Dopamine receptor type-5 in the primary cilia has a dual chemo- and mechano-sensory role.

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Running Title: vascular hypertension in polycystic kidney disease

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SUPPLEMENT MATERIAL

I. Materials and Methods

Animal and cell culture

In our studies, we used vascular endothelial cells that were previously generated and characterized for various surface and intracellular markers[1,2]. Cells were grown on a glass surface that had been coated with rat type I collagen and sterilized under UV light. Cells were grown to a confluent monolayer in Dulbeccos's Modified Eagle Medium with 2% fetal bovine serum at 39 ºC and a constant 5% CO_2 and 95% O_2 mixture for at least 2 to 3 days before the experiments.

Qualitative PCR analysis

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

Cilia analysis and measurement

Primary cilia were observed with fluorescence and scanning electron microscopes. For fluorescence microscopy, cells or femoral arteries were first fixed with 4% paraformaldehyde in 2% sucrose solution for 10 minutes at room temperature. Dopamine receptor-type 5 (EMD/MercSciences; 1:2500 dilution, 72 hours at 4 °C) and type 3 (Calbiochem; 1:5,000 dilution, 72 hours at 4 °C)-specific antibodies were used. Acetylated-α-tubulin (Sigma clone 6-11B-1; 1:10,000 dilution) was used both as a cilia marker and to measure cilia length. The cover slip was then mounted on the microscope slide with mounting media containing dapi. When femoral artery was used, a segment of about 2 mm was cut open. The open lumen containing endothelia was then covered with microscope cover slip. Images were observed in an inverted Nikon Ti-U microscope and analyzed with Metamorph 7.0. All image analyses were performed by capturing series of Z-stack and compiled for a more accurate measurement.

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

siRNA transfection

To examine cellular function of dopamine, various siRNAs were designed to knock down dopamine receptor type 5. Dividing cells were transfected with lipofectamine (Invitrogen), scramble siRNA, siRNA1 (5'-AUC AUG UGG ACA UAG GCA GCA GCG A-3'), siRNA2 (5'-AUG ACC AGC AAU GCC ACG AAG AGG U-3'), or siRNA3 (5'-CAC ACU AGG ACG UUG CCG AGC AAG G-5'). All siRNAs were conjugated with GFP to monitor transfection efficiency, and 24 nM was used with transfection efficiency of about 95%.

Cytosolic calcium measurement

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

Protein detection

Protein concentration was first measured using BSA kit (Pierce) with the linear regression of the standards of 0.999. A total protein of 50 or 150 mg was analyzed with a standard Western blot. The following antibodies and dilutions were used in our analyses: phospho-cofilin (Cell Signaling, 1:100), anti-cofilin (Cell Signaling, 1:250), dopamine receptor type5 (Calbiochem, 1:200), α-tubulin (Abcam, 1:5,000), actin (Sigma, 1:500) and GAPDH (Cell Signaling, 1:1,500). Expression levels were quantified using NIH's ImageJ software.

Pharmacological treatment

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

Statistical Analysis

All quantifiable experimental values are expressed as mean±SEM, and values of $p<0.05$ were considered significant. All comparisons between two groups were performed with student's t-test with 2 samples assuming unequal variance. Comparisons of three or more groups were done using ANOVA, followed by Tukey's posttest. Data analysis was performed using Sigma Plot software version 11.

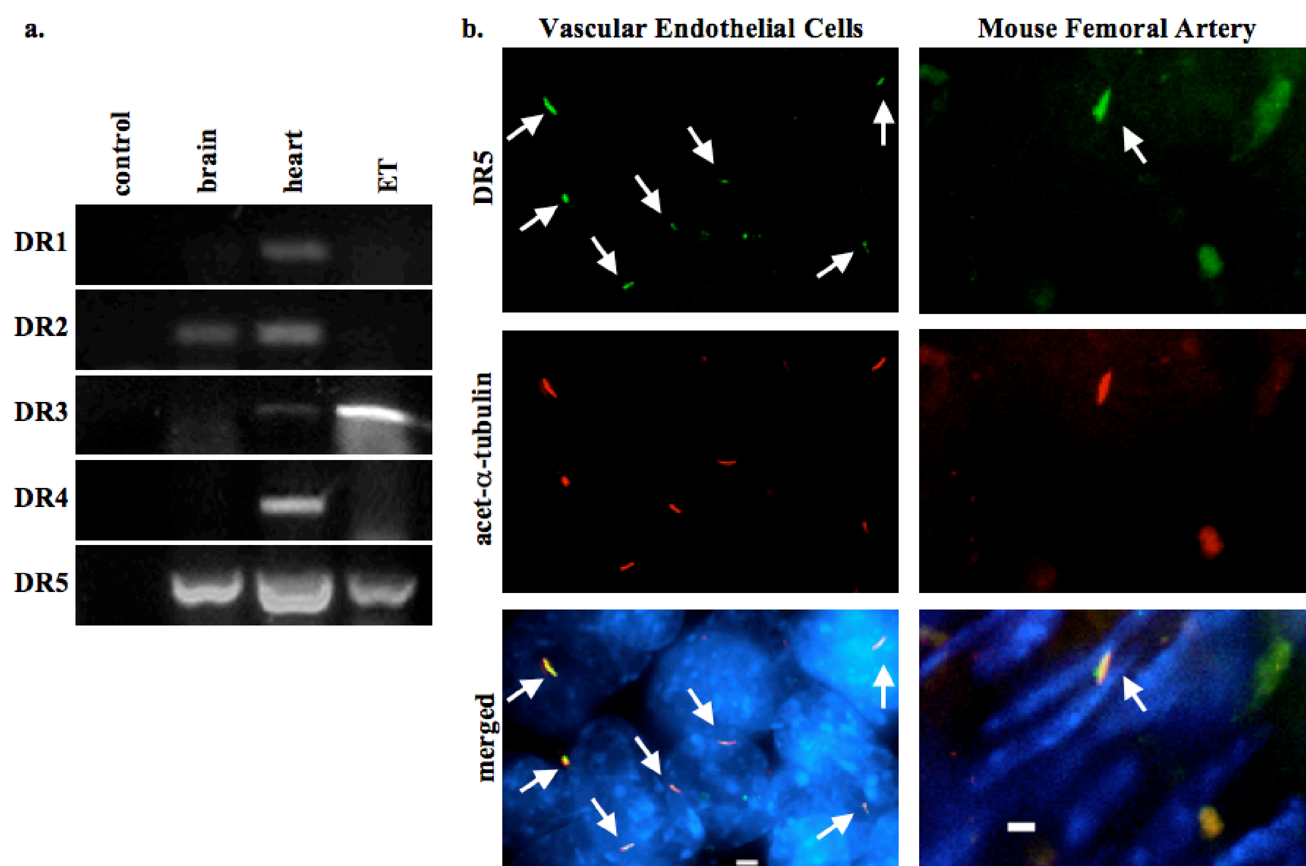
II. Online References

1. AbouAlaiwi WA, Takahashi M, Mell BR, Jones TJ, Ratnam S, Kolb RJ, Nauli SM. Ciliary polycystin-2 is a mechanosensitive calcium channel involved in nitric oxide signaling cascades. *Circulation research.* 2009;104:860-869.

2. Nauli SM, Kawanabe Y, Kaminski JJ, Pearce WJ, Ingber DE, Zhou J. Endothelial cilia are fluid shear sensors that regulate calcium signaling and nitric oxide production through polycystin-1. *Circulation.* 2008;117:1161-1171.

III. Online Figures

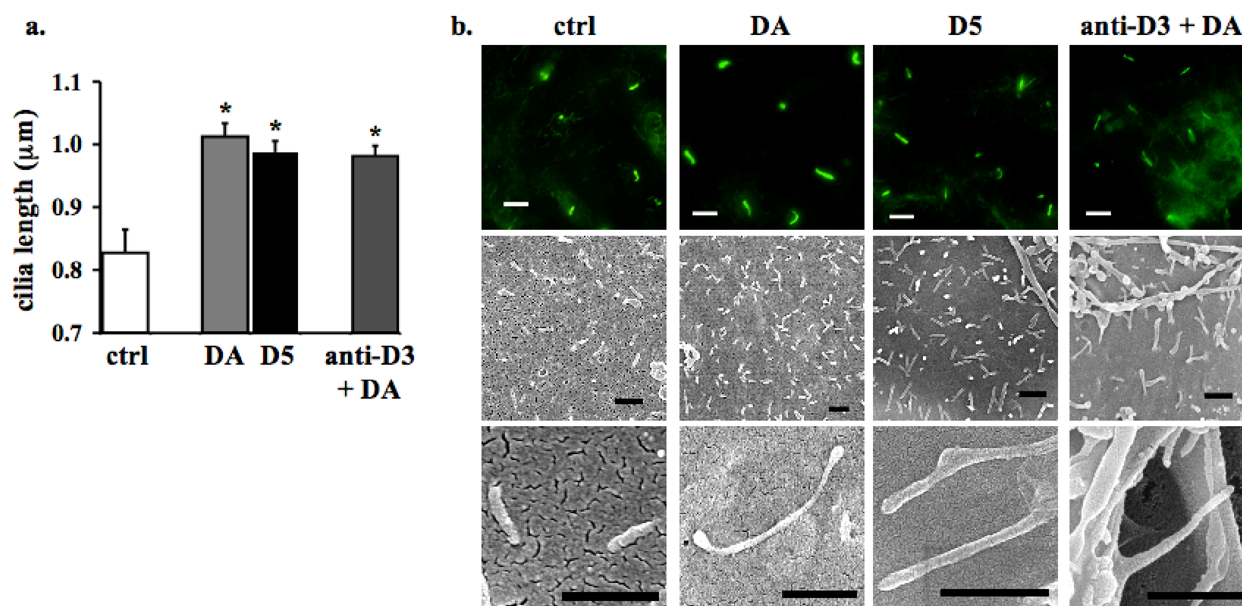
Online Figure 1



<u>Supplemental Figure 1. Dopamine receptor is expressed in endothelial cells.</u>

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

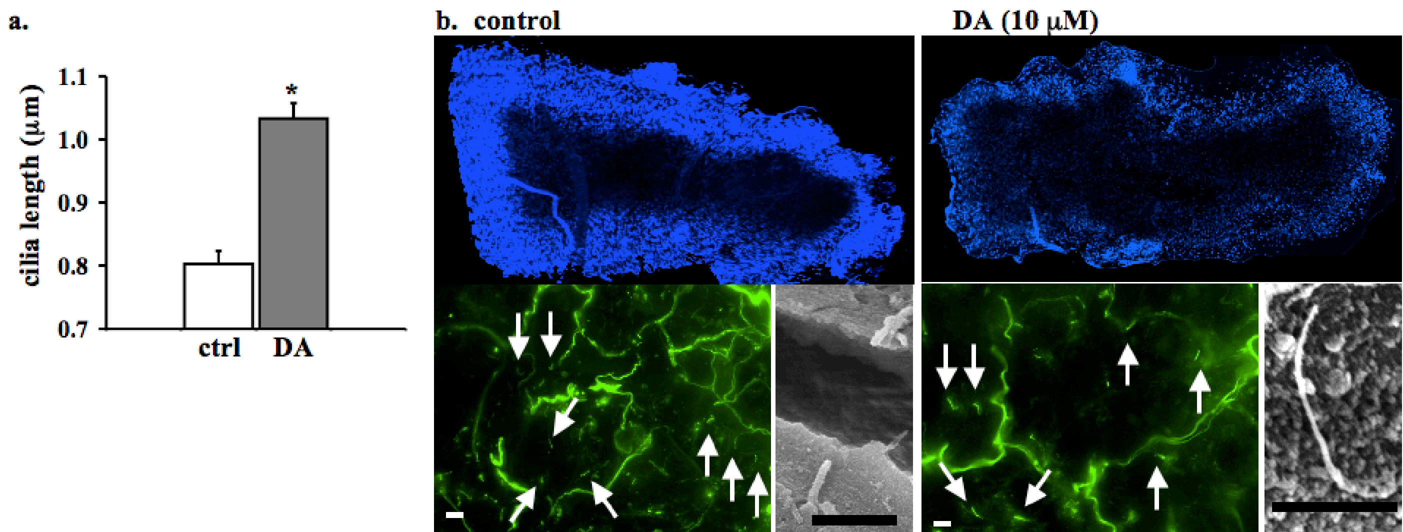
Online Figure 2



Supplemental Figure 2. Dopamine increases ciliary length in cultured endothelial cells.

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

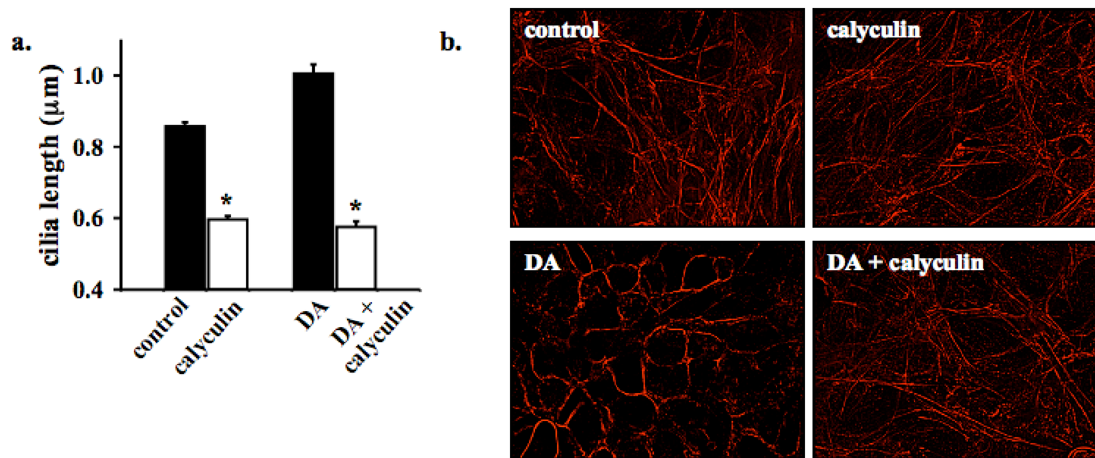
Online Figure 3



Supplemental Figure 3. Dopamine increases ciliary length in endothelia of femoral artery.

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

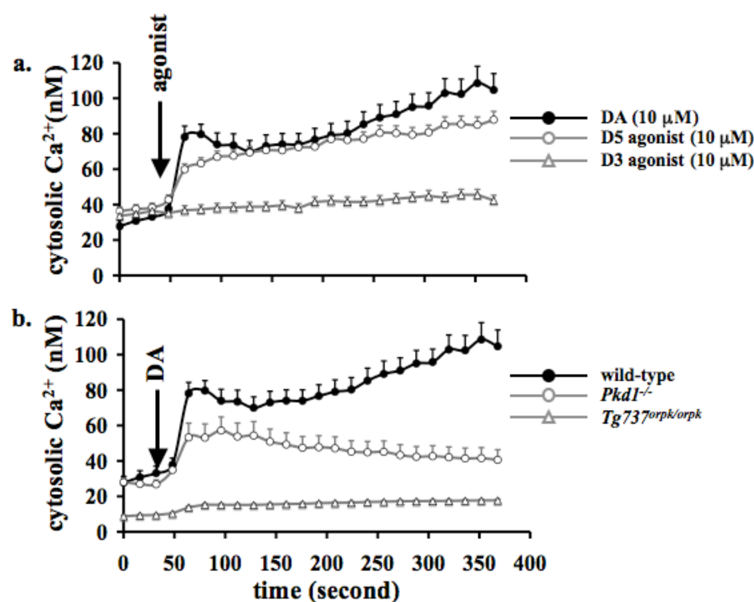
Online Figure 4



Supplemental Figure 4. Protein phopatase-1 plays an important role in dopamine-induced cilia length and actin rearrangement.

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

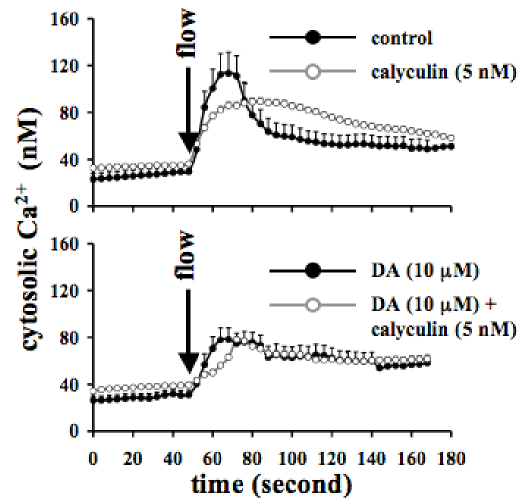
Online Figure 5



Supplemental Figure 5. Chemosensory function of DR5 depends on proper cilia structure.

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

Online Figure 6



<u>Supplemental Figure 6. Protein phopatase-1 plays a minor role in cilia function.</u>

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