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Review

Putative roles of cilia in polycystic kidney disease ☆

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ABSTRACT

The last 10 years has witnessed an explosion in research into roles of cilia in cystic renal disease. Cilia are membrane-enclosed finger-like projections from the cell, usually on the apical surface or facing into a lumen, duct or airway. Ten years ago, the major recognised functions related to classical "9 + 2" cilia in the respiratory and reproductive tracts, where co-ordinated beating clears secretions and assists fertilisation respectively. Primary cilia, which have a "9 + 0" arrangement lacking the central microtubules, were anatomical curiosities but several lines of evidence have implicated them in both true polycystic kidney disease and other cystic renal conditions: ranging from the homology between *Caenorhabditis elegans* proteins expressed on sensory cilia to mammalian polycystic kidney disease (PKD) 1 and 2 proteins, through the discovery that *orpk* cystic mice have structurally abnormal cilia to numerous recent studies wherein expression of nearly all cyst-associated proteins has been reported in the cilia or its basal body. Functional studies implicate primary cilia in mechanosensation, photoreception and chemosensation but it is the first of these which appears most important in polycystic kidney disease: in the simplest model, fluid flow across the apical surface of renal cells bends the cilia and induces calcium influx, and this is perturbed in polycystic kidney disease. Downstream effects include changes in cell differentiation and polarity. Pathways such as hedgehog and Wnt signalling may also be regulated by cilia. These data support important roles for cilia in the pathogenesis of cystic kidney diseases but one must not forget that the classic polycystic kidney disease proteins are expressed in several other locations where they may have equally important roles, such as in cell-cell and cell-matrix interactions, whilst it is not just aberrant cilia signalling that can lead to de-differentiation, loss of polarity and other characteristic features of polycystic kidney disease. Understanding how cilia fit into the other aspects of polycystic kidney disease biology is the challenge for the next decade. This article is part of a Special Issue entitled: Polycystic Kidney Disease.

1. Cilia and first links to PKD

Cilia are finger-like projections from the cell surface bounded by modified cell membrane [1]. They contain a microtubule cytoskeleton in the ciliary axoneme, comprising either 9 peripheral doublets and 2 central microtubules (9 + 2 arrangement) or just the 9 peripheral pairs (9 + 0; Fig. 1). van Leeuwenhoek provided the first description of cilia around 300 years ago and, until recently, most vertebrate cilia research focussed on 9 + 2 motile functions in multi-ciliated epithelia such as the respiratory and reproductive tracts. Nevertheless, a series of observers in the mid-late twentieth century reported solitary non-motile cilia and these were named 'primary cilia' by Sorokin in 1968 (see historical review by [2]); electron microscopy confirmed a 9 + 0 microtubule layout but roles of these non-motile cilia remained obscure until the last decade.

In 1999, Barr and Sternberg were investigating mating behaviour in *Caenorhabditis elegans* when they identified the *lov-1* gene, that is required for sensory response and vulva location [3]. This encodes a transmembrane protein with homology to human PKD1 and it is expressed in adult male sensory neuron cilia in the rays, hook and head; moreover the *C. elegans* homologue of PKD2 also localises to these neurons and later work suggests that they function in the same male sensory behavioural pathway [4]. In 2000, Murcia and colleagues linked PKD to cilia in the Oak Ridge Polycystic Kidney (orpk) mouse: they observed that the *Tg737* gene is required for left-right axis determination, and the reason this is impaired in mutants is because they fail to express the central cilium in ventral node cells in early development [5]; the node is an early organising centre that determines laterality, which is variously known as Kupffer's vesicle, and Hensen's node in different organisms. They named the product of *Tg737*, Polaris; an excellent choice in retrospect, based on the increasing evidence linking cilia and PKD not just to left-right body symmetry but also epithelial polarity during development.

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2. Structure and assembly of primary cilia

Primary cilia are highly specialised, dynamic structures, consisting of cytoplasm and cell membrane supported by longitudinal microtubules. They are rapidly assembled and disassembled at different stages of the cell cycle and the microscopical process was well-described by Sorokin almost 50 years ago: as the cell enters G0, the centrosome migrates towards the cell surface, the mother centriole attaches to a Golgi derived vesicle mediated in part by the distal appendages of the centriole, also known as transition fibres, and the axoneme of the primary cilium grows from the cell surface into the lumen [6,7]. The primary cilium is resorbed as cells re-enter the cell cycle and divide, but regrows as each daughter cell becomes quiescent. Structurally, lengthening the cilium is analogous to constructing the first crane on a building site with extra components being transported up the crane, then added to the tip to make it higher; this is reiterated multiple times until the crane reaches full height. Transport of the proteins needed to build the cilium occurs by the same processes as required to build flagella, hence it is termed intraflagellar transport (IFT) and speeds of around 1 μm per second have been recorded [8]. Movement towards the tip of the cilium is termed anterograde IFT and depends upon kinesin-2 microtubule motors, whilst the reverse retrograde transport requires dynein [9]. Disruption of transport in either direction perturbs cilia formation: defective anterograde movement blocks development whereas disruption of the dynein motor results in short, stumpy cilia [10]. Moreover, kidney-targeted inactivation of kinesin-2 not only inhibits renal ciliogenesis but also induces cystic kidney disease [11]. Cilia are also shorter than normal in the *orpk* mouse [12]. Cilia assembly may also be regulated by microRNAs because targeted disruption of the miRNA enzyme dicer in the collecting ducts disrupts cilia development and is associated with cysts [13].

Although the cilium and rest of the cell are both covered by the cell membrane, there is evidence that there is regulated movement of both proteins and lipids into the cilium which means that membrane composition is different in these two areas [14]. A ciliary necklace [7] and "ciliary pore complex"[15] have been reported in various species, functioning as barriers through which only selected proteins are allowed passage into the ciliary compartment. The corollary of this is that selective transport will increase the concentration of specific proteins within cilia which may facilitate targeted interactions—a kind of molecular meeting place within the organelle. More recently,

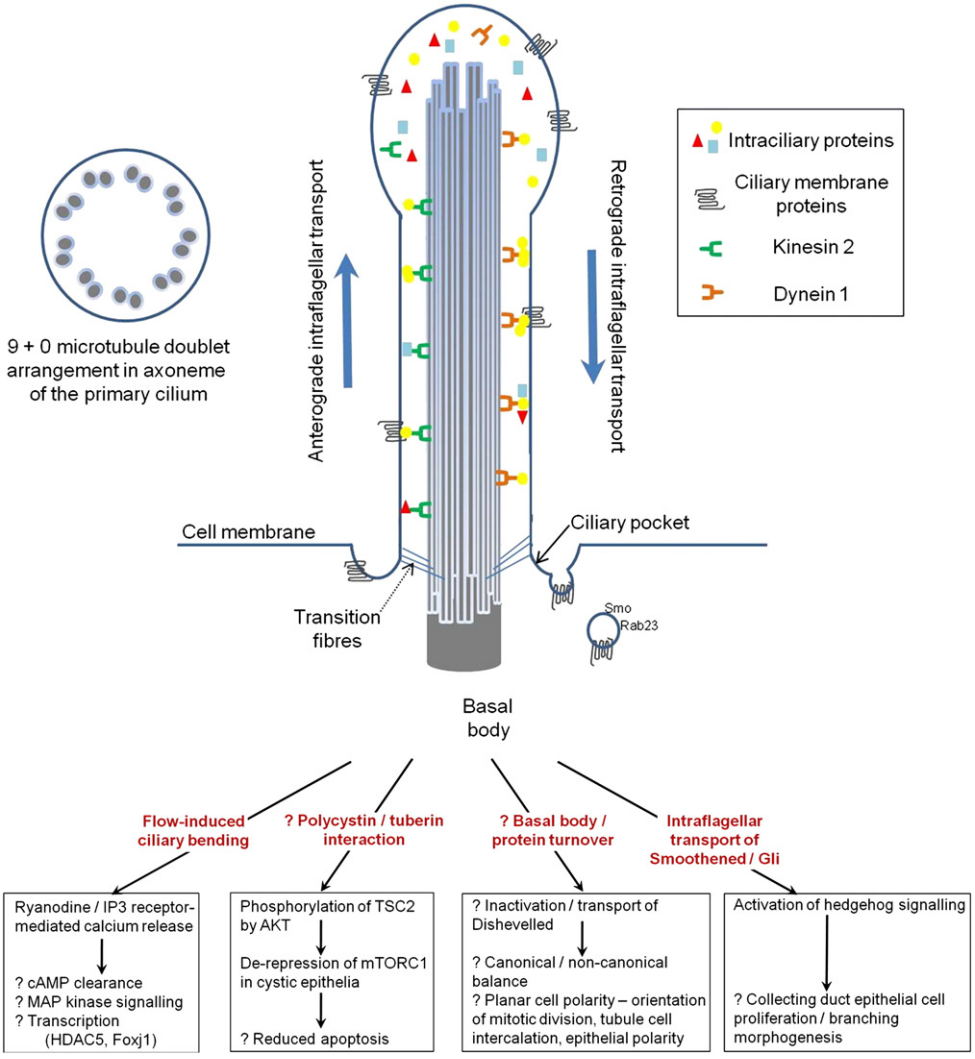

Fig. 1. Schematic representation of a primary cilium demonstrating the 9 + 0 microtubule axonemal structure. Anterograde and retrograde transport via kinesin 2 and dynein 1 is shown on the left and right of the cilium respectively. Also shown are the ciliary pocket, transition fibres and basal body (see text for details), and as well as vesicles fusing with and budding from the basal membrane. These vesicles carry receptors, such as Smo, and are regulated by small GTPases, such as Rab23. Note, most illustrations outline a uniformly narrow cilium but scanning electron microscopy reveals many different morphologies including this one where the end is slightly distended (also see Figs. 2 and 3). Putative roles of cilia in contributing to PKD are summarised below the diagram (see text for details).

Molla-Herman and colleagues described the ciliary pocket, a depression of the plasma membrane in which the primary cilium is rooted [16]: the pocket shares many morphological features with the flagellar pocket of Trypanosomatids, which is a trafficking-specialised membrane domain at the base of the flagellum. The pocket is observed around virtually all primary cilia in mouse retinal pigment epithelial cells but it cannot be absolutely essential for function since it is not universal in kidney cells [16]. A critical component of this diffusion barrier at the base of the cilium may be Septin 2, a member of the guanosine triphosphatase family conserved from yeast [17]. Loss of this factor disrupts ciliary membrane protein localisation, sonic hedgehog signalling and ciliogenesis [17]. Septin localisation is, at least in *Xenopus* embryos, controlled by the planar cell polarity protein Fritz, and mutations in human Fritz may contribute to some cases of Bardet–Biedl and Meckel–Gruber syndromes, conditions in which cystic kidney disease can also occur [18,19]. Moreover, several of the Bardet–Biedl syndrome genes (known as the BBSome) are crucial for targeting proteins to the cilium [20].

3. Ciliopathy—a new class of disease

It is not just in classical PKD, however, that malformed or malfunctioning cilia have been linked to renal cyst development: Ciliopathy is a whole new disease classification that has 'evolved' over the last 5 years, with significant genetic and molecular discoveries in areas such as the Bardet–Biedl and Nephronophthisis groups [21,22]. Full coverage of these is beyond the scope of this review but the unifying feature of ciliopathies is that the mutated proteins are all normally expressed in either primary cilia or centrosomes. This is not necessarily just in the kidney but can also include non-renal sites such as the photoreceptor cells in the eye, which are modified cilia. Widespread distribution of these affected proteins explains the wide variety of phenotypes in addition to renal cystic disease which include situs inversus (as one might expect from defective node cilia function), retinitis pigmentosa and retinal degeneration, hepatic disease, polydactyly and mental retardation [23].

4. Cilia, fluid flow, calcium and PKD

Polycystin-1 and -2 localise to the primary cilium of diverse cell types, but vertebrates lacking functional polycystins do not have structurally abnormal cilia. We have now generated original data showing that this is true for both humans (Fig. 2) and several of the animal models such as the congenital polycystic kidney mouse (*cpk*; Fig. 3). This suggests that polycystins may modulate sensory functions of cilia, rather than cilia formation. The first hint that this role may be mediated by sensing fluid flow and calcium influx came from non-renal studies looking at laterality determination: loss of *Pkd2* causes randomisation of organ laterality both in mice and zebrafish, which is thought to result from abnormal signal transduction in peripheral monocilia of the node (the mammalian "laterality organ"[24–26]. Mechanical bending of individual cilia using micropipettes induces calcium influx [27], hence it was postulated that consistent directional fluid flow generated by cilia in the node bends these cilia to induce polycystin-dependent calcium signalling in the paraxial mesoderm to the left of the node [25,28–30]. Moving to the kidney, fluid flow also induces calcium influx in cultured Madin-Darby Canine Kidney cells (derived from collecting duct epithelia), and this requires intact cilia, as well as Polycystin-1 and -2; this influx leads to calcium-induced calcium release from the endoplasmic reticulum mediated via the ryanodine and inositol triphosphate receptors [24,31–33].

How might bending of cilia and aberrant calcium signalling lead to cyst formation? One suggestion is that lower intracellular calcium in mutant renal epithelial cells may lead to reduced clearance of intracellular cAMP (for review see [34]), which is supported by elevated levels of cAMP in renal cystic epithelia [35]. Downstream effects may include activation of map kinase signalling, leading to increased cell proliferation and abnormal fluid secretion, both contributing to cyst formation. Fluid flow may also regulate gene expression directly, because it causes phosphorylation and nuclear export of the transcriptional co-repressor HDAC5, leading to upregulation of *Mef2c*; this may be relevant, because formation of renal cysts can be rescued in some strains following knockdown of *Mef2c*[36]. Furthermore, *Foxj1a*, a master regulator of cilia formation, and its downstream targets are upregulated following cystic distension of zebrafish pronephric ducts and in *Ift88*$^{-/-}$ renal cystic epithelia, leading to increased ciliary beating [37]. Therefore, fluid flow may modulate cilia function, which could further contribute to the progression of renal cyst formation.

5. Cilia and mTOR

The link between mTOR signalling, PKD and ciliary biology began with the identification of the tuberous sclerosis (TSC) genes, *TSC1* and *TSC2* [38,39], which encode Hamartin and Tuberin, respectively.

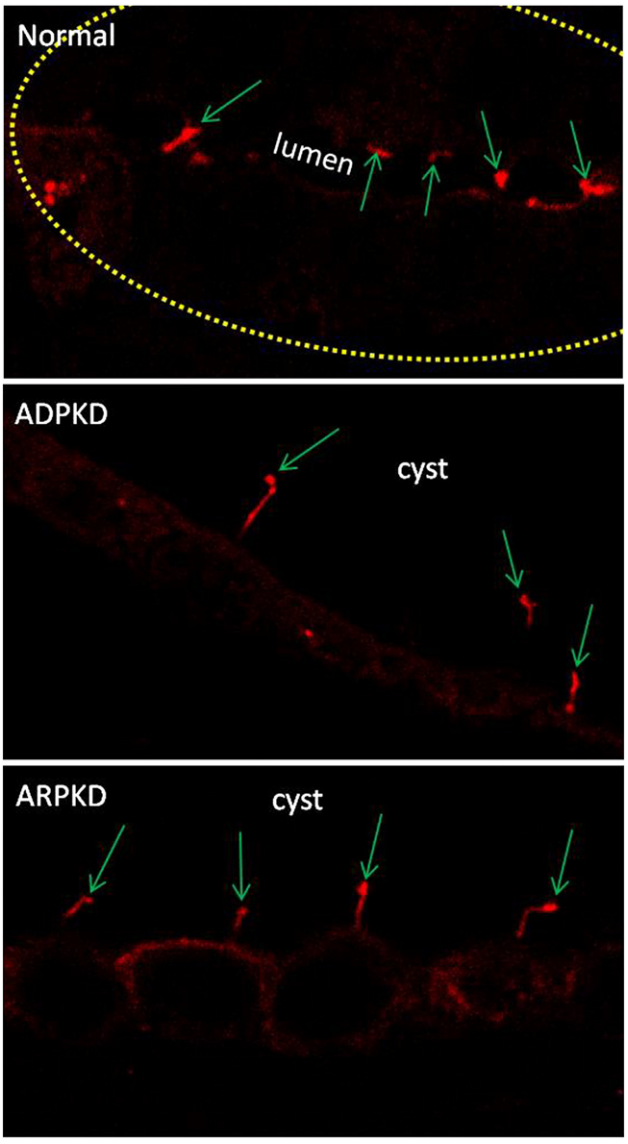

Fig. 2. Confocal microscopy immunostained for acetylated-α-tubulin of A) normal human kidney tubule, B) human ADPKD sample and C) human ARPKD sample. A) Outside of tubule is outlined in yellow to aid visualisation; clear apical cilia (green arrows), although morphology is difficult to distinguish because the lumen is not dilated and the cilia overlap. B) and C) Several apical cilia projecting into the cyst lumen.

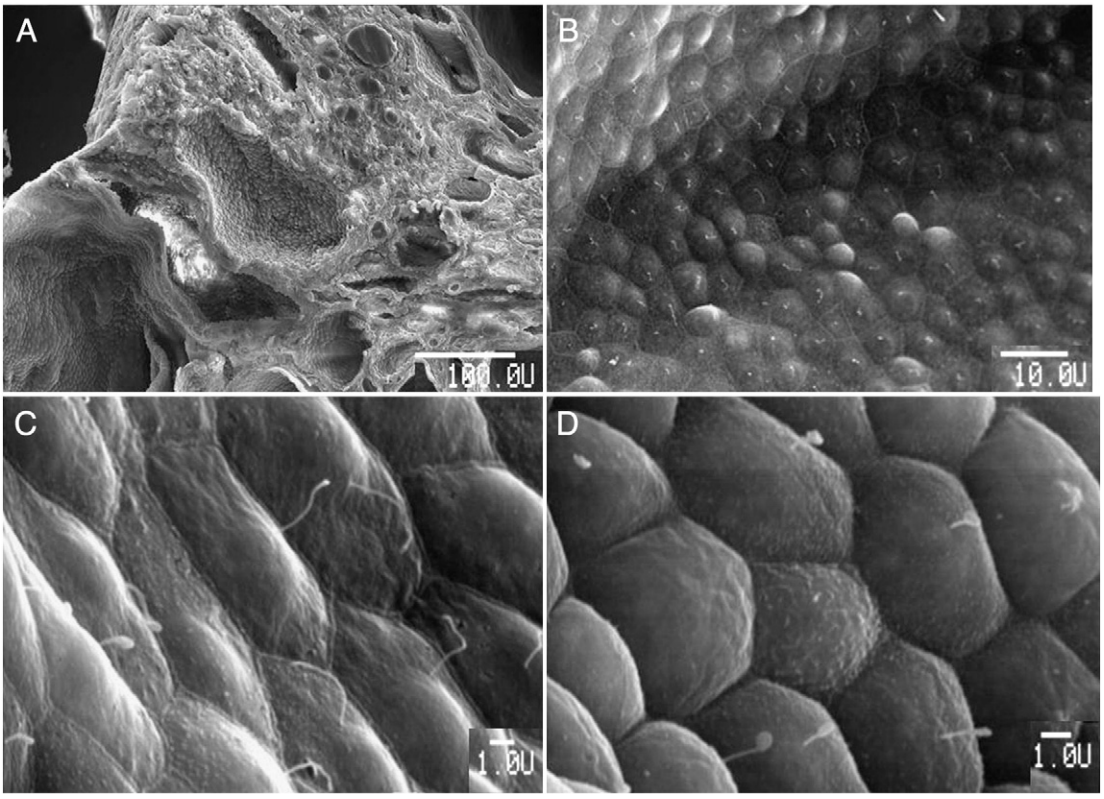

Fig. 3. Scanning electron microscopy of *cpk* cystic kidneys at low (A), medium (B) and high magnification (C and D). Note the different length and morphology of the cilia within diverse epithelia.

Subsequently, Polycystin-1 and TSC1 were shown to localise to the cilium and basal body [24,40–42]. TSC is an autosomal dominant condition characterised by the formation of hamartomas in multiple tissues and organs. Renal cysts form in ~30% of patients with TSC, while a minority of these patients develop severe PKD. The *TSC2* and *PKD1* genes lie adjacent to one another on human chromosome 16p13.3, and contiguous deletions of both genes have been identified in patients with this early onset ADPKD [43]. This observation suggests that TSC and PKD genes interact functionally in the pathogenesis of PKD, which is supported by the observation that loss of one allele of *Pkd1* worsens the renal cystic phenotype of $Tsc1^{+/-}$ and $Tsc2^{+/-}$ mice [41].

TSC1 and 2 form a complex with Rheb, a small GTPase belonging to the Ras superfamily. This TSC complex is inhibited in response to PI3K/Akt signalling. PI3K (phosphatidyl-inositol 3-kinase) is activated following binding of various ligands to their cognate receptor, including members of the tyrosine kinase receptor family. Activation of PI3K leads to the conversion of phosphatidyl-inositol 2-phosphate (PIP_2) into phosphatidyl-inositol 3-phosphate (PIP_3), which in turn leads to phosphorylation of Akt (forming pAkt) by protein dependent kinase-1 (PDK1). At the centre of this cascade is mTOR complex 1 (mTORC1), which is composed of mTOR and Raptor. mTORC1 activates a variety of cellular processes, such as growth and proliferation, via activation of serine 6-kinase (S6K). TSC1 and 2 heterodimerise to form a Rheb GTPase-activating protein, which is inhibitory to mTORC1, and phosphorylation of TSC2 by pAkt promotes mTOR signalling by inhibiting the TSC/Rheb complex. Therefore TSC1 and 2 regulate mTOR signalling by modulating activity of the PI3K/Akt kinase signalling cascade.

mTORC1 is also implicated in renal cyst formation, because readouts of mTOR signalling, such as phospho-S6K and -mTOR, are elevated in epithelial cells lining cysts in kidney samples taken from patients with ADPKD and ARPKD, and from mouse models of renal cystic disease [44–47]. Furthermore, rapamycin, a specific inhibitor of mTORC1, causes regression of renal cysts in several rodent models of PKD, suggesting a role for mTOR signalling in pathogenesis [44,48–50]. mTOR signalling is also elevated in renal cystic epithelia of mice with mutations in genes implicated in ciliogenesis, including *Tg737*/*Ift88* and *Ofd1*, and regression of renal cysts in these mice following rapamycin treatment suggests that cilia may regulate mTOR signalling during cyst formation [44,51].

The C-terminal cytoplasmic tail of Polycystin-1 regulates the subcellular localisation of Tuberin via a direct physical interaction, and modulates phosphorylation of TSC2 by AKT [44,45]. Because Polycystin-1 and TSC1 localise to the cilium and basal body, respectively, and cilia formation is abnormal in both mouse embryonic fibrobalsts and renal tubular epithelium lacking either *Tsc1* or *Tsc2*[40,41,45], regulation of mTOR signalling by polycystins may relate to functions that converge on the cilium. Indeed, bending of cilia has recently been shown to down-regulate mTOR signalling, independent of both calcium influxes and Akt [52]. Regulation of mTOR signalling may be independent of cilia in some circumstances, however, because Dere et al. reported that HEK-293 and hTERT RPE-1 cells lacked cilia in their culture conditions [45].

6. Cilia, Wnt and cell polarity

Diverse recent studies confirm that knockdown of ciliogenesis genes disrupts Wnt signal transduction in mouse, zebrafish and frog. A number of Wnt signalling pathways have been described, which can be grouped into canonical or non-canonical pathways, but signalling is typically initiated by interaction of different Wnt ligands with specific Frizzled (Fz) receptors, followed by recruitment of the intracellular protein, Dishevelled (Dvl), and activation of specific co-receptors [53,54]. While canonical Wnt signalling results in activation of β-catenin-mediated transcription, non-canonical pathways are, by

definition, β-catenin-independent. The Wnt/Ca2+ non-canonical pathway may be linked to calcium influx via bending the cilium but a better studied pathway is Wnt/planar cell polarity (PCP) which regulates cell migration and polarity.

In the canonical pathway, mice overexpressing β-catenin in renal epithelia develop cysts, owing to defects in cell turnover and aberrant localisation of ion channels [55]. The cytoplasmic tail of Polycystin-1 interacts with β-catenin and may modulate Wnt signalling, although there are conflicting results over whether this is stimulatory or inhibitory [56,57]. Secreted Frizzled-related protein 4, which specifically inhibits canonical Wnt signalling, is upregulated in human ADPKD and several mouse models of renal cystic disease, and genetic variation of *DKK3*, encoding a Wnt/β-catenin antagonist, may modify the severity of ADPKD [58,59]. Therefore, canonical Wnt signalling is directly implicated in renal cystic disease.

Wnt/PCP signalling is activated by ligands, such as Wnt11 and Wnt9b, which are essential for normal kidney development. Wnt11 is expressed in ureteric ampullae, where it activates Gdnf/Ret signalling and branching morphogenesis [60]. Furthermore, loss of Wnt9b, or the PCP protein Fat4, leads to renal cystic disease associated with abnormal polarity of renal epithelia, randomised orientation of cell division and abnormal elongation of tubules [61,62]. Epithelial cell intercalation drives elongation of renal tubules in embryonic development, whereas direction of cell division is more important postnatally since the mitotic spindles are orientated in the direction of tubule elongation after birth. Both of these processes are disrupted in mouse models of renal cystic disease, including mice with mutations in *Hnf1β*, *Tsc1/2* and *Pkd1*[41,63]. Several ciliopathy genes have been implicated in the regulation of Wnt/β-catenin and Wnt/PCP signalling pathways, and work in zebrafish and frogs has controversially suggested that these components may regulate the balance between canonical and non-canonical signalling, possibly through regulation of Dvl, which is common to both pathways; furthermore, this balance may be regulated by fluid flow [9,64]. Therefore, cilia may coordinate Wnt signalling pathways, and deregulated wnt signalling may contribute to cyst formation.

7. Cilia and hedgehog signalling

Cilia play a central role in hedgehog (Hh) signal transduction. The canonical Hh signalling cascade elicits quantitative transcriptional responses within cells according to the extracellular concentration of secreted Hh morphogen (for review see [65]). Hh signalling is relatively simpler than other signalling cascades, with only three essentially equivalent morphogens (Sonic (Shh), Indian (Ihh) and Desert (Dhh) hedgehog) that bind predominantly to only a single receptor, Patched 1 (Ptch1) [66]. Unlike most other signalling pathways, this Hh receptor does not induce intracellular signal transduction upon binding of ligand, but instead serves as an inhibitor of a second transmembrane protein, Smoothened (Smo), in the absence of morphogen. Ligand binding leads to derepression of Smo, allowing it to accumulate in cilia and to activate a series of events that lead to the conversion of the Gli transcription factors, Gli2 and Gli3, from transcriptional repressors to transcriptional activators [65].

Recent evidence suggests that transport of Smo along the ciliary axoneme causes it to undergo conformational change, and leads to release of Gli2 and Gli3 from cilia into the nucleus [67–69]. Therefore, cilia are essential for proper regulation of Hh signalling, and various mutants that exhibit defective ciliogenesis show abnormal gradients of Hh pathway activity (i.e. elevated Hh signalling in tissues not normally eliciting a Hh response, and reduced signalling in tissues normally showing high levels of Hh signal transduction; e.g. the Joubert syndrome gene, *Arl13b* [70]). Therefore, abnormal Hh signalling may result from defective ciliogenesis, observed in renal cystic epithelia. It is likely that cilia represent a site at which many components of the Hh and other signalling pathways become concentrated, making it a 'molecular meeting place' that promotes targeted interactions owing to the juxtaposition of the ciliary axoneme and specialised membrane.

Abnormal Hh signalling in humans is associated with abnormal renal development. In particular, ectopic and cystic kidneys are commonly found in Smith–Lemli–Opitz syndrome (SLOS), which is caused by mutations in *DHCR7*, which may affect Hh signalling via defective cholesterol biosynthesis [71–74]. Reduced Hh signalling is also associated with ectopic kidneys and formation of renal cysts in mice [75], although it is not clear whether renal cyst formation in these mice is a direct result of abnormal signalling within the kidney itself. Other experiments have suggested a role for elevated Hh signalling within renal epithelia in renal cyst formation. *Ihh*, which in contrast to *Shh* is normally expressed in outer medullary and cortical collecting duct epithelia [76], was identified as the most highly upregulated gene in a renal explant model of corticosteroid-induced renal cyst formation [77]. Cyst formation in this model was reduced following treatment with the Smo antagonist cyclopamine, implicating canonical Hh signalling in pathogenesis.

Bearing in mind the essential role of cilia in regulating the Hh cascade, these data linking Hh signalling in renal cystic diseases suggest that Hh effects may be mediated by ciliary functions. However, these are observations rather than definite causal links, so a thorough analysis of Hh signalling in models of PKD with normal and abnormal cilia is necessary.

8. Cilia are unlikely to be the only cause of cystic kidneys

In this review, we have presented a 'cilia-centric' view of renal cystic disease. There is overwhelming evidence that cilia are involved in many cystic diseases, but we would question whether they are the sole cause of PKD pathogenesis. There are three main lines of evidence supporting critical cystogenic roles for cilia: 1) the protein products of Polycystins and ciliopathy genes localise to cilia and mutations in these genes are associated with renal cystic disease accompanied by functional or structural abnormalities in cilia; 2) both polycystin and ciliopathy gene products regulate signal transduction cascades, and these pathways are demonstrably abnormal in cystic epithelia; and, 3) rescue of these cilia-related pathways inhibits cyst formation.

Localisation of the Polycystins to the cilium and ciliopathy genes within the cilium is tantalising evidence that disruption of cilia is important cyst formation, but these molecules are all expressed in other sites too. Polycystins and fibrocystin, for example are found in focal adhesion complexes, and there is a significant body of PKD work on aberrant cell-matrix and matrix-matrix interactions [78,79] which cannot all be due to malfunctioning cilia. Similarly, although ciliopathy genes are expressed in the cilium by definition, it is now clear that these genes also regulate a variety of cellular processes external to the cilium, including cell division and cytoskeletal organisation [80]; although some of these functions are via the basal body and centriole [81] which are part of the "cilium complex," it is difficult to conceive that all of the effects are cilia mediated when expression is so much more diverse than this organelle. What if the ciliary expression of some of these factors is just coincidental, because they are randomly distributed throughout the plasma membrane? This might explain why, if you look hard enough, almost every protein implicated in PKD appears to be expressed in the cilium. Distribution cannot be completely random, however, because there is definitely restricted passage and targeting of some molecules to the cilium [14,17,20]. The evidence linking aberrant formation of cilia to cystic kidney disease is compelling in the *orpk* mouse and several of the *IFT* mutants [5,11,12] although not all of these (or the other ciliopathies) get devastating PKD as is seen with the human *PKD* mutations. There is some high quality work on functions such as flow-mediated calcium influx [24], but these studies are technically difficult and many laboratories do not have the technology to dissect more subtle flow-

related effects. One of the problems in studying cilia and PKD is that it is not possible to specifically ablate cilia without disrupting other cellular processes. Moreover, cilia may have a different structure and modified functions in normal and cystic epithelia, which makes it difficult to distinguish cause from effect. As an example, cilia are morphologically normal in precystic epithelial cells of *Ofd1* mutant mice but are shorter in cystic epithelia [51]; so is it the change in cilia length that stimulates cyst formation or is it the cystic dilatation and changes within the cystic epithelia that disrupt cilia structure secondarily? Similarly, in TSC and other models, mTOR signalling is upregulated in cystic but not precystic epithelia [41], and there is a suspicion that rapamycin may be more effective in already formed cysts rather than at earlier stages. This effect has been attributed to increased apoptosis in cystic epithelia [44] and it may help explain the lack of success of recent human rapamycin trials where there was a modest reduction in kidney volume without rescue of renal function [82,83].

Overall, the last decade has undoubtedly implicated cilia in renal cyst biology but the challenge for the next decade is to determine how cilia-related effects tie in to all of the other facets of PKD biology in order to devise rational treatments for human disease.

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