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Mechanosensory role of vascular endothelial primary cilia in the development of hypertension in polycystic kidney disease

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Mechanosensory Role of Vascular Endothelial Primary Cilia in the Development of Hypertension in Polycystic Kidney Disease

by

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

Master of Science Degree in

Pharmaceutical Sciences

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An Abstract of

Mechanosensory Role of Vascular Endothelial Primary Cilia in the Development of Hypertension in Polycystic Kidney Disease

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Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common life-threatening hereditary genetic disease. Early cardiovascular complications, including hypertension, have been witnessed in the majority of the ADPKD patients, and have been attributed as the primary cause of mortality. PKD originates from defective primary cilia which have a mutation in either the Pkd1 or Pkd2 gene. These two genes encode for polycystin-1 and polycystin-2 respectively, and lack of their ciliary localization has already been established as an essential element in PKD pathophysiology. Endothelial primary cilia in the inner surface of blood vessels sense blood flow and convert it into intracellular biochemical signals. Vascular endothelial cilia have been demonstrated to have a polycystin-1 and polycystin-2 mediated mechanosensory mechanism. Intracellular nitric oxide (NO) is one of the most important regulators of blood pressure; *in-vitro* studies showed that vascular endothelial ciliary polycystin-1 and polycystin-2 controls NO biosynthesis by a calcium signaling pathway. In this study, we focused on the role of vascular endothelial cilia in the initiation and the development of hypertension in PKD. In addition to studying vascular phenotypes of the mutant Pkd1 animal model, we also

extended our investigation in the analysis of the mechanism of hypertension in PKD by looking into the transient receptor potential channels, which have been evidenced for mechanosensory activity in vascular endothelium.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADPKD …….Autosomal Dominant Polycystic Kidney Disease

ARPKD …….Autosomal Recessive Polycystic Kidney Disease

DAPI …….4', 6-diamidino-2-phenylindole

PC-1…….. Polycystin-1

PC-2 ……..Polycystin-2

PKD …….. Polycystic Kidney Disease

WT ……Wild Type

ORPK…….Oak Ridge Polycystic Kidney

PKD1……Polycystic Kidney Disease-1

TRP……..Transient Receptor Potential

NO……Nitric Oxide

Chapter 1

Introduction

1.1. Polycystic Kidney Disease

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common life-threatening hereditary genetic disease. ADPKD affects more the 12.5 million patients worldwide and approximately 600,000 patients in the USA (1). ADPKD is characterized by progressive enlargement of cyst-filled kidneys followed by destruction of functional parenchyma. Studies have shown an average increase of total kidney volume by 204 ± 246 ml over three year period, and reduction in Glomerular Filtration Rate (GFR) of 4.3 ± 8.1 ml/min per year in participants with baseline kidney volume above 1500 ml. Average cyst volume increment was found to be 218 ± 263 ml during the same period. 45% to 70% patients face end stage renal disease by the age of 65, eventually leading to death (2, 3). The most common causes of death of PKD patients have been found to be infection, uremia and cardiac diseases (4). Unfortunately, current therapies have unsuccessfully hindered the progression of cyst growth or controlled volume expansion of the kidney.

The search for cure of ADPKD demands a better understanding of the pathophysiology of this disease. The discovery of PKD1 and PKD2 genes have advanced the quest significantly and unlocked new areas of research. PKD can be inherited as either an autosomal dominant trait (ADPKD) or an autosomal recessive trait (ARPKD). ADPKD is very common and occurs in both children and adults, while ARPKD is less common and occurs mostly in newborns and children. Mutation in either the PKD1 gene on chromosome 16 or the PKD2 gene on chromosome 4 has been attributed as the cause of ADPKD. The PKHD1 gene on chromosome 6 is responsible for ARPKD. PKD1 and PKD2 genes are widely expressed, so their mutation affects extra-renal tissues as well (5).

Although the principal characteristic of ADPKD is kidney cyst and renal failure, substantial cyst growth has been observed in liver (75%), ovaries (40%), choroid plexus, and pancreas. Prevalence of cardiovascular diseases such as hypertension (78%), diverticulosis (70%), cardiac valve disorders (25%) and intracranial aneurysms (10%) in ADPKD patients are also very common. These cardiovascular phenotypes typically have an earlier onset than progressive renal failure and renal insufficiency, and account for at least one-third of the deaths by ADPKD (1, 4).

1.2. Cilia

Cilia are hair-like structures projected from the apical surface of most mammalian cells. Extensive research in past decades has converted cilia from a non-functional oddity to an organelle with fundamental sensory capabilities and center of different human diseases. A cilium is a structure that is divided into five distinct blocks - ciliary membrane, soluble compartment, axoneme, ciliary tip and basal body. Ciliary membrane is continuous with cell membrane but has a different lipid bilayer composition. It accommodates membrane receptors and ion channels to facilitate ciliary mechanosensation and chemosensation. The soluble matrix, known as cilioplasm, is fluid material, and consists of numerous ciliary proteins supporting signaling activities. The characteristic microtubular structure is called the axoneme and is responsible for the long structure and transport of cellular component in and out of cilia. The ciliary tip is composed of proteins too and the basal body is the mother centriole from which a cilium protrudes (6).

Cilia are mainly classified as non-motile (primary) and motile cilia. Both of these subtypes are composed of highly organized microtubule arrangements with accessory elements such as inner and outer dynein arms and radial spokes. For a long time, motile cilia were the center of all attention in regard to their functions, such as moving mucus in the respiratory tract or moving eggs in the fallopian tubes, were readily observable. Although non-motile primary cilia have been observed as early as 1867 in vertebrates, and later in almost all mammalian cells, they were always considered a non-functional remnant of evolution. Recent studies have proved otherwise, delineating essential mechanosensory and chemosensory activities of primary cilia (6-8).

1.3. Motile cilia

The crucial structural difference between motile cilia and non-motile cilia is the arrangement of microtubules. Microtubules are formed from α- and β- monomers of tubulin (8) or αβ tubulin heterodimers (9). Motile cilia have a "9+2" microtubule pattern which are furnished with outer dynein arms, inner dynein arms, and radial spokes. These are defined protein complexes and outer dynein arms are closer to the ciliary membrane and inner dynein arms are closer to the central apparatus of microtubules. Dynein arms are molecular motors which produce force to cause sliding of microtubule doublets against one another. These asynchronous sliding results in a synchronous beat with a normal beat frequency of 10-20 Hz capping at 100 Hz. Motile cilia move fluid in relative to the cell body. In single-celled organisms, it helps movement of cells through water and, in mammals it helps to move water across cell surface (8, 9).

1.4. Non-motile (Primary) cilia

Non-motile 9+0 cilia are almost ubiquitous in the cells of body. These so called primary cilia are found in epithelial cells such as kidney tubule, bile duct, thyroid and endocrine pancreas, and also in endothelial cells, neurons, osteoblasts, fibroblasts and others (6, 9). Unlike motile cilia, these primary cilia are solitary appendages on cells. Until recently, functional studies of primary cilia have been restricted in photoreceptor visual transduction and olfaction. The basic axonemal doublet structure of cilia is maintained in primary cilia too, but they lack central microtubule complex and dynein motors. This

deficiency of dynein motors makes primary cilia immotile and do not beat actively. Other aspects of primary cilia are similar to motile cilia, such as having the same ciliary necklace which separates the selective protein of the ciliary membrane from cell membrane. The composition of ciliary membrane is also similar to 9+2 motile cilia. The ciliogenesis process for both types utilizes the same transport mechanisms and analogous IFT (Intra-Flagellar Transport) proteins. Loss of motility in primary cilia does not reduce other functions such as meachanosensation and chemosensation, and for decades their importance has been demonstrated in cell cycle and cell differentiation (10).

1.5. Nodal cilia

There is a third type of specialized cilia in the embryonic node. These are called nodal cilia which are solitary in cells and consist of 9+0 axonemal structure like primary cilia. Surprisingly, these cilia possess dynein arms and are motile. Embryonic nodal cilia are crucial for appropriate left-right positioning of organs within the body. The exact mechanism of such left-right positioning by nodal cilia is unknown but several mechanisms have been proposed which are related to the mechanosensation of primary cilia (6, 9).

1.6. Mechanosensory role of cilia in PKD

Primary cilia protrude from the apical surface of the kidney epithelium into the tubule lumen. In 2000, Pazour, et al. first demonstrated the role of primary cilia in PKD by developing $Tg737^{Orpk/Orpk}$ mouse model for human ARPKD. In this model, the mouse homolog of IFT88 protein was mutated resulting in renal epithelial cells with very short or no cilia(11). They also indicated that cilia may play a crucial role in the development of PKD. Kramer-Zucker, et al. and Lin, et al. substantiated the significance of cilia in normal kidney function by disrupting an IFT-associated kinesin motor, KIF3a (12, 13). The role of primary cilia in cyst formation still remained elusive. Later, it was found that mutations which are not involved with the genes encoding IFT proteins also caused PKD. Mutation in the PKD1 and PKD2 genes, which encode polycystin-1 and polycystin-2 respectively, caused PKD although ciliary structure was normal. Polycystin-1 is a transmembrane receptor and polycystin-2 is a calcium channel (14). Restoration of polycystins in the absence of normal cilia structure also caused cyst formation. So, it was confirmed that mutations in ciliary polycystin-1 and polycystin-2 are responsible for cyst formation in PKD and their function to maintain normal Ca^{2+} influx is necessary for renal function(15, 16). Primary cilia can be bent mechanically by fluid flow and cilia bending results in an increase of intracellular Ca^{2+} (17, 18). Ciliary polycystin-1 and polycystin-2 mediate this mechanosensitive activity of primary cilia and mutation leading to either of these is the primary source of cyst formation in PKD (14).

1.7. Hypertension in PKD

Hypertension is a chronic medical condition characterized by high blood pressure where arterial blood pressure is elevated.(19) Endothelial dysfunction is one of the mechanisms suggested for vascular hypertension.(20) Vascular endothelial cells in the inner surface of blood vessels can sense changes in blood flow and convert the mechanical stimuli into a biochemical cascade (21, 22).

Although PKD is the predominantly studied cilia-related disorder (ciliopathy), cardiovascular complications such as hypertension are the leading cause of death for PKD patients. Due to the extreme pathology of the renal system in PKD patients, the cardiovascular prevalence in PKD has unfortunately not been well studied. Although almost 75% of PKD patients manifest hypertension, the exact molecular mechanism is still unknown (1).

As pathophysiology of ADPKD is mostly attributed to ciliary dysfunction and because of the omnipresence of primary cilia, cilia are implicated for extra-renal disorders too. Primary cilia are present in vascular endothelial cells, and they act as mechanosensitive organelles (23, 24). The same mutation in PKD1 and PKD2, which cause epithelial cilia to lose its mechanosensitivity, with subsequent renal cyst formation, may also be accountable for lack of mechanosensitivity of vascular endothelial cilia. It has already been demonstrated that primary cilia of vascular endothelia specifically sense changes in fluid-shear stress. Bending of primary cilia by shear force results in an intracellular Ca^{2+} signaling pathway involving calmodulin (CaM), calcium-dependent protein kinase (PKC) and endothelial nitric oxide synthase (eNOS), leading to nitric oxide (NO) biosynthesis

and vasodilation(14, 23-28). Following these *in-vitro* studies, it was proposed that mutation in PKD1 and PKD2 leading to dysfunction of vascular endothelial cilia may play significant role in development of hypertension in PKD.

1.8. Transient Receptor Potential channels

Polycystin-1 and Polycystin-2 are members of the protein superfamily named Transient Receptor Potential (TRP) channels. The TRP superfamily consists of 28 members encoded by separate genes in the mammalian genome. The first member of this family was identified in Drosophila and was found necessary for Phospholipase-C (PLC) dependent visual transduction. In the mutant flies, response to light was transient, instead of sustained, and hence the name transient receptor potential was coined. The TRP superfamily is subdivided into six families depending on DNA and protein sequence homology: TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPA (Ankyrin), TRPP (Polycystin) and TRPML (MucoLiptin). Most of these 28 cation channels are non-selective and allow passage of both monovalent and divalent ions (29, 30).

TRP proteins have six transmembrane hydrophobic domains; the pore for ion flow is formed between the fifth (S5) and the sixth (S6) domain. There is one N-terminal intracellular hydrophobic domain (h1) in most members. TRPP and TRPML have an extended intracellular loop between S1 and S2. TRP channels form both hetro- and homotetramers where these subunits contribute to shared selectivity filter and ion-conducting pore as seen in K^+ channels. Ion flux through six transmembrane channels is controlled by allosteric interaction among the subunits. The structure which has the most

proximity among different TRP families is the amino acid sequences lining the pore. The S5, S6 and the proximal C-terminal tail are very similar in all the 28 members, although they have other significant differences in structure and function. It is assumed that structural determinants of gating information are more varied than the core region itself (31).

Activation methods of TRP channels cannot be assigned exclusively to specific families. TRPV, TRPA and TRPM have members with thermosensitivity, while TRPV, TRPC and TRPM have members which are stimulated by exocytosis. Most of the TRP channels respond to multiple stimuli such as heat, shear stress, pro-inflammatory agents, exocytosis, temperature, light, chemicals etc (29, 31).

TRP channels have emerged as mechanosensory proteins recently. TRPs have also been identified to localize in different cilia (30, 32-35). A few TRPVs can act as mechanosensory mediator after stimulation by shear stress, and within the TRPV subfamily mechanosensation has mostly been attributed to TRPV-1, TRPV-2 and TRPV-4(30, 36). Presence of TRPV4 in endothelial cells has been confirmed in numerous studies and (36, 37) Mendoza et al. showed that mechanical activation of TRPV-4 is crucial for endothelium dependent vasodilation. Endothelial TRPV-4 channel controls vascular functions by producing NO upon Ca^{2+} influx.(38, 39) Polycystin-2 is also a TRP channel (TRPP-2) and it forms a polymodal complex with TRPV-4 localized in renal epithelial primary cilia. In absence of TRPV-4 in renal epithelia, flow induced calcium transient was seen to be diminished even after the presence of Polycystin-2.(35) Though TRPV1, TRPV2, TRPV3 and TRPV4 have been found in vascular endothelial cells with clear roles in vascular functions, investigation regarding the expression and function of

TRPV5 and TRPV6 in endothelium is too sparse. (40, 41) TRPV5 and TRPV6 are highly selective TRP Ca^{2+} channels with a P_{ca}/P_{Na} ratio over 100. All these suggest that TRPVs, localized in vascular endothelial primary cilia, may have important function in blood induced shear stress sensation by cilia.

1.9. Mechanism of the development of hypertension in PKD

It has been well-established that sensory polycystins are required for cells to sense fluid-shear stress (42-51). At least in endothelial cells, it has reported that NO is produced upon cilia activation. Using cultured endothelial cells, it has also been shown that having sensory polycystins is not sufficient to sense fluid-shear stress in cilia-less cells (24). Unfortunately, these experiments were only performed in cultured endothelial cells *in-vitro* because the traditional transgenic mice are prenatally lethal in utero. Thus, the functions of endothelial polycytins *in-vivo* remain elusive. We hypothesize that vascular endothelial polycystins are integral to the shear stress induced mechanosensitivity of endothelial cells, and inactivation of polycystins impair stress-induced vascular endothelial blood pressure regulation. Using conditional mouse models, our current investigation will focus on the physiological and cellular roles of endothelial polycystin-1 *in-vivo*.

Although hypertension is prevalent in PKD, and development of hypertension has been correlated with sensory intracellular Ca^{2+} influx via polycystins, the exact molecular mechanism is yet to be discovered. Endothelial cilia house numerous other proteins and cation channels which should have significant role in the mechanosensitive activity of vascular endothelial cilia. TRP channels have been emerged as mechanosensory proteins

by diverse researches. TRPs have also been identified to localize in different cilia (30, 32-35). So, our *in-vitro* study will concentrate on the expression of TRPV channels in vascular endothelial and renal epithelial cells and their ciliary localization.

Chapter 2

Materials and Methods

The Institutional Animal Care and Use Committee (IACUC) of The University of Toledo approved all of the procedures for animal use in accordance with the guidelines of the Institutional Animal Care and Use Committee at the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

2.1 Animal Breeding and Conditional Knockout of PKD1

To achieve *Pkd1* conditional knockout mouse model, specifically in vascular endothelia, we utilized *Tie2Cre* system that allowed us to inactivate the gene of interest at a specific age. *Pkd1* homozygous and *Tie2Cre* hemizygous models were acquired from our laboratory, and they have been previously characterized using biochemical methods (52, 53). $Pkd1^{flox/flox}$ mice have loxP sites on both sides of exon 2-4 of Pkd1. We crossbred $Pkd1^{flox/flox}$ mice with *Tie2Cre* transgene expressing mice to generate the $Pkd1^{flox/flox}$ *Tie2Cre* model. Gene deletion was achieved with the chemical tamoxifen (Santa Cruz Biotechnology, sc-208414) to initiate the Cre recombinase to delete the gene flanked by *loxP* sequence. Two-Three days old mice were injected with 50 μL of 10 mg/mL tamoxifen in sunflower oil intraperitoneally, every day for a total of five consecutive

days. Previous time-dependent analysis indicated that complete gene deletion is achievable with this method (52, 53).

Breeding was conducted by monogamous mating. Homozygous *Pkd1* mice were bred with hemizygous *Tie2Cre* mice to generate heterozygous *Pkd1* mice containing hemizygous *Tie2Cre*. In-breeding this second generation strain generated our desired *Pkd1*$^{flox/flox}$ *Tie2Cre* model. Subsequent generations of our model were achieved by in-breeding *Pkd1*$^{flox/flox}$ *Tie2Cre* strain.

2.2. Confirmation of Genotype

To confirm the genotype of our animals, DNA analysis was done using polymerase chain reaction (PCR) which is one of the most sensitive and versatile techniques in molecular biology. Mice pups of 16-18 days of age were ear-tagged, and a tail-snip of 1 mm – 2 mm was collected from each of the pups. To extract DNA from tail tissue, 180 µL lysis buffer (100mM Tris Hcl pH 8.5, 0.5M EDTA, 10% SDS, 5M NaCl) and 20 µL 1mg/ml proteinase-K inhibitor were added to every tissue sample and kept at 55 °C in a water bath overnight. Tissue and buffer mixtures were vortexed and centrifuged for 5 minutes at 10,000 rpm and the supernatants containing DNA were collected to perform PCR.

Table-1: List of Primers for genotyping by PCR

Primer		Primer Sequence (5'-3')
Pkd1-flox	forward	TTGCTGCCAGCTCTGTGAT
	reverse	CACAGCGGTAGGAAGAGGAG
Tie2Cre	Cre1	CGAGTGATGAGGTTCGCAAG
	Cre2	TGAGTGAACGAACCTGGTCG

Table-2: PCR condition

Step	**Temp °C**	**Time (minutes)**	**Cycle**
1	95	10:00	Temperature
2	94	01:00	Cycle-1 (35X) Step-1
3	60	00:30	Cycle-1 (35X) Step-2
4	72	00:30	Cycle-1 (35X) Step-3
5	72	10:00	Temperature
6	4	Hold	Temperature

Primer sequences for PCR are listed in **Table-1**. After extraction, DNA samples were diluted 1:11 with 2X taq buffer, containing taq DNA polymerase and dNTPs, and specific primers. An automated thermocycler was used to carry out the reactions for 35 cycles with specific denaturing, annealing and extension temperatures **(Table-2)**. Then, PCR amplification samples were run in 1.5%-2.5% agarose gel, depending on the base pairs of mutant allele, containing ethidium bromide, as ethidium bromide makes the entire sample fluorescent.

2.3. Tail-cuff method

Each group of strains, induced mutants and non-induced controls were bred, housed and studied concomitantly to minimize environmental effects. To measure blood pressure of subject animals, we utilized the tail-cuff method (CODA system, Kent Scientific) which uses Volume Pressure Recording (VPR) to measure blood pressure determining tail blood volume. Six-week old mice were acclimatized to the technique for at least for 1 day prior to data recording. Conscious restrained mice were warmed at 36-37 °C and data were collected using CODA software. This software runs five acclimation cycles prior to collecting data, which increases data accuracy. Maximum occlusion pressure was set at 250 mmHg as this is sufficiently higher than normotensive and hypertensive mouse blood pressure. Twenty five recording cycles were set up for experiments and data were collected only from the recordings with at least 6 stable readings. Data from sessions resulting in less than 6 stable readings were discarded and, these sessions were repeated

at a different time. Maintaining these standards allowed us to keep fluctuations, due to the confinement induced stress of animals, the minimum.

2.4. Cell culture

Three different vascular endothelial and two kidney epithelial mouse cell lines were cultured for our *in-vitro* studies. These cell lines were a generous donation from Dr. Surya Nauli (14, 24, 28). Endothelial cell lines are Wild-type (EtWT), $Tg737^{orpk}$ (EtOrpk) cells which lack the ciliary structure, and $Pkd1^{-/-}$ (EtPkd1) cells which do not express polycystin-1 due to mutation is Pkd1 gene. Epithelial cell lines are Wild-type (MekWT) cells, and $Pkd1^{del34}$ (MekDel34) which do not express polycystin-1. These adherent cells were cultured in Dulbeccos's Modified Eagle Medium with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin at 37 ºC. A constant 5% CO2 and 95% O2 mixture air were provided during cell growth.

2.5. Immunofluorescence microscopy

Immunofluorescence studies are excellent tools to visually demonstrate cellular and subcellular localization of proteins and cation channels. Prior to immunofluorescence experiments, cells were serum starved for 24 hours for differentiation. 80%-90% confluent cells, grown on a glass cover slip, were subjected to experiments. Cells were fixed with 4% paraformaldehyde (Thermo-Fisher Scientific) containing 2% sucrose, and permeabilized with 0.1% Triton-X (Thermo-Fisher Scientific). To reduce unspecific

binding, primary antibodies [mouse acetylated-α-tubulin (1:10,000, Sigma, Inc.), rabbit anti-TRPV4 (1:1,000, Alomone Labs.), rabbit anti-TRPV5 (1:1,000, Alomone Labs), rabbit anti-TRPV6 (1:1,000, Alomone Labs)] were diluted to desired concentration in Phosphate Buffer Solution (PBS) containing 10% FBS. All secondary antibodies [anti-mouse FITC fluorescence secondary antibody (1:500, Vector Lab, Inc.), anti-rabbit TexasRed fluorescence secondary antibody (1:500, Vector Lab, Inc.)] were diluted in 10% FBS too, to decrease background fluorescence. Cells were washed three times with PBS after each antibody incubation. Washed and treated cells were mounted with DAPI nuclear stain. Images were taken with Nikon TE2000 using Metamorph software.

2.6. Immunoblotting

Cell lysate was prepared using a standard technique. Briefly, adherent cells with 80%-90% confluency were rinsed with PBS, then harvested and transferred to microcentrifuge tube. Fresh radioimmunoprecipitation assay (Ripa) buffer containing protease inhibitor were added to the cells. Cells were placed on ice, gently shaken for 1 hour, and cetrifuged for 15 min at 10,000 g (accuSpin Micro 17, Fisher scientific, Inc.). Total cell lysate was transferred into another microcentrifuge tube and subjected to western blot. Briefly, protein samples were analyzed using a standard 4–10 % gradient sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Antibodies against TRPV4, TRPV5, TRPV6 (Alomone Labs), and GAPDH (Cell signaling, Inc.) were used at dilutions of 1:1000, 1:1000, 1:1000 and 1:10,000, respectively. All images were quantified with ImageJ software.

2.7. Statistical analysis

All images were analyzed using Metamorph software. All quantitative data are displayed as mean ± SD. Statistical analysis using student t-test has been performed to compare the blood pressure of mutant and control groups and to compare protein expression level between wild type and mutant cells. All statistical results were considered significant at a significance level of $p<0.05$.

Chapter 3

Results

3.1. Systolic, diastolic and mean blood pressure (BP) is significantly higher in *Pkd1* mutants

Ciliary polycystin-1 plays a crucial role in development and progression of PKD. Polycystins are also postulated to have significant role in the development of hypertension in PKD, though *in-vitro* studies. (23, 24) In an attempt to deduce the role of polycystin-1 in PKD associated hypertension through *in-vivo* experiments, we generated a conditional knockout mouse model of *Pkd1*, the gene responsible of polysystin-1 synthesis, where the knockout was specific to vascular endothelial cells, and studied the blood pressure of 6 weeks old mutant mice and compared it with age matched controls.

We found systolic blood pressure of *Pkd1* mutant mice to be significantly higher than non-induced control group (**Table-3)**. **Figure-1** shows the graphical representation of this data and their statistical significance. Diastolic BP was also found to be higher in *Pkd1* mutant group that the control **(Table-4). Figure-2** shows the graphical representation of diastolic BP data and their statistical significance. Our study also showed an increase in the Mean arterial BP of *Pkd1* mutant group when compared with non-induced control group. **(Table-4)**

In addition to comparing the entire mutant group with the entire control group, we subdivided each group according to gender and compared the mutants and controls of each gender. Systolic and mean arterial blood pressures were found significantly different irrespective of the mix of genders. Diastolic blood pressure is significantly different only when the entire group was analyzed. Comparison of gender specific groups of diastolic BP did not show any significant difference ($p > 0.05$).

Table-3: Average systolic BP of six-week old mice (mmHg ± SD)

	All	Male	Female
Control	123±6 (n=7)	128±4 (n=3)	119±5 (n=4)
Pkd1	135±6 (n=8)	139±6 (n=4)	132±3 (n=4)

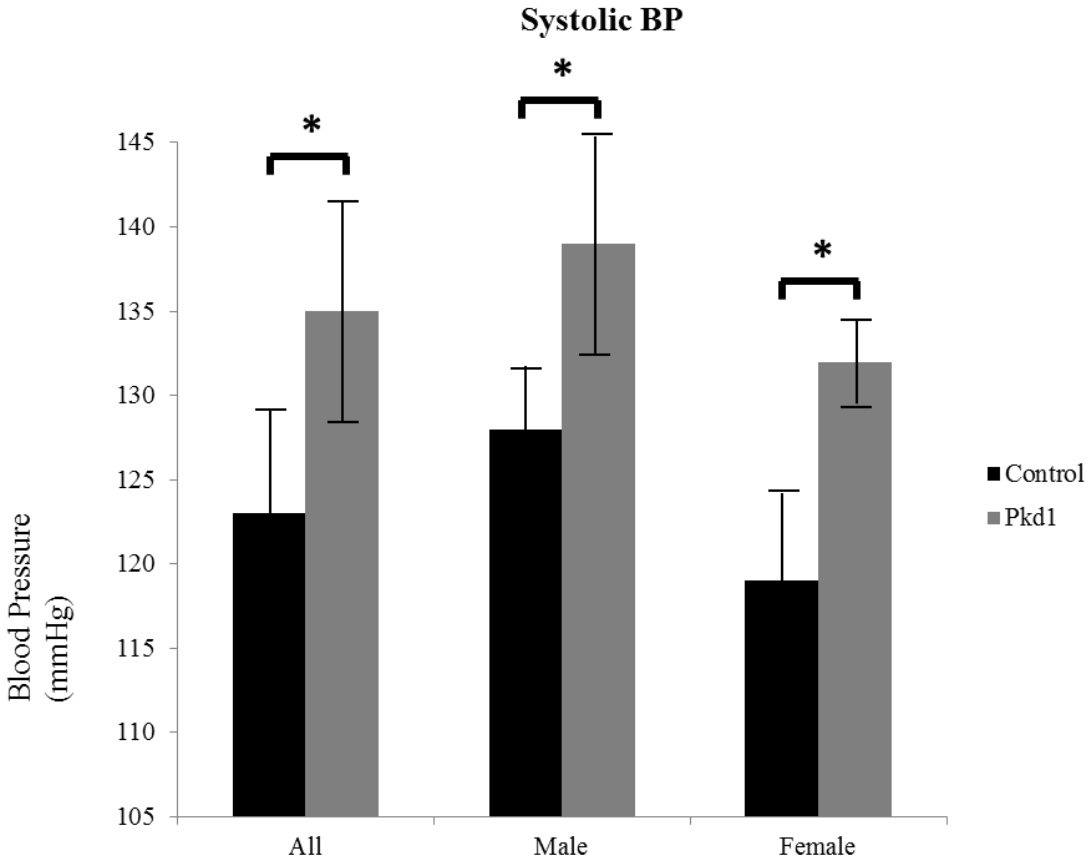

Figure-1: Comparison of average systolic BP .
* Significantly different. (P< 0.05)

Table-4: Average diastolic BP of six-week old mice (mmHg ± SD)

	All	Male	Female
Control	79±6 (n=7)	82±7 (n=3)	76±5 (n=4)
Pkd1	89±5 (n=8)	92±3 (n=4)	87±7 (n=4)

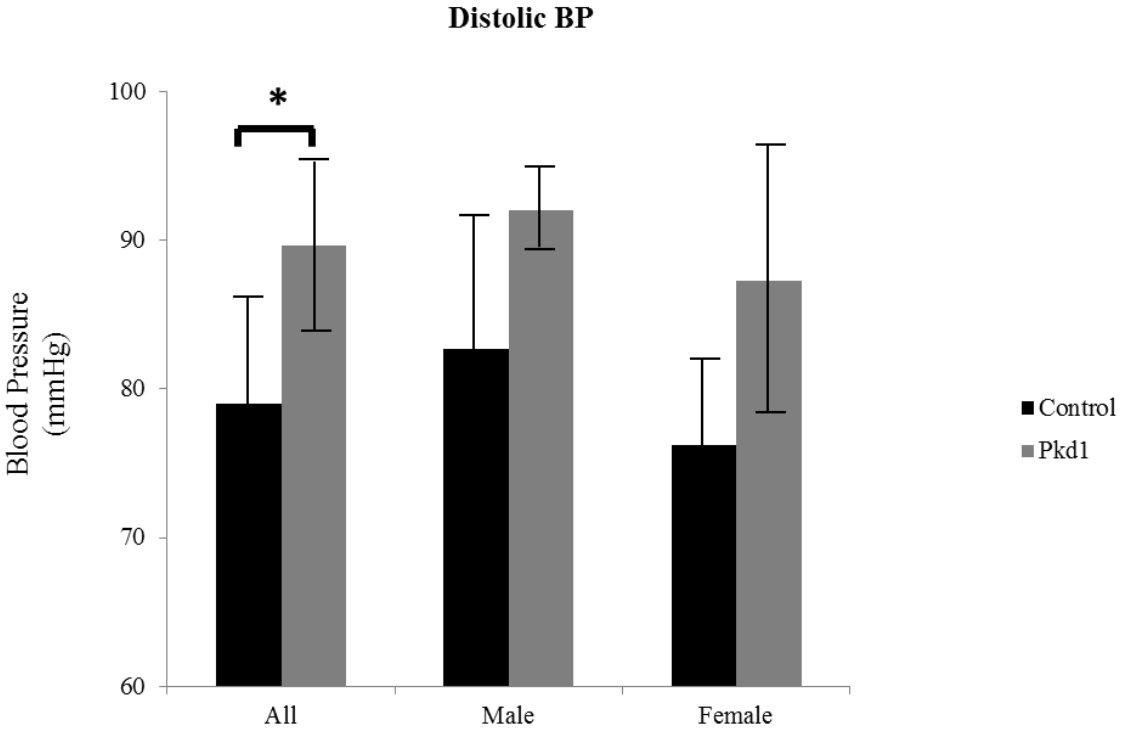

Figure-2: Comparison of average diastolic BP .
* Significantly different. (P< 0.05)

Table-5: Average mean arterial BP of six-week old mice (mmHg ± SD)

	All	Male	Female
Control	93±6 (n=7)	97±5 (n=3)	90±5 (n=4)
Pkd1	104±4 (n=8)	107±3 (n=4)	102±5 (n=4)

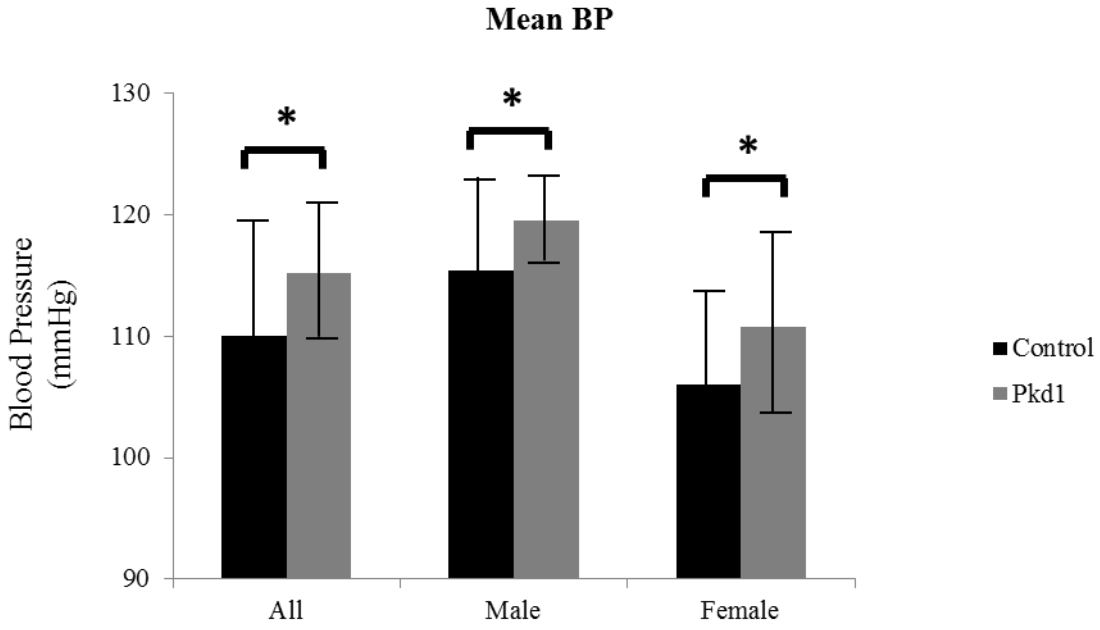

Figure-3: Comparison of average mean BP .
* Significantly different. (P< 0.05)

3.2. TRPV4 and TRPV5 are localized in primary vascular endothelial and renal epithelial cilia

To study the ciliary localization of TRPV4, TRPV5 and TRPV6 we conducted immunostaining experiments with all five types of cells. TRPV4 was found to be localized at the basal body of the cilia of EtWT, EtOrpk and EtPkd1 cells. No difference was observed between in the nature of localization between wild-type and mutant cells' cilia. TRPV4 was also observed the basal body of endothelial cilia. **(Figure-4)**

Our data indicated localization of TRPV5 at the basal body of EtWT, EtOrpk and EtPkd1 cells. TRPV5 was also found to localize at the basal body of endothelial cilia. No difference of localization was observed between wild-type and mutant cells. **(Figure-5)** Our experiments did not reveal any co-localization of TRPV6 in vascular endothelial cilia. TRPV6 was not found to co-localize with renal epithelial cilia. **(Figure-6)**

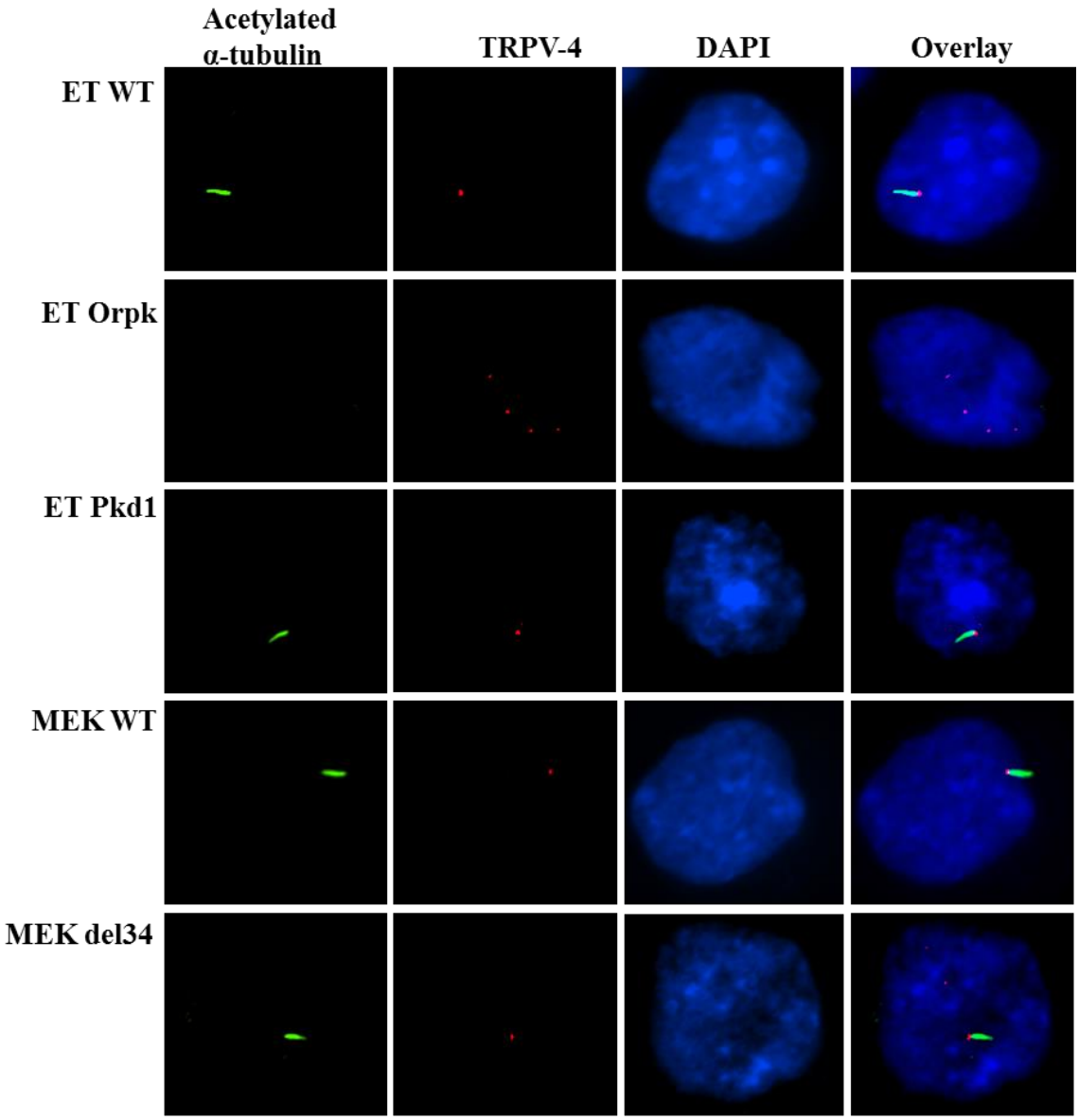

Figure-4: Ciliary colocalization of TRPV4. .(Magnification 60X)

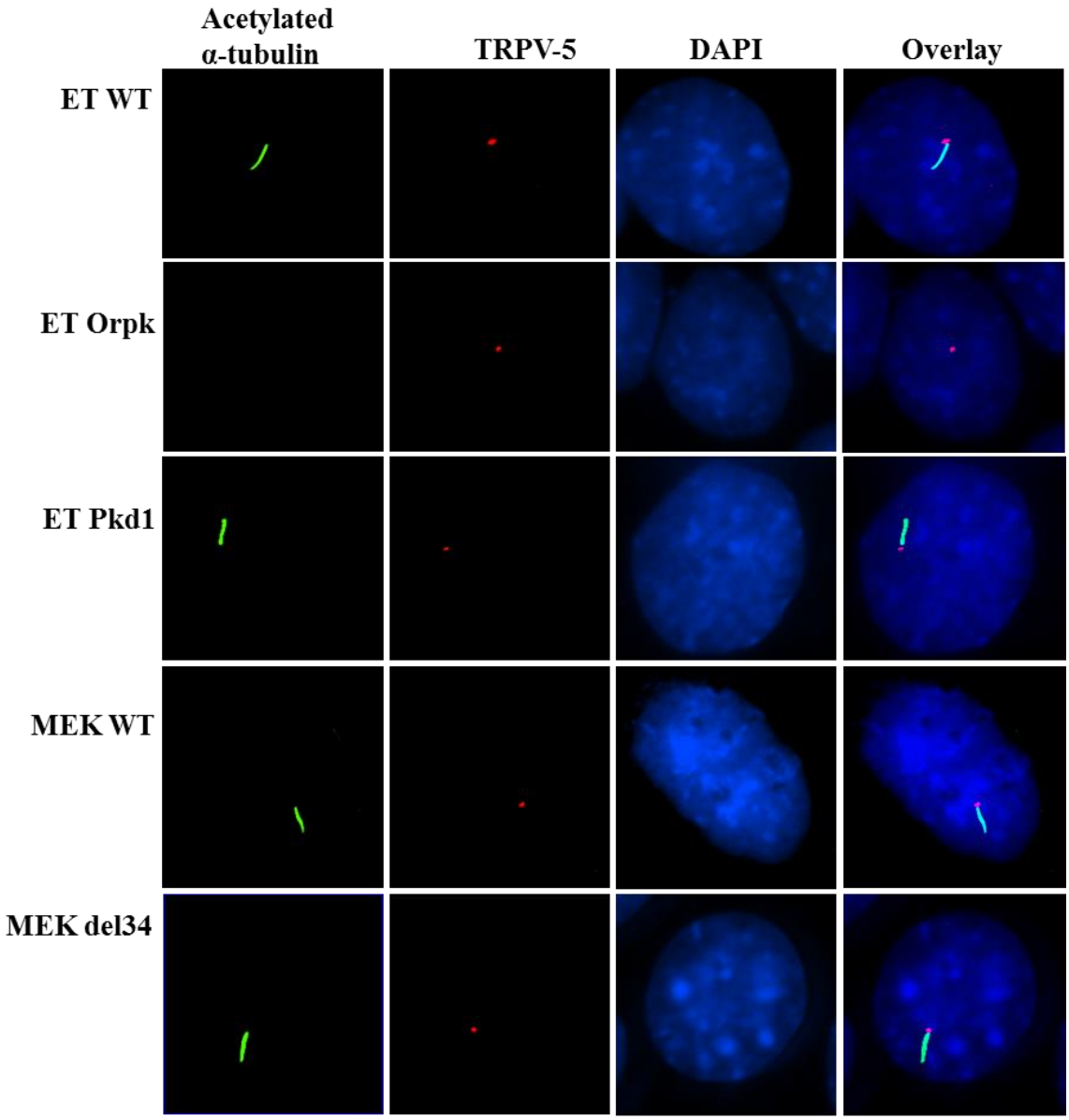

Figure-5: Ciliary colocalization of TRPV5 (Magnification 60X)

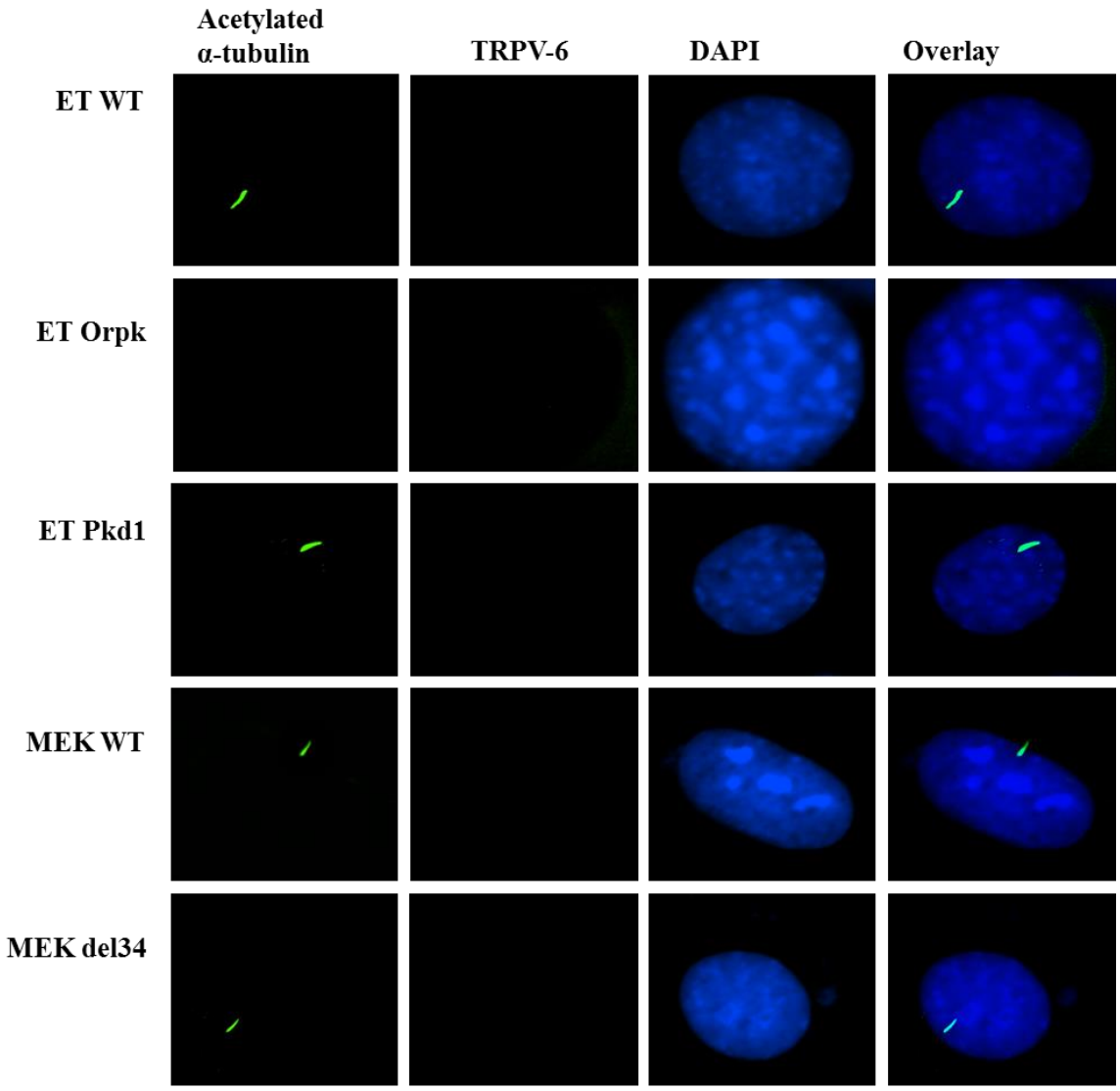

Figure-6: TRPV6 is not colocalized in cilia (Magnification 60X)

3.3. TRPV4 and TRPV5 are expressed in vascular endothelial and renal epithelial cells

To study the expression of TRPV4, TRPV5 and TRPV6 in vascular endothelial and renal epithelial cells we carried out protein analysis and quantification by western blot. TRPV4 expressed in all three types of endothelial (WT, Orpk, Pkd1) and two types of epithelial (WT, Del34) cells. There was no significant difference in the expression level of TRPV4 between EtWT and EtOrpk, but expression level was significantly higher in EtPkd1 cells than EtWt. Difference in the Expression level of TRPV4 between epithelial wild-type and epithelial mutant cells were not statistically significant.

TRPV5 was found to be expressed in all three types of endothelial (WT, Orpk, Pkd1) and two types of epithelial (WT, Del34) cells. There was no significant difference in the expression level of TRPV5 when wild-type and mutant cells were compared.

Our experiments did not find any expression of TRPV6 in neither endothelial (WT, Orpk, Pkd1) nor epithelial cell (WT, Del34) lines.

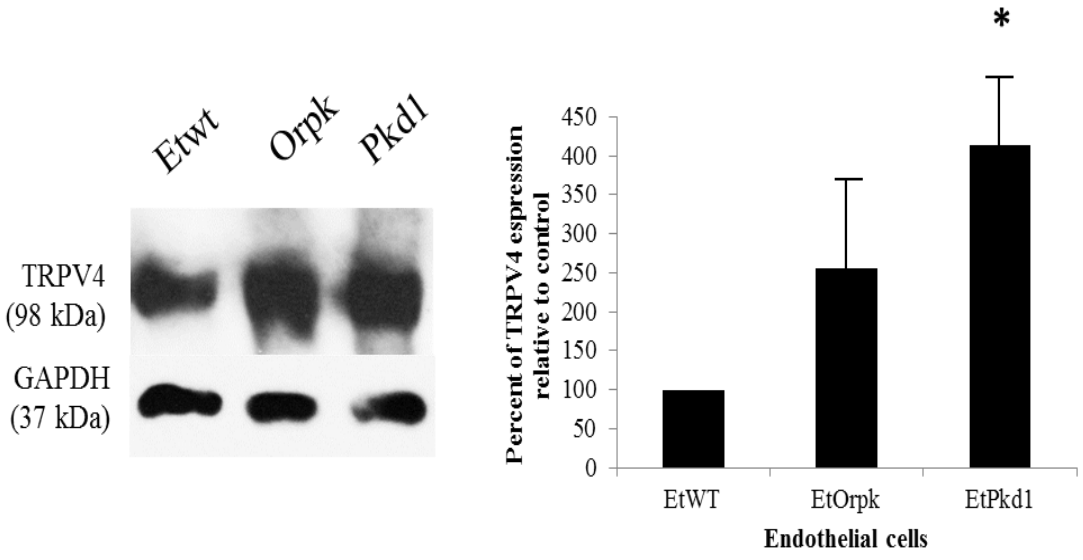

Figure-7: Expression of TRPV4 in endothelial cells. (n=3)
* Significantlly different. ($P<0.05$)

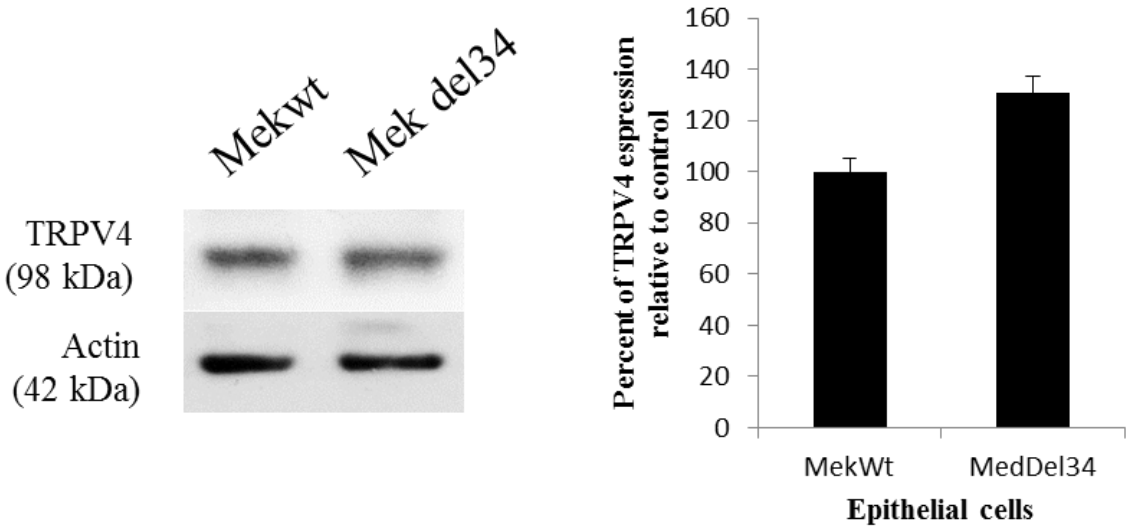

Figure-8: Expression of TRPV4 in epithelial cells. (n=3)

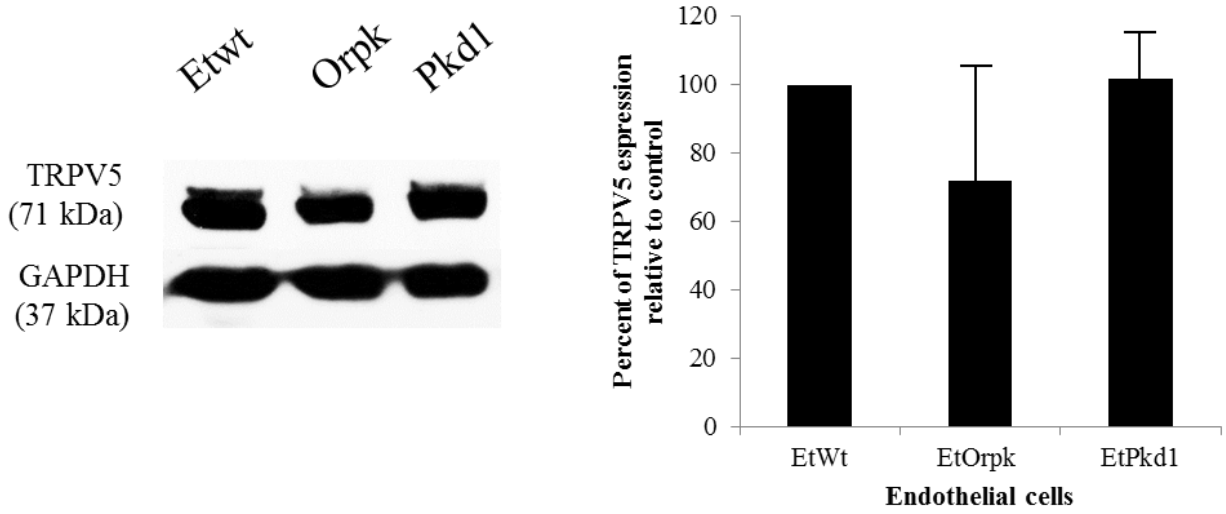

Figure-9: Expression of TRPV5 in endothelial cells. (n=4)

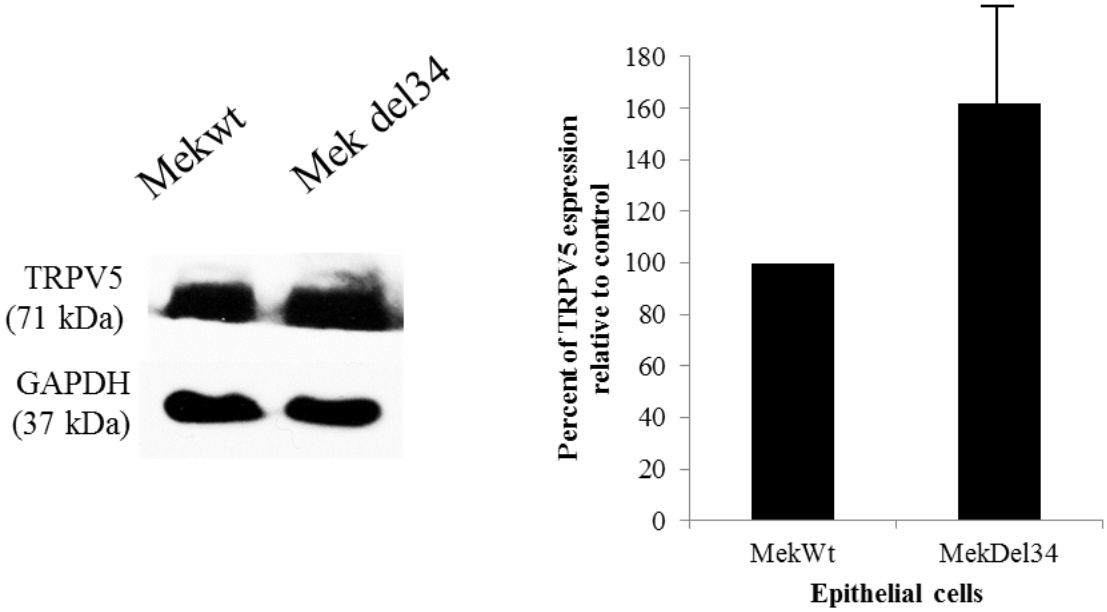

Figure-10: Expression of TRPV5 in epithelial cells. (n=3)

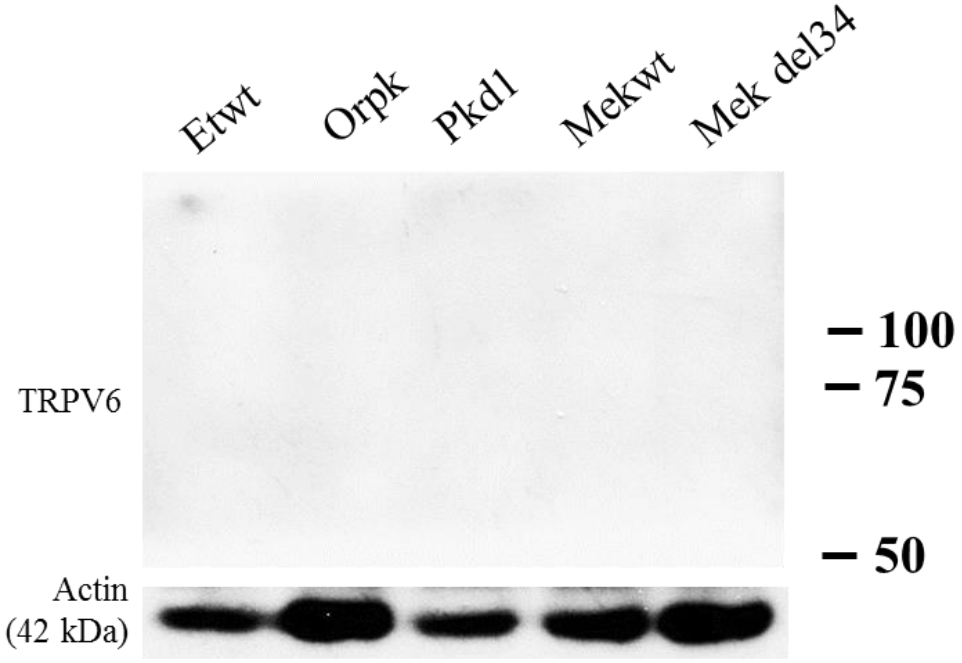

Figure-11: Expression of TRPV6 in endothelial and epithelial cells.

Chapter 4

Discussion

Polycystin-1 is a well know mechanosensitive protein and its role in PKD is already well-established (14, 28). Polycystin-1 regulates calcium influx through activation of polycystin-2 into the cell upon sensation of fluid flow and its absence deprives the epithelial cells of the kidney of mechanosensative response after fluid flow sensation; which ultimately results in cyst formation and PKD. As hypertension is a very common phenomenon in PKD patients and oftentimes it is observed even before clinical symptoms of PKD are established, the overall quality of life of the patients are downgraded considerably because of hypertension. If Pkd1 is mutated in epithelial cells, it is also possible that Pkd1 is mutated in vascular endothelial cells too. In our *in-vivo* study we specifically knocked out *Pkd1* from vascular endothelia which allowed us to focus our investigation on endothelial mutation only, but not epithelial mutation. Genotype of every mouse was confirmed prior to any experimentation. VPR technology enabled us to obtain reading easily from our dark-skinned mice, C57BL/6, and provided us six with different blood pressure parameters: systolic and diastolic blood pressure, heart rate, mean blood pressure, tail blood flow, and tail blood volume (54, 55). Though this system is compatible with both anaesthetized and non-anaesthetized mice, to maintain minimum interference with rodent physiology, we experimented only with non-anaesthetized mice. Although tail-cuff method to measure blood pressure in rodent has

been questioned because of the confinement of subjects during measurement, which may affect the readings, it has been shown in diverse research that tail-cuff method present valid and accurate data when performed appropriately(56, 57).

Our data suggests a significant increase in the systolic, diastolic and mean blood pressure in Pkd1 mutant mice than control group. Endothelial ciliary polysystin-1 mediates shear stress induced fluid flow sensation by calcium influx and through nitric oxide production. (24) Nitric oxide is a well-known vasodilator, so any defect in polysystin-1 should result in altered vasodilation, in other word, defective blood pressure control. High prevalence of hypertension in PKD might result from this inability of endothelial cells to sense blood flow and subsequent disturbances in blood pressure control. This was previously hypothesized by *in-vitro* studies and our research confirms that endothelial polycystin-1 is crucial to maintain a normal blood pressure, as polycystin-1 mutant showed elevated blood pressure reading. Hypertension in mice can be defined as systolic blood pressure higher than 140 mmHg, (58) and our reading suggest an average of 135±6 mmHg systolic blood pressure in *Pkd1* mice, and we used only one age group of mice, we cannot confirm if these animals developed hypertension or not; but it is convincingly clear from the result of our study that expression of polycystin-1 in vascular endothelia is crucial for normal blood pressure maintenance.

Previous studies suggested the involvement of vascular ciliary Polycystin-1 in hypertension (59). However, our study was limited to polycystin-1 only, as our animals faced complete knockout of polycystin-1 from endothelia, not cilia selective knockout. Future studies with conditional knockout of cilia from vascular endothelia will provide more insight into the role of ciliary polysystin-1 in hypertension. Also, the impact of

conditional knockout of cilia or polycystin-1 from kidney epithelial cells on blood pressure can be investigated. Further detail age grouped study is required to substantiate the role of endothelial ciliary polycystin-1 in PKD associated hypertension.

Polycystin-1 is also called TRPP-1 protein and there are 27 other members in that protein family. Among the six members of TRPV sub-family, only the first four members have already been extensively studied for their expression in different vascular cells, including endothelium, and have been found to be expressed (30). TRPV5 and TRPV6 are the two least studied proteins although they are very selective to calcium. Therefore, we studied the expression of these two proteins and their ciliary localization. The result of our study indicated the expression of TRPV5 in vascular endothelial cells. TRPV6 was not found to be expressed in vascular endothelial or renal epithelial cells. As our experiments with TRPV6 did not involve any positive control, we cannot conclude that TRPV6 is not expressed in vascular endothelia or renal epithelia, but our multiple experiments with TRPV6 did not result in showing any expression.

TRPV4 has already been shown to express in vascular endothelial and kidney epithelial cells (36, 37). Moreover, it was found to localize in epithelial cilia, along with Polycystin-2, where it forms a heterodimer with polycystin-2, and TRPV4 in epithelial cells are proved to have mechanosensitive role (35, 36). Our results match perfectly with the previous data. In addition, we characterized the expression of TRPV4 in cilia-mutant and Pkd1 mutant cells too.

Our data indicate an overexpression of TRPV4 in endothelial Orpk and Pkd1 cells when compared with the wild-type cells. Being a mechanosensitive protein, TRVP4 might have

roles to play in altered mechanosensitiviy of Orpk and Pkd1 cells, however further investigations are required to confirm the suggestion. Except TRPV4 in endothelial cells, all our characterization of TRPV4 and TRPV5 did not show any significant difference in expression between wild-type and mutant cells. But it has been confirmed for the first time that, TRPV4 and TRPV5 are expressed in the cilia mutant and Pkd1 mutant endothelial and epithelial cells

In addition, we studied the ciliary localization of TRPV4, TRPV5 and TRPV6 in both types of cells. We are the first to show localization of TRPV5 in vascular primary endothelial cilia at the basal body. We have also shown TRPV4 to localize at the basal body of cilia. Being a potent calcium channel and colocalizing with cilia, TRPV5 might have roles to play in cilia mediated calcium influx into the cells and might be involved in other ciliary activities. Further functional study is required to develop a correlation between ciliary TRPV5 and mechanosensitivity.

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