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Molecular mechanisms underlying polycystic kidney disease: From the smallest bricks to the big scenario

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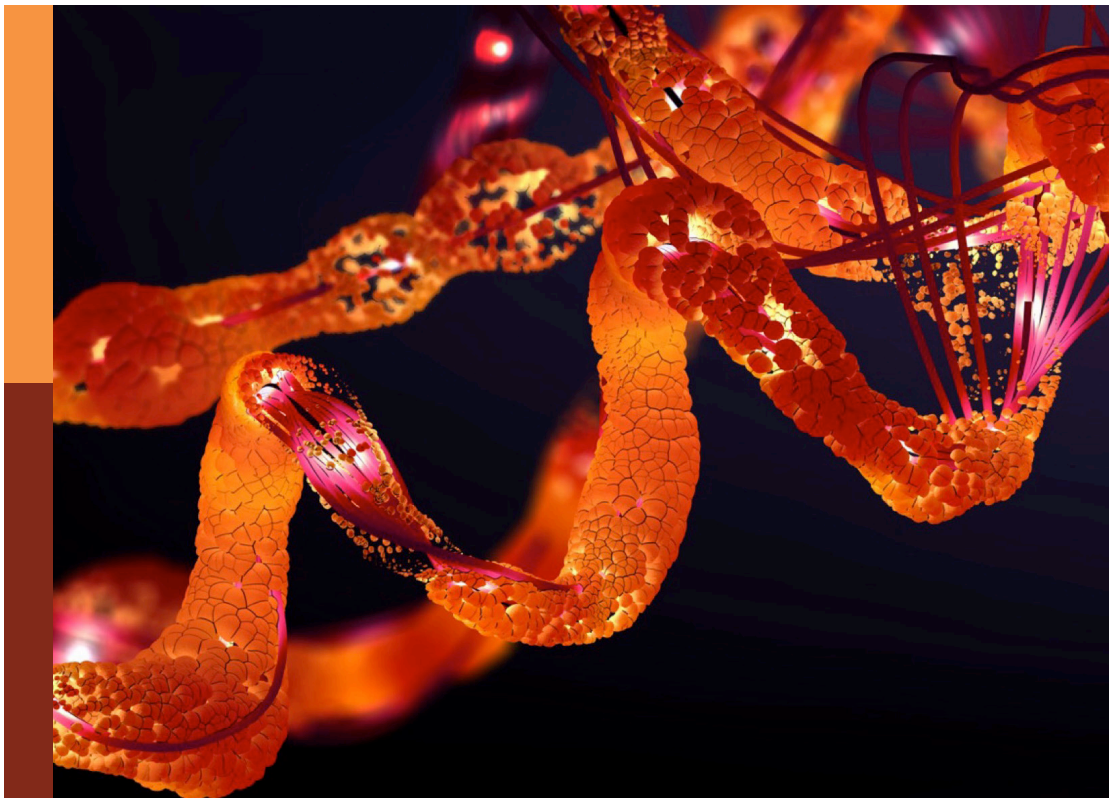

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Molecular mechanisms underlying polycystic kidney disease: From the smallest bricks to the big scenario

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Editorial: Molecular mechanisms underlying polycystic kidney disease: from the smallest bricks to the big scenario

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KEYWORDS

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Editorial on the Research Topic
Molecular mechanisms underlying polycystic kidney disease: from the smallest bricks to the big scenario

Polycystic Kidney Disease (PKD) is a genetic disorder characterized by the development and progressive enlargement of fluid-filled cysts in the kidney. It is caused by mutations in one of two genes, *PKD1*, encoding polycystin-1 (PC-1), or *PKD2*, encoding polycystin-2 (PC-2), causing Autosomal Dominant Polycystic Kidney Disease (ADPKD), or mutations in the *PKHD1* gene, encoding fibrocystin, leading to Autosomal Recessive Polycystic Kidney Disease (ARPKD) (Hughes et al., 1995; Mochizuki et al., 1996; Bergmann et al., 2004). Cyst growth alters renal structure and leads to kidney enlargement, causing impaired function and potentially lethal organ failure.

Due to its complex genetic background and its outcome on renal function and other organs, efforts to understand PKD require a multifaceted approach. The present Research Topic contains 9 noteworthy articles describing recent progress and emerging insights from PKD research, shedding light on novel therapeutic approaches and promising targets for intervention.

ADPKD prevalence is reported to be between 1 in 400 and 1 in 1,000 births, resulting in kidney failure in 50% of patients by 60 years of age (Bergmann et al., 2018). ARPKD instead is a much rarer disease characterized by a perinatal, pediatric onset with kidney impairment described in 60% of patients by 20 years of age (Bergmann et al., 2018). Most ARPKD children are hypertensive in the first year of life. Conversely, a significantly lower percentage of ADPKD patients develop hypertension during childhood, but this percentage is probably underestimated. In the review by Lucchetti et al., the authors analyze the available pediatric studies and experience-based observations on cardiovascular impairment associated with both PKD forms. Since early onset hypertension (before the age of 35 years) represents a risk factor for fast progression of chronic kidney disease (CKD), the authors underline that an early hypertension treatment may slow down the progression of the disease and reduce cardiovascular complications.

PKD is listed among ciliopathies—disorders affecting primary cilia proteins (Ta et al., 2020). Polycystins (PCs) localize to the primary cilium in the kidney tubular epithelial cells

and loss of PCs function results in loss of lumen diameter control leading to luminal expansion and cyst formation (Luo et al., 2023). Specifically, polycystin function is believed to embody an inhibitory activity that suppresses the cilia-dependent cyst activation (CDCA) signal (Luo et al., 2023). In this respect, in the review by Walker et al., the authors discuss the current model of the CDCA mechanism in ADPKD and consider the possible roles of ciliary and extraciliary polycystins in regulating CDCA. Moreover, they hypothesize the existence of cilia-localized components of CDCA (cCDCA) and cilia-localized cyst inhibition (CLCI) signals, proposing TULP3 cargoes as potential cilia-localized components that determine cystogenesis in kidneys during development and in adult mice.

Recent studies have highlighted that PC1's capacity to modulate G protein signaling may play a crucial role in preventing the development of renal cysts, although the exact mechanisms are still being investigated (Fedeles et al., 2014; Wu et al., 2016; Parnell et al., 2018). PC1 may be involved in the control of GPCR-mediated signaling pathways based on the structural and functional similarities between polycystin-1 and the family of cell adhesion GPCRs, such as the presence of a conserved GPCR proteolysis site (GPS) (Maser and Calvet, 2020). The evidence for PC1 GPCR-like activity, the role of GPS cleavage in controlling PC1 GPCR function, and the possible interaction between PC1 GPCR-like activity and the regulation of polycystin complex channel properties have been reviewed by Maser et al.

PKD progression can be exacerbated by the presence of renal innate immune cells (Zimmerman et al., 2020). This interesting aspect is examined in the review by Agborbesong et al., focusing on epigenetic regulation, inflammation, and cell death as molecular mechanisms underlying ADPKD. It draws attention to the intricate interplay that drives cyst formation and disease progression, involving *PKD* gene alterations, epigenetic changes, inflammatory responses, and different forms of cell death. The inflammasome system responds to stimuli such as cellular damage by activating Caspase-1, and producing essential mediators of the inflammatory pathway, including IL-1β and IL-18. In the original research article by Swenson-Fields et al., the authors demonstrate that Caspase-1 knockout markedly reduced the onset of PKD in female mice, indicating sex-specific immunological responses, showing for the first time that the activated Caspase-1/inflammasome promotes cyst expansion and disease progression in PKD, particularly in females.

Currently, there is no cure for PKD other than renal transplantation (Dennis et al., 2023). Tolvaptan is the only drug approved by FDA proven to slow eGFR decline in ADPKD patients at the risk of rapid disease progression. Widespread use of tolvaptan is limited by the substantial aquaretic effects that it produces and by the potential for liver toxicity (Zhou and Torres, 2023). Recent advances in understanding the pathophysiology of PKD have led to new approaches to treatment via targeting different signaling pathways (Zhou and Torres, 2023).

The original research article by Hallows et al. investigates the potential therapeutic effects of bempedoic acid (BA), an ATP citrate-lyase (ACLY) inhibitor. The authors demonstrate that BA inhibited cyst growth and improved mitochondrial function *in vitro*, and reduced disease severity *in vivo*, suggesting BA as a promising therapy for PKD, having beneficial effects alone and associated with tolvaptan.

The review article by Zhou and Torres explores the emerging therapies for ADPKD with a focus on cAMP signaling. It discusses the role of cAMP and PKA signaling in ADPKD pathogenesis and the potential of targeting downstream pathways beyond cAMP production for therapeutic interventions. Over the past years, several *in vitro* and animal studies have shown that metabolic reprogramming might be a general feature of PKD (Hopp et al., 2022). Glucose metabolism is defective in ADPKD, with cystic cells reprogrammed to favor aerobic glycolysis. In addition to glucose, altered amino acid metabolism, reduced fatty acid oxidation, and dysregulated lipid metabolism have also been identified as key features of PKD (Hopp et al., 2022). In the mini-review by Bacaj and Pokai, the authors discuss metabolism-based approaches for ADPKD treatment, highlighting the role of metabolic reprogramming in cyst growth, specifically upregulated mTOR and c-Myc pathways, and the potential for targeting these pathways as therapeutic approaches.

Obesity and overweight are very common in ADPKD patients and represent independent risk factors for the disease advancement. In this regard, Iliuta et al. examine the shared pathobiology between ADPKD and obesity, emphasizing the role of reduced AMPK activity and increased mTOR signaling. The pharmacological activation of AMPK is discussed as a promising approach to treat both ADPKD and obesity-related kidney disease.

In conclusion, the present Research Topic provides an overview of the ongoing efforts to unravel the complex interaction of molecular signaling pathways associated with PKD progression, exploring innovative therapeutic approaches to improve patient outcomes.

Author contributions

AD: Writing–original draft, Conceptualization. MC: Writing–review and editing, Conceptualization. GV: Writing–review and editing, Conceptualization.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Cilia-Localized Counterregulatory Signals as Drivers of Renal Cystogenesis

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Primary cilia play counterregulatory roles in cystogenesis—they inhibit cyst formation in the normal renal tubule but promote cyst growth when the function of polycystins is impaired. Key upstream cilia-specific signals and components involved in driving cystogenesis have remained elusive. Recent studies of the tubby family protein, Tubby-like protein 3 (TULP3), have provided new insights into the cilia-localized mechanisms that determine cyst growth. TULP3 is a key adapter of the intraflagellar transport complex A (IFT-A) in the trafficking of multiple proteins specifically into the ciliary membrane. Loss of TULP3 results in the selective exclusion of its cargoes from cilia without affecting their extraciliary pools and without disrupting cilia or IFT-A complex integrity. Epistasis analyses have indicated that TULP3 inhibits cystogenesis independently of the polycystins during kidney development but promotes cystogenesis in adults when polycystins are lacking. In this review, we discuss the current model of the cilia-dependent cyst activation (CDCA) mechanism in autosomal dominant polycystic kidney disease (ADPKD) and consider the possible roles of ciliary and extraciliary polycystins in regulating CDCA. We then describe the limitations of this model in not fully accounting for how cilia single knockouts cause significant cystic changes either in the presence or absence of polycystins. Based on available data from TULP3/IFT-A-mediated differential regulation of cystogenesis in kidneys with deletion of polycystins either during development or in adulthood, we hypothesize the existence of cilia-localized components of CDCA (cCDCA) and cilia-localized cyst inhibition (CLCI) signals. We develop the criteria for cCDCA/CLCI signals and discuss potential TULP3 cargoes as possible cilia-localized components that determine cystogenesis in kidneys during development and in adult mice.

Keywords: polycystic kidney disease, cystogenesis, primary cilia, polycystin 1 and 2, tubby-like protein 3, cilia-dependent cyst activation, cilia-localized cyst inhibition, intraflagellar transport

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INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the formation of numerous fluid-filled cysts (Igarashi and Somlo, 2002; Harris and Torres, 2009) from all tubule segment origins. The cysts destroy normal parenchyma and cause kidney failure in more than half of the patients by the age of 60 (Gabow, 1993). ADPKD is caused primarily by mutations in *PKD1* or *PKD2*, which encodes polycystin-1 (PC1) or polycystin-2 (PC2), respectively (polycystins or PCs

collectively) (The European Polycystic Kidney Disease Consortium, 1994; Mochizuki et al., 1996). PC1 is a 4302 aa 11-transmembrane (TM) protein (Hughes et al., 1995) with a GPCR autoproteolysis-inducing (GAIN) domain located upstream of the first TM domain (Ponting et al., 1999; Arac et al., 2012). PC1 undergoes autoproteolytic cleavage at a G protein-coupled receptor (GPCR) proteolysis site (GPS) within the GAIN domain—a key post-translational modification of the protein (Qian et al., 2002; Yu et al., 2007; Chapin et al., 2010; Cai et al., 2014; Kim et al., 2014; Kurbegovic et al., 2014; Gainullin et al., 2015; Padovano et al., 2020). PC2 belongs to the transient receptor potential (TRP) channel superfamily of proteins (Koulen et al., 2002) and can form a homotetramer that functions as a non-selective cation channel (Shen et al., 2016; Grieben et al., 2017). PC1 and PC2 form a stable complex with a 1:3 stoichiometry (Yu et al., 2009; Zhu et al., 2011; Su et al., 2018). Recent studies have shown that PC1/PC2 complex has an ion channel function with properties that are distinct from that of the homomeric PC2 channel (Wang et al., 2019; Ha et al., 2020). Moreover, the PC1 subunit directly contributes to the channel pore and thereby affects its ion channel function (Wang et al., 2019). Polycystins transduce intracellular calcium signals in response to extracellular stimuli (Nauli et al., 2003; Delmas, 2004; Nauli and Zhou, 2004) by unknown mechanisms. Multiple downstream cellular pathways, such as extracellular regulated kinase (Shibazaki et al., 2008; Ma et al., 2013), mTOR (Shillingford et al., 2006; Boletta, 2009; Distefano et al., 2009), cAMP (Torres et al., 2004; Wang et al., 2018), WNT (Kim et al., 2016), Ca^{2+} (Koulen et al., 2002; Nauli et al., 2003; Cantiello, 2004; Anyatonwu et al., 2007; Kim et al., 2016) and G-protein signaling (Parnell et al., 1998; Hama and Park, 2016; Parnell et al., 2018; Zhang et al., 2018) are dysregulated in polycystic kidneys with mutated polycystins. How these downstream pathways are mechanistically linked to cystogenesis caused by a lack of polycystins remains largely unknown. Although current therapies target many of these pathways, they have achieved limited effects and showed significant side effects that restrict their usage (Torres et al., 2010; Torres and Harris, 2014; Torres et al., 2017; Torres et al., 2020). Many therapies are targeted toward preventing the progression of the disease rather than halting disease initiation. Indeed, distinguishing the changes that arise from initiation of, and are proximate to, cystogenesis from those that are secondary to the progression of cysts has been challenging. Key upstream signals that are regulated by polycystins and determine kidney cystogenesis in ADPKD remain unknown, preventing the development of more effective therapies.

COMPLEX ROLE OF THE PRIMARY CILIUM IN CYSTOGENESIS

An important clue into the role of the polycystins in tubular homeostasis came from the findings that both proteins localize to the primary cilium (Pazour et al., 2002; Yoder et al., 2002; Nauli et al., 2003; Nauli and Zhou, 2004). The primary cilium is a small hair-like projection from the apical surface of almost all cell types (Rosenbaum and Witman, 2002; Wheatley, 2005). Primary cilia function as sensory antennae in most vertebrate cells playing fundamental roles in cell cycle control, cellular differentiation, and polarity (Goetz and Anderson, 2010; Anvarian et al., 2019).

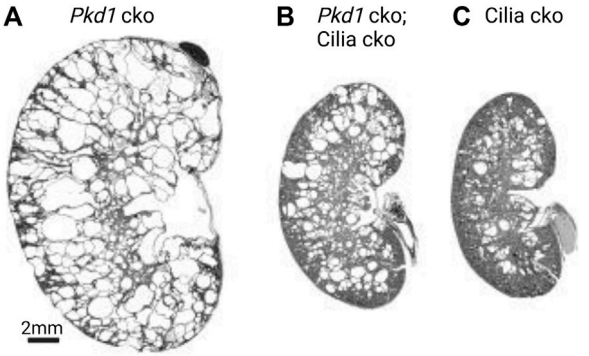

FIGURE 1 | Complex roles of cilia in cystogenesis. Cysts from PC1 loss are severe and only partially suppressed from cilia loss. Images adapted from (Ma et al., 2013) with permission. Postnatal day 24 (P24) kidneys from **(A)** *Pkd1* cko (*Pkhd1-cre;Pkd1*$^{fl/fl}$), **(B)** *Pkd1* cko; cilia cko (*Pkhd1-cre; Kif3a*$^{fl/-}$*;pkd1*$^{fl/fl}$), and **(C)** cilia cko (*Pkhd1-cre;Kif3a*$^{fl/-}$) mice. Abbreviations: cko, conditional knockout.

In the kidney, primary cilia jut out into the tubular lumen and are thought to sense urine flow-induced shear stress or ion composition (Nauli et al., 2003; Praetorius et al., 2003).

The ciliary localization of polycystins has led ADPKD to be categorized as a ciliopathy, a genetic disease caused by defective cilium-resident proteins and ciliary dysfunction. In fact, kidney cyst formation during development appears to be common pathogenesis for many other ciliopathies. These results suggest that cilia regulate tubular homeostasis and that ciliary dysfunction is a key upstream pathogenic step leading to tubular dilatation and cystogenesis (Pazour, 2004; Nauli et al., 2006). It has been suggested that ciliary polycystins may sense mechanical stimuli by mediating calcium entry into cilia, and loss of the mechanically induced cilia-initiated calcium signaling may underlie cystogenesis (Nauli et al., 2003). This view has recently been challenged by the Clapham laboratory who demonstrated that primary cilia are not calcium-responsive mechanosensors (Delling et al., 2016).

Genetic studies have shown a complex role of primary cilia in cystogenesis. Loss of cilia, by deletion of the intraflagellar transport complex-B proteins (Davenport et al., 2007; Jonassen et al., 2008) or kinesin-II (Lin et al., 2003), causes mild fibrocystic disease with slow cyst growth in the kidney. This finding suggested that cilia inhibit cyst formation in normal renal tubules. By comparison, loss of the polycystins caused significantly more severe cystogenesis marked by early and rapid cyst growth while keeping intact ciliary structure (Lu et al., 1997; Boulter et al., 2001; Piontek et al., 2004), for example see **Figure 1A**. In a pioneering study, the Somlo laboratory has shown that concomitant loss of polycystins and cilia results in an intermediate phenotype (**Figure 1B**), closer to that of loss of cilia alone (**Figure 1C**) (Ma et al., 2013). Therefore, polycystins play an important role in cilia signaling to suppress tubular dilation and cystogenesis. Furthermore, the extent of the suppression was directly related to the length of time between

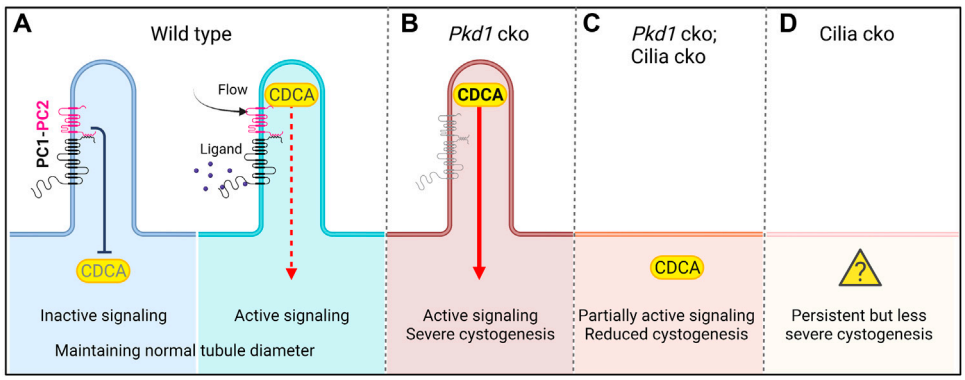

FIGURE 2 | Cilia-dependent cyst activation (CDCA) model for cystogenesis in ADPKD (adapted from Ma, 2021). **(A)** The CDCA in the wild-type cell is suppressed by polycystins and is activated through an unknown mechanism, possibly ligand binding to polycystins or flow bending the cilium. **(B)** In *Pkd1* cko, CDCA is derepressed and constitutively activated leading to severe cystogenesis. **(C)** If *Pkd1* and cilia are co-ablated, the CDCA cannot be upregulated. The cystogenic signal is thus diminished compared with single *Pkd1* cko, resulting in reduced cystogenesis. In this model, the CDCA signal must retain a partial "leaky" function in the cytoplasm to explain how *Pkd1*-cilia double mutants display significant cystic changes. **(D)** In cilia single mutants, there is persistent mild cystogenesis. Here the CDCA must have a leaky function despite the presence of intact polycystins and lack of cilia (**"?"**). Created in BioRender.

the initial loss of the polycystins and the subsequent involution of cilia—the disease worsened when this time interval was lengthened (Ma et al., 2013). The suppression of cyst growth by the loss of cilia was found to occur in all segments of the renal tubules following both early and late *Pkd1* or *Pkd2* gene deletion. The genetic epistasis data indicate that primary cilia play dual and opposing roles in cyst development dependent on the presence of polycystins: they inhibit cyst formation in the normal renal tubule but promote cyst growth when polycystins are lacking.

PC1/PC2 REPRESSED CILIA-DEPENDENT CYST ACTIVATION SIGNAL

Based on the above-mentioned genetic epistasis data from *Pkd1*/*Pkd2* and cilia conditional knockout (cko) mice, the Somlo laboratory has proposed an unidentified cilia-dependent cyst activation (CDCA) signal(s) (Ma et al., 2013; Ma et al., 2017). The CDCA signal is dependent on intact cilia for activity and is normally inhibited by the functioning of polycystins (**Figure 2A**). The function of polycystins in suppressing CDCA might be part of a normal physiological or homeostatic cilia-dependent signaling pathway promoting functional tubule adaptation to either chemical or mechanical signals (Ma et al., 2013). Loss of polycystins in the presence of intact cilia—the condition for cyst initiation in ADPKD—leads to upregulation of CDCA, perhaps *via* ciliary translocation, and constitutive activation (**Figure 2B**). This leads to uncontrolled lumen diameter expansion and cyst growth (Ma et al., 2013; Ma et al., 2017; Ma, 2021). Ciliary localization of CDCA is required for full activation in *Pkd1* cko; the CDCA signal is impeded when cilia are co-ablated, thus resulting in a reduction of the cystic burden in the *Pkd1*-cilia double mutants (**Figure 2C**).

The molecular nature of CDCA components and effectors are unknown. The CDCA mechanism of cyst growth is active in all tubular segments following both early and late ADPKD gene inactivation (Ma et al., 2013). The CDCA signal(s) is likely distinct from MAPK/ERK (Shibazaki et al., 2008; Distefano et al., 2009), mTOR (Pema et al., 2016), cAMP (Torres and Harris, 2014; Bergmann et al., 2018), and phosphorylated cAMP Response Element-Binding Protein (pCREB) signaling (Kakade et al., 2016) at a cellular level (Ma et al., 2013), which are altered in cystic kidneys. These pathways are unlikely to be central to the CDCA mechanism because they show restricted nephron segment specificities and later activation (Ma et al., 2013). These pathways may be activated only in certain cell types or conditions to actively promote cyst growth, making them unlikely to be universally applicable targets for reducing cyst growth. A recent study has shown that signaling through the Hedgehog pathway is not required for cystic phenotype caused by loss of function of *Pkd1* (Ma et al., 2019). In fact, neither activation nor repression of the Hedgehog signaling components (*Smo*, *Gli2*, and *Gli3*) influenced the progression of polycystic kidney disease in mouse *Pkd1* models of developmental or adult-onset of ADPKD. These results suggested that the Hedgehog signaling pathway does not contribute to the CDCA or other ciliary signals that drive renal cystogenesis.

ROLES OF CILIARY AND EXTRACILIARY POLYCYSTINS IN REGULATING CILIA-DEPENDENT SIGNALING

PC1 and PC2 localize to multiple subcellular compartments in the cell body in addition to cilia where they can exert their various functions (Boletta and Germino, 2003; Kottgen and Walz, 2005; Chapin and Caplan, 2010). This raises the question as to which of the polycystins pools may be involved in regulating the cilia-dependent signaling. Ciliary polycystins may inhibit the CDCA within the cilia, but direct evidence is lacking. Recent data suggest a critical role of the ciliary polycystins in this role. Walker et al.

(2019) have analyzed Pkd2lrm4 mutant model, a missense (E442G) mutant variant that encodes a channel-functional (Yoshiba et al., 2012) but non-cilia localizing (Grimes et al., 2016; Walker et al., 2019) form of PC2. The mutant mice developed a *Pkd2* null-like phenotype characterized by embryonic kidney cysts (Walker et al., 2019). This finding suggests that ciliary exclusion of PC2 is sufficient to cause kidney cystogenesis in a mouse model of ADPKD. Similarly, $PC2^{W414G}$, a human pathogenic variant, retains its channel activity but fails to traffic to the cilia (Cai et al., 2014). Cai et al. (2014) examined the ciliary localization of a cohort of human missense pathogenic variants of PC1 and PC2 and found that ~70% of them exhibit defects in ciliary trafficking. These data suggest that ciliary polycystins may be necessary to prevent kidney cyst formation and functional polycystins remaining in the cell body is not sufficient to counter the cystogenic signal from the cilium. The molecular mechanism by which the ciliary pool of polycystins inhibits the CDCA is unknown.

A critical role of the ciliary PC1 is further suggested by the finding of a GPS cleavage resistant mouse PC1 mutant with an amino acid substitution L3040H within the GAIN/GPS domain (Cai et al., 2014). This mutant did not reach cilia and its expression by a BAC transgene could not rescue embryonic cystogenesis and lethality in mouse *Pkd1* mutant background. However, the PC1 mutant (L3040H) expressed in the cell body remains completely Endoglycosidase H (Endo H) sensitive implying that this mutant is retained in the ER and defective in the trafficking (Cai et al., 2014). It is thus unclear whether loss-of-function observed for the PC1-L3040H mutant may be a secondary consequence of the unfolding of the GAIN domain or global structural disruption, making it difficult to conclude a role of the extraciliary pool of PC1. Interestingly, studies of the hypomorphic *Pkd1* knock-in model, $Pkd1^{V/V}$, provided evidence for a role of the extraciliary pools of polycystins in regulating the cilia-dependent signals (Yu et al., 2007). The $Pkd1^{V/V}$ mouse contains a single amino acid substitution T3041V at the GAIN/GPS domain, which blocks autoproteolytic cleavage of PC1 (Qian et al., 2002; Yu et al., 2007; Trudel et al., 2016). The resulting non-cleavable $PC1^{V}$ mutant is excluded from cilia without disrupting the cilia (Kim et al., 2014). The $Pkd1^{V/V}$ mice escaped renal cystogenesis and lethality during embryonic stages that are seen in *Pkd1* null models but started cystic dilation in distal nephron segments and collecting ducts at birth, culminating in death at ~3 weeks of age (Yu et al., 2007). The lack of ciliary access of this mutant PC1 form supports a mechanism that a CDCA signal is generated in intact cilia that are devoid of polycystins. Molecular analyses of the uncleavable $PC1^{V}$ mutant protein showed that this mutant acquires Endo H resistance and thus can exit the ER and is functional for intracellular trafficking (Kim et al., 2014; Kurbegovic et al., 2014; Trudel et al., 2016). We recently showed that this extraciliary PC1 can form a functional ion channel complex with PC2 in *Xenopus* oocytes (Wang et al., 2019). The delayed and restricted cystogenesis in the $Pkd1^{V/V}$ model thus suggested that the extra-ciliary PC1 likely suppresses CDCA or its effector pathway(s), albeit less effectively than the wild-type protein. It remains to be seen whether the anti-cystogenic role of $PC1^{V}$ in the cell body is mediated through its function in the mitochondria (Padovano et al., 2017) or *via* interaction with the cell matrix (Lee et al., 2015) or by the ion channel function of the polycystin complex at extraciliary membranes (Wang et al., 2019; Ha et al., 2020; Vien et al., 2020).

CYST FORMATION IN CILIA MUTANTS: CILIA-REGULATED SIGNALS THAT SUPPRESS CYSTOGENESIS INDEPENDENT OF POLYCYSTINS

The concomitant ablation of cilia significantly suppressed rapid cyst growth in *Pkd1* cko compared with *Pkd1* cko single mutants. However, instead of completely resolving renal cyst development, this suppression was only partial, resulting in an intermediate phenotype of cyst growth, closer to that of loss of cilia alone (Ma et al., 2013). This was described by Ma et al. (2013), which shows that *Pkd1*-cilia double cko produced phenotypes more severe than cilia cko but much less severe than *Pkd1* cko when measured by the kidney to body weight ratio, cystic index, and serum urea nitrogen. The intermediate phenotype was consistent for both *Pkd1* and *Pkd2* mutants when in combination with a cilia cko, suggesting that this is a consistent phenomenon for the interaction between polycystins and cilia. This observation highlights several gaps in the CDCA model as it stands. First, cilia single knockouts cause significant cystic changes in the presence of polycystins, which should otherwise inhibit the CDCA to prevent cystogenesis in normal tubules (Fig. 1C). Without cilia, the cilia-dependent signal should be abolished and the cyst inhibiting role of polycystins should prevail. Second, cyst growth continues to persist in *Pkd1*-cilia double mutants (Fig. 1B); however, loss of cilia should prevent CDCA from initiating cystogenesis if the cyst activation signal solely originates in the cilium. Moreover, neither concomitant reduction in dose (by half) of PC1 nor transgenic overexpression of PC1 were found to have an impact on the cystic burden in cilia mutants (Ma et al., 2013). These data indicate that cysts from cilia single knockouts arise independently of PC1.

The above observations have additional implications. First, and according to the model (Ma et al., 2017; Ma, 2021) (**Figure 2**), the CDCA signal has to retain a partial "leaky" function or quiescent state in the cytoplasm to explain how *Pkd1*-cilia double mutants display significant cystic changes. Second, in cilia single mutants the CDCA has to be activated in absence of cilia and in the presence of intact polycystins to develop cysts (**Figure 2D**, **"?"**). An alternative parsimonious interpretation would be that persistent cyst growth in *Pkd1*-cilia double mutants arises from additional ciliary roles independent of polycystins. Cilia-defective mutants are likely to cause fibrocystic kidney disease phenotype by mechanisms (Jonassen et al., 2008; Choi et al., 2011) that are likely divergent from loss of polycystins (Shibazaki et al., 2008). The cilia-regulated signal(s) could normally suppress cystogenesis, parallel to the CDCA. Such signal(s) would require localization to cilia for full activity and cannot be generated if cilia are ablated as in cilia-single and *Pkd1*-

cilia double mutants to suppress cystogenesis. We annotate this polycystin-independent ciliary component as cilia-localized cyst inhibition (CLCI) signal(s).

Overall, these considerations suggest a more complex cilia-regulated mechanism in cyst growth involving a combination of positive and negative regulatory signals in ADPKD. These counterregulatory signals generated within the cilium are in a finely tuned balance to facilitate functional tubule adaptation to physiological inputs in normal kidneys. Dysregulation of these ciliary signals may underlie cystic kidney diseases.

CILIA-DEPENDENT SIGNALING WITHIN CILIA: CHALLENGES OF ASSESSMENT

Several difficulties have limited our ability to dissect the crucial ciliary signals. First, ciliary signals cannot be identified in experimental models that lack cilia. Ablation of cilia, an important cellular compartment, disrupts a great number of cellular pathways, including cell cycle regulation and cell polarity (Anvarian et al., 2019). The crude disruption of cilia, therefore, obscures our ability to determine the nature of the ciliary signals that are pertinent to cystogenesis. Identification of these ciliary signals will necessitate approaches that retain intact cilia. Second, uncoupling ciliary signals causing tissue phenotypes, such as cystogenesis, from the downstream pathways affected is difficult (Mukhopadhyay et al., 2017). Third, the small size of the cilium with respect to the cell (Delling et al., 2013) makes ciliary perturbations difficult to detect. Fourth, ciliary proteins, including PC1 and PC2 (Pisitkun et al., 2004; Hogan et al., 2009; Wang et al., 2015; Hardy and Tsiokas, 2020; Hu and Harris, 2020; Lea et al., 2020) have additional extraciliary roles necessitating approaches that selectively target ciliary pools of signaling proteins.

The components of the CDCA signal may be present in cilia and cytoplasm (Ma et al., 2017; Ma, 2021), but their relative contributions remain unclear. A recent transcriptomic study using *Pkd1* single and *Pkd1*-cilia double mutant kidneys has identified non-ciliary cyclin-dependent kinase 1 as a driver of cyst cell proliferation from *Pkd1* inactivation but did not find changes in ciliary drivers in cystogenesis (Zhang et al., 2021). The lack of detection of the ciliary drivers in the study may reflect heterogenous cystic mechanisms between polycystin and cilia loss. Alternatively, changes in ciliary signaling or trafficking of ciliary components may be too small to be detected at the global transcriptional level and are unlikely to be detected by the whole kidney analyses.

TUBBY-LIKE PROTEIN 3 IS A KEY ADAPTER OF THE IFT-A COMPLEX IN TRAFFICKING MULTIPLE PROTEINS TO THE CILIARY MEMBRANE

The tubby family member, Tubby-like protein 3 (TULP3) is a key adapter of the intraflagellar transport complex A (IFT-A) in the trafficking of multiple proteins specifically into the ciliary membrane. The IFT-A holo-complex is generally considered to be regulating the retrograde trafficking of cargoes including IFT-B complex in the cilia (Piperno et al., 1998; Iomini et al., 2001; Iomini et al., 2009). TULP3 interacts with the IFT-A core (consisting of IFT140/122/144 subunits) (Mukhopadhyay et al., 2010; Behal et al., 2012) through its N-terminus to enable trafficking of itself and TULP3 bound cargoes into cilia (Mukhopadhyay et al., 2010; Badgandi et al., 2017). Thus, the IFT-A core complex has an additional function in the pre-ciliary trafficking of TULP3 and cargoes, in addition to its established role in retrograde trafficking in cilia. TULP3 mediates ciliary trafficking by a 3-step mechanism: 1) capture of membrane cargo by the tubby domain in a $PI(4,5)P_2$-dependent manner, 2) ciliary delivery by IFT-A core-binding to TULP3 N-terminus, and 3) release into PIP_2-deficient ciliary membrane (**Figure 3**) (Badgandi et al., 2017). Loss of TULP3 results in the selective exclusion of its cargoes from cilia without affecting their extraciliary pools and without disrupting cilia or IFT-A complex integrity. Therefore, studying TULP3 provides a unique opportunity to investigate potential ciliary components that regulate cystogenesis from intact cilia.

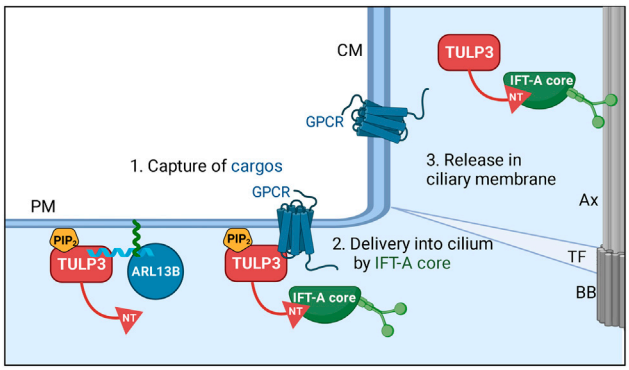

FIGURE 3 | Model for TULP3 and IFT-A—mediated trafficking of cargoes into cilia. TULP3 tubby domain is anchored to the plasma membrane by $PI(4,5)P_2$. The tubby domain captures short CLS (cilia localization signal) peptide regions in diverse cargoes. The N-terminus (NT) of TULP3 binds to the IFT-A core subunits (IFT140, IFT122, IFT144) and recruits the tubby domain-bound cargoes to cilia. After reaching cilia, the lack of $PI(4,5)P_2$ in the ciliary membrane could dislodge the cargoes from the tubby domain. The cargoes are very diverse and include transmembrane proteins and membrane-associated proteins such as ARL13B. The N-terminus amphipathic helix in ARL13B that binds the tubby domain in TULP3 is shown in blue. ARL13B is anchored to the membrane by palmitoylation (green) inside the helix. Abbreviations: Ax, Axoneme; TF, transition fiber; BB, Basal body; CM, ciliary membrane; PM, plasma membrane. Created in BioRender.

The IFT-A core and peripheral subunit mutants also affect the ciliary localization of multiple TULP3 cargoes (Fu et al., 2016; Takahara et al., 2018; Picariello et al., 2019; Kobayashi et al., 2021; Quidwai et al., 2021) indicating that IFT-A is required for their ciliary trafficking. Substitution of Ift140 subunit in *Chlamydomonas* with a WD-40 repeat deleted Ift140 fragment restores flagella partially, as opposed to lack in *Ift140* knockout. Instead, trafficking of multiple lipidated proteins, including ARL13 and other farnesylated and myristoylated proteins, are affected. Thus IFT-A could have an evolutionary function in the pre-ciliary trafficking of cargoes to cilia.

Recent genetic studies of *Tulp3* provided compelling evidence for cilia-localized signals in determining cyst growth. *Tulp3*

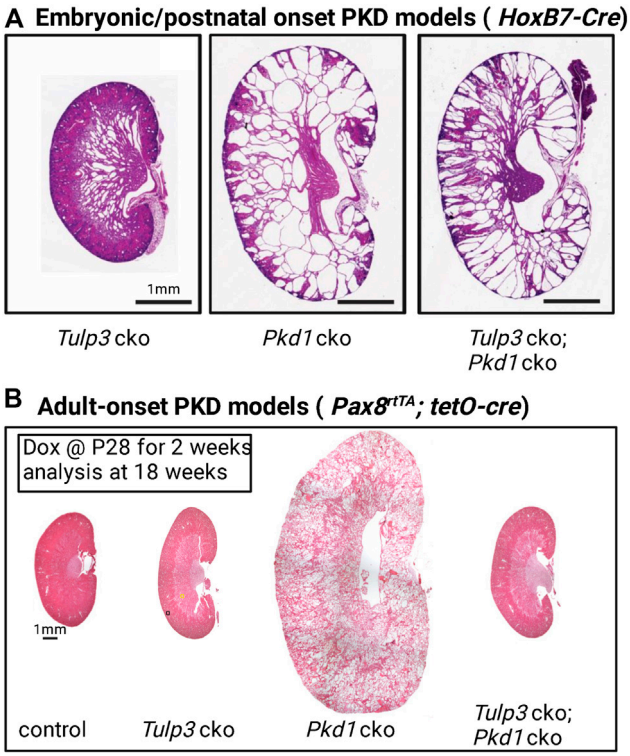

FIGURE 4 | *Tulp3* and *Pkd1* double knockout models of PKD. **(A)** Kidneys from postnatal day 5 (P5). *Tulp3* cko (*HoxB7-cre; Tulp3*$^{fl/fl}$), *Pkd1* cko (*HoxB7-cre; Pkd1*$^{fl/fl}$), *Tulp3* cko;*Pkd1* cko (*HoxB7-cre; Tulp3*$^{fl/fl}$*;Pkd1*$^{fl/fl}$). cko: conditional knockout. Scale bar, 1 mm. Images adapted from (Hwang et al., 2019). **(B)** Adult-onset models. *Pax8*^{rtTA}*; tetO*-cre doxycycline inducible mice were given doxycycline (Dox) starting at P28 for 2 weeks and analyzed at 18 weeks. Control (mice without *tetO*-cre), *Tulp3* cko (*Pax8*^{rtTA}*; tetO-cre*; *Tulp3*$^{fl/fl}$), *Pkd1* cko (*Pax8*^{rtTA}*; tetO-cre; Pkd1*$^{fl/fl}$), *Tulp3* cko;*Pkd1* cko (*Pax8*^{rtTA}*; tetO-cre; Tulp3*$^{fl/fl}$*;Pkd1*$^{fl/fl}$). Images adapted from (Legue and Liem, 2019) with permission.

depletion has been shown to be deleterious but also protective in developmental (Hwang et al., 2019) and adult-onset ADPKD models in mice (Legue and Liem, 2019). These results are described in detail below and they provide a unique approach to test potential TULP3 cargoes as possible components of the cilia-localized signals that determine cystogenesis.

TULP3/IFT-A CILIARY CARGOES AS CILIA-LOCALIZED CYST INHIBITION SIGNAL(S) DURING DEVELOPMENT

Conditional knockout (cko) of *Tulp3* in renal tubules in mice using *Ksp1-Cre* or *HoxB7-Cre* caused cyst formation during development approximating ciliary loss (Hwang et al., 2019) (**Figure 4A**). This result indicated that a subset of TULP3-trafficked ciliary cargoes suppresses cystogenesis in normal tubules, and that lack of these molecules in the cilia underlies the disease in the *Tulp3* cko. Furthermore, *Tulp3* cko mutants showed cystic changes indistinguishable from *Tulp3*-cilia double cko mutants (Hwang et al., 2019). This result implies that TULP3/IFT-A is necessary to traffic most, if not all, crucial components of cilia-regulated cyst inhibitory signals to cilia.

Cystic kidney disease in *Tulp3* cko was slower to develop and less severe than that caused by loss of *Pkd1*. However, concomitant *Tulp3* cko had a distinctive effect on cystogenesis of *Pkd1* cko than from concomitant ciliary loss. Unlike loss of cilia, concomitant *Tulp3* cko did not inhibit cystogenesis upon PC1 loss but rather caused earlier lethality than *Pkd1* cko alone (Hwang et al., 2019), suggesting that *Tulp3* inactivation accelerated loss of renal function in the *Pkd1* cko. This genetic epistasis between *Tulp3* and *Pkd1* implied that some TULP3 ciliary cargoes suppress cystogenesis independently of polycystins during kidney development and that dysregulation of these signals may significantly contribute to the cyst growth in ADPKD.

A recent multi-group study led by the Bergmann laboratory found biallelic *TULP3* mutations in patients with progressive fibrocystic kidney disease, degenerative liver fibrosis, and hypertrophic cardiomyopathy with atypical fibrotic patterns in histopathology (Devane et al., 2022). Another multi-group study led by the Harris laboratory found monoallelic loss-of-function *IFT140* mutations in patients with mild polycystic kidney disease with limited kidney insufficiency. The authors analyzed the United Kingdom Biobank cystic kidney disease group and found probands with *IFT140* loss-of-function variants as the third most common group after *PKD1* and *PKD2* (Senum et al., 2022). Conditional knockout of another IFT-A core subunit, *Ift144*, does not suppress cystogenesis from *Pkd1* loss during embryogenesis, despite causing shortened or no cilia (Yu et al., 2022). Rather cyst number is increased arguing for a role of IFT144 cargoes in CLCI signaling (Yu et al., 2022). Although IFT140 and IFT144 are core IFT-A complex subunits (Mukhopadhyay et al., 2010; Behal et al., 2012) and regulate retrograde trafficking in cilia (Piperno et al., 1998; Iomini et al., 2001; Iomini et al., 2009), the mild polycystic kidney disease phenotype in patients with *IFT140* mutations and the lack of cyst suppression in *Pkd1; Ift144* double cko with respect to *Pkd1* cko could partly arise from pre-ciliary function of the IFT-A core in trafficking TULP3 and its cargoes.

Based on these results, we propose that a subset of TULP3/IFT-A ciliary cargoes generate **CLCI** signal(s) and can be defined experimentally by the following criteria:

(i) It is trafficked to cilia.
(ii) It could be a ciliary cargo of TULP3.
(iii) Lack causes cystic changes but milder than *Pkd1/2* cko.
(iv) Concomitant cko with *Pkd1/2* cko enhances cystic kidney phenotype during development.

TULP3/IFT-A CILIARY CARGOES AS CILIA-LOCALIZED CDCA SIGNAL(S) IN ADULT KIDNEYS

Recent epistasis data of *Tulp3* and *Pkd1* in adult mouse kidneys have provided compelling evidence for a critical role of *Tulp3* in the trafficking of the ciliary component(s) of the CDCA signal in *Pkd1* cko mice. The Liem laboratory showed that in adult mice, concomitant loss of *Tulp3* completely suppressed cystogenesis in *Pkd1* cko in adult mice at 18 weeks (**Figure 4B**) (Legue and Liem, 2019). *Tulp3* cko by itself at this age caused no cystogenesis

(**Figure 4B**). However, later at 42 weeks, *Tulp3* cko did cause limited cystogenesis (Legue and Liem, 2019).

These results suggest that TULP3 traffics cilia localized cyst promoting signal(s) into cilia, which are suppressed by polycystins in normal tubules but are derepressed in adult-onset *Pkd1* cko. These signals are equivalent to the ciliary components of the CDCA. Overall, these findings provide strong evidence for a cilia-localized component(s) of the CDCA signal and delineate those as a subset of Tulp3/IFT-A ciliary cargoes in adult kidneys. To highlight their cilia-specificity and distinctiveness in action within the cilia, we term the cilia-localized component ciliary CDCA or cCDCA.

The *Pkd1* cko late-onset model offers a cyst suppressor system to test the cCDCA candidates from among the TULP3/IFT-A ciliary cargoes. The TULP3-dependent cCDCA signal(s) may be identified experimentally by the following criteria:

(i) It is trafficked to cilia.
(ii) It is a ciliary cargo of TULP3.
(iii) Lack causes no cystic changes in early adulthood.
(iv) Concomitant cko rescues adult-onset PKD in *Pkd1/2* cko at early adulthood.

The Tran laboratory found a result similar to *Tulp3* for the IFT-A peripheral subunit *Ift139/Thm1*. Concomitant loss of this IFT-A subunit suppresses cystogenesis from PC1 or PC2 loss in adult kidneys while retaining the cilia (Wang et al., 2022). TULP3 entry into cilia is not affected in *Thm1* mutants, as only the IFT-A core is required for TULP3 trafficking (Mukhopadhyay et al., 2010; Hirano et al., 2017). However, TULP3 is accumulated in cilia or in ciliary tips upon loss of IFT139 and other IFT-A core subunits, from defects in the retrograde ciliary trafficking (Mukhopadhyay et al., 2010; Hirano et al., 2017). Whether mechanisms similar to that from loss of TULP3 underlie the suppression of cystogenesis from IFT139 loss in adult-onset ADPKD is unclear. It is possible that TULP3 cargoes functioning as cCDCA signals could show abnormal accumulation in end-state ciliary levels from defects in retrograde trafficking out of cilia in the *Thm1* mutants, thereby affecting or disrupting the function of the cCDCA signals.

REGULATION OF CILIA-LOCALIZED SIGNALS IN EARLY-VS. LATE-ONSET CYSTOGENESIS BY TULP3

Recent studies of *Tulp3* mouse models have indicated complex counterregulatory cilia localized signals that positively (cCDCA) and negatively (CLCI) impact cyst growth (**Figure 5A**). Given the differences in dependency of these signals on polycystins for activity, the CLCI and cCDCA are likely distinct molecular entities. If TULP3/IFT-A can traffic the components for both CLCI and cCDCA signals to cilia in the absence of polycystins, why does concomitant *Tulp3* cko result in such different effects on cyst growth following early and late *Pkd1* gene inactivation?

One possibility is that TULP3/IFT-A traffics different cargoes in developing vs. adult kidneys (**Figures 5B,C**), perhaps to meet specific needs and tasks of the kidneys that likely differ at each stage. TULP3 inactivation would result in the ciliary exclusion of a different set of cargoes in the two stages to account for the different effects. During kidney development, TULP3/IFT-A may predominantly traffic CLCI components to cilia but few cCDCA components, which are activated following the loss of polycystins (**Figure 5B**). There could be redundancy between TULP3 and Tubby (or other members of the family) in trafficking cCDCA components to cilia in the developing kidney. This may allow the signal(s) to be regulated by a broader number of inputs perhaps required during development when renal tubules are forming, elongating, and branching. However, unlike in adult kidney epithelia, *Tubby* is expressed at very low levels in the developing embryonic and perinatal kidney compared to *Tulp3* (https://www.ebi.ac.uk/gxa/experiments/E-MTAB-6798/Results). In the adult kidney, TULP3/IFT-A might predominantly traffic the cCDCA component(s) unique to the adult state, but few CLCI components (until the later adult stages) (**Figure 5C**). The CLCI signal(s) might no longer be present or may be less effective to inhibit cyst growth at this stage when terminal tubule differentiation and maturation are complete.

Alternatively, there may be no fundamental differences in the TULP3/IFT-A regulated trafficking of cilia-signaling components between the two stages. The cCDCA may instead be trafficked by TULP3/IFT-A during development as in adult kidneys, but this regulation may be obscured by the rapid cyst growth following early inactivation of *Pkd1*. Conditional inactivation of *Tulp3* causes a stop to the ongoing ciliary trafficking of the cCDCA components. However, the already elevated cCDCA may have delayed turnover from the cilia to halt rapid cyst growth following early *Pkd1* inactivation. Residual activity of cCDCA is likely sufficient to drive considerable cyst growth that is rapidly ongoing, thus preventing a rescue of cyst growth in the *Tulp3-Pkd1* double cko during development. This scenario is consistent with the previous finding that severity of cyst growth is highly sensitive to the length of time between the initial loss of the polycystins and the subsequent involution of cilia (Ma et al., 2013). Therefore, TULP3 may regulate cCDCA in addition to the CLCI during development, but fast cyst progression in absence of Pkd1 could make such regulation difficult to detect. Genetic epistasis between TULP3 cargo and *Pkd1* mutants of varying severities will be required to unmask either cCDCA or CLCI signal during development (see next Section). In comparison, cysts grow at a much slower rate following *Pkd1* cko in adult age. The elevated level of cCDCA may drop below a threshold that is required to promote cyst growth within a time interval that is not sufficient to drive cyst growth to a significant extent.

Such counterregulatory ciliary roles are the foundational basis of the Hedgehog (Hh) pathway (Anvarian et al., 2019; Kopinke et al., 2021). We and others (Zhang et al., 2001; Huangfu et al., 2003; Norman et al., 2009; Qin et al., 2011; Mukhopadhyay et al., 2013; Somatilaka et al., 2020) have shown that cilia play an essential role by localizing both positive (e.g., Smoothened) (Corbit et al., 2005; Rohatgi et al., 2007) and negative regulators of Hh pathway, e.g., GPCR Gpr161 (Mukhopadhyay et al., 2013) and adenylyl cyclases (Somatilaka et al., 2020). They modify Gli-transcriptional

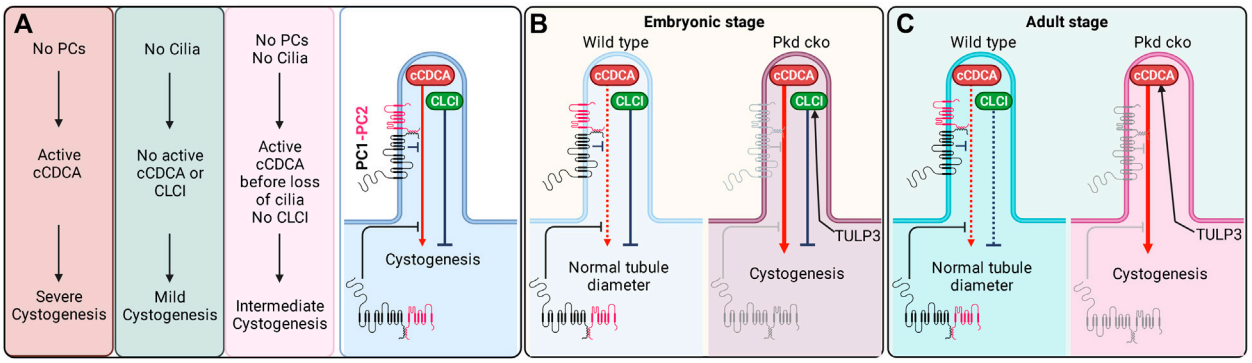

FIGURE 5 | Dual roles of cilia in cystogenesis. **(A)** The genetic data (**Figures 1**, **4**) implies **(i)** cilia-localized cilia-dependent cyst activation (**cCDCA)** signal(s) inhibited by PC1/2 in both the cell body and cilium and **(ii)** independent cilia-localized cyst inhibition (**CLCI**) signal(s). **(B)** In the embryonic stages, the cCDCA is normally weak and suppressed by polycystins. However, in the absence of polycystins, cCDCA is enhanced. TULP3 likely traffics components of the CLCI. **(C)** In the adult stage, the inhibitory arm is weaker and a lack of polycystins is sufficient to lead to strongly activated cCDCA. TULP3 likely traffics components of the cCDCA. Created in BioRender.

factors into repressors or activators by activating or repressing cAMP-regulated protein kinase A, strictly in a cilia-dependent manner. Even the Gli transcriptional factors localize (Haycraft et al., 2005) and transit through cilia in this process (Ocbina and Anderson, 2008; Kim et al., 2009).

DISSECTING TULP3 CARGOES AS POTENTIAL CLCI AND cCDCA SIGNALS BY GENETIC EPISTASIS

The genetic epistasis approaches with TULP3 cargoes and *Pkd1* mutants of varying severity during kidney development or in adult kidneys would inform whether TULP3 cargoes function as likely CLCI or cCDCA signals. A decrease in the severity of *Pkd1* mutant cystic phenotype upon concomitant loss of a TULP3 cargo would argue for this cargo to function as a cCDCA signal. An increase in severity of *Pkd1* mutant cystic phenotype upon concomitant loss of a TULP3 cargo would argue for this cargo to function as a CLCI signal.

Tulp3 deletion alone causes milder cystogenesis than *Pkd1* loss. Concomitant *Tulp3* cko in *Pkd1* cko animals did not inhibit cystogenesis but caused earlier lethality than *Pkd1* cko alone, suggesting that *Tulp3* inactivation accelerated loss of renal function in the *Pkd1* cko. Therefore, it is highly likely that TULP3 traffics cilia-localized cyst inhibition (CLCI) signal(s) during kidney development. Alternatively, Tulp3 could additionally regulate a cCDCA signal during development, but fast cyst progression in absence of *Pkd1* could make such regulation difficult to detect. Genetic epistasis between TULP3 cargo and developmental models of *Pkd1* mutants of varying severities could unmask either signal.

Lack of TULP3 in adult-onset models does not cause cystogenesis at 18 weeks. Cystogenesis in adult-onset *Pkd1-Tulp3* double cko mice is fully suppressed at this stage. Intact kidney epithelial cilia in *Tulp3* mutants argue for cilia-generated signaling rather than gross ciliary morphology defects in such suppression. Thus, it is highly likely that TULP3 traffics the ciliary component of CDCA (cCDCA) signal(s) predominantly in adult kidneys. Mild cystogenesis from *Tulp3* deletion in adult-onset models occurs much later at 42 weeks, suggesting low CLCI activity of TULP3 cargoes only at older ages. Genetic epistasis between *Tulp3* cargo mutants and *Pkd1* mutants could therefore unmask cCDCA signal(s).

The complete lack of a TULP3 cargo using a conditional knockout strategy does not equate to *Tulp3* cko that shows a selective loss of the corresponding TULP3 cargo from cilia alone without affecting the extraciliary pools. In certain cases, TULP3 cargoes that are selectively deficient in trafficking to cilia without affecting the functionality can be generated by mutating ciliary localizing signals targeted by TULP3 (e.g., for the GPCR cargo GPR161 (Hwang et al., 2021) or by targeting sequences that affect ciliary localization by TULP3 independent mechanisms (e.g., the RVxP motif for the lipidated protein ARL13B (Gigante et al., 2020), also a TULP3 cargo).

Although TULP3 loss does not affect ciliogenesis, complete lack of certain cargoes, such as ARL13B (Li et al., 2016; Seixas et al., 2016) or ARL13B-regulated INPP5E (Hakim et al., 2016), developmentally can cause ciliary disruption. These indirect effects of TULP3 cargoes on ciliary morphologies should be accounted for when performing genetic epistasis approaches between *Pkd1* mutants and TULP3 cargo cko mutants.

KNOWN TULP3 AND IFT-A CARGOES AS POTENTIAL CLCI AND CCDCA SIGNALS

Here we discuss TULP3-trafficked ciliary cargoes that might regulate both CLCI signal(s) independent of polycystins during development and cCDCA signal(s) repressed by polycystins in adult kidneys.

ARL13B

We and others recently showed that ARL13B is a TULP3 cargo in kidney epithelia *in vivo* (Hwang et al., 2019; Legue and Liem, 2019). ARL13B is a ciliary GTPase that regulates intraciliary trafficking of lipidated cargoes (Humbert et al., 2012). Inhibiting

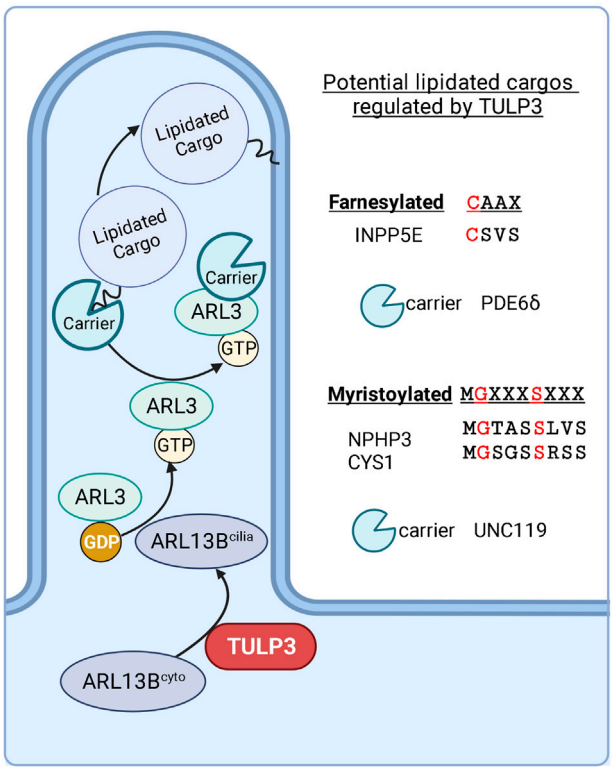

FIGURE 6 | Potential lipidated cargoes of TULP3. TULP3 directs the trafficking of ARL13B to cilia. ARL13B regulates the release of farnesylated and myristoylated cargoes in the ciliary compartment *via* ARL3. Created in BioRender.

ARL13B would block multiple lipidated cargoes bound for cilia. In further support of the TULP3-regulated CLCI, developmental deletion of *Arl13b* causes mild fibrocystic kidney disease in mice models (Li et al., 2016; Seixas et al., 2016), similar to cilia loss. The zebrafish *arl13b* (*scorpion*) allele has nephric duct dilatation phenotypes, and analysis of phenotypic rescue using *arl13b* variants in this model suggests that ciliary localization is essential for *in vivo* function of ARL13B (Sun et al., 2004; Duldulao et al., 2009).

ARL13B Regulated Cargoes

ARL13B functions as a GEF for ARL3 (Gotthardt et al., 2015; Ivanova et al., 2017). $ARL3^{GTP}$ regulates the ciliary localization of farnesylated proteins (e.g., 5' phosphatase INPP5E), and myristoylated proteins (e.g., NPHP3 and Cystin-1) by releasing them from their binding partners PDE6δ (Humbert et al., 2012) and UNC119B (Wright et al., 2011), respectively (**Figure 6**). The full list of ARL3-regulated lipidated cargoes in cilia is unknown. Residues flanking farnesylation site (CAAX box) regulate PDE6δ selectivity to cargoes (Fansa et al., 2016). The direct binding between TULP3 and certain cargoes, such as that between TULP3 and INPP5E (Humbert et al., 2012), could also factor in TULP3 mediated trafficking of these cargoes. Using IMCD3 *Tulp3* ko cell lines, we recently showed that ARL13B and INPP5E were most affected, whereas NPHP3 and Cystin-1 were comparatively less affected (Palicharla et al., 2021). We also demonstrated a significant difference in the kinetics of loss of ARL13B and ARL3-dependent lipidated proteins from the *Tulp3* cko kidney cilia: ARL13B is almost completely lost by P0, INPP5E by P5 and NPHP3 by P24. The percentage of ciliated cells and ciliary length in the collecting ducts was unchanged in *Tulp3* cko mice (Hwang et al., 2019). The farnesylated protein LKB1, which is ARL3 independent for trafficking to cilia, is not TULP3 regulated. The effectiveness for complete depletion of INPP5E in cilia in *Tulp3* cko might be related to direct binding between ARL13B and INPP5E and a requirement of such binding for effective ciliary retention of Inpp5e (Humbert et al., 2012; Qiu et al., 2021). NPHP3 is concentrated in the proximal ciliary inversin compartment by binding to NEK8 and ANKS6 that are required downstream of Inversin for NPHP3 localization (Bennett et al., 2020). Such binding might promote some retention of NPHP3 even in the absence of TULP3. Lack of *Arl3* (Schrick et al., 2006) or ARL3-dependent lipidated proteins, *Inpp5e* and *Nphp3* in developmental cko models (Wright et al., 2011; Humbert et al., 2012) or *Cystin-1* mutants (Ricker et al., 2000; Hou et al., 2002; Omori et al., 2006), causes fibrocystic disease. Nonetheless, these cilia localization experiments in *Tulp3* cko provide a potential road map for testing the most affected ARL13B regulated cargoes, such as INPP5E, as potential CLCI candidates.

GPCRs

Multiple class A rhodopsin family GPCRs that are trafficked to cilia are TULP3 cargoes. One of these GPCRs, GPR161, is known to be ciliary in the IMCD3 cells (Mukhopadhyay et al., 2013) and expressed highly in the kidney CCD cells (Mehta et al., 2022). However, lack of GPR161 during kidney development does not cause cystogenesis (Hwang et al., 2019). Thus at least this GPCR is not a CLCI signal. Tolvaptan, an antagonist to the GPCR V2R, is the only FDA-approved drug to slow eGFR decline in patients (Torres et al., 2017; Torres et al., 2020). In addition to its prevailing basolateral plasma membrane localization, V2R was also reported to localize to the cilia (Raychowdhury et al., 2009; Sherpa et al., 2019). V2R is a Gαs coupled GPCR (Ausiello et al., 1987; Fenton et al., 2007), and Tolvaptan should prevent the cAMP level increase from Vasopressin. However, Tolvaptan treatment paradoxically increased cAMP levels in cilia in a V2R-independent manner (Sherpa et al., 2020), suggesting that such a nonspecific increase does not correspond to the role of V2R in the downstream signaling (Wang et al., 2005; Reif et al., 2011).

Polycystins

An hypomorphic *Tulp3* mutant (*K407I*) shows cystogenesis in the embryonic kidney, but cilia are maintained (Legue and Liem, 2019). Ciliary disruption (Lin et al., 2003; Jonassen et al., 2008; San Agustin et al., 2016) or loss of ARL13B (Li et al., 2016; Seixas et al., 2016) or that of INPP5E (Hakim et al., 2016) does not cause cystogenesis during embryogenesis. Thus, the effects of the hypomorphic mutant are supposedly not only from lack of ARL13B/INPP5E trafficking to cilia. Polycystins themselves are also regulated in ciliary localization by TULP3 (Kim et al., 2014; Badgandi et al., 2017; Hwang et al., 2019), and PC2 is also partially reduced in ciliary levels in kidney epithelial

cilia in the *Tulp3 K407I* mutant (Legue and Liem, 2019), which might partly explain this conundrum. PC2 trafficking to cilia is also regulated by the RVxP motif at its N-terminal end (Geng et al., 2006) while PC1 trafficking to cilia may be regulated by a similar RVxP motif at its C-terminal end (Ward et al., 2011; Su et al., 2015). The lack of the RVxP motif reduces proximity between TULP3 and PC2 (Hwang et al., 2019) but the coordination between TULP3 and the RVxP motif in the trafficking of PC2 is unclear. The *Pkd2lrm4* (E442G) mutant in the first extracellular loop is unable to localize to cilia despite an intact RVxP (Walker et al., 2019), suggesting that the RVxP motif alone is not sufficient to cause a ciliary localization (Pearring et al., 2017). TULP3's role in regulating polycystins abundance in cilia should not affect genetic epistasis analyses between potential TULP3 cargoes and polycystins to unravel cCDCA/CLCI signals.

Adenylyl Cyclases

Both *AC5* (Wang et al., 2018) and *AC6* (Rees et al., 2014) deletion individually reduces renal cyclic AMP and cyst growth in an orthologous mouse model of polycystic kidney disease, suggesting their role in ADPKD. A recent omics-level study of cAMP signaling components in kidney tissue showed that AC6 and AC5 are predominantly present in collecting ducts and less strong in proximal tubules (Mehta et al., 2022). AC5/6 are cilia localized. The Igarashi laboratory showed that AC5/6 are in a complex with protein A-kinase anchoring protein 150 (AKAP150) and protein kinase A (Choi et al., 2011). The authors proposed that PC2 interacts with the AC5/6 complex through its carboxy terminus. A cAMP-specific member of the phosphodiesterase family, PDE4C, is also located in renal primary cilia and interacts with the AKAP150 complex. An IFT-A core subunit IFT144 mutant ($Ift144^{dmhd}$) shows reduced AC3 in the neural tube cilia (Liem et al., 2012). AC3 is one of the predominant ACs in the neurulation (Somatilaka et al., 2020). Whether such a role of IFT144 in AC3 trafficking encompasses other ciliary ACs and affects kidney expressed AC5 and AC6 is currently unknown. Besides, whether such a role of IFT144 in AC trafficking involves TULP3 interactions with the core subunits of IFT-A is also unknown. Recent optogenetic and/or chemogenetic techniques for manipulating the ciliary cAMP (Hansen et al., 2020; Truong et al., 2021) could be pivotal in determining if ciliary cAMP signaling plays a role in initiating cystogenesis.

CONCLUDING STATEMENT

What could be the molecular output propagated in cilia by CLCI and cCDCA signals? Based on our results showing early depletion of ARL13B and INPP5E from *Tulp3* cko kidney epithelial cilia (Hwang et al., 2019; Palicharla et al., 2021), they are strong contenders for CLCI signals. INPP5E is a 5′phosphatase that generates PI(4)P from $PI(4,5)P_2$ (Kisseleva et al., 2000). If INPP5E is the CLCI signal, by generating a PI(4)P exclusive ciliary membrane domain distinct from the plasma membrane rich in $PI(4,5)P_2$, it could regulate ciliary components. The CLCI signal could be activating a transcription factor in cilia that is regulatable by the ciliary microenvironment. Such an example is seen in Hh pathway where GLI2 and GLI3 transcription factors are modified in cilia upon Hh addition (Haycraft et al., 2005). Some of the Gli-similar proteins (Lichti-Kaiser et al., 2012), at least two of which, GLIS3 (Kang et al., 2009) and GLIS2 (Attanasio et al., 2007), are ciliary, and show mild cystic changes perinatally upon deletion (Kang et al., 2009) or in the sensitized background of partial *Kif3a* loss (Lu et al., 2016). The cCDCA signal could similarly be activating a transcription factor in cilia that is regulatable by ciliary cAMP. An important feature of such a transcription factor would be that it localizes to cilia (in addition to the nucleus) but its deletion would not cause adult-onset cysts. Another feature would be its regulation of cell proliferation, like GLI2 in Hh-induced proliferation of cerebellar granule cells by CyclinD1/N-Myc (Yin et al., 2020). Nonetheless, understanding how ciliary signals transduce and amplify downstream cellular effects could provide important leads to understanding cystogenesis.

AUTHOR CONTRIBUTIONS

FQ and SM had the idea for the article. FQ, RW, and SM performed the literature search and drafted the article. AM, VP, and S-HH critically read and revised the work. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Molecular Mechanisms of Epigenetic Regulation, Inflammation, and Cell Death in ADPKD

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Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disorder, which is caused by mutations in the *PKD1* and *PKD2* genes, characterizing by progressive growth of multiple cysts in the kidneys, eventually leading to end-stage kidney disease (ESKD) and requiring renal replacement therapy. In addition, studies indicate that disease progression is as a result of a combination of factors. Understanding the molecular mechanisms, therefore, should facilitate the development of precise therapeutic strategies for ADPKD treatment. The roles of epigenetic modulation, interstitial inflammation, and regulated cell death have recently become the focuses in ADPKD. Different epigenetic regulators, and the presence of inflammatory markers detectable even before cyst growth, have been linked to cyst progression. Moreover, the infiltration of inflammatory cells, such as macrophages and T cells, have been associated with cyst growth and deteriorating renal function in humans and PKD animal models. There is evidence supporting a direct role of the PKD gene mutations to the regulation of epigenetic mechanisms and inflammatory response in ADPKD. In addition, the role of regulated cell death, including apoptosis, autophagy and ferroptosis, have been investigated in ADPKD. However, there is no consensus whether cell death promotes or delays cyst growth in ADPKD. It is therefore necessary to develop an interactive picture between PKD gene mutations, the epigenome, inflammation, and cell death to understand why inherited PKD gene mutations in patients may result in the dysregulation of these processes that increase the progression of renal cyst formation.

Keywords: ADPKD, epigenetics, post-translational modifications, inflammation, cell death

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1 INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common genetic disorder of the kidney, caused by mutations in the *PKD1* and *PKD2* genes that encode for transmembrane proteins polycystin-1 (PC1) and polycystin-2 (PC2), respectively (Grantham, 2008). The disease is characterized by the formation and enlargement of fluid filled cysts in the kidneys, and patients with ADPKD eventually develop renal insufficiency and end-stage kidney disease (ESKD), requiring dialysis or kidney transplants (Alam and Perrone, 2010; Grantham et al., 2011). Disease development is associated with hypertension, hematuria, and urinary tract infections (Bajwa et al., 2001; Ecder and Schrier, 2009; Torres and Harris, 2009). In addition, extra-renal complications include cyst formation in other epithelial organs, including the liver and pancreas (Harris et al., 1993). To date, there is only one Food and Drug Administration (FDA) approved treatment, Tolvaptan, for

ADPKD (Torres et al., 2012). However, the long-term use of Tolvaptan causes side effects, such as thirst, polyuria, and liver injury (Torres et al., 2012). Therefore, there is an urgent need to develop more effective and safe treatments with the better understanding of molecular mechanisms of ADPKD.

Mutations in the *PKD1* and *PKD2* genes correlate with the development of ADPKD. Individuals with an inherited PKD gene mutation develop detectable renal cysts by 30 years of age (Nicolau et al., 1999). On average, *PKD1* gene mutations lead to ESKD at ~54 years compared to ~74 years for *PKD2* (Hateboer et al., 1999). A striking feature of ADPKD is the variability in the phenotype, with the disease severity, the on-set of ESKD, and the spectrum of extra-renal manifestations being highly variable between patients, and even within members of the same family (Milutinovic et al., 1992; Fick et al., 1993; Zerres et al., 1993). Owing to the research focus in our lab over the past 15 years, we will in this review, discuss three molecular mechanisms that may contribute to the disease variability and progression of ADPKD, including epigenetic mechanisms, inflammation, and cell death.

First, epigenetics is broadly defined as a genomic mechanism that reversibly influences gene expression without affecting the DNA sequence (Berger et al., 2009). Epigenetic regulation has been proposed as a potential mechanism to explain disease variability, including ADPKD (Villota-Salazar et al., 2016). We and others found an abnormal upregulation of epigenetic modifiers in kidneys from *Pkd1* animal models and in ADPKD patients (Li, 2011; Bowden et al., 2021). Moreover, inhibition of specific epigenetic factors reduces cyst growth and improves kidney function in preclinical studies, enforcing the role of epigenetic mechanisms in ADPKD (Zhou et al., 2013; Zhou et al., 2014; Zhou et al., 2015; Li et al., 2017c).

Second, we found that the progression of PKD can be influenced by the presence of inflammatory factors such as tumor necrosis factor alpha (TNF-α) (Li et al., 2008) and macrophage migration inhibitory factor (MIF) (Chen et al., 2015) in the cyst fluid. Inhibiting or reducing inflammation by decreasing macrophages for example, has been demonstrated to reduce cyst burden and improve renal function, thereby displaying beneficial effects both on cyst burden and disease progression in preclinical PKD animal models (Swenson-Fields et al., 2013). The upregulation of genes associated with immune and inflammatory responses have also been identified by microarray analysis of ADPKD kidneys (Schieren et al., 2006; Song et al., 2009). Increased T cells (component of the adaptive immunity), specifically localized to cystic lesions, correlate with disease severity. In particular, the role of $CD8^+$ T cells in inhibiting ADPKD disease progression has been demonstrated (Kleczko et al., 2018). These studies support the involvement of the inflammatory response, and to a broader scope, the innate and adaptive immune systems in the pathogenesis of ADPKD, and suggest that immunotherapy, such as the reactivation of T cells, might represent a novel therapeutic strategy. Third, we and others have reported that regulatory cell death, including apoptosis (Fan et al., 2013b), ferroptosis (Zhang et al., 2021c) and autophagy (Shillingford et al., 2006), plays a critical role in ADPKD animal models. However, there is a controversy as to whether regulated cell death promotes or delays cyst growth in ADPKD.

In this review, we discuss the roles and molecular mechanisms underlying epigenetics associated with DNA methylation and histone modifiers, inflammation, and programmed cell death in the regulation of disease progression in ADPKD. We debate on the short comings and controversies in the field and how these may impact the discovery of novel mechanisms and treatment options. In addition, we summarize the therapeutic implications and outcomes associated with the therapy of epigenetic, inflammation and cell death. Finally, we provide perspectives on how a better understanding of the diverse mechanisms involved in cyst growth may be applied for combined therapeutic strategies in ADPKD.

2 THE ROLES AND MECHANISMS OF EPIGENETIC REGULATION IN ADPKD

Epigenetic alterations which ultimately influence key signaling pathways, have recently been suggested to affect the pathogenesis of ADPKD (Li, 2015). Epigenetic mechanisms including, but not limited to DNA methylation and histone modification, act to regulate accessibility of the DNA by transcription factors to control gene expression (Stuppia et al., 2015). Epigenetic mechanisms play a role in cellular growth and differentiation during development, and as cells mature, these epigenetic modifications change to accommodate the role of the cell. These modifications, including any disease-causing epigenetic changes may be inherited (Robertson, 2005; Greer and Shi, 2012). In addition to regulating the chromatin state, histone modifiers are known to alter gene expression and protein function by post-translational modifications (Miller and Grant, 2013; Sadakierska-Chudy and Filip, 2015). Evidence for alterations in the epigenetic control of gene expression and protein function in ADPKD is accumulating (Li, 2011; Kerr et al., 2019; Li and Li, 2021) and emerging data regarding DNA methylation and histone/lysine modifiers in cystogenesis and ciliogenesis are discussed below.

2.1 DNA Methylation and ADPKD

DNA methylation is a stable and heritable epigenetic mark that involves the addition of a methyl group to cytosine residues on the genome by a group of enzymes, named DNA methyl transferases (DNMTs) (Bird, 2002). The DNMT family has four members, DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT3A and DNMT3B are referred to as *de novo* methyl transferases, with DNMT3L acting as a stimulator of their catalytic activity (Jeltsch, 2006; Zhang and Xu, 2017). Together, they establish *de novo* methylation patterns which are maintained faithfully during cell replication by DNMT1, hence often referred to as maintenance methyl transferase (Schubeler, 2015). While this modification does not affect the DNA nucleotide sequence, it can modify the availability of the genome to the transcriptional machinery thereby affecting gene expression (Razin and Cedar, 1991). In general, methylation within the gene promoter is typically associated with gene repression and though not well understood, methylation

TABLE 1 | Summary of DNA methylation studies in ADPKD.

Sample type	Gene methylation status	Conclusion	Method	Reference
Kidney tissue	*PKD1* gene body hypermethylation	Reduced expression of *PKD1* gene and genes related to cystogenesis in ADPKD.	MIRA-seq	Woo et al. (2014)
Kidney tissue	*MUPCDH* gene promoter hypermethylation	Reduced gene expression and potential novel biomarker	MIRA-seq	Woo et al. (2015)
Kidney tissue	*PKD1* gene body hypermethylation	Differentially hypomethylated fragments of the genome associated with ADPKD.	RRBS	Bowden et al. (2018)
iPSC	No change in DNA methylation pattern in promoters of *PKD1* and *PKD2* genes. DMRs observed between control and PKD mutant iPSCs	Methylation pattern was indicative of PKD-specific epigenetic memory	MeDIP-seq	Kenter et al. (2020)
Kidney cysts	N/A[a]	DMRs in individual cysts matched whole kidney tissue; a subset of loci showed marked DNA methylation heterogeneity	RRBS	Bowden et al. (2020)
Blood	*PKD1* promoter hypermethylation	Inversely correlated with *PKD1* gene expression	MS-HRM	Hajirezaei et al. (2020)

[a]*Coverage was too little for* PKD1 *gene methylation analysis.*

Abbreviations used: iPSC, induced pluripotent stem cells; DMRs, differentially methylated regions; N/A, none applicable; MIRA-seq, methylated-CpG island recovery assay with parallel sequencing; RRBS, reduced representation bisulfite sequencing; MeDIP-seq, methylated DNA immunoprecipitation sequencing; MS-HRM, methylation-sensitive high-resolution melting.

within the gene body is typically associated with sustained or increased gene expression (Saxonov et al., 2006; Shen et al., 2007; Brenet et al., 2011). However, these could deviate from the norm as is the case of ADPKD, as discussed below. DNA methylation has been heavily implicated in human diseases. In cancers, for example, dysregulation of DNA methylation has been reported and inhibitors for DNA methyl transferases (DNMTs), have been developed and approved for the treatment of certain neoplasias, including chronic myelomonocytic leukemia (CMML) and myelodysplastic syndromes (MDS) (Jones et al., 2016). In ADPKD, cysts are believed to arise independently, however, the molecular alterations that underlie cyst formation are poorly understood. Recent studies have identified global methylation patterns of ADPKD patient kidneys and individual cysts, providing evidence of a role for DNA methylation in cystogenesis.

In a pioneering study, the global DNA methylation in ADPKD patient kidneys compared to non-ADPKD kidneys (Woo et al., 2014) was analyzed by methylated-CpG island recovery assay with parallel sequencing (MIRA-seq). This study found that 11,999 genomic fragments, out of the 15 million examined, were differentially hypermethylated, accounting for 91% of all methylation changes. However, only 1,228 genomic fragments were hypomethylated, accounting for 9% of all methylation changes. In addition, this study found that hypermethylation of the *PKD1* gene body (exon 43) in ADPKD patient samples, negatively correlated with the *PKD1* gene expression, suggesting that epigenetic silencing of the *PKD1* gene is involved in kidney cyst development (Woo et al., 2014) (**Table 1**). As such, Woo et al. theorized that if hypermethylation of the ADPKD genome resulted in cyst growth, then inhibition of the DNMTs could be targeted for therapeutic purposes. In agreement with Woo et al., a second study, utilizing Reduced Representation Bisulfite Sequencing (RRBS) also reported that the *PKD1* gene body was hypermethylated in ADPKD patient kidneys (**Table 1**). However, contrary to Woo et al., this study found that hypermethylation of the *PKD1* gene body was associated with an increase in *PKD1* gene expression rather than a decrease (Bowden et al., 2018). In addition, unlike Woo et al., this study showed a 2% difference in the methylation status of the genome, with ADPKD patient kidneys being hypomethylated (Bowden et al., 2018). Utilizing methylation-sensitive high-resolution melt (MS-HRM) analysis, a third study demonstrated that hypermethylation of the *PKD1* promoter inversely correlated with gene expression in ADPKD patient blood (Hajirezaei et al., 2020) (**Table 1**). Fourth, analyzing global methylation patterns of individual cysts derived from the same ADPKD patient (**Table 1**) revealed that approximately 15% of analyzed fragments exhibited inter-cyst variation in DNA methylation pattern. While the CpG islands and gene body regions demonstrated elevated levels of methylation variation, the intergenic regions had comparatively stable methylation levels within cysts from the same ADPKD patient (Bowden et al., 2020).

The potential use of DNMT inhibitors (demethylating agents) for therapeutic purposes in cancers have been acknowledged and well documented. As such, the similarities, and associations between cancer cells and ADPKD suggest that DNMT inhibitors that slow the progress of tumors would have similar effects on cyst growth in ADPKD. At present, the demethylating agents used in clinics are cytotoxic, mutagenic and exhibit lack of specificity towards genes, limiting their clinical application. With the slow progression of ADPKD disease, the long-term use of such drugs may proof harmful. Furthermore, the effect of demethylating agents at pharmacological dosages may depend on the nature and/or extent of the epigenetic changes. ADPKD disease results from different mutations and presents with variable phenotypes, suggesting that the methylation status and subsequent molecular mechanisms may vary. Therefore, understanding epigenetics may help provide new mechanistic insights on cyst development and growth so that broad spectrum and tolerable epigenetic therapy may be developed for ADPKD disease. Advancements in genome-wide technologies have made it possible to analyze genomic methylation levels in ADPKD. Although these studies provide valuable information that point out changes in DNA methylation of the *PKD1* gene, variations have been observed in the methylation status which might be

caused by the differences in techniques used for analysis (**Table 1**). Therefore, use of Whole-Genome Bisulfite Sequencing (WGBS) may be more appropriate to provide a full, unbiased description of the extent of DNA methylation in ADPKD kidneys (Bowden et al., 2021). So far, majority of the methylation analysis studies conducted in ADPKD used kidney tissues from patients. It is important to mention that with the nature of the ADPKD disease (fluid-filled cystic kidneys), it is not practical to obtain kidney biopsies. This suggests that the data presented in the field, arises from kidney tissues obtained at ESKD. We speculate that from the initiation and on-set of cyst growth to ESKD, there may have been changes in the DNA methylation status that are not captured during these analyses. To overcome this problem, blood and/or urine samples from which genomic DNA may be obtained for analysis could be collected from ADPKD patients as the disease progresses. It is our belief that obtaining the DNA methylation status at multiple stages of the disease may provide a more comprehensive epigenetic landscape, which would lay out the foundation for future mechanistic insights and development of therapy in ADPKD. It is important to note that while blood and urine samples are readily accessible, the DNA methylation patterns identified in specific genes obtained from the blood and urine-derived genomic DNA, may not reflect the DNA methylation patterns in the genome of renal cystic epithelial cells and kidneys.

2.2 Histone Modifications and ADPKD

In the nucleus, DNA is organized and packaged around histone proteins, which control how accessible the DNA is to the transcription machinery (Cutter and Hayes, 2015). A range of post-translational modifications of these histone proteins (histone "tails"), play a vital role in gene expression. These post-translational modifications determine how tight or loose the histones are packaged, which in turn determines how freely DNA can be transcribed (Shen and Casaccia-Bonnefil, 2008). Several types of histone modifications are known including acetylation, and methylation, phosphorylation, ubiquitination, and sumoylation (Strahl and Allis, 2000; Lunyak and Rosenfeld, 2008; Tan et al., 2011; Graff and Tsai, 2013). Histone modifications at the N-terminal tails on amino acids such as lysine, arginine, serine, threonine, and tyrosine, are catalyzed by specific enzymes that act (Shen and Casaccia-Bonnefil, 2008). Acetylation of histones, catalyzed by histone acetyltransferases (HATs), results in active gene transcription, while deacetylation, catalyzed by histone deacetylases (HDACs) results in reduced levels of gene transcription (Vidali et al., 1988; Kuo and Allis, 1998; Turner, 2000). Histone methylation, regulated by histone methyl transferases (HMTs), results in either the activation or repression of gene transcription, depending on the targeted amino acid residue on the histone tail and/or the number of methyl groups added (mono-, di-, or tri-methylation) (Pedersen and Helin, 2010; Sawan and Herceg, 2010). Removal of methyl groups from the histone tails is catalyzed by histone demethylases (Pedersen and Helin, 2010). In ADPKD, there is accumulating evidence of the dysregulation of enzymes involved in histone acetylation/deacetylation and methylation/demethylation in cystic kidneys (Li, 2011; Li et al., 2017b; Bowden et al., 2021). To the best of our knowledge, there is little to no evidence regarding histone phosphorylation, ubiquitination, and sumoylation in ADPKD. Below, we summarize the role of histone modifying enzymes in ADPKD, with a focus on acetylation and methylation.

2.2.1 Histone Deacetylases in ADPKD

Growing evidence suggest that HDACs are important regulators of PKD genes and/or the signaling pathways that are involved in cystogenesis (Li, 2011). First, it has been proposed that polycystin signaling activates p53, which in turn, in cooperation with HDACs, controls *PKD1* gene expression (Van Bodegom et al., 2006). This study found that the tumor suppressor protein/transcription factor, p53, was a negative regulator of *PKD1*, and that inhibition of HDAC activity rendered the *PKD1* promoter overly sensitive. Second, HDAC5 was identified as a target of the PKD1-dependent fluid stress-sensing in renal epithelial cells (Xia et al., 2010). This study reported that polycystin-1 (PC1) facilitates calcium influx into the cell and subsequent phosphorylation of HDAC5 by protein kinase C. These studies suggested a role for HDACs in the regulation of cystogenesis in ADPKD. Subsequent studies further found that treatment with HDAC inhibitors decreased cyst growth in PKD mutants. In another study, the HDAC class I and II deacetylase inhibitor trichostatin A (TSA), and the Class I HDAC inhibitor, valproic acid, were found to effectively reduce cyst formation, body curvature and laterality in *Pkd2* mutant zebra fish morphants (Cao et al., 2009). Valproic acid also reduced cyst growth in a *Pkd1* mouse model. HDAC6 is upregulated in *Pkd1* mutant mouse cells and was found to activate factors associated with cyst growth, such as EGFR (Liu et al., 2012). Inhibition of HDAC6 with tubacin attenuated cyst growth and improved kidney function through cAMP signaling by preventing Ca^{2+} efflux from the endoplasmic reticulum (Yanda et al., 2017). Additionally, the Class III HDAC SIRT1, was also upregulated in *Pkd1* mutant mouse cells and kidneys to promote cyst growth and treatment with the SIRT1-specific inhibitor, EX-527, reduced cyst growth in *Pkd1* mouse models (Zhou et al., 2013).

The primary cilium is a pivotal organelle for the pathogenesis of cystic kidney diseases, which presents on almost all eukaryotic cells (Eggenschwiler and Anderson, 2007). The cilium is a microtubule-based organelle that functions as a mechano- and chemo-sensor (Nauli et al., 2003). Signaling receptors expressed on the ciliary membrane mediate extracellular sensory signals, creating a response inside the cells (Eggenschwiler and Anderson, 2007; Gerdes et al., 2009; Hildebrandt et al., 2011). Polycystin-1 (PC1) and polycystin-2 (PC2) locate to the cilia and PC2 functions as a Ca^{2+} ion channel (Qian et al., 1997; Yoder et al., 2002; Wang et al., 2019). Furthermore, mutations in cilia-related genes or ablation of cilia result in cystic kidney diseases (Pazour and Rosenbaum, 2002; Yoder et al., 2002). In addition to their role in regulating cell proliferation associated signaling pathways in ADPKD, HDACs also regulate primary cilia structure. HDAC6 is reported to regulate primary cilia disassembly by deacetylating α-tubulin and subsequent studies demonstrated that inhibition of HDAC6 with tubacin, prevents primary cilia resorption (Pugacheva et al., 2007; Zilberman et al.,

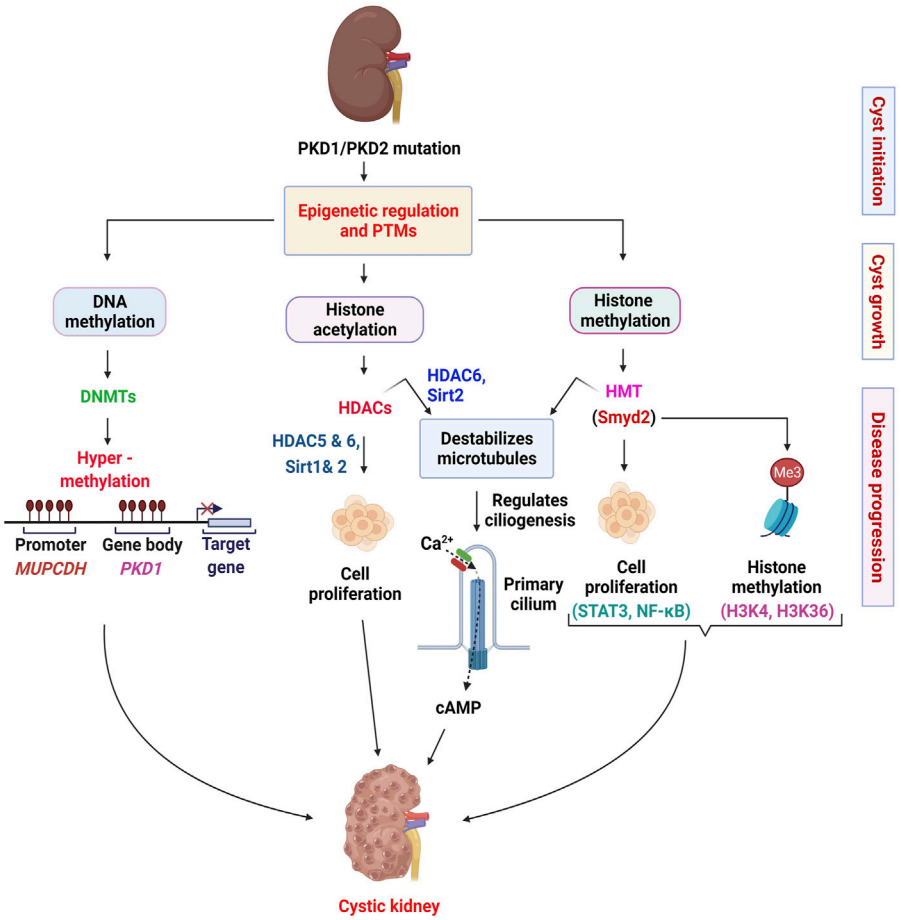

FIGURE 1 | Epigenetic mechanisms implicated in the pathogenesis of ADPKD. In this scheme, we summarize the roles of DNA methyl transferases (DNMTs), histone deacetylases (HDACs), and histone methyl transferases (HMTs) in renal epithelial cells. We indicate the roles of DNMTs in regulating the transcription of *PKD1* and *MUPCDH* genes. In general, we indicate the HDACs and the HMT involved in regulating cell proliferation associated pathways. We also depict the role of HDACs and HMTs in regulating ciliogenesis through deacetylation of α-tubulin (HDAC6 and SIRT2) and methylation of α-tubulin (Smyd2). The involvement of calcium signaling in these processes is possible but uncertain. The various stages of ADPKD (cyst initiation, cyst growth and disease progression) require different epigenetic controls and therefore may require different therapeutic approaches.

2009; Ran et al., 2015). The Class III HDAC SIRT2 was also found to regulate primary cilia disassembly and inhibition of SIRT2 by nicotinamide prevented this process (Zhou et al., 2014). Furthermore, inhibition of SIRT2 was found to reduce cyst growth in *Pkd1* mouse kidneys (Zhou et al., 2014). Since HDAC6 and SIRT2 are increased in ADPKD, these studies suggest the involvement of HDAC6 and SIRT2 in the regulation of cystogenesis through cilia-dependent signaling in ADPKD. In sum, these studies suggest that HDACs contribute to ADPKD pathogenesis by regulating both PKD-mediated and cilia-dependent signaling pathways (**Figure 1**).

2.2.2 Histone Methyl Transferases and ADPKD

Histone methylation commonly occurs in specific arginine and lysine residues at the N- terminal tails of histones (Smith and Denu, 2009). Each arginine methylation can exist in either the mono-methylated, di-methylated symmetrical and asymmetrical methylated states (Byvoet et al., 1972; Bedford and Richard, 2005), whereas each lysine has three possible methylation states: mono-methylated, di-methylated, or tri-methylated states (Murray, 1964; Hempel et al., 1968; Nguyen et al., 2010). Differences in residue methylation and modification states correlate with either gene transcription activation or repression (Zhang and Reinberg, 2001; Li et al., 2007). Lysine methylation at H3 lysine 4 (H3K4) and H3K36 for example are associated with transcriptional activation (Ringrose and Paro, 2004). In contrast, methylation at H3K9 and H3K27 are associated with transcriptional repression (Heard et al., 2001; Schotta et al., 2002; Sims et al., 2003; Ringrose and Paro, 2004). To date, there is limited evidence to support a role for histone methylation in ADPKD pathogenesis. Recently, our group found that the histone/lysine methyl transferase SMYD2, one of the SET and MYND-containing lysine methyl transferases (SMYD), contributed to cyst growth in ADPKD (Li et al., 2017b). SMYD2 can methylate both H3K4 and H3K36 (Brown et al., 2006; Abu-Farha et al.,

2008) and non-histone proteins, including p53/TP53 and RB1 (Huang et al., 2006; Cho et al., 2012).

First, we found that SMYD2 expression is increased in *Pkd1* mutant mouse renal epithelial cells and kidneys as well as in ADPKD patient kidneys (Li et al., 2017b). Utilizing *Pkd1* knockout mice and the SMYD2 inhibitor, AZ505, we showed that SMYD2 is a critical mediator of renal cyst growth in ADPKD. In addition, we found that SMYD2 promotes cyst growth in ADPKD via the methylation of H3K4 and H3K36. In particular, SMYD2 regulated cystic epithelial cell proliferation and survival through STAT3 and NF-κB. SMYD2-mediated methylation of STAT3 and NF-κB is important for the activation of these two pathways. We proposed that SMYD2, via two positive feedback loops: SMYD2/STAT3/SMYD2 and SMYD2/NF-κB/SMYD2, promotes cyst development in ADPKD (**Figure 1**). Second, we determined that SMYD2 is an α-tubulin methyl transferase that together with cyclin-dependent kinases 4 and 6 (CDK4/6), regulates ciliogenesis in renal epithelial cells (Li et al., 2020b). The cross-talk between CDK4/6 and SMYD2 is important for the regulation of ciliogenesis and targeting CDK4/6-SMYD2 signaling affects not only ciliogenesis but also cilia-dependent hedgehog signaling activation in *Pkd1* mutant renal epithelial cells (Li et al., 2020b) (**Figure 1**). This was the first study to shed light on the contribution of an epigenetic regulator of histone methylation on cyst growth and cilia biogenesis, thereby linking the "tubulin code" (a concept that describes how post-translational modifications that mark subsets of microtubules in the cytoskeleton direct microtubule-based functions) (Park et al., 2016), and cilia-dependent signaling to histone methylation and cyst growth in ADPKD.

The study of epigenetics and associated post-translational modifications have increasingly become an area of interest in ADPKD. The culminative efforts from different research teams have led to the identification of an increasingly complex network of epigenetic mechanisms associated with cystogenesis (**Figure 1**). Thus far, studies in the field have focused on identifying dysregulated epigenetic modifiers and characterizing their roles and mechanisms in cystogenesis (Zhou et al., 2013; Zhou et al., 2014). Advances were made in the category of histone modifiers, with the identification of the first lysine methyl transferase, Smyd2 and how it is involved in the regulation of cystogenesis and ciliogenesis (Li et al., 2017b; Li et al., 2020b). These studies provided new molecular mechanisms of the disease and provided a novel target for therapeutic purposes. With the advancements made in technology, the field has witnessed an exponential burst in studies aimed at characterizing the epigenome of ADPKD kidneys (Woo et al., 2014; Woo et al., 2015; Bowden et al., 2018; Bowden et al., 2020). These studies have shed light on the role of DNA methylation in the pathogenesis of ADPKD. However, more studies are required to establish a consensus of DNA methylation markers and changes in ADPKD. In addition, how the PKD mutation affects epigenetic mechanisms remains unstudied. Also, the use of epigenetic patterns as markers for cell composition and origin of ADPKD cysts remains unclear. To address these questions, techniques such as whole-genome bisulfite sequencing (WGBS) and single-cell epigenomics sequencing could be applied. Though expensive, the use of WGBS would provide a complete and unbiased description of the extent of DNA methylation in ADPKD kidneys. Single-cell epigenomics sequencing on the other hand would be an effective way to identify the origin, composition, and differentially activated epigenetic mechanisms during the development and progression of ADPKD. Together, these techniques have the potential to identify specific molecular targets that would be more appropriate for ADPKD therapy.

2.3 Therapeutic Targets and Therapeutic Implications

The pharmacological control of epigenetic signatures has become a new frontier in different diseases, including cancer. However, the ubiquitous effects of epigenetic changes on pathways limit any potential clinical application in disease treatment. Hypo- and hyper-methylated states in DNA have been associated with ADPKD and thus they represent a potential therapeutic target. DNA methylation is catalyzed by the DNA methyl transferases (DNMTs), and this process potentially contributes to the suppression of gene transcription. This makes it challenging to design drugs whose mechanism of action relies on reactivation of abnormally silenced suppressor genes. There exist multiple classes of DNMT inhibitors (DNMTi) such as nucleoside analog inhibitors azacitidine, and decitabine, however, there is limited information on their efficacy in humans. Thus far, these inhibitors, in combination with chemotherapy have been employed for the treatment of cancers. However, with reports of their cytotoxicity, their use is short-term. With the slow progressive nature of ADPKD pathogenesis, long-term use of DNMTi may result in extensive cytotoxicity.

Changes in histone acetyl groups have also been recognized as epigenetic marks of ADPKD. For histone acetyltransferases (HATs) and histone deacetylases (HDACs), a correlation with cystic burden and severity has been demonstrated in ADPKD mouse models. The activity of HATs may be modulated by bromodomain and extra-terminal motif-containing proteins (BET). In this regard, Zhou et al. demonstrated that targeting the BET bromodomain (BRD) protein, Brd4 with its inhibitor JQ1 (a thieno-triazolo-1,4-diazapine) slows renal cyst growth in *Pkd1* mutant mice (**Table 2**). With respect to potential modifiers of histone deacetylation, trichostatin A and valproic acid function as HDAC inhibitors (HDACi), and niacinamide acts as Sirtuin (SIRT) inhibitor. Even though HDACi are approved for the treatment of hematological malignancies, their beneficial application in ADPKD is limited to preclinical studies (**Table 2**). The Sirtuin inhibitor niacinamide on the other hand, is currently undergoing clinical trial for its potential use in the treatment of ADPKD (**Table 2**).

Alterations in histone methylation patterns contribute to the epigenetic control of RNA transcription from DNA. Through the transfer of methyl group to lysine or arginine residues, histone methylation, like DNA methylation, is associated with transcriptional repression. However, exceptions exist depending on the methylated residues. Because of the ubiquitous function of the histone-lysine N-methyl transferase

TABLE 2 | Summary of clinical trials and preclinical studies targeting epigenetic factors in ADPKD.

Drug	Mediator	Status	Clinical outcome or animal model	Reference
Niacinamide	SIRT1	Phase 2	TKV, eGFR, pain score, urine MCP-1	NCT02558595
Valproic acid	HDAC	Preclinical	*Pkd1* mutant mice	Cao et al. (2009)
JQ1	Brd4	Preclinical	*Pkd1* mutant mice	Zhou et al. (2015)
AZ505	Smyd2	Preclinical	*Pkd1* mutant mice	Li et al. (2017b)

Abbreviations used: TKV, total kidney volume; eGFR, estimated glomerular filtration rate; MCP-1, monocyte chemoattractant protein-1.

enzyme SMYD2, AZ505 may be a promising agent with histone methylation inhibitory properties. However, since SMYD2 plays diverse roles in different cells and organs by regulating distinct substrates, side effects may be unavoidable if SMYD2 inhibitors are used as therapeutic targets in ADPKD.

Despite compelling evidence, the role of epigenetic mechanisms in ADPKD remains unclear. In recent years, research from our lab and others have made substantial contributions towards understanding the mechanisms of epigenetic modifiers in ADPKD disease progression. DNA methyltransferase enzymes and histone modifiers are known to differentially affect the functioning of diverse pathways in cells and organs. One can speculate that oral or intravenous administration of drugs targeting these modifiers may have side effects. Therefore, the use of drug carriers with different affinities for target cells or organs such as kidneys in the case of ADPKD, for the delivery of inhibitors may be a direction of future research.

3 THE ROLES AND MECHANISMS OF RENAL INFLAMMATION IN ADPKD

The roles of inflammatory response in the pathogenesis of ADPKD has become a central focus in the past decade (Li et al., 2008; Swenson-Fields et al., 2013; Chen et al., 2015; Sadasivam et al., 2019; Zimmerman et al., 2020). ADPKD patients are susceptible to exogenous pathogens due to multi-organ decline caused by loss of renal function, resulting in immune cells proliferation and cytokine secretion. As such, the renal inflammatory response in ADPKD patients has been recognized as a non-initial and secondary effect of cyst progression for a long time (Chen et al., 2015). However, because non-infectious inflammation is present in the early and progressive stages in most *Pkd1* mutant mouse models and ADPKD patients, it is necessary to understand the roles and underlying mechanisms that drive inflammatory response in ADPKD, which may facilitate the development of novel therapeutic strategy for ADPKD treatment. In this section, we discuss how inflammatory response functions in the pathogenesis of ADPKD, and the therapeutic potential associated with these mechanisms.

The two immune systems, innate immune system, and adaptive immune system, which are unique in many aspects, are synergistically mobilized in response to endogenous or exogenous stimulus according to the patterns to recognize the pathogens and response timelines. Innate immune response, also termed non-specific and natural immune response, comes in as the first line of defense against invading pathogens or in response to altered endogenous molecules. This sometimes provides the initiating signal for adaptive immune response, composed of innate immune cells and innate immune associated molecules, including macrophages, dendritic cells, natural killer (NK) cells, natural killer T (NKT) cells, gamma-delta T cells (γδ T cells), eosinophil, neutrophil, mast cells and complement family proteins (Warrington et al., 2011; Netea et al., 2020). The adaptive immune response, also known as acquired or specific immune response, is composed of specialized, systemic cells and processes that eliminate pathogens or prevent their growth through antibodies and cytotoxic T cells. The adaptive immune system relies on the canonical T cells, and B cells by producing antibodies or effector T cells, upon stimulation with antigens presented by antigen-presenting cells (APC) and recognition of T cells receptor (TCR) or B cell receptor (BCR) (Lanzavecchia et al., 1999; Avalos and Ploegh, 2014; Gaudino and Kumar, 2019). NKT and γδ T cells also express TCRs and can also be categorized as adaptive immune cells. Based on their activation patterns, NKT and γδ T cells can also be characterized as innate immune cells. Due to the non-infectious environment during kidney development and the progression of polycystic kidney diseases, the categories of kidney resident immune cells are less complex. According to their origins, all the innate and adaptive immune cells are categorized into three main cell types—granulocytes, mononuclear phagocytes, and lymphocytes. Cells differentiated from granulocytes and mononuclear phagocytes are attributed to innate immune response, while lymphocytes, which include B cells and T cells engage in adaptive immune response. The granulocytes, including eosinophils, neutrophils, and mast cells, are responsible for allergic inflammation. As such, they are detected in peripheral blood and rarely detected in kidneys (Hume, 2008; Malech et al., 2014; Zimmerman et al., 2020). Thus, we will focus on the dominant immune cell types in the kidneys, including macrophages, NK cells, NKT cells, γδ T cells and canonical T cells, to understand their functional roles in the progression of ADPKD.

3.1 The Innate Immune Response in the Pathogenesis of ADPKD

3.1.1 The Roles of Macrophages in ADPKD

Kidney macrophages derive from circulating monocytes and resident macrophages. In general, the tissue-resident macrophage population is derived from the yolk sac and fetal liver during development but is complimented by circulating

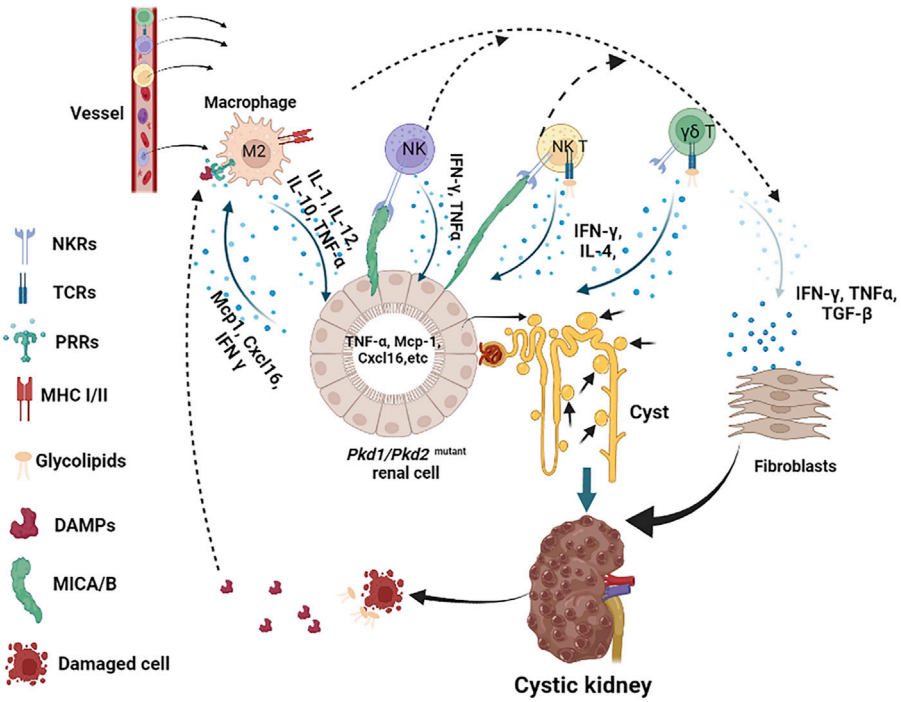

FIGURE 2 | Innate immune response cells and molecules in the pathogenesis of ADPKD. In this scheme, we describe the major innate immune cells in the pathogenesis of ADPKD, including macrophages (referred to M2), NK, NKT and γδ T cells, and the major molecules that participate in this process, including DAMPs, glycolipids, and cytokines. We indicate the main receptors on immune cells, such as MHC I/II on macrophages, TCRs and NKRs on NK, NKT and γδ T cells. *Pkd1* deficient renal cells release Mcp-1 and Cxcl16, which recruit macrophages to the kidney, other cytokines also attract NK, NKT and γδ T cells to infiltrate to kidney as well. Activated macrophages release TNF-α and other cytokines that stimulate renal cell proliferation and induce stress response, resulting in the accumulation of DAMPs in fluid or intestinal or induction of MICA/B on surface of renal cells. Upon recognition of DAMPs and glycolipids released by damaged or dying cells, or MICA/B presented on the surface of cystic cells, activated macrophages, NK, NKT and γδ T cells produce cytokines, such as TNF-α, IFN-γ, and TGF-β etc, to further promote cystic renal cell proliferation or renal fibrosis in ADPKD.

monocytes in response to stimulus (Epelman et al., 2014). Macrophages can be recruited by chemokines, such as monocyte chemoattractant protein-1 (MCP-1), to the damage sites or infectious tissues, switching from monocytes to macrophage. In different organs, macrophages may be attributed unique names, such as Kupffer cells in liver and alveolar macrophage or dust cell in lung (Laskin et al., 2001).

Accumulated evidence exists to support a role for interstitial macrophages in promoting cyst growth in human ADPKD and rodent cystic models (Karihaloo et al., 2011; Swenson-Fields et al., 2013). Treatment with liposomal clodronate to deplete phagocytic cells delayed cyst growth and improved renal function in *Pkd1*$^{fl/fl}$*:Pkhd1-Cre* mice compared to vehicle treated animals (Karihaloo et al., 2011). Another study reported that M2-like macrophages are abundant in ADPKD patient and mouse kidneys, and depletion of these macrophages led to a milder cystic phenotype and an improved renal function in *Pkd1* mutant mouse kidneys (Swenson-Fields et al., 2013). In general, classical M1-macrophage activation indicates oxidative and pro-apoptotic features, whereas the M2-macrophage activation has proliferative, remodeling and pro-fibrotic effects. This study emphasized a role of M2 macrophages in promoting the progression of ADPKD, highlighting the complexity and differences between M1 and M2 macrophages in this disease.

The renal interstitial macrophages are mainly recruited and derived from circulating monocytes to kidneys during the disease progression, supporting the hypothesis that chemokines and other factors that attract the infiltration of macrophages should also contribute to ADPKD progression. It has been reported that cultured *Pkd1*-deficient cells express elevated levels of macrophage chemoattractants, including Mcp1 and Cxcl16 (**Figure 2**), and both of these factors are able to stimulate macrophage migration, suggesting that they may contribute to the recruitment of macrophages to cystic kidneys (Karihaloo et al., 2011). To further understand the mechanisms involved in the recruitment of macrophage in PKD kidneys, we identified the role for the macrophage migration inhibitory factor (MIF) in the recruitment of macrophages to pericystic regions and MIF also regulated other signaling pathways to promote cyst growth in *Pkd1* mutant mice (Chen et al., 2015).

The activation of macrophages is a complicated process, dependent on synergistically coordinated signals from cytokines, ligands, and the corresponding receptors on macrophages (Mosser and Edwards, 2008). In the canonical

pathway for macrophage activation and function in response to infection or injury, IFN-ɣ is the most potent macrophage-activating factor and is mainly triggered by viral or parasite infection and released by other immune cells or pathogen affected cells (Muller et al., 2018; Kang et al., 2019; Zhang et al., 2021). Macrophages can also be activated through pattern recognition receptors (PRRs) by an engagement with pathogen-associated molecular patterns (PAMPs) or the damage-associated molecular patterns (DAMPs). PAMPs are presented or released by pathogens, while DAMPs are derived from injured or dying cells (Amarante-Mendes et al., 2018; Roh and Sohn, 2018; Li and Wu, 2021). DAMPs are mainly related to altered self-molecules, including high-mobility group box 1 (HMGB1), S100 proteins, and heat shock proteins (HSPs), etc., reported to be abnormally expressed in diseases, but not or limited in normal situations (Roh and Sohn, 2018). Due to the non-infectious environment of the kidneys during early-stage progression of ADPKD, DAMPs may be the main stimulus for the activation of macrophages compared to PAMPs. The serum levels of HMGB1 are increased in ADPKD patients (Nakamura et al., 2011; Nakamura et al., 2012), while S100A8 and A9 were found to be remarkably increased in both ADPKD patients and mouse models (Lee et al., 2015). Many factors are known to induce the release of DAMPs, including cellular stressors, such as nitric oxide (NO), reactive oxygen species (ROS) and oxidized mitochondrial DNA. Under cellular stress, these factors are released into the cytosol and are responsible for the activation of macrophages (Malysheva et al., 2007; Muralidharan and Mandrekar, 2013; Minton, 2017).

Activated macrophages could either function against inflammatory (M1 macrophage) or further promote inflammatory response (M2 macrophage), respectively, to eliminate infected or injured cells, and assist in the activation of adaptive immune cells, or promote cell proliferation, cell remodeling and fibrosis through different cytokines (Mosser and Edwards, 2008; Arango Duque and Descoteaux, 2014; Krzyszczyk et al., 2018). Activated macrophages can produce cytokines, including IL-1, IL-12, IL-10, TNF-α, etc. (**Figure 2**). Among those cytokines, TNF-α, which is present in the cystic fluid of human ADPKD kidneys, can disrupt the localization of polycystin-2 to the plasma membrane and primary cilia through a TNF-α induced scaffold protein FIP2, to promote cyst formation in organ cultures and in *Pkd2* mutant mice (Li et al., 2008). The cyst fluid TNF-α may be secreted by activated macrophages or *PKD* mutant cystic renal epithelial cells or both in ADPKD kidneys. These studies suggest that activated macrophage-mediated inflammation plays a role in promoting and/or inducing cystogenesis in the presence of cytokines during cyst expansion. In addition, DAMPs-PRRs activated macrophage may exert its effect through the formation of inflammasomes. Inflammasomes are a group of multimeric protein complexes that consist of a sensor molecule such as PRR, the adaptor protein ASC and caspase 1. Activated inflammasomes play a significant role by releasing IL-1β and IL-18, which are proteolytically activated by caspase 1 (Erlich et al., 2019; Zheng et al., 2020).

3.1.2 The Roles of NK Cells, NKT Cells and γδ T Cells in ADPKD

Natural killer (NK) cells, also called large granular lymphocytes (LGL), are the main innate immune cells that show strong cytolytic function against physiologically stressed cells (tumor and virus-infected cells) and represent 5%–20% of all circulating lymphocytes in humans (Perera Molligoda Arachchige, 2021). NKT and γδ T cells are very similar in many aspects, including: 1) NKT cells and γδ T cells both arise in the thymus, undergo T cell receptor (TCR) gene rearrangement and express CD3 molecule, either CD4 or CD8 molecules, or double negative of CD4/CD8, which is different from conventional T cells (Huang et al., 2014; Krijgsman et al., 2018), and 2) both NKT cells and γδ T cells acquire the expression of the natural killer receptor (NKR) NK1.1 during maturation, including inhibitory NKR (KIRs) and activating NKR (Sawa-Makarska et al.), which is the major difference between NKT cells, γδ T cells and conventional T cells (Krijgsman et al., 2018). Thus, NKT and γδ T cells are at the interface between the innate and adaptive immune system (Dranoff, 2004). The recognition patterns of NKT cells and γδ T cells are more like NK cells rather than conventional T cells, making them pass for innate immune cells instead of adaptive immune cells.

As innate immune cells, NK cells are major effectors of the innate immune system to kill target cells. The role of NK cells in renal fibrosis has been reported, where the accumulation of NK cells in the tubulointerstitial compartment of fibrotic kidneys was correlated with the severity of fibrosis (Law et al., 2017). There is no report regarding the roles of NK cells in the pathogenesis of ADPKD, however, the fact that renal fibrosis is one of the major features of ADPKD, suggests that NK cells may also contribute to renal fibrosis in ADPKD kidneys, and warrants investigation. NKT and γδ T contribute to the main portion of double negative T cell (CD4/CD8 negative, DN), reported to be increased in human ADPKD kidneys compared to controls (Sadasivam et al., 2019). The exact roles of NKT cells in ADPKD are not clear, however, it has been reported that kidney injury induces the activation of NKT cells, and causes hematuria and nephritic casts by damaging glomerular endothelial cells in a perforin-dependent manner through secretion of IFN-γ and other mechanisms and result in kidney dysfunction (Turner et al., 2018). This suggests a role for NKT cells during cyst expansion mediated by kidney injury. Taken together, NK cells, NKT cells and γδ T cells may play a vital role in the progression of ADPKD and need further investigation.

Next, we wanted to address the activation of NK cells, NKT cells and γδ T cells in ADPKD in the absence of pathogens. The fact that NKT cells and γδ T cells harbor two systems of receptors gives them the ability to activated via T cell-like mechanisms or NK cell-like mechanisms. TCRs on NKT and γδ T cells can recognize glycolipids in the context of CD1 family molecules (Liu and Huber, 2011; Pellicci et al., 2020), which is different from conventional T cells, mainly relying on MHC molecules to present peptides antigens. The representative molecule CD1d primarily expressed by antigen-presenting cells (APC), including macrophages, B cells and Dendritic cells, can

present both exogenous and endogenous glycolipids in the context of CD1d to activate NKT cells and γδ T cells. Glycolipids include exogenous microbial- and non-microbial-derived glycolipids, and endogenous glycolipids. The latter is mainly released by apoptotic cells or damaged cells or expressed by malignant cells, but rarely detected on normal cells, including gangliosides and sulfatide, phospho-glycerolipids and sphingomyelin (Podbielska et al., 2011; Krijgsman et al., 2018). In ADPKD kidneys, metabolic glycerolipids derived from abnormally proliferative cells or released from DNA damage induced dead cells might be the main glycolipids antigens to stimulate the activation of NKT cells and γδ T cells. In addition to TCRs, NKT cells and γδ T cells also express NK cell receptors. As such, they are activated in a manner comparable to NK cells. Upon stimulation, the outcomes of NK cells, NKT cells and γδ T cells are dependent on the balance between inhibitory and activating signals obtained via the major inhibitory receptors, killer Ig-like receptors (KIRs), and killer cell activating receptors (Sawa-Makarska et al.), respectively (Long et al., 2013). KIRs, provide inhibitory signals upon binding with classical MHC molecules to maintain silence against normal cells (Kumar, 2018). KARs recognize a variety of MHC-like molecules, such as the canonical KAR, NKG2D, which recognizes MHC class I-like molecules A and B (MICA/B) and unique long-binding proteins, which are usually not expressed or lowly expressed in normal cells but robustly expressed on malignant or stress-induced cells, termed as "stress protein" (Long and Rajagopalan, 2002). Thus, these cells play a vital role to maintain homeostasis.

Although the expression of MIC-A/B or other ligands of NKT cells and γδ T cells receptors has not been reported on the surface of cystic cells in ADPKD, the fact that the MICA gene contains an NF-κB-binding site which is necessary and sufficient for transcriptional transactivation of MICA in response to TNFα in primary endothelial cells (ECs) (Lin et al., 2012), suggests a potential of MICA being expressed on cystic renal epithelial cells in ADPKD kidneys. In addition, it has been reported that the regulatory promoter module of MICA/B contains heat shock elements resembling those of HSP70 genes, suggesting that HSP70 and its family proteins also have the potential to stimulate the expression of MICA/B on stress-induced cells (Elsner et al., 2007; Schilling et al., 2015). As an important DAMPs, increased HSP70 also has the potential to induce the activation of NK cells, NKT and γδ T cells through the induction of MICA/B. Loss of self-MHC molecules or abnormal expression of MICA/B in cystic cells induced by HSPs and cyst fluid TNF-α, NK cells, NKT cells and γδ T cells might be activated and produce lots of cytokines. Similarly, upon activation via TCRs, NKT and γδ T cells could also rapidly expand and secrete a range of cytokines, mainly including IFN-ɣ and IL-4 (Coquet et al., 2008; Krijgsman et al., 2018), whereby IFN-ɣ could act as the most potent cytokine to stimulate the activation of macrophages. This would induce a feed-forward loop between these cells in ADPKD kidneys (**Figure 2**). Taken together, the engagement of receptors of innate immune cells and the potential ligands expressed on cystic cells or released by damaged cells may activate innate immune cells and contribute to the progression of ADPKD (**Figure 2**).

3.2 The Adaptive Immune Response in the Pathogenesis of ADPKD

The adaptive immune response modulated by CD4 or CD8 T cells, and B cells, are responsible for cellular immunity and humoral immunity, respectively. The major difference between innate and adaptive immunity is the specificity of antigen recognition mediated by the TCRs or B cell receptor (BCR). The composition of conventional T cells (TCR αβ), referred to as T cells, are more complicated. T cells including Th1, Th2, CD4/CD25 regulator T cells, and Th17 cells, etc., all belong to CD4 T cells (Zhu et al., 2010). Activated CD8 T cells are mainly cytotoxic T cells, mediating a direct cell killing towards the target cells and releasing of cytokines, such as IFN-ɣ (Bhat et al., 2017; Nicolet et al., 2020). Activated CD4 T cells function significantly different from CD8 T cells, according to the specific phenotype, including the transcription activation or inhibition, and cytokines secretion (Luckheeram et al., 2012). Since the sterile-immune response is the main situation in ADPKD, there is no specific immunogen or pathogen to activate the adaptive immune cells, thus adaptive immune response is more likely a secondary response in ADPKD kidneys.

Reports indicate that both renal $CD4^+$ and $CD8^+$ T cell numbers are elevated, and correlate with disease severity in the $Pkd1^{RC/RC}$ mouse, but with selective activation of $CD8^+$ T cells, as analyzed by flow cytometry analysis. In addition, immunodepletion of $CD8^+$ T cells worsen ADPKD pathology in one to 3 months *C57Bl/6* $Pkd1^{RC/RC}$ mice. Furthermore, the expression of T cell recruiting chemokines, CXCL9/CXCL10, which were secreted by cystic epithelial cells and renal interstitial cells, were significantly increased in kidneys of $Pkd1^{RC/RC}$ mice compared to those in kidneys from wildtype mice (Kleczko et al., 2018). These results suggested a protective role of $CD8^+$ T cell in ADPKD and implied that the increase in T cells was as a result of extrarenal recruitment rather than the amplification of resident T cells. Besides CD8 T cells, there is no report defining the roles of CD4 T cells in ADPKD. A study found the CD4 T regulatory cells worsen chronic kidney disease (CKD) or end-stage kidney disease (ESKD) (Hartzell et al., 2020), suggested that CD4 T regulatory cells might also promote the progression of ADPKD.

Studies have reported increases in B cell numbers in ADPKD, however, little is known on the roles of B cells in disease progression. Furthermore, the mechanisms of activation of adaptive immune cells are still largely unknown. It has been proposed that DNA damage response mediated ubiquitous cell proliferation across the cystic kidneys, is the cause of the increases in T and B cells in ADPKD kidneys. Reports indicate that loss of polycystin-1 (PC1) impairs DNA damage response and induces cell proliferation of PC1 deficient cells in the kidneys (Zhang et al., 2021b). Because PC1 and PC2 are also expressed in lymphocytes, the intrinsic rate of DNA damage and the susceptibility to DNA damage agents are also increased in the peripheral blood lymphocytes from ADPKD

TABLE 3 | Prospective immunotherapy strategies in the treatment of ADPKD.

Category	Targets	Methods and outcomes	References
Targeting abnormal immune cells	$CD8^+$ T cells	Immunodepletion of CD8 T cells worsens ADPKD phenotype	Kleczko et al. (2018)
	$CD4^+$ Treg cells	Antibody against CD25 attenuate the progression of ADPKD.	Onda et al. (2019)
	Macrophages	Exhaustion of macrophages delayed cyst growth	Swenson-Fields et al. (2013)
	Macrophages	Genetic deletion of MIF delayed cyst growth	Chen et al. (2015)
	NK, NKT and γδ T cells	Neutralization with antibodies against KAR NKG2D prevents the activation of NKG2D-expressing cells	Lodoen et al. (2003), Steigerwald et al. (2009)
Targeting cytokine secretion	IFN-γ	Neutralization of IFN-γ with antibodies inhibits the proliferation	Prencipe et al. (2018)
	TNF-α	Neutralization of TNF-α might overactivation of inflammation in ADPKD.	Li et al. (2008)
Targeting cytokine regulators	Caspase 1	Targeting caspase 1 with its inhibitor could suppress the inflammasome activation and reduce IL-1β and IL-18	Flores et al. (2020), Liang et al. (2020)
	NLRP3	Targeting NLRP3 with its inhibitor could reduce IL-1β and IL-18	Zahid et al. (2019)
Targeting the regulatory machinery of immune response	CBP/p300 coactivators, KMTsetc.	CBP/p300 coactivators regulates the transcription of TNF-α and its family members	Falvo et al. (2000), Granja et al. (2006)
	Set7	Stress-mediated induction of histone methyltransferase Set7, leads to promoter modification on MCP-1 through Set7-mediated H3K4 methylation	Batista and Helguero (2018)

patients (Aguiari et al., 2004; Li et al., 2013). Thus, PC1 deficiency may also induce cell proliferation in lymphocytes in an immune-recognition independent manner. In addition, activated innate immune cells could produce cytokines, such as IFN-ɣ, TNF-α TGF-β and IL-1β to regulate the amplification of adaptive cells and adaptive immune response, especially T cells. For example, by activating inflammasome, innate immune cells including macrophage, produce IL-1β, which is a pro-inflammatory cytokine that stimulates T cell activation, resulting in T cell differentiation under different conditions (Wan and Flavell, 2007; Croft, 2009; Van Den Eeckhout et al., 2020).

3.3 Prospective in Immune Therapy in the Treatment of ADPKD

Immunotherapies to restore the dysregulated immune response in ADPKD by either the inactivation of the overactivated cell types or the activation of the protective cell types would benefit the patients. Several immunotherapeutic strategies have been evaluated in *Pkd1* mutant mouse models (**Table 3**).

(1) Targeting abnormal immune cells: First, it was reported that immunodepletion of CD8 T cells worsens ADPKD phenotype, suggesting that activation of CD8 T cells should attenuate the progression of ADPKD. Second, it has been found that CD4 Treg cells promote cyst growth, suggesting that specifically targeting CD4 Treg cells with antibody against CD25 rather than CD4 to delete Treg cells might be better than targeting other subsets of CD4 T cells, since CD25 was the first surface marker used to identify Tregs (Onda et al., 2019). Third, it has been confirmed that induced exhaustion of macrophages or genetic deletion of MIF delayed cyst growth and improved renal function (Swenson-Fields et al., 2013; Chen et al., 2015), supporting the hypothesis that targeting macrophages and factors associated with macrophage recruitment and function is a potential strategy for the treatment of ADPKD. With regards to other immune cell types such as NK, NKT and γδ T cells, the neutralization with antibodies against activating receptors may have beneficial effect. One possibility is to target KAR NKG2D with an antibody, which can prevent the activation of NKG2D-expressing cells (here referring to NK, NKT and γδ T cells) and cytokine secretion by inducing rapid internalization of antigen-antibody complex upon binding to NKG2D (Lodoen et al., 2003; Steigerwald et al., 2009).

(2) Targeting cytokine secretion: It has been reported that neutralization of IFN-γ with antibodies inhibits the proliferation and activation of immune cells in virus-infection model (Prencipe et al., 2018), and may be a strategy to be tested in ADPKD mouse models and patients. The neutralization of TNF-α might also provide another possibility to reduce overactivation of inflammation in ADPKD (Li et al., 2008).

(3) Targeting cytokine regulators: Caspase 1 is responsible for the release of both IL-1β and IL-18. Targeting caspase 1 with its inhibitor could suppress the inflammasome activation and downstream effects, which has already been tested in Alzheimer's disease models and ischemia-associated blood-brain barrier dysfunction (Flores et al., 2020; Liang et al., 2020). In addition to the regulation of caspase 1, inhibitors targeting NLRP3, another component of inflammasome, have been evaluated in other disease associated cell models (Zahid et al., 2019).

(4) Targeting the regulatory machinery of immune response: As discussed above, epigenetic mechanisms contribute to ADPKD progression. Epigenetic regulation plays a role in renal inflammation in kidney diseases by regulating the expression of cytokines through chromatin modifications at the transcriptional levels. For example, the transcription of TNF-α and its family members is associated with several epigenetic regulators responsible for histone

TABLE 4 | Diverse types of cell death in ADPKD.

Cell death type	Basic features	Biochemical features	Morphological features	Detection methods
Apoptosis	Type1 programmed cell death Reversible	Activation of caspases, oligonucleosomal DNA fragmentation	Plasma membrane blebbing, nuclear condensation and fragmentation, apoptotic bodies	TUNEL, DNA ladder, DNA content analysis, apoptosis enzyme-linked immunoassay, annexin binding assay, LDH activity assay, mitochondrial membrane potential assay
Autophagy	Type2 programmed cell death Reversible	Increased lysosomal activity, LC3I to LC3II transformation	Formation of double-membraned autolysosomes	Western blotting or Fluorescence Microscopy of LC3 (marker protein for autophagosomes) and p62 (autophagy substrate)
Ferroptosis	Reversible	Iron and ROS accumulation, inhibition of xCT and reduced GSH.	Increased density of outer cell membrane, ruptured outer mitochondrial membrane	Iron assay kit, GSSG/GSH Quantification kit, Glutamine assay kit

Abbreviations used: ROS, reactive oxygen species; xCT, light-chain subunit of SLC7A11 (system xc− cystine/glutamate antiporter); GSH, glutathione; GSSG, oxidized glutathione; LDH, lactate dehydrogenase.

acetylation and methylation, including the CBP/p300 coactivators (Falvo et al., 2000; Granja et al., 2006). It has also been reported that stress-mediated induction of histone methyltransferase Set7, leads to promoter modification on MCP-1 through Set7-mediated H3K4 methylation (Batista and Helguero, 2018). These studies suggest that epigenetic regulation should also be involved in renal inflammation in PKD, providing an alternative strategy for the combination of immuno- and epigenetic therapy in ADPKD treatment.

In summary, both innate and adaptive immune responses participate in the pathogenesis of ADPKD through unique mechanisms. The roles of macrophages have been extensively investigated in PKD in the past decade. The roles of T cells in PKD have also been examined. So far there is not any clinical immunotherapeutic strategy for the treatment of ADPKD, all these immunotherapeutic strategies listed above are prospective immunotherapy according to the current studies in this field. (**Table 3**). Next, we may focus on the roles and mechanism of NK, NKT and γδ T cells in the regulation of PKD progression. In addition, the interaction of innate immune system and adaptive immune system should also be determined. Furthermore, the effect of immunotherapy should be assessed in pre-clinical and clinical trials. Overall, synergistical network of innate and adaptive immune cells, cytokines, immune related components, as well as the upstream regulatory factors and downstream effectors, make inflammation an important process in the pathogenesis of ADPKD. Epigenetic mechanisms contribute to the regulation of inflammatory response, and eventually regulate cell proliferation and cell death. In turn, the cytokines and other inflammatory factors regulate the expression and activity of these epigenetic factors, and regulate cell fates as well, such as proliferation and death. Thus, the integration of inflammation, epigenetic regulation and cell death should be investigated, in order to identify useful mechanisms to develop novel therapeutic strategies for the treatment of ADPKD in the future.

4 THE ROLES AND MECHANISMS OF REGULATED CELL DEATH IN ADPKD

Cell death is an essential process to maintain homeostasis in the human body and is involved in diverse physiological processes including embryonic development and elimination of harmful or unnecessary cells (Norbury and Hickson, 2001; Fuchs and Steller, 2011; Tower, 2015). There are distinct types of regulated cell death, including apoptosis, autophagy, necrosis, and the most recently identified ferroptosis (**Table 4**). Thus far, three types of cell deaths, including apoptosis, autophagy and ferroptosis have been associated with the pathogenesis of ADPKD, and accumulated evidence suggests that targeting cell death pathways may be a potential therapeutic strategy for ADPKD treatment (Capuano et al., 2022).

4.1 The Mechanisms of Regulated Cell Death

4.1.1 The Mechanism of Apoptosis

Apoptosis is the process of programmed cell death characterized by membrane shrinkage, chromatin condensation, nuclear fragmentation (pyknosis) and eventually the formation of apoptotic bodies (Elmore, 2007; Obeng, 2021). Apoptosis functions at all stages of human life, including embryonic development and aging to eliminate unwanted cells, and as a defense mechanism to remove injured cells that have been damaged beyond repair (Ke et al., 2018). There are two main pathways in apoptosis, the intrinsic and the extrinsic pathways (**Figure 3**), which are initiated and executed by two groups of caspases (cysteine-aspartic proteases), including the initiator caspases (caspases 2, 8, 9, 10) and the executioner caspases (caspases 3, 6, 7) (Zhou and Li, 2015). Caspases are a family of protease enzymes which can cleave their substrates at aspartic acid residues (Alnemri et al., 1996; Julien and Wells, 2017). The activation by initiator caspases can be induced by external death ligands or the release of cytochrome c from mitochondria, which

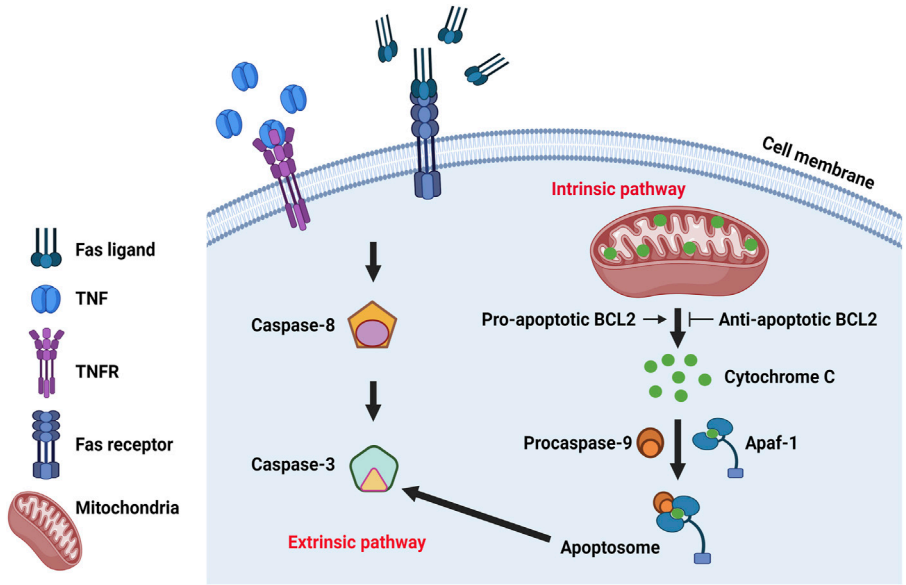

FIGURE 3 | The molecular mechanism of apoptosis. Classic regulatory pathways of apoptosis include extrinsic pathway and intrinsic pathway. Intrinsic pathway is mitochondria dependent and regulated by BCL2 protein family which can influence the release of cytochrome C from the mitochondria. Cytochrome c can bind to the cytosolic protein Apaf-1 and promote the formation of an apoptosome to recruit and activate caspase-9, which in turn activates caspase-3 and leads to cell apoptosis. Extrinsic pathway is initiated by the transmission of death signals from the cell's surface through the binding of ligands and the death receptors, resulting in the aggregation and recruitment of initiator caspases that subsequently activates executioner caspase 3, leading to apoptosis.

initiate the apoptotic signals and directly cleave and activate executioner caspases for the execution of apoptotic program (Duclos et al., 2017).

The intrinsic pathway is mitochondria-dependent, initiated when an injury occurs within the cell and mainly regulated by proteins of the BCL2 (B-cell lymphoma 2) family, which are evolutionarily conserved with shared Bcl-2 homology domains, including BH1, BH2, BH3, and BH4 (Boletta et al., 2000). BCL2 family proteins can be divided into three types: pro-apoptosis (Bcl-2, Bcl-xL, etc.), anti-apoptosis (BAX, BAK, etc.) and regulatory (BAD, BIK, BIM, etc.) members (Reed, 1998; Kale et al., 2018). Diverse types of BCL2 family contain different BH domains. Anti-apoptotic proteins usually contain BH1 and BH2 domains and pro-apoptotic proteins usually contain a BH3 domain which is essential for dimerization with other proteins of the Bcl-2 family and crucial for their killing activity. Some pro-apoptotic proteins also contain BH1 and BH2 domains (Bax and Bak). The BH3 domain may also be present in some anti-apoptotic proteins, such as Bcl-2 or Bcl-x(L) (Kale et al., 2018). The balance between pro- and anti-apoptotic Bcl-2 family members is essential to control the activity of caspases (Swanton et al., 1999; Marsden et al., 2002; Hatok and Racay, 2016; Roufayel, 2016). BCL2 family proteins are found in the mitochondrial membrane, where the pro-apoptotic proteins can promote the release of cytochrome c from mitochondria while anti-apoptotic proteins can inhibit its release (Adams and Cory, 1998; Korsmeyer, 1999; Morales-Cruz et al., 2014; Pena-Blanco and Garcia-Saez, 2018). Cytochrome c can bind to the cytosolic protein Apaf-1 and promote the formation of an apoptosome to recruit and activate caspase-9, which in turn can activate caspase-3 and leads to cell apoptosis (Bratton and Salvesen, 2010; Elena-Real et al., 2018).

The extrinsic pathway which begins outside the cell, is initiated by the transmission of death signals from the cell's surface to intracellular signaling pathways through the binding of specific death receptors to their ligands. This results in the aggregation and recruitment of initiator caspases that subsequently activates executioner caspase 3, leading to apoptosis (Elmore, 2007; Green and Llambi, 2015). Several death ligands and their corresponding death receptors have been identified, including Fas ligand and Fas receptor (FasL/FasR), tumor necrosis factor (TNF) and its receptor 1 (TNF-α/TNFR1), Apo3L/DR3, Apo2L/DR4 and Apol2L/DR5 (Walczak, 2013). TNFRs are death receptors that belong to members of the TNF receptor superfamily with shared cysteine-rich extracellular domains and a cytoplasmic death domain (Micheau and Tschopp, 2003; Dostert et al., 2019). The classical extrinsic pathway is mediated by FasL/FasR and TNF-α/TNFR1 (Nagata, 1999; Rath and Aggarwal, 1999; Yanumula and Cusick, 2022). The binding of FasL with FasR results in the recruitment of the adaptor protein FADD (Fas-associated death domain) (Wajant, 2002; Caulfield and Lathem, 2014) which then associates with procaspase-8 to form the death-inducing signaling complex (DISC), leading to the activation of procaspase-8 (Kischkel et al., 1995; Wajant, 2002). Conversely, TNF binds to TNFR resulting in the transient recruitment of TRADD (TNF-related apoptosis inducing ligand), TNF receptor-associated factor 2 (TRAF2), TRAF5, cellular inhibitor of apoptosis 1 and 2 (cIAP1/2) and receptor interacting protein 1 (RIP1) to form pro-survival complex I (Micheau and Tschopp, 2003; Shi and Sun, 2018) which can activate nuclear factor κB

(NF-κB) and JNK pathways to regulate the expression of pro-survival genes, including the cellular FLICE-like inhibitory protein (cFLIP) (Liu et al., 1996; Dhanasekaran and Reddy, 2008). With the deubiquitination of RIP1, TRADD and RIP1 become disassociated from complex I, then RIP1 associates with FADD and caspase-8 to form complex II, the so-called death complex to trigger cell death (Amin et al., 2018).

4.1.2 The Mechanism of Autophagy

Autophagic cell death is another form of programmed cell death characterized by the large-scale accumulation of vacuolated-like structures called autophagosomes (Kroemer and Levine, 2008; Garg et al., 2015). Autophagy is an important physiological process that has been associated with ADPKD pathology and is regulated by known PKD associated signaling pathways. For example, the mammalian target of rapamycin (mTOR) inhibits autophagy, while the AMP-activated protein kinase (AMPK) is known to activate autophagy.

Autophagy process includes five steps: initiation, elongation, maturation, fusion, and degradation. The activation and phosphorylation of Unc-51 like autophagy activating kinase (ULK1) protein complex, inhibited by the mTOR pathway (including mTORC1 and mTORC2) and activated by the AMPK pathway, initiates autophagy. Under normal conditions, mTORC1 inhibits the activation of the ULK1 complex by phosphorylating Ser 757 of ULK1 and interrupting the interaction between ULK1 and AMPK. Upon stimulation by cellular or environmental stresses, inhibition of mTORC1 results in the dephosphorylation of ULK1. Meanwhile, AMPK directly activates ULK1 through phosphorylation of Ser 317 and Ser 777 in ULK1 (Kim et al., 2011). Phosphorylation of ULK1 initiates the process of autophagy as described above. Activated ULK1 then phosphorylates other components of the ULK1 complex (including FIP200, ATG13 and ATG101), and recruits the PI3KC3 complex (including BECN1, Vps15, Vps34, NRBF2, AMBRA1, Atg14) to coordinate the nucleation and biogenesis of autophagosome (Meijer and Codogno, 2006; Mizushima, 2010; Lin and Hurley, 2016). ATG9 is the only transmembrane protein in the ATG protein family that functions as a membrane carrier to deliver lipids to the forming autophagosome from several cellular membranes, including the plasma membrane, mitochondria, recycling endosomes and Golgi complex (Matoba et al., 2020; Sawa-Makarska et al., 2020). The process of expansion and maturation of the autophagosome membrane involves two ubiquitin-like conjugation systems: the conjugation of ATG12 to ATG5, and the conversion of LC3 I to LC3 II (Mizushima, 2020). The conjugation of Atg12 to Atg5 occurs at Lys130 through the activation of E1 enzyme Atg7 and the E2-like Atg10 (Mizushima et al., 1998; Otomo et al., 2013). The Atg12-Atg5 conjugate then forms a large protein complex with Atg16, acts as the E3 ligase for the conjugation of LC3 I to PE (phosphatidylethanolamine). Atg4 cleaves the C-terminal arginine of pro-LC3 to form LC3 I. The conversion of LC3 I to LC3 II is also a ubiquitin-like conjugation reaction. E1, E2, and E3-like enzymes are Atg7, Atg3, and Atg12-Atg5-Atg16 (Tanida et al., 2004). LC3 II is a characteristic marker of autophagic membranes and can recruit selective cargo to the autophagosome via its interaction with cargo receptors (Kabeya et al., 2000; Tanida et al., 2004). The maturation of the autophagosome leads to the autophagosome-lysosome fusion. The lysosome is a double-membrane cell organelle that contains digestive enzymes. The enzymes contained in the lysosome ensure the degradation of the cargo and cargo receptors which are recycled to be used again during cellular metabolism (Kriegenburg et al., 2018).

4.1.3 The Mechanism of Ferroptosis

Ferroptosis is an iron dependent form of cell death which can be induced by small molecules such as erastin and Ras-selective lethal small molecule 3 (RSL3) (Dolma et al., 2003; Yang and Stockwell, 2008), and can be inhibited by specific inhibitors such as ferrostatin-1 (Fer-1), liproxstatin-1 and vitamin E. In addition, iron chelators and lipophilic antioxidants can prevent ferroptosis (Xie et al., 2016). The failure of glutathione-dependent antioxidant defenses initiates ferroptosis, leading to uncontrolled lipid peroxidation and eventually cell death (Li et al., 2020a; Zhang and Li, 2022). Ferroptosis is regulated by Glutathione/GPX4 (glutathione peroxidase 4) signaling pathways, iron metabolic signaling pathway and lipid metabolic signaling pathway as indicated in **Figure 4** (**Figure 4**) (Li et al., 2020a). GPX4 is a selenoenzyme which can convert GSH (glutathione) into oxidized glutathione (GSSG) and reduces membrane cytotoxic lipid hydroperoxides to maintain cellular redox homeostasis and prevent the iron (Fe^{2+})-dependent formation of toxic lipid reactive oxygen species (ROS) (Seiler et al., 2008). Inhibition of GPX4 can lead to the accumulation of lipid peroxides and induction of ferroptosis (Yang and Stockwell, 2008; Yang et al., 2014).

Iron is one of the most abundant transition metals and is an essential element for many living organisms. Because iron-dependent oxidative damage is characteristic for ferroptosis, iron metabolism is controlled by ferroptosis in many aspects. The metabolism of iron includes iron uptake (transferrin receptor), iron export (ferroportin), iron storing (ferritin), and ferritinophagy, selective autophagy of ferritin mediated by lysosome and NCOA4 (nuclear receptor coactivator 4). Excess heme and non-heme iron can directly induce ferroptosis (Li Q. et al., 2017), and both heme and non-heme iron-containing enzymes, such as ALOXs, NOXs, and CYP can promote lipid peroxidation in ferroptosis. During ferroptosis, a phenomenon in which increased labile iron is released by the cell is referred to as ferritinophagy. NCOA4 is a selective cargo receptor which functions in ferritinophagy. The genetic inhibition of NCOA4 inhibited ferritin degradation and suppressed ferroptosis. In contrast, overexpression of NCOA4 increased ferritin degradation and promoted ferroptosis (Hou et al., 2016). Iron chelators are drugs that can remove extra iron from the body, blocking ferroptotic cell death both *in vitro* and *in vivo*. Ferritin is regulated by ATG5-ATG7 and NCOA4 pathways, as well as IREB2 (Iron Responsive Element Binding Protein 2). In addition, p62-Keap1-NRF2 and HSPB1 (Heat Shock Protein Family B Member 1) signaling pathways can also regulate iron metabolism.

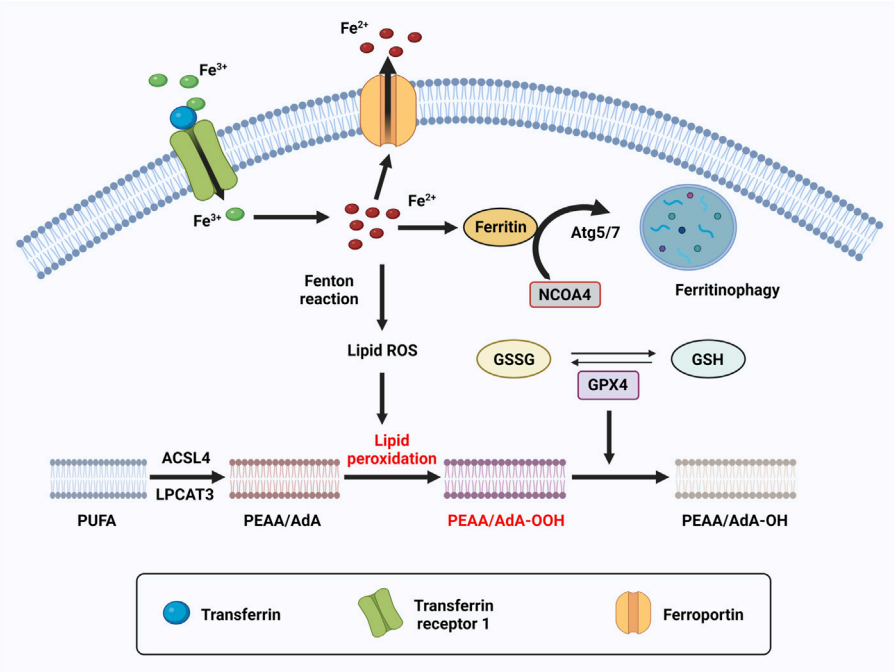

FIGURE 4 | The molecular mechanism of ferroptosis. Ferroptosis is an iron dependent form of cell death. The metabolism of iron includes iron uptake (transferrin receptor), iron export (ferroportin), iron storing (ferritin), and ferritinophagy. Ferritinophagy is a selective autophagy of ferritin which mediated by Atg5/7 and NCOA4. Excess can directly induce ferroptosis through lipid peroxidation. GPX4, converts PEAA/AdA-OOH to PEAA/AdA-OH and inhibits ferroptosis. This reaction occurs through the use of glutathione (GSH) as a substrate.

Lipid peroxidation is a process of oxidative lipid degradation that eventually leads to ferroptosis. During this process, ROS such as oxygen free radicals attack lipids, especially polyunsaturated fatty acids (PUFAs). PUFAs are preferentially incorporated into phospholipids such as phosphatidylethanolamines (PEs) and transformed into PUFA-PEs by two enzymes, ACSL4 (Acyl-CoA synthetase long-chain family member 4) for synthesizing PEs and LPCAT3 (lysophosphatidylcholine acyltransferase 3) for lipid remodeling. Therefore, blocking the expression of ACSL4 and LPCAT3 results in the suppression of esterification of PEs reducing the accumulation of lipid peroxide substrates in cells, thus inhibiting ferroptosis (Kagan et al., 2017).

4.2 The Roles of Regulated Cell Death in ADPKD

4.2.1 Apoptosis in ADPKD

Aberrant apoptosis and apoptotic pathways were first detected in human ADPKD, the congenital mouse model of ARPKD (*cpk*), and the *pcy* mice (Woo, 1995). In the past decades, an increasing number of studies have demonstrated that apoptosis plays an essential role in the regulation of cystogenesis in ADPKD. However, whether apoptosis is increased or decreased in ADPKD kidneys, and whether induction of apoptotic cell death promotes, or delays cyst growth remains controversial.

First, studies found that apoptosis is elevated in rodent ADPKD models. In the *Han:SPRD-Cy (Cy)* rat model which closely resembles ADPKD, apoptosis is increased and correlates with the upregulation of caspase protein levels and activity and the downregulation of anti-apoptotic Bcl2 family proteins (Ecder et al., 2002; Tao et al., 2005b). In *cpk* mice, excessive apoptosis occurs in the interstitium while seldom evident in the cystic epithelium or noncystic tubules. The expression of various caspases, including bax and bcl-2, are also upregulated indicating that apoptotic cell death contributes to cyst formation (Ali et al., 2000). In Madin-Darby canine kidney (MDCK) cells, apoptosis was increased and overexpression of PC1 displayed resistance to thrombin/Gα12-stimulated apoptosis while PC1-silenced MDCK cells displayed enhanced thrombin-induced apoptosis (Boletta et al., 2000). In *Pkd1* mutant mice, *Pkd1* deletion in renal stromal cells by Foxd1-driven Cre showed epithelial changes and progressive cystogenesis accompanied by excessive apoptosis and proliferation (Nie and Arend, 2017). *Pkd1* conditional knockout mice also showed increased expression levels of pro-apoptotic markers and downregulation of the anti-apoptotic marker (Rogers et al., 2016). In *Pkd1*-deleted MEFs, enhanced cytochrome c release and increased apoptosis were detected which caused increased sensitivity to ROS (Zhang et al., 2015). In *Pkd2* mutant models, haploinsufficiency and loss of heterozygosity at the *Pkd2* locus results in both increased proliferation and apoptosis (Belibi et al., 2004; Wilson and Goilav, 2007; Kim et al., 2009; Wegierski et al., 2009). In addition, *Pkd2*-overexpressing transgenic mice showed typical renal cyst growth with an increase in both proliferation and apoptosis (Park et al., 2009; Zhang et al., 2015). Besides, in $Pkd1^{+/-}$ pigs, both intrinsic and extrinsic apoptosis increased at ages of 1 month and

3 months. This provides more evidence for the correlation of apoptosis in ADPKD among different mammalian species (Wang et al., 2021).

Second, different studies have either reported no significant change or decreased apoptosis in rodent ADPKD models. In *Pkd1*$^{flox/flox}$*:Ksp-Cre* mice, increased proliferation which correlated with increased cystic kidney, was observed, however, no significant changes in apoptosis were reported (Shibazaki et al., 2008). Meanwhile, apoptotic cells were rarely detected in kidneys of *Pkd1* knockdown mice and in kidneys from *Pkd1*$^{flox/flox}$*:Ksp-Cre* neonates. In proximal tubule derived cell lines lacking PC1 which spontaneously formed cysts, the overall number of apoptotic nuclei in kidney tissues was very low and did not differ significantly between cystic and normal kidneys (Wei et al., 2008). In the kidneys from *Pkd1* conditional knockout and *Pkd1* hypomorphic *Pkd1*$^{nl/nl}$ mice, apoptotic cells were rarely detected (Fan et al., 2013a). In *Pkd1*$^{-/-}$ E15.5 MEKs (mouse embryonic kidneys), apoptosis was rare and negligible as detected by TUNEL assay (Zhou et al., 2013). In *Pkd1*$^{fl/fl}$*:Smyd2*$^{+/+}$*:Ksp-Cre* neonates, apoptosis was rare in kidneys and knockout of Smyd2 induced cyst-lining epithelial cell death in kidneys from *Pkd1*$^{fl/fl}$*:Smyd2*$^{fl/fl}$*:Ksp-Cre* neonates (Li et al., 2017b). The controversial role for apoptosis in PKD is due to confounding factors including: 1) the use of variable animal models versus human PKD, 2) comparison at various stages of the disease, early versus late, 3) comparison between cyst lining epithelium versus normal appearing tubules. It is evident that both cell apoptosis and proliferation are dysregulated in ADPKD, and both may contribute to the general mechanism for cyst growth. Moreover, PKD kidneys are disproportionately enlarged. If apoptosis were therefore a predominant factor in the regulation of cyst growth, one would expect the kidneys to eventually involute. This therefore suggests that apoptosis is not the primary factor of cystogenesis (Zhou and Li, 2015).

4.2.2 Autophagy in ADPKD

Like the role of apoptosis in ADPKD, the role of autophagy in ADPKD is also controversial. The disruption of autophagy has been identified in diverse ADPKD animal models. However, both increased and decreased autophagy are reported. In *cpk* mice and *Han:SPRD* rats, autophagy is increased as autophagosomes were found by electron microscopy in the tubular cells lining the cysts, and enhanced autophagy components LC3-II and beclin-1 were also observed (Belibi et al., 2011). In PC1 deficient cells, basal autophagy was enhanced (Decuypere et al., 2021). However, in *Pkd1* mutant mice, the expression level of autophagy genes was decreased although LC3-II protein level showed no change (Belibi et al., 2011; Chou et al., 2018). mTOR pathway is hyperactivated and suppressed autophagic flux is detected in the heart and kidneys of *Pkd1* mutant mouse models (Atwood et al., 2020a; Atwood et al., 2020b). In the *Pkd1* mutant zebrafish model, the mTOR pathway is abnormally activated and autophagy is inhibited (Zhu et al., 2017). In *Pkd1*$^{flox/-}$*:Ksp-Cre* mice, autophagy-related protein ULK1 and the ratio of LC3 II/LC3 I decreased, indicating autophagy is inhibited (Liu et al., 2020). In *Pkd1 transgenic* mice, the expression levels of autophagy-related genes, including Atg5, Atg12, Ulk1, Beclin-1, and Sqstm1 (p62) were down-regulated. However, the p62 protein level in cystic lining cells was increased, indicating impaired degradation of the protein by the autophagy-lysosome pathway (Chou et al., 2018). In human ADPKD patient samples, autophagy did not change though LC3 was highly increased in cystic ADPKD patient kidneys. Meanwhile, the enhanced LC3 expression in PKD mouse models enlarged renal cysts and enhanced renal failure (Lee et al., 2020).

Dysregulation of the autophagy pathway has been associated with disease progression in ADPKD, however, the mechanisms involved in the PC1- and PC2-mediated regulation of autophagy remains unclear. Treatment with hyperosmolar concentrations of sorbitol or mannitol induces PC2 dependent autophagy and downregulation of PC2 prevents inhibition of hyperosmotic stress-induced mTOR pathway activation in HeLa and HCT116 cell lines (Pena-Oyarzun et al., 2017). PC2 can bind to BECN1, a component of PI3KC3 complex and is essential to initiate autophagy, through CC1 domain, which is in the carboxy-terminal tail of PC2. Note that the PC2-BECN1 complex is required for the induction of autophagy (Pena-Oyarzun et al., 2021). In *Pkd2* knockout mice, the activation of autophagic flux was suppressed and overexpression of PC2 could increase autophagic flux (Criollo et al., 2018). While PC2 seems to be essential for stress-induced autophagy, PC1 also functions in the process of autophagy. Dysfunction of PC1 and/or PC2 in cell lines lead to reduced intracellular Ca^{2+} signaling, increased mTOR activity, increased cAMP levels and increased proliferation (Seeger-Nukpezah et al., 2015). PKD mutant cells can be more resistant against nutrient stress and delay cell death by maintaining autophagy modulated through PC1 in a PC2-dependent manner. In *PC1* knockout mice, starvation-induced autophagic response is enhanced. Although knockdown of PC2 in *PC1* knockout cells did not significantly alter autophagy level, *PC2* knockout cells showed reduced protein level of PC1 and the PC1 band seemed to be at a lower molecular weight, suggesting that the stability of PC1 protein during starvation is dependent on PC2 expression (Decuypere et al., 2021).

4.2.3 Ferroptosis in ADPKD

The dysregulation of ferroptosis was detected in animal models of ADPKD and human ADPKD. The expression of antioxidant enzymes GPX and SOD and their activity were decreased in *cpk* mice and *Han:SPRD* rats (Maser et al., 2002) while lipid peroxidation is increased in human ADPKD patients (Schreiber et al., 2019), suggesting that ferroptosis plays an important role in ADPKD. Lipid peroxidation correlated with cyst growth and the activation of TMEM16A (anoctamin 1), a chloride bicarbonate transmembrane channel, inducing growth of renal cysts by lipid peroxidation while the direct inhibition of TMEM16A or inhibition of lipid peroxidation delayed cyst development in *Pkd1* mutant mice (Schreiber et al., 2019). Treatment with two potent inhibitors of TMEM16A, niclosamide and benzbromarone, significantly reduced renal cyst size in *Pkd1*$^{-/-}$ mice compared to control mice (Cabrita et al., 2020). Recently, we reported the decreased expression of the negative regulator of ferroptosis GPX4, and the increased expression of iron importers TfR1 and DMT1 in *Pkd1* mutant

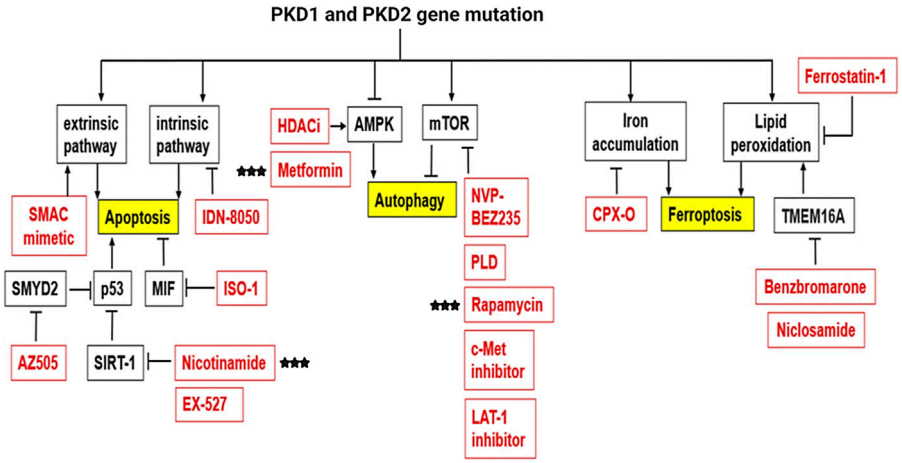

FIGURE 5 | Cell death pathway and the drug targets in ADPKD. In ADPKD, mutation of PKD1/PKD2 causes dysregulation of cell death, including apoptosis, autophagy and ferroptosis. Apoptosis is regulated through extrinsic pathway and intrinsic pathway. Treatment with SMAC mimetic activates apoptosis through an extrinsic dependent pathway to delay cyst growth, while treatment with pan-caspases inhibitor IDN-8050 inhibits apoptosis through an intrinsic pathway which also reduces cyst size. Treatment with SIRT-1 inhibitors, including nicotinamide and EX-527, and Smyd2 inhibitor, AZ505, as well as MIF inhibitor, ISO-1, induced apoptosis in a p53 dependent manner and delay cyst growth. Autophagy is regulated through PKD associated AMPK and mTOR pathways. Treatment with inhibitors of mTOR pathway, including NVP-BEZ236, PLD and rapamycin as well as c-Met and LAT-1 inhibitors, delays cyst growth, and treatment with AMPK inducers, metformin and HDACi, also slows cyst growth. Ferroptosis is an iron dependent cell death characterized with the increase of lipid peroxidation. Treatment with CPX-O, a chelator of iron, inhibited cystogenesis. Treatment with ferroptosis inhibitor, Ferrostatin-1, delays cyst growth and cystic cell proliferation. TMEM16A can induce lipid peroxidation, and treatment with TMEM16A inhibitor, niclosamide and benzbromarone, significantly reduced renal cyst size in ADPKD animals (Three black stars indicate drugs in clinical trials).

mouse models. This alteration in expression levels resulted in high iron levels, low GSH and GPX4 activity, increased lipid peroxidation, and a propensity for ferroptosis. 4HNE (4-hydroxynonnenal), a lipid peroxidation product, is also increased in *Pkd1* null cells and is reportedly responsible for the promotion of proliferation of *Pkd1* mutant cells through the activation of Akt, S6, Stat3, and Rb (Zhang and Li, 2022). These findings indicated that ferroptosis is highly associated with ADPKD progression.

4.3 Therapeutic Regulation and Implication of Regulated Cell Death in ADPKD

4.3.1 Apoptosis-Based Therapeutic Strategies

In ADPKD, apoptosis may function as a double-edged sword. While apoptosis occurs in the normal-appearing, non-cystic tubules, loss of renal tissue may result in the functional progressive deterioration of kidney (Woo, 1995), increased apoptosis in ADPKD may inhibit hyperproliferation thus prevent the progression of PKD.

Targeting apoptosis to attenuate cyst growth is quite complex (**Figure 5**). First, the increase in apoptosis in ADPKD suggests that inhibition of apoptosis should delay cyst progression. Short-term treatment with the pan-caspase inhibitor IDN-8050 resulted in the inhibition of caspase-3 and caspase-7 activity in kidneys of *Cy/Cy* rats (Tao et al., 2005b) and decrease of apoptosis. This inhibited renal enlargement and cystogenesis and attenuated the loss of kidney function (Tao et al., 2005a). Deletion of caspase-3 resulted in the increased caspase-7 and decreased anti-apoptotic protein Bcl-2 levels, and prolonged the survival of *cpk* mice (Tao et al., 2008). In *Han:SPRD* rats, treatment with 2-hydroxyestradiol (2-OHE) reduced apoptosis and resulted in decreased cyst growth and preservation of kidney (Anderson et al., 2012). Furthermore, in two ARPKD mouse models (*jck* juvenile cystic kidney mice and *cpk* mice), treatment with cyclin-dependent kinase (CDK) inhibitor roscovitine resulted in long-lasting arrest of cystogenesis and decreased apoptosis. The decreased expression of pro-apoptotic protein Apaf1 and caspase-2, and increased expression of Bcl-2 and Bcl-xL were also detected. The may be caused by the inhibition of Cdk5, which is responsible for anti-apoptotic effects in neurodegenerative diseases, thereby resulting in effective reduction of apoptosis in cystic kidneys (Di Giovanni et al., 2005; Bukanov et al., 2006). The Glc-Cer synthase inhibitor, Genz-123346, also slowed cyst growth by decreasing the proliferation and apoptosis in *jck* mice (Natoli et al., 2010).

Second, there is evidence to support that induction of apoptosis contributes to delayed cyst growth in ADPKD. As described above, TNF-α is an apoptosis ligand and accumulates at elevated levels in ADPKD cyst fluid (Li et al., 2008). While treatment with TNF-α alone did not induce apoptosis in either *Pkd1* wild-type or null MEK cells even at high concentration (due to the activation of NF-κB which inhibited the activity of caspase 8) (Fan et al., 2013a), treatment with TNF-α combined with the SMAC (second mitochondria-derived activator of caspases) mimetic induced apoptosis in primary cultures of mural epithelial cells from human ADPKD cysts and *Pkd1* null MEK cells, suggesting that SMAC-mimetic induces TNF-α–dependent cell death (Fan et al., 2013a). Importantly, treatment with SMAC-mimetic reduced cyst growth in *Pkd1* mutant mice (Fan et al.,

2013a). After this fundamental study, subsequent studies provided more evidence to support a beneficial role for the induction of cystic renal epithelial cell apoptosis. Treatment with SMAC-mimetic also showed cyst reduction in a drosophila model of ADPKD (Millet-Boureima et al., 2019). In *Pkd1* mutant model, the deacetylase sirtuin 1 (SIRT1) is upregulated and treatment with pan-sirtuin inhibitor (nicotinamide) or a SIRT1-specific inhibitor (EX-527) delayed cyst growth through the deacetylation of p53, which released its inhibition on apoptosis in *Pkd1* mutant mice (Zhou et al., 2013). A randomized double blinded small clinic trial with the enrollment of 36 patients was conducted to determine the effect of nicotinamide in ADPKD patients. Although nicotinamide is safe and well-tolerated in ADPKD patients, there was no beneficial effect in the inhibitor treated group compared to placebo treated group (El Ters et al., 2020). SMYD2, a lysine methyltransferase, also upregulated in *Pkd1* mutant renal epithelial cells and kidneys, was shown to inhibit p53-dependent cystic renal epithelial cell apoptosis through the methylation of p53. Treatment with specific SMYD2 inhibitor AZ505 delayed cyst growth in *Pkd1* mutant kidneys by increasing p53 mediated apoptosis (Li et al., 2017b). Macrophage migration inhibitory factor (MIF) reported to be upregulated in cyst-lining epithelial cells of *Pkd1* mutant mouse kidneys, accumulates in cyst fluid of human ADPKD kidneys. Treatment with ISO-1, a MIF inhibitor, efficiently slowed cyst growth, accompanied with an increase in apoptosis (Chen et al., 2015). Genetic ablation of apoptosis regulatory genes also contributes to attenuation of cyst growth in ADPKD. Homozygous deletion of ILK (integrin-linked kinase) gene, whose product is a scaffold protein associated with multiple cellular functions including cell proliferation, resulted in increased caspase-3-mediated apoptosis and reduced cyst size of $Pkd1^{fl/fl}$;*Pkhd1-Cre* mice (Raman et al., 2017). In *cpk* mice, haploinsufficiency of Pax2 attenuated progressing cyst growth via increased p53 mediated apoptosis. *Pax2* is known to suppress p53 transcription by binding to cis-acting regulatory sequences (Stuart et al., 1995). Apoptosis in *Pax2* heterozygous kidneys of *cpk* mice may be regulated in part by increased levels of p53 (Ostrom et al., 2000). MicroRNAs (miRNAs are short noncoding RNAs that acts as sequence-specific inhibitors of gene expression (O'Brien et al., 2018 #95). miR-21 is upregulated in renal cysts through cAMP signaling pathway and represses proapoptotic genes to inhibit cystic cell apoptosis and promotes PKD progression. Deletion of miR-21 showed attenuation of cyst growth and induction of apoptosis in kidneys from an orthologous model of ADPKD (Lakhia et al., 2016). These studies indicated a beneficial role for the induction of apoptosis in ADPKD treatment.

4.3.2 Autophagy-Based Therapeutic Strategies

Direct targeting of dysfunctional autophagy in PKD animal models has been determined effective in slowing cyst growth. In *Pkd1* mutant zebrafish, knocking down the core autophagy protein Atg5 caused inhibition of autophagy and promoted cystogenesis, while treatment with a specific inducer of Beclin-1 peptide activated autophagy and also slowed cyst growth (Zhu et al., 2017). However, oral supplement with trehalose, a natural autophagy enhancer, showed no effect on the progression of ADPKD in *Pkd1* miRNA transgenic mice and could not restore impaired autophagy, suggesting that an oral supplement of trehalose may not affect the progression of ADPKD (Chou et al., 2018).

mTOR pathway plays a significant role in the regulation of autophagy and targeting mTOR pathway has been indicated effective for ADPKD (Zafar et al., 2010; Li et al., 2017a). Rapamycin has been used as an autophagy inducer that functions through inhibition of mTOR pathway. The effect of rapamycin in suppressing the aberrant epithelial proliferation of ADPKD kidney has been identified in rodent ADPKD animal models. Moreover, rapamycin was used in human studies, and while there was a reduction in TKV (total kidney volume) in rapamycin treated groups compared to placebo group, the decrease in TKV was not significant (Kim and Edelstein, 2012; Braun et al., 2014). Phospholipase D (PLD) is an enzyme of the phospholipase superfamily and its product phosphatidic acid (PA) regulate mTOR activity. The activity of PLD was elevated in PKD cells and targeting PLD with small molecule inhibitors could reduce cell proliferation and enhance the sensitivity of PKD cells to rapamycin which indicated that combining PLD inhibitors and rapamycin synergistically inhibited PKD cell proliferation (Liu et al., 2013). Two large randomized clinical trials with rapamycin in ADPKD were undertaken. However, the effect of rapamycin were unimpressive and the treatment presented an increased side-effect profile, which might be associated with the short-term administration of rapamycin and the lack of randomization (Kim and Edelstein, 2012). Other drugs targeting mTOR pathway such as NVP-BEZ235, a mTOR inhibitor, also inhibited proliferation and normalized kidney morphology in ADPKD (Liu et al., 2018). Defective ubiquitination of c-Met caused hyperactivation of mTOR in PKD and treatment with a c-Met inhibitor resulted in the inhibition of mTOR activity and blocked cystogenesis in Pkd1-null mice model of ADPKD (Qin et al., 2010). Branched-chain amino acids (BCAAs), including leucine, is an activator of mTOR pathway. Treatment with BCAA in *Pkd1* conditional knockout mice accelerated disease progression and upregulation of mTOR pathway. L-type amino acid transporter 1 (LAT-1) is a transporter of neutral amino acids, including BCAAs. Treatment with LAT-1 inhibitor reduces mTOR signaling and attenuation of cyst growth indicated that LAT-1 inhibitor is a potential therapeutic agent for ADPKD (Yamamoto et al., 2017).

Targeting AMPK pathway is also involved in the treatment of ADPKD. Metformin is an AMPK inducer and treatment with metformin slowed cyst formation in both *in vitro* and *ex vivo* models of renal cystogenesis (Takiar et al., 2011; Chang et al., 2017). In a clinical trial, it was also determined that treatment with metformin improved disease progression. In this study, it was found that metformin effects on ADPKD progression are mediated through the activation of the AMPK pathway (Seliger et al., 2018). In addition, treatment with inhibitors of histone deacetylases (HDACi) delayed cyst growth in $Pkd1^{-/-}$ models, partially by activating AMPK pathway (Sun et al., 2019). Furthermore, inhibition of miR-25-3p enhanced autophagy by

increasing ULK1 expression and the ratio of LC3 II/LC3 I in kidneys from *Pkd1*$^{flox/-}$;*Ksp-Cre* mice (Liu et al., 2020). These studies further support that targeting specific autophagy regulators may attenuate cyst growth, as such, they provided novel targets for the treatment of ADPKD (**Figure 5**).

4.3.3 Ferroptosis-Based Therapeutic Strategies

Lipid peroxidation correlated with cyst growth and the activation of TMEM16A (anoctamin 1), a chloride bicarbonate transmembrane channel, inducing growth of renal cysts by lipid peroxidation while the direct inhibition of TMEM16A or inhibition of lipid peroxidation delayed cyst development in *Pkd1* mutant mice (Schreiber et al., 2019). Treatment with two potent inhibitors of TMEM16A, niclosamide and benzbromarone, significantly reduced renal cyst size in *Pkd1*$^{-/-}$ mice compared to control mice (Cabrita et al., 2020). Ferrostatin-1 is a synthetic antioxidant and also works as a lipid peroxidation inhibitor but much more efficiently. Treatment with Ferrostatin-1 inhibits ferroptosis and the proliferation of *Pkd1* mutant renal epithelial cells and kidneys (Zhang et al., 2021c). Ciclopirox (CPX) or its olamine salt (CPX-O) inhibits the activity of iron-dependent enzymes through chelation of iron. Treatment with CPX-O in PKD mice inhibited cystogenesis as seen by the decrease of cyst index and cystic cell proliferation, and the improvement of renal function, suggesting that CPX-O may delay cyst growth through affecting ferroptosis process in *Pkd1* mutant mice (Radadiya et al., 2021). In conclusion, targeting ferroptosis may be a new strategy for ADPKD treatment but still need clinical trials to identify (**Figure 5**).

In summary, the regulated cell death, including apoptosis, autophagy and ferroptosis, has been investigated in ADPKD, and there is evidence to suggest their involvement in cyst progression (**Figure 5**). In-spite of these studies, the role of regulated cell death in regulating cystogenesis in ADPKD remains unclear. Moreover, the controversial reports add to the complexity of programmed cell death in general, and how it associates to ADPKD. A major unresolved concern in the field is whether induction of regulated cell death promotes or delays cyst growth. Thus far, the answer to this question is based on two confounding factors including: 1) the type of cell death induced, and 2) the stage of the disease. While targeting regulated cell death appears to be an attractive approach for ADPKD therapy, we believe that further investigation is required to help identify more specific targets for the development of more precise therapy for ADPKD.

5 CONCLUSION AND PERSPECTIVE

In the last decade, an understanding of common mechanisms involved in ADPKD disease, including epigenetic modifications, inflammation, and regulated cell death, have pointed to alternative approaches for potential therapeutic intervention, with promising results emerging from multiple pre-clinical studies. The elucidation of these different mechanisms has uncovered key concerns regarding disease progression. First, the disease model and experimental techniques used, and second, the stage of the disease during which samples are collected, determine the conclusions drawn and how the data obtained is extrapolated for therapeutic purposes. Third, there exits feedback mechanisms and cross-talks between dysregulated pathways in ADPKD which create a more complex network. For example, epigenetic modifications contribute to renal damage by regulating multiple cellular processes and signaling pathways including inflammation and cell death pathways, while inflammation and programmed cell death can contribute to the renal damage progression by inducing epigenetic modifications. Therefore, efforts towards creating a comprehensive picture of the epigenetic landscape and understanding how epigenetic mechanisms affect inflammation and regulated cell death pathways should lay out the foundation for future drug development against ADPKD.

The treatment of ADPKD is a long-term effort, owing to the progressive nature of the disease. Therefore, it is important and necessary to find more selective and less toxic drugs that will be effective for long-term treatment. As such, a better understanding of the mechanisms involved in ADPKD disease progression is essential. To achieve this goal, we should develop and apply the techniques of single cell epigenomics, including single-cell DNA methylome sequencing, single-cell assay for transposase-accessible chromatin with sequencing (scATAC-seq), single-cell ChIP-sequencing and single-cell Hi-C, in our future research to better understand how epigenetic mechanisms are involved in ADPKD progression. In addition, CRISPR–Cas9 screens may be applied to identify transcriptional or epigenetic factors that modulate the immune sensitivity of cystic cells or immune activity of immune cells. A better understanding of immunosuppression mechanisms in cystic kidneys should also facilitate the identification of novel immunotherapeutic targets and the development of an immunotherapeutic strategy for ADPKD treatment. Furthermore, to establish a connection of epigenetic mechanisms and renal inflammation with regulated cell death in ADPKD and to investigate the roles of other types of regulated cell death, such as pyroptosis (a highly inflammatory cell death usually caused by microbial infection), necroptosis (a regulated inflammatory mode of cell death that mimics features of both apoptotic and necrotic cell death) and oxeiptosis (a ROS-induced caspase-independent apoptosis-like cell death) in ADPKD should facilitate the identification of more specific targets for a more precise therapy for ADPKD. We believe that an effective and long-term treatment of ADPKD can be achieved by simultaneously or serially targeting different signaling pathways.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Shared pathobiology identifies AMPK as a therapeutic target for obesity and autosomal dominant polycystic kidney disease

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Autosomal dominant polycystic kidney disease (ADPKD) is the most common Mendelian kidney disease, affecting approximately one in 1,000 births and accounting for 5% of end-stage kidney disease in developed countries. The pathophysiology of ADPKD is strongly linked to metabolic dysregulation, which may be secondary to defective polycystin function. Overweight and obesity are highly prevalent in patients with ADPKD and constitute an independent risk factor for progression. Recent studies have highlighted reduced AMP-activated protein kinase (AMPK) activity, increased mammalian target of rapamycin (mTOR) signaling, and mitochondrial dysfunction as shared pathobiology between ADPKD and overweight/obesity. Notably, mTOR and AMPK are two diametrically opposed sensors of energy metabolism that regulate cell growth and proliferation. However, treatment with the current generation of mTOR inhibitors is poorly tolerated due to their toxicity, making clinical translation difficult. By contrast, multiple preclinical and clinical studies have shown that pharmacological activation of AMPK provides a promising approach to treat ADPKD. In this narrative review, we summarize the pleiotropic functions of AMPK as a regulator of cellular proliferation, macromolecule metabolism, and mitochondrial biogenesis, and discuss the potential for pharmacological activation of AMPK to treat ADPKD and obesity-related kidney disease.

KEYWORDS

autosomal dominant polycystic kidney disease, energy metabolism, obesity, metabolic dysregulation, AMPK

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common Mendelian kidney disease (lifetime prevalence of at least 1:1,000 births), accounting for almost 5% of all end-stage kidney disease (ESKD) in developed countries (Harris and Torres, 2009; Lanktree et al., 2018). Mutations in *PKD1* (encoding polycystin 1 or PC1) and *PKD2* (encoding polycystin 2 or PC2) account for 70%–85% and 15%–30% of genetically resolved cases, respectively (Rossetti et al., 2007; Barua et al., 2009; Cornec-Le Gall et al., 2013; Heyer et al., 2016; Hwang et al., 2016). ADPKD is characterized by the slow expansion of innumerable cysts in the kidneys with inflammation and fibrosis as defining features associated with advanced kidney failure in a high proportion of affected individuals (Grantham, 2008; Harris and Torres, 2009). Currently, only tolvaptan, an antagonist of vasopressin V2 receptors is approved to treat ADPKD (Irazabal et al., 2011; Torres et al., 2012; Torres et al., 2017). However, tolvaptan is an expensive drug with serious side effects such as significant polyuria, nocturia, and potential liver toxicity (Irazabal et al., 2011). Hence, there is a pressing need for novel interventions that are safe and effective for patients with ADPKD.

The kidneys are among the most metabolically active organs in the body: although they only represent 0.5% of the human body mass, they consume 10% of the body's oxygen (Nowak and Hopp, 2020). Consequently, the modulation of key metabolic pathways has generated much interest in the field of ADPKD. The pathophysiology of ADPKD was shown to be strongly linked to metabolic dysregulation, which may occur secondary to defective polycystin function (Distefano et al., 2009; Zheng et al., 2009). Metabolic reprogramming, mediated through novel or repurposed drugs, or through dietary changes (e.g., caloric restriction), holds the promise of improving the course of cystic disease (Nowak and Hopp, 2020; Menezes and Germino, 2019; Podrini et al., 2020; Haumann et al., 2020; Pickel et al., 1093; Warner et al., 2016). Recently, overweight and obesity were demonstrated to be independent predictors of ADPKD progression (Nowak et al., 2018; Nowak et al., 2021). This suggests that weight loss could provide a strategy for controlling cyst growth. In this narrative review, we highlight the shared pathobiology between ADPKD and overweight/obesity. In particular, we focus on the interaction between mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) signaling, and their biological functions in the kidneys. mTOR and AMPK are two diametrically opposed sensors of energy metabolism that play a key role in regulating cell growth and proliferation (Steinberg and Kemp, 2009). We summarize the current knowledge on the function of AMPK as a regulator of cellular energy metabolism and modulator of tissue inflammation, and the preclinical and clinical evidence supporting pharmacological activation of AMPK to treat ADPKD and improve obesity-related kidney disease.

Obesity is clinically associated with more severe autosomal dominant polycystic kidney disease

The World Health Organization (WHO) defines overweight and obesity in adults as a body mass index (BMI) ≥25 kg/m^2 and ≥30 kg/m^2, respectively. The global prevalence of obesity in adults was 13% in 2016 (15% of women and 11% of men), a threefold increase since 1975 (https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight, updated 9 June 2021). Worryingly, between 1975 and 2016, the global prevalence of obesity in children and adolescents also increased from 0.7 to 5.6% in girls and from 0.9 to 7.8% in boys (Abarca-Gómez et al., 2017). The rate of increase in BMI since 2000 has slowed down in high-income countries, but has accelerated in some regions of the developing world (NCD Risk Factor Collaboration, 2016). Epidemiologic studies have highlighted high BMI as a risk factor for numerous chronic conditions, including cardiovascular disease, diabetes mellitus, chronic kidney disease (CKD), cancer, and musculoskeletal disorders (The GBD 2015 Obesity Collaborators Ashkan Afshin et al., 2017). Obesity results from energy intake exceeding energy expenditure, which leads to adipose tissue expansion (Rosen and Spiegelman, 2006). Contributors to this energy imbalance are numerous, including genetic, epigenetic, physiological, environmental, sociocultural, and behavioral factors (Fall et al., 2017; Gadde et al., 2018).

Although it was noted almost 20 years ago that the number of patients with ADPKD and concomitant obesity has been increasing, only recently has the impact of high BMI on ADPKD been explored (Schrier et al., 2003). Nowak et al. (2018) observed that, in 441 non-diabetic subjects with early-stage ADPKD, more than half of the cohort was overweight or obese. Importantly, BMI was independently associated with a greater annual percent change in total kidney volume (TKV). Obesity, compared to normal weight, was also independently associated with a greater decline in eGFR (Nowak et al., 2018). A later study confirmed some of these observations using patients at high risk of rapid progression from the Tolvaptan Efficacy and Safety in Management of Autosomal Dominant Polycystic Kidney Disease and Its Outcomes (TEMPO) 3:4 trial (Nowak et al., 2021).

Obesity commonly co-occurs with the metabolic syndrome. The latter is characterized by a cluster of cardiometabolic risk factors including abdominal obesity, high blood pressure, elevated fasting blood glucose, and dyslipidemia (i.e., high triglycerides and low HDL cholesterol) (Alberti et al., 2009). Pietrzak-Nowacka et al. (2009) found that hypertension was the main component of the metabolic syndrome to correlate with ADPKD, whereas BMI and waist-to-hip ratio did not differ compared to age- and gender-matched controls. Fasting glucose levels were higher in patients with ADPKD, but the difference in blood glucose did not persist after an oral glucose tolerance test. However, these findings must be interpreted with caution,

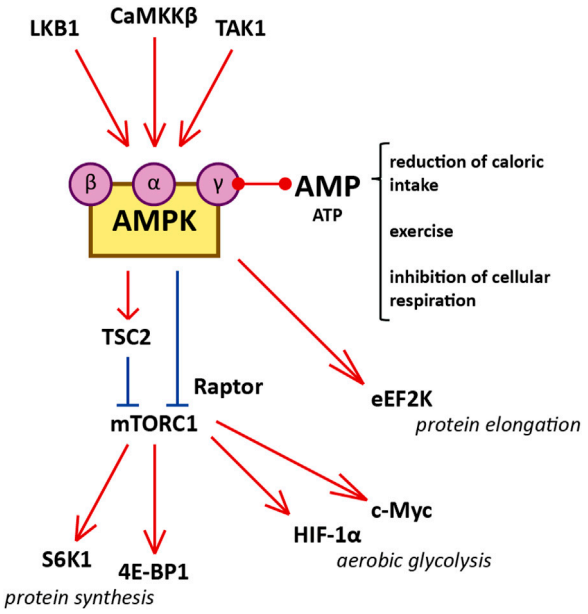

FIGURE 1
Diagram illustrating the interaction between AMPK and mTORC1, and cellular functions of mTORC1 that influence cystogenesis in ADPKD. Red represents activation and blue represents inhibition. AMPK is a heterotrimer consisting of a catalytic α-subunit, a regulatory β-subunit, and a regulatory γ-subunit assembled in a 1:1:1 ratio. Reduced caloric intake, physical exercise, and interference with electron transport in the mitochondria increase the AMP/ATP ratio. Binding of AMP to the regulatory γ-subunit of AMPK allows the catalytic α-subunit to be phosphorylated at residue Thr172 by one of the three AMPK kinases (LKB1, CaMKKβ, and TAK1). Certain molecules, such as salicylate, can activate AMPK directly by binding the β-subunit. After phosphorylation by the AMPK kinases, AMPK inhibits mTORC1 1) by phosphorylating the Raptor component of the complex (at residues Ser722 and Ser792) and 2) by phosphorylating TSC2 (at residues Thr1227 and Ser1345). The inhibition of mTORC1 suppresses protein synthesis by downregulating S6K1 and 4E-BP1. mTORC1 enhances the translation of c-Myc and HIF-1α, both of which contribute to aerobic glycolysis. Additionally, AMPK inhibits protein translation and elongation by phosphorylating and activating eEF2K.

as BMI was not adjusted for kidney and liver volume. Reed et al. (2012) described larger kidney volumes in patients with ADPKD and type 2 diabetes, compared to those with ADPKD alone. However, since BMI was higher on average in the group with type 2 diabetes, it remains unclear whether it was the dysglycemia or elevated BMI that contributed to the results.

Several signaling pathways relevant to obesity, such as AMPK, are also known to play a major role in the pathobiology of ADPKD. The correlation between BMI and cystic disease progression may be explained by a number of mechanisms including metabolic dysregulation related to nutrient availability, hormonal changes such as hyperinsulinemia, low-grade chronic inflammation, or any combination thereof. Examining the pathways common to both obesity and ADPKD may help generate hypotheses to support future mechanistic studies (Nowak et al., 2018).

mTORC1 plays a major role in the pathophysiology of autosomal dominant polycystic kidney disease

The mTOR protein is a serine/threonine kinase central to two different complexes: mTOR complex 1 (mTORC1), which includes six protein components, and mTOR complex 2 (mTORC2), which includes seven. mTORC1 responds to available amino acids and oxygen, growth factors, DNA homeostasis, and cellular energy levels to activate cell proliferation by enhancing protein synthesis, stimulating the cell cycle, and inhibiting autophagy. On the other hand, mTORC2 is involved in the organization of the actin cytoskeleton and regulates mTORC1 function (Laplante and Sabatini, 2012; Yoon, 2017; Boutouja et al., 2019). mTORC1 is downregulated by AMPK, which phosphorylates and inactivates the Raptor component of the complex, and phosphorylates and activates tuberous sclerosis complex 2 (TSC2), a negative regulator upstream of mTORC1 (Figure 1). (Boutouja et al., 2019) mTORC1 signaling promotes protein synthesis by phosphorylating the translational regulators S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1) (Laplante and Sabatini, 2012). Moreover, mTORC1 is a potent suppressor of autophagy *via* phosphorylation and inhibition of unc-51-like kinase 1 (ULK1) (Laplante and Sabatini, 2012). mTORC1 is activated in the kidney proximal tubules of obese mice fed a high-fat diet (HFD), and long-term lipid overload was recently shown to induce lysosomal dysfunction, impaired autophagy, and susceptibility to kidney injury (Yamahara et al., 2013; Yamamoto et al., 2017). Enhanced mTORC1 signaling was repeatedly confirmed in the epithelium of kidney cysts, stimulating cell proliferation and inhibiting autophagy, which are both involved in the pathogenesis of ADPKD; multiple preclinical studies of mTORC1 inhibitors sirolimus (rapamycin) and everolimus showed some benefit in rodent models (Table 1). (Tao et al., 2005; Shillingford et al., 2006; Wahl et al., 2006; Wu et al., 2007; Zafar et al., 2009; Shillingford et al., 2010; Zafar et al., 2010; Song et al., 2020)

Interestingly, mTORC1 signaling also stimulates aerobic glycolysis, also known as the Warburg effect, by enhancing the translation of critical mediators such as c-Myc and hypoxia-inducible factor 1α (HIF-1α), which upregulate many glycolytic genes (Figure 1). (Osthus et al., 2000; Thomas et al., 2006; Lee et al., 2020) Aerobic glycolysis refers to the predominant cellular use of glycolysis to generate ATP, as opposed to the more metabolically efficient oxidative phosphorylation, even in the presence of abundant oxygen

TABLE 1 *In vitro* and animal studies investigating metabolic agents of interest in the treatment of ADPKD.

Agent	Mechanism of action	Study	Experimental model	Evidence
Sirolimus	mTOR inhibitor	Riwanto et al. (2016)	Human ADPKD cells	↓mRNA expression of glycolytic genes and ↓lactate
		Tao et al. (2005)	Han:SPRD rat	↓cyst burden, ↑kidney function
		Wahl et al. (2006)	Han:SPRD rat	↓cyst burden, ↑kidney function
		Shillingford et al. (2006)	*orpk*-rescue mouse	↓cyst burden
		Zafar et al. (2009)	Han:SPRD rat	↓cyst burden, ↑kidney function
		Zafar et al. (2010)	[a]*Pkd2*$^{WS25/-}$ mouse	↓cyst burden
		Shillingford et al. (2010)	[a]*Pkd1*$^{cond/cond}$:*Nestin*^{cre} mouse	↓cyst burden, ↑kidney function, ↓fibrosis
Everolimus	mTOR inhibitor	Wu et al. (2007)	Han:SPRD rat	↓cyst burden, ↑kidney function
2DG	Glycolysis inhibitor/ AMPK agonist	Rowe et al. (2013)	• [a]*Pkd1*$^{flox/-}$:*Ksp-Cre* mouse; • [a]*Pkd1*$^{V/V}$ mouse	↓cyst burden ↓cyst burden
		Riwanto et al. (2016)	Han:SPRD rat	↓cyst burden, ↑kidney function
		Chiaravalli et al. (2016)	[a]*Pkd1*$^{\Delta C/flox}$*TmCre* mouse (inactivation of *Pkd1* at two time points)	↓cyst burden (both medium- and long-term models), ↑kidney function and ↓inflammation (long-term model)
		Lian et al. (2019)	[a]Inducible deletion of PKD1 in minipig	↓cyst burden, ↑kidney function
Metformin	AMPK agonist	Riwanto et al. (2016)	Human ADPKD cells	↓mRNA expression of glycolytic genes and ↓lactate
		Takiar et al. (2011)	• MDCK cells cultured with forskolin/ IBMX[i] • Embryonic C57/B6 mouse kidneys cultured with cAMP[i] • [a]*Pkd1*$^{flox/-}$:*Ksp-Cre* mouse[i] • [a]*Pkd1*$^{flox/-}$:*pCX-CreER* mouse	↓cyst size ↓cyst area ↓cyst burden ↓cyst burden
		Lian et al. (2019)	[a]Inducible deletion of PKD1 in minipig	↓cyst burden, ↑kidney function
		Chang et al. (2017)	[a]Morpholino knock-down of *pkd2* in zebrafish embryo	↓pronephric cyst burden and ↓inflammation
		Pastor-Soler et al. (2022)	[a]Homozygous R3277C point mutation in mouse *Pkd1*	↓cyst burden, ↑kidney function
Salsalate	AMPK agonist	Leonhard et al. (2019)	[a]iKsp-*Pkd1*^{del} conditional knock-out mouse	↓cyst burden, ↑kidney function
Fenofibrate	PPARα agonist	Hajarnis et al. (2017)	[a]*Pkd2* knock-out mouse	↓cyst burden
		Lakhia et al. (2018)	[a]*Pkd1*$^{RC/RC}$ mouse	↓cyst burden, ↑kidney function, ↓inflammation

2DG: 2-deoxy-D-glucose; cAMP: cyclic AMP; IBMX: 3-isobutyl-1-methylxanthine; MDCK: Madin-Darby canine kidney.
[a]Orthologous animal model.

(Vander Heiden et al., 2009). Aerobic glycolysis is an important metabolic feature of proliferating cells (such as cancer cells) that facilitates replication of the cellular biomass (nucleotides, amino acids, and lipids) by providing specific molecular components (e.g., reduced nicotinamide adenine dinucleotide phosphate or NADPH) (Vander Heiden et al., 2009). Importantly, the Warburg effect is also a feature of cystic epithelia in ADPKD. Sirolimus reduced the expression of glycolytic genes in human ADPKD cells (Riwanto et al., 2016). Studies in rodents and minipigs showed that inhibiting glycolysis with 2-deoxy-D-glucose (2DG) improved cystic disease (Table 1) (Rowe et al., 2013; Rowe and Boletta, 2014; Chiaravalli et al., 2016; Riwanto et al., 2016; Lian et al., 2019).

However, in studies of patients with ADPKD, treatment with sirolimus and everolimus has produced disappointing results (Table 2). In randomized controlled trials of either sirolimus or everolimus, beneficial effects on TKV were short-lived or confounded by flaws in study design (e.g., small sample size or short follow-up) (Perico et al., 2010; Serra et al., 2010; Walz et al., 2010; Ruggenenti et al., 2016). In two of these studies, side effects from sirolimus prompted investigators to reduce the delivered doses below those intended, therefore potentially jeopardizing the effectiveness of the drug (Perico et al., 2010; Serra et al., 2010). A sirolimus trial including patients with advanced CKD was terminated after 1 year due to accelerated disease progression and multiple side effects (Ruggenenti et al., 2016). Lower-dose sirolimus might be a viable alternative, with evidence of fewer side effects (Braun et al., 2014). In addition, the study protocol for a randomized controlled trial of pulsed (weekly) oral sirolimus was published previously, although at this time the results remain unknown (*NCT02055079*) (Riegersperger et al., 2015). In conclusion, pharmacological inhibition of mTORC1 at present is not a promising clinical option for the treatment of ADPKD.

TABLE 2 Clinical studies investigating metabolic agents of interest in the treatment of ADPKD.

Agent	Mechanism of action	Study	Evidence	Advantages/Disadvantages of the therapy
Sirolimus	mTOR inhibitor	Serra et al. (2010) RCT Perico et al. (2010) RCrT Braun et al. (2014) RCT Ruggenenti et al. (2016) RCT Ongoing trial: NCT02055079 (status unknown)	Inconclusive (low number of patients, short follow-up, no difference in TKV)	• Multiple side effects • Accelerated disease progression in patients with advanced CKD
Everolimus	mTOR inhibitor	Walz et al. (2010) RCT	Inconclusive (effect on TKV not maintained)	• Multiple side effects
Metformin	AMPK agonist	Sorohan et al. (2019) SA Perrone et al. (2021) RCT Brosnahan et al. (2022) RCT Ongoing trials: NCT03764605 (status unknown); NCT04939935 (not yet recruiting)	Good tolerability, studies not designed to assess change in kidney function	• Well-known, inexpensive drug • G-I side effects • Risk of lactic acidosis with lower kidney function

CKD: chronic kidney disease; RCT: randomized controlled trial; RCrT: randomized crossover trial; G-I: gastro-intestinal; SA: single-arm pilot study; TKV: total kidney volume.

AMP-activated protein kinase is a potential therapeutic target in both obesity and autosomal dominant polycystic kidney disease

AMPK, an important integrator of multiple pathways, is a therapeutic target of particular interest. AMPK functions as a major molecular sensor of cellular energy, responding to energy depletion by switching on catabolic pathways and switching off anabolic pathways (Hardie et al., 2012). As summarized in a recent review, AMPK plays a central role in the pathophysiology of ADPKD by modulating multiple biological processes, including cellular proliferation, autophagy, solute transport, metabolism and mitochondrial homeostasis in the kidney, all of which are important for cystogenesis (Figure 2). (Song et al., 2020)

AMPK is a heterotrimer consisting of a catalytic α-subunit, a regulatory β-subunit, and a regulatory γ-subunit assembled in a 1:1:1 ratio (Hallows et al., 2010; Hardie et al., 2012). Subunits have multiple isoforms (α1, α2, β1, β2, γ1, γ2, and γ3), each encoded by a different gene with tissue-specific expression and activity (Steinberg and Kemp, 2009). Isoform frequency in the kidney is species-dependent; for instance, the β1 subunit is present in 93, 96, and 64% of kidneys of C57BL/6J mice, Wister Han rats, and humans, respectively (Fraser et al., 2005; Salatto et al., 2017). AMPK is activated by rising concentrations of AMP relative to ATP. Upon binding to AMP, AMPK is phosphorylated in its catalytic loop (at residue Thr172) by one of the three upstream AMPK kinases (Figure 1): liver kinase B1 (LKB1), Ca^{2+}/calmodulin-activated protein kinase kinase β (CaMKKβ), or Tak1 kinase (TAK1) (Steinberg and Kemp, 2009; Hallows et al., 2010; Hardie et al., 2012).

It has been suggested that AMPK signaling may be suppressed with obesity (Figure 3) (Steinberg and Kemp, 2009). AMPK activity is reduced in the heart, liver, and skeletal muscle in genetic models of rodent obesity (Barnes et al., 2002; Yu et al., 2004; Wangyun and Unger, 2005; Sriwijitkamol et al., 2006; Steinberg et al., 2006). On the other hand, AMPK activity is not always altered in the tissues of animals with HFD-induced obesity, nor is it altered in the skeletal muscle of obese humans (Steinberg et al., 2004; Bandyopadhyay et al., 2006; Martin et al., 2006). Nevertheless, AMPK activity is strongly downregulated in the adipose tissue of obese humans (Galic et al., 2011). This inhibition of AMPK may be related to low-grade chronic inflammation and high levels of glucose and insulin, which collectively suppress kinase activity through several distinct mechanisms (Steinberg et al., 2006; Valentine et al., 2014; Zhao et al., 2018; Jiang et al., 2021). With respect to lipid metabolism, AMPK inhibits acetyl-CoA carboxylase 1 and 2 (ACC1 and ACC2), thereby increasing fatty acid oxidation and suppressing fatty acid synthesis; AMPK also inhibits hydroxy-3-methylglutaryl-CoA reductase (HMGCR), therefore suppressing cholesterol synthesis (Steinberg and Kemp, 2009; Steinberg and Carling, 2019). This may be of special importance to the pathogenesis of lipid-induced nephrotoxicity, a type of ectopic lipid accumulation associated with obesity (Figures 2, 3) (van Herpen and Schrauwen-Hinderling, 2008). Indeed, AMPK was demonstrated to be an important pathway in HFD-associated kidney disease, described by the authors as glomerulomegaly with increased mesangial matrix, inflammation, and albuminuria. Specifically, HFD feeding in mice markedly decreased phosphorylation of glomerular AMPKα. Treating C57BL/6J mice with the AMPK

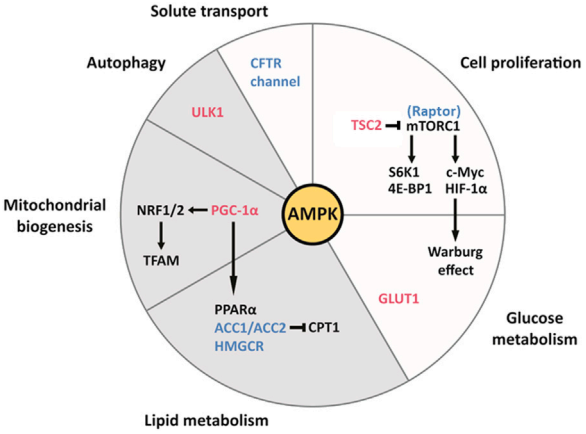

FIGURE 2
The pleiotropic effects of AMPK on cell function and metabolism in the kidney, and their relevance to ADPKD and obesity-related kidney disease. AMPK activates the downstream effectors colored in red and inhibits those colored in blue. Each slice of the pie chart summarizes pathways involved in the pathogenesis of ADPKD (white and grey) and lipid-induced nephrotoxicity seen with obesity (grey only). Clockwise from the top. AMPK inhibits mTORC1 by directly inhibiting the Raptor component of the complex and by activating TSC2, which inhibits mTORC1. mTORC1 is highly active in cyst cell linings and drives cellular proliferation through its downstream effectors S6K1 and 4E-BP1. By enhancing the translation of c-Myc and HIF-1α, mTORC1 contributes to the Warburg effect (aerobic glycolysis), which supports the replication of the cellular biomass necessary for proliferation. Additionally, AMPK enhances the translocation of the transmembrane transporter GLUT1 to the plasma membrane of baby hamster kidney (BHK) cells, which contributes to glucose uptake in response to insulin (Baldwin et al., 1997). AMPK inhibits ACC1/ACC2 and HMGCR, therefore suppressing fatty acid and cholesterol synthesis, respectively. ACC production of malonyl-CoA inhibits carnitine palmitoyl-transferase 1 (CPT1), which is required for the entry of fatty acyl-CoA into the mitochondria. Therefore, by inhibiting ACC, AMPK also stimulates fatty acid oxidation (Steinberg and Carling, 2019). Because of the intrinsic defect in fatty acid oxidation seen in ADPKD, which is associated with downregulation of the PPARα gene network, the intracellular accumulation of fatty acids may contribute to cystogenesis. Furthermore, lipid-induced nephrotoxicity appears to result from suppression of AMPK activity by fatty acid overload, leading to activation of ACC1 and HMGCR, and to suppression of PPARα expression. AMPK promotes mitochondrial biogenesis via PGC-1α, which activates NRF1 and NRF2, both of which regulate TFAM, a crucial transcription factor for mitochondrial DNA transcription and replication. By phosphorylating PGC-1α, AMPK activates PPARα. Defects in mitochondrial morphology and function are a hallmark of ADPKD. Furthermore, HFD can reduce the kidney expression of NRF1 and TFAM, thereby compromising mitochondrial biogenesis, in addition to inhibiting PGC-1α (Wang et al., 2018). AMPK phosphorylates ULK1, which initiates autophagy. The latter process is essential to maintain the homeostasis of renal tubular epithelial cells and appears to be defective in ADPKD. Lipid overload stimulates autophagy in the kidney proximal tubule and long-term lipid-induced autophagic activation can stress the lysosomal system, resulting in lysosomal dysfunction, impaired autophagy, and susceptibility to kidney injury. AMPK inhibits the CFTR channel responsible for intra-cystic fluid secretion.Figure adapted from Song et al. (2020)

activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) for 1 week restored AMPK activity, preventing kidney hypertrophy and macrophage infiltration (Declèves et al., 2011). However, it should be noted that AICAR has a number of AMPK-independent effects, and can lower blood pressure and blood glucose; as such, these results should be interpreted with caution. Interestingly, in a later study, HFD-induced renal injury was further described as vacuolated proximal tubular cells with loss of the brush border, suggesting tubular damage (Declèves et al., 2014). Glucagon-like peptide-1 (GLP-1) receptor agonist liraglutide was shown to restore AMPK signaling, and reduce glomerular size, glomerulosclerosis, and interleukin levels in obese Sprague-Dawley rats fed a HFD (Wang et al., 2018). Therefore, obesity-related kidney disease associated with lipid deposits appears to be mediated by suppressed AMPK activity, affecting both the glomerular and tubular compartments of the kidney.

Following the initial preclinical research examining the role of mTORC1 in ADPKD, several studies focused on its inhibitor AMPK. More than 10 years ago, metformin, an AMPK agonist,

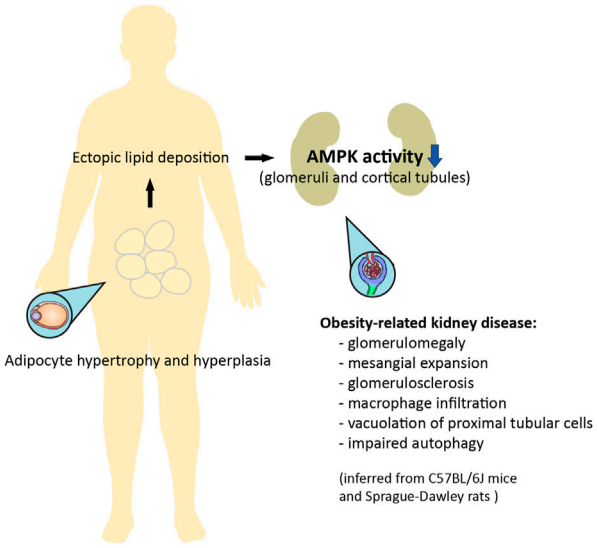

FIGURE 3
Ectopic lipid deposition in obesity can result in nephrotoxicity mediated by downregulation of AMPK activity. Adipocyte hypertrophy and hyperplasia is associated with an increase in circulating lipids, which leads to ectopic lipid deposition. Obesity-related kidney disease, as inferred from rodent models, is mediated by inhibited AMPK activity in the glomeruli and cortical tubular epithelium, and is characterized by glomerular enlargement, mesangial expansion, glomerulosclerosis, inflammation, evidence of tubular damage, and impaired autophagy (Declèves et al., 2011; Declèves et al., 2014; Wang et al., 2018). Some of these features can be improved with pharmacological activation of AMPK.

was used to suppress cystogenesis in an *in vitro* model, an *ex vivo* model, and two mouse models of ADPKD (Table 1). Metformin was shown to reduce mTOR signaling in the kidneys of the two mouse models (Takiar et al., 2011). The authors concluded that AMPK activation may alleviate cystic disease by two main mechanisms: by inhibiting the pro-proliferative mTOR pathway and the cystic fibrosis transmembrane conductance regulator (CFTR) channel that mediates fluid secretion into cysts (Takiar et al., 2011). In a subsequent study, AMPK phosphorylation was demonstrated to be reduced in fibroblasts isolated from *Pkd1* knock-out mouse embryos compared to wild-type cells (Rowe et al., 2013). In addition to its inhibitory effect on mTORC1, AMPK may potentially suppress cell proliferation in cysts by phosphorylating and activating eukaryotic elongation factor 2 kinase (eEF2K), which plays an essential role in inhibiting protein synthesis (Steinberg and Kemp, 2009). The distribution of AMPK in the kidney further supports its role in cystogenesis, as phosphorylated AMPK was detected on the basolateral surface of the rat collecting duct, which in the adult kidney is a major site of cyst formation (Fraser et al., 2005; Harris and Torres, 2009). While the molecular cause for suppressed AMPK activity in ADPKD remains unclear, *Pkd1* knock-out mouse embryonic fibroblasts (MEFs) displayed increased activity of the extracellular signal-regulated kinase (ERK). In the same experiment, ERK inhibited TSC2, which may contribute to mTORC1-mediated cell proliferation (Distefano et al., 2009). Although ERK has been postulated to suppress the LKB1-AMPK axis in *Pkd1* knock-out MEFs, this pathway remains to be confirmed (Rowe et al., 2013).

Pharmacological agonists of AMP-activated protein kinase show promise in the treatment of autosomal dominant polycystic kidney disease

Several drugs that activate AMPK may have applications in the treatment of APDKD. Metformin, an oral agent commonly used to treat type 2 diabetes, activates AMPK indirectly by inhibiting complex I of the electron transport chain, leading to a drop in cellular ATP and an increase in AMP (Rena et al., 2017). In an *in vitro* model of kidney cystogenesis, metformin inhibited the expression of glycolytic genes and lowered lactate production (Riwanto et al., 2016). Several animal studies have demonstrated that metformin improves cystic disease (Table 1). In two *Pkd1* knock-out mouse models, metformin ameliorated cystic kidney disease (Takiar et al., 2011). Moreover, in MEFs isolated from *Pkd1* knock-out embryos, phosphorylated AMPK was reduced compared to wild-type cells; treating the mutant cells with metformin or AICAR restored AMPK activity (Rowe et al., 2013). In a minipig model and a zebrafish model, metformin similarly activated AMPK, thereby reducing cyst formation (Chang et al., 2017; Lian et al., 2017; Lian et al., 2019). Importantly, metformin improved disease severity in the *Pkd1* RC/RC mouse model, which approximates the slow progression of cystic disease in humans (Pastor-Soler et al., 2022). Pilot metformin studies in human populations with ADPKD did not show a benefit on eGFR, although the drug was well tolerated in both a single-arm study and two randomized controlled trials (Table 2). (Sorohan et al., 2019; Perrone et al., 2021; Brosnahan et al., 2022) Two additional trials of metformin in ADPKD are ongoing in Italy and Australia (*NCT03764605* and *NCT04939935*, respectively).

Canagliflozin, an SGLT2 inhibitor used to treat type 2 diabetes, has the off-target effect of activating AMPK indirectly by inhibiting mitochondrial respiration (Leonhard et al., 2019). By contrast, salicylate derived from the pro-drug salsalate activates AMPK directly by binding to its β-subunit. Salsalate, unlike aspirin, interacts little with the cyclooxygenase pathway, which limits the occurrence of gastrointestinal and bleeding side effects (Anderson et al., 2014). In a collaboration between our group and Leiden University, salsalate alone or in combination with metformin was shown to decrease kidney weight and improve survival from ESKD in an adult-onset conditional *Pkd1* knock-out mouse model. Conversely, treatment was ineffective with metformin alone, canagliflozin alone, or metformin in combination with canagliflozin (Leonhard et al., 2019). In this study, the method of administration of metformin was different (oral) from that used by Takiar et al. (2011) (intra-peritoneal), but the dosage was identical; the relatively low oral bioavailability of metformin (~30–60%) likely limited its action in the kidneys (Padwal et al., 2011). Although the same oral metformin dosage was used in the *Pkd1* RC/RC mouse study, the longer duration of treatment in the latter may explain the improvement seen in kidney disease (Pastor-Soler et al., 2022). To our knowledge, there are currently no clinical trials of salsalate in ADPKD.

Lastly, AMPK-activating resveratrol, a polyphenol that also activates sirtuin 1 (SIRT1), was used to reverse diabetic nephropathy and prevent lipid-induced nephrotoxicity in db/db mice (Kim et al., 2013). Nonetheless, because treatment with a pan-sirtuin inhibitor (nicotinamide) and a SIRT1-specific inhibitor (EX-527) delayed cyst growth in *Pkd1* knock-out mouse embryonic kidneys, *Pkd1* conditional knock-out postnatal mice, and *Pkd1* hypomorphic mice, it is unclear whether sirtuin 1 is an interesting drug target in combined obesity and ADPKD (Zhou et al., 2013). In conclusion, AMPK-activating drugs such as metformin and salsalate are clinically easier to tolerate than mTORC1 inhibitors, making them more promising candidates to control cystogenesis in ADPKD. However, more clinical trials are needed.

AMP-activated protein kinase interacts with other processes relevant to the pathophysiology of autosomal dominant polycystic kidney disease

In addition to modulating cell proliferation and fluid secretion, AMPK regulates other processes that have been shown to be abnormal in ADPKD, such as mitochondrial structure and function, the peroxisome proliferator-activated receptor α (PPARα) pathway, and the innate immune response (Figure 2). (Song et al., 2020)

The kidney displays mitochondrial abnormalities in both obesity and autosomal dominant polycystic kidney disease

Strong evidence indicates that AMPK promotes mitochondrial biogenesis by activating peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α) (Steinberg and Kemp, 2009). As a transcriptional coactivator, PGC1α interacts with nuclear respiratory factors 1 and 2 (NRF1 and NRF2), which regulate mitochondrial transcription factor A (TFAM), the latter playing a crucial role in maintaining mitochondrial DNA transcription and replication. (Figure 2). (Kelly and Scarpula, 2004; Reznick and Shulman, 2006)

Mitochondrial dysfunction, presumably related to insulin resistance, is present in obesity, affecting tissues that participate in nutrient metabolism such as the adipose tissue, liver, and skeletal muscle (Bournat and Brown, 2010; Montgomery and Turner, 2015). The progressive enlargement of white adipose tissue in mice and humans can impair blood flow and result in hypoxia, which can lead to macrophage recruitment and infiltration (Wood et al., 2009). In turn, inflammation, in addition to excessive food intake, may increase the production of reactive oxygen species, causing mitochondrial dysfunction, although there is also evidence the latter may in fact precede adipose tissue inflammation (Lahera et al., 2017; de Mello et al., 2018; Woo et al., 2019). Increased mitochondrial fission, defective mitochondrial biogenesis and oxidative capacity, and intracellular triglyceride overload have been described in the adipose tissue, liver, and skeletal muscle of obese rodents and humans (Bournat and Brown, 2010; de Mello et al., 2018). In the kidney specifically, mitochondrial abnormalities have been inconsistently described in *ob/ob* mice and mice with HFD-induced obesity (Declèves et al., 2014; Szeto et al., 2016). Liraglutide restored AMPK and PGC-1α signaling in the kidneys of HFD-fed rats, and inhibited the formation of mitochondrial reactive oxygen species (Wang et al., 2018). There is also evidence that SS-31, an antioxidant that protects the structure of mitochondrial cristae, may prevent the renal toxicity, including mitochondrial damage, caused by fatty acid accumulation (Szeto et al., 2016).

The role of mitochondria in the pathophysiology of ADPKD has been covered in depth in several recent reviews (Padovano et al., 2018; Menezes and Germino, 2019; Nowak and Hopp, 2020; Podrini et al., 2020). Briefly, mitochondrial abnormalities can be found in *Pkd1* knock-out kidney epithelial cells isolated from mice, in mouse and rat models of polycystic kidney disease, and in kidney tissues from patients with ADPKD (Ishimoto et al., 2017; Lin et al., 2018). Morphological changes on electron microscopy include swelling and fragmentation of the mitochondria, and damaged cristae. These morphological changes in mitochondria, suggestive of defects in mitophagy, are very analogous to those observed in mice deficient for AMPK in muscle and brown adipose tissue (Bujak et al., 2015; Mottillo et al., 2016). Moreover, the mitochondrial DNA copy number decreases from an early stage of ADPKD, in parallel with kidney mRNA and protein expression of PGC-1α (Ishimoto et al., 2017). Several studies have highlighted that the impairment of mitochondrial metabolic processes such as fatty acid oxidation and oxidative phosphorylation drives cyst formation, in part *via* reduced PPARα expression (Menezes et al., 2012; Rowe et al., 2013; Menezes et al., 2016; Hajarnis et al., 2017; Padovano et al., 2017; Podrini et al., 2018). Because of defective fatty acid oxidation, a higher fat intake aggravated the kidney cyst burden in a non-orthologous rat and two orthologous mouse models of polycystic kidney disease (Jayapalan et al., 2000; Menezes et al., 2016). Since PC1 and PC2 appear to affect both the morphology and function of mitochondria, mitochondrial dysfunction might be one of the original molecular events preceding metabolic dysregulation in ADPKD (Padovano et al., 2017; Lin et al., 2018; Kuo et al., 2019).

Peroxisome proliferator-activated receptor α activation ameliorates lipid-induced toxicity in the kidney

AMPK phosphorylates, thereby activating, PGC-1α, which acts as a transcriptional coactivator of PPARα (Figure 2). (Vega et al., 2000; Steinberg and Kemp, 2009) The AMPKα subunit can also coactivate PPARα independently of its kinase activity (Bronner et al., 2004). This ligand-activated transcription factor binds to fatty acids to regulate essential metabolic responses to fasting, including ketogenesis in the liver and fatty acid utilization in multiple tissues such as the kidney (Kersten et al., 1999; Guan, 2002; Wang, 2010). In a mouse model of HFD-induced glomerular injury, treatment with the PPARα agonist fenofibrate increased the expression of lipolytic enzymes, reducing lipid accumulation and oxidative stress in the glomeruli, and preventing glomerular fibrosis (Tanaka et al., 2011). The network comprising PPARα and its gene targets is known to be downregulated in mouse models of ADPKD and human cysts, and PPARα downregulation

is in part responsible for the intrinsic defect in fatty acid oxidation seen in ADPKD (Hajarnis et al., 2017; Lakhia et al., 2018; Lakhia, 2020). One mechanism underlying PPARα downregulation may be post-transcriptional inhibition by the microRNAs miR-17–92 and miR-21, which are upregulated in mouse and human ADPKD (Lakhia et al., 2016; Hajarnis et al., 2017). Using fenofibrate improved the phenotype of both aggressive and slowly progressive mouse models of ADPKD, and ameliorated the tubulointerstitial fibrosis associated with reduced fatty acid oxidation (Table 1). (Kang et al., 2015; Hajarnis et al., 2017; Lakhia et al., 2018) However, although fibrates are commonly used in the treatment of dyslipidemia, their long-term safety in kidney disease remains unclear (Jun et al., 2012; Melissa et al., 2020).

Chronic inflammation is a feature of obesity and autosomal dominant polycystic kidney disease

Both obesity and ADPKD are associated with a state of low-grade chronic inflammation. While other immune components participate in the inflammatory phenotype in both conditions, macrophages have been most extensively studied and play a dominant role (Zimmerman et al., 2020). In obesity, the upregulation of monocyte chemoattractant protein 1 (MCP-1) in fat depots with resulting accumulation of macrophages is crucial to induce inflammation in the adipose tissue (Oh et al., 2012; Amano et al., 2014). In humans, increased circulating MCP-1 correlates with higher levels of other inflammatory markers such as C-reactive protein (CRP) and interleukin-6 (IL-6), but also obesity, waist circumference, and the homeostatic model assessment for insulin resistance (HOMA-IR) score (Kim et al., 2006). Studies in obese mice support the causal role for MCP-1-mediated macrophage accumulation in adipose tissue in promoting insulin resistance (Kamei et al., 2006; Kanda et al., 2006; Weisberg et al., 2006; Aouadi et al., 2013; Kim et al., 2016). Interestingly, macrophage AMPKβ1 appears to blunt the inflammatory response to saturated fatty acids by increasing fatty acid oxidation (Galic et al., 2011).

In patients with ADPKD, the inflammatory markers MCP-1 and macrophage migration inhibitory factor (MIF) are elevated in the urine and cyst fluid (Zheng et al., 2003; Meijer et al., 2010; Kim and Tam, 2011; Chen et al., 2015). Urinary MCP-1 correlates with TKV and predicts progression to CKD stage 3 (Meijer et al., 2010; Chapman et al., 2012; Messchendorp et al., 2018; Segarra-Medrano et al., 2020). In an inducible knock-out model of *Pkd1*, MCP-1 upregulation, independently of tubular injury, caused monocyte infiltration. Monocytes differentiated into pro-inflammatory M1-like macrophages that accumulated around cysts, causing oxidative damage and tubular injury; a switch to alternatively activated M2-like macrophages then coincided with increased cyst proliferation (Cassini et al., 2018). In fact, most interstitial macrophages in the kidneys of patients with ADPKD were found to be M2-like, and human cyst cells were shown to promote M2-like macrophage polarization *in vitro* by secreting soluble factors including lactates (Swenson-Fields et al., 2013; Yang et al., 2018). M2-like macrophages, normally involved in tissue repair, are likely locked in a maladaptive cycle of pro-proliferative and pro-fibrotic activity (Weimbs, 2018). Previous work reported that global macrophage depletion with liposomal clodronate slowed kidney disease progression in non-orthologous and orthologous mice (Karihaloo et al., 2011; Swenson-Fields et al., 2013; Yang et al., 2018). Moreover, attenuating macrophage accumulation in *Pkd1*-deficient mice with *Mcp1* knock-out or an antagonist of the MCP-1 receptor (also known as CCR2) reduced cyst growth and tubular injury (Cassini et al., 2018; Viau et al., 2018).

Importantly, the proinflammatory cytokine MIF is associated on the one hand with increases in MCP-1, tumor necrosis factor α (TNF-α), and macrophage accumulation, and on the other hand with increased glucose uptake and glycolytic gene expression, inhibition of AMPK activity, and activation of ERK and mTOR signaling (Chen et al., 2015). Its deletion or pharmacologic inhibition has reversed these abnormalities and slowed disease progression in multiple *Pkd1*-deficient murine models (Chen et al., 2015). As an additional link between kidney inflammation and metabolic disease, LKB1 forms a complex with PC1 to suppress the expression of MCP-1 in tubular epithelia; the tubular deletion of *Lkb1* or *Pkd1* restores MCP-1 expression and causes monocyte/CCR2+ macrophage recruitment (Viau et al., 2018). This is notable because ciliary LKB1 colocalizes with and activates AMPK (Boehlke et al., 2010; Mick et al., 2015). Thus, similarly to MIF, LKB1 appears to be an upstream regulator of both inflammatory and metabolic changes in ADPKD.

Conclusion

The pathobiology of ADPKD is strongly linked to metabolic dysregulation, which may be secondary to mitochondrial abnormalities induced by defective polycystin function. Pathways common to both obesity-related kidney disease and ADPKD suggest that obesity with ectopic lipid deposition in the kidneys may contribute to the progression of cystic disease through various mechanisms, notably through inhibition of AMPK activity. Overweight and obesity are frequent in patients with ADPKD, and weight loss should be beneficial for both conditions (Pickel et al., 1093; Nowak et al., 2018). Further work will be needed to understand if and how weight loss can attenuate cystogenesis on a molecular level. In addition to dietary changes, pharmacological activation of AMPK may attenuate lipid-related kidney injury as well as the cystic burden. The repurposing of metabolic drugs to treat patients with ADPKD has seen limited development thus far.

Nevertheless, based on promising *in vivo* data, it is reasonable to believe that well-known and relatively safe pharmacological agents such as metformin or salsalate, both of which activate AMPK, could attenuate lipid-induced nephrotoxicity and reduce cystic disease severity. Given the slow progression of human ADPKD, using pharmacological agents that are safe and tolerable will be critical.

Author contributions

I-AI wrote the different versions of the manuscript, with help from LP and guidance from XS, YP, GS, and H-KS. LP, XS, AH, JS, H-KS, GS, and YP helped to revise the manuscript. All authors approve the final version of this manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Emerging therapies for autosomal dominant polycystic kidney disease with a focus on cAMP signaling

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Autosomal dominant polycystic kidney disease (ADPKD), with an estimated genetic prevalence between 1:400 and 1:1,000 individuals, is the third most common cause of end stage kidney disease after diabetes mellitus and hypertension. Over the last 3 decades there has been great progress in understanding its pathogenesis. This allows the stratification of therapeutic targets into four levels, gene mutation and polycystin disruption, proximal mechanisms directly caused by disruption of polycystin function, downstream regulatory and signaling pathways, and non-specific pathophysiologic processes shared by many other diseases. Dysfunction of the polycystins, encoded by the PKD genes, is closely associated with disruption of calcium and upregulation of cyclic AMP and protein kinase A (PKA) signaling, affecting most downstream regulatory, signaling, and pathophysiologic pathways altered in this disease. Interventions acting on G protein coupled receptors to inhibit of 3′,5′-cyclic adenosine monophosphate (cAMP) production have been effective in preclinical trials and have led to the first approved treatment for ADPKD. However, completely blocking cAMP mediated PKA activation is not feasible and PKA activation independently from cAMP can also occur in ADPKD. Therefore, targeting the cAMP/PKA/CREB pathway beyond cAMP production makes sense. Redundancy of mechanisms, numerous positive and negative feedback loops, and possibly counteracting effects may limit the effectiveness of targeting downstream pathways. Nevertheless, interventions targeting important regulatory, signaling and pathophysiologic pathways downstream from cAMP/PKA activation may provide additive or synergistic value and build on a strategy that has already had success. The purpose of this manuscript is to review the role of cAMP and PKA signaling and their multiple downstream pathways as potential targets for emergent therapies for ADPKD.

KEYWORDS

cAMP signaling, protein kinase A (PKA), ADPKD (autosomal dominant polycystic kidney disease), PKD, vassopressin, tolvaptan

Introduction

Autosomal dominant polycystic kidney disease (ADPKD), the most common inherited renal cystic disease, has an estimated genetic prevalence between 1: 400 and 1:1,000 individuals and is responsible for 5%–10% of kidney failure world-wide (Bergmann et al., 2018). It is characterized by development of numerous tubular diverticula which become cysts after detaching. Unrelenting cyst growth causes kidney enlargement and functional decline, associated with hypertension, cyst hemorrhage, gross hematuria, nephrolithiasis, cyst infection, pain, and reduced quality of life. The total annual costs attributed to ADPKD in the United States were estimated to be $7.3 to $9.6 billion in 2018 (Cloutier et al., 2020). Direct healthcare costs accounted for $5.7 billion (78.6%), mostly driven by renal replacement therapy ($3.2 billion; 43.3%). Indirect costs accounted for $1.4 billion (19.7%), mostly driven by productivity loss due to unemployment ($784 million; 10.7%) and reduced productivity at work ($390 million; 5.3%).

Progress in the understanding of polycystic kidney disease

Over the last 3 decades there has been great progress in the understanding of ADPKD (1). It is caused by mutations in *PKD1* and *PKD2* and disruption of the encoded proteins (polycystin-1 and polycystin-2). Polycystin 1 is an adhesion type G protein coupled receptor, characterized by the presence of many adhesive extracellular domains, a G protein autoproteolytic site, and an intracellular G protein binding domain (Figure 1). The cleavage at the autoproteolytic site generates a N-terminal fragment and a stalk that binds to extracellular loops of the C-terminal domain and regulates Gα- and Gβγ-protein signaling, possibly inhibiting cAMP production (Maser and Calvet, 2020). Polycystin-2 is a transient receptor potential protein and interacts with polycystin-1 in a three to one ratio to form heterotetramer channels permeable to calcium. It has been proposed that the C-lectin in the N-terminal fragment of PC1 interacts with the top domain of PC2 to activate the channel (Ha et al., 2020). G-protein and calcium signaling reciprocally interact, G proteins regulating intracellular calcium dynamics and

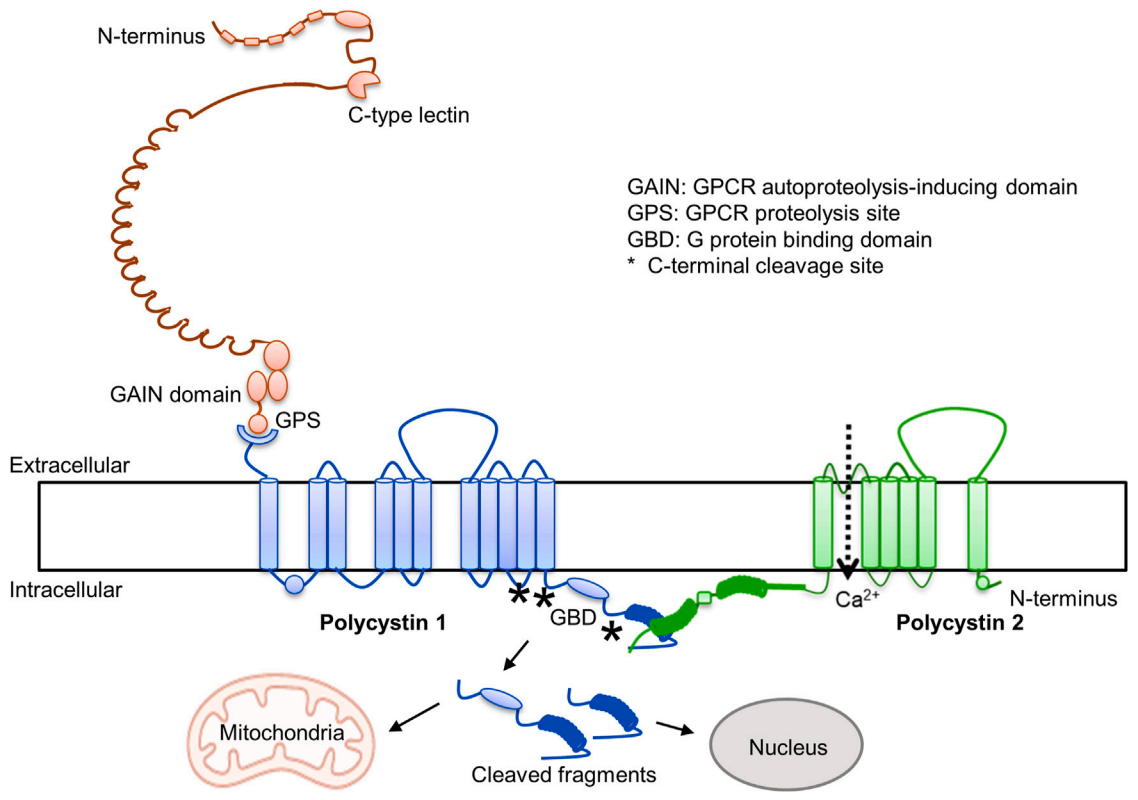

FIGURE 1
Polycystin 1 and polycystin 2.

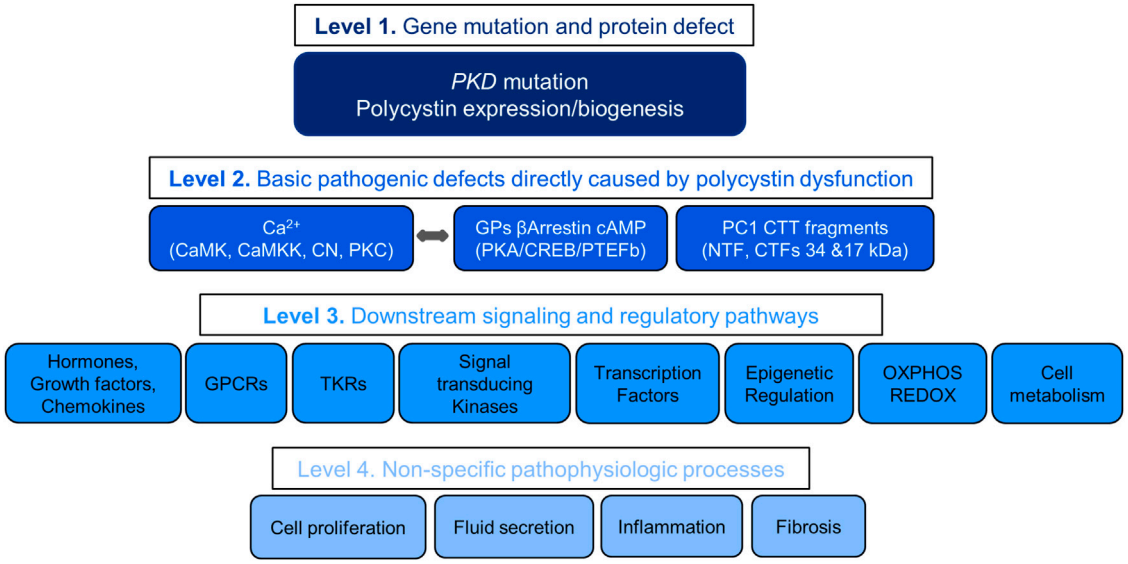

FIGURE 2
Levels of Therapeutic targets.

intracellular calcium regulating cAMP synthesis and degradation (Sussman et al., 2020). In addition, the C-terminal tail of polycystin-1 undergoes regulated cleavage with formation of cleaved fragments, containing mitochondrial and nuclear targeting sites, that translocate to nuclei and mitochondria may also regulate downstream signaling (Padovano et al., 2020). Dysregulation of G protein and intracellular calcium signaling, and possibly of signaling regulated by polycystin-1 C-terminal tail cleaved fragments, alter multiple pathways and pathophysiologic processes and promote cystogenesis.

Stratification of therapeutic targets

Understanding the pathogenesis of ADPKD allows the stratification of therapeutic targets into four levels with decreasing likelihood of therapeutic efficacy (Figure 2). The first includes the gene mutations and protein disruptions. The second, the basic pathogenic mechanisms directly caused by the disruption of polycystin function. The third, downstream signaling and regulatory pathways. The fourth, non-specific pathophysiologic processes shared by many other renal and non-renal diseases. Redundancy of mechanisms, numerous positive and negative feedback loops, and possibly downstream counteracting effects may account for the limited, although statistically significant effectiveness of many treatments in preclinical studies. Few of over 150 compounds effective in rodent models of PKD have been tested in clinical trials, mostly with negative results. Failure in clinical trials has in part been due to toxicity of the compounds (limiting the dosing feasible in human trials) and possibly development of somatic mutations or reprogramming of the epigenome, transcriptome or kinome of the cystic cells.

Central role of cAMP in the pathogenesis of ADPKD

Overwhelming evidence supports the central role of cAMP in ADPKD, possibly by promoting cyst initiation and most definitely by stimulating proliferation of the cystic epithelium and fluid secretion into the cysts, thus promoting cyst growth.

Cyst initiation

Enhanced cAMP and protein kinase A (PKA) signaling disrupts tubulogenesis. Epithelial tubulogenesis requires canonical Wnt/β-catenin signaling at early inductive stages and noncanonical Wnt/planar cell polarity signaling later. PKA is known to enhance Wnt/β-catenin signaling through phosphorylation of glycogen synthase kinase 3b (stabilizing β-catenin) and phosphorylation of β-catenin (promoting its transcriptional activity) (Li et al., 2000; Taurin et al., 2006). Sustained PKA-dependent canonical Wnt signaling blocks a post-epithelialization morphogenetic step (conversion of the renal vesicle to the S-shaped body) in spinal cord-induced metanephric mesenchyme, resulting in disorganized epithelial clusters and large dilations (Gallegos et al., 2012). Overexpression of constitutively active PKA catalytic subunits can also act as a

negative regulator of planar cell polarity signaling and block convergent extension during Xenopus gastrulation (Song et al., 2003). Deletion of polycystin-1 increased cAMP and switched tubule formation by principal-like MDCK cells to cyst formation, and pharmacological elevation of cAMP in polycystin-1-competent cells caused cyst formation, impaired plasticity, nondirectional migration, and mis-orientation strongly resembling the phenotype of polycystin-1-deficient cells (Scholz et al., 2022). Mis-orientation of developing tubule cells in metanephric kidneys upon loss of polycystin-1 was also phenocopied by pharmacological increase of cAMP in wildtype kidneys. These observations suggest that cAMP triggers the initiation of cyst formation and not only promotes cyst enlargement in ADPKD.

Proliferation of the cystic epithelium

cAMP stimulates the proliferation of ADPKD cells but inhibits the proliferation of normal human kidney cells (Yamaguchi et al., 2003; Yamaguchi et al., 2004; Yamaguchi et al., 2006). The proliferative effect on ADPKD cells is due to aberrant crosstalk between intracellular calcium and cAMP. Intracellular calcium is reduced in the cystic epithelium and cAMP is increased. Restoration of intracellular calcium with a channel activator or low levels of a calcium ionophore converts the proliferative to an antiproliferative effect. In contrast, decreasing the intracellular calcium converts the antiproliferative to a proliferative response in the normal epithelium. How intracellular calcium determines whether cell proliferation is stimulated or inhibited by cAMP is not well understood. It has been proposed that a reduction in intracellular calcium de-represses BRAF causing cAMP activation of MEK-ERK and increased cell proliferation (Yamaguchi et al., 2004). Collecting duct specific expression of constitutively active BRAF induces cystogenesis in wildtype, and accelerates cyst growth, inflammation, and fibrosis in slowly progressive models of PKD (Ramalingam et al., 2021).

Fluid secretion into the cysts

Fluid secretion into the cysts is driven by cAMP-dependent chloride secretion, involving the basolateral Na-K-Cl cotransporter (NKCC1) and the apical cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (Sullivan et al., 1998; Grantham, 2003). The stimulation of chloride driven fluid secretion by cAMP is common to many secretory epithelia including normal collecting ducts and kidney tubules. Extracellular chloride entry by the basolateral NKCC1 (Kim et al., 1999; Gonin et al., 2001; Ortiz, 2006) raises its intracellular chloride concentration above the electrochemical gradient for chloride efflux. In the presence of cAMP agonists, CFTR channels are activated by PKA dependent phosphorylation and chloride flows into the cysts. The transepithelial transport establishes an electrical gradient for sodium transport through the paracellular pathway.

Importance of vasopressin

The evolution of vasopressin-related peptides dates back more than 700 million years, prior to the appearance of kidneys and pituitary gland (Juul, 2012). The evolutionary emergence of vasopressin, arginine vasopressin receptor 2 (Avpr2), and urine-concentrating mechanisms paralleled the development of loops of Henle and renal medulla and of nephron heterogeneity (short- and long-looped nephrons) in mammals. Lack of nephron heterogeneity in homozygous Brattelboro rats lacking vasopressin and induction of anatomic changes, i.e., hypertrophy and elongation of the thick ascending limb of Henle's loop and the inner stripe of the outer medulla by the administration of the vasopressin V2 receptor (V2R) agonist 1-deamino-8-d-arginine vasopressin suggest a mechanistic coupling of these evolutionary changes (Trinh-Trang-Tan et al., 1987).

Prolonged and sustained elevation of circulating vasopressin by osmotic stimulation or through continuous infusion of vasopressin for at least 3 days induces a proliferative response in cells expressing V2Rs (thick ascending limb of Henle and collecting duct) that was blocked by V2R but not by V1a or V1b receptor antagonists (Naito et al., 2001; Alonso et al., 2009). These observations suggest that prolonged V2R stimulation can induce to a cAMP-dependent proliferative phenotype in distal tubular and collecting duct cells (Figure 3).

Vasopressin acting on V2Rs is the main agonist of adenylyl cyclase in freshly dissociated collecting ducts (Yasuda and Jeffries, 1998). The kidneys are continuously exposed to the tonic action of vasopressin to avoid dehydration. This exposure is further enhanced in PKD owing to a concentrating defect distal to cAMP generation and PKA activation (Gattone et al., 1999; Seeman et al., 2004). Levels of cAMP are increased in cystic tissues (Yamaguchi et al., 1997). These observations together with the importance of cAMP for cyst initiation and progression provided a strong rationale for strategies to inhibit its production (Gattone et al., 1999; Gattone et al., 2003). Moreover, the almost exclusive localization of V2Rs on collecting ducts, connecting tubules, and thick ascending limbs of Henle (Mutig et al., 2007), the main sites of cystogenesis (Verani and Silva, 1988) predicted few off-target toxicities.

Preclinical trials targeting the vasopressin V2R and other GPCRs

Cyst development was markedly inhibited in PCK rats lacking circulating vasopressin (generated by crosses of PCK

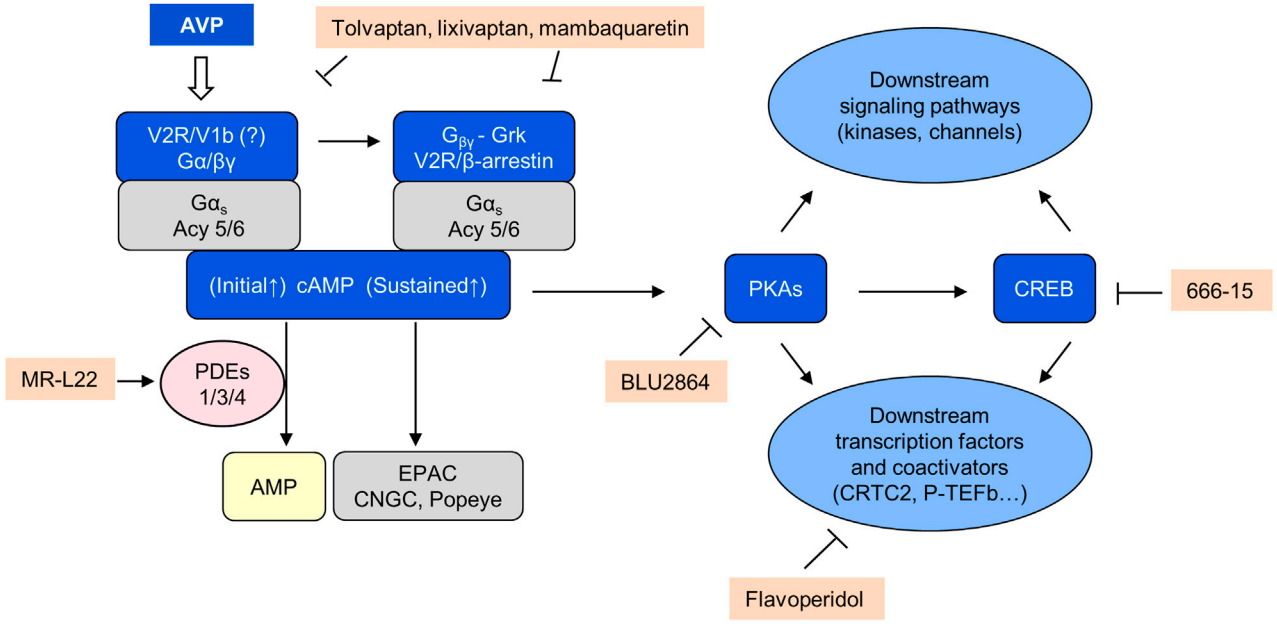

FIGURE 3
AVP/cAMP/PKA signaling.

and Brattleboro rats), an effect reversed by the administration of the V2R agonist 1-deamino-8-d-arginine vasopressin (Wang et al., 2008). Suppression of vasopressin by high water intake sufficient to achieve a 3.5-fold increase in urine output attenuated the progression of PKD in PCK and LPK rats but not in *Pkd1*$^{RC/RC}$ mice (Nagao et al., 2006; Hopp et al., 2015b; Sagar et al., 2019). Vasopressin V2 receptor antagonists (mozavaptan, tolvaptan, lixivaptan and mambaquaretin-1) ameliorated PKD in multiple orthologous (*Pkd2*$^{WS25/-}$, *Pkd1*$^{RC/RC}$ mice; PCK rat) and non-orthologous (pcy mouse) rodent models (Gattone et al., 2003; Torres et al., 2004; Wang et al., 2005; Zittema et al., 2016; Ciolek et al., 2017; Di Mise et al., 2019; Wang et al., 2019; Arroyo et al., 2021). Sustained suppression of vasopressin V2R was critical for the protective effect (Aihara et al., 2014).

Somatostatin, a hormone secreted by cells of the nervous system, gastrointestinal tract, and pancreatic islets, acts on five GPCRs (SSTR1 to 5) coupled to Gi proteins, inhibits cAMP generation in MDCK cells and rat collecting ducts, and antagonizes vasopressin effects in the toad urinary bladder and dog collecting ducts (Winkler et al., 1982; Friedlander and Amiel, 1986; Parnell et al., 2022). It also inhibits the secretion of several hormones and growth factors such as growth hormone, IGFI and vascular endothelial growth factor that promote cyst growth. Consistent with these observations, somatostatin analogs ameliorated PKD and polycystic liver disease in several orthologous models (*Pkd1*$^{RC/RC}$ and *Pkd2*$^{WS25/-}$ mice, and PCK rats) (Masyuk et al., 2007; Masyuk et al., 2013). Furthermore, tolvaptan and pasireotide, a synthetic analogue of somatostatin, had an additive effect inhibiting the development of the renal cystic disease in *Pkd1*$^{RC/RC}$ mice as characterized by decreased kidney weight to body weight ratio, cystic and fibrotic volume, and cAMP Level (Hopp et al., 2015a).

Other GPCRs coupled to Gαs proteins also may contribute to the development of PKD and their blockade has been protective in animal models. The β3-adrenergic receptor (β3-AR) is expressed the loops of Henle and cortical collecting ducts and is found at high levels in murine and human polycystic kidneys (Procino et al., 2016). Selective activation of β3-AR increases cAMP levels in isolated mouse renal tubules and activates key proteins involved in transepithelial water and solute movement. β3-AR blockade with a selective antagonist (SR59230A) decreases cAMP levels and ameliorates the cystic phenotype (Schena et al., 2021). TGR5 is overexpressed in cystic kidneys and livers and its activation by bile acids increases cAMP and proliferation. The TGR5 agonist, oleanolic acid, worsened renal and hepatic cystogenesis in PCK rats, whereas genetic elimination of *Tgr5* ameliorated the fibropolycystic liver disease of *Pkhd1* knockout mice (Masyuk et al., 2017). PGE_2 acting on EP2 or EP4 receptors promote cystogenesis in 3D-culture which is abolished by selective EP2 and EP4 antagonists (Lannoy et al., 2020). Unexpectedly, selective EP2 (PF-04418948) or EP4 (ONO-AE3-208) antagonists aggravated the cystic disease in two (hypomorphic and inducible) *Pkd1* mouse models, possibly related to unexpected pro-inflammatory effects (Lannoy et al., 2020).

Clinical trials indirectly or directly targeting GPCRs

Only 3 drugs are currently used in clinical practice to specifically treat PKD or polycystic liver disease (all targeting cAMP signaling), and only one has been approved by the FDA, EMA, and other regulatory agencies (tolvaptan).

Two large randomized clinical trials of the V2 receptor antagonist tolvaptan, TEMPO 3:4 in patients with CKD 1 and 2 and REPRISE in patients with an eGFR between 25 and 65, led to its approval for patients with rapidly progressive ADPKD (Torres et al., 2012; Torres et al., 2017). TEMPO 3:4 was enriched for patients with rapidly progressive disease and showed a reduction in kidney growth of 49% over 3 years. Both trials showed a reduction in the rate of decline of GFR. TEMPO 3:4 showed a reduction in kidney pain.

TEMPO 4:4, an open label extension of TEMPO 3:4, showed that the benefit of 3:4 was sustained and a single center longer follow-up study showed that it was cumulative over time (Torres et al., 2018a; Edwards et al., 2018). Two additional studies have been reported in the last year. An open label extension of REPRISE showed effectiveness of tolvaptan at very advanced stages of CKD (Torres et al., 2021). A pediatric randomized double-blind trial showed that the rates of kidney growth and eGFR decline were substantially less in the tolvaptan than in the placebo group, without reaching statistical significance, possibly due to the small size of the groups, heterogeneity of study patients, and short duration of follow-up (Mekahli et al., 2021).

Recently, the results of a randomized clinical trial of high prescribed water intake show no significant effect on the rate of kidney growth or decline of kidney function, but was inconclusive because the target 24 h urine osmolality was achieve in only half of the patients without any difference in the plasma copeptin levels between the groups (Rangan et al., 2022).

Three large trials have assessed the efficacy of somatostatin analogs for PKD, Aladin 1 and DIPAK mainly in patients with CKD 3 and Aladin 2 mainly in patients with CKD 3B and 4 (Caroli et al., 2013; Meijer et al., 2018; Perico et al., 2019). All showed a significant treatment effect slowing the rate of kidney growth. Two studies showed no significant effect on the rate of eGFR decline, one study showed a non-significant slowing trend after the first year, and another showed a reduced risk for doubling the serum creatinine or reaching ESKD. All the studies, plus additional trials on PLD, showed a significant treatment effect on liver growth (van Keimpema et al., 2009; Hogan et al., 2010; Hogan et al., 2012; Hogan et al., 2020).

Given the central and proximal role of cAMP, the efficacy of tolvaptan and somatostatin analogs in preclinical and clinical trials, and the inability of these drugs to completely block renal cAMP production, targeting other links in the cAMP signaling pathway seems logical. Currently the mechanisms by which cAMP promotes the development and progression of ADPKD are not completely understood and opportunities for targeting cAMP signaling in ADPKD have not been exhausted and remain feasible. Furthermore, binding of vasopressin or somatostatin to their GPCRs may also affect signaling pathways independent from cAMP.

Complexity of GPCR signaling

GPCRs consist of an extracellular amino-terminal domain, seven transmembrane spanning α-helices, and an intracellular carboxyl tail (Katritch et al., 2013; Zhang et al., 2015; Hilger et al., 2018; Chan et al., 2019; Sussman et al., 2020). Ligand binding to GPCRs rearranges its transmembrane helices and facilitates coupling to heterotrimeric G-proteins. These are composed of Gα subunits (Gαs, Gαi/o, Gαq/11 or Gα12/13) and Gβγ-dimers. Ligand binding promotes exchange of GDP bound to the Gα subunit for GTP. GTP-bound Gα dissociates from the receptor and from Gβγ, and Gα and Gβγ separately mediate downstream G protein signaling. Gαs and Gαi bind directly to adenylyl cyclase increasing or decreasing cAMP production. Gαq and Gα11 activate phospholipase C. Phospholipase C hydrolyses membrane lipid phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol, each initiating a signal transduction cascade. Gα12 and Gα13 activate Rho. Gβγ subunits interact with many effector proteins and have many biological functions. GPCR-bound Gβγ recruits GPCR kinases (GRK) that phosphorylate specific serine and threonine residues of agonist-activated GPCRs within its carboxy terminal domain and promote the binding of β-arrestins.

Relevance of β-arrestins as negative and positive regulators of vasopressin and G protein signaling

The canonical β-arrestin function is the negative regulation of G protein signaling by several mechanisms (Jean-Charles et al., 2017; Peterson and Luttrell, 2017). β-arrestins bind to phosphorylated residues in the GPCR C-terminal tail and in the transmembrane core (third intracellular loop). Since the binding site in the GPCR core overlaps with the G-protein-binding site, β-arrestin recruitment hinders further G protein activation. β-arrestins couple GPCRs to clathrin and adaptor protein-2, which mediate endocytosis of GPCRs and desensitization. Furthermore, β-arrestins may limit GPCR signaling by acting as a scaffold for cyclic nucleotide phosphodiesterases and diacylglycerol kinases.

More recent evidence suggests that some internalized GPCR/β-arrestin complexes result in sustained activation of adenylyl-cyclase and/or β-arrestin dependent activation of Src, extracellular signal regulated kinase (ERK1/2), c-Jun-N-terminal kinase (JNK), and p38 MAPK (Shenoy et al., 2006;

Pakharukova et al., 2020; Kim et al., 2022). The extent of GRK-mediated phosphorylation determines the stability of GPCR/β-arrestin complexes. GPCRs with few phosphorylation sites (e.g., β2 adrenergic receptor) interact with β-arrestin in endosomes with low affinity and rapidly recycle back to the plasma membrane. GPCRs with many phosphorylation sites (e.g., vasopressin V2 receptor or V2R) exhibit sustained high-affinity interactions with β-arrestin in endosomes and slowly recycle or traffic to lysosomes for degradation (Luttrell et al., 2001; Beautrait et al., 2017; Jean-Charles et al., 2017; Thomsen et al., 2018; Baidya et al., 2020). When β-arrestins bind only through the C tail to promote internalization, the receptor core region is exposed and the GPCR can interact simultaneously with both G proteins and β-arrestins and induce G-protein and/or β-arrestin signaling. Thus, β-arrestins can act as negative or positive regulators of G protein signaling at the plasma membrane or endosomes, respectively. Once activated GPCRs can selectively promote the activation of G-protein or β-arrestin signalling, a phenomenon known as functional selectivity or ligand bias.

The affinity of the agonist for the receptor and the stability of the agonist/GPCR interaction also affects the balance between plasma membrane and endosomal signaling. Both vasopressin and oxytocin bind to the V2R. Vasopressin binds tightly to the V2R, which results in prolonged internalization and endosomal G protein signaling, whereas oxytocin binds to V2R with lower affinity and dissociates from the receptor soon after internalization, which results in predominant plasma membrane G protein signaling (Thomsen et al., 2018). While vasopressin promotes cAMP generation at the plasma membrane and sustained cAMP accumulation at endosomes after β-arrestin mediated receptor internalization (Figure 3), oxytocin only induces a transient generation of cAMP at the plasma membrane and does not cause β-arrestin binding or V2R internalization. Consistent with these effects, vasopressin has strong antidiuretic and antinatriuretic effects as opposed to oxytocin which has weak effects only measurable in the absence of natural vasopressin. The selective β-arrestin/β2-adaptin inhibitor barbadin prevents the vasopressin-promoted endocytosis of the V2 receptor, cAMP accumulation, and ERK1/2 activation (Beautrait et al., 2017).

Several observations suggest that V2R/β-arrestin signaling may be important in PKD. First, the effect of vaptans on PKD is moderate compared to that of genetic elimination of circulating vasopressin (Wang et al., 2008). This may be because vaptans inhibit the V2R-dependent G-protein signaling but exhibit a partial agonist activity on β-arrestin recruitment and MAPK activation. Second, although secretin, like vasopressin, activates adenylyl cyclase in the outer medulla and decreases urine output in wild-type and Brattleboro rats, genetic elimination of the secretin receptor and administration of exogenous secretin to PCK or PCK/Brattleboro rats or to *Pkd2*$^{-/WS25}$ mice did not significantly affect PKD (Wang et al., 2012). This may be because the internalization of the secretin receptor is unaffected by GRK mediated phosphorylation or by the expression of dominant negative β-arrestin (Walker et al., 1999). Recruitment of β-arrestins to GPCRs after agonist activation is to large extent dependent on β/γ signaling. Third, inhibition of β/γ signaling using gallein or knocking out the G-protein β subunit, likely inhibiting β-arrestin signaling, corrected the phenotype of Xenopus *pkd1* morphants and inhibited cystogenesis of *Pkd1*$^{-/-}$ cells in 3D culture (Zhang et al., 2018). Finally, expressions of β-arrestin 1 and/or 2 are increased in murine polycystic and human ADPKD kidneys (Xu et al., 2018).

At present, the role of β-arrestins in the pathogenesis of PKD remains unexplored. Interestingly, vaptans block cAMP generation but moderately stimulate β-arrestin signaling, whereas the mambaquaretin-1, a peptide from green mamba venom, inhibits both, cAMP generation and β-arrestin signaling (Ciolek et al., 2017). A study comparing the effectiveness of vaptans and mambaquaretin-1 has not been done.

Adenylyl cyclases as therapeutic targets in ADPKD

cAMP is produced by nine membrane-bound adenylyl cyclase (AC) isoforms (ACs1–9) activated by GαsPCRs or one soluble AC (AC10) (Dessauer et al., 2017; Bassler et al., 2018) activated by bicarbonate and calcium. ACs 6 and 5 mRNA and protein levels are increased, whereas AC3 levels are decreased in ADPKD compared with NHK kidneys and cells. Calcium directly inhibits AC5 and AC6, whereas calcium/calmodulin activates and CaMKII inhibits AC3. All three contribute to the synthesis of cAMP in response to vasopressin (Strait et al., 2010). AC6 knockout mice have a concentrating defect, whereas AC3 knockout mice are normal, casting doubt on the relevance of AC3 in the regulation of water permeability of the collecting duct (Roos et al., 2012; Kittikulsuth et al., 2014). The cAMP increase in response to vasopressin *in vitro* is blunted in ADPKD compared to NHK cells and is mediated by AC3 (Pinto et al., 2012). AC3 inhibition does not affect AVP-induced cAMP production in NHK cells. The different response is thought to be due to reduced intracellular calcium, since it is reproduced in NHK cells treated with a calcium chelator or a calcium channel blocker. The relevance of these observations to *in vivo* conditions is uncertain. Mice with a collecting duct-specific double knockout of PC1 and AC6 have markedly decreased kidney and renal cyst volumes, improved renal function, reduced activation of the B-Raf/ERK/MEK pathway, and increased survival compared to mice with collecting duct-specific knockout of PC1 alone (Rees et al., 2014). In contrast, mice with a conditional *PC2/AC6* double knockout have no decrease in liver cyst volume compared to *Pkd2* knockout alone, whereas AC5 siRNA and/or inhibitors inhibit cAMP production and pERK1/2 expression by PC2 deficient

cholangiocytes, growth of PC2 deficient biliary organoids, and liver cystic area and cell proliferation in conditional *Pkd2* knockout mice. A third study showed that knockdown of either AC5 or AC6 attenuated the increase in cAMP levels in PC2 deficient renal epithelial cells (Wang et al., 2018) and that *AC5*/*Pkd2* double mutant mice had less kidney enlargement, lower cyst index, and improved kidney function compared to *Pkd2* mutant mice.

Adenylyl cyclase inhibition has not been directly tested in animal models of PKD. Nevertheless, it might have contributed to the effect observed with some drugs directed against other targets. Metformin inhibits complex 1 of the mitochondrial respiratory chain (El-Mir et al., 2000), lowering ATP production and increasing AMP (Owen et al., 2000). AMP in turn inhibits adenylyl cyclase and activates AMPK. The inhibition of adenylyl cyclase (Miller et al., 2013) and activation of phosphodiesterase PDE4B by AMPK-mediated phosphorylation (Johanns et al., 2016) have been found to lower cAMP in response to metformin. Statins may lower cAMP through the downregulation of Gα s protein (Kou et al., 2012). Demeclocycline, and to a lesser extent doxycycline and other tetracycline antibiotics, have been shown to reduce urinary concentrating ability and have been used to treat the syndrome of inappropriate antidiuretic hormone in humans (Kortenoeven et al., 2013). In part, this is due to their capacity to reduce the expression of AC5, AC6 and AC3 without affecting their protein stability. By this mechanism, they could have a beneficial effect on PKD. Treatment with doxycycline aimed at inhibiting metalloproteinases indeed inhibited epithelial cell proliferation and cystic disease progression in PCK rats (Liu B. et al., 2012). However, the nephrotoxicity of these drugs when used at high doses may limit their potential for the treatment of PKD; for example, doxycycline at a high dose was found to aggravate cyst growth and fibrosis in pcy mice (Osten et al., 2009).

cAMP phosphodiesterases as therapeutic targets in ADPKD

Phosphodiesterases control cAMP accumulation by promoting cAMP degradation. PDE 1, 3 or 4 control cAMP pools that regulate cell proliferation, fluid secretion, and cystogenesis in human cyst-derived epithelial cells *in vitro*, zebrafish and mouse models (Wang et al., 2010; Pinto et al., 2016; Sussman et al., 2016; Ye et al., 2016; Wang et al., 2017). Therefore, it seems likely that PDE activators would have a beneficial effect. Unfortunately, development of PDE activators has almost been non-existent, in contrast with great interest on the development and clinical applications of PDE inhibitors.

Long forms of PDE4 are activated by PKA phosphorylation as a feedback mechanism to terminate physiological cAMP mediated signaling. Small molecule compounds that mimic the effect of PKA phosphorylation and inhibit forskolin induced accumulation of cAMP have been developed by Mironid (Omar et al., 2019). These compounds inhibit *in vitro* cystogenesis of IMCD3 cells in matrigel and have been shown to lower kidney cAMP and ameliorate PKD in $Pkd1^{RC/RC}$ mice to a degree comparable to tolvaptan with less effect on urine output (Henderson et al., 2020).

It has been proposed that reduced cytosolic calcium is responsible for inhibition of the calcium dependent PDE1 and increased cAMP levels in cystic tissues and for the proliferative response of these cells to cAMP. Both provide a rationale for treatments increasing intracellular calcium. TRPV4 activators significantly alleviated cystic disease development in PCK rats (Gradilone et al., 2010; Zaika et al., 2013). Administration of triptolide, a drug that has been proposed to activate the PC2 channel by a poorly understood mechanism, has an inhibitory effect on the development of PKD in $Pkd1^{-/-}$ embryos and in kidney specific *Pkd1* knockout mice and in Han:SPRD rats (Leuenroth et al., 2007; Leuenroth et al., 2008; Leuenroth et al., 2010; Jing et al., 2018). Activation of the calcium-sensing receptor has been shown to increase intracellular calcium and decrease cAMP and mTOR in *PKD1* deficient cells (Di Mise et al., 2018). Calcimimetic agents ameliorated PKD in Cy/+ Han:SPRD rats and pcy mice, but not (except for reduced fibrosis) in PCK rats and $Pkd2^{WS25/-}$ mice, possibly because the potential beneficial effect of the drug was offset by marked hypocalcemia (Gattone et al., 2009; Wang et al., 2009; Chen et al., 2011). More recently, a lower dose of calcimimetic R-568 decreased intracellular cAMP level by the activation calcium-sensing receptor, reduced cyst progression in PCK rats and $Pkd1^{RC/RC}$ mice, and had an additive effect when given in combination with the V2 receptor antagonist lixivaptan (Di Mise et al., 2021). On the other hand, interventions that lower intracellular calcium aggravate the development of PKD. The administration of verapamil aggravated cyst development in Cy/+ Han:SPRD rats (Nagao et al., 2008). Treatment of zebrafish with CaV1.2 morpholinos induced pronephric duct cysts (Jin et al., 2014). Lentiviral transfection of CaV1.2 shRNA aggravates the cystic phenotype in $Pkd1^{+/-}$ mice (Jin et al., 2014).

PKA as a direct therapeutic target

Direct inhibition of PKA is appealing because PKA can also be activated in PKD independently from cAMP by mechanisms associated with oxidant conditions, NFkB activation and TGFb activation (Zhong et al., 1997; Dulin et al., 2001; Zhang L. et al., 2004; Brennan et al., 2006; Yang H. et al., 2008; Lignitto et al., 2011; Zhou et al., 2012; Lignitto et al., 2013; Liu et al., 2014; Rinaldi et al., 2015; Hama et al., 2017; Sun et al., 2017). PKA is a tetramer with two catalytic subunits that remain inactive while bound to two regulatory subunits (Skalhegg and Tasken, 2000; Tasken and

Aandahl, 2004; Yu et al., 2012; Baro Graf et al., 2020). When cAMP binds to the regulatory subunits the catalytic subunits are released and become active. Knocking out *Prkar1a* (coding for RIα, the most ubiquitous and important regulatory subunit) results in constitutive PKA activation (Bossis and Stratakis, 2004; Kirschner et al., 2005). Breeding of floxed *Prkar1a*, *Pkhd1*-Cre, and *Pkd1*$^{RC/RC}$ mice was used to generate mice with kidney specific constitutively active PKA on a *Pkd1* hypomorphic background and on a wild-type background (Ye et al., 2017). Constitutive kidney specific PKA activation stimulated multiple downstream signaling pathways (Src, Ras, ERK1/2, mTOR, GSK3β, β-catenin) and transcription factors (CREB, Stat3, Pax2) and markedly aggravated PKD. By itself constitutive kidney specific PKA activation on a wild-type background stimulated the same downstream signaling pathways and transcription factors and was sufficient to induce cystogenesis and fibrosis without much kidney enlargement. The presence of kidney and liver cysts in patients with the Carney complex, an autosomal dominant multitumoral disease caused by a heterozygous mutation in *PKAR1A*, supports the relevance of these observations for human ADPKD (Ye et al., 2017). More recently, mice with a conditionally expressed *Prkar1a* mutation (that makes RIα unable to release catalytic subunits in the presence of cAMP), *Pkhd1*-Cre and *Pkd1*$^{RC/RC}$ mice were used to generate mice with a constitutive kidney specific downregulation of PKA and their controls (Wang et al., 2022). Constitutive kidney specific PKA downregulation inhibited the same downstream signaling pathways and transcription factors activated by PKA constitutive activation and attenuated PKD.

Although these results provide a strong rationale for the utilization of PKA inhibitors to treat PKD, this has not been feasible because available PKA inhibitors are poorly selective and unsuitable for *in vivo* studies (Murray, 2008). Recently, highly specific and potent PKA inhibitors have been developed by Blueprint (Schalm et al., 2022). One of these compounds ameliorated the development of PKD in the *Pkd1*$^{RC/RC}$ with less increase in urine output compared to tolvaptan (Wang et al., 2022).

Signal transduction kinases regulated by PKA

Many signal transduction kinases have been shown to play a role in the pathogenesis of PKD. Cyclic AMP/PKA signaling may directly or indirectly regulate most of them (Figure 4).

MAPK pathway

The classical MAPK signalling pathway is implicated in growth-factor and tyrosine kinase receptor-mediated cell proliferation. It consists of a cascade of three consecutive phosphorylation steps exerted by MAP3Ks (RAF proteins), MAP2Ks (MEK1/2), and MAPKs (ERK1/2) (Cuarental et al., 2019). Activated ERK1/2 translocate to the nucleus, where they phosphorylate and stabilize several transcription factors that are involved in the early phases of the G1–S cell cycle transition and the upregulation of glucose transporters and several rate-limiting glycolytic enzymes. In wild-type renal epithelial cells, PKA phosphorylates and inhibits Raf1 and MAPK signaling. In ADPKD cells or with calcium deprivation (lowering extracellular calcium concentrations or using calcium channel blockers) PKA increases the expression and activity of B-Raf in a Ras and Src dependent manner bypassing the inhibition of Raf-1 (Yamaguchi et al., 2004). It was proposed that calcium deprivation causes this phenotypic switch by inhibiting of PI3K/Akt signaling which in turn increases the levels of B-Raf levels via adjustments in synthesis and/or turnover rate.

Targeting Raf/MEK/ERK has given inconsistent results, possibly due to redundancies with other pathways. PLX5568 (Buchholz et al., 2011a), a Raf kinase inhibitor, attenuated cyst enlargement *in vitro* and in cy/+ Han:SPRD rats, but had no effect on kidney/body weight ratio or kidney function and promoted hepatic and renal fibrosis. Sorafenib, a Raf kinase inhibitor with activity against vascular endothelial growth factor receptor and platelet-derived growth factor receptor kinases, inhibited cAMP-dependent activation of B-Raf/MEK/ERK signaling, cell proliferation, and growth of ADPKD cysts *in vitro*. In contrast, sorafenib stimulated pERK1/2 and proliferation of *Pkd2* knockout cells *in vitro* and augmented ERK activation, cell proliferation and hepatic cystogenesis in *Pkd2* knockout mice (Yamaguchi et al., 2010; Spirli et al., 2012). Assays of Raf kinase activity showed that sorafenib inhibited B-Raf in wild-type and *Pkd2* knockout cells and inhibited Raf-1 in wild-type cells, but stimulated Raf-1 in *Pkd2* knockout cells. Pre-treatment with a PKA inhibitor or co-treatment with octreotide abolished the paradoxical activation and inhibited cyst growth. The MEK inhibitor PD184352 ameliorated PKD in pcy mice (Omori et al., 2006), but the MEK inhibitor UO126 had no effect on cystogenesis in *Pkd1* knockout mice at doses sufficient to reduce phospho-ERK1/2 in cystic kidneys (Shibazaki et al., 2008).

Non-receptor tyrosine kinase Src

PKA phosphorylates c-Src on serine-17 to regulate its activity. Being a common link downstream from cAMP/PKA and the tyrosine receptor kinases pathway, Src is an attractive therapeutic target for ADPKD. Bosutinib, a Src/Abl inhibitor, was found to ameliorate PKD in bpk and *Pkd1* heterozygous mice and in PCK rats (Sweeney et al., 2008; Elliott et al., 2011). A phase II clinical trial (NCT01233869) showed a reduction in the rate of kidney growth but has not been further pursued for the treatment of ADPKD because of lack of effect on kidney function and high

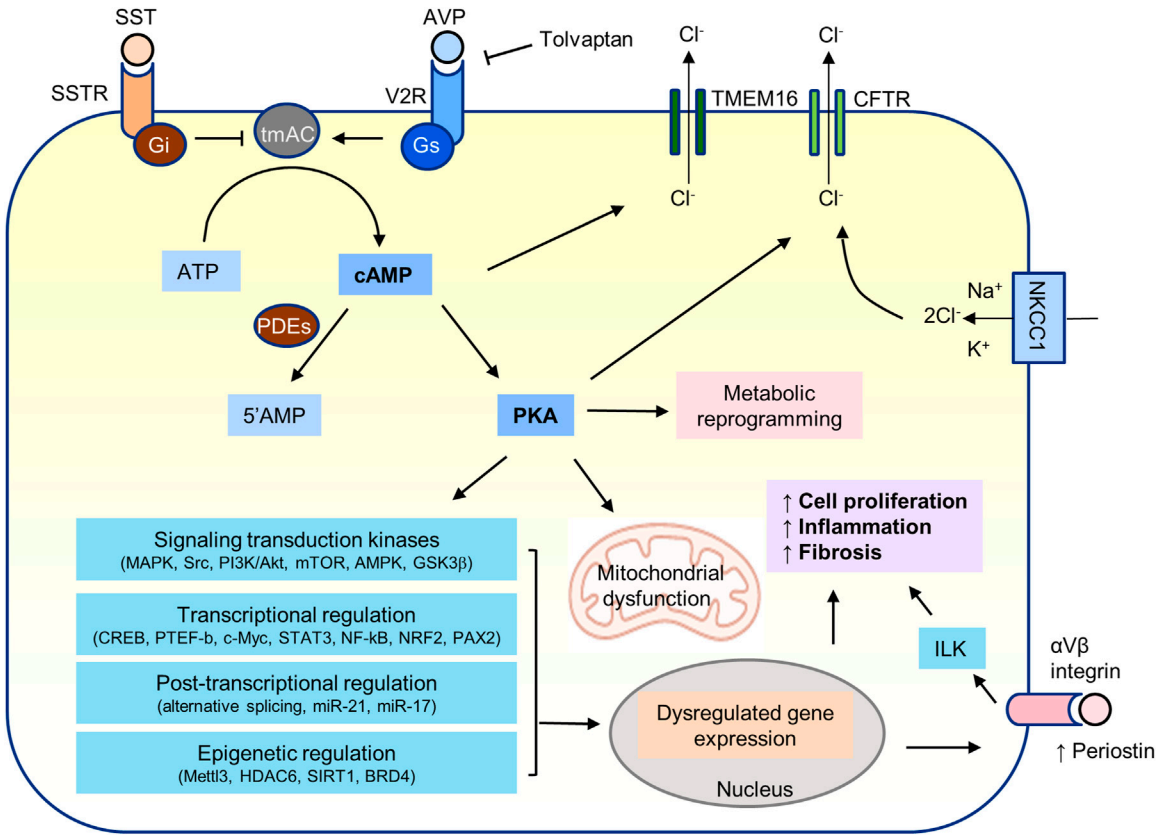

FIGURE 4
cAMP/PKA signaling in PKD.

rate of discontinuation due to frequent adverse events (Tesar et al., 2017). Tesevatinib, an inhibitor of Src and multiple tyrosine kinase receptors (EGFR, ERBB2, and VEGFR2), ameliorated PKD and liver cystic disease in bpk mice and PCK rats. Phase I clinical trials have been completed and a phase II trial is ongoing, but no results have been published.

Phosphoinositide 3-kinase and AKT serine/threonine kinase

PI3Ks are activated by receptor tyrosine kinases and phosphorylate membrane-associated phosphatidylinositol-4,5-bisphosphate to yield phosphatidylinositol-3,4,5-triphosphate (PIP3) (Margaria et al., 2020). PIP3 activates 3-phosphoinositide-dependent protein kinase 1 (PDK1), which in turn phosphorylates and activates Akt. The activity of PI3K is opposed by PTEN (Phosphatase and Tensin Homolog), which dephosphorylates PIP3 back to PIP2. PI3K and Akt stimulate cell proliferation via tuberin phosphorylation and activation of mTORC1. PI3K and Akt families consist of multiple members, eight (divided in three classes) and three respectively, with distinct functions. While cystic tissues from patients and rodent models of PKD exhibit enhanced phosphorylation of PI3K and Akt and PI3K/AKT signaling may promote cystogenesis (Wahl et al., 2007; Conduit et al., 2020), inhibition of PI3K/Akt signaling has also been proposed to be responsible for the proliferative response of the cystic epithelium to cAMP (Yamaguchi et al., 2004). Furthermore, class II PIK3C2A has been shown to inhibit cystogenesis by promoting correct cilia formation and targeting of polycystin-2 (Franco et al., 2016). Both genetic and pharmacologic downregulation of PKA have been shown to inhibit Akt phosphorylation in *Pkd1*$^{RC/RC}$ mice, possibly through interference in one or more pathways associated with AKT activation or inhibition of PTEN downstream from PKA (Wang et al., 2022).

Mammalian target of rapamycin

mTOR functions as two distinct PKD multi-protein kinase-signaling complexes, mTORC1 and mTORC2 (Margaria et al., 2020). Downstream from PKA, ERK mediated phosphorylation

and Akt activate mTORC1 in PKD (Rowe et al., 2013). Activated mTORC1 phosphorylates and activates 4E-BP (eukaryotic translation initiation factor 4E-binding protein) and S6K (S6 kinase) and controls mRNA translation, mitochondrial activity and biogenesis, and metabolic reprogramming (Hsieh et al., 2012; Thoreen et al., 2012; Morita et al., 2013). ERK-dependent inhibition of LKB1 (liver kinase B1) inhibits AMPK, which may further enhance mTORC1 signaling. At doses and blood levels achievable in humans, mTORC1 inhibiting rapalogs (sirolimus and everolimus) ameliorated PKD in cy/+ Han:SPRD rats, a model affecting proximal tubules, but not in PCK rats, a model affecting the distal nephron and collecting duct (Shillingford et al., 2006). Mice tolerate much higher doses and blood levels than rats and humans. These high doses of rapalogs were consistently effective in orthologous and non-orthologous mouse models. However, the results of clinical trials have been mostly discouraging (NCT00346918; NCT00491517; NCT00414440) (Serra et al., 2010; Walz et al., 2010), likely because achievable blood levels capable of inhibiting mTOR in peripheral blood mononuclear cells do not inhibit mTOR in the kidney or because mTORC1 inhibition triggers a compensatory activation of mTORC2, PI3K and Akt (Canaud et al., 2010).

AMP-activated protein kinase

AMPK is a sensor of the cellular energy status reflected by the ratio AMP and ADP to ATP. AMP and ADP activate AMPK through an allosteric effect that facilitates its phosphorylation LKB1 and maintain AMPK in an active state by blocking its dephosphorylation by phosphatases. AMPK stimulates oxidative phosphorylation and fatty acid oxidation and inhibits aerobic glycolysis via activation of the tuberin/hamartin complex and inhibition of mTORC1. Drugs and xenobiotics that activate AMPK may be beneficial in PKD by inhibiting cell proliferation and chloride driven fluid secretion (Hallows et al., 2003). Metformin activates AMPK indirectly by inhibiting mitochondrial respiration (El-Mir et al., 2000). The results of preclinical studies of metformin for PKD have given inconsistent results. Metformin has been reported to be protective on *in vitro* and *ex vivo* renal cystogenesis, and *in vivo* in two rapidly progressive and in one slowly progressive *Pkd1* mouse models (Takiar et al., 2011; Pastor-Soler et al., 2022). In contrast metformin was detrimental in hypomorphic *Pkd1* miRNA transgenic mice (Chang et al., 2022). Another study compared two indirect (metformin and canagliflozin) and one direct (salsalate) AMPK activators in an inducible adult onset *Pkd1* knock-out mouse model (Leonhard et al., 2019); only salsalate had a protective effect. In the PCK, rat metformin ameliorated the liver disease but had no effect on the kidney disease (Sato et al., 2021). Oral metformin treatment was reported to slow PKD progression in a miniature pig model (Lian et al., 2019). Surprisingly, mouse models with a global or kidney specific expression of constitutively active AMPK, with a gain-of-function mutation in the AMPKγ1 subunit, resulted in an early-onset polycystic kidney phenotype with collecting duct cysts, compromised renal function, increased cAMP levels, ERK activation and hexokinase I expression (Wilson et al., 2021). Another study described cystic kidneys and impaired kidney function in mice expressing an activating mutation in the γ2 subunit of AMPK (Yang et al., 2016). Possibly, the timing (initiating versus progressing phases of the disease) and level of AMPK activation may account for these conflicting results.

The results of two phase II double-blinded randomized placebo-controlled trials, the TAME PKD (Trial of Administration of Metformin in PKD, NCT02656017) at Tufts University and University of Maryland in 97 adult patients with eGFR >50 ml/min/1.73 m^2 treated for 2 years (Perrone et al., 2021) and the second (Feasibility Study of Metformin Therapy in ADPKD, NCT02903511) in the University of Colorado in 51 adult patients with eGFR 50–80 ml/min/1.73 m^2 treated for 1 year (Brosnahan et al., 2022) have been recently reported. Both studies found that the administration of metformin was safe and tolerable. Changes in total kidney volume and eGFR in these underpowered studies were not significantly different between the groups.

Glycogen synthase kinase β

GSK3β is a ubiquitously expressed and constitutively active serine/threonine protein kinase (Beurel et al., 2015). In normal mouse kidneys, GSK3β positively regulate cAMP generation in response to AVP (Rao et al., 2010). Mice with renal collecting duct-specific gene knockout of GSK3β have reduced adenylate cyclase activity, cAMP generation, and ability to concentrate urine. A positive feed-forward mechanism has been described whereby cAMP and CREB-mediated signaling stimulate GSK3β expression and GSK3β in turn enhances cAMP generation and CREB activity (Kakade et al., 2016). In mouse models of PKD, GSK3β expression increases progressively with age in parallel with the increase in renal cAMP levels. Collecting duct-specific gene knockout of GSK3β or treatment with the specific GSK3β inhibitor TDZD-8 (4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione) ameliorated PKD in cpk and kidney specific *Pkd1* knockout mice (Tao et al., 2015). This was accompanied by a significant reduction cyclin-D1 and c-Myc, whereas β-catenin levels were increased. The increase in β-catenin levels, which may be due to the inhibition of GSK3b that negatively regulates its cytoplasmic accumulation, suggests that β-catenin may not be critical for proliferation of the cyst-lining epithelium in these animal models.

Regulation of ion channels relevant to polycystic kidney disease

Cystic fibrosis transmembrane conductance regulator

Active transport of chloride from the basolateral to the apical side is the driving force for fluid secretion into the cysts (Figure 4) (Sullivan et al., 1998; Grantham, 2003; Jouret and Devuyst, 2020). The energy is generated by the sodium pump (Na, K-ATPase) in the basolateral membrane of cyst epithelial cells. Chloride enters from the basolateral side through the sodium-potassium-chloride cotransporter (NKCC1) and uses the gradient established by the sodium pump to bring potassium and chloride into the cells. PKA-induced phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) in the apical membrane opens the channel and allows the flow of chloride ions down an electrochemical gradient into the cyst, generating increased transepithelial electron activity that, in turn, drives sodium ions through paracellular pathways. Cyclic AMP and PKA signaling also promote the surface expression of NKCC1 in the thick ascending limb of Henle and of functional Na,K-ATPase units in cortical collecting duct principal cells (Kim et al., 1999; Gonin et al., 2001; Ortiz, 2006).

CFTR blockers (glibenclamide, NPPB, genistein) have been shown to reduce cyst growth and cAMP stimulated chloride currents in MDCK cyst in collagen gel (Magenheimer et al., 2006; Lu et al., 2010). CFTR thiazolidinone inhibitors stabilize the channel in the closed state and inhibited cystogenesis *in vitro*, *ex vivo* (metanephric kidney organ culture), and *in vivo* (rapidly progressive kidney-specific *Pkd1* knock-out mice (Yang B. et al., 2008; Snyder et al., 2011)). Steviol has been reported to promote CFTR degradation by the proteasome, inhibit MDCK cystogenesis *in vitro* and in kidney specific *Pkd1* knockout mice treated with high intraperitoneal doses (Yuajit et al., 2013; Yuajit et al., 2014; Nantavishit et al., 2018). The proton pump inhibitor Lansoprazole has also been recently proposed to reduce kidney cysts *in vitro* (MDCK cells) and *in vivo* (PCK rat) via activation of the liver X receptor and subsequent down-regulation of CFTR (Nantavishit et al., 2018). Treatment of *Pkd1* knockout mice with the CFTR regulator VX-809 (Lumacaftor) increased the localizations of CFTR in the basolateral membrane of cyst lining cells and those of the sodium proton exchanger 3 and the epithelial sodium channel in the apical membrane, thus promoting net resorption of cyst fluid, reducing cyst growth, and protecting kidney function (Yanda et al., 2018). A phase 2, placebo-controlled randomized controlled trial (NCT04578548) to investigate the safety and tolerability of the CFTR inhibitor GLPG2737 in ADPKD patients at risk for rapidly progressive disease((U.S.), 2020) is ongoing.

TMEM16A (anoctamin-1)

Recent studies suggest that the calcium-dependent chloride channel TMEM16A may also promote chloride driven fluid secretion into the cysts (Buchholz et al., 2011b; Buchholz et al., 2014; Kraus et al., 2016; Schreiber et al., 2019). It is upregulated in the apical membrane of human cyst-lining cells and may be activated by ATP in the cyst fluid acting on P2Y receptors (Buchholz et al., 2011b). Apyrase (ATP scavenger) and suramin (P2 receptor inhibitor) reduced cAMP-driven fluid secretion in MDCK cysts while increasing extracellular ATP potentiated cAMP-mediated cyst growth, suggesting a synergistic interaction between CFTR and TMEM16A. TMEM16A inhibitors and morpholinos inhibited cyst growth in metanephric kidney cultures (Buchholz et al., 2014). Tubule-specific knockout of TMEM16A and TMEM16A inhibitors ameliorated PKD in an adult *Pkd1* orthologous mouse model (Cabrita et al., 2020). In contrast, the effect of deleting of CFTR together with *Pkd1* was not statistically significant in a small number of adult mice (5 and 7) (Talbi et al., 2021). The presence of TMEM16A was reported to be necessary for the expression of CFTR at the plasma membrane. It has been suggested that TMEM16A activation, linked to local hypoxia and stabilization of the hypoxia-inducible transcription factor-1α (HIF-1α) promoting the expression of P2YR, may be more important at advanced than at early stages of PKD (Kraus et al., 2018).

PKA regulation of extracellular matrix

Alterations in focal adhesion complexes, basement membranes, and extracellular matrix contribute to the pathogenesis of PKD. Focal adhesion complexes contain integrin αβ heterodimer receptors, which link the actin cytoskeleton to basement membrane laminin αβγ heterotrimers, collagens and matrix proteins. Abnormal expression of these proteins accelerates cyst growth through activation of integrin signaling (Daikha-Dahmane et al., 1997; Joly et al., 2003; Shannon et al., 2006; Wu et al., 2009).

Periostin is a secreted matricellular protein that binds to αVβ3 and αVβ 5 integrins and is expressed during development and tissue remodeling. In a microarray analysis of cultured human ADPKD cyst epithelial cells, periostin mRNA was markedly overexpressed compared with normal human kidney cells (Wallace et al., 2008). Periostin overexpression, which is important for the development of myocardial infarction induced myocardial fibrosis, can be prevented by siRNA or shRNA CREB downregulation both *in vitro* and *in vivo* (Xue et al., 2022). It seems likely that the overexpression of periostin in cystic kidneys is also driven by CREB likely downstream from PKA signaling (Figure 4).

ADPKD cells secret periostin across luminal and basolateral plasma membranes. Periostin binds to αVβ3 and αVβ integrins, activates the integrin-linked kinase (ILK), a component of focal adhesion plaques, and promotes *in vitro* cyst growth (Wallace et al., 2008). Pharmacologic inhibition or shRNA knockdown of ILK prevented periostin-induced Akt/mTORC1 signaling and ADPKD cell proliferation *in vitro*. Knocking out periostin (Postn) inhibited mTOR signaling, cell proliferation and interstitial fibrosis, and ameliorated PKD in pcy mice (Wallace et al., 2014). Lowering the expression of ILK had similar effects in rapid and slowly progressive kidney-specific *Pkd1* knockouts. Whereas heterozygous knockdown of ILK in collecting ducts of wild-type mice had no effect on renal morphology or function, complete ILK knockout caused caspase-3 mediated anoikis, dilated cortical tubules with apoptotic cells, interstitial fibrosis, and death by 10 weeks of age (Raman et al., 2017). Because complete knockout of ILK in collecting duct cells caused renal injury, long-term use of an ILK inhibitor may not be feasible.

Transcriptional regulation downstream from PKA

Many transcription factors implicated in the pathogenesis of PKD are regulated by cAMP and PKA signaling among other pathways (Figure 4).

Cyclic AMP response element-binding protein

Under basal conditions the regulatory and catalytic PKA subunits colocalize in cytoplasmic puncta (Zhang et al., 2020). In stimulated cells, cAMP generated by internalized agonist-bound Gαs protein-adenylyl cyclase complexes in endosomes diffuses to adjacent puncta releasing the catalytic subunits that enter nuclei and phosphorylate substrates involved in transcriptional regulation. PKA phosphorylates and activates the cAMP responsive element binding protein (CREB) family of transcription factors, CREB1, cAMP responsive element modulator (CREM), and activating transcription factor 1 (ATF-1) (Mayr and Montminy, 2001; Rosenberg et al., 2002). Phosphorylated CREB recruits the coactivators CREB binding protein (CBP) or p300 and bind to cAMP-response elements (CREs) in the genome to drive transcription of target genes. CBP and p300 are histone acetyltransferases that enhance the ability of CREB to activate transcription by relaxing the chromatin structure at gene promoter regions and creating scaffolds for recruitment of RNA polymerase II complexes to the promoter.

CREB is phosphorylated and hyperactive in ADPKD (Ye et al., 2017). An integrative analysis using cleavage under targets and release using nuclease (CUT&RUN), RNA-sequencing, and rescue of differentially expressed genes by treatment with a selective CREB inhibitor (665-15) identified the genomic loci bound to phosphorylated CREB in cystic epithelial cells (Liu et al., 2021). Function enrichment analysis of CREB direct targets revealed prominent enrichment of genes related to cell proliferation and inflammation-related pathways, including ribosome biogenesis, metabolism of RNA, the tight junction, metabolism of polyamines, the cell cycle, and the immune response. Treatment with 666-15 and overexpression of a dominant-negative inhibitor of CREB (A-CREB) were protective in mice with an inducible *Pkd1* knockout (Liu et al., 2021). Novel CREB inhibitors with higher solubility and bioavailability have been actively under development and shown promise in preclinical studies (Sapio et al., 2020).

Positive transcription elongation factor b and CREB regulated transcription coactivator 2

PTEFb and CRTC2 are also critical to regulate transcription mediated by CREB and other transcription factors. Both are activated by PKA. Under basal conditions PTEFb is kept inactive in a silencing P-TEFb/HEXIM1/7SK snRNP complex (Yik et al., 2003; Michels et al., 2004). P-TEFb is hyperactivated in mouse and human ADPKD kidneys (Sun Y. et al., 2019). PKA phosphorylates HEXIM1 and releases PTEFb which is then recruited to target genes by direct interaction with transcription factors. These include transcription factors of great importance in the pathogenesis of PKD such as c-Myc, Stat3, and NFkB. Constitutive activation of PTEFb induces pronephric cysts in zebrafish and the pTEFb inhibitor flavopiridol ameliorates the cystic disease in rapid and slowly progressive *Pkd1* mouse models. PKA also activates P-TEFb through the activation of CRTC2 (Mi et al., 2022). Under basal conditions CRTC2 is phosphorylated by salt inducible kinase 1 (SIK1) and retained in the cytoplasm through phosphorylation-dependent interactions with 14-3-3 proteins. PKA phosphorylation inactivates SIK1 and allows the translocation of CRTC2 to the nucleus where it forms liquid-liquid condensates and activates P-TEFb by disrupting the inhibitory 7SK snRNP complex. Genetic depletion of CRTC2 suppresses cyst growth in an orthologous ADPKD mouse model.

c-Myc proto-oncogene

c-Myc is overexpressed in the kidneys of human as well as of virtually all orthologous and non-orthologous animal models of PKD (Kurbegovic and Trudel, 2020). The induction of PKD by renal overexpression of c-Myc in transgenic mice and the amelioration of the disease by its genetic or pharmacologic

downregulation demonstrate a causal connection between c-Myc and cystogenesis. The precise molecular mechanism(s) responsible for c-Myc activation in ADPKD is not known but may involve cAMP/PKA signaling. C-Myc is regulated by multiple mechanisms at the transcriptional, translational, and post-translational levels. PGE2 acting on an EP4R/GS/AC/cAMP/PKA/CREB signaling pathway increases c-Myc expression at both mRNA and protein levels and proliferation of hepatocellular carcinoma cells (Xia et al., 2014). PKA has also been shown to protect c-MYC from proteasome-mediated degradation through phosphorylation at Ser-279 in prostatic carcinoma cells (Padmanabhan et al., 2013).

Signal transducer and activator of transcription 3

STAT3 is a member of the STAT family of transcriptions factors. STAT3 activation mediates promotes migration of neutrophils, B-lymphocytes, dendritic cells and macrophages, and inflammation (Strubl et al., 2020). It is strongly activated in renal cyst-lining cells in human ADPKD and several PKD mouse models (Strubl et al., 2020). In addition to members of the Janus family of protein kinases (JAK) and several tyrosine kinase receptors (EGFR, PDGFR and c-Met), the non-receptor tyrosine kinase Src phosphorylates and activates STAT proteins promoting their nuclear translocation (Wang et al., 2000; Silva, 2004). Cyclic AMP/PKA signaling was found to enhance the effect of the cleaved PC1 tail Src and STAT3 activation (Talbot et al., 2014). In the PCK rat model, activation of STAT3 in renal cystic cells depended on vasopressin V2R signaling. Genetic inhibition of vasopressin expression or treatment with a pharmacologic V2R inhibitor strongly suppressed STAT3 activation and reduced renal cyst growth (Wang et al., 2008). Two STAT3 inhibitors, pyrimethamine and S3I-201, also inhibited cyst growth in a neonatal and an adult *Pkd1* model (Takakura et al., 2011). Curcumin, a compound with a broad spectrum of activity that ameliorates PKD, also inhibits STAT3 (Leonhard et al., 2011).

Nuclear factor κB

The NF-κB family of transcription factors consists of NF-κB1 (also named p50), NF-κB2 (also named p52), RelA (also named p65), RelB and c-Rel, which form different combinations of homo- or heterodimers (Barnabei et al., 2021). These dimers (e.g., p65:p50) are retained in the cytoplasm while bound to IκB proteins. Pro-inflammatory stimuli (e.g., TNFα, lipopolysacharide, etc) activate IκB kinases (IKKs) that promote IκB phosphorylation, ubiquitination and proteolysis. Released NF-κB dimers can then translocate into the nucleus, bind to promoter sequences of inflammation-related genes, and recruit chromatin modifying coactivator complexes or components of the general transcriptional machinery (Bhatt and Ghosh, 2014). Phosphorylation of NF-κB subunits by multiple kinases may either enhance or downregulate the transcription of target genes (Christian et al., 2016). PKA dependent phosphorylation may either inhibit or promote NF-κB activity (Gerlo et al., 2011). PKA has been demonstrated to phosphorylate p50 at S337, which is critical for DNA binding, *in vitro* and *in vivo*. PKA also phosphorylates p65 at S276. In resting cells the PKA catalytic subunit is bound in an inactive state to cytosolic IκBα:p65 complexes. Following IKK complex activation and degradation of IκBα, the active PKA catalytic subunit is liberated and phosphorylates p65 at S276 in a cAMP-independent process. S276 phosphorylation triggers a conformational change in p65 that promotes its interaction with CBP/p300 and increases p65 transcriptional activity (Christian et al., 2016). Several studies support a role for NF-κB in cyst formation. *Pkd1*$^{-/-}$ cells and kidneys exhibit increased phosphorylation and nuclear localization of the NF-κB subunit p65 and NF-κB driven overexpression of Pax2 (paired box 2), Wnt7a and Wnt7b (Qin et al., 2012). Inhibition of NF-κB or Wnt7b ameliorates the cystic disease in organ culture models and *Pkd1* mutant mice. The increased expression of Wnt7a, Wnt7b and Pax2 downstream from NF-κB after the addition of 8-Br-cAMP does not prove but is consistent with cAMP-PKA signaling enhancing the activity of NF-κB in PKD.

Nuclear factor erythroid 2–related factor 2

Nrf2 is an important regulator of antioxidant and anti-inflammatory mechanisms (Guerrero-Hue et al., 2020; Ito et al., 2020). Under physiological conditions the Kelch-like ECH-associated protein 1 (Keap) binds to Nrf2, facilitates its ubiquitination and proteasomal degradation, and prevents its translocation to the nucleus (Zhang D. D. et al., 2004). Reactive oxygen species disrupt the interaction and facilitate the nuclear translocation, transcriptional upregulation of antioxidant enzymes and transcriptional downregulation of inflammatory cytokines. A second mechanism to prevent Nrf2 signaling is phosphorylation by GSK3β that also facilitates ubiquitination and proteasomal degradation (Lu et al., 2019). In PKD, at least in advanced stages, the expression of KEAP and GSK3β are increased and Nrf2 expression is low (Lu et al., 2020). Deletion of Nrf2 further aggravates the severity of the PKD. In contrast, activation of Nrf2, either by Sulforaphane that disrupts the Nrf2-KEAP interaction, or by a compound that inhibits GSK3β, ameliorates PKD (Lu et al., 2020). A subanalysis of ADPKD patients in a clinical trial of the Nrf2 activator bardoxolone for CKD (Phoenix) showed that bardoxolone increased eGFR during a three-month follow-up (Pergola et al., 2019). A phase III clinical trial of bardoxolone in ADPKD is ongoing.

Paired box 2

Pax 2 upregulation contributes to the proliferative phenotype of the cystic epithelium. Pax2 is highly expressed in developing collecting ducts and the cystic epithelium (Dressler and Woolf, 1999). Heterozygous Pax2 mutants, mice and humans, have a phenotype due to a loss of gene dosage consistent with haploinsufficiency. A reduction of Pax2 gene dosage slowed the progression of PKD in two different mouse models of PKD (*Pkd1* mutant and cpk) (Ostom et al., 2000; Stayner et al., 2006). Conversely, transgenic overexpression of Pax2 results in cyst formation in kidneys (Dressler et al., 1993; Stayner et al., 2006). Addition of 8-Br-cAMP increased the expression of Pax2 in $Pkd1^{-/-}$ explants but not in Pkd1 WT kidneys. Constitutive activation or inhibition of PKA in $Pkd1^{RC/RC}$ mice increase or decrease Pax2 expression respectively. These observations suggest the expression of Pax2 in PKD is at least in part downstream from PKA. Pax2 has recently been shown to be part of a H3K4 methyltransferase complex, which is known to activate gene expression (Patel et al., 2007).

Post-transcriptional regulation downstream from PKA

Alternative splicing

Alternative splicing is controlled by intronic and exonic regulatory elements called intronic or exonic splicing enhancers or silencers. PKA phosphorylates arginine/serine-rich splicing factors and has been implicated in the regulation of alternative splicing (Figure 4) (Li et al., 2009). The control of alternative splicing by PKA and CaMKIV acting on the same RNA elements may be another point of convergence between cAMP and calcium signaling in PKD. The role of alternative splicing has been studied in cancer and many other diseases but not in PKD (Climente-Gonzalez et al., 2017; Goncalves et al., 2017). In cancer the proliferative or anti-proliferative responses to cAMP have been reported to depend on the relative expression of two B-Raf splicing variants (95 and 62 kD) (Papin et al., 1998; Fujita et al., 2002). cAMP stimulates cell proliferation in cells that express mostly the 95-kD isoform, whereas it is inhibitory in cells that do not express B-Raf or express mostly the 62-kD isoform. The relative expression of these two isoforms depends not only on cell type but also on cellular density (predominantly 95-kD isoform in subconfluent cells and 62-kD isoform in confluent cells) and may work as a molecular switch to activate and inhibit ERK and cell proliferation (Takahashi et al., 2004). Interestingly, the protective effect of two V2R antagonists in PKD models was accompanied by a significant reduction in the ratio of the 95- and 62-kD isoforms (Wang et al., 2005).

Non-coding RNAs

The importance of non-coding RNA for the regulation of the expression of mRNAs is now well recognized. MicroRNAs (miRNAs) are ~22 nucleotides double-stranded non-coding RNAs (ncRNAs) that function as inhibitors of post-transcriptional mRNA expression. Numerous miRNAs are aberrantly expressed in murine and human forms of ADPKD (Ramalingam et al., 2020).

miR-21 is amongst the most upregulated miRNA in response to tissue injury (Ramalingam et al., 2020). It is thought to mediate repair and regeneration, but sustained miR-21 activation is maladaptive and can propagate injury and tissue fibrosis. cAMP-PKA signaling transactivates the miR-21 gene via conserved CREB motifs in the miR-21 promoter region (Figure 4) (Lakhia et al., 2016). miR-21 is overexpressed in PKD (Lakhia et al., 2016; Woo et al., 2017). miR-21 deletion attenuates PKD progression in the *Pkd2*-KO mouse model. The V2R antagonist mozavaptan inhibits the cAMP-PKA pathway and reduces miR-21 expression in ADPKD mouse models. The validated targets of miR-21 are well-known players of survival and inhibitors of apoptosis (Buscaglia and Li, 2011). Pdcd4 (programmed cell death 4) and Ppara are among the many genes downregulated by miR-21 (Lakhia et al., 2016). Pdcd4 mediates apoptosis and Ppara promotes fatty acid oxidation. Repression of apoptosis and fatty acid oxidation are thought to play a role in the pathogenesis of PKD.

miR-17~92 is a polycistronic miRNA cluster, which consists of six individual miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-l, and miR-92a) (Ramalingam et al., 2020). It is essential for the development of the kidneys and many other organs, but its expression declines with maturation, its inducible deletion in adult mice and kidney specific deletion after nephrogenesis have no impact (Ramalingam et al., 2020). c-Myc transactivates miR-17~92 via conserved binding sites in the miR-17 promoter region. Transgenic upregulation of c-Myc or miR-17~92 in wild-type mice causes PKD (Patel et al., 2013). Genetic deletion of miR-17~92 attenuates PKD in multiple mouse models (Patel et al., 2013). Anti-miRs against the individual miRNA families of the six-member miR-17~92 cluster were developed to identify the family that is the crucial pathogenic driver in PKD. Anti-miR-17, but not antimiR-18, anti-miR-19 or anti-miR-25 LNAs, attenuated cyst growth in *Pkd1* knock-out mice (Yheskel et al., 2019). Anti-miR-17 derepressed many mRNA targets including Ppara, *PKD1*, and *PKD2*. These observations have provided the scientific rationale for targeting the miR-17 family for ADPKD treatment.

RGLS4326, a single-stranded, phosphorothioate oligonucleotide of only 9 nucleotides in length that bears complementarity to the miR-17 seed sequence, was synthesized for investigation in clinical trials (Lee et al., 2019). RGLS4326 displaces miR-17 from translationally active polysomes and thereby de-represses multiple miR-17 mRNA

targets. RGLS4326 treatment attenuates cyst growth in multiple PKD mouse models and human *in vitro* ADPKD models. A Phase 1b, open-label, adaptive design dose-ranging study to evaluate ADPKD biomarkers, pharmacokinetics, safety, tolerability, and pharmacodynamics of RGLS4326 administered via SC injection to patients with ADPKD was terminated early to prioritize a different compound.

Epigenetic mechanisms downstream from PKA

Epigenetic regulation is the process that modifies gene expression in the absence of changes in genome sequence. The main mechanisms of epigenetic regulation are DNA and RNA methylation, and histone modification. Three main histone modifications are phosphorylation, methylation, and acetylation. Recent evidence suggests that epigenetic regulators play important roles in cyst growth in ADPKD. Epigenetic regulators have been reported to be downstream targets of cAMP/PKA/CREB signaling, and/or modulate this pathway (Figure 4).

DNA methylation

DNA methylation is a major epigenetic mechanism that a methyl group was transferred onto the C5 position of the cytosine of CpG sites to form 5-methylcytosine (5 mC). This process is mediated by DNA methyltransferases (DNMTs), including DNMT3a, DNMT3b, and DNMT1 in mammals. DNMT3a and DNMT3b are associated with *de novo* DNA methylation during embryogenesis, whereas DNMT1 is involved in maintenance of methylation pattern (Moore et al., 2013). DNA methylation within a gene promoter typically suppresses gene expression through inhibiting the binding of transcription factors or recruiting histone deacetylases that results in chromatin condensation and gene inactivation (Moore et al., 2013). Methylation of CpGs within the gene body leads to increased expression of a gene (Jones, 2012).

The genome-wide DNA methylation status in kidneys from ADPKD and non-ADPKD individuals has been analyzed by performing methylated-CpG island recovery assay with parallel sequencing (MIRA-seq). The hypermethylation within the gene-body regions of *PKD1* and other genes associated with iron transport and cell adhesion were identified in ADPKD (Woo et al., 2014). The hypermethylation of *PKD1* led to its downregulation. Additionally, the methylation of *PKD1* promoter inversely correlates with its gene expression in peripheral blood of ADPKD patients (Hajirezaei et al., 2020). Inhibition of DNA methylation with 5-aza-2′-deoxycytidine increased the expression of *Pkd1* and decreased the cyst formation of MDCK cells (Woo et al., 2014). The CpG island within promoter region of MUPCDH gene was hypermethylated, which resulted in downregulation of MUPCDH and abnormal cell proliferation (Woo et al., 2015). The hypermethylation of MUPCDH promoter is correlated with the increased rate of total kidney volume change in ADPKD (Woo et al., 2015). In contrast, global hypomethylation was found in genomic DNA from ADPKD kidneys versus non-ADPKD kidneys by performing reduced representation bisulfite sequencing (RRBS) (Bowden et al., 2018). The hypermethylation was identified with 3′ end of *PKD1* gene body, but not associated with decreased expression of *PKD1* mRNA. A recent study investigated the DNA methylation changes of cystic epithelia within independent cysts by RRBS (Bowden et al., 2020). This study demonstrated that the greatest amount of variation occurs in fragments within CpG islands and gene bodies, but not within intergenic fragments across the ADPKD kidney. These regions with variation were defined as inter-cyst variants (ICVs). A proportion of the IVC associated genes were differentially methylated in ADPKD versus non-ADPKD kidney tissue. This work provided evidence that different DNA methylation changes contributes to the development of each cyst.

cAMP signaling has been reported to induce cardiac hypertrophy through regulating DNA methylation (Fang et al., 2015). Increased intracellular cAMP by the stable cAMP analog DBcAMP or PDE inhibitor caffeine and theophylline increased the expression of DNMTs and increased the global DNA methylation in HL-1 cardiomyocytes. Inhibition of DNMT activity with 5-azacytidine decreased global DNA methylation induced by DBcAMP.

DNA hydroxymethylation is a novel epigenetic modification of DNA. 5-methycytosine (5 mC) is converted to 5-hydroxymethylcytosine (5hmC) that is catalyzed by TET family proteins. TET proteins are Fe(II)-dependent and 2-oxoglutarate-dependent methylcytosine dioxygenases. 5hmC acts as an intermediate in the reaction of DNA demethylation or a signal for chromatin factors (Guibert and Weber, 2013). cAMP treatment increased the generation of 5hmC in multiple cell types (Camarena et al., 2017). cAMP promoted Fe(II) release to the intracellular labile Fe(II) pool (LIP) by the acidification of endosomes. This effect was confirmed by stimulation of GPCR adenylate, and by cyclase activators and PDE inhibitors. The DNA hydroxymethylation induced by cAMP was correlated with a majority of differentially transcribed gene. In addition to cAMP/PKA targeted transcription factors, cAMP modulates transcriptome by promoting DNA demethylation. DNA hydroxymethylation and demethylation have not been investigated in ADPKD. Whether cAMP contributes to cystogenesis via regulating DNA methylation and hydroxymethylation needs to be investigated in the future.

RNA methylation

RNA methylation regulates the stability of mRNA and its translation to protein. The N6-methyladenosine (m6A)

methylation of mRNA (m6A) is medicated by methyltransferase-like 3 (Mettl3). The levels of Mettl3 and m6A were upregulated in kidneys of mouse and human ADPKD (Ramalingam et al., 2021). Knockout of Mettl3 delayed cyst growth in three orthologous ADPKD mouse models. Overexpression of Mettl3 in a kidney specific transgenic mouse model developed tubular cysts. C-Myc and Avpr2 were identified as Mettl3 targets by methylated RNA immunoprecipitation sequencing (MeRIP-seq). Mettl3 promotes renal cystic epithelial cell proliferation by regulating the expression of c-Myc and Avpr2. m6A levels on c-Myc and Avpr2 mRNAs were upregulated in mouse PKD kidneys versus control kidneys, which were decreased in PKD1 and Mettl3 double knockout kidneys. The Mettl3 induced the phosphorylation of CREB via the upregulation of Avpr2 protein and activation of cAMP. In bone marrow mesenchymal stem cells, deletion of Mettl3 decreased cAMP accumulation induced by PTH and its downstream phosphorylation of CREB (Wu et al., 2018). One recent study reported that CREB upregulated the expression of miR-373 that suppressed Mettl3 in chondrocytes (Zhang et al., 2022). Whether cAMP/PKA/CREB signaling directly regulates the transcription of Mettl3 remains unclear.

Histone deacetylases

The acetylation of histone tail is catalyzed by histone acetyltransferases (HATs). Histone acetylation mainly increases transcriptional activity via promoting the accessibility of transcriptional regulatory proteins to relaxed chromatin. Deacetylation of histone is medicated by histone deacetylases (HDACs). Deacetylation of histone leads to heterochromatin and inhibition of gene transcription. HDACs can also bind to and catalyze non-histone proteins. Human HDACs are divided into four categories: Class I HDAC (HDAC1, HDAC2, HDAC3, and HDAC8), Class II proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), Class III HDAC (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7), and Class IV HDAC (HDAC11) (Seto and Yoshida, 2014). Class I, II and IV HDACs are zinc-dependent deacetylase, while class III HDACs require NAD+ as the cofactor for deacetylase activity (Seto and Yoshida, 2014).

Trichostatin A (TSA), a class I and class II HDAC inhibitor, and valproic acid (VPA), a class I specific HDAC inhibitor, have been found to suppress kidney cyst formation by performing a chemical modifier screening in PKD zebrafish models (Cao et al., 2009). TSA ameliorates cyst formation through regulating different factors, such as Id2/p21 and Rb/E2F1 pathways(Fan et al., 2012), AMPK pathway, and autophagy (Sun L. et al., 2019). Additionally, HDAC5 has been identified as one of the targets of polycystin-dependent fluid stress sensing in renal epithelial cells (Xia et al., 2010). TSA modulates renal epithelial cell differentiation by targeting HDAC5 (Xia et al., 2010). Quisinostat, a second-generation class I and class II HDAC inhibitor, has been discovered to affect the viability of ADPKD cells with minimal effect on normal human kidney cells via a high-throughput screening platform of cancer drugs (Asawa et al., 2020).

The expression and activity of HDAC6 are upregulated in PKD mutant cells and kidneys (Liu et al., 2012b; Cebotaru et al., 2016). Targeting of HDAC6 with different specific inhibitors, including tubastatin, tubsin and ACY-1215 reduced cyst growth in PKD mouse models by inhibiting cystic renal epithelial cell proliferation (Cebotaru et al., 2016; Yanda et al., 2017a; Yanda et al., 2017b). Inhibition of HDAC6 reduced intracellular cAMP and intracellular calcium level by increasing ATP-stimulated calcium release and reducing the release of calcium from the endoplasmic reticulum (Yanda et al., 2017a; Yanda et al., 2017b). Treatment with tubacin decreased CFTR chloride currents activated by cAMP in MDCK cells (Cebotaru et al., 2016). Inhibition of HDAC6 by ACY-1215 reduced hepatic cystogenesis in polycystic liver disease model, PCK rats (Lorenzo Pisarello et al., 2018). A combination of ACY-1215 and the somatostatin receptor analogue, pasireotide synergistically reduced liver cyst growth in PCK rats. ACY-1215 and pasireotide decreased cAMP levels and cell proliferation in cultured cystic cholangiocytes. These findings support that the activation of HDAC6 promotes kidney and liver cyst growth and is an upstream regulator of cAMP in PKD. Isoproterenol activated cAMP/PKA via GPCR and inhibited downstream c-Raf/MEK/ERK signaling, and consequently increased the expression of HDAC6 in human lung cancer cells (Lim and Juhnn, 2016). Whether the activation of cAMP/PKA/CREB signaling in cystic renal epithelial cells regulates the expression and activity of HDAC6 to form a positive feedback loop has not been studied.

Several HDAC inhibitors have been approved by the FDA for the use in T cell lymphoma and multiple myeloma. The efficacy of the combination of HDAC inhibitor and different chemotherapy regimen in these hematological neoplasms were studied in several clinical trials (Bondarev et al., 2021). HDAC inhibitor alone or the combination of HDAC inhibitor and tolvaptan may be novel therapeutic strategies in ADPKD patients.

Sirtuin 1 (SIRT1) is the most conserved mammalian NAD+ dependent histone deacetylase. Activation of cAMP/PKA pathway by forskolin, an adenylyl cyclase activator, epinephrine, or a beta 2 adrenergic receptor agonist clenbuterol increased the deacetylase activity of SIRT1 via the phosphorylation of its serine 434 (Gerhart-Hines et al., 2011). The activation of SIRT1 increased PGC-1a deacetylation that results in the increased fatty acid oxidation (Gerhart-Hines et al., 2011). The transcription of SIRT1 is directly stimulated by the activation of cAMP/PKA/CREB as a response to humoral factors (glucagon and norepinephrine) released during fasting (Noriega et al., 2011). Seven putative half CREB-binding sites are identified

in the proximal promoter region of SIRT1 gene. Stimulation of cAMP by forskolin, glucagon, and norepinephrine increased the SIRT1 mRNA abundance in hepatic cells (Noriega et al., 2011). A recent study in *C. elegans* reported that the activation of PKA by hydralazine was mediated by its binding and stabilization of a catalytic subunit of PKA, which was independent of cAMP (Dehghan et al., 2019). PKA activated the downstream SIRT1 and SIRT5, leading to improved mitochondrial function and metabolic homeostasis (Dehghan et al., 2019). Furthermore, CREB promotes SIRT1 transcription via binding to its promoter in neuronal cells (Fusco et al., 2012). CREB recruits SIRT-1 to DNA, which leads to the increased expression of CREB target genes, such as Peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1α) and neuronal NO synthase (Fusco et al., 2012).

The expression of SIRT1 was upregulated in *Pkd1* mutant mouse renal epithelial cells and kidney tissues, and human ADPKD cells (Zhou et al., 2013; Warner et al., 2016). SIRT1 expression is regulated by c-Myc and induced by TNFa. Conditional double knockout of SIRT1 and Pkd1 delayed cyst growth in $Pkd1^{flox/flox}$:Ksp-Cre mice (Zhou et al., 2013). Inhibition of SIRT1 with a pan-sirtuin inhibitor nicotinamide and a specific inhibitor EX-527 slowed cyst growth in three PKD mouse models (embryonic model $Pkd1^{-/-}$, $Pkd1^{flox/flox}$:Ksp-Cre, and hypomorphic $Pkd1^{nl/nl}$). The upregulated SIRT1 increased cystic renal epithelial cell proliferation through deacetylation of Rb, and inhibited apoptosis via deacetylation of p53. The SIRT1 activator resveratrol treatment increased cyst formation, and FK866, an NAD synthetic enzyme Nampt inhibitor, decreased cyst formation in the cystogenic assay of MDCK cells (Warner et al., 2016). The expression of SIRT2 was upregulated in *Pkd1* knockout mouse kidney cells. SIRT2 is a NAD+ dependent protein deacetylase that interacts with HDAC6 and deacetylates a-tubulin (North et al., 2003). SIRT2 regulates cilia formation and disassembly and contributes to aberrant centrosome amplification and polyploidy in PKD (Zhou et al., 2014).

These preclinical studies have provided the rationale to support that SIRT1 is an attractive therapeutic target of ADPKD. Nicotinamide (Niacinamide), also known as vitamin B3, is a water-soluble amide derivative of nicotinic acid and a dietary supplement. Nicotinamide inhibits sirtuins at high doses. In a small randomized, double blinded, placebo-controlled trial, nicotinamide is safe and well-tolerated in patients with ADPKD but does not show difference in the change in height-adjusted total kidney volume over 12 months (El Ters et al., 2020). Due to the limitations of small sample size and short duration of intervention, further studies will be needed to validate nicotinamide as a clinical therapy for ADPKD. EX-527 (Selisistat), a SIRT1 specific inhibitor, ameliorated PKD in three ADPKD mouse models. EX-527 has been found to be well-tolerated and safe in healthy human subjects (Curry et al., 2021). Two phase III clinical trials of EX-527 for treatment of Huntington's disease and endometriosis-mediated IVF failure respectively are ongoing. EX-527 is a potential therapy for ADPKD patients that needs to be studied.

Bromodomain-containing protein 4

BRD4, a member of bromodomain and extraterminal (BET) proteins, is an epigenetic reader that binds to acetylated lysine residue on histones. BRD4 activates gene transcription through the recruitment of p-TEFb and multiprotein mediator complex. BRD4 has been found to be involved in transcriptional activation by cAMP/PKA/CREB pathway. CREB typically activates the transcription of its target genes by recruitment of coactivators to promoter proximal binding sites. CREB regulates the expression of pancreatic beta cell-specific genes through hyperacetylation of promoter-distal super-enhancer region (Van de Velde et al., 2019). CREB recruits histone acetyltransferase p300 and CREB binding protein (CBP) to super-enhancer region, subsequently increases in histone acetylation and facilitates the recruitment of the coactivators CREB-regulated transcription coactivator 2 (CRTC2) and BRD4 (Van de Velde et al., 2019). D1 dopamine receptor (D1R) is a GPCR that activates cAMP/PKA signaling in neuron cells. BRD4 is recruited to dopamine-induced genes in response to cAMP/PKA signaling, and thereby increases the transcription of D1R-induced genes in striatal neurons (Jones-Tabah et al., 2022). Similarly, the activation of GPCR a1-adrenergic receptor (a1-AR) activated cAMP/PKA that increased BRD4 occupancy at promoters and super-enhancers of hypertrophic genes in cardiomyocytes (Martin et al., 2020).

Active P-TEFb is a crucial regulator for stimulating RNA polymerase II elongation. About half of P-TEFb associates with 7SK snRNA and HEXIM1 to form an inactive P-TEFb/HEXIM1/7SK snRNP complex. P-TEFb is hyperactivated that is mediated by PKA induced phosphorylation of HEXIM1 (Sun Y. et al., 2019) and cAMP induced nuclear translocation and condensate formation of CRTC2 (Mi et al., 2022) in ADPKD. Furthermore, P-TEFb is activated by BRD4. BRD4 interacted with P-TEFb, converted the 7SK/HEXIM1-bound P-TEFb into the BRD4-associated form. BRD4-associated P-TEFb functions as active form to promote transcription.

BRD4 was upregulated in *Pkd1* mutant mouse renal epithelial cell and tissues (Zhou et al., 2015). Inhibition of BRD4 with its inhibitor JQ1 delayed cyst growth and preserved kidney function in two PKD mouse models ($Pkd1^{flox/flox}$:*Pkhd1*-Cre and $Pkd1^{nl/nl}$). BRD4 has been identified as an upstream regulator of the expression of c-Myc in cystic renal epithelial cells. BRD4 promotes cystic renal epithelial cell proliferation through regulating c-Myc/p21 pathway. BRD4 has been reported to activate the transcription of target genes that are important for cell

proliferation and apoptosis in a variety of cancers (Shorstova et al., 2021). Multiple clinical trials of BET inhibitors (BETi) in different cancers are ongoing (Shorstova et al., 2021). Apabetalone is a novel oral small molecule inhibitor of BRD4. Apabetalone reduced the incidence of major adverse cardiovascular events and showed favorable renal outcomes in patients with diabetic kidney disease and coronary artery disease in a phase 3 clinical trial (Kulikowski et al., 2018; Ray et al., 2020). Targeting BRD4 with apabetalone might be a potential therapeutic strategy for ADPKD.

PKA, mitochondria and oxidative stress

Recent studies have identified functional and structural mitochondrial abnormalities in cyst lining cells of kidneys of patients with or mouse models of ADPKD. Mitochondrial oxidative phosphorylation is the main cellular source of ATP and reactive oxygen species. Reactive oxygen species can exert signaling roles or pathological effects depending on their levels. Mitochondria consist of an outer mitochondrial membrane, an intermembranous space, an inner mitochondrial membrane with folds called cristae, and a mitochondrial matrix.

Mitochondria contain three distinct cAMP-PKA signaling compartments, one tethered to outer membrane by different AKAPs, the second in the intermembrane space with PKA I tethered to the internal membrane by SKIP (sphingosine kinase-interacting protein), and the third confined to the mitochondrial matrix (Di Benedetto et al., 2021). cAMP and PKA signalling in the matrix and intermembrane space are mainly involved in the regulation of oxidative phosphorylation, whereas cAMP signaling in the outer membrane is involved in the regulation of mitochondrial dynamics, mitophagy, and apoptosis.

Oxidative phosphorylation consists of the transfer of two electrons from NADH (nicotinamide adenine dinucleotide) and FADH2 (flavin adenine dinucleotide) by enzymatic complexes I to IV (mitochondrial respiratory chain) within the inner mitochondrial membrane, coupled to proton extrusion at the level of complexes I, III, and IV into the intermembranous space, generation a proton gradient across the inner mitochondrial membrane, and phosphorylation of ADP to ATP by a process driven by the backflow of protons into the matrix involving ATP synthase (Zhao et al., 2019). Under physiological conditions, 0.2%–2% of the electrons leak out of complexes I, II and III and interact with oxygen to produce superoxide or hydrogen peroxide (Irazabal and Torres, 2020). PKA-dependent phosphorylation of the electron transport chain modulates oxidative phosphorylation (Figure 4) (Bouchez and Devin, 2019). PKA phosphorylates several subunits of complex I and is involved in the assembly and enzymatic activity of this complex. PKA also phosphorylates several subunits of complex IV increasing complex IV activity.

Structural mitochondrial abnormalities and reductions of mitochondrial copy number develop early in animal models of PKD and become more marked with disease progression (Cassina et al., 2020; Kahveci et al., 2020). Cells with a heterozygous *PKD1* mutation exhibit increased mitochondrial basal respiration and ATP production but decreased spared capacity with no difference in maximal mitochondrial respiration (Ishimoto et al., 2017). In contrast, cells with a homozygous *PKD1* mutation exhibit reduced basal respiration, ATP production, maximal respiration, spare capacity, and proton leakage. PKA activation may be responsible for the increased mitochondrial respiration in the cells with a heterozygous *PKD1* mutation since it was inhibited by the PKA inhibitor H-89. Increased nonmitochondrial respiration was found in both, cells with homozygous or heterozygous *PKD1* mutations. Oxidative stress may contribute to the accentuation of the structural and functional mitochondrial alterations with disease progression. Treatment with the mitochondrion-specific antioxidant MitoQ reduced mitochondrial superoxide production and proliferation of cyst-derived *PKD1* heterozygous cells. The mechanism(s) responsible for the early functional and structural mitochondrial alterations in PKD may only be partially understood. Cyclic AMP was found to be increased and calcium decreased in heterozygous *PKD1* cells. It was proposed that the increased cAMP-PKA activity was likely responsible for the early increase in mitochondrial respiration and superoxide generation, while reduced intracellular calcium acting on various regulators of PGC-1α expression (nitric oxide synthase, p38 MAPK and calcineurin) was responsible for its downregulation. Furthermore, downregulation of PGC-1α activates GSK3β and inhibits Nrf2 thus increasing the susceptibility of cystic kidneys to oxidative stress (St-Pierre et al., 2006).

Mitochondria form a dynamic network in the cytoplasm subject to biogenesis, fission, fusion and mitophagy (Mishra and Chan, 2016). Cyclic AMP and PKA signaling affects many of these processes. Mitochondrial biogenesis denotes the generation of new from existing mitochondria. It relies on nuclear transcription factors that regulate the transcription of most mitochondrial proteins, including those required for the transcription of the mitochondrial genome. PGC-1α regulates mitochondrial biogenesis by its ability to increase the expression and activity of NRF2 and subsequently of mitochondrial transcription factor A that promotes mitochondrial DNA replication (St-Pierre et al., 2006). PGC-1α also induces the expression of uncoupling protein-2, an inner mitochondrial membrane protein that dissipates the mitochondrial membrane potential, uncouples electron transport from ATP synthesis and reduces reactive oxygen species production (Vergnes et al., 2020). Downregulation of PGC-1α in PKD inhibits mitochondrial biogenesis.

Mitochondrial fission and fusion determine the size and number of mitochondria. Fission is regulated by dynamin-related protein 1 (Drp1) that recruits dynamin-2 to constriction points on the outer mitochondrial membrane (Di Benedetto et al., 2018). PKA-dependent phosphorylation of Drp1 retains it in the cytosol inhibiting fission. Inhibition of fission causes abnormal elongation of mitochondria. Nevertheless, both PKA dependent inhibition and promotion of fission have been reported (Chang and Blackstone, 2007; Cribbs and Strack, 2007; Cereghetti et al., 2008; Wikstrom et al., 2014; Di Benedetto et al., 2018). Inhibition of fission by PKA may favor fusion which is under the control of mitofusin 1 and 2 and optic atrophy 1. PKA also phosphorylates the mitochondrial membrane protein mitofilin, the clearance of damaged mitochondria (mitophagy) which is initiated by PTEN-induced putative kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin (Akabane et al., 2016).

PKA and inflammation

Activation of inflammatory pathways NFκB and STAT3, inhibition of anti-oxidative and anti-inflammatory Nrf2 signaling, and oxidative stress are associated with the upregulation of multiple inflammatory cytokines (TNF-α, IL-1, IL-6) and chemokines (MCP1) that promote cyst growth, interstitial inflammation and fibrosis. Cyclic AMP and PKA signaling may contribute to the development of interstitial inflammation and fibrosis by multiple mechanisms beyond the regulation of STAT3, NF- κB and Nrf2. Vasopressin has been shown to stimulate V2R/cAMP/PKA/ERK/YAP-mediated cell signaling in tubular epithelium, promote the expression and release of connective tissue growth factor (CCN2) and other YAP targets (PAI-1, AREG and MCP-1), and increase the number and activity of interstitial myofibroblasts and fibrosis in ADPKD kidneys (Figure 4) (Dwivedi et al., 2020). Renal tubule-specific YAP gene deletion and pharmacologic YAP inhibition using verteporfin attenuated the cystic disease in *Pkd1* knockout mice. Consistent with this observation, the administration of tolvaptan suppresses the excretion of MCP-1 in the urine (Grantham et al., 2017). This may account for the beneficial effect of tolvaptan at advanced stages of ADPKD (Torres et al., 2018b; Torres et al., 2021). Furthermore, cAMP has been shown in other tissues to induce monocyte recruitment in a manner dependent on PKA and MCP1/CCR2 signaling and to promote reprogramming of bone-marrow-derived macrophages to a M2 phenotype as seen by increased Arginase-1/CD206/Ym-1 expression and IL-10 levels (M2 markers (Polumuri et al., 2021)) through a PKA/C/EBPb/CREB dependent pathway (Negreiros-Lima et al., 2020).

PKA and metabolic reprogramming

In the presence of oxygen, mammalian cells convert glucose to pyruvate (glycolysis) which enters the mitochondria to be completely oxidized through the mitochondrial tricarboxylic acid cycle where oxygen is the final acceptor in the electron transport chain (oxidative phosphorylation). Under anaerobic conditions (anaerobic glycolysis), stabilization of HIF-1α leads to the activation of glucose transporters, stimulation of enzymes promoting glycolysis (hexokinase, phosphofructokinase and pyruvate kinase), lactate dehydrogenase A, which enhances the flux of glucose through the glycolytic pathway while attenuating entry of pyruvate into the TCA cycle, and pyruvate dehydrogenase kinase, which inactivates the mitochondrial pyruvate dehydrogenase further inhibiting the entry of pyruvate into the tricarboxylic acid cycle and oxidative phosphorylation.

Rapidly proliferating cells repress oxidative metabolism and enhance glycolytic flux even under aerobic conditions (aerobic glycolysis (Vander Heiden et al., 2009; Lunt and Vander Heiden, 2011)). This metabolic reprogramming is required to support cell proliferation of rapidly growing tissues and is a feature of ADPKD, cancer and fetal development. It is characterized by inhibition of oxidative phosphorylation and fatty acid oxidation, stimulation of glycolysis and the pentose phosphate pathway, dependence on glutamine to provide intermediates to support the TCA cycle and lipid synthesis. The main goal of this metabolic reprogramming is the accumulation of glycolytic intermediates for biosynthetic purposes. Although glycolysis generates only 2 ATP molecules per molecule of glucose, as compared to 34 molecules of ATP per molecule of glucose generated by oxidative phosphorylation, the high glycolytic flux compensates for the lower efficiency.

mTOR and c-Myc are thought to play a central role in the metabolic reprogramming (Guertin and Sabatini, 2007; Duvel et al., 2010; Dejure and Eilers, 2017). In ADPKD, mTOR and c-Myc are consistently upregulated in PKD kidneys and repress oxidative metabolism, markedly enhance glycolytic flux (through upregulation of glucose transporters and the key glycolytic enzymes HK and PFK) and lactic acid production and export (through upregulation of LDH-A and monocarboxylic acid transporter 4, MCAT4 (Rowe et al., 2013; Podrini et al., 2020)). Nevertheless, mTOR and c-MYC are activated by PKA/MEK/ERK signaling and therefore activation of cAMP/PKA signaling may be a driver of metabolic reprogramming in some tissues. Furthermore, CREB has been implicated in metabolic reprogramming by targeting some critical glycolytic enzymes and PKA/CREB signaling has been found to drive metabolic reprogramming in some cancers (e.g., hepatocellular and prostatic carcinomas (Figure 4) (Moon et al., 2011; Sun et al., 2021)).

Because of their dependence on glycolysis and on glutamine PKD cells are particularly sensitive to glucose and glutamine deprivation. Inhibition of glycolysis with 2-deoxyglucose, a glucose analog that is not metabolized by cells, reduced cell proliferation in human PKD cells and kidney cyst growth in

various murine and in a pig model (Chiaravalli et al., 2016; Riwanto et al., 2016; Lian et al., 2019). Furthermore, diet interventions such as caloric restriction, which attenuate PKD in several animal models may act through a similar mechanism (Kipp et al., 2016; Warner et al., 2016). Inhibition of glutaminase-1, which transforms glutamine into glutamate then catabolized into α-ketoglutarate, slowed cyst growth in *Aqp2*-Cre;*Pkd1*$^{flx/flx}$ but not in *Pkhd1*-Cre; *Pkd1*$^{flx/flx}$ mice (Flowers et al., 2018; Soomro et al., 2018).

Discussion

Autosomal dominant polycystic kidney disease (ADPKD), with an estimated genetic prevalence between 1:400 and 1: 1,000 individuals, is the third most common cause of end stage kidney disease after diabetes mellitus and hypertension (Bergmann et al., 2018). Over the last 3 decades there has been great progress in understanding its pathogenesis. A wealth of evidence supports that upregulation cAMP signaling promotes cystogenesis in ADPKD. Reduced polycystin function is thought to cause dysregulation of intracellular calcium, activation of adenylyl cyclases 5 and 6, inhibition of phosphodiesterase 1, and upregulation of cAMP and PKA signaling (Gattone et al., 2003; Torres et al., 2004; Wang X et al., 2005; Wang et al., 2008; Aihara et al., 2014). Upregulation of cAMP and PKA signaling may to a large extent be responsible for the disruption of tubulogenesis and initiation of cystogenesis (Song et al., 2003; Gallegos et al., 2012; Scholz et al., 2022) and for the progression of the cystic disease by stimulating fluid secretion (Sullivan et al., 1998; Grantham, 2003) and, in the setting of reduced intracellular calcium, epithelial cell proliferation (Yamaguchi et al., 2003; Yamaguchi et al., 2004; Yamaguchi et al., 2006). Previous studies have shown that constitutive activation of PKA not only causes a marked aggravation of PKD in mice with a hypomorphic *Pkd1* mutation but also induces a cystic phenotype in mice with a wild-type genetic background (Ye et al., 2017). Cyclic AMP analogs activating PKA, but not those activating Epac (exchange protein directly activated by cAMP), promote cystogenesis in *Pkd1*$^{RC/RC}$ metanephric organ cultures supporting the dominant role of cAMP/PKA signaling rather than Epac signaling in cystogenesis(Ye et al., 2017). Furthermore, constitutive inhibition of PKA attenuated PKD in *Pkd1*$^{RC/RC}$ mice (Wang et al., 2022). A recent study has shown that genetic inhibition of CREB suppresses cyst growth in ADPKD mouse models and that CREB orchestrates the expression of a cystogenesis associated transcriptome (Liu et al., 2021).

Interventions acting on G protein coupled receptors to inhibit of cAMP production have been effective in preclinical trials and have led to the approval of a vasopressin V2R antagonist (tolvaptan) for the treatment of rapidly progressive ADPKD (Torres et al., 2012; Torres et al., 2017). Their efficacy, however, is less than it can be achieved by the genetic elimination of circulating vasopressin in PCK rats, which causes massive polyuria and nearly completely abolishes the development of PKD but is not feasible in patients (Wang et al., 2008). Indeed the dosing of tolvaptan is limited by its powerful aquaretic effect. The proximal and central role of cAMP in the pathogenesis of ADPKD, the efficacy of tolvaptan and somatostatin analogs in preclinical and clinical trials, and the inability of these drugs to completely block renal cAMP production, provide a compelling rationale for exploring other targets in the cAMP signaling pathway. V2R antagonists affect PKA activity indirectly by lowering the production and tissue levels of cAMP. The tissue levels of cAMP can also be decreased by accelerating its degradation. Indeed, a PDE4 activator has been found to decrease kidney cAMP levels and attenuate the cystic disease to a degree comparable to that observed with tolvaptan with less polyuria in *Pkd1*$^{RC/RC}$ mice (Henderson et al., 2020). PKA activation in ADPKD can occur independently from cAMP. A novel PKA inhibitor also attenuated PKD in *Pkd1*$^{RC/RC}$ mice with only mild polyuria (Wang et al., 2022). CREB activation can also occur independently from PKA. A CREB inhibitor attenuated the cystic disease in ADPKD mouse models. Cyclic AMP/PKA/CREB upregulation affects many other downstream regulatory, signaling, and pathophysiologic pathways altered in ADPKD, as discussed in this review. Interventions targeting some of these downstream pathways may provide additive or synergistic value. In ADPKD, like in cancer and many other diseases, combinatory strategies with multiple drugs may be needed to achieve optimal results and tolerability. Currently the mechanisms by which cAMP promotes the development and progression of ADPKD are not completely understood and opportunities for targeting cAMP signaling in ADPKD are far from exhausted and remain feasible. A better understanding of cAMP/PKA/CREB/downstream signaling in ADPKD is likely lead to novel treatments that build on a strategy that has already been successful.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The GPCR properties of polycystin-1- A new paradigm

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Polycystin-1 (PC1) is an 11-transmembrane (TM) domain-containing protein encoded by the *PKD1* gene, the most frequently mutated gene leading to autosomal dominant polycystic kidney disease (ADPKD). This large (> 462 kDal) protein has a complex posttranslational maturation process, with over five proteolytic cleavages having been described, and is found at multiple cellular locations. The initial description of the binding and activation of heterotrimeric Gαi/o by the juxtamembrane region of the PC1 cytosolic C-terminal tail (C-tail) more than 20 years ago opened the door to investigations, and controversies, into PC1's potential function as a novel G protein-coupled receptor (GPCR). Subsequent biochemical and cellular-based assays supported an ability of the PC1 C-tail to bind numerous members of the Gα protein family and to either inhibit or activate G protein-dependent pathways involved in the regulation of ion channel activity, transcription factor activation, and apoptosis. More recent work has demonstrated an essential role for PC1-mediated G protein regulation in preventing kidney cyst development; however, the mechanisms by which PC1 regulates G protein activity continue to be discovered. Similarities between PC1 and the adhesion class of 7-TM GPCRs, most notably a conserved GPCR proteolysis site (GPS) before the first TM domain, which undergoes autocatalyzed proteolytic cleavage, suggest potential mechanisms for PC1-mediated regulation of G protein signaling. This article reviews the evidence supporting GPCR-like functions of PC1 and their relevance to cystic disease, discusses the involvement of GPS cleavage and potential ligands in regulating PC1 GPCR function, and explores potential connections between PC1 GPCR-like activity and regulation of the channel properties of the polycystin receptor-channel complex.

KEYWORDS

ADPKD, polycystin-1, polycystin-2, receptor-ion channel complex, GPS cleavage, tethered peptide agonist, heterotrimeric G proteins

1 Background

1.1 PKD genes and centrality of polycystin-1

Autosomal dominant polycystic kidney disease (ADPKD) is caused by mutations of the *PKD1* or *PKD2* genes, which encode the proteins polycystin-1 (PC1) and polycystin-2 (PC2), respectively. PC1 and PC2 are integral membrane proteins proposed to co-exist as a heterotetrameric receptor-like/ion channel complex. Both proteins are found in multiple cellular locations, including the ER, plasma, and primary ciliary membranes. Together, PC1 and PC2 are thought to play an important role in cellular ion homeostasis and signal transduction, possibly in response to ligand binding and mechanical stimuli (Nigro and Boletta, 2021).

Mutations in additional genes have also been reported that account for a small fraction of ADPKD cases. These genes and their protein products include *GANAB*/glucosidase II alpha subunit, *DNAJB11*/DnaJ homolog (hsp40) subfamily B member 11, *ALG9*/alpha-1,2-mannosyltransferase, and *IFT140*/intraflagellar transport 140 (Cornec-Le Gall et al., 2018a; Lemoine et al., 2022). The *GANAB* and *DNAJB11* products are ER-resident proteins involved in protein transport, folding and quality control. The *IFT140* protein is in a complex responsible for retrograde transport in the primary cilium and is involved in ciliary entry of GPCRs. Of the ADPKD genes, mutation of *PKD1* is by far the most predominant cause of the disease (~78%), followed by mutation of *PKD2* (15%) and *IFT140* (~2%) (Senum et al., 2022). Interestingly, the protein products of *PKD2*, *GANAB*, *DNAJB11*, and the genes *SEC63* and *PRKCSH*, which are mutated in autosomal dominant polycystic liver disease, are necessary for the proper biogenesis or trafficking of PC1 (Fedeles et al., 2011; Besse et al., 2017; Cornec-Le Gall et al., 2018b; Besse et al., 2019; Hu and Harris, 2020). Such findings reveal the key importance of PC1 in the pathogenesis of the cystic diseases caused by each of these genes and underscore the need to better understand the structure-function relationships and the central role of this complicated protein.

1.2 The structural and functional complexity of Polycystin-1

The *PKD1* gene was identified over a quarter of a century ago (The European Polycystic Kidney Disease Consortium, 1994; The International Polycystic Kidney Disease Consortium, 1995; Hughes et al., 1995). The PC1 protein sequence of 4,302 residues was proposed to have multiple membrane-spanning domains flanked by an extensive N-terminal extracellular region (ECR) and a much shorter cytosolic C-terminal tail (C-tail) (Figure 1A). These early sequence analyses suggested the possibility of 7–13 membrane-spanning domains, however it was not until the sequencing of the pufferfish *Pkd1* gene that the field began to settle on an 11-TM domain conformation (Sandford et al., 1997). Biochemical approaches utilizing N-linked glycosylation analyses subsequently confirmed the integral membrane status,

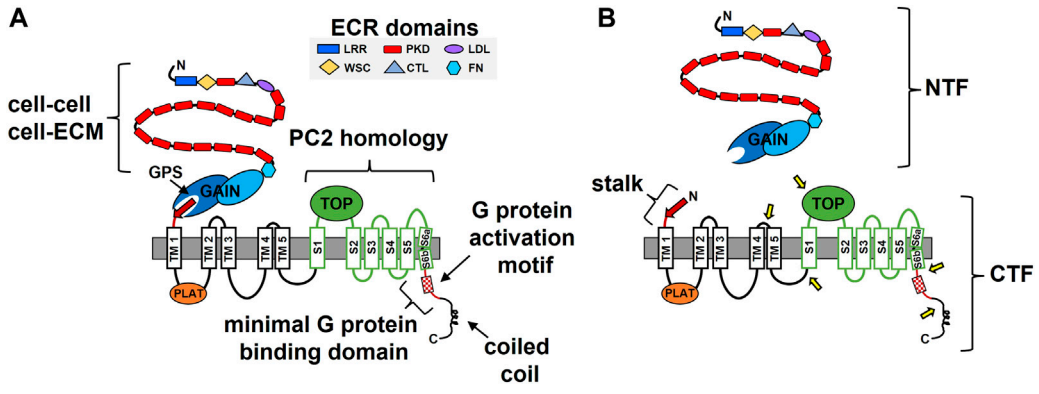


FIGURE 1
The structure-function features of polycystin-1 (PC1). **(A)** Domains identified in the N-terminal extracellular region (ECR) and within the membrane-associated portion of PC1 are indicated along with identified functional roles. ECM, extracellular matrix; GPS, GPCR proteolysis site; GAIN, GPCR autoproteolysis inducing; TM, transmembrane, S, transmembrane segment; PLAT, polycystin/lipoxygenase/α toxin; TOP, Tetragonal Opening of Polycystins; LRR, leucine-rich repeats; CTL, C-type lectin; WSC, cell-wall integrity and stress-response component; FN, fibronectin-like. The region of PC1 with sequence and structural homology to the ion channel polycystin-2 (PC2) is indicated in green. **(B)** The cleavage products of PC1. Shown are the N-terminal fragment (NTF) and C-terminal fragment (CTF) that result from auto-catalyzed GPS cleavage and separation from each other. Separation of the NTF and CTF subunits exposes the stalk consisting of the final, 13th beta strand (red arrow) of the intact GAIN domain and a linker, which then constitutes the N-terminus of the CTF. Approximate locations of the protease-mediated cleavage sites within the CTF are indicated by the yellow arrows.

topology, and 11-TM structure of PC1 (Boletta et al., 2001; Nims et al., 2003). A cryo-EM-based structure of the membrane-integrated portion of PC1 in complex with PC2 was solved in 2018 (Su et al., 2018). This work provided final proof of an 11-TM structural conformation for PC1 with a > 3,000 residue N-terminal region and a < 200 residue cytosolic C-tail. Importantly, the region of PC1 encompassed by the last 6 TM domains, which was originally noted to share homology with the sequence of PC2 (Mochizuki et al., 1996), was found to have an ion channel-like structure (Su et al., 2018) (Figure 1). Using the nomenclature adapted from ion channels, this region of PC1 consists of a voltage-sensing domain (S1-S4), a potential pore-forming unit (S5-S6), and a large extracellular loop between S1 and S2 named the Tetragonal Opening of Polycystins (TOP) domain (Figure 1A). Such observations are consistent with a proposed ion channel subunit function for PC1 (Hanaoka et al., 2000) (see more below).

The PC1 ECR consists of multiple functional domains (Figure 1). One unique domain whose structure resembles an Ig-fold and is repeated 16 consecutive times was subsequently named the PKD repeat (Sandford et al., 1997). Atomic force microscopy analyses with bacterially-expressed PKD repeats demonstrated their mechanical strength, which was altered by changes in the solvent or by including ADPKD missense mutations, consistent with a suggested role in mechano- or force-sensing (Forman et al., 2005; Qian et al., 2005; Ma et al., 2009; Ma et al., 2010). PKD repeats also have the ability to interact with each other and have been shown to mediate cell-cell interactions (Ibraghimov-Beskrovnaya et al., 2000; Streets et al., 2003). Other ECR domains with homology to leucine-rich repeats (LRRs), a C-type lectin domain (CTL), and a cell-wall integrity and stress-response component (WSC) suggest a role for PC1 in cell adhesion, which was supported by *in vitro* binding studies demonstrating interactions between the LRR and CTL domains and various purified components of the ECM (Weston et al., 2003). Recently, the CTL and WSC domains were reported to bind secreted Wnts (Kim et al., 2016) and the LRR domain was implicated in activation of the ion channel activity of PC2 (Ha et al., 2020). The membrane proximal portion of the PC1 ECR was noted to have homology with the sea urchin Receptor-for-Egg-Jelly (suREJ) protein involved in the sperm acrosome reaction (Moy et al., 1996) and was thereby called the REJ module (Sandford et al., 1997). This region was found to consist of a fibronectin-like fold and a unique structure called the GPCR autoproteolysis inducing (GAIN) domain, which undergoes autocatalytic proteolytic cleavage at a conserved GPCR proteolysis site (GPS) (Arac et al., 2012; Xu et al., 2013). GPS cleavage of PC1 creates an extracellular N-terminal fragment (NTF) and a membrane embedded C-terminal fragment (CTF) (Figure 1B), which remain non-covalently associated (Qian et al., 2002), and likely play important roles in PC1 function (see Section 3 for further details).

In addition to GPS cleavage, the membrane-associated portion of PC1 undergoes protease-mediated cleavage at multiple sites (Figure 1B). These sites are located within the loops between TM4-TM5, TM5-S1, S1-S2 (TOP domain) and in the last TM domain and C-tail (Chauvet et al., 2004; Woodward et al., 2010; Talbot et al., 2011; Lea et al., 2020). The C-terminal fragments produced from these cleavage events have been observed in either cell culture, kidney tissue, or urinary exosomes, and a variety of roles have been ascribed for some of them (e.g., as regulators of transcription, store-operated calcium entry, cytokine expression, and mitochondrial function) (Lal et al., 2008; Woodward et al., 2010; Talbot et al., 2011; Azevedo et al., 2018). Notably, the two C-tail cleavage fragments are able to undergo nuclear translocation *via* an intrinsic nuclear translocation signal or a transcription factor binding partner, respectively (Chauvet et al., 2004; Talbot et al., 2011).

Multiple functional roles have been described for domains or motifs located within the membrane-associated portion of PC1. The polycystin/lipoxygenase/α toxin (PLAT) domain, which comprises most of the first intracellular loop, regulates the membrane trafficking of PC1 by its ability to bind phosphatidylserine, PI_4P, and β-arrestin (Xu et al., 2016). Binding of β-arrestin to 7-TM GPCRs is typically induced by GRK phosphorylation of the GPCR following its activation of heterotrimeric G proteins and can result in downregulation of G protein signaling or can promote β-arrestin-mediated signaling (Jiang et al., 2022). For PC1, β-arrestin-binding is regulated by phosphorylation at a nearby PKA site (S3164), and when bound by β-arrestin, PC1 is removed from the membrane (Xu et al., 2016).

A short sequence within the C-tail that was capable of stimulating GTPase activity of Gαi/o when tested as a synthetic peptide was named the G protein activation motif and led to a proposed GPCR-like function for PC1 ((Parnell et al., 1998); see Section 2 for more). Two different motifs involved in ciliary targeting of PC1 have been described within the C-tail: KVHPSST at the C-terminus (Ward et al., 2011) and a sequence that overlaps with the G protein activation motif and the binding sites for protein phosphatase 1 and calmodulin (Parnell et al., 2012; Doerr et al., 2016; Luo et al., 2019). Finally, the membrane-distal portion of the PC1 C-tail was discovered to contain a coiled-coil domain that interacts with the PC2 C-tail (Qian et al., 1997; Tsiokas et al., 1997) and other protein partners (Hardy and Tsiokas, 2020), most of which have roles that remain to be determined.

As one might expect from its structural complexity, a multitude of functions have been proposed for PC1. In addition to a role in cell adhesion based on its ECR domains, PC1 is reported to functionally interact with cadherins and to be localized to multiple plasma membrane domains, including adherens junctions, desmosomes, focal adhesions, and the primary cilium (Huan and van Adelsberg, 1999; Scheffers

et al., 2000; Yoder et al., 2002). Interactions between PC1 and cytoskeletal elements have been described, as have PC1-dependent effects on cell polarity, cell migration, and planar cell polarity (Castelli et al., 2013; Yao et al., 2014; Castelli et al., 2015; Nigro et al., 2015). A number of early studies involving ectopic expression of various PC1 C-terminal expression constructs implicated a role in cellular signaling for PC1. These included an ability to activate signaling pathways to AP-1 (involving Cdc42, Rac-1, PKC, JNK, and heterotrimeric G proteins) (Arnould et al., 1998; Parnell et al., 2002), TCF (*via* β-catenin stabilization) (Kim et al., 1999a), and NFAT (*via* Gαq, PLC, and intracellular calcium) (Puri et al., 2004). An ability of the PC1 C-tail to bind and activate G proteins led to an early proposal that PC1 functions as an atypical GPCR (see Section 2). Later, full-length PC1 was shown to activate a p21 gene promoter (*via* JAK2/STAT1) in a PC2-dependent manner (Bhunia et al., 2002) and to regulate tubule versus cyst formation in 3D collagen gel assays. Roles for PC1 in the modulation of a variety of signaling pathways have been proposed, including Wnt signaling (Kim et al., 1999a), STAT regulation (Weimbs et al., 2013), and YAP/TAZ activity (Nigro et al., 2019), among others.

1.3 Polycystin-1 as an ion channel subunit

PC1 is thought to form a membrane receptor-ion channel complex with PC2 that is responsive to ligand- or mechanical-activation and that plays a role in cellular ion homeostasis. This concept is based on the homology between PC1 and the ion channel structure of PC2 and demonstration of PC1/PC2 interaction and complex formation (Newby et al., 2002). Studies in transfected CHO cells revealed unique, PC1 and PC2 co-dependent, calcium-permeable cation currents (Hanaoka et al., 2000). However, the idea of a joint ion channel function for these two proteins did not become widely recognized until the ability of PC1/PC2 to sense fluid shear stress and modulate intracellular calcium levels was demonstrated in cells (Nauli et al., 2003; Alenghat et al., 2004; Nauli et al., 2008; MacKay et al., 2022). Most recently, endothelial cell-specific *Pkd1* and *Pkd2* knockout mice were used to demonstrate regulation of vasodilation by the PC1/PC2 complex (MacKay et al., 2022). Formation of an ion channel-like structure consisting of PC1 and PC2 was finally validated by solving the molecular structure of the membrane-associated portions of the two proteins together (Su et al., 2018). The cryo-EM structure revealed a heterotetrameric complex consisting of three PC2 subunits and one PC1 subunit, as originally proposed (Yu et al., 2009; Zhu et al., 2011). Although the putative pore loops of the PC1 subunit were not visible, an ion conduction pore formed by the final two TM domains of each subunit was discernable. Electrophysiological studies in *Xenopus* oocytes recently demonstrated a direct role of the PC1 subunit in the ion channel activity of the PC1/PC2 complex (Wang et al., 2019). Co-expression of PC1 together with a gain of function (GOF) mutant of PC2 resulted in ion channel properties that differed from the homotetrameric PC2 GOF channel, including an increased permeability for Ca^{2+}. Missense mutations within the putative pore region of PC1 resulted in significant alterations in ion permeability and current characteristics of the PC1/PC2 GOF complex demonstrating a direct role for PC1 in formation of the pore and thus in ion channel activity. This work also showed that GPS cleavage of the PC1 subunit was not required for channel activity and that complexes formed with the CTF or the six C-terminal TM domains of PC1 were also capable of ion conductance.

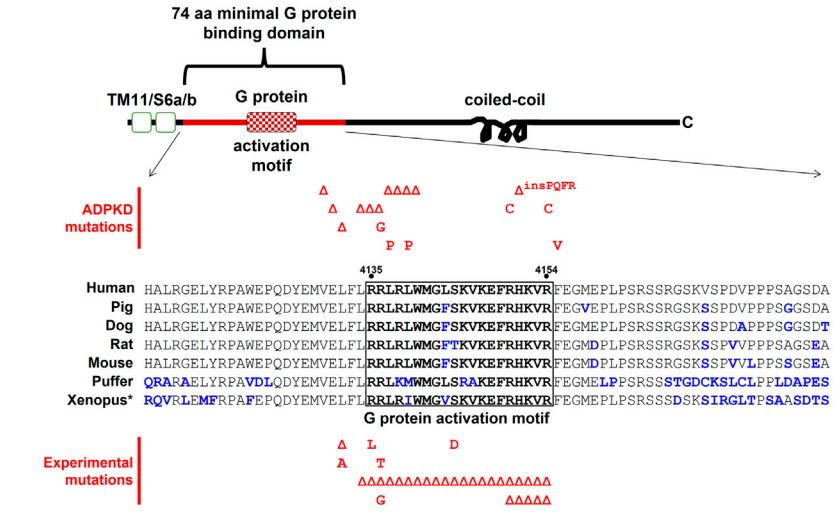
Relatively little is known regarding the regulation of this unusual PC1/PC2 ion channel complex. Binding of Wnts to the CTL and WSC domains of the PC1 ECR results in ligand-mediated activation of the PC1/PC2 ion channel complex (Kim et al., 2016). In an intriguing twist, the LRR domain within the PC1 ECR was shown to bind to N-glycans of the PC2 TOP domain and to activate the ion conductance of the complex (Ha et al., 2020). In this latter work, the PC1 NTF was proposed to act as a soluble ligand that activates the PC1/PC2 receptor-ion channel complex. Currently, there is only a single study suggesting that PC1-mediated G protein signaling regulates PC1/PC2 channel activity (Parnell et al., 2018). In contrast, an earlier study proposed that channel activation occurred *via* conformational rearrangements of PC1 (Delmas et al., 2004). As such, this is an important aspect of polycystin function that remains to be clarified by further investigation.

In summary, while cellular adhesion, signal transduction, and ion channel activity have all been identified as PC1 functions, how these functions are interconnected and which specific function whose loss initiates cystogenesis remains unresolved. The focus of this article is on the evidence that suggests a critical function of PC1 is to regulate heterotrimeric G protein signaling.

2 Evidence for polycystin-1 GPCR function

2.1 Polycystin-1 interacts with heterotrimeric G proteins

The first evidence that PC1 could interact with heterotrimeric G proteins came from *in vitro* binding studies utilizing GST fusion proteins consisting of various portions of the C-tail of mouse PC1 (Parnell et al., 1998). Pull-down and co-immunoprecipitation assays demonstrated interactions between these C-terminal fusion proteins and heterotrimeric Gα and Gβ subunits from various sources, including heterotrimeric complexes purified from bovine brain and from rat brain lysates. These experiments also identified a membrane-proximal, minimal binding region of 74 amino acids required for stable interactions between



	In Vitro				*In Vivo*	Reference
Mutation	G protein binding	G protein dependent signaling	PC2 binding	Other		
L4132Δ	ND	↓	No effect	†	Embryo lethal; cystogenic	Parnell et al., 2018
L4132A	ND	No effect	No effect	ND	ND	Parnell et al., 2018
R4135L	ND	↓	No effect	ND	ND	Parnell et al., 2018
R4136G	ND	↓	No effect	ND	ND	Parnell et al., 2018
R4136T	ND	↓	No effect	ND	ND	Parnell et al., 2018
S4144D	ND	No effect	No effect	¥	ND	Doerr et al., 2016
L4134-4154Δ21**	ND	↓	ND	£	ND	Chauvet et al., 2004
R4150-R4154Δ5	↓	ND	ND	¢	Fails to rescue *Nbc1* localization	Zhang et al., 2018

FIGURE 2
The cytosolic C-terminal tail of PC1 is shown schematically with sequence alignment of the 74 aa minimal G protein binding domain (corresponding to human aa 4,111–4,184). Small deletion and missense ADPKD-associated mutations that fall within this region and score as "Pathogenic" or "Likely Pathogenic" (as determined by the ADPKD Variant Database <pkdb.mayo.edu>) are shown above the sequence alignment. Experimentally-generated mutations designed to test effects on G protein signaling are shown below the alignment. Additional references for these ADPKD-associated mutations can be found at (Afzal et al., 1999; Perrichot et al., 1999; Garcia-Gonzalez et al., 2007; Rossetti et al., 2007; Reed et al., 2008; Tan et al., 2009; Audrezet et al., 2012; Rossetti et al., 2012). ND, not determined; †, decreased PC1/PC2 channel activity; ¥, calmodulin-binding disrupted, decreased PC1/PC2 channel activity and flow-dependent channel response, no effect on ciliary localization, decreased energy metabolism; £, decreased nuclear localization; ¢, C-tail nuclear localization unaltered. **Xenopus* sequences are from *X. tropicalis*; **construct expressed as a soluble protein. Sequence accession numbers: human AAC37576; pig CBZ01637; dog AAM22956; rat AAG33986; mouse AAC53207; puffer XP_011610747; *Xenopus* XP_017952982.

PC1 and heterotrimeric G proteins that is highly conserved among vertebrates (Figure 1A, Figure 2). This minimal G protein binding domain contains a polybasic stretch of 20 amino acid residues that possess guanine-nucleotide exchange factor activity. Exchange factor activity was demonstrated by assays using purified heterotrimeric G proteins and a synthetic 20 amino acid peptide spanning this so-called G protein activation motif (Parnell et al., 1998). The minimal binding domain is distinct from the membrane-distal portion of PC1's C-tail, which contains the coiled-coil domain responsible for interactions with PC2 (Qian et al., 1997) (Figure 1A, Figure 2). Several engineered mutations affecting PC1 function, as well as ADPKD-associated mutations, have been generated within and near the G protein activation motif (discussed further in sections 2.2, 2.3).

Additional studies have reported interactions between Gαi1, Gαi3, Gαs, and Gα12 using various approaches. Kwak et al. (2018) demonstrated an interaction between PC1 and Gαi3 by both co-immunoprecipitation and FRET between transiently-expressed constructs. Yuasa et al. (2004) found that, when expressed as a GST-fusion protein, the PC1 C-tail pulled down Gα12 subunits from transfected MDCK cell lysates, and endogenously expressed Gαs and Gαi1 subunits. Stable

interactions were also detected between PC1 and $G\alpha12^{Q229L}$ (Aragay et al., 1995), a mutant form of Gα12 that exists in a constitutively active state due to an inability to hydrolyze GTP. Yu et al. (2010) demonstrated stable interactions between PC1 and Gα12, and subsequently Yu et al. (2011) identified mutations within Gα12 that completely disrupted interactions with PC1. Importantly, this work also identified the previously characterized 74 amino acid minimal G protein binding domain within the C-tail of PC1 as essential for the PC1/Gα12 interaction. An endogenous interaction between PC1 and Gαi2 in mouse embryonic fibroblasts was also suggested by studies utilizing the *Pkd1*$^{HA/HA}$ mouse model and an approach involving SILAC coupled with immunoprecipitation and mass spectrometry (Nigro et al., 2019). Stable interactions between the C-tail of PC1 and various Gα protein subunits were also demonstrated by surface plasmon resonance in a screen of bacterially-expressed PC1 C-tail constructs (from *Xenopus*) and all Gα subunits found in the *Xenopus* embryonic pronephros expressed in reticulocyte lysates. High-affinity interactions were detected both ways between the C-tail and Gnas, Gna14, Gnai1, and Gnai2, as well as mouse Gna12 (Zhang et al., 2018). These binding affinities were comparable to those found for Gα subunits and other GPCRs (Komolov et al., 2006), and binding was completely disrupted by deletion of 5 amino acids from the previously identified G protein activation motif (Figure 2).

The minimal binding domain for G proteins also overlaps with other previously described regions of interest, including binding sites for calmodulin (Doerr et al., 2016) and protein phosphatase 1 (Parnell et al., 2012), a protein kinase A phosphorylation site (Parnell et al., 1999), and sequences for ciliary (Luo et al., 2019), mitochondrial (Lin et al., 2018), and nuclear (Chauvet et al., 2004) localization, suggesting that G proteins may be involved in multiple PC1 functions. The distal portion of the C-terminal tail of PC1, beyond the minimal G protein binding domain, has also been shown to interact with RGS7 (Kim et al., 1999b). RGS7 is a member of the negative Regulator of G protein Signaling family capable of stimulating the GTPase activity of Gα subunits, resulting in their inactivation (Dohlman and Thorner, 1997). This interaction was identified genetically by a yeast two-hybrid screen and physically *via in vitro* binding assays and co-immunoprecipitation of transiently-expressed components. Co-expression of the C-tail of PC1 altered the cellular localization of RGS7 and prevented its degradation in transfected cells, further suggesting a physical interaction between the two proteins.

2.2 Polycystin-1 regulates heterotrimeric G protein signaling

In addition to direct interactions between PC1 and heterotrimeric G protein subunits, numerous lines of evidence have suggested that PC1 regulates heterotrimeric G protein-dependent signaling in cellular assay systems. In MDCK cells ectopically expressing PC1, resistance to apoptosis was shown to be dependent on PI3Kβ activation *via* heterotrimeric G proteins, by using pertussis toxin (Boca et al., 2006). PC1-mediated activation of c-Jun-N-terminal kinase (JNK) and AP-1 promoter-reporter activity was inhibited by Gβγ-sequestering βARK-ct, dominant-negative Gαi2, and the Gα12/13 dominant-negative inhibitor p115RhoGEF (Parnell et al., 2002). In this study, PC1-dependent JNK and AP-1 activity was augmented by co-transfection of WT Gα subunits, including Gαi1, Gαi2, Gαi3, Gα12/13, and Gαq. Gαq also potentiated activation of PC1-dependent NFAT promoter-reporter activity (Puri et al., 2004). Mutation of critical amino acids within the G protein activation motif, including ADPKD patient-associated mutations [see Figure 2 and (Afzal et al., 1999; Perrichot et al., 1999; Garcia-Gonzalez et al., 2007; Rossetti et al., 2007; Reed et al., 2008; Tan et al., 2009; Audrezet et al., 2012; Rossetti et al., 2012)], also reduced PC1-mediated basal and G protein-augmented activation of AP-1 promoter reporter activity (Parnell et al., 2018). Of note, a single amino acid ADPKD patient mutation L4132Δ (Afzal et al., 1999) was found to block basal and augmented AP-1 activity as well as PC1/PC2 channel activity in electrophysiological studies in CHO cells (Parnell et al., 2018). This mutation is one of several ADPKD-associated small deletion mutations (see Figure 2), interspersed over amino acids 4,130–4,140, that are predicted to disrupt the amphipathic helical structure of the 20 amino acid G protein activation motif. Within this cluster of mutations are two proline substitutions that are likely to break the localized helical structure of the motif. Notably, an experimentally engineered substitution L4132A, which would presumably allow retention of the amphipathic nature of the activation motif, did not block basal AP-1 activation. Deletion of the entire G protein activation motif in the context of a soluble C-terminal tail fragment of PC1 blocked both AP-1 activity and nuclear translocation of the soluble fragment (Chauvet et al., 2004). These results suggest that PC1 regulates cellular signaling pathways, including PC2 channel activity, by activating heterotrimeric G protein signaling.

In additional electrophysiological studies, expression of PC1 in sympathetic neurons that do not otherwise express PC1 resulted in modulation of Ca^{2+}- and GIRK-channel activity. PC1-mediated channel modulation could be prevented by inhibitors of G protein signaling, including Gβγ-sequestering Gα transducin, non-hydrolyzable GDP-β-S, and pre-treatment of cells with pertussis toxin and N-ethylmaleimide. In this assay system, G protein-dependent channel regulation was antagonized by co-assembly of PC1 with co-expressed PC2 (Delmas et al., 2002). A later study by this same group showed that structural rearrangement of PC1 simultaneously but independently stimulated the channel activity of PC2 itself and G protein-dependent signaling (Delmas et al., 2004). These results suggest that G protein-dependent

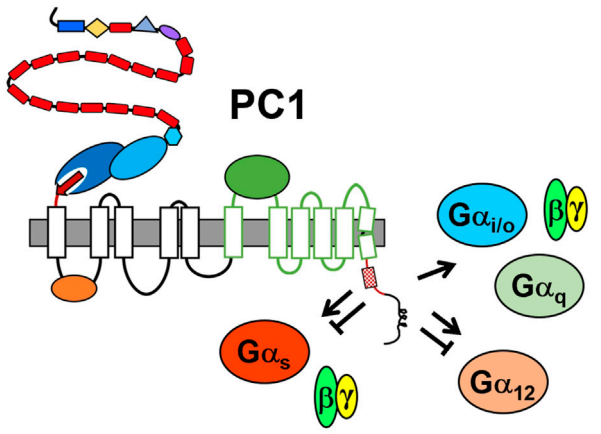


FIGURE 3
Summary of PC1-mediated regulation of heterotrimeric G protein signaling. The minimal G protein binding domain is represented by the membrane-proximal portion of the C-tail, i.e., red line with hatched box representing the G protein activation motif. Other structural domains are as identified in Figure 1. Arrow indicates activation; the bar-headed line indicates inhibition. There is evidence that PC1 both positively and negatively regulates Gαs and Gα12 families.

signaling and channel activity of the PC1/PC2 complex are coordinately regulated, potentially *via* a ligand-mediated structural rearrangement of PC1.

Co-transfection of PC1 and PC2 with activator of G protein signaling 3 (Ags3) increased PC1/PC2 channel activity, and this activity could be inhibited by co-transfection of Gβγ-sequestering βARK-ct (Kwon et al., 2012). Co-transfection of PC1 with TRPC4β increased TRPC4β-dependent channel currents and increased the amount of Gαi3 in complex with TRPC4β. These currents were inhibited by a dominant-negative Gαi3 (Kwak et al., 2018). These results suggest that PC1-mediated activation of heterotrimeric G protein signaling can regulate the activity of numerous channel proteins. Interestingly, GPS-cleavage-deficient PC1 mutants were incapable of TRPC4β activation (Kwak et al., 2018), suggesting a potential link between GPS cleavage and G protein activation (see Section 3).

Several lines of evidence have also suggested that PC1 negatively regulates G protein signaling. Activation of Gα12 in MDCK cells induces JNK and stimulates apoptosis. This Gα12-stimulated activity is enhanced by silencing of PC1 and is inhibited by over-expression of PC1 (Yu et al., 2010). PC1-dependent inhibition of Gα12-mediated apoptosis is abrogated by deletion of the minimal G protein binding domain and by Gα12 mutations that uncouple binding between Gα12 and PC1 (Yu et al., 2010; Yu et al., 2011). PC1 silencing or Gα12 activation also promoted increased shedding of E-cadherin and nuclear localization of β-catenin in an ADAM10-dependent fashion (Xu et al., 2015), and altered expression of N-cadherin from early-to late-isoforms in MDCK cells (Wu et al., 2016). In an assay of mouse proximal tubular cells grown in matrigel, loss of PC1 resulted in cyst formation, but treatment of these cells with a small-molecule inhibitor of Gβγ subunits, gallein, inhibited cell proliferation and promoted tubule formation (Zhang et al., 2018). Thus, PC1 appears to have the potential to both positively and negatively regulate heterotrimeric G protein signaling (see Figure 3).

2.3 Evidence from animal models

Several of the studies offering cell-based evidence for PC1-mediated regulation of G protein signaling are complemented by experiments performed in animal models. In Parnell et al. (2018) the ADPKD patient mutation L4132Δ was introduced into the mouse *Pkd1* gene. This single amino acid deletion mutation, which blocked PC1/G protein mediated activation of promoter-reporter activity as well as PC1/PC2 channel activity in cellular assays, also resulted in a severe loss of PC1 function as evidenced by a cystic embryonic kidney phenotype and embryonic lethality in $Pkd1^{\Delta L/\Delta L}$ embryos, and rapid cyst formation in newborn $Pkd1^{\Delta L/fl}$ following Hoxb7 Cre-mediated excision of the floxed allele. These results suggest that the ΔL mutation, thought to interfere with the local structure of the C-tail G protein activation motif, prevents an essential function of the PC1 protein, namely G protein activation. In other experiments, Kwon et al. (2012) determined the consequence of Ags3 knockout in the context of the hypomorphic $Pkd1^{V/V}$ mouse. In cell-based assays, PC1/PC2 channel activity was increased by co-transfection of Ags3 in a Gβγ-dependent manner. Likewise, cystic disease in the $Pkd1^{V/V}$ mouse was exacerbated by homozygous deletion of Ags3. In contrast, however, Yu et al. demonstrated that various Gα12-dependent signaling outputs, including JNK and apoptosis, were upregulated in the absence of PC1 and downregulated by its over-expression (Yu et al., 2010; Yu et al., 2011). Wu et al. (2016) subsequently demonstrated that genetic deletion of Gα12 completely blocked renal cystogenesis in $Pkd1^{fl/fl}$ mice with Mx1 Cre-driven deletion of *Pkd1*, suggesting that Gα12 is required for the development of renal cysts following loss of PC1 function. Finally, Zhang et al. (2018) demonstrated that treatment of *Xenopus* embryos with the Gβγ subunit inhibitor gallein, which inhibited cell proliferation and cyst formation in *Pkd1*-deficient proximal tubular cells, also prevented cystic phenotypes in *Xenopus Pkd1* morphants. A cystic phenotype could also be induced in *Xenopus* by morpholinos directed against cAMP-activating *Gnas*, and cystic *Xenopus* phenotypes were rescued by deletion of a Gβ subunit or by expression of the PC1 C-tail. However, a C-tail construct with a mutation within the G protein binding domain was not capable of rescuing the *Pkd1* morphant phenotype. Refer to Figure 2 for a summary of the effects of mutations within the

minimal G protein binding domain observed in *in vitro* and *in vivo* experimental systems.

While these various cellular and *in vivo*-based experiments describe potential links between PC1 and G protein dependent signaling, it is important to note that a limitation of these studies is that they do not distinguish between effects that are dependent on a direct interaction between PC1 and heterotrimeric G proteins versus G protein-dependent effects that are downstream of PC1-dependent signaling. Additional experimentation will likely be required to resolve these questions.

3 Polycystin-1 as a novel adhesion GPCR

3.1 Adhesion G protein-coupled receptors, G protein-coupled receptors proteolysis site cleavage and the GAIN domain

One of the defining and generally conserved structural features of the PC1 protein family identified early on (Ponting et al., 1999) is the presence of a GPCR proteolysis site, or GPS, that is now known to be part of a larger, evolutionarily conserved structure named the GAIN domain (Arac et al., 2012). The GAIN domain and its unusual properties (see below) are found only in the PC1 and adhesion GPCR families (Promel et al., 2013). Adhesion GPCRs play important functions in planar cell polarity, neuronal development, and tumor cell biology among others (Maser and Calvet, 2020; Lala and Hall, 2022). Unlike other GPCR families they typically have extremely large, extracellular N-terminal regions. Furthermore, the ECRs of adhesion GPCR proteins are composed of multiple types of "adhesive" domains (e.g., LRR, Ig-like, lectin) that are often involved in cell-cell and cell-matrix interactions. Together with the GAIN domain, these ECR properties and functions represent additional features shared between adhesion GPCRs and PC1.

The GAIN domain and GPS motif were named due to their involvement in a proteolytic reaction that occurs at a conserved [HL$\downarrow^{T}/_{S}$] tripeptide (where ↓ indicates the cleavage site) within the GPS motif. The GPS motif is an ~50 residue sequence characterized by conserved tryptophan and 2-4 disulfide bond-forming cysteine residues. This motif is located in the extracellular N-terminal region of both adhesion GPCRs and PC1 in close proximity to the first TM domain and is part of a larger (~300 residue) GAIN domain (Arac et al., 2012). The prototypical GAIN domain consists of two subdomains, A and B, and is composed of 8 alpha helices and 13 beta strands, in which strands 9–13 make up the GPS motif. Cleavage at the GPS occurs *via* an autocatalytic cis-proteolytic reaction facilitated by nucleophilic residues surrounding the cleavage site. GPS cleavage generates a C-terminal, membrane-embedded fragment, the CTF, and an N-terminal, extracellular fragment, the NTF. The NTF and CTF subunits remain non-covalently attached through numerous hydrophobic and H-bond interactions between the final 13th beta strand and strands 6, 7 and 9 within the C-terminal B subdomain. Since this seminal discovery, the GAIN domain structures of GPR56/ADGRG1 and GPR126/ADGRG6 (Salzman et al., 2016; Leon et al., 2020) have also been solved and revealed only slight variations, primarily in size, in the overall composition and structure of this domain.

3.2 Mechanisms of G protein activation by adhesion GPCRs

As befits their complex structural organization, the regulation of G protein signaling by individual adhesion GPCRs has been shown to involve multiple mechanisms. Several groups reported that expression constructs encoding the CTF subunit alone, beginning with the first residue following GPS cleavage, were capable of activating heterotrimeric G proteins in a constitutive manner [reviewed in (Maser and Calvet, 2020)]. When compared to the NTF/CTF heterodimer, CTF-mediated activation was much greater for a number of adhesion GPCRs. These observations were originally interpreted to suggest that the associated NTF subunit had a role in inhibiting signaling by the CTF. Thereafter, two different groups showed that the constitutive signaling activity of the CTF subunit was dependent on the presence of the short, N-terminal 'stalk' preceding the first TM domain (Liebscher et al., 2014; Stoveken et al., 2015). This requirement for the stalk for activation of adhesion GPCRs was demonstrated by the inability of CTF constructs with deletion of the stalk to signal. The ability of soluble, synthetic peptides derived from the stalk sequence to rescue G protein signaling by the stalk-deleted mutants provided additional support for the stalk-dependent mechanism. To fit these and preceding observations, the 'tethered cryptic ligand/agonist' model for activation of G protein signaling by adhesion GPCRs was proposed (Liebscher et al., 2014; Stoveken et al., 2015).

In the tethered cryptic agonist model, GPS cleavage followed by dissociation of the NTF results in exposure of the stalk/tethered agonist (TA) previously buried within the GAIN domain. Exposure (de-cryption) of the largely hydrophobic stalk was proposed to favor its subsequent interaction with the membrane-embedded 7-TM helical bundle of the CTF, presumably leading to conformational changes which would drive heterotrimeric G protein binding and activation. Since proposal of this mechanism, the CTF stalk has also been referred to as the tethered peptide ligand, TA, or Stachel sequence (Stachel being German for *stinger*). Proponents of the TA model envisioned that the NTF might be removed from the CTF subunit *via* mechanical means or its interactions with an adhesion ligand.

Activation of signaling by the CTF as a direct consequence of NTF dissociation has since been demonstrated by replacing the GAIN domain and the GPS cleavage site with the recognition site for an exogenous protease such as thrombin or enterokinase. Protease treatment of cells expressing these chimeric adhesion GPCRs was shown to result in the exposure of the TA and led to activation of signaling (Mathiasen et al., 2020; Frenster et al., 2021; Lizano et al., 2021). Interaction with ECM binding partners followed by activation of signaling has also been demonstrated for a number of adhesion GPCRs, as has the application of mechanical stimulation by vibration, shaking, or shear stress (Petersen et al., 2015; Wilde et al., 2016; Yeung et al., 2020). Studies in *Drosophila* have shown a role for the adhesion GPCR latrophilin/dCIRL in mechanosensing by chordotonal neurons that involves regulation of TRP channel activity (Scholz et al., 2015; Scholz et al., 2017). Furthermore, there are links between missense mutations in EMR2/ADGRE2 and defects in VLGR1/ADGRV1 with familial vibratory urticaria and hearing loss, respectively (McMillan and White, 2010; Naranjo et al., 2020; Kusuluri et al., 2021). Such observations support a general view that the structural conformation of adhesion GPCRs is especially conducive to mechano-responsive signaling functions [reviewed in (Lin et al., 2022)].

Following proposal of the cryptic TA mechanism, its general applicability was challenged by observations of non-cleavable and heterodimeric, NTF/CTF-associated adhesion GPCRs that were still capable of signaling (e.g., GPR114/ADGRG5) (Wilde et al., 2016). Interestingly, for some cleavage-defective adhesion GPCRs, G protein signaling remained dependent on the TA (Bohnekamp and Schoneberg, 2011; Promel et al., 2012; Wilde et al., 2016; Scholz et al., 2017). A unifying paradigm for these observations was recently provided by experiments that utilized biorthogonal click-labeling to identify solvent-exposed TA residues (Beliu et al., 2021). Together with molecular dynamics simulations, this study revealed an inherent conformational flexibility within the GAIN domain. Two flexible loops or flaps were identified in the GAIN domain which appear to open and thereby allow portions of the TA sequence to become accessible for interaction with the TM bundle. It was postulated that 'flexing' of the GAIN domain might be modulated by the engagement of specific ligands to the GAIN domain itself (e.g., synaptamide), or to an adhesion domain within the ECR. Some groups have used the binding of synthetic ligands, such as antibodies directed at ECR domains or at ectopic N-terminal epitope tags, as a means to activate adhesion GPCRs (Salzman et al., 2017; Bhudia et al., 2020; Huang et al., 2020; Mitgau et al., 2022). Recently, the cryo-electron micrograph (EM) structures of the CTF subunit for a number of adhesion GPCRs were published by four groups (Barros-Alvarez et al., 2022; Ping et al., 2022; Qu et al., 2022; Xiao et al., 2022). These structures confirmed the originally proposed interaction of the TA in a hydrophobic binding pocket formed by the 7-TM helical bundle. Furthermore, the cryo-EM structure of a full-length, GPS cleavage-defective adhesion GPCR, GPR110/ADGRF1, revealed that its TA was able to bind in the 7-TM pocket (Qu et al., 2022). This structural evidence demonstrates the flexibility of the GAIN domain and supports this mechanism as another means of adhesion GPCR activation by its TA.

3.3 GPS cleavage is critical for Polycystin-1 function and prevention of cystogenesis

PC1 GPS cleavage occurs both *in vitro* and *in vivo* (Qian et al., 2002; Yu et al., 2007) to yield ~300 kDal NTF and ~130 kDal CTF subunits (Figure 1B), which can be found non-covalently associated or as separate subunits. GPS cleavage is ubiquitous, but incomplete, as shown by the presence of both full-length uncleaved and cleaved NTF/CTF forms of PC1 in multiple tissues and cell lines (Yu et al., 2007; Castelli et al., 2013; Kurbegovic et al., 2014). The relative proportion of cleaved versus uncleaved PC1 isoforms varies between tissue types and at different developmental stages (Castelli et al., 2013; Kurbegovic et al., 2014). Such observations suggest that this autocatalytic event can be regulated by additional factors, perhaps *via* conformational changes of the GAIN domain induced by the binding of ligands to various domains of the ECR. In support of this idea, the ER-resident protein, Sec63, involved in translocation of integral membrane and secreted proteins, has been implicated as being necessary for GPS cleavage of PC1 (Fedeles et al., 2015), as has the presence of PC2 (Chapin et al., 2010; Gainullin et al., 2015). It is also possible that the non-cleaved, NTF/CTF-associated or -dissociated isoforms of PC1 carry out distinct functions.

Approximately 30% of the missense mutations identified in *PKD1* are located within the GAIN domain or near the GPS motif. Studies in cultured cells have shown that many of these mutations reduce or prevent GPS cleavage and inhibit the ability of PC1 to both activate certain signaling pathways and induce tubulogenesis of MDCK cells in 3D collagen gels (Qian et al., 2002; Arac et al., 2012; Qian and Li, 2015). GPS cleavage may also be necessary for the proper maturation and trafficking of PC1 to the primary cilium (Cai et al., 2014; Kim et al., 2014; Su et al., 2015). Two *Pkd1* mouse models, each with defective GPS cleavage of PC1 due to a different missense mutation, have demonstrated that cleavage is essential for preventing cyst formation (Yu et al., 2007; Cai et al., 2014). Despite the likely importance of PC1 GPS cleavage, the mechanisms affecting cleavage and the role that GPS cleavage isoforms play in various PC1 functions, including the modulation of G protein signaling (Kwak et al., 2018), remain relatively unknown.

3.4 The adhesion GPCR-like signaling activation mechanism for polycystin-1

Although PC1 has been referred to as a novel or atypical adhesion GPCR based on the GAIN domain and GPS cleavage,

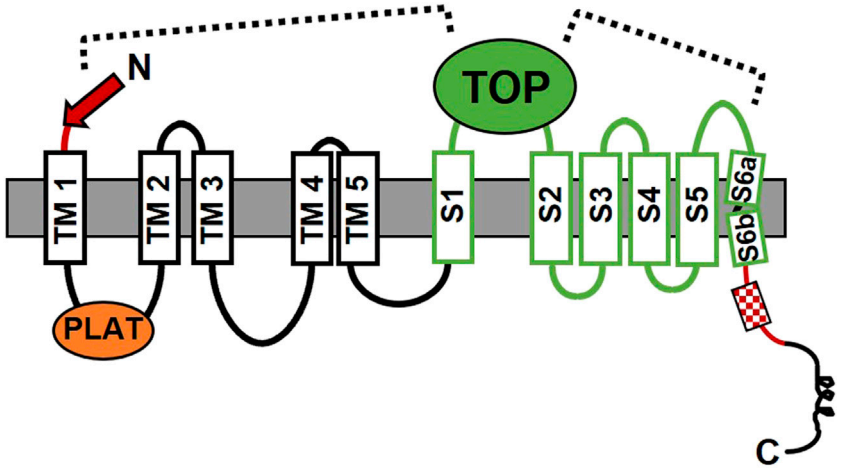


FIGURE 4
The proposed allosteric mechanism for stalk-mediated activation of signaling by the CTF form of PC1. The model is supported by the results from molecular dynamics simulations and mutagenesis-signaling studies (Pawnikar et al., 2022). The dashed lines indicate regions of the PC1 CTF protein where important correlated residue motions or residue-residue interactions were identified as being involved in activation of signaling by the stalk, i.e., between the N-terminal stalk (red arrow) and TOP domain, and between the TOP domain and the putative pore loop between S5 and S6 TM domains. Domains are as described in Figure 1; Section 1.

TA-dependent signaling had not been reported until recently. In work from Pawnikar et al. (2022), transient transfection of wild-type (WT) or stalk-mutant CTF expression constructs of PC1 revealed a requirement for the stalk/TA in the activation of a promoter-luciferase signaling reporter. The stalk-mutant constructs included a CTF lacking the first 21 residues of the stalk (ΔstalkCTF) and three additional proteins each with an ADPKD-associated missense mutation within the stalk- G3052R, R3063C and R3063P (ADPKD Variant Database, https://pkdb.mayo.edu). The CTF stalk mutants G3052R, R3063C, and Δstalk displayed significantly reduced reporter activity in comparison to WT CTF. In another study, the CTF form of PC1 was shown to activate the NFAT reporter to a much greater extent than full-length PC1, and synthetic peptides derived from the CTF stalk sequence were able to stimulate NFAT reporter activation by ΔstalkCTF (Magenheimer et al., 2021). Work by Kwak et al. (2018) has shown that GPS cleavage of PC1 is required for activation of TRPC4 *via* Gαi3 in endothelial cells. Altogether, such observations are consistent with the PC1 CTF stalk possessing a TA-like activity that can mediate signaling by PC1.

A potential mechanism for the stalk TA-mediated activation of the PC1 CTF was uncovered by molecular dynamics simulations using computer models of the WT and stalk-mutant CTF proteins, ΔstalkCTF, G3052R, R3063C and R3063P (Pawnikar et al., 2022). Highly correlated residue motions between the stalk-TOP and TOP-pore loop domains were observed for WT CTF (Figure 4) that were significantly lower in the stalk mutants, suggesting these domains were important for stalk TA-mediated signaling. Key residue-residue interactions between these regions were identified for WT CTF that appeared to be absent in simulations with the stalk mutants. Low-energy conformational states differed between WT and stalk-mutant CTF proteins and revealed that most of the key residue interactions identified in WT CTF were broken or absent in the stalk mutants. The importance of these residue-residue interactions was corroborated in functional cell signaling assays in which NFAT reporter activation was decreased for CTF expression constructs with single residue substitutions designed to disrupt key interactions. Such results are consistent with the proposal that an allosteric transduction pathway connecting the stalk-TOP-pore loop domains was responsible for stalk TA-mediated activation of signaling by the PC1 CTF. While consistent results were obtained in the studies described above, it is important to point out that both approaches involved examination of PC1 CTF alone, i.e., not in complex with PC2. The cryo-EM structure of PC1/PC2 complex revealed inter-subunit interactions between the TOP domains and the TOP domain with extracellular loops (Su et al., 2018). As such, the molecular mechanism for activation of signaling by the PC1/PC2 complex may differ from that of CTF alone.

It is tempting to speculate, based on the shared similarities between the ECRs of PC1 and adhesion GPCRs, that stalk TA-mediated activation of signaling by PC1 could be stimulated by its cell adhesion or mechanosensing properties. For example, components of the ECM interacting with the LRR, C-type lectin, or WSC domains of PC1 could serve as activating ligands. Similarly, trans-cellular (or even cis-cellular) interactions between the PKD repeats within the ECR of separate PC1 molecules, or shear stress might also serve as stimulatory signals (Ibraghimov-Beskrovnaya et al., 2000). In this scenario, these processes would remove the NTF, or alter the conformation

of the GAIN domain, leading to exposure of the stalk TA of PC1. Once exposed, interaction of the stalk with the TOP domain would result in a signaling-active conformation of the CTF. So far, only Wnts and fluid shear stress have been identified as activating factors for PC1 using ion channel activity of the PC1/PC2 complex as a functional readout (Nauli et al., 2003; Kim et al., 2016). While the involvement of G protein signaling was ruled out in the case of shear stress (Nauli et al., 2003; Kim et al., 2016), the ability of Wnt9b binding to activate GPCR signaling by PC1 has been suggested in other work (Gresko et al., 2019).

Notably, the mechanism proposed for stalk TA-activated signaling by PC1 differs substantially from that recently revealed by the cryo-EM studies of adhesion GPCRs (Barros-Alvarez et al., 2022; Ping et al., 2022; Qu et al., 2022; Xiao et al., 2022). Whereas the PC1 CTF may be activated by interaction of the stalk TA with the extracellular TOP domain, for the adhesion GPCRs, the N-terminal end of the TA is inserted within the TM helical bundle. This disparity could be due to differences in their stalk/TA sequences and/or in the size and structure (and additional functions) of their extracellular loops. Furthermore, it is likely that the mechanism of TM signal transmission will also differ given the 11-TM versus 7-TM structural conformations of PC1 and adhesion GPCRs. Much work remains to be done to reveal the means of stalk TA-mediated TM signal transduction, and G protein selection and binding for PC1 (discussed further in Section 4), and it is anticipated that such insights will be novel and beneficial for our understanding of both atypical and canonical GPCRs.

4 Controversies regarding polycystin-1 G protein-coupled receptor function

A primary controversy that remains is the mechanism by which PC1 affects G protein function. Wu et al. demonstrated that *Pkd1* knockout led to increased Gα12 activation, and that genetic deletion of Gna12 in mice blocked cystogenesis induced by conditional deletion of *Pkd1* (Wu et al., 2016). In this model, PC1 is hypothesized to sequester Gα12 subunits that are putatively pro-cystogenic. In another study, Zhang et al. (2018) demonstrated a PKD phenotype in the pronephric Xenopus kidney following loss of Gαs, and that inhibition of Gβγ signaling antagonized this phenotype, suggesting that loss of Gαs leads to unregulated, cystogenic Gβγ signaling. This result suggests that PC1 inhibits PKD phenotypes in *Xenopus* by binding and sequestering Gαs in the heterotrimeric complex. These studies are in contrast with Parnell et al. (1998) who showed that PC1 contains a motif that activates guanine nucleotide exchange, and that a PC1 ADPKD patient mutation that disrupts G protein-dependent signaling in cellular assays results in severe cystic disease when introduced into a mouse model (Parnell et al., 2018). However, while these results may seem discordant, there are several potential explanations that may be able to reconcile these different models of PC1 function.

For one, PC1 appears to be promiscuous in its ability to interact with heterotrimeric G proteins, and may have differential effects on the activity of the various families or be affected by the context in which they interact. Paradigms for this model include the β_2-adreneric receptor, which can activate either Gαs or Gαi, depending on the PKA phosphorylation status of the receptor (Lefkowitz et al., 2002), or the vasopressin V_2 receptor, which has been shown to activate Gαs-dependent signaling and to inhibit Gα12 signaling in response to ligand-mediated activation (Okashah et al., 2020). In a similar manner, PC1 may activate a subset of G proteins under certain circumstances while binding and sequestering another subset of G proteins under other circumstances. It is also important to note that NAAIRS-based substitution of Gα12 did not identify its helix 5, which typically comprises ~70% of the interaction surface between Gα subunits and GPCRs (Inoue et al., 2019), as a PC1 binding determinant. Instead, this analysis identified PC1-binding determinants in regions unique to Gα12, suggesting that different binding and regulatory properties may exist between PC1 and specific Gα families (Yu et al., 2011). Given this potential for both positive and negative regulatory mechanisms of interaction between PC1 and diverse Gα family members (Figure 3), it will be essential to determine the effects of knocking out other Gα family members on cystic disease initiation and progression.

Regardless of the mechanism by which PC1 regulates G protein signaling, it is important to note that all studies that describe any sort of interaction between PC1 and heterotrimeric G proteins ascribe central importance to the minimal G protein binding domain originally identified by Parnell et al. (1998). Given the centrality of this domain it is also essential to consider whether its presence (or absence) in model systems may affect experimental outcomes and interpretations. Ablation of Gα12 was seen to antagonize cystic disease in a mouse model with complete loss of the PC1 C-tail and G protein binding domain. However, perturbation of G protein signaling in other cystic models with intact C-terminal PC1 tails may yield different results than those observed in *Pkd1* conditional models that do not express any PC1. For instance, the hypomorphic $Pkd1^{RC}$ model is cystic due to decreased expression levels of PC1 (Hopp et al., 2012), but the protein retains an intact C-tail presumably capable of interacting with heterotrimeric G proteins and regulating their signaling properties *via* activation and/or sequestration. Likewise, cleavage mutants such as $Pkd1^{T3041V}$ (Yu et al., 2007) or signaling mutants such as $Pkd1^{\Delta L}$ (Parnell et al., 2018) may potentially retain the ability to bind, but not signal to heterotrimeric G proteins. This line of thinking also begs the question of whether the expression levels of PC1 determine the mechanism by which it regulates different families of heterotrimeric G proteins. This question is particularly relevant given current interest in therapeutic

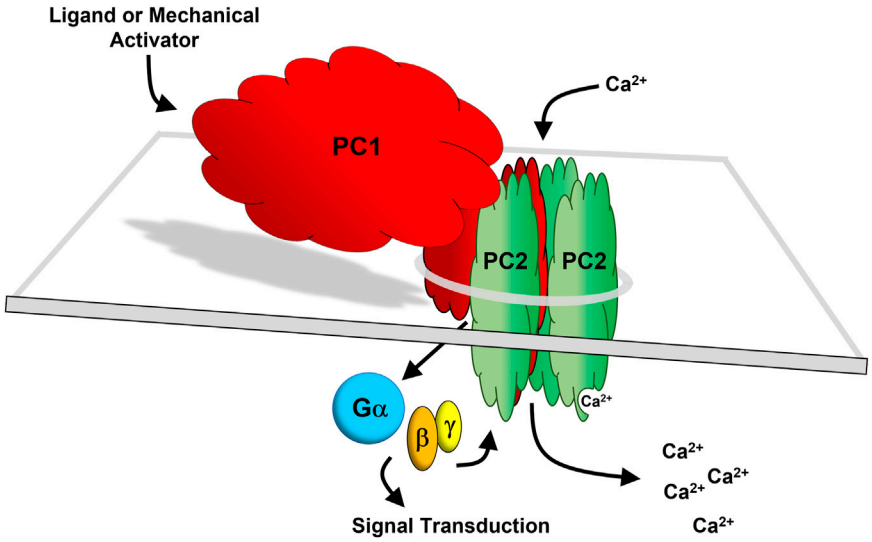


FIGURE 5
The PC1/PC2 hybrid receptor-channel complex. PC1 (red) and PC2 (green) are depicted as forming a heteromeric four-subunit complex comprised of three subunits of PC2 and one subunit of PC1 (Yu et al., 2009; Zhu et al., 2011; Wang et al., 2019). The last 6 TM domains of PC1 have homology with PC2. In this model, it is envisioned that PC2, being a transient receptor potential (TRP) channel (TRPP2) conducts a cation current together with PC1 acting as the fourth subunit of the channel. PC1 may function as a ligand-activated or mechanosensitive ionotropic receptor which transduces a signal to the channel subunits *via* heterotrimeric G protein activation (Parnell et al., 2018). Additionally, PC1 functioning as a G protein-dependent metabotropic receptor may independently activate downstream signal transduction.

approaches to ADPKD that involve re-expression of PC1 (Dong et al., 2021) or increasing *PKD1* and *PKD2* protein levels by blocking miR-17 (Lee et al., 2019; Lakhia et al., 2022). Since G protein activation is a catalytic event it would not require a large number of PC1 molecules to initiate signaling *via* activating mechanisms. In contrast, regulation of signaling *via* sequestration would be limited by the number of PC1 molecules available to interact. Thus, a thorough analysis of the role of G protein signaling in PC1 function may require the testing of a broader spectrum of PC1 mutants.

5 Time for a new paradigm

As described in previous sections, PC1 appears to function as a ligand-activated and/or mechanosensitive adhesion GPCR and an ion channel subunit that forms a heterotetrameric channel with PC2. GPCRs are inherently metabotropic since they work through second messenger signaling mechanisms. In contrast, ionotropic receptors gate ions upon receptor activation. Based on current evidence, it would appear that PC1 may possess both properties, suggesting that PC1 represents a new paradigm, as a *hybrid* metabotropic-ionotropic receptor-channel protein. As shown in Figure 5, PC1 and PC2 are envisioned to form a heteromeric four-subunit channel complex comprised of three subunits of PC2 and one subunit of PC1 (Yu et al., 2009; Zhu et al., 2011; Wang et al., 2019). In this model, PC1 acts as the fourth subunit of the channel while also functioning as a ligand-activated or mechanosensitive ionotropic receptor that transduces a signal to the channel subunits through PC1-dependent heterotrimeric G protein activation (Parnell et al., 2018). In addition, PC1 may also act as a ligand activated or mechanosensitive metabotropic receptor that can directly activate other downstream signaling events through heterotrimeric G protein activation.

Is there a precedent for metabotropic-ionotropic receptor coupling? One example is glutamatergic signaling in the central nervous system where ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) function in concert (Reiner and Levitz, 2018). There are 18 known iGluRs comprising the AMPAR, KAR, Glud, and NMDAR families and 8 different mGluRs divided into Groups I, II, and III. Both types of receptors multimerize and both bind glutamate in their ligand binding domains. This iGluR and mGluR coupling may result from a direct interaction between their C-terminal tails and/or through scaffolding proteins. It is also likely that there is crosstalk between their respective downstream signaling effectors. For example, iGluRs and mGluRs can cooperate to activate Ca^{2+} signaling through different mechanisms, where iGluR causes Ca^{2+} influx while mGluR causes ER Ca^{2+} release to raise intracellular Ca^{2+}. In comparison, PC1 and PC2 may function together more intimately as one multi-subunit complex to regulate Ca^{2+} signaling through distinct but complementary mechanisms. Where the iGluRs and mGluRs segregate these coordinated metabotropic and ionotropic functions in different protein complexes, the PC1 protein may function to carry out both metabotropic and ionotropic functions as a single subunit of the

PC1/PC2 receptor-channel complex, where both GPCR and cation channel functions are intrinsic to the PC1 subunit.

Another example of metabotropic-ionotropic receptor coupling involves the Latrophilin/CIRL adhesion GPCR family (Johnson, 2017; Scholz et al., 2017). In this case, two different receptors, the CIRL adhesion GPCR and the NOMPC ion channel cooperate to sense and respond to the same signal–mechanical stress. In neuronal cells expressing these proteins, the NOMPC membrane channel lies anchored to the cytoskeleton and thus is poised to sense and respond to extracellular mechanical forces transmitted through cytoskeletal mechanisms. The 7-TM CIRL protein senses mechanical forces through its extensive extracellular domain interacting with the ECM, transmitting mechanical signals that activate Gαi, which then inhibit adenylate cyclase and lower cAMP to modify the channel function of NOMPC. Thus, in this case two separate proteins, an adhesion GPCR and an ion channel, coordinate the cellular response to mechanical force. In contrast, PC1 alone, as an adhesion GPCR and ion channel subunit may be able to carry out both metabotropic and ionotropic functions as one subunit of the heterotetrameric PC1/PC2 receptor-channel complex.

6 Conclusions and future directions

It is of high priority to determine how PC1 functions, including whether PC1 responds to ligand binding or mechanical forces, or both. PC1 contains multiple potential binding motifs that could engage in ligand binding or that could interact with the ECM. At present, all known adhesion GPCRs have 7-TM domains (typical of all canonical GPCRs) and are thought to undergo intracellular "cis" signaling. Cis receptors signal within the same cell on which the receptor resides. Adhesion GPCRs also have additional GPCR-independent trans-cellular "trans" functions mediated by interactions with adhesion receptors on other cells, such as integrins or teneurins (Dunn et al., 2019; Sando et al., 2019; Li et al., 2020; Sreepada et al., 2022). Trans receptors signal by binding receptors on other cells. Thus, it is possible that PC1, as an adhesion GPCR, also does both, and it will be important to dissect these functions and determine the cell and tissue context for each of these multiple possible signaling modalities.

A related question is whether PC1 always functions as an integral subunit of the heteromeric PC1/PC2 receptor-channel complex, or whether it also functions separately as an isolated adhesion GPCR to carry out a PC1-specific signaling role in all cells or in a more limited tissue-specific or developmental context. While it is likely that PC2 can function as a homomeric channel without PC1, it seems less likely that PC1 can function alone, given that the C-terminal 6-TM channel-forming and TOP domains might need to interact with PC2 subunits. However, arguing against this are the many studies where over-expressed PC1, C-terminal domain, or C-tail fragments of PC1 have been shown to activate G protein signaling in a constitutive manner (Delmas et al., 2002; Parnell et al., 2002; Puri et al., 2004) and additional work supporting PC2-independent functions of PC1 (Viau et al., 2020).

As a final thought, it will be informative to examine PC1 in the broader context of the PC1 (and PC2) orthologs. There are four known *PKD1* family paralogs in addition to *PKD1* (PC1). These are human *PKDREJ*, *PKD1L1*, *PKD1L2*, and *PKD1L3*, which unlike *PKD1* have restricted tissue expression (Gunaratne et al., 2007; Kashyap et al., 2019). The protein products of all four are predicted to have 11 TM domains with the last 6 TM domains having ion channel homology. Two undergo GPS cleavage, the exceptions being the products of *PKD1L1* and *PKDREJ*, which do not have the conserved GPS $HL^{T}/_{S}$ tripeptide sequence (Butscheid et al., 2006; Field et al., 2011). Both products of *PKD1L1* and *PKD1L2* appear to bind G proteins and thus may have GPCR function (Yuasa et al., 2004), and the *PKDREJ* protein is reported to modulate G protein signaling (Sutton et al., 2006). In addition, there are ten sea urchin (*S. purpuratus*) REJ domain-containing proteins (Gunaratne et al., 2007), some with 11 TM domains that include 6-TM ion channel homology and a GPS cleavage site. Interactions are known to occur between the PC1-like and PC2-like proteins. For example, PC2L1 forms ion channels with both PC1 and PC1L2 (Murakami et al., 2005; Bui-Xuan et al., 2006; Petracca et al., 2016). Important functions for these complexes include left-right asymmetry development in the early embryo (Field et al., 2011; Kamura et al., 2011); formation of a calcium permeable channel on primary cilia (DeCaen et al., 2013); and an unidentified but important role in sour taste perception (Huang et al., 2006; Ishimaru et al., 2006; LopezJimenez et al., 2006). In addition, the complex formed by PC1L3 and PC2L1 shares similar assembly mechanisms and ion channel function as the PC1/PC2 complex (Yu et al., 2012), suggesting that the other PC1-like proteins may also have intrinsic channel function. Taken together, these observations suggest that the PC1 family of bi-functional receptor ion-channel proteins will undoubtedly be found to have many unique biological roles during development, and in tissue and organ physiology, and in human disease.

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RM wrote and edited the manuscript. JC wrote and edited the manuscript. SP wrote and edited the manuscript.

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Conflict of interest

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Beneficial effects of bempedoic acid treatment in polycystic kidney disease cells and mice

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ADPKD has few therapeutic options. Tolvaptan slows disease but has side effects limiting its tolerability. Bempedoic acid (BA), an ATP citrate-lyase (ACLY) inhibitor FDA-approved for hypercholesterolemia, catalyzes a key step in fatty acid/sterol synthesis important for cell proliferation. BA is activated by very long-chain acyl-CoA synthetase (FATP2) expressed primarily in kidney and liver. BA also activates AMPK. We hypothesized that BA could be a novel ADPKD therapy by inhibiting cyst growth, proliferation, injury, and metabolic dysregulation *via* ACLY inhibition and AMPK activation. *Pkd1*-null kidney cell lines derived from mouse proximal tubule (PT) and collecting duct (IMCD) were grown in 2D or 3D Matrigel cultures and treated ± BA, ± SB-204990 (another ACLY inhibitor) or with Acly shRNA before cyst analysis, immunoblotting or mitochondrial assays using MitoSox and MitoTracker staining. *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* C57BL/6J mice were induced with doxycycline injection on postnatal days 10 and 11 (P10-P11) and then treated ± BA (30 mg/kg/d) ± tolvaptan (30–100 mg/kg/d) by gavage from P12-21. Disease severity was determined by % total-kidney-weight-to-bodyweight (%TKW/BW) and BUN levels at euthanasia (P22). Kidney and liver homogenates were immunoblotted for expression of key biomarkers. ACLY expression and activity were upregulated in *Pkd1*-null PT and IMCD-derived cells vs. controls. Relative to controls, both BA and SB-204990 inhibited cystic growth in *Pkd1*-null kidney cells, as did *Acly* knockdown. BA inhibited mitochondrial superoxide production and promoted mitochondrial elongation, suggesting improved mitochondrial function. In ADPKD mice, BA reduced %TKW/BW and BUN to a similar extent as tolvaptan vs. untreated controls. Addition of BA to tolvaptan caused a further reduction in %TKW/BW and BUN vs. tolvaptan alone. BA generally reduced ACLY and stimulated AMPK activity in kidneys and livers vs. controls. BA also inhibited mTOR and ERK signaling and reduced kidney injury markers. In liver, BA treatment, both alone and together with tolvaptan, increased mitochondrial biogenesis while inhibiting apoptosis. We conclude that BA and ACLY inhibition inhibited cyst growth *in vitro*, and BA decreased ADPKD severity *in vivo*. Combining BA with

tolvaptan further improved various ADPKD disease parameters. Repurposing BA may be a promising new ADPKD therapy, having beneficial effects alone and along with tolvaptan.

KEYWORDS

ACLY, AMPK, ADPKD, metabolism, bempedoic acid, ETC-1002, ACSVL1

Introduction

Autosomal dominant polycystic kidney disease (ADPKD), the most common genetic cause of end-stage kidney disease (ESKD), affects every ethnicity with a prevalence of ~1: 500–1,000 and ~600,000 patients in the U.S. alone (Gabow, 1993; Chebib and Torres, 2016). Patients with ADPKD present with enlarging cystic lesions in the kidney and often the liver as well, leading to a progressive decline in kidney function that is associated with ESKD in half of ADPKD patients by age 50–60 (Chebib and Torres, 2016). Most ADPKD patients have loss-of-function mutations in the multifunctional proteins polycystin-1 or -2 (PC1 and PC2, encoded by the genes *PKD1* and *PKD2*) (Chebib and Torres, 2016). ADPKD therapeutic options to specifically address the decline in glomerular filtration rate (GFR) are very limited. The only current FDA-approved drug for ADPKD is tolvaptan, a vasopressin 2 receptor (V2R) antagonist. This drug slows disease progression in patients at risk for rapid progression towards end-stage kidney disease ESKD (Torres et al., 2012). However, tolvaptan has the dose-dependent side effect of polyuria and a risk of hepatotoxicity that requires monthly monitoring of liver function tests (Woodhead et al., 2017; Blair, 2019). Thus, there is a clear need for additional ADPKD therapies targeting different cellular pathways dysregulated in ADPKD that could potentially be used alone or in combination with tolvaptan.

There is growing recognition that ADPKD cyst-forming PC1-deficient cells have major metabolic derangements that likely contribute to kidney tubular epithelial cyst formation and expansion. Specifically, compared to control kidney tubular epithelial cells, ADPKD cells display increased aerobic glycolysis (the Warburg effect), impaired fatty acid oxidation, increased cellular proliferation, and reduced AMP-activated protein kinase (AMPK) activity (Rowe et al., 2013; Menezes et al., 2016). Earlier, we helped pioneer the use of the AMPK activator metformin to inhibit ADPKD kidney cyst growth in mouse models of *Pkd1* knockout (Takiar et al., 2011).

AMPK is a ubiquitous metabolic sensor that regulates many cellular processes (Hallows, 2005; Steinberg and Kemp, 2009; Steinberg and Carling, 2019). The role of AMPK in the protection of kidney function has been studied in many models of acute and chronic kidney disease (Rajani et al., 2017). Of note, kidney AMPK activity is generally decreased in both humans and mice with chronic kidney disease (CKD) (Dugan et al., 2013; Li et al., 2015). The renoprotective role of AMPK in CKD is thought to occur through activation and induction of several effector pathways including autophagy, fatty acid oxidation, antioxidant pathways, (Decleves et al., 2011) and *via* inhibition of the inflammatory cascade (Peairs et al., 2009). In response to metabolic and other cellular stresses, AMPK activation helps maintain cellular energy balance by restoring ATP levels through regulation of metabolic enzymes, promoting cellular energy efficiency, and inhibiting pro-growth anabolic pathways.

Our group recently demonstrated that the AMPK activator metformin ameliorates relevant disease parameters in a hypomorphic PKD mouse model that closely mimics human ADPKD (Pastor-Soler et al., 2022). We have also been involved in the TAME-PKD study where metformin was found to be safe and tolerable in ADPKD patients (Perrone et al., 2021). Of note, metformin doses that inhibit cyst growth in pre-clinical ADPKD models may not be as tolerable or clearly efficacious in ADPKD patients (Perrone et al., 2021; Brosnahan et al., 2022). Moreover, along with the various beneficial effects of metformin, including inhibition of cyst fluid secretion, cell proliferation, and cAMP production (Takiar et al., 2011; Miller et al., 2013), metformin inhibits Complex I of the mitochondrial respiratory chain (Owen et al., 2000), which may hamper the promotion of defective mitochondrial oxidative metabolism in ADPKD. Thus, novel drugs targeting complementary pathways in ADPKD that could potentially synergize with tolvaptan or metformin may afford lower effective drug dosing and have better efficacy against the disease when used in combination in patients.

Here we explored targeting and inhibiting the enzyme ATP-citrate lyase (ACLY) to determine its effects on relevant disease parameters *in vitro* and in a conditional *Pkd1* knockout mouse model of ADPKD. ACLY is a key metabolic enzyme that promotes lipid and cholesterol biosynthesis by generating acetyl-CoA from cytosolic citrate and has been recently identified as a new therapeutic target for lowering cholesterol in patients with atherosclerotic cardiovascular disease (Huynh, 2019). In addition, ACLY inhibition has emerged as a new therapeutic strategy for cancer, where ACLY inhibition blocks lipid synthesis and cellular proliferation (Zhao et al., 2016). As with tumor growth, cystic growth in ADPKD relies on such mechanisms that support enhanced cellular proliferation. Importantly, ACLY has also been reported to bind to and inhibit the AMPK-β_1 subunit (Lee et al., 2015), suggesting a mutual antagonism between ACLY and AMPK.

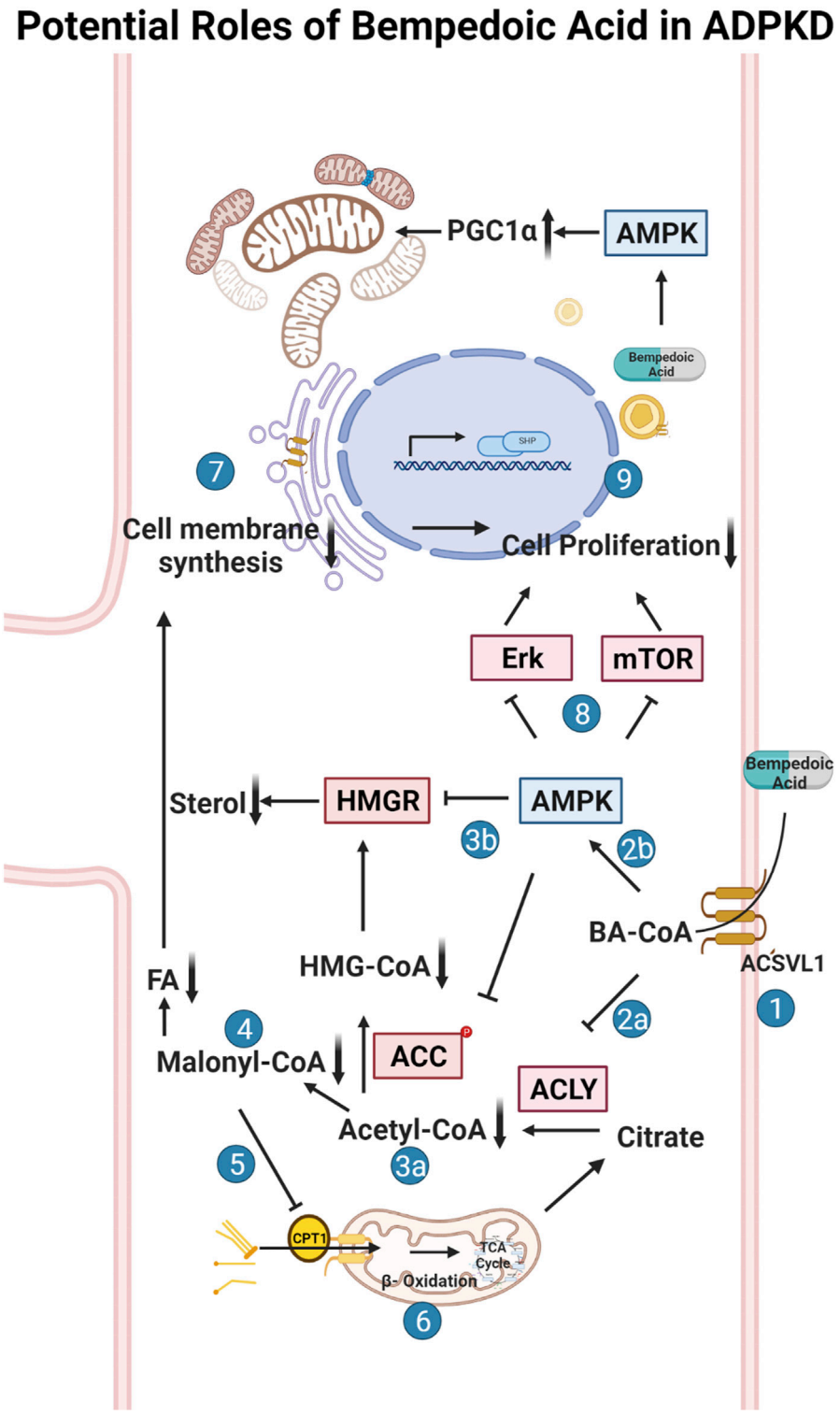

FIGURE 1
Potential roles of bempedoic acid (BA) in ADPKD. Schematic flow diagram of the effects of bempedoic acid (BA; a.k.a. ETC-1002) on various metabolic pathways and cellular proliferation *via* inhibition of ATP-citrate lyase (ACLY) and activation of AMP-activated protein kinase (AMPK). 1. The pro-drug BA gets converted to its active form (BA-CoA) *via* Very Long-Chain Acyl-CoA Synthetase (ACSVL1; a. k.a. FATP2), whose long isoform is only substantially expressed in liver and kidney tissues (Steinberg et al., 1999; Pinkosky et al., 2016). BA-CoA simultaneously inhibits ACLY (2a) and activates AMPK (2b) in cells. Inhibition of ACLY, which converts citrate to acetyl-CoA and oxaloacetate, results in decreased cytosolic acetyl-CoA
(*Continued*)

FIGURE 1 (Continued)
production (3a). Activated AMPK inhibits HMG-CoA reductase (HMGR) and acetyl-CoA carboxylase (ACC) (3b), which along with decreased acetyl-CoA production, inhibits the formation of malonyl-CoA (4) and HMG-CoA and thus inhibits both sterol and fatty acid (FA) synthesis. Decreased levels of malonyl-CoA, which is an inhibitor of carnitine palmitoyltransferase-1 (CPT-1), promotes fatty acid (FA) uptake into mitochondria *via* CPT-1 (5) and thus promotes FA beta oxidation in mitochondria *via* the tricarboxylic acid (TCA) cycle (6). Decreased sterol and FA synthesis inhibits the synthesis of cellular membranes (7), which along with AMPK-dependent inhibition of various cellular pathways (incl. the mammalian target of rapamycin (mTOR) pathway) (8), causes inhibition of cellular proliferation and thus inhibition of cyst growth and expansion in ADPKD (9). The schematic was created using BioRender software available at BioRender.com.

TABLE 1 Antibodies used for western blot.

Antigen	Manufacturer	Cat. #	Host	Dilution	Incubation	
					Time	Temperature
pACC(Ser79)	Cell Signaling	3661	Rabbit polyclonal	1:1,000	O/N	4°C
tACC	Cell Signaling	3676	Rabbit polyclonal	1:1,000	O/N	4°C
pACLY(Ser455)	Cell Signaling	4331	Rabbit polyclonal	1:1,000	O/N	4°C
tACLY	Cell Signaling	4332	Rabbit polyclonal	1:1,000	O/N	4°C
pAMPKα(Thr172)	Cell Signaling	2531	Rabbit polyclonal	1:1,000	O/N	4°C
tAMPKα	Cell Signaling	5831	Rabbit polyclonal	1:1,000	O/N	4°C
pERK(Thr202/Tyr204)	Cell Signaling	9101	Rabbit polyclonal	1:1,000	O/N	4°C
tERK	Cell Signaling	4696	Mouse monoclonal	1:1,000	O/N	4°C
Cleaved Cas3(Asp175)	Cell Signaling	9661	Rabbit polyclonal	1:1,000	O/N	4°C
PGC-1α	Santa Cruz	SC-517380	Mouse monoclonal	1:500	O/N	4°C
pP70S6K(Thr389)	Santa Cruz	SC-11759R	Rabbit polyclonal	1:500	O/N	4°C
NGAL	Abcam	ab63929	Mouse polyclonal	1:1,000	O/N	4°C
KIM-1	R&D systems	AF1817	Goat polyclonal	1:800	O/N	4°C
FATP2	Proteintech	14048-1-AP	Rabbit polyclonal	1:1,000	O/N	4°C
Revert 700 total protein stain kit	LI-COR	926–11010			5 min	RT

O/N, Overnight; p, Phosphorylated or phosphor; RT, Room Temperature; T, Total.

Bempedoic acid (BA; also known as ETC-1002) inhibits ACLY and is approved by the FDA as an adjunct to diet and maximally tolerated statin therapy for the treatment of adults with atherosclerotic cardiovascular disease who require additional lowering of LDL-C. BA also activates AMPK in mice (Pinkosky et al., 2016). BA exists as a pro-drug that gets converted to its active form by an enzyme (Very long-chain acyl-CoA synthetase; ACSVL1 or FATP2) whose tissue expression is primarily limited to kidney and liver (Pinkosky et al., 2016), the two principal organs affected in ADPKD. We thus hypothesized that BA treatment could be beneficial in ADPKD by inhibiting kidney cyst growth, inflammation, injury, and metabolic dysregulation *via* simultaneous ACLY inhibition and AMPK activation with limited off-target effects. The rationale for the use of BA in ADPKD involves correcting the dysregulated metabolism and excessive cell proliferation in ADPKD and is summarized schematically in Figure 1. Herein, we tested the potential beneficial effects of BA on 3D cyst growth and mitochondrial function in *Pkd1*-null kidney cells *in vitro*, and of BA alone and in combination with on key parameters of disease severity in kidneys and liver in conditional *Pkd1* knockout mice.

Materials and methods

Reagents and chemicals

All reagents and chemicals used were purchased from Sigma (St. Louis, MO, United States) or Thermo Fisher (Pittsburgh, PA, United States) unless otherwise stated. Pharmaceutical grade bempedoic acid (BA) and tolvaptan were obtained from Esperion Therapeutics, Inc. (Ann Arbor, MI, United States) and Otsuka Pharmaceuticals (Japan), respectively. Please see Table 1 for detailed information on the antibodies and conditions that were used for immunoblotting.

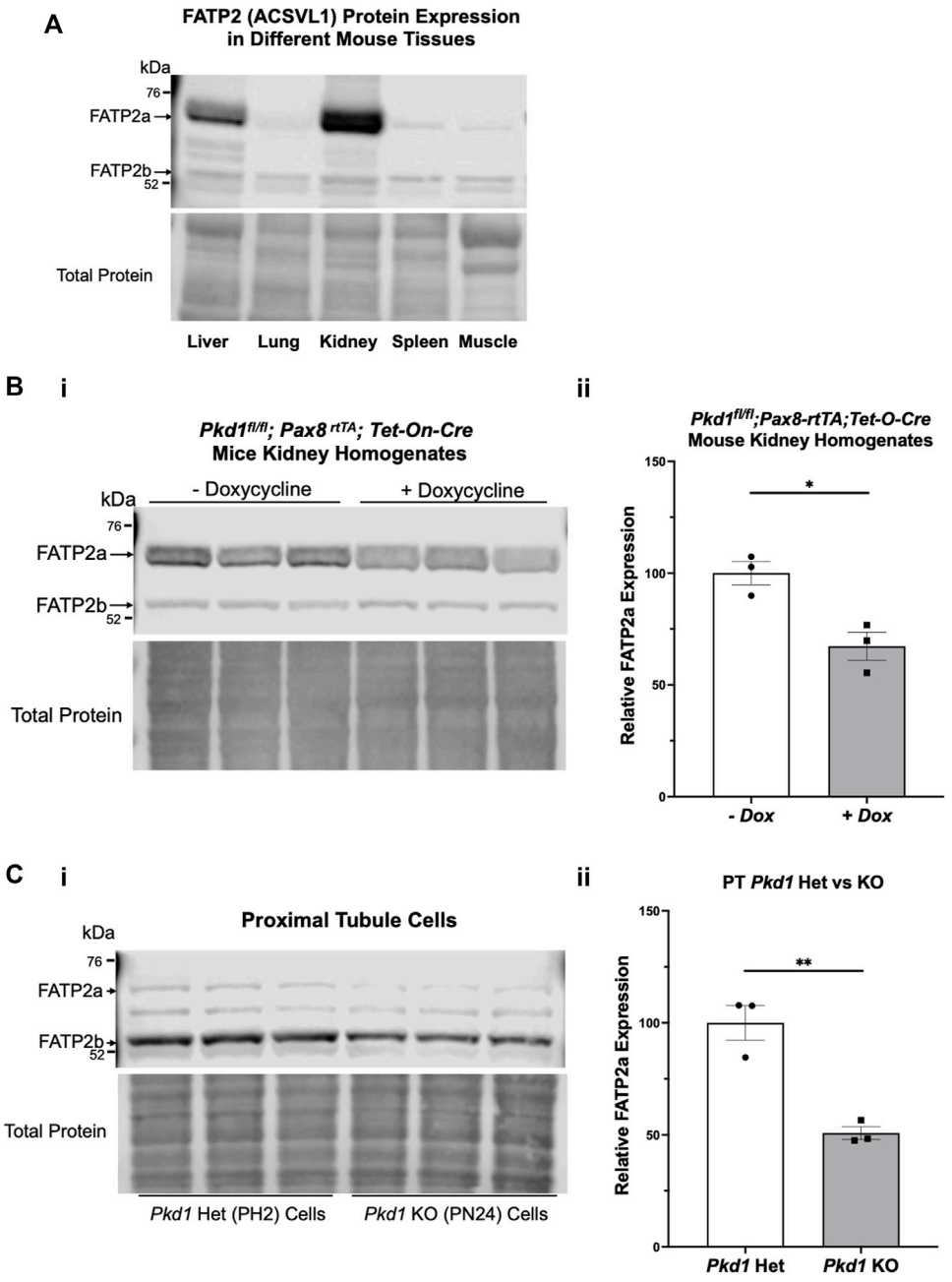

FIGURE 2
The bempedoic acid-activating enzyme ACSVL1 (FATP2) is expressed in different mouse tissues and PKD kidneys and kidney cell lines. **(A)**. Upper, immunoblotting of various mouse tissues reveals expression of two distinct FATP2 isoforms. The short splice variant (FATP2b), which lacks the acyl-CoA synthetase domain required for conversion of BA to its active metabolite BA-CoA, is expressed in all tissues tested. However, the full-length long form (FATP2a at ~70 kDa) has significant expression only in liver and kidney tissue, thus providing specificity of BA pro-drug conversion to its active form only in these tissues. Lower, staining for total protein as a loading control. **(B)** FATP2 immunoblotting of representative *Pkd1*$^{fl/fl}$;*Pax8-rtTA;Tet-O-Cre* mouse kidneys treated with or without doxycycline to induce tubule-specific *Pkd1* gene inactivation at P10-P11. i Upper, the immunoblot revealed expression of FATP2a in all mouse kidneys at the time of euthanasia (P22). Lower, staining for total protein as a loading control. ii Densitometric analysis of the FATP2a band normalized to total protein revealed a ~35% reduction of the expression of the active enzyme in *Pkd1*-null mouse kidneys (*$p < 0.05$). **(C)** Both FATP2a and FTAP2b isoforms are also expressed in immortalized mouse kidney epithelial cells that were derived from PT. i Upper; We observed generally lower FATP2a expression in *Pkd1* KO cells (right) than in controls (left). Lower, staining for total protein as a loading control. ii. Densitometric quantification of the FATP2a levels, normalized to total protein indicates that the *Pkd1*-null PT cells express approximately 50% less FATP2a than the heterozygous *Pkd1*$^{+/-}$ cells (**$p < 0.01$). Three representative lysate immunoblots are shown for each condition.

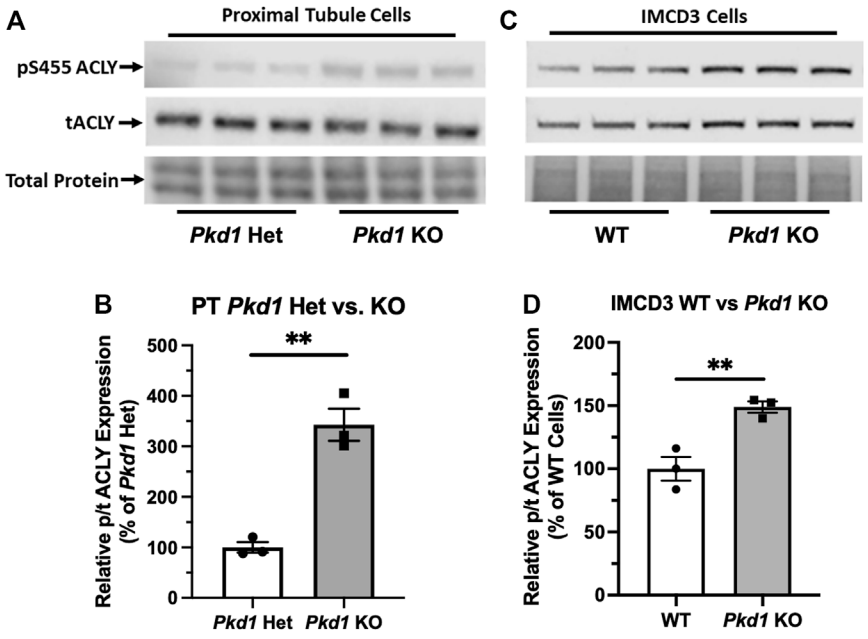

FIGURE 3
ACLY activity is increased in Pkd1−/− kidney epithelial cells as compared to controls. **(A)** Immunoblotting of proximal tubule (PT)-derived mouse epithelial cells probed for total ACLY expression (tACLY, upper) and activated ACLY, as detected using a phospho-specific antibody directed against the Akt phosphorylation site Ser455 (pS455 ACLY, middle), in PN24 cells with *Pkd1* expression knocked out at both alleles as compared with PH2 cells heterozygous for *Pkd1* deletion. Lower, total protein staining as loading control. **(B)** Densitometric quantitation of the pS455 ACLY/tACLY ratio shows a significant ~250% increase in ACLY activation in the *Pkd1*-null cells as compared to *Pkd1*-het controls (**$p < 0.01$; unpaired *t*-test; $n = 3$). **(C)** Immunoblotting of inner medullary collecting duct (IMCD)-derived mouse epithelial cells for expression of tACLY (middle) and pS455 ACLY (upper), in *Pkd1*-null (ID1-3E5) cells as compared with WT (IMCD3). Lower, total protein staining as loading control. **(D)** Densitometric quantitation revealed a significant ~50% increase in the pS455 ACLY/tACLY ratio in the ID1-3E5 cells relative to control IMCD3 cells (**$p < 0.01$; unpaired *t*-test; n = 3).

Cell culture models

Pkd1-Null (PN24) and *Pkd1*-Het (PH2) cells were a kind gift of Dr. Stefan Somlo and were originally derived by microscopically dissecting and then dissociating proximal tubules (PTs) from $Pkd1^{flox/-}$:TSLargeT (ImmortoMouse) mice. Parental $Pkd1^{flox/-}$ cells from a clone showing epithelial properties were then transiently transfected with a plasmid encoding Cre recombinase and cloned again by limiting dilution, giving rise to daughter cells that either expressed Cre and therefore had undergone transformation to $Pkd1^{-/-}$ or that had not expressed Cre and retained the parental $Pkd1^{flox/-}$ genotype, as described previously (Joly et al., 2006). In this manuscript, we also refer to the PH2 cells as $Pkd1^{+/-}$ cells for simplicity. These cells were cultured in medium composed of equal volumes of DMEM and Ham's F-12 plus 7.5 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 2 nM triiodothyronine, 5 μg/ml insulin, and 2% (vol/vol) FBS. Cells were maintained under permissive conditions (33°C with 10 U/ml γ-interferon) in a humidified 5% CO_2-95% air incubator with medium changes every other day and passaged at least twice weekly. To induce differentiation, cells were kept under non-permissive conditions (37°C without γ-interferon) for 5–7 days prior to use in experiments. Mouse inner medullary collecting duct (IMCD) cell lines were also generously provided by Dr. Stefan Somlo. Wild-type (Wt)-IMCD3 (Cas9) is a control cell line for IMCD3-CRISPR-knockout cells (ID1-3E5 $Pkd1^{-/-}$) (Decuypere et al., 2021). Both cell lines were cultured in regular DMEM/F12 medium containing 5% FBS in a 37°C humidified 5% CO_2-95% air incubator with medium changes every other day and passaged approximately twice weekly.

Generation of ATP citrate-lyase knockdown cell lines

A non-silencing lentiviral shRNA (pGIPZ) was used as a control and three different ACLY shRNA lentiviral constructs (pGIPZ) were obtained from Horizon Discovery (Waterbeach, Cambridge, United Kingdom). Recombinant lentiviral particles were produced by transient transfection of 293T cells according to the manufacturer's protocol. The PT-derived $Pkd1^{-/-}$(PN24) and $Pkd1^{+/-}$ (PH2) cells were infected with the cell culture supernatant containing lentiviral particles for 48 h. These cells were then selected in puromycin to generate stable cell lines with non-silencing and ACLY-specific shRNA. Cell lines were validated for diminished ACLY expression by Western blot analysis.

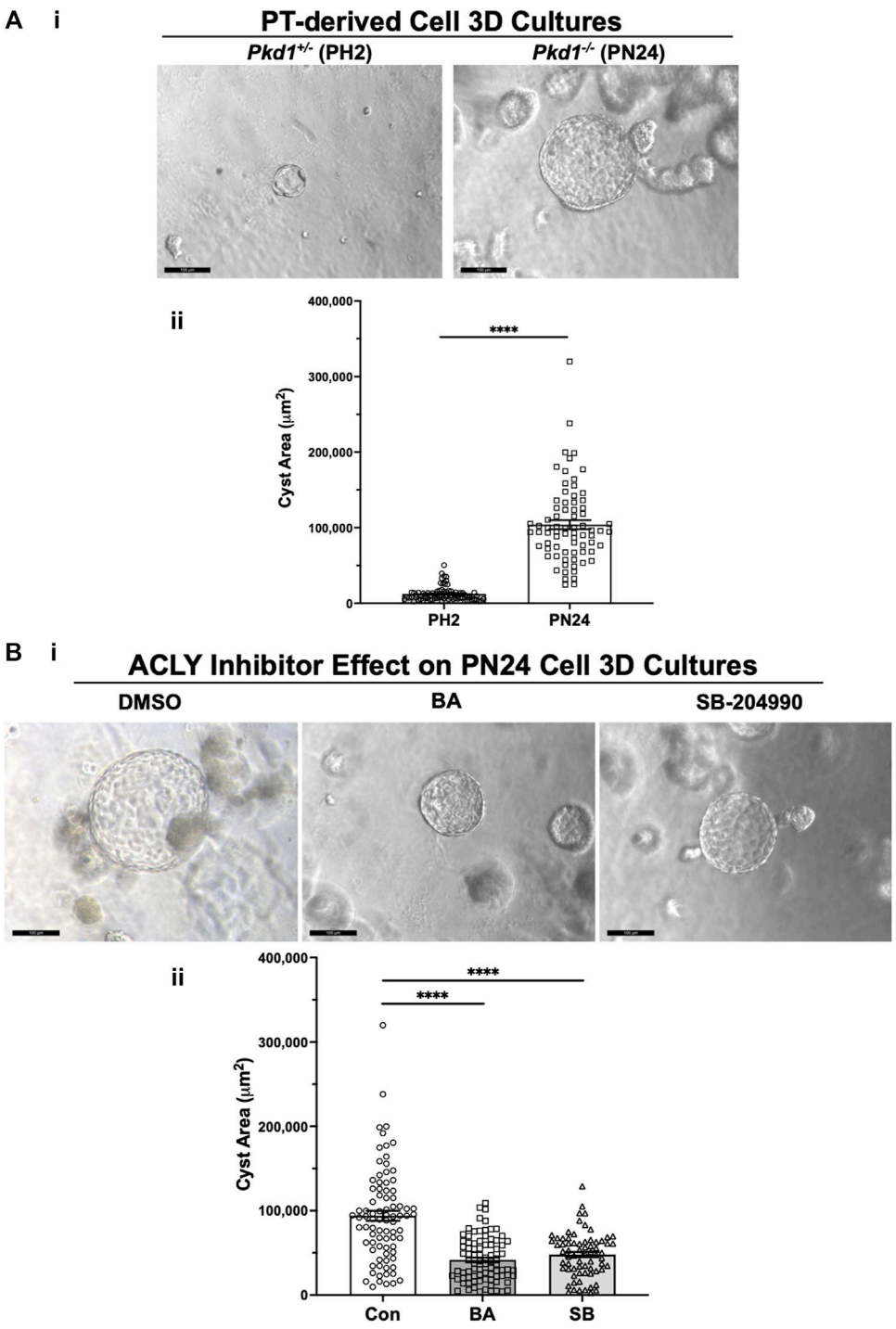

FIGURE 4
ACLY inhibitors reduce cyst size in 3D cultures of PT-derived *Pkd1*$^{-/-}$ (PN24) kidney cells. **(A)** PT-derived *Pkd1*$^{-/-}$ cells (PN24) and *Pkd1*$^{+/-}$ (PH2) cells were grown for 10–12 days in Matrigel supplemented with forskolin + IBMX after 1 day, before imaging and cyst size analysis. i Representative light microscopy images of cystic structures from PH2 vs. PN24 3D cultures (scale bar = 100 μm). ii Summary data reveal that PT-derived *Pkd1*$^{-/-}$ (PN24) cells developed dramatically bigger cystic structures than *Pkd1*$^{+/-}$ (PH2) (n = 74–82, in a total of 4 independent experiments (****p < 0.0001). **(B)** ACLY inhibitors BA and SB-204990 inhibit cyst growth of PT-derived *Pkd1*$^{-/-}$ (PN24) kidney epithelial cells in 3D culture. Cells were cultured for a total of 10–12 days in Matrigel supplemented with forskolin + IBMX after 1 day, and then treated with either vehicle (DMSO; CON), 100 μM BA or 30 μM SB-204990 for the last 3 days of culture before imaging and cyst size analysis. i Representative images of cystic structures in 3D culture of PN24 cells DMSO (left) vs. BA (middle) vs. SB-204990 treatment (right; scale bar = 100 μm). ii Summary data reveal that in PN24 cells treated with BA or SB-204990 the cyst area relative to CON is dramatically reduced (n = 69–84, represent 3-5 independent experiments; ****p < 0.0001 for the indicated comparisons).

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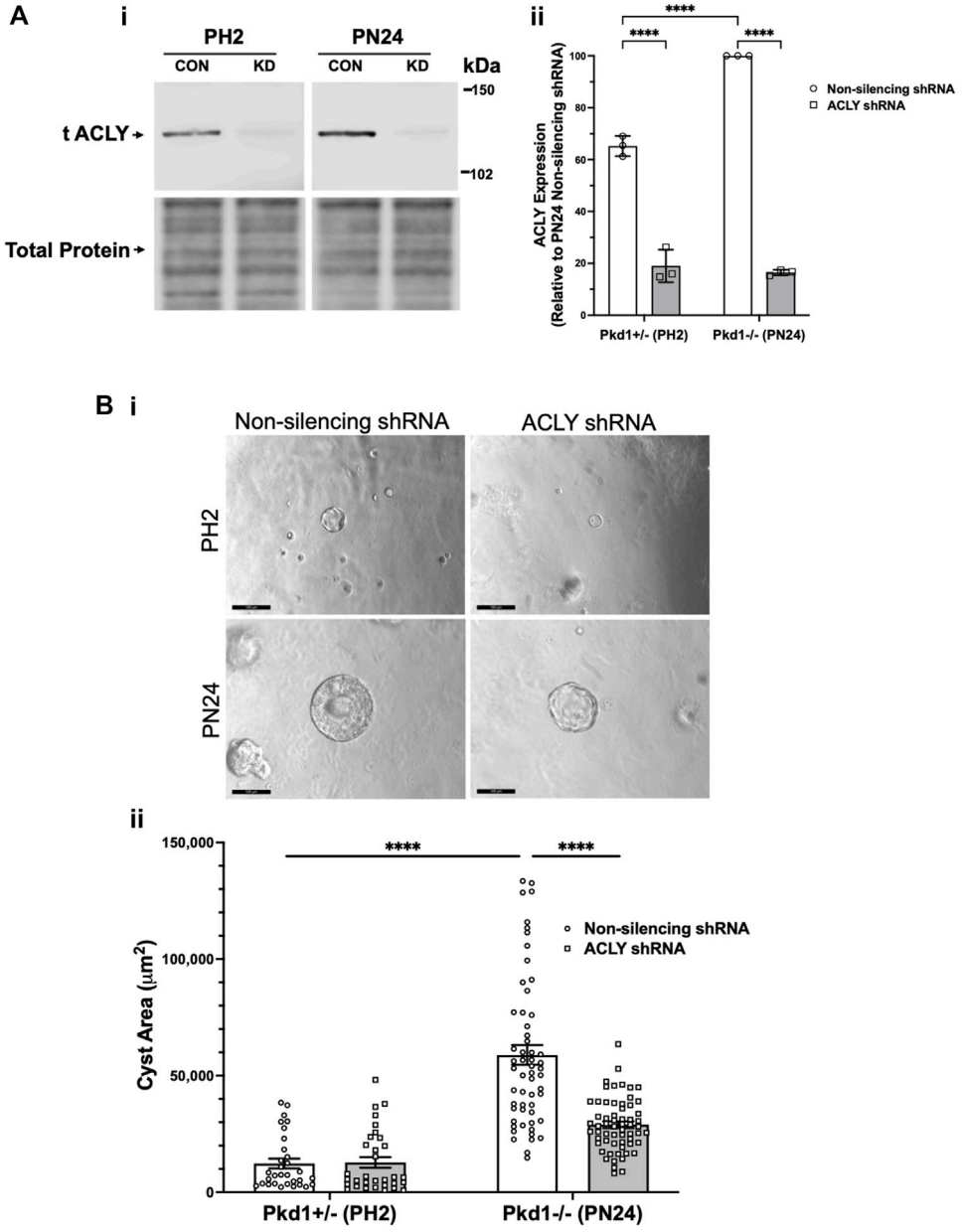

FIGURE 5
Acly knockdown inhibits cystic growth in 3D cultures of PT-derived kidney epithelial cells. **(A)** Stable PT-derived cell lines expressing either non-silencing control shRNA or shRNA against Acly were generated and checked for ACLY protein expression by immunoblotting analysis. i Representative immunoblotting of ACLY protein expression in the different cell lines. ii Summary data reveal that *Pkd1*$^{+/-}$ (PH2) cells expressing non-silencing control shRNA have ~35% reduced ACLY expression compared with ACLY expression in *Pkd1*$^{-/-}$ (PN24) cells expressing non-silencing control shRNA. There were means of 71% and 83% knockdown of ACLY expression in PH2 and PN24 cells, respectively, expressing shRNA against ACLY relative to ACLY expression in cells expressing non-silencing control shRNA (n = 3, ****$p < 0.0001$ for the indicated comparisons). **(B)** *Pkd1*$^{-/-}$ (PN24) cells developed significantly larger cysts than *Pkd1*$^{+/-}$ (PH2) cells, and shRNA-mediated Acly knockdown inhibited cyst growth of *Pkd1*$^{-/-}$ (PN24) kidney epithelial cells in 3D culture. Cells were cultured for a total of 12 days in Matrigel supplemented with forskolin + IBMX after day 1, and cysts were imaged and cyst size was analyzed as described in Materials and Methods. i Representative images of cystic structures in the different cell lines. ii Summary data reveal *Pkd1*$^{-/-}$ (PN24) cells developed significantly larger cysts than *Pkd1*$^{+/-}$ (PH2) cells (n = 31–57 cysts analyzed from 3 biological replicate experiments; ****$p < 0.0001$). Cells expressing shRNA directed against ACLY dramatically reduced mean cystic areas relative to those of cells expressing non-silencing control shRNA (n = 57–61 cysts analyzed from 3 biological replicate experiments, ****$p < 0.0001$).

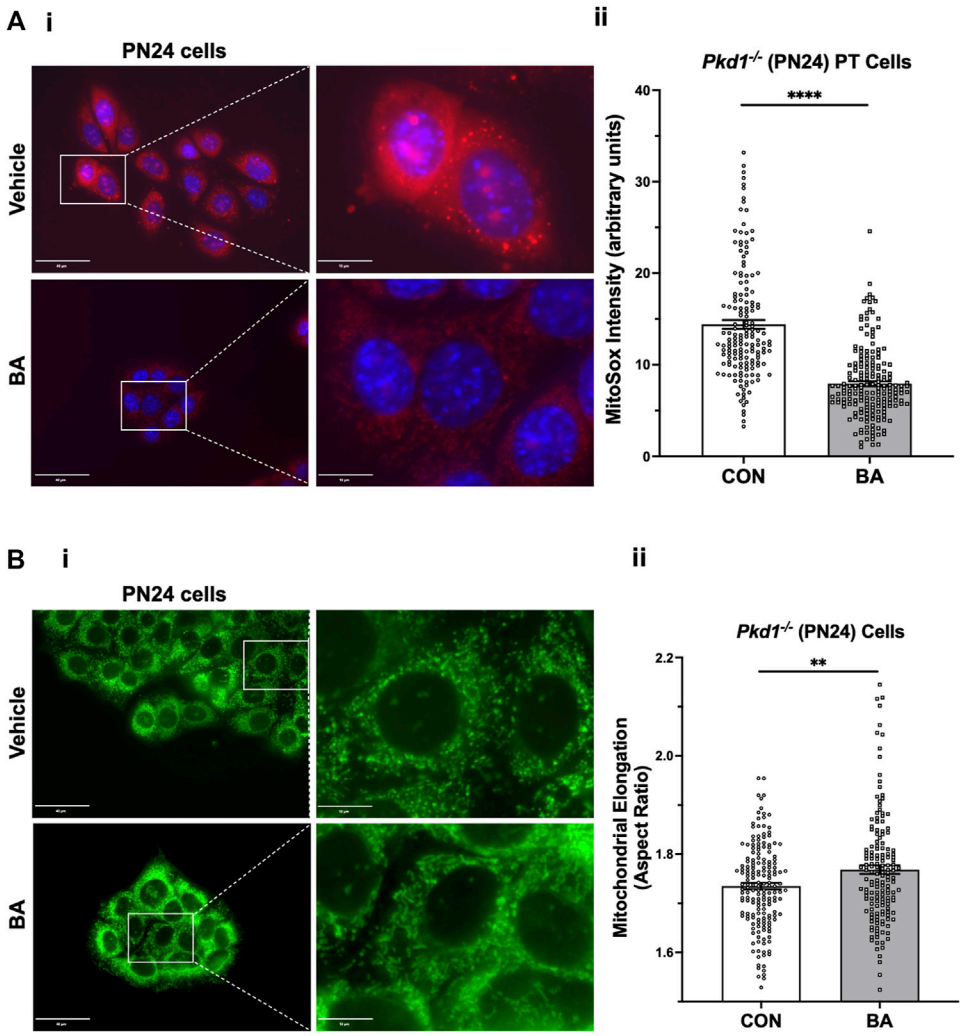

FIGURE 6
Bempedoic acid (BA) inhibits mitochondrial superoxide production and promotes mitochondrial elongation in *Pkd1*$^{-/-}$ cells. **(A)** To analyze the effect of BA on mitochondrial superoxide production, PT-derived *Pkd1*$^{-/-}$ (PN24) cells were stained with MitoSOX™ Red mitochondrial superoxide indicator (red) and Hoechst 33342 nuclear stain (*blue*). i Representative epifluorescence micrograph images are shown of PN24 cells in the absence (top) or presence (bottom) of BA treatment (100 μM) for 24 h. Left panel scale bars = 40 μm. Right panels show enlargement of inset areas in the left panels (right panel scale bars = 10 μm). ii Summary data reveal that BA treatment dramatically decreased mitochondrial superoxide production in PN24 cells (n = 159–188 cells analyzed from four biological replicates; ****$p < 0.0001$). **(B)** BA treatment significantly increased mitochondrial elongation of *Pkd1*$^{-/-}$ PN24 cells. i Representative images of MitoTracker Green-stained cells in the absence (top) or presence (bottom) of BA treatment (100 μM) for 24 h. Left panel scale bars = 40 μm. Right panels show enlargement of inset areas in the left panels (right panel scale bars = 10 μm). ii Summary data reveal that BA treatment significantly increased mean cellular mitochondrial elongation in PN24 cells (mean cellular mitochondrial elongation values on n = 159–177 cells analyzed from four biological replicates, as described in Materials and Methods; **$p < 0.01$).

3D culture

Matrigel™ from BD Biosciences (BD #356234) was thawed overnight at 4°C prior to adding 50 μl to each well of an 8-well glass chamber slide (Lab-Tek #155409) and spreading evenly in the well using a P-200 tip. The slides were then placed in a cell culture incubator to allow the Matrigel™ to solidify for at least 15 min. During this time, PH2 *Pkd1*$^{+/-}$ cells or PN24 or ID1-3E5 *Pkd1*$^{-/-}$ cells were trypsinized and mixed into a stock of culture medium containing 2% Matrigel™ (assay medium) at a density of 6,000 cells per 400 μl of this medium. 400 μl of this mixture was then plated on top of the solidified Matrigel™ in each well of the chamber slide on day 0. Cells were then allowed to grow in a 5% CO_2 humidified incubator at 37°C and fed with assay medium every other day for 12 days. Starting on day 1 after seeding, 3D cell cultures were treated with 10 μM forskolin plus 100 μM isobutylmethylxanthine (IBMX), in the absence or presence of 100 μM BA or 30 μM SB-204990 for the last 3 days of culture before imaging and cyst size

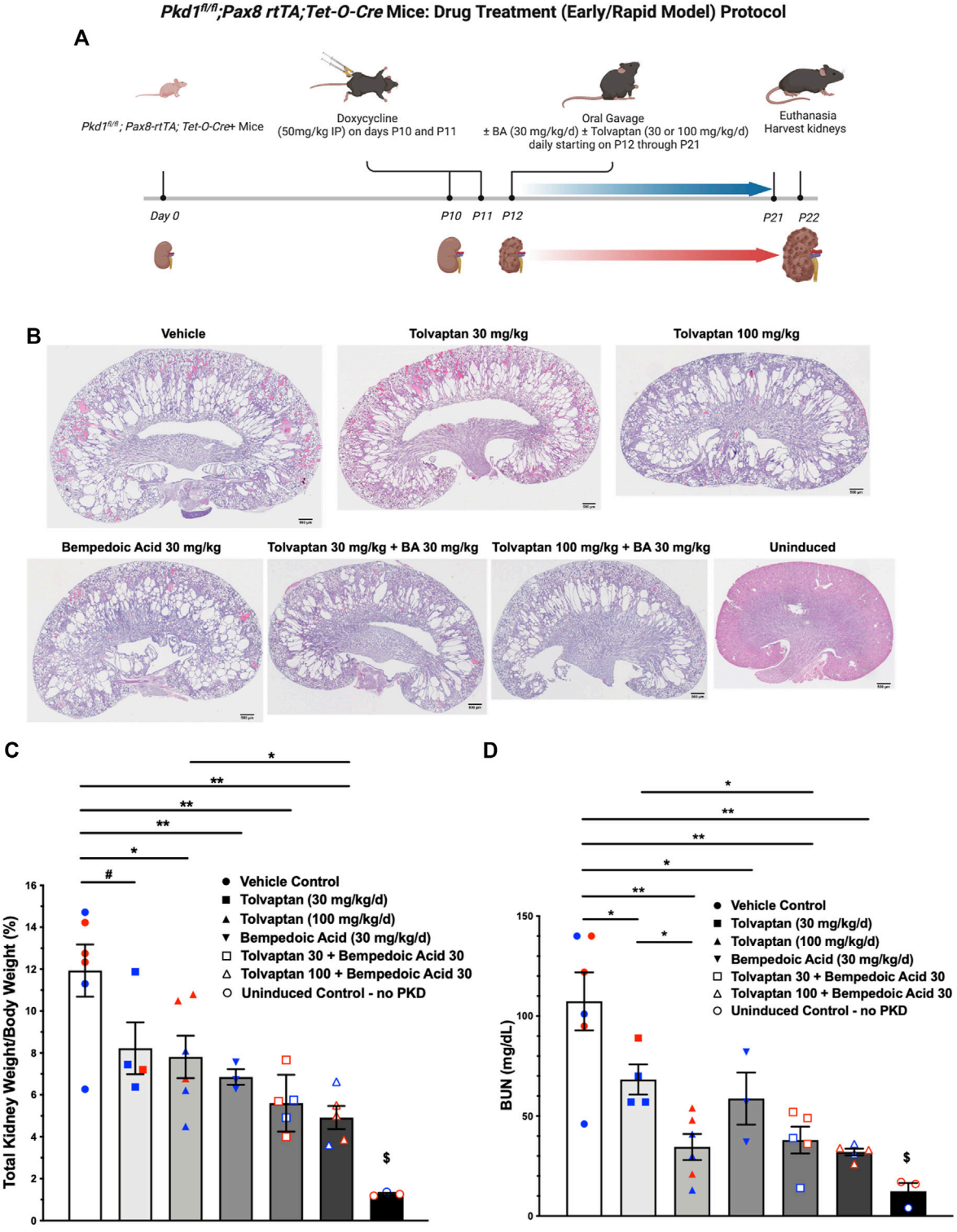

FIGURE 7
Bempedoic acid (BA) treatment alone and in combination with tolvaptan reduces PKD disease severity in an early/rapid induced *Pkd1* gene inactivation ADPKD mouse model. As measures of disease severity *in vivo*, *Pkd1*$^{fl/fl}$; *Pax8-rtTA; Tet-O-Cre* mice were induced with doxycycline to inactivate *Pkd1* and then treated ± BA ± tolvaptan for 10 days prior to euthanasia, as shown schematically **(A)**, and then total kidney weight/body weight ratio (TKW/BW) and blood urea nitrogen (BUN) levels by iStat were evaluated as described in Materials and Methods. **(B)** Representative H&E-stained sagittal kidney sections under the different treatment conditions are shown. BA (30 mg/kg/d) reduced mean (±SE) TKW/BW **(C)** and
(*Continued*)

FIGURE 7 (Continued)
BUN **(D)** to a similar extent as tolvaptan (30 and 100 mg/kg/d). Addition of BA to tolvaptan caused further reductions in TKW/BW and BUN vs. tolvaptan alone (*$p < 0.05$, **$p < 0.01$, and $^{\#}0.05 < p < 0.10$ for the indicated comparisons). $^{\$}$Significantly different from all other treatment conditions. Data obtained from male and female mice were combined for each treatment condition as mice were studied at ages before reaching reproductive capability. Blue and red data points shown in **(C)** and **(D)** correspond to male and female mice, respectively. The schematic protocol was created using BioRender software available at BioRender.com.

analysis. DMSO was the vehicle control. For the studies shown in Figure 5, PH2 or PN24 cells stably transduced to express either non-silencing or *Acly* shRNA constructs were used. For analysis, cysts (fluid-filled cell structures) were thresholded from background, and the cross-sectional area of each cyst grown in 3D Matrigel culture was calculated using the ImageJ Analyze Particles plug-in software (NIH).

Mitochondrial morphology and superoxide quantification

To analyze mitochondrial morphology and superoxide production as indirect measures of mitochondrial health, PT- and IMCD-derived *Pkd1*$^{-/-}$ cell lines were treated with vehicle or BA (100 μM) and then stained with MitoTracker™ Green FM (M7514), MitoTracker™ Deep Red FM (M22426) or MitoSOX™ Red mitochondrial superoxide indicator (M36008), along with the nuclear stain Hoechst 33,342 (H3570; ThermoFisher Scientific Inc. Pittsburgh, PA, United States). Briefly, PN24 or ID1-3E5 cells were seeded onto 4-well chamber slides in the above-described media prewarmed to 37°C. Staining and washes were performed 2 days after plating and 24 h after the indicated treatments according to the manufacturer's protocols. Cells were imaged using a Leica DMi8 live cell imaging fluorescence microscope using a ×40 oil objective at zoom 1.6. Automated quantification of mitochondrial morphology was done on MitoTracker-stained cells using ImageJ software. The aspect ratio (length/width) was used as a measurement of mitochondrial elongation. We compiled the mean aspect ratio of >1,000 mitochondria in each of the selected cells and then compared the mean values (±SEM) from each of the cells analyzed across the two conditions (± BA treatment). MitoSOX Red fluorescence was used to assess mitochondrial superoxide production. The fluorescence intensity of each randomly selected cell was quantified using ImageJ software. Results are reported as compiled mean cellular values ±SEM from four independent biological replicate experiments for PN24 cells (with n = 159–188 cells analyzed) in Figure 6 and one experiment for ID1-3E5 cells (n = 26–31 cells analyzed) in Supplementary Figure S2.

Autosomal dominant polycystic kidney disease mouse models

All animal procedures followed NIH guidelines for the care and use of laboratory animals and were approved by the University of Southern California's Institutional Animal Care and Use Committee. Male and female *Pkd1*$^{fl/fl}$*;Pax8-rtTA;Tet-O-Cre* transgenic mice in the C57BL/6J background were obtained as a generous gift from the Baltimore PKD Core Center and were used as an ADPKD model for *in vivo* studies, as described previously (Ma et al., 2013). Genotyping was confirmed between postnatal days 5 and 7 (P5-P7) and *Pkd1* inactivation was induced with IP doxycycline injection (50 mg/kg) on P10 and P11 to induce rapidly progressive cyst development. Mice were then treated in the absence or presence of BA (30 mg/kg/d) with or without cotreatment with tolvaptan (30–100 mg/kg/d) by daily oral gavage from P12-21. These drugs (or vehicle) were formulated into micelle suspensions comprised of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy-poly (ethylene glycol 2000) (DSPE-PEG (2000)-maleimide, Avanti Polar Lipids, Alabaster, AL, United States) to improve solubility prior to daily gavage injection by syringe into the mouse pups. Drug-loaded micelles were self-assembled *via* thin film evaporation using previously described methods (Huang et al., 2020). Briefly, 18 mg of BA (and/or up to 66 mg of tolvaptan) and 100 μM of DSPE-PEG (2000)-methoxy were dissolved in methanol and evaporated with nitrogen gas to form thin films. The resulting thin films were dried overnight under vacuum and hydrated in PBS at 80°C for 30 min, and drug-loaded micelles were stored at 4°C and used within 3 days of formulation. Mice were euthanized at the end of the experiment at P22 to harvest kidneys and livers and obtain blood to assess various measures of disease severity.

Whole blood chemistry measurements

We used the Abbott i-STAT handheld blood analyzer equipped with Chem 8 + cartridges for measurements blood urea nitrogen (BUN) from mixed venous blood at the time of euthanasia at P22 (Tinkey et al., 2006). Briefly, mice were anesthetized with isoflurane, and approximately 100 μl of blood was obtained from the submandibular venous plexus using a single-use lancet (Golde et al., 2005). The blood was collected in a heparinized vial and quickly added to the i-STAT cartridge, and results were obtained within two minutes.

Kidney weight measurements, tissue preparation and microscopy

After clamping the renal pedicle at P22 the left kidney was quickly removed, rapidly weighed, sectioned coronally in two

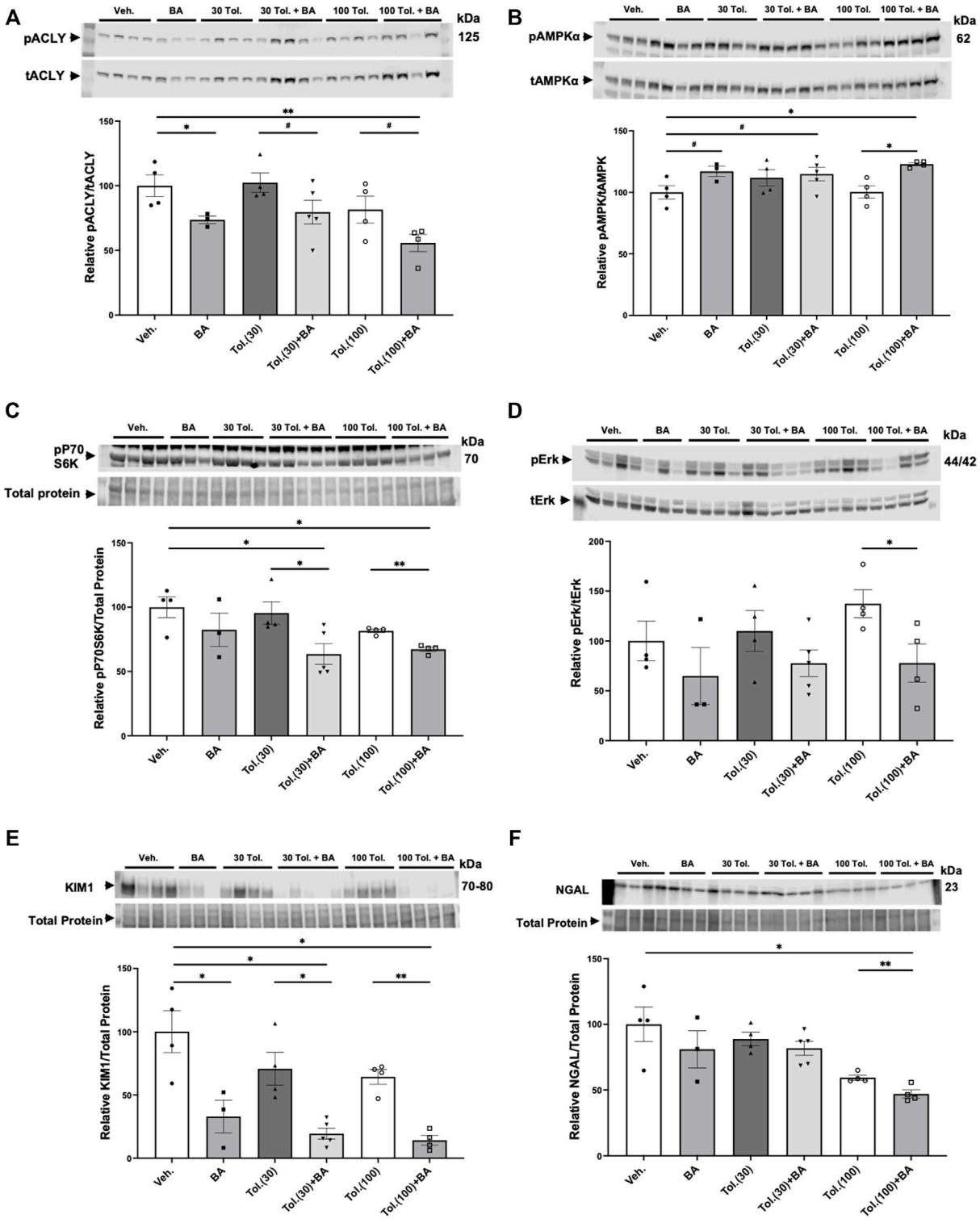

FIGURE 8
Effects of bempedoic acid and tolvaptan treatment in PKD mice on protein expression of key signaling and injury markers in kidney homogenates. Immunoblots were performed on kidney tissue homogenates from *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* mice with early/rapid induced *Pkd1* gene inactivation with or without concurrent treatment with BA (30 mg/kg/d) and/or tolvaptan (30 or 100 mg/kg/d). Densitometric intensities of immunoblot bands from each lane were normalized to the total protein signal from that lane as a loading control [shown in middle panels of **(C)**, **(E)**, and **(F)**] or normalized to

(*Continued*)

FIGURE 8 (Continued)
the total non-phosphorylated protein signal [shown in middle panels of **(A)**, **(B)**, and **(D)**]. Kidney homogenates were probed for: **(A)** phosphorylated ACLY (pSer455 ACLY; pACLY) and total ACLY (tACLY); **(B)** phosphorylated AMPKα (pThr172 AMPK; pAMPKα) and total AMPKα (tAMPK); **(C)** mTOR pathway activation (pP70S6K); **(D)** phosphorylated ERK (pERK) and total ERK (tERK); and the kidney injury markers **(E)**. KIM1, and **(F)**. NGAL. Representative immunoblots are shown in upper and middle panels, and summary quantitation of mean (±SE) relative protein expression levels are shown in lower panels ($^{\#}0.05 < p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$ for the indicated comparisons).

parts and placed in microcentrifuge tubes and frozen in liquid nitrogen. The right kidney was also removed after clamping of the renal pedicle, quickly weighed, and rapidly cut coronally. One-half of this right kidney was fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS, and the other half was placed in RNAlater (Qiagen, Waltham, MA) for future preparation of cDNA. A section of liver was also obtained for each mouse and placed immediately in liquid nitrogen.

After overnight fixation at 4°C, the kidney tissues were washed in PBS, quenched in NH_4Cl and further washed in PBS. The samples were then placed in 10% neutral buffered formalin (VWR, Radnor, PA, United States) at 4°C for 16–18 h. The fixed kidney samples were then dehydrated in serial alcohols, then cleared with xylene, embedded in paraffin, and cut into 4-μm sections on a rotary microtome at the Keck School of Medicine of USC Norris Pathology Core. The fixed tissues were stained with hematoxylin and eosin (H&E) for histological evaluation. We then obtained images using an Olympus IX73 inverted microscope using a Plan Achromat × 2 objective for a total magnification of × 20 (numerical aperture of 0.06 and working distance of 5.8 mm).

Electrophoresis and immunoblotting analysis

Kidney and liver lysates were prepared from frozen tissues, homogenized, centrifuged, proteins quantitated, and samples electrophoresed and transferred to nitrocellulose membranes, as described previously (Pastor-Soler et al., 2022). The membrane was first stained with Revert 700 total protein stain solution (LI-COR) and then washed prior to imaging at 700 nm for total protein quantitation per lane, as per the manufacturer's recommendations. After destaining, the membrane was then blocked and probed with primary and secondary antibodies of interest. Quantification of immunoblot images was performed by densitometry and analyzed using Image Studio Lite Ver 5.2 software (LI-COR, United States). Please refer to Table 1 for information on antibodies and blotting conditions.

Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad, La Jolla, CA, United States) to obtain the mean values and SEM for each treatment group. In most experiments, significance was determined using two-tailed, unpaired Student's t-tests assuming unequal variances for the groups, or one-way ANOVA with post-hoc Tukey corrections for multiple comparisons. Individual data points are shown in the bar graphs of figures, along with the mean (±S.E.) for each treatment condition. p values < 0.05 were considered significant.

Results

As outlined above, the purpose of this initial study was to test as a proof of concept the potential role of the ACLY inhibitor and AMPK activator BA as a novel therapeutic for ADPKD using *in vitro* and *in vivo* ADPKD model systems. A particular feature of BA that may lend itself specifically to ADPKD therapy is its expected activity limitation primarily to kidney and liver, the two organs predominantly affected in the disease, where the BA pro-drug can get converted to its active metabolite *via* local expression of very long-chain acyl-CoA synthetase (FATP2 or ACSVL1; Cf. Figure 1, step 1), as described below.

Very long-chain acyl-CoA synthetase is detected in mouse kidney, liver, and in key model cell lines for the study of autosomal dominant polycystic kidney disease

To determine whether our kidney cell lines and conditional *Pkd1* KO mouse model would be useful tools to evaluate the potential beneficial effects of BA in reducing cyst size or number in ADPKD, we first evaluated FATP2 protein expression in cells and tissues by immunoblot. Specifically, two bands were detected using an anti-FATP2 primary antibody that detects both a shorter FATP2 splice variant (FATP2b) and the long form of FATP2a at ~70 kDa (Melton et al., 2011). The FATP2b variant lacks the protein domain required for the conversion of BA to its active metabolite (BA-CoA) and was expressed in all mouse tissues tested (Figure 2A, lower band). However, the FATP2a form was previously reported to have significant expression only in liver and kidney tissue, thus providing specificity of BA pro-drug conversion to its active form only in these organs (Steinberg et al., 1999; Pinkosky et al., 2016). Consistent with these earlier studies, we detected the full-length FATP2a primarily in liver and

kidney tissue homogenates, with minimal expression in other tissues (Figure 2A, upper panel, upper band). Importantly, FATP2 immunoblotting of representative *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* mouse kidney homogenates treated with or without doxycycline to induce tubule-specific *Pkd1* gene inactivation revealed expression of both FATP2 isoforms in both induced and uninduced mouse kidneys (Figure 2B). Quantification of the immunoblot showed that the kidneys of induced mice, the mice that developed ADPKD upon doxycycline injection, expressed less FATP2a than the uninduced mice. We also detected both FATP2a and FTAP2b isoforms in immortalized mouse kidney epithelial cells derived from both PT (Figure 2C) and IMCD (Supplementary Figure S1). Of note, densitometric quantification of the immunoblots revealed significant decreases in FATP2a expression in both *Pkd1-null* kidney tissue and cells compared to controls (Figure 2; Supplementary Figure S1). Altogether, these results confirm that the ADPKD cell lines and ADPKD mouse model used in our studies express the enzyme required to convert the BA pro-drug to its active compound and help support a rationale to evaluate the effects of BA on cyst growth in cell culture and *in vivo*.

Pkd1 knockout in kidney cell lines is associated with an increase in active ATP-citrate lyase when compared to control parental cell lines

Due to the abundance of ACLY expression in non-cystic PT cells, it was challenging to evaluate differences in ACLY expression and activity by immunoblot of total kidney homogenates. To assess whether cell lines that recapitulate ADPKD cystic disease *ex vivo* express ACLY, one of the targets of BA, we tested the expression of this enzyme by immunoblotting PT- and IMCD-derived model cell lines in cells grown in 2D. All four cell lines tested, parental PT-derived *Pkd1*$^{+/-}$ (PH2) cells, PT-derived *Pkd1*$^{-/-}$ (PN24) cells (Joly et al., 2006) and IMCD-derived WT-IMCD3 and ID1-3E5 *Pkd1*$^{-/-}$ cells (Decuypere et al., 2021) express ACLY (total ACLY or tACLY; Figure 3A,C). In addition, these cell lines also expressed an active form of ACLY phosphorylated at a target site of Akt (also known as Protein Kinase B; PKB) at Ser-455 (pS455 ACLY). We then normalized the levels of active pS455 ACLY to tACLY in the same samples. We found a significant increase of active ACLY in the *Pkd1*$^{-/-}$ cells from both PT and IMCD origin, compared to parental controls (Figure 3B,D). These results suggest that ACLY activity is increased in ADPKD epithelial cells with cyst-forming capability, compared to cells that are heterozygous for *Pkd1* KO or wild-type at the *Pkd1* locus. Moreover, we surmised that the higher ACLY activity in these ADPKD cell models potentially contributes to cystic growth and disease progression, and thus that targeting this enzyme for inhibition by BA *in vivo* may be beneficial in reducing cyst size in ADPKD mouse models.

Bempedoic acid treatment and the ATP-citrate lyase inhibitor SB-204990 dramatically inhibited cystic growth in Pkd1-null kidney cells lines grown under 3D cyst-forming conditions

We generated cysts from PT-derived *Pkd1*$^{+/-}$ (PH2) control cells and *Pkd1*$^{-/-}$ (PN24) ADPKD model kidney epithelial cell lines by growing the cells in Matrigel for a total of 12 days and treating these cells with forskolin plus a phosphodiesterase inhibitor (IBMX) from day 2–12. This treatment enhances cyst formation in these commonly used cell culture models (Cabrita et al., 2020). Once cysts appeared by day 9, we exposed the PN24 cultures to either vehicle control (DMSO), the AMPK activator and ACLY inhibitor BA, or the ACLY inhibitor SB-204990 (Chu et al., 2010) for the last 3 days of the experiment. Under light microscopy, we observed only minimal to very small cystic structures in PH2 control cultures (Figure 4Ai, left) as compared to PN24 (*Pkd1*$^{-/-}$) ADPKD cultures, which resulted in much larger cystic structures (Figure 4Ai, right). We quantified the cystic area from those micrographs using ImageJ software, and as shown in Figure 4Aii, there was a dramatic increase in cyst size in the *Pkd1*-null cells (PN24) as compared to the *Pkd1*$^{+/-}$ heterozygous cells under cyst-inducing conditions. Moreover, in PN24 (*Pkd1*$^{-/-}$) ADPKD cultures there was a substantial decrease in cyst size in the cultures treated with BA (Figure 4Bi, middle) or SB-204990 (Figure 4Bi, right) as compared with vehicle control (Figure 4Bi, left). Using the same technique as in Figure 4Aii, in ImageJ software, we quantified the cystic area from those micrographs. As summarized in Figure 4Bii, there was a significant reduction in cyst area in the BA- and SB-204990-treated cultures as compared with those exposed to vehicle control. We similarly tested whether these two ACLY inhibitors could reduce cyst area in IMCD-derived *Pkd1*$^{-/-}$ (ID1-3E5) cells grown in 3D Matrigel cultures treated with IBMX and forskolin and found that BA and SB-204990 both significantly reduced cyst size compared with vehicle (DMSO) (Supplementary Figure S2).

Acly knockdown inhibited cystic growth in Pkd1-null kidney cell 3D cultures

To examine the role of ACLY more directly in governing ADPKD cyst growth *in vitro*, we generated stably transduced PH2 and PN24 cell lines using the pGIPZ lentiviral system to express either shRNA directed against mouse *Acly* (KD cells) or a non-silencing shRNA control (Figure 5). ACLY protein

expression in both the PN24 (*Pkd1*$^{-/-}$) and PH2 (*Pkd1*$^{+/-}$) cells was dramatically inhibited to ~20%–30% of levels in the corresponding non-silencing controls, and baseline ACLY expression was significantly reduced in the PH2 cells relative to PN24 cells (Figure 5A). We then performed 3D cyst growth assays in these cells as described above. While PH2 cells generated only small cystic structures whose size was not significantly affected by *Acly* KD, cyst growth in the PN24 *Acly* KD cells was significantly inhibited relative to that in the PN24 non-silencing control cells (Figure 5B). Taken altogether, these experiments further confirmed our hypothesis that BA treatment and ACLY inhibition has the promise of a therapeutic effect *in vivo* by inducing a significant reduction in cyst size in ADPKD *in vitro* 3D cell culture models.

Bempedoic acid inhibits mitochondrial superoxide production and promotes mitochondrial elongation in autosomal dominant polycystic kidney disease kidney-derived cell lines

Normal mitochondrial function is regulated in part by the interplay of two targets of BA, AMPK and ACLY (Cf. Figure 1). These key metabolic enzymes appear to be dysregulated in ADPKD, where there is upregulation of ACLY activity (Figure 3) and downregulation of AMPK activity (Rowe et al., 2013; Song et al., 2020). Moreover, when mitochondria are unable to efficiently utilize fatty acids for oxidative metabolism (Cf. Figure 1 - step 6) with decreased electron transfer in the respiratory chain, there is increased reactive oxide species (ROS) production leading to accumulation of superoxide (Lenaz, 2001). Mitochondrial dysfunction with increased oxidative stress is a key feature of ADPKD (Chang and Ong, 2018). Moreover, it has been shown that AMPK activation may improve mitochondrial function in ADPKD and overall disease severity (Song et al., 2020). BA would thus appear to be a good candidate drug to induce simultaneous ACLY inhibition and AMPK activation as regulators of mitochondrial function.

To examine whether BA-mediated cyst reduction is associated with improved mitochondrial function in ADPKD, we first tested the effects of BA on mitochondrial superoxide production in PT-derived *Pkd1*$^{-/-}$ (PN24) cells (Figure 6A). These cells were loaded with MitoSOX™ Red, a mitochondrial superoxide indicator, and incubated in the presence of BA or vehicle for 24 h. Our results support the hypothesis that BA improves mitochondrial function, as demonstrated by reduced fluorescence signal from MitoSOX™ Red in cells treated with BA compared to the vehicle control. We found a similar reduction in MitoSOX™ Red intensity in IMCD-derived *Pkd1*$^{-/-}$ (ID1-3E5) cells treated with BA relative to vehicle control (see Supplementary Figure S3A). These findings indicate that there is reduced mitochondrial superoxide production with BA treatment relative to controls in both PT-derived *Pkd1*$^{-/-}$ (PN24) or IMCD-derived *Pkd1*$^{-/-}$ (ID1-3E5) cells.

Mitochondrial morphology is another important indicator of mitochondrial oxidative function. Specifically, mitochondrial elongation facilitates cristae formation and assembly of respiratory complexes to enhance oxidative phosphorylation in cells (Li et al., 2017). Germino and colleagues demonstrated that mitochondrial elongation is defective in ADPKD model cell lines with decreased elongation in *Pkd1*-null cells compared to wild-type controls (Lin et al., 2018), as reviewed in (Padovano et al., 2018). Here we tested the effects of BA treatment on mitochondrial elongation (or aspect ratio) using MitoTracker™ staining in both PT-derived PN24 cells (Figure 6B) and IMCD-derived ID1-3E5 cells (Supplementary Figure S3B). Quantification of mitochondrial length divided by width was performed on the IF images using ImageJ. We found that BA treatment significantly increased mitochondrial elongation of both *Pkd1*-null cell lines (Figure 6Bii; Supplementary Figure S3Bii). These findings are consistent with the hypothesis that BA promotes mitochondrial oxidative function in *Pkd1*-deficient cells.

In vivo studies testing the effects of bempedoic acid and tolvaptan on PKD disease severity in an early/rapid induced Pkd1 gene inactivation autosomal dominant polycystic kidney disease mouse model

The use of tolvaptan, the only FDA-approved drug currently to treat ADPKD progression in patients with certain characteristics, is limited by its side effects, such as polyuria and thirst, potential hepatotoxicity, and its availability and cost (Blair, 2019). Future ADPKD treatment strategies may involve combination therapies including tolvaptan and other drugs that target complementary dysregulated cellular signaling pathways, potentially conferring synergistic or additive benefits, and allowing lower drug doses than those used in ADPKD monotherapy. To evaluate the effects of BA and tolvaptan *in vivo* we used an early and rapid model of ADPKD progression, the *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* mouse. The disease was induced *via* doxycycline administration at P10-P11 to inactivate *Pkd1*. Subsequently, the mice were treated with either vehicle, BA, BA plus tolvaptan or tolvaptan alone through P21 prior to euthanasia at P22, a timeline summarized in Figure 7A. These mice progress to ESKD at around the time of euthanasia (Ma et al., 2013). We first measured changes in the % total kidney weight/body weight (%TKW/BW) and blood urea nitrogen (BUN) levels at the time of euthanasia as markers of disease severity in this ADPKD model. Representative kidney section micrographs stained by H&E under the different treatment conditions are shown in Figure 7B. As

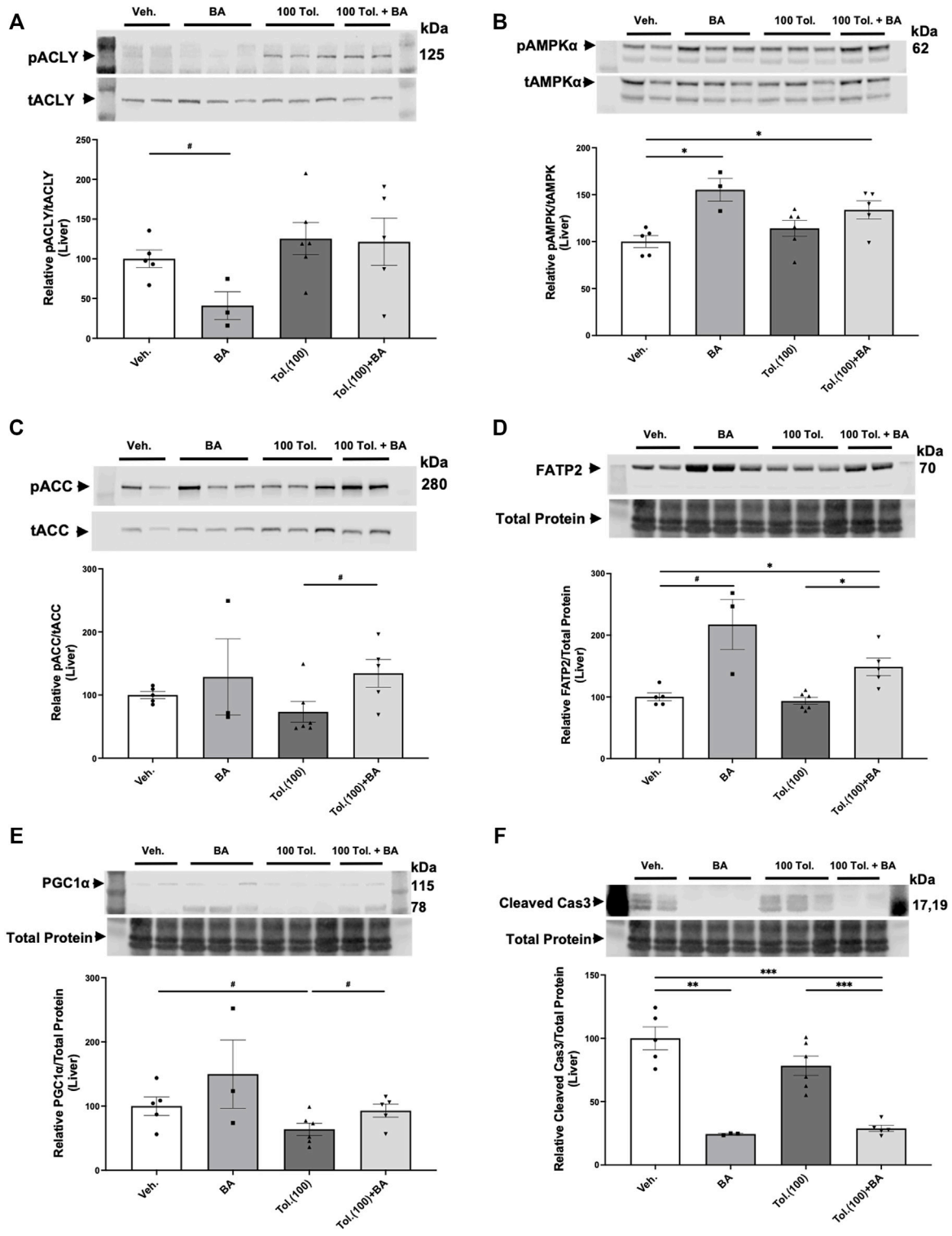

FIGURE 9
Effects of bempedoic acid (BA) and tolvaptan treatment in PKD mice on protein expression of key signaling markers in liver homogenates. Immunoblots were performed on liver tissue homogenates from *Pkd1*$^{fl/fl}$*; Pax8-rtTA; Tet-O-Cre* mice with early/rapid induced *Pkd1* gene inactivation with or without concurrent treatment with BA (30 mg/kg/d) and/or tolvaptan (100 mg/kg/d). Densitometric intensities of immunoblot bands from each lane were normalized to the total protein signal from that lane as a loading control [shown in middle panels of **(D)**, **(E)** and **(F)**] or normalized to the total non-phosphorylated protein signal [shown in middle panels of **(A)**, **(B)**, and **(C)**]. Liver homogenates were probed for: **(A)** phosphorylated ACLY (pSer455 ACLY; pACLY) and total ACLY (tACLY); **(B)** phosphorylated AMPKα (pThr172 AMPK; pAMPKα) and total AMPKα (tAMPK); **(C)** phosphorylated ACC (pACC) and total ACC; **(D)**. FATP2 expression; **(E)**. the mitochondrial biogenesis marker PGC-1α; and **(F)**. the apoptosis marker cleaved caspase 3. Representative immunoblots are shown in upper and middle panels, and summary quantitation of mean (±SE) relative protein expression levels are shown in lower panels ($^{\#}0.05 < p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or as stated for the indicated comparisons).

shown in Figure 7C, there were graded reductions of the %TKW/BW that occurred with the BA and/or tolvaptan treatments. Specifically, BA (30 mg/kg/d) reduced %TKW/BW vs. vehicle at euthanasia (6.9 vs. 11.9%; $p < 0.01$). Similarly, tolvaptan (100 mg/kg/d) reduced %TKW/BW to 7.8% vs. vehicle ($p < 0.05$). Addition of BA (30 mg/kg/d) to tolvaptan (100 mg/kg/d) caused a further reduction in %TKW/BW (4.9%; $p < 0.05$) vs. tolvaptan alone. As shown in Figure 7D, there were also graded reductions of BUN levels at euthanasia with BA and/or tolvaptan treatments. BA treatment was associated with a reduced levels of BUN vs. vehicle (59 vs. 107 mg/dl; $p < 0.05$). Tolvaptan treatment is associated with lower levels of BUN relative to vehicle at 30 (68 mg/dl; $p < 0.05$) and 100 mg/kg/d (35 mg/dl; $p < 0.01$). Again, addition of BA to tolvaptan at 30 mg/kg/d led to further significant reduction in BUN (38 mg/dl; $p < 0.05$). In summary, these findings indicate that both BA and tolvaptan reduce kidney growth and improve kidney function in this early onset ADPKD mouse model, and there are additive benefits with combination therapy.

Effects of bempedoic acid and tolvaptan treatment in PKD mice on protein expression of key signaling and injury markers in kidney homogenates

Kidneys from the conditional *Pkd1* knockout mice were harvested at the time of euthanasia to analyze the effects of treatment with BA and tolvaptan on relevant target proteins (Figure 8). As expected, BA treatment generally reduced ACLY activity, as indicated by the ratio of pSer455 ACLY (pACLY) to total ACLY (tACLY; Figure 8A) and stimulated AMPK activity, as indicated by the ratio of pThr172 AMPKα (pAMPK) to total AMPKα (tAMPK; Figure 8B) in kidney tissue homogenates relative to vehicle controls or treatment with tolvaptan alone. BA also tended to inhibit mTOR and ERK pathway signaling, which are upregulated in ADPKD (Saigusa and Bell, 2015), as evidenced by decreased phosphorylation of P70S6K (Figure 8C) and ERK (Figure 8D), respectively, relative to paired controls in the absence of BA. Finally, BA also sharply reduced expression of the PT kidney injury marker KIM1 (Figure 8E) and, to a lesser extent, tended to inhibit the distal kidney injury marker NGAL (Figure 8F) relative to controls. These BA effects occurred both alone and in combination with tolvaptan.

Effects of bempedoic acid and tolvaptan treatment in autosomal dominant polycystic kidney disease mice on protein expression of key signaling markers in liver homogenates

The liver is a main target for BA in its inhibition of sterol synthesis and its other metabolic effects (Pinkosky et al., 2016). As idiosyncratic hepatotoxicity is a concern in ADPKD patients treated with tolvaptan (Blair, 2019), we also examined protein expression of several markers in liver homogenates from the conditional *Pkd1* knockout mice to analyze the effects of treatment with vehicle control, BA (30 mg/kg/d) in the presence or absence of high-dose tolvaptan (100 mg/kg/d; Figure 9). BA treatment tended to cause the expected decrease in ACLY activity (pACLY/tACLY) in liver tissue (Figure 9A) while increasing AMPK activity, as evidenced by increased pAMPK/tAMPK (Figure 9B). BA also tended to increase phosphorylation of acetyl-CoA carboxylase (pACC/tACC), a canonical target of AMPK (Figure 9C). Interestingly, BA treatment generally caused an enhancement in the expression in liver of FATP2, the fatty acid transporter that is required for activation of the BA pro-drug (Pinkosky et al., 2016), both alone and in combination with tolvaptan (Figure 9D). This effect of BA treatment enhancing FATP2 expression was also found in kidney tissue homogenates (Supplementary Figure S4). Of note, BA also appears to promote mitochondrial biogenesis as measured by enhanced expression of PGC-1α in the ADPKD livers, while high-dose tolvaptan tended to decrease PGC-1α (Figure 9E). Finally, BA treatment dramatically decreased cleaved caspase-3, a key marker for apoptosis, in ADPKD mice livers, both alone and in combination with tolvaptan (Figure 9F). This finding suggests that BA may serve a protective role in preventing apoptosis, a marker of hepatocyte injury and death, in the setting of high-dose tolvaptan treatment.

Discussion

There are few therapeutic options for people living with ADPKD to arrest disease progression. As it has become recognized that ADPKD is a metabolic disease with dysregulated mitochondrial function (Padovano et al., 2018), identifying new therapies that target dysregulated metabolism are especially attractive. In searching for new metabolic targets to ameliorate the disease and prevent its progression, we found that ACLY activity was increased in PT- and IMCD-derived *Pkd1* KO cells relative to controls (Figure 3). As BA was recently FDA-approved for treatment of hypercholesterolemia, we considered that repurposing this drug could be compelling to test as a novel ADPKD therapeutic. Consistent with previous work (Steinberg et al., 1999; Pinkosky et al., 2016), we confirmed that mouse kidney and liver tissue, along with ADPKD cells, express the FATP2 (ACSVL1) enzyme that mediates the activation of BA (Figure 2). Thus, BA should only be converted to its active form in kidney and liver, the two organs primarily affected with cysts in ADPKD. This targeting specificity would be advantageous in ADPKD to reduce potential side effects in other organs, as ADPKD patients may need to be treated for several decades. Indeed, off-target side effects have historically limited the use of certain promising experimental drugs that would appear to have

therapeutic benefits in ADPKD (e.g., rapamycin and analogues) (Serra et al., 2010).

For this initial study to explore the potential utility and efficacy of BA as a new therapy for ADPKD, we opted to test its effects first in PKD cell lines and in an inducible ADPKD mouse model that develops early, severe kidney disease. We found that both BA and a distinct ACLY inhibitor SB-204990 inhibited 3D cystic growth in PT- and IMCD-derived *Pkd1*-null epithelial cells (Figure 4; Supplementary Figure S2). Moreover, ACLY knockdown similarly inhibited 3D cystic growth in PT-derived *Pkd1*-null epithelial cells (Figure 5). In *Pkd1*-null cell lines, BA treatment also reduced mitochondrial superoxide production, a marker of cellular oxidative stress, and increased mitochondrial elongation, a marker of mitochondrial oxidative function (Figure 6; Supplementary Figure S3). To test whether BA is effective in ADPKD to reduce cyst size *in vivo*, we used an early *Pkd1* conditional KO mouse model of rapidly progressive kidney cystic disease, similar to that described previously (Ma et al., 2013). In this mouse model, we found that BA treatment reduced kidney size and function (BUN) to a similar extent as tolvaptan relative to untreated controls, and addition of BA to tolvaptan caused a further reduction in both markers of cystic disease severity and kidney function when compared with tolvaptan alone (Figure 7). The apparent additive benefits of combination BA plus tolvaptan therapy could have important clinical implications if confirmed in human clinical ADPKD trials as it may afford lower dosing to achieve efficacy and with fewer side effects.

Treatment with BA also inhibited key cellular signaling pathways associated with ADPKD cellular proliferation (mTOR and ERK) (Saigusa and Bell, 2015) and sharply reduced the kidney injury marker KIM1 and, to a lesser extent, NGAL (Figure 8). Finally, BA therapy in combination with tolvaptan in the *Pkd1* conditional KO mouse model dramatically inhibited apoptosis (cleaved caspase-3) and tended to increase mitochondrial biogenesis (PGC-1α) in liver tissue relative to tolvaptan treatment alone (Figure 9), which suggests that BA combination therapy with higher dose tolvaptan could help mitigate tolvaptan-associated hepatotoxicity. Taken altogether, the findings of this study suggest that BA may be considered a promising new therapeutic candidate for ADPKD, which deserves additional exploration.

Interestingly, BA is a pro-drug that requires activation by the very long-chain acyl-CoA synthetase, a fatty acid transporter also known as ACSVL1 or FATP2, which adds a CoA moiety to free fatty acids in kidney and liver cells (Pinkosky et al., 2016). Our initial intent for FATP2 immunoblotting was to ascertain whether the BA-activating enzyme FATP2a was expressed in cells with *Pkd1* knockout, and therefore whether the pro-drug BA could be useful for *in vitro* cystogenesis assays. Interestingly, we found that there was a reduction of FATP2a protein levels in both *Pkd1*-null cells and kidney tissue relative to controls (Figure 2; Supplementary Figure S1). Moreover, treatment of *Pkd1*-null cells with BA increased the levels of FATP2a expression compared to control cells treated with vehicle (Figure 9D; Supplementary Figure S4). Although in-depth characterization of the mechanism for FATP2 regulation by BA is beyond the scope of our current study, we speculate that expression of FATP2a, as a fatty acid transporter, may be determined by differences in the levels or activities of long-chain fatty acids in the *Pkd1*-null cells as compared to controls. Such differences may reflect the profound metabolic changes induced by *Pkd1* knockout, which are perhaps reversed by treatments that ameliorate cystic disease.

Further studies will be important to explore the potential benefits of BA in more relevant mouse and/or other animal models of ADPKD (e.g., slower onset disease models, including the hypomorphic $Pkd1^{RC/RC}$ mouse model (Hopp et al., 2012), and in other inducible and *Pkd2* mutant animal models). Employing animal models with slower disease trajectories that encompass the animals' reproductive ages will also allow exploration of potential sex differences with respect to disease parameters and BA treatment effects in the setting of ADPKD. In the ADPKD model presented here, mice develop early disease and are treated before having reproductive capability, with euthanasia occurring at P22, so sex differences were likely not relevant. It is also currently unclear what are the specific downstream targets or relevant ADPKD cellular signaling pathways of BA. Administration of this drug to mice with more chronic cystic disease will allow a more careful and comprehensive exploration of its effects on cellular pathways and its role in regulating disease progression.

Of note, as BA is already FDA-approved and is generally well-tolerated even in patients that lack hypercholesterolemia (Banach et al., 2020; Powell and Piszczatoski, 2021), initiating human clinical trials for ADPKD may become feasible relatively soon. Importantly, as BA targets different cellular signaling pathways than tolvaptan and other emerging therapies, it will be important to test for benefits of BA both alone and in combination with tolvaptan and other drugs that target complementary cellular signaling pathways in future clinical trials.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the IACUC of the University of Southern California.

Author contributions

KH, HL, BS, EC, SP, and NMP-S conceived and designed research. HL, BS, SS, PH, JP, JW, and VM performed experiments. KH, HL, BS, SS, PH, JP, VM, and NMP-S analyzed data. KH, HL, BS, SS, PH, JP, VM, SP, and NMP-S interpreted results of experiments. KH, HL, BS, and NMP-S prepared figures. KH, HL, and NMP-S drafted manuscript. KH, HL, BS, SP, and NMP-S edited and revised the manuscript.

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Conflict of interest

The authors declare that this study received funding from Esperion Therapeutics. The funder had the following involvement in the study: data review and paper review as per author contributions of SP.

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Supplementary material

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Caspase-1 and the inflammasome promote polycystic kidney disease progression

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We and others have previously shown that the presence of renal innate immune cells can promote polycystic kidney disease (PKD) progression. In this study, we examined the influence of the inflammasome, a key part of the innate immune system, on PKD. The inflammasome is a system of molecular sensors, receptors, and scaffolds that responds to stimuli like cellular damage or microbes by activating Caspase-1, and generating critical mediators of the inflammatory milieu, including IL-1β and IL-18. We provide evidence that the inflammasome is primed in PKD, as multiple inflammasome sensors were upregulated in cystic kidneys from human ADPKD patients, as well as in kidneys from both orthologous (*PKD1*$^{RC/RC}$ or RC/RC) and non-orthologous (*jck*) mouse models of PKD. Further, we demonstrate that the inflammasome is activated in female RC/RC mice kidneys, and this activation occurs in renal leukocytes, primarily in CD11c+ cells. Knock-out of *Casp1*, the gene encoding Caspase-1, in the RC/RC mice significantly restrained cystic disease progression in female mice, implying sex-specific differences in the renal immune environment. RNAseq analysis implicated the promotion of MYC/YAP pathways as a mechanism underlying the pro-cystic effects of the Caspase-1/inflammasome in females. Finally, treatment of RC/RC mice with hydroxychloroquine, a widely used immunomodulatory drug that has been shown to inhibit the inflammasome, protected renal function specifically in females and restrained cyst enlargement in both male and female RC/RC mice. Collectively, these results provide evidence for the first time that the activated Caspase-1/inflammasome promotes cyst expansion and disease progression in PKD, particularly in females.

Moreover, the data suggest that this innate immune pathway may be a relevant target for therapy in PKD.

KEYWORDS

Caspase-1, hydroxychloroquine, IL-1β, IL-18, inflammasome, MYC, polycystic kidney disease, YAP

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenic kidney disease, with an estimated prevalence of around 1 in 1000 individuals (Lanktree et al., 2018), and is the fourth leading cause of renal failure (Spithoven et al., 2014). ADPKD is caused primarily by inherited mutations in one of two genes, *PKD1* (~78% of cases) and *PKD2* (~15%) (Cornec-Le Gall et al., 2018). The disease progresses slowly and variably, resulting in renal failure for roughly half of patients with ADPKD by the sixth decade (Cornec-Le Gall et al., 2018). The disease is accompanied by acute and chronic pain for most (60%) patients (Gabow, 1993; Bajwa et al., 2004). Currently, there is only one FDA-approved treatment for ADPKD, the vasopressin V2-receptor antagonist, Jynarque (tolvaptan) (Torres, 2018). However, because of adverse side effects of tolvaptan, there remains a need to develop safe, effective new therapies for PKD.

ADPKD is characterized by numerous renal cysts derived from the nephron that expand continuously over the patient's lifetime, causing massive enlargement of the kidney. These expanding cysts, many of which originate in the collecting duct, compress and distort the surrounding parenchyma, including the microvasculature, causing chronic ischemic and obstructive renal injury. Massive fibrosis develops in these kidneys, further contributing to the compressive injury, which ultimately results in compromised renal function (Grantham, 2003; Grantham et al., 2011). Cystic changes are also accompanied by ongoing inflammation and the presence of innate and adaptive immune cells in large numbers, some of which have been shown to modulate disease progression (for review, see (Zimmerman et al., 2020). Using rodent models of PKD, we and others have used experimental methods to deplete renal mononuclear phagocytes, including infiltrating and resident macrophages and dendritic cells, to demonstrate that these cells contribute to cyst cell proliferation and cyst expansion (Karihaloo et al., 2011; Swenson-Fields et al., 2013; Yang et al., 2018).

To discover new treatment strategies for ADPKD, we sought to understand the pathophysiologic mechanisms that promote disease progression, reasoning that this approach could identify common key molecular processes that could be therapeutic targets. A number of seemingly disparate stimuli have been shown to accelerate cystic disease in PKD model rodents. These include exposure to commensal microbes or microbial products (Werder et al., 1984; Gardner et al., 1986; Gardner et al., 1987), renal ischemia-reperfusion (IR) injury (Patel et al., 2008; Takakura et al., 2009; Kurbegovic and Trudel, 2016), and the deposition of renal calcium oxalate (CaOx) or calcium phosphate (CaP) crystals (Torres et al., 2019). One feature, that is, common to these stimuli is that each has been shown to activate the Caspase-1 inflammasome (Werder et al., 1984; Gardner et al., 1986; Gardner et al., 1987; Melnikov et al., 2001; Shigeoka et al., 2010; Mulay et al., 2013). Potential effects of Caspase-1/inflammasome activation on PKD progression have not previously considered.

The Caspase-1 inflammasome is a multi-protein scaffold, the sole known purpose of which is activation of Caspase-1. Once activated, Caspase-1 cleaves multiple cellular substrates, including gasdermin D, pro-IL-1β, and pro-IL-18. Cleaved gasdermin D promotes the formation of plasma membrane pores, which then allows the release of cleaved, active IL-1β and IL-18, and may also trigger pyroptosis (Carty et al., 2019). There are far-reaching inflammatory sequelae of IL-1β and IL-18 release. IL-1β in particular is a master regulatory cytokine that influences many cell types to promote the transcription of hundreds of genes, including those encoding both inflammatory cytokines and chemokines (Weber et al., 2010; Anders, 2016). This amplifies inflammation further by promoting the infiltration and activation of neutrophils, dendritic cells, monocytes, and lymphocytes.

Inflammasome activation has been well characterized mainly in innate immune cells. Components of the inflammasome have been found to be expressed in other renal cells, including endothelial cells, mesangial cells, podocytes, and tubular epithelial cells, but convincing evidence demonstrating the hallmarks of canonical inflammasome activation in these cells when isolated from primary sources (i.e., cleavage of Caspase-1 and extracellular release of IL-1β) is lacking or inconsistent (Anders, 2016). The inflammasome assembles as an innate immune response triggered by both microbial products (microbe-associate molecular patterns, MAMPs), produced by both commensal and pathogenic microbes, and also host-derived cellular products released in response to stress, tissue injury, or cell death (damage-associated molecular patterns, DAMPs). MAMPs include bacterial products such as toxins, peptidoglycans, flagellin, outer membrane components (e.g., LPS) and nucleic acids, including those from RNA and DNA viruses (Anders and Muruve, 2011). DAMPs include products present in all intracellular compartments (e.g., ATP, heat shock proteins, histones, HMGB1, mtDNA), as well as extracellular matrix breakdown products (e.g., biglycan, hyaluronan, fibrinogen) and crystals of all types (e.g., urate, cholesterol,

CaOx, CaP, and adenine) (Roh and Sohn, 2018). Stimuli known to promote PKD progression, i.e., exposure to commensal microorganisms, renal IR, and the induction of CaOx and CaP renal crystals, all generate MAMPs and DAMPs that have been shown to promote Caspase-1 inflammasome formation and activation and play a role in mediating the resulting inflammatory renal injury and/or systemic effects (Li et al., 1995; Melnikov et al., 2001; Wang et al., 2005; Mulay et al., 2014).

MAMPs and DAMPs engage germ-line encoded pattern recognition receptors (PRRs) present both on the plasma membrane and in the cytosol to initiate formation of the inflammasome complex, typically by promoting self-oligomerization through homotypic molecular interactions. These oligomers then recruit the adaptor protein, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and pro-Caspase-1, which then self-cleaves to become active. Some PRRs can be activated by both MAMPs and DAMPs, thereby promoting signaling pathways common to both types of stimuli. These PRRs include the toll-like receptors (TLRs) present on membranes, as well as the cytosolic inflammasome sensors, which include the NLRP (nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin, domain containing) group of proteins, the most well-known of which is NLRP3 (Anders et al., 2004; Anders and Muruve, 2011). Other sensors known to activate Caspase-1 in both humans and mice also include other NLRP-related proteins (Nlrp1, 2, 3, 4, 6,12, and Nlrc4), IPAF, a sensor for bacterial flagellin, AIM2, a sensor for dsDNA, and MEFV (aka Pyrin), a specific MAMP sensor (Broz and Dixit, 2016; Christgen et al., 2020). NLRP3 is best known among the inflammasome sensors because it is activated in response to myriad MAMPs and DAMPs and contributes to a wide variety of inflammatory diseases (Mangan et al., 2018).

Most DAMPs and MAMPs, including those that engage the inflammasome sensors, act first by interacting with TLRs, especially TLR2, 4, and 6 (Roh and Sohn, 2018, which are among the many TLRs expressed by both immune cells and non-immune cells, including renal tubular epithelial cells (Anders, 2004 #6). Binding of TLRs results in the activation of NF-kB-mediated transcription, which acts directly or indirectly to drive the upregulation of both inflammasome sensors and inflammasome components (e.g., *NLRP1*, *NLRP3*, and *Casp1*) to facilitate formation and activation of the inflammasome complex in a process known as "priming" (Lee et al., 2015; Christgen et al., 2020). TLR-activated NF-kB can also directly drive expression of some pro-inflammatory cytokines and chemokines (Liu et al., 2017).

As renal cysts expand and promote injury in PKD, DAMP generation likely occurs. A primary DAMP for both human and mouse PKD is likely to be extracellular ATP (eATP). eATP is released by cells in response to a wide range of stimuli including mechanical stress, cell membrane damage, inflammation and hypoxia (Anders and Muruve, 2011). Notably, renal epithelial cells from patients with ADPKD or from PKD mouse models have been shown to release 5 times higher levels (high-nanomolar to micromolar quantities) of extracellular ATP than cells from healthy controls (Wilson et al., 1999; Schwiebert et al., 2002). In addition, high levels of ATP have been identified in the cyst fluids of PKD kidneys from both human and rodents (Wilson et al., 1999; Palygin et al., 2018). eATP is a well-known stimulator of the NLRP3 inflammasome and acts by binding the ionotropic P2X7 receptor, which is highly expressed in immune cells and is also present in epithelial cells (Di Virgilio et al., 2018; Kopp et al., 2019). Signaling by eATP *via* this receptor stimulates K+ efflux, which is a required step for NLRP3 inflammasome activation (Munoz-Planillo et al., 2013). Whether these elevated levels of ATP promote activation of the NLRP3 inflammasome in PKD has not been studied.

Our hypothesis is that, in addition to mediating effects of experimental stimuli that promote cystic disease, the Caspase-1 inflammasome is likely to be operative during the natural course of PKD, promoting its progression. In these studies, we test this hypothesis using both animal models and analysis of human ADPKD tissue. We demonstrate for the first time that the canonical Caspase-1/inflammasome is primed in both human ADPKD and in both orthologous and non-orthologous mouse models of disease and is activated primarily in immune cells. Further, we provide evidence that activated Caspase-1 promotes PKD progression in female mice and that it can be effectively targeted to ameliorate disease.

Results

Inflammasome components and products are elevated in kidneys from autosomal dominant polycystic kidney disease patients

To determine whether inflammasome priming occurs in PKD, the relative transcript levels of ten inflammasome sensors known to promote Caspase-1/inflammasome activation, as well as *CASP1* and *IL1B*, were assessed first in the kidneys of ADPKD patients vs. non-cystic human kidneys (NHK) (Figures 1A–D). Transcripts encoding multiple sensors, including NLRP1, NLRP3, NLRP12, AIM2, MEFV, NLRC4, and *CASP1* and *IL1B* were significantly elevated in kidneys from ADPKD patients relative to NHK. In addition, Western blot of the NLRP3 sensor showed increased levels of this protein in these cystic tissues (Figure 1E). We were unable to unambiguously detect Caspase-1 protein (uncleaved or cleaved) on western blots of whole kidney extracts, however (data not shown), which may be due to technical issues with the antibodies or may reflect the expression of this protease in a restricted subset of renal cells [http://humphreyslab.com/SingleCell/ (Wu et al., 2019; Kirita et al., 2020)].

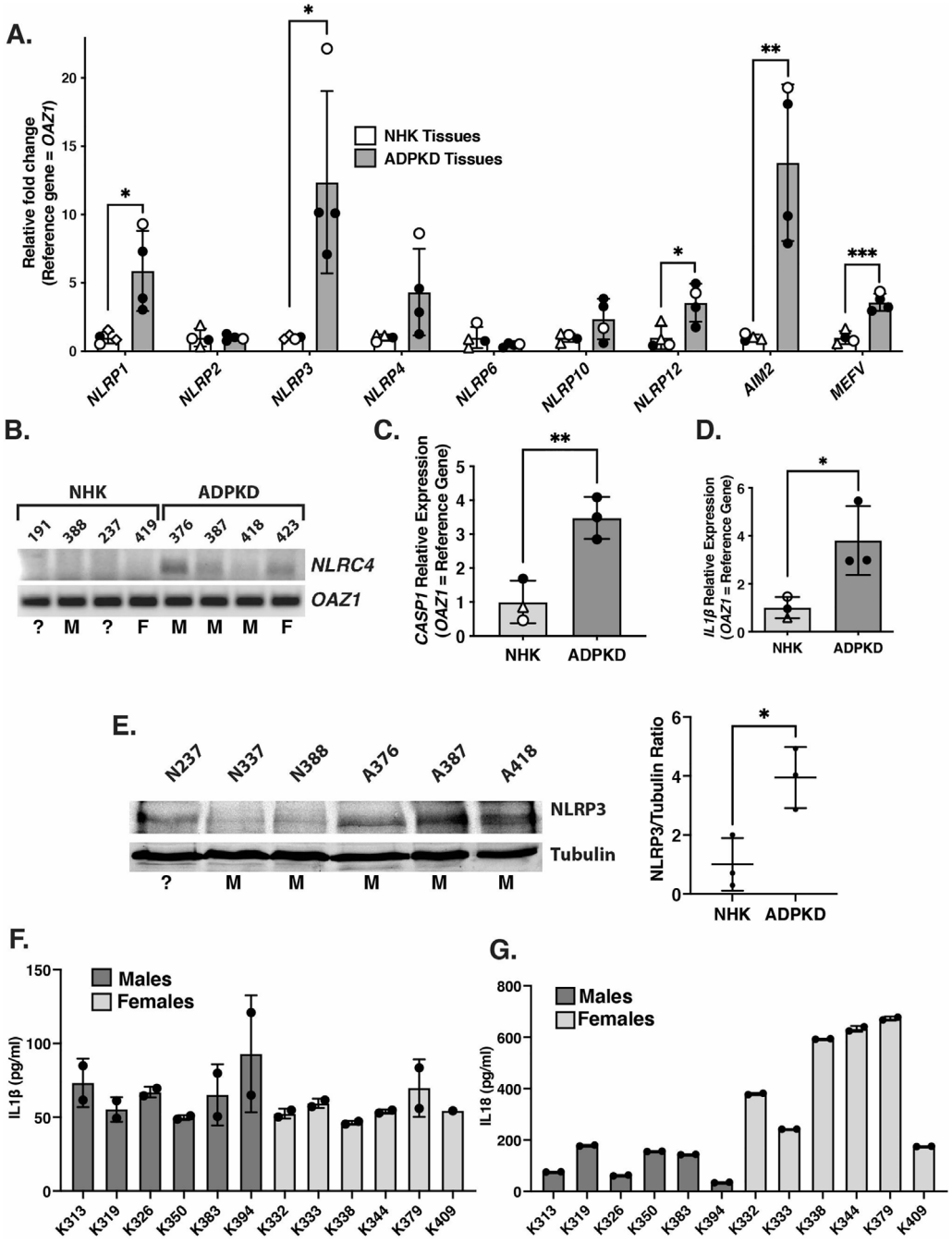

FIGURE 1
Expression of inflammasome sensors and components are elevated in kidneys from ADPKD patients. **(A)** qRT-PCR of transcripts encoding inflammasome sensors from kidneys of patients with ADPKD or from non-cystic human kidneys (NHK). Open circles = females; closed circles = males; open triangles = unknown sex. **(B)** Semi-quantitative PCR products of *NLRC4* and reference gene (*OAZ1*) from cDNAs of transcripts from the same kidney samples as in **(A)**. Assigned kidney numbers are shown for each NHK and ADPKD sample. M = male; F = female; ? = unknown sex. **(C,D)** qRT-PCR of transcripts *CASP1* **(C)** and *IL1β* **(D)** from ADPKD and NHK kidney tissues samples. Symbols indicating sex are the same as in **(A)**. **(E)** Western blot of NLRP3 and tubulin from NHK and ADPKD kidney protein samples. Assigned kidney numbers and sex are shown. On right is graph showing the relative NLRP3/Tubulin protein levels measured from the blot. **(F,G)** Concentrations of IL-1β **(F)** and IL-18 **(G)** in duplicate samples of cyst fluids collected from individual male and female patients with ADPKD measured by ELISA.

To further evaluate inflammasome activation in the kidneys of ADPKD patients, we measured the levels of IL-1β and IL-18, which are primary products of activated Caspase-1, in the cyst fluids collected from 12 different patients (Figures 1F,G). This analysis revealed measurable levels of both cytokines (ranging from 46 to 93 pg/ml for IL-1β and 34–673 pg/ml for 1L-18) in all samples. These findings are similar to those obtained in previous studies (Gardner et al., 1991; Parikh et al., 2012). While there are non-canonical Caspase-1-independent mechanisms of generating the extracellular presence of IL-1β and IL-18, these mechanisms operate primarily under inflammatory conditions in which neutrophils are the primary infiltrate (Afonina et al., 2015; Netea et al., 2015), which is not the case in cystic kidneys of ADPKD patients (TAF, unpublished). Thus, in spite of our inability to detect cleaved Caspase-1 protein in whole kidney extracts, our collective results showing both inflammasome priming and the presence of IL-1β and IL-18 suggest that inflammasome activation is likely occurring in the kidneys of ADPKD patients.

We evaluated the potential contribution of cystic epithelial cells from the kidneys of ADPKD patients to inflammasome activation. In previous studies, ADPKD cyst nephrons were shown to have elevated transcript expression of a number of inflammasome components, including IL-1β and IL-18 (de Almeida et al., 2016). In addition, renal tubular epithelial cells have been shown to express both NLRP3 and Caspase-1 transcripts and proteins, although whether these cells are capable of inflammasome activation and release of IL-1β is questionable (for reviews (Anders, 2016; Kim et al., 2019). Using human ADPKD cyst cells and tubular epithelial cells isolated from NHK grown in culture, we found a number of transcripts encoding sensors and inflammasome components that were elevated in cyst cells, suggesting that priming of the inflammasome had occurred (Supplementary Figure S1A–D). Notably the pattern of elevated transcripts only partially overlapped that found in whole human kidney tissues. NLRP3 protein was detected in these cells but, unlike renal tissues, there was no difference in the levels present in ADPKD *vs.* NHK cells (Supplementary Figure S1E). In addition, little to no IL-1β was detected in the conditioned media from either cell type (Supplementary Figure S1F). These results indicate that while ADPKD cyst cells in culture are primed (or partially primed) inflammasome activation is not ongoing.

We tested whether ADPKD cyst cells could activate the NLRP3 inflammasome using established methods to maximally prime and trigger this activation (Guzova et al., 2019). ADPKD cyst cells were treated with agonists Pam3CSK4 and LPS specific for the membrane receptors, TLR2 and TLR4, respectively, which are known to be present on these cells and to interact with same MAMPs and many DAMPs present *in vivo* to bring about priming (Roh and Sohn, 2018; Christgen et al., 2020). These cells were then treated with a potassium ionophore, nigericin, known to rapidly and potently force activation of the NLRP3 inflammasome in primed cells, causing Caspase-1 cleavage and release of IL-1β (Guzova et al., 2019). While treatment of ADPKD cyst cells with these MAMPs was sufficient to promote further upregulation of *CASP1* and *IL1B* transcripts, there was no change in the level of Caspase-1 protein, and the addition of potassium ionophore did not result in cleavage of Caspase-1 or the release of IL-1β (Supplementary Figure S1G–J). In contrast, similar treatment of a control monocytic cell line (THP-1 cells) performed in parallel resulted in Caspase-1 cleavage and IL-β release, as expected (Supplementary Figure S1I,K). These results suggest that while inflammasome priming mechanisms can occur in ADPKD cyst cells, their ability to activate the NLRP3 inflammasome appears unlikely. Thus, inflammasome activation in human ADPKD kidneys is likely occurring in cells other than the cyst epithelial cells. We examined this hypothesis further using an orthologous mouse model of PKD.

Inflammasome components are elevated in kidneys from orthologous and non-orthologous mouse models of polycystic kidney disease

To begin analysis of inflammasome activation in an animal model of PKD, the relative expression levels of inflammasome sensors and components were assessed in the kidneys of an orthologous mouse model of ADPKD, $Pkd1^{RC/RC}$ (or "RC/RC" mice). These mice have a knock-in *Pkd1* missense allele, *Pkd1* (p.R3277C) or "RC," that matches an allele found in a human family with cystic disease (Hopp et al., 2012). On the C57BL/6 background these RC/RC mice develop detectable renal cysts by 3 months of age, which slowly and progressively enlarge with time. At 6 months, RC/RC mice exhibit many well-developed renal cysts, although no loss of kidney function has yet occurred (Arroyo et al., 2021). As in the kidneys of human ADPKD patients, there was significant upregulation of transcripts for multiple sensors in the RC mice kidneys relative to WT, including those encoding NLRP1a, NLRP3, and AIM2 (Figure 2A). These kidneys also showed elevated expression of *Casp1* transcripts and trended toward an upregulation of *Il1b* as well (Figures 2B,C). There was also increased expression of the NLRP3 sensor protein in RC/RC *vs.* WT kidneys, similar to that seen in renal tissue of human ADPKD patients (Figure 2D). As for human tissue, we were unable to detect Caspase-1 protein in whole kidney extracts by western blotting (data not shown). Regardless, these results suggest that inflammasome priming is ongoing in RC/RC mice at this stage.

To evaluate whether the mutations in *Pkd1* (RC/RC mice) or *PKD1* and *PKD2* (humans) were specifically responsible for the elevated expression of inflammasome components, the relative transcript levels encoding sensors, Caspase-1, and IL-1β were

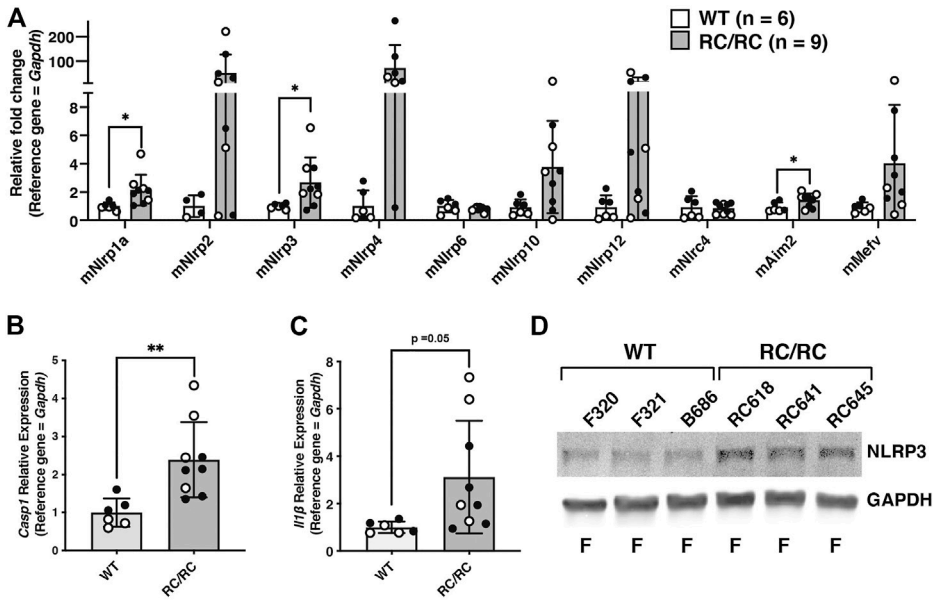

FIGURE 2
Expression of inflammasome sensors and components are elevated in kidneys from RC/RC mice. **(A)** qRT-PCR of transcripts encoding inflammasome sensors from kidneys of RC/RC or WT mice at 6 months of age. Open circles = females; closed circles = males. qRT-PCR of transcripts *Casp1* **(B)** and *Il1b* **(C)** from RC/RC and WT kidney tissues samples. Symbols indicating sex are the same as in **(A)**. **(D)** Western blot of NLRP3 and GAPDH from WT and RC/RC kidney protein samples. Assigned kidney numbers and sex are shown.

assessed in the kidneys of mice with juvenile cystic kidney (*jck*) disease and the non-cystic heterozygotes from the same breeding group at PN38. PKD in *jck* mice arises due to a homozygous mutation in the gene *Nek8*, which encodes a cilia-associated kinase (Liu et al., 2002). While there is formation and growth of renal cysts in the early life of *jck* mice, they do not begin to lose kidney function until after ~ PN38 (Smith et al., 2006). Results from qRT-PCR showed significant upregulation of transcripts encoding the sensors NLRP3, NLRP10, and MEFV, as well as those encoding Caspase-1 and IL-1β, in the kidneys of *jck* mice relative to the noncystic controls (Supplementary Figure S2A–C). These results indicate that inflammasome priming, and potentially activation, is also ongoing in the kidneys of *jck* mice, similar to that seen for RC/RC mice and patients with ADPKD. These results also suggest that *PKD1* and *PKD2* mutations are not directly responsible for inflammasome priming and activation.

CD11c+ macrophages and dendritic cells are responsible for inflammasome activation in RC/RC cystic kidneys

Inflammasome activation has been reported to occur in a number of different cell types, although it is primarily associated as an activity of immune cells (Anders, 2016). In cystic PKD kidneys, inflammasome activity has not been previously assessed. However, single cell RNA sequencing in mouse kidneys injured by other means (ischemia-reperfusion, unilateral ureter obstruction, and allograft rejection) has demonstrated that Caspase-1 is most upregulated in monocytes, macrophages, and dendritic cells [http://humphreyslab.com/SingleCell/(Wu et al., 2018; Wu et al., 2019; Kirita et al., 2020)]. Given this observation and our inability to detect Caspase-1 cleavage in whole RC/RC kidney extracts, we examined whether inflammasome activation was occurring in the renal immune cells of RC/RC mice.

Initially, we assessed inflammasome activity in total renal leukocyte populations. For these experiments, we prepared single-cell suspensions from individual kidneys of female WT or cystic RC/RC mice and used Ficoll gradients to enrich for leukocytes. Using this method, we obtained an average of ~ 2–3 × 10^6 leukocyte-enriched cells for each single RC/RC kidney for further purification, whereas only ~1/3 of this number was typically obtained from WT kidneys (data not shown). Flow cytometry analyses revealed ~70%–90% $CD45^+$ cells in these preparations from both RC/RC and WT (data not shown). $CD45^+$ is a tyrosine phosphatase expressed on the plasma membrane of all mature hematopoietic cells, except erythrocytes and platelets (Nakano et al., 1990). $CD45^+$ cells include macrophages, dendritic cells, T and B lymphocytes, and innate lymphoid cells. The $CD45^+$ cells were isolated from the

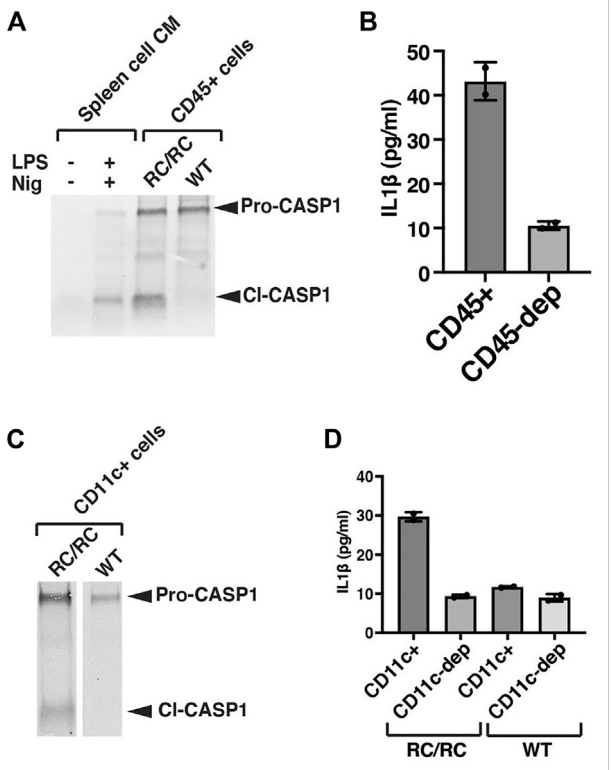

FIGURE 3
CD11c+ macrophages and dendritic cells are responsible for inflammasome activation in RC/RC cystic kidneys. **(A)** CD45$^+$ MACS (magnetic cell separation) cells were purified from single kidneys of female RC/RC or WT mice prior to immunoblot analysis (100,000 cells/lane) using an antibody specific for Caspase-1. This experiment was carried out on 4 separate occasions with similar results. As control samples, single cells were prepared from mouse spleens and either treated or not *in vitro* with LPS and nigericin to stimulate inflammasome activation. Conditioned media from these cells were collected and concentrated prior to immunoblot analysis. **(B)** CD45$^+$ cells were purified from single kidneys of female RC/RC mice by MACS column as in **(A)** and equal numbers (100,000) of these cells and those left after passing through the column (CD45-depleted, dep) were seeded in 96-well plates and incubated overnight prior to the collection of conditioned media and the measurement of IL-1β by ELISA. This experiment was carried out on 3 separate occasions with similar results. **(C)** CD11c+ MACs cells were purified from single kidneys of female RC/RC and 2 kidneys/experiment from female WT mice and subjected to immunoblot analysis (100,000 cells/lane) as in **(A)**. **(D)** CD11c+ cells purified by MACS column as in **(C)**, and equal numbers (100,000) of these cells and those left after passing through the column (CD11c-depleted, dep) were seeded in 96-well plates and incubated overnight prior to the collection of conditioned media and the measurement of IL-1β by ELISA. This experiment was carried out on 3 separate occasions with similar results.

Ficoll-enriched preparations using anti-CD45 antibody-coupled magnetic beads and were assessed for the presence of Caspase-1 by western blot. Uncleaved pro-Caspase-1 was readily detectable in equal numbers of CD45$^+$ cells from both RC/RC and WT kidneys. However, only those cells isolated from RC/RC kidneys showed the presence of the cleaved, activated form of this enzyme (Figure 3A). These results suggest that the inflammasome is activated in the CD45$^+$ leukocytes of RC/RC but not WT kidneys.

To determine whether these RC/RC renal leukocytes are producing IL-1β, CD45$^+$ cells were isolated from cystic kidneys and cultured overnight, and IL-1β was measured in the conditioned media (CM) by ELISA (Figure 3B). In parallel, equal numbers of leukocyte-enriched cells following removal of the CD45$^+$ population (CD45-depleted) were also cultured. Post-isolation flow analyses in these experiments demonstrated high efficiency of the depletion: >90% of the cells isolated with the anti-CD45 magnetic beads were positive for CD45, but only 1%–4% of the CD45-depleted cells were positive (data not shown). As show in Figures 3A,B relatively high-level of IL-1β (43 pg/ml in this experiment) was measured in the CM of CD45$^+$ isolated cells compared to that from the CD45-depleted cells (11 pg/ml), which was below the lowest IL-1β standard (15.6 pg/ml) in this assay.

In efforts to more narrowly identify those leukocytes in which the inflammasome is activated in RC kidneys, we focused on CD11c + cells. CD11c is a marker both of dendritic cells and macrophage populations in the kidney (Weisheit et al., 2015; Viehmann et al., 2018), and these populations have been found to be primarily responsible for renal inflammasome activation in the setting of kidney injury types other than PKD (Mulay et al., 2013). Treatment of mice with clodronate lipsosomes, such as that used previously to restrain cystic disease progression in mouse models of PKD, has been shown to deplete renal CD11c + cells (Mulay et al., 2013), suggesting potential pro-cystic effects of these cells. Flow analysis of the leukocyte-enriched populations from RC/RC and WT kidneys showed around 30%–40% of these cells were CD11c+ (data not shown).

Renal CD11c+ cells were isolated from the Ficoll-enriched cell populations of female RC/RC and WT mice with magnetic anti-CD11c beads and assessed for Caspase-1 cleavage and IL-1β production. There was a reduced efficiency of isolation of CD11c+ cells compared to that obtained with the anti-CD45 antibody reagents: ~70%–85% of the cells isolated by anti-CD11c beads from both RC/RC and WT kidneys were CD11c +, while ~5%–15% of the CD11c-depleted cells were CD11c+ (data not shown). As expected, there was a high percentage of CD45$^+$ cells present in both the anti-CD11c bead-isolated and CD11c-depleted cells (greater than 95% and 70%–90% respectively, not shown). Western blots of Caspase-1 showed that uncleaved Caspase-1 was present in anti-CD11c-isolated cells from both RC/RC and WT kidneys, but only RC/RC kidneys showed cleaved Caspase-1 in these preparations (see Figure 3C for an example). In addition, while elevated levels of IL-1β were produced by renal anti-CD11c

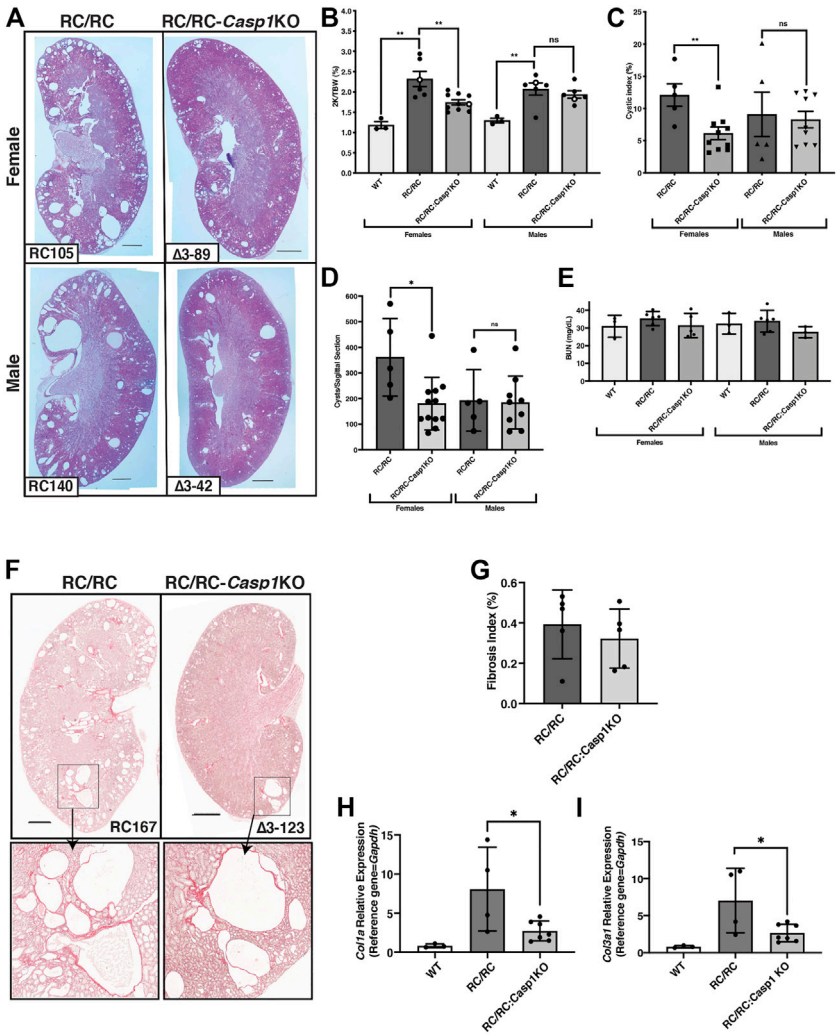

FIGURE 4
Casp1-deficiency restrains cystic disease progression in female RC/RC mice. **(A)** Formalin-fixed average sized kidneys from 6 m old RC/RC and RC/RC:*Casp1*KO mice were sectioned and stained with hematoxylin and eosin. Scale bars = 1 mm. **(B)** The two-kidney/total body weight percentage (2K/TBW %) was determined for each animal analyzed and plotted as a function of genotype for females and males. Open symbols indicate data points from those mice from which the H&E images shown in **(A)** were taken. The cystic index **(C)** and the number of cysts per sagittal section **(D)** for RC/RC and RC/RC:*Casp1*KO mice were determined and plotted as a function of gender and genotype. **(E)** Blood urea nitrogen (BUN) was determined for each mouse tested and plotted as a function of gender and genotype. **(F)** Sections from kidneys of female RC/RC and RC/RC:*Casp1KO* mice were stained with picrosirius red and illuminated by bright field to visualize collagen type I-α1 (*Col1α1*) and collagen type III-α1 (*Col3α1*). Scale bars = 1 mm. Enlarged images of the boxed regions are shown below as indicated by the arrows. **(G)** The fibrosis index was determined from picrosirius red-stained kidney sections from the RC/RC and RC/RC:*Casp-1*KO female mice. Quantitative RT-PCR of RNA isolated from WT, RC/RC, and RC/RC:*Casp-1*KO female mice, showing the relative expression of the fibrosis markers *Col1α1* **(H)** and *Col3α1* **(I)**.

isolated cells from RC mice, very little IL-1β was detected in equal numbers of CD11c+ isolated cells from WT mouse kidneys or by the CD11c-depleted cells from either mouse type (see Figure 3D for an example), despite the high percentage of $CD45^+$ cells in this depleted population. These experiments indicate that inflammasome activation in the kidneys of RC/RC mice is occurring predominantly in leukocytes, and that CD11c+ cells are likely the primary immune cells responsible for this activation.

Caspase-1 deficiency in RC mice restrains cystic disease progression in females but not males

Since multiple types of kidney insults that promote inflammasome activation also are known to promote cystic disease progression in rodent models of PKD, we hypothesized that a genetic deficiency of *Casp1* in RC/RC mice to restrict inflammasome activation might restrain cyst

expansion during the natural course of the disease. Using TALENs technology the *Casp1* gene was mutated to create several deletions (Supplementary Figure S3A–C), one of which was bred to homozygosity in the RC/RC mouse. This mutation (Del3, Supplementary Figure S3B) which has a 28 bp deletion that includes the coding region for the active site cysteine residue of Caspase-1 and the splice donor of exon 6, results in a knockout (KO) genotype for expression of the gene (Supplementary Figure S3D).

WT mice, RC/RC mice, and RC/RC mice homozygous for *Casp1* KO (RC/RC:*Casp1*KO) were euthanized at 6 months of age and assessed for parameters of cystic disease progression. These included microscopic examination of H&E-stained, formalin-fixed renal mid-sagittal sections and determination of the 2 kidney/total body weight (2K/TBW) ratios, cystic index, cyst number, and renal function, as estimated by serum blood urea nitrogen (BUN). H&E-stained sections of average-sized kidneys for both females and males showed robust cystic disease in RC/RC mice that appeared to be restrained in the RC/RC:*Casp1*KO mice, particularly in females which showed apparent smaller and fewer cysts (Figure 4A). As shown previously for this strain, the 2K/TBW was elevated in both male and female RC mice compared to WT at this age. The RC/RC:*Casp1*KO mice showed reduced 2K/TBW compared to RC/RC mice but only in females (Figure 4B). Similarly, the cystic index and number of cysts/sagittal sections were also reduced in female RC/RC:*Casp1*KO mice but not the males (Figures 4C,D). The BUN of the RC/RC mice at this age was not elevated, as has been shown previously (Arroyo et al., 2021), and these values were unaltered in the RC/RC:*Casp1*KO mice (Figure 4E).

We assessed the *Casp1* KO effects on renal fibrosis initially by collagen (type1-α1 and type III-α1) staining of sections from fixed samples with picrosirius red (Figure 4F) Because of the predominantly female effect of *Casp1* KO on cyst number and kidney size, we concentrated this analysis on female samples. There was little fibrotic area in the RC/RC kidneys at this age as has been shown previously (Arroyo et al., 2021), and quantitation of the fibrosis indices of these samples showed no significant difference between the RC/RC and RC/RC:*Casp*1KO kidneys (Figure 4G). To detect more subtle potential effects of *Casp1* KO on fibrosis pathways in these kidneys, the relative transcript expression of *Col1α1* and *Col3α1* were determined by qRT-PCR (Figures 4H,I). The expression of these fibrosis transcript markers was elevated in the RC/RC kidneys when compared to WT and was significantly reduced by *Casp1* KO. These results suggest that early renal fibrotic pathways in the female RC/RC mice at this age are restrained by *Casp1* KO.

Sections of these female mouse kidneys were also stained for the proliferation marker, Ki67, which showed very few positive cells (Supplementary Figure S4A). Quantification of the Ki67 + cells lining the cysts and those in the interstitium was carried out and showed no significant difference between the RC/RC and RC/RC:*Casp1*KO samples (Supplementary Figure S4B,C). Finding no differences in proliferation, particularly for the cyst-lining cells, despite the differences in cystic index and cyst numbers, is likely due to the overall low numbers of proliferating cells in these kidneys, coupled with the inadequacies of a snap-shot assessment at a single time point.

Gene expression profiles of RC/RC and RC/RC:*Casp1*KO kidneys

To elucidate the cellular pathways influenced by *Casp1* KO to restrain cyst formation and cyst growth in PKD, gene expression profiling following RNASeq was carried out using both male and female kidneys from WT, RC/RC, and RC/RC:*Casp1*KO mice (see Data Availability Statement). Hierarchical cluster analysis of expressed genes from these kidneys reveals distinct expression profiles (Figure 5A). Notably, many of the gene expression changes seen in RC/RC mice compared to WT are reversed in RC/RC:*Casp1*KO compared to WT. Examination of kidney gene expression in individual mice reveals a similar theme (Supplementary Figure S5), although: 1) female RC/RC mice had a somewhat different pattern of upregulated and downregulated genes, compared to males; and 2) at least a subset of genes downregulated by *Casp1* KO in females appeared to be more variably affected by *Casp1* KO in males. Due to the small number of mice in each group, subsequent RNAseq analyses were performed using the combined male/female data, unless otherwise noted, though key genes were assessed and validated using qRT-PCR in individual males and females separately.

The differentially expressed genes (DEGs) that were upregulated in RC/RC vs. WT kidneys included genes in pathways that have been previously reported in PKD (Chen et al., 2008; Song et al., 2009; Happe et al., 2011; Pandey et al., 2011; Menezes et al., 2012; Dweep et al., 2013; de Almeida et al., 2016; Chatterjee et al., 2017; Malas et al., 2017; Cai et al., 2018; Kunnen et al., 2018; Terabayashi et al., 2020). Upregulated DEGs mapped to Gene Ontology or KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways that are associated with proliferation and growth, such as cAMP, WNT, Hedgehog, Hippo, and TGFβ, as well as pathways involved in epithelial-to-mesenchymal transition and fibroblast proliferation and activation (Supplementary Table S1). Strikingly, there were numerous upregulated genes mapped by Gene Ontology analysis to more than 270 different pathways related to various innate and adaptive immune responses, including immune cell activation, function, and migration and response to cytokines (Supplementary Table S2). Pathways related to pyroptosis and IL-β and IL-18 production, secretion, and response are including among these inflammatory pathways.

Gene expression profiles were compared between RC/RC and RC/RC:*Casp1*KO kidneys to discover those pathways altered by the absence of *Casp1* and particularly those that might influence

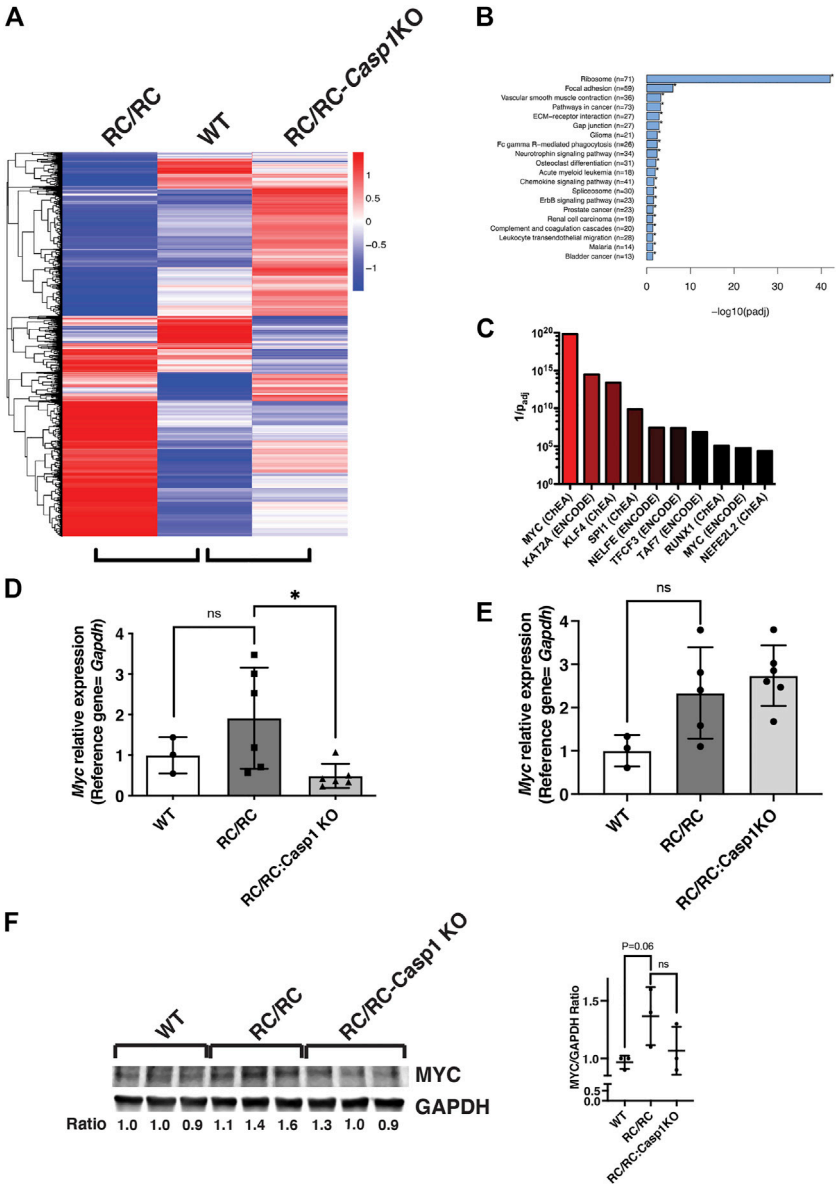

FIGURE 5
The MYC pathway in RC/RC mice is downregulated by *Casp1* KO in females but not males. **(A)** RNA was isolated from kidneys of 6 month-old RC/RC, WT, and RC/RC:*Casp1*KO mice (2 males and 2 females per group). RNAseq was performed, and hierarchical cluster analysis of differentially expressed genes was used to compare gene expression patterns from each group. Hierarchical clustering analysis was carried out with the log10(FPKM+1) of union differential expression genes of all comparison groups. Differential expression analysis between two conditions/groups was done by using the DESeq2 R package, with the significance criterion being $p_{adj} < 0.05$. Color descending from red to blue indicates log10(FPKM+1), from large to small. **(B)** ClusterProfiler software was used to perform KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis from the RNAseq data (males and females). Shown are pathways significantly enriched among all the 3162 DEGs in RC/RC vs. RC/RC:*Casp1KO* kidneys. Pathways are ranked by -log10 (p_{adj}). Shown in parentheses are the number of genes mapped to each pathway. **(C)** Genes significantly downregulated in male and female RC/RC:*Casp1KO* kidneys compared to RC/RC kidneys were analyzed using the web-based tool ENRICHR (see text for references). Transcription factors that are present in ENCODE and ChEA databases were matched with consensus target genes from within this gene set. The *y*-axis represents $1/p_{adj}$. **(D and E)** qRT-PCR of *Myc* transcripts from kidneys of 6 month-old WT, RC/RC, and RC/RC:*Casp1*KO female **(D)** and male **(E)** mouse kidneys (*$p < 0.05$; *t* test). **(F)** Western blots of MYC and GAPDH from whole protein samples isolated from the kidneys of 6 month-old WT, RC/RC and RC/RC:*Casp1*KO female mice (3 each). The ratio of MYC/GAPDH is shown beneath each sample and is plotted in the graph to the right.

cystic disease progression. KEGG pathway analysis of all the 3164 DEGs in RC/RC vs. RC/RC:*Casp1KO* kidneys showed that the ribosome pathway was the most altered (Figure 5B and Supplementary Table S3). This pathway included 71 DEGs, mostly encoding ribosome proteins, all of which were downregulated (70 significantly) in the RC/RC:*Casp1*KO kidneys compared to those of RC/RC. A reduction in the expression of these proteins would be expected to diminish ribosome biogenesis and slow growth, which may contribute to the restrained cystic disease in these mice.

Caspase-1 knockout in female but not male RC mice restrains MYC and YAP pathways, which are central mediators of kidney pathogenesis in polycystic kidney disease.

Since ribosomal genes are downregulated by *Casp1* KO, we focused the next stage of analysis on DEGs that were downregulated in the RC/RC:*Casp1KO* kidneys compared to RC/RC. These DEGs were expected to be associated with cystic disease-promoting pathways that are downregulated by *Casp1* KO. This gene set, which comprises 1405 DEGs, was used to perform ChIP enrichment analysis (ChEA) utilizing a publicly available, online tool, Enrichr (Lachmann et al., 2010; Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021), to identify transcription factors that might regulate these genes. In this analysis, MYC was identified as strongly associated with DEGs downregulated in the RC/RC:*Casp1KO* kidneys (Figure 5C and Supplementary Table S4). MYC is a transcription factor, that is, known to promote expression of ribosomal proteins and ribosome biogenesis. Importantly, MYC is also known to be a central node responsible for the promotion of tubular epithelial cell proliferation and cystogenesis in PKD (Kurbegovic and Trudel, 2020). qRT-PCR assessment of renal *Myc* expression in female mouse kidneys showed an uptrend in the levels of this transcript in RC/RC vs. WT mice at this disease stage, which was significantly reversed by *Casp1* KO (Figure 5 D). In male mouse kidneys, while there was also an uptrend in *Myc* expression in RC/RC vs. WT mice similar to that found in females, there was no effect on the levels of this transcript elicited by *Casp1* KO (Figure 5E). The levels of MYC protein in whole kidneys of female mice were assessed by Western blot, which showed increased levels of this protein in RC/RC vs. WT kidneys and reduced levels (2 out of 3 cases) in the RC/RC:*Casp1*KO vs. RC/RC kidneys (Figure 5F). These results suggest that KO of *Casp1* in female RC/RC mice may dampen the MYC pathway in these PKD animals.

MYC is a transcriptional target of the oncoprotein and transcription coactivator YAP, and the YAP-MYC signaling axis has been found to be a mediator of cystic kidney pathogenesis in a human-orthologous PKD mouse model (Cai et al., 2018). It was of interest then to determine whether the DEGs that were downregulated in the RC/RC:*Casp1*KO vs. RC/RC kidneys were enriched in YAP targets as well as MYC targets. Comparison of these downregulated genes with YAP targets previously identified by ChIP-seq in mouse embryonic stem cells (Lian et al., 2010) revealed that these targets comprised ~38% of the 1405 DEGS downregulated in the RC/RC:*Casp1*KO kidneys vs. RC/RC (Supplementary Table S5). This list includes key YAP targets upregulated in human ADPKD cystic tissue compared to minimally cystic tissue, such as *Axl*, *Ctgf*, *Cyr61*, and *Myc* (Cai et al., 2018). qRT-PCR analysis showed that multiple YAP targets in females, including these and *Fxyd1*, were upregulated in the RC/RC vs. WT mice and, as with *Myc*, were downregulated in the RC/RC:*Casp1*KO vs. RC/RC mice

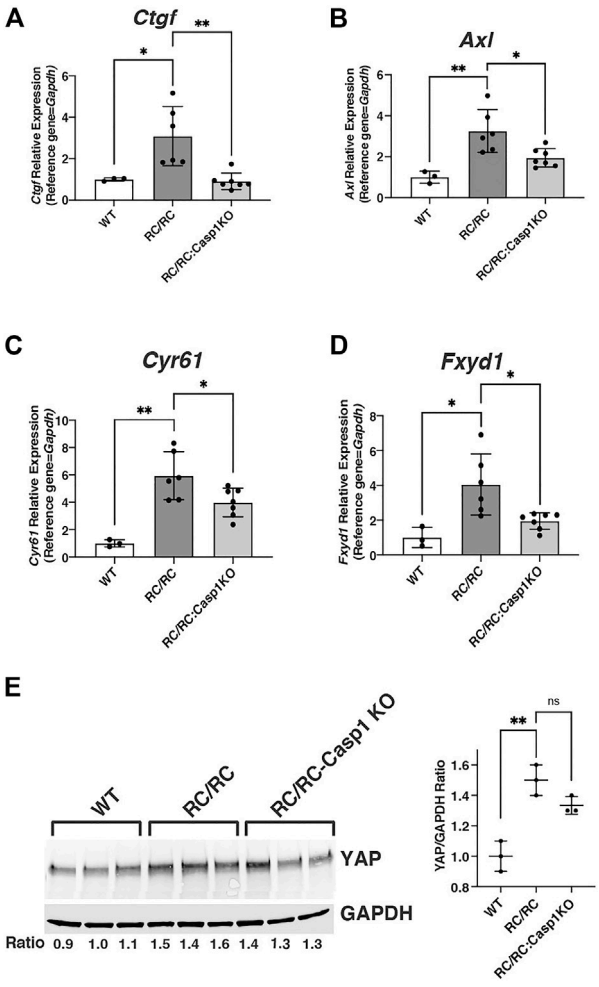

FIGURE 6
YAP pathway in RC/RC female mouse kidneys is downregulated by *Casp-1* KO. **(A–D)** qRT-PCR of YAP transcript targets from WT, RC/RC and RC/RC:*Casp-1*KO female mouse kidneys: **(A)** *Ctgf*; **(B)** *Axl*; **(C)** *Cyr61* and **(D)** *Fxyd1*. **(E)** Western blots of YAP and GAPDH from protein samples of WT, RC/RC, and RC/RC:*Casp1*KO female kidneys (3 each).

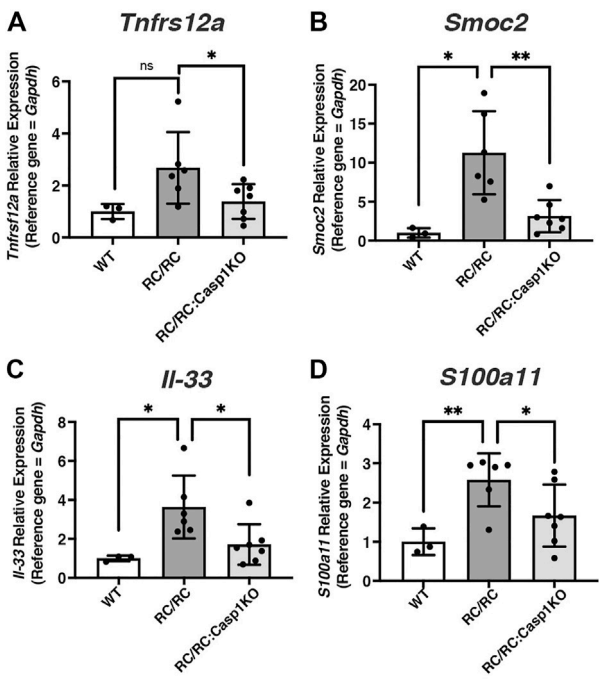

FIGURE 7
The expression of coordinately regulated YAP/MYC targets upregulated RC/RC female mouse kidneys is diminished by *Casp1* KO. **(A–D)** qRT-PCR of YAP/MYC coordinately regulated transcript targets from WT, RC/RC, and RC/RC:*Casp1*KO female mouse kidneys: **(A)** *Tnfrs12a*; **(B)** *Smoc2*; **(C)** *Il-33;* and **(D)** *S100a11.*

(Figures 6A–D). In males, two of the YAP targets, *Ctgf* and *Axl* were assessed and while there was an uptrend in these targets in RC/RC vs. WT mice, there was no effect on the levels of these target transcripts in the RC/RC:*Casp1*KO vs. RC/RC mice (Supplementary Figure S6A,B). Assessment of YAP protein in female mouse kidneys showed elevated levels of this protein in RC/RC compared to WT and reduced levels (2 out of 3 cases) in the RC/RC:*Casp1*KO vs. RC/RC kidneys (Figure 6E). Collectively these results suggest that deficiency of Caspase-1 in this PKD model restrains the full activation of both MYC and YAP pathways in females but not males, correlating with the female-specific effects of this deficiency in restraining cystic disease progression.

In a recent study YAP was found to activate MYC-dependent transcription cooperatively to promote a full proliferative response through integration of both mitogenic and mechanical signals (Croci et al., 2017). The differentially expressed genes that responded to MYC and YAP together but not, or less so, to either alone were identified and were linked mainly to cell proliferation. Given the known contribution of both MYC and YAP to PKD disease progression and the elevated levels of these proteins in cystic tubular cells (Happe et al., 2011; Trudel, 2015; Cai et al., 2018), we explored the possibility that YAP/MYC transcriptional cooperation may be occurring in PKD kidneys, especially in females, and that inflammasome activation may influence this phenomenon. Expression of these known coordinately regulated genes was assessed in our dataset from WT, RC/RC, and RC/RC:*Casp-1* KO, kidneys. This analysis revealed 65 coordinately YAP/MYC-regulated targets that were upregulated in RC/RC vs. WT and downregulated in RC/RC:*Casp1*KO vs. RC/RC (Supplementary Table S6). Validation of this observation was carried out, initially in females, by qRT-PCR for several of these genes, including those encoding TNF receptor super family member 12A (*Tnfrs12a*), SPARC related modular calcium binding 2 (*Smoc2*), Interleukin 33 (*Il33*), and S100 calcium binding protein A11 (*S100a11*) (Figures 7A–D). In males, while there was an uptrend in the single coordinate YAP/MYC target analyzed in RC/RC vs. WT mice, *Tnfrs12a*, there was no effect on the levels of this target transcript in the RC/RC: *Casp1*KO vs. RC/RC mice as seen in females (Supplementary Figure S6C). Other YAP/MYC-regulated targets were not assessed in males.

Collectively, these results suggest that deficiency of *Caspase-1* in the RC/RC PKD model restrains the activation of both MYC and YAP pathways and likely their coordinately regulated pathways in females but not males. Given the demonstrated critical roles of the YAP and MYC pathways to PKD cystogenesis, it is possible that the restraint of these pathways in female RC/RC mice deficient for *Caspase-1* contributes to the female-specific restraint of cystic disease progression in these mice.

Hydroxychloroquine constrains inflammasome activation and restrains cystic disease progression in RC mice

Given the encouraging data showing ameliorative effects of *Casp1* depletion in PKD, at least in females, we sought to identify FDA-approved medications with the ability to inhibit inflammasome activation that could potentially be used for long-term treatment in PKD patients. Hydroxychloroquine (HCQ) is widely used to treat chronic inflammatory illnesses, such as lupus and rheumatoid arthritis (Ponticelli and Moroni, 2017; Schrezenmeier and Dorner, 2020), and is low-cost with uncommon side effects. While the mechanisms of HCQ's immunomodulatory effects are incompletely understood, in studies of the well-known anti-inflammatory effects of HCQ, this drug was shown to dampen ATP-induced inflammasome activation *in vitro* and *in vivo* (Eugenia Schroeder et al., 2017). eATP is likely to be a relevant NLRP3 inflammasome-activating DAMP in PKD because: 1) as we show here, renal expression of the NLRP3 sensor appears to be a common PKD feature, suggesting that priming of this inflammasome has occurred; and 2) abundantly elevated levels of eATP are produced by tubular epithelial cells from PKD kidneys compared to control

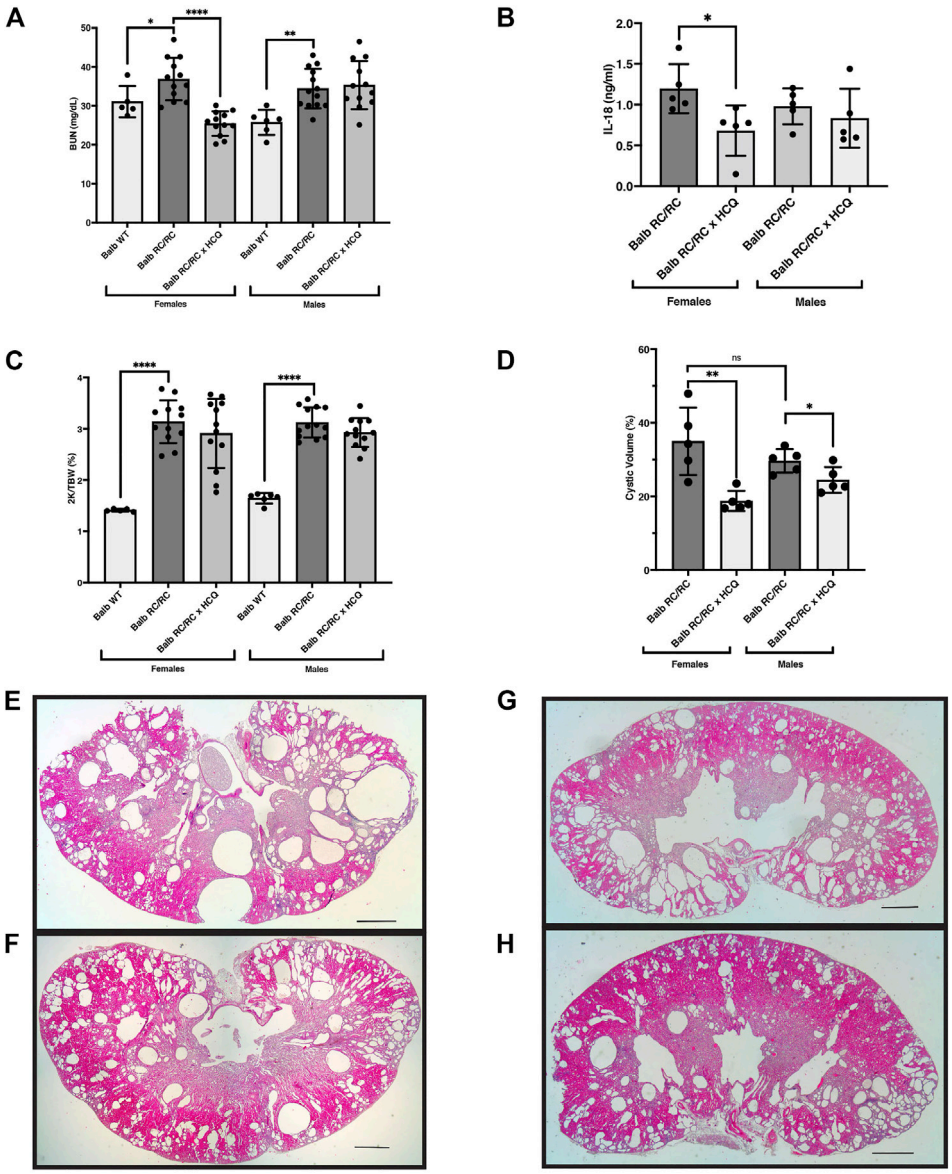

FIGURE 8
Hydroxychloroquine treatment of RC/RC mice protects kidney function in females and restrains cyst growth in females and males. Shown are measured parameters of PKD progression and serum from RC/RC mice on the BALB/c background that were treated with hydroxychloroquine, and control untreated mice of the same age. **(A)** Blood urea nitrogen (BUN) was determined for each mouse and plotted for females and males. **(B)** Serum IL-18 was measured for the five mice with BUNs closest to the mean for each RC/RC sample type and plotted for females and males. **(C)** The two-kidney/total body weight percentage (2K/TBW %) was determined for each mouse and plotted for females and males. **(D)** The cystic volume as a percentage of total kidney volume was determined for the five mice with BUNs closest to the mean for each RC/RC sample type and plotted for females and males. **(E,F)** Formalin-fixed average sized kidneys from HCQ-treated and untreated RC/RC mice were sectioned and stained with hematoxylin and eosin. **(E)** Untreated female. **(F)** HCQ-treated female. **(G)** Untreated male. **(H)** HCQ-treated male. Scale bars = 1 mm.

cells, giving rise to the elevated levels in these diseased kidneys (Wilson et al., 1999; Schwiebert et al., 2002; Palygin et al., 2018). Thus, it seemed reasonable to hypothesize that treatment of PKD mice with HCQ, an inhibitor of ATP-dependent inflammasome activation, might restrain cystic disease progression, similar to our findings in the Caspase-1 KO RC/RC female mice.

Before we tested this hypothesis, we examined the effects of HCQ on inflammasome production of IL-1β and IL-18 in cell-based experiments. In LPS-primed human THP-1 monocytes, HCQ diminished the ATP-induced release of both IL-1β and IL-18 in a concentration dependent manner (Supplementary Figure S7A,B). Similarly, using LPS-treated mouse primary spleen cells,

HCQ diminished the ATP-induced release of IL-1β (Supplementary Figure S7C,D). These data support previous findings in the literature and the authors' conclusions regarding the inhibitory effects of HCQ on inflammasome activation (Eugenia Schroeder et al., 2017).

To test the effects of HCQ in PKD, we treated RC/RC mice (Balb/C background) with HCQ dissolved in the drinking water. The RC/RC mice in the Balb/C background are amenable to assessing the efficacy of short-term treatment regimens on renal function, which is why they were chosen for this study. The Balb/C RC/RC mice begin to show reduced kidney function, evidenced by elevated BUN, earlier than the C57Bl/6 RC/RC mice (typically within a few months) (Arroyo et al., 2021). However, one disadvantage of the cystic disease progressing earlier in the Balb/C RC/RC mice is that, after weaning, changes in the 2K/TBW in the following few months are minimal in the Balb/C RC/RC mice. This reduces the usefulness of this measurement as a parameter of PKD progression in short-term studies begun after weaning.

With this limitation in mind, we examined the effects of HCQ provided in the drinking water from the time of weaning until 4 months of age. To assess renal function, serum BUN in Balb/C RC/RC control mice and those treated with HCQ was measured. As shown in Figure 8A, HCQ treatment resulted in a significant reduction in BUN in the females, whereas there was no difference in this parameter in males, similar to the observed gender-specific effects on cystic index of *Casp1* KO. To confirm the inhibitory effects of HCQ on inflammasome activation in these mice we measured the serum-levels of IL-18 in five RC/RC mice in both treated and untreated groups having BUNs closest to the mean of each group. Similar levels of this cytokine were found in untreated male and female RC/RC mice. In the HCQ-treated mice, there was significant reduction of this cytokine in the sera of treated females relative to untreated females, whereas in males there was a downward trend of IL-18 levels in the treated group, but it did not reach significance (Figure 8B). As expected, there was no difference in the 2K/TBW in HCQ-treated *versus* untreated mice for either females or males (Figure 8C). To determine the relationship between the protective effects of HCQ and cystic disease, measurements of kidney cystic volumes were carried out. Interestingly, there was a significant reduction of cystic volumes in the HCQ-treated vs. untreated mice in both females and males (Figure 8D), though the effect was especially prominent in females. Stained histological sections of average-sized kidneys corroborated these data, as both females and males showed robust cystic disease in the untreated mice that appeared to be restrained in the HCQ-treated mice (Figures 8E–H).

Discussion

The inflammasome is a key component of the innate immune response, and activation of the Caspase-1/inflammasome has been shown to be common to many processes known to accelerate cyst formation and expansion, including renal IR, exposure to commensal microbes/microbial products, and deposition of renal crystals. Evidence is presented here for the first time that the Caspase-1/inflammasome is primed in PKD kidneys of both humans and mice and is activated in the cystic kidneys of female RC/RC mice, a human-orthologous PKD model mouse strain. Renal CD11c+ cells were identified in these mice as the predominant immune cells demonstrating the hallmarks of Caspase-1 inflammasome activation (cleavage of pro-Caspase-1 and production of extracellular IL-1β). We also show for the first time that knockout of Caspase-1 slows cyst expansion and disease progression specifically in females suggesting a significant sex difference in the inflammatory environment of these mice. Evidence is also provided that the presence of Caspase-1 in female but not male mice supports MYC/YAP pathway activation in the kidney, which has been shown to play a central role in PKD progression (Trudel, 2015; Cai et al., 2018; Kurbegovic and Trudel, 2020). Finally, we show that treatment of RC/RC mice with the anti-inflammatory drug HCQ, which can inhibit the NLRP3/Caspase-1 inflammasome among its effects, both protects kidney function in females and restrains cystic growth in both females and males, suggesting its potential use in the treatment of ADPKD patients.

In initial studies we found that the expression of multiple cellular sensors that act upstream of Caspase-1/inflammasome activation are elevated in cystic kidneys of both human ADPKD and PKD model mice, suggesting that the inflammasome priming process has occurred. The pattern of inflammasome sensor expression was found to be different between human and mouse PKD kidneys. The human ADPKD kidneys examined are end-stage, so the set of DAMPs contained within these kidneys are likely to be more extensive and probably include MAMPs, since microbial products are routinely detected in these kidneys (Miller-Hjelle et al., 1997). The specific set of DAMPs present in the two PKD model mice, *jck* and RC/RC, are also likely to be different. While these mice are exposed to the same external conditions (identical housing and in the same room), the stage of the cystic disease progression at the time of evaluation for these experiments is not identical, so differences in the number of injured or dying cells releasing DAMPs are likely. In addition, there may be genetic differences influencing the expression of specific damage patterns found in these mice. Elevation of the NLRP3 sensor expression is a common feature among the PKD kidneys (human and mouse) included in this study. This feature, along with the known elevated levels of extracellular ATP in PKD kidneys (Wilson et al., 1999; Schwiebert et al., 2002), suggests that activation of this inflammasome is a likely to be a common feature also.

Within the cystic kidneys of female RC/RC mice, immune cells, particularly CD11c+ cells, were found to be the primary cell showing evidence of inflammasome activation. CD11c+ (also known as Integrin alpha X, *Itgax*) is a cell surface marker found

in populations of both macrophages and dendritic cells, so it is likely that inflammasome activation was present in both of these populations, and that they contributed to the Caspase-1-dependent pro-cystic functions in these mice. Since renal CD11c+ cells can be depleted by clodronate liposomes (Mulay et al., 2013; Gottschalk and Kurts, 2015), it is likely that cells expressing activated Caspase-1/inflammasomes were depleted in earlier studies using this drug to suppress cystic disease in PKD mice (Karihaloo et al., 2011; Swenson-Fields et al., 2013; Yang et al., 2018).

Transcriptome analysis of cystic kidneys by RNAseq in this study suggests a potential mechanistic connection between the Caspase-1/inflammasome and cystic disease. Namely, the inflammasome appears to promote YAP/MYC pathways in females specifically. This possibility was supported further by immunoblots of renal YAP and MYC and qRT-PCR of their individual and coordinately regulated target genes. Both of these transcription factors have been previously implicated in PKD pathogenesis. Since the initial discovery of elevated renal MYC levels in a non-orthologous model of PKD (Cowley et al., 1987), this protein and MYC-stimulated pathways have been found to be elevated in all models of PKD that have been examined, as well as in human ADPKD (Trudel, 2015; Kurbegovic and Trudel, 2020). Overexpression of *Myc* in mouse kidneys is sufficient to induce tubular cell proliferation and cystogenesis (Trudel et al., 1991), and genetic deficiency of renal *Myc* in a human-orthologous mouse model of PKD dramatically restrained cyst formation (Cai et al., 2018). *Myc* is also a transcriptional target of YAP, which appears to promote the transcription of *Myc* directly (Cai et al., 2018). Elevated levels of YAP and YAP target genes have been found in human ADPKD kidneys and in multiple orthologous mouse models of PKD (Happe et al., 2011). Genetic deficiency of YAP and its transcriptional coactivator, TAZ, reduced Myc expression and suppressed cyst formation in *Pkd1*-deficient mouse kidneys (Cai et al., 2018). In these studies, a pathway flowing from YAP to MYC was shown to be an important contributor to cystogenesis in the *Pkd1* mutant mouse kidneys.

Crosstalk between innate immune pathways and the Hippo-YAP pathway have been documented previously. The release of inflammatory cytokines, including TNFα, IL-6, and most notably, the inflammasome-generated cytokine IL-1β (Taniguchi et al., 2015; Liu et al., 2020; Wang et al., 2020; Caire et al., 2022), have been shown to stabilize levels of YAP protein and activate its target transcriptional pathways. The effects of activated YAP appear to be cell-type specific. In tubular epithelial cells, this pathway typically results in cell proliferation and contributes to regeneration of epithelial cells after injury (Taniguchi et al., 2015; Xu et al., 2016; Chen et al., 2018). Activation of YAP in mononuclear immune cells by inflammatory cytokines appears to promote inflammatory pathways further, including the upregulation of inflammatory cytokines/chemokines (Liu et al., 2020; Caire et al., 2022). While it seems likely that the reduced renal levels of YAP and YAP target genes, including *Myc,* that we show here in female RC/RC: *Casp1* KO mice contribute to their suppressed cystic disease, the specific contribution of this pathway to direct effects on tubule cell proliferation or to the inflammatory environment influencing cyst growth has not yet been studied.

In the studies reported here, knockout of *Casp1* slowed cyst expansion and disease progression, but the effects were statistically significant only in females. There were no apparent differences in disease severity between males and females. The reason for the gender-specific effects of *Casp1* deletion is uncertain. It is possible that there are female-specific influences on inflammasome activation. In humans, the baseline expression of multiple Caspase-1 inflammasome sensors, including *NLRP1*, *NLRC4,* and *MEFV*, was found to be elevated in macrophages derived from peripheral blood mononuclear cells of healthy females compared to males, suggesting that estrogen might play a role in promoting macrophage inflammasome priming and activation (Yang et al., 2015). Such an effect might contribute to the sex differences found in the incidence of those autoinflammatory conditions that occur primarily in females and to which the inflammasome contributes, including rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome, and multiple sclerosis (Orstavik, 2017; Govindarajan et al., 2020; Li et al., 2020). In contrast to these diseases, however, cystic disease in human ADPKD and in multiple PKD mouse models is not found primarily in females and is typically worse in males compared with females (Schrier et al., 2014). Regardless, a female-specific upregulation of multiple Caspase-1 inflammasome sensors in the immune cells of the RC/RC mice could sensitize these cells to Caspase-1/inflammasome activation.

It is also possible that males exhibit a dominance of Caspase-1-independent, inflammatory pathways. This hypothesis is supported by the results of treatment with HCQ (Figure 8). Previous studies have shown that HCQ can inhibit the NLRP3 inflammasome in immune cells (Eugenia Schroeder et al., 2017; Tang et al., 2018), and our data support these results (Supplementary Figure S7). Males and female RC/RC mice have similar, elevated levels of serum IL-18, however, HCQ lowered serum levels of IL-18 in females but not significantly in males. In males the production of this cytokine is likely occurring *via* non-canonical inflammatory mechanisms (Afonina et al., 2015; Netea et al., 2015). Such Caspase-1-independent inflammatory pathways in RC/RC males may be related to the reported amplified response to renal ischemic injury that occurs in males (Kher et al., 2005), which could be relevant in PKD because of the ongoing ischemic and mechanical injury arising from vascular compression and cyst expansion (Grantham et al., 2011). Thus, an amplified, non-canonical inflammatory response predominating in male RC/RC mice, coupled with inflammasome sensitization in females, could reasonably

account for the female-dominant ameliorative effects of *Casp1* KO.

HCQ treatment also had gender-specific effects on the RC/RC mice, i.e., female-specific protection of kidney function that correlated with female-specific reduced serum levels of IL-18. However, HCQ significantly restrained cyst expansion in both males and females, though the effect was more robust in females. The minimal effects on serum IL-18 levels in HCQ-treated males in this study suggest that the restrained cystic expansion in HCQ-treated males is arising from other HCQ effects outside of those on inflammasome activation.

HCQ is an anti-malarial agent, which is also commonly used to treat multiple autoinflammatory diseases, including those to which the NLRP3 inflammasome is known to contribute: rheumatoid arthritis, systemic lupus erythematosus, and Sjögren's syndrome (Nirk et al., 2020). The drug has multiple cellular effects in addition to NLRP3 inflammasome inhibition that are likely to influence the inflammatory environment and could affect PKD progression (Nirk et al., 2020). The mechanism of inhibition of ATP-induced NLRP3 inflammasome by HCQ in THP-1 macrophages was shown to due to the inhibition of Ca^{2+}-activated K^+ channels, including the KCa3.1 (KCNN4) channel, and consequent inhibition of K^+ efflux (Eugenia Schroeder et al., 2017). Notably, the KCa3.1 channel is also expressed in tubular epithelial cells and was shown to play an important role in cAMP-dependent chloride secretion and cyst growth in *vitro* studies of ADPKD cyst cells (Albaqumi et al., 2008). Similar HCQ inhibition of this channel *in vivo* could restrain fluid secretion and cyst growth in PKD mice, in addition to its inhibitory effects on the NLRP3 inflammasome. Also, HCQ is known to impair endosome acidification, and, since multiple TLRs require acidified endosomes for their activation, this effect results in the impairment of TLR-mediated production of cytokines, including TNFα, IL-6, and IFN-γ (Nirk et al., 2020). Actions of HCQ such as these could be responsible for the HCQ-mediated suppression of cyst growth in male RC/RC mice, and they could contribute to the effects in females. Regardless of the gender-specific effects of HCQ treatment on PKD progression in these mice, the reduction in cystic volume of both sexes in this study suggests that HCQ might be effective in the treatment of patients with ADPKD.

In sum, we have provided evidence that the Caspase-1/inflammasome is activated during the course of PKD in RC/RC mice, is an important driver of PKD progression in females and may be a viable therapeutic target. Several relevant questions remain. Is Caspase-1/inflammasome activation a common feature of all renal injuries that promote polycystic kidney disease? The answer is uncertain. Treatment of PKD mice with the nephrotoxin 1,2-dichlorovinyl-cysteine (DCVC) to induce renal injury accelerates cyst formation significantly (Happe et al., 2009). However, effects of this nephrotoxin on Caspase-1/inflammasome activation have not been examined.

Another question to be explored is whether renal activation of Caspase-1/inflammasome in PKD mice always leads to accelerated cyst formation and disease progression. Also, it is worth considering whether activated Caspase-1 is sufficient to promote *de novo* cyst formation in the absence of mutations that cause PKD. Such a scenario is suggested by the studies of (Kurbegovic and Trudel, 2016), who showed that renal IR, a Caspase-1-activating process, was sufficient to promote *de novo* cyst formation in WT mice. Additionally, it was recently uncovered that patients with either hereditary hypophosphatemic rickets with hypercalciuria or CYP24A1 deficiency show a high incidence of renal cysts (Hanna et al., 2021; Hanna et al., 2022). A characteristic feature of these disorders is renal stones, which would suggest the likely presence of activated Caspase-1. It may be that the pro-cystic effects of activated Caspase-1 demonstrated in this paper may also be present in the absence of mutations that cause PKD. Experiments to test this possibility are currently underway.

Materials and methods

An expanded version of the methods is included in Supplementary Materials and Methods. Detailed methods for production of the RC/RC:*Casp1KO* mouse, HCQ treatment of RC/RC mice, histology, immunohistochemistry, quantitative RT-PCR, determination of cystic volume, preparation and isolation of primary immune cells, flow cytometry, and cell culture are included. All animal experiments were approved by KUMC Institutional Animal Care and Use Committee, and the use of human tissue was approved by the KUMC Institutional Review Board. Data are presented as the mean $^{\pm}$ s.d. and were compared using the two-tailed *t*-test calculated with Prism (v9.2, GraphPad, La Jolla, CA). The *p*-values of <0.05 were considered significant, as indicated in the figures.

Data Availability Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE207957.

Ethics statement

The studies involving human participants were reviewed and approved by KUMC Institutional Review Board. The patients/participants provided their written informed consent to participate

in this study. The animal study was reviewed and approved by KUMC Institutional Animal Care and Use Committee.

Author contributions

KS-F designed the studies, drafted the manuscript, was primary experimentalist, and performed data analysis. TF designed the studies with KS-F, drafted and revised the manuscript, and performed data analysis. CW performed experiments provided critical reagents and contributed to data analysis. DW provided critical reagents and contributed to data analysis. MB contributed to data analysis. MV, JS, and PR provided critical resources and contributed to data analysis. ML, SF, AH, JM, AR, EW, and KJ performed experiments. All authors approved the final version of the manuscript.

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Conflict of interest

Author MV was employed by the company Resilio Therapeutics LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.971219/full#supplementary-material

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Metabolism-based approaches for autosomal dominant polycystic kidney disease

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Autosomal Dominant Polycystic Kidney Disease (ADPKD) leads to end stage kidney disease (ESKD) through the development and expansion of multiple cysts throughout the kidney parenchyma. An increase in cyclic adenosine monophosphate (cAMP) plays an important role in generating and maintaining fluid-filled cysts because cAMP activates protein kinase A (PKA) and stimulates epithelial chloride secretion through the cystic fibrosis transmembrane conductance regulator (CFTR). A vasopressin V2 receptor antagonist, Tolvaptan, was recently approved for the treatment of ADPKD patients at high risk of progression. However additional treatments are urgently needed due to the poor tolerability, the unfavorable safety profile, and the high cost of Tolvaptan. In ADPKD kidneys, alterations of multiple metabolic pathways termed metabolic reprogramming has been consistently reported to support the growth of rapidly proliferating cystic cells. Published data suggest that upregulated mTOR and c-Myc repress oxidative metabolism while enhancing glycolytic flux and lactic acid production. mTOR and c-Myc are activated by PKA/MEK/ERK signaling so it is possible that cAMPK/PKA signaling will be upstream regulators of metabolic reprogramming. Novel therapeutics opportunities targeting metabolic reprogramming may avoid or minimize the side effects that are dose limiting in the clinic and improve on the efficacy observed in human ADPKD with Tolvaptan.

KEYWORDS

ADPKD (autosomal dominant polycystic kidney disease), tolvaptan, metabolic reprograming, therapeutic approaches, metabolism & obesity, GLP-1, glucagon

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited cause of kidney disease with an estimated prevalence between 1:400 and 1:1,000 (Torres et al., 2007; Chow and Ong, 2009; Harris and Torres, 2009). In ADPKD, enlarging fluid-filled cysts develop in both kidneys, eventually leading to kidney failure. Besides kidney cysts that can be very painful, ADPKD can present with extra-renal manifestations such as development of cysts in the liver, pancreas, spleen and epididymis, abnormal heart valves and brain aneurysm (Perrone et al., 2015). Common features of ADPKD are flank and abdominal pain, urinary tract infections, hypertension, and kidney stones (Gabow, 1990; Torres et al., 2007). ADPKD is predominantly caused by mutations in either PKD1 or PKD2 genes encoding for two ciliary proteins, Polycystin 1 (PC1) and Polycystin 2 (PC2) (Harris and Torres, 2009; Takiar and Caplan, 2011). These mutations within epithelial cells of the kidney interfere with multiple pathways located within the cilia and promote proliferation, de-differentiation and fluid secretion resulting in growth of these cells into cysts. Due to the slow progression and the intrafamilial difference in

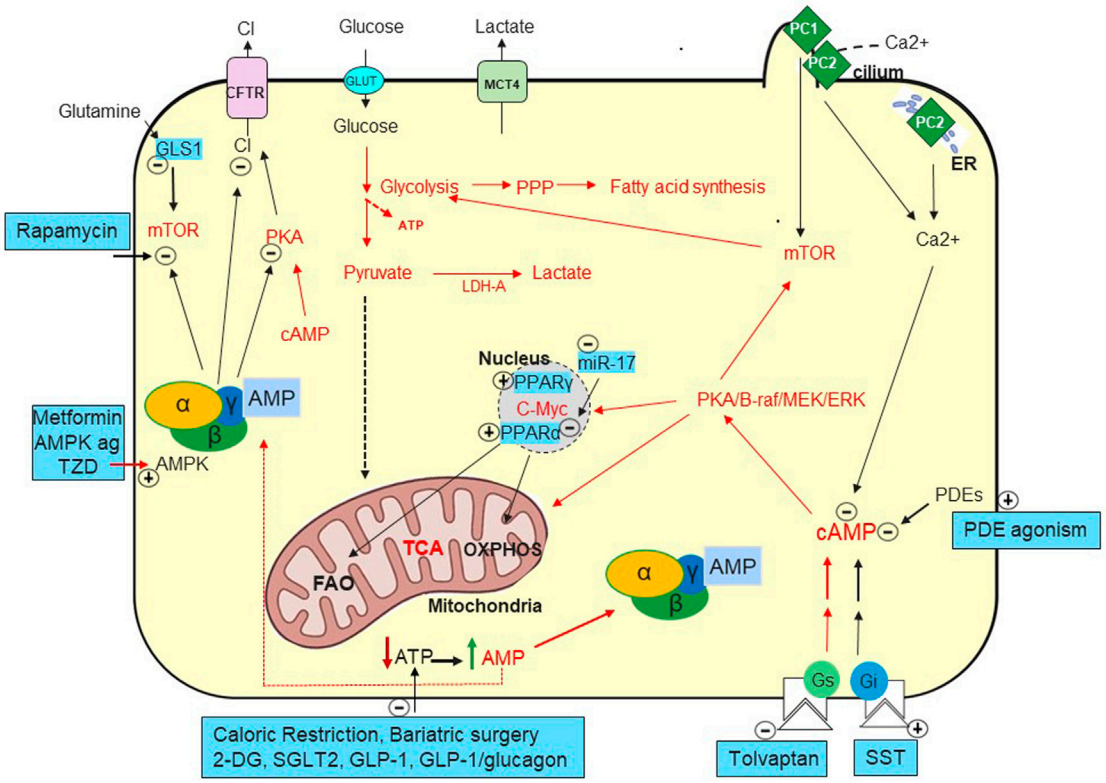

FIGURE 1
Metabolic Pathways in ADPKD. Increased cAMP due to vasopressin (Chebib et al., 2015) but likely also to decreased phosphodiesterases (PDEs) (Pinto et al., 2016) play a key role in generating fluid-filled cysts. Defects in PC1 and PC2 mediated calcium ion influx in the primary cilia and/or in the endoplasmic reticulum (ER) (Nauli et al., 2003; Padhy et al., 2022). Decreased intracellular calcium seems to convert the antiproliferative to proliferative effect of cAMP (Yamaguchi et al., 2003) causing activation of MEK-ERK and increased cell proliferation. cAMP activates PKA and stimulates chloride secretion through the cystic fibrosis transmembrane conductance regulator (CFTR) (Sullivan et al., 1998). Tolvaptan, a vasopressin V2 receptor antagonist, was approved to preserve kidney function by targeting cAMP (Chebib and Torres, 2021). Several somatostatin analogues (SST) are being investigated to lower cAMP with Ocreotide-LAR being approved in ADPKD in Italy (Capuano et al., 2022a). Mammalian target of rapamycin (mTOR) and c-Myc are upregulated in ADPKD and suppress oxidative metabolism while enhancing glycolytic flux, lactate production and export (LDH-A and MCT4) (Rowe et al., 2013; Podrini et al., 2020).

disease severity, it has been suggested that defective clearance of precipitated microcrystals may promote cyst formation and drive kidney injury when Pkd1 or Pkd2 are mutated (Torres et al., 2019a). An increase in cyclic adenosine monophosphate (cAMP) and a simultaneous dysregulation in intracellular calcium in the cystic epithelium seems to play a key role in generating and maintaining fluid-filled cysts (Yamaguchi et al., 2003; Belibi et al., 2004; Di Mise et al., 2018). cAMP activates protein kinase A (PKA) and stimulates epithelial chloride secretion through the cystic fibrosis transmembrane conductance regulator (CFTR) (Sullivan et al., 1998). A vasopressin V2 receptor (V2R) antagonist, tolvaptan, was recently approved to preserve kidney function in ADPKD by lowering vasopressin-mediated cAMP increase (Chebib and Torres 2021). However, considering the potential drawbacks of Tolvaptan i.e., side effects, poor tolerability, and high cost it is important to identify additional pathways and novel therapeutic interventions. Recently, alterations of metabolic pathways (metabolic reprogramming) in ADPKD have shown that the abnormal cystic growth utilize aerobic glycolysis, glutaminolysis and reducing oxidative phosphorylation (OXPHOS) (Padovano et al., 2018; Podrini et al., 2020). This review will review and discuss potential therapeutic approaches targeting metabolism-based pathways in ADPKD.

Current approved therapies for ADPKD lowering cAMP

Tolvaptan

Tolvaptan is a vasopressin-2-receptor (V2R) antagonist approved to slow kidney function decline in adult patients with rapidly progressive ADPKD by reducing cAMP levels (Chebib and Torres 2021, Figure 1). Tolvaptan reduces TKV (Total kidney volume TKV, prognostic biomarker for risk assessment in ADPKD, Fick-Brosnahan et al., 2002) and renal function decline. However, its clinical use is limited by poor tolerability due to aquaretic symptoms, potential liver failure and high cost (Chebib and Torres 2021; Müller et al., 2022). Recently Lixivaptan, a selective vasopressin V2 receptor antagonist which was predicted to have a lower risk of

hepatotoxicity compared to tolvaptan was discontinued (https://investors.centessa.com/news-releases/news-release-details/centessa-pharmaceuticals-makes-strategic-decision-discontinue). In the kidney, the V2 receptor is mainly expressed in the distal nephron potentially limiting the area of action of V2R antagonists (Mutig et al., 2007; Sparapani et al., 2021). Tolvaptan has been shown to exhibit a partial agonist activity on β-arrestin recruitment whose expression is increased in human ADPKD kidneys (Xu et al., 2018). These data suggest that there is space for safer and tolerated best in class cAMP lowering approaches in ADPKD either targeting additional pathways regulating cAMP (PKA inhibition, PDE activation), biased V2R antagonists or combination with targets that may provide an additive or synergistic effect such as the calcium-sensing receptor (Di Mise et al., 2021; Zhou and Torres, 2022).

Octreotide-LAR

Octreotide long-acting release (octreotide-LAR) is a somatostatin analogue that lowers the annual slope of TKV increase with no effect on renal function worsening. It was approved in Italy for the treatment of adult ADPKD patients at high risk of progression with eGFR ranging from 15 to 30 ml/min/1.73 m2 based on less frequent doubling of serum creatinine or ESKD compared to placebo. Side effects reported for somatostatin analogues include cholelithiasis and risk of cholecystitis, alopecia and increases in blood glucose (Perico et al., 2019; Griffiths et al., 2020). Hepatic cyst infections were also reported in patients treated with Lanreotide which required hospital admission and antibiotics. It has been suggested that the small size of the trials involving somatostatin analogues can explain the inconclusive renoprotective effects (Meijer et al., 2018; Capuano et al., 2022b). While awaiting publication of the results of Lanreotide in ADPKD (LIPS, NCT02127437) a plausible explanation for the apparent different effects across analogues is the affinity for the five somatostatin receptors (SSTR1 to 5) (Suwabe et al., 2021; Bais et al., 2022; Ruggenenti et al., 2022).

Metabolic reprogramming in ADPKD

Glutamine metabolism and aerobic glycolysis

The idea of metabolic reprogramming first came from the Warburg effect in cancer cells where OXPHOS is inhibited and cells tend to utilize aerobic glycolysis to produce energy (Koppenol et al., 2011). Metabolic reprogramming does not include only the Warburg effect, but also other metabolic changes. Rowe first suggested that mutations in Pkd1 result in a defective glucose metabolism with decreased gluconeogenesis and increased aerobic glycolysis to supply energy and promote proliferation (Rowe et al., 2013, Figure 1). 2-deoxyglucose (2DG), which is transported into the cells but cannot undergo glycolysis, inhibited the proliferation of $Pkd1^{-/-}$ cells and prevented disease progression in ADKPD models (Rowe et al., 2013; Chiaravalli et al., 2016; Riwanto et al., 2016; Lian et al., 2019). Recently, Soomro provided evidence that alteration in glutamine metabolism play a role in cyst growth (Soomro et al., 2020). During glutaminolysis the enzyme glutaminase (GLS, Figure 1) converts glutamine to glutamate then converted to a TCA cycle intermediate, alpha-ketoglutarate to generate ATP for cyst growth (Soomro et al., 2020). Podrini confirmed the defective glucose metabolism and characterized other altered metabolic pathways in mouse kidney without Pkd1 such as increased pentose phosphate pathway (PPP), glutamine uptake and decreased TCA cycle and fatty acid oxidation (FAO) (Podrini et al., 2020). The authors also generated data supporting targeting asparagine synthetase to interfere with glutaminolysis in conjunction with glycolysis to slow $PKD1^{-/-}$ cell growth and survival (Podrini et al., 2020). Decreased FAO also appears to contribute to disease exacerbation as increased c-MYC upregulates miR-17 in mouse cystic kidneys inhibiting PPARα and leading to FAO inhibition to support proliferation of ADPKD cells. Anti-miR-17 restored PPARα and improved FAO, ameliorating ADPKD (Lee et al., 2019, Figure 1). Considering that a single miRNA specie can regulate hundreds of targets, it is unclear if the beneficial effect is mediated by PPARα (Mohr and Mott, 2015). Nevertheless, the PPARα agonist fenofibrate showed increased FAO and reduced cystic volume in preclinical ADPKD (Lakhia et al., 2018). An anti-miR17 oligonucleotide is in Phase 1b in ADPKD patients to de-repress multiple miR-17 mRNA targets including Pkd1 and Pkd2 (Lakhia et al., 2022; https://www.prnewswire.com/news-releases/regulus-therapeutics-announces-first-patient-dosed-in-phase-1b-multiple-ascending-dose-mad-clinical-trial-of-rgls8429-for-the-treatment-of-autosomal-dominant-polycystic-kidney-disease-adpkd-301665896.html).

Mammalian target of rapamycin (mTOR)

The mechanisms that account for elevated mTOR activity in ADPKD are not fully understood but it appears that cAMP/PKA/ERK and AKT are upstream regulators (Distefano et al., 2009; Rowe et al., 2013; Margaria et al., 2020). Animal studies demonstrate that mTOR inhibition improves cystic disease and kidney function (Pema et al., 2016; Su et al., 2022), however, metanalysis of clinical data with ADPKD patients receiving rapamycin, sirolimus, or everolimus did not support a significant influence on renal progression (Lin et al., 2019). In these trials, it is not clear if mTOR inhibition was achieved in the kidney or whether mTORC1 inhibition triggers a compensatory activation of mTORC2 limiting the beneficial effects of mTORC1 (Canaud et al., 2010). Recently Janssen announced the acquisition of Anakuria Therapeutics and its first-in-class ADPKD candidate, AT-20494, a small molecule inhibitor of mTORC1 (https://www.fdanews.com/articles/206455-janssen-acquires-anakuria-therapeutics-nets-early-phase-polycystic-kidney-disease-candidate).

AMP-activated protein kinase (AMPK)

AMPK is activated under conditions of metabolic and other cellular stresses (Steinberg and Hardie, 2022). AMPK activation during low energy states leads to upregulation of energy generating processes and inhibition of energy-intensive processes involved in

cyst expansion such as indirect inactivation of mTORC1 (Inoki et al., 2003; Gwinn et al., 2008) and inhibition of CFTR chloride channel, thus suppressing epithelial fluid and electrolyte secretion (Caplan, 2022).

Metformin

Metformin, a drug approved for T2D and polycystic ovary syndrome, may serve as a therapy for ADPKD. Treatment of kidney epithelial cells leads to stimulation of AMPK and subsequent inhibition of both mTOR and CFTR (Takiar et al., 2011). However, mixed results in animal models of PKD (Takiar et al., 2011; Leonhard et al., 2019; Lian et al., 2019; Chang et al., 2022; Pastor-Soler et al., 2022) and increased plasma lactate levels observed in Pkd1 miRNA transgenic mice, call for a careful examination of the risk benefit of metformin especially in patients with later stage of ADPKD (Chang et al., 2022). The Trial of administration of Metformin in PKD (TAME PKD, NCT02656017; Seliger et al., 2020) in 97 non-diabetic ADPKD adults with eGFR>50 ml/min per 1.73 m^2 (Ong and Gansevoort, 2021; Perrone et al., 2021) suggests that metformin is safe in patients in the early stages of ADPKD (although only 35% completed the study at the maximal dose resulting in dose reductions). Results of the exploratory secondary endpoints were, however, inconclusive, with non-significant trends for eGFR slope and htTKV. A definitive answer should come from the IMPEDE PKD trial where a slow-release formulation of 2000 mg/d Metformin will be tested in 1164 patients with rapid progressive ADPKD over 2 years with estimated completion in 2026 (NCT04939935).

Direct AMPK activation

While metformin inhibits renal cyst growth in mouse models, it remains unclear whether its metabolic effects are related to its capacity to activate AMPK (Takiar et al., 2011; Chang A. R. et al., 2017), and it may have tolerability issues. Hence potent selective AMPK activation may be required in ADPKD. Recently, the AMPK activator PF-06409577 demonstrated inhibition of mTOR pathway-mediated proliferation of cyst-lining epithelial cells and reduced CFTR-regulated cystic fluid secretion (Su et al., 2022). Given the potential for cardiac hypertrophy of AMPK following chronic administration it will be important to define the precise isoform selectivity required (Myers et al., 2017). Recently the FDA granted Orphan Drug Designation to Poxel's AMPK activator PXL770 for the treatment of patients with ADPKD (https://www.poxelpharma.com/en_us/news-media/press-releases/detail/224/poxel-announces-pxl770-granted-orphan-drug-designation-from).

Cholesterol reducing agents

Statins

Statins (HMG-CoA reductase inhibitors) are widely prescribed to lower cholesterol in humans (Ginsberg and Tuck, 2001). Among the additional effects that make statins attractive for use in ADPKD (Belibi and Edelstein, 2010) is activation of AMPK (Sun et al., 2006) and cAMP lowering (Kou et al., 2012). Statins have been shown to improve early-onset ADPKD (TKV improvement) in children and young adults (Cadnapaphornchai et al., 2014). Recently, Baliga conducted targeted metabolomics in plasma samples from a phase III trial designed to test the efficacy of pravastatin on ADPKD progression in children and young adults on the ACE inhibitor (Baliga et al., 2021). The authors demonstrated changes in metabolites involved in metabolic reprogramming however statin treatment for 36 months had limited effect on disease progression (Baliga et al., 2021). While these results are overall encouraging, a larger randomized trial in young people with ADPKD is required. In the absence of such data, no consensus was reached on the use of statins in this population (Gimpel et al., 2019). An ongoing trial evaluating 2 years treatment with pravastatin in 150 adults with early stage ADPKD (NCT03273413) should also clarify the s inconclusive results in adult ADPKD (van Dijk et al., 2001; Fassett et al., 2010; Brosnahan et al., 2017; Xue et al., 2020).

Bempedoic acid

Bempedoic acid (BA) antagonizes the ATP citrate-lyase (ACLY) enzyme upstream of HMGCoA reductase and is approved as an adjunct to diet and statin therapy in familial hypercholesterolemic patients who require additional lowering of LDL-C (Huynh, 2019; Ruscica et al., 2022). In animal models BA also activates AMPK (Pinkosky et al., 2016; Hallows et al., 2022) and reduces cystic growth, TKW and BUN (Hallows et al., 2022). BA seems to have a reduced risk of muscle-related side effects reported with statins (Ruscica et al., 2022) although a recent meta-analysis concluded that statins cause a small risk of muscle symptoms that are outweighed by the known cardiovascular benefits of statins (Cholesterol Treatment Trialists' Collaboration, 2022). No major safety concerns were identified for BA in a randomized controlled phase III trial during the intervention period when added to statin therapy, but the incidence of AEs leading to discontinuation was higher in the BA group, as was the incidence of gout (Ray et al., 2019). Bempedoic acid was generally well-tolerated following a single oral dose in subjects with renal impairment (Amore et al., 2022). Because ACLY has been reported to inhibit the AMPK-β1 subunit (Lee et al., 2015), future studies should conclusively demonstrate that the beneficial effects of BA in ADPKD are mediated by AMPK activation and clarify the AMPK subunit involved.

Weight loss and insulin resistance

Similar to the general population, the prevalence of overweight and obese ADPKD patients is increasing. In rodent models of ADPKD, caloric restriction has shown to slow kidney growth and improve kidney function (Kipp et al., 2016; Warner et al., 2016). It has been suggested that these improvements involve mTOR signaling inhibition, AMPK activation, and a reduction in IGF-I supporting restoration of metabolic reprogramming. Accordingly, a clinical trial evaluating the effect of weekly caloric reduction achieved with either caloric restriction or intermittent fasting in

29 overweight/obese individuals with ADPKD was recently completed (Hopp et al., 2021; https://clinicaltrials.gov/ct2/show/NCT03342742). The trial was designed as a weight loss intervention based on the prior epidemiological observation that ADPKD progression is faster with higher BMI (Nowak et al., 2018). The investigators demonstrated the feasibility of 1-year daily caloric restriction (DCR) and intermittent fasting (IMF) in a cohort of overweight or obese patients with ADPKD. Weight loss occurred with both DCR and IMF, however, weight loss was greater, and adherence and tolerability were better with caloric restriction (Hopp et al., 2021). The study was a pilot and feasibility study, so the sample size was small, and a control group was not included but, according to the investigators, similar annual kidney growth in both groups was observed that was qualitatively low compared to historical controls. Cessation of kidney growth was observed in participants who achieved clinically meaningful weight loss (Nowak et al., 2018; Nowak et al., 2021). A larger 2-year phase 2 trial with a direct comparison of caloric restriction to a control group, powered for a primary endpoint of change in htTKV (NCT04907799) is currently recruiting with an expected completion in 2026 (https://clinicaltrials.gov/ct2/show/NCT04907799).

Ketogenic diet

Ketosis improves the phenotype of animal models of ADPKD (Torres et al., 2019b). From a mechanism of action perspective, it is possible that Ketone bodies may promote metabolic reprogramming by decreasing glucose availability and increasing fatty acids (Hall et al., 2016). Initial data from clinical trials are becoming available. A self-enrolled survey of ADPKD patients who have self-administered ketogenic diet for at least 6 months reported weight loss and blood pressure lowering together with improvement in PKD symptoms and eGFR in a subgroup of patients. Caution should be applied to the interpretation of this retrospective study since only half of the patients were able to comply with the diet and the side effects reported suggest potential long-term tolerability and safety issues (kidney stones, increased cholesterol) (Strubl et al., 2021). In a follow-up study, Oehm demonstrated the feasibility of a short-term ketogenic intervention in 10 ADPKD patients (RESET-PKD 72h fast or 14 days of a KD) where TLV was decreased while no changes in TKV were observed (Oehm et al., 2022a; Oehm et al., 2022b). Despite the challenges identified, large-scale trials such as the ongoing KETO-ADPKD (NCT04680780) study will address the feasibility and the therapeutic potential of longer-term ketogenesis interventions in ADPKD (Ong and Torra, 2022). Adherence (ketone concentrations), feasibility and secondary outcomes including TKV, and BMI will be evaluated. Based on these findings, Bruen and collaborators have designed a plant-focused ketogenic diet (Ren.Nu diet) for ADPKD based on the theory that a diet high in carbohydrate and animal protein might accelerate disease progression (Bruen et al., 2022). A preliminary beta test was conducted for 12 weeks in 24 ADPKD patients and with the obvious limitations of the study (no control, selection bias, self-reporting). Preliminary data suggest reasonable adherence and feasibility (Bruen et al., 2022).

Bariatric surgery

Bariatric surgery is an effective option to achieve sustained weight loss and improving hypertension and diabetes. Gastric bypass and sleeve gastrectomy result in 20%–30% weight loss (Brajcich and Hungness, 2020) and is expected to impact metabolic reprogramming. Therefore, it is important to understand the benefits and risks of bariatric surgery in ADPKD patients. While evidence suggests important trends for bariatric surgery and overall kidney related outcomes in patients with CKD, there exist several renal risks, including acute kidney injury, and risks of nephrolithiasis, oxalate nephropathy that will need to be considered in a comprehensive risk benefit assessment profile in ADPKD patients (Chang M. Y. et al., 2017).

Glucagon-Like Peptide-1 (GLP-1) receptor agonism

Several GLP-1 receptor agonists have been approved for the treatment of T2D and obesity and are being considered for liver and kidney complications (Müller et al., 2019; Brown et al., 2021; Newsome et al., 2021). One attractive feature of new generation GLP-1 analogues is the propound weight loss (>10%) achieved in obese and diabetic patients (Frías et al., 2021). Importantly, GLP-1 exerts its effects by binding to GLP-1R and activating adenylate cyclase, which leads to the generation of cAMP, so it will be important to assess the expression of GLP-1R on the cystic epithelium and the potential impact of GLP-1 agonism on cAMP (Körner et al., 2007; Müller et al., 2019).

GLP-1R/glucagon receptors dual agonism

Dual agonism at the GLP-1 and glucagon receptors has shown superior weight lowering effect to selective GLP-1 agonism (Pocai et al., 2009; Day et al., 2012). Because glucagon lowers mTORC1 and stimulates AMPK (Baum et al., 2009; Welles et al., 2020) and ketogenesis (Pocai, 2012; Torres et al., 2019a), the simultaneous agonism of GLP-1R and glucagon receptors constitute a potential approach for ADPKD. A recent observational study in ADPKD patients with higher endogenous glucagon did not provide evidence for a protective role of glucagon in ADPKD (Knol et al., 2021). Mechanistic studies are needed to determine the relationship between glucagon and ADPKD and evaluate the expression of the receptor in kidney cysts. Future studies will clarify if the body weight lowering effect together with the other reported desirable actions of GLP-1 agonists have potential in ADPKD.

Thiazolidinediones (TZD)

TZD are Peroxisome Proliferator Activator Receptor gamma (PPARγ) agonists approved for T2D. Preclinical studies have tested TZD and found reduced progression of cystic disease (Blazer-Yost et al., 2010; Flaig et al., 2016). A small phase 1b clinical trial was designed to investigate safety and tolerability of low-dose (15 mg) pioglitazone (Blazer-Yost et al., 2021). Concerns about fluid retention, bone loss and weight gain have reduced their use in

the clinic and need to be considered in an appropriate risk/benefit assessment in ADPKD patients (Lebovitz, 2019).

Sodium–glucose cotransporter (SGLT2) inhibition

SGLT2 inhibitors (SGLT2i) prevent the reabsorption of filtered glucose from the tubular lumen resulting in glucose lowering and additional benefits of weight loss and blood pressure reduction (DeFronzo et al., 2021). While the mechanisms contributing to these beneficial effects are unknown, SGLT2i switch metabolism to a ketotic state and increase plasma glucagon potentially regulating PKA and mTOR pathways (Saponaro et al., 2018). ADPKD patients are more prone to urinary tract infections and were excluded from renal studies as genitourinary infection is a potential risk of SGLT2i (Afsar et al., 2022). In two PKD animal models, SGLT2 inhibition did not reduce cyst growth (Kapoor et al., 2015; Rodriguez et al., 2015). However, because of the beneficial effects of SGLT2 inhibition on kidney function, vascular function, and mortality (DeFronzo et al., 2021), a clinical trial is ongoing in ADPKD patients (NCT05510115).

Discussion

ADPKD is the leading genetic cause of ESKD. Recent advances in understanding the mechanisms leading to cyst formation and progression has led to the approval of tolvaptan (Torres et al., 2017). Treatment of ADPKD still represents a challenge due to the poor tolerability and the unfavorable safety profile of Tolvaptan. Thanks to new scientific discovery and preclinical models, new targets are being investigated. Metabolic defect in ADPKD support cell proliferation of rapid growing tissues leads to cystic epithelial proliferation and growth. Upregulated mTOR and c-Myc play a major role in repressing oxidative metabolism and FAO while enhancing glycolytic flux, lactic acid production, PPP and glutaminolysis downstream of cAMP/PKA (Rowe et al., 2013; Podrini et al., 2020). It will be important to expand upon the role of dysregulated metabolism as metabolic defects in cells within the kidney microenvironment may also contribute to ADPKD progression. A better understanding of the human pathways regulated by approved therapies (Tolvaptan and Octreotide-LAR) and initial results from ongoing trials with metabolic drugs should provide valuable human data and help expedite the development of new ADPKD therapeutics. Recently, the trial in ADPKD with Venglustat (Glucosylceramide synthase inhibitor; Natoli et al., 2010) was discontinued as it did not reduce TKV growth rate (https://www.sanofi.com/en/media-room/press-releases/2021/2021-06-01-05-00-00-2239122). Trial testing metformin in ADPKD should be completed by 2026 and provide valuable information (Testa and Magistroni, 2020). Weight loss targeting metabolic alterations has the potential to be a disease modifying intervention in ADPKD, but behavioral dietary interventions are limited by long-term adherence (Quiroga and Torra, 2022) so it will be important to explore the potential of bariatric surgery and pharmacological approaches. Therefore, it is tempting to speculate that interventions targeting downstream events such as metabolic reprogramming may retain and improve on the tolerability and efficacy reported with Tolvaptan offering potential new therapeutic opportunities. Ongoing trials may help in answering some of these questions.

Author contributions

IB: writing original draft. AP: writing original draft, reviewing and editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Autosomal dominant and autosomal recessive polycystic kidney disease: hypertension and secondary cardiovascular effect in children

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Autosomal dominant (ADPKD) and autosomal recessive (ARPKD) polycystic kidney disease are the most widely known cystic kidney diseases. They are significantly different from each other in terms of genetics and clinical manifestations. Hypertension is one of the main symptoms in both diseases, but the age of onset and secondary cardiovascular complications are significantly different. Most ARPKD children are hypertensive in the first year of life and need high doses of hypertensive drugs. ADPKD patients with a very early onset of the disease (VEO_{ADPKD}) develop hypertension similarly to patients with ARPKD. Conversely, a significantly lower percentage of patients with classic forms of ADPKD develops hypertension during childhood, although probably more than originally thought. Data published in the past decades show that about 20%–30% of ADPKD children are hypertensive. Development of hypertension before 35 years of age is a known risk factor for more severe disease in adulthood. The consequences of hypertension on cardiac geometry and function are not well documented in ARPKD due to the rarity of the disease, the difficulties in collecting homogeneous data, and differences in the type of parameters evaluated in different studies. Overall, left ventricular hypertrophy (LVH) has been reported in 20%–30% of patients and does not always correlate with hypertension. Conversely, cardiac geometry and cardiac function are preserved in the vast majority of hypertensive ADPKD children, even in patients with faster decline of kidney function. This is probably related to delayed onset of hypertension in ADPKD, compared to ARPKD. Systematic screening of hypertension and monitoring secondary cardiovascular damage during childhood allows initiating and adapting antihypertensive treatment early in the course of the disease, and may limit disease burden later in adulthood.

KEYWORDS

ARPKD, ADPKD, hypertension, cardiovascular disease, children

1 Background

Autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD) are the most well-known cystic kidney diseases. They belong to the group of ciliopathies, but are significantly different from each other in terms of genetics and clinical manifestations. In a not too distant past, they were named

"adult" and "infantile" polycystic kidney diseases, respectively. Nowadays, these terms have been abandoned because they do not describe accurately the natural history of the diseases. Most ARPKD children are hypertensive in the first year of life and need very early high doses of antihypertensive drugs. A significantly lower percentage of ADPKD patients develop hypertension during childhood, but this percentage is probably underestimated. The PROPKD score, which has been validated based on outcome measures, indicates that early onset hypertension (before the age of 35 years) is a risk factor for fast progression of chronic kidney disease (CKD). The purpose of this review is not to compare ARPKD with ADPKD, but to review of the available pediatric studies (Table 1 and Table 2) and experience-based observations on cardiovascular aspects of both conditions.

2 Autosomal dominant polycystic disease (ADPKD)

2.1 Introduction

ADPKD is the most common genetic kidney disease in adulthood, characterized by multiple and bilateral kidney cysts. Progressive enlargement of cysts starts early in life, causing kidney volume enlargement, leading to progressive decline of kidney function and ultimately end stage kidney disease (ESKD), usually around 50–60 years of age, depending on the underlying genetic defect. The disease is caused by pathogenic variants in the *PKD1* gene, located on chromosome 16p13.3 and in the *PKD2* gene, located on chromosome 4q21, encoding for polycystin-1 (PC1) and polycystin-2 (PC2), respectively. *PKD1* is responsible for the more severe phenotype and is involved in approximately 85% of cases. Most cases managed by pediatrician are secondary to *PKD1* variants or to rare digenic conditions, since these manifest earlier in life compared to *PKD2* variants. Cyst formation requires mutations of both alleles in either *PKD1* or *PKD2* genes. Since every cell carries one germ line mutation (first hit), a second somatic mutation in the normal allele (second hit) is needed to lead to the formation of cysts from previously normal epithelial cells of renal tubules and biliary ducts. The phenotypic expression of the disease is variable. Several factors explain this variability. In particular, the phenotypic expression depends on the type of variant, ranging from hypomorphic to variants that cause complete loss of function, which impacts on the amount of residual functioning polycistin proteins. The lower the levels, the faster the cyst formation and growth (Fedeles et al., 2014).

ADPKD is a systemic disorder with many clinical manifestations, including hypertension, left ventricular hypertrophy (LVH), heart valve disease, hepatic cysts, urinary tract infections, proteinuria, hematuria, kidney stones, intracranial and extracranial aneurisms (Perrone et al., 2001; Ecder, 2013; Chapman et al., 2015; Gimpel et al., 2019). Cardiovascular manifestations are the main extra-renal complications of ADPKD. With improvements in renal replacement therapy and kidney transplantation over the past decades, cardiovascular complications have become the most common cause of morbidity and mortality in adult patients with ADPKD (Fick et al., 1995; Helal et al., 2012).

In the past two decades, considerable progresses have taken place in our knowledge of the pathogenesis and clinical manifestation of ADPKD in children. As pediatricians, we now know that ADPKD is not only an adult disease and that some renal and extra-renal manifestations may appear during childhood, albeit sometimes they remain underdiagnosed (Fick et al., 1994; Fick-Brosnahan et al., 2001; Cadnapaphornchai, 2013).

Hyperfiltration and hypertrophy of unaffected nephrons may mask renal impairment, preserving normal glomerular filtration for many years (De Rechter et al., 2017). Approximately 60% of ADPKD patients have kidney cysts by the age of 5 years (Gabow et al., 1997). A subset of patients, termed Early Onset ADPKD (EO_{ADPKD}), suffer from a severe form of the disease that manifest with significant symptoms between 1.5 and 15 years of age. These patients have early hypertension and rapid progression of CKD (MacDermot et al., 1998; Shamshirsaz et al., 2005). Another very small group of patients, named Very Early Onset ADPKD (VEO_{ADPKD}), develop oligohydramnios and hyperechoic enlarged kidney *in utero* (MacDermot et al., 1998). In these patients, two hypomorphic biallelic variants in the *PKD1* gene are often identified resulting in a very severe phenotype and their clinical picture mimic that of ARPKD (Bergmann, 2019).

2.2 Pathogenesis and prevalence of hypertension

Arterial hypertension is the most frequent initial manifestation in ADPKD and is observed in 50%–75% of adults (Chapman et al., 2010a). Since most patients become symptomatic in adulthood the vast majority of the literature on this subject has been produced in adult patients. Altogether, studies have shown that hypertension usually develops around 30–35 years of age (i.e., earlier than essential hypertension) and precedes almost always the onset of CKD. These studies have also shown that hypertension may accelerate progression towards ESKD (Ecder and Schrier, 2001; Chapman et al., 2010a) and promotes the development of LVH (Gabow et al., 1992; Fick et al., 1995).

The pathogenesis of hypertension in ADPKD is multifactorial, involving the renin-angiotensin-aldosterone system (RAAS), the sympathetic nervous system, and endothelial dysfunction (De Rechter et al., 2017; Krishnappa et al., 2017).

The activation of the RAAS system plays a fundamental role. Progressive increase in the size of cysts causes compression of renal arterioles, leading to localized ischemia and hypoxia, which, in turn, activates the RAAS system (Chapman et al., 1990; Loghman-Adham et al., 2004). RAAS activation may then stimulate the sympathetic nervous system, increasing plasma catecholamines to higher levels in comparison to those observed in patients with essential hypertension (Schrier, 2009).

In addition, studies on cells derived from renal cysts, renal mesangium, and from the muscular layer of renal arteries have shown increased synthesis of endothelin 1 and over-expression of the Endothelin Receptor Subtype A in ADPKD, contributing to the development of arterial hypertension and gradual loss of kidney function (Hocher et al., 1998; Krishnappa et al., 2017). Sodium retention and reduced nitric oxide synthesis also appears to play a role. Low or absent levels of *PKD1* and *PKD2* gene expression have been associated with reduced nitric oxide synthesis, which results in impaired vascular response to stress and activation of the RAAS system (De Rechter et al., 2017; Krishnappa et al., 2017).

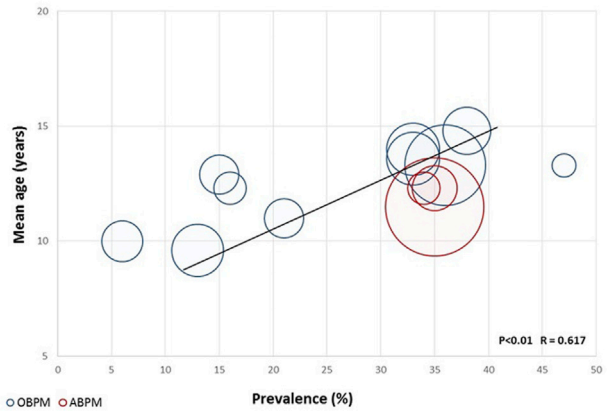

FIGURE 1
Prevalence of hypertension in ADPKD children according to age and measuring method.

Arguably, many adult nephrologists suggest patients to test their children for possible ADPKD only after the age of 18 years, since the disease usually becomes symptomatic during adulthood. Current guidelines on the subject are limited and this matter remains controversial. The experience gained in other renal conditions indicates that delayed treatment of hypertension, including during childhood, can increase significantly cardiovascular morbidity (Gimpel et al., 2019).

As expected, the prevalence of hypertension in ADPKD increases with age in all studies, reaching >90% after the age of 50 years. Pediatric nephrology units that treat large number of children with ADPKD have observed a significant prevalence of hypertension in their patients (Figure 1).

Some early prevalence data were reported in 2010 by Mekhali et al., who observed a prevalence of hypertension of 15% in 47 patients (Mekahli et al., 2010). In the following years, other studies have reported prevalence data ranging 6%–44%, which are significantly higher than data reported in the general pediatric population (3%–5%) (Fick et al., 1994; Seeman et al., 2003; Kelleher et al., 2004; Cadnapaphornchai et al., 2008; Mekahli et al., 2010; Selistre et al., 2012; Cadnapaphornchai, 2013).

In 2016, Marlais performed a systematic review and a meta-analysis to better define the prevalence of hypertension in children and young adults with ADPKD. The analysis of 14 studies included 928 patients and revealed a mean prevalence of hypertension of 20%, even after removing the studies with a high risk of selection biases. A positive significant correlation between the mean age of the cohort and the prevalence of hypertension was observed across eleven of the fourteen selected studies. This meta-regression analysis confirms studies in adults, showing that onset of hypertension starts in a majority of patients during the third decade of life and increases rapidly thereafter. The authors also analyzed the prevalence of proteinuria in eight of the analyzed studies and report a prevalence of proteinuria in 20% of patients, although usually not severe (Marlais et al., 2016). No correlation was observed between the prevalence of proteinuria and the prevalence of hypertension. This meta-analysis had limitations due to the possible selection of more severe cases in tertiary centers that published their findings and to the fact that hypertension was not the primary outcome in any of the selected studies. In addition, cohorts were heterogeneous in terms of size, age, method for diagnosing hypertension (Marlais et al., 2016). One of the first study that used ambulatory blood pressure monitoring (ABPM) was published in 1997 and suggested that the prevalence of hypertension may have been be underestimated (Seeman et al., 1997). In most cases, hypertension was diagnosed based on office blood pressure measurements (OBPM) or home blood pressure measurements (HBPM), which may miss borderline forms of hypertension and do not allow diagnosing isolated nocturnal hypertension. ABPM represents the gold standard for detecting hypertension in children and in adults (Lurbe et al., 2016; Flynn et al., 2017). It is more accurate than office blood pressure in diagnosing hypertensive or pre-hypertensive patients (Stergiou et al., 2005; de Almeida et al., 2007) and allow detecting night-time hypertension or lack of nocturnal dipping (de Almeida et al., 2007; Brady et al., 2008). These latter two conditions may represent risk factors for the development of secondary organ damage and have been described in ADPKD patients. In addition, ABPM data correlate better with LVH than OBPM and HBPM (Valero et al., 1999; Brady et al., 2008). Only few studies have assessed hypertension in ADPKD children using ABPM (Zeier et al., 1993; Seeman et al., 1997; Seeman et al., 2003; Massella et al., 2018; Marlais et al., 2019).

In 2003, Seeman reported a prevalence of hypertension of 35% in 62 children evaluated by ABPM. In almost 30% of them, hypertension was nocturnal. Two-thirds of children with normal blood pressure by OBPM had hypertension by ABPM. A positive correlation between ABPM values, kidney volume, and the number of cysts was observed (Seeman et al., 2003).

In 2018, Massella published data from a European multicenter retrospective study on 310 ADPKD patients under the age of 18 with normal kidney function. The study showed that 21% of patients were hypertensive throughout the entire 24 h cycle. This rate increased to 35% when considering patients who were not found hypertensive by ABPM but were receiving treatment for hypertension. Nearly 18% of patients had isolated nocturnal hypertension and nearly half of patients lacked significant dipping at night. Logistic regression analysis showed a significant positive correlation between the number of cysts and daytime, night-time, and 24 h blood pressure values. Kidney length was significantly associated with night-time and isolated nocturnal hypertension (Massella et al., 2018).

Recently, Seeman et al. published a longitudinal study conducted on 69 ADPKD patients and 40 healthy subjects with an average follow-up of 6.3 years. Their data show an increase in the prevalence of hypertensive patients from 20% at the beginning of the study to 38% at the end of the study. During the observation period, there was no significant decline in kidney function or increase in proteinuria, but a significant increase in kidney size and in the number of renal cysts (Seeman et al., 2021) (Summary of the literature in Table 1).

2.3 Cardiovascular consequences of hypertension

The heart is the main target organ of hypertension in terms of secondary damage.

From the adult literature, we have learned that arterial hypertension can causes LVH and arteriosclerosis with an

TABLE 1 Published literature on hypertension in ADPKD children.

	Patient	Age (mean ± SDS)	OBPM HTN (%)	ABPM HTN (%)	Non-dipper (%)
Ivy et al. (1995)	83	9.6 ± 0,5	13	-	-
Seeman et al. (1997)	32	12.3 ± 4.7	16	34	12.5
Seeman et al. (2003)	62	12.3 ± 4.3	-	35	-
Shamshirsaz et al. (2005)	199	13.3	36	-	-
Stergiou et al. (2005)	17	13.3 ± 2.9	47	-	-
Cadnapaporchai et al. (2008)	85	13.6 ± 0.8	33	-	-
Cadnapaporchai et al. (2009)	85	14 ± 1	33	-	-
Mekahili et al. (2010)	47	12.9 ± 5.1	15	-	-
Selistre et al. (2012)	52	10 ± 4	6	-	-
Massella et al. (2018)	295	11.5 ± 4.1	-	35	52
Marlais et al. (2019)	47	11	21		35
Seeman et al. (2021)	69	14.8 ± 4.7	38	-	-

Abbreviations: OBPM, Office Blood Pressure Measurement; ABPM, ambulatory blood pressure measurement; HNT, hypertension. Hypertension: defined as blood pressure >95th centile for age, height and gender or if in anti- HTN treatment.

increase in cardiovascular mortality. Hypertension is not the only factor that induces LVH. Other factors, such as anemia, increased body mass index, excessive sodium intake, and increased activity of the RAAS system may also be involved (Gabow et al., 1992; Fick et al., 1995). Studies on adult patients with ADPKD have evaluated LVH by ultrasound or magnetic resonance imaging. Despite limitations due to the use of different instrumental techniques and different formulas for calculating the left ventricular mass index (LVMi), all these studies are in agreement in showing that the LVMi correlates with blood pressure values, kidney function, and total kidney volume (TKV) (Chapman et al., 1997; Ecder, 2013; Kuo and Chapman, 2020).

Several adult studies have also shown that intensive blood pressure control can reduce LVH and cardiovascular morbidity (Schrier et al., 2002; Chapman et al., 2010b; Schrier et al., 2014; Torres et al., 2014). In particular, the HALT-A study has shown that intensive blood pressure control (target 95–110/60–75 mmHg) in patients with ADPKD and relatively preserved kidney function (estimated Glomerular Filtration Rate (eGFR) > 60 ml/min), resulted in a significant reduction in LVMi, albuminuria, and TKV increase, compared to standard blood pressure control (target 120–130/70–80 mmHg) (Chapman et al., 2010; Schrier et al., 2014). Similar conclusions were also reached in the HALT-B study that enrolled patients with more advanced kidney failure (eGFR 25–60 ml/min) (Torres et al., 2014). No significant differences were observed when comparing the efficacy of angiotensin converting enzyme inhibitor (ACE-i) monotherapy with a scale-up combination therapy with ACE-i and angiotensin II receptor blockers (ARBs), suggesting that benefits were related to achievement of a lower target blood pressure, rather than using a higher dose of blood pressure medications.

In 1993, Zeier et al. published one of the first pediatric studies on this subject, including 24 young ADPKD patients, 12 children (age 5.7–13.3 years) and 12 adolescents and adults (age 15.2–24.9 years), and 24 controls matched for age, sex and body surface area. All patients had normal kidney function. The authors studied left ventricular mass (LVM) by real-time directed M-mode echocardiography and observed a significantly higher LVMi across all ages without overt LVH. Blood pressure, measured by ABPM, was normal in children but was significantly higher in young adults, compared to controls. Left ventricular systolic function was normal in the two groups (Zeier et al., 1993).

In 1995, Ivy compared 83 ADPKD children with 66 unaffected siblings from 66 ADPKD families. A significantly higher prevalence of mitral valve prolapse (MPV) was found in affected patients compared to unaffected controls (12% vs. 3%). Patients with MPV were older and had more severe renal disease (>10 cysts). In addition, a significantly higher prevalence of hypertension was observed in affected children. In respect to LVMi, the authors observed a non-statistically significant trend towards higher values in ADPKD children (p = 0.07). When the LVMi was compared with systolic and diastolic blood pressure values in affected and unaffected children, a significant positive relationship between systolic blood pressure and LVMi (R = 0.43, $p < 0.0001$) was observed in ADPKD children. Hypertensive patients had significantly higher LVMi compared to normotensive patients. The latter group has LVMi values similar to normotensive controls (Ivy et al., 1995).

In 2008, Cadnapaphornchai assessed the impact of blood pressure in the clinical outcomes of 85 ADPKD children and young adults. Patients were divided into three groups according to their blood pressure status: hypertension (HBP, systolic and diastolic pressure higher than 95th percentile), borderline hypertension (BBP; 75–95th percentile), normotensive patents (NBP; less than 75th centile). The authors compared LVMi, TKV, microalbuminuria, and kidney function in these three groups. No significant differences were observed in kidney function and microalbuminuria. Compared to patients with normal blood pressure, both hypertensive and borderline

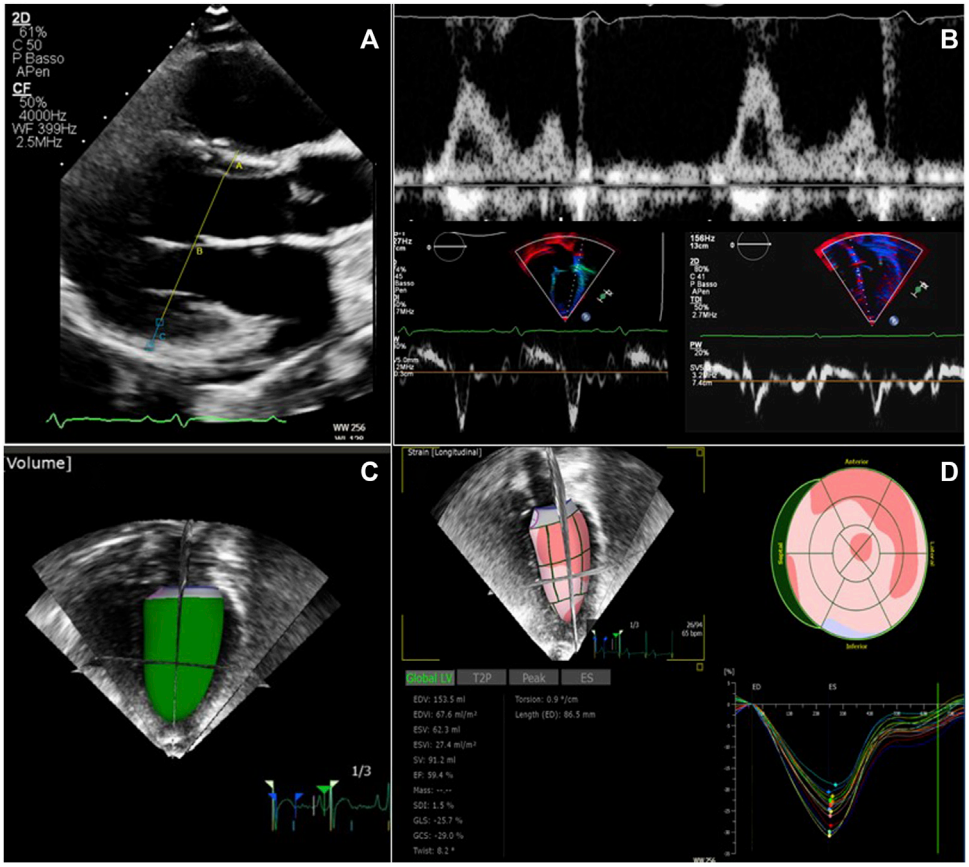

FIGURE 2
Echocardiographic examination in patients with ADPKD and ARPKD. **(A)** Left ventricular mass calculation from two-dimensional parasternal long axis view in end-diastole. **(B)** Diastolic function evaluation merging data from transmitral inflow velocities (upper image) and tissue Doppler velocities (lower images) from both the lateral and the septal left ventricular wall. **(C)** Real time three-dimensional left ventricular volume estimate **(D)** Global longitudinal strain analysis from speckle tracking imaging, using real time three dimensional acquisition.

hypertensive patients had higher LVMi. Of note, overt LVH was not present in any subgroup, but a statistically significant increase in LVMi was detected in patients with borderline pressure with still normal TKV (Cadnapaphornchai et al., 2008).

Data from the same cohort were subsequently published after 5 years of follow-up, during which patients received treatment with ACE-i. The RAAS blockade appeared to stabilize LVMi and prevent deterioration of kidney function in normotensive or borderline hypertensive ADPKD children, but not in patients that were hypertensive. The authors hypothesized that once hypertension was established, treatment with ACE-i alone may no longer be sufficient to prevent cardiovascular disease (Cadnapaphornchai et al., 2009).

Seeman et al. also observed higher average LVMi in children with ADPKD, compared to healthy controls [mean 30.4 ± 6.6 g/m2.7 vs. 26.2 ± 6.2 g/m2.7, $p = 0.01$], but no overt LVH (Seeman et al., 2021).

The above-cited studies have only analyzed the left ventricular geometry. Yet, LVM *per se* is not a measure of heart function and increased ventricular mass is not always associated with heart failure, although it is a contributing factor (Fick et al., 1995; Chapman et al., 1997; Ecder, 2013; Chebib et al., 2017; Chen et al., 2019; Kuo and Chapman, 2020). Very few data on cardiac function are available in ADPKD children.

In 1998, Bardaji et al. studied the ventricular function by transmitral pulsed Doppler flow and observed in addition to higher LVMi, evidence of early diastolic dysfunction (Bardaji et al., 1998).

Some illustrative images of advanced echocardiography are shown in Figure 2.

Another aspect of cardiovascular disease in ADPKD patients concerns arterial dysfunction. Endothelium dependent dilation (EDD), stiffening of the large elastic arteries, such as the aorta and carotid arteries, and increased carotid intima-media thickness (cIMT) are important independent predictors of cardiovascular events. In adult subjects with ADPKD, these parameters are abnormal, independently from the progression of kidney disease and before the onset of hypertension (Borresen et al., 2007).

Some studies have demonstrated that vascular dysfunction begins very early in the course of the disease, including during childhood. In 2017, Nowak et al., studied vascular dysfunction in 15 ADPKD children and young adults (age range 6–22 years) with normal kidney function, and in 15 controls. All subjects had normal blood pressure (<140/90 mmHg if adults or <95th percentile), but 8/15 (46%) patients with ADPKD were treated with ACE-is. The authors observed a significant decrease in the EDD in ADPKD subjects, as measured by brachial artery flow mediated dilation. The

carotid-femoral pulse wave velocity (PWVcf), a measurement of arterial stiffness, was on average 14% higher in ADPKD children and young adults. The carotid augmentation index (cAIx) and carotid systolic blood pressure were also higher compared to the control group, while cIMT was similar (Nowak et al., 2017). In 2018, Karava et al. analyzed 21 ADPKD subjects aged 6–19 years and observed increased LVMi in 2 patients (9.5%), PWVcf in 4 patients (19%), and increased cIMT in 8 patients (38.1%). Anti-hypertensive therapy was not associated with higher PWVcf or cIMT (Karava et al., 2018).

Studies in young adults with ADPKD have also shown early vascular dysfunction independently from blood pressure values. Borresen et al. reported in 2007 that the reflection of the pulse wave was amplified in young normotensive ADPKD patients, demonstrating early arterial damage (Borresen et al., 2007). In 2009, Azurmendi et al. reported increased cIMT in patients with albumin/creatinine ratio >6.8 mg/g, regardless of the hypertension status. A linear correlation was observed between blood pressure levels and LVMi, but not with cIMT or PWVcf values (Azurmendi et al., 2009).

Marlais et al. compared in 2019 a large population of ADPKD children under the age of 18 with age-matched healthy controls. No significant differences in PWVcf were observed. This data differs from previous reports and differences are probably related to the younger age of patients in their cohort, suggesting that vascular changes develop only during late childhood and early adulthood. On average, children with ADPKD had higher blood pressure and LVMi, although overt LVH was not observed; 35% of patients lack nocturnal dipping (Marlais et al., 2019).

More recently, Seeman et al. studied the vascular function in ADPKD children and observed higher pulse pressure amplification in ADPKD subjects compared to controls, but no significant differences in the PWVcf (Seeman et al., 2021).

2.4 Final remarks

Taken together, current data indicate that the prevalence of hypertension in ADPKD children ranges 6%–47%. When considering only studies that have used ABPM to assess blood pressure, hypertension was observed in approximately one-third of patients. The main mechanism driving hypertension is the activation of the RAAS system, but other factors contribute to the elevation of blood pressure, including over-expression of endothelin receptors, increased synthesis of endothelin 1, sodium retention, and reduced synthesis of nitric oxide in the vascular endothelium. Most hypertensive children with ADPKD do not develop overt LVH, although their LVMi is on average higher compared to normotensive patients and to control subjects. Data on vascular dysfunction are scanty and findings are not uniform. They raise however, intriguing hypotheses on the pathogenesis of vascular damage in ADPKD that deserve further studies. Of note, children may be the ideal subjects to perform these studies because they may show early signs of vascular dysfunction independently from the development of hypertension.

Several limitations apply when analyzing the literature on ADPKD in children in respect to the prevalence of arterial hypertension. The majority of studies are retrospective. The size and age of cohorts are often very different. Most studies were carried out in third-level centers that tend to recruit more severe patients and more motivated families. This may cause overestimating the prevalence of hypertension and cardiovascular consequences. In addition, the methodologies used to assess blood pressure and cardiovascular damage vary in different studies.

Nevertheless, the available data support regular measurement of blood pressure once a year in children with ADPKD or at risk of ADPKD, as suggested by expert opinion publications (Gimpel et al., 2019). Measuring blood pressure is minimally invasive and should be part of the routine pediatric check-up. These measurements can be willingly accepted by children of affected patients that have not yet undergone diagnostic procedures to rule out ADPKD. Although the vast majority of ADPKD children do not have LVH, one can hypothesize that early initiation with anti-hypertensive therapies may limits future development of cardiovascular organ damage. It is unclear if this will also modify the evolution of cystic disease. The European Pediatric Registry on Polycystic Dominant Kidney (ADPedKD Registry) may in the future answer this question. In other chronic kidney diseases, pediatric nephrologists have learned that early treatment of hypertension may slow down the progression of CKD and reduce cardiovascular complications. In this respect, pediatricians may be in a privileged position to impact significantly on the outcome of ADPKD patients by acting very early during the course of the disease.

3 Autosomal recessive polycystic kidney disease (ARPKD)

3.1 Introduction

Although ARPKD is a rare disease, it is the foremost cystic disease in early childhood. This ciliopathy is caused by biallelic variants of the *PKHD1* gene that encodes for the nephrocystin protein. In the kidneys, nephrocystin localizes to the sensory *cilium* in the cortical and medullary collecting ducts and thick ascending limbs of the loop of Henle. In addition, nephrocystin is expressed in the biliary and pancreatic tracts. Compared to ADPKD, recessive inheritance causes early onset and more severe symptoms, although significant phenotypic variability is observed. Severe perinatal disease has been associated with biallelic null variants, but biallelic missense variants do not exclude very early onset of a severe disease. A recent genotype-phenotype correlation study including 304 children with ARPKD has shown that the location of variants in different domains of the *PKHD1* gene plays an important role in determining the phenotype (Burgmaier et al., 2021).

The two organs involved in ARPKD are the kidney and the liver. The latter usually present with congenital hepatic fibrosis, which is sometimes associated with Caroli disease. Enlarged kidneys with non-obstructive fusiform dilations of collecting ducts are the main characteristics of renal involvement. Since the expression of the disease begins very early, most cases are identified *in utero* or immediately after birth. Kidney failure may develop prenatally causing oligohydramnios, which in turn impairs normal lung development. Pulmonary hypoplasia in newborns with severe antenatal ARPKD is the main cause of death in the first year of life (Roy et al., 1997; Guay-Woodford, 2014; Guay-Woodford et al., 2014). However, clinical expression is extremely variable in terms of severity and age of onset of symptoms, for both renal and hepatic phenotype.

TABLE 2 Published literature on hypertension in ARPKD children.

	Cole et al. (1987)	Kaplan et al. (1989)	Zerres et al. (1996)	Roy et al. (1997)	Rhona Capisonda et al. (2003)	Guay-Woodford and Desmond (2003)	Bergmann et al. (2005)	Dell et al. (2016)	Chinali et al. (2019)	Seeman et al. (2022)
Patients	17	55	115	52	31	166	186	22	27	36
Follow-up (y)	14	37	6	43	10	12	6	-	-	12
Age at diagnosis	1°y	42% < 1 m	51% < 1°m	85% ≤ 1°y	55% < 1°m	73% < 1°m	54% < 1°m	0,1°y (mean)	-	0,2°y (median)
		42% 1-12 m	23% 1-12°m	15% > 1°y	19% 1-12°m	11% 1-12°m	16% 1-12°m			
		16% > 1 y	26% > 1 y		26% > 1°y	16% > 1°y	30% > 1°y			
ESKD	29%	-	10%	33% by 15°y	16%	13%	29% by 10°y	-	21%	0%
HTN	100%	65%	71%	-	55%	65%	-	86%	74%	86% (ABPM)
Anti- HTN treatment	-	-	70%	39% by 1°y	55%	65%	76%	86%	63%	86%
				60% by 15°y						
Age start drug	-	-	180°d (mean)	-	50 % by 5°y	16°d (mean)	3°y (median)	-	-	-
					58% by 15°y					
Hyponatremia	-	54%	25%	-	10%	26%	-	-	-	-
Patient survival rate	1°y 88%	1 y 79%	1°y 89%	-	1°y 87%	1°y 79%	1°y 85%	-	-	-
		10 y 51%	3°y 88%		9°y 80%	5°y 75%	5°y 84%			
		15 y 46%					10°y 82%			
Death rate in 1°y	12%	24%	9%	26%	13%	8%	15%	-	-	-

Abbreviations: y, year; d, day; m, months; ESKD, end stage kidney disease; HNT, hypertension. Hypertension: defined as blood pressure >95th centile for age, height and gender or if in anti-HTN treatment.

Early diagnosis and improvements in neonatal intensive care have significantly increased the survival rate of patients with early clinical expression. Non-etheless, 30%–40% of patients still die from pulmonary hypoplasia and respiratory failure (Kaplan et al., 1989; Deget et al., 1995; Roy et al., 1997; Rhona Capisonda et al., 2003; Guay-Woodford et al., 2014). Perinatal survival rates are also be influenced by medical and/or parental decisions to withhold treatment in oliguric infants with severe pulmonary distress. Patient survival increases markedly after the first month of life (Bergmann et al., 2005; Dell, 2011), in particular regarding patients who survived to 1 year of age, 82% of them were alive at 10 years (Kaplan et al., 1989; Bergmann et al., 2005). Historical data reported conflicting results on the correlation between kidney size and kidney function. In 2021, data from the European Recessive Polycystic Kidney Registry (ARegPKD) demonstrated an inverse correlation between TKV adjusted for length in the first 18 months of life and kidney survival from prenatal life to adolescence (Burgmaier et al., 2021b).

3.2 Pathogenesis and prevalence of hypertension

As for ADPKD, hypertension is a main symptom of ARPKD that on average develops much earlier. The estimated prevalence of hypertension in published studies ranges from 33% to 75% (Deget et al., 1995; Zerres et al., 1996; Guay-Woodford and Desmond, 2003; Bergmann et al., 2005; Dell, 2011; Guay-Woodford et al., 2014). Hypertension develops even in the first weeks of life and is often difficult to control, requiring early combined therapy with different antihypertensive drugs (Deget et al., 1995; Zerres et al., 1996; Guay-Woodford et al., 2014; Chinali et al., 2019; Seeman et al., 2022). Hypertension is not always associated with kidney failure. In patients with preserved kidney function, it usually precedes the development of CKD (Zerres et al., 1996; Guay-Woodford and Desmond, 2003).

In 1987, Cole et al. were among the first investigators to describe high prevalence of severe early-onset hypertension in patients with ARPKD (Cole et al., 1987). At that time, the disease was not very well studied. In the following years, several publications have studied the outcome of children with ARPKD and have reported high prevalence of arterial hypertension, although blood pressure was not a primary outcome measurement of these investigations.

In 1989, Kaplan et al. observed a 65% prevalence of hypertension in 55 patients with ARPKD. In the same study, they monitored plasma renin and serum sodium levels, and concluded that in ARPKD, arterial hypertension is probably not driven by renin but by an increase in intravascular volume, as suggested by hyponatremia, which they observed frequently, especially in

younger children (Kaplan et al., 1989). A few years later, Deget et al. reported a similar prevalence of hypertension, but observed that the age of onset was extremely variable, ranging from the first months of life to adolescence (Deget et al., 1995).

Soon after, Zerres et al. published a multicenter study in which 115 patients were recruited in 34 pediatric nephrology centers. More than half of the cohort (i.e., 77/115 children) needed antihypertensive treatment during the observation period, and 50% were hypertensive at diagnosis. Overall, that study showed a very high prevalence of early-onset hypertension; 64% of patients had started anti-hypertensive therapy within the first year of life. At the last observation, one-third of patients had not achieved satisfactory blood pressure control (Zerres et al., 1996).

In 1997, Roy et al. reported somewhat lower rates of early-onset hypertension in another cohort followed from the first month of life. In their cohort, the percentage of patients requiring antihypertensive therapy increased from 39% at 1 year of age to 60% at the age of 15 years (Roy et al., 1997). A higher prevalence was observed by Rhona Capisonda et al., who studied 31 ARPKD patients aged 0–14 years at diagnosis. When first evaluated, 55% of patients were hypertensive. The prevalence increased to 85% in patients who had survived the neonatal period, and approximately half of patients needed more than one medication to achieve adequate blood pressure control. The age of onset of hypertension ranged 4 days to 3 years. Interestingly, the pharmacological needs to achieve blood pressure control seemed to decrease over time, even in patients with very early-onset hypertension, a finding that indirectly emerges also from the analysis of other studies. Similarly to the data reported by Kaplan et al., the authors described in 3 infants a positive association between hyponatremia and hypertension (Rhona Capisonda et al., 2003). Hyponatremia is a frequent finding in ARPKD children. It is not associated with increased urinary sodium losses and develops before progression to CKD, probably as a consequence of altered free-water clearance and possibly increased sodium reabsorption, resulting in hypervolemia, especially in infants who are exposed to a lower osmotic load (Kaplan et al., 1989; Zerres et al., 1996; Guay-Woodford and Desmond, 2003; Guay-Woodford et al., 2014; Wicher et al., 2021).

In 2003, Guay-Woodford et al. published data from the North American ARPKD Clinical Database. The study included 209 patients stratified by year of birth; 21% and 79% of patients were born before and after 1990, respectively. The prevalence of hypertension and the risk of developing hypertension were higher in patients born before 1990 (80% vs. 65%). The interpretation of this finding is unclear and may be altered by biases. The median age at diagnosis was significantly lower in more recent patients, most likely reflecting improvements in the diagnosis and care of sick newborns and infants over the years. The age of onset of hypertension and of CKD was significantly lower in infants requiring mechanical ventilation, reflecting more severe disease. In patients born after 1990 that survived the first month of life, the age of onset of hypertension and of CKD were positively correlated, although this finding does not necessarily imply a direct cause-and-effect relationship between these two variables. In addition, 96% of patients with early-onset hypertension had hyponatremia, supporting the hypothesis that hypertension in ARPKD may be the result of a dysregulation in the sodium reabsorption mechanism in the ectatic collecting ducts, but they also recognize that this mechanism has not been demonstrated by other studies (Guay-Woodford and Desmond, 2003).

In a 6-year follow-up study published by Bergmann et al., in 2015 to assess genotype-phenotype correlations, the prevalence of hypertension was 43% at the beginning of observation period and 73% at the end of follow-up. Anti-hypertensive treatment was started at an average age of 3 years; half of patients were receiving treatment at the age of 6 months (Table 2) (Bergmann et al., 2005).

As for ADPKD, most studies in ARPKD children have assessed hypertension with office blood pressure measurements. More recently, ABPM has also been used in children with ARPKD. Seeman et al. have published a retrospective study on 36 children with ARPKD, of whom 29 had performed at least two ABPM recordings. The median age at the first ABPM was 4.4 years and the average interval between the first and the last recording was 5 years. Confirming previous reports (Dell et al., 2016), the initial evaluation showed hypertension in 94% of patients, of which only one-third were well controlled with antihypertensive therapy (Seeman et al., 2022). At the last evaluation, the prevalence of hypertension remained stable (86%), but two-third of patients were now adequately treated. Blood pressure did not correlate with kidney length nor with glomerular function. The prevalence of hypertension was similar when using ABPM or OBPM (94% and 86%), probably because values were markedly elevated with both methods. The improvement of blood pressure control overtime may be related to physician tendency to use more aggressive blood pressure therapy in older children, but may also reflect a natural tendency of blood pressure to improve spontaneously in older children with ARPKD (Rhona Capisonda et al., 2003).

The pathophysiology of hypertension in ARPKD is still incompletely understood. Volume overload secondary to CKD may represent the main factor driving blood pressure increase (Kaplan et al., 1989; Rhona Capisonda et al., 2003). In 2005, Rohatgi et al. have analyzed the fluid composition of renal cysts obtained from a murine model of ARPKD and from nephrectomized kidneys of patients with ARPKD. They observed increased reabsorption of sodium in murine epithelial cells, but not in human cells (Rohatgi et al., 2005). Some years after, data from the ARPKD mouse model suggest a local paracrine activation of the intra-renal RAAS mediated by up-regulation of RAAS genes in immature tubular structures, without significant systemic activation (Goto et al., 2010). Additional hypotheses include up-regulation of the epidermal growth factor/epidermal growth factor receptor axis, activation of non-classic components of the RAAS (angiotensin 1-7, ACE2 and Angiotensin II type 2 receptor) and increased cyclic adenosine monophosphate (cAMP) activity, which, in addition to its role in cystogenesis, can stimulate overexpression of RAAS genes (Goto et al., 2010).

Despite several studies have reported low circulating renin levels in children with ARPKD, RAAS inhibitors are the most frequently medications used to treat hypertension and are efficient in most patients.

3.3 Cardiovascular consequences of hypertension

Studies on cardiac involvement in ARPKD children are very limited and data are not homogeneous due to differences in the

techniques that have been used to assess cardiac geometry and function.

In 2007 Phillips et al. have studied cardiac geometry in an ARPKD mouse model (Lewis polycystic kidney) in which kidney cysts develop from the age of 3 weeks, hypertension at 6 weeks of age, and LVH at 24 weeks of age, indicating a clear temporal sequence between progression of kidney disease, hypertension, and cardiac damage (Phillips et al., 2007).

Dell et al. have studied the cardiac geometry as a secondary outcome measure in pediatric patients with ARPKD and children with mild-to-moderate CKD secondary to renal dysplasia and urinary tract obstruction. No significant differences in the prevalence of LVH and hypertension (defined with ABPM) were observed. However, a significantly greater percentage of ARPKD children received blood pressure medications compared to children with other renal diseases (Dell et al., 2016).

Seeman et al. have also evaluated cardiac geometry in ARPKD children using two-dimensional echocardiography. They observed LVH in 30% of patients, including some patients that had good blood pressure control (Seeman et al., 2022).

We have studied 27 ARPKD children at an initial age of 3.8 years. Of these, 92% were treated with antihypertensive drugs, which had been started before 6 months of life in 48% of patients. Compared to age- and sex-matched healthy children, patients with ARPKD had on average significantly higher LVMi and showed a significantly higher prevalence of LVH, mainly of the concentric type (Chinali et al., 2019). Multivariate analysis showed a significant association between LVMi and blood pressure, confirming the predominant role of arterial hypertension in inducing LVH (Mitsnefes et al., 2016). We also analyzed the ejection fraction (EF), the longitudinal strain, the circumferential strain, and the midwall fractional shortening (FSmw). These latter parameters allow identifying geometry independent subclinical systolic dysfunction (Narayanan et al., 2009). The EF was normal in children with ARPKD, but 22% had abnormal FSmw and/or circumferential strain values, indicating subclinical systolic dysfunction. Of note, FSmw did not correlate with blood pressure, with the EF and with kidney function. ACE-i/ARBs were weakly associated with improvements in FSmw over time, supporting the use of these drugs to treat myocardial dysfunction and suggesting a non-hemodynamic effects of the RAAS system on cardiac remodeling, as already proposed by others (Schlaich and Schmieder, 1998).

Some illustrative images of advanced echocardiography are shown in Figure 2.

Altogether and despite the small number of patients included in the few available studies, current data indicate that children with ARPKD have significantly higher LVMi and develop concentric LVH in a significant proportion of cases. In addition, many have evidence of subclinical cardiac dysfunction, similarly to children with CKD secondary to other causes (Chinali et al., 2015), but differently form adult hypertensive subjects that for the most part develop selective alteration of the longitudinal strain (Narayanan et al., 2009; Sengupta et al., 2013). Incomplete correlation between cardiac abnormalities and blood pressure values suggest that LVH in ARPKD may not be driven solely by arterial hypertension.

3.4 Final remarks

Compared to ADPKD, ARPKD is a much rarer disease. Therefore, fewer data are available on cardiovascular consequences. Furthermore, younger age and more severe disease of patients at diagnosis renders more difficult assessing blood pressure using ABPM and performing sophisticated cardiac investigations. Overall, severe, early-onset hypertension is more frequent in ARPKD children. The prevalence of hypertension ranges 55%–100% in different studies. The pathogenesis of hypertension is not fully elucidated and may not be entirely due to the activation of the RAAS. However, ACE-i and ARBs remain the mainstay of treatment, together with calcium channel blockers. Frequently, more than one drug is needed to treat hypertension, although blood pressure control tends to improve spontaneously over time. Approximately one-third of patients surviving the neonatal period have abnormal cardiac geometry. More studies are needed to establish the role of genotype in the severity of cardiovascular changes.

4 Conclusion

ARPKD and ADPKD are severe ciliopathies that are characterized by marked cardiovascular involvement. In both diseases, early detection of hypertension and of cardiac anomalies allows limiting cardiovascular damage and long-term complications. To which extent very early treatment of cardiovascular symptoms impact on the evolution of kidney diseases remains uncertain but deserves more attention in the future. In the meantime, pediatricians can play a crucial role in balancing prevention and excessive medicalization, in particular in children with ADPKD.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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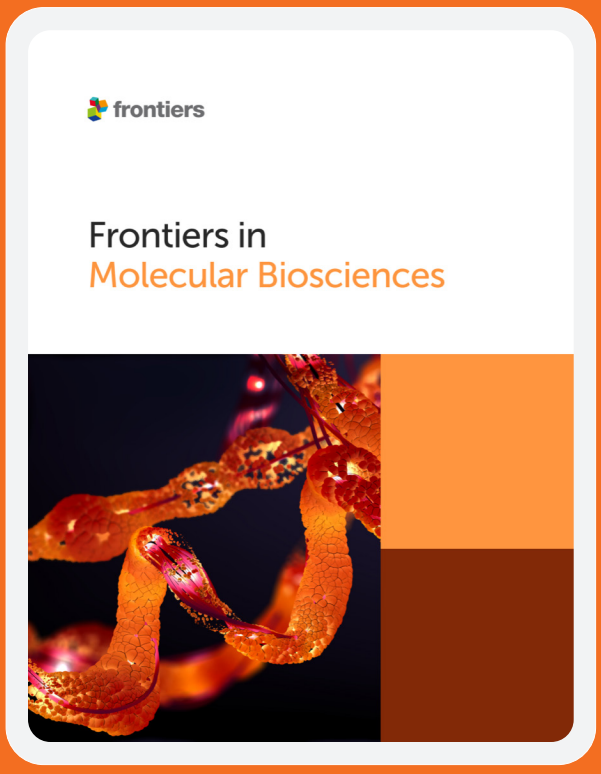

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