Published in final edited form as:
Hypertension. 2011 August ; 58(2): 325–331. doi:10.1161/HYPERTENSIONAHA.111.172080.

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

Shakila Abdul-Majeed and **Surya M. Nauli**
Departments of Medicinal and Biological Chemistry, Pharmacology, and Medicine, The University of Toledo, Toledo, OH 43614

Abstract

Polycystic kidney disease (PKD) is characterized by cardiovascular irregularities, including hypertension. Dopamine, a circulating hormone, is implicated in essential hypertension in humans and animal models. Vascular endothelial primary cilia are known to function as mechano-sensory organelles. Though both primary cilia and dopamine receptors play important roles in vascular hypertension, their relationship has never been explored. To determine the roles of the dopaminergic system and mechanosensory cilia, we studied the effects of dopamine on ciliary length and function in wild-type (WT) and mechano-insensitive polycystic mutant cells (*Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$). We show for the first time that mouse vascular endothelia exhibit dopamine receptor-type 5 (DR5), which co-localizes to primary cilia in cultured cells and mouse arteries *in vivo*. DR5 activation increases cilia length in arteries and endothelial cells through cofilin and actin polymerization. DR5-activation also restores cilia function in the mutant cells. In addition, silencing DR5 completely abolishes mechano-ciliary function in WT cells. We find that DR5 plays very important roles in ciliary length and function. Furthermore, the chemosensory function of cilia can alter the mechanosensory function through changes in sensitivity to fluid-shear stress. We propose that activated ciliary DR5 has a functional mechanosensory role in endothelial cells.

Keywords

Dopamine receptors; polycystic kidney disease; primary cilia; vascular endothelia

Introduction

Primary cilium is a small hair-like projection present on the apical membrane of most cells. By virtue of its shape and location, the primary cilium is able to act as an antenna, sensing and transmitting information from the extracellular matrix to the cell interior. To assist with its unique sensory roles, a high density of specialized proteins such as receptors, ion channels, kinases, phosphatases, secondary messengers and other signaling modules are localized in the ciliary membrane, cilioplasm, or at the ciliary base[1]. These proteins enable the primary cilia to act as chemosensors and mechanosensors.

Dysfunctional cilia have been associated with a large number of diseases such as polycystic kidney disease (PKD) and various other diseases, which have been collectively referred to as “ciliopathies”. Improper structure and function of the primary cilia has been reported in

Corresponding author: Surya M. Nauli, Ph.D., The University of Toledo, Health Science Campus; HEB 274, 3000 Arlington Ave.; MS 1015, Toledo, OH 43614, Phone: 419-383-1910, Fax: 419-383-1909, Surya.Nauli@UToledo.Edu.

Conflict of Interest
N/A

patients suffering from PKD[2–4]. In addition to renal cyst formation, PKD is also characterized by non-cystic manifestations such as hypertension, left ventricular hypertrophy, cardiac valve abnormalities, intracranial aneurysms and abdominal wall hernias, among others[5,6]. Furthermore, hypertension in PKD has been associated with abnormal mechanosensory cilia function and structure[7,8].

Dopamine is an endogenous neuronal hormone, known to produce a wide range of cardiovascular and renal effects. Various subtypes of dopamine receptors are known to be present in different parts of the cardiovascular system[9]. Hence, dopamine is known to regulate systemic blood pressure, renal hemodynamics and electrolyte balance. In humans, activation of dopamine receptors within the blood vessels can cause vasodilation[10]. Most importantly, circulating dopamine mediates vasodilation through both endothelium-dependent (60%) and endothelium-independent (40%) mechanisms[11]. Within the vascular endothelial cells, dopamine receptors type-1 (DR1) and -5 (DR5) are involved in endothelium-dependent relaxation[12]. Because of this, any abnormality in dopamine metabolism and/or receptor function has been implicated in essential hypertension in humans[13–15] and animal models[16–18].

Despite the fact that cilia and dopamine play critical roles in hypertension, their relationship has not been explored. All we know from the clinical study is that PKD patients with borderline hypertension are better managed with DOPA (a dopamine precursor) than with angiotensin converting enzyme (ACE) inhibitors[19]. Our current studies show for the first time that the dopaminergic system regulates sensory cilia structure and function. Activation of ciliary dopamine receptor increases cilia length. To examine the relationship between dopamine and cilia within PKD, we further used mechano-*in*sensitive *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ endothelial cells, previously derived from *Pkd* mouse models[7,8]. We show that ciliary dopamine activation can restore mechanosensory cilia function in response to fluid-shear stress. We propose that localization of dopamine receptor to cilia plays important chemosensory and mechanosensory roles in vascular endothelial cells.

Materials and Methods

The use of endothelial cells and other biohazard reagents was approved by the Institutional Biosafety Committee of The University of Toledo. The use of animal tissues was approved by The University of Toledo animal care and use committee. The details of this section on pharmacological agents, sequences for primers and siRNAs are available online at http://hyper.ahajournals.org (online supplement).

Results

DR5 localizes to and regulates length of primary cilia

We show for the first time that dopamine receptor (DR)-type 5 is localized to the primary cilia of cultured endothelial cells and femoral artery *in vivo*. Using well-characterized mouse endothelial cells, expressions of DR type-3 and 5 (DR3 and DR5) are detected at the transcript level (SuppFig1a). Subcellular localization of these receptor subtypes was studied three-dimensionally using DR3- and DR5-specific antibodies (Fig1a). DR5 is localized to primary cilia of wild-type and *Pkd1*$^{-/-}$ endothelial cells. DR5 is also localized in short, stubby cilia of *Tg737*$^{orpk/orpk}$ cells. DR5 cilia localization was observed widely in a monolayer of endothelial cells and also in endothelia of femoral artery *in vivo* (SuppFig1b). No specific localization of DR3 was observed in the cilia (not shown).

Dopamine treatment for 4 or 16 hours increases cilia length in a dose-dependent manner (Fig1b). Concentration of dopamine to induce maximal increase in cilia length is optimal at

10 μmol/L for both 4 and 16 hours. Activation of DR5 is sufficient to increase cilia length (SuppFig2a). To further confirm that DR3 activation does not play a role in cilia length regulation, we used DR3 inhibitor in the presence of dopamine. Observation with immunofluorescence and electron microscopy techniques shows that DR5 activation, either with dopamine or DR5-specific agonist, increases in cilia length (SuppFig2b). To further verify this finding, we isolated and treated mouse femoral arteries with either vehicle or 10 μmol/L dopamine for 16 hours (SuppFig3a). As expected, dopamine increases cilia length *ex vivo* comparable to that of cultured cells. Because the femoral artery contains smooth muscle cells, which also have primary cilia[20,21], the artery was laid down in such a way that only the first layer of cells from the intima was observed through both immunofluorescence and electron microscopy techniques (SuppFig3b).

To understand the functional relevance of ciliary DR5 in PKD, we examined DR5 activation in *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ endothelial cells (Fig1b). Interestingly, cilia length is also increased significantly in *Pkd1*$^{-/-}$ cells treated with dopamine. Because of their small and stubby cilia, we were not able to accurately determine the cilia length measurement in *Tg737*$^{orpk/orpk}$ cells. However, it is surprising that the length of cilia in *Tg737*$^{orpk/orpk}$ cells tends to be longer or occasionally restored as seen in wild-type cells. In all genotypes, receptor activation with dopamine does not show an apparent subcellular redistribution of DR5 (not shown).

Dopamine increases cilia length through cellular actin differentiation via cofilin dephosphorylation

Inhibition of actin polymerization has been shown to play an important role in ciliogenesis[22–24]. Furthermore, dephosphorylated or activated form of cofilin has been shown to inhibit actin polymerization[25,26]. To examine this possibility in our system, we measured phosphorylated cofilin before and after treatment with dopamine for 15 and 60 minutes (Fig2a). Supporting our idea, a significant decrease of phosphorylated cofilin is observed in dopamine-treated cells (Fig2b). Throughout our Western blot analyses, we also consistently observed the expression level of total actin to be greater in *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ than in wild-type cells. Please note that we denoted the total actin as globular actin (G-actin) and filamentous actin (F-actin) because we reduced and monomerized F-actin during our sample preparation. Thus, we next analyzed F-actin only to further understand the effects of dopamine in actin polymerization (Fig2c). To our surprise, dopamine induces actin re-arrangement in all cell types. Although the effect on *Tg737*$^{orpk/orpk}$ cells is not as substantial, dopamine induces redistribution of stress actin fibers to cortical actin. This actin redistribution has been associated with shear-induced cellular differentiation[27], a characteristic of mechanical-induced cilia activation[28].

To further confirm the roles of cofilin in regulating cilia length, we used calyculin to increase the basal phosphorylation level of cofilin by blocking protein phosphatase-1 (PP1). Thus, calyculin would be constitutively inactivated. Blocking cofilin sufficiently and significantly decreases cilia length in the presence or absence of dopamine (SuppFig4a). When the F-actin was analyzed, the association between actin rearrangement and cilia length was further confirmed (SuppFig4b). We found that calyculin could block dopamine-induced actin re-arrangement.

Activation of ciliary dopamine receptor partially restores mechanosensory function of endothelial cilia in *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ cells

Because primary cilia have been proposed to be chemosensory organelles[29,30] and to further verify the functional specificity of DR5 in the cilia, we challenged wild-type endothelial cells with dopamine, DR5-and DR3-specific agonists (SuppFig5a). Our data show that DR3

activation has no functional implication, at least in cytosolic calcium increases. Most important is that the agonists-induced cytosolic calcium studies validate the involvement of DR5 in cilia function. We also challenged *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ cells with dopamine (SuppFig5b). As in wild-type cells, the chemosensory role of dopamine receptor in the cilia was verified in these mutant cells. Due to shorter cilia and thus lower DR5 expression level to cilia, a much smaller increase in cytosolic calcium was observed in *Tg737*$^{orpk/orpk}$ cells.

Vascular endothelial cilia have also been proposed to function as mechanosensory organelles[31,32]. To examine the correlation between cilia length and function, we performed fluid-shear stress experiments to analyze cilia function in wild-type cells treated with vehicle or 10 μmol/L of DA for 16 hours (Fig3a). We found that the averaged area under the curve in the presence and absence of dopamine were not significantly different. Thus, while dopamine significantly increases cilia length in wild-type endothelial cells, its effect on cilia function is minimal. Because dopamine also increases cilia length in *Pkd1*$^{-/-}$ as well as *Tg737*$^{orpk/orpk}$ cells, we next examined whether dopamine could have an effect on the mechanosensory function of cilia. Unexpectedly, the mechanosensory role in *Pkd1*$^{-/-}$ cells was restored in the presence of dopamine (Fig3b). More surprisingly, the mechanosensory role in *Tg737*$^{orpk/orpk}$ cells was also restored in the presence of dopamine (Fig3c).

Ciliary DR5 is a mechanosensory receptor

To further confirm DR5 as a mechanosensory receptor, we transfected wild-type endothelial cells with various GFP-tagged siRNAs, thereby knocking down DR5 level in the cells. Protein analysis shows substantially repressed DR5 expressions in siRNA treated cells compared to untreated cells or transfected cells with scramble siRNA (Fig4a). As expected, no DR5 was detected in the cilia of DR5-specific siRNA treated cells (Fig4b). Furthermore, DR5 knockdown cells have significantly shorter cilia, confirming the role of dopamine in regulating cilia length (Fig4c). Consistent with our data showing that dopamine regulates cilia length through actin distribution, dopamine-induced actin distribution is abolished in DR5 knockdown cells (Fig4d). To examine the mechanosensory function of cilia, we next challenged the cells with fluid-shear stress (Fig4e). Cells treated with siRNA became insensitive to fluid flow, supporting the role of DR5 as a mechanosensory protein in vascular endothelial cells. Unlike the regulatory system of cilia length, however, blocking cofilin through PP1 antagonist does not have any substantial effect on mechanosensory cilia function (SuppFig6). This indicates that regulatory pathways of cilia length and function may be differentially regulated.

Discussion

For the first time, we show that dopamine receptor type 5 (DR5) is localized to primary cilia in wild-type as well as in ciliary abnormal *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ endothelial cells. We further provide evidence supporting the ciliary roles of DR5 as chemo- and mechano-sensors. In addition, we show that endothelial cells can alter their sensitivity to fluid-shear stress through chemosensory function of cilia. The regulation of cilia structure involves dephosphorylated cofilin, which controls cellular cytoskeleton actin filament rearrangement. This cellular actin differentiation is required for extension of cilia length, including in *Tg737*$^{orpk/orpk}$ cells. Most interesting is that activation of ciliary DR5 would promote and restore cilia function, especially in *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ endothelial cells. We propose that ciliary DR5 within the vascular endothelia can provide a substantial implication in cilia-related diseases, such as hypertension and polycystic kidney disease (PKD).

In the present study, we find that DR5 is specifically localized to vascular endothelial cilia in cultures and arteries *in vivo*. Dopamine through activation of DR5 increases cilia length in a concentration-dependent manner within 4 hours in cultures and mouse femoral arteries *ex*

vivo. We did not perform *in vivo* study in mice, because the dose-response study *in vivo* is still largely a challenge due to a drop in blood pressure caused by dopamine. More specifically, dopamine induces vasodilation *in vivo*, compromising an overall collapse of the cardiovascular system. Regardless, the ciliary DR5 in cultured cells or blood vessels is a functional receptor, because specific activation of DR5 shows responses in both ciliary length and cytosolic calcium increase.

Recently, we have proposed that endothelial cilia are mechanosensory organelles that play a major role in pathogenesis of hypertension[7,8]. Abnormalities in dopamine synthesis or dopamine receptor function have also been implicated in essential hypertension in humans[13–15] and animal models[16–18]. For example, mouse genetic model with aberrant DR5 exhibits severe hypertension with unopposed sympathetic activity[18]. Furthermore, it was previously unknown how hypertensive PKD patients could be managed more successfully with dopamine precursor than other anti-hypertensive therapies[19].

To examine functional relevance of DR5 within PKD, we used *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ endothelial cells previously derived from *Pkd* mouse models. These mutant cells have abnormal mechanosensory cilia function and structure, respectively[7,8]. Similar to wild-type cells, activation of DR5 also increases cilia length in *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ endothelial cells. Because most *Tg737*$^{orpk/orpk}$ endothelial cells have very short stubby cilia, if any, we were not able to quantify the magnitude of cilia length increase. Nonetheless, the ciliogenesis in *Tg737*$^{orpk/orpk}$ cells implies that a mechanism other than the intraflagellar transport exists. Most importantly, this mechanism can be activated with dopamine to increase cilia length.

A primary cilium is structurally composed of acetylated and detyrosinated microtubules, which connect to the sub-membraneous actin network at its basal body. Ciliogenesis in *Tg737*$^{orpk/orpk}$ cells has been observed in the presence of cytocalasin D, an actin polymerization inhibitor[22–24]. Pharmacological agents which disrupt microtubule polymerization, like nocadazole, promote the formation of actin stress fibers. On the other hand, agents that stabilize microtubules, such as taxol, inhibit assembly of actin filaments. Thus, any activation or inhibition of actin polymerization could affect the microtubule-based cilium.

Patients with PKD exhibit significant connective tissue abnormalities involving vascular and airway smooth muscle cells[21], suggesting a possible disruption of cellular actin dynamics. In addition, vascular cells derived from a mouse Pkd model exhibit higher levels of cofilin when activated by the adrenergic receptor agonist[33], which further confirms the role of actin dynamics in cilia dysfunction model. Cofilin, a small ubiquitous protein that binds to actin cytoskeleton, can promote the rate of monomer disassociation and sever actin filament, thereby inhibiting actin-polymerization[25,26].

To further examine the roles of activated cofilin by dopamine, we also measured phosphorylated cofilin in our cells. Compared to wild-type cells, higher basal levels of inactivated cofilin in *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ cells were consistently observed. This probably results in inhibition of cilia length and/or function. Consistent with this view, a consistently higher level of total actin is needed for F-actin polymerization in the mutant cells. We further show that dopamine-induced cofilin activation can promote cortical F-actin formation, which has been used as a differentiation marker in neurons[34]. Overall, our data show that dopamine can induce cofilin activation, through dephosphorylation by PP1[35]. Consistent with this view, when we inhibited PP1 with calyculin, cilia length was significantly decreased. Calyculin, downstream to dopamine, is also able to reverse the increase in cilia length and actin rearrangement observed in cells treated with dopamine. In

addition, no significant change in intracellular calcium response was observed in the presence of fluid-flow shear stress, reinforcing the cellular functions of actin reorganization in ciliogenesis and DR5 in the mechanosensory property of primary cilia.

Not only do our data show the functionality of DR5 as a chemosensor in cilia, our studies also demonstrate the role of DR5 as a ciliary mechanosensor. We and others have shown that *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ cells lack mechanosensory cilia function[36,37]. To our surprise, dopamine not only induces an increase in cilia length, but it also restores the mechanosensory roles in both *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ cells. Interestingly, in wild-type cells with normal mechanosensory cilia function, activation of DR5 does not initiate a significant change in cilia function, although cilia length is significantly increased. We propose that activated DR5 in the cilia can have a functional sensory role in endothelial cells.

To further investigate the mechanosensory capacity of DR5 in wild-type cells, we next knocked down DR5 expression. Inhibition of DR5 expression in cilia was achieved using different siRNA constructs, which happened to have a similar efficacy to that verified by our Western blot and immunolocalization studies. Dopamine-induced cofilin activation was ceased in siRNA-transfected cells as evidence from the discontinuation of F-actin rearrangement. Most surprising is the loss of mechanosensory function in DR5 knockdown cells. The length of the cilia in knockdown cells was similar to the length of those treated with calyculin. However, while the cilia in DR5-knockdown cells no longer possessed their mechanosensory capabilities, cells treated with calcyculin, which still exhibited DR5 co-localized to the cilia retained their mechanosensory abilities. Thus, we propose that ciliary DR5 in endothelial cells have dual chemosensory and mechanosensory roles.

Primary cilia have been proposed to regulate cardiovascular functions, including blood pressure[7,8]. Given the facts that DR5 mutations in mice[17,18] or abnormal dopaminergic system in humans[13–15] leads to hypertension, we suggest that dysfunctional DR5 is associated with cellular function of cilia. As such, dysfunction in cilia and DR5 could result in a similar hypertension phenotype as observed in PKD patients[5,6] and patients associated with dopaminergic system[13–15]. Thus, the chemosensory and mechanosensory roles of primary cilia are equally important for the maintenance of proper cardiovascular homeostasis and vascular tone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Authors would like to thank Maki Takahashi and Blair Mell for their technical support. Charisse Montgomery is acknowledged for her editorial assistance.

Sources of Funding

NIH DK080640, DK080640-01S1 and DK080640-02S1

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Perspectives

Previous clinical study indicates that hypertension in PKD patients is better managed with a dopamine precursor[19]. However, it was not immediately understood why and how it is more useful than other anti-hypertensive agents. We show here that activation of peripheral dopamine receptor can regulate cilia length and restore cilia function in PKD. Overall, our study helps explain dopamine receptor agonism as a potential therapeutic option in hypertensive PKD patients.

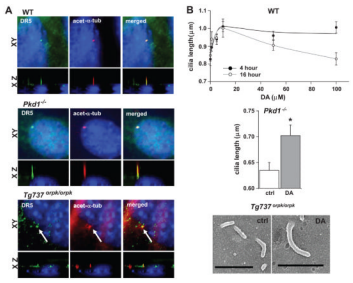

Figure 1. Dopamine receptor-type 5 (DR5) localizes to and regulates length of primary cilia

a. Immunolocalization study using specific antibody to DR5 confirms ciliary expression in monolayer wild-type (WT) and mechano-*in*sensitive (*Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$) vascular endothelial cells. The XY and XZ fluorescence images show localization of DR5 (green) to the cilia (acetylated-α-tubulin; red). Arrows indicate abnormal cilia formation in *Tg737*$^{orpk/orpk}$ cells. **b.** Dopamine treatment for 4 or 16 hours increases ciliary length in a dose dependent manner in wild-type cells. Dopamine also increases cilia length in *Pkd1*$^{-/-}$ cells as shown in the bar graph or induces cilia formation in *Tg737*$^{orpk/orpk}$ cells as shown in the representative electron micrographs. Asterisks denote $p<0.05$. Bar = 1μm. N>3 independent experiments.

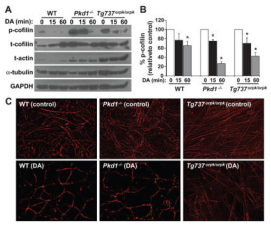

Figure 2. Dopamine increases cilia length through cellular actin differentiation via cofilin dephosphorylation

a. Wild-type (WT), *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ cells were analyzed for the ratio of phosphorylated cofilin (p-cofilin) to total cofilin (t-cofilin) before and after 10 μmol/L dopamine (DA) treatment for 15 or 60 minutes. Both α-tubulin and GAPDH were used as loading controls, while total actin (t-actin) consistently expresses at a higher level in the mutant cells. **b.** Dopamine significantly decreases levels of phosphorylated cofilin in wild-type, *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ cells. **c.** Dopamine induces cellular differentiation as evidenced from cytoskeletal actin rearrangement. Stress actin fibers and cortical actin formation are respectively presented before (control) and after 60 minutes dopamine treatment. N=3 independent experiments. Asterisks denote $p<0.05$.

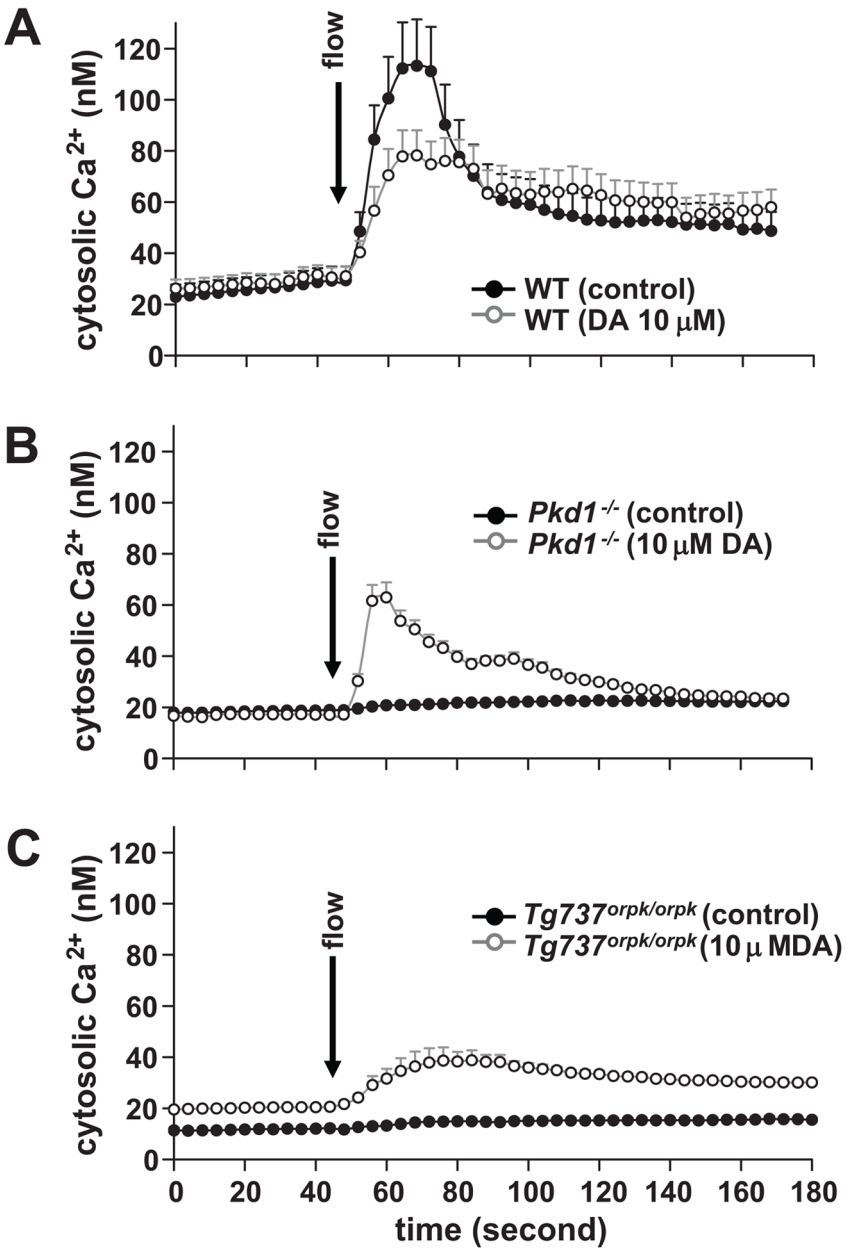

Figure 3. Activation of ciliary dopamine receptor partially restores mechanosensory function of endothelial cilia in *Pkd1*$^{-/-}$ and *Tg737*$^{orpk/orpk}$ cells

a. Mechanosensory function of cilia is determined by studying changes in intracellular calcium in response to fluid-flow shear stress. Untreated wild-type (WT) cells and cells treated with dopamine (DA) for 16 hours do not exhibit significant changes in cytosolic calcium in response to fluid-flow shear stress. **b.** *Pkd1*$^{-/-}$ cells are incapable of responding to fluid-flow shear stress and hence an increase in intracellular calcium is not observed in *Pkd1*$^{-/-}$ in response to fluid flow. However, after treatment with dopamine for 16 hours, *Pkd1*$^{-/-}$ cells regain their ability to sense fluid flow, with a significant increase in intracellular calcium. **c.** *Tg737*$^{orpk/orpk}$ cells, which have short or stubby cilia, are incapable of sensing fluid flow shear stress. After dopamine treatment, these cells exhibit a small but

significant rescue of the ciliary function. N=5–9 independent experiments; each represents an average of 100–150 cells.

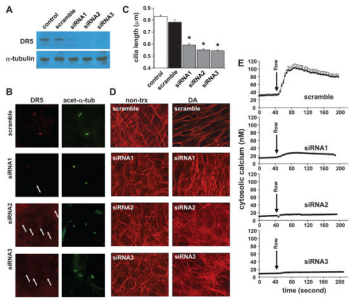

Figure 4. Ciliary DR5 is a mechanosensory receptor

a. The presence of dopamine receptor type 5 (DR5) was analyzed by western blot in wild-type vascular endothelial cells transfected with lipofectamine only (control), scramble RNA, siRNA1, siRNA2 or siRNA3. α-tubulin was used as a loading control. **b.** The presence of ciliary dopamine receptor was verified in transfected cells, and acetylated-α-tubulin was used as a ciliary marker. White arrows indicate the absence of dopamine receptor in the cilia. **c.** Cilia length decreases in siRNA transfected cells compared to non-transfected (control) or scramble siRNA transfected cells. Asterisks denote $p<0.05$. **d.** Cytoskeletal actin filament was analyzed in the transfected cells before treatment (non-trx) and after 16 hour-dopamine treatment (DA). **e.** Mechanosensory function of cilia was analyzed in the transfected cells. N>3 independent experiments; each represents an average of 100–150 cells.