Shear stress regulated signaling in renal epithelial cells and polycystic kidney disease

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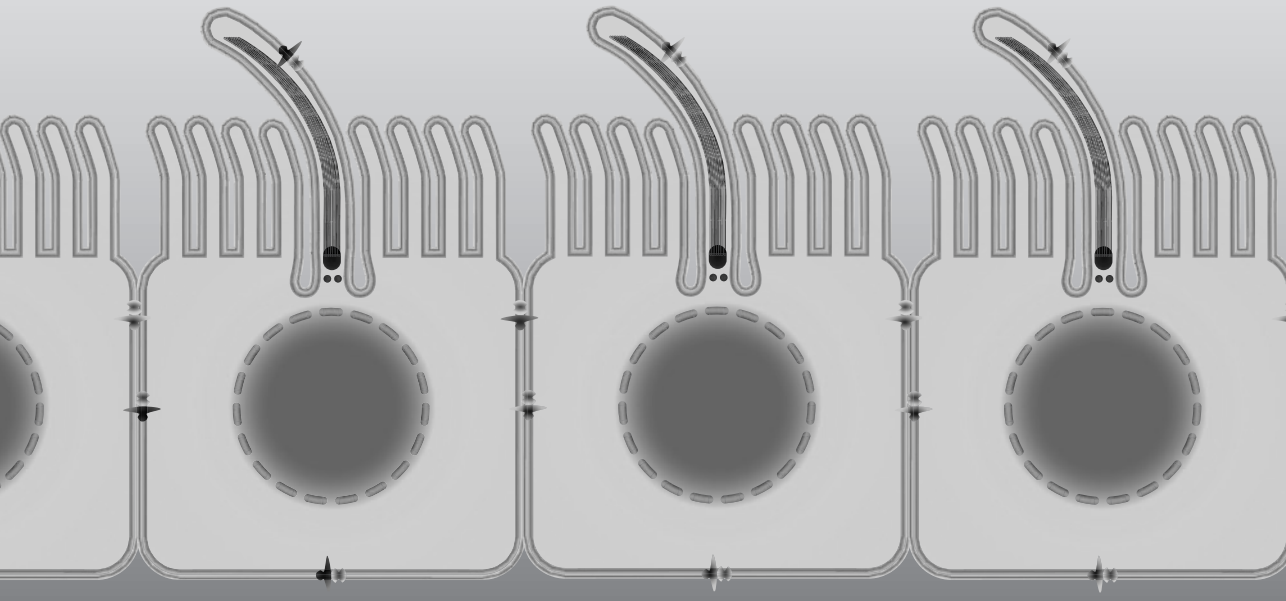
CHAPTER 6

Summarizing discussion



Shear stress regulated signaling is essential during embryonic development, tissue homeostasis and cellular function. Defects in fluid shear sensing and mechanotransduction can cause or modulate a broad range of diseases including ciliopathies and ADPKD[1-7]. In the last two decades, shear stress was more extensively investigated in renal epithelial cells to evaluate the role of mechano-sensing in cellular function and disease phenotypes[8-10]. However, cellular processes and signaling pathways that are altered by shear stress in renal epithelial cells are still not completely understood.

In this thesis, a comprehensive evaluation of shear stress regulated signaling in proximal tubular epithelial cells is presented, followed by a comparison of signaling pathways altered in ADPKD. Fluid flow within the lumen of nephrons exposes renal epithelial cells to mechanical forces like shear stress. Renal epithelial cells experience a relatively steady flow velocity and shear stress in a physiological range between 0.05 - 1 dyn/cm^2, where proximal tubular epithelial cells (PTECs) experience the highest range of shear stress[10-14]. However, variations in hydrodynamic forces and shear stress are common in kidney diseases, including ADPKD, due to hyperfiltration, tubular dilation and obstruction[15]. In this thesis, we applied a shear stress ranging from 0.25 to 2 dyn/cm^2, to mimic physiological and more pathological (hyperfiltration) levels of shear stress.

In **chapter 2** we presented fluid shear induced TGF-β/ALK5 signaling in both wild-type and *Pkd1*$^{-/-}$ PTECs. This was evidenced by phosphorylation and nuclear accumulation of p-SMAD2/3, as well as altered expression of downstream target genes and EMT markers. ALK4/5/7 inhibitors blocked SMAD2/3 mediated signaling, indicating that autocrine signaling is involved, which was reported for endothelial cells as well[16]. This was further supported by shear induced mRNA expression of *Tgfb1* and *Tgfb3*. In addition, TGF-β neutralizing antibodies inhibited the flow response, while an activin ligand trap (sActRIIB-Fc) was ineffective, indicating that the flow response was TGF-β/ALK5 dependent. However, it still remains unclear how fluid shear activates TGF-β ligands, but a previous study suggests that TGF-β can be released from its latency-associated peptide (LAP) by shear stress, probably by forces exerted on α_v-β_6-integrins via the actin cytoskeleton[17,18]. In addition, a recent publication reported increased apical endocytosis upon shear stress exposure in PTECs[19]. Endocytosis may be important for the shear response in PTECs, since TGF-β/ALK5 signaling is mediated via clathrin dependent endocytosis[20,21].

In contrast to the ALK4/5/7 inhibitor, the MEK-inhibitor further enhanced shear induced expression of several SMAD2/3 target genes. Several studies report that TGF-β ligands can activate canonical (SMAD2/3) and non-canonical (MAPK/ERK) TGF-β pathways and the downstream responses can be modulated at several levels[22,23]. Activated ERK proteins can both enhance and repress SMAD2/3 dependent gene transcription, depending on timing

and amount of ERK activation[24,25]. Integration of TGF-β and MAPK/ERK signaling cascades is complex, biological context dependent and therefore difficult to predict.

The flow response appeared to be slightly but significantly stronger in *Pkd1*$^{-/-}$ cells, suggesting that *Pkd1* restrains shear induced TGF-β/ALK5 signaling. Additionally, when comparing physiological (0.25-1 dyn/cm^2) with pathological (2 dyn/cm^2) levels of shear, we showed that flow induced target gene expression in PTECs was increased by the amount of shear stress. These data suggest enhanced TGF-β/ALK5 signaling upon *Pkd1* gene disruption and hyperfiltration, which might contribute to epithelial cell plasticity, fibrosis and kidney diseases progressions, where hyperfiltration is a common feature[15]. In contrast, cilia ablation did not inhibit flow mediated SMAD2/3 signaling in *Pkd1*^{wt} cells. Even more, expression of early responsive SMAD2/3 target genes was enhanced upon deciliation, suggesting that to a certain extent primary cilia suppress TGF-β/ALK5 signaling. Additionally, our data indicate that fluid shear stress in PTECs is also regulated by other mechano-sensors, located at different locations of the cell membrane, which needs additional investigation in the future.

In **chapter 3** we further evaluated the cellular response of PTECs upon shear stress exposure, using RNA sequencing. In agreement with the previous chapter we showed that expression of numerous components of TGF-β and MAPK signaling pathways were up-regulated by shear stress. In addition, functional enrichment-analysis revealed shear induced Wnt and p53 signaling, as well as several other cytokine signaling pathways, including PDGF, FGF, HB-EGF and CXC. It needs more extensive investigation whether these increased cytokine signaling pathways are caused by autocrine or paracrine loops, as reported for TGF-β in this thesis. Nevertheless, increased expression of cytokines suggests activation of inflammatory cells upon shear *in vivo*. Inflammation is a common phenomenon during progression of kidney diseases, where shear stress is fluctuating due to changes in glomerular filtration rate, tubular hyperfiltration and obstruction[26]. We further concluded that TGF-β and MAPK/ERK are master regulators of shear-induced gene expression, because inhibitors of these pathways modulate a wide range of mechanosensitive genes. This can be caused by several interactions between TGF-β and MAPK signaling[23-25,27]. However, the main down-regulated pathway, *i.e.* JAK/STAT, is independent of TGF-β and MAPK/ERK.

What is clear from chapter 3 is that the cellular response to shear is modified at several levels. This is indicated by altered expression of genes involved in cell-matrix, cytoskeleton and glycocalyx remodeling, but also glycolysis and cholesterol metabolism are altered. Increased expression of cell-cell/cell-matrix interaction genes suggests reinforcement of epithelial cell morphology[11,28,29]. However, high pathological shear stress of 5 dyn/cm^2 can result in loss of epithelial cell morphology[30], indicating that physiological relevant levels of shear are required to maintain epithelial integrity. Flow down-regulation of genes involved

in various metabolic pathways is mediated via AMPK, which was previously shown for endothelial cells[31-35]. AMPK is an important kinase in energy metabolism and plays a central role in fluid flow induced down-regulation of mTORC1 activity in renal epithelial cells, which is dependent on bending of the primary cilium[36,37].

Our results revealed shear induced expression of genes involved in glycocalyx remodeling. This can be important for mechanotransduction of shear stress by amplifying the drag force exerted on renal epithelial cells. However, in this thesis we did not investigate if the glycocalyx itself is involved in mechano-sensing in PTECs. Nonetheless, we investigated the role of cilia in the shear stress response. Cilia ablation abolished shear induced expression of a subset of genes, but genes involved in TGF-β, MAPK and Wnt signaling were hardly affected, suggesting that other mechano-sensing structures or complexes play a prominent role in the shear stress response of renal epithelial cells. We finally showed that altered signaling due to increased fluid shear stress may be relevant for renal physiology and pathology, as indicated by elevated gene expression at pathological levels of shear stress compared to physiological shear.

RNA sequencing gene expression profiles of shear stress treated *Pkd1*$^{-/-}$ PTECs were compared with *Pkd1*^{wt} controls in **chapter 4**. Functional enrichment analysis revealed that shear regulated genes in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs are involved in the same signaling pathways, including MAPK, PI3K-AKT, Hippo, Wnt, TNF, p53, FoxO, TGF-β, calcium, JAK-STAT and mTOR signaling. These data indicate that *Pkd1* is not directly involved in shear dependent activation of these pathways. However, expression of a number of genes was significantly more activated by shear stress in *Pkd1*$^{-/-}$ PTECs, suggesting that *Pkd1* is partially restraining shear regulated signaling, which was shown for TGF-β/ALK5 related signaling in chapter 2 as well.

To study shear stress dependent signaling in ADPKD, we compared changes in gene expression in *Pkd1*$^{-/-}$ PTECs during shear with *in vivo* transcriptome analysis of kidneys in *Pkd1*^{del} mice at three early pre-cystic time-points. *Pkd1* gene disruption was specifically induced in 40-50% of the renal epithelial cells, while there was still fluid flow present in *Pkd1*^{del} mice at pre-cystic stage[38-40]. Upon *Pkd1* gene disruption, there was a clear overlap in altered pathways and cellular processes between cells and mice, which was attributed to altered expression of identical or paralogous genes. A number of altered flow-induced pathways upon *Pkd1* gene disruption are also implicated in ADPKD, including PI3K-AKT, Hippo, MAPK, p53, calcium, Wnt, JAK-STAT and TGF-β signaling. This suggests that these pathways are already modified at pre-cystic stage and may contribute to the onset of disease. Several genes involved in endocytosis exhibited altered expression in *Pkd1*^{del} mice and *Pkd1*$^{-/-}$ PTEC cells, while this process was also increased by *in vitro* fluid shear exposure, reported

in this thesis and previous publications[19,41,42]. Enhanced endocytosis upon shear is mediated via cilium dependent Ca^{2+} increase, and subsequent calmodulin mediated activation of Cdc42[43]. Increased endocytosis in ADPKD was recently published in transcriptomic analyses of ADPKD models as well[44]. In addition, the involvement of endocytosis in several growth factor signaling cascades, like TGF-β and MAPK[20,45], makes this finding more interesting. Altered endocytosis by shear stress or *Pkd1* gene disruption suggests that this process may contribute to imbalanced cellular signaling or disease progression, although additional research is required to confirm the causality.

As discussed before, several cellular processes are affected by fluid shear stress and *Pkd1* gene disruption, including TGF-β signaling. Furthermore, the TGF-β pathway is proposed to be the initiator of fibrosis, which is a common clinical feature of ADPKD[46-48]. This is supported by a study showing nuclear translocation of p-SMAD2 in cyst lining epithelial cells of *Pkd1* mutant mice[49]. Therefore, we focused on different interventions to target the TGF-β signaling pathway in **chapter 5**. Genetic disruption of TGF-β type I receptor (*Alk5*) in *Pkd1*^{del} mice only showed minor to no reduction of SMAD2/3 target gene expression or cyst progression. In contrast, primary cells from *Pkd1-Alk5* conditional knock-out (cKO) mice showed attenuated SMAD2 phosphorylation upon TGF-β stimulation compared to single *Pkd1* cKO mice. This may suggest that renal epithelial TGF-β/ALK5 signaling is not the driving factor during cyst progression *in vivo*, although it cannot be excluded that several other renal cell types contribute to PKD phenotype via TGF-β signaling. Alternatively, ALK5-independent pathways may be involved in disease progression.

One of these alternative TGF-β pathways is activin signaling via the activin type I (ALK4) and type II receptors[50]. Activin ligands are formed by two homo- or heterodimers of inhibin-β subunits of which four are characterized in mammalians (βA, βB, βC, βE). We found that *Inhba* and *Inhbb* gene expression was increased in *Pkd1*^{del} mice. The protein products of these genes are important for embryonic development, injury repair and tissue homeostasis, while inhibin-βC or βE knockout mice didn't display any phenotype[51]. However, activin C might have an antagonistic function to compete with activin A signaling. Activins are implicated in several diseases and can stimulate cell migration, differentiation and fibrosis. Therefore, activin inhibition was proposed as possible therapeutic strategy to retard disease progression[52].

In our study we used a soluble activin receptor type II B fusion protein (sActRIIB-Fc), which is a ligand trap to sequester activins. We treated two young and one adult PKD mouse model with sActRIIB-Fc to assess if inhibition of activin signaling could be a therapeutic strategy for PKD. Overall cyst progression was significantly reduced in sActRIIB-Fc treated PKD mice compared to PBS treated littermates. The highest dose of sActRIIB-Fc treatment was in most

analyses not significant compared to the lower dose, although there was a dose dependent trend visible. This suggests that the lower dose of sActRIIB-Fc is already sufficient to reduce cyst progression in PKD mouse models. In addition, we found decreased SMAD2 expression and phosphorylation, as well as reduced SMAD2/3 target gene expression and collagen deposition, indicating that sActRIIB-Fc is sequestering SMAD inducing ligands, like activins. However, other TGF-β superfamily ligands can also be sequestered by sActRIIB-Fc, but with lower affinity, including GDF11 and myostatin[53,54]. RNA sequencing data from our lab showed that myostatin (*Mstn*) is not expressed in kidneys of the PKD mouse models and *Gdf11* is low expressed and not changed in PKD mice (chapter 4 and Malas *et al.*)[55]. In addition, the same data showed clearly higher expression of *Inhbb* compared to *Inhba* in PKD kidneys, while *Inhbc* and *Inhbe* were not expressed. This suggests that reduced cyst formation upon sActRIIB-Fc treatment might act by sequestering activin B. However, subtype specific activin type II receptor ligand traps or antibodies needs to be used in the future to confirm this hypothesis. In conclusion, activin inhibition showed promising results to slow PKD progression in three ADPKD mouse models. In addition, clinical trials assessing the safety of soluble activin receptor ligand traps reported that these compounds are well tolerated[56-58], although some clinical trials were discontinued due to safety or efficacy concerns[59,60]. Nevertheless, future research needs to address if the use of soluble activin receptor ligand traps is safe and effective during long term treatment of ADPKD patients.

GENERAL DISCUSSION

In the first chapters of this thesis we presented various cellular processes and signaling pathways that were altered by shear stress and we revealed that several factors can modify the fluid shear response in PTECs. Clearly, pathological shear can enhance the flow induced response in PTECs, but *Pkd1* gene disruption can also elevate shear induced expression of several genes involved in MAPK, TGF-β and Wnt signaling. It has been hypothesized that critical polycystin-1 levels are required to restrain cellular and cilia- or shear-related signaling in order to prevent polycystic kidney disease[61]. Moreover, strong variations in hydrodynamic forces and fluid shear are common in kidney diseases, including ADPKD, due to cyst growth, tubular dilation, obstruction and hyperfiltration, which occur to compensate for lost glomeruli and tubules[15]. Furthermore, shear stress is increased after unilateral nephrectomy[40,62], which can accelerate cyst progression in *Pkd1*^{del} mice[39]. In addition, long-term pathological shear exposure can result in tubulointerstitial lesions and fibrotic deposition, which is commonly seen after renal mass reduction or during progression of renal diseases[8,63-65]. Overall, this leads to the hypothesis that pathological shear stress and *Pkd1* gene disruption can both lead to imbalanced cellular signaling, which probably contribute to renal cyst formation and fibrosis. This hypothesis is supported by results in this thesis, showing that several processes

and pathways are cooperating to regulate the shear stress response in PTECs and to maintain cellular physiology. TGF-β, MAPK and Wnt signaling are elevated upon pathological shear or *Pkd1* gene disruption, while these pathways are associated to fibrosis, differentiation and uncontrolled cell proliferation in kidney diseases. Furthermore, several of these pathways are also perturbed in pre-cystic *Pkd1*^{del} mouse models (shown in chapter 4), indicating that imbalanced signaling is already occurring early upon *Pkd1* disruption at pre-cystic stage. In addition, a recent study by our group revealed clustered cyst formation in an adult slow onset PKD mouse model, which resembles the human ADPKD phenotype[39]. Renal tubules are compressed and PKD-related signaling is increased in nephrons near existing cysts[66]. Tubular compression or obstruction will likely affect local mechanical forces and shear stress on epithelial cells and may lead to altered signaling and local cyst formation. Of course, cellular processes and signaling pathways that drive cyst formation will depend on many factors in addition to functional PKD protein levels, including shear stress, renal injury, inflammation and cellular metabolism.

To date, it is still not completely clear which mechano-sensing structures and complexes are involved in fluid shear response of renal epithelial cells or several other cell types. In the last two decades, the function of the primary cilium was extensively investigated, but we lack knowledge about the role of the microvilli and glycocalyx in renal epithelial cells[42]. The glycocalyx is particularly important for the shear stress response in endothelial cells, but in renal epithelial cells the glycocalyx can also amplify the frictional force on the cell membrane and the microvilli. In this thesis, we revealed that shear altered expression of a number of genes was cilium dependent, while several other genes were still induced by fluid shear after cilia disruption. This indicates that additional mechano-sensing structures or complexes are involved in the shear stress response of renal epithelial cells. However, the involvement of other mechano-sensors, *i.e.* microvilli, the glycocalyx, tight junction proteins and integrin's, and possible interactions between these complexes were not addressed in this thesis, which needs more extensive investigation in future research.

Obviously, the broad range of human ciliopathies and the diversity of their phenotypes, including polycystic kidneys, have focused the research on the primary cilium function. Several studies have proposed that fluid flow bending of primary cilia induces a rapid Ca^{2+} influx, mediated by the polycystin1-2 complex, followed by release of intracellular Ca^{2+} stores and subsequent cytosolic Ca^{2+} increase[67-71]. This process modulates several signaling cascades, including cAMP signaling. In ADPKD, aberrant ciliary polycystin function is the proposed mechanisms leading to reduced Ca^{2+} signaling, which induces cAMP, trans-epithelial fluid secretion and MAPK/ERK mediated cell proliferation[72-74]. However, recent studies question the direct role of primary cilia and polycystins in the Ca^{2+} response, since they show cytosolic calcium response prior to the ciliary calcium increase, whereas other

calcium transporters are involved[75-80]. In chapter 4, we showed shear altered expression of genes involved in Ca^{2+} signaling, while *Pkd1*^{wt} and *Pkd1*$^{-/-}$ PTECs display both overlap and differences in Ca^{2+} signaling related gene expression. However, we did not detect differences in the shear induced cytoplasmic Ca^{2+} influx, when comparing *Pkd1*^{wt} and *Pkd1*$^{-/-}$ PTECs using a Fura-2 AM reporter in a microscopic flow system (unpublished data). This suggests that polycystin-1 is not primarily involved in the shear stress induced Ca^{2+} response in renal epithelial cells. Moreover, a recent study reported that polycystin-2 is a monovalent cation channel within the primary cilium of renal epithelial cells, with a preference for K^+ and Na^+ over the divalent Ca^{2+} ions[81]. Ciliary localization of polycystin-2, nor its channel activity were altered in the absence of polycystin-1, although polycystin-1 and 2 may still interact at other cellular locations or via indirect mechanisms[81]. In this thesis, we lack data of *Pkd2*$^{-/-}$ models, which is definitely required to refine the role of the polycystins in renal physiology and pathology, like ADPKD, whereas the molecular mechanisms of cyst formation may be distinct.

Despite numerous studies about fluid shear mediated Ca^{2+} influx, the mechanism of Ca^{2+} signaling is not entirely clear and still under debate, whereas the direct involvement of cilia and the polycystins is being criticized[75-79,81,82]. Therefore, it is of indisputable importance to further investigate the role of primary cilia, polycystins and other mechano-sensing complexes in the shear response of renal epithelial cells. It is likely that shear stress sensing and mechanotransduction are different during development and cellular repair or tissue homeostasis, emphasizing the importance to select appropriate cellular models. This will hopefully reveal the complex mechanism of mechano-sensing and the relevance for disease progression, including ciliopathies and ADPKD. However, the cellular mechanism that initiate cyst formation might be distinct between ciliopathies and ADPKD, since a study by Ma *et al.* revealed that cilia disruption suppressed cyst growth in ADPKD models[83]. They proposed that functional polycystin levels are essential to restrain cilia-dependent cyst activation, although additional research is required to refine this mechanism[79]. Our results can support this hypothesis, since cilia ablation partially suppressed shear induced signaling, while *Pkd1* disruption seems to enhance specific shear induced signaling pathways, including TGF-β and Wnt signaling. Recent data, including data in this thesis, suggest that *Pkd1* has the function to restrain shear regulated signaling instead of being a mechano-sensing activator. Accordingly, *Ma et al*. previously proposed a role for *Pkd1* in restraining an unknown cilia-dependent signaling pathway involved in cyst formation[79]. Therefore, it is of utmost importance to further elucidate the functions of cilia and ciliary located proteins, like the polycystins and IFT proteins, as well as their role outside the cilia. This is needed to establish a definite mechanism of cyst formation and disease progression, including ADPKD and other ciliopathies. Additionally, this should be supported by future research to identify the mechanism of mechanotransduction and the involved mechano-sensors using

PKD and ciliopathy models. In chapter 4 we showed a broad overlap in cellular processes and pathways that were altered upon *Pkd1* gene disruption, between *in vitro* and *in vivo* experiments. Despite the overlap there were clearly some differences. The most notable divergence was presented in chapter 5, which showed that inhibition of activin signaling was able to reduce cyst formation in *Pkd1*^{del} mice, but *in vivo Alk5* ablation was ineffective. This seems in contrast with the *in vitro* situation, where shear stress induced signaling is (partially) TGF-β/ALK5 dependent and is increased at pathological shear or upon *Pkd1* disruption. Obviously, the *in vitro* study is not fully representative for the *in vivo* situation, where several other cells types and cytokines in the nephrons are involved and cellular signaling will depend on the overall biological context. In chapter 5, genetic disruption of *Pkd1* and *Alk5* in mice was specifically induced in renal epithelial cells. So, TGF-β/ALK5 signaling can still be present in other cell types of *Pkd1-Alk5* cKO mice, like fibroblasts or inflammatory cells. Activin expression can be induced in macrophages and other inflammatory cells by several cytokines, like TGF-β, EGF, PDGF, TLRs, TNFα, INF-γ and interleukins[84,85]. In addition, macrophages are found to accumulate near cysts and can stimulate cyst growth, while macrophage depletion inhibited cyst growth[66]. So, this suggests that different cell-types may be involved during cyst formation and PKD-progression is biological context dependent.

In this thesis, we found several processes and pathways that were altered both by fluid shear stress and *Pkd1* gene disruption. Many of these signaling pathways are implicated in ADPKD as well and numerous potential therapies are targeting these pathways[86]. However, more than 20 years after the discovery of *PKD1* and *PKD2* as genetic cause of ADPKD, the exact cellular function of the polycystins still remains unclear. Our data indicate that polycystin-1 is not a direct mechano-sensor, but it restrains shear stress induced gene expression via an unknown mechanism. Additional research is required to identify the cellular function of polycystins and the mechanism of mechanotransduction. This is needed to refine the mechanism of cyst formation in ADPKD and other ciliopathies, which could identify potential targets for therapy. Nevertheless, we showed that inhibition of activin signaling is a promising therapy to slow cyst progression in *Pkd1*^{del} mice. Although other treatment strategies have been tested successfully to reduce PKD progression in pre-clinical studies, the efficacy in human patients is sometimes minimal or absent, which was reported for several mTOR inhibitors[87-89]. Therefore, it has been suggested to combine therapies and target multiple signaling pathways affected in ADPKD[66,90,91]. These combined therapies should reestablish the balance in cellular signaling of renal epithelial cells and maintain cellular homeostasis within physiological boundaries. However, additional research is required to evaluate the safety and efficacy of combined therapies, which will be challenging because ADPKD patients will require life-time treatment to postpone end-stage renal disease.

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