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An extracellular vesicle based hypothesis for the genesis of the polycystic kidney diseases

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Abstract

Autosomal dominant polycystic kidney (ADPKD) disease is the commonest genetic cause of kidney failure (affecting 1:800 individuals) and is due to heterozygous germline mutations in either of two genes, *PKD1* and *PKD2*. Homozygous germline mutations in *PKHD1* are responsible for autosomal recessive polycystic kidney (ARPKD) disease a rare (1:20,000) but severe neonatal disease. The products of these three genes, *PKD1* (polycystin-1 (PC1 4302(3)aa)), *PKD2* (polycystin-2 (PC2 968aa)) and *PKHD1* (fibrocystin (4074aa)) are all present on extracellular vesicles (EVs) termed, PKD-exosome-like vesicles (PKD-ELVs). PKD-ELVs are defined as 100 nm PC1/PC2/CD133 and fibrocystin positive EVs which are shed into the urine from the apical plasma membrane of proximal tubule (PT) cells. PKD-ELVs are therefore ectosomes and are distinct from classical exosomes from the multivesicular body. PC1, PC2, fibrocystin and exosomal polycystin-1 interacting protein (EPIC, CU062) form a higher order ion channel complex termed the polycystin complex (PCC) on the surface of the PKD-ELV. We hypothesize that the PCC is involved in the generation of the PKD-ELV and is a structural component thereof. The PCC has also been detected on the primary cilium, a hair like 9+0

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Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement
Marie C. Hogan: Writing – review & editing, Writing – original draft, Conceptualization. **Christopher J. Ward:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

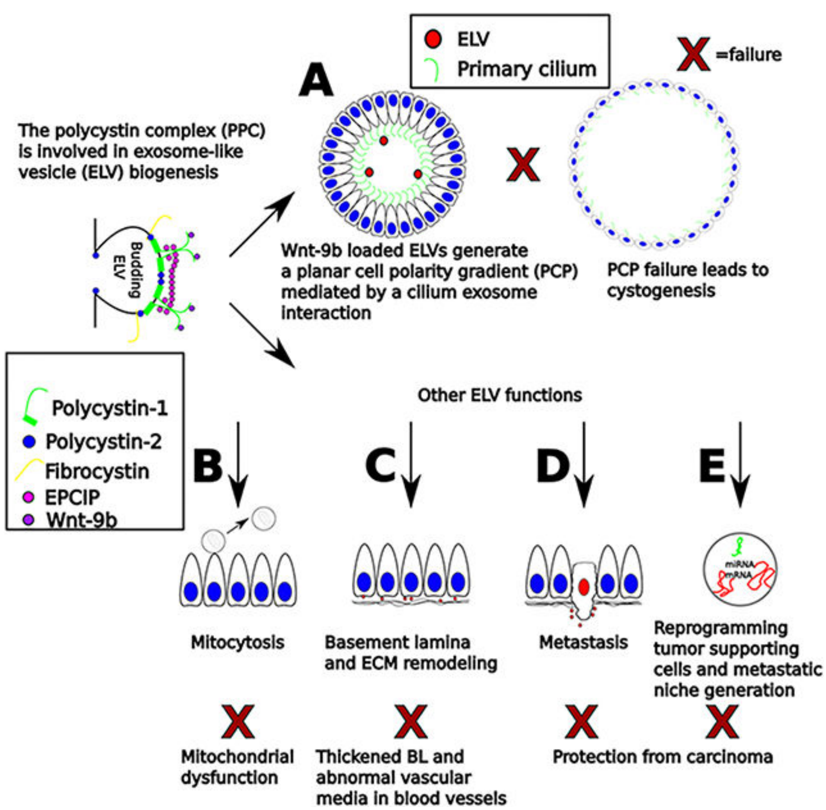
Institutional review
Urine and human primary kidney epithelial cells were de-identified through the KUMC biospecimens core, PI: Dr. Darren Wallace. This core complies with federal regulations and was approved by the institutional review board at KUMC. This core (PKD Repository: HSC #13674; approval on July 9, 2014) adheres to Section 164.514(b)2 of the HIPAA Privacy Rule, which provides the standard for de-identification of protected health information (Safe Harbor method). Informed consent was confirmed for each individual that donated urine or tissue in accordance with the Declaration of Helsinki.

Informed consent
Informed consent was obtained for all individuals enrolled in this study. Data was deindentified using the Safe Harbor method.

Appendix A. Supplementary data
Supplementary material related to this article can be found online at https://doi.org/10.1016/j.vesic.2024.100048.

microtubule based structure present on all cells except hepatocytes. In kidney epithelial cells, the primary cilium protrudes into the lumen of the tubule where it regulates planar cell polarity (PCP) and tubule lumen diameter. Here we present a theory that explains the presence of the PCC on PKD-ELVs and primary cilia as well as other cryptic aspects of ADPKD and ARPKD. We suggest that the fundamental role of the PCC is to assemble PKD-ELVs on the plasma membrane and then shed them into the extracellular space or the lumen of the tubule. The resultant PKD-ELVs can have multiple functions in different biological contexts. One of the roles of the resultant PKD-ELVs is to generate a planar cell polarity (PCP) signaling gradient along kidney tubules in developing or regenerating kidney. This is mediated via an adhesion event between the PKD-ELV and primary cilium. Defects in the primary cilium or PKD-ELV assembly lead to cystogenesis, the major feature of ADPKD. The other important role for the PCC dependent PKD-ELV is the detection, packaging and extrusion of defective mitochondria. The PKD-ELV is also critical in the transfer of mRNA and miRNAs between cells and as a vector for extracellular proteinases and hyaluronidases involved in tissue remodeling. A PKD-ELV centric view of polycystic disease (EV theory) can explain the requirement for primary cilium function in ADPKD (where the primary cilium is the PKD-ELV receptor), the observation of defective mitochondria in the disease, the abnormalities detected in the extracellular matrix (ECM) as well as the resistance to carcinoma noted in ADPKD patients and individuals carrying *PKHD1* mutations, see graphical abstract.

Graphical Abstract



Keywords

Extracellular vesicle; Primary cilium; Polycystin-1; Polycystin-2; Fibrocystin; Autosomal dominant polycystic kidney disease; Autosomal recessive polycystic kidney disease; PKD1; PKD2; PKHD1

1. Introduction

Polycystic kidney disease is responsible for a significant health and economic burden. ADPKD is mainly due to germline mutations in one copy of either the *PKD1* (OMIM:601313) or *PKD2* (OMIM:173910) gene with minor contributions from an expanding set of genes involved in ER quantity control/folding and primary cilium assembly.[1–7] *PKD1* is mutated in about 75% of ADPKD pedigrees and is the more severe disease leading to kidney failure in the 5th decade of life. *PKD2* is mutated in 18% of pedigrees and presents as a milder disease with kidney failure occurring in the 7th decade.[8,9] The other minor genes are responsible for about 1% of cases with about 7%–10% of individuals having 'no mutation detected'. These latter cases are likely new germline or somatic mutations that occurred early in embryogenesis or are due to deep intronic mutations or possibly loci that have not been identified yet.

ADPKD is characterized by the slow but relentless growth of fluid filled cysts, which are derived mainly from the collecting duct (CD). Cystogenesis is associated with fibrosis, inflammation, apoptosis and cellular proliferation leading to the destruction of the surrounding nephrons and their associated vasculature.

Autosomal recessive polycystic kidney disease (ARPKD) is due to homozygous germline mutation in *PKHD1*. In its most severe manifestation, cysts are observed *in utero* by ultrasound scan.[10] Kidneys can be up to 10 times normal in size with global dilatation of the CDs. Hepatic fibrosis and kidney failure in ARPKD make it the commonest reason for combined kidney–liver transplant in early childhood.

1.1. The structure of the polycystins and fibrocystin

The *PKD1*, *PKD2* and *PKHD1* genes encode the proteins polycystin-1 (PC1)(P98161 PKD1_HUMAN 4302(3)aa), polycystin-2 (PC2)(Q13563 PKD2_HUMAN 968aa) and fibrocystin (P08F94 PKHD1_HUMAN 4074aa), Fig. 1. These proteins interact with each other in 2MDa ion channel complex.[11] PC1 is a 4302(3) aa 11 transmembrane (TM) spanning protein that is autoproteolytically cleaved at 3048 aa to generate a large N-terminal ectodomain (450kDa) and a membrane spanning C-terminus of 150kDa Fig. 1.[1,12–15] The bulk of the ectodomain is composed of 17 PKD repeats, a β-sandwich of 80–90 aa which can interact with itself or EPCIP.[16–18] PC2 is a 968aa six TM spanning protein and a member of the transient receptor potential P (TRPP) family of ion channel subunits.[2,19] The last six TM regions of PC1 and the TMs of PC2 from a cation channel with three PC2 and one PC1 subunit.[20] Fibrocystin is a large 4074 aa type-1 membrane protein with an ectodomain composed of 12 IPT/TIG (an immunoglobulin-like fold) domains, one PA14 (protective antigen (anthrax toxin) 14) domain, two G8 (a domain with eight conserved glycines) domains and two blocks of parallel β-helix (PbH). PbHs form a rigid helix with a triangular cross section and are often carbohydrate binding or structural components of a higher order assembly. Fibrocystin has a pro-protein convertase cleavage site at 3617..3620, Fig. 1.[21–23]

1.2. The polycystin complex (PCC) is present on PKD-ELVs and forms a multimolecular complex around the extracellular vesicle

PC1, PC2 and fibrocystin are present together on a CD133+ subfraction of human urinary EVs, termed PKD-exosome-like vesicles (PKD-ELVs). PKD-ELVs are 100 nm diameter bilipid membrane bound ectosomes budded from the plasma membrane of PT cells. PKD-ELVs are distinct from classical exosomes that are of multivesicular body origin as they are low in the classical tetraspanin markers CD9, CD63 and CD81 but are a rich source of the ectosome marker CD133, a pentaspanin protein abundant in the plasma membrane of PT cells.[24] PKD-ELVs resolve at a refractive index (η=1.3530) on a 5%–30% sucrose D_2O gradient. Podocyte ectosomes rich in podocin and podocalyxin resolve at a higher density and large EVs from the CD, rich in aquaporin-2, resolve at a lighter density on this gradient.[25,26]

Proteomic analysis of sucrose/D_2O gradient purified PKD-ELVs from 13 individuals with proven *PKD1* mutations (average age 29.5 ± 5.5 years) compared to age similar subjects without kidney disease (average age 30.1 ± 5.0) showed that 9/2008 proteins assayed (0.32%) had a statistically significant reduction, including PC1 (reduced to 54% of the normal level) and PC2 (reduced to 53%).[26] Fibrocystin was also reduced in PKD-ELVs from *PKD1* individuals but did not reach statistical significance. This study showed that a small secreted glycoprotein, EPCIP, was also significantly reduced.[18] Further work using blue native gel (BNG) technology showed that PC1, PC2 and fibrocystin exist in a 2MDa complex and that the ectodomains of PC1 and fibrocystin can be released from the PCC by increasing levels of the denaturing detergent sodium dodecyl sulfate (SDS).

Analysis of the localization of the PCC shows that in freshly isolated non confluent non immortalized PT cells, PC1, PC2, fibrocystin and EPCIP are present in the focal adhesions (FAs).[18] PC1 is also a major membrane protein on tetraspanin-4 (TSPN4)+ retraction fibers (RFs), actin based filaments left on the substratum by migrating cells. RFs extrude TSPN4+ vesicles, 'migrasomes', that are intensely positive for both PC1 and particularly EPCIP, Fig. 2A. These structures become positive for mitochondrial proteins if the cell is challenged with mitochondrial decoupling agent, Fig. 3 (white arrows show PC1, EPCIP and mitochondrium positive migrasomes on the substratum).[27] In confluent PT cells, PC1 and EPCIP are ER proteins which rapidly change their distribution when mitochondria are decoupled, see Section 1.6.1.

1.3. The role of PCC positive EVs in experimental systems

At the moment, there is little direct data as to the signaling function of PKD-ELVs in mammals. The nematode worm *C. elegans* has homologous versions of the *PKD1* and *PKD2* genes/proteins termed location of vulva-1 (LOV-1) and PKD2. Both genes are required for male mating behavior, the recognition of the hermaphrodite and especially the identification of the vulva for mating. The protein products are present on the male specific neuronal cilia and on extracellular vesicles released into the environment. These LOV-1/PKD2 positive EVs have a potent effect on the behavior of isolated male worms (increased male-self contact or tail-chasing behavior) and interact with the hermaphrodite upon mating.[28]

In the vertebrate embryonic node, a transient ciliated structure that determines left/right (L/R) axis formation, EVs shed are from the cells lining the pit of the node and are transported to left side of the node by nodal flow where they interacted with the immotile ‘picket fence’ primary cilia. This induces a PC2 dependent asymmetric Ca^{++} flux and is the first signaling event in symmetry breaking in the vertebrate embryo.[29,30] Our previous work showed that epitope tagged fibrocystin + PKD-ELVs can bind to the primary cilium of CD derived IMCD3 cells.[31] The actual signaling function of these PKD-ELVs is subject to debate, see below.

1.4. The polycystin complex is present on primary cilia and the primary cilium has a critical role in cystogenesis

The primary cilium is a membrane covered 9+0 microtubule based structure which protrudes from the surface of the cell into the extracellular space for connective tissue cells or the lumen of the tubule in the case of epithelial cells. The structure is an extension of the mother centriole and is composed of at least 200 proteins.[32] The primary cilium is involved in signaling with the classical hedgehog pathway requiring primary cilium function in vertebrates. The Wnt, platelet derived growth factor pathways (PDGF) and notch signaling pathways are also in part dependent on the primary cilia, reviewed in Ref. 33.

The primary cilium is a critical organelle in cystogenesis as mutations in the genes for intraflagellar transport (IFT) proteins induce PKD. The Tg737-mutant (Oak Ridge Polycystic Kidney (ORPK) mouse model) has a hypomorphic IFT88 allele and develops recessive PKD. IFT88 (part of the IFT-B1 complex) is required for flagellum and primary cilium assembly in *Chlamydomonas reinhardtii* and mice, respectively. These observations show that the primary cilium is central to the development of polycysts in the kidney.[34] The localization of PC1, PC2, fibrocystin and cystin-1 (Cys1, the product of the *cpk* cystogene) to the primary cilium further supports the key role of this organelle in the genesis of PKD.[35,36]

We confirmed the presence of the PCC on the primary cilium together with the EPCIP (PC1 ligand). The PC1, PC2 and EPCIP staining appeared to be vesicular in nature with EVs applied to the shaft of the acetylated-α-tubulin + primary cilium. Deconvolution confocal microscopy showed that the PC1 staining resolved as 100 nm rings on the 200 nm diameter primary cilium implying that the vesicles were hollow, Fig. 2C. On these primary cilium attached EVs, EPCIP appeared as an outer ring of staining on a core of PC1 staining generating a ‘bull’s eye’ appearance.[18]

Our hypothesis predicts that the critical role of the primary cilium in PKD is that of a PKD-ELV receptor. The primary cilium extends into the lumen of the tubule and interacts with PKD-ELVs secreted from cells proximal in the tubule, especially PT cells. The PKD-ELVs are then cleared from the cilium into the cell where they transduce a signal.

Thus, the **primary cilium hypothesis** suggests that defects in primary cilium signaling alone are responsible for phenotypes observed in ADPKD. On the other hand, the **EV hypothesis** stresses the role of the PKD-ELV, which has multiple functions, one of which is to transduce a PCP signal through the primary cilium.

1.5. Function of the polycystin complex (PCC) in planar cell polarity and cystogenesis

The EV hypothesis is predicated on the idea that the PCC is involved in EV biogenesis — in particular the PKD-ELV subtype, Fig. 4. The PCC generates a basal physical structure, a PKD-ELV, which can be modified for diverse functions all of which are defective in ADPKD. In ADPKD the most important function is the generation of an non-canonical Wnt/PCP gradient along the tubule. As tubules elongate during embryogenesis or repair, cell divisions orientate in parallel with the long axis of the tubule and convergent extension both narrows and extends the tubule. Non canonical Wnt signaling is critical in this phenomenon, and is mediated by Wnt-9b. Wnt-9b signaling is non cell autonomous. However, Wnts are lipid modified (Wnt-9b has palmitic acid added to S216, by similarity, by porcupine (PORCN)) and are insoluble in aqueous solutions lacking detergent. Thus, it is unlikely that Wnt can signal without a carrier.[37] Wnt-9b is known to interact with the N-terminal Leucine Rich Repeat (LRR) and cell wall integrity and stress response component (WSC) of PC1 and can gate the PC1/PC2 Ca^{++} channel.[38] We predict that in developing and regenerating kidney Wnt9b is in a complex with PC1 on the PKD-ELV and that the PKD-ELV acts as a Wnt carrier generating a PCP gradient along the kidney tubule. Disruption of PKD-ELV production, due to a decrease or absence of the PCC (PC1, PC2 or fibrocystin), leads to a failure of PCP. Convergent extension fails, the mitotic spindles misalign and tubular dilatation then initiates leading to cystogenesis.

1.6. Other roles for PKD-ELVs that give rise to primary cilium independent phenotypes in PKD

If the EV theory is correct then it could explain multiple phenotypes that appear independent of primary cilium function. For example;

1. There are defects in mitochondrial morphology and function in ADPKD.
2. Enhanced fitness (resistance to toxic insults) is observed in *PKD1*$^{+/-}$, *PKD2*$^{+/-}$ or *PKHD1*$^{+/-}$ hepatocytes (hepatocytes lack primary cilia).[39]
3. There is resistance to carcinoma in individuals with heterozygous *PKD1* and *PKHD1* mutations.
4. There are alterations in the structure of the basement lamina (BL) which is abnormally thickened under cyst lining epithelia and there is abnormal remodeling of ECM in the *tunica media* of intracranial arteries.

1.6.1. The role of the polycystin complex (PCC) in extrusion of senescent mitochondria (mitocytosis)—

Migrating cells treated with agents that induce mitochondria to produce reactive oxygen species (ROS) respond by extruding the malfunctioning mitochondria in tetraspanin-4 (TSPN4)+ EVs termed migrasomes in a process termed mitocytosis.[27] We have shown that migrasomes are PC1+/TSPN4+.[18]

ADPKD cyst lining cells accumulate morphologically and biochemically abnormal mitochondria. Biochemical defects include compromised glucose entry into the tricarboxylic acid cycle (TCA) under aerobic conditions, a 'Warburg effect', a defect in β-oxidation of fatty acids and an enhanced utilization of the glutamine/glutamate/α-ketoglutaric/TCA shunt.[40–43] These phenomena can be explained if they are a manifestation of the retention of defective senescent mitochondria. In this scenario there is a failure of PCC dependent mitochondrial extrusion in ADPKD and ARPKD. Cells that lack or have low levels of PC1 or PC2 will accumulate mitochondria that have multiple defects.

Our experiments with freshly isolated primary human kidney epithelial cells challenged with the mitochondrial inner membrane H^+ gradient decoupling agent carbonyl cyanide m-chlorophenyl hydrazone (CCCP) showed that PC1, PC2 and EPCIP interact in a higher order complex in the ER that is associated with swollen and disrupted mitochondria.[18] In untreated subconfluent PT cells, PC1, PC2 and EPCIP are localized to the focal adhesions (FAs), RFs and migrasomes. These proteins also have a diffuse ER localization in both subconfluent and confluent cells. Initially, after 1–2 h of CCCP treatment, intense foci of PC1 become apparent around clusters of swollen mitochondria, Figs. 2B and 3A. The nidi then expand into PC1+ rings (up to 6 μm in diameter) with disintegrating mitochondria in the ring wall (we call these 'fairy rings' (FRs) as the PC1 appears like the green grass and mitochondria are similar to the mushrooms in these fungal phenomena. FRs are likely the source for PKD-**ELVs**), Fig. 2B. The center of the ring is EPCIP positive. After >4 h, and only in confluent cells, large 4μm to 8μm EVs appear on the apical surface of the cell, Fig. 3B. Confocal and EM microscopy show that these contain disintegrating mitochondria and that on the surface of this structure PC1, PC2 and EPCIP (CU062) interact with one another in a network that extends across the surface of the extruding vesicle, Fig. 4A.[18] The PCC can thus be imagined as a hairnet wrapped around a balloon (the PKD-ELV) and is likely a structural multiprotein complex involved in molding the membrane and acting as a platform for extracellular signaling molecules (for example, Wnts).

1.6.2. Increased somatic fitness in hepatocytes induced by PKD1, PKD2 and PKHD1 mutations—Defective mitocytosis may confer a selective advantage to cells with mutant PCC genes. Compromised mitocytosis allows cells to retain mitochondria that would otherwise be extruded in WT cells and then survive a reactive oxygen species (ROS) insult. In short, the ROS damaged mitochondria in PCC mutants, are able to supply enough ATP and NADH for the cell to survive.

Hepatic cirrhosis is characterized by the clonal expansion of nests of relatively normal hepatocytes surrounded by walls of fibrosis and abnormal ECM deposition (mainly collagen). Cirrhosis is commonly due to chronic hepatic insults such as problematic alcohol use, chronic viral infection (hepatitis B and C) and non-alcoholic steatohepatitis. Whole exome analysis of regenerating hepatocytes in the cirrhotic nodules shows that *PKD1* is the most commonly mutated gene (in 12.9% nodules) and *PKHD1* the 10th most common (in 6.5% nodules).[44] Mutations in these genes are rarely observed in normal tissue or in DNA from hepatocellular carcinomas. Mutations in *PKD2* were also observed in cirrhotic nodules.

To investigate the enhanced fitness of hepatocyte clones with somatic mutations, Zhu et al. used mice homozygous fumarylacetoacetate hydrolase (*Fah*) mutations. *Fah* mutants accumulate fumarylacetoacetate the second last product in the tyrosine catabolism pathway. Fumarylacetoacetate and its derivative succinylacetone are toxic ketones that give rise to ROS that induce liver degeneration. Homozygous mice can be protected from liver failure with the drug nitisinone which blocks 4-hydroxyphenylpyruvate dioxygenase an earlier step in tyrosine catabolism and prevents the build up of toxic fumarylacetoacetate and succinylacetone. Hydrodynamic transfection of *Fah*$^{-/-}$ livers with a transposase plasmid and a transposon encoding *Fah* allowed *Fah*$^{-/-}$ mice to survive the removal of nitisinone from their diet. In the Zhu et al. experiments, livers were transfected with a library of CRISPRs that targeted the genes implicated in the genesis of somatic fitness (the CRISPRs and Cas9 are on the same plasmid as the *Fah* gene, 882 gRNAs targeting 147 genes). *Fah* complemented, CRISPR mutated hepatocytes then competed to repopulate the liver. Genes that when mutated confer enhanced fitness were then represented by a greater number of copies of the integrated CRISPR gRNA per liver. The relative abundance of gRNAs was determined by PCR amplification of the gRNA encoding section of the vector from total mouse liver genomic DNA and quantified using count data from high throughput DNA sequencing. This generated a rank order of gene targets (gRNAs) that represented the relative fitness conferred by mutating the genes in question. *Pkd1* was consistently in the top 10 of protective gRNAs identified in all experiments.[44]

Further studies showed that *Pkd1*$^{fl/+}$ mice transduced with a Cre adeno associated virus were resistant to carbon tetrachloride (CCl_4) compared to WT animals. In *Pkd1* disrupted mice, there were lower serum alanine and asparate aminotransferase enzyme levels at 24 h post CCl_4 challenge and a decreased necrotic area after 48 h of treatment. This phenomenon appeared not to involve cell proliferation but to be due to resistance to the toxic insult *per se*.

Alcohol, viral infections, fumarylacetoacetate and CCl_4 all generate ROS in the liver. The EV hypothesis suggests that the hepatic resistance to ROS cannot be due to a primary cilium signaling pathway as hepatocytes are one of the few cell types that lack primary cilia.[39] Instead, we suggest that in response to ROS the injured hepatocytes induce mitocytosis and extrude damaged mitochondria. This process is so efficient in WT hepatocytes that it causes direct cell death by removing the mitochondrium generated pools of ATP, NADH and NADPH necessary for survival. Clones lacking WT levels of the PCC due to somatic mutations in the *PKD1*, *PKD2* and *PKHD1* genes have an advantage because mitocytosis is less efficient. Thus, mitochondria that would have otherwise been removed, are retained and produce enough ATP and NADH to survive the chemical insult.

1.6.3. Resistance to carcinoma in individuals with ADPKD and those carrying PKHD1 mutations—The relative risk of developing carcinoma in ADPKD is 0.84 (CI:0.77–0.91).[45] Thus, germline mutations in *PKD1* are protective against malignancy despite the massive neoplastic field change present in kidney cysts. The incidence of colonic cancer is also decreased in individuals carrying the common T36M *PKHD1* mutation, *PKHD1*$^{+/-}$ individuals.[46] We detected 15:3,603 (0.42%) T36M mutations in controls versus 1:3,767 (0.027%) colorectal cancer individuals, indicating that heterozygous *PKHD1* mutations are protective.

Somatic mutations in the Adenomatous Polyposis Coli (*APC*) gene are the commonest changes observed in sporadic colon cancer in humans. The inducible colorectal carcinoma (CRC) mouse has bialellic floxed *Apc* genes which are inactivated by the actuation of a tamoxifen activated estrogen receptor Cre fusion gene (*CreERT2*). The *CreERT2* gene is driven by a colonic epithelia specific *Cdx2* promoter. Administration of tamoxifen induces Cre mediated disruption of both *Apc* genes. *Cdx2ERT-Apc*$^{fl/fl}$ *Pkd1*$^{+/+}$ mice developed more numerous and larger colonic tumors than mice with *Cdx2ERT-Apc*$^{fl/fl}$ *Pkd1*$^{+/fl}$ or *Cdx2ERT-Apc*$^{fl/fl}$ *Pkd1*$^{fl/fl}$ genotypes.[47] Thus, disruption of the murine *Apc* gene concomitantly with *Pkd1* compromises initiation and growth of colonic tumors.

Resistance to carcinoma may be due to defects in EV production necessary for, bystander cell reprogramming and metastatic niche production. Alternatively, EV dependent direct spread may be compromised as EVs are necessary for PCP or are involved in carrying proteinases and hyaluronidases necessary for local invasion, see Discussion, Section 2.

1.6.4. Thickened basement lamina (BL) and defects in the extracellular matrix (ECM) of intracranial arteries—

The BL underlying cysts is grossly thickened and the ECM in the *tunica media* of intracranial arteries is often abnormal leading to an increased incidence of berry aneurysms in ADPKD.[48,49] Both phenomena cannot be readily explained by a simple primary cilium hypothesis. The PCC may be involved the generation of EVs that carry surface bound metalloproteinases involved ECM remodeling, the ADAMs (a disintegrin and metalloproteinase) and ADAMTS (ADAM with thrombospondin motifs)[50] and database in Ref. 26. Furthermore, PKD-ELVs have membrane bound hyaluronidase, cell migration-inducing and hyaluronan-binding protein (CEMIP2, TMEM2), which is the major extracellular hyaluronidase in vertebrates. Hyaluronic acid is an important extracellular connective tissue component with a high turn over.[26,51] EV can also have sialidases and heparanases on their extracellular surfaces, reviewed in Ref. 52. Defects in the production of PKD-ELVs may lead to a disordered turnover and thus accumulation of BL proteins or inappropriate remodeling of ECM in the arterial media.

1.7. Evolutionary data suggesting a role for PC1, PC2 and fibrocystin in extracellular vesicle biogenesis

Deconvolution confocal microscopy suggests that the PCC forms a multiprotein complex on the surface of the PKD-ELV, Fig. 4A. We examined the various protein domains found in the PCC and investigated their function across the archaeal, bacterial and eukaryotic lineages. We focused on situations where these domains may be involved in membrane manipulation or where they might have a structural role.

The PKD domain which makes up 34.7% and 49% of the PC1 ORF and ectodomain, respectively, is a rare domain in multicellular organisms but is over-represented in the structural proteins making up the surface-layer (S-layer) of archaea. The S-layer of archaeal cells forms a tough external protein barrier encompassing the entire cell and is thought to protect the plasma membrane of the microorganism from the immediate and often hostile environment (for example a hydrothermal vent). The S-layer is composed of a regular pseudo-crystalline array of proteins that may also act as selective filter. The best understood

S-layer is derived from the methanogen *Methanosarcina mazei*, which has two surface layer proteins one composed of two seven-bladed β-propeller domains and 12 PKD domains and another protein which has four PKD repeats and 9 PbHs, similar to those observed in fibrocystin. PKD and PbH repeat containing proteins are also common in the S-layer of other archaea such as *Methanosarcina acetivorans* often acting as 'standoffs' between the plasma membrane and the S-layer *per se*.[53] Similarly, in PKD-ELVs and migrasomes PC1 and fibrocystin are so abundant that they may have a similar structural role supporting a macromolecular complex on the surface of the EV.

The fission yeast *Schizosaccharomyces pombe* has a close homolog of PC2, the TRP channel Pkd2p. Pkd2p localizes to the cleavage furrow of the dividing cell, regulates the ring closure during cytokinesis and is required for the separation of the daughter cells.[54] In PKD-ELVs and migrasomes, PC2 may have a similar role in manipulating the membrane and budding off EVs. This would be coordinated with PC1 and the genesis of the putative extracellular coat (an S-layer analogue) covering the vesicle. Thus, we think that the PCC is a multiprotein complex involved in manipulation and curving of a cholesterol rich plasma membrane.

2. Discussion

A primary cilium based theory explains the defects in PCP and the development of polycysts in ADPKD and ARPKD. Non canonical Wnt signaling sets up primary cilium dependent PCP along the developing or regenerating tubules which then lengthen by Wnt-9b mediated convergent extension. In the absence of a functional primary cilium or Wnt-9b the orientation of cellular division and convergent extension becomes disordered leading to tubular dilatation and ultimately cysts. Wnt signaling is not usually cell autonomous and the Wnt proteins themselves are lipid modified heparin binding proteins with poor water solubility. This raises the issues of how they transmit a signal. In drosophila, *wingless* protein, the prototypical Wnt, is transported through the cells of the imaginal disc on the outer leaflet of EVs termed, argosomes.[55] In mammals it is known that Wnt-9b interacts with the N-terminus of PC1 and can gate the PC1/PC2 channel complex.[38] Here, we suggest that PCC generated EVs carry Wnts and that the PCP signaling event occurs on the primary cilium of the receiving cells.

The observation of enhanced somatic fitness seen in hepatocytes with mutations in the PCC genes and the resistance to carcinoma shown in individuals with ADPKD or carrying heterozygous *PKHD1* mutations initially appeared to be paradoxical. These findings can be unified by EV theory that suggests that EVs generated by the PCC assembly pathway have different functions in different contexts. Somatic fitness may be due to compromised mitocytosis whereas the resistance to carcinoma may be due to the inability to produce sufficient miRNA containing EVs to reprogram bystander cells to become tumor supportive. For example, tumor derived EVs carrying miRNA-125b can induce normal fibroblasts to become supportive tumor associated fibroblasts (TAFs).[56,57] Another possibility is that EVs from individuals with PCC mutations are incapable of generating EVs necessary for the Wnt/PCP pathway involved in metastasis.[58] EVs have also been implicated in angiogenesis and the preparation of distant metastatic niches.[59–61] Yet another possibility is that PCC deficient cells generate few proteinase bearing EVs so that direct invasion of the local tissue is compromised. The thickened BL and the various arterial and valvular ECM pathologies in ADPKD may also be due to defects in assembly and secretion of proteinase and hyaluronidase bearing EVs.

3. Conclusion

The finding that PC1, PC2 and fibrocystin are present both on PKD-ELVs and primary cilia combined with the observations that ADPKD is associated with a variety of non cystic phenotypes raises important questions about the pathogenesis of the disease. Individuals with ADPKD are resistant to carcinoma development, have profound defects in mitochondrial function in cystic cells and yet somatic mutations in hepatocytes confer enhanced fitness to chemical insults. These observations can be explained by EV theory.

The central tenet of EV theory is that the PCC is involved in the fabrication and genesis of a subset of EV termed PKD-ELVs. In this scenario, PC2 is involved in the budding of EVs (much like the function of its close homolog in *Schizosaccharomyces pombe*) and both PC1 and fibrocystin are structural components on the extracellular aspect of the EV. EV theory is a testable idea that suggests a variety of experiments. For example, if we could find a gene/ protein (for example EPCIP) that is involved solely in PCC mitocytosis, a knockout mouse would be expected to have morphologically and biochemically abnormal mitochondria, be CCl_4 resistant and completely non cystic. This would be an important experiment that could distinguish between primary cilium and EV theory. Another possibility would be to make an inducible kidney specific *flox Wnt9B* mouse. This mouse should develop normally. If the adult kidney is injured and then the *Wnt9B* gene inactivated we would expect a cystic phenotype but no mitochondrial phenotype. This is because EV theory suggests that Wnt-9b is a passenger on a signaling PKD-ELV and not involved in mitocytosis.

The EV hypothesis explains a range of phenotypes observed in ADPKD and ARPKD that are unlikely to be due primary cilium signaling. In short, we think that EV theory can explain the global ADPKD and ARPKD phenotypes in a parsimonious manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The corresponding author, Christopher J. Ward, will supply any data or reagents mentioned in this paper upon email request. This may require a material transfer agreement (MTA) from KUMC.

Abbreviations used in paper:

ADAM	A disintegrin and metalloproteinase
ADAMTS	ADAM with thrombospondin motifs
APC	Adenomatous Polyposis Coli
CC	Coiled-coil
CCCP	Carbonyl cyanide m-chlorophenyl hydra-zone
CD	Collecting duct
CEMIP2	Cell migration-inducing and hyaluronanbinding protein
CreERT2	Tamoxifen activated estrogen receptor Cre fusion gene
EPCIP	Exosomal polycystin-1 interacting protein
Fah	Fumarylacetoacetate hydrolase
FR	Fairy ring
G8	A domain with eight conserved glycines
GAIN	G-protein-coupled receptor (GPCR) autoproteolysis-inducing
IFT	Intraflagellar transport
IPT	Immunoglobulin-like fold
LDL-A	LDL-receptor class A
LRR	Leucine rich repeat
PA14	Protective antigen (anthrax toxin) 14
PbH	Parallel-β-helix
PC1	Polycystin-1
PC2	Polycystin-2
PKD	polycystin repeat
PKD1	Polycystic kidney disease type-1
PKD2	Polycystic kidney disease type-2

PKD-ELVs	PKD-exosome-like vesicles
PKHD1	Polycystic kidney and hepatic disease type 1
PLAT	Polycystin-1, Lipoxygenase, Alpha-Toxin
PT	Proximal tubule
REJ	Receptor egg jelly
RF	Retraction fiber
ROS	Reactive oxygen species
S-layer	Surface-layer
TM	Transmembrane
TMEM2	Transmembrane protein 2
TRPP	Transient receptor potential P
TSPN4	Tetraspannin-4
Wnt	Wingless int-1
WSC	Wall stress component

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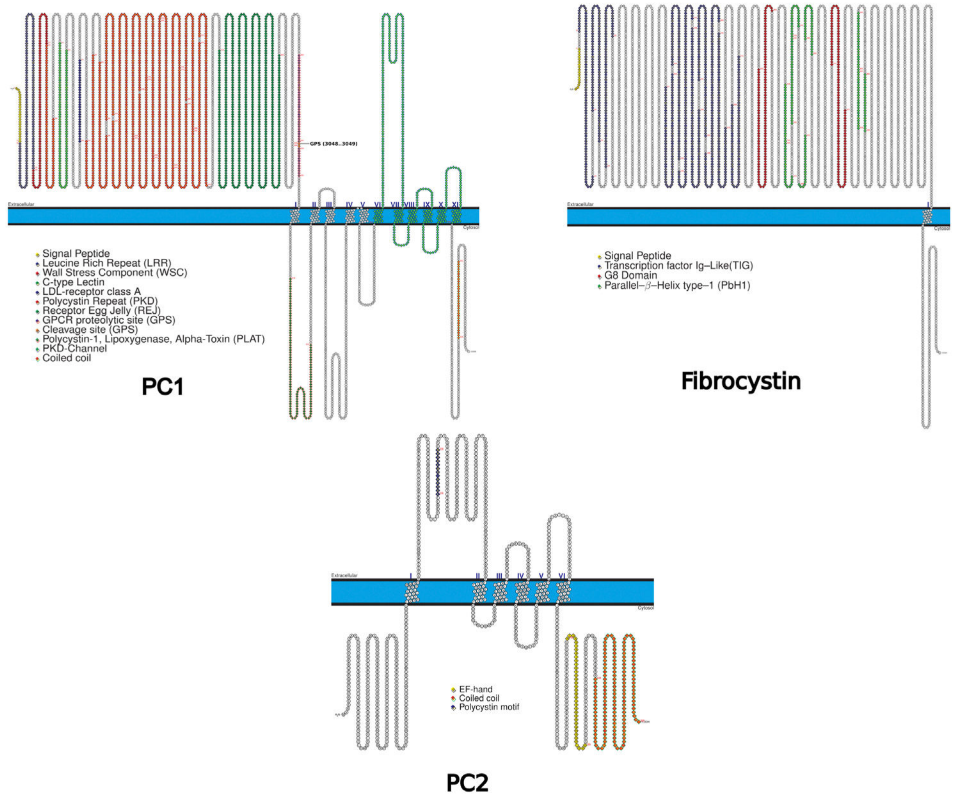

Fig. 1.

The structure of PC1, PC2 and fibrocystin: PC1 spans the membrane 11 times and has a large ectodomain composed of a mosaic of different domains, its overall length is 4302(3) aa and undergoes an autoproteolytic event at 3048 aa. The last six TM spans of PC1 have shared ion channel homology with PC2. PC2 is an ion channel subunit also referred to as TRPP2. Fibrocystin is 4074 aa long and spans the membrane once, a type-I membrane protein. The ectodomain is cleaved at a proprotein cleavage site a 3617..3620. The ectodomain is composed of transcription factor Ig (TIGS)/IPT (Ig-like, plexins, transcription factors) domains in the N-terminal portion and PbH domains in the C-terminal half of the ectodomain. There is also a lectin-like protective antigen 14 (PA14) domain at the N-terminus of the protein. See supplemental figure 1 for larger version.

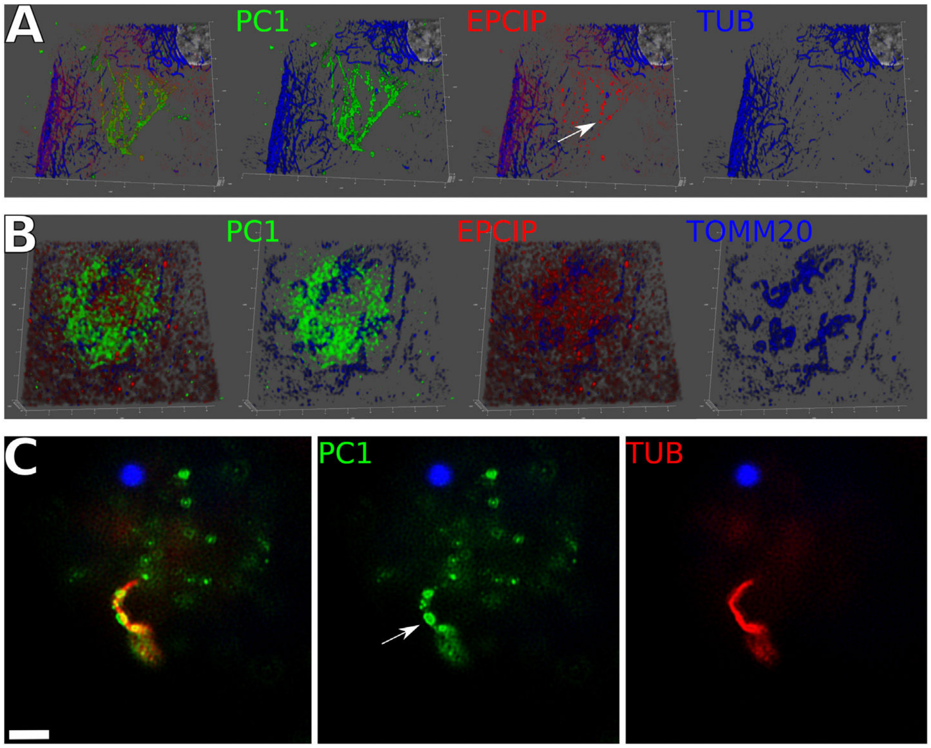

Fig. 2.

Deconvolution confocal microscopy, 3D reconstructions: The distribution of PC1 and EPCIP in PT and CD cells at various stages of confluence: (A) WT human PT cells migrating on a fibronectin substratum. PC1 (7e12, green) highlighting tubulin negative retraction fibers (RFs) (actin based), which are studded with EPCIP (CJW_2D11, red) positive migrasomes (white arrow). The cell cytoskeleton is stained for acetylated-α-tubulin (6–11B-1), which is absent in the RFs. (B) Fully confluent PT cells treated with 2μM CCCP. Note the ring of PC1 staining (green), the core of EPCIP (red) and the disintegrating TOMM20+ mitochondria embedded in the fairy ring (FR) (blue). (C) A fully polarized WT primary CD cell serum starved for two days. The attached PKD-ELVs are visualized with PC1 (green) and primary cilia with acetylated-α-tubulin (red). Note that the PKD-ELVs are hollow and therefore vesicular in nature, white arrow. Scale bar 1μm. These are new images from experiments performed in Ref. 18.

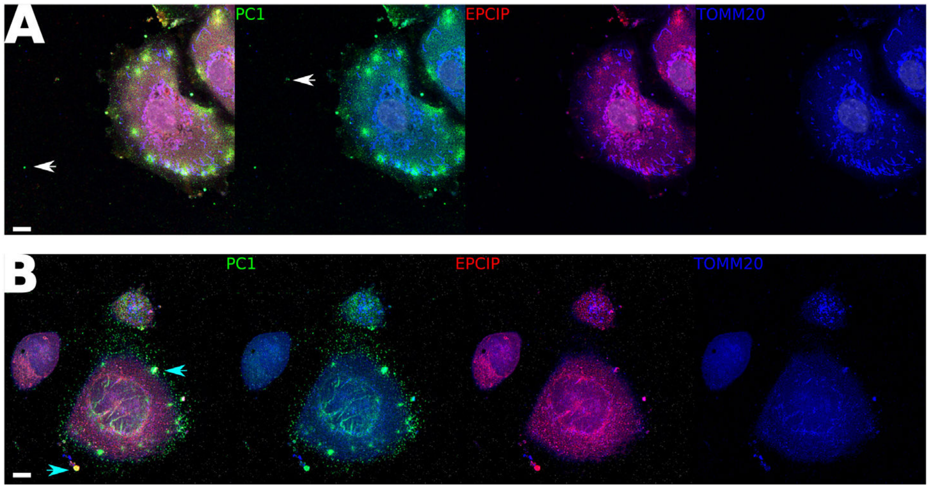

Fig. 3.

Mitochondria are extruded from cells in time dependent fashion upon treatment with CCCP. Response of human primary PT cells to 2μM CCCP with time: (A) At four hours of treatment with CCCP PC1 (green) + FR are visible with an EPCIP (red) center. These are associated with disintegrating TOMM20+ (blue) mitochondria. Outside the cell are migrasomes with a core of TOMM20+ positive material and a shell of PC1, and sometimes EPCIP (white arrow). (B) At nine hours of treatment mitochondria have mainly disintegrated and there are prominent blebs containing mitochondrial material surrounded by a coat of PC1 and EPCIP, (blue arrow). Scale bar 5μm.

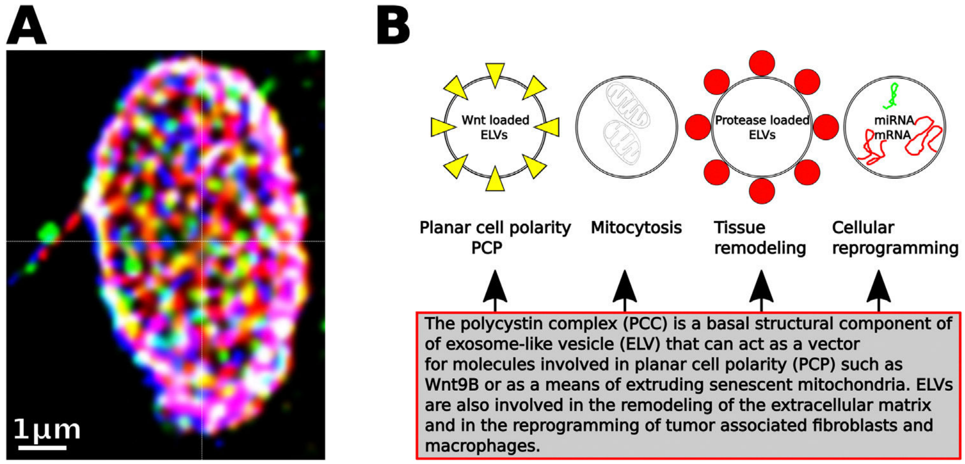

Fig. 4.

A structural role for the PCC in the generation of PKD-ELVs explains many of the phenotypes observed in ADPKD: (A) High resolution deconvoluted confocal image of a budding apical vesicle. PC1 (7E12), blue, EPCIP (CJW_2D11), red, and PC2 (CJW_9D5), green. Note the lattice work of higher order structures on the EV. (B) PKD-ELVs generated by the PCC may have multiple functions, the transport of morphogens (Wnt-9b), mitocytosis, the transport of ECM modifying proteinases and hyaluronidases and the transfer of mRNA and miRNAs between cells. These are new images from experiments performed in Ref. 18.