Regulation of calcium signaling by primary cilia and its role in polycystic kidney disease pathogenesis

Xingjian Jin
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A Dissertation

Entitled

Regulation of Calcium Signaling by Primary Cilia and Its Role in Polycystic Kidney Disease Pathogenesis

By

Xingjian Jin

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biomedical Sciences

Dr. Surya M. Nauli, Committee Chair

Dr. Bina Joe, Committee Member

Dr. Ivana de la Serna, Committee Member

Dr. Ming-Cheh Liu, Committee Member

Dr. David R. Giovannucci, Committee Member

Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

May 2014
The primary cilium is a solitary organelle present in the majority of mammalian cells. The cilium has been implicated in a variety of cellular events. By virtue of its structure and cellular location, the most important and well-known function of primary cilium is sensation. Projected from the apical membrane of a cell to extracellular environment, the cilium senses stimulations from the extracellular matrix and translates each cue into second messengers in intracellular signaling pathways. Among these messengers, calcium is proposed to be the most important. Thus, dysfunctional cilia might lead to abnormal calcium signaling and consequently, several diseases, collectively referred as “ciliopathies”. Our study has two goals. First, we aim to show that the cilium is an independent cellular compartment for calcium signaling evoked by both mechanical and chemical stimulation. To date, though many studies have related cilia to calcium signaling, there is no direct evidence showing where and how the calcium influx initially occurs. Without such evidence, the critical first step of cilia-mediated calcium signaling
remains speculative. To test our hypothesis, we developed a novel imaging and cell culture system to visualize calcium signaling within an individual cilium. Then we apply different stimulations to cell and observe subsequent responses. Using this advanced system we show for the first time that following extracellular stimulation, calcium flux into the cilia body, or cilioplasm. However, cells have different responses to different stimulations. When fluid shear-stress is applied, calcium enters into the cilioplasm and such calcium signals are further amplified in the cytoplasm. In contrast, in the case of chemical stimulation, such as fenoldopam, a dopamine receptor-type 5 (DR5) agonist, calcium signals are contained exclusively in the cilioplasm. Also, we examine the effects of different signaling pathway inhibitors or siRNA on ciliary calcium responses. Echoes to previous studies, we find that the flow-induced calcium signaling is dependent on polycystin-2 (PC2) calcium channel, whereas Fenoldopam-induced calcium influx is through L-type calcium channel (Cav1.2). Additionally, we find that the flow-induced calcium signaling cascade is regulated by ryanodine receptor through calcium induced calcium release. Our second aim is to identify the role of Cav1.2 inhibition in the pathogenesis and progression of polycystic kidney disease (PKD). Lower intracellular calcium level has been reported in cells isolated from cyst linings. However, if other calcium channels than PC2 can affect intracellular calcium level and implicate in PKD pathogenesis is unknown. In clinical practice, L-type calcium channel blockers have been well prescribed for hypertensive patients. It’s noteworthy that such drugs are generally avoided in hypertensive PKD patients for their putative adverse effects on PKD. However, how L-type calcium channel blockers accelerate cyst formation remains unclear. In our study, we observe significant decreases of already lowered intracellular
calcium level in PKD cells with Cav1.2 knockdown. Such reduction further causes irregular AKT and ERK phosphorylation. As we expected, abnormal mitogenesis signaling leads to more severe mitosis catastrophe and higher proliferation rate. Consequently, when Cav1.2 is inhibited in PKD animal models, much larger cysts are observed at an earlier stage. We thus propose that Cav1.2 work in concert with PC2 as cilia related calcium channel to maintain intracellular calcium homeostasis and loss of Cav1.2 in PKD cells can lead to abnormal mitosis and cell division, which facilitate the formation of cysts.
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Chapter 1

Introduction

1.1 Cilia

Cilia are hair-like structures that project from the apical membrane of the cell surface. The microtubule cytoskeleton of cilia is called ciliary axoneme, which is a continuation of symmetric nine-fold structure of the mother centriole, or basal body of cilia. Cilia are generally categorized into two types, non-motile cilia or primary cilia and motile cilia, based on their differences in cytoskeleton (Figure 1-1). The axoneme of primary cilia typically has a ring of nine outer microtubule doublets (called a 9+0 axoneme), and the axoneme of a motile cilium has two central microtubule doublets in addition to the nine outer doublets (called a 9+2 axoneme) [1]. The axoneme of both primary cilia and motile cilia are composed of stable, highly acetylated α-tubulin. Primary cilia usually do not have dynein, which is responsible for the movement of motile cilia. Solitary 9 + 0 primary cilia can be found on many mammalian cells, including kidney epithelia, chondrocytes, fibroblasts, and neurons. In contrast, motile cilia are usually present in large numbers and can be found in many organs including trachea, brain and Fallopian tubes [1, 2]. Contrary to motile cilia whose role has been well identified and accepted,
primary cilia have for decades been considered vestigial organelles without specific biological functions.

However, we now know that cilia are loaded with a variety of proteins, collectively called ciliary proteins, which in turn enable cilia to function in many cellular processes [2-4]. Due to their ubiquitous nature and importance in sensation and cell signaling, abnormal
cilia have been related to many fatal physiological disorders, or ciliopathies (Figure 1-2).

Therefore, it becomes extremely critical to understand this ubiquitous organelle and relevant signaling pathways.

1.2 Primary cilium and its biological functions

A primary cilium is composed of an axoneme of 9 doublet microtubules rods, and protrudes into extracellular space. Many IFT motor proteins have been identified along the axoneme, which suggests a significant role of axoneme in protein transportation.

![Figure 1-2 The cilia/basal body hypothesis of renal cystic disease and related disorders.](Hildebrandt F et al., 2007)
machinery within the cilia. The axoneme is sheathed with a lipid bilayer, where a large number of receptors and ion channels are localized [4, 5]. A cilium arises from the mother centriole or centrosome (Fig 1-1C). It is also noteworthy that the distal tubule of mother centriole/basal body is separated from the cell membrane by a specific structure called ciliary necklace. The ciliary necklace is thought to regulate ciliary proteins entering and existing a cilium [6].

Primary cilia, which are found on virtually all mammalian cells in the body, do not assemble the central microtubule complex, and therefore, they do not actively beat. However, in the past 10 years, many researches have suggested that cilium is an important sensory organelle that detects extracellular cues and transmit these signals to cell interior, resulting in changes in gene expression and cellular events.

A cilium, like the nucleus, mitochondria, Golgi apparatus etc., should also be viewed as an independent entity [7]. Many researches have related cilia to variety of signaling transduction including but not limited to photo- [7-9], chemo- [10, 11], osmo- [12], gravitational- [13, 14], mechano-sensor [15]. Therefore, it is not surprising that a high density of specialized proteins such as signal modules, receptors, ion channels, kinases, phosphatase are found localizing to ciliary compartment.

Among all the proposed functions, chemo- and mechano-sensation are the most important ones given the fact that majority of the cilia in the body are in a micro-aqueous environment where many different ligands can be found and subtle change in fluid flow could cause the cilia bending. For example, dopamine receptor type 5 (DR5) is a type of GPCR that couples with Gs protein. DR5 has been shown to localize in the cilia and regulate morphology of them (Fig 1.3) [11, 16]. Another example is epidermal growth
factor receptor (EGFR). It has been reported to localize in the primary cilium and regulate cell proliferation through ciliary calcium channel [17-19]. Due to its flexible nature, a cilium can be bent by fluid flow (Figure 1-4). Such morphology change has been shown to have biological significance. In animal model ift88TGN737Rpw mouse (AKA ORPK mouse), cilia are completely deficient. This animal model generally develops PKD at very young age. Cells isolated from cyst lining of such animal do not possess the ability to sense the flow. In addition to this study, various other groups also established the role of primary cilium as mechanosensor in many cell types [20-23]. Diseases that relate to abnormality of such function include PKD, hypertension, BBS, cystic liver disease, obesity and so on. Interestingly, ciliary response to flow is also selective and the amplitude is dependent on the flow rate. Taken together, it is becoming very critical to elucidate the signaling pathways that are implicated in ciliary sensation.

1.3 Cilia-regulated calcium signaling

A family of calcium channels have been identified in primary cilium [5]. In this regard, much work of cilia research has been focused on cilia-mediated calcium signaling. Many previous studies have reported that upon cilia-related stimulation, such as fennoldopam (DR5 agonist) treatment and fluid flow, changes in intracellular calcium concentration are often observed prior to other cellular events [11, 21-23].

Calcium signaling regulates a large number of signaling pathways and is widely considered as a multipurpose intracellular signaling molecule. Calcium signaling is important for gene expression, neurotransmitter release, synaptic transmission, muscle contraction, metabolism, proliferation, fertilization, and many other processes [24-26]. Cytosolic calcium is buffered
by the intraorganellar store and the extracellular content. Within the intraorganellar store, there are two calcium release channels that predominantly coordinate intracellular calcium signaling. These receptor ion channels include inositol 1,4,5-trisphosphate
(IP3R) and ryanodine (RyR) receptors. Stimulation, such as fluid flow, has been shown to activate these two receptors [27-29].

1.3 Cilia-regulated calcium signaling

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On the other hand, a protein-heterodimer is well identified as a major factor that could initiate the ciliary calcium signaling. It consists of two proteins, PC1 and PC2. PC1 and PC2 physically interact through their C-terminal cytoplasmic domains (Fig 1.9). Mechanical stimulation such as fluid flow can change the physical conformation of PC1-PC2 complex, and subsequently, PC2 will allow extracellular calcium to enter and trigger calcium induced calcium release (CICR). Additionally, the altitude of calcium increase varies corresponding to different ranges of fluid flow rates [23]. These observances make many researchers speculate that the calcium increase evoked by ciliary specific stimulations might consist of two well-coordinated phases with different sources and mechanisms: 1. An early phase due to modulated calcium influx into cilium body which is dependent on the type and strength of the stimulations 2. A later phase where calcium signal is further amplified by downstream effectors such as IP3R and RyR. However, a systematic understanding of this model is yet to be established owing to the absence of direct evidence that cilium is able to serves as an independent compartment for calcium signaling following particular stimulations. The challenge is constituted of inability of

![Figure 1.4 Cilia can be bent by fluid flow.](image)

Cilia are sensory organelles that 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. This biomechanical properties play a very important role in vestibular organs than support body fluid perfusion. (Alaiwi WA et al., 2009)
commonly used calcium imaging methods to visualize calcium signaling in cilium due its sub-micron diameter.

1.4 Calcium signaling and Autosomal Dominant Polycystic Kidney Disease (ADPKD)

ADPKD is an inherited systemic disorder that predominantly affects the kidneys, but may affect other organs including the liver, pancreas, brain, and arterial blood vessels. ADPKD occurs worldwide and affects about 1 in 400 to 1 in 1000 people. Approximately 50% of people with this disease will develop end stage renal failure and require dialysis or kidney transplantation making it the most common and fatal ciliopathy.

ADPKD is hallmarked by the presence of fluid-filled cysts in the kidneys (Figure 1-5), and expansion and cysts will further damage the renal tissue nearby, which leads to kidney failure at late stage. Defects in two genes are thought to be responsible for
ADPKD. In 85% of patients, ADPKD is caused by mutations in the gene PKD1 on chromosome 16 (encoding PC1); in 15% of patients mutations in PKD2 (encoding PC2) are causative [30].

Given the facts that cilium has a major role in regulating calcium homeostasis at cellular level and mutation of calcium channel complex PC1-PC2 is responsible for ADPKD, the most common ciliopathy, it becomes intriguing to examine the role of cilia regulated calcium signaling in ADPKD. Lower intracellular calcium concentration in primary cells isolated from cyst lining of human patients or PKD animal models has been observed and reported by several independent research groups. Restoring intracellular calcium level in

![Figure 1-6 Effect of verapamil (VP) on renal cyst development in female and male Cy/p rats. (Nagao S et al., 2008)](image)

human PKD cells also rescues abnormal cell proliferation phenotypes in one of the
studies [31-35]. Besides PC1/PC2 calcium channel complex, a previous study indicated that Cav 1.2 could also be involved in cilia regulated calcium signaling. Cav 1.2 inhibition by verapamil cause larger cysts in rodent PKD model (Figure 1-6) [36].

The cAMP regulated Ras/Raf/MEK/ERK signaling pathway has been proposed to be a possible mechanism which incorporates calcium ion as a important regulator (Figure 1-

![Proposed signal transduction pathways for Ca2+ regulation of cAMP-dependent B-Raf signaling to MEK/ERK and cell proliferation.](image)

Normal cell proliferation is controlled by growth factors binding to receptor tyrosine kinase with sequential activation of Ras→Raf-1→MEK→ERK to induce cell proliferation. There is a phenotypic difference between normal kidney cells and PKD cells in the cAMP effect on proliferation. (A) In NHK cells, basal intracellular Ca2+ levels, controlled by a variety of Ca2+ entry mechanisms, maintain the activity of phosphatidylinositol 3-kinase (PI 3-K) and Akt, preventing cAMP-dependent activation of B-Raf. B-Raf kinase activity is inhibited by Akt phosphorylation of an inhibitory site. cAMP agonists, e.g., arginine vasopressin, inhibit Raf-1 through a protein kinase A–dependent mechanism. Thus, ERK activation and cell proliferation are controlled by a balance of signals (positive and negative) that affect Raf-1 in NHK cells. (B) In PKD cells, a reduction in intracellular Ca2+ levels, as a result of a loss of PC1/PC2 function, decreases PI 3-K activity, relieving B-Raf inhibition by Akt. cAMP then signals through B-Raf to activate MEK and ERK and to stimulate cell proliferation. (Yamaguchi T et al. 2006)
The evidence that the level of cAMP and phosphorylation of MEK/ERK is abnormal in PKD cells supports this model [37, 38]). Under normal conditions, cAMP inhibits MAPK and cell proliferation in ADPKD or other abnormal calcium derived conditions, cAMP promotes MAPK activity and thus accelerates cells growth. These differences in cAMP function directly correlate to changes in intracellular calcium level and are caused by defective cilia. However, it is still unclear if other ciliary localized calcium channels (such as Cav1.2) are also implicated in this mechanism, given the fact that pharmacological inhibition of Cav1.2 causes abnormal MEK/ERK activation and facilitate cysts formation in vivo.

Due to the strong correlation between cilia regulated calcium signaling and ADPKD, decoding the mechanism by which downstream cellular events are systematically modulated by ciliary calcium signaling will provide novel targets for future therapies to treat ADPKD and other ciliopathies in the clinic.

1.5 Hypotheses and experiments outline

A. Cilium is an independent compartment for calcium signaling.

B. Cav1.2 is a secondary calcium channel and work in concert of PC2 to modulate cilia regulated calcium signaling.

To test our hypothesis, we have designed following experiments:

I. Design a imaging system that enables visualize cilia from the side.
II. Incorporate specially designed GECI to cells and examine its localization and sensitivity to determine if it is suitable for calcium imaging within individual cilium.

III. Apply stimulations including fluid flow, Fenoldopam (FD), Thrombin, Ionomycin to cells and examine calcium transduction.

IV. Combine different inhibition with above treatment to elucidate possible effectors that are responsible for calcium influx and/or signal amplification.

V. Generate cell lines that are lack of functioning PC1 and PC2.

VI. Permanently knockdown Cav1.2 in above mutant cell lines.

VII. Examine AKT, ERK activity, cell proliferation, cell division, intracellular calcium level, cilia morphology in above genetically manipulated cells.

VIII. Knock down Cav1.2 in zebra fish and examine their renal phenotype.

IX. Knock down Cav1.2 in wild type and PKD animal models and examine their renal phenotype.
Chapter 2

Cilioplasm is a cellular compartment for calcium signaling in response to mechanical and chemical stimuli

Xingjian Jin¹, Ashraf M. Mohieldin², Brian S. Muntean², Jill A. Green³, Jagesh V. Shah⁴, Kirk Mykytyn³, Surya M. Nauli¹,²


¹Department of Medicine, College of Medicine, and ²Department of Pharmacology, College of Pharmacy, The University of Toledo, Toledo, OH 43606.
³Department of Pharmacology, College of Medicine, The Ohio State University, Columbus, OH 43210
⁴Department of Systems Biology, Harvard Medical School and Renal Division, Brigham and Women's Hospital, Boston, MA 02115.

2.1 Abstract

Primary cilia with a diameter of ~200 nm have been implicated in development and disease. Calcium signaling within a primary cilium has never been directly visualized and has therefore remained a speculation. Fluid-shear stress and dopamine receptor type-5 (DR5) agonist are among the few stimuli that require cilia for intracellular calcium signal transduction. However, it is not known if these stimuli initiate calcium signaling within the cilium, or if the calcium signal originates in the cytoplasm. Using an integrated single-cell imaging technique, we demonstrate for the first time that calcium
signaling triggered by fluid-shear stress initiates in the primary cilium and can be distinguished from the subsequent cytosolic calcium response through the ryanodine receptor. Importantly, this flow-induced calcium signaling depends on the ciliary polycystin-2 calcium channel. While DR5-specific agonist induces calcium signaling mainly in the cilioplasm via ciliary CaV1.2, thrombin specifically induces cytosolic calcium signaling through the IP$_3$ receptor. Furthermore, a non-specific calcium ionophore triggers both ciliary and cytosolic calcium responses. We suggest that cilia not only act as sensory organelles but also function as calcium signaling compartments. Cilium-dependent signaling can spread to the cytoplasm or be contained within the cilioplasm. Our study also provides the first model to understand signaling within the cilioplasm of a living cell.
2.2 Introduction

Primary cilia are microtubule-based organelles found on the apical surface of most mammalian cell types. The sensory functions of primary cilia in the kidney are generally characterized into mechano- [23, 32, 34, 39-44] and chemosensation [11, 45-47]. The hypothesis of mechanosensory cilia is based on the observation that cilia bend when subjected to fluid flow [48], resulting in changes in cytosolic calcium levels [41]. Moreover, many laboratories have shown that fluid-shear stress and a few pharmacological agents depend on primary cilia for intracellular signaling, including calcium signaling [11, 23, 32, 34, 39-47]. Due to the size of primary cilia with a diameter of ~200 nm, however, there has never been a direct visualization of any signaling occurring within the primary cilium. Worth mentioning is that most primary cilia are oriented perpendicular to the cell surface, which makes visualizing ciliary signaling in live cells extremely challenging, in addition to the tiny diameter of primary cilia.

Because abnormal primary cilia have been associated with a long list of clinical pathologies [49], it is extremely crucial to examine signaling pathways in the cilia of distinct living cells. Measuring a specific signal explicitly within a cilium would allow a precise interpretation of the ciliary functions. In addition, it would provide important insight into the dynamics of signaling between the cilium and cell body. In other words, many cilium-dependent responses have only been detected at the cellular level or in fixed tissues, resulting in uncertainty of how ciliary signal transduction propagates from the cilium as a sensory organelle to the cell body as a whole.
In the present study, we adopted a new integrated single-cell imaging technique to
distinctively visualize the cilium (cilioplasm) and the cell body (cytoplasm). For the first
time, we provide differential visual evidence of the cilium-dependent and cilium-
independent signaling mechanisms in a living cell. Our data demonstrate that fluid-shear
stress and dopamine receptor-type 5 (DR5) agonist generate calcium fluxes in the
cilioplasm through distinct ciliary calcium channels polycystin-2 and CaV1.2,
respectively. While thrombin induces calcium signaling mainly in the cytoplasm,
ionomycin generates non-specific calcium increases in cilioplasm and cytoplasm.

2.3 Materials and Methods

Plasmid Construct

We used a genetically encoded indicator (GECl), G-CaMP3 to enable us to monitor
intracellular calcium [50]. Of note is that none of the calcium fluorophores (e.g. Fura-2,
Fluo-2, Fluo-4 and Asante Calcium Red) can be loaded into the cilioplasm due to the
restriction in the ciliary transport or diffusion constraint within ciliary necklace [51]. The
CTS-G-CaMP3 construct was generated using PCR to fuse the first 68 amino acids of the
intracellular C-tail of fibrocystin (Pkhd1 C1-68) to the N-terminus of the calcium
indicator, G-CaMP3. G-CaMP3 was obtained from Addgene (Addgene plasmid 22692).
The sequences of the fusion primers are 5’-AGT CAT GCT AGC CAT GCT GCT CTG CAT
TCC CTG-3’ and 5’-CAG GGA ATG CAG AGC ATG GCT AGC ATG ACT-3’. In
addition, primers were designed to have an EcoRI digestion site at the 5’ end and a
BamHI digestion site at the 3’ end of the insert. The insert was subcloned into
pcDNA3.1 (-) using these restriction sites.
Cell transfection

Cells were then transfected with the CTS-G-CaMP3 construct (0.5 μg/mL) using Xtreme6 transfection system (Roche, Inc.). In some cases, cells were co-transfected with scramble siRNA (5’- UUC UCC GAA CGU GUC ACG U-3’) or Pkd2 siRNA (5’-GCU CCA GUG UGU ACU ACU ACA-3’) using DharmaFECT Duo Transfection reagent (Thermo Scientific, Inc.). All siRNAs were conjugated with Cy5 to monitor transfection efficiency. The siRNA (20 nM or 30 nM) provided transfection efficiency of about 80%.

Immunostaining

LLCPK cells were immunostained using standard techniques. Briefly, cells were rinsed with 1× phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde containing 2% sucrose for 10 minutes and permeabilized with 1% Triton-X in PBS for 5 minutes. Acetylated-α-tubulin (1:10,000; Sigma, Inc.), CaV1.2 (1:100; Alomone Lab, Inc.), dopamine receptor-type 5 (1:2,500; Calbiochem, Inc.) or thrombin receptor (1:500; Santa Cruz, Inc.) antibody was used followed by fluorescence secondary antibody (1:500; Pierce, Inc.). Cells were counterstained with 4.6-diamidino-2-phenylindol DAPI (Vector Laboratories, Inc.). Three-dimensional images were taken with Nikon TE2000 and reconstructed using Metamorph software, as previously described [11].

Precision microwire

A special order for the precision microwire can be obtained from H.P. Reid, Inc. in Palm Coast, FL. This wire was first coated with type I collagen, UV sterilized and mounted on a microscope coverslip (No. 1). Cells would preferentially grow on the collagen, i.e. on
the wire. As needed, such wire could be gently rotated to observe the confluency of the cells around the wire. Due to its steadiness and non-toxic nature, the wire is an excellent set-up for any experimental manipulations. In our case, this includes application of fluid-shear stress and treatment of various pharmacological agents to the wire.

Live-Cell Imaging Analysis

LLCPK cells were grown at 39°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum on the precision microwire. After cells were transfected with the CTS-G-CaMP3 for 2 days, serum was withdrawn from the cell culture medium to promote ciliation. The wire-containing cells were then moved to a custom-designed perfusion chamber made of a standard 6-cm culture plate. This culture plate would basically support the inlet and outlet of fluid perfusion. More importantly, the plate would allow a tight seal of the microscope coverslip, in which the precision microwire was mounted on. Once settled on the microscope stage, the wire containing cells was equilibrated for 15-30 minutes.

In addition to the differential side-view imaging platform, we utilized customized high-resolution differential interference contrast (DIC) microscopy to distinctively visualize the cilium (cilioplasm) and the cell body (cytoplasm). The DIC and fluorescence images were captured using Nikon Ti-U microscope. The No. 1 microscope coverslip was used, because it is best suited for our customized 1.4 numerical aperture oil condenser lens and 60X objective lens with near-infrared resolution prism. These modifications provide sharp-contrast images suitable for live-cell imaging studies on small organelles, such as
cilia. For greater sensitivity, we used Photometrics Coolsnap ES2, 12 bit, 20 MHz Digital Monochrome Camera with IEEE-1394 interface. For better focusing, the microscope was equipped with XY-axis motorized flat-top inverted stage (Prior Nanopositioning Piezo Z), automatic focusing RFA Z-axis drive (digital autofocus PIFOC), and custom-designed vibration isolation platform (Isolator Table). For a better-controlled environment, the body of the microscope was enclosed inside a custom built chamber to control CO2, humidity, heat and light. External stimuli were applied as described previously [15]. DIC images will be automatically adjusted for optimal brightness/contrast, exposure, and focus. Live images of CTS-G-CaMP3 were streamed at the excitation and emission wavelengths of 495 and 515 nm, respectively.

Protein analysis

LLCPK cells were homogenized in immunoprecipitation buffer (Boston Bioproducts, Inc.) according to the manufacturer’s instructions. Equal amounts of protein extracts (50 µg/lane) were mixed with sample buffer and then separated on 7% SDS-PAGE gels. Antibodies used for Western blotting included rabbit polyclonal anti-polycystin2 (1:300; Santa Cruz Biotech, Inc.), monoclonal rabbit anti-mouse GAPDH antibody (1:10,000; Cell Signaling, Inc.) and HRP-conjugated goat anti-rabbit antibody (1:10,000; Thermo Scientific, Inc.). GAPDH was used as a loading control.

Pharmacological agents

All drugs were freshly prepared in Hank’s balanced salt solution. For each drug, their optimal concentrations have been previous determined from the dose-response curve [11,
23]. Fenoldopam (DR5-specific agonist; *Hospira, Inc.*) or ionomycin (non-specific ionophore; *Sigma Aldrich, Inc.*) was applied to the cell at final concentration of 1 μM. Hexapeptide thrombin receptor agonist (*Biopharm Laboratories, Inc.*) was used at concentration of 1 μM with specific activity of about 1 unit/mL. Caffeine (60 mM; *Fisher Scientific, Inc.*) and 2-APB (10 μM; *Tocris, Inc.*) were used to inhibit intraorganellar ryanodine and IP₃ receptors, respectively. Verapamil (1 μM; *Sigma Aldrich, Inc*) was used to inhibit CaV1.2 calcium channel. These inhibitors were incubated with the cells for an hour.

**Data analysis**

Experiments were repeated on at least three different sets of cell populations. For each population, three individual cells were randomly selected for analysis. For immunostaining analysis, a minimum of six coverslips (cell populations) was used for each experiment. All images were analyzed using Metamorph software. For calcium analysis, fluorescence signal intensity was first subtracted from the background fluorescence. For quantification, ciliary and cytosolic fluorescence intensities were differentially measured at each time point. To obtain proper and unbiased measurements, signal intensity was normalized to baseline fluorescence. Next, the signal intensity measured in pixel was further normalized with the total area of the measurement. To verify homogeneity of variance within each data set (homoscedasticity), heterogeneous variances were analyzed. If detected, the data distributions were normalized via log transformation to produce normally distributed data sets for all analyses. All quantifiable data were reported as mean ± SEM. Comparisons between means were performed using
ANOVA with post hoc comparisons via Dunnet, and statistical significance implies $p < 0.05$. All data analysis was done using GraphPad Prism v.5.

2.4 Results

Precision microwire allows differential visualization of CTS-G-CaMP3 in the cilioplasm and cytoplasm. None of the calcium-sensitive fluorophores are loaded into the cilioplasm (data not shown). To visualize calcium signaling within the cilium, we therefore fused G-CaMP3 with a highly conserved cilia-targeting sequence (CTS) that was identified in the fibrocystin protein [52]. To test the specificity of our CTS-G-CaMP3 construct, we also examined the localization of G-CaMP3 lacking the CTS. Whereas G-CaMP3 shows only cytosolic localization, CTS-G-CaMP3 localizes to both the cytosol and cilia of fixed LLCPK cells, as indicated colocalization with the ciliary marker acetylated-α-tubulin (Figure 2-1). To allow us to observe cultured cells from the side, we adopted a differential side-view imaging platform using precision microwire to overcome the issues with optical distortions of traditional 2-dimensional live-cell imaging. Renal epithelial cells were grown on a specially made Tungsten wire with a purity of greater than 99.9% and a standardized diameter of 100 μm. Using precision microwire, we are able to perform a differential side-view imaging of individual cells (Figure 2-2a). To determine if our CTS-G-CaMP3 construct is expressed and localizes to cilia in cells grown on the intended platform, we re-examined the G-CaMP3 fluorescence signal in live LLCPK cells (Figure 2-2b). Live-cell imaging demonstrates that the non-transfected cells have
no specific fluorescence signal. As expected, G-CaMP3-transfected cells show a fluorescence signal only in the cytoplasm, but the CTS-G-CaMP3 signal can be observed in the cilioplasm and cytoplasm. The localization of CTS-G-CaMP3 in cilia and cytosol may be a consequence of high expression levels of CTS-G-CaMP3; it may also be due to the fact that fibrocystin is expressed in both cilia and cytosol [53]. Nevertheless, the nature of this subcellular localization allows us to measure both ciliary and cytosolic calcium levels differentially and simultaneously.

Flow-induced ciliary calcium depends on polycystin-2 and propagates to the cytoplasm via the ryanodine receptor.

To determine whether bending of cilia results in increased ciliary calcium, we applied fluid-shear stress of 0.7 dyne/cm², which has been previously shown to activate cilium-dependent intracellular calcium signaling [23, 34]. This magnitude of shear stress also allows optimal physical bending of a cilium (Figure 2-3; Movie 1). In the presence of fluid-shear, the bending of the cilium induces an increase in ciliary calcium levels (Movies 2 and 3). The increase in ciliary calcium is then followed by an increase in cytosolic calcium. While inhibiting IP3 receptor with 2-APB (10 μM) does not have any
Figure 2-1 CTS-G-CaMP3 is co-localized with acetylated-α-tubulin in primary cilia.

LLCPK cells were fixed, permeabilized and immuno-stained with the ciliary marker acetylated-α-tubulin (acet-α-tub). Non-transfected cells show no green fluorescence signal and are used as a negative control (upper row). Cells transfected with G-CaMP3 show green fluorescence signal only in the cell body (middle row). CTS-G-CaMP3 transfected cells show green fluorescence signal in the cilium (lower row), as indicated by the ciliary marker (red fluorescent). Merged images of green and red signals are shown with dapi (nuclear DNA staining). Bar=4 μm.
Figure 2-2 CTS-G-CaMP3 is localized to the cilium in live cells.

A live LLCPK cell was imaged from the side using high-resolution differential interference contrast (DIC) and fluorescence microscopy. a. A Tungsten precision microwire with a diameter of 100 μm was coated with collagen (upper row). Cells were seeded and selectively grew around the wire (lower row). b. A non-transfected cell displays no fluorescence signal (control; upper row). Background autofluorescent from the microwire can be occasionally seen. A cell transfected with G-CaMP3 shows fluorescence only in the cell body (middle row). A CTS-G-CaMP3 transfected cell shows fluorescence signal in the cell body and cilium (lower row). Black Bar = 50 μm; white Bar=4 μm.
Figure 2-3 Flow-induced calcium signaling originates from the cilium.

DIC and fluorescence images were captured simultaneously. Representative DIC images are to show the bending of cilia by fluid-shear stress. Representative calcium signaling in the cilioplasm and cytoplasm was then differentially observed for each group. Quantitation analysis from 6 independent experiments was averaged and plotted in the line graphs for cilioplasm and cytoplasm. Fluid-shear induces calcium signaling in the cilioplasm followed by signaling in the cytoplasm. Representative fluorescent images from two separate flow experiments are shown.

major effect on flow-induced ciliary or cytosolic calcium increase (Figure 2-4), blocking the ryanodine receptor with caffeine (10 □M) distinctively abolishes cytosolic calcium, while ciliary calcium is still increased by fluid-flow (Figure 2-5). Our study also shows that polycystin-2 is involved in flow-induced intracellular calcium increases in both cilioplasm and cytoplasm (Figure 2-6). We first examined the effect of Pkd2 siRNA on polycystin-2 expression (Figure 2-6). Either 20 or 30 nM of Pkd2 siRNA is sufficient to repress the overall expression of polycystin-2.
As indicated by our Cy5-tagged Pkd2 siRNA, we have a transfection frequency of about 80%. This Cy5-tagged strategy also allows us to selectively pick a successfully transfected cell for live imaging. Overall quantitation

![Graph](image)

**Figure 2-4 2-APB doesn’t affect flow-induced calcium signaling.**

DIC and fluorescence images were captured simultaneously. Representative DIC images are to show the bending of cilia by fluid-shear stress. Representative calcium signaling in the cilioplasm and cytoplasm was then differentially observed for each group. Quantitation analysis from 3 independent experiments was averaged and plotted in the line graphs for cilioplasm and cytoplasm. Pre-incubation with 2APB for 30 minutes has a minimal effect on ciliary or cytosolic calcium.

indicates that polycystin-2 and ryanodine receptor are differentially and distinctively involved in subcellular calcium signaling in response to fluid-shear stress (Figure 2-7).

Differential subcellular calcium signaling provides a new paradigm of chemosensory role of renal epithelial cilia.
Our group and others recently identified DR5 as a chemosensory receptor in cilia of endothelial and fibroblast cells [11, 54]. We therefore hypothesized that DR5-specific agonist, fenoldopam, can also trigger calcium signaling within the cilioplasm of renal epithelia. To test this hypothesis, we challenged the cells with 1 μM of fenoldopam (Figure 2-8; Movies 4 and 5). We found that fenoldopam specifically increases ciliary calcium levels with minimal calcium increase in the cytoplasm. To further understand the calcium channel involved in this response, we screened various inhibitors that would block DR5-induced ciliary calcium, including Pkd2 siRNA. Surprisingly, 1 μM of...
verapamil sufficiently inhibits the effect of fenoldopam on ciliary calcium signal (Figure 2-9). Worth mentioning is that verapamil is generally known as a potent inhibitor of L-type calcium channel in the heart [55].
Figure 2-6 *Pkd2* knockdown reduced the calcium influx in cilia.

a. Cells transfected with 20 or 30 nM *Pkd2* siRNA showed a significant decrease in polycystin-2 (PC2) expression, compared to those with a scrambled siRNA (N=4).

b. Knockdown by *Pkd2* siRNA significantly reduced both ciliary and cytoplasmic calcium signal. DIC and fluorescence images were captured simultaneously. Representative DIC images are to show the bending of cilia by fluid-shear stress. Representative calcium signaling in the cilioplasm and cytoplasm was then differentially observed for each group.
Figure 2-7  Statistical analysis on all the treatments in the presence of fluidflow

2APB treatment had no effects on both cilary and cytoplasmic calcium level. Caffeine didn’t affect calcium influx in cilia but abolished calcium release in cytoplasm. Pkd2 knockdown abolished calcium increase in both cilia and cytoplasm.

Figure 2-8 Fenoldopam-induced calcium signaling can occur in the cilioplasm. Representative DIC and calcium images in each panel show that the primary cilium remained in focus throughout our studies, and calcium signaling in the cilioplasm and cytoplasm was differentially quantified for each group. Quantitation analysis of 6 independent experiments was averaged and plotted in the line graphs for cilioplasm and cytoplasm. Fenoldopam (FD)-induced calcium signaling is more apparent in the cilioplasm than cytoplasm.
Figure 2-9 Fenoldopam-induced calcium signaling can be abolished by verapamil.

Quantitation analysis of 3 independent experiments was averaged and plotted in the line graphs for cilioplasm and cytoplasm. Fenoldopam (FD)-induced calcium signaling was abolished by verapamil treatment. (N=3)

Figure 2.10 Thrombin (TH)-induced calcium signaling occurred only in the cytoplasm.

Quantitation analysis of 3 independent experiments was averaged and plotted in the line graphs for cilioplasm and cytoplasm. Thrombin-induced calcium signaling is observed only in cytoplasm. (N=3)
Figure 2-11 Thrombin-induced calcium signaling can be abolished by 2APB
Quantitation analysis of 3 independent experiments was averaged and plotted in the line graphs for cilioplasm and cytoplasm. Thrombin-induced calcium signaling was abolished by APB treatment. (N=3)

Figure 2-12 Ionomycin (IO) induced non-specific calcium signaling in both cilioplasm and cytoplasm
Quantitation analysis of 4 independent experiments was averaged and plotted in the line graphs for cilioplasm and cytoplasm. Ionomycin (IO) induced non-specific calcium signaling in both cilioplasm and cytoplasm.
We previously confirmed that calcium signaling in response to thrombin or ionomycin is not a cilia-mediated signal transduction [23]. We thus challenged a cell with thrombin, a specific agonist for G-protein-coupled thrombin receptor, in the absence (Figure 2-10; Movies 6 and 7) or presence (Figure 2-11) of 10 μM 2APB. While 1 μM of thrombin increases cytosolic calcium, the effect of thrombin is blocked by 2-APB, indicating the IP3 receptor is required for the thrombin-induced signal transduction pathway. Unlike thrombin which has no effect on ciliary calcium, 1 μM of ionomycin non-specifically increases calcium levels in both the cilioplasm and cytoplasm (Figure 2-12; Movies 8 and 9). Of note is that ionomycin as a non-selectivity calcium ionophore can function as our positive control in verifying our new experimental set-up.

Overall quantitation indicates that 1) fenoldopam increases ciliary calcium, and verapamil significantly represses this calcium change, 2) thrombin increases cytosolic calcium, and 2APB significantly represses this increase, and 3) ionomycin increases a much greater cytosolic than ciliary calcium (Figure 2-13).
The L-type voltage-dependent calcium channel and dopamine receptor-type 5 are localized to primary cilia of renal epithelia.

To elaborate more on the result of our chemical screening and the relationship between verapamil and fenoldopam (Figure 2-9), we examined the possibility of the L-type calcium channel (CaV1.2) and dopamine receptor-type 5 (DR5) in the cilia. We here report for the first time that CaV1.2 is localized to renal epithelial cilia (Figure 2-14a). Whereas DR5 is mainly localized to the primary cilia in renal epithelial (Figure 2-14b),

**Figure 2-14** The L-type calcium channel CaV1.2 and dopamine receptor-5 are localized to primary cilia of kidney epithelial cells.

LLCPK cells immuno-stained with L-type calcium channel (CaV1.2; a), dopamine receptor type-5 (DR5; b) or thrombin receptor (THR; c).
thrombin receptor is localized throughout the apical membrane (Figure 2-14c). The immunolocalization data support the overall functional studies defining distinct subcellular calcium signaling within a single cell.

2.5 Discussion

Our present studies integrate three technologies to allow direct measurement of ciliary signaling in an individual living cell. These technologies include precision microwire, calcium sensor CTS-G-CaMP3 construct and modified microscopy system. This integrated single-cell analysis offers the first evidence of calcium signaling in the primary cilium of a living renal epithelium. Our data further show that cilium-dependent signaling can occur through mechanical and chemical stimulations (Figure 2-15). This signaling provokes extracellular calcium influx into the cilioplasm, because fluid media without extracellular calcium fails to increase ciliary calcium (data not shown). Consistent with our previous study [23], fluid shear-induced ciliary calcium through polycystin-2 propagates to and is amplified in the cytoplasm through the ryanodine receptor. We also provide evidence that DR5-mediated ciliary calcium signaling is mediated by the L-type calcium channel CaV1.2. Interestingly, DR5-induced ciliary calcium retains within cilioplasm, suggesting that the ciliary calcium is differentially regulated between mechanical and chemical stimuli.

Primary cilia are sensory organelles that translate extracellular stimuli into intracellular responses. Fluid-shear stress and the dopamine receptor type-5 (DR5) agonist are among the few stimuli that require cilia for intracellular calcium signal transduction. Cells
without cilia are thus unable to respond to fluid-shear or DR5 agonist [39, 40, 42, 43, 56]. However, it was not known if these stimuli initiated calcium signaling within the cilium, or if the calcium signal originates in the cytoplasm. We therefore selected fluid-shear and fenoldopam as cilium-dependent stimuli. We also used two cilium-independent stimuli to validate the ciliary calcium signaling. Thrombin was selected, because it distintively and consistently showed cytosolic calcium signaling in our initial screenings. This was further supported by our previous study, in which thrombin was used as a control in our cilia-mutant renal epithelial cells [23]. Ionomycin was also selected, because it has a unique property as a non-specific calcium ionophore. While thrombin requires G protein-coupled thrombin receptor to exert its effect, ionomycin does not activate any receptor. We thus believe that these four stimuli represent a balanced approach to study ciliary signaling, although there are definitely more stimuli in which ciliary roles are worth-examined in future studies.
Our studies utilized a novel model to visualize calcium signaling within both cilioplasm and cytoplasm. More importantly, this is the very first visualization of ciliary signaling in the cilioplasm of a single-living cell. Our experimental setup was designed to permit side-view of a living cell, allowing us to examine mechanical stimulus in the cilium and cytosol simultaneously (Movies 1, 2 and 3). We often observed two types of cytosolic calcium. The peak of cytosolic calcium could occur after the stimulus (bending) was
relieved (Type I; Movie 2), or the peak of cytosolic occurred while the stimulus was occurring (Type II; Movie 3). The temporal and spatial differences between the cilioplasm and cytoplasm were therefore significant in that the peak of cytosolic calcium was delayed by 2.1±0.6 seconds after ciliary calcium.

It is imperative to note that ryanodine receptor blockage does not inhibit sensory function of primary cilia as we previously thought [23]. Because our current single cell analysis provides a much higher temporal and spatial resolution, we are able to determine that a minute calcium spark within the cilioplasm was not affected by inhibiting ryanodine receptor indicating two possible independent yet intertwined events within the cilioplasm and cytoplasm.

We found that the baseline calcium levels in both the cilioplasm and cytoplasm are very stable, with no obvious fluorescence fluctuations in our single-cell studies. Our studies also show that the chemosensory capacity of renal epithelia can occur specifically within the cilioplasm (Movies 4 and 5) and cytoplasm (Movies 6 and 7), as confirmed by ionomycin control (Movies 8 and 9). It is worth mentioning that the fenoldopam-induced ciliary signal can also be observed in the proximity of the ciliary base within the cytoplasm and the thrombin-induced cytoplasmic signal can be sporadically detected at the base of a cilium. This suggests that a proportion of calcium ions are able to diffuse across the size-exclusion permeability barrier within the ciliary necklace or transition zone [57].
For the first time, we also present DR5 and CaV1.2 localizations to primary cilia of kidney epithelial cells. Although the presence of voltage-dependent channels in non-excitable cells used to be controversial, CaV1.2 mRNA has been detected and reported in renal tissue and isolated renal epithelia [58, 59]. The cilia localization further supports our functional study that CaV1.2 activation can be activated by ciliary DR5 and that blocking of CaV1.2 with verapamil can inhibit the effect of fenoldopam in primary cilia. We thus propose that the calcium signaling-induced by fenoldopam on DR5 requires CaV1.2.

In summary, the present study elucidates the dynamic process of calcium signaling in the cilium and cytosol. Although many laboratories have independently predicted that flow- or DR5-induced signaling is a cilium-mediated process [39, 40, 42, 43, 56], our present study provides the first opportunity to directly visualize calcium signaling within primary cilia. Our experimental approach will allow future studies to better understand the roles of ciliary proteins in cellular signaling and the possible downstream pathways associated with ciliary signaling and cilia-related diseases.

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Supplemental Materials (Movies)

**Movie 1. Fluid-shear stress induces cilium bending**

The movie was taken with a high-resolution, high-speed differential interference contrast microscope. The cell was challenged with fluid-shear stress (flow). Number represents time in seconds.

**Movie 2. Fluid-shear stress induces calcium signaling in the cilioplasm and cytoplasm.**

Using high-speed excitation wavelength exchanger for the DG4/DG5 system, a movie of fluorescence was captured simultaneously with Movie 1. Color bar indicates calcium level, where black-purple and yellow-red colors represent low and high calcium levels, respectively. Number represents time in seconds.

**Movie 3. Fluid-shear stress induces calcium signaling in the cilioplasm followed by the cytoplasm.**

A movie of fluorescence changes in an experiment independent from Movie 2. Color bar indicates calcium level, where black-purple and yellow-red colors represent low and high calcium levels, respectively. Number represents time in seconds.

**Movie 4. The cilium and cell body remain in focus in a cell treated with fenoldopam.**

The movie was taken with a high-resolution, high-speed differential interference contrast microscope. The cell was challenged with fenoldopam (FD). Number represents time in seconds.
**Movie 5.** Fenoldopam induces calcium signaling specifically in the cilioplasm.

Using high-speed excitation wavelength exchanger for DG4/DG5 system, a movie of fluorescence changes was captured simultaneously with Movie 4. Color bar indicates calcium level, where black-purple and yellow-red colors represent low and high calcium levels, respectively. Number represents time in seconds.

**Movie 6.** The primary cilium and cell body remain in focus in a cell treated with thrombin.

The movie was taken with a high-resolution, high-speed differential interference contrast microscope. The cell was challenged with thrombin (TH). Number represents time in seconds.

**Movie 7.** Thrombin induces calcium signaling specifically in the cytoplasm.

Using high-speed excitation wavelength exchanger for DG4/DG5 system, a movie of fluorescence changes was captured simultaneously with Movie 6. Color bar indicates calcium level, where black-purple and yellow-red colors represent low and high calcium levels, respectively. Number represents time in seconds.

**Movie 8.** The primary cilium and cell body remain in focus in a cell treated with ionomycin.
The movie was taken with a high-resolution, high-speed differential interference contrast microscope. The cell was challenged with ionomycin (IO). Number represents time in seconds.

**Movie 9.** Ionomycin induces calcium signaling in both the cilioplasm and cytoplasm.

Using high-speed excitation wavelength exchanger for DG4/DG5 system, a movie of fluorescence changes was captured simultaneously with Movie 8. Color bar indicates calcium level, where black-purple and yellow-red colors represent low and high calcium levels, respectively. Number represents time in seconds.

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Chapter 3

L-type Calcium Channel Modulates Polycystic Kidney Phenotype

Xingjian Jin¹#, Brian S. Muntean²#, Munaf S. Aal-Aaboda², Qiming Duan³, Jing Zhou⁴, Surya M. Nauli¹,²

#: these authors contribute equally.

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¹Department of Physiology and Pharmacology, The University of Toledo, Toledo, Ohio
²Department of Medicinal Chemistry, The University of Toledo, Toledo, Ohio
³Department of Biochemistry and Cancer Biology
⁴Department of Medicine, Harvard Medical School, Massachusetts

Running title: primary cilia and cystic kidney

Corresponding author:
Surya M. Nauli, Ph.D.
The University of Toledo
Department of Pharmacology; MS 1015
3.1 Abstract

In polycystic kidney disease (PKD), abnormal proliferation and genomic instability of renal epithelia have been associated with cyst formation and kidney enlargement. We recently showed that L-type calcium channel (Cav1.2) is localized to primary cilia of epithelial cells. Previous studies have also shown that low intracellular calcium level was associated with the hyperproliferation phenotype in the epithelial cells. However, the relationship between calcium channel and cystic kidney phenotype is largely unknown.

In this study, we generated cell lines with somatic deficient Pkd1 or Pkd2. The expression of Cav1.2 was also inhibited by lentiviral knockdown in wild-type and mutated epithelial cell lines. Although Cav1.2 knockdown did not change division and growth patterns in wild-type epithelium, it led to hyperproliferation and polyploidy in mutant cells. Lack of Cav1.2 in Pkd mutant cells also decreased the intracellular calcium level. This contributed to a decrease in CaM kinase activity, which played a significant role in regulating Akt and Erk signaling pathways. Consistent with our in vitro results, Cav1.2 knockdown in zebrafish and Pkd1 heterozygous mice facilitated the formation of kidney cysts. Consequently, larger cysts were developed more quickly in Pkd1 heterozygous mice. Overall, our findings emphasized the importance of CaV1.2 expression in kidneys with somatic Pkd mutation. We further suggest that CaV1.2 could serve as a modifier gene to cystic kidney phenotype.
3.2 Introduction

Polycystic kidney disease (PKD) is one of the most common genetic diseases. It is characterized by formation and expansion of multiple fluid-filled cysts in the kidneys. Many previous studies have suggested that the abnormality of kidney cystic cells is contributed by interrupted calcium homeostasis [31, 60-62], probably due to the abnormal calcium channel polycystin-2 (PC2) or its regulatory protein polycystin-1 (PC1)

Both PC1 and PC2 are localized to primary cilia of renal epithelia and are required for calcium signaling [16, 23, 34]. Cells isolated from the cyst lining of human patients and PKD animal models have been shown to lack normal flow-sensitive calcium signaling and have lower intracellular calcium concentration. This deficient calcium level has been thought to be critical for pathogenesis of PKD [61]. In cystic kidney-derived cells, the aberrant intracellular calcium level has been shown to cause cAMP-dependent activation of the MEK/ERK pathway and increased cell proliferation [62-64]. In contrast, cAMP often inhibits the MEK/ERK pathway in normal kidney cells. Calcium concentration has long been thought to be a major factor that differentiates these two phases of cAMP. Furthermore, PKD phenotypes could be partially rescued by recovering intracellular calcium level, whereas reduction of that could reproduce PKD phenotypes [31, 61].

One of the major complications of PKD is hypertension, occurring in 50 to 70 percent of cases before any significant reduction in glomerular filtration rate, with an average onset at 30 years of age [65]. Abnormalities in the renin-angiotensin system and vascular
endothelial cilia have been proposed as the mechanisms of hypertension [32, 40, 66]. Because hypertension in PKD is still poorly understood, the hypertensive therapy has not been much different than therapy for hypertension in other chronic kidney diseases. L-type calcium channel blockers have been tested in PKD patients to control their blood pressure [67, 68]. Considering the fact that calcium level is lower in cystic kidney epithelia [31, 60-62], understanding the roles of L-type calcium channel in PKD becomes scientifically and clinically imperative. When heterozygous Han:SPRD Cy rats were treated with a serial of L-type calcium channel blockers to inhibit its largest subunit (Cav1.2), larger cysts were observed at a much earlier stage in treated- than in non-treated groups [60]. However, the molecular mechanism of CaV1.2 in the kidney remains largely unknown. In the present study, we examined the roles of CaV1.2 in the cilia and in cystic kidney formation. Our study suggested a potential role of CaV1.2 as a modifier gene in cystic renal epithelial cells.
3.3 Materials and Methods

Cell culture

Mouse kidney epithelial cells (wild-type, *Pkd1*+/− and *Pkd2*+/−) were isolated from embryonic day 15.5 kidneys from a cross of *Pkd1*+/− or *Pkd2*+/− mice that also carried a temperature-sensitive simian virus 40 (SV40) large T-antigen transgene. Thus, the resulting cell lines were conditionally immortalized, and the expression of the SV40 large T-antigen was regulated by temperature and IFN-γ. All cell lines were cloned by positive selection for renal epithelial marker (wheat germ agglutinin, WGA). Cell lines were grown at 33°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with IFN-γ. To study cilia function, a constant laminar flow generating a shear-stress of 7 dyne/cm² was applied for 18 hours. Such force has been shown to be able to activate flow-sensitive cell signaling mediated by primary cilia [15].

Fluorescence automated cell sorting

Fully differentiated cells were rinsed with HBSS (Hank's balanced salt solution), and approximately 10⁵ viable cells were analyzed for expression of epithelial marker. Cells were incubated with FITC-conjugated WGA antibody (10 μg/ml; Vector Laboratories) and subjected to a robust analysis using FACStar-PLUS with a laser excitation of 200 mm.

Lentivirus production and infection

HEK-293T cells were co-transfected with lentivirus packaging plasmids mix and one of four different constructs to block *CaV1.2* expression. These constructs were labeled 1
(5'-GTC CAG CAC ACC TCC TTC AGG AAC CAT AT-3’), 2 (5'-TCA GAA GTG CCT CAC TGT TCT CGT GAC CT-3’), 3 (5'-TCA GAA GTG CCT CAC TGT TCT CGT GAC CT-3’) or scrambled (5’-AAA CCC ATG AGA GAC CTT TTA GAA GAT T-3’). Lentivirus was harvested at 48 and 72 hours after the initial transfection. The titration was determined to be ~10^7 with the method described previously [69]. One ml of each shRNA-lentivirus was added to cells grown on the 10-cm cell culture dishes. The efficiency of infection was determined with Accuri Flow Cytometer by monitoring the percentage of GFP positive cells. Gene silencing was further confirmed by Western Blot, and only the shRNA lentivirus with higher knockdown efficiency was selected for future use. Infected cell lines were maintained with cell growth medium described above supplemented with 10 µg/ml of puromycin to maintain homogenous population. For in vitro applications, lentivirus was concentrated to a titration of 10^9-10^10 with an established method [70].

**Immunoblot analysis**

A total protein of 30 mg was analyzed with a standard Western blot. The following antibodies and dilutions were used in our analyses: phospho-ERK (Cell Signaling, 1:1000), anti-ERK (Cell Signaling, 1:1000), phosphor-AKT (Cell Signaling, 1:200), anti-AKT (Cell Signaling, 1:1000), anti-Cav1.2 (Alomone, 1:500) and GAPDH (Cell Signaling, 1:1,500). In some cases, wild-type cells were also treated with CaM kinase II inhibitor W7 (10 µM) for 18 hours prior to Western blot analysis. Band intensity was quantified using NIH’s Image J software.
Immunofluorescence studies

Cells were fixed with 2% sucrose plus 3% paraformaldehyde, permeabilized with 0.5%
(vol/vol) Triton-X, and incubated with rabbit Antibodies at 1:200 dilution. To stain
Cav1.2, rabbit polyclonal anti-Cav1.2 (1:200) was applied to cells and incubated
overnight at 4 °C. Mouse antibody to acetylated α-tubulin (1:10,000; Sigma Aldrich) was
used as a ciliary marker, and cells were counterstained with 4′,6-diamidino-2-
phenylindole (DAPI; Vector Laboratories) to label the nuclei. Respective secondary
antibodies were used at a dilution of 1:500.

Chromosomal analysis

For flow cytometry analysis, cells were harvested and washed with cold phosphate
buffered solution (PBS). Cells were then re-suspended at 1-2 x 10⁶ cells/ml. One ml of
cell aliquot was placed in a 15 ml polypropylene. Three ml of ethanol were added to the
cell aliquot for fixation at -20 °C overnight. After two washes with PBS, propidium
iodide staining solution and RNase A were added to cell pellet for three hours at 4 °C.
Cells were analyzed with Accuri Flow Cytometer, as previously described [71, 72].

For chromosomal counting, cells were plated for at least one day and grown to 80%
confluence. They were then grown for an additional 2 h after incubating with 0.05 μg/ml
colcemid solution for 30 min at 37 °C in the dark. Next, cells were collected and
incubated with 0.56% KCl hypotonic solution for 45 min at 37 °C, followed by fixation
with 3:1 methanol:acetic acid. The cells were dropped onto pre-cleaned glass slides and
allowed to dry. Dried slides were counterstained with DAPI for microscopy analysis. At least 50 chromosome spreads were counted from each cell analysis, as previously described [73].

**Calcium measurement**

Cells were cultured on glass-bottom dishes and perfused 18 hours prior to experiment. After two washes with HBSS, cells were incubated with 5 μM of Fura-2 AM (Telf Labs) for 45 minutes in the dark. After incubation, cells were washed and left in HBSS for 15 min to allow Fura2-AM to hydrolyze. We have previously described details on experimental set-up and calcium calculation [15].

**Animal models**

Adult wild-type AB zebrafish were obtained from the Zebrafish International Resource Center (Eugene, OR) and used for breeding. Embryos were injected with 1 mM antisense translation blocking morpholino oligos (GeneTools) at the 1-2 cell stage. Zebrafish embryos were then cultured at 28.5°C in sterile egg water [55]. The following morpholino sequences were used: control MO: 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′, cav1.2 MO: 5′-ACA TGT TTT TGC TTT CAT TTA CCA T-3′.

Previously generated Mx1Cre:Pkd1<sup>wt/flx</sup> mice at two months old were used in our studies [74]. In addition, only males were selected because they were shown to be more sensitive to CaV1.2 inhibition [36]. Polyinosine-polyctosine (plpC, 62.5 μg) was injected intraperitoneally at seven days of age for five consecutive days. Pre-
concentrated lentivirus (IFU=10^{9-10}/ml) was injected to animals at day 10 after birth subcutaneously. At two months of age, animals were euthanized and their kidneys were removed for analysis. Kidney samples were first cut in half. One half was used for immunoblotting, while the other half was for hematoxylin and eosin (H&E) staining.

Statistical analysis

All data are reported as mean±standard error of mean with statistical power greater than 0.8 at p<0.05. Data were then analyzed utilizing ANOVA test followed by Tukey’s post-test. Analysis of data was performed with Prism GraphPad 5 software.
3.4 Results

CaV1.2 was expressed in Pkd cell lines.

To confirm the presence of CaV1.2 in renal epithelial cells and determine whether it was altered among different Pkd cell lines, we performed immunoblotting for CaV1.2 in wild type, Pkd1−/− and Pkd2−/− renal epithelial cells; all cell lines expressed renal epithelial marker, wheat Germ Agglutinin (data not shown).

**Figure 3-1:** CaV1.2 was up-regulated in Pkd1−/− and Pkd2−/− cell lines.

a. Immunoblot analysis was performed to detect CaV1.2 expression in wild-type (WT), Pkd1−/− and Pkd2−/− cell lines. CaV1.2 was overexpressed in Pkd cells compared to wild-type cells. GAPDH was used as loading control. b. Overexpression of Cav1.2 was also confirmed by immunostaining. CaV1.2 was localized to cilia in wild-type cells. In Pkd cell lines, CaV1.2 was not detected in the cilia. Acetylated-α-tubulin (acet-a-tub) was used as cilia marker. N=3; * indicates significant difference from the control wild-type group.
Our immunoblot studies indicate that CaV1.2 was expressed in all cell lines but was two folds higher in Pkd compared to wild-type cells (Figure 3-1a). We most recently showed that CaV1.2 interacts with PC2 in the myocyte cilia [75].
To explore how PC1 or PC2 deficiency affected CaV1.2 distribution in renal epithelial cilia, we also studied CaV1.2 localization with immunofluorescence. Cav1.2 was mainly localized to primary cilia in wild-type cells but was found to be randomly distributed in the cytoplasm of Pkd cells (Figure 3-1b).

_CaV1.2 knockdown shortened cilia length in Pkd epithelial cells._

To study the loss-of-function effects of Cav1.2, we transfected renal epithelial cells with lentivirus carrying different shRNA sequences specific for unique regions of mRNA coding for CaV1.2. Among the three sequences tested, shRNA sequence #3 produced the highest knockdown efficiency (Figure 3-2a). This sequence was thus selected for use in all of our future studies. To explore whether loss of CaV1.2 could play a role in cilia formation, we examined the cilia length in all cell groups (Figure 3-2b). We observed no change in cilia length in wild-type cells. In contrast, knockdown of CaV1.2 significantly shortened cilia length in Pkd groups. This observation is intriguing because shorter cilia were often related to less sensitivity to extracellular cues, such as fluid-flow shear stress [11, 45]. Also noteworthy is that Pkd1<sup>−/−</sup> and Pkd2<sup>−/−</sup> cell lines had longer cilia compared to wild-type cells.

_CaV1.2 regulated flow-induced cytosolic calcium level in Pkd epithelial cells._
Despite its unclear physiological function in renal epithelial cells, CaV1.2 has been proposed to be a physiologically relevant calcium channel in the kidney [59]. To examine if CaV1.2 regulated cytosolic calcium concentration in physiological condition, we challenged both CaV1.2 knockdown and control groups of wild-type and Pkd cells with continuous fluid-flow at 0.7 dye/cm for 18 hours (Figure 3-3). There was no difference in calcium levels in wild-type cells between control and CaV1.2 knockdown. On the other hand, the already low calcium concentrations in Pkd cell lines were further lowered in those cells with CaV1.2 knockdown.

![Figure 3-3: CaV1.2 knockdown decreased cytosolic calcium levels in Pkd1−/− and Pkd2−/− cell lines.](image)
CaM kinase II activity was regulated by CaV1.2.

Calcium ions serve as a very important second messenger in many signaling pathways. Therefore, we proposed that altered cytosolic calcium homeostasis could lead to changes in a signaling cascade. Because Akt has been shown to be abnormal in tissue samples isolated from PKD patients [71, 72, 76], we first examined phosphorylated Akt levels (Figure 3-4a). CaV1.2 knockdown did not change the phosphorylation level of Akt in wild-type cells, whereas it significantly reduced Akt phosphorylation in Pkd cells. Phosphorylation ERK was consequently increased in CaV1.2 knockdown Pkd cells, consistent with the fact that inactivated Akt could not inhibit ERK phosphorylation [77].
Because calcium is an important regulator for CaM kinase II, which also phosphorylates and activates Akt [78], we treated wild-type cells with W7, a CaM kinase II inhibitor. As predicted, Akt phosphorylation was decreased in wild-type control cells. We thus propose that CaV1.2 played an important role in Akt and Erk activities through cytosolic calcium and CaM kinase II (Figure 3-4b).

Effects of Cav1.2 knockdown on cell proliferation and polyploidy
PKD is characterized by abnormal cell proliferation and polyploidy [71, 72]. Therefore, we explored if CaV1.2 knockdown affected these two factors. Our studies reinforced previous results that both Pkd1 and Pkd2 were involved in abnormal cell proliferation and polyploidy (Figure 3-5). Importantly, our studies further indicated that although CaV1.2 knockdown had no effects on wild-type cells, it significantly accelerated cell division and polyploidy formation in both Pkd1 and Pkd2 cells. To further confirm our flow cytometry analysis, we randomly selected 30 individual cells with large nuclei from

**Figure 3-6: Chromosome counting confirm the higher polyploidy degree in Pkd cells when treated with Cav1.2 shRNA**
each group to directly count the number of chromosomes (Figure 3-6). Chromosome numbers remained relatively normal in wild-type cells without or with the Cav1.2 knockdown. Although Pkd cells were polypoidy, Cav1.2 knockdown induced more polypoidy (≥4N). Of note is that many of Pkd cells with Cav1.2 knockdown had three times the normal number of chromosomes (≥6N).

Cav1.2 modulated cystic kidney formation in zebrafish and Pkd1 mouse model. We have recently shown that abnormal cell division could induced polyploidy, which could further promote cystic kidney formation in zebrafish and mice [71, 72]. To investigate the pathophysiological role of CaV1.2 in cyst formation, we simply used the
Figure 3-5: CaV1.2 knockdown accelerated cell proliferation and polyploidy in Pkd1−/− and Pkd2−/− cells

Flow cytometry analysis was carried out using propidium iodide (PI) to measure DNA content. Cell proliferation and polyploidy formation were more pronounced in Pkd cells with CaV1.2 knockdown. N=5 for each group; # indicates significant difference from the wild-type (WT); * indicates significant difference between scramble and CaV1.2 shRNA groups.
morpholino knockdown approach in zebrafish. Supporting our hypothesis of the role of CaV1.2 in cyst formation, we consistently observed dilatated nephrons in CaV1.2 morpholino-knockdown fish. Unlike in fish, however, knockdown of CaV1.2 in
wild-type mice did not produce a cystic kidney. In $Pkd1^{+/−}$ mice ($Mx1Cre:Pkd1^{wt/flox}$), we surprisingly observed severe cystic kidneys and higher kidney to body weight ratios compared to control as young as two months old (Figure 3-8).

Figure 3.8: CaV1.2 modulates cystic kidney formation in mouse.

Lentiviral infection carrying scramble or CaV1.2 shRNA was used in the wild-type (WT) or $Pkd1$ heterozygous mice. Cystic kidney formation and significantly higher kidney to body weight ratio could be easily observed from the isolated kidney.
3.5 Discussion

It has been well accepted that calcium ions play a significant role as a second messenger in translating extracellular stimulation into intracellular signaling [79]. The renal epithelium, one of the main tissues for sensing cues from the extracellular environment, is also dependent on calcium signaling to modulate cellular events such as mitosis, proliferation, fluid and ion transportation, cell volume regulation and many others. Although many calcium channels have been studied in the renal epithelium, not much effort has been given to study voltage-dependent calcium channels in the kidney. This was probably due to the belief that unlike neurons, myocytes or muscle cells, the renal epithelium is not an excitable cell. Thus, any changes in epithelial membrane potential would be considered insignificant for voltage-operated channels.

Interestingly, independent research groups have identified voltage-dependent calcium channels, including L-type calcium channel (CaV1.2), in renal epithelial cells [59, 80, 81]. Based on their electrophysiological and pharmacological properties, voltage-dependent calcium channels are categorized into four groups (L, N, T, and P). The L-type Ca\(^{2+}\) channels are composed of multiple subunits (\(\alpha 1, \alpha 2, \beta, \delta, \) and \(\gamma\)). Some of these subunits express ubiquitously within the kidney, including proximal tubules, medullary collecting ducts, cortical thick ascending limbs, distal convoluted tubules, and cortical collecting ducts [59]. Given the abundance of CaV1.2 within renal epithelial cells, it is very intriguing to understand its functionality in the kidney.
In the present study, we reported for the first time that localization of CaV1.2 to primary cilia was interrupted in *Pkd* cell lines. This indicates that polycystins could regulate CaV1.2 ciliary localization. Consistent with this view, we have recently shown that CaV1.2 and PC2 interact in very specific domains [75]. Furthermore, we observed a significantly higher expression level of Cav1.2 in *Pkd* cells. Because polycystin-2 calcium channel (PC2) was also malfunctioned in *Pkd* cells, we speculated that CaV1.2 could function in harmony with PC2. We thus hypothesized that if PC2 was not working properly, CaV1.2 might be up-regulated to make up the loss. In accord with this view, the loss of CaV1.2 in normal wild-type kidney cells did not have much effect on the intracellular calcium level. However, when CaV1.2 expression was suppressed in *Pkd* cells, a lower intracellular calcium level was significantly observed.

Considering that the CaV1.2 expression level was significantly higher in *Pkd* cells than in wild-type cells, it is logical to speculate that CaV1.2 functions as a secondary calcium-permeable channel to the polycystins complex. In the presence of polycystins, the expression of CaV1.2 was low, and so was its activity. However, when polycystins are not functioning, CaV1.2 expression was up-regulated, probably to compensate for the loss of the PC2 calcium channel. Therefore, inhibition of CaV1.2 in *Pkd* cells could shut down this compensatory mechanism and consequently reduce the already lower intracellular calcium concentration. Consistent with previous studies [31, 36], such a discrepancy in cytosolic calcium levels leads to increased Erk activity in PKD. Our studies also showed that the Erk activity could also be reproduced by inhibition of CaM kinase activity in wild-type cells. In wild-type cells, inhibiting CaM kinase II resulted in
a low phosphorylated Akt level and consequently a higher Erk phosphorylation. We therefore hypothesize that CaM kinase II plays a central role in the association between intracellular calcium and downstream effectors, such as Akt and Erk.

On the cellular level, we observed that the length of primary cilia was longer in *Pkd* cells compared to control wild-type cells. We have recently shown that longer cilia tend to increase the sensitivity of renal epithelial cells to fluid-shear stress [11, 45]. Thus, longer cilia in *Pkd* cells might be caused by a compensatory response to further extend the cilia length so the cells would be more sensitive to extracellular stimulation. Equally interesting, decreased CaV1.2 expression in *Pkd* cells also resulted in shorter cilia. This might be a result of a disrupted ciliary assembly that requires intracellular calcium. It is also possible that CaV1.2 could interact with intraflagellar molecules to facilitate cilia length maintenance.

Our studies also indicated that CaV1.2 modulated cell proliferation and polyploidy formation in *Pkd* cells. By contrast, it did not have any effect on wild-type cells. The differential effects of CaV1.2 between wild-type and *Pkd* cells would very likely result from insufficient intracellular calcium in *Pkd* cells. This in turn leads to abnormal CaM kinase II, Akt and Erk pathways. Our *in vivo* studies confirmed the roles of CaV1.2 in kidney cyst formation. Renal cysts were consistently formed in wild-type zebrafish with *cav1.2* morpholino knockdown, unlike in wild-type mice with *CaV1.2* shRNA knockdown. This is probably due to less redundancy in genetic makeup for zebrafish compared to mice. Regardless, our studies offer a unique correlation between the
expression of CaV1.2 and cystic kidneys. Taken together, we propose that CaV1.2 could function as a genetic modifier for Pkd, the loss of which aggravates the cystic kidney phenotype.

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Chapter 4

Summary

A large body of evidence has shown that many cell types can respond to a variety of extracellular stimulation by transient or long-lasting calcium influx Jones and Nauli [79]. In renal epithelial cells particularly, consensus is being reached that such responses are due to the chemo and mechano-sensitivity of primary cilia [82]. However, major criticism to this theory still exist due to the lack of evidences that directly show the spatiotemporal details of the calcium signaling initiated by primary cilia. To test if primary cilia can transduce calcium influx from the extracellular environment upon stimulation, we combined a novel 3-dimension cell culture system with genetically encoded calcium indicator (GECI) technique and successfully visualized calcium signaling within primary cilia and cytoplasm. Complementing many previous reports by independent researches, our study showed that cilia function as an independent calcium signaling compartment when stimulated by extracellular cues including fluid flow and pharmacological reagents. More importantly, our strategy allowed us to test several previously proposed hypotheses regarding cilia related calcium influx. For instance, by comparing the effects of different pharmacological inhibitors on ciliary calcium signaling, we were able to create a working hypothesis that depicts the calcium signal transduction and amplification in cilioplasm
and cytoplasm. Another example is that Polycystin-2 (PC-2) contained calcium permeable channel properties demonstrated by previous structural analysis and other indirect evidence. In our study, such hypothesis was confirmed because genetic suppression of PC-2 largely abolished calcium signaling in cilia and cytoplasm. We thus made the observation that drug-induced ciliary calcium signaling tends to localize exclusively in cilia, whereas fluid flow induced ciliary calcium signaling could be transduced to cytoplasm. This lead us to speculate that the transition zone in the vicinity of cilia basal body could play a role in controlling calcium ion transport by conformational changes upon different stimulation.

As described in Chapter 3, Cav1.2 L-type calcium channel (Cav1.2) was previously shown in renal epithelial cells [59, 80, 81]. Several lines of evidence also suggested that blockage of Cav1.2 by CCB might also lead to aggravation of PKD in animal model [36, 83, 84]. Though similar incidences in PKD patients were absent, a considerable number of reports demonstrated that CCB failed to provide the same renal-protective effects as ACE inhibitors in PKD patients [68, 85-88]. We first confirmed the presence of Cav1.2 in normal, PKD1-/- and PKD2-/- mouse renal epithelial cells. Interestingly, Cav1.2 seemed to be up-regulated in the latter two PKD cell lines. This observation suggested a possible compensatory mechanism that responds to the loss of PC-1/PC-2 calcium channel complex. In order to study the loss-of-function effect of Cav1.2 on all three epithelial cell lines, we utilized lentiviral mediated shRNA knockdown to suppress the Cav1.2 expression, which enable us to inhibit the activity of Cav1.2 without any possible side effects by CCB. Unexpectedly, Cav1.2 inhibition did not cause effects in normal
epithelial cells, whereas it lead to a series of cellular changes in PKD cell lines which included altered intracellular calcium concentration, higher proliferation rates, and unstable mitosis. Consequently, the irregularities in cell division in PKD cell lines became more apparent. Consistent with these in vitro studies, Reduction of Cav1.2 caused no apparent effects on wild type (WT) animals, but led to the formation of large cysts at an early stage of life in PKD heterozygous mice. Thus we proposed that Cav1.2 functioned as secondary calcium channel in cilia. Therefore, the function of CaV1.2 was only significant under certain abnormal conditions such as the loss of PC-2 and/or PC-1. It is noteworthy that Cav1.2 suppression in WT zebrafish rendered severe kidney cysts along with other ciliopathies including hydrocephalus. One possible explanation for these disparate results in mice and zebrafish is that the roles of Cav1.2 and PC-2 could be altered evolutionarily so that Cav1.2 could play a more important role in lower organisms such as zebrafish. Nonetheless, our study of Cav1.2 provides the first evidence that Reduction of Cav1.2 can lead to abnormal cellular events via lowered calcium concentration and altered AKT-mediated ERK signaling in PKD cell lines. In light of the fact that heterozygous PKD animals developed more severe kidney cysts after knockout of Cav1.2, we should give urgent attention to Cav1.2 in order to discover its potential role in restoring calcium homeostasis in patients with germ line mutations in PKD genes. Ultimately, a more customized management for hypertensive PKD patients could be possible regarding the use of CCB.
Chapter 5

Future study

As described in Chapter 2, by combining a unique three-dimension cell culture method with a Genetic Encoded Calcium Indicator (GECI), we were able to examine calcium signaling in cilioplasm and cytoplasm spatiotemporally. Considering that the primary cilium is a key organelle that regulates intracellular calcium homeostasis in renal epithelial cells [23, 42], it is becoming imperative to study how dysfunctional cilia lead to abnormal calcium signaling with our system. Particularly, we want to study several cilia-localized proteins, which if mutated, could lead to PKD phenotypes in cells and/or animal models. For example, we identified in Chapter 2 and 3 that Cav1.2 L-type calcium channel (Cav1.2) physically localizes in primary cilia, interacts with Polycystin-2 (PC-2), and mutation could lead to more severe phenotypes in vitro and in vivo. Therefore, Cav1.2 is a very good candidate to be studied with our calcium signaling imaging platform regarding how it affects the cellular response to different extracellular stimulation. Another possible direction will be studying the primary ciliary calcium signaling of endothelial cells with our system. A large body of evidence has shown that cilia modulate the calcium homeostasis in endothelium [32, 89, 90], and thus mutation in
PKD patients can potentially contribute to hypertension, aneurysm, and other non-renal complications. To be able to depict the signaling pathways implicated in these pathogeneses, it is exceptionally important to determine how cilia transduce the calcium signals into cytoplasm. Thus, our system could be a perfect tool to study this process spatiotemporally.

In our second project (as described in Chapter 3) we showed that mitosis was instable and growth rate was high in PKD cell lines treated with Cav1.2 shRNA. As many previous studies reported, instable mitosis and higher growth rates were regularly observed in typical PKD animal models, and are thought to be main causes of cysts formation [91-94]. However, these cellular abnormalities are not sufficient to answer the very intriguing question of what causes the extraordinarily large cysts in early age heterozygous animal models in our study. Renal cysts that we found in treated PKD animals were morphologically different than typical cysts as they usually measure 0.5 to 3 mm in diameter, whereas ours were 12 mm in diameter on average. Because abnormal fluid secretion has been shown to contribute to the enlargement of cysts [64, 94-97], our next step is to study whether Cav1.2 affect the fluid secretion. It is becoming a consensus that cysts lining epithelial cells require abnormally activated Aquaporin-2 (Aqp-2) and cystic fibrosis transmembrane conductance regulator (CFTR) to facilitate fluid secretion and these two channels are both downstream effector of PKA in a cAMP dependent fashion [64, 94-97]. Our results that showed a much lower intracellular calcium concentration in PKD cell lines with Cav1.2 shRNA perfectly echo this theory, as calcium is a major regulator of cAMP concentration. Thus, we plan to examine the activity of Aqp-2 and CFTR in different renal epithelial cell lines with and without Cav1.2 knockdown. As a
good complement to this proposed study, we also like to analyze the composition of cysts in our PKD animal models, and to compare the results to that of typical cysts. Additionally, blood urea nitrogen (BUN), a key index for kidney function would also be interesting to test. Besides studies focusing on mechanisms contributing to the atypical cysts observed in our study, it is also interesting to determine the specific origin of these cysts. Many reports indicated that cysts formed at different stages of life could have unique origins. For instance, the majority of cysts formed during embryonic development have a proximal tubules origin, whereas cysts initiated after birth are often found to be distal tubules or collecting ducts oriented [98-100]. Considering that our heterozygous animals were injected with shRNA on day 1 postnatal, we expect to see a distal tubule and/or collecting ducts origin.
Reference


46. Siroky, B.J.; Ferguson, W.B.; Fuson, A.L.; Xie, Y.; Fintha, A.; Komlosi, P.;
Yoder, B.K.; Schwiebert, E.M.; Guay-Woodford, L.M.; Bell, P.D., Loss of
primary cilia results in deregulated and unabated apical calcium entry in arpkd
collecting duct cells. *American journal of physiology. Renal physiology* 2006,
290, F1320-1328.

47. Xu, C.; Shmukler, B.E.; Nishimura, K.; Kaczmarek, E.; Rossetti, S.; Harris, P.C.;
Wandinger-Ness, A.; Bacallao, R.L.; Alper, S.L., Attenuated, flow-induced atp
release contributes to absence of flow-sensitive, purinergic ca2+ signaling in
human adpkd cyst epithelial cells. *American journal of physiology. Renal
physiology* 2009, 296, F1464-1476.

growth and cilia function in endothelial cells. *Cellular and molecular life sciences* :
*CMLS* 2012, 69, 165-173.

49. Abdul-Majeed, S.; Nauli, S.M., Dopamine receptor type 5 in the primary cilia has

50. Praetorius, H.A.; Praetorius, J.; Nielsen, S.; Frokiaer, J.; Spring, K.R., Beta1-
integrins in the primary cilium of mdck cells potentiate fibronectin-induced ca2+
signaling. *American journal of physiology. Renal physiology* 2004, 287, F969-
978.

51. Rondanino, C.; Poland, P.A.; Kinlough, C.L.; Li, H.; Rbaibi, Y.; Myerburg,
al., Galectin-7 modulates the length of the primary cilia and wound repair in


87. AbouAlaiwi WA, Ratnam S, Booth RL, Shah JV, Nauli SM (2011) Endothelial cells from humans and mice with polycystic kidney disease are characterized by


Appendix A

Supplementary data for Chapter 2

**Movie 1.** Fluid-shear stress induces cilium bending

(http://link.springer.com/content/esm/art:10.1007/s00018-013-1483-
1/file/MediaObjects/18_2013_1483_MOESM1_ESM.mov)

The movie was taken with a high-resolution, high-speed differential interference contrast microscope. The cell was challenged with fluid-shear stress (flow). Number represents time in seconds.

**Movie 2.** Fluid-shear stress induces calcium signaling in the cilioplasm and cytoplasm.

(http://link.springer.com/content/esm/art:10.1007/s00018-013-1483-
1/file/MediaObjects/18_2013_1483_MOESM2_ESM.mov)

Using high-speed excitation wavelength exchanger for the DG4/DG5 system, a movie of fluorescence was captured simultaneously with Movie 1. Color bar indicates calcium
level, where black-purple and yellow-red colors represent low and high calcium levels, respectively. Number represents time in seconds.

**Movie 3.** Fluid-shear stress induces calcium signaling in the cilioplasm followed by the cytoplasm.

([link](http://link.springer.com/content/esm/art:10.1007/s00018-013-1483-1/file/MediaObjects/18_2013_1483_MOESM3_ESM.mov))

A movie of fluorescence changes in an experiment independent from Movie 2. Color bar indicates calcium level, where black-purple and yellow-red colors represent low and high calcium levels, respectively. Number represents time in seconds.

**Movie 4.** The cilium and cell body remain in focus in a cell treated with fenoldopam.

([link](http://link.springer.com/content/esm/art:10.1007/s00018-013-1483-1/file/MediaObjects/18_2013_1483_MOESM4_ESM.mov))

The movie was taken with a high-resolution, high-speed differential interference contrast microscope. The cell was challenged with fenoldopam (FD). Number represents time in seconds.

**Movie 5.** Fenoldopam induces calcium signaling specifically in the cilioplasm.

([link](http://link.springer.com/content/esm/art:10.1007/s00018-013-1483-1/file/MediaObjects/18_2013_1483_MOESM5_ESM.mov))
Using high-speed excitation wavelength exchanger for DG4/DG5 system, a movie of fluorescence changes was captured simultaneously with Movie 4. Color bar indicates calcium level, where black-purple and yellow-red colors represent low and high calcium levels, respectively. Number represents time in seconds.

**Movie 6.** The primary cilium and cell body remain in focus in a cell treated with thrombin.


The movie was taken with a high-resolution, high-speed differential interference contrast microscope. The cell was challenged with thrombin (TH). Number represents time in seconds.

**Movie 7.** Thrombin induces calcium signaling specifically in the cytoplasm.


Using high-speed excitation wavelength exchanger for DG4/DG5 system, a movie of fluorescence changes was captured simultaneously with Movie 6. Color bar indicates calcium level, where black-purple and yellow-red colors represent low and high calcium levels, respectively. Number represents time in seconds.
**Movie 8.** The primary cilium and cell body remain in focus in a cell treated with ionomycin.


The movie was taken with a high-resolution, high-speed differential interference contrast microscope. The cell was challenged with ionomycin (IO). Number represents time in seconds.

**Movie 9.** Ionomycin induces calcium signaling in both the cilioplasm and cytoplasm.


Using high-speed excitation wavelength exchanger for DG4/DG5 system, a movie of fluorescence changes was captured simultaneously with Movie 8. Color bar indicates calcium level, where black-purple and yellow-red colors represent low and high calcium levels, respectively. Number represents time in seconds.