SUPPLEMENT MATERIAL

I. Materials and Methods

Cell isolation and culture

Primary vascular endothelial cells were obtained from *Pkd2* mouse aortas and interlobar arteries of nine human ADPKD kidneys. Generation of *Pkd2* mice has been described previously[1]. After isolation from the rest of the tissues, arteries were briefly perfused with phosphate buffered saline (PBS, pH 7.4). To dissociate the endothelial cells from the vessel, arteries were subsequently perfused with trypsin and incubated for 20-30 minutes at 37 ºC. Endothelial cells were then plated in Dulbecco's Modification of Eagle's Media (DMEM; *Cellgro, Inc.*) containing 2-15% of fetal bovine serum (FBS; *HyClone, Inc.*). This cell isolation method, which provides a relatively pure culture of endothelial cells, has been previously described[2]. A successful cell isolation typically provides endothelial characteristics, which include cell growth, morphology, and surface markers. In some cases, established mouse aortic endothelial cells and human umbilical vein endothelial cells (HUVEC; *GlycoTech*, *Inc*.) were used to confirm our findings.

Immunofluorescence, immunoblotting and immunoprecipitation

Confluent cells grown to full differentiation were fixed with 4% paraformaldehyde containing 4% sucrose. For the mouse femoral artery, tissues were then embedded into optimal cutting temperature solution (*TissueTek, Inc.*) and cut at 10 μm thickness. Acetylated-α-tubulin (clone 6-11B-1; *Sigma, Inc.*) was used at dilution of 1:10,000, polycystin-2 (clone H-280; *Santa Cruz Biotehcnology, Inc*.) at 1:50, eNOS (*ABcam, Inc*) at 1:100, and CD144 (clone F-8; *Santa Cruz Biotehcnology, Inc*.) at 1:20 followed by the corresponding secondary fluorescence antibodies. CD144, also known as vascular endothelial-cadherin or VE-cadherin, is a major endothelial adhesion molecule involved in controlling cellular junctions, vascular homeostatsis, inflammation, and angiogenesis[3].

The expression levels of polycystin-2 were confirmed with Western blot at dilution of 1:100, CD144 at 1:100, eNOS at 1:200, α-tubulin (*ABcam, Inc*) at 1:1,000, Akt (clone 40D4; *Cell Signaling Technology, Inc.*) at 1:1,000, and β-actin (clone AC-40 from *Sigma, Inc.*) at dilution of 1:1,000. Because we were not able to perform immunoblot on polycystin-1, the immunoprecipitation for polycystin-1 (clone P-15; *Santa Cruz Biotehcnology, Inc*.) was carried out at dilution of 1:5 and blotted for both polycystin-1 and polycystin-2 at 1:50. The immunoprecipitation for polycystin-2 was performed in a similar manner.

Knockdown and RT-PCR

We used RNA interference (siRNA) approach to enable us to assess polycystin-2 functions in established mouse endothelial cells, HUVEC and isolated arteries. The double stranded RNA (dsRNA) oligonucleotides targeting *Pkd2* mouse and/or *PKD2* human mRNA were used based on the NCBI database with accession number NM_008861 for mouse and NM_000297 for human (**Online Table I**). One of these RNA oligonucleotides was then transfected into cells (50 nM) or artery (100 nM) according to protocol from *Santa Cruz Biotechnology, Inc*. A scramble unrelated dsRNA sequence with random sequence or no dsRNA was used as a negative control. All transfection reagents were purchased from *Santa Cruz Biotechnology, Inc*. Because we can control cellular proliferation in our system through simian virus 40 (SV40) gene expression[2], cells or tissues were treated with 0.75 μg/L interferon-γ to induce proliferation for transfection. Transfection was carried out for eight hours by incubating dsRNA with the cells / tissues at 37 ºC in a CO_2 incubator. To examine the success of the transfection, polycystin-2 mRNA and protein levels were analyzed after thirty six hours. For mRNA analysis, polycystin-2 (forward: 5'-GCGAG GTCTC TGGGG AAC-3'; reverse: 5'-

TACAC ATTGG AGCTC ATCAT GC-3'), polycystin-1 (forward: 5'-GTGAA ATAAA CCTGA GTGGG AAC-3'; reverse: 5'-GCGAC ATACT CCTCA CCACA-3'), and α-tubulin (forward: 5'-GCCAA CCAGA TGGTG AAATG-3'; reverse: 5'-GGTAC TCTTG GTCTT GATGG-3') primers were designed to be compatible for both human and mouse studies.

Calcium and NO measurements

Cytosolic calcium and intracellular NO fluorescence were measured using Nikon TE2000-U microscope, connected with photometric Coolsnap EZ 20 MHz monochrome camera and high speed excitation wavelength changer for DG4/DG5 system controlled by MetaFluor / MetaMorph software (*Molecular Devices, Corp.*). For better focusing, the microscope was equipped with XY-axis motorized flat top inverted stage, Nikon automatic focusing RFA Z-axis drive, and custom designed vibration isolation platform. For a better controlled environment, the body of the microscope was enclosed inside a custom built chamber to control CO_2, humidity, heat and light.

To examine cytosolic calcium, cells or arteries were loaded for 30 minutes at 37 ºC with 5 μM Fura-2 AM (*Invitrogen, Inc.*). After being washed to remove excess Fura-2 AM, cells or arteries were placed and observed under Nikon microscope. Pairs of intracellular calcium images were captured every five seconds at the excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm. These images were captured through Fura-2 filter kit that contains 25mm 340/380 exciter filters, a dichroic mirror, and a wide band 510 nm emission filter. A more detail protocol has been described previously[2].

Endothelial cells lining the lumen of blood vessels are known to control the vascular contractility through production of NO. Because NO gas escapes easily from the cells to the media, we measured both intracellular production and extracellular release of NO. Whereas NO-specific dye, DAF-FM, was used to measure intracellular NO, extracellular NO was measured indirectly with nitrite-specific dye, DAN. To examine intracellular NO biosynthesis, cells were loaded for 30 minutes at 37 ºC with 20 μM DAF-FM (*Invitrogen, Inc.*) as described previously[2]. Intracellular NO was then measured every five seconds at the excitation and emission wavelengths of 495 and 515 nm, respectively. The measurement was obtained from images that were captured through a 25mm 495 exciter filter, a dichroic mirror, and a 25mm 515 emission filter.

To examine extracellular NO release, the perfusate media was collected and assayed with nitrate / nitrite fluoremetric assay kit (*Cayman Chemical, Corp*). It is known that NO gas is converted easily to nitrite (NO_2^-) and nitrate (NO_3^-). Because the relative proportion of this conversion is too variable to predict with certainty, all of the NO_3^- in the media need to be reduced to NO_2^- prior to the extracellular NO measurement. Therefore, this extracellular NO assay provides a measurement of total NO_2^- and NO_3^- in the media through a two-step process. The first step involves conversion of NO_3^- to NO_2^- by a nitrate reductase enzyme. The second step involves the addition of NO_2^--specific dye, diaminonaphthalene (DAN). DAN and NO_2^- react easily, resulting in highly fluorescence chemical, naphthotriazole (NAT). Further alkaline treatment of NAT would enhance its fluorescence characteristics with excitation of 360 nm and emission of 430 nm. For each 96-well plate measurement, a replicated standard curve with a known NO_3^- concentration was generated by first converting it to NO_2^-. The NAT fluorescence was then analyzed with multi-detection microplate reader (Spectramax M5; *Molecular Devices, Corp.*) at the optimal excitation and emission wavelengths of 360 and 430 nm, respectively.

Regardless of the methods of measurement, the profiles of NO inside and outside the cells were very similar. More specifically, they showed an instant peak increase in response to shear stress, followed by an immediate drop to baseline level.

Cell and artery perfusion

For cell perfusion, cells were placed in a FCS2 chamber with electrical enclosure heater (*Biotechs, Inc.*). The FCS2 chamber has a width of 1.4 cm and height of 0.05 cm, and cells were perfused with Dulbecco's Phosphate Buffer Saline (DPBS with pH 7.2; *MediaTech, Inc.*) at a flow rate of 550 μL/sec, which resulted in a shear stress of about 7.2 dyne/cm^2. Because different cell types could have different biophysical properties of cilia, we assayed optimal fluid-shear stress for cilia activation. The 7.2 dyne/cm^2 was determined to be optimal shear for vascular endothelial cilia, because it provided maximum cytosolic calcium increase and NO production[2].

Because hypertension occurs frequently and is an early manifestation of ADPKD patients[4,5], we also isolated and used interlobar endothelial cells from ADPKD kidneys. Based on our previous study related to shear stress in normal and ADPKD kidneys[6], we calculated that a shear stress of 0.2 to 20 dyne/cm^2 is still within a reasonable range for human kidneys. The same shear stress value of 7.2 dyne/cm^2 was therefore used in the human endothelial cells.

To confirm that our biophysical calcium and biochemical NO read-outs are biologically and technically relevant, we applied various inhibitors to block the molecular functions that are supposedly involved in shear-induced NO production[7-11]. In some experiments, cells were perfused in the presence of 1 mM of EGTA. In other experiments, cells were incubated with L-NAME (10 μM), LY-294,002 (10 or 30 μM), calphostin C (0.3 or 1.0 μM), W7 (10 μM), Akt inhibitor II (10 μM), or wortmannin (1 or 10 μM) for 30 minutes before the experiments. The sub-maximal concentrations of each inhibitor were used in these studies to obtain more reproducible blockage data.

For arterial perfusion, the abdominal aorta of an adult mouse was isolated and cleaned from connective tissues. After incubation with dsRNA (scrambled or mouse siRNA1) in a sterile, heat and humidity-regulated environment for at least 24 hours, the aorta was then submerged freely in DPBS or placed inside a glass capillary tube containing DPBS. The capillary (*Kimble, Inc.*) has an outer diameter of 1.5 mm with a glass thickness of 0.2 mm. This thickness did not interfere with our imaging system and thus allowed us to monitor fluorescence intensity. Assuming that the artery has a uniformed diameter averaging 1.2 mm, a flow rate of 164 μL/sec would result in a shear stress of about 7.2 dyne/cm^2. To investigate mechanosensory polycystin-2 function in more detail, we perfused the isolated artery that had been transfected with either scrambled or *Pkd2* siRNA (mouse siRNA1, **Online Table I**). In some experiments, the aorta was pre-incubated for 30 minutes and perfused with DPBS containing 2 to 4 units / mL of apyrase.

In some cases, isolated arteries and endothelial cells were challenged with 10 μM ATP in the presence or absence of 1 mM EGTA. Unless otherwise stated, all chemicals were purchased from *Sigma, Inc*. except for Akt inhibitor, which was obtained from *Calbiochem*.

Data analysis

For calcium and NO imaging study, a total of 50 cells within a cell population was randomly analyzed and their changes in fluorescence intensity were averaged (N=1). The experiments were repeated on different sets of cell populations, and the numbers of experiments were considered sufficient with a statistical coefficient variant of ≤20%. All quantifiable data points are reported as mean±SEM. Comparisons between two groups were carried out using student-*t* test, and the difference between groups was determined statistically significant at $p<0.05$.

II. Online Table I

Mouse *Pkd2*		
code	position	sequence
siRNA1	890	5'-ACGGCATGAT GAGCTCCAAT GTGTA-3'
siRNA2	1176	5'-TCAGGACCTG CGAGATGAAA TTAAA-3'
siRNA3	1484	5'-AACCTGTTCT GTGTGGTCAG GTTAT-3'
siRNA4	4337	5'-GGTTTTTGTG TCTGTCAAAG ACAG-3'
Human *PKD2*		
code	position	sequence
siRNA1	782	5'-ACGGCATGAT GAGCTCCAAT GTGTA-3'
siRNA2	1366	5'-AACCTGTTCT GTGTGGTCAG GTTAT-3'
siRNA3	2668	5'-GACGCCGTGA TCGTGAAGCT AGAGA-3'
siRNA4	4946	5'-TGGTATTATT AAAAAGACAT TACAT-3'

Legend for Supplement Table I: siRNA sequence for mouse *Pkd2* (NM_008861; gi: 31543486) and human *PKD2* (NM_000297; gi: 33286447)

Previous studies have demonstrated that the mechanosensitive protein polycystin-1 localizes to the cilia of human cells[6,12,13]. To address the polycystin-2 role independently from polycystin-1 function, we employed mouse *Pkd2* and human *PKD2* siRNA approach using normal mouse and human endothelial cells. We targeted different sites of mouse *Pkd2* and human *PKD2* mRNA. As also observed in mouse cells (**Figure 2**), the inhibition of polycystin-2 in human cells (**Figure 4**) depends on the target probes. Please also note that siRNA probes 1 and 3 for mouse are the same as probes 1 and 2 for human; both probes were also previously used by another laboratory[14]. This further indicates that the efficiency of siRNA approach depends on both target probes and cell origins (**Figures 2** and **4**). For example, siRNA1 that has the same target sequence for mouse and human mRNA shows inhibition efficacy in mouse, but not in human, cells. More importantly, these studies confirmed our notion from the experiments of ADPKD endothelial cells that regardless of the functionality of polycystin-1, proper expression level and subcellular localization of polycystin-2 were required in mediating fluid shear sensing in endothelial cells.

III. Online Figure I

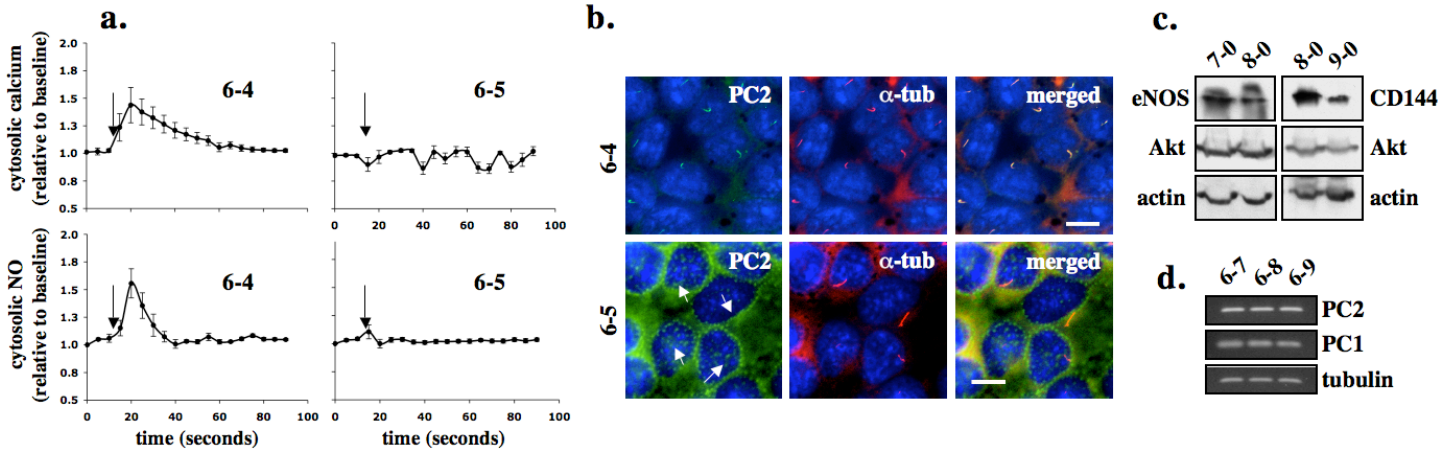

Figure Legend: Effects of fluid-shear stress in vascular endothelial cells of an ADPKD patient

Vascular endothelial cells were isolated from several interlobar arteries of an ADPKD kidney. **a.** Endothelial cells (6-4 and 6-5) from segmental arteries 4 and 5 of patient #6 were cultured and challenged with fluid shear stress, and their cytosolic calcium and NO changes were recorded. Black arrows indicate the start of fluid flow. **b.** The primary culture was then subjected to immuno-localization studies for polycystin-2 (PC2). Acetylated-α-tubulin (α-tub) was used as a ciliary marker, and nuclear marker (dapi) is shown in the merged images. White arrows indicate the absence of polycystin-2. **c.** Pooled endothelial cells obtained from patients #7 (7-0), #8 (8-0), and #9 (9-0) were subjected for immunoblot studies to further confirm our endothelial isolation technique. CD144, eNOS, and Akt were used as endothelial markers, and β-actin was used as a loading control. **d.** Endothelial cells isolated from interlobar segment 7, 8, and 9 of patient #6 were analyzed for the presence of some portions of N-terminal *PKD1* and *PKD2* transcripts. Cells from passage #2, 3 and 4 were used. Bar=5 μm.

Note that although we were not able to further analyze the human cells due to the short passages of primary cultures, our western analysis from pooled endothelial cells of patients #7, 8 and 9 confirms our cell isolation technique. We could obtain vascular cells that consistently showed the presence of various endothelial markers, such CD144, eNOS, and Akt. Because we could not propagate enough cells from a single isolation to analyze expression levels of polycystin-1 and -2, we performed RT-PCR to examine *PKD1* and *PKD2* transcripts. The RT-PCR shows that at least in interlobar segments 7, 8, and 9 from ADPKD patient #6, *PKD1* and *PKD2* transcripts could be detected within nucleotides 562-754 and 657-806, respectively.

IV. Online References

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