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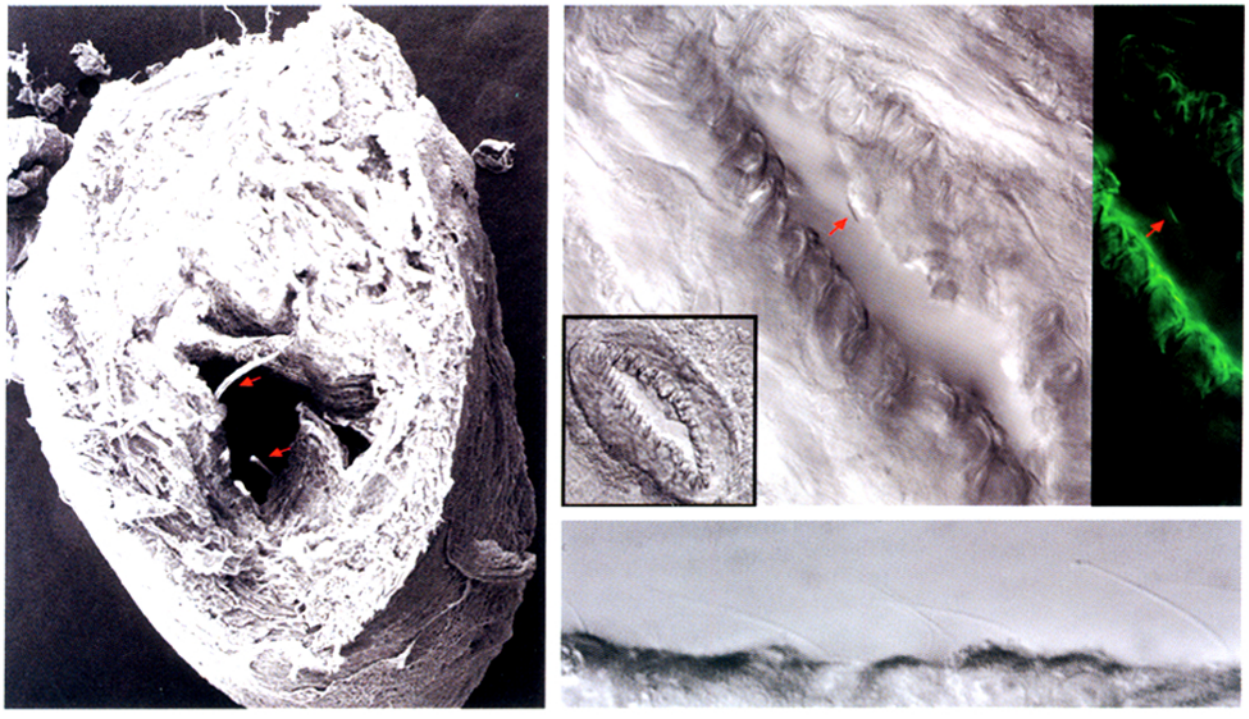
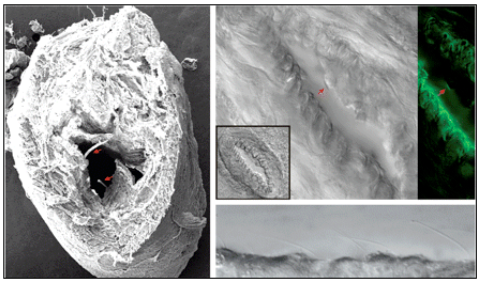


About the Cover



On the cover: Endothelial cilia (arrows) from mouse embryonic aorta and adult femoral artery are shown in scanning electron micrograph and high-resolution differential interference contrast image, coupled with immunofluorescence for acetylated-α-tubulin, a marker for cilia. Cilia projected toward apical membrane are bent in response to fluid-shear stress, as shown here in cell culture. See related article, page 860.

Ciliary Polycystin-2 Is a Mechanosensitive Calcium Channel Involved in Nitric Oxide Signaling Cascades

Wissam A. AbouAlaiwi, Maki Takahashi, Blair R. Mell, Thomas J. Jones, Shobha Ratnam, Robert J. Kolb, Surya M. Nauli

Abstract—Cardiovascular complications such as hypertension are a continuous concern in patients with autosomal dominant polycystic kidney disease (ADPKD). The *PKD2* encoding for polycystin-2 is mutated in ≈15% of ADPKD patients. Here, we show that polycystin-2 is localized to the cilia of mouse and human vascular endothelial cells. We demonstrate that the normal expression level and localization of polycystin-2 to cilia is required for the endothelial cilia to sense fluid shear stress through a complex biochemical cascade, involving calcium, calmodulin, Akt/PKB, and protein kinase C. In response to fluid shear stress, mouse endothelial cells with knockdown or knockout of *Pkd2* lose the ability to generate nitric oxide (NO). Consistent with mouse data, endothelial cells generated from ADPKD patients do not show polycystin-2 in the cilia and are unable to sense fluid flow. In the isolated artery, we further show that ciliary polycystin-2 responds specifically to shear stress and not to mechanical stretch, a pressurized biomechanical force that involves purinergic receptor activation. We propose a new role for polycystin-2 in transmitting extracellular shear stress to intracellular NO biosynthesis. Thus, aberrant expression or localization of polycystin-2 to cilia could promote high blood pressure because of inability to synthesize NO in response to an increase in shear stress (blood flow). **(*Circ Res*. 2009;104:860-869.)**

Key Words: biophysical force ■ endothelia ■ mechanotransduction ■ primary cilium ■ shear stress

Autosomal dominant polycystic disease (ADPKD) (Online Mendelian Inheritance in Man 173900) is characterized by bilateral cyst formation in the kidneys. The only effective treatments presently available for ADPKD patients are renal dialysis and transplantation. Although the cystic kidney phenotype is the hallmark of ADPKD, a wide range of cardiovascular complications also affect a large number of ADPKD patients. In particular, hypertension occurs frequently and is an early manifestation of ADPKD.[1–4] Aside from hypertension, other vascular complications may include left ventricular hypertrophy, cerebral aneurysm, thoracic/abdominal aortic aneurysm, and prolapse of the mitral valve.[1,2] Although the etiology of these cardiovascular abnormalities is currently unclear, ADPKD patients are generally placed on antihypertensive therapy.

ADPKD is a genetic disease caused by the mutation of either *PKD1* or *PKD2*, which encode for polycystin-1 or polycystin-2, respectively. Whereas polycystin-1 is an 11-transmembrane protein with a long extracellular domain, polycystin-2 is a cation channel with 6-transmembrane domains and belongs to a superfamily of transient receptor potential (TRP) ion channels. To advance the understanding of the cellular and molecular mechanisms of ADPKD, several *Pkd2* mouse models have been generated. They, too, present degrees of cardiovascular abnormalities.[5] In particular, expression of polycystin-2 has been detected in vascular systems of mice[6,7] and humans.[7,8] In addition to focal hemorrhage, the *Pkd2* mouse exhibits progressive total body edema, a feature of cardiac failure.[5]

Polycystin-2 is a calcium channel whose activity and/or localization is modulated by polycystin-1.[9] Through antibody mediated inhibition of polycystin-2, we have previously shown that polycystin-2 may function as a mechanosensitive calcium channel in renal epithelial cells.[10] Polycystin-2 functioning as a fluid flow sensor has been further suggested in mouse nodal cells.[11] In the present study, we expand this observation and investigate the roles of polycystin-2 in sensing mechanical fluid shear stress in *Pkd2* knockdown and knockout mouse aortic and human endothelial cells. Mutations in *PKD2* have been suggested to contribute to vascular hypertension,[3,4] probably because of failure to convert an increase in mechanical blood flow into cellular nitric oxide

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(NO) biosynthesis to control the vascular tone, ie, blood pressure. To examine polycystin-2 roles in hypertension, we also measured intracellular NO in endothelial cells generated from ADPKD patients. We show that endothelial cells depend on polycystin-2 and a cascade of intracellular signaling molecules to synthesize NO in response to fluid shear stress.

Materials and Methods

Signed and informed consent to collect disposed ADPKD human kidneys was obtained from the patients, and kidney collection protocols were approved by the Department for Human Research Protections of the Biomedical Institutional Review Board of The University of Toledo. The use of animal tissues was approved by The University of Toledo animal care and use committee.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Polycystin-2 Is Localized to Endothelial Cilia and Functions in Mechanosensing

We previously showed that cilia are microsensory compartments that house sensory molecules and that polycystin-1 is among the sensory molecules.[12] Because polycystin-1 and -2 interact with each other,[9,13] we examined whether polycystin-2 could also localize to endothelial cilia like polycystin-1. We show here, for the first time, that polycystin-2 is localized to the cilia of the vascular endothelia in the femoral artery from an adult mouse (Figure 1a). Polycystin-2 is colocalized with acetylated α-tubulin, a well-recognized marker for cilia. Using high-resolution differential interference contrast imaging, we also show the presence of cilia in the arterial lumen. To study the mechanosensory function of polycystin-2, we first identified its presence in previously characterized endothelial cells from mouse embryonic aorta.[12] Polycystin-2 is clearly present in the cilia of cultured endothelial cells (Figure 1b). The cells were further validated to retain endothelial marker, CD144.

Because endothelial cell lines with *Pkd2* mutation do not exist, we used a small interfering (si)RNA approach to inhibit the expression level of polycystin-2. We designed several siRNA probes to target a series of *Pkd2* mRNA sites (supplemental Table I). The efficiency of transfection was verified by examining the transcript and expression levels of polycystin-2 (Figure 2a and 2b). We noted that the efficiency of siRNA approach on *Pkd2* depends largely on the siRNA probes; siRNA1 and siRNA4 appear to be more effective than siRNA2 and siRNA3. To assay mechanosensory cilia function, we perfused the cells with an optimal shear stress of 7 dyn/cm^2. This magnitude of shear stress provides the greatest increase in cytosolic calcium and NO production in endothelial cells, as determined previously.[12] When changes in cytosolic calcium in response to fluid flow were examined, cells transfected with siRNA1 and siRNA4 were less responsive toward fluid shear (Figure 2c). To confirm our calcium readout, we also monitored changes in cytosolic NO as an indication of NO biosynthesis (Figure 2d). Although variations in cytosolic calcium and NO were observed within a cell population, we consistently observed that in 3 independent experiments, cells transfected with siRNA1 or siRNA4 were less responsive to fluid shear (Figure 2e). Their calcium and NO responses were significantly repressed compared to corresponding calcium and NO in control groups.

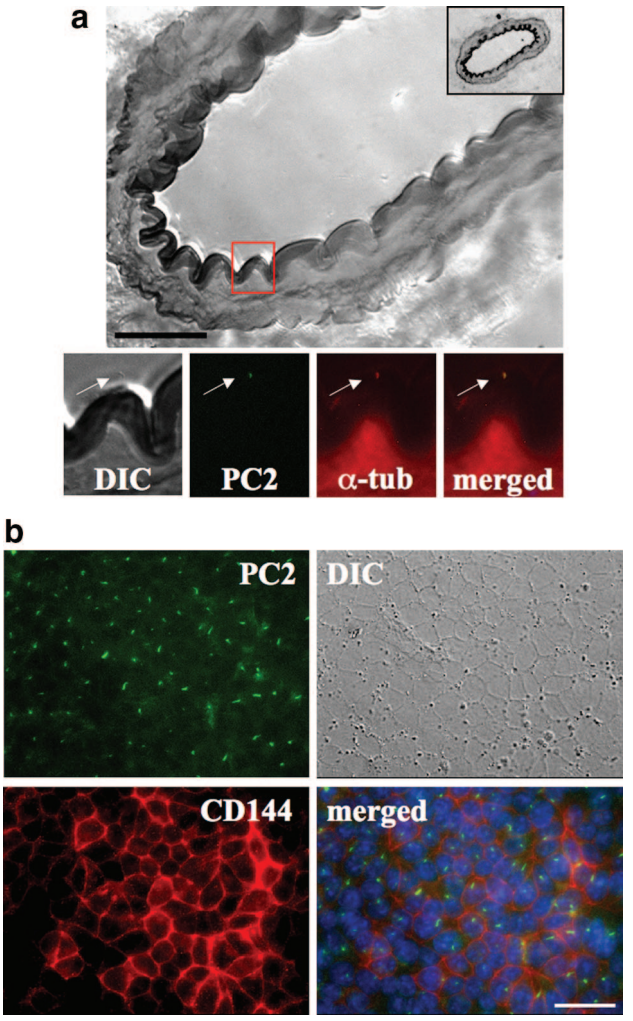

Figure 1. Polycystin-2 localization in vivo and in vitro. Localization of polycystin-2 (PC2) was examined with immunofluorescence staining. a, High-resolution differential interference contrast (DIC) image shows a section of femoral artery with a thickness of 10 μm. The inset in the top right corner shows the full section of the artery. The red box magnifies an endothelial cell, which shows localization of PC2 to endothelial cilia. Acetylated α-tubulin (α-tub) was used as a ciliary marker. b, Cultured endothelial cells also show the presence of polycystin-2 in cilia, and VE-cadherin (CD144) was used as an endothelial marker. Nuclear marker (DAPI) is shown in the merged images. Scale bar=25 μm.

Ciliary Polycystin-2 Is Functionally Relevant in Human Endothelial Cells

To examine the clinical relevance of polycystin-2, we isolated endothelial cells from interlobar arteries of nine ADPKD kidneys. Interestingly, we observed either a normal or null response within a diseased kidney. For example, within 5 successful endothelial isolations from a kidney of patient 5, endothelial cells from segment 7 consistently showed neither calcium nor NO responses (Figure 3a). On the other hand, cells from other segments responded to fluid shear stress by showing cytosolic calcium increases and NO biosynthesis.

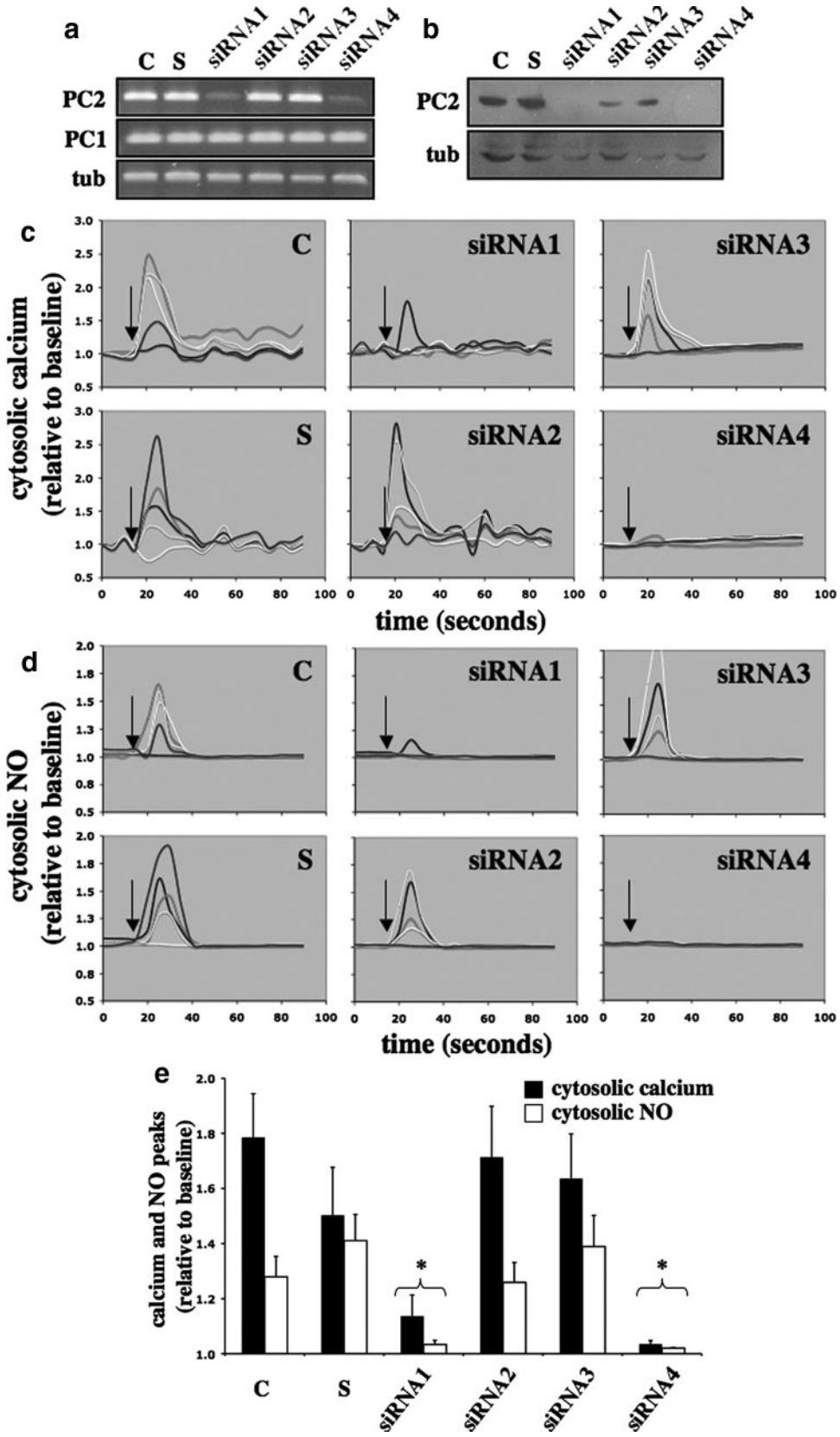

Figure 2. Effects of polycystin-2 expression in mouse endothelial cells. The expression level of polycystin-2 corresponds to the cytosolic calcium increase and NO biosynthesis in response to fluid shear stress. Mouse endothelial cells were transfected with Lipofectin only (control, C), scramble siRNA (S), siRNA1, siRNA2, siRNA3, or siRNA4 of polycystin-2. a, The transfected cells were collected to examine the transcript levels of polycystin-2 (PC2) and polycystin-1 (PC1), and α-tubulin (tub) was used as a control. b, The transfected cells were analyzed for polycystin-2 expression, and the membrane was reblotted for α-tubulin as a loading control. c, Transfected cells were challenged with fluid shear stress of 7 dyn/cm^2 (indicated by arrows), and 5 individual responses were randomly selected and analyzed for changes in cytosolic calcium levels. d, Biosynthesis of NO in response to shear stress was plotted in 5 individual cells. e, Peaks of cytosolic calcium and NO in response to shear stress were averaged. Asterisks indicate statistically significant against control at $P<0.05$ (N=3).

Surprisingly, the ciliary expression of polycystin-2 correlates with our fluid shear assays (Figure 3b). Similar findings from patient 6 are also presented (Figure I in the online data supplement). Although ADPKD kidneys that we obtained more likely had *PKD1* mutations than *PKD2* mutations, ≈85% compared to 15% of the ADPKD cases, respectively, we could confidently suggest that the failure of 5–7 endothelial cells (from patient 5, segment 7) to respond to fluid shear

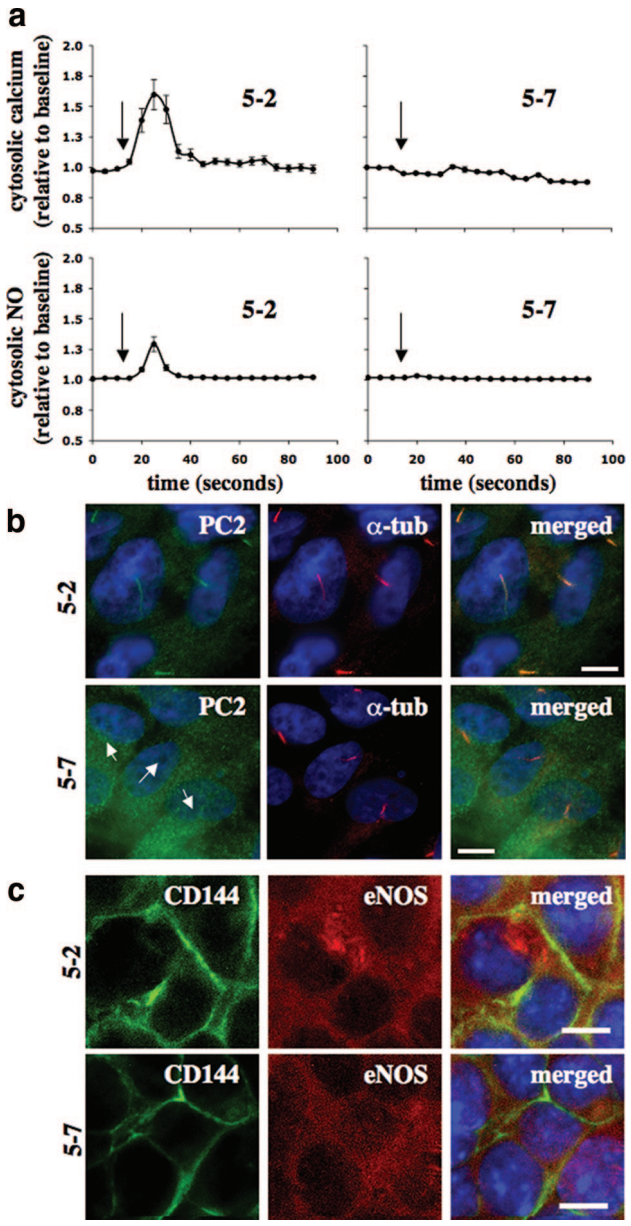

Figure 3. Effects of fluid shear stress in vascular endothelial cells of an ADPKD patient. Vascular endothelial cells were isolated from several interlobar arteries of an ADPKD kidney. a, Endothelial cells (5-2 and 5-7) from segmental arteries 2 and 7 of patient 5 were cultured and challenged with fluid shear stress, and their cytosolic calcium and NO changes were recorded. Arrows indicate the start of fluid flow. b, The primary culture was then subjected to immunolocalization 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. Arrows indicate the absence of polycystin-2. c, Immunofluorescence studies indicated the presence of endothelial markers CD144 and eNOS in both 5-2 and 5-7 cells. N=3 with passages 2, 3, and 4. Scale bar=5 μm.

stress is attributable, in part, to an absence of ciliary polycystin-2. In addition, we could show that 5–2 and 5–7 cells possessed endothelial markers CD144 and endothelial NO synthase (eNOS) (Figure 3c). Although we were not able to further analyze these cells because of the short passages of primary cultures, our Western blot analysis from pooled endothelial cells of patients 7, 8, and 9 confirms our cell isolation technique (supplemental Figure I).

To delineate the roles of polycystin-2 in human cells independently from polycystin-1 function, we used the siRNA approach on cultured human umbilical vein endothelial cells. Several siRNA probes were made against a series of *PKD2* mRNA sites (supplemental Table I). The efficiency of transfection was verified by quantifying the transcript and expression levels of polycystin-2 (Figure 4a and 4b). Similar to results from the mouse *Pkd2*, we noted that the efficiency of siRNA approach on human *PKD2* depends largely on the siRNA probes; siRNA2 and siRNA3 appear to be more effective than siRNA1 and siRNA4. To assay mechanosensory cilia function, we perfused the cells and measured changes in cytosolic calcium and NO in response to fluid flow. Cells transfected with siRNA2 and siRNA3 did not respond to fluid shear (Figure 4c). To complement our calcium readout, we also monitored changes in cytosolic NO (Figure 4d). Although variations in cytosolic calcium and NO were observed in individual cells, especially with siRNA4, we consistently observed that in 3 independent experiments, cells transfected with siRNA2 or siRNA3 did not respond or showed less response to fluid shear (Figure 4d). Their calcium and NO responses were statistically repressed, compared to corresponding calcium and NO in control groups.

Shear-Induced NO Biosynthesis Depends on Ciliary Polycystin-2 Calcium Channel

Together with mouse (Figure 2) and human (Figures 3 and 4) endothelial cells, we report here, for the first time, that endothelial cells ability to sense fluid shear stress depends on the expression level and/or ciliary localization of polycystin-2. In addition, a "2-hit" mechanism has been suggested in ADPKD.[14,15] This mechanism explains that patients would inherit a germ line mutation from one of the parents, and a random second somatic mutation is required to facilitate the disease phenotypes. To examine this possibility in vascular hypertension in PKD and to further verify our data, especially those obtained from human ADPKD patients, we used a *Pkd2* mouse model to compare fluid sensing ability of $Pkd2^{+/-}$ and $Pkd2^{-/-}$ primary endothelial cells. Unlike $Pkd2^{+/-}$ cells, $Pkd2^{-/-}$ endothelial cells did not respond to fluid shear stress (Figure 5a). The $Pkd2^{+/-}$, but not $Pkd2^{-/-}$, cells show cytosolic calcium increases in response to fluid shear. Extracellular and intracellular measurements of NO were in agreement with the results from the calcium readout. The subcellular ciliary localization of polycystin-2 was also examined (Figure 5b). As expected, we never observed ciliary polycystin-2 in $Pkd2^{-/-}$ cells. To examine differential expressions of polycystin-1 in $Pkd2^{+/-}$ and $Pkd2^{-/-}$ endothelial cells, we performed immunoprecipitation studies. When polycystin-1 was immunoprecipitated, no apparent difference was observed in polycystin-1 expressions between $Pkd2^{+/-}$ and $Pkd2^{-/-}$ endothelial cells (Figure 5c, i). Because we could reblot for polycystin-2 in $Pkd2^{-/-}$ cells, this study further indicates that polycystin-1 interacts with polycystin-2 in vascular endothelial cells. We next immuno-

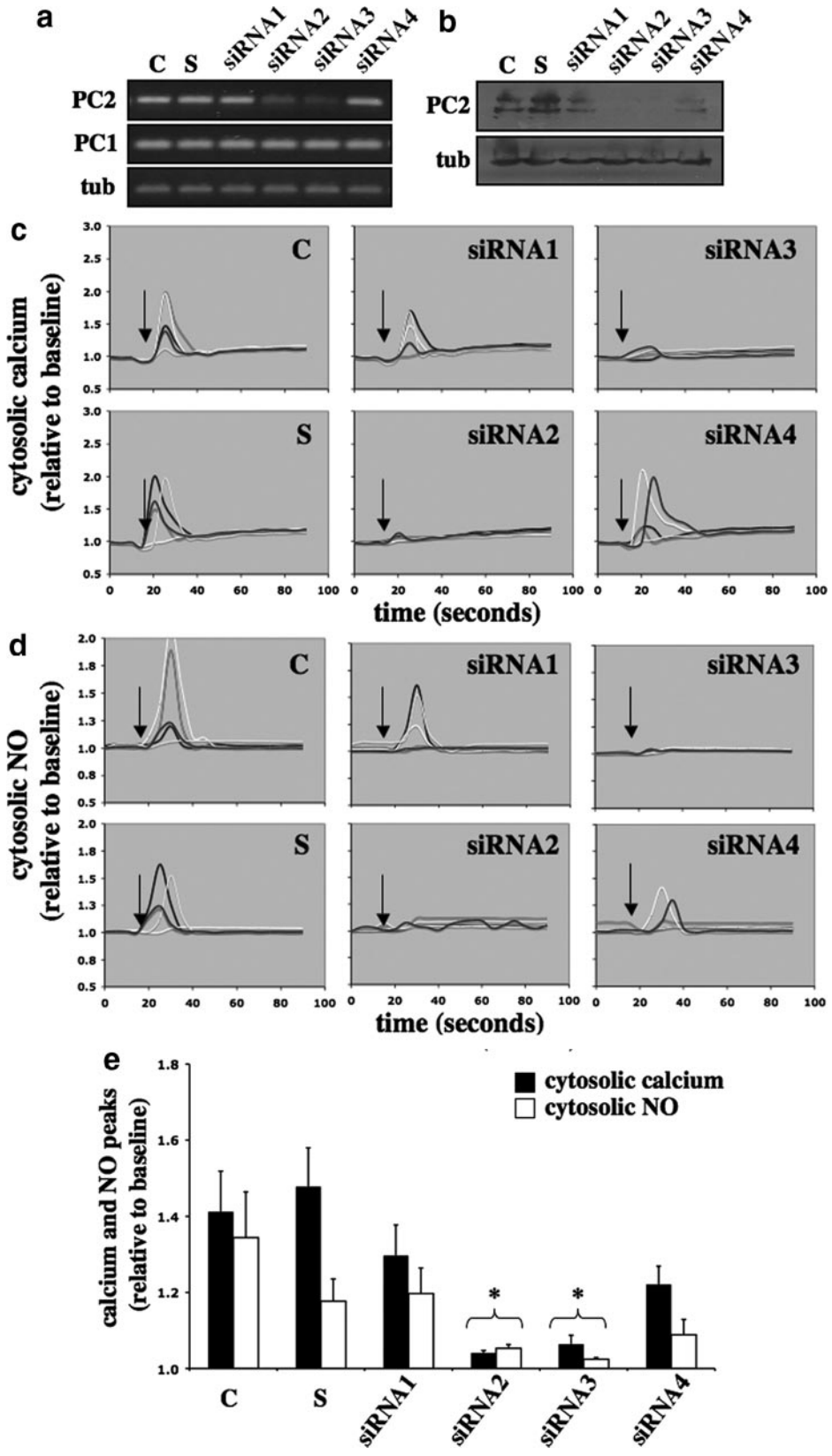

Figure 4. Effects of polycystin-2 expression in human endothelial cells. The expression level of polycystin-2 corresponds with the cytosolic calcium increase and NO biosynthesis in response to fluid shear stress. Human umbilical vein endothelial cells were transfected with Lipofectin only (control, C), scramble siRNA (S), siRNA1, siRNA2, siRNA3, or siRNA4 of polycystin-2. a, The transfected cells were collected to examine the transcript levels of polycystin-2 (PC2) and polycystin-1 (PC1), and α-tubulin (tub) was used as a control. b, The transfected cells were analyzed for polycystin-2 expression, and the membrane was reblotted for α-tubulin as a loading control. c, Transfected cells were challenged with fluid shear stress of 7 dyn/cm^2 (indicated by arrows), and 5 individual responses were randomly selected and analyzed for changes in cytosolic calcium levels. d, Biosynthesis of NO in response to shear stress was plotted in 5 individual cells. e, Peaks of cytosolic calcium and NO in response to shear stress were averaged. Asterisks indicate statistically significant against control at $P<0.05$ (N=3).

precipitated polycystin-2 and blotted for both polycystin-1 and -2 to demonstrate that polycystin-1 and -2 interaction could be confirmed reversibly (Figure 5c, ii). In all cases, polycystin-2 expression was not detected in *Pkd2*$^{-/-}$ endothelial cells. We further showed that *Pkd2*$^{+/-}$ and *Pkd2*$^{-/-}$ primary endothelial cells within passages 2, 3, and 4 consistently retain endothelial markers, such as eNOS, CD144, and Akt (Figure 5d).

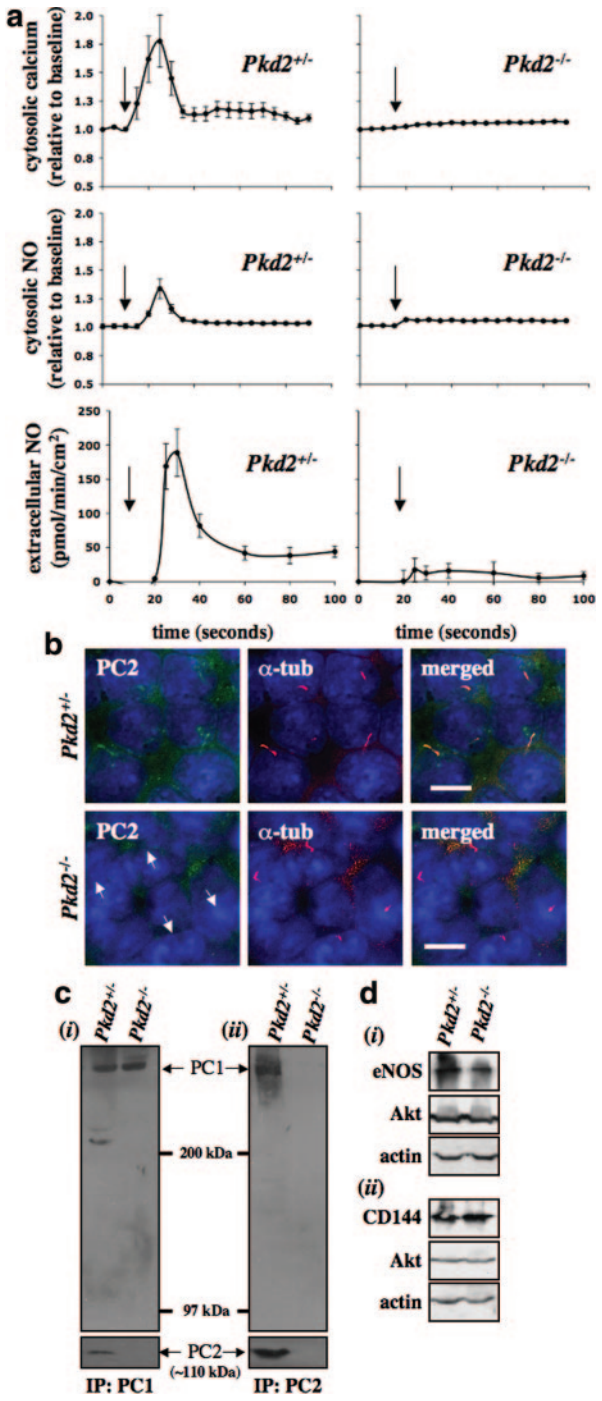

Figure 5. Polycystin-2 required in fluid-flow sensing. Vascular endothelial cells were isolated from *Pkd2*$^{+/-}$ and *Pkd2*$^{-/-}$ embryonic aortas. a, The endothelial cells were cultured and challenged with fluid shear stress, and their cytosolic calcium increase, intracellular NO production, and extracellular NO release were measured. Arrows indicate the start of fluid flow. b, The primary culture was then subjected to immunolocalization 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. Arrows indicate the absence of polycystin-2. c, Immunoprecipitation studies for polycystin-1 (i) and -2 (ii) confirmed interaction of polycystin-1 and -2 in vascular endothelial cells and absence of polycystin-2 in *Pkd2*$^{-/-}$ cells. d, The presence of endothelial markers eNOS (i), CD144 (ii), and Akt (i and ii) confirmed the cell type used in our study, and β-actin was used as a loading control. N=3 for calcium and NO measurements with passages 2, 3, and 4. Scale bar=5 μm.

Polycystin-2–Dependent NO Production Involves a Cascade of Signaling Molecules

In this study, we propose that ciliary polycystin-2 is a shear-sensitive calcium channel that is required to activate a biochemical cascade for NO production. To confirm that our biophysical calcium and biochemical NO readouts are biologically and technically relevant, we applied various inhibitors to block the molecular functions that are supposedly involved in shear-induced NO production.[16] Removing extracellular calcium with EGTA abolished both calcium and NO readouts in wild-type endothelial cells, indicating that extracellular calcium influx is a prerequisite for both cytosolic increase in calcium and NO production (Figure 6). We also confirmed that eNOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) could block shear-induced NO biosynthesis but not cytosolic calcium increase. To explore calcium-dependent mechanisms of NO production, we used calphostin C and W7 to inhibit calmodulin and calcium-dependent protein kinase (PK)C and calmodulin, respectively. When these calcium-binding proteins were inhibited, calcium, but not NO, readout showed an increase, indicating that PKC and calmodulin act downstream of the calcium pathway and that inhibition of either molecule will block NO synthesis.

To explore the possibility of Akt or PKB contribution to shear stress–induced NO production,[16] we treated wild-type cells with Akt inhibitor II. Inhibition of Akt/PKB resulted in blockage of NO readout but did not alter calcium signaling. In addition to calmodulin, phosphoinositide 3-kinase (PI3K) is also a major regulator for the Akt/PKB pathway.[16] To further examine the roles of PI3K in Akt/PKB function, we treated the cells with either LY-294,002 or wortmannin (not shown). Interestingly, neither of these inhibitors significantly inhibited calcium signaling or NO production in response to fluid shear stress. Together, our data suggest that calcium is an important messenger for relaying extracellular fluid flow stimuli to intracellular NO production through ciliary polycystin-2 calcium channel.

Ciliary Polycystin-2 Is a Shear Stress–Specific Molecule

To investigate mechanosensory polycystin-2 function in more detail, we perfused isolated artery that had been transfected with either scrambled or *Pkd2* siRNA. Artery with scrambled siRNA was either used as a control or further treated with apyrase. In a freely placed artery, a flow rate of 164 μL/sec resulted in cytosolic calcium increases (Figure 7a). In a control artery, a continuous fluid flow resulted in sustained increase in cytosolic calcium (Figure 7a and 7c). Interestingly, an artery that had been pretreated with apyrase and was perfused with apyrase showed an increase in cytosolic calcium, but with a very different calcium profile than observed in the control group. A smaller but similar calcium profile than in the control group was observed in the artery transfected with *Pkd2* siRNA. Because, at a higher microscopic magnification, we observed that the freely placed artery was moved as a result of the motion from the luminal fluid perfusate, we predicted that the movement would result in stretching-like motion on the arterial wall. Consistent with this idea, we hypothesize that the luminal wall stretching

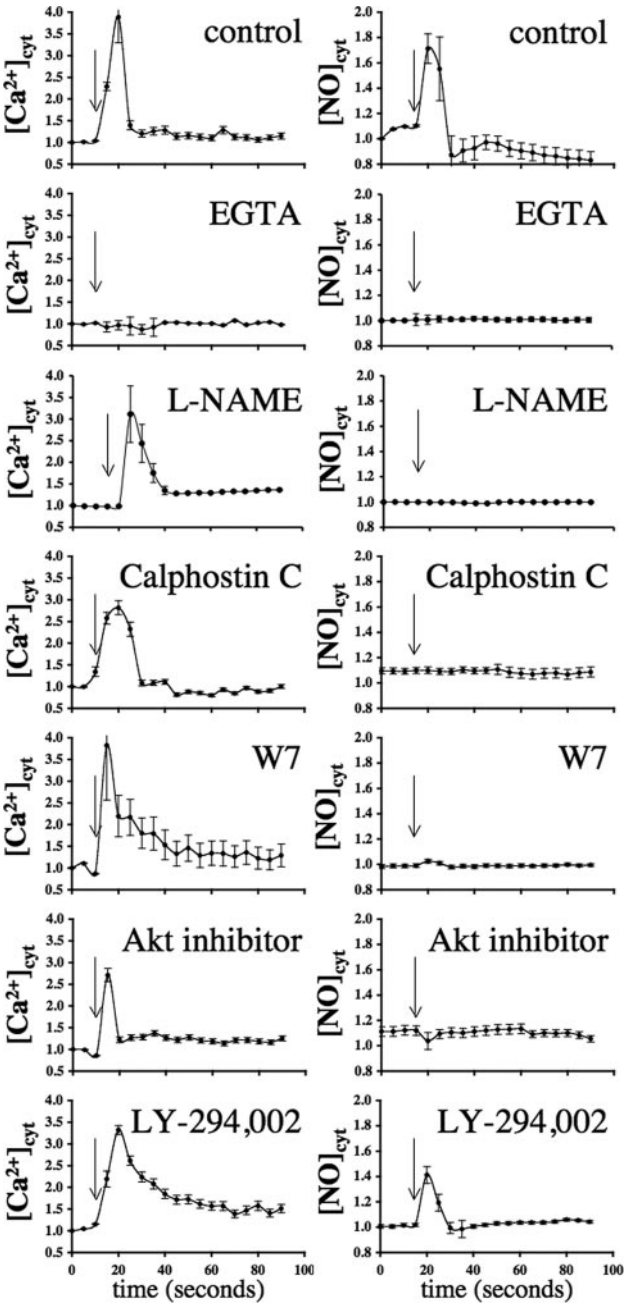

Figure 6. Molecular cascade involved in shear stress–induced calcium and NO signaling. The left and right graphs represent the response to fluid shear stress in mouse endothelial cells for cytosolic calcium ($[Ca^{2+}]_{cyt}$) and NO ($[NO]_{cyt}$), respectively. Effects of various inhibitors on response to fluid shear stress, as indicated by arrows, are presented in the line graphs. The extracellular calcium chelator EGTA abolished both calcium and NO increases, indicating extracellular calcium influx is required in shear-induced NO biosynthesis. NO synthase inhibitor L-NAME indicated that shear-induced NO production involves a rapid activation of NO synthase. Whereas PKC (calphostin C), calmodulin (W7), and Akt inhibitors played a role in mechanical fluid sensing, PI3K inhibitor (LY-294,002) did not affect shear-induced NO production. N≥4.

would result in sustained cytosolic calcium increase, a mechanism that would involve ATP release.[17,18] Furthermore, it is worth mentioning that the calcium profiles in apyrase-treated arteries and in isolated endothelial cells are very similar (Figures 2 through 6), indicating that apyrase might have diminished the stretch-induced calcium response in a freely placed artery.

To further confirm this possibility, we carefully inserted an artery into a glass capillary tube (Figure 7b). The aorta inside the capillary tube had very limited room for perfusate pressure-induced arterial stretching or expending. In this capillary-enclosed setting, neither control nor treated arteries showed a sustained increase in cytosolic calcium in response to a similar flow rate of 164 μL/sec (Figure 7c). Most important is that the *Pkd2* siRNA artery did not show a significant increase in cytosolic calcium, although it still responded to ATP (not shown). To verify these findings, we challenged both $Pkd2^{+/-}$ and $Pkd2^{-/-}$ endothelial cells with ATP in the presence and absence of EGTA (Figure 7d). Because $Pkd2^{-/-}$ endothelial cells were able to respond to ATP and because *Pkd2*-depleted arteries could respond to mechanical fluid flow in freely placed but not in capillary-enclosed settings, we propose that polycystin-2 functions as a mechanical channel and has a specific role in fluid shear sensing. We, therefore, propose that ciliary polycystins are only few examples of a large family of sensory proteins that a cell may have. Thus, depending on its sensory proteins, an endothelial cell could have different mechanisms to detect a range of mechanical stimuli.

Discussion

Dysfunction of many ciliary proteins has been linked to a list of human diseases, from cystic kidney and obesity to blindness and mental retardation. Although many ciliary functions have been proposed,[19] their mechanical function as microsensory compartments has been the most described.[20–22] In our present study, we suggest that polycystin-2 is a ciliary calcium channel that functions as one of the sensory machineries in endothelial cells. Our study also indicates that abnormality in polycystin-2 expression, localization and/or function is related to the inability of endothelial cells to generate NO in response to fluid shear stress. We further propose that failure to produce NO in response to shear stress is clinically relevant to the development of hypertension, particularly in PKD patients.

In the present study, we show, for the first time, that polycystin-2 is localized to endothelial cilia in cell culture and in vivo. We studied polycystin-2 extensively, using an siRNA approach and genetic model in mouse and human vascular endothelial cells. Although our siRNA approach using mouse endothelial cells did not provide similar inhibition levels of polycystin-2 expression, the transcript and expression levels were well correlated with the overall endothelial cell response to fluid shear. To confirm that polycystin-2 function is clinically relevant, we isolated interlobar endothelial cells from ADPKD kidneys. For each diseased kidney, however, we observed a mixed response from different arterial segments. This result is consistent with our previous findings whereby not all ADPKD kidney epithelial cells are irresponsive to fluid shear stress.[23] We and others have found that only epithelial cells isolated from cyst-linings that do not show polycystin-1 or -2 localization to cilia are abnormal in flow sensing.[23,24] In agreement with

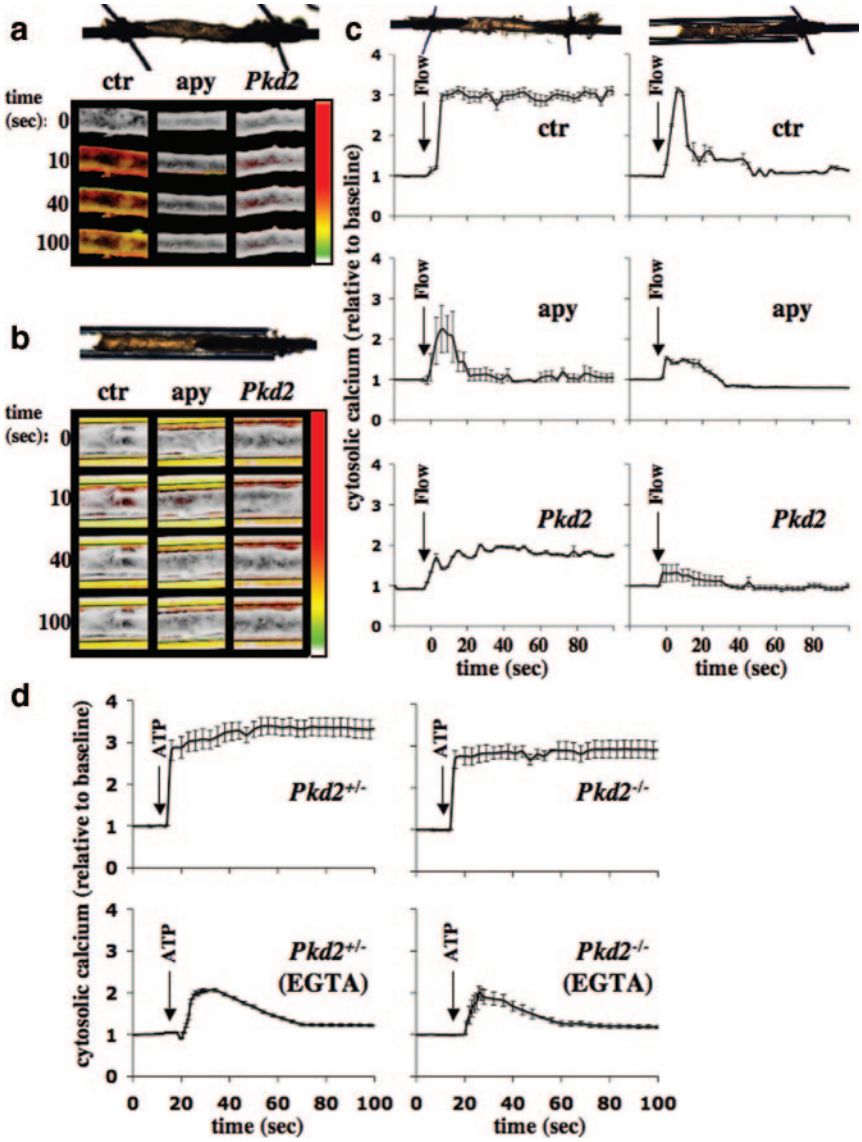

Figure 7. Integration of polycystin-2 and purinergic signaling in isolated artery. To differentiate mechanical forces generated by perfusate, an isolated artery was freely placed to provide unrestrained movement, or the artery was capillary-enclosed to limit stretching motion. Artery transfected with scrambled siRNA is denoted as control (ctr), whereas artery transfected with scrambled siRNA and pretreated and perfused with apyrase is designated as apyrase (apy). Isolated artery was also transfected with *Pkd2* siRNA (*Pkd2*). a, In the freely placed artery, as illustrated by the top image, calcium imaging studies show different cytosolic calcium profiles among control, apyrase, and *Pkd2*-treated groups. b, In the capillary-enclosed artery, as illustrated by the top image, flow-induced cytosolic calcium increase was diminished in *Pkd2*, compared to control or apyrase-treated arteries. Changes in cytosolic calcium was pseudocolored, white/green represents a low level, and yellow/red denotes a higher level of cytosolic calcium. In all cases, a similar fluid-flow rate of 164 μL/sec was initiated after time 0 second. c, Five responsive areas within the artery, if any were present, were randomly selected and analyzed for changes in cytosolic calcium levels. d, Both $Pkd2^{+/-}$ and $Pkd2^{-/-}$ endothelial cells were able to respond to 10 μmol/L ATP in the presence or absence of 1 mmol/L EGTA. N=3 arteries for each group and treatment, and N>2 for cells with passage 2, 3, or 4.

this notion, our data suggest that ciliary localization of polycystin-2 is required in fluid shear sensing. Moreover, we have also shown that ciliary localization of polycystin-2 could depend on functional polycystin-1 to cilia in human and mouse cells.[10,23] Therefore, mutation(s) in *PKD1* may alter subcellular ciliary localization of polycystin-2. Nonetheless, proper ciliary localization and function of polycystin-2 are requirements for fluid sensing in the endothelial cells.

We further hypothesize that vascular endothelia also require a "second-hit" in ADPKD in a similar manner to renal epithelia.[14,15] This implies that a germ-line mutation (heterozygous) may not be sufficient to cause any clinical symptoms such as hypertension, but another random somatic mutation (homozygous) is required. To examine this possibility, we used a *Pkd2* mouse model to compare $Pkd2^{+/-}$ and $Pkd2^{-/-}$ endothelial cells in response to fluid flow. In fact, there was only 1 study that assessed sensory polycystin-2 function using *Pkd2* mouse model.[11] Regardless, the results support our hypothesis that unlike $Pkd2^{-/-}$ cells, $Pkd2^{+/-}$ endothelial cells maintain responsiveness to fluid flow. More importantly, our studies confirm that polycystin-2 is an important shear-sensitive calcium channel in endothelial cells.

Although polycystin-1 and -2 have been shown to interact at the COOH termini,[9,13] there is no study in vascular endothelial cells examining polycystin-1 and -2 interaction. Through coimmunoprecipitation studies, we confirmed that in endothelial cells, both polycystins interact to one another reciprocally. There were no apparent changes in polycystin-1 level between $Pkd2^{+/-}$ and $Pkd2^{-/-}$ endothelial cells. In lieu of these results, we propose that polycystin-1 mechanosensor interacts with polycystin-2 calcium channel, and this polycystin complex localizes in the microsensory compartment, cilium. An abrupt increase in blood pressure would result in fluid shear increase, followed by activation of cilia and polycystin complex to generate NO.

Throughout our studies, we used 2 different readouts to confirm the fluid shear sensing ability of the endothelial cells. Whereas the calcium readout is biophysically pertinent to basic science, NO is biochemically more relevant to the

etiology of hypertension. Interestingly, we observed that if a *Pkd2* knockdown or knockout cell shows a negative calcium readout, the NO readout is also negative and vice versa. To test the hypothesis that increases in cytosolic calcium are a prerequisite signaling event for NO biosynthesis, we used EGTA to chelate extracellular calcium. In the absence of extracellular calcium, the cytosolic calcium and NO increases were abolished, indicating that fluid shear sensing involves extracellular calcium influx, which in turn is required for NO production. To further verify our flow assay on the signaling event for NO biosynthesis, we used L-NAME to inhibit eNOS. As expected, L-NAME inhibited NO production but not calcium signaling in response to fluid flow. Because eNOS has a specific phosphorylation site for PKC,[16] whose activity depends on calcium, we used calphostin C to demonstrate that PKC is required for shear-induced eNOS activation.

Because eNOS activation depends biochemically on calmodulin as a cofactor, we used W7 to inhibit calmodulin function. Our data shows that similar to L-NAME, W7 inhibited NO production but not calcium signaling. Not only was calmodulin a cofactor for eNOS, calcium–calmodulin complex has also been shown to activate Akt/PKB activity.[16] To investigate whether Akt/PKB is involved in eNOS activity, we applied Akt inhibitor II in our system. Our data indicate that Akt/PKB is also involved in regulation of eNOS activation in response to fluid shear. In addition to calmodulin, Akt/PKB is also regulated by PI3K, which has been shown to be involved in shear stress–induced NO release.[16] However, PI3K did not seem to play a major role in shear-induced eNOS activation, at least in our system. Collectively, our study suggests that endothelial cells require functional mechanosensory cilia and a list of intermediate machineries to produce NO in response to fluid shear stress. Upon sensing this mechanical signal, polycystin-2 promotes extracellular calcium influx that, in turn, activates PKC and binds to calmodulin; the calcium–calmodulin complex then increases Akt/PKB activity. Activation of eNOS by calmodulin, PKC, and Akt/PKB initiates an immediate NO synthesis.

Biomechanical forces in the blood vessel can be observed in the many forms, including stretch resulting from muscle distention caused by blood pressure and shear stress resulting from drag force generated by blood flow. To differentiate these mechanical forces, we designed a capillary-enclosed system that would allow an isolated artery to experience shear stress only. In a step increase in fluid flow, the capillary-enclosed artery showed a short burst increase in cytosolic calcium, similar to those seen in perfused cultured cells. On the other hand, the conventional freely placed artery, which induced stretch and increased in arterial diameter, showed a sustained increase in cytosolic calcium in response to fluid flow. Stretch-induced ATP release has been shown in many systems,[17] including in endothelial cells.[18] To investigate this purinergic involvement in our system, the artery was first treated with apyrase to hydrolyze any nucleoside triphosphates or diphosphates. Our data show that apyrase-treated artery has a very different calcium profile, indicating that ATP may play a role in stretch-induced calcium increase in freely placed artery.

To verify that $Pkd2^{-/-}$ endothelial cells did not have abnormal response to ATP, we challenged both $Pkd2^{+/-}$ and $Pkd2^{-/-}$ cells with ATP in the absence and presence of extracellular calcium chelator, EGTA. Cells from both genotypes demonstrated similar calcium profiles in response to ATP, with or without EGTA. It is worth noting that EGTA abolished flow-induced, but not in ATP-induced, calcium changes, demonstrating the complexity of mechanosignal transduction systems in vasculature. Consistent with this idea, shear stress has been shown to potentiate ATP-induced cytosolic calcium increase.[25] To further establish the mechanosignaling complexity, we have previously demonstrated that although $Pkd1^{-/-}$ endothelial cells failed to respond to shear stress, they were able to respond to other mechanical and pharmacological stimuli.[12] Similarly, $Pkd2^{-/-}$ endothelial cells lost their responsiveness to shear stress but not to ATP. This confirms our data from mouse and human endothelial cells that polycystin-2 has a specific shear-sensing role in vascular endothelial cilia.

All in all, our present study helps to explain the hypertensive phenotype seen in patients with ADPKD. We show that polycystin-2 in cilia plays crucial roles in the mediation of fluid shear-sensing, as well as the transduction of these mechanical signals into changes in calcium signaling and NO synthesis in endothelial cells. Hence, ciliary polycystin-2 may play crucial roles in the regulation of cardiovascular homeostasis. In view of the data presented here, we propose that abnormal ciliary polycystin-2 functions can lead to compromised fluid sensing which will further impair synthesis of NO, a mediator for other downstream signaling pathways in smooth-muscle relaxation.

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Disclosures

None.

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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

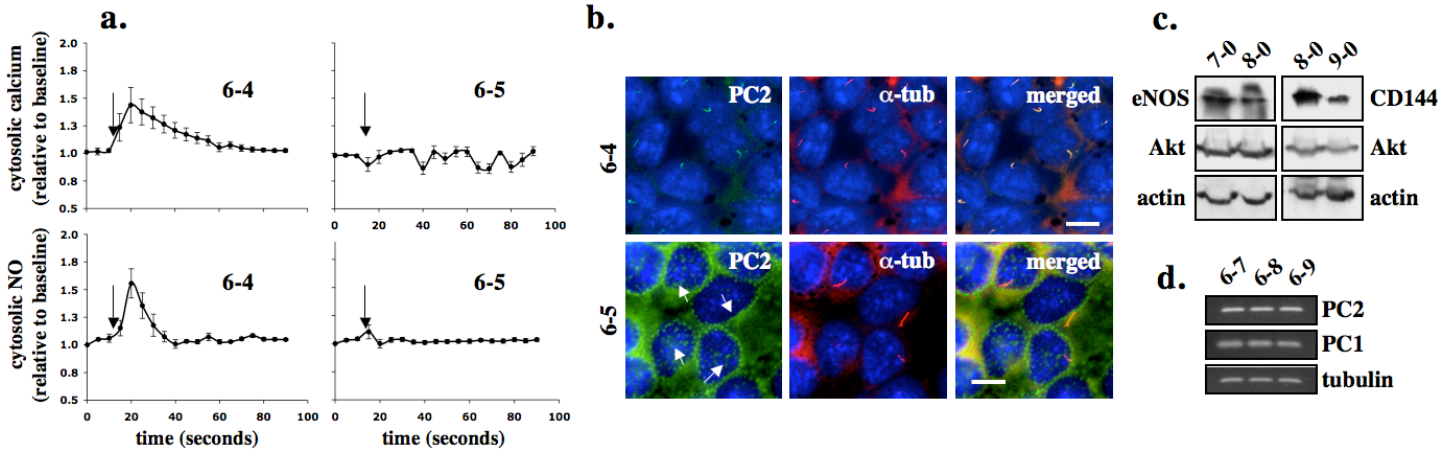

Figure Legend: Effects of fluid-shear stress in vascular endothelial cells of an ADPKD patient

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.

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