Molecular mechanisms involved in renal injury-repair and ADPKD progression

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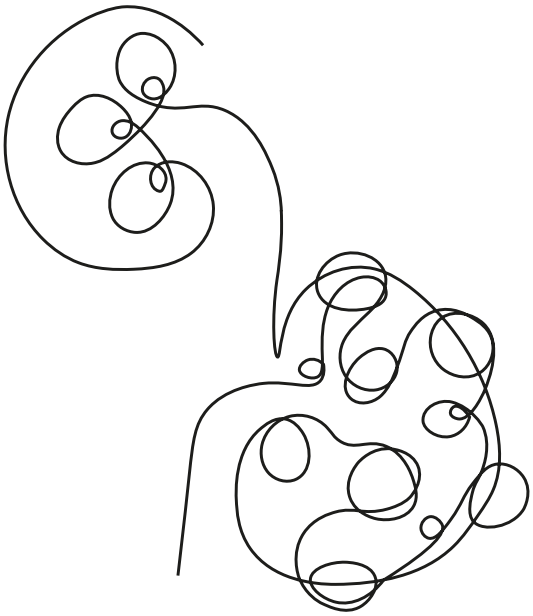

CHAPTER 1

General Introduction

Adapted from:

Molecular pathways involved in injury-repair and ADPKD progression

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1. Autosomal Dominant Polycystic Kidney Disease

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a heritable genetic disorder with a prevalence of <5/10.000 in the European Union[1]. The major hallmark of ADPKD is the formation of many fluid-filled cysts in the kidneys, which ultimately impairs the normal renal structure and function, leading to end-stage renal disease (ESRD)[2,3]. Additionally, extrarenal manifestations such as liver and pancreas cysts and cardiovascular abnormalities are also present[2].

In the majority of the cases, ADPKD is caused by a mutation in either of two genes: *PKD1* in 85% of the case; *PKD2* in 15% of the cases. *PKD1/2* genes encode for Polycystin 1 (PC1) and Polycystin 2 (PC2), respectively[2]. PC1 is a very large membrane protein of 4303 amino acids, with a long extracellular N-terminal, eleven transmembrane domains, and a small intracellular C-terminal[4]. PC2 is a much smaller transmembrane protein of 968 amino acids, with six transmembrane domains, and intracellular N- and C-terminal[5]. The polycystins are localised at various location in a renal epithelial cell. Particularly, PC1 expression has been observed at the apical and basal side of the epithelial cells, the primary cilium, and several lateral junctions. PC2, instead, have been observed mainly at the primary cilium, basolateral membrane and endoplasmic reticulum[6]. The exact functions of these two proteins are still not completely understood. PC1 may function as a receptor able to respond to both mechanical and chemical signals and transducing them to downstream signalling. Indeed, the intracellular portion of the protein can be cleaved and translocate to the nucleus where it interacts with several transcription factors like β-catenin and STATs[7-10]. PC2 seems to be a non-selective cation channel and might be regulating the intracellular Ca^{2+} concentration, influencing several signalling pathways[4,6]. However, the molecular mechanisms that lead to cyst initiation and progression after the loss of functional levels of PC1/2 are still not understood.

Conversely, the pathophysiology of the disease progression is mainly known (Figure 1). In most cases, ADPKD patients carry a germline mutation in one allele of *PKD1/2* genes. Throughout life, due to somatic mutations in the unaffected allele (second hit mutations) or to stochastic fluctuations in the gene dosage of *PKD1/2* (haploinsufficiency), the level of expression of PC1/2 drops below a critical threshold[11]. As a consequence, renal epithelial cells are more prone to cyst formation. Interestingly, the time between the critical reduction of PC1/2 and cyst initiation can be influenced by the biological context. As evidenced by several studies, differences in timing and location of gene inactivation, the metabolic status, the genetic context and introduction of renal injury can influence cyst formation and progression[12]. Once cysts have been formed, proliferation and fluid secretion contribute to the cyst size increase, which eventually cause stress on the surrounding tissue resulting

in local injury and fibrosis[13]. In the advanced stages of the disease, cystic kidneys are characterised by local injury, production of growth factors and cytokines, infiltrating cells and progressive fibrosis, which ultimately lead to loss of renal function[11].

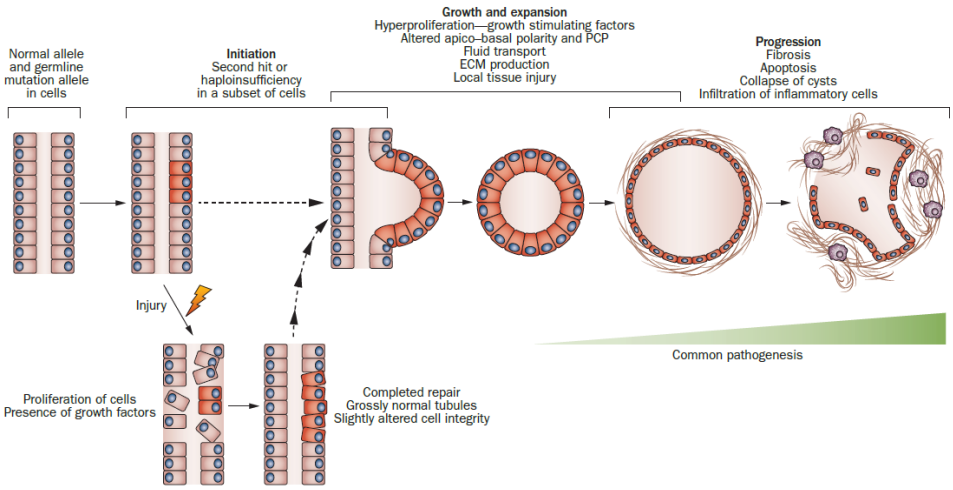

Figure 1. Disease progression in ADPKD

A single *PKD1* or *PKD2* allelic mutation is inherited in patients affected with ADPKD. Later in life, due to a second hit mutation in the unaffected allele or to stochastic variation in gene expression, the level of PC1/2 expression drops under a critical threshold. As a result, epithelial cells are more prone to initiate cyst formation. The time between the critical reduction of PC1/2 levels and the initiation of the cysts is variable and can be influenced by other events, for example, renal injury. After injury, the tissue repair occurs in the absence of sufficient levels of PC1/2 resulting in an abnormal tubular epithelium. The structurally altered epithelial cells are more prone to cyst formation, accelerating disease progression. After cysts are formed, increased proliferation and altered fluid secretion help the cyst to grow and expand, compressing the surrounding tissue. Mechanical stress, as well as secretion of cytokines and growth factors, generate additional injury locally, which contributes to cyst formation and fibrosis deposition. In the more advanced stages of the disease, the tissue is increasingly fibrotic, with visible cellular infiltrates and loss of normal parenchyma. Image from Happé *et al.* [11]

2. Renal injury and repair mechanisms

Following a renal insult, the kidneys are able to repair the injury themselves by inducing proliferation of surviving tubular epithelial cells[14]. During this regeneration phase, tubular epithelial cells are lost or show an aberrant morphology (e.g., loss of brush border and flattening of proximal tubular epithelial cells). Also, infiltration of inflammatory cells is observed[15]. All these events ensure a proper repair of kidney structures and function. However, in some cases the damage is too extensive, or the injury insult persists leading to tissue remodelling, progressive fibrosis and loss of renal function. This fibrotic phase is characterised by chronic inflammation, expansion of alpha-smooth muscle actin (αSMA) positive cells, capillary rarefaction and hypoxia, which fuel the deposition of extracellular

matrix (ECM) and, at the same time, perpetuate local injury leading eventually to chronic kidney injury (CKD) and ESRD[16] (Figure 2a).

3. Renal injury and progression of ADPKD

The link between cyst progression and injury has been already suggested by Weimbs, who postulated that a possible role for PC1 is to sense renal injury via changes in luminal fluid flow. As a result, PC1 activates molecular pathway transducers, such as mTOR and STAT6, leading to increased proliferation and repair of the injured kidney tissue. In ADPKD, reduced levels of PC1 might trigger the activation of proliferation even in the absence of injury, resulting in cyst growth and expansion[17]. However, especially in adult kidneys, deletion of *Pkd1* alone does not immediately translate in cyst formation, which occurs only after a lag period. Another event, such as renal injury, must occur to start cyst formation[17]. In line with this idea, several research groups employing different kinds of renal injury (e.g., nephrotoxic compound, ischemia-reperfusion or unilateral nephrectomy) demonstrated how acute kidney injury (AKI) was able to speed-up cyst progression in mice[18-22]. Additionally, cyst expansion causes mechanical stress to the surrounding tissue and vessels together with the secretion of cytokines and growth factors, activating pathways involved in cyst progression and resulting in a snowball effect that supports more cyst formation[13]. Interestingly, various mechanisms normally active during the injury-repair phase such as proliferation, inflammation, cell differentiation, cytokines and growth factors secretion, are also activated during PKD progression, and largely overlap with the mechanisms at play during renal development[17,22-24]. In fact, renal epithelial cells in ADPKD kidneys often appear "dedifferentiated" with reduced expression of the epithelial marker E-cadherin, which is compensated by increased expression of the mesenchymal marker N-cadherin. The switch in cadherins seems to be a direct effect of the missing interaction of PC1/2 with the E-cadherins at the adherens junctions. As a consequence, the cells lose the proper polarisation resulting in a less differentiated phenotype and alterations in cellular functions[25,26]. Moreover, ADPKD cells re-express genes normally expressed during developmental stages and silenced in adult tissues, in line with the partial dedifferentiated phenotype observed[27]. Altogether, these events can ultimately contribute to disease progression. In a normal situation, reactivation of the aforementioned pathways following renal injury allows remodelling of the tissue and a proper organ repair. Instead, in a context of PKD-related gene mutations, aberrant or chronic activation of these developmental pathways and repair/remodelling mechanisms results in exacerbation of the disease (Figure 2b).

In the following paragraphs, I will discuss some of the main pathways involved in injury-repair and ADPKD.

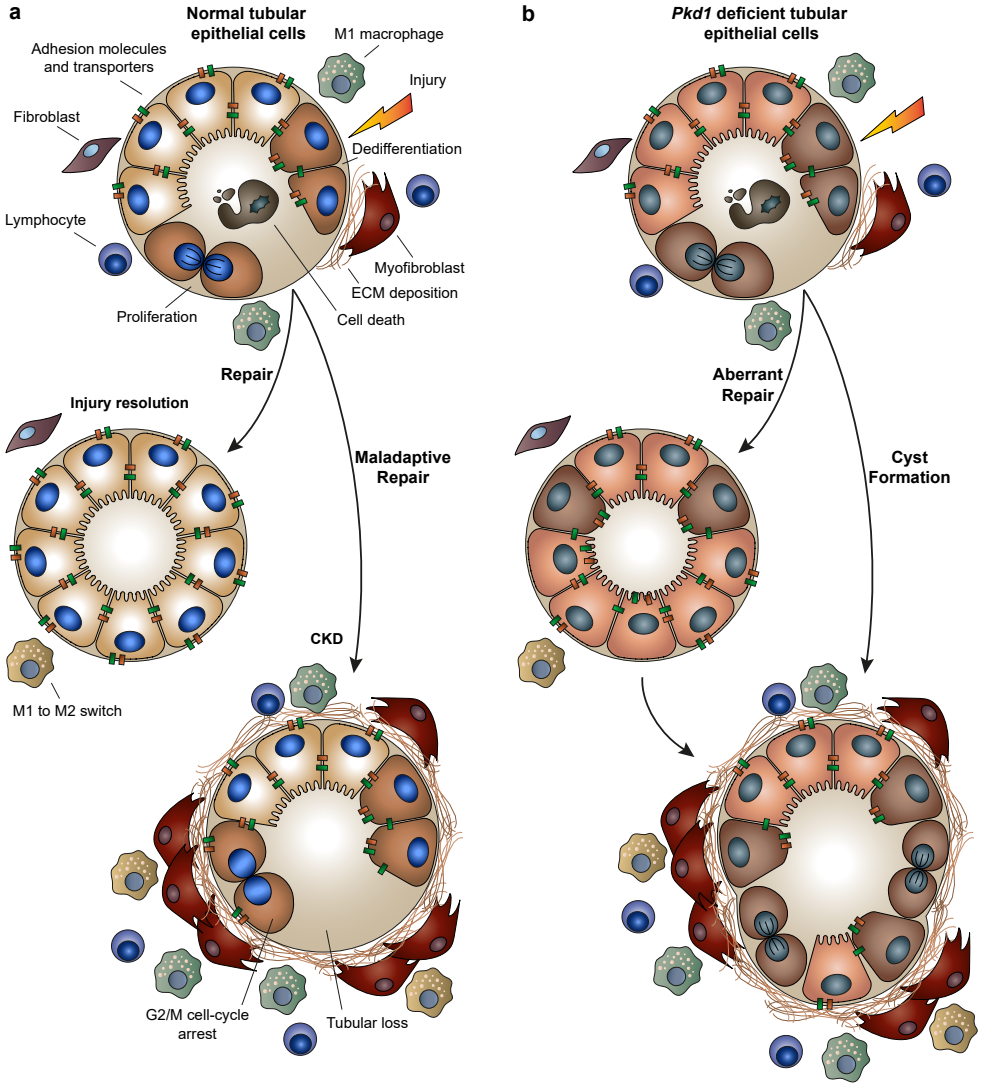

Figure 2. The evolution of injury in normal and *Pkd1* deficient kidneys

a) After injury, the renal epithelium can regenerate the damaged and lost tissue. This phase is characterised by the dedifferentiation and proliferation of epithelial cells, as well as the recruitment of leucocytes and the activation of fibroblasts in myofibroblasts. When all these processes work harmoniously, the tissue is restored and the injury resolved. The infiltrating macrophages undergo an M1-like to M2-like switch and help in the resolution of the inflammatory response and renal growth. However, in case of severe damage or chronic activation of the inflammatory signalling, it is possible to have maladaptive repair. Proliferating cells may arrest in G2/M phase and start to produce pro-inflammatory and pro-fibrotic molecules that fuel a chronic inflammation with progressive collagen deposition and loss of the normal parenchymal structure, leading to chronic kidney disease (CKD). **b)** After injury, *Pkd1* deficient kidneys can repair the tissue damage or can develop cysts, depending on how intense the injury insult was, and/or the genetic makeup of the organism. However, even in case of tissue repair, *Pkd1* deficient kidneys show aberrant repair with altered cell polarity and cell differentiation, providing a potential explanation for the increased speed of cyst formation observed after injury.

3.1 Injury-repair and fibrosis

3.1.1 Renal injury

Repair after renal injury relies on the surviving epithelial cells, which can dedifferentiate to be able to spread and migrate to cover exposed tubular tracts and to proliferate in order to restore the integrity of the tissue. Indeed, injured kidneys are strongly positive for proliferation markers such as Ki67 and proliferative cell nuclear antigen (PCNA)[28,29]. Once the integrity of the tubules is restored, cells need to differentiate back into fully mature epithelial cells to re-establish the proper function of the organ. It has been proposed that laminin-integrin interactions might drive re-differentiation of the epithelium. In particular, laminin-5 and $\alpha_3\beta_1$-integrin expression are increased after ischemic kidney injury[30]. Interestingly, laminin-5 expression is also increased in the ECM of ADPKD kidneys. Stimulation with purified laminin-5 can activate the extracellular-signal-regulated kinase (ERK) in traditional cell cultures and can stimulate proliferation and cyst formation in three-dimensional cultures[31]. Moreover, a hypomorphic mutation in laminin-5 in mice causes cyst formation, both in the cortex and medulla, showing that defects in ECM components are sufficient to cause PKD[32].

After renal injury, expression levels and activity of transforming growth factor-beta (TGF-β) are increased and play a role in maintaining the injured tubule in a dedifferentiated state. This is necessary for cell proliferation and repair of the tubule[33]. The TGF-β superfamily of proteins comprises a group of highly conserved secreted morphogens, which regulate a variety of developmental and homeostatic processes. Upon binding of the TGF-β family members to their receptors, a series of phosphorylation events are triggered. The signalling cascade ends up with the phosphorylation and subsequent activation of the SMAD transcription factors, which translocate to the nucleus where they can drive gene expression[34]. Thus, TGF-β can suppress the expression of epithelial markers and increase the expression of mesenchymal markers, such as αSMA and vimentin, leading to partial dedifferentiation, i.e. epithelial-to-mesenchymal transition (EMT). Although EMT is a physiological event during renal development and useful adaptive response to injury, sustained EMT can result in increased matrix deposition and cytokine production that lead to CKD. Moreover, TGF-β can stimulate ECM deposition by acting directly on the transcription of fibronectin, proteoglycans, collagens and integrins. At the same time, TGF-β antagonises matrix degradation by stimulating proteases inhibitors production[35]. In line with these findings, several *in vivo* and *in vitro* experiments have shown that TGF-β might be the mediator of AKI-to-CKD progression[36].

3.1.2 PKD

EMT and excessive ECM deposition are also characteristic features of ADPKD.

Immunohistochemical analyses of human kidneys in advanced stages of ADPKD showed increased αSMA^{+} myofibroblast and interstitial fibrosis, loss of epithelial markers in favour of mesenchymal ones in tubules, and increased TGF-β-SMAD signalling[37]. All these events suggest that local injury and TGF-β regulated EMT might play a role in ADPKD progression. Increased TGF-β and nuclear phospho-SMAD2 staining are often observed in cyst-lining epithelium and interstitial cells surrounding cysts, both in mice and humans[38]. Shear stress, induced by fluid flow, on wildtype and *Pkd1*$^{-/-}$ tubular epithelial cell cultures activates the TGF-β downstream targets SMAD2/3, which is prevented by administration of TGF-β-neutralizing antibodies and by inhibitors of the TGF-β-binding type-I-receptor ALK5 (activin receptor-like kinase 5). This indicates that autocrine TGF-β signalling is activated upon shear stress in renal epithelial cells[39-41]. This response was higher in *Pkd1*$^{-/-}$ cells because of more TGF-β-production. In addition, it has been shown that TGF-β can restrict cystogenesis in a three-dimensional culture of both murine and human cells[42,43]. However, in mice, conditional ablation of *Alk5* together with *Pkd1* in renal epithelium did not result in amelioration of PKD progression, while sequestering of activin A and B, other members of the TGF-β family, via administration of the soluble activin receptor IIB, lead to amelioration of PKD progression in three different mouse models[44]. Paracrine effects on interstitial cells, rather than autocrine effects might be critical in PKD, and it seems that there is context-dependent effect of TGF-β family members. Indeed, it is known that TGF-β can both inhibit and promote cell growth, drive differentiation but also dedifferentiation of cells, and can be helpful in injury but can also be the major driver of fibrosis. Although the signalling process is essentially the same, the context, cell types and the cofactors involved shape the outcome of the signalling[34]. Hence, further studies to investigate the role and the possible use of TGF-β as a therapeutic target in ADPKD are needed.

The cells mostly responsible for ECM deposition, are myofibroblasts. Myofibroblasts can originate from different precursor cells, but the most important seem to be renal fibroblasts, resident macrophages and other cells of hematopoietic origin that migrate into the kidney[45]. These cellular transitions, together with direct injury-induced damage to the vasculature, can contribute to the loss of capillaries surrounding the renal tubules resulting in local hypoxia, a known driver of fibrotic response in CKD[46]. In ADPKD, cyst formation and expansion is associated with altered vascular architecture. In particular, peritubular microvasculature shows signs of regression of larger capillaries together with flattened arterioles and atresic venules. At the same time, cysts are surrounded by a dense but disorganised capillary network, which forms a sort of "vascular capsule"[47]. The observed vascular alteration can be the result of expanding cysts, exerting mechanical compression of intrarenal vasculature and impairing its function. This could also be directly related to reduced expression of the polycystins in the vasculature where they have crucial roles in mechanosensation, fluid-shear stress sensing, signalling and maintaining structural integrity[48-52]. Regardless of

the primary mechanism, a final common outcome is the development of local hypoxia, which in turn induces expression of hypoxia-inducible factor-1 alpha (HIF-1α) in the cystic epithelium, and HIF-2α in interstitial cells[53] (Figure 3). Particularly, HIF-1α seems to have a central role in cyst growth *in vivo*, because deletion of *Hif1a* in a conditional kidney-specific *Pkd1* mutant mouse model was able to reduce fibrosis and improve PKD progression[54]. Additionally, gene expression studies of renal cells from cystic human and murine kidneys found a consistent hypoxia gene expression profile suggesting that hypoxia has an important role in ADPKD progression[23,55]. In fact, on one hand, hypoxia in ADPKD contributes to the

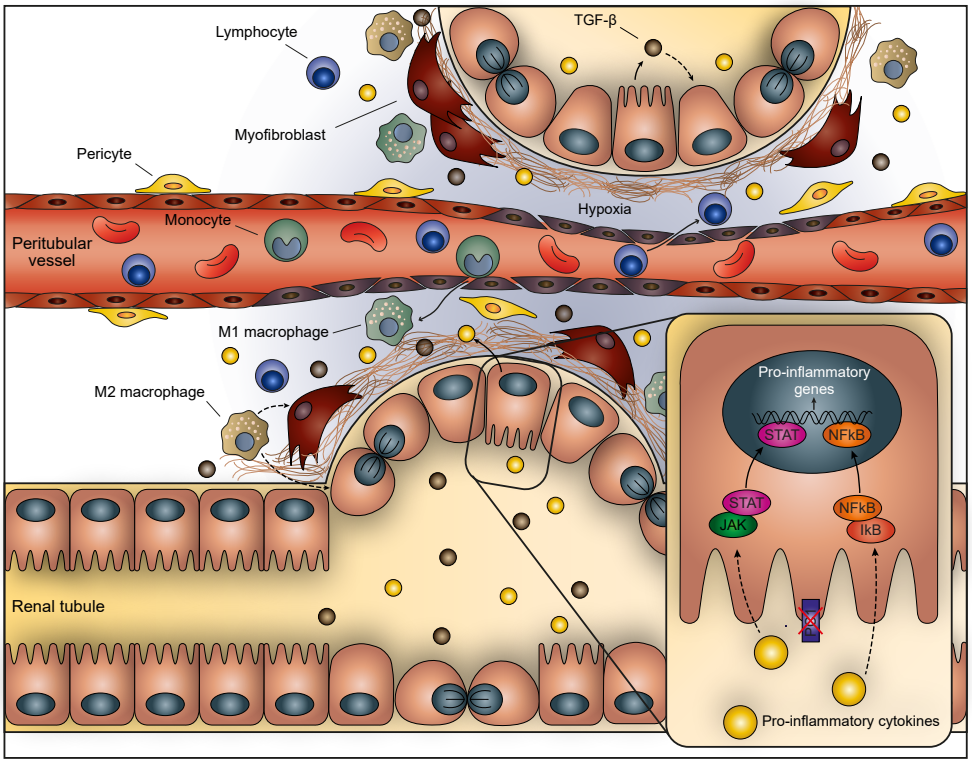

Figure 3. Effect of cyst expansion on the surrounding tissues

Cyst expansion causes mechanical stress to the surrounding tissue and vessels. As a result, injury-related mechanisms are activated, and cytokines and growth factors are secreted in the renal tubules and the surrounding interstitium. TGF-β secretion drives tubular cell dedifferentiation, recruitment of infiltrating cells to the cyst site, and activation of αSMA$^+$ myofibroblasts with increased extracellular matrix deposition. M2-like macrophages accumulate around the cysts where they secrete anti-inflammatory and pro-fibrotic molecules that stimulate tubular cells proliferation and myofibroblasts activation. Pro-inflammatory stimuli and accumulation of fibrosis lead to pericyte dissociation resulting in microvasculature rarefaction and local hypoxia, which exacerbates the fibrotic response. Inflammatory cytokines, such as TNF-α, IL-1β and INF-γ, as well as the deletion of PC1, activate two major inflammatory pathways in renal epithelial cells: NF-κB and JAK-STAT. As a result, pro-inflammatory molecules are produced and released, attracting and activating even more infiltrating cells, which aggravate the local injury and ultimately contribute to cyst progression.

hypervascularisation of cysts, increasing the cysts' nutrient intake, which is necessary to sustain their growth. On the other hand, it contributes to interstitial fibrosis by driving pro-fibrotic responses, ultimately leading to organ failure and contributing to ADPKD progression.

3.2 Inflammation

3.2.1 Renal injury

Following renal injury, both innate and adaptive immune responses intervene to respond to the tissue damage. Injured tubular epithelial cells release pro-inflammatory cytokines and chemokines, growth factors and adhesion molecules, such as interleukin-1 (IL-1), tumour necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1) and TGF-β, which help with the recruitment and activation of immune cells[15]. This early pro-inflammatory response is crucial to clear the tissue from dead cells and cellular debris. At the same time, immune cells also secrete chemoattractant cytokines and growth factors in a self-perpetuating feedback loop that recruits and activates surrounding cells, stimulates angiogenesis and contributes further to the injury response. Two important pathways activated by this response are the nuclear factor-κB (NF-κB) pathway and the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. The NF-κB pathway is activated by the pro-inflammatory cytokines secreted after injury (e.g., TNF-α, MCP-1), which bind to their specific ligands on the tubular cells resulting in the phosphorylation of the NF-κB inhibitor, IκB, and subsequent nuclear translocation of NF-κB complex[56]. Also JAK-STAT pathway is activated by pro-inflammatory cytokines (e.g., IL-6 and interferon-gamma/ INF-γ), which activate JAK that in turn, activates STAT proteins leading to their translocation into the nucleus[57]. The final effect of the activation of these two pathways is the transient transcription of pro-inflammatory genes that encodes for cytokines and growth factors, which sustain the recruitment of leucocytes to the site of injury. To counterbalance this first inflammatory phase, infiltrating leucocytes can also secrete anti-inflammatory and pro-fibrotic factors that lead to activation of myofibroblasts and ECM deposition. When these processes occur harmoniously together, and in collaboration with tubular epithelial cells, the injury can be successfully healed. Conversely, in case of persistent or extensive injury, chronic activation of this pro-inflammatory response together with the chronic production of anti-inflammatory and pro-fibrotic factors can result in a maladaptive repair and progressive fibrotic renal disease[58].

3.2.2 PKD

ADPKD cannot be defined as an inflammatory disorder. However, renal histology analysis of patients with ADPKD showed apparent interstitial inflammation and fibrosis in both minimally and severely cystic kidneys[59,60]. Also, pro-inflammatory molecules, such as TNF-α,

MCP-1, osteopontin and IL-1β can be found in the urine and cyst fluid of human patients[61-63]. Moreover, several studies described the accumulation of infiltrating inflammatory cells, such as macrophages and T cells, in the renal interstitium and urine of ADPKD patients[59,64,65] (Figure 3). Particularly, macrophages seem to have a key role in cyst progression. Transcriptome analysis in the congenital polycystic kidney (*cpk*) mutant mice (non-orthologous mice with a mutation in the *Cys1* gene), which are a model of cystic renal disease, showed that the most upregulated genes in the more progressive stages of disease were associated with the innate immune system, and particularly with the alternative macrophage activation pathway (M2-like macrophages)[66]. Furthermore, accumulation of M2-like macrophages around cyst was observed in several orthologous animal models for ADPKD[67,68]. Interestingly, accumulation of macrophages could already be observed at early stages and specifically around PC1 and PC2 deficient tubules[13,68]. Depletion of macrophages leads to the reduction of cyst-lining cell proliferation, lower cystic-index (percent of kidney occupied by cysts) and improved renal function in different mouse models[67,68]. Additionally, deletion of macrophage migration inhibitory factor (*Mif*) or pharmacological inhibition of MIF, which is upregulated in cyst-lining cells and is responsible for macrophage recruitment, resulted in reduced MCP-1-dependent macrophage accumulation in the cystic kidneys and subsequent delay of cyst growth in several PKD mouse models[69]. Since comparable results were observed in both orthologous (with a *Pkd1/2* mutant gene) and non-orthologous models (with mutations in genes other than *Pkd1/2*), it is plausible that M2-like macrophages have a common role in cyst development regardless of the genetic mutation that is causing it. Indeed, M2-like macrophages are able to stimulate tubular cells proliferation after injury, promoting tissue repair[70]. However, *in vitro* M2-like macrophages stimulated formation and proliferation of microcysts, and *in vivo* they might have increased the tubular proliferation observed after injury in a model of adult-induced cyst formation[68,71]. These results imply that M2-like macrophages in PKD have a detrimental role more than a protective one; thus, therapies that can target specifically this population in ADPKD patients might be beneficial.

3.2.2.1 Inflammatory cytokines

Among all the different inflammatory cytokines, TNF-α seems to have a particularly relevant role in cyst formation. High levels of TNF-α are found in cysts' fluids of ADPKD patients, and gene expression is increased in murine cystic kidneys, where it positively correlates with age and cyst size[61,72,73]. *In vitro*, stimulation of inner medullary collecting duct cells with TNF-α was accompanied with the altered subcellular localisation of PC2 and disruption of PC1-PC2 interaction. Moreover, both wild-type and *Pkd2*$^{+/-}$ embryonic kidney explants treated with TNF-α developed several cyst-like structures. *In vivo*, intraperitoneal injections of TNF-α increased the incidence of cyst formation in *Pkd2*$^{+/-}$ mice of 8.5 weeks of age. Additionally, also TNF-α receptor -I and -II, and TNF-α converting enzyme (TACE) are enriched in human ADPKD cyst fluids, where they contribute to accumulation and stabilisation of bioactive

TNF-α[74,75]. In fact, inhibition of TACE in *bpk* mice (non-orthologous model with mutation in the *Bicc1* gene) resulted in amelioration of PKD[76].

Another important inflammatory chemokine in ADPKD progression is MCP-1. MCP-1 is a pro-inflammatory cytokine that attracts monocytes at the site of injury[77]. Expression and urinary excretion of MCP-1 is increased already at early stages of the disease in rodent PKD models and human ADPKD patients[62,69,78]. Particularly, analysis of the urinary MCP-1 (uMCP-1) in patients from the TEMPO 3:4 trial, showed that uMCP-1 correlated with renal function and that tolvaptan treatment was able to reduce uMCP-1 levels[79]. Recently, *Cassini et al*. demonstrated that *Mcp-1* expression is increased after *Pkd1/2* deletion already in pre-cystic kidneys and prior to macrophage infiltration[80]. Increased MCP-1 levels led to the recruitment of pro-inflammatory macrophages (M1-like macrophages), which caused direct damage to tubules. Subsequently, these macrophages differentiated to M2-like macrophages, which stimulate tubules proliferation and cyst growth[80]. Genetic deletion of *Mcp-1* together with *Pkd1*, as well as administration of an MCP-1 receptor inhibitor, reduced macrophage infiltration, cyst growth and improved renal function and survival[80]. Therefore, targeting macrophage recruitment and activation might be a promising therapeutic approach in ADPKD. However, administration of an inhibitor of MCP-1 synthesis in a non-orthologous model of PKD in rats was able to reduce interstitial inflammation but did not affect cyst formation, questioning the importance of the MCP-1-recruited inflammatory infiltrates in cysts initiation[81].

3.2.2.2 Inflammatory cytokine related signalling pathways

The major inflammatory pathways activated in PKD are NF-κB and JAK-STAT. NF-κB complex proteins are regulators of transcription of several genes among which inflammatory genes like TNF-α, IL-1β and MCP-1[82]. Increased NF-κB activity has been described in several rodent models for PKD and human ADPKD[83-85]. Particularly, the expression of NF-κB proteins was described specifically in cyst-lining cells from early stages until more progressive ones both in human and rodent PKD model[85]. Interestingly, *in vitro* activation of NF-κB was observed following overexpression or depletion of *Pkd1* or *Pkhd1*, suggesting that upregulation of the NF-κB pathway might be an early feature of ADPKD[83,86,87]. Comparison of transcription profiles of AKI with those of rapidly progressive cystic kidneys revealed an extensive overlap of genes between injury and cyst progression, with the most enriched being NF-κB targets[23,88]. In agreement with these findings, NF-κB pathway inhibition using anti-inflammatory compounds successfully ameliorated PKD progression in animal models, indicating that NF-κB is a viable target for therapy[60,89-91]. However, more studies are needed to characterise the role of this pathway in inflammation in the context of ADPKD. Another link between inflammation and ADPKD is the JAK-STAT pathway. After an injury in normal cells, changes in fluid flow lead to proteolytic cleavage of the C-terminal tail of PC1, which is released

from the membrane and can translocate to the nucleus[7]. Here, PC1 tail can interact with JAK-activated STATs and other transcriptional coactivators (e.g., EBNA2 coactivator P100 and STAT6), participating in gene regulation and transient activation of pro-inflammatory cytokines and chemokines, which in turn recruit leucocyte to the injured tubules[9,10,92]. These findings suggest that PC1 regulation of STATs plays an important role in the transduction of mechanical stimuli from the cilia to the nucleus. Thus, in ADPKD local injury or defective ciliary signalling related to PC1/2 mutations interfere with the normal cilia-to-nucleus transduction and leads to persistent activation of JAK-STAT signalling. As a consequence, the production of pro-inflammatory and pro-fibrotic mediators is increased, ultimately contributing to driving cyst progression. Indeed, STAT3 and STAT6 have been described by several groups to be activated in cyst-lining cells in different PKD mouse models, and inhibition of STAT3 or STAT6 was able to ameliorate the cystic phenotype[93-96]. Consistent with these results, gene expression profiling in human and mouse cystic kidneys found JAK-STAT pathway and NF-κB pathway among the highest upregulated signalling pathways[23,55]. Thus, inhibition of the inflammatory response via modulation of NF-κB and/or JAK-STAT pathways seems to be a promising therapeutic strategy in PKD.

3.3 Growth factors

3.3.1 Renal injury

Recovery from renal injury requires that the damaged tubular cells are replaced with new ones ensuring that the structure and function of the nephrons are restored. For this purpose, the cells that participate in the repair process produce growth factors (GFs), which modulate metabolism, proliferation and differentiation, and allow the tissue to adapt to the injury and finally resolve it. Indeed, there are several lines of evidence showing that administration of epidermal growth factor (EGF), insulin-like growth factor (IGF) and hepatocyte growth factor (HGF) ameliorate the outcome of acute kidney injury, although for some of the GFs the evidence are still controversial[97-101]. Nevertheless, once the injury is repaired, these stimuli should stop. In the case of maladaptive repair, persistent and/or aberrant expression of GFs can lead to the development of fibrosis. Several other GFs have been implicated in fibrosis and CKD progression, i.e. TGF-β1, TGF-α, connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF)[35,102-105].

3.3.2 PKD

Several of these GFs are also upregulated in ADPKD. Probably the best described are TGF-β1, which we already covered in a previous paragraph, and EGF (Figure 4). EGF and EGF family ligands (e.g., TGF-α and heparin-binding EGF/HB-EGF) play an important role in renal cyst expansion. This is based on the fact that cystic epithelial cells have higher expression of

EGF, TGF-α and HB-EGF[106,107]. Transgenic overexpression of *Tgf-α* in mice resulted in cyst formation and accelerated PKD progression in *pcy* mice (non-orthologous model with mutation in the *Nphp3* gene)[108,109]. Moreover, these ligands are found abundantly in cyst fluid of ADPKD patients, and stimulation of cultured epithelial cells with cysts fluid promotes cyst formation and expansion *in vitro*[110]. Interestingly, cystic epithelial cells isolated from ADPKD patients are more responsive to the proliferative stimulus of EGF[111]. Additionally, the EGF family ligands receptors (EGFR) are expressed at the basal side of normal adult tubular epithelial cells, while in ADPKD kidneys, they are also localised on the apical side[112,113]. As a consequence, the cystic epithelium establishes an autocrine loop where EGF is synthesised, released into the cyst lumen and utilised by the same cyst-lining epithelial cells, thereby driving their proliferation and cyst expansion. Further evidence is provided by *in vivo* experiments. *Orpk* mice (non-orthologous model with a mutation in *Tg737* gene) with an EGFR mutation that results in reduced EGFR tyrosine kinase activity showed a significant reduction of collecting tubular cysts compared to mice without this mutation. These findings paved the road to therapies that target the tyrosine kinase activity of the EGFR to ameliorate PKD progression[114]. Indeed, treatment with an inhibitor of EGFR tyrosine activity in *Han:SPRD-Cy/+* rats (a non-orthologous model with a mutation in *Anks6* gene), or combination treatment of an EGFR inhibitor together with the reduction of TGF-α in a mouse model of ARPKD (Autosomal Recessive PKD), were successful in reducing cyst formation and increasing survival[115,116]. However, these molecules affect not only the cystic epithelium but all the proliferating epithelia with broad adverse effects, which are not compatible with the life-long treatment necessary in ADPKD where tolerability is a major concern.

Also, other GFs, including IGF1, HGF, VEGF, PDGF, FGF and CTGF have been described to be involved in ADPKD. Increased gene expression of IGF1 and other IGF family members is observed in murine and human renal cysts and is associated with hyperproliferation of *Pkd1* mutant cystic cells[55,117]. HGF and its receptor, the tyrosine kinase receptor c-Met, are overexpressed in cyst-lining epithelial cells in human ADPKD, and levels of HGF are increased in proximal cysts fluid[118]. *In vitro*, *Pkd1*$^{-/-}$ cells showed defective ubiquitination of c-Met after HGF stimulation with a subsequent c-Met-dependent increase of the PI3K/Akt/mTOR signalling pathway[119]. This suggests that, as for IGF-1, also HGF and its receptor may contribute to epithelial cystic cells growth in an autocrine manner.

For other GFs, the effect is a bit broader and also extends to the interstitial cells. VEGF is an angiogenic cytokine that plays pivotal roles in the maintenance of the vascular networks. In rodent and human kidneys, VEGF is lowly expressed in the epithelium of the glomerulus and in collecting ducts[120,121]. On the contrary, in ADPKD VEGF and VEGF receptor-1 are expressed in some of the cysts and dilated tubules epithelial cells, which are also able to secrete VEGF when grown *in vitro*[121,122]. Consistently, increased levels of VEGF are detected in serum and

cystic fluids of *Cy/+* rats[121]. Overexpression of VEGF in mice using a transgenic mouse model (Pax8-rtTA/(tetO)$_7$VEGF) resulted in dose-dependent cyst formation and activation and proliferation of interstitial fibroblasts[102]. However, epithelial cells-secreted VEGF acts also in an autocrine fashion on cell proliferation. In fact, administration of ribozymes targeting mRNA of VEGF receptor 1 and 2 reduced the expression of the receptors in tubular cells with subsequent inhibition of proliferation of cystic epithelial cells[121]. Additionally, administration of VEGFC, a member of VEGF family normally downregulated in PKD, to *Pkd1*$^{nl/nl}$ mice led to the normalisation of the pericystic vascular vessels, reduction of M2-like macrophages infiltration and, in *Cys1*$^{cpk/cpk}$ mice, increase in life span[101]. Altogether, these results suggest that tubular cells in ADPKD aberrantly express VEGF, which in turn stimulates tubular cells,

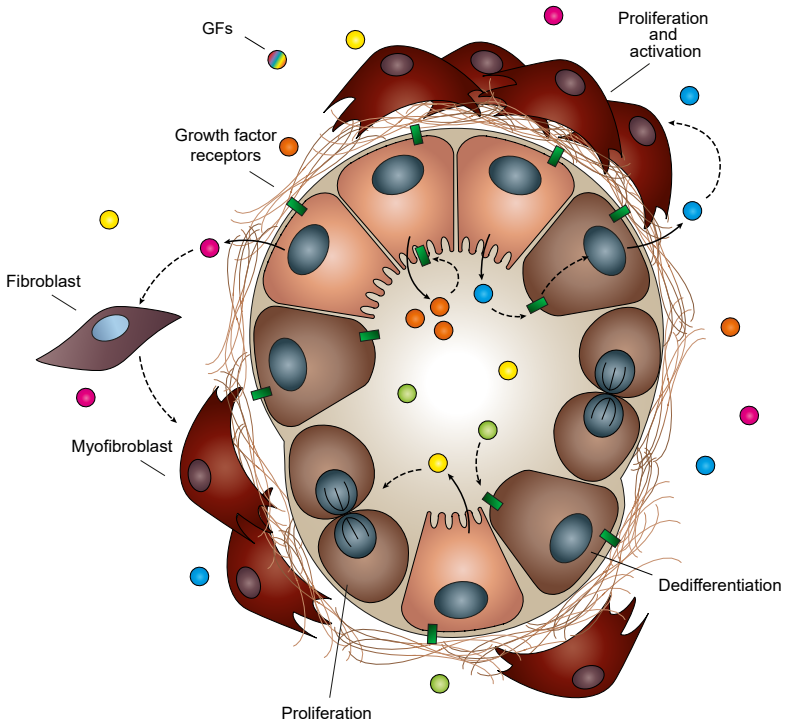

Figure 4. Growth factors (GFs) affecting cyst progression

After injury, secretion of GFs helps to replace the damaged tubular cells. In ADPKD, GFs are produced and secreted in the lumen of the tubules. For example, EGF and other EGF family ligands are overexpressed in cystic epithelial cells and accumulate in the cystic fluids. At the same time, EGF receptors are mislocalised at the apical side of cells in the cystic epithelium. As a result, EGF secreted in the lumen of the cysts can interact with its receptor establishing an autocrine and paracrine loop that drives proliferation and cyst expansion. Another well-known GF in PKD is TGF-β. Cystic epithelial cells often show increased TGFβ expression and nuclear phopspho-SMAD2 staining. TGFβ promotes dedifferentiation of epithelial cells into a more mesenchymal type, as well as ECM deposition and activation of myofibroblasts, contributing to the cystic phenotype. Some GFs like CTGF, but also PDGF and FGF, can be secreted in the interstitial space surrounding the cysts. Here, they can stimulate resident fibroblasts to differentiate into active myofibroblasts, but also sustain myofibroblasts proliferation and activation, with a subsequent extracellular matrix deposition, further contributing to cysts growth.

interstitial fibroblasts and endothelial cells, contributing to disease progression. Thus, targeting VEGF signalling cascade in PKD seems to be a viable therapeutic option. However, treatment of *Cy/+* rats with an anti-VEGF-A antibody led to exacerbation of the cystic disease and severe kidney injury, highlighting the need for more studies to better characterise the role of the different VEGF molecules and their receptors in kidney injury and cyst growth[123].

Other GFs involved in ADPKD, such as PDGF, FGF and CTGF, showed an effect mainly on interstitial cells (Figure 4). PDGF expression, especially PDGF-B, was found in cyst-lining epithelial cells using immunohistochemistry in a human ADPKD kidney[124]. However, PDGF did not show a mitogenic effect on epithelial cells *in vitro* but was able to stimulate the proliferation of ADPKD-derived fibroblasts *in vitro* more effectively than with healthy-derived fibroblasts[125]. A similar effect was observed upon FGF stimulation, which caused ADPKD-derived fibroblasts to proliferate more, produce and release more FGF and elicit a more consistent and lasting tyrosine phosphorylation signalling cascade compared to normal renal fibroblasts[126]. CTGF is most known for its role as a driver of interstitial fibrosis mediating, at least in part, the TGF-β pro-fibrotic program[127]. In normal kidneys, it is expressed mainly in the glomerulus, but in injured tubules and cystic kidneys, its expression is increased particularly at more advanced stages of the disease, in areas of focal fibrosis and in interstitial cells surrounding the cystic epithelium[128-130]. In addition to its role in renal fibrosis, CTGF can also participate in the recruitment of inflammatory cells by activating the NF-κB pathway[131]. Thus, CTGF is a common factor in renal fibrosis, both in injury and ADPKD, and might contribute to the progression of the cystic disease towards the end-stage. Anti-CTGF therapies using a human monoclonal antibody that targets CTGF have successfully improved fibrosis in several animal models and have been tested in clinical trials for pulmonary fibrosis, pancreatic cancer and diabetic kidney disease without notable adverse effects[127]. No data is available about a possible effect on PKD progression, but based on its positive effect on fibrosis and good tolerability, it is plausible to think that anti-CTGF drugs might be a useful adjuvant therapy in ADPKD.

Overall, GFs secreted by cystic tubular epithelial cells trigger surrounding epithelial and interstitial cells to produce proliferative and profibrotic factors, which regulate cyst growth and interstitial fibrosis observed in ADPKD progression.

3.4 Reactivation of developmental pathways

Gene expression analysis of human and rodent kidneys after injury and during CKD compared to healthy control kidneys unveiled aberrant expression of genes that belong to the Notch, wingless-related integration site (Wnt), hedgehog (Hh) and Hippo pathways[132-134]. Although these evolutionarily conserved pathways are regulated and signal through different

routes, they have in common a role in renal development, and illustrate the reactivation of developmental pathways in the injury-repair response[24,135]. However, prolonged activation of these developmental pathways due to chronic or repetitive injury may lead to a maladaptive response and CKD[136,137]. Also in ADPKD, gene expression analysis found signs of cell dedifferentiation and upregulation of developmental and mitogenic signalling pathways[55]. As observed after injury, genes belonging to Notch, Wnt, Hh and Hippo pathways are upregulated in ADPKD, in line with the idea that injury-repair and ADPKD progression share common molecular mechanisms[55].

3.4.1 Notch signalling pathway

3.4.1.1 Renal injury

Notch pathway controls cell proliferation, differentiation and cell fate[138]. During renal development, Notch2 downregulates Six2, a transcription factor expressed in nephron progenitor cells, by suppressing its upstream regulator, Pax2. This leads to the reduction of the progenitor pools in favour of the differentiation of proximal tubular cells[139,140]. Persistent activation of Notch signalling is associated with kidney fibrosis, which is ameliorated with the administration of Notch inhibitors[141]. Interestingly, a well-characterized Notch signalling partner is TGF-β, a known driver of fibrosis. Indeed, TGF-β can directly regulate downstream targets of Notch, and the targets *Hes* and *Hey*, and can induce the expression of Notch ligand *Jag1*. At the same time, the increased level of Notch can stimulate TGF-β expression, creating a positive-feedback loop that sustains renal fibrosis[133,142,143].

3.4.1.2 PKD

Notch signalling genes are enriched in ADPKD, in line with the dedifferentiation and increased proliferation of tubular epithelial cells[144-146]. Protein expression analysis of Notch signalling components in mouse and human ADPKD kidneys revealed increased expression in cysts lining epithelium. Particularly, Notch3 activation was increased, and *in vitro* inhibition of Notch resulted in reduced proliferation and cyst formation in 3D culture of primary human ADPKD cells[82]. Thus, modulation of Notch signalling may be an interesting therapeutic approach to prevent fibrosis and cyst growth. However, there is evidence showing that reduction of Notch signalling during nephrogenesis leads to proximal tubular cysts due to loss of oriented cell division, suggesting that the effect of this pathway on cyst progression might be more complex[145].

3.4.2 Wnt signalling

3.4.2.1 Renal injury

Wnt signalling pathways regulate a range of cellular processes including proliferation,

migration and polarity, thereby contributing to organ homeostasis. They are normally classified in canonical, which involves activation and nuclear translocation of β-catenin, and noncanonical, which is β-catenin-independent and includes the Wnt/planar cell polarity (PCP) route. In kidney morphogenesis, Wnt orchestrates the mesenchyme-to-epithelial transition necessary for nephrogenesis[147]. The involvement of aberrantly activated canonical Wnt pathway with renal fibrosis has been extensively shown in different injury animal model, as reviewed elsewhere[148].

3.4.2.2 PKD

In the context of ADPKD, transgenic mice with increased β-catenin activation present with renal cyst development[149-151]. Moreover, PC1 interacts with β-catenin at the plasma membrane, at cell-cell contacts and in the nucleus, suggesting that PC1 may have an important regulatory role on Wnt signalling[8,26,152]. Once this regulation is lost, for example due to *Pkd1* mutation, the resulting aberrant Wnt pathway activation might contribute to cyst formation. Indeed, one of the major downstream targets of the Wnt signalling, c-MYC, is upregulated in ADPKD, especially in the cystic tubular epithelium[153]. Specific overexpression of *c-Myc* in renal epithelial cells mimicked human ADPKD, and both direct and indirect inhibition of c-Myc *in vivo* resulted in amelioration of the cystic phenotype, placing this protein in a central position in PKD progression[153]. Interestingly, renal injury caused by ischemia-reperfusion (IRI) was associated with activation of Wnt signalling and increase in *c-Myc* expression both in transgenic *Pkd1* mice and non-transgenic control mice, in line with the idea that injury activates pathways involved in PKD progression[22].

Also noncanonical Wnt signalling has been implicated in cyst formation, in particular, the PCP route. PCP orchestrate cell polarity within the plane of epithelial cells and is essential to establish proper cell function and organ architecture[154]. Alteration of oriented cell division (OCD) has been associated with cyst formation[18,155]. However, a recent publication demonstrated that, although altered OCD is a feature of expanding cysts, it is not sufficient nor necessary for cyst initiation after *Pkd1/2* mutation, thus challenging the role of PCP in cyst formation[156]. In chapter 2, we investigate the role of Four-jointed box-1, a component of the PCP route.

3.4.3 Hh signalling pathway

3.4.3.1 Renal injury

The Hh signalling pathway controls embryonic development and tissue homeostasis. Deregulation of this pathway during kidney morphogenesis is associated with severe malformations, indicating that Hh signalling plays a critical role in this process[157]. In mammals, there are three Hh homologous. One of them, sonic hedgehog (Shh) is induced

early after renal injury in tubular epithelial cells and has been implicated in the pathogenesis of fibrosis and CKD by acting on interstitial fibroblasts leading to their activation and ECM deposition. Moreover, Hh signalling pathway can interact and cooperate with other key pathways known to be drivers of fibrosis, such as TGF-β, canonical Wnt and Notch[136,158].

3.4.3.2 PKD

The connection between Hh signalling and ADPKD is complex and mainly via cilia-dependent signalling. Mutations in ciliary genes lead to cystic renal disease, but also aberrant Hh signalling[159-161]. Also, the loss of a functional component of the Hh pathway, Glis2, resulted in the development of nephronophthisis in human and mice[162]. In the context of PKD, downregulation of Hh signalling is accompanied by reduced proliferation and cyst formation in *Pkd1* mutant mice and human primary ADPKD cell cultures[163,164]. However, a recent study using a conditional mouse model lacking *Pkd1* in combination with three Hh signalling members (*Smo*, *Gli2* and *Gli3*) demonstrated that Hh pathway is not required for cyst formation in mouse models of developmental or adult-onset of ADPKD[165]. Thus, it seems that the Hh pathway does not have a causative role for the disease *in vivo*, but it might contribute to disease progression due to its effect on renal fibrosis.

3.4.4 Hippo signalling pathway

3.4.4.1 Renal injury

The Hippo pathway regulates tissue growth and development. Unlike the pathways mentioned above, which are activated by the binding of specific ligands, a diversity of upstream Hippo pathway regulators have been identified. Identified upstream signals include cell polarity, cell junctions, cytoskeleton, mechanical forces, GPCR ligands and stress signals[166]. The core components of the Hippo pathway are a group of kinases (mammalian Ste20 like kinases 1/2 or MST1/2 and large tumour suppressor 1/2 or LTS1/2), which are responsible for the phosphorylation of the final effectors Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ). When the Hippo pathway is activated, YAP and TAZ are phosphorylated and restrained into the cytoplasm; when the Hippo pathway is inactive, YAP and TAZ are unphosphorylated and can translocate to the nucleus where they can bind with a series of transcription factors, such as TEAD 1-4 but also SMADs and β-catenin, and regulate the transcription of a wide range of genes involved in cell proliferation, apoptosis and migration[167,168]. In renal development, mutations of the core kinases or the final effector YAP, lead to disruption of nephrogenesis[169,170]. Interestingly, deletion of the orthologous protein TAZ in a developmental mouse model does not impair nephrogenesis but results in renal cyst formation. This indicates that these two proteins, although having largely redundant functions, also have distinct roles[171]. In kidney injury, nuclear accumulation of YAP and TAZ is observed in both in epithelial and interstitial cells in

several models of injury. In particular, Yap expression is observed in dedifferentiated tubular cells in AKI-to-CKD transition, confirming that the Hippo pathway has a role in injury-repair mechanism[137]. Moreover, after injury, ECM production causes the tissue stiffness to increase, providing the driving cue for fibroblast TGF-β activation. The mechanosensitive response to TGF-β-induced activation of renal fibroblasts is mediated by YAP/TAZ, which interact with SMAD2/3, translocate to the nucleus and drive transcription of profibrotic genes[172].

3.4.4.2 PKD

Increased nuclear localisation of YAP and TAZ has been described in several diseases, among which ADPKD and nephronophthisis[129,173]. Moreover, zebrafish mutant for *Pkd2* and *Scrib*, a member of the SCRIB complex involved in the establishment and maintenance of cell polarity, showed increased nuclear YAP. Interestingly, expression of cytoplasmic but not nuclear YAP could rescue the phenotype, suggesting that cytoplasmic YAP has a role in the suppression of cyst formation[174]. Knock-out of *Yap* in a *Pkd1* mutant mouse model was able to reduce PKD progression mildly, and the effect was even increased by concomitant knock-out of *Taz*. In particular, YAP target, c-MYC, was found to critically contribute to kidney cystogenesis, implicating the Hippo pathway in the pathogenesis of PKD[175]. Interestingly, expression of *Ctgf*, a known YAP/TAZ target[176], which is also induced by TGF-β[127], was increased in *Pkd1* mutant mice but only in those presenting clear signs of fibrosis, suggesting that a certain level of signal crosstalk between TGF-β and Hippo pathways is occurring in the PKD context as well[129]. For its role in modulating cell proliferation and cell migration and fibrosis, Hippo pathway regulation has been proposed as a possible strategy to ameliorate ADPKD progression by acting on two major aspects of the disease. However, administration of YAP-specific antisense oligonucleotides (ASOs) in adult *Pkd1* mutant mouse model did not result in a reduction of cyst growth (data presented in chapter 3). Such results, together with the cystic effect of TAZ deletion in a developmental mouse model, suggest that the role of these proteins and the effect of targeting them in PKD is complex and need further characterisation[171].

3.5 Transcription factors (TFs) and epigenetics in renal injury and PKD

Injury-repair is a complex mechanism that involves several cell types and requires the modulation of a plethora of signalling pathways. Thus, a perfect time- and space-regulated transcription program is paramount for the good outcome of the tissue injury response. For this reason, a series of transcription factors (TFs) and epigenetic changes intervene to orchestrate all the different steps we discussed above, which ultimately lead to organ repair[177]. Altered TFs expression or epigenetic regulation can interfere with injury-repair and lead to CKD[178] and orchestrate and modulate signalling in PKD.

Gene expression analysis in ADPKD revealed dysregulation of TFs, many of which are involved in key processes of kidney development. Interestingly, from a meta-analysis study that identified a set of 1515 genes dysregulated in PKD emerged that 92 of them are TFs, and that about 35% of the identified TFs are known to be involved in injury-repair mechanisms (further shown in chapter 5). Mutations in *Pkd1* are also associated with other epigenetic changes, such as increased expression of DNA methyltransferases (DNMTs), histone deacetylases (HDACs) and bromodomain proteins[179]. For example, SET and MYND domain 2 (SMYD2) protein is a lysine methyltransferase upregulated in PKD. Inhibition of SMYD2 was able to delay cysts growth via interfering with SMYD2-dependent activation of STAT3 and the p65 subunit of NF-κB[180]. Treatment with HDACs inhibitors has also been proven effective in delaying cyst growth and preserving renal function in several *Pkd1/2* mutant mouse models, pointing to epigenetic modifiers drugs as promising candidates for PKD treatment[181-186]. Moreover, epigenetic changes such as hypomethylation of the *Pkd1* gene-body have been described in cystic tissues from ADPKD patients[187,188]. These modifications can interfere with the normal expression of *Pkd1* and might be responsible for disease progression.

4. Conclusions

Altogether, the current knowledge suggests that injury-repair mechanisms are part of ADPKD progression. The two events are so intertwined that it is difficult to dissect them. Indeed, injury can cause or accelerate cyst formation, but at the same time, cyst enlargement is a source of local injury, establishing an injury-like cyst milieu that exacerbates renal function decline. Further investigations are required to be able to separate a direct effect of the polycystins on the cyst initiating dysfunctional molecular mechanisms, from the secondary effects of disease progression and cyst expansion.

5. Aim and outline of the thesis

There is a consistent body of literature that describes the strong connections between the injury-repair mechanisms and ADPKD progression. The scope of this thesis is to identify and investigate molecular pathways involved in injury-repair and ADPKD progression to better characterise the steps in disease progression, and provide new cues for future studies and therapeutic approaches.

In **chapter 2,** we investigate the role of Four-jointed box protein 1 (FJX1) in injury and ADPKD progression. FJX1 is a Golgi kinase implicated in the regulation of two important dysregulated pathways in ADPKD: planar cell polarity (PCP) and Hippo signalling. In a previous study performed in our group, FJX1 was found aberrantly expressed during both the injury-repair

phase and PKD progression in mice, suggesting a possible role for FJX1 in cyst formation and progression. Specifically, we investigated if genetic deletion of FJX1 might influence PCP or Hippo pathway regulation, and result in a modification of the normal PKD progression. We did not find any evidence for differential regulation of PCP or Hippo pathway. However, we observed an effect of FJX1 on fibrosis and cellular infiltrates.

In **chapter 3,** we investigate further the role of the Hippo pathway in PKD progression. Hippo pathway is a highly conserved signalling pathway that regulates organ size. Several of the molecular mechanism modulated by Hippo pathways are also central to cyst growth. Indeed, in a previous study, we observed increased expression of one of the downstream effectors of the Hippo pathway, YAP, in the nucleus of the cystic epithelium. Therefore, in chapter 3 we hypothesise that reducing YAP level in *Pkd1* KO mice might ameliorate the cystic phenotype. We decided to take an approach based on antisense oligonucleotides (ASO) that target specifically YAP transcripts leading to a significant reduction of expression in the kidneys. We found no effect on cyst progression. We also investigated the effect of *Yap* or *Taz* knock-out on cyst formation *in vitro* using a 3D cyst assay.

In **chapter 4,** we use a combined approach based on RNAseq analysis of in house generated *Pkd1*-mutant mouse model and a meta-analysis of publicly available PKD expression profile to identify a list of genes normally dysregulated in PKD. Moreover, we investigated the link between PKD progression and injury-repair mechanisms. Finally, we employed different *Pkd1*-mutant mice, with or without toxic renal injury, to validate the findings.

In **chapter 5,** we elaborate on the work presented in chapter 4 using computational analysis. We primarily focus on the transcription factors (TFs) altered during both PKD progression and injury-repair. We validated our computational analysis with wet-lab experiments, including qPCR, immunohistochemistry, and chromatin immunoprecipitation.

Lastly, a general overview of the results described in the previous chapters and suggestions for future research are discussed in **chapter 6**.

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