivo, compromising an overall collapse of the cardiovascular system. Regardless, the ciliary DR5 in cultured cells or blood vessels is a functional receptor, because specific activation of DR5 shows responses in both ciliary length and cytosolic calcium increase.

Recently, we have proposed that endothelial cilia are mechanosensory organelles that play a major role in the pathogenesis of hypertension[7,8]. Abnormalities in dopamine synthesis or dopamine receptor function have also been implicated in essential hypertension in humans[13–15] and animal models[16–18]. For example, mouse genetic model with aberrant DR5 exhibits severe hypertension with unopposed sympathetic activity[18]. Furthermore, it was previously unknown how hypertensive PKD patients could be managed more successfully with a dopamine precursor than other antihypertensive therapies[19].

To examine the functional relevance of DR5 within PKD, we used $Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$ endothelial cells derived previously from Pkd mouse models. These mutant cells have abnormal mechano-sensory cilia function and structure, respectively[7,8]. Similar to wild-type cells, activation of DR5 also increases cilia length in $Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$ endothelial cells. Because most $Tg737^{orpk/orpk}$ endothelial cells have very short, stubby cilia, if any, we were not able to quantify the magnitude of cilia length increase. Nonetheless, the ciliogenesis in $Tg737^{orpk/orpk}$ cells implies that a mechanism other than the intraflagellar transport exists. Most importantly, this mechanism can be activated with dopamine to increase cilia length.

A primary cilium is structurally composed of acetylated and detyrosinated microtubules, which connect to the submembranous actin network at its basal body. Ciliogenesis in Tg737$^{orpk/orpk}$ cells has been observed in the presence of cytocalasin D, an actin polymerization inhibitor[22–24]. Pharmacological agents that disrupt microtubule polymerization, like nocodazole, promote the formation of actin stress fibers. On the other hand, agents that stabilize microtubules, such as Taxol, inhibit assembly of actin filaments. Thus, any activation or inhibition of actin polymerization could affect the microtubule-based cilium.

Patients with PKD exhibit significant connective tissue abnormalities involving vascular and airway smooth muscle cells[21], suggesting a possible disruption of cellular actin dynamics. In addition, vascular cells derived from a mouse Pkd model exhibit higher levels of cofilin when activated by the adrenergic receptor agonist[33], which further confirms the role of actin dynamics in the cilia dysfunction model. Cofilin, a small ubiquitous protein that binds to actin cytoskeleton, can promote the rate of monomer disassociation and sever actin filament, thereby inhibiting actin polymerization[25,26].

To further examine the roles of activated cofilin by dopamine, we also measured phosphorylated cofilin in our cells. Compared with wild-type cells, higher basal levels of inactivated cofilin in Pkd1$^{-/-}$ and Tg737$^{orpk/orpk}$ cells were consistently observed. This probably results in inhibition of the cilia length and/or function. Consistent with this view, a consistently higher level of total actin is needed for F-actin polymerization in the mutant cells. We further show that dopamine-induced cofilin activation can promote cortical F-actin formation, which has been used as a

differentiation marker in neurons[34]. Overall, our data show that dopamine can induce cofilin activation, through dephosphorylation by protein phosphatase 1[35]. Consistent with this view, when we inhibited protein phosphatase 1 with calyculin, cilia length was significantly decreased. Calyculin, downstream to dopamine, is also able to reverse the increase in cilia length and actin rearrangement observed in cells treated with dopamine. In addition, no significant change in intracellular calcium response was observed in the presence of fluid-flow shear stress, reinforcing the cellular functions of actin reorganization in ciliogenesis and DR5 in the mechano-sensory property of primary cilia.

Not only do our data show the functionality of DR5 as a chemo-sensor in cilia, our studies also demonstrate the role of DR5 as a ciliary mechano-sensor. We and others have shown that $Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$ cells lack mechano-sensory cilia function[36,37]. To our surprise, dopamine not only induces an increase in cilia length, but it also restores the mechanosensory roles in both $Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$ cells. Interestingly, in wild-type cells with normal mechano-sensory cilia function, activation of DR5 does not initiate a significant change in cilia function, although cilia length is significantly increased. We propose that activated DR5 in the cilia can have a functional sensory role in endothelial cells.

To further investigate the mechano-sensory capacity of DR5 in wild-type cells, we next knocked down DR5 expression. Inhibition of DR5 expression in cilia was achieved using different siRNA constructs, which happened to have a similar efficacy to that verified by our Western blot and immunolocalization studies. Dopamine-induced cofilin activation was ceased in siRNA-transfected cells, as

evidence from the discontinuation of F-actin rearrangement. Most surprising is the loss of mechano-sensory function in DR5 knockdown cells. The length of the cilia in knockdown cells was similar to the length of those treated with calyculin. However, whereas the cilia in DR5-knockdown cells no longer possessed their mechano-sensory capabilities, cells treated with calcyculin, which still exhibited DR5 colocalized to the cilia, retained their mechano-sensory abilities. Thus, we propose that ciliary DR5 in endothelial cells has dual chemo-sensory and mechano-sensory roles.

Primary cilia have been proposed to regulate cardiovascular functions, including blood pressure[7,8]. Given the fact that DR5 mutations in mice[17,18] or abnormal dopaminergic system in humans[13–15] lead to hypertension, we suggest that dysfunctional DR5 is associated with cellular function of cilia. As such, dysfunction in cilia and DR5 could result in a similar hypertension phenotype, as observed in PKD patients[5,6] and patients associated with the dopaminergic system[13–15]. Thus, the chemo-sensory and mechano-sensory roles of primary cilia are equally important for the maintenance of proper cardiovascular homeostasis and vascular tone.

3.6. Perspectives

Previous clinical study indicates that hypertension in PKD patients is better managed with a dopamine precursor[19]. However, it was not immediately understood why and how it is more useful than other antihypertensive agents. We show here that activation of peripheral dopamine receptor can regulate cilia length and restore cilia function in PKD. Overall, our study helps explain dopamine receptor agonism as a

potential therapeutic option in hypertensive PKD patients.

3.7. Acknowledgments

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3.9. Disclosures

None.

3.10. References

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Chapter 4

Calcium-mediated mechanisms of cystic expansion

Shakila Abdul-Majeed[1], Surya M. Nauli [1,2 †]

[1]Department of Medicinal & Biological Chemistry, [2]Department of Pharmacology,

University of Toledo, Health Science Campus, HEB 274, 3000 Arlington Avenue,

MS 1015, Toledo, OH 43614, USA.

†Address correspondence and reprint requests to

Surya M. Nauli
College of Pharmacy
University of Toledo, Health Science Campus, HEB 274, 3000 Arlington Avenue,
MS 1015, Toledo, OH 43614, USA.
Tel: (419) 383 1921
Fax: (419) 383 xxxx
Email: surya.nauli@utoledo.edu

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Keywords primary cilia, mechanosensor, signaling, shear stress

4.1. Abstract

In this review, we will discuss several well-accepted signaling pathways toward calcium-mediated mechanisms of cystic expansion. The second messenger calcium ion has contributed to a vast diversity of signal transduction pathways. We will dissect calcium signaling as a possible mechanism that contributes to renal cyst formation. Because cytosolic calcium also regulates an array of signaling pathways, we will first discuss cilia-induced calcium fluxes, followed by Wnt signaling that has attributed to much-discussed planar cell polarity. We will then look at the relationship between cytosolic calcium and cAMP as one of the most important aspects of cyst progression. The signaling of cAMP on MAPK and mTOR will also be discussed. We infer that while cilia-induced calcium fluxes may be the initial signaling messenger for various cellular pathways, no single signaling mediator or pathway is implicated exclusively in the progression of the cystic expansion.

4.2. Introduction

Polycystic kidney disease (PKD) is characterized by formation of fluid filled cysts. For the past decade, many ideas and much hard work have been put forth to understand the disease, although the mechanisms of cyst formation and expansion remain speculations. Based on transmittance, PKD can be simply classified into acquired and hereditary forms. The acquired form of PKD can be found in patients who have had acute renal failure with subsequent dialysis. The majority of PKD cases, however, are transmitted hereditarily from the parents. The two most common hereditary forms of PKD are autosomal dominant PKD (ADPKD) and autosomal

recessive PKD (ARPKD). The genes mutated in ADPKD include PKD1 and PKD2, whereas ARPKD is caused by mutation in PKHD1 gene (OMIMs: #601313, 613095, 263200). The prevalence of ADPKD and ARPKD is 1 in 1000 and 1 in 20,000 live births, respectively.

The products encoded by these PKD genes are called cysto-proteins (Fig. 4.1), which include polycystin-1 (PKD1), polycystin-2 (PKD1), and fibrocystin (PKHD1). Though the mechanism of cyst formation is still a mystery, abnormal function of these proteins results in cyst formation. In particular, cysto-proteins interact with one another (Fig. 1). Thus, aberrant functions in any of these cysto-proteins may result in similar pathogenic phenotypes in the kidney, liver, pancreas and possibly other organs. In addition, these three proteins are localized within the same subcellular domain in the cell. Because these proteins are localized and have distinct functions in the primary cilia, ciliary hypothesis has been developed to explain a unifying pathogenic concept for PKD.

In this review, we will discuss calcium signaling as a possible mechanism that contributes to the functions of these cysto-proteins. Dysfunction of any of these proteins may thus interrupt calcium signaling pathways, which may promote abnormal downstream signal transductions of various signaling molecules participating in renal cyst formation.

4.3. Calcium signaling by primary cilia

When the PKD genes were discovered and cloned [1–3], their functions in cation

transport immediately became of considerable interest in understanding the

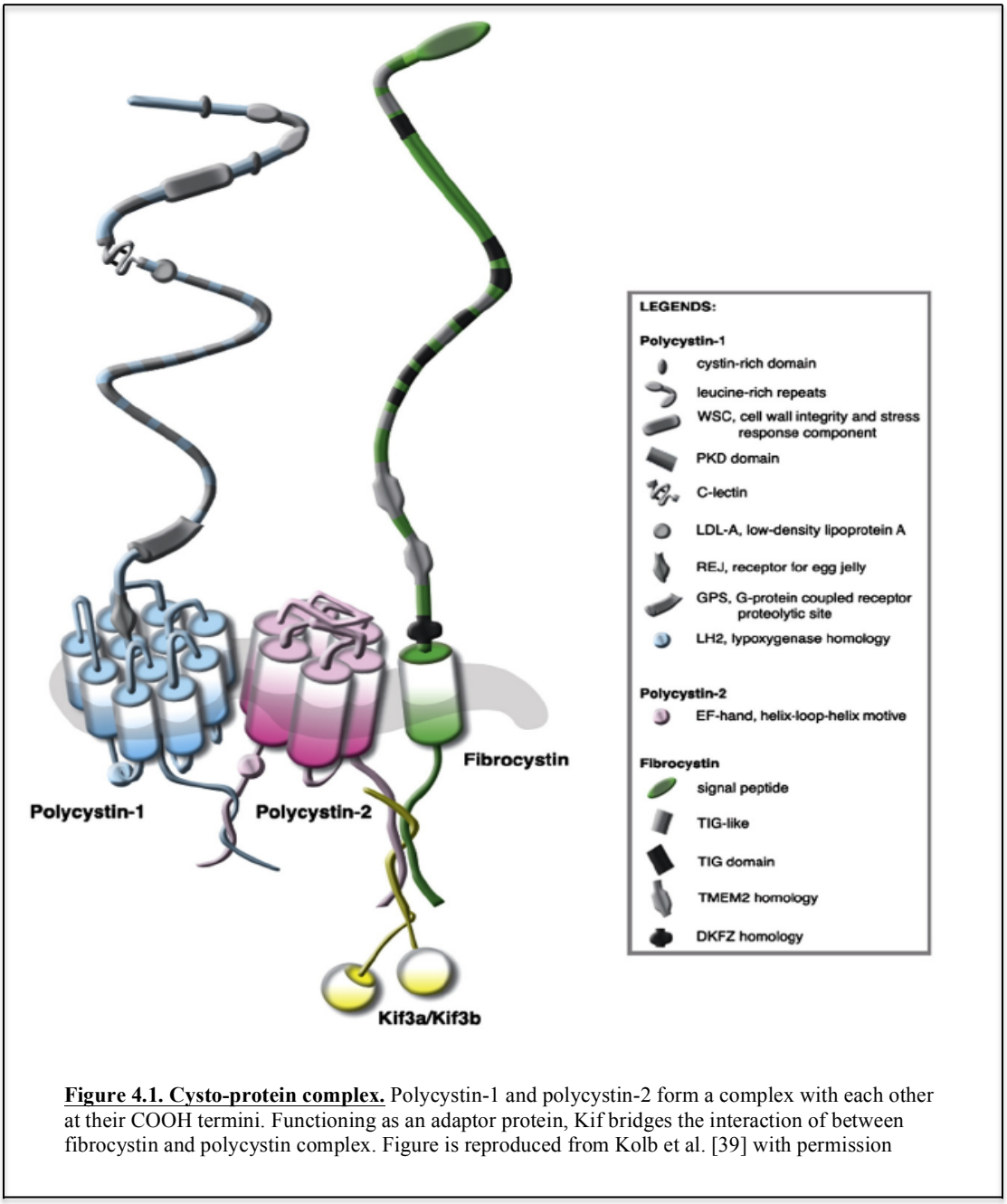

Figure 4.1. Cysto-protein complex. Polycystin-1 and polycystin-2 form a complex with each other at their COOH termini. Functioning as an adaptor protein, Kif bridges the interaction of between fibrocystin and polycystin complex. Figure is reproduced from Kolb et al. [39] with permission

molecular functions of the cysto-proteins. In particular, sequence analysis of polycystin-2 showed putative homologies with other known calcium channels [4]. In addition to their physical and functional interactions with other calcium-regulated proteins [5-7], interactions of cysto-proteins with other calcium channels also

provided further insights into the modulation of intracellular calcium signaling [8–12]. Thus, polycystin-2 has long been predicted to regulate cytosolic calcium [13,14], including modulating intraorganellar calcium release [15] and extracellular calcium influx [16].

To function as a calcium channel, polycystin-2 depends on its interaction with polycystin-1 [16,17]. Likewise, proper function of fibrocystin depends on the indirect interaction with the polycystin complex [18–20]. In addition, activation of polycystin-2 has been found to depend on its interaction with mammalian diaphanous-related forming 1 (mDia1). mDai1 is able to regulate polycystin-2 depending on the membrane potential or voltage levels in the cells. At resting potentials, mDai1 in an autoinhibited state binds to polycystin-2 thereby inhibiting its channel activity. However, at positive potentials, GTP-bound mDia1 releases and thereby allows activation of polycystin-2 [21].

Localization of cysto-proteins to primary cilia further confirms the roles of polycystins and fibrocystin in intracellular calcium signaling. In addition, it further elaborates the molecular functions of cysto-proteins as regulators for intracellular calcium signaling. Most important are the mechanosensory functions of cysto-proteins that have been independently described in the mouse and human kidney epithelia [20,22–27], vascular endothelia [28–30], osteochondrocytes [31,32], cholangiocytes [33,34] and developing nodes [35–37]. It is nowgenerally accepted that localization of these cysto-proteins to the primary cilia is important and necessary to initiate the first signaling cascade of intracellular calcium [38–40]. This signaling pathway may further provide other complex downstream signaling

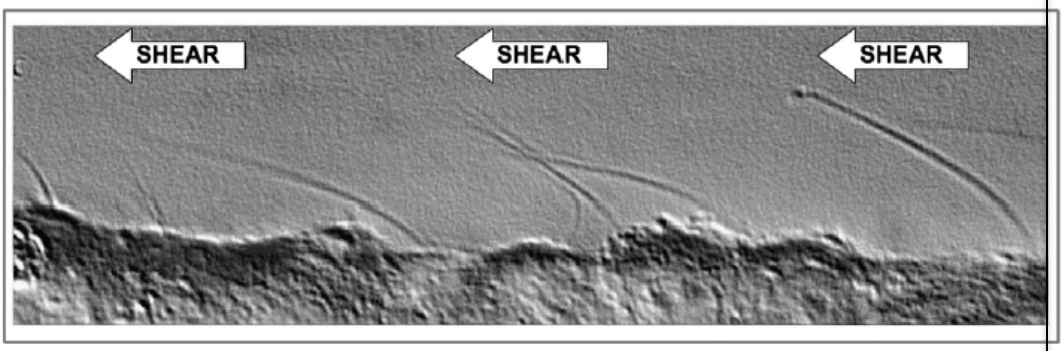

Figure 4.2. Primary cilia in signal transduction system. Cilia sense fluid-shear stress on the apical membrane of the cells. Fluid flow that produces enough drag-force on the top of the cells will bend sensory cilia. Bending of cilia will activate the cysto-proteins, resulting in extracellular calcium influx. Figure is reproduced from AbouAlaiwi et al. [38] with permission.

pathways.

In general, primary cilia are mechanosensory compartments that house many sensory proteins, including the cysto-proteins. Shear stress that produces enough drag force on the cell surface is able to bend the primary cilium (Fig. 4.2). Subsequently,

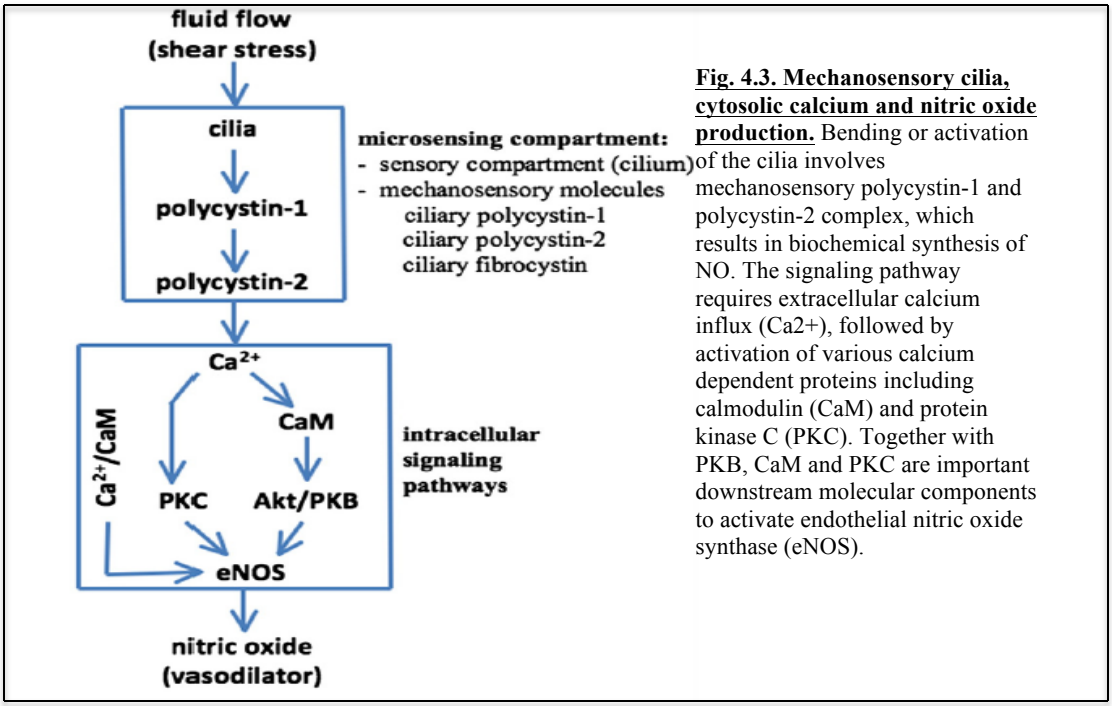

Fig. 4.3. Mechanosensory cilia, cytosolic calcium and nitric oxide production. Bending or activation of the cilia involves mechanosensory polycystin-1 and polycystin-2 complex, which results in biochemical synthesis of NO. The signaling pathway requires extracellular calcium influx (Ca2+), followed by activation of various calcium dependent proteins including calmodulin (CaM) and protein kinase C (PKC). Together with PKB, CaM and PKC are important downstream molecular components to activate endothelial nitric oxide synthase (eNOS).

activation of the polycystins and other interacting proteins in this complex may result in cytosolic calcium increase. This paradigm was established based mainly on in vitro studies where cultured kidney cells were challenged with a fluid-flow shear

stress [41,42]. The ex vivo experiments using isolated gastrulation stage node, perfused tubules and arteries have further confirmed the mechanosensory function of the primary cilium [28,36,43]. In the vascular artery [28], the influx of extracellular calcium initiates the biochemical cascades that lead to production of nitric oxide vasodilator through endothelial nitric oxide (eNOS). This activation of eNOS depends on the contribution or activity of calmodulin, phosphoinositide kinase-3, protein kinase B and calcium-dependent protein kinase (Fig. 4.3).

In the next sections, we will discuss the downstream pathways that depend, directly or indirectly, on the initial calcium signaling Because of the complexity in calcium signaling, we will discuss only those pathways that have possible relevance to renal cyst expansion.

4.4. Signaling by Wnt

Wnt signaling pathways are involved in many aspects of cell development, such as cell polarity determination, cell adhesion, growth, motility, and many others. The Wnt pathway involves a daunting number of secreted Wnt ligands and Frizzled receptors that regulate a large number of Wnt signaling molecules. In the simplest terms, Wnt signaling can activate three distinct pathways (Fig. 4.4): (1) *β-catenin dependent canonical pathway,* (2) *β-catenin independent non-canonical or PCP (planar cell polarity) pathway,* and (3*) Wnt calcium pathway,* which can influence both the canonical and noncanonical pathways [44].

Many regulatory proteins involved in Wnt signaling are localized in the primary cilium and base of the cilium, also known as basal body. It is thus speculated that

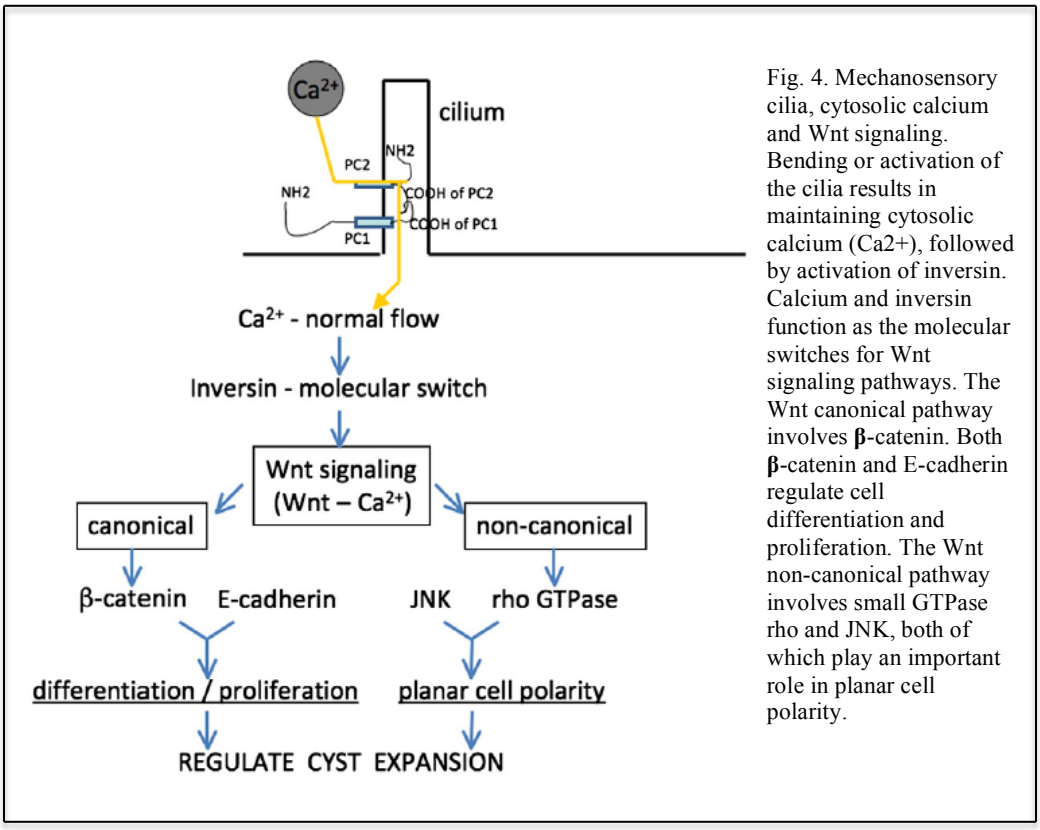

Fig. 4. Mechanosensory cilia, cytosolic calcium and Wnt signaling. Bending or activation of the cilia results in maintaining cytosolic calcium (Ca2+), followed by activation of inversin. Calcium and inversin function as the molecular switches for Wnt signaling pathways. The Wnt canonical pathway involves **β**-catenin. Both **β**-catenin and E-cadherin regulate cell differentiation and proliferation. The Wnt non-canonical pathway involves small GTPase rho and JNK, both of which play an important role in planar cell polarity.

flow-induced cytosolic calcium influx switches off the canonical Wnt pathway and activates the non-canonical Wnt/ calcium signaling pathway (Fig. 4). This molecular switch is regulated by inversin, which is a ciliary protein that can turn differentWnt signaling pathways on and off [45]. Of note is that abnormalities in inversin function result in polycystic kidney phenotype. The zebrafish cystic kidney gene seahorse has also been found to be involved in a variety of cilia-mediated processes such as body curvature, kidney cyst formation, left-right asymmetry, and others including PCP signaling and inhibition of the canonical Wnt signaling [46]. Seahorse seems to be essential for a functional non-canonical Wnt signaling. It associates with Dishevelled

(Dsh), the divergence point for the canonical Wnt and PCP pathways. The Seahorse gene encodes a highly conserved five leucine-rich repeats (LRR) and a leucine-rich repeat cap, from Drosphila to humans. One of the leucine rich repeat proteins, LRRC50, has been found to be conserved in both zebrafish and humans. Lrrc50 in expressed in all ciliated tissues in zebrafish, resulting in ciliary dysfunction in lrrc50 mutants. In humans and dog kidney cells, LRRC50 has been shown to localize at the mitotic spindle and cilium, implying it to be a ciliary protein in vertebrates [47].

It is generally accepted that Wnt signaling pathway is not regulated properly in polycystic kidney disease (Fig. 4.4). In the canonical Wnt pathway, β-catenin in the nucleus mediates many gene induction events, and any deregulation of this pathway can result in uninhibited proliferation of cells [48,49]. It is speculated that flow-induced cytosolic calcium influx is required to turn off the canonical Wnt pathway and activate the non-canonical Wnt/calcium signaling pathway. As such, over-activation of canonical Wnt pathway, by over-production of an activated form of β-catenin for an example, would result in polycystic kidney phenotype [50]. This view is consistent with the profiling gene expression study [51]. A consistently high level of Wnt signaling is observed in cystic tissues from ADPKD patients, but not in tissues which exhibited low level or no cyst formation from the same patients. Furthermore, abnormalities in polycystins enhance activity of Wnt signaling pathway [52–55]. E-cadherin is one of the interacting Wnt signaling molecules, and it plays an essential role in intercellular cell junction assembly. It is required for epithelial polarity and tubule formation. Disruption of E-cadherin could lead to abnormal levels of β-catenin and impeding renal epithelial polarization [54,56].

Furthermore, the protein complex of polycystins, E-cadherin, and β-catenin is interrupted in cyst lining epithelial cells in ADPKD patients [57]. In addition, the levels of β-catenin in the developing hearts and kidneys of $Pkd1^{-/-}$ mouse embryos compared to wild type embryos are decreased [53]. Overall, the data suggest that interruption in Wnt signaling pathway would result in less differentiated epithelial cells, yielding to proliferation and acceleration of cyst expansion [58].

Planar cell polarity, which involves non-canonical Wnt signaling, has recently been the most discussed topic towards the understanding of renal cysts. Planar cell polarity is principally involved in the development of tissue architectures along a parallel axis, other than the apical–basolateral axis of a renal tubule (Fig. 4.5). Oriented cell division is thought to be necessary for the elongation of the developing nephron. Abnormalities in the planar cell polarity, as reflected by the mitotic spindle, have been observed in various PKD mouse models [52,59–63]. In these studies, cell division or mitotic spindle orientation within the tubular axis were measured. However, it is currently unclear whether such an alignment can be considered a process toward planar cell polarity [64]. In Drosophila, for example, mitotic spindle alignment is achieved only after centrosomes have been properly aligned [65]. Thus, there is a cell-cycle checkpoint for centrosomal positioning. The question remains whether such a checkpoint is disrupted in PKD. Within the non-canonical Wnt signaling, there is also a huge body of evidence indicating that JNK signaling system is involved in PKD [66–70]. The strongest evidence of non-canonical Wnt signaling,

however, came from a study involving Rho small G protein. In the mouse model,

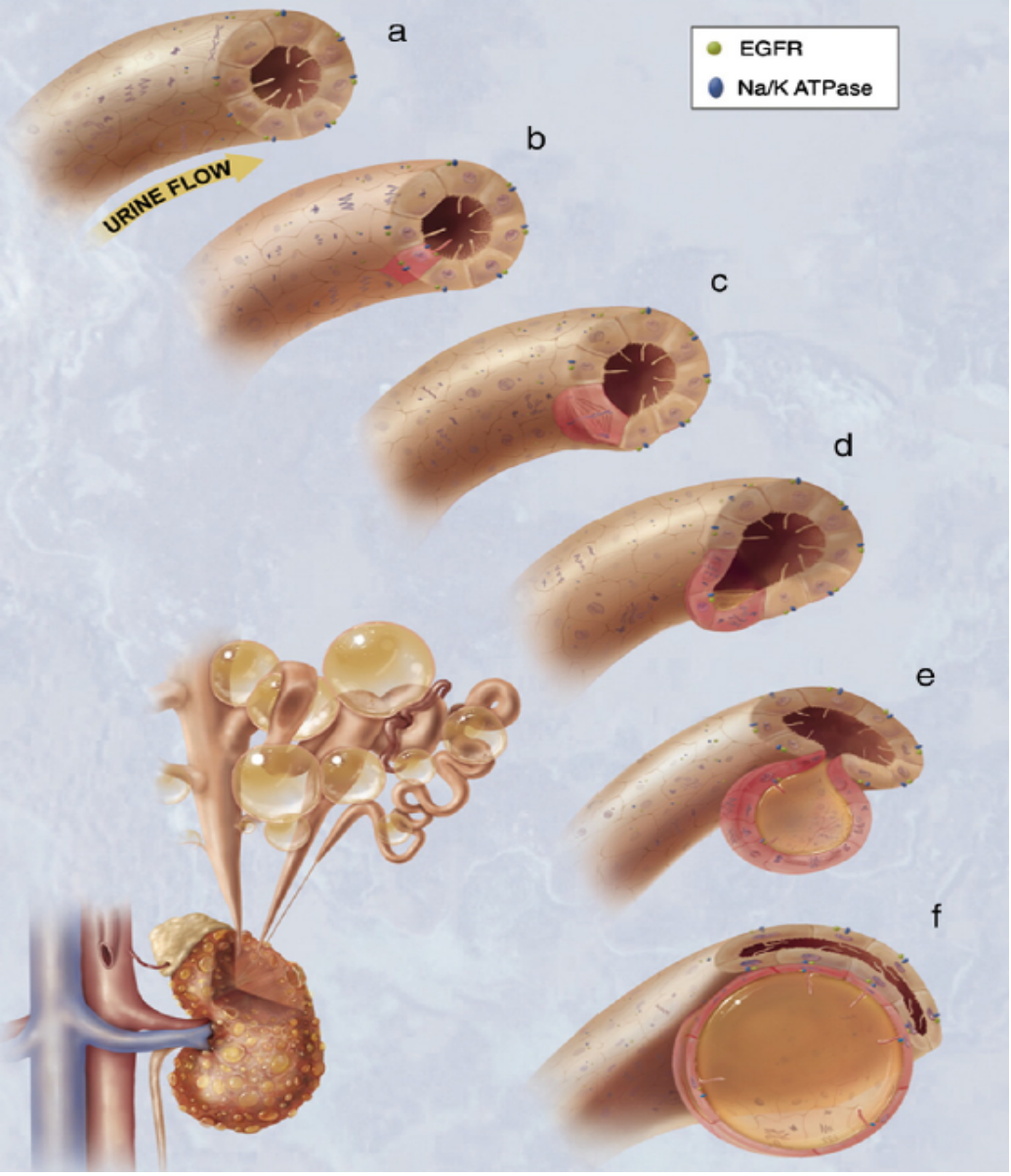

Figure 4.5. Planar cell polarity and cystic kidney disease. The illustration depicts the mechanism of mitotic spindle formation as an index of planar cell polarity in cyst expansion. **(a)** Each cilium senses changes in urine flow. This message provides critical calcium signals to the cell. **(b)** Abnormalities in any of the cysto-proteins results in abnormal ciliary function. **(c)** The functional abnormality in ciliary sensing results in disorientation of the mitotic spindle, and hence the cell will lose planar cell polarity. **(d)** Direction of cell division becomes randomized. This will result in increasing tubular diameter, rather than tubular elongation. **(e)** Budding of a cyst from the renal tubule occurs. The abnormal localizations of epidermal growth factor receptor (EGFR) and Na+/K+ ATPase pump are typical characteristics of polycystic kidneys. (f) The cyst is eventually enlarged and expanded further. Multiple cysts within a kidney are illustrated on the bottom left corner. Figure is reproduced from Kolb et al. [39] with permission.

mutation in the key regulator of Rho small G protein results in cystic kidney phenotype [71]. Taken together, interruption in Wnt signaling pathway would result

in cyst formation due to planar cell polarity defects, and mitotic spindle orientation might be a contributing factor in the process.

4.5. Signaling by cAMP/MAPK

Cyclic adenosine monophosphate, or cAMP, has been identified as one of the most important players in cyst progression in both ADPKD and ARPKD (Fig. 4.6). Two

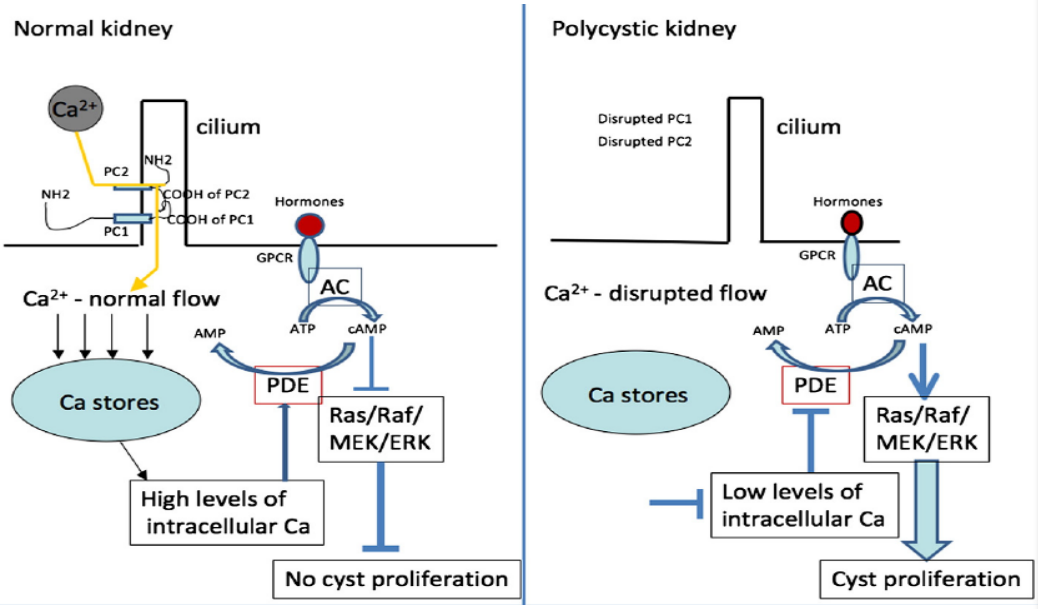

Fig. 4.6. Cytosolic calcium and cAMP signaling. Under normal conditions, the carboxy terminal tails of polycystin-1 and polycystin-2 interact, which enables calcium entry into the cell. This further stimulates the release of calcium from the calcium stores inside the cell. In the presence of high, steady state levels of calcium, calcium-stimulated phosphodiesterases are capable of degrading cAMP into AMP, thereby controlling the cAMP levels in the cell. The low levels of cAMP are unable to stimulate MAPK pathway. In polycystic kidneys, however, the resulting abnormality in cysto-proteins causes a lower level of intracellular calcium. Hence, the activity of phosphodiesterases is not stimulated and cAMP level increases, leading to activation of B-Raf of MAPK pathway.

major processes that contribute to the expansion of the renal cysts in polycystic kidney disease are cell proliferation and fluid secretion. cAMP is intimately involved in accelerating both of these processes [72–75]. Tissues from the kidneys, liver, and vascular smooth muscles of PKD animal models exhibit increased levels of cAMP [76–78].

cAMP is known to be one of most important players in effecting hormonal activation of intracellular pathways and is intimately involved in cell proliferation in almost all cell lines. However, cAMP does not produce the same effects in all cell lines. Though cAMP has been used for several years as an anti-proliferative agent [79,80], cAMP has also been known to stimulate cell proliferation by activating the mitogen activated protein kinase (MAPK) pathway (Fig. 4.6). For example, cAMP is anti-proliferative in normal tissues, but it stimulates cell proliferation in cystic epithelial cells [73,81,82].

The change in cAMP related phenotype–inhibition of cell proliferation in normal cells and stimulation of cell proliferation in cystic cells–is associated with the amount of intracellular calcium in PKD tissues and cells [82,83]. The imbalance in the cytosolic calcium due to the disruptions in the cysto-proteins promotes abnormal function of phosphodiesterase (Fig. 4.6). Phosphodiesterase 1 isoforms (PDE1a, PDE1b and PDE1c) are present in especially high levels in the kidneys [84]. Most importantly, the activity of this phosphodiesterase is regulated by intracellular calcium and cAMP. In PKD cells with a low level of intracellular calcium, phosphodiesterase activity is down-regulated. This results in aberrant conversion of cAMP to AMP. The resulting increase in cytosolic cAMP further stimulates the MAPK pathways, which promotes cyst expansion through higher cell proliferation.

In general, cystic epithelia in polycystic kidneys have exhibited high levels of both cAMP and MAPK activity compared to normal cells, resulting in cell proliferation in polycystic kidneys and an inhibition of cell proliferation in normal cells [85,86]. In normal epithelial cells, cAMP agonists inhibit MAPK pathway by blocking

activation of Raf-1 (Raf-C) through cAMP-dependent protein kinase (PKA). On the other hand, cAMP was found to stimulate the MAPK pathway in PKD cells, thereby stimulating cell proliferation. This difference has also been observed to result from an increased affinity of cAMP for B-Raf rather than A-Raf and Raf-1 (C-Raf) [82,86]. As mentioned earlier, this change in cAMP-related signaling is also attributed to the level of intracellular calcium. The high levels of cAMP, combined with low levels of calcium fluxes in polycystic kidneys, could further result in a decrease of PI3K/Akt activity, thereby stimulating B-Raf activation and hence activation of the MAPK pathway of Ras/B-Raf/MEK/ERK [83,87,88].

4.6. Signaling by mTOR

The Ras/Raf/ERK pathway plays another important role in polycystic kidney disease by regulating the mammalian target of rapamycin (mTOR) pathway through molecular signaling of tuberin (Fig. 4.7). Tuberin, which is also regulated by Akt, is a GTPase activating protein (GAP). Tuberin regulates the activity of Rheb, a small G-protein belonging to the Ras super family. GTP-bound Rheb is active, while GDP-bound Rheb is inactive. Hamartin and tuberin form a heterodimer which converts Rheb-GTP to Rheb-GDP, thereby inactivating Rheb. Rheb activates mTOR pathway. Hence, tuberin inactivates GTP-bound Rheb and inhibits the mTOR pathway [89–91].

The respective protein products of TSC1 and TSC2, hamartin and tuberin, regulate formation of primary cilia [92,93]. Most importantly, however, analysis of normal and diseased cells from ADPKD patients indicates that the cyst lining epithelial

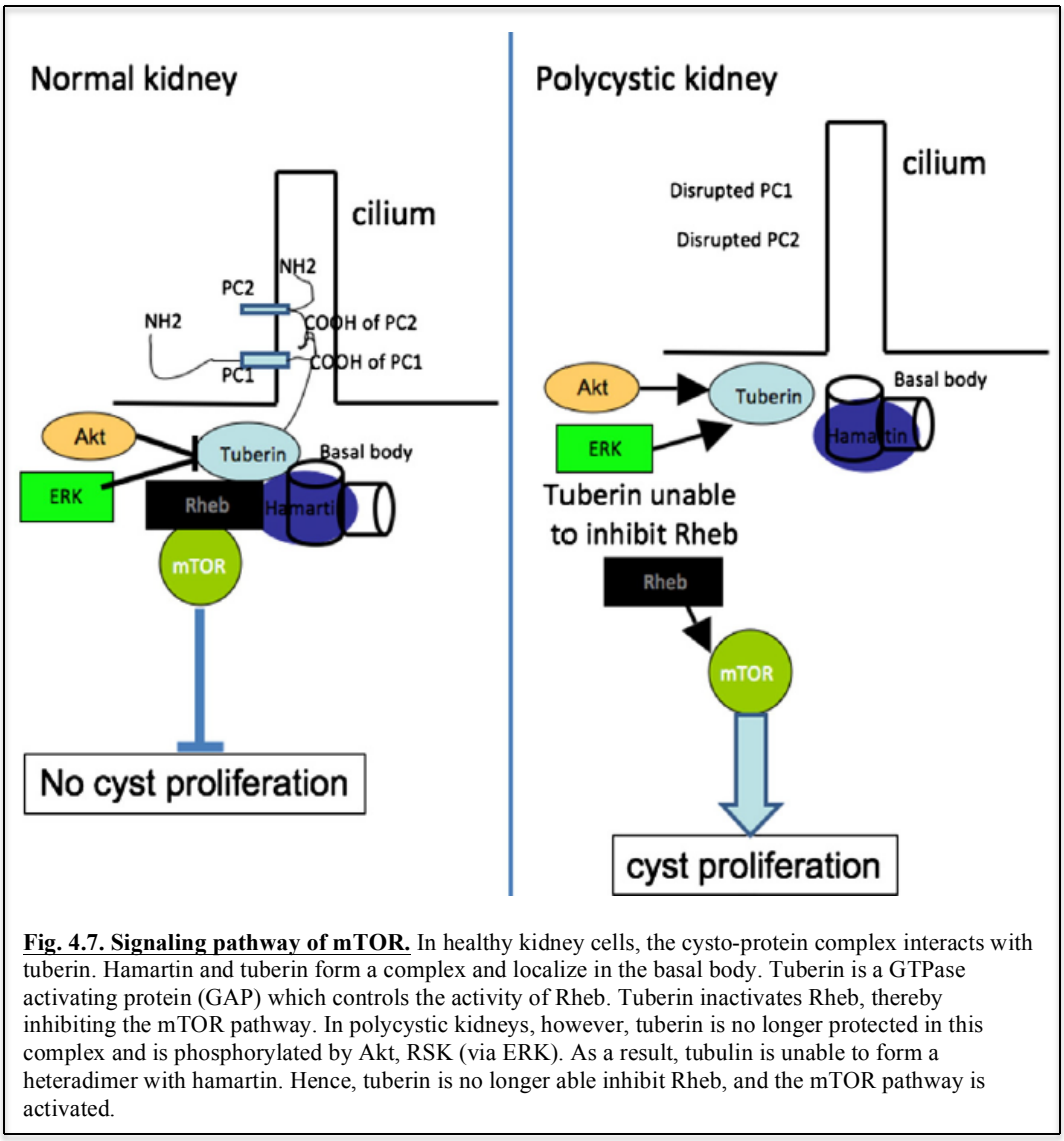

Fig. 4.7. Signaling pathway of mTOR. In healthy kidney cells, the cysto-protein complex interacts with tuberin. Hamartin and tuberin form a complex and localize in the basal body. Tuberin is a GTPase activating protein (GAP) which controls the activity of Rheb. Tuberin inactivates Rheb, thereby inhibiting the mTOR pathway. In polycystic kidneys, however, tuberin is no longer protected in this complex and is phosphorylated by Akt, RSK (via ERK). As a result, tubulin is unable to form a heteradimer with hamartin. Hence, tuberin is no longer able inhibit Rheb, and the mTOR pathway is activated.

cells exhibit higher levels of mTOR signaling compared to the surrounding normal epithelium [94]. When mTOR pathway is inhibited with rapamycin, many murine models of ADPKD and ARPKD show a decrease in renal cyst expansion [94–100]. The consensus is that the cytoplasmic tail of polycystin-1 directly or indirectly interacts with both mTORC1 complex as well as tuberin, the protein product of Tsc2 which itself regulates mTORC1. Membrane localization of polycystin-1 and hamartin are therefore able to bind to tuberin, keeping it near the plasma membrane.

Thus, membrane bound polycystin-1 is capable of controlling mTORC1 pathway and hence extensive cell proliferation. Disruption of polycystin-1would imply the activation of themTORC1 cascade yielding cell proliferation and subsequent cyst formation [101,102].

In addition, the cysto-proteins are known to contribute to the activation of the Akt/PKB pathway [101,103,104]. Thus, the interaction of Akt and tuberin can further provide an additional regulation of mTOR pathway by the cysto-proteins. Although the role of calcium in mTOR signaling has still not been properly studied in polycystic kidney disease, there is a possibility that calcium-ERK-mTOR pathway could exist. In particular, calcium is known to be associated with cAMP/ERK activity (Fig. 4.6), and ERK has been implicated in mTOR signaling (Fig. 4.7). There is no doubt that further studies are needed to dissect the contributions of calcium, ERK and mTOR in cystogenesis.

Interestingly, clinical trials of mTOR inhibitors in ADPKD patients have not yielded much anticipated results [105–107]. The two mTOR inhibitors tested in ADPKD patients were sirolimus and everolimus. Sirolimus was tested on 100 patients (18 to 40 years) exhibiting early stages of the disease, while everolimus was tried on 433 patients in advanced-stage II and III, with a renal baseline volume of 1500mL.

Sirolimus did not show any decrease in total kidney size in humans, though it showed promising results in mouse models. However, the animals were treated with a dose of 5 mg/kg of body weight [94], a dosage that is unsafe for humans, who were administered 2 mg sirolimus for 18 months. No difference in GFR was observed as these patients exhibited the initial stages of the disease [105]. Everolimus, on the

other hand, slowed the increase in the total kidney volume without any effect on GFR. After a brief transient period, patients exhibited a rapid decline in GFR [106]. These studies imply that reducing the cyst size need not necessarily improve renal function in these patients.

4.7. Prospective

Cilia function and structure are important and necessary to maintain the architecture of kidney tissue. Abnormalities in the structure or function of cilia results in PKD. The mechanosensory cilia are crucial in maintaining intracellular calcium signaling. Many cell types, including renal epithelia, use cytosolic calcium as a second messenger to further regulate other cellular homeostasis through a very complex signal transduction system. This signaling system includes Wnt, cAMP-MAPK, Akt, mTOR, and other pathways that are not discussed in this review.

In understanding this complexity, an important lesson is that no single signaling mediator is implicated exclusively in the progression of the cystic expansion. Rather, all of these signaling pathways are intimately connected, thereby regulating the progression of the disease. Nonetheless, only by understanding such a complicated system do we have better insights to attain the most effective way to retard progression of polycystic kidney disease.

4.8 Acknowledgments

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Chapter 5

Summary

The first study (Chapter 2) shows that pharmacological agents can regulate ciliary length. cAMP analog and protein kinase activators such as protein kinase A and C, are able to increase cilia length, while protein kinase C inhibitor and MAPK inhibitor decrease cilia length. The increase in cilia length correlates to a restructuring of actin cytoskeleton.

Though actin and microtubules have distinct roles in cellular systems, an intact microtubule cytoskeleton is required to maintain the polarized distribution of actin-dependent protrusions at a migrating edge (Vasiliev.J.M., 1970, Rodriguez, 2003) in fibroblasts. Such interactions have been observed in diverse cell types such as epithelia, fibroblasts, neurons, oocytes etc. In yeasts, the plus end of microtubules attach to sites of an actin cortex for proper positioning of the mitotic spindle (Gundersen, G.G., 2002). Similar to our results, research in the past one year has shown a strong correlation between actin dynamics and ciliogenesis (Kim et al, 2010, Pitaval et al, 2010, Sharma et al, 2011, Engel et al, 2011).

In addition, we find that cofilin, an actin binding protein that regulates actin dynamics, is involved in controlling cilia length. Inhibiting cofilin via protein

phosphatase-1 is able to curb the increase in cilia length observed in the presence of cAMP and PKA and PKC activators. The pharmacological agents we have used, activate Ras/MEK/ERK pathway, which has been known to activate both actin dynamics and motor/linker proteins involved in intraflagellar transport.

Though the ras/MEK/ERK pathway is able to control the cilia length, no correlation was found between this pathway and cilia function. Our functional studies indicate cAMP and PKA to be the important players controlling cilia function, in addition to increasing cilia length. Thus, this study concludes that the molecular interactions between cilia function and cilia length can be independent of one another.

Thus, with the results obtained so far imply that the first 6 hypotheses that we had assumed at the beginning of our study holds, and that cilia length can be manipulated using pharmacological agents via ERK pathway. Actin restructuring plays an important role in ciliogenesis; and cilia function could be independent of cilia length. Though the effect of cAMP and PKA activators has been shown by previous research, this is the first research that shows the role of PKC and MAPK kinase on both cilia length and cilia function. In addition, this is the first study that deals with mechanisms involved in regulating cilia function.

Our second study, chapter 3, deals with hypertension observed in polycystic kidney disease (PKD) patients. In this study, we show, for the first time, that dopamine receptor -5 (DR5) co-localizes to the primary cilium in endothelial cells. And, this co-localization is observed in wild type cells as well as *PKD* mutated cells. This

work was described as "***Elegant and highly interesting work. Methods are appropriate and findings novel and relevant"*** by all the three reviewers.

Though the primary characteristic of PKD is the formation of fluid filled cysts in the kidneys, hypertension is the leading cause of death in PKD patients. Using endothelial cells derived from two *PKD* mice models ($Pkd1^{-/-}$ and $Tg737^{orpk/orpk}$), we show that dopamine receptor 5 plays an important role in controlling both cilia length and cilia function in endothelial cells. In the first mutant model, the protein polycystin-1, a mechano-sensor, localized to the primary cilia is dysfunctional, though the ciliary structure is normal. The second cell line, $Tg737^{orpk/orpk}$ have disrupted cilia due to mutations in the intraflagellar transport gene. These cells do not exhibit cilia, or at max, exhibit short, stubby cilia. Thus, the dysfunctionality of the primary cilium arises due to two different reasons, though the end result is the same, that is, these cilia are incapable of sensing fluid flow.

DR5 localizes to the primary cilia of all these three cell lines. However, we find the DR5 is inactive in the two mutated cell lines. However, treating these two mutated cell lines with 10μmol/L dopamine for 16 hours, significantly increases the cilia length, and most surprisingly, also stimulates the ciliary mechanosensory abilities. And, again, as in the first study, we find actin dynamics to play a significant role in increase in cilia length.

The pertinent question in this paper is, "is it the increase in cilia length in the mutated cell lines that restores the ciliary mechanosensory role or the activation of DR5 by incubation with dopamine". We find that activation of DR5 to be the main cause of the revival of ciliary mechanosensory role. We base this conclusion on the

fact that, when we transfect wild type cells with siRNA and prevent the expression of DR5 in the cilia, the cilia length decreases significantly and more importantly, ciliary mechanosensation is lost. When we inhibit the growth of cilia by inhibiting protein phosphatase-1, thereby inhibiting actin rearrangement; the cilia length decreases significantly. The cilia length is now comparable to those observed when DR5 expression is inhibited. However, these cells still have DR5 co-localized to the cilia and the mechanosensory ability of the cilium is not compromised. This indicates that an active DR5 co-localized to the primary cilium in endothelial cells plays an important role as both, a chemo sensor and a mechano sensor. This study therefore, helps to explain dopamine receptor agonism as a potential therapeutic option in hypertensive PKD patients.

Chapter 6

Future Studies

Though reviews in the past on cilium have indicated that cilium could play such a role, no concrete studies have yet been performed to study the correlation between cilium and hypertension. Based on our second study, chapter 3, we find that cilia could play an important role in hypertension, at least with in polycystic kidney disease. In addition, to these two studies, in collaboration with Dr. Bina Joe, Department of Physiology/Pharmacology, University of Toledo, we have studied the primary cilia in hypertensive ADAMTS16$^{+/+}$ S rats and normotensive ADMATS16$^{-/-}$ S rats using immunofluorescence and scanning electron microcopies. We find that the normotensive rats exhibited much larger cilia than the hypertensive rats (manuscript in process).

Based on these studies, two sets of experiments have been planned and set in motion to study the correlation between cilia and hypertension.

1. Investigate the relation between dopamine receptor-5 and hypertension in polycystic kidney mouse model, *in-vivo.*

2. Determine the presence of cilia and measure cilia length in congenic breeds of S rats that are normotensive, hypertensive, and severely hypertensive.

For the first set of experiments, we will use transgenic mice models in which *Pkd1* and *Ift88* genes are mutated, using tissue-specific "Cre-Lox" gene inactivation technique. The mutated and control mice will be treated with DR1/5 agonist and their blood pressure, heart rate, heart structure; sodium and dopamine excretion in urine will be compared against control mice. The mice will then be sacrificed and their vasculature and kidneys will be studied for cilia presence and length. We have started breeding the mice for this experiment. In addition, I am currently being trained to insert a telemetry probe into the left carotid artery of mice, reaching till the tip of the aortic arch to determine the blood pressure when the animal is in a free environment.

To ensure the correlation between cilia and hypertension exists across a variety of species, we will perform the second set of experiments in rats. Here, the animals have been generated by mitotic recombination (not altered through genetic engineering) and their blood pressures have been studied in detail (Saad et al, 2007). This experiment will also overcome any problems that may be encountered using transgenic strains, as in the first set of experiments.

Based on studies in our lab and Dr. Joe's labs, we hope to find normotensive animals to express more and longer cilia than hypertensive animals. In addition, we hope treating the polycystic kidney mutants with DR1/5 agonist, should improve their blood pressure, along with heart rate and structure.

Chapter 7

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