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**Title:** Molecular mechanisms involved in renal injury-repair and ADPKD progression
**Issue Date:** 2020-09-10
CHAPTER 6

Summarizing discussion
Autosomal Dominant Polycystic Kidney Disease (ADPKD) progression involves a complex interaction of different molecular pathways, ultimately leading to cyst growth and loss of kidney function. The exact mechanism behind cyst formation is still not clearly understood. Moreover, we know some of the molecular pathways involved in cyst initiation and progression, but we do not know at which stage of the disease they play a role.

In this thesis, we investigated the molecular pathways involved in renal injury-repair mechanisms and ADPKD. According to the currently available literature, injury-repair and ADPKD are two extremely intertwined mechanisms, which not only are characterised by activation of similar molecular pathways but are also able to influence each other. In fact, injury is able to accelerate cyst formation and progression, and cyst growth can cause injury to the surrounding tissue. Thus, the introduction of injury in the context of ADPKD can help to characterize the steps of disease progression, particularly in the early phases of cyst initiation, and direct future research to new possible therapeutic targets.

In chapter 2, we investigated the role of FJX1 in PKD and injury. We showed that the lack of Fjx1 in Pkd1/Fjx1 double KO mice was able to limit the effect of injury on disease progression. Indeed, double KO mice with toxic renal injury lived significantly longer compared to single Pkd1 KO mice with renal injury. Analysis of the renal tissues revealed that differences in cyst initiation and progression could not explain this. In fact, 2 KW/BW ratios and cystic index at mild stages of disease were comparable in double KO and Pkd1 KO mice, suggesting that Fjx1 does not play a role in cyst initiation and progression. Consistently, the examination of the two Fjx1 downstream pathways, the planar cell polarity and Hippo pathways, which are believed to be involved in cyst progression, did not show significant changes in the two genotypes.

Interestingly, even though cyst growth was comparable, we observed reduced injury marker expression and fibrosis in double KO mice compared with Pkd1 KO. Based on the current knowledge, cyst growth and expansion cause compression of the surrounding tubules and vessels leading to local injury, ECM deposition and activation of pathways involved in ADPKD pathogenesis. Consequently, there is an increased likelihood of additional cyst formation and accumulation of fibrosis, which ultimately result in the organ function decline. However, it was always difficult to establish whether inflammation and fibrosis are responsible for, or just a consequence of cyst formation. In chapter 2, we showed a separation between cyst progression and fibrosis in the absence of Fjx1 expression. Indeed, we confirmed that injury could accelerate cyst formation in Pkd1 mutant mice, regardless of the presence or absence of Fjx1, suggesting that some of the pathways involved in response to injury are crucial in cyst formation and progression. At the same time, we observed that the inflammatory and fibrotic responses, which follow the initial injury event and the chronic local injury
induced by cyst growth and compression, are in part dependent on \textit{Fjx1} expression and do not influence cyst formation significantly. However, they did impact the functionality of the cystic kidneys and the survival of the animal. Altogether, our results suggest that \textit{Fjx1} regulates pathways related to the fibrotic response and that these are critical in the advanced stages of the disease, more than in cyst initiation and expansion. Such results are particularly interesting, as they demonstrate how modulation of the injury response can help mitigate disease progression and might be considered as a part of a therapeutic approach in PKD.

Among the possible pathways that might be responsible for the reduced inflammatory and fibrotic response in double KO mice, TGFβ and WNT pathways are particularly interesting. TGFβ and WNT pathways have been extensively described for their role in renal fibrosis\textsuperscript{13-16}. We found reduced expression of \textit{Tgfb1} and three targets genes (\textit{Pdgfb}, \textit{Fn1} and \textit{Col1a1})\textsuperscript{17-19} in double KO mice compared to \textit{Pkd1} KO. Similarly, WNT targets \textit{Axin2}, \textit{Cd44}, \textit{Ccnd1} and \textit{Myc} were lower in double KO mice\textsuperscript{20-24}. Future investigation is needed to define the mechanistic link between FJX1 and the TGFβ/WNT pathway. A clarification of the underlying molecular mechanism might open the path for future therapies not only in the context of ADPKD but also of other chronic kidney diseases.

In chapter 3, we explored the option of modulating one of the FJX1 downstream pathways, the Hippo pathway, to halt PKD progression. In particular, we decided to target the pathway effector YAP, as we observed increased nuclear localization of YAP in cyst lining epithelial cells\textsuperscript{2}. The advantage of our approach lies in the use of a mouse model that develops cyst in all the kidney segments recapitulating the situation in humans closely. In addition, we used Antisense Oligonucleotides (ASO) as a therapeutic strategy, which could be reasonably easily translated into the clinic. Although we were able to achieve a reduction of about 70% of \textit{Yap} expression in the kidneys, we did not see any improvement of the cystic phenotype. Thus, our results suggest that YAP does not play a critical role in cystic proliferation. However, we could not exclude that TAZ might be compensating for YAP reduction, leaving open the option that targeting both YAP and TAZ might be a better approach to cyst growth inhibition. \textit{In vitro} experiments revealed that \textit{Taz} KO cells did not show altered cyst formation and cyst growth compared to wild-type cells. In line with our \textit{in vitro} findings, \textit{Taz} deletion mice developed a mild cystic phenotype, even in the absence of \textit{Pkd1} KO\textsuperscript{6,25}. Moreover, TAZ and Polycystin 1 (PC1) can directly interact and participate in common signalling routes\textsuperscript{26}. Hence, reduction or depletion of TAZ levels might worsen disease progression. This advises against the possibility of targeting TAZ in PKD.

Another consideration is that YAP and TAZ have mostly overlapping yet also unique functions\textsuperscript{27}. This is supported by the partly distinct expression pattern of YAP and TAZ in
the different segments of the kidneys and by the impossibility to generate a Taz KO line in mIMCD3 cells, suggesting that YAP and TAZ dynamics may differ in different segments of the nephron. Nevertheless, the modulation of YAP levels might affect TAZ functions and vice versa. Indeed, it has been reported that YAP inversely regulates TAZ protein levels, meaning that reducing YAP levels might result in overactivation of TAZ\textsuperscript{28}. However, such dynamics are not completely clear and should be addressed in future research.

To complicate the picture further, YAP and TAZ are at the crossroad of several signalling pathways, such as TGFβ and WNT pathways. When phosphorylated, YAP and TAZ are restrained in the cytoplasm of the cell, where they can interact with SMADs and β-catenin and regulate their localization and transcriptional activity\textsuperscript{29}. In our study, we observed increased expression of some of the downstream targets of TGFβ and WNT pathways in Yap ASO treated mice. In detail, we observed a significant increase in the expression of Myc in Yap ASO treated mice, and a similar trend for Axin2, both WNT pathway targets. Among the TGFβ pathway targets, we found increased expression of alpha-smooth muscle actin (Acta2) and vimentin (Vim); we also observed a consistent trend for collagen 1 alpha-1 (Col1a1), fibronectin (Fn1), plasminogen activator inhibitor-1 (Pai1) and matrix metalloptidase 2 (Mmp2). Therefore, our results show that reduction of YAP results in increased activation of the WNT pathway target MYC, known to be a critical player in PKD\textsuperscript{30,31}, and of some of the TGFβ targets involved in fibrosis, a well-known biological process involved in PKD progression\textsuperscript{32}. Since activation of TGFβ and WNT pathways has been described in PKD\textsuperscript{33,34}, the effect of YAP/TAZ modulation on these signalling routes, and how it affects PKD progression, must be addressed before pursuing this line of therapy.

An additional critical take-home message is the importance to use the right set-up in the study of new possible targets and therapeutic approaches. For example, we observed that Yap KO in cells was able to impair cyst formation in 3D cyst assays. This may suggest YAP as a perfect candidate for PKD treatment. However, characterization of the mutant cell lines revealed that Yap KO resulted in impaired expression of integrins, which are important for the interaction of the cells with the ECM and the correct establishment of the cystic structures. As a consequence, Yap KO cells were able to form cysts only sporadically. However, the sporadic cysts could grow normally, suggesting that proliferation was not affected. Consistently, in vivo, we did not observe any effect on proliferation after Yap ASO treatment.

Although our findings cannot exclude that the Hippo pathway is involved in cyst growth (possibly via YAP independent routes), we believe that a therapeutic intervention for PKD based on the modulation of YAP levels might not be feasible.
In chapter 4, we generated a robust PKD gene expression signature using a combination of a meta-analysis of PKD expression profiles mined from the literature and our newly generated expression data. This approach allowed us to overcome single study biases related to experimental or technological variations and to come up with a list of genes likely involved in PKD. Moreover, based on the assumption that PKD progression and renal injury-repair mechanisms are strongly linked together\textsuperscript{1,35-37}, we characterized the overlap between injury-repair related genes and the PKD signature and found 35% overlap. Even more, injury-repair genes were involved in 65% of the molecular functions connected to PKD progression, confirming the strong link between PKD and injury-repair mechanisms.

From the comparison of our signature with an independent PKD dataset obtained from \textit{Pkd1} mutant mice at different stages of the disease\textsuperscript{38}, we could see significant enrichment of the PKD Signature genes throughout disease progression. Interestingly, we observed a major contribution of the genes involved in injury-repair mechanisms in the more severe stages of the PKD. In contrast, the genes only involved in PKD and not in injury-repair were more enriched in the early stages. Therefore, we can zoom in on genes consistently dysregulated in PKD, and, at the same time, obtain insights into the temporal and mechanistic importance of the different genes identified.

Additionally, we compared our PKD Signature with a study focused on macrophage populations in renal injury, as macrophages have a critical role in response to injury but also in PKD progression\textsuperscript{39-42}. We were able to observe enrichment for genes related to the different macrophages populations in the PKD Signature, with a larger contribution of injury-repair genes than of the non-injury ones. Nevertheless, we could not discriminate the origin of the expression between the macrophages and the epithelial cells as our study used total kidney lysates to generate the expression profile. In the future, generation and integration of datasets from single-cell sequencing in cystic kidneys might help to clarify this point and gain more information about the role of macrophages and other cell populations in PKD.

With this study, we provide an extensive analysis of expression profiles in PKD with a particular focus on the effect of renal injury in the progression of the disease. We believe that this work can provide a proof of principle on how to use this knowledge to expand our understanding of the PKD progression and discover attractive drug targets and molecular processes for therapy.

In chapter 5, we further explore the PKD Signature outlined in chapter 4. Mainly, we decided to focus on the Transcription Factors (TFs) in the signature. TFs control the expression of genes involved in a variety of biological functions, which intervene in the establishment and maintenance of cell states, both in physiological and pathological situations. Dysregulation
of TFs activity can be at the base of the development of a broad range of diseases. Using computational approaches, we interrogated the signature in different ways. First of all, we defined the list of TFs dysregulated in PKD using MsigDB, and identified those with an involvement in injury-repair. Several of the TFs identified were already known in PKD, proving the validity of our approach. At the same time, we identified many other TFs never described in PKD before, which might be interesting candidates for future studies. Subsequently, employing the ChEA 2016 database of TFs targets, we predicted TFs that are relevant to PKD based on the enrichment of their targets in the PKD Signature. This method allowed us to identify TFs that were missing in the signature, maybe because their expression level is not changed in PKD. Nevertheless, their activity is likely changed, as the expression of their targets is altered in PKD progression. At the same time, knowing which TFs and their targets were deregulated in the different stages of the disease, gave us insight into which molecular mechanisms might be affected. Finally, pathway analysis of the identified TFs using Genetrail2 and Wikipathways revealed enrichment for pathways like the TGF-β pathway, oxidative stress, cellular metabolism, interleukins signalling, adipogenesis and estrogen signalling and apoptosis, which have been shown to be involved in PKD43-46.

To validate our approach, we focused on two TFs for further wet-lab experiments. We selected STAT3 and RUNX1 as they showed the most significant change in expression, both in PKD progression and injury. We confirmed that the expression of the TFs and their putative targets were altered in kidneys from Pkd1 KO mice compared to Wt. Moreover, we set-up and performed a ChIP assay and confirmed an increase in the binding activity of STAT3 and RUNX1 to the promoter region of their target genes in cystic kidneys compared to Wt kidneys. Immunohistochemical analysis revealed that STAT3 and RUNX1 are virtually not expressed in healthy kidneys, both in human and mice. However, their expression is visibly increased in cystic kidneys and after renal injury, confirming our computational analysis. Increased expression of STAT3 has been described before in several ADPKD mouse model, in human cystic tissues and also after renal injury47-49. Indeed, we were not surprised to find it back in our analysis, and we consider it a proof of our approach reliability. Additionally, previous in vitro evidence suggested that STAT3 might be directly activated by cleaved PC1, although the exact mechanism linking Pkd1 deletion and STAT3 activation in cystic tubules was still elusive49,50. A recent study in Pkd1 KO mice demonstrated that STAT3 activation occurs after macrophage recruitment by secretion of CCL2 early after gene deletion. However, they observed that tubular STAT3 was only partially responsible for cyst growth, but was central in the establishment of a feedback loop that limits immune cells infiltration in cystic kidneys51. The results provided in this work denote that a clear mechanistic evaluation of interesting targets is paramount in the development of targeted therapies in ADPKD. RUNX1 involvement in ADPKD has never been described before. Recently, RUNX1 was proposed as a regulator of TGF-β-induced renal tubular EMT and fibrosis52. TGF-β signalling is involved
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in ECM deposition and cyst progression\(^4^1\), making it plausible for RUNX1 to play a role in ADPKD. Still, we need to obtain more insight into the molecular mechanisms behind the involvement of RUNX1 in PKD before being able to select it as a therapeutic target.

With this work, we further dug into the PKD Signature and showed how this could be used to better understand the role of TFs in different steps of disease. Further analyses are needed to clarify the molecular mechanisms behind the contribution of these TFs to PKD progression and cyst formation.

Concluding remarks

To this day, the scientific literature reports a variety of dysregulated molecular mechanisms in ADPKD progression. However, a clear hierarchical overview of these events is still missing, thus making it difficult to separate the early effects of PKD gene disruption on cyst initiation, from the secondary effects of disease progression and cyst expansion. Moreover, we need to keep in mind that different regions of the kidney might be experiencing different steps of progression at any time. Inevitably, we need to deepen our knowledge of the steps of disease progression and the molecular mechanisms behind them. Our functional annotation of the genes in the PKD signature allowed us to identify biological targets that are most relevant in the different phases of the disease, from the initial moderate phase to the more advanced and severe one. The knowledge we generated is just a starting point for future studies that need to evaluate in detail the actual significance of the identified mechanisms in PKD.

We know that by inducing injury and the subsequent repair phase, we can accelerate cyst initiation and growth. However, I believe that injury-repair must be considered more as a means to understand the initial phases of cystogenesis more than a possible target for therapy. Indeed, even though injury-repair mechanisms are involved in PKD progression, some of the biological functions they control are actually employed to cope with the alterations taking place during disease progression.

In my opinion, particular attention should be given to the elucidation of the early stages of cyst formation. However, this is not an easy task. At this stage, the renal tissue is still mostly unaffected. As a consequence, the major input to all kind of -omics data analysis is coming from the healthy tissue, while the signal coming from the diseased tissue is lost in the noise. Thus, to gather information about these early stages, we need to employ different models and more sensitive methodologies. For example, organoids (3D cyst assay and renal organoid) might offer a simplified framework to study the early events that occur after \( Pkd1/2 \) inactivation. However, a significant limitation of these systems is that they cannot take
into account the contribution of infiltrating cells, nor that of the fluid flow, both extremely important in PKD. The implementation of spatial transcriptomics methods gives another possibility. Single-cell RNA sequencing is a powerful technique that can be employed to get insight into the contribution of the different cell types in the kidneys. However, since cells need to be dissociated to perform this technique, the information relative to the positions and interactions of the different cells is lost. A possible way to overcome this is the use of a newly developed technique called Slide-seq\textsuperscript{53}. With Slide-seq, the RNA can be transferred from a tissue section to a support covered in barcoded beads allowing to generate a spatially resolved gene expression profile. This kind of approach might be extremely useful in the quest to understand the molecular mechanism of ADPKD progression.
References


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