The handle http://hdl.handle.net/1887/19155 holds various files of this Leiden University dissertation.

**Author:** Happé, Hester  
**Title:** Cyst initiation, cyst expansion and progression in ADPKD  
**Date:** 2012-06-27
Summarizing discussion
Autosomal dominant polycystic kidney disease (ADPKD) is characterized by large fluid-filled cysts and progressive deterioration of renal function necessitating renal replacement therapy. In this thesis different phases of ADPKD were studied. The major findings of this study are incorporated in the schematic representation of the proposed sequence of events in cystogenesis and disease progression shown in figure 1. The initiation phase, with hyper proliferative epithelial cells as a hallmark, is followed by a phase of cyst growth and cyst expansion. Later on, proliferation of cystic epithelia ceases and interstitial fibrosis and inflammation become apparent.

Cyst initiation
In chapter 3 the initiation of cyst formation was studied. In contrast to patients with ADPKD, a heterozygous mutation in Pkd1 does hardly lead to cyst formation in mice. Using a tamoxifen-inducible, kidney epithelium-specific Pkd1-deletion mouse model, our group showed that inactivation of the Pkd1 gene induces rapid cyst formation in developing kidneys and a slow onset of disease in adult mice. Therefore, we hypothesized that the proliferative status of the renal tissue at the time of gene disruption is important for the rate at which cysts are formed after Pkd1 conditional deletion. In chapter 3 we investigated this hypothesis and showed that
Chapter 7

Pkd1-gene disruption at PN40 leads to a disease progression rate intermediate to that of neonatal and adult mice as expected from the renal proliferation levels, which are intermediate to that of neonatal and adult mice. We also showed that DCVC-induced renal epithelial injury significantly accelerates the progression of renal cyst formation. The data in chapter 3 confirm our hypothesis that cyst formation after Pkd1-gene disruption is dependent on the proliferative status of the renal epithelial cells, as renal epithelial injury is followed by a repair phase that includes proliferation to restore the tubular epithelial architecture (Fig. 1).

After the increased proliferation during tissue regeneration, proliferation decreased to basal levels in Pkd1-deletion mice just as in DCVC-treated controls. However, in severe cystic kidneys, 10–14 weeks after injury, proliferation increased again. This biphasic response suggests that unrestricted cell proliferation after injury subsequent to Pkd1-gene deletion is not the underlying mechanism for cyst formation.

Similar to embryogenesis and renal development, tissue repair reactions require properly regulated cell proliferation to restore epithelial architecture. Defects in oriented cell division were observed during tubular elongation in renal development in non-orthologous animal models for PKD.2, 3 In addition, loss of the Planar Cell Polarity (PCP) gene Fat4 leads to renal cysts formation.4 Altogether; these data suggest a potential role for aberrant PCP or oriented cell division.

In order to investigate whether defects in PCP during tissue regeneration could be involved in accelerated cyst formation as in adult Pkd1-deletion mice, we analyzed the position of centrosomes after repair of injury at a pre-cystic stage. The use of centrosome position as a readout system for PCP was discussed in chapter 2 and 4.

As expected, in the majority of cells of control mice (+DCVC), centrosomes are positioned close to the center of the apical membrane. In mutants with and without DCVC, however, a significantly different pattern in the distribution of centrosome position was observed. Centrosomes were positioned further away from its normal position in the center of the apical membrane above the middle of the nucleus. From these data, we conclude that improper positioning of centrosomes is an early event after Pkd1-gene disruption. Additionally, gene expression of several PCP-related genes was investigated and showed differential expression in pre-cystic kidneys of Pkd1 conditional knockout mice. The expression pattern of Four-jointed-1 (Fjx1) is particularly interesting. Our results suggested that Four-jointed is needed during tissue repair. In Pkd1-deletion mutants, however, the expression of Fjx1 decreased upon Pkd1-gene disruption, suggesting defects in PCP signaling. It is believed that when PCP signaling is aberrant, canonical Wnt signaling is activated.5 Indeed signs of activated canonical Wnt signaling were observed.

Since in untreated Pkd1-deletion mice centrosome position and the expression of canonical Wnt targets were also altered, we conclude that these are early responses to Pkd1-gene disruption (Fig. 1). We speculate that these early alterations create a permissive condition for accelerating cyst formation and that additional factors are required. Abnormal integrity of the newly formed cells, resulting from the absence of Pkd1 expression during repair, might be such a factor. It is likely
that polycystins are required during repair for the proper formation of these protein complexes. Without \textit{Pkd1}, cellular structure, geometry and cohesion will be modified (Fig. 1). In agreement with this hypothesis, Silberberg et al. showed that polycystins are required for assembling cell-cell contacts.\textsuperscript{6} Additionally, polycystin-1/-2 levels are elevated after ischemic injury.\textsuperscript{7,9} Concurrently with the publication of our study, a report was published also showing the accelerating effect of injury on cyst formation in \textit{Pkd1} conditional knockout mice but without investigating planar cell polarity. However, in this study ischemic reperfusion injury was used instead of a nephrotoxic compound. Here, the cyst accelerating event is termed “third hit”, where the first hit is the germ line mutation in the first \textit{Pkd1} allele and the second a somatic mutation in the remaining “normal” allele, following the two hit hypothesis. The term “third hit”, however, suggests that this hit is also a genetic hit, while it is in fact a modifying factor related to tubular epithelial proliferation. Recently, more papers were published regarding the role PCP signaling in ADPKD, including a study in which cyst formation was found without aberrant PCP signaling in pre-cystic stages and aberrant PCP signaling but without cyst formation.\textsuperscript{10-12} Additionally a recent paper suggested that cyst formation is more likely to be caused by non-canonical Wnt signaling, PCP signaling, than by the related canonical Wnt signaling. Here it was shown that in a \textit{Pkd1} and \textit{Pkd2} model no β-catenin activity was reported after establishment of urinary flow or in cystic epithelia.\textsuperscript{13} How this study should be interpreted is unclear and requires further research, since many studies (including ours) showed that increased canonical Wnt signaling is associated with cyst formation. The results of all of these studies show that the puzzle is more complex and results and mechanisms may depend on the models used as was also discussed in chapter 2.

**Hippo signaling and cyst growth**

The Hippo signaling pathway is emerging as an important pathway, not only in organ size control but also in cancer. The product of the neurofibromatosis type II tumor suppressor gene (\textit{NF2}), Merlin, is a regulator of the Hippo pathway. In addition, increased YAP expression and nuclear accumulation were shown in multiple types of human cancer.\textsuperscript{14-17} This pathway is subject of investigation in chapter 5, as in chapter 3 we showed that during the repair phase of tubular epithelial injury, when PCP signaling is needed, renal mRNA expression of the PCP-pathway component Four-jointed (Fjx1), was increased in controls, but not in kidneys of the \textit{Pkd1}-deletion mice. Here, the expression of \textit{Fjx1} decreased after \textit{Pkd1}-gene disruption and persisted at relatively low levels at pre-cystic stages. Interestingly, later on, in cystic kidneys \textit{Fjx1} expression dramatically increased. The above mentioned roles of the Hippo pathway, together with the opposing expression pattern of \textit{Fjx1} during epithelial repair and at cystic stages, triggered us to investigate the activity of the Hippo pathway during these processes. In chapter 5, we provide for the first time evidence for altered activation of the Hippo signaling pathway in cyst growth in ADPKD and other cystic diseases. In different mouse models for
ADPKD, nuclear localization of YAP is observed only in cystic epithelia and dilated tubules but not in tubules of pre-cystic kidneys. Increased amounts of YAP-positive nuclei are observed in morphological aberrant tubules, dilated tubules, and cysts but not in normal-looking pre-cystic tubules. This suggests that aberrant Hippo signaling is not an initiating event in cyst formation but accompanies progressive cystic growth.

Our findings, showing altered Hippo signaling during cyst growth, are in line with previous studies reporting renal cyst formation caused by disturbed Hippo signaling; for example, deregulation of YAP in zebrafish results in pro-nephric cyst formation.\textsuperscript{18, 19} In addition, mice lacking TAZ (Wwtr1), the YAP paralog, show renal cystic disease.\textsuperscript{19-21}

The results in chapter 5 strengthen the concept that during epithelial repair, Four-jointed is involved in PCP signaling, while in cystic kidneys it is related to cyst growth. Decreased expression of \textit{Fjx1} is a direct effect of \textit{Pkd1} inactivation, while increased \textit{Fjx1} and YAP target expression can be regarded as an effect of altered signaling during cyst growth, an indirect effect of \textit{Pkd1} inactivation (Fig. 1).

Although both under- and over-expression of YAP, as well as TAZ deficiency, can result in cyst formation in animal models, in renal cystic disease it seems to be associated with an increase in nuclear YAP, as shown in the different mouse models for ADPKD. This is confirmed by the results in patients with ADPKD and ARPKD, demonstrating nuclear localization of YAP in epithelia in the majority of cysts. In addition, we showed that, nuclear translocation of YAP in cystic epithelia was accompanied by the up-regulation of several postulated targets of YAP in cystic kidneys. Additionally, increased nuclear YAP was observed not only in cystic epithelia in ADPKD and ARPKD, but also in cystic epithelia in different renal tumors and in liver cysts derived from bile ducts of hypomorphic \textit{Pkd1} mutants. It is therefore tempting to speculate that altered Hippo signaling is a general feature in cystogenesis.

Polycystic kidneys have been elegantly called ‘neoplasia in disguise’.\textsuperscript{22} Indeed, many pathways involved in neoplastic growth are also involved in cyst formation. It is not surprising that a number of drugs developed for the treatment of cancer have been shown to be effective in attenuating cyst growth in PKD mouse models.\textsuperscript{23-28} This supports the idea that targeting the Hippo pathway could be effective in inhibiting cyst expansion and slowing down progression towards end-stage renal failure in PKD. An interesting future experiment will be to explore whether in vivo absence of YAP during cyst growth indeed attenuates cyst expansion.

Recently YAP and TAZ were found to be transcription factors involved in relaying mechanical signals exerted by ECM rigidity and cell shape. This regulation requires Rho GTPase activity and tension of the actomyosin cytoskeleton, but is independent of the Hippo signaling pathway.\textsuperscript{29} Interestingly, \textit{Pkd1} and \textit{Pkd2} are also thought to be involved in mechano-transduction and are known to play a role in arterial pressure sensing.\textsuperscript{30} In addition, the Rho GTPase Cdc42 is up-regulated in pre-cystic kidneys of \textit{Pkd1} conditional knockout mice.\textsuperscript{10} It would be interesting to investigate whether the role of YAP in cyst growth is dependent on the Hippo signaling cascade with or without crosstalk with PCP signaling through Four-jointed, mechano-transduction, or both.
Disease progression

Chapter 3 and chapter 5 describe processes involved in cyst initiation and cyst growth, while chapter 6 deals with aspects involved in disease progression.

Many mouse and rat models exist for studying the formation and subsequent growth of cysts in polycystic kidney disease. However, few models have been described representing the phenotype observed in ADPKD patients with advanced stage disease. Clearly, animal models with a phenotype that resembles the human pathology are needed to predict accurately the effect of potential therapeutics on disease progression in ADPKD patients.

The Pkd1

 mouse model is a model for PKD in which the variation of the phenotype depends on the genetic background. Here we report that on a controlled 50%/50% genetic background of B6 and Ola (B6Ola), Pkd1

 mice develop large cystic kidneys within 4 weeks and are able to survive up to a year. In contrast, to the models homozygous for a Pkd1 inactivating allele, B6Ola-Pkd1

 mice, express low levels of Pkd1, are viable, and exhibit grossly normal kidney structure, but with dilated proximal tubules at birth. Although expression of the full length Pkd1 transcript is strongly reduced, a 3'-transcript is still produced and it cannot be excluded that this may have an effect on renal development and survival.

In B6Ola-Pkd1

 mice, tubular dilatation and the first cysts could be observed at post-natal day 1. The first cysts are mostly from proximal tubular origin while later on the majority of cysts appeared to be derived from distal tubules, or collecting ducts. Interestingly, B6Ola-Pkd1

 mice show an initial increase in renal volume, followed by a decrease in renal and cystic volume. The partial regression in renal volume was accompanied by “collapse” of cysts, which was paralleled by an increase in fibrosis and influx of inflammatory cells (Fig. 1). In addition, apoptosis in cystic epithelia was highest during the period of regression in renal volume, suggesting that altered balance between apoptosis and proliferation, favoring apoptosis, may play a role in the regression and “collapse” of cysts.

Previously, it was already suggested that apoptosis plays an important role in the pathogenesis of PKD. Apoptosis was found to be abundantly present in cystic as well as non-cystic epithelia in polycystic kidneys of PKD mouse and patients and was suggested to contribute to the loss of normal renal tubules. Although, apoptosis may not be involved in cystogenesis, it may play an important role in the progression of the disease and the progressive deterioration of renal function.

The regression of renal volume in our mouse model is a striking phenomenon that is also observed in ARPKD patients that survive the neonatal period. However, careful investigation of renal tissue of ADPKD patients revealed regressing or collapsing cysts, as well as cyst remnants. It can be expected that in classical forms of ADPKD, the phenomenon of collapsing cysts will not occur extensively.

Renal volume as clinical end-point used in clinical trials for ADPKD was questioned after disappointing results of clinical trials with mTOR inhibitors. It was suggested that renal volume might not be the best end-point in advanced PKD. Indeed we only observed a minor effect...
on renal volume after low dose sirolimus treatment in the $B6Ola-Pkd1^{nl,nl}$ model. In line with our data, regarding renal volume regression, and increased fibrosis and inflammatory infiltrates, a study revealed that extent of fibrosis and inflammation correlates better with disease progression to renal failure than renal volume. It would be worthwhile investigating the applicability of this method as clinical end-point in clinical trials or maybe even in daily clinical practice. Models, in which almost all nephrons develop cysts simultaneously in a relative short time span, are useful to study cyst initiation and progression. However, models closely resembling advanced stage human ADPK are of outmost importance, especially to better predict the effect of new therapeutics.

With regard to the characteristic aspects of human ADPKD disease progression, fibrosis and inflammation, the $B6Ola-Pkd1^{nl,nl}$ model shows great similarity with the human ADPKD renal pathology. Even more, when onset and degree of nephrons involved is taken into account, the model mostly resembles patients with two incomplete penetrant alleles of PKD1 and PKD2, showing a severe early onset renal cystic phenotype. It would be interesting to identify the course of the disease renal volume changes in these patients.

The $B6Ola-Pkd1^{nl,nl}$ model not only resembles the phenotype of human ADPKD; it also shows altered activity of canonical Wnt signaling, TGF-β signaling, the mTOR pathway and Hippo signaling, all of them are found to be altered in models for PKD and, importantly, in renal tissue ADPKD patients.

In conclusion, the renal cystic phenotype of $B6Ola Pkd1^{nl,nl}$ mice seems to reflect the phenotype of patients with early onset PKD as result of two hypomorphic mutations. In addition, this model showed that regression of cysts and renal volume occurs, implying that renal volume may not be a good predictor of progression to renal failure and end stage renal disease.

**General discussion**

In this thesis it was shown that injury induced proliferation is involved in cyst formation. Altered PCP and Wnt signaling create a permissive condition in which requires an additional factor setting cyst formation in motion. Abnormal integrity of the newly formed cells, resulting from the absence of Pkd1 expression during repair, might be such a factor (Fig. 1). As the base of this aberrant cellular integrity lies within the epithelial repair it would be interesting to investigate the process of repair more carefully, as in our study no differences at the end of repair between controls Pkd1 heterozygotes and conditional Pkd1-deletion mice were observed, while others did for Pkd1 and Pkd2 mutant mice with other types of injury. Possibly, differences can be observed in earlier phases, before repair approaches completion.

In ADPKD patients injury induced proliferation may occur as well. The injury causing factors can be of exogenous as well as endogenous origin. Especially when there are already cysts present...
surrounded by relatively normal renal parenchyma. These cysts compress the surrounding tissue and are a source for oxidative stress and injury, causing proliferation. When the disease progresses more, inflammatory cells as well as the presence of fibrotic tissue become a source of injury and proliferation. This accelerates the cyst formation in healthy tubules and in its turn provides more sources of injury and proliferation.

Then we showed that the Hippo signaling pathway is also implicated in cyst growth. This pathway is known to be implicated in organ size control, tumorigenesis and recently also tissue regeneration and stem cell self renewal. Although a complex network of signaling pathways is involved in cyst growth, finding treatment for ADPKD may benefit from the great promise that the Hippo-YAP pathway holds as a target for anti-cancer therapy and regenerative medicine.

Finally disease progression was studied and showed that the \textit{B6Ol-a-Pkd1} mouse model showed regression of renal and cystic volume followed by fibrosis and inflammation. This was not seen in the conditional \textit{Pkd1}-deletion model. In these mice fibrosis is hardly observed probably due to the rapid cyst formation in all most all nephrons synchronously, leading to renal failure. Furthermore, in these mice \textit{Pkd1} deletion is only achieved in renal epithelial cells while in \textit{B6Ol-a-Pkd1} mice all cells express low levels of \textit{Pkd1}, including fibroblast and inflammatory cells. As altered activation of Cdc42, a protein involved in cell migration, was found to be involved in cystogenesis in \textit{Pkd1} mutant mice\textsuperscript{10}, it would interesting to investigate the effect of \textit{Pkd1} deletion in fibroblast and inflammatory cells and their role in ADPKD disease progression. In addition, macrophage depletion studies in mice with PKD provide evidence for an accelerating role of macrophages in growth of renal cysts\textsuperscript{60}. However, in the \textit{Pkd1} model used only renal epithelial cells were depleted of polycystin-1, while the \textit{Pkd2} model all cells have reduced polycystin-2 expression. As inflammation and interstitial fibrosis, in which macrophages play an important role, are important in ADPKD disease progression, therapies also targeting macrophage recruitment into the kidney may be beneficial in the treatment of ADPKD disease progression.

Due to the similarities with the human ADPKD renal pathology the \textit{B6Ol-a-Pkd1} mice provide a suitable model to study the effect of potential therapeutic drugs on ADPKD disease progression. In addition this model showed that renal volume is a poor clinical end point as surrogate for disease progression.

Taken together cyst formation, cyst growth and ADPKD disease progression are complex processes regulated by many signaling networks. This not only provides many targets for therapeutic intervention but is also a difficulty at the same time. Aiming for a single therapeutic target will most likely be not successful, and therapeutic intervention in ADPKD requires a multi-target therapy.
References


