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Shakila Abdul-Majeed
University of Toledo

Surya M. Nauli
Chapman University, nauli@chapman.edu

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Calcium-mediated mechanisms of cystic expansion

Shakila Abdul-Majeed and **Surya M. Nauli**
Department of Pharmacology, College of Pharmacy, and Department of Medicine, College of Medicine, The University of Toledo, Toledo, OH 43614

Abstract

In this review, we will discuss several well-accepted signaling pathways toward calcium-mediated mechanisms of cystic expansion. The second messenger calcium ion has contributed to a vast diversity of signal transduction pathways. We will dissect calcium signaling as a possible mechanism that contributes to renal cyst formation. Because cytosolic calcium also regulates an array of signaling pathways, we will first discuss cilia-induced calcium fluxes, followed by Wnt signaling that has attributed to much-discussed planar cell polarity. We will then look at the relationship between cytosolic calcium and cAMP as one of the most important aspects of cyst progression. The signaling of cAMP on MAPK and mTOR will also be discussed. We infer that while cilia-induced calcium fluxes may be the initial signaling messenger for various cellular pathways, no single signaling mediator or pathway is implicated exclusively in the progression of the cystic expansion.

Introduction

Polycystic kidney disease (PKD) is characterized by formation of fluid-filled cysts. For the past decade, many ideas and much hard work have been put forth to understand the disease, although the mechanisms of cyst formation and expansion remain speculations. Based on transmittance, PKD can be simply classified into acquired and hereditary forms. The acquired form of PKD can be found in patients who have had acute renal failure with subsequent dialysis. The majority of PKD cases, however, are transmitted hereditarily from the parents. The two most common hereditary forms of PKD are autosomal dominant PKD (ADPKD) and autosomal recessive PKD (ARPKD). The genes mutated in ADPKD include *PKD1* and *PKD2*, whereas ARPKD is caused by mutation in *PKHD1* gene (OMIMs: #601313, 613095, 263200). The prevalence of ADPKD and ARPKD is 1 in 1,000 and 1 in 20,000 live births, respectively.

The products encoded by these PKD genes are called cysto-proteins (Figure 1), which include polycystin-1 (*PKD1*), polycystin-2 (*PKD1*), and fibrocystin (*PKHD1*). Though the mechanism of cyst formation is still a mystery, abnormal function of these proteins results in cyst formation. In particular, cysto-proteins interact with one another (Figure 1). Thus, aberrant functions in any of these cysto-proteins may result in similar pathogenic phenotypes in the kidney, liver, pancreas and possibly other organs. In addition, these three proteins are localized within the same subcellular domain in the cell. Because these proteins

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

are localized and have distinct functions in the primary cilia, ciliary hypothesis has been developed to explain a unifying pathogenic concept for PKD.

In this review, we will discuss calcium signaling as a possible mechanism that contributes to the functions of these cysto-proteins. Dysfunction of any of these proteins may thus interrupt calcium signaling pathways, which may promote abnormal downstream signal transductions of various signaling molecules participating in renal cyst formation.

Calcium signaling by primary cilia

When the PKD genes were discovered and cloned [1-3], their functions in cation transport immediately became of considerable interest in understanding the molecular functions of the cysto-proteins. In particular, sequence analysis of polycystin-2 showed putative homologies with other known calcium channels [4]. In addition to their physical and functional interactions with other calcium-regulated proteins [5-7], interactions of cysto-proteins with other calcium channels also provided further insights into the modulation of intracellular calcium signaling [8-12]. Thus, polycystin-2 has long been predicted to regulate cytosolic calcium [13,14], including modulating intraorganellar calcium release [15] and extracellular calcium influx [16].

To function as a calcium channel, polycystin-2 depends on its interaction with polycystin-1 [16,17]. Likewise, proper function of fibrocystin depends on the indirect interaction with the polycystin complex [18-20]. In addition, activation of polycystin-2 has been found to depend on its interaction with mammalian diaphanous-related forming 1 (mDia1). mDai1 is able to regulate polycystin-2 depending on the membrane potential or voltage levels in the cells. At resting potentials, mDai1 in an autoinhibited state binds to polycystin-2 thereby inhibiting its channel activity. However, at positive potentials, GTP-bound mDia1 releases and thereby allows activation of polycystin-2 [21].

Localization of cysto-proteins to primary cilia further confirms the roles of polycystins and fibrocystin in intracellular calcium signaling. In addition, it further elaborates the molecular functions of cysto-proteins as regulators for intracellular calcium signaling. Most important are the mechanosensory functions of cysto-proteins that have been independently described in the mouse and human kidney epithelia [20,22-27], vascular endothelia [28-30], osteochondrocytes [31,32], cholangiocytes [33,34] and developing nodes [35-37]. It is now generally accepted that localization of these cysto-proteins to the primary cilia is important and necessary to initiate the first signaling cascade of intracellular calcium [38-40]. This signaling pathway may further provide other complex downstream signaling pathways.

In general, primary cilia are mechanosensory compartments that house many sensory proteins, including the cysto-proteins. Shear stress that produces enough drag force on the cell surface is able to bend the primary cilium (Figure 2). Subsequently, activation of the polycystins and other interacting proteins in this complex may result in cytosolic calcium increase. This paradigm was established based mainly on *in vitro* studies where cultured kidney cells were challenged with a fluid-flow shear stress [41,42]. The *ex vivo* experiments using isolated gastrulation stage node, perfused tubules and arteries have further confirmed the mechanosensory function of the primary cilium [28,36,43]. In the vascular artery [28], the influx of extracellular calcium initiates the biochemical cascades that lead to production of nitric oxide vasodilator through endothelial nitric oxide (eNOS). This activation of eNOS depends on the contribution or activity of calmodulin, phosphoinositide kinase-3, protein kinase B and calcium-dependent protein kinase (Figure 3).

In the next sections, we will discuss the downstream pathways that depend, directly or indirectly, on the initial calcium signaling. Because of the complexity in calcium signaling, we will discuss only those pathways that have possible relevance to renal cyst expansion.

Signaling by Wnt

Wnt signaling pathways are involved in many aspects of cell development, such as cell polarity determination, cell adhesion, growth, motility, and many others. The Wnt pathway involves a daunting number of secreted Wnt ligands and Frizzled receptors that regulate a large number of Wnt signaling molecules. In the simplest terms, Wnt signaling can activate three distinct pathways (Figure 4): **1)** *β-catenin dependent canonical pathway*, **2)** *β-catenin independent non-canonical or PCP (planar cell polarity) pathway*, and **3)** *Wnt-calcium pathway*, which can influence both the canonical and non-canonical pathways[44].

Many regulatory proteins involved in Wnt signaling are localized in the primary cilium and base of the cilium, also known as a basal body. It is thus speculated that flow-induced cytosolic calcium influx switches off the canonical Wnt pathway and activates the non-canonical Wnt/calcium signaling pathway (Figure 4). This molecular switch is regulated by inversin, which is a ciliary protein that can turn different Wnt signaling pathways on and off [45]. Of note is that abnormalities in inversin function result in polycystic kidney phenotype.

The zebrafish cystic kidney gene *seahorse* has also been found to be involved in a variety of cilia-mediated processes such as body curvature, kidney cyst formation, left-right asymmetry, and others including PCP signaling and inhibition of the canonical Wnt signaling [46]. *Seahorse* seems to be essential for a functional non-canonical Wnt signaling. It associates with Dishevelled (Dsh), the divergence point for the canonical Wnt and PCP pathways. The *Seahorse* gene encodes a highly conserved five leucine-rich repeats (LRR) and a leucine-rich repeat cap, from *Drosphila* to humans. One of the leucine-rich repeat proteins, LRRC50, has been found to be conserved in both zebrafish and humans. *Lrrc50* in expressed in all ciliated tissues in zebrafish, resulting in cilary dysfunction in *lrrc50* mutants. In humans and dog kidney cells, LRRC50 has been shown to localize at the mitotic spindle and cilium, implying it to be a ciliary protein in vertebrates [47].

It is generally accepted that Wnt signaling pathway is not regulated properly in polycystic kidney disease (Figure 4). In the canonical Wnt pathway, β-catenin in the nucleus mediates many gene induction events, and any deregulation of this pathway can result in uninhibited proliferation of cells [48,49]. It is speculated that flow-induced cytosolic calcium influx is required to turn off the canonical Wnt pathway and activate the non-canonical Wnt/calcium signaling pathway. As such, over-activation of canonical Wnt pathway, by over-production of an activated form of β-catenin for an example, would result in polycystic kidney phenotype [50]. This view is consistent with the profiling gene expression study [51]. A consistently high level of Wnt signaling is observed in cystic tissues from ADPKD patients, but not in tissues which exhibited low level or no cyst formation from the same patients. Furthermore, abnormalities in polycystins enhance activity of Wnt signaling pathway [52-55].

E-cadherin is one of the interacting Wnt signaling molecules, and it plays an essential role in intercellular cell junction assembly. It is required for epithelial polarity and tubule formation. Disruption of E-cadherin could lead to abnormal levels of β-catenin and impeding renal epithelial polarization [54,56]. Furthermore, the protein complex of polycystins, E-cadherin, and β-catenin is interrupted in cyst-lining epithelial cells from ADPKD patients [57]. In addition, the levels of β-catenin in the developing hearts and kidneys of *Pkd1*$^{-/-}$ mouse embryos compared to wild type embryos is decreased [53]. Overall, the data suggest that interruption in Wnt signaling pathway would result in less

differentiated epithelial cells, yielding to proliferation and acceleration of cyst expansion [58].

Planar cell polarity, which involves non-canonical Wnt signaling, has recently been the most discussed topic toward understanding of renal cysts. Planar cell polarity is principally involved in the development of tissue architectures along a parallel axis, other than the apical-basolateral axis of a renal tubule (Figure 5). Oriented cell division is thought to be necessary for the elongation of the developing nephron. Abnormalities in the planar cell polarity, as reflected by the mitotic spindle, have been observed in various PKD mouse models [52,59-63]. In these studies, cell division or mitotic spindle orientation within the tubular axis were measured. However, it is currently unclear whether such an alignment can be considered a process toward planar cell polarity [64]. In Drosophila, for example, mitotic spindle alignment is achieved only after centrosomes have been properly aligned [65]. Thus, there is a cell-cycle checkpoint for centrosomal positioning. The question remains whether such a checkpoint is disrupted in PKD.

Within the non-canonical Wnt signaling, there is also a huge body of evidence indicating that JNK signaling system is involved in PKD [66-70]. The strongest evidence of non-canonical Wnt signaling, however, came from a study involving Rho small G protein. In the mouse model, mutation in the key regulator of Rho small G protein results in cystic kidney phenotype [71]. Taken together, interruption in Wnt signaling pathway would result in cyst formation due to planar cell polarity defects, and mitotic spindle orientation might be a contributing factor in the process.

Signaling by cAMP / MAPK

Cyclic adenosine monophosphate, or cAMP, has been identified as one of the most important players in cyst progression in both ADPKD and ARPKD (Figure 6). Two major processes that contribute to the expansion of the renal cysts in polycystic kidney disease are cell proliferation and fluid secretion. cAMP is intimately involved in accelerating both of these processes [72-75]. Tissues from the kidneys, liver, and vascular smooth muscles of PKD animal models exhibit increased levels of cAMP [76-78].

cAMP is known to be one of most important players in effecting hormonal activation of intracellular pathways and is intimately involved in cell proliferation in almost all cell lines. However, cAMP does not produce the same effects in all cell lines. Though cAMP has been used for several years as an anti-proliferative agent [79,80], cAMP has also been known to stimulate cell proliferation by activating the mitogen activated protein kinase (MAPK) pathway (Figure 6). For example, cAMP is anti-proliferative in normal tissues, but it stimulates cell proliferation in cystic epithelial cells [73,81,82].

The change in cAMP related phenotype - inhibition of cell proliferation in normal cells and stimulation of cell proliferation in cystic cells - is associated with the amount of intracellular calcium in PKD tissues and cells [82,83]. The imbalance in the cytosolic calcium due to the disruptions in the cysto-proteins promotes abnormal function of phosphodiesterase (Figure 6). Phosphodiesterase 1 isoforms (PDE1a, PDE1b and PDE1c) are present in especially high levels in the kidneys [84]. Most importantly, the activity of this phosphodiesterase is regulated by intracellular calcium and cAMP. In PKD cells with a low level of intracellular calcium, phosphodiesterase activity is down-regulated. This results in aberrant conversion of cAMP to AMP. The resulting increase in cytosolic cAMP further stimulates the MAPK pathways, whichpromotes cyst expansion through higher cell proliferation.

In general, cystic epithelia in polycystic kidneys have exhibited high levels of both cAMP and MAPK activity compared to normal cells, resulting in cell proliferation in polycystic

kidneys and an inhibition of cell proliferation in normal cells [85,86]. In normal epithelial cells, cAMP agonists inhibit MAPK pathway by blocking activation of Raf-1 (Raf-C) through cAMP-dependent protein kinase (PKA). On the other hand, cAMP was found to stimulate the MAPK pathway in PKD cells, thereby stimulating cell proliferation. This difference has also been observed to result from an increased affinity of cAMP for B-Raf rather than A-Raf and Raf-1 (C-Raf) [82,86]. As mentioned earlier, this change in cAMP-related signaling is also attributed to the level of intracellular calcium. The high levels of cAMP, combined with low levels of calcium fluxes in polycystic kidneys, could further result in a decrease of PI3K/Akt activity, thereby stimulating B-Raf activation and hence activation of the MAPK pathway of Ras/B-Raf/MEK/ERK [83,87,88].

Signaling by mTOR

The Ras/Raf/ERK pathway plays another important role in polycystic kidney disease by regulating the mammalian target of rapamycin (mTOR) pathway through molecular signaling of tuberin (Figure 7). Tuberin, which is also regulated by Akt, is a GTPase activating protein (GAP). Tuberin regulates the activity of Rheb, a small G-protein belonging to the Ras super family. GTP-bound Rheb is active, while GDP-bound Rheb is inactive. Hamartin and tuberin form a heterodimer which converts Rheb-GTP to Rheb-GDP, thereby inactivating Rheb. Rheb activates mTOR pathway. Hence, tuberin inactivates GTP-bound Rheb and inhibits the mTOR pathway [89-91].

The respective protein products of *TSC1* and *TSC2*, hamartin and tuberin, regulate formation of primary cilia [92,93]. Most importantly, however, analysis of normal and diseased cells from ADPKD patients indicates that the cyst lining epithelial cells exhibit higher levels of mTOR signaling compared to the surrounding normal epithelium [94]. When mTOR pathway is inhibited with rapamycin, many murine models of ADPKD and ARPKD show a decrease in renal cyst expansion [94-100]. The consensus is that the cytoplasmic tail of polycystin-1 directly or indirectly interacts with both mTORC1 complex as well as tuberin, the protein product of *Tsc2* which itself regulates mTORC1. Membrane localization of polycystin-1 and hamartin are therefore able to bind to tuberin, keeping it near the plasma membrane. Thus, membrane bound polycystin-1 is capable of controlling mTORC1 pathway and hence extensive cell proliferation. Disruption of polycystin-1 would imply the activation of the mTORC1 cascade yielding cell proliferation and subsequent cyst formation [101,102].

In addition, the cysto-proteins are known to contribute to the activation of the Akt/PKB pathway [101,103,104]. Thus, the interaction of Akt and tuberin can further provide an additional regulation of mTOR pathway by the cysto-proteins. Although the role of calcium in mTOR signaling has still not been properly studied in polycystic kidney disease, there is a possibility that calcium-ERK-mTOR pathway could exist. In particular, calcium is known to be associated with cAMP/ERK activity (Figure 6), and ERK has been implicated in mTOR signaling (Figure 7). There is no doubt that further studies are needed to dissect the contributions of calcium, ERK and mTOR in cystogenesis.

Interestingly, clinical trials of mTOR inhibitors in ADPKD patients have not yielded a much anticipated results [105-107]. The two mTOR inhibitors tested in ADPKD patients were sirolimus and everolimus. Sirolimus was tested on 100 patients (18 to 40 years) exhibiting early stages of the disease, while everolimus was tried on 433 patients in advanced-stage II and III, with a renal baseline volume of 1500 mL. Sirolimus did not show any decrease in total kidney size in humans, though it showed promising results in mouse models. However, the animals were treated with a dose of 5 mg/kg of body weight [94], a dosage that is unsafe for humans, who were administered 2 mg sirolimus for 18 months. No difference in GFR

was observed as these patients exhibited the initial stages of the disease [105]. Everolimus, on the other hand, slowed the increase in the total kidney volume without any effect on GFR. After a brief transient period, patients exhibited a rapid decline in GFR [106]. These studies imply that reducing the cyst size need not necessarily improve renal function in these patients.

Prospective

Cilia function and structure are important and necessary to maintain the architecture of kidney tissue. Abnormalities in the structure or function of cilia results in PKD. The mechanosensory cilia are crucial in maintaining intracellular calcium signaling. Many cell types, including renal epithelia, use cytosolic calcium as a second messenger to further regulate other cellular homeostasis through a very complex signal transduction system. This signaling system includes Wnt, cAMP - MAPK, Akt, mTOR, and other pathways that are not discussed in this review.

In understanding this complexity, an important lesson is that no single signaling mediator is implicated exclusively in the progression of the cystic expansion. Rather, all of these signaling pathways are intimately connected, thereby regulating the progression of the disease. Nonetheless, only by understanding such a complicated system do we have better insights to attain the most effective way to retard progression of polycystic kidney disease.

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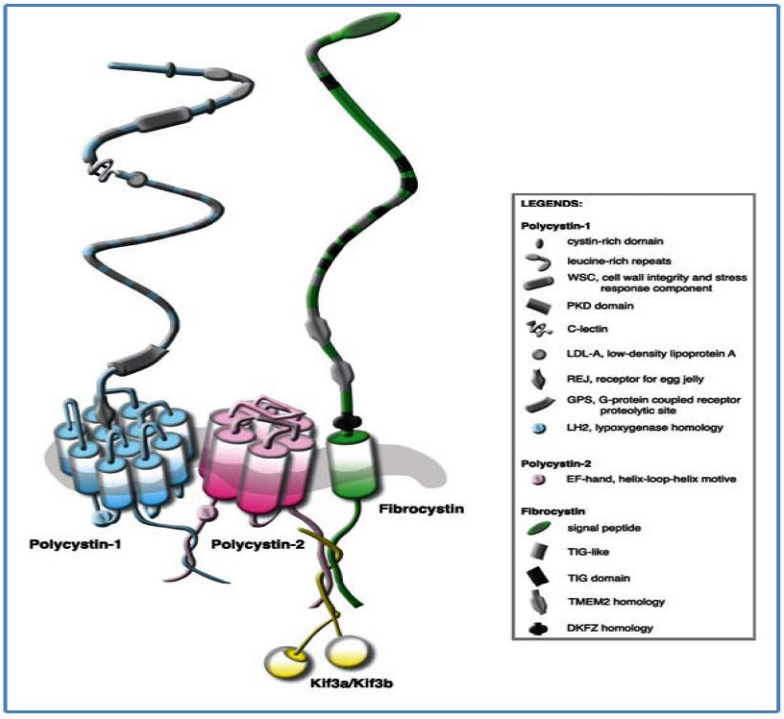

Figure 1. Cysto-protein complex

Polycystin-1 and polycystin-2 form a complex with each other at their COOH termini. Functioning as an adaptor protein, Kif bridges the interaction of between fibrocystin and polycystin complex. Figure is reproduced from *Kolb, et al* with permission [39].

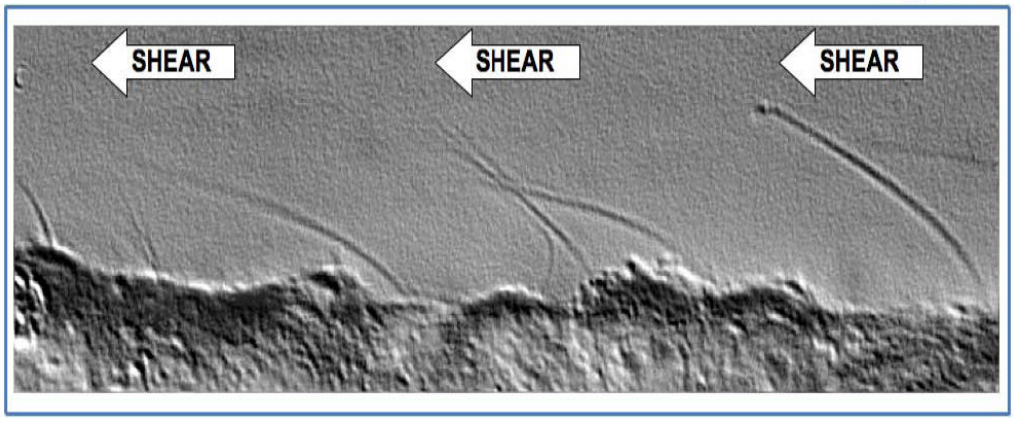

Figure 2. Primary cilia in signal transduction system

Cilia sense fluid-shear stress on the apical membrane of the cells. Fluid flow that produces enough drag-force on the top of the cells will bend sensory cilia. Bending of cilia will activate the cysto-proteins, resulting in extracellular calcium influx. Figure is reproduced from *AbouAlaiwi, et al* with permission [38].

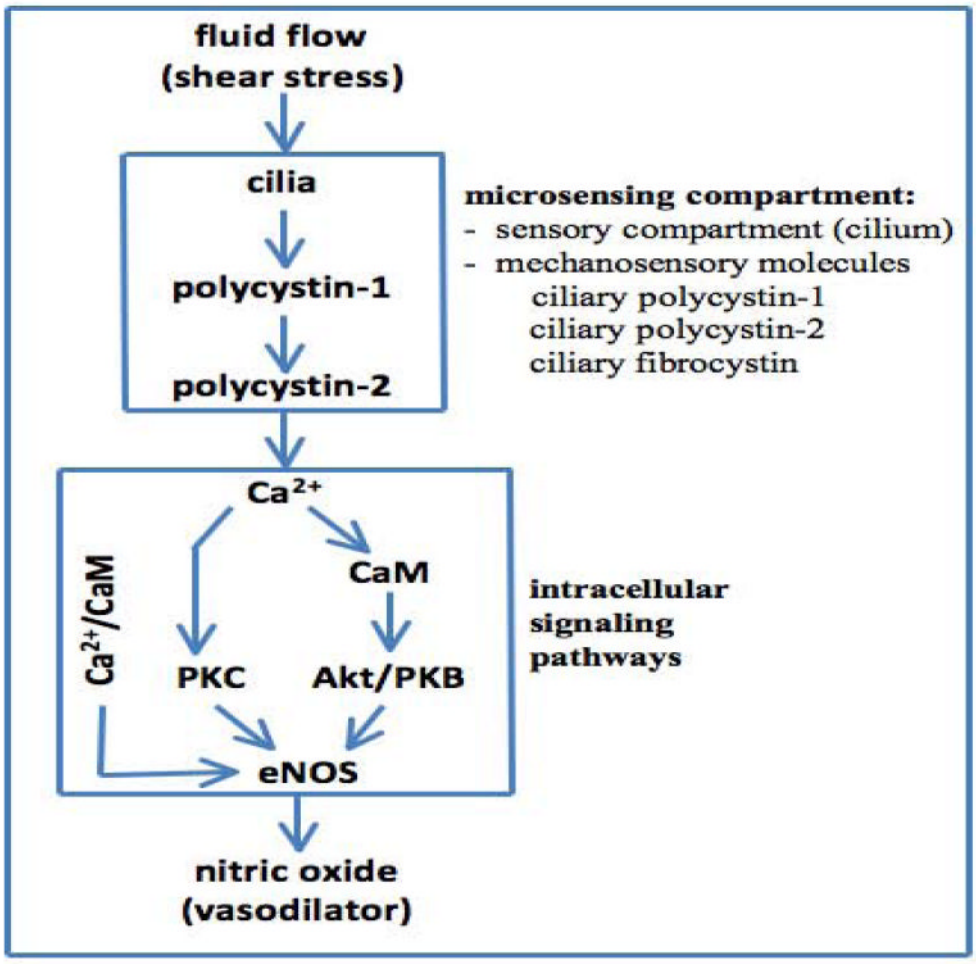

Figure 3. Mechanosensory cilia, cytosolic calcium and nitric oxide production

Bending or activation of the cilia involves mechanosensory polycystin-1 and polycystin-2 complex, which results in biochemical synthesis of NO. The signaling pathway requires extracellular calcium influx (Ca^{2+}), followed by activation of various calcium-dependent proteins including calmodulin (CaM) and protein kinase C (PKC). Together with PKB, CaM and PKC are important downstream molecular components to activate endothelial nitric oxide synthase (eNOS).

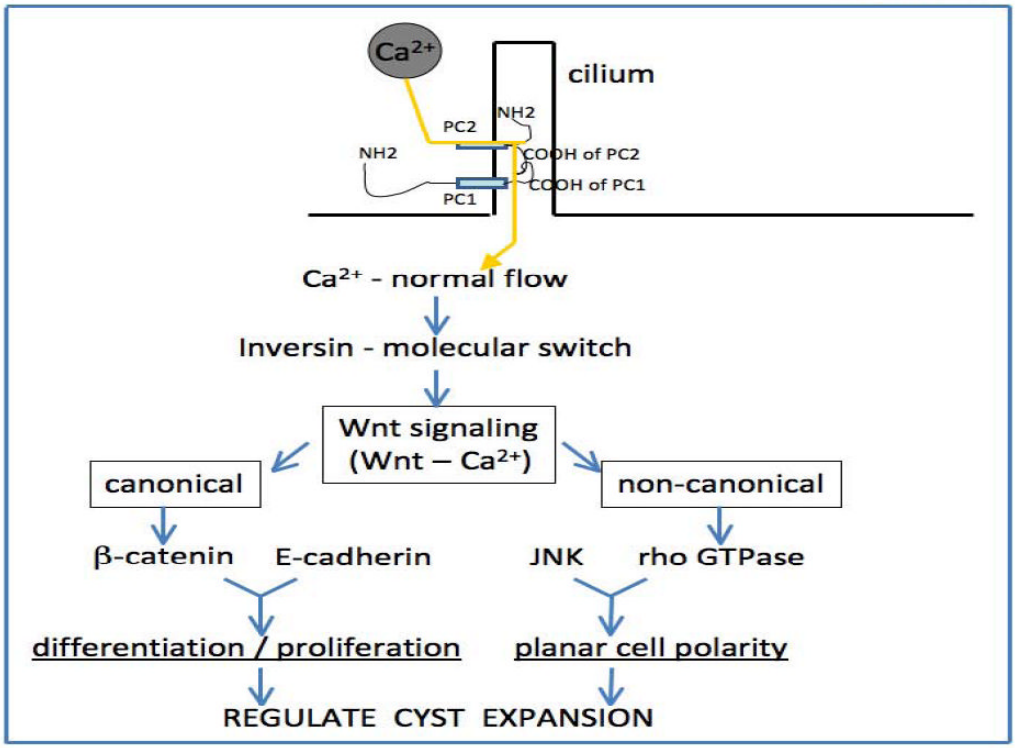

Figure 4. Mechanosensory cilia, cytosolic calcium and Wnt signaling

Bending or activation of the cilia results in maintaining cytosolic calcium (Ca^{2+}), followed by activation of inversin. Calcium and inversin function as the molecular switches for Wnt signaling pathways. The Wnt canonical pathway involves β-catenin. Both β-catenin and E-cadherin regulate cell differentiation and proliferation. The Wnt non-canonical pathway involves small GTPase rho and JNK, both of which play an important role in planar cell polarity.

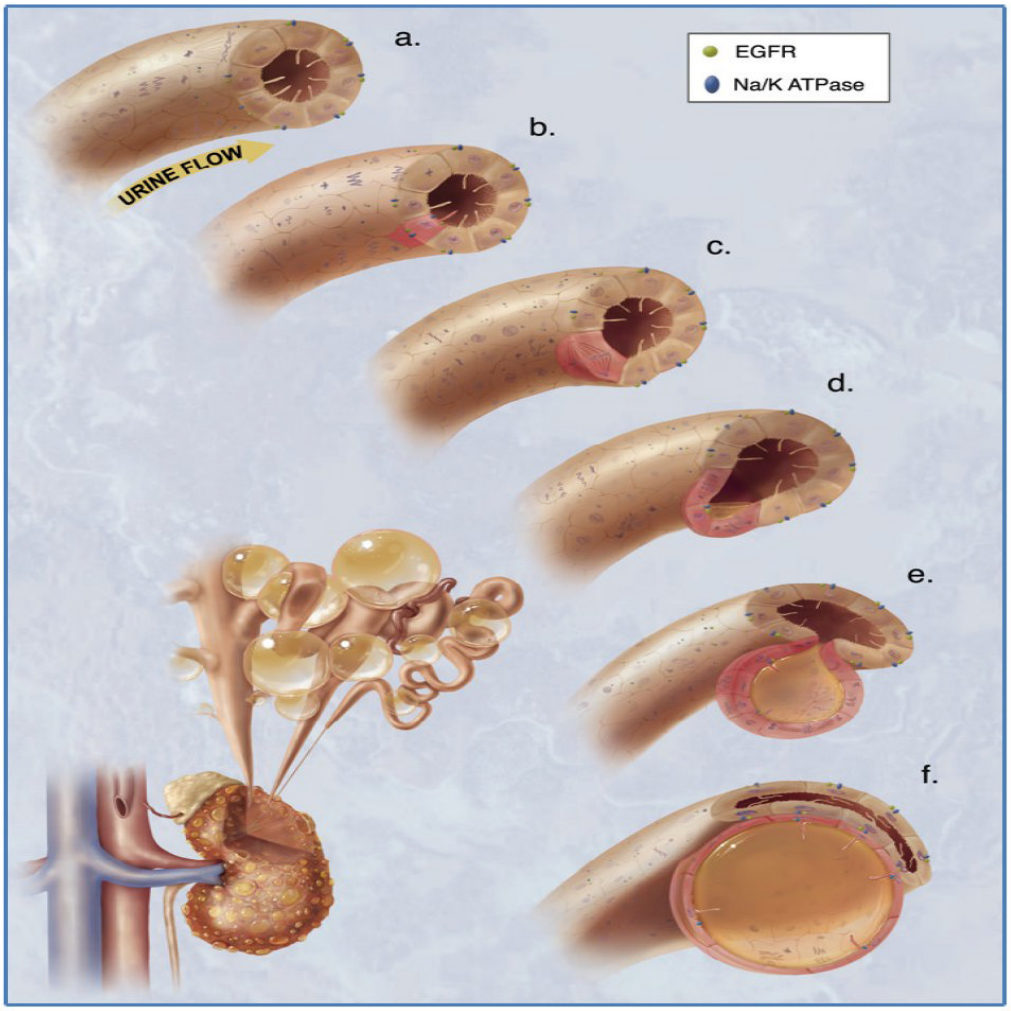

Figure 5. Planar cell polarity and cystic kidney disease

The illustration depicts the mechanism of mitotic spindle formation as an index of planar cell polarity in cyst expansion. **a**. Each cilium senses changes in urine flow. This message provides critical calcium signals to the cell. **b**. Abnormalities in any of the cysto-proteins results in abnormal ciliary function. **c**. The functional abnormality in ciliary sensing results in disorientation of the mitotic spindle, and hence the cell will lose planar cell polarity. **d**. Direction of cell division becomes randomized. This will result in increasing tubular diameter, rather than tubular elongation. **e**. Budding of a cyst from the renal tubule occurs. The abnormal localizations of epidermal growth factor receptor (EGFR) and Na^+/K^+ ATPase pump are typical characteristics of polycystic kidneys. **f**. The cyst is eventually enlarged and expanded further. Multiple cysts within a kidney are illustrated on the bottom left corner. Figure is reproduced from *Kolb, et al* with permission [39].

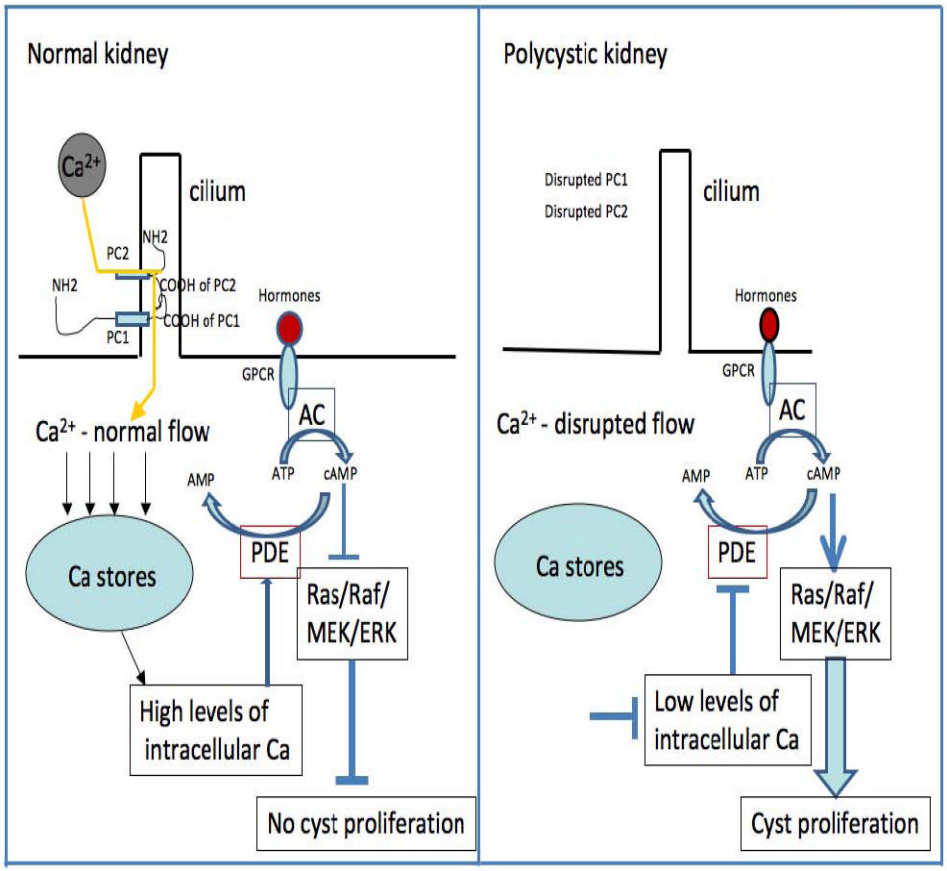

Figure 6. Cytosolic calcium and cAMP signaling

Under normal conditions, the carboxy terminal tails of polycystin-1 and polycystin-2 interact, which enables calcium entry into the cell. This further stimulates the release of calcium from the calcium stores inside the cell. In the presence of high, steady state levels of calcium, calcium-stimulated phosphodiesterases are capable of degrading cAMP into AMP, thereby controlling the cAMP levels in the cell. The low levels of cAMP are unable to stimulate MAPK pathway. In polycystic kidneys, however, the resulting abnormality in cysto-proteins causes a lower level of intracellular calcium. Hence, the activity of phosphodiesterases is not stimulated and cAMP level increases, leading to activation of B-Raf of MAPK pathway.

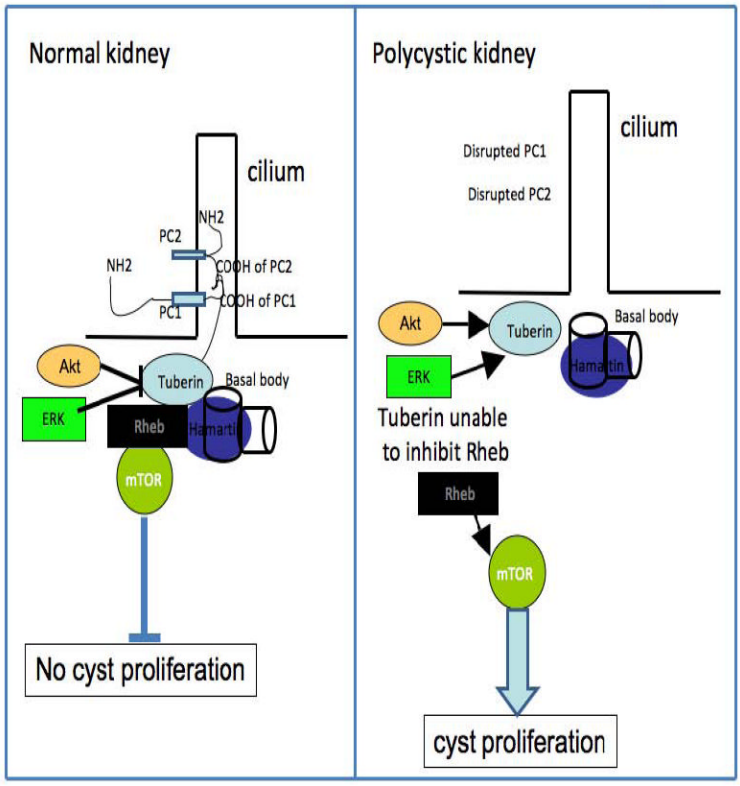

Figure 7. Signaling pathway of mTOR

In healthy kidney cells, the cysto-protein complex interacts with tuberin. Hamartin and tuberin form a complex and localize in the basal body. Tuberin is a GTPase activating protein (GAP) which controls the activity of Rheb. Tuberin inactivates Rheb, thereby inhibiting the mTOR pathway. In polycystic kidneys, however, tuberin is no longer protected in this complex and is phosphorylated by Akt, RSK (via ERK). As a result, tubulin is unable to form a heteradimer with hamartin. Hence, tuberin is no longer able inhibit Rheb, and the mTOR pathway is activated.