THE ROLE OF MACROPHAGES IN THE PROGRESSION OF POLYCYSTIC KIDNEY DISEASE

BY

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Abstract

Polycystic kidney disease (PKD) is one of the most common potentially fatal genetic disorders. The most common form, autosomal dominant polycystic kidney disease (ADPKD), affects approximately 12.5 million individuals worldwide, and approximately 600,000 individuals in the US, over half of whom will develop end-stage renal disease (ESRD). Mutations in either the *PKD1* or *PKD2* genes are the primary cause of this disease, although, there is increasing evidence that non-genetic factors, including kidney injury and inflammatory responses, play a role in PKD progression.

Infiltrating macrophages play an important part in kidney injury and repair as well as in chronic renal inflammatory diseases. Whether macrophages play a role in ADPKD cyst progression however, has remained largely unknown. Recent data from our lab and others have shown that macrophages accelerate disease progression by promoting ADPKD cell proliferation, a driving force behind cyst expansion and subsequent renal failure. In this study, we addressed the hypothesis that macrophages are recruited to the kidneys in response to chronic injury that is induced by progressively expanding cysts. Once in this environment, the injured renal cells stimulate the macrophages to undergo a phenotypic switch that, in turn, allows the macrophages to express pro-proliferative factors that are ultimately detrimental to the kidneys.

Results presented in this study implicate MCP-1 as the predominant contributor to macrophage recruitment to the kidneys; while our MCP-1 knockout mouse model demonstrated a significant increase in survival of mice affected with PKD. In addition, we uncovered signaling through CXCR2 as a potential pathway in the programmed-macrophage-induced pro-proliferative response in ADPKD cyst cells. Experiments on

the effect of WNT5A, the expression of which is upregulated in the presence of macrophages, on ADPKD cyst cell progression pointed towards its importance in the promotion of growth of ADPKD cyst cells. This study highlights the significance of macrophage-induced cell proliferation in the progression of PKD and uncovers several components that can be targeted for the design of drug therapies.

Dedication

This dissertation is dedicated to my parents, Mahmoud Salah and Salwa Ayyad, and my loving husband, Wael Mourad. Their love, support and encouragement are the driving force behind all of my successes.

Acknowledgments

It takes a scientific village to develop a graduate student, and I am ever grateful and indebted to the many individuals who I have come across throughout my graduate career, that have been absolutely instrumental in guiding me through to this point. Their scientific, emotional, intellectual, spiritual and technical contribution and support have shaped me both as a scientist and as an individual, and have helped carry me over seemingly insurmountable obstacles.

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I was blessed to be in a lab comprised of individuals of various scientific and life backgrounds, that made it fun coming to lab every day. I thank Jackie for keeping me

sane and grounded through the tough times when nothing ever seemed to work, and perhaps equally important, for accompanying me to our countless trips to Starbucks. Her stepping up for me in the lab when I was sick or during dissertation writing without second thought is a testament to her helpful and kind personality. I also thank Carolyn Vivian and Jessica Rossol-Allison in the lab for sharing their immense technical expertise and scientific knowledge with me, and for all the fun and entertaining lab discussions we would have. I would also like to thank Tyler Stephenson for his assistance with almost everything we asked of him. I also thank the many undergrads and summer and students who passed through our lab for keeping the lab fun and lively.

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Chapter One

Introduction

Polycystic Kidney Disease (PKD)

Polycystic Kidney Diseases are a group of life-threatening kidney disorders characterized by multiple fluid-filled cysts found primarily in kidneys as well as in other organs[1]. Reduced renal function, mainly due to structural damage of the kidney tissue and microvasculature, is brought on by the progressive enlargement of these cysts[2] and eventually leads to end-stage renal disease (ESRD) for most patients. The rate of disease progression, age of onset, and severity of symptoms vary widely among this group of diseases.

PKD can be acquired by either spontaneous developmental abnormalities or as a result of natural or environmental factors that include aging, drugs, hormones and dialysis treatment[3, 4]. However, PKD is most commonly the result of inherited germ-line mutations in single genes. Inherited PKDs include autosomal dominant and autosomal recessive polycystic kidney disease (ADPKD and ARPKD, respectively), nephronophthisis and medullary cystic disease complex[5, 6]. Of these, Autosomal Dominant Polycystic Kidney Disease (ADPKD) accounts for the vast majority of genetically inherited cases of PKD and is the most studied[7]. It is the primary focus of the studies described herein.

Autosomal Dominant Polycystic Kidney Disease

ADPKD is a common genetic disease, occurring in up to 1 in every 500 individuals, 50% of whom will require renal replacement therapy by age 60 as a result of

end-stage renal disease (ESRD)[1, 8-11]. There are no specific treatments for ADPKD, but those ADPKD patients with ESRD account for approximately 10% of all patients requiring renal replacement therapies[12], costing more than $2 billion per year in the U.S. alone. The slow-expanding cysts, characteristic of ADPKD, can also distort the kidney and result in numerous complications prior to onset of ESRD, for example, hypertension, hematuria, polyuria, and flank pain. ADPKD patients are usually prone to recurrent urinary tract infections and renal stones as well[8]. Furthermore, patients can exhibit extrarenal abnormalities in other organs (i.e., liver, intestines and pancreas) as a result of their disease. Connective tissue abnormalities can lead to complications such as intracranial aneurysms, cardiac valve abnormalities, aortic dissection and abdominal wall hernias[13].

Since ESRD develops later in life for ADPKD patients, treatments that slow cyst growth could potentially delay the onset of ESRD long enough to avoid the need for renal replacement therapy and improve the quality of life for many adult patients. To develop rationally targeted treatments for ADPKD, a thorough understanding of the basic biology of the genes involved and disease pathogenesis is needed.

The primary mutation responsible for 85-90% of ADPKD cases is in *PKD1*, a large gene on Ch 16 that encodes a 4302-amino acid, transmembrane protein polycystin-1 (PC1)[14]. Mutations in another protein, polycystin-2 (PC2), a gene product of *PKD2* (Ch 4), are responsible for most, if not all, of the remaining cases of ADPKD. Polycystin-2 is also an integral membrane protein and shares 25% homology to PC1[1, 15]. As implied by its name, ADPKD results from the loss of a single allele of either of these genes. Mutations in either gene are generally loss of function mutations;

penetrance is essentially 100%, though there is variability in disease presentation and severity among PKD patients including between family members[16].

The one common manifestation is the presence of multiple cysts within the renal parenchyma. These cysts arise from tubular segments, especially in the collecting ducts, and can be considered benign neoplasms characterized by epithelial cell hyperproliferation and abnormal fluid secretion, both of which contribute to the formation and expansion of cysts[17-19]. Renal cysts form via abnormal proliferation of tubule epithelial cells, which, rather than promoting elongation of the renal tubule segment, causes the formation of sac-like protrusions that begin to expand out radially[20]. The majority (75%) of these protrusions that arise from tubule segments eventually detach from their parent tubule after reaching approximately 2 mm in diameter to form isolated spherical cysts, which continue to expand throughout the patients' life span[21]. While ADPKD cyst formation begins early in fetal life, cysts are generally only detectable by ultrasound in patients around 20 years of age, and many afflicted individuals remain asymptomatic past their child-bearing years. This is due to the slowly progressive nature of the disease.

As cysts expand over the course of years, they begin to exert pressure on surrounding lymphatics, capillaries and neighboring non-cystic tubules. This leads to chronic damage of the surrounding parenchyma. Ultimately, the injury caused by expanding cysts, compounded by obstruction of surrounding tubules, culminates to disrupt renal integrity and function, promote fibrosis and eventually results in ESRD[20]. Recapitulating the response observed in non-cystic kidneys following injury, injured ADPKD kidneys produce chemokines and cytokines, which attract fibroblasts,

macrophages, and other immune cells. These interstitial infiltrates are important for effecting repair of injured non-cystic kidneys. In this dissertation, we explore the hypothesis that, in ADPKD kidneys, infiltration of these cells, especially macrophages, exacerbates the disease in a failed attempt at repair.

The Polycystins

Expression and Localization

Polycystin-1 and polycystin-2 are collectively referred to as the polycystins (Figure 1.1). PC1 is a large integral membrane protein with receptor-like structural characteristics[22] that is widely expressed in organs such as kidney, liver, brain, pancreas, small intestine, lungs and heart[23]. It is expressed on the plasma membrane at focal adhesions, tight junctions and adherens junctions, desmosomes, in the shaft and basal body of primary cilia, and possibly the endoplasmic reticulum and nuclei[1, 16, 24, 25]. PC1 is also detected in urinary exosomes, which are small (50-100nm) vesicles secreted by the renal epithelial cells into the urine[26].

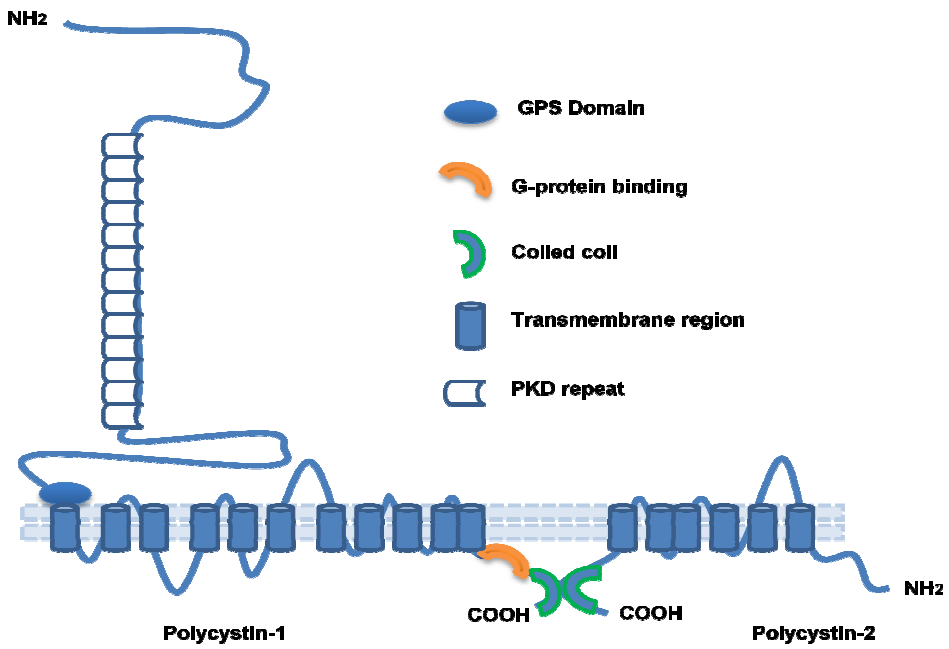


Figure 1.1: Structure of polycystins. Polycystin-1 has 11 transmembrane domains with a large extracellular domain, and a short cytoplasmic C-terminal tail. The coiled coil domain in the C-terminal end of PC1 interacts with the C-terminal tail of Polycystin-2. Polycystin-2 has cytoplasmic N and C-terminus. Redrawn from reference (25).

Although the last six transmembrane domains of PC1 are significantly homologous to the domains of Na+ and Ca^{2+} channels and transient receptor potential (TRP) channels, PC1 overexpression alone does not yield any measurable channel activity[24]. PC2, on the other hand, is a Ca^{2+}-regulated, non-selective cation channel that is expressed predominantly in the ER and in the Golgi[27]. PC2 is also expressed in the plasma membrane, where it forms a complex with PC1[28-30], as well as on the primary cilium[31, 32].

The subcellular localization of both PC1 and PC2 is believed to be clinically significant. It has become apparent that many inherited cyst-forming diseases are the result of mutations in genes that encode proteins that normally localize to the non-motile primary cilia. ADPKD, and these other renal cyst-forming diseases, can be described as

ciliopathies[33, 34]. Primary cilia are microtubule-based sensory structures that extend from the basal bodies or centrosomes of epithelial cells. They have been highly conserved throughout evolution and are capable of detecting a wide variety of physical and chemical stimuli[35]. In renal tubular epithelial cells, fluid flow leads to physical bending of the primary cilium. It is thought that the large extracellular domains of PC1 present on the cilium are capable of sensing the fluid flow and subsequently activate the associated PC2 calcium channels. This induces an influx of extracellular calcium into the cells, causing release of intracellular Ca2+ stores with consequential effects on the cells[32], which will be described later in this chapter.

The identification of PKD as a ciliopathy came from mouse studies using the *orpk* mouse model of PKD. Mutations in *Tg737*, which leads to the renal cystic disease in these mice, also caused stunted primary cilia in their renal epithelial cells[36]. Ift88, the *Chlamydomonas reinhardtii* orthologue of Tg737, is known to be required for intraflagellar transport and assembly of motile cilia[37]. It was subsequently discovered that the polycystins and fibrocystins, mutations in which lead to ADPKD and ARPKD respectively, also localize to the primary cilia along with other renal cystic disease-associating genes[31].

Structure and Function

PC1 is an integral membrane protein with a large extracellular N-terminal domain (~3K amino acids), eleven transmembrane domains (~1K amino acids) and a small C-terminal cytosolic domain of about 200-225 amino acids, which undergoes cleavage at both of its N- and C-terminal domains[38-40]. Partial cleavage of the N-terminal tail of PC1 occurs at the extracellular G protein-coupled receptor proteolytic site (GPS) domain,

resulting in an N-terminal, extracellular fragment that is non-covalently linked to the C-terminal fragment on the membrane[38, 41]. Missense mutations at the GPS domain that prevent PC1 cleavage have been reported in ADPKD patients. This same mutation in the GPS cleavage domain of PC1 was unable to rescue PC1-null cells *in vitro*[38, 42, 43]. This suggests that in order to be fully functional, PC1 N-terminal cleavage at the GPS domain must be able to occur. Not all PC1 proteins present in the cells undergo the cleavage at the GPS however, and the full length un-cleaved protein co-exists with the cleaved version in cells in equal ratios[38]. The C terminal tail of PC1 (PC1 CTT), on the other hand, contains phosphorylation sites for different tyrosine and serine/threonine kinases[44] as well as a G protein interaction domain[45]. The C-terminal region is also where PC1 interacts with PC2 through a coiled-coiled domain[25] (Figure 1.1).

C-terminal cleavage of PC1 releases a soluble ~ 35kD fragment that was first described by Chauvet *et al*[39]. This study showed that nuclear translocation of the PC1 CTT can lead to the activation of the activating protein-1 (AP-1) pathway. Co-transfection experiments using full length PC2 and PC1 CTT led to the retention of PC1 CTT outside the nucleus and a reduction in AP-1 activity, suggesting that PC2 prevents PC1 CTT from entering the nucleus[39].

PC1 CTT cleavage and nuclear translocation is also associated with the mechanosensory effects of cilia, since both unilateral ureteral ligation, which reduces fluid flow, and inactivation of the kinesin family member 3A (*Kif3a*) gene, a subunit of kinesin-II that is essential for cilia formation, led to an increase in the nuclear translocation of PC1 CTT in mouse models. These results demonstrate that reduced fluid flow or loss of sensitivity to fluid flow in renal epithelial cells can cause an

accumulation of PC1 CTT in the nucleus and subsequent transcription of its target genes[39]. A second, more distal cleavage of the PC1 CTT releases a 15-kD fragment that was found to interact with the coactivator p100, as well as the transcriptional activator STAT6 (signal transducer and activator of transcription 6). This cleavage and subsequent translocation of both the PC1 tail and STAT6 to the nucleus was increased after stopping fluid flow[40].

Consistent with its function as a permeable, non-selective cation channel, PC2 directly responds to local increases in Ca^{2+} concentration by releasing intracellular stores of calcium[46, 47]. PC2 also interacts with two other calcium channels, the ryanodine receptor and the inositol 1,4,5-trisphosphate receptor (IP3R), to modulate cytoplasmic calcium levels indirectly[48, 49]. Calcium levels in primary ADPKD renal cyst epithelial cells were found to be significantly lower than non-cystic renal cells when steady-state levels of calcium were measured[50]. Interestingly, disruption of either of the polycytin genes results in perturbations in calcium regulation. Hanaoka *et al.* demonstrated that polycystin-1 and -2 interact to produce new calcium-permeable non-selective cation currents. However, neither polycystin-1 nor -2 was capable of producing these currents individually[28]. It is not clear whether this is due to the functional properties of the PC1-PC2 complex, or simply due to PC2's intrinsic channel properties. As will be described in a later section, calcium regulation by the polycystins is an important modulator of ADPKD cyst cell growth.

Polycystins and Mammalian Kidney Development

Polycystins play an important role in the epithelial cells that give rise to the nephrons during mammalian kidney development. These cells are derived from two

distinct sources. The first originate from the Wolffian duct, where they branch and invade the metanephric mesenchyme (MM) to form the collecting duct system. The second are generated by a mesenchymal-to-epithelial transition (MET) of a group of cells from the MM that form around the ureteric bud (UB), known as mesenchymal aggregates, creating the renal vesicles (RVs) that give rise to the majority of the nephron from the glomerulus to the distal tubule through the process of nephrogenesis. During different stages of nephrogenesis, the renal vesicle develops into the comma-shaped body and the S-shaped body, with the latter fusing with the UB to form a single, continuous epithelial tubule[51, 52] .

To produce a functional kidney, proper coordination of collecting duct and nephron tubule development, morphogenesis and polarity is essential and relies on reciprocal interactions between several intracellular signaling molecules. Polycystin-1 is highly expressed in the cells that give rise to nephrons, as well as in other tissues that correlate with phenotypic abnormalities found in PKD patients, such as in the brain, liver, pancreas, heart, and intestine [23, 53, 54]. Polycystin-1 is predominantly expressed in basal-membrane focal adhesions of migrating epithelia derived from the ureteric bud in kidneys from human fetuses starting at 8 weeks of gestation. Expression of polycystin-1 begins decreasing in 16-week-old fetuses and throughout adult kidney development. It also becomes spatially restricted to just the lateral cell–cell adherens junctions in the medullary collecting tubules[8].

The importance of polycystins in coordination of kidney developmental processes was demonstrated by loss-of-function experiments performed during renal development. In *Pkd1*$^{-/-}$ knockout mice, cyst formation was not observed in tubules until later

embryonic stages of development, a time period correlating with tubular elongation and architectural maintenance, and after completion of nephrogenic condensation and epithelialization. These results were consistent with peaks in polycystin expression in renal epithelia appearing during the same time period, suggesting the importance of polycystin in maintaining renal tubular architecture[55]. Similar results were obtained in *Pkd2*$^{-/-}$ knockout mice, further supporting the role of polycystins in the proper maintenance and elongation of nephron segments[56]. In humans, multiple renal cysts have also been found to occur *in utero* in PKD patients, indicating the importance of the polycystins during early kidney developmental events[57].

Signaling in PKD

Major Signaling Pathways Contributing to PKD Progression

The two major processes responsible for cyst expansion involve abnormal cell proliferation and fluid secretion[58]. An important factor leading to both of these responses in ADPKD is the second messenger, adenosine 3', 5' cyclic monophosphate (cAMP). Yamaguchi *et al.* made the observation that primary human ADPKD cyst cells showed a proliferative response to cAMP in culture, whereas non-cystic human kidney (NHK) cells displayed an anti-mitogenic response to the same cAMP stimulation[59]. The stimulatory effect of cAMP on proliferation of ADPKD cells in this study was associated with activation of the extracellular signal-regulated kinase (ERK) pathway, as inhibitors of upstream components, including protein kinase A (PKA) and mitogen-activated protein (MEK), blocked the proliferative response of ADPKD cells to cAMP[59]. Investigation of pathway components located between PKA and ERK later revealed that ERK activation

was caused by cAMP dependent activation of B-Raf (v-raf murine sarcoma viral oncogene homolog B1) specifically in ADPKD cyst cells, but not in NHK cells[60]. Since disruption of the polycystins affects regulation of intracellular calcium levels, Yamaguchi *et al.* hypothesized that this abnormal polycystin function in ADPKD cyst cells may affect calcium signaling, therefore causing the cAMP growth-stimulated phenotype[61]. This hypothesis was elegantly tested and confirmed by this group when treatment of non-cystic cells with either calcium channel blockers or with agents that lowered extracellular calcium, to mimic the calcium-deficient PKD cell state, caused them to switch to a PKD-like phenotype. This conversion from a normal cAMP growth-inhibited phenotype to an abnormal cAMP growth-stimulated phenotype was associated with activation of B-Raf and ERK upon stimulation with cAMP. These studies determined that disruption of calcium in non-cystic, cAMP-inhibited cells de-represses the B-Raf/ERK pathway, therefore converting these cells to a cAMP growth-stimulated phenotype that resembles that of PKD cells[60, 61] (Figure 1.2).

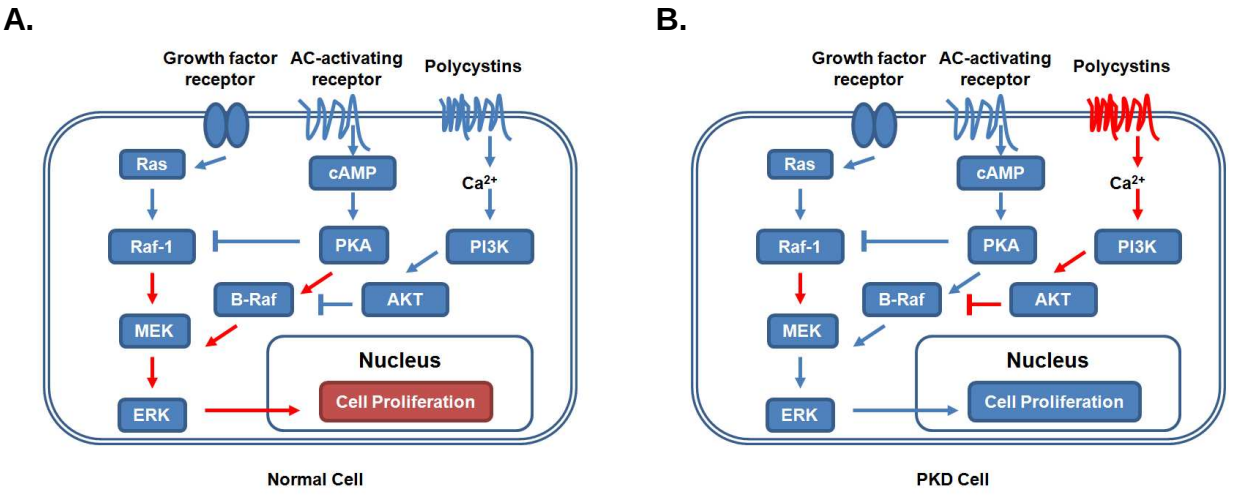


Figure 1.2: Signal transduction pathways in (A) NHK cells and (B) PKD cells. Blue lines, active pathways; red lines, diminished pathways. AC, adenylate cyclase; ERK, extracellular signal-regulated kinase; MEK, MAP kinase kinase; PC, polycystin; PI3K, Phosphatidylinositol-3′-kinase; PKA, protein kinase A. Data from references (60, 61); adapted from reference (62).

Later studies further revealed that the anti-diuretic hormone, arginine vasopressin (AVP), acting through cAMP, is a major growth-stimulating factor responsible for proliferation of the cyst lining cells[63]. These studies demonstrated the importance of AVP in the actual process of cyst initiation using the Battleboro rat, which lacks AVP. When these rats were crossed with an ARPKD orthologous rat model (PCK; *Pkhd1*$^{-/-}$ rats), not only were the kidneys smaller in the double-knockout mice, but the number of cysts developing overall were significantly lower than in *Pkhd1* null rats with completely functional AVP. Importantly, administration of vasopressin analog (desmopressin) to these double-null mice at 12 weeks of age resulted in numerous and enlarged cysts that were comparable to regular PCK mice by 20 weeks of age, whereas their un-treated counterparts remained unaffected[64]. These studies underline the importance of cAMP and its agonists as inducers of cyst initiation and as positive regulators of cell proliferation.

Not only is cAMP an important promoter of ADPKD cyst cell proliferation, but it also contributes to the cyst-growth process due to its role in chloride secretion and fluid accumulation in the cysts. cAMP-dependent fluid secretion is believed to occur through the activation of cystic fibrosis transmembrane conductance regulator (CFTR), which is expressed in the apical membrane of cyst-lining epithelial cells[65-67]. When *Pkd1* mutant kidneys with wild-type *Cftr* alleles were stimulated with cAMP in organ culture, they developed numerous large cysts, whereas kidneys from *Pkd1*:*Cftr* double-null mice had no evidence of cyst formation[68]. Thus, in this model cyst formation in response to cAMP is completely dependent on CFTR. Furthermore, small-molecule inhibitors of CFTR

were found to slow cyst growth in *in vitro* and *in vivo* models of PKD, and patients who have both cystic fibrosis and PKD exhibit a milder form of the disease[68].

Other Signaling Pathways Activated in PKD

Although alterations in intracellular calcium/cAMP downstream signaling and the resulting abnormal proliferative response to these signals in PKD play a central role in the pathogenesis of PKD, the polycystins have also been found to modulate a host of other various signaling pathways through interactions with a wide variety of proteins[25, 69]. A few of these interactions, modulating growth regulation, activation of G proteins or Wnt signaling pathways, will be described in this section.

Growth Regulation

Mammalian target of rapamycin (mTOR), which regulates protein translation, cell proliferation and cell growth[70-72], has been shown to be negatively regulated by PC1. The mTOR suppressor, tuberin (the gene product of TSC2), is tethered to the membrane through interaction with PC1 CTT. Tuberin suppression of mTOR only takes place when it is membrane-bound because, in this state, it is available to bind with the small GTPAse Rheb and its activating partner, TCS1, which affect the kinase activity of mTOR[70]. In normal kidneys with complete PC1 function, mTOR remains mostly inactive and its downstream effectors, effectors- S6K1, S6K2 (ribosomal kinases) and 4EBP1 and 4E-BP2 (eukaryotic initiation factor 4E-binding proteins) responsible for stimulation of protein synthesis and cell proliferation activity, are suppressed. In the absence of PC1 however, tuberin is no longer tethered to the membrane and is instead partitioned to the cytosol allowing mTOR to become activated[70, 72].

This data was consistent with results by Shillingford *et al.* that previously demonstrated an increase in activated mTOR in the cyst-lining epithelium of ADPKD patients[70]. Furthermore, the immunosuppressant drug, rapamycin, which is a specific inhibitor of the mTOR pathway, has been shown to slow the progression of cystic disease in several different rodent models of PKD, suggesting that the activation of the mTOR pathway may be common to all renal cystic diseases[70-72]. Unfortunately, clinical trials of single agent mTOR inhibitors have been disappointing thus far[73, 74], although suboptimal dosing and delayed timing of treatment may have contributed to the lack of effects[75].

The polycystins have also been found to negatively regulate cell cycle progression through their involvement in the janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway. In the presence of an intact PC1 CTT, PC2 has been shown to interact with JAK2, leading to activation of STAT1 and STAT3 via PC1. As a result, cyclin-dependent kinase inhibitor 1 (p21) expression and activity are increased, which in turn inhibits the cyclin-dependent kinase, Cdk2, and thus cell cycle progression is also decreased[76]. PC2 has also been shown to directly interfere in p21 regulation by de-repressing the p21 repressing helix-loop-helix proteins, Id2 and E47, by preventing their nuclear localization and subsequent transcriptional activation of p21[77].

Liang *et al.* was able to uncover another direct PC2-dependent mechanism of reducing cell growth via activation of pancreatic ER-resident eIF2a kinase (PERK). Once activated, PERK in turn increases phosphorylation of eukaryotic translation elongation initiation factor 2a (eIF2a), leading to a decrease in cell proliferation[78].

G-protein Activation

PC1-mediated activation of heterotrimeric G proteins have been shown to stimulate signaling pathways that are growth modulatory and, thus, potentially disease-relevant in PKD. Parnell *et al.* discovered a highly conserved trimeric G protein activation domain in the PC1 CTT [45]. They were able to demonstrate that PC1 activated G-protein α-subunits, which in turn led to the activation of c-Jun N-terminal kinase (JNK) and AP-1 pathways. They found that the activation of JNK/AP-1 pathway is mediated specifically by signaling through Gα (including G_i, G_q, and G_{12} families), as well as G$\beta\gamma$ subunits[79]. The pathway connecting PC1 to G proteins and JNK was corroborated by another group. In these studies they demonstrated that PC1 specifically interacts with $G\alpha_{12}$, negatively regulating its ability to promote JNK-mediated apoptosis in MDCK cells[80, 81]. Notably, their work showed more selectivity in PC1-G protein interaction ($G\alpha_{12}$ but not $G\alpha_{13}$). The reason for this discrepancy is not known, but their distinct experimental conditions, including cellular context, likely account for the differences.

The JNK/AP-1 pathway is involved in many cellular processes including differentiation, apoptosis, and inflammation[82]. Several components of this pathway, including protein kinase C α (PKCα) and small G proteins (Cdc42 and Rac1), were found to mediate this JNK/AP-1 signaling[83]. Interestingly, activity of AP-1 components such as ATF-2, c-jun and c-fos are increased in ADPKD patients and in a hypomorphic *Pkd1* mouse model. Taken together, these data indicate that PKD1-induced JNK/AP-1 activation is mediated via trimeric G protein subunits signaling through small GTP-binding proteins, stimulating a signaling cascade that may influence pathogenesis of PKD[84].

Further evidence for PC1 signaling through G-proteins comes from studies demonstrating that PC1 is capable of activating phospholipase C (PLC) via the $G\alpha_q$ subunit[85]. This interaction was shown to lead to the activation of the calcineurin/NFAT (nuclear factor of activated T-cells) pathway, which is known to regulate apoptosis as well as cellular growth and differentiation[86]. When exogenous PC1 was expressed, nuclear accumulation of NFAT was increased, while co-expression of $G\alpha_q$ further enhanced this effect. The pattern of NFAT expression was also found to be coincident with the presence of PC1 in renal tubular epithelial cells of developing and adult mice, suggesting a possible functional relationship between the two[85]. If PC1 can regulate NFAT target genes, then these data suggest that PC1 deficiency during conditions when PC1 is required, such as during renal development or in response to adaptive signals in the adult kidney, would be expected to cause the mis-expression of these target genes and thereby contribute to cyst growth in ADPKD.

Canonical and non-canonical Wnt signaling

PC1 has been found to regulate the Wnt signaling pathway, implying that this pathway may play some role in ADPKD pathogenesis. Wnts are secreted signaling molecules that play an important role in many developmental processes including organogenesis, embryonic induction, generation of cell polarity, cell proliferation and the specification of cell fate, while their misregulation has been associated with several diseases. Wnts are classically categorized as canonical (β-catenin-dependent), or non-canonical (β-catenin-independent) ligands, depending on the downstream signaling pathway activated. When a canonical Wnt binds its cognate Frizzled receptor with lipoprotein receptor-related protein LRP5/6 co-receptor, β-catenin is stabilized in the

cytosol and translocates to the nucleus, where it binds a member of the T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) family and leads to transcription of proliferative genes[87]. Non-canonical Wnts, including *WNT5A*, activate β-catenin-independent pathways, which include numerous effector molecules including JNK, ROCK, PKC, and NFAT signaling cascades, which mediate widely different functions such as planar cell polarity, convergent extension movements, or neuronal and epithelial cell migration among others[88, 89].

Experiments performed to demonstrate a role for canonical Wnts in ADPKD have generated contradictory results. Using a TCF/Lef-dependent reporter as a read-out for β-catenin stabilization, Kim *et al.* were able to conclude that PC1 C-tail overexpression activates canonical Wnt signaling[90]. In addition, overexpression of either an activated mutant form of a β-catenin[91] or c-myc[92], a β-catenin/TCF-regulated oncogene, induces a renal cystic phenotype in transgenic mice. C-myc has also been previously found to be overexpressed in ADPKD[93, 94] and several other renal cystic diseases[95, 96].

On the other hand, Lal *et al*. demonstrated that the PC1 CTT co-localizes with and binds to β-catenin in the nucleus, thus inhibiting its signaling rather than activating it[97]. Miller *et al.* using PKD-2/WS25 mice, which display a later onset of polycystic kidney disease, as well as *Pkd1* null mutant mice, found no evidence of TCF/Lef activity in cyst-lining cells in either mouse model[98]. Similar results were obtained when another group mated inversin mutant mice, which develop severe cystic kidney disease during late embryogenesis and perinatally, to TCF/Lef reporter mice. As with the Miller group, no reporter activity was observed in cyst-lining epithelia in these mice[99]. The discrepancy between these data have been attributed to the TCF/Lef-LacZ mice

used[100], and the verdict is still out on the exact role of β-catenin signaling in PKD pathogenesis.

There has also been evidence to suggest that non-canonical, or β-catenin-independent, Wnt signaling may play a role in cystogenesis. One of the pathways involved in non-canonical Wnt signaling includes the Planar Cell Polarity (PCP) pathway. PCP contributes to both convergent extension (CE), a process of the intercalation of adjacent cells during development of various tissues, as well as oriented cell division (OCD), the alignment of the axis of cell division during mitosis. Based on findings that several rodent models of cystic kidney disease show tubule epithelial cells with altered planes of division, Fischer and colleagues came to the conclusion that these defects in OCD were causative for cystogenesis[101]. However, more extensive analysis by Nishio *et al.* using several inducible cystic disease murine models, including *Pkd1*$^{-/-}$ and *Pkd2*$^{-/-}$, revealed that loss of OCD is only evident after tubules have dilated and was not present or responsible for cyst initiation[102]. These data suggest that, although alterations in PCP may not induce cystogenesis, defective OCD due to abnormal PCP may promote expansion of nascent cysts and those that have already initiated.

Modifiers of PKD

Genetic Modifiers of Disease

Mutations in either of the polycystins result in essentially identical ADPKD disease manifestations, although patients with PC2 mutations tend to have increased renal survival and fewer overall complications, owing to later disease onset and

generally milder disease manifestations[1]. So far, approximately 314 different loss-of-function mutations in the *PKD1* gene have been identified in 400 families, whereas 91 truncating mutations in *PKD2* from 166 families have been described. Missense mutations have also been identified and make up an additional 25% of mutations in the polycystin genes[18].

A “two-hit” mechanism has been theorized as a method of cyst development in ADPKD patients. This is because analysis of cyst epithelial cells[103, 104] as well as evidence presented by animal models of ADPKD[56], have demonstrated that complete loss of function of a PKD protein, combined with somatic inactivation of the unaffected allele, is required for cysts to develop. However, Rossetti *et al.* have discovered that gene dosage via incompletely penetrant PKD alleles also plays a role in cyst initiation in PKD. They identified hypomorphic *PKD1* and *PKD2* alleles in ADPKD patients and found that the presence of these heterozygous hypomorphic alleles resulted in milder cystic disease. A more moderate to severe PKD phenotype was established when homozygosity of the hypomorphic allele, or compound heterozygosity with another hypomorphic allele existed[105, 106]. Furthermore, either overexpression or lowering of *Pkd1* protein levels in murine models is sufficient to cause cystic disease, suggesting that dysregulation of polycystins is what leads to PKD rather than loss of function *per se*. Dosage levels attributable to *PKD* genetic and allelic variability, as well as other possible gene modifier and environmental effects, likely underlie the large phenotypic variability among affected individuals.

Other Modifiers of PKD Progression

Other factors that may influence the rate of cyst growth and/or susceptibility to somatic mutations include the genetic background of the affected host. This can occur as a result of disruptions to the same pathway or a separate synergistic pathway that is important for cystogenesis. For example, haploinsufficiency of both PC1 and the previously described downstream signaling target protein, tuberin, enhances the severity of disease[107]. Another example is that of the AVP knockout rat, described previously, whose PKD disease progression is significantly reduced despite complete *Pkhd1* deficiency. Once these double-knockout mice were administered with desmopressin, an AVP analog, multiple cysts developed that were equal to or greater than those of the *Pkhd1* knockout rats alone[64]. This suggests that another "hit" in addition to loss of both alleles is necessary for cystogenesis. Other factors may also constitute a "third hit" and affect disease progression, including the development stage of the kidneys affected and renal injury, which will be described in detail in the upcoming section.

Developmental Stage and Injury as Modifiers of PKD Progression

Several lines of evidence indicate that the developmental stage of the kidney influences the initiation and progression of cystic disease. In a seminal set of experiments, Germino and colleagues showed that inactivation of the *Pkd1* gene, using an inducible knockout model, induced rapid renal cyst formation and disease progression, but only if the gene was deleted in early developing kidneys, i.e., prior to PN 13[108]. In contrast, *Pkd1* knockout induced in mice past PN 13 resulted in slow and variable disease onset and progression. Similarly, loss of genes required for primary

cilia function, including *ift88* and *Kif3a*, leads to a rapid cystic phenotype only in the young mouse, whereas appearance of cysts is significantly delayed when these genes are inactivated during adulthood[109, 110]. For example, in the *ift88* knockout model, renal cysts are only observed after 6 months of gene inactivation in the adult mouse[109]. The mechanisms underlying this striking effect are not completely understood; however, one characteristic of this early developmental period is that there is abundant epithelial cell proliferation that diminishes over a time frame that temporally correlates with the loss of susceptibility to induction of rapid cystic disease.

Another modifier of disease that may share some common pathogenic mechanisms is kidney injury. Takakura and colleagues showed that renal injury resulting from ischemia/reperfusion (IR) accelerated PKD progression in an inducible *Pkd1* knockout adult mouse model[111]. Other models of renal injury also accelerated PKD progression. For example, injury induced by the nephrotoxicant 1,2-dichlorovinyl-cysteine (DCVC) after *Pkd1*-gene inactivation was also found to accelerate cyst formation significantly in adult mice[112]. Other experiments showed that *Pkd1* or *Pkd2* haplo-insufficiency alone was sufficient for microcyst formation and increases in tubule dilation after acute injury[113, 114]. Further evidence was provided by acute kidney injury experiments in adult *Kif3a* mutant mice[110]. In addition, unilateral nephrectomy, which leads to hypertrophy of the remaining kidney, performed on the *ift88* ciliary protein conditional knockout model triggered accelerated renal cyst formation in 8-week old adult mice[115]. Taken together, these studies suggest that injury in a cyst-prone background is sufficient to accelerate the onset of cystogensis.

As with developmental stage, the mechanisms by which injury promotes cystic disease progression are not fully understood. However, one common feature is that in the kidney post-injury, there is a hyperproliferative state that is a reflection of the repair response. This repair response is thought to produce a more favorable condition for cystogenesis in a polycystin-deficient background[108, 116]. This hyperproliferative state is similar to that seen in early kidney development.

These experiments suggest that initiation or acceleration of cystic disease progression in PKD may be the result of the loss of polycystins “predisposing” cells to cyst formation in response to a mitogenic response[62, 117]. Thus, it is possible that any pre- or post-developmental event in a kidney with abnormal polycystin or ciliary function that introduces a mitogenic response may potentially constitute a “third hit” resulting in cystogenesis. Moreover, these findings imply that, in the adult kidney, an essential role of the polycystins, or other ciliary proteins, is to dampen or halt post-injurious hypertrophic and proliferative signals. Reminiscent of this role is their function in the developing kidney to inhibit proliferation after renal maturation is complete. The idea of re-introducing their anti-proliferative response post-injury is supported by the observation that tubule cells continue to form cysts in their absence. Continued cyst growth as a result of this impaired inhibition process leads to further injury and continuous activation of the innate renal epithelial repair program in an endless cycle of cyst-promoting injury response, all culminating to eventual renal destruction[118].

Macrophages

Macrophages and Their Phenotypes

In recent years, macrophages have been shown to play a central role in the repair of damaged tissue post-injury. Renal macrophages are mononuclear cells that are members of the renal mononuclear phagocytic system (MPS). They are mostly derived from bone-marrow hematopoietic stem cells (HSCs) that give rise to monocytes. Circulating monocytes are released into the blood system, where they further differentiate into macrophages and/or dendritic cells[119]. Depending on environmental conditions, renal bone-marrow derived mononuclear phagocytic cells (MPCs) play a host of roles that relate to homeostasis, inflammation and surveillance against injury and repair within the kidney[120]. For example, under steady-state conditions, MPCs are believed to differentiate into resident macrophages, which are responsible for phagocytosis of apoptotic tissue and replenishment of cells lost to injury via growth factor production to maintain tissue homeostasis. These resident macrophages may be replenished via circulating monocytes, although they are also capable of self-renewal and proliferation[121].

The plasticity of macrophages allows them to switch phenotypes into diverse functional states that can be artificially divided into either an M1 or M2 state, depending on their response to different *in vivo* environments or *in vitro* stimuli[122, 123]. M1 macrophages (in the mouse, $F4/80^{+}Cd11c^{-}MR^{-}$) are “classically activated” by stimuli from cytokines, such as IFNγ (*IFNG*), LPS, TNFα, or GM-CSF and produce nitric oxide (NO). M2 macrophages ($F4/80^{+}Cd11c^{-}MR^{+}$) are “alternatively activated” by IL-4 and IL-13 stimulation and express high levels of arginase-1, mannose receptor (MR), and IL-

10[124-126]. M2 macrophages are further divided into, but not limited to, the following subsets (Table 1.1): M2a, M2b, and M2c based on their gene expression profiles[126]. The M2a subtype is generated by IL-4 or IL-13 stimulation; M2b by IL-1R ligands or exposure to immune complexes in addition to LPS, and the M2c subtype by IL-10, TGF-β, and glucocorticoid hormones. These M2 subsets generally demonstrate an IL-2^{low}, IL-23^{low}, and IL-10^{high} phenotype. They have been found to promote growth due to their ability to re-purpose arginine metabolism to ornithine and polyamines[127].

Type	M1	M2a	M2b	M2c
Stimulation/activation	IFN-gamma TNF-alpha LPS	IL4/IL13	ICs LPS LTR/IL-1R	IL-10 TGF-beta GCs
Expression	CD86 CD80 MHC II↑ IL-1R I TLR2 TLR4 iNOS (converts arginine to L-citrulline and NO)	CD163 MHC II SR CD206 ↑ (MR↑) TGM2↑ Decoy R IL-1R II *Mouse only:* *Ym1* *Fizz1* *Arg-1*	CD86 MHC II	CD163 TLR1 TLR8
Cytokines: Produced by macrophage sub-sets	TNF-alpha IL-1beta IL-6 IL-12 IL-23	IL-10 TGF-beta IL-1ra	IL-1 IL-6 IL-10 TNF-alpha	IL-10 TGF-beta
Putative Function(s)	Potent microbicidal properties Promotes Th1 responses	Exert immune regulatory functions Drive Th2 responses	Exert immune regulatory functions Drive Th2 responses	Suppress immune responses Promote tissue remodeling

Table 1.1: Macrophage Polarization Table: Classically activated (M1) and Alternatively activated (M2) subset phenotypes. *(Arg-1, arginase-1; FIZZI, resistin-like molecule-alpha (Relm-alpha); GCs, glucocorticoids; ICs, Immune complexes ; IL1-ra, IL-1 receptor antagonist; LIF, Leukocyte inhibitory factor; TGM2: Transglutaminase 2; TNF-alpha: transforming growth factor-beta; MR (CD206), Mannose Receptor; NO, Nitric oxide; iNOS, Nitric oxide synthase; SR, scavenger receptor; VEGF, vascular endothelial growth factor; Ym1 (also known as chitinase-3-like protein-3 (Chi3l3)).*

This classification of macrophages into discrete M1 and M2 types is based on stimulation of macrophages in an *in vitro* setting. However, since macrophages *in vivo* normally exist under more complicated environmental influences, they are unlikely to

exhibit identical characteristics with artificially stimulated M1 or M2 macrophages[123, 128]. A full-characterization of macrophages present *in vivo* and in any particular disease state has not been achieved due to difficulties resulting from macrophage plasticity and the rate at which they change phenotypes when placed into a new environment. Nonetheless, “M1-like” macrophages found in *in vivo* settings, including the kidneys, that share similar markers with the classically defined M1 macrophages have been shown to play an important role in the inflammatory response during chronic or acute injury, while “M2-like” macrophages have been shown to promote the tissue repair and regeneration of cells post-injury[128, 129].

Macrophages and Kidney Injury

Analyses of postmortem kidney specimens in cases of acute tubular injury reveal an accumulation of inflammatory cells in the injured kidney[130]. This is recapitulated by rodent models of renal ischemia/reperfusion (IR), where injury leads to an immediate influx of leukocytes, including neutrophils, lymphocytes, and macrophages, into the interstitium[131]. Shortly after injury, leukocyte-attracting chemokines, including monocyte chemoattractant protein-1, stromal cell-derived factor-1, IL-6 and IL-8, are expressed by surrounding cells, promoting infiltration of leukocytes into the kidney[132, 133]. As a result, natural-killer (NK) T-cells and neutrophils are the first to infiltrate the interstitium - within hours after reperfusion - and contribute to renal tubular injury. This is followed by the infiltration of monocytes 24 h post reperfusion, where they subsequently differentiate into macrophages, initially of the M1-like phenotype. During the following up to 7-day renal “recovery” phase, the macrophages continue to increase in number and undergo a phenotypic switch to M2-like macrophages to aid in the repair process[134].

In rodent models of IR, macrophages are one of the predominant cell groups to accumulate around the injured renal tissue post-reperfusion[135]. Studies have shown that kidney damage is reduced and kidney function improved in rodent models when monocytes and macrophages are depleted using liposomal clodronate prior to I/R [135, 136]. On the other hand, when macrophages are genetically ablated during the "recovery" phase post IR, when M2-type macrophages predominate, effects on kidney outcomes were found to be deleterious in several other studies[137-139].

These data suggest that macrophages can play different roles in injury at different phases. Lee *et al.* examined this hypothesis by systemically ablating macrophages both before and after IR in their study. They found that liposomal clodronate ablation of monocytes and macrophages prior to IR was protective for the kidney, whereas ablation during the "repair" phase post IR resulted in more renal injury. They took these findings further by re-introducing macrophages, programmed *in vitro* to express either an M1 phenotype or an M2 phenotype, into the kidneys after IR to determine whether the phenotype of the recruited macrophages is what determines the extent of renal injury. They found that the pro-injurious M1 macrophages did indeed exacerbate injury, whereas the M2-primed macrophages did not[140]. These results demonstrate the important role that macrophages play in the repair response post-injury and that targeting of specific macrophage phenotypes may be a therapeutic tool in cases of renal injury.

Inflammation in PKD

Interstitial inflammation is an established characteristic of PKD. Interstitial infiltrates have been detected in both early and advanced stages of PKD (Zeier M,

1992). Macrophages are present at high densities in tissue sections obtained from chronic kidney disease patients[141-144] including human ADPKD and ARPKD tissue samples, where the majority of macrophages present were found to be of the M2-like phenotype[144].

Renal interstitial macrophages have also been found in both orthologous and non-orthologous animal models of ADPKD. For example, in both the inducible knockout *Pkd1*$^{fl/fl}$*;Pkhd1-Cre* mouse, as well as in the *Pkd2*$^{WS25/-}$ mouse, higher numbers of F4/80+ macrophages have been recorded when compared to their control littermates [145]. We have found an abundance of predominantly M2-like macrophages in the cystic kidneys of the non-orthologous *cpk* mouse, which phenotypically resembles human ARPKD[144]. Over-expression of PC2 in a transgenic mouse model of PKD revealed inflammatory macrophage-like cells surrounding cysts and in the renal cortex with H&E staining[146]*.* Also of note is that the expression levels of immune cell markers, including many macrophage markers, in PKD mouse models, such as the *cpk* mouse, positively correlates with disease progression[147]. For example, when profiling the renal gene expression in the *cpk* mouse, Mrug *et al.* discovered that the genes with the most over-expression levels were macrophage-associated genes, such as *Arg1* and *Ccr2*, and that severely affected kidneys from *cpk* mice expressed higher levels of those markers than mildly affected mice[148].

These data show that immune cell infiltration correlates with disease progression; however, whether immune cell infiltrates play a role in cystogenesis *per se* is an unanswered question and one for which there is conflicting data. Decades ago, it was demonstrated that exposure of germ-free PKD model mice to commensal

microorganisms or bacterial products accelerates the onset of renal cystic disease[149-151]. Werder and colleagues found that a mouse model of PKD grown in germ-free environments outlived their cystic counterparts grown under ambient environments[151]. Gardner *et al.* performed similar studies in a model of chemically-induced PKD. Consistent with the Werder study findings, renal cyst growth in this model was lower in rats grown in germ-free conditions compared to those in ambient environments. Furthermore, after deconditioning (removal from the germ-free environment), cystogenesis developed more rapidly than in rats housed conventionally from birth[149]. The studies suggest that the presence of commensal microbes is sufficient to stimulate the onset of cystogenesis. Gardner *et al.* took the studies further by co-treating the germ-free rats with injected *E. coli* endotoxin, which was found to be sufficient to stimulate cystogenesis. The effects on peripheral leukocyte counts and renal morphology following injection were assessed in this study and a correlation between renal damage severity and higher counts of leukocytes and lymphocytes in the peripheral blood were observed. Whether these infiltrating cells play an important role in triggering the onset of cystic disease or in disease progression following endotoxin injection in these experiments is a formal possibility that has yet to be tested.

Supporting evidence for the primary role that one immune cell type, macrophages, may play in cystogenesis comes from experiments observing the initial appearance of macrophages relative to onset of disease. In the Han:SPRD rat model of PKD, macrophages have been observed at a stage when there were only very minimal cystic dilations in the kidneys[152], suggesting a pre-cystic involvement of macrophages in PKD. In contrast, however, other studies have hinted that the appearance of

macrophages is only secondary to PKD progression. For example, despite the fact that cystic changes in the *pcy* mouse model, which resembles human ARPKD, were observed as early as in fetal and newborn mice, Takahashi *et al.* were able to observe macrophages and lymphocytes in the cortex and medulla no earlier than 18 weeks post-partum[153]. Similarly, in the LPK rat model of ARPKD, renal cysts develop predominantly in the collecting duct at around week 3, whereas interstitial macrophages were not detected until later time points[154]. These studies suggest that interstitial infiltration may be a secondary result of expanding cysts in these animal models. In sum, it is not clear what role inflammation may play in cyst initiation. Careful time-course experiments looking at the initial appearance of these infiltrates in relation to cyst development would be required to make a definitive determination about a potential causative role.

The Role of Macrophages in PKD Progression

Renal interstitial inflammation and macrophage infiltration temporally correlate with PKD cyst growth and disease progression, as described in the previous section. Recently, a direct role for these inflammatory cells in disease progression has been described. To determine the role, if any, that macrophages played in the progression of PKD, we examined the effects of clodronate-induced depletion of macrophages in the *cpk* model of PKD. Liposomal encapsulation of clodronate, a biphosphate that causes apoptotic cell death, has been shown to have high selectivity for activated macrophages when compared to other phagocytic cell types *in vitro*[155] and is commonly used to deplete macrophages *in vivo*[156, 157]. Depletion of macrophages using this method resulted not only in a decrease in the number of renal macrophages, but, importantly, a

decrease in the cystic index, Ki67-positive renal cell proliferation and improved renal function when compared to vehicle-treated controls[144].

Next, we studied the *in vitro* relationship between cystic renal epithelial cells and macrophages and the reciprocal effects these cell types have on each other, in the context of PKD. We were able to demonstrate that soluble factors produced by each cell type can contribute to a change in behavior of the other cell type. Specifically, we showed that both NHK and ADPKD primary human epithelial cells are able to influence the conversion of macrophages to a phenotype that exhibits M2-like qualities. In turn, these macrophages produce soluble factors that are able to induce proliferation of the renal epithelial cells as well as epithelial cyst growth *in vitro*. These results corroborated concurrent studies performed in an orthologous mouse model of PKD[145]. Collectively, these studies suggest that macrophage depletion attenuates disease progression and does so regardless of the underlying genetic abnormalities responsible for the PKD.

Goals of this Study

ADPKD is one of the most common potentially fatal genetic illnesses. PKD is the fourth leading cause of renal failure in the US, resulting in tremendous suffering and costing at least $2 billion per year. Recently, an important trial of Tolvaptan, a vasopressin type 2 receptor antagonist that lowers cAMP production in renal tubules of collecting duct origin, showed promising results for ADPKD patients. However, the FDA did not approve the drug due to concerns about toxicity. Thus, there are currently no specific therapies for PKD.

There is a clear need for specific treatments for PKD, including those that target cAMP and other pathogenic pathways. As summarized in this chapter, there is ample evidence that non-genetic factors, including kidney injury and inflammatory responses, can play a potentially important role in PKD progression. In this work, I have sought to identify and elucidate interactions between the cystic kidney and infiltrating macrophages that are pathologic and thus may be viable targets for therapy. We have shown that macrophages are recruited to cystic kidneys and converted to a pathogenic phenotype by specific renal epithelial factors. We have also demonstrated that these macrophages can accelerate disease progression by promoting cyst expansion and ADPKD cell proliferation, which is the driving force behind cyst enlargement and subsequent renal failure. In particular, I have sought to **identify renal epithelial recruitment factors, as well as pro-proliferative macrophage factors and the underlying mechanisms by which they promote PKD in order to uncover specific targets for therapeutic intervention to hinder proliferation and slow disease progression.**

In Chapter two, the potential pathologic role of monocyte chemoattractant-1 (MCP-1) in PKD is addressed. This factor is the major monocyte recruitment factor secreted by cystic renal epithelial cells from ADPKD patients, and inhibition of MCP-1 signaling can prolong survival in PKD model mice. In Chapter three, I show that "programming" macrophages by human cystic epithelial cells promotes secretion of several cytokines that are known to promote epithelial cell proliferation. Blockade of signaling from at least one of these factors, GRO-α, can inhibit macrophage-stimulated proliferation of human PKD cysts cells and thus, may provide a potential new target for

therapeutic treatments designed to slow cyst expansion and disease progression. Finally, in Chapter four, the potential pathogenic role of *WNT5A*, a non-canonical Wnt family member, whose expression is upregulated in ADPKD cells as compared to non-cystic human kidney (NHK) cells is discussed. I also show that *WNT5A* expression in ADPKD cells is stimulated by incubation with macrophages *in vitro*, and depletion of this signaling molecule inhibits cyst cell proliferation.

Chapter Two

Genetic Deficiency of Monocyte-Chemoattractant-1 (MCP-1) Attenuates Polycystic Kidney Disease Progression

Abstract

ADPKD is a progressive renal disease that is characterized by the presence of multiple kidney cysts that expand throughout the patient's life leading to gradual decline in renal function and end-stage renal disease in the majority of sufferers. Our lab has recently demonstrated the facilitative role that macrophages play in cyst expansion and polycystic kidney disease progression. In this study, we find that primary human ADPKD cyst epithelial cells produce a robust chemoattractant activity for monocytes, the cells from which macrophages derive. We identify MCP-1 (also known as CCL2) as the major contributor to this chemoattractant activity *in vitro* and show that its genetic ablation in *cpk* mice, an *in vivo* model of PKD, prolongs their life-span. Our data identify MCP-1 as a potential target of therapy for slowing PKD progression.

Introduction

Polycystic kidney diseases (PKD) are a group of inheritable disorders, characterized by the development of multiple fluid-filled renal cysts[7]. The most common form, autosomal dominant polycystic kidney disease (ADPKD), is caused by mutations in the ciliary proteins, PC1 and PC2, which are encoded by the polycystin genes, *PKD1* and *PKD2*, respectively[15, 158]. Autosomal recessive polycystic kidney disease (ARPKD)

is much less common but is also caused by mutations in a cilia-associated gene, *PKHD1*, which encodes the fibrocystin protein[159].

Interstitial renal infiltrates composed of macrophages and other inflammatory cells are a hallmark of cystic kidneys in PKD and have been observed in both human[141-144] and animal models[144-146]. For example, an increased concentration of macrophages in human kidneys from both ADPKD and ARPKD patients as well as in cystic kidneys of *cpk* mice, a model of ARPKD, was recently observed by our lab. These macrophages were of a distinct, wound-healing phenotype (M2-like), capable of stimulating the proliferation and microcyst formation of ADPKD cyst lining epithelial cells in culture[144]. Chemical depletion of these macrophages in the *cpk* mouse model by our lab[144], as well as in an orthologous *Pkd1* model by another group[145], restrained cyst expansion and improved renal function. A reduction in cortical cystic index (cortical cyst area/total cortical area) and reduced cyst epithelial cell proliferation index in the kidneys of these mice suggest that the presence of macrophages promote cyst enlargement by stimulating the proliferation of cyst lining epithelial cells *in vivo*[144, 145]. Together, these data demonstrate the contribution of macrophages to PKD progression, regardless of the genetic cause of the disease. Therefore, therapies that block the recruitment of these cells are likely to prevent the proliferative effects on cystic kidneys and could be effective in slowing PKD progression.

Monocyte chemoattractant-1 (MCP-1 or CCL2) is a pro-inflammatory chemokine that recruits immune cells to sites of inflammation, injury, or infection[160, 161]. It has been detected in cyst fluid of ADPKD patients as well as in their urine, where levels were significantly higher compared to non-ADPKD individuals. Notably, increased MCP-1

levels in these ADPKD patients were also found to correlate with worse renal function. Interestingly, despite these higher levels in urine and cyst fluid, serum MCP-1 levels remained normal, indicating that the kidneys may be the main source of MCP-1 expression. Further, a 10-fold increase in concentration of MCP-1 was measured in cyst fluid compared to urine, suggesting a cyst lumen or cyst epithelial cell origin, although this has yet to be formally demonstrated[162].

Elevated MCP-1 levels were also found in animal models of PKD. Murine cells obtained from *Pkd1*$^{-/-}$ kidneys displayed higher levels of MCP-1 transcripts compared to cells heterozygous for *Pkd1* mutation[145]. In the non-orthologous Han:SPRD rat model of PKD, *Cy*$^{-/-}$ homozygous mutants also displayed the highest level of MCP-1 mRNA compared to heterozygous rats, who, in turn, had a still higher level of expression than their wild-type counterparts. In these rats, MCP-1 was found to be localized to the cyst epithelial cells[163], corroborating data demonstrating that cultured human ADPKD cells can secrete MCP-1[162]. Not surprisingly, the levels of MCP-1 expression in these rats correlated with an increase in the number of observed macrophages[163].

In this study, we find that ADPKD cyst cells, as well as cells obtained from the cystic kidneys of mice with PKD, produce a robust monocyte chemoattractant activity, and that MCP-1 accounts for a large majority of this activity. We also demonstrate, for the first time, that MCP-1 within the human kidney localizes predominantly to tubule epithelial cells in both ADPKD and ARPKD, where it is overexpressed relative to epithelial cells of non-cystic kidneys. The effects of MCP-1-deficiency on PKD progression are tested here using *cpk* mice, a rapidly progressing PKD model that normally results in early death due to renal insufficiency. The outcomes from these

studies indicate that reduction of MCP-1 levels significantly extends the life-span of these animals. These results suggest that therapies which block MCP-1 or its receptor, which would be expected to block macrophage infiltration, may slow cystic disease progression in PKD.

Experimental Procedures

Cell culture: Primary cultures of ADPKD cyst cells and NHK (non-cystic human kidney epithelial) cells were supplied by the PKD Biomaterials Research Core laboratory at the University of Kansas Medical Center and cultured in "ADPKD cyst cell media" (DMEM/F-12 [Cellgro 15-090-CV, Mediatech Inc.; Manassas, VA] supplemented with 5% FBS, 15 mM HEPES, 5µg/ml insulin, 5µg/ml transferrin, and 5 ng/ml sodium selenite [ITS, BD Biosciences; Bedford, MA] plus penicillin (100 U/ml), streptomycin (130 µg/ml) (Pen/Strep). THP-1 monocytes were maintained in RPMI-1640 media (R8758, Sigma-Aldrich, St. Louis, MO) containing 10% FBS, 200 µM L-glutamine and Pen/Strep.

Conditioned Media (CM) from primary human ADPKD cyst cells: CM was obtained from ADPKD cells (3.7×10^6 cells/6-well plate) incubated in ADPKD cyst cell media for 24 hr followed by washing once with 1 X PBS and replacing with "basic media" (DMEM [D6429; Sigma] containing 0.1% FBS, 2 mM additional glutamine and Pen/Strep) for 1 d. CM was then collected from these cells and cellular debris removed by centrifugation at 600 x g for 10 min.

Preparation of primary cells from cystic cpk *mouse kidneys for producing CM:* Cystic kidneys from PN16 *cpk* mice were collected and placed in ice-cold PBS containing 2X

Pen/Strep for transfer. Under sterile conditions, kidneys were placed in a culture dish and diced with a razor to generate fragments of ~2 mm^2, which were then transferred into a 50 ml tube containing 15 ml cold DMEM with Pen/Strep. The diced tissue was digested by adding 5 ml collagenase (4 mg/ml; Worthington Collagenase Type 4; LS004189), quickly warmed by incubation in a 37℃ water bath for 5 min and then further incubated at 37℃ for 35 min with shaking (150 rpm). The collagenase was neutralized by the addition of 20 ml DMEM containing 10% FBS. The partly digested tissue was strained using a 40 µm stainer and then cultured with ADPKD cyst cell media for 3 days to allow attachment of kidney cells. The tissue was removed by aspiration, and the kidney cells were allowed to grow until confluent (3-4 d). Media was replaced with "complete media" (DMEM [D6429; Sigma] containing 10% FBS, 2 mM additional glutamine and Pen/Strep), and cells were incubated for 3 d and CM collected.

Migration and Neutralizing Assays: Cell migration assays were performed using 24-well transwells with 8 µm pore-size uncoated polycarbonate membranes (Costar, Cat. No. 3422). Lower wells contained 600 µl complete media only (to measure chemokinesis), purified human MCP-1 diluted in complete media as a positive control or CM made by incubating confluent cultures of primary cells from ADPKD cysts or from cystic kidneys of *cpk* mice with complete media for 3 d. CM was diluted 5-fold in complete media for migration assays. Upper wells contained 100 µl of THP-1 monocytes (1×10^6 cells/ml) in complete media. After overnight incubation (18 hr) media and non-migratory cells were removed by aspiration from upper well followed by the addition of EDTA (20 µl of 20mM EDTA made up in PBS) and incubation for 20 m at 4^oC. PBS (100 µl) was then added to each upper well before removal of the inserts and collection of migrated cells

into microcentrifuge tubes. Cells were pelleted by centrifugation (600 x g, 5 min), and, following removal of media, cell pellets were frozen at -80℃ and the relative cell numbers quantified using the Cyquant Cell Proliferation Assay Kit (Molelcular Probes). Values for the total number of cells seeded were obtained by quantifying, in parallel, pelleted frozen 100 µl aliquots of the original cell suspension.

Neutralization of MCP-1 in the CMs was carried out by pre-incubating the species-appropriate anti-MCP-1 blocking antibodies (R&D Systems; No. MAB479 for mouse; No. MAB679 for human) or the isotype-matched Ig controls (mouse IgG2b for human CMs and rat IgG2b for mouse CMs; eBioscience No. 14-4732-85 and No. 14-4031-85, respectively) with CMs for 1 h at 37℃ with agitation prior to the direct use of these CMs in migration assays. Antibodies were used at increasing concentrations to achieve maximal blockade with the anti-MCP-1 Ig (2 µg/ml and 3 µg/ml).

Quantitative RT-PCR: Total RNA was isolated from cells (ADPKD or NHK) using RNeasy Miniprep kit (Qiagen, Valencia, CA). 1.5 µg RNA was used as template for cDNA synthesis using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) in a 20 µl reaction. Real-time qRT-PCR was performed on Stratagene Mx3005P using Sybr Green Master Mix (#330529; Qiagen). The thermal cycler conditions were 95 ℃ for 10 m X 1, then 40 cycles of 95 ℃ for 30 s, 55 ℃ for 1 min, and 72 ℃ for 1 min, and completed with 1 cycle of 95 ℃ 1 min, 55 ℃ for 30 s, and 95 ℃ for 30 s. Expression of human gene products was normalized to human *HPRT* using the delta-delta C(T) method[164].

Blood Urea Nitrogen (BUN) Measurement: Blood was collected at the time of sacrifice. Serum was isolated from each sample (centrifugation 2500 X g for 8 min at 4℃,

followed by another spin of the supernatant for 4 min), and blood urea nitrogen (BUN) was measured using QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA).

Immunohistochemistry: De-paraffinized sections were steamed in 0.01 M citrate buffer (pH 6.0) for 20 min (steamer #HS900, Black & Decker, Madison, WI). Sections were then incubated in 3% H_2O_2 followed by serum from the host animal in which the relevant secondary antibody was generated. Samples were incubated with an MCP-1 antibody (Cat. No. HPA019163, Sigma-Aldrich, St. Louis, MO) overnight at 4℃. Appropriate secondary antibodies (ImmPRESS, Vector Laboratories, Burlingame, CA) were then applied for 30 min at room temperature prior to visualization by light microscopy.

Results

Conditioned media from primary human and mouse PKD cells exhibit monocyte chemoattractant activity in vitro.

Tubule epithelial cells are known to produce a number of macrophage recruitment factors in tubulointerstitial diseases[128]. To assess the chemoattractant activity produced by epithelial cells in PKD cells, we collected conditioned media (CM) from primary cyst-lining cells obtained from different ADPKD patient kidneys, as well as from the primary cells grown from several *cpk* mouse cystic kidneys. The monocyte chemoattractant activity of these CMs was measured in a transwell migration assay of THP-1 cells, a human monocyte cell line. To set up the migration assay, THP-1 monocytes were seeded in the top chamber of a transwell culture dish containing inserts with uniform-sized pores, and the PKD cell CMs for each type were seeded in the bottom chamber. Parallel controls were also set up to include complete media

(negative control) or complete media containing human recombinant MCP-1 (positive control). Monocytes were allowed to migrate through the pores toward the control media or the various CM preparations for 18 h, and migrated cells were quantified and compared to controls. Our results showed enhanced monocyte chemoattractant activity in the CMs from all cell preparations compared to controls, varying from ~10-21 and ~9-12 fold enhancement in ADPKD cyst cell and *cpk* kidney cell CMs, respectively (Figure 2.1). This demonstrated the robust production of monocyte chemoattractant factors from both primary human and mouse PKD cells.

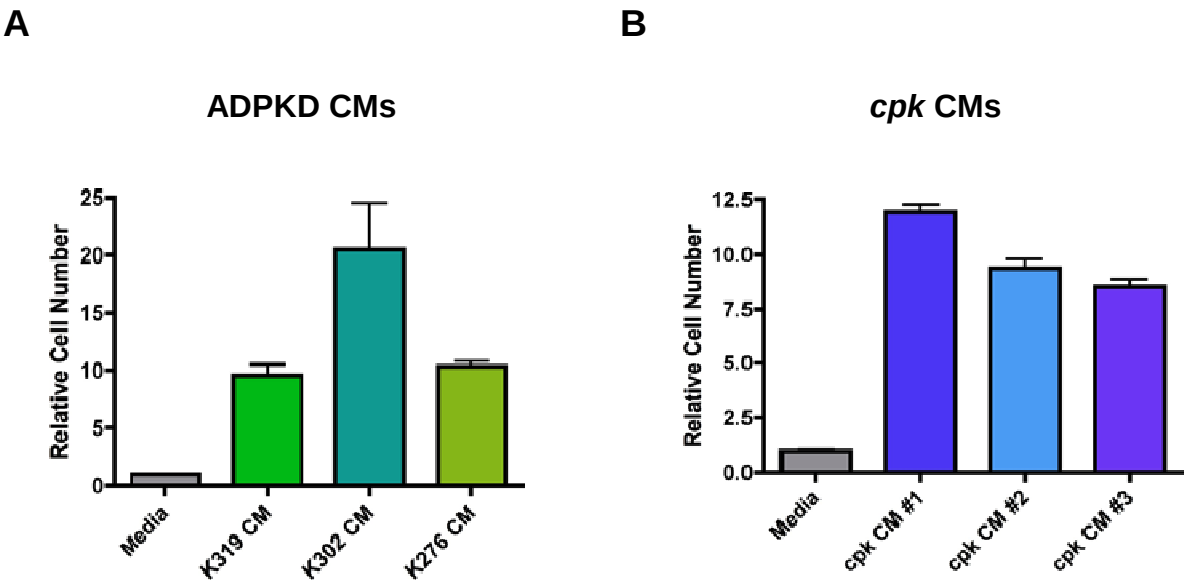


Figure 2.1: Conditioned media from human and mouse PKD exhibits monocyte chemoattractant activity *in vitro*. A: CMs collected from primary human ADPKD cyst cells as well as from, B: renal epithelial cells of *cpk* mice demonstrate chemoattractant activity toward THP-1 monocytes (n=3 kidneys, each).

MCP-1 *is the primary contributor to monocyte chemoattractant activity from* PKD cell *conditioned media.*

To determine whether MCP-1 was contributing to the chemoattractant activity of the PKD cell CMs, parallel transwells were set up that included the addition of increasing concentrations of blocking antibodies against MCP-1 (or isotype control Ig) to CMs prior to placement of these samples in the lower chambers. As shown in Figure 2.2, anti-MCP-1 neutralizing antisera resulted in a striking inhibition of monocyte migration to the ADPKD CM (Figure 2.2A). Antibody titration revealed the maximal effective concentrations of neutralizing antibody (2 and 3 ug/ml) reduced the mean migration by 74 and 68%, respectively (Figure 2.2A, upper panel). Similar concentrations of antibody were found to maximally reduce monocyte migration toward a *cpk* cystic kidney cell CM, in which case a mean migration reduction of 96 and 87% were found using 2 and 3 ug/ml, respectively, of the neutralizing antibody (Figure 2.2A, lower panel). Analysis of the combined results of these experiments showed that the monocyte chemoattractant activity present in ADPKD cyst cell and *cpk* cell CMs could be maximally reduced by 76 ± 2 and 78 ± 9%, respectively, using the anti-MCP-1 neutralizing antibody (Figure 2.2B). These results indicate that MCP-1 is responsible for a large majority of the macrophage recruitment factors made by the cells from these cystic kidneys.

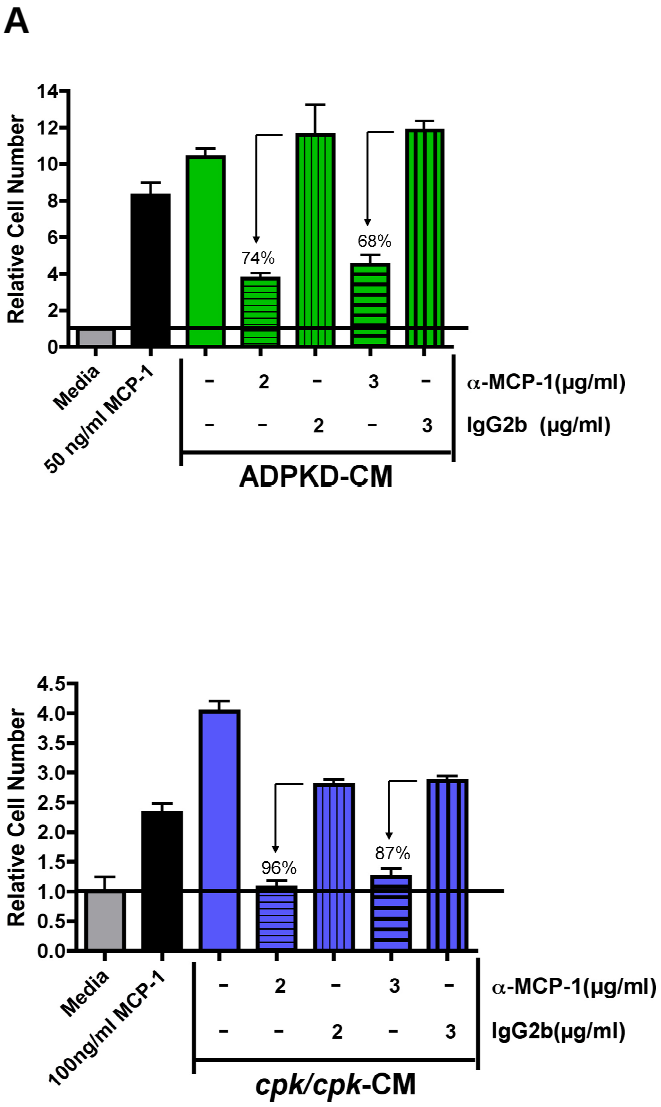


Figure 2.2: MCP-1 is the primary contributor to monocyte chemoattractant activity from PKD conditioned media *in vitro*. A: Neutralization of MCP-1 in CMs collected from primary human ADPKD cyst cells (top) as well as from renal epithelial cells of *cpk* mice (bottom) demonstrate primary contribution of MCP-1 to chemoattractant activity toward THP-1 monocytes at maximal effective blocking concentrations. B: Mean percentage of macrophage chemoattractant activity attributable to MCP-1 in ADPKD CM (left) and CM from cpk kidneys (right) n=3 for each CM.

MCP-1 is overexpressed in human and mouse PKD tissue and is localized to the tubule epithelial lining of cysts in human ADPKD and ARPKD sections.

While both ADPKD cyst cells and NHK cells have been shown to produce MCP-1 when cultured *in vitro*[162], the relative RNA expression levels of MCP-1 in these cells has not been assessed. qRT-PCR analysis of RNA isolated from primary human ADPKD

cyst cells and NHK cells revealed an ~ 3-fold upregulation of MCP-1 transcripts in ADPKD cells (Figure 2.3A).

Immunohistochemistry of ADPKD, ARPKD and NHK sections were performed to assess the relative levels of MCP-1 protein, as well as to establish localization. In both ADPKD and ARPKD kidney sections, there was robust staining of MCP-1 that localized predominantly to tubule epithelial cells of both cysts and normal tubules (Figure 2.3B, top panel). However, only a low level of staining, which also localized to tubule epithelial cells, could be detected in sections of non-cystic kidneys (Figure 2.3B, bottom panel). These data establish the cyst epithelial cell origin of MCP-1 in human PKD that had been suggested by previous studies[162, 163].

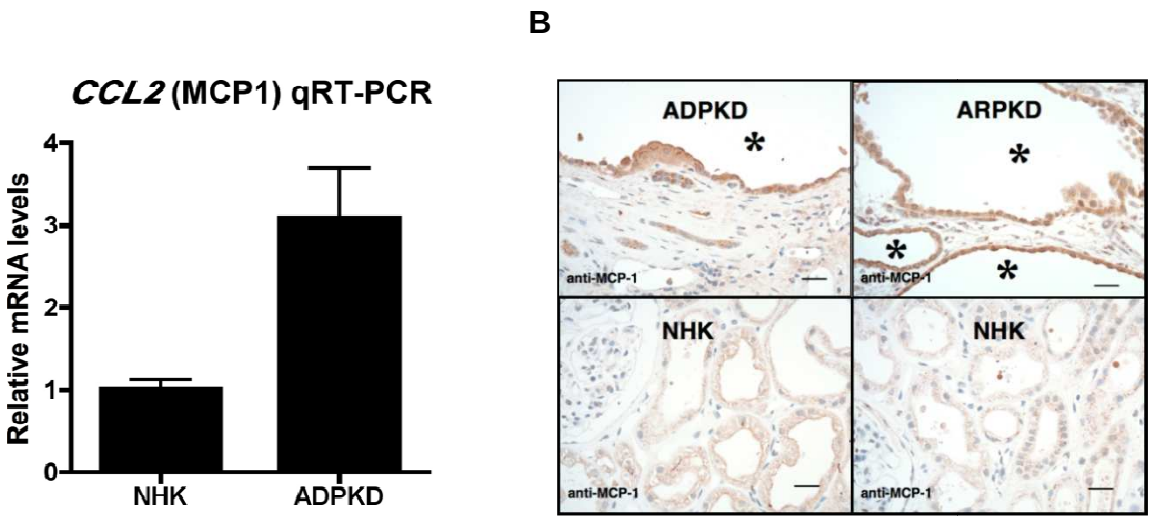


Figure 2.3: MCP-1 is overexpressed in human ADPKD and ARPKD tissue, and is localized to the tubule epithelial lining of cysts in human PKD sections. A: mRNA was isolated from cultured cells as indicated in the figure and qRT-PCR was performed. B: IHC for MCP-1 was performed on ADPKD (n=7), ARPKD (n=2) or NHK kidneys (n=2). Sections were processed in parallel. Representative images are shown. Scale bar= 25 µm; *=cystic space.

Genetic knockout of MCP-1 *prolongs cpk mouse longevity*

Cpk mice were used to determine the *in vivo* effects of MCP-1 deficiency on PKD progression. These mice, which provide a non-orthologous model of ARPKD, develop cystic disease due to a homozygous mutation in *Cys1*, which encodes the cilia-associated protein, cystin. *Cpk* mice are born with renal cysts that rapidly expand leading to renal failure and death, typically by 3 weeks[31, 165-167]. C57/Blk6 *cpk* mice were crossed with C57/Blk6 MCP-1-null mice (Jackson Labs) in a breeding strategy to generate progeny that were either null (*Mcp-1*$^{-/-}$, n=10), heterozygous (*Mcp-1*$^{+/-}$, n=18), or wild-type (*Mcp-1*$^{+/+}$, n=20) at the MCP-1 locus. Mice were monitored regularly for end-stage symptoms, and survival was recorded for each genotype. Remarkably, there were significant differences in survival among all groups. As previously reported[31, 165-167], *cpk* mice with wild-type expression of MCP-1 showed rapid progression of disease, and all died within 3 weeks (Figure 2.4, black). Cystic mice with MCP-1 knockout showed a significant increase in survival (average=45 days), up to ~18 weeks in one case, a lifespan that is over 600% longer than that of the average *cpk*$^{-/-}$ mouse that is wild type at the MCP-1 locus (average=21 days) (Figure 2.4, red). Notably, heterozygous loss of MCP-1 also showed a significant improvement in survival (average=25 days) compared to wild type MCP-1, up to 5 weeks in one case (Figure 2.4, blue).

The differences in survival imply that loss of MCP-1 slows cystic progression and likely preserves renal function. While this has not yet been fully assessed, preliminary measurements of renal function have been collected for mice at PN13. Average renal function at postnatal day 13 (PN 13), as measured by BUN, showed no differences

between double-knockout mice compared to *cpk* mice wild-type at the MCP-1 locus (data not shown). Ongoing studies are being carried out to re-assess BUN, macrophage numbers, as well as changes in the cortical cystic index, in kidneys from these mice at earlier time points (PN 10). A decrease in the number of macrophages observed in addition to reduced BUN and cortical cystic index numbers, correlating with increasing levels of MCP-1 deficiency, is considered likely. If so, these results would support the notion that MCP-1 contributes to PKD progression by recruiting macrophages to cystic kidneys, further enhancing their cystogenesis.

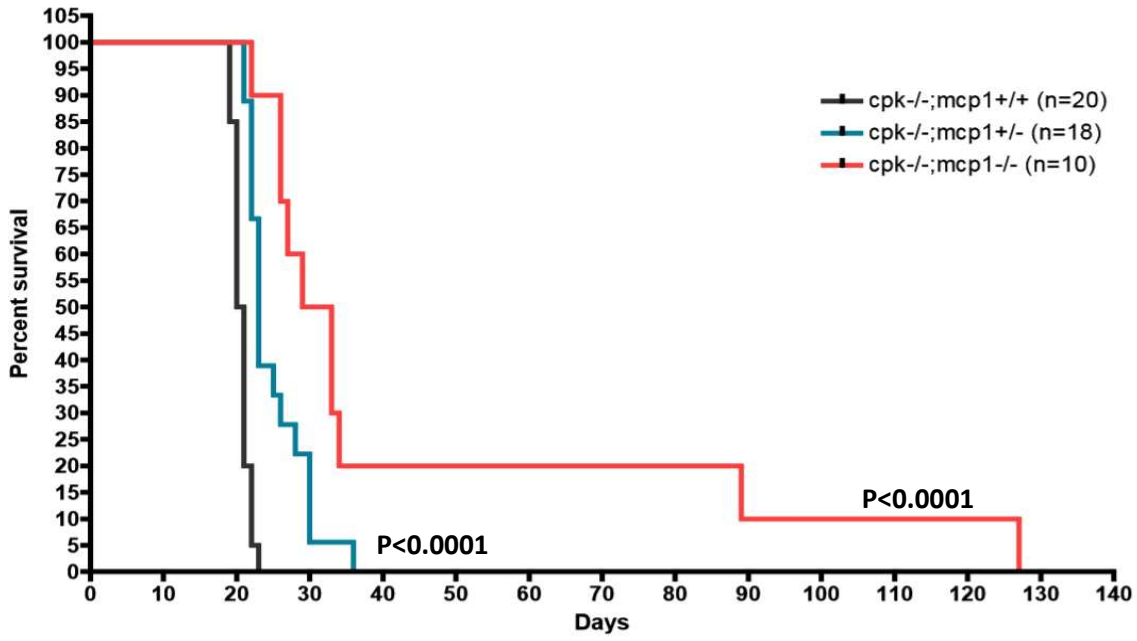


Figure 2.4: Genetic knockout of MCP-1 prolongs cpk mouse longevity and improves average renal function *in vivo*. MCP-1 deficiency in C57/Blk6 *cpk* mice increased survival rates in a dose dependant manner. $Cpk^{-/-};mcp\text{-}1^{+/+}$ (n=20) versus $Cpk^{-/-};mcp\text{-}1^{+/-}$ (n=18) or $Cpk^{-/-};mcp\text{-}1^{-/-}$ (n=10), P<0.0001. $Cpk^{-/-};mcp\text{-}1^{+/-}$ versus $Cpk^{-/-};mcp\text{-}1^{-/-}$, P=0.01.

Discussion

Studies in several animal models of renal damage as well as in various types of human renal disease provide increasing evidence for a major role of MCP-1 in the progression of renal failure[168]. High expression levels of renal MCP-1 have been detected in several chronic kidney diseases such as lupus nephritis[169], glomerular disorders[170], obstructive nephropathy[171] as well as in ADPKD[162]. In a mouse model of diabetic nephropathy, loss of MCP-1 resulted in a significant reduction in the number of atrophied tubules, correlating with a decrease in creatinine levels[172]. Administration of antibodies to MCP-1 in a mouse model of glomerulonephritis was found to decrease the extent of tissue damage and improve renal dysfunction, correlating with a decrease in infiltrating macrophages[173]. Since increases in levels of MCP-1 expression, associated with higher numbers of interstitial macrophages[163], have been found in renal epithelial cells of animal models of PKD, we sought to elucidate the role of MCP-1 in PKD progression in this study.

Our data is the first to demonstrate the negative contribution of MCP-1 to survival in PKD model mice. The upregulation of MCP-1 likely leads to the influx of macrophages to the kidneys, promoting disease progression. This could explain why MCP-1 deficiency would ameliorate disease progression in a similar manner to direct macrophage depletion[144, 145], hence prolonging longevity (Figure 2.4). Notably, the data demonstrate variability in days survived between double mutant mice (as early as 26 days to as long as 127 days). This could be explained by differences in genetic polymorphisms that may exist even between individual mice of seemingly identical backgrounds. A more plausible reason, however, could be explained by differences in

individual extrinsic factors affecting each mouse (e.g., infections, injuries, etc.). The inability of MCP-1 deficient cells to communicate this signal of distress and attract immune cells to the site of injury prevents a necessary immune response that may also be inadvertently affecting the immediate well-being of these mice. While no overt deleterious effects were detected in these mice as a result of MCP-1 deficiency, the presence of sub-clinical infection/inflammation is possible. Depending on the severity of the environmental effects, loss of MCP-1 may be more detrimental to the overall health of these mice in a manner that outweighs the slower concurring benefits of resolution from PKD. This theory is also supported by our data demonstrating that *cpk* mice wild-type at the MCP-1 locus exhibit very tight survival figures (between 19 and 21 days). All of the mice used in this study were housed in a conventional environment that was clean but not sterile. It may be that more dramatic and consistent results would be obtained from an identical study of mice housed in sterile conditions. These varying environmental effects combined with an MCP-1 deficient background may also explain the lack of improvement in BUN values at PN 13 obtained between *cpk* mice wild-type for MCP-1 compared to the double-knockout mice (data not shown), since infection and/or inflammation in other areas of the body may mask kidney specific improvements in renal function.

The time-point at which the sera from these mice were obtained (PN 13), may also explain the lack of improvement in BUN levels. In *cpk* mice, there are three postnatal stages of cyst development. The first stage (P1-P6, or week 1) involves proximal and collecting tubule segmental dilations, whereas the second stage (P7-P13, or week 2) consists of continued rapid growth of cortical and medullary collecting duct

cysts. After postnatal day 13 (week 3 and beyond), almost all of the kidney parenchyma is replaced by collecting duct cysts[174-176]. These changes are accompanied by a decrease in kidney function as measured by BUN[175]. Therefore, analyses of BUN at earlier time points (i.e. stage II) may reveal changes in kidney function before substantial damage has occurred. This may also have the additional benefit of limiting overall environmental exposure for these mice. Thus, earlier time-point experiments, as well as housing mice in a more environmentally controlled setting, will likely decrease the variability within the MCP-1 deficient groups. Nonetheless, it is important to note that regardless of these variances, overall, the double-knockout mice survived significantly longer than their wild-type ($P<0.0001$) and even heterozygous ($P=0.01$) counterparts. One double-mutant mouse survived as long as 127 days, a phenomenon that has never been previously described for untreated and otherwise genetically unaltered C57/Blk6 *cpk* mice, to the best of our knowledge.

Although previous studies of ADPKD kidneys have suggested the cyst epithelial cell origin of MCP-1[162, 163], our experiments are the first to demonstrate MCP-1 localization to the epithelial cells lining cysts of human ADPKD kidneys (Figure 2.3B). This is consistent with the theory that MCP-1 is produced by these renal epithelial cells in response to renal injury or stress occurring in tubules undergoing cystogenesis. Interestingly, we have found that MCP-1 was also upregulated in surrounding non-cystic tubules, suggesting that either this phenomenon is intrinsic to polycystin deficiency in these cells, or that the tubules are responding to, and further propagating, stress signals, whether autocrine or paracrine, perhaps as a result of the physical compression imposed by surrounding cysts. Based on our results comparing MCP-1 mRNA

expression levels between primary human cultured NHK and ADPKD cells, it may be that *Pkd1/2* defects can have a direct effect on MCP-1 production (Figure 2.2A). This is in contrast to results by Zheng *et al.*, where no differences in secreted MCP-1 concentrations between these cell types were observed[162]. However, as suggested in these studies, this may be due to mechanical or structural changes that occur as the cells are transferred into a tissue culture environment, which then elicit a cellular injury response[162]. Nonetheless, our results do not rule out the possibility that upregulation of MCP-1 mRNA expression in ADPKD cyst cells was triggered by environmental influences surrounding these cells prior to their removal from the kidney. Thus, the MCP-1 response, which was maintained after culture, may not be directly related to *Pkd1* deficiency, but rather a sustained result of extrinsic factors present in its pre-culture state.

Studies monitoring direct changes in MCP-1 expression as mediated by loss of PC1 or PC2 may shed further light into the causative role the polycystins may play in regulating MCP-1 expression. MCP-1 mRNA expression has been shown to be induced by activation of the extracellular regulated kinase (ERK) in response to shear stress in vascular endothelial cells as well as in renal epithelial cells[177, 178]. This shear-stress mediated ERK activation and subsequent MCP-1 mRNA expression is triggered by the polycystins, expressed on the primary cilium of renal epithelial cells, in response to fluid flow[177, 178]. Flores *et al.* showed that cells deficient for PC2, however, were unable to induce MCP-1 mRNA expression in response to shear stress by preventing the transport of pERK into the nucleus[179]. This suggests that polycystin 2 deficiency hinders the upregulation of MCP-1 mRNA levels, rather than increasing them as we have

detected in our PKD cells. This implies that the increase in levels of MCP-1 mRNA in our study is unlikely due to polycystin, or at least PC2, deficiency. Nonetheless, it is still unclear what effects overexpression of the polycystins, which also leads to PKD[24], may have on MCP-1 mRNA expression levels.

Although genetic mutations in *Pkd2* may not upregulate MCP-1 production, they are likely to increase the susceptibility to inflammation, but only following a "second hit" event. This is evident in mouse models of acute kidney injury (AKI), where the number of macrophages post-injury was significantly higher in *Pkd2* heterozygous mouse kidneys compared to wild-type injured mice, despite lower levels of *Pkd2* mRNA expression in the heterozygotes. Notably, macrophage numbers were not significantly different between heterozygotes and wild-types prior to injury in the kidneys of these mice[114]. This suggests that *Pkd2* heterozygosity alone is insufficient to promote inflammation, but likely predisposes the kidney to a greater inflammatory response post-injury.

If MCP-1 is the major chemoattractant in PKD, as our data suggest, monitoring the timing at which macrophages appear during different stages of cystogenesis might aid in the determination of whether genetic mutations or environmental factors are responsible for inducing the expression of this cytokine in PKD. Studies looking at the PCK (*Pkhd1*$^{-/-}$) rat model of ARPKD, in which cystogenesis was prevented through loss of a "second hit" mitogenic stimulus (i.e., simultaneous genetic knockout of vasopressin), showed no evidence of interstitial inflammation in the double-mutant kidneys, despite the loss of the PKD causing gene[64]. These results suggest that mutated *Pkhd1* genes alone are incapable of inducing an inflammatory response, in the

form of MCP-1 overexpression or otherwise. Rather, immune cell infiltration is likely a secondary result of tissue damage and injury in this model. Whether or not mutations in other genes causing PKD influence inflammatory responses has yet to be studied.

Regardless of how MCP-1 expression is being mediated in PKD cells, our data support a role for MCP-1 in promoting disease progression. This is likely through attracting cyst-growth-promoting macrophages to PKD kidneys, as abnormalities in monocyte recruitment in MCP-$1^{-/-}$ mice have been observed[180]. However, there is also evidence to support a non-inflammatory signaling role for MCP-1, which our data does not rule out, as of yet, as the cause for improved PKD mouse survival. Besides its potent chemoattractant property, MCP-1 has been shown to increase cytotoxic lymphocyte and natural killer cell activity[181], affect Th cell phenotypes[182], alter the phenotype of vascular smooth muscle cells[183], activate monocyte cytokine secretion[184], as well as affect cellular responses of vascular endothelial cells (ECs) via direct activation of MAP kinases[185]. In human tubular epithelial cells, MCP-1 has also been found to activate transcription factors involved in growth responses[186]. Further studies ruling out involvement of these other non-inflammatory signaling properties of MCP-1, as well as establishment of a direct link between MCP-1 deficiency and macrophage inhibition in PKD animals will be necessary to delineate the exact role that MCP-1 plays in prolonging *cpk* mouse longevity.

Since MCP-1 mediates its chemoattractant effects through its interaction with its receptor, CCR2, animal studies are currently underway in our lab testing the effectiveness of MCP-1 receptor antagonists on murine PKD disease progression. Results of these studies may be complicated due to the receptor's ability to bind other

ligands, as well as the ability of MCP-1 to bind other receptors[187, 188]. CCR2 is a G protein-coupled receptor that binds multiple ligands, including MCP-1, MCP-2, MCP-3, and MCP-4. However, the relative contribution of each of these ligands to CCR2-mediated *in vivo* function remains unclear[189, 190]. MCP-1 has also been shown to stimulate CCR4 activity in a basophilic cell line[191] as well as induce its expression and regulate its activity in human osteoclasts[192]. Nonetheless, functional redundancies in mice deficient for MCP-1 or CCR2 with regards to the inflammatory response in disease models *in vivo*[193] provide strong support for targeting this ligand/receptor pair in chronic inflammatory states[194], such as in the case of PKD. Therefore, targeting of MCP-1 using currently available receptor antagonists, alone or in combination with other PKD therapies currently being tested, could aid in ameliorating disease progression for PKD patients.

Chapter Three

Identification and Characterization of Macrophage Factors that Promote ADPKD Cyst Cell Proliferation.

Abstract

We have recently shown that renal macrophages can promote PKD progression in an animal model of disease. We also showed that the mechanism likely underlying this phenomenon is a reciprocal interaction between infiltrating macrophages and cyst epithelial cells: cyst cells stimulate macrophage differentiation to an M2-like phenotype and macrophages promote cyst cell proliferation. In this chapter, we demonstrate that the induction of the M2-like state enhances macrophage production of pro-proliferative factors, implying that the "programming" of macrophages by cyst cells is an important step in disease pathogenesis. Further, we show that soluble, protease-sensitive factor(s) are responsible for the pro-proliferative activity. To identify programmed macrophage-secreted factors, we performed proteomic and RNAseq analyses, which revealed upregulation of several macrophage cytokines, including GRO-α. Blockade of the GRO-α receptor, CXCR2, partially inhibited the pro-proliferative effect. In parallel efforts, we examined downstream signaling pathways within ADPKD cells that are activated by programmed macrophages. This analysis demonstrated activation of the PKA and MEK/ERK signaling pathways in the proliferative effect on ADPKD cyst cells. Targeting of these factors or components may be a viable strategy to slow PKD progression.

Introduction

Macrophages play an important role in the pathophysiology of kidney disease. Macrophages are monocyte-derived cells that exhibit phenotypic plasticity influenced by environmental stimuli and have as such been classified into distinct functional phenotypes. M1 macrophages are those that develop following incubation *in vitro* with the Th_1-type cytokines, IFNγ, LPS, TNFα, or GM-CSF, while M2 macrophages are those that develop following exposure to the Th_2-type cytokines IL-4 and/or IL-13. Since renal macrophages *in vivo* exist under complex environmental influences and stimuli, they are likely to exhibit distinct characteristics when compared with these artificially defined M1 or M2 macrophages[123, 128]. M1-like macrophages in the kidney play an important role in the inflammatory response during chronic or acute injury, while M2-like macrophages promote the tissue repair and fibrosis processes post-injury[128, 129]. Animal models of acute kidney injury demonstrate that these M2-like macrophages differentiate from infiltrating monocytes responding to tissue damage. They are capable of stimulating tubular cell proliferation, acting as wound-healing agents to promote repair post injury[140, 195]. Renal M2-like macrophages are also found to predominate relative to other macrophage types in several chronic kidney injury diseases[143].

Our lab has demonstrated that in ARPKD and ADPKD, the vast majority of the interstitial macrophages present in cystic kidneys were M2-like based on histological analysis[144]. Moreover, we and others have demonstrated a role for these macrophages in promoting polycystic kidney disease (PKD) progression[144, 145]. In these studies, systemic depletion of macrophages in a non-orthologous model of ARPKD, as well as in an orthologous model of ADPKD, resulted in the restraint of cyst expansion that was

associated with a reduction in proliferation of cyst epithelial cells. Furthermore, co-culture of macrophages with ADPKD cyst epithelial cells *in vitro* was found to promote M2-like differentiation of the macrophages and proliferation of the cyst cells[144, 145]. Stimulation of cyst cell proliferation was found to be mediated by soluble factor(s) secreted by the macrophages[144]. Thus, identification and characterization of the macrophage factors responsible for the proliferative response in PKD cells may provide new targets of therapy that could be used to treat patients and aid in slowing disease progression.

In this study, we propose to 1) characterize the proliferative factor(s) produced by programmed macrophages and determine whether programming affects their production; 2) identify potential candidates using expression and proteomic analysis; 3) uncover signaling pathways within ADPKD cyst epithelial cells that are stimulated by the programmed macrophage factor(s) to induce the proliferative response.

Experimental Procedures

Cell culture: Primary cultures of ADPKD cyst cells and THP-1 monocytes were maintained as previously described (Chapter 2).

Conditioned Media (CM): ACM was obtained from ADPKD cells (3.7 x 10^6 per 15cm tissue culture plate) incubated for one day in ADPKD growth media (above) for 24 hours following by washing and incubating for 3 d in XVIVO[10] (Bio Whittaker, Walkersville, MD) supplemented with 15mM HEPES. THP-1 monocytes were differentiated into macrophages by treatment for 3 d with 200 nM PMA (phorbol myristate acetate, P1585, Sigma-Aldrich) in THP-1 in XVIVO[10] and, after washing with 1 X PBS, media was

replenished with XVIVO[10] alone for 3 days. Next, media was washed with PBS and changed to either fresh XVIVO[10] alone (to produce TCM), or with ACM (to produce pTCM) for 2 d. At the end of the 2 d, brief washing in PBS was carried out followed by the addition of "basic media" (DMEM (D6429, Sigma-Aldrich) supplemented with 200 μM L-glutamine, Pen/Strep plus 0.1% FBS) for 24 hr to generate TCM and pTCM, respectively. Basic media was used alone in all proliferation assays as negative control. Modified methods were taken for preparation of pTCM for mass spectrometry analysis. All steps were identical to above, except, DMEM used in "basic media" was exchanged for phenol-red-free DMEM (D2902, Sigma-Aldrich) for the final step to generate the pTCM.

CM fractionation: pTCM was separated by the VIVASPIN 6 ultrafiltration system's molecular weight cut-off columns (Sartorius AG, Goettingen, Germany) to generate 4 flow-through samples (6ml each) consisting of <10, <30, <50 and <100 kDa protein fractions, per manufacturers' protocol, and each fraction was then used in subsequent proliferation assays.

Gel filtration: pTCM was also filtered through per manufacturers' protocol Sephadex G-50 columns (G5080, Sigma-Aldrich) to determine active fraction. Briefly, after equilibrating with PBS, the column was eluted into four fractions of pTCM each and assayed for proliferation. BSA at 5mg/ml was similarly fractioned and each fraction was assayed for protein concentration using a standard Bradford protein determination assay.

CM heat-inactivation and proteinase K treatment: To heat-inactivate pTCM, samples were boiled for 20 min followed by immediate cooling on ice to RT prior to treatment of

cells. Proteinase K (PK) (P9290, Sigma-Aldrich) was added to pTCM at a final concentration of 0.5 mg/ml (0.05 U) and allowed to incubate with rotation for 2 hr at 4^{o}C. The pTCM plus PK mixture was spun down at 2,000 x g for 5 min and supernatant was passed through a 0.22um filter. FBS was added at a final concentration of 0.1% to generate the PK-treated pTCM sample. For control experiments, Proteinase K Inhibitor (539470, Calbiochem) was added at a final concentration of 50uM prior to incubation of PK with pTCM.

Cytokine array: Cytokines in pTCM and TCM were detected using an antibody capture array [Human XL Cytokine Array (ARY022, R&D Systems, MN], which allows quantitative detection of 102 different secreted proteins. The manufacturer's protocol was modified to allow detection using near-red fluorescence on the Odyssey® Infrared Imaging System (LI-COR). Briefly, the steps in protocol ARY005 were followed with replacement of Buffer 4 and Buffer 5 in the protocol with Buffer 6 and Buffer 4 from the ARY022 kit, respectively.

Proliferation assays: Primary ADPKD cyst epithelial cells (1 x 10^{4}/well) were seeded into 24-well tissue culture plates and incubated overnight followed by 24 hr incubation in ATP-depleting, starvation media (glucose-free DMEM [11966, Life Technologies] containing 2% FBS,10 mM deoxyglucose and Pen/Strep3). For each assay described, control experiments were performed by incubation of ATP-depleted cells for 3 d with DMEM (D6429, Sigma-Aldrich) supplemented with 200 μM L-glutamine, Pen/Strep containing 0.1% FBS (low-serum media). Treatment of cells with pTCM alone (positive control) or with the addition of reagents being tested was concurrently performed as described for each experiment. Following 3 d incubation at 37^{o}C, media was removed

and cells were frozen at -80^{o}C prior to lysis in buffer containing CyQUANT® GR dye (Life Technologies), according to manufacturer's directions. Quantitative measurement of fluorescence was carried out using a SynergyTM 2 microplate reader (BioTEK Instruments, Inc., Winooski, VT).

Western blots: Western blot analyses were performed per standard protocol on whole cell extracts of ADPKD cyst cells as described for each experimental reagent.

Materials and Antibodies: H89 and SL-327 were obtained from Sigma-Aldrich (St. Louis, MO) and SB225002 from Tocris (Ellisville, MO, USA). GRO-α (MAB275) antibody used in immunodepletion experiments were obtained from R&D Systems (Abingdon, UK). All other antibodies described were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at 1:1000 dilutions.

RNA isolation: Total RNA was isolated from programmed and unprogrammed THP-1 cells using RNeasy Miniprep kit (Qiagen, Valencia, CA). 1.0 µg RNA was used as template for cDNA synthesis using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) in a 20 µl reaction.

Results

Part I: Characterization of Programmed Macrophage Proliferative Factor(s)

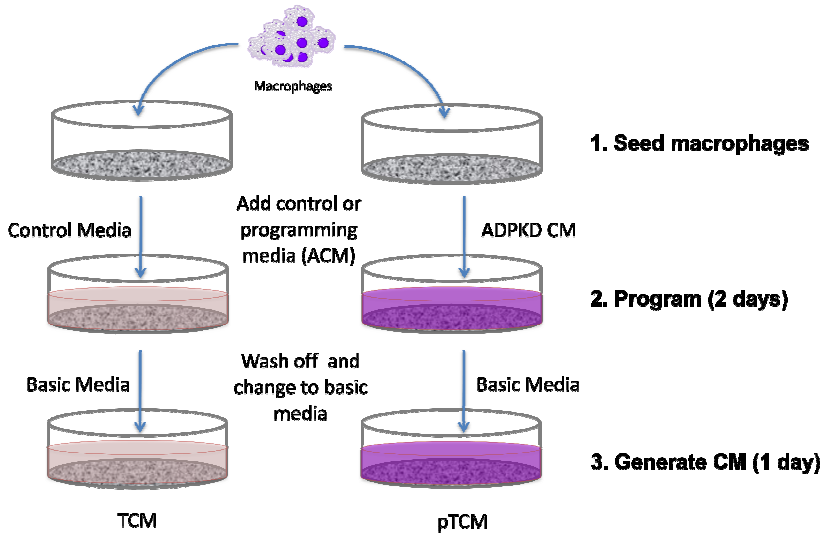
Soluble programmed-macrophage factor(s) promote ADPKD cyst cell proliferation

We have previously shown that macrophages have the capacity to promote proliferation of both ADPKD cyst cells in 2D and 3D co-culture, and, using transwell assays, we showed that macrophage-secreted factor(s) are responsible for the activity[144]. To facilitate identification of the factors, we first determined whether the

proliferative activity was stable in solution. We collected conditioned media (CM) from human THP-1 macrophages and used it in a proliferation assay to assess effects on ADPKD cyst cell growth. After incubation with the THP-1 conditioned media (TCM) for 3 days, the number of ADPKD cyst cells was quantified and compared to untreated cells cultured in basal media. Treatment with TCM induced a significant proliferative response in the ADPKD cells compared to un-treated cells (Figure 3.1, middle bar). We next assessed the influence of macrophage differentiation on secretion of proliferative factors. To accomplish this, THP-1 cells were first programmed by incubation with ADPKD cyst cell CM for 2 days. Then, the media was changed and programmed THP-1 conditioned media (pTCM) was collected after an additional 24h (Figure 3.1A). As shown in Figure 3.1, the programming step significantly enhanced secretion of proliferative activity, with pTCM-stimulated proliferation ranging between ~20-40% higher than that in TCM treated cells (Figure 3.1B).

These results confirm that the pro-proliferative factor(s) produced by the macrophages are soluble and show that the activity is stable enough to be transferred to ADPKD cells in culture to induce its proliferative effects, a property that will facilitate further identification and characterization. Furthermore, the data show that the programming interaction between macrophages and ADPKD cells significantly enhances macrophage secretion of pro-proliferative factor(s).

A



B

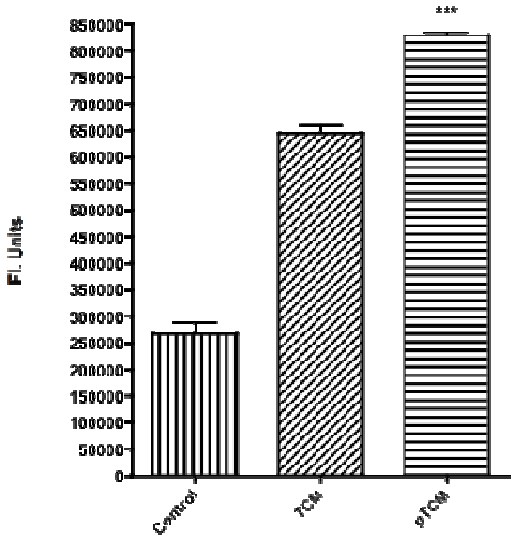


Figure 3.1: Conditioned media from programmed human THP-1 macrophages promotes proliferation *in vitro*. A: After differentiation of THP-1 macrophages, media was washed with PBS and changed to either fresh XVIVO[10] alone (control), or with ACM (programmed) for 2 d. At the end of the 2 d, brief washing in PBS was carried out followed by the addition of "basic media" for 24 hr to generate TCM and pTCM, respectively. B: CM collected from control THP-1 macrophages (TCM) promotes proliferation of human ADPKD cyst cells ($P<0.0001$) after a 3 day incubation period, compared to control media alone. Pre-programming of macrophages with ADPKD cyst cell CM to generate pTCM further stimulates the proliferative effect ($P<0.0001$, compared to un-programmed TCM). (n=3 experiments).

Soluble programmed-macrophage pro-proliferative factor(s) are heat- and proteinase K-sensitive

To determine whether a protein is responsible for the pro-proliferative activity, pTCM was treated with either activated proteinase K or with proteinase K that was first inactivated with a proteinase K inhibitor (negative control) prior to incubation with ADPKD cells. After 3 days, the levels of proliferation were assessed in these cultures. Proteinase K completely eliminated the proliferative effect on ADPKD cells, while proliferative activity was the same in untreated pTCM and pTCM treated with inactivated proteinase K (Figure 3.2A). Similarly, heat treatment (100℃) of the pTCM abolished the proliferative activity (Figure 3.2B). These results demonstrate that the soluble proliferative factor is heat- and proteinase K-sensitive and therefore likely a protein.

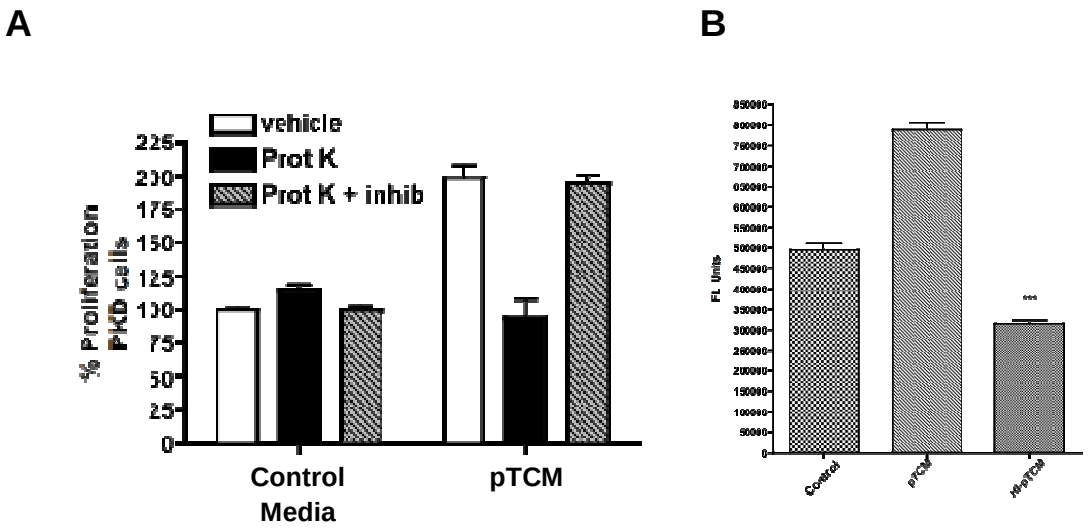


Figure 3.2: Proliferative pTCM factor is a protein. A: pTCM factor is proteinase K (PK) sensitive. Primary ADPKD cyst epithelial cells (1 x 10^4/well) were seeded into 24-well tissue culture plates, serum starved and treated with either control media/pTCM alone, with PK, or with preinactivated PK. Cells were analyzed for changes in proliferation after 3 d (n=2). PK inhibits proliferative effects, whereas inhibition of PK activity prior to treatment of ADPKD cyst cells restores proliferative response. B: Heat-inactivation of pTCM (Hi-pTCM) was performed by heating pTCM (100^{o}C) for 20 min followed by immediate cooling on ice. Proliferation assay was performed as with PK experiments. Hi-pTCM inhibits proliferative effect on ADPKD cyst cells (n=3).

Soluble programmed-macrophage factor(s) have apparent molecular weight of less than 50 KDa

To further characterize our protein factor, we sought to determine the approximate molecular weight. To do so, two methods of size determination were undertaken. First, size-exclusion gel filtration chromatography of pTCM was carried out using a Sephadex G-50 spin column, which has a molecular weight (MW) cutoff of ~30KDa and an effective fractionation range of ~1500-30,000 Da. pTCM was loaded onto G-50 and 5 fractions (including the void) collected successively. Fractions were incubated with ADPKD cells and proliferative activity assessed. As shown in Figure 3.3, nearly all of the activity was in the void volume and first fraction (Figure 3.3A, top). This elution pattern is similar to that of bovine serum albumin (BSA, MW=66.5 KDa) under identical conditions (Figure 3.3A, bottom). These data suggest one or more factors or complexes with an apparent molecular weight >~30 KDa are responsible for the proliferative activity.

To further define the MW of our protein factor, the ultrafiltration system, VIVASPIN 6 was also used. Columns in this system have polyethersulfone (PES) membranes of varying pore sizes, each capable of separating proteins in solution based on their molecular weight as they are spun through the column. Using this system, four pTCM samples were fractionated separately using columns with 4 molecular weight cut-offs (10, 30, 50 and 100 kDa). Filtrates from each were subsequently collected and incubated with ADPKD cyst cells in a proliferation assay. No significant activity was detected in the fractions <10 and <30KDa; however, significant activity was detected in the <50 and <100 KDa fractions (Figure 3.3B), which is consistent with the gel filtration

results. These data were reproduced in four separate experiments; however, in two out of the four experiments, a small amount of activity was also detected in the <30 KDa filtrate fraction (data not shown). This could be explained by inconsistent segregation between individual membranes, or perhaps the active factor exists in a complex or oligomer that influences its apparent molecular weight. Nonetheless, the majority of the proliferative activity was found in the 30-50 KDa fractions, suggesting that the MW for the macrophage protein factor(s) or complex falls within that range. Of note, even the most active fraction (<50KDa) did not reach proliferative activity at the level achieved by incubation of ADPKD cyst cells with pTCM alone (Figure 3.3B). This is most likely related to technical limitations, as the handling process, including long centrifugation times required for separation, can cause partial loss of activity.

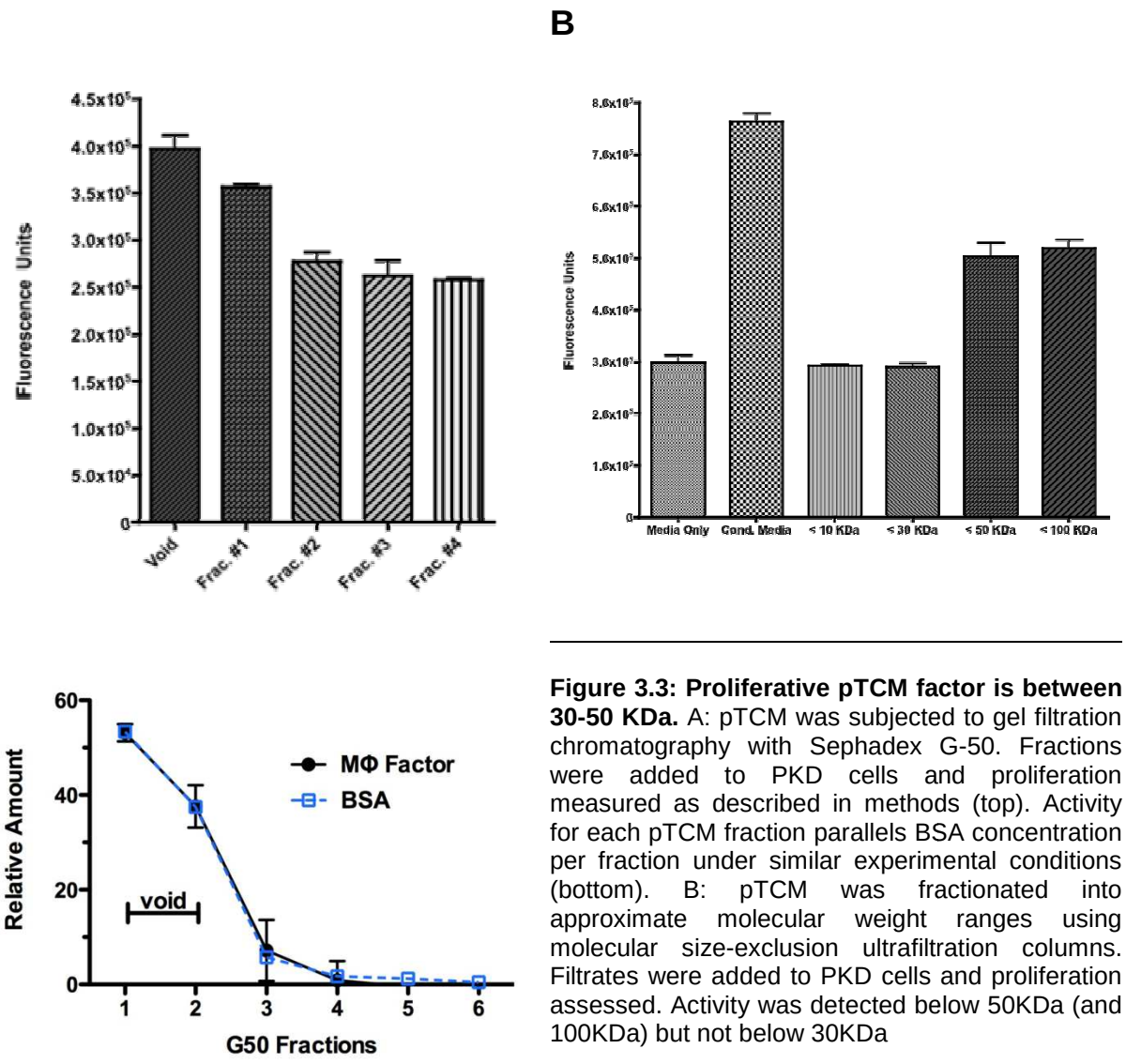


Figure 3.3: Proliferative pTCM factor is between 30-50 KDa. A: pTCM was subjected to gel filtration chromatography with Sephadex G-50. Fractions were added to PKD cells and proliferation measured as described in methods (top). Activity for each pTCM fraction parallels BSA concentration per fraction under similar experimental conditions (bottom). B: pTCM was fractionated into approximate molecular weight ranges using molecular size-exclusion ultrafiltration columns. Filtrates were added to PKD cells and proliferation assessed. Activity was detected below 50KDa (and 100KDa) but not below 30KDa

Part II: Identification of potential programmed macrophage factor(s)

Mass spectrometry analyses identifies candidate factor(s)

To identify potential programmed macrophage proliferative factors, pTCM was fractionated using the 50KDa Vivaspin 6 MW cut-off ultrafiltration column to exclude molecules above ~50 KDa from the filtrate. The filtrate fraction containing factors less than 50 KDa was then analyzed using mass spectrometry for peptide sequence

identification. This analysis was complicated by solubility issues in the core lab, and a substantial part of the sample was lost on both repeats of the experiment. Nevertheless, several candidate proteins were identified (see Table 3.1).

Candidate Factor	**MW**
Isoform alpha-enolase of a-enolase	47.1
Interstitial collagenase isoform 2	46.2
Chitinase-3-like protein 1 [+*]	**42.6**
Pyruvate kinase	40.2
Chitotriosidase-1	40.2
Fructose-bisphosphate aldolase A	39.4
Isoform 1 of Annexin A2	38.6
Cathepsin L1 [+]	**37.5**
Tartrate-resistant acid phosphatase type 5	36.6
CAPG [+]	**36.2**
Cathepsin Z [+]	**33.8**
Cyclophilin A [*]	**18**
MIF [*]	**12.5**

Table 3.1: List of factors identified in mass spectrometry analysis. Select candidates were identified for testing (bold). +=denotes testing with inhibitor against specific protein; *=denotes testing with recombinant protein.

Several factors identified from the mass spectrometry candidate list were excluded from further testing due to recognition as being non-secreted factors. The presence of these factors in the CM is likely due to inadvertent cell lysis during processing of pTCM for mass spectrometry analysis. The remainder of the candidates

(Table 3.1, **bold**) were subjected to further testing. In some instances purified, recombinant forms were assessed in ADPKD cell proliferation assays, and in other cases neutralization antibodies and/or inhibitors of known candidate receptors or downstream signaling targets/components (denoted in Table 3.1) were used in proliferation assays. However, these experiments did not reveal any potential candidates likely to contribute to the pTCM-induced proliferation of ADPKD cyst cells (data not shown). It is possible that the factor is in low enough abundance and below the limit of mass spectrometric detection. Partial loss of the sample, as mentioned earlier, may have also contributed to our inability to detect a proliferative factor.

RNA-sequencing and cytokine array analysis of secretome reveals candidate factor(s)

In addition to mass spectrometry, we simultaneously sought to identify mRNAs that are differentially upregulated in programmed macrophages using RNAseq. For this experiment, three separate samples of THP-1 macrophages were programmed for 3 days by ADPKD cyst cell-CM (ACM) obtained from three separate kidneys. The same ACM was used to program a parallel well of macrophages, which were used to confirm the induction of secreted pro-proliferative activity (not shown). Three additional sets of un-programmed macrophages were also set-up as controls. RNA was isolated from each of these samples and analyzed at the University of Kansas Sequencing Core. RNAseq analysis showed upregulation of 53 genes in programmed macrophage ($p<0.05$). Of these, 22 are known to encode proteins that can be secreted. The list of candidates with their respective fold changes are summarized in Table 3.2.

Gene ID	Name	Fold Change
ACHE	acetylcholinesterase	9.17304044
CAP1	CAP, adenylate cyclase-associated protein 1	5.089486133
CCL24	chemokine (C-C) ligand 24 (eotaxin-2)	2.886557572
CCL4	chemokine (C-C) ligand 4 (MIP 1-beta)	3.451898395
CCR7	chemokine (C-C) receptor 7	4.41168566
CD84	CD84 (leukocyte antigen)	2.390313755
CSF2	colony stimulating factor 2	5.331383638
CTSB	cathepsin B	3.677817774
CXCL1	chemokine (C-X-C) ligand 1 (Gro-alpha; KC)	2.039350853
CXCL2	chemokine (C-X-C) ligand 2 (MIP 2a; GRO-b))	3.37000329
FJX1	four jointed box 1	3.360602716
GYS1	glycogen synthase 1	2951.024126
HLA-DQA2	major histocompatibility complex class II DQa2	3.521817132
IER3	immediate early response 3	1.841847991
IGFBP3	insulin-like growth factor binding factor 3	2.221940216
IL1B	IL-1 beta	2.172972626
IL23A	IL-23 alpha subunit	2.7504163
INHBB	inhibin, beta B	2.484576564
INHBE	inhibin, beta E	2.722911325
LYPLA2	lysophospholipase II	556.3812532
MMP7	matrix metalloproteinase 7 (matrilysin)	2.629120015
NPR1	natriuretic peptide receptor 1	2.145181921
SERPINE2	serpin peptidase inhibitor, clade E, member 2 (PAI-1)	1.981530262

Table 3.2: RNA-sequencing data summarizing secreted genes from THP-1 macrophages which were upregulated >1.5-fold after ACM-programming.

Unfortunately, q value calculation did not confirm statistical significance for any of the differentially expressed transcripts. This is most likely due to variation in samples. Since only a limited number of candidates were identified by this approach, we decided to use a limited antibody array to validate possibilities. The 22 possible targets include cytokines known to be produced by macrophages. Thus, we prepared TCM and pTCM and compared them using the Human XL Cytokine Array (ARY022, R&D Systems, MN), which quantitatively measures 102 different human cytokines, including 7 candidates from the RNAseq data (CCL4, CSF2, CXCL1/GRO-α, CXCL2, IL1β, IL23A, IGFBP-3).

A list of cytokines whose secretion was upregulated in pTCM (above 1.5 fold) is summarized in Table 3.3 and graphed in Figure 3.4. Of these, two candidates were also found to be overexpressed by RNAseq, namely, GRO-α and IL-1β.

Name	Fold Change
GRO-α	**9.886800823**
IL-1β	**2.05963567**
Angiogenin	1.791012897
TNF-α	1.780837461
CD30	1.637781668

Table 3.3: List of cytokines up-regulated in pTCM based on 1.5 fold increase or higher, compared to TCM. Cytokines also over-expressed in RNA-sequencing analysis are in bold text

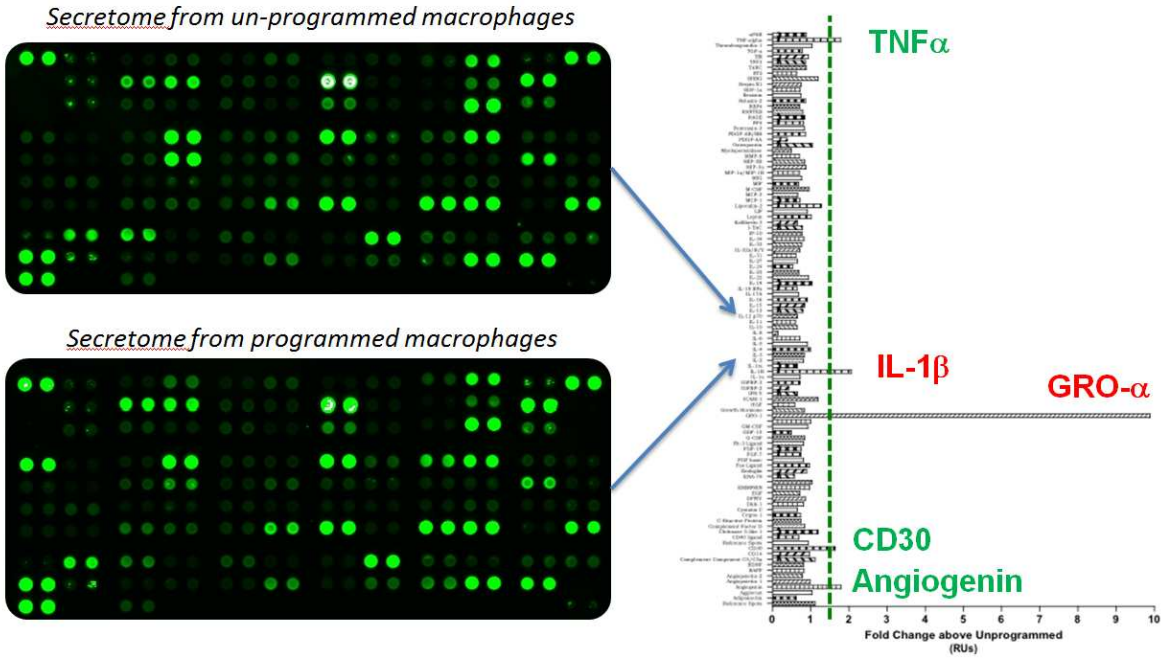


Figure 3.4: Cytokine array identifies factors upregulated in pTCM. TCM and pTCM was collected and tested for the presence of a set of cytokines (102 total) using cytokine arrays. Graph depicts all cytokine expression levels. Cytokines upregulated at least 1.5 fold in pTCM are indicated next to their respective graph bars. Cytokines found to also be overexpressed in RNA-sequencing analysis are highlighted in red.

Analysis of candidates identifies CXCR2 as potential signaling target

To determine whether GRO-α (also known as CXCL1) might be responsible, in part or in whole, for the pTCM-induced proliferative effect, we looked to block its only receptor, CXCR2, using a selective inhibitor (SB225002). ADPKD cyst cells were pre-treated with this inhibitor for 30 min, followed by co-treatment with pTCM plus the inhibitor. After 3 days of incubation, a 35% reduction in proliferation compared to pTCM alone was found, while the drug had no effect on proliferation of cells incubated in control media alone (Figure 3.5). While the effect is small, it is important to recall that the measured difference in this assay is only between programmed and unprogrammed

macrophages, which as mentioned previously, corresponds to ~20-40% of enhanced proliferation of ADPKD cells. Since inhibition of the GRO-α receptor resulted in ~35% inhibition of proliferation, it is possibly responsible for most of the proliferative effects induced by programming of macrophages to a more M2-like phenotype. Experiments to compare the effects of this drug on proliferation in the presence of pTCM are underway. In any event, these results point to the likely contribution of CXCR2 to pTCM-induced ADPKD cyst cell proliferation *in vitro*.

We next assessed the specific contribution of GRO-α to the pro-proliferative activity in pTCM. Our attempts to block GRO-α activity with a neutralizing antibody did not result in a decrease in proliferation of ADPKD cyst cells compared to Ig control pTCM (data not shown). Therefore, further testing using a second neutralizing antibody will be carried out to rule out the direct contribution of GRO-α to the pTCM-induced proliferative effect. Alternatively, it is possible that another ligand is responsible for the pro-proliferative activity stimulated by activation of CXCR2 (see Discussion). Other chemokines with known pro-proliferative effects in other cells, including IL-8 and ENA-78, share the CXCR2 receptor with GRO-α[196]. However, neither ENA-78 nor IL-8 was upregulated in the cytokine array (Figure 3.4).

Experiments determining the contribution of IL-1β, also upregulated in the cytokine array as well as in the RNA sequencing analyses, on proliferation of ADPKD cyst cells are also ongoing. It is also worth noting, that TNF-α, which is also upregulated in the cytokine array, has been found to mediate a pathway promoting ADPKD[197]. Interestingly, TNF-α has also been shown to upregulate expression of GRO-α in melanoma cells[198]. However, pre-incubation of pTCM with a TNF-α neutralizing

antibody prior to treatment of ADPKD cyst cells failed to prevent the pro-proliferative response in our experiments (data not shown). Therefore, it is unlikely that the TNF-α in our pTCM is directly or indirectly responsible for this pro-proliferative response.

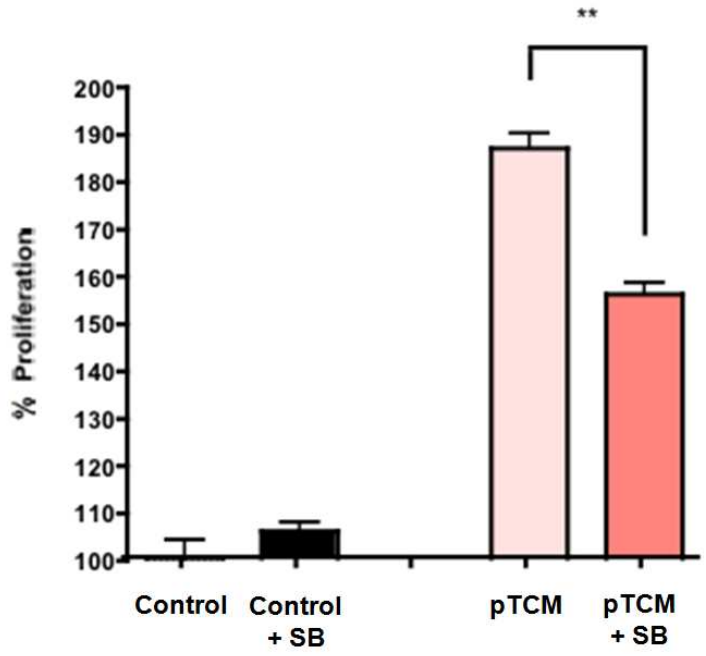


Figure 3.5: Inhibition of CXCR2 signaling reduces pTCM-induced proliferation of ADPKD cyst cells. ADPKD cyst cells were pre-treated with CXCR2 antagonist, SB225002 (200nM), for 1 hour then incubated with pTCM in the presence or absence of inhibitor for 3 day proliferation assay. Treatment with inhibitor reduces proliferative effect of pTCM by ~35% ($P<0.001$), whereas there was no significant effect on proliferation of cells incubated in control media. (n=3)

Part III: Identification of the potential downstream signaling components stimulated by programmed macrophage factor(s)

The PKA Inhibitor, H89, prevents pTCM-induced CREB activation and ADPKD cyst cell proliferation in vitro

To identify potential downstream signaling targets stimulated by pTCM, we began by analyzing activation of some pathways thought to be important in PKD[25]. Treatment of ADPKD cells by pTCM did not reveal significant changes in targets of the mTOR (S6-Kinase phosphorylation) or Wnt/ β-catenin signaling (β-catenin stabilization) pathways (data not shown). As described in Chapter One, increased cyclic adenosine 3',5'-monophosphate (cAMP) levels contribute to cystogenesis by stimulating chloride and fluid secretion as well as cell proliferation[65, 66]. To examine potential effects of pTCM on cAMP signaling, we assessed phosphorylation of the transcription factor cAMP response element-binding protein (CREB), at Ser133, which is a primary target of the cAMP-dependent protein kinase (PKA)[199]. Western blot analysis revealed robust activation of CREB in ADPKD cyst cells upon treatment with pTCM within 20 min. Furthermore, incubation with the PKA inhibitor, H89 (5 uM), completely blocked this pTCM-induced CREB activation (Figure 3.6A).

To discern whether H89-mediated inhibition of CREB activation functionally correlated with a decrease in ADPKD cyst cell proliferation, treatment of these cells with pTCM in the presence and absence of this PKA inhibitor (5 uM) was carried out, and effects on proliferation were measured at the end of the 3-day incubation period. Similar to that observed for CREB phosphorylation, H89 completely blocked the pTCM-

stimulated ADPKD cell proliferation (Figure 3.6B, compare third and fourth bars), while this concentration of drug had no effect on basal proliferation of PKD cells (Figure 3.6B, compare first and second bar).

These data indicate a contribution of H89 targets in pTCM-induced cell proliferation. In addition to PKA (Ki=135 nM), H89 also inhibits other kinases, including S6K1, MSK1, and ROCK-II with Ki values in the nM range (80 nM, 120 nM, and 270 nM, respectively). However, S6K1 and MSK1 activation were not detected after pTCM treatment (not shown). ROCK activation is commonly associated with cell migration, but testing with a more selective ROCK inhibitor (Y27632)[200] is underway. In any event, the correlation with proliferation with CREB phosphorylation (Figure 3.6) implies a potential link to PKA.

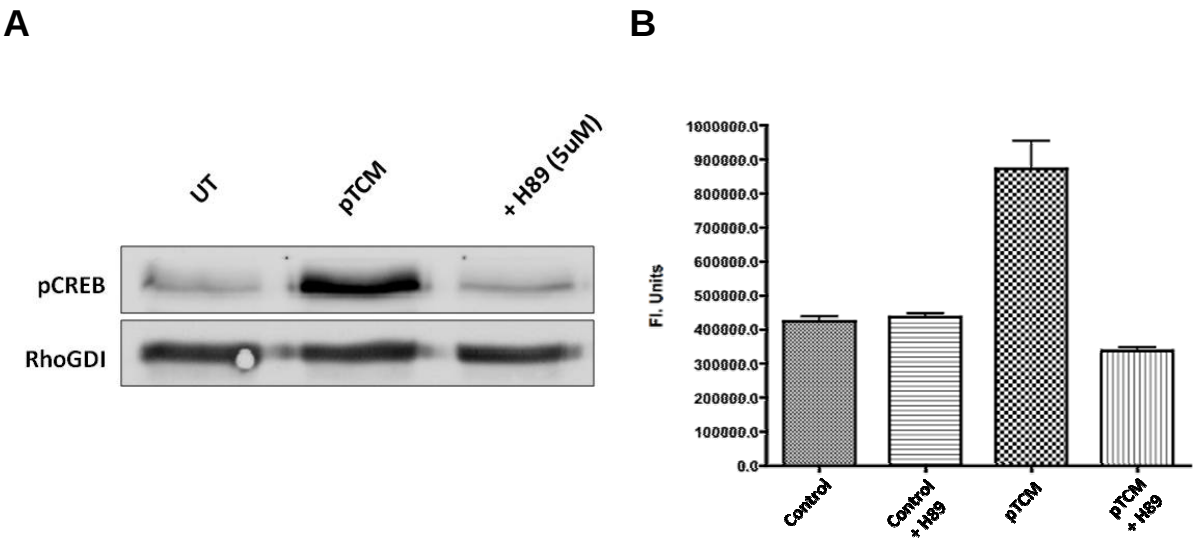


Figure 3.6: PKA Inhibitor, H89, prevents pTCM-induced CREB activation and ADPKD cyst cell proliferation *in vitro*. Cells were pre-treated with PKA inhibitor, H89 (5 uM), for one hour then incubated with pTCM in the presence or absence of inhibitor or vehicle for A: 20 min before collection for western blot analysis, or B: for 3 day proliferation assay. Treatment with inhibitor prevents CREB activation (A), and completely reduces proliferative effect (B) ($P<0.001$).

MEK Inhibitor, SL327, prevents pTCM-induced ERK and CREB activation, and ADPKD cyst cell proliferation in vitro

cAMP induced cell proliferation of ADPKD cyst cells or wild-type tubular epithelial cells with low intracellular Ca^{2+} stores is mediated, at least in part, through mitogen-activated protein kinase/extracellularly regulated kinase (MAPK/ERK) signaling in a PKA, Src- and Ras-dependent manner[61]. To examine the effects of pTCM-induced ADPKD cyst proliferation on ERK activation, we stimulated cells with pTCM for 20 min and assessed for phosphorylation of ERK using western blot analyses. pTCM treatment of ADPKD cyst cells was indeed able to induce ERK activation in this assay (Figure 3.7A).

Next, we used a selective inhibitor of MEK-1 and MEK-2, SL-327 (Sigma-Aldrich, St. Louis, MO), to assess effects of MEK/ERK inhibition on pTCM-induced proliferation of ADPKD cyst cells as well as on CREB activation, since, in addition to PKA, ERK has also been shown to promote the Ser133 phosphorylation of CREB[201-204]. Western blot analysis revealed a decrease in both ERK and CREB activation (Figure 3.7A). Interestingly, despite complete loss of ERK activity at the lowest concentration of the inhibitor used (10 uM), total inhibition of proliferative activity was achieved only at the highest concentrations of the inhibitor (40 uM). However, the dose-dependent decrease in pTCM-induced proliferative effects correlated with a decrease in CREB phosphorylation (Figure 3.7B). Further assessment of the contribution of ERK phosphorylation to pTCM-induced CREB activation as well as cell proliferation is ongoing.

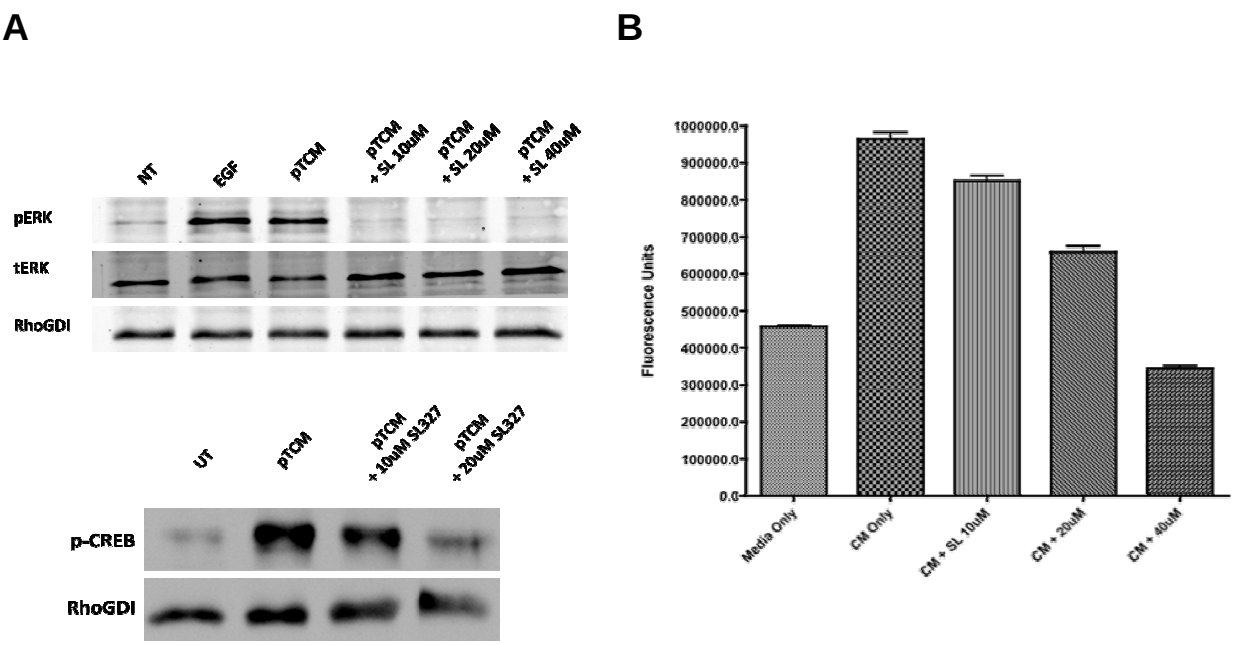


Figure 3.7: MEK Inhibitor, SL327, prevents pTCM-induced ERK and CREB activation, and ADPKD cyst cell proliferation *in vitro*. Cells were pre-treated with MEK inhibitor, SL-327 (10, 20 and 40 uM), for one hour then incubated with pTCM in the presence or absence of inhibitor at each dose for A: 20 min before collection for western blot analysis, or B: for 3 day proliferation assay. Treatment with inhibitor reduces both ERK (A, top) and CREB (A, bottom) activation, and completely inhibits proliferative effect at highest concentration (B) ($P<0.001$).

Discussion

In malignant neoplastic diseases, tumor-associated macrophages (TAMs) can make up a large portion of the tumor mass and have been demonstrated to display an M2-like phenotype, which is achieved through programming by the tumor microenvironment[205, 206]. TAMs generally promote tumor progression, in part by stimulating tumor cell proliferation[205, 206], and their presence in large numbers in the tumor environment is often associated with a bad prognosis[207-210]. Thus, therapeutic targeting of TAMs is an active area of cancer research.

PKD can also be considered a neoplastic disease (though non-malignant), characterized by abnormal growth of fluid-filled cysts[211]. Similar to malignant disease, interstitial renal infiltrates composed largely of macrophages are a hallmark of cystic kidneys in PKD in both human[141-144] and animal models[144-146]. Moreover, we have shown that, like TAMs, the vast majority of macrophages in human PKD kidneys express M2-like markers, and our *in vitro* data suggest that interaction with cyst epithelial cells induces this M2-like phenotype[144]. In this chapter we demonstrate that induction of this phenotype enhances the secretion of macrophage protein factor(s) that stimulate cyst cell proliferation. Since proliferation of the tubule epithelial cells lining the cysts in ADPKD kidneys is the primary driving force behind disease progression, these macrophage factors could be prime targets for therapy that could be used to halt or hinder disease progression.

Using a variety of approaches, two prime candidate factors have emerged: GRO-α (CXCL1) and IL-1β. In considering these two molecules, it is important to note that the M1/M2 designation is an oversimplification in the process of distinguishing macrophages and their secretomes as either “detrimental” (M1)- or “healing” (M2)-types. There is actually a graded spectrum of environmentally-defined activation patterns of macrophages, including those expressing properties of both stereotypical M1 and M2 phenotypes. Therefore, it was not a complete surprise when our combined RNA sequencing and cytokine array results both identified GRO-α and IL-1β, which in some contexts are regarded as M1 cytokines. Indeed, both GRO-α and IL-1β were previously described to demonstrate pro-tumoral properties. For example, GRO-α, has been shown to be involved in the processes of angiogenesis, inflammation, wound-

healing as well as tumorigenesis[212]. It has also been shown to stimulate proliferation of human melanoma cells[213] and was originally identified in tumorigenic human and hamster cells[214]. Its receptor, CXCR2, whose inhibition in our studies resulted in a reduction of the macrophage pro-proliferative response in ADPKD cysts cells, has also been demonstrated to promote tumor survival, angiogenesis and metastasis[215, 216]. These data and the fact that a GRO-α neutralizing antibody did not attenuate proliferation stimulated by pTCM mean that either the antibody was not effective or another CXCR2 ligand promotes proliferation. Interestingly, IL-1β has been shown to upregulate expression of ENA-78, a distinct CXCR2 ligand, in kidney epithelial cells[217]. IL-1β also has been shown to stimulate GRO-α expression in glial cells[218]. The contribution of these two molecules to macrophage-induced ADPKD cell proliferation is under current investigation.

Another part of our study focused on identifying pathways stimulated by the pTCM that may be contributing to the proliferation of ADPKD cyst cells *in vitro*. Several pathways have been identified to be aberrantly expressed in cyst cells from human and animal PKD cells and have been the focus of investigation for PKD therapies[25]. Given that macrophages act to promote renal epithelial cell proliferation, it is likely that macrophages induce their effects through one or more of these proliferative pathways. Experiments were unsuccessful in identifying changes in the mTOR or Wnt/B-catenin pathways upon pTCM stimulation. We also examined potential effects on p38 and p90RSK, but no activation was detected (not shown). Thus, our focus shifted to identifying changes to components of the cAMP signaling cascade. We looked at the effect of PKA inhibition on ADPKD cyst cell proliferation using ERK and CREB (a target

of both PKA and ERK) activation as pathway readouts. We found that both targets were activated upon pTCM stimulation, and that inhibition using either PKA or MEK inhibitors resulted in a loss of proliferative activity. However, even though the MEK inhibitor (SL327) completely abolished ERK activation assessed at 20 min at low drug concentrations, this was accompanied by only a slight reduction in the proliferation of cells. The dose-dependent decrease in proliferation was instead correlated with a reduction in CREB phosphorylation. This may indicate a minor contribution of ERK to PKA-induced ADPKD cyst cell proliferation (and CREB activation) or ERK activation not captured in this single time point experiment. The CREB inhibition at high SL327 may also reflect off target effects of the drug. A summary of a potential pTCM-induced PKA-dependent pathway is illustrated in Figure 3.8.

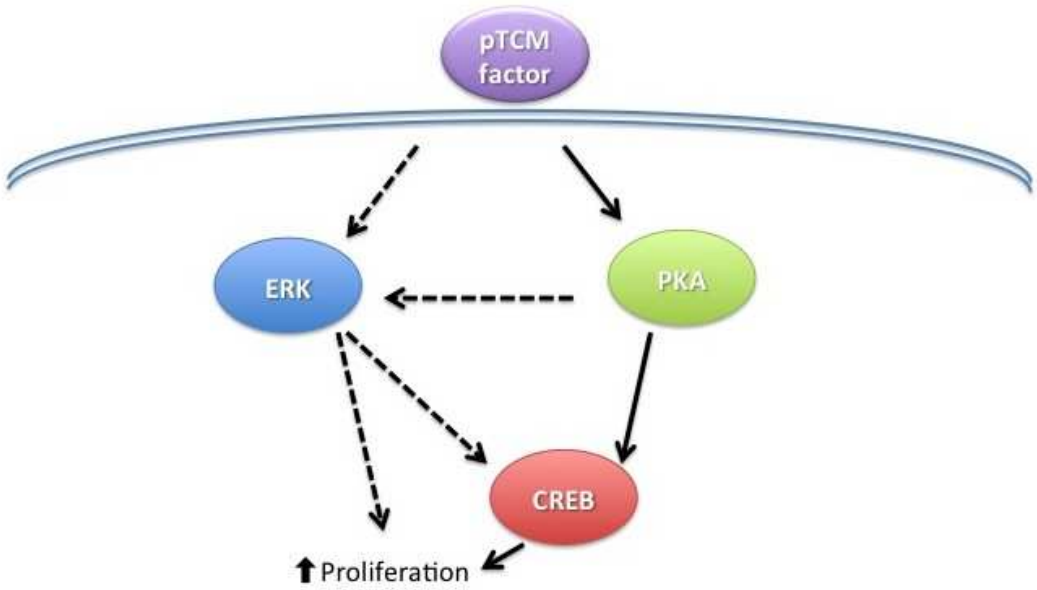


Figure 3.8: Schematic illustrating possible pathway scenarios contributing to pTCM-induced proliferation of ADPKD cyst cells. pTCM stimulates CREB activation and proliferation in ADPKD cyst cells in a PKA-dependent manner. pTCM-induced ERK activation may be caused directly or indirectly via PKA. ERK activation may subsequently stimulate ADPKD cyst cell proliferation directly or in a CREB-dependent manner.

The data do suggest a role for CREB activation in proliferation. This molecule sits at a hub with many different signaling inputs and has been tied to cell proliferation and survival, especially in tumors[219]. Unfortunately, the direct contribution of CREB activation to pTCM-induced ADPKD cyst cell proliferation using a CBP/CREB interaction inhibitor resulted in cell death even at minor concentrations (data not shown), and conclusions could therefore not be drawn from these experiments. Nonetheless, these results indicate a correlation between CREB activation and pTCM-induced ADPKD cyst cell proliferation. The effects of ERK inhibition using alternate inhibitors will be assessed to determine whether PKA stimulation directly contributes to CREB activation, or if it does so indirectly through ERK phosphorylation. Further testing to confirm pTCM-induced cAMP stimulation and subsequent cell proliferation will also be necessary.

Interestingly, GRO-α, and its receptor, CXCR2, have also been implicated in the activation of the Ras-ERK signaling pathway, downstream of the epidermal growth factor receptor (EGFR) in ovarian cancer cells[220]. Activation of tyrosine kinase receptors (TKR), including EGFR, by ligands present in cystic fluid have also contributed to MAPK/ERK signaling activation and cell proliferation[25]. It is possible that GRO-α from the pTCM signals through a similar pathway in ADPKD cyst cells, particularly since treatment of these cells with pTCM also leads to EGFR activation (data not shown). Unfortunately, treatment of ADPKD cells with TKR inhibitors in our study have proven detrimental to their survival and, therefore, conclusions could not be drawn from these results (data not shown). We are currently investigating the effect of exogenous GRO-α treatment on ADPKD cyst cell proliferation. If successful, inhibition of CXCR2 and Ras-

ERK signaling components will be carried out in the presence of recombinant GRO-α as well as with pTCM to determine the effects, if any, that GRO-α and CXCR2 contribute to the activation of the MAPK/ERK pathway and ADPKD cyst cell proliferation.

It is also possible that GRO-α mediates its pro-proliferative effects via CXCR2 in a Ca^{2+}/Calmodulin-dependent manner. CXCR2 is a G-protein coupled receptor, which induces Ca^{2+} flux in certain cell types[221, 222]. In ADPKD cells, Ca^{2+}/CaM stimulated adenylate cyclase 3 (AC3) appears to be a dominant isoform of AC that responds to vasopressin[223]. Experiments examining the effects of CaM inhibition on pTCM-induced proliferation and activation of downstream targets of cAMP signaling in ADPKD cyst cells would determine this link.

Our study characterizes programmed macrophage factors as soluble, stable, protein molecules capable of inducing proliferation of ADPKD cyst cells *in vitro*. A candidate factor, GRO-α, and its receptor, CXCR2, have been identified as potential effectors of this proliferative response. Furthermore, we have identified pTCM-induced activation of certain downstream components of the cAMP signaling cascade upon pTCM stimulation, supporting their targeting as potential therapies against PKD progression.

Chapter Four

The Role of WNT5A in Autosomal Dominant Polycystic Kidney Disease Progression

Abstract

Polycystic kidney diseases (PKD) are inherited disorders characterized by fluid-filled cysts primarily in the kidneys. Wnts, a group of secreted glycoproteins, including WNT5A, are involved in many developmental processes, whereas their deregulation has been shown to contribute to a multitude of diseases, including cancers of the breast, colon and skin, human birth defect disorders like spina bifida, the most common human neural tube closure birth, as well as skeletal defects. We have identified elevated levels of WNT5A in cyst epithelial cells in renal tissue sections from ADPKD patients and an upregulation of *WNT5A* transcripts in primary cyst epithelial cells compared to non-cystic human kidney (NHK) cells. This increase in ADPKD *WNT5A* transcript levels was further enhanced in the presence of macrophages. Treatment of ADPKD cyst cells with exogenous WNT5A resulted in increased proliferation of the cells compared to controls. Furthermore, knockdown of endogenous *WNT5A* transcript in ADPKD cyst cells using shRNA attenuated their growth. Taken together, our results suggest a role for WNT5A in ADPKD cyst cell growth and identify WNT5A as a potential target of therapy to slow down disease progression.

Introduction

ADPKD affects approximately 600,000 individuals in the US, 60% of whom will require some form of renal replacement therapy. Genetic mutations in the polycystin-1 (PKD1) or polycystin-2 (PKD2) genes are primarily responsible for the onset of ADPKD. It is a systemic disorder characterized by the development of multiple fluid-filled renal cysts in addition to several extrarenal manifestations. At a cellular level, ADPKD kidneys show increased cyst cell proliferation, increased fluid secretion, extracellular matrix abnormalities, as well as interstitial inflammation and fibrosis[7].

As such, ADPKD is a chronic kidney disease and shares many common features with ischemic injury or ureteral obstruction, both forms of acute kidney injury (AKI). For example, in these forms of AKI kidney epithelial cells have been found to exhibit partial de-differentiation, thickening of the basement membrane, excessive extracellular matrix deposition, and increased rates of both apoptosis and proliferation, all of which are characteristics present in ADPKD kidneys[224-228]. Furthermore, severe tubule dilation, characteristic of ADPKD, also occurs after ureteral obstruction injury, partially the result of increased rates of proliferation[229]. In addition, infiltrating inflammatory cells characteristic of human ADPKD and mouse models of PKD are also a hallmark feature after ureteral obstruction and ischemia-reperfusion (IR)-induced injury[230, 231]. The subsequent and gradual development of fibrosis observed in PKD is also observed after experimental ureteral obstruction[228].

AKI has also been shown to cause renal cyst development in rodent studies. Significant dilation of the tubules as well as cyst formation are observed long term in wild-type rodents post-injury[232, 233]. Interestingly, AKI has also been identified as a

"third-hit" responsible for promotion of cystogenesis in adult mouse models of PKD[111, 112]. These studies suggests that overactive injury repair processes following significant renal damage can inadvertently lead to or accelerate cyst growth, and that identification of these processes may uncover pathways that can be targeted for therapy to slow down disease progression in PKD.

Several members of the Wnt signaling pathway have been found to be upregulated in the kidneys of animal models in the context of renal injury and repair[137, 234, 235]. Wnts are secreted signaling molecules that play an important role in many developmental processes including organogenesis, while their misregulation has been associated with several diseases including Alzheimer's, diabetes and kidney disease[236-238]. Currently, 19 different Wnt ligands and 10 Wnt receptors (Frizzleds) have been discovered in mammals[88]. Wnts can be categorized as either canonical, β-catenin-dependent, or non-canonical, β-catenin-independent ligands, depending on the downstream signaling pathways activated. The binding of a canonical Wnt to its cognate Frizzled receptor and lipoprotein receptor-related protein LRP5/6 co-receptor, results in the stabilization of β-catenin in the cytosol, allowing it to subsequently translocate to the nucleus. β-catenin then binds a member of the T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) family, stimulating the transcription of several proliferative genes[87]. On the other hand, non-canonical Wnts activate β-catenin-independent pathways, which include Wnt/calcium pathway[239] and the planar cell polarity (PCP) pathway[240].

Studies have revealed that ablation of β-catenin in renal epithelium post-injury leads to aggravated AKI[241]. Lin *et al.*, found that infiltrating macrophages post-injury produced functionally active Wnt ligands, including *Wnt7b*. Canonical Wnt7B has also

been shown to play an important role during kidney development by regulating epithelial differentiation, proliferation and polarization. Genetic ablation of Wnt7b from macrophages prior to kidney injury in mouse models resulted in incomplete repair, suggesting the critical role that macrophages and their secreted factors play in tissue regeneration post-injury[137].

Among the non-canonical Wnts, WNT5A plays an important role in early development. Wnt5a homozygous mutant mice exhibit orofacial abnormalities, dwarfism, dysplasia of the lungs and genitals, shortened tails and limbs, and die perinatally[242]. *Wnt5a* is expressed in the interstitial mesenchyme of the developing mouse kidney[243] and Wnt5a conditional knockout in nascent mesoderm exhibit renal abnormalities including the formation of duplex ureters and kidneys[244]. In addition, *Wnt5a* has been shown to be upregulated in mouse kidneys after acute kidney injury[137], suggesting a role in kidney repair. However, the specific functions of Wnt5A during the repair process post-injury, is unclear.

In this study, we analyze the expression and role of WNT5A in the chronic kidney injury model of ADPKD. We begin by observing the upregulation of *WNT5A* RNA and protein levels in ADPKD cyst cells. This expression was found to be further upregulated in the presence of macrophages, cells which we have shown act to promote tubule cell proliferation and cyst expansion. We also demonstrate that WNT5A has an effect on ADPKD cell proliferation as well as on microcyst expansion *in vitro*. Knockdown of *WNT5A* in these ADPKD cells using shRNA lentiviruses targeting *WNT5A* transcripts had attenuates their proliferation. This study provides direct evidence that WNT5A

promotes the proliferation of ADPKD cyst cells and could contribute to cystogenesis in ADPKD.

Experimental Procedures

Cell culture: Primary cultures of ADPKD cyst cells were supplied by the PKD Biomaterials Research Core laboratory at the University of Kansas Medical Center and cultured in “ADPKD cyst cell media” as described in Chapter Two.

Immunohistochemistry: Immunohistochemistry of de-paraffinized kidney sections were performed as described previously (Chapter Two).

Generating shRNA lentiviruses: For the generation of lentivirus knockdown of *WNT5A* in ADPKD cyst cells, eight plasmids from the GIPZ WNT5A shRNA Transfection Starter Kit (Open Biosystems, US), which are programmed to constitutively co-express the targeting shRNA as well as GFP, were each co-transfected into HEK293T cells in addition to packaging vectors using a second generation plasmid system (psPAX2 and pMD2.G; Addgene plasmids 12,259 and 12,260, respectively, Cambridge, MA) by Lipofectamine reagent (Invitrogen, US), according to manufacturer instructions. Media from transfected cells was replaced after 2 days and then collected, combined and centrifuged after 2 and 4 days post-transfection for use in infection experiments.

Infection with shRNA lentiviruses: ADPKD cells (2 X 10^5 cells/ well of a 6-well culture plate) were seeded and incubated at 37 ℃ for 1 d. Lentiviruses carrying *WNT5A* targeting shRNA or non-silencing shRNA in media containing 8ug/ml polybrene were added to separate wells at a multiplicity of infection (MOI) of 10. Virus containing media

was then removed the next day and replaced with media containing 10% FBS. After 24 hr cells were trypsinized and subjected to cell sorting to select for GFP-expressing cells.

Proliferation assay: Proliferation of ADPKD cells was measured using the CyQUANT® GR dye (Life Technologies) according to the manufacturer's protocol. Briefly, *WNT5A* knockdown and control ADPKD cells (2 X 10^3 cells/well of a 96-well culture plate) were incubated for 0, 1, 3 and 5 d either in low serum media or high serum media (containing 10% FBS) at 37 ℃. For each time point, media was r emoved from culture plates, which were then frozen at -80 ℃ to facilitate cell lysis upon the addition of CyQUANT® lysis buffer. After addition of dye, quantitative measurement of fluorescence was carried out using a SynergyTM 2 microplate reader (BioTEK Instruments, Inc.; Winooski, VT).

RNA isolation and qRT-PCR: Total RNA was isolated from cells (ADPKD cells knocked-down for *WNT5A*, ADPKD cells infected with non-silencing shRNA lentivirus, and uninfected ADPKD and NHK cells) after 5 days of culture using the RNeasy Miniprep kit (Qiagen, Valencia, CA). One µg RNA was used as template for cDNA synthesis using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative RT-PCR was performed on Mx3005P using Sybr Green Master Mix (Agilent, Palo Alto, CA). The thermal cycler conditions were 95 ℃ for 10 m X 1, then 40 cycles of 95 ℃ for 30 s, 55 ℃ for 1 m, an d 72 ℃ for 1 min, and completed with 1 cycle of 95 ℃ 1 m, 55 ℃ for 30 s, and 95 ℃ for 30 s. Expression of human *WNT5A* gene expression was be normalized to human *Gapdh.*

Results

WNT5A *is upregulated in ADPKD cyst cells and its expression is stimulated by macrophage co-culture in vitro.*

Microarray gene expression analysis revealed upregulation of the non-canonical WNT5A in ADPKD cells relative to NHK (not shown; Darren Wallace, personal comm). To verify these data, RNA samples were collected from primary epithelial cyst cells obtained from kidneys of ADPKD patients and non-cystic human kideys (NHK) cells. *WNT5A* expression levels were found to be increased approximately 2.5 fold in ADPKD cyst cells using qRT-PCR analysis (Figure 4.1A).

To determine whether the level of WNT5A was also elevated at the protein level, sections of kidneys from ADPKD patients were stained for WNT5A using immunohistochemistry. This revealed WNT5A levels to be highly upregulated in the epithelial walls lining the cysts (Figure 4.1B).

Since we have previously demonstrated the proliferative effect of macrophages on ADPKD cyst cells[144], we sought to determine whether *WNT5A* expression in tubule epithelial cells was affected by the presence of macrophages. RAW 264.7 murine macrophages were co-cultured with both NHK and ADPKD cysts cells for 24 hours, after which RNA was isolated and *WNT5A* expression levels analyzed from each cell type using qRT-PCR. *WNT5A* expression in both epithelial cell types was found to be further upregulated in the presence of macrophages, up to 6.5 fold in co-cultured ADPKD cyst cells compared to ADPKD cyst cells alone (Figure 4.1A).

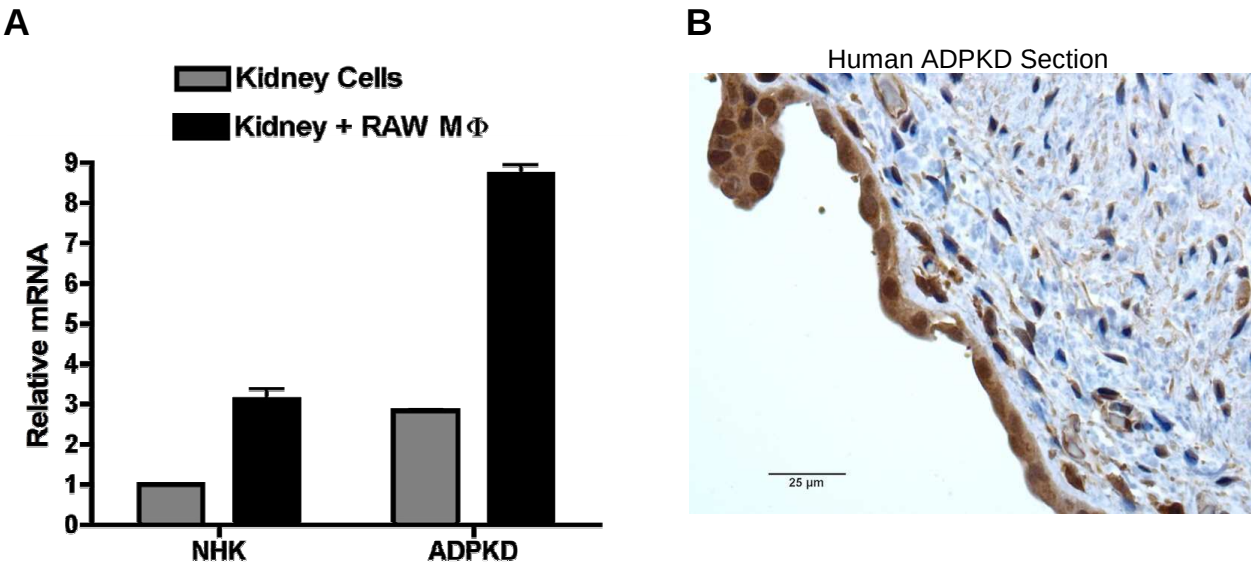


Figure 4.1: WNT5A is upregulated in ADPKD and its expression is stimulated by macrophages *in vitro.* A: qRT-PCR of NHK and ADPKD cyst cells. ADPKD cyst cell expression is 2.5 fold higher than in NHK cells. *WNT5A* expression is further upregulated after 24 hour co-culture with RAW macrophages in both cell types. B: Immunohistochemistry using human WNT5A antibody reveals epithelial cyst cell specific expression pattern (representative image of n=3).

WNT5A promotes ADPKD cell proliferation and microcyst expansion in vitro*.*

To determine the effects of WNT5A on ADPKD cell proliferation, ADPKD cells were incubated with Wnt5a conditioned media (WCM), made from mouse L-cells stably transfected with Wnt5a, for three days. WCM was found to stimulate ADPKD cell proliferation compared to control conditioned media (CCM) made from control L-cells (Figure 4.2A). To confirm the specificity of this response, Wnt5a was depleted from WCM using specific antisera directed against Wnt5a and effects on ADPKD cell proliferation were compared to WCM containing control IgG. Depletion of Wnt5a in the WCM obviated the proliferative response, whereas no effect was seen in the control-depleted samples (Figure 4.2A).

We also assessed effects of WNT5A on microcyst growth in a 3D collagen culture system. ADPKD cells were seeded in a collagen matrix incubated in media supplemented with either Forskolin (Fsk), Epidermal Growth Factor (EGF) or both to initiate microcyst formation, in the presence or absence of purified recombinant WNT5A. After incubation for 10 days, cyst area per well was determined and compared with each sample. Similar to the pro-proliferative macrophage response observed in Chapter 2, WNT5A was also found to induce significant expansion of ADPKD microcysts, especially in the presence of cysts initiated by Fsk and EGF (Figure 4.2B) with no change in total number of cysts per well (data not shown). These results are the first to demonstrate a pro-proliferative role for WNT5A in ADPKD cells. Since *WNT5A* is upregulated in the presence of macrophages, this raises the possibility that WNT5A may act as one of the potential mediators of the pro-proliferative effect induced by macrophages in ADPKD cells.

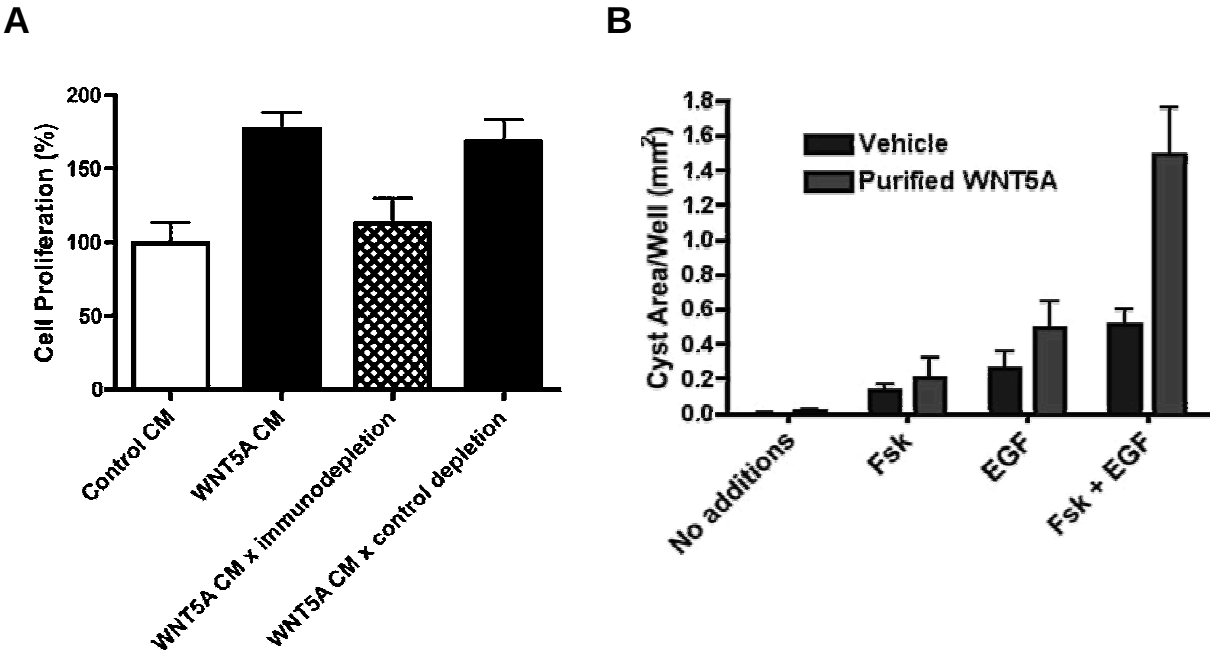


Figure 4.2: WNT5A promotes ADPKD cell proliferation and microcyst expansion *in vitro.* A: WCM stimulate ADPKD cell proliferation compared to control CCM. Wnt5a was depleted from WCM using specific antisera directed against Wnt5a and effects on ADPKD cell proliferation were compared to WCM containing control IgG. Depletion of Wnt5a in the WCM obviated the proliferative response, whereas no effect was seen in the control-depleted samples (n=3). B: ADPKD cells seeded in a collagen matrix with media supplemented with either Forskolin (Fsk), Epidermal Growth Factor (EGF) or both to initiate microcyst formation, in the presence or absence of purified recombinant WNT5A. After incubation for 10 days, cyst area per well was determined and compared with each sample. WNT5A induces significant expansion of ADPKD microcysts, particularly in the presence of cysts initiated by Fsk and EGF.

WNT5A *knockdown in ADPKD cyst cells attenuate cyst cell growth* in vitro*.*

After observing the proliferative effect of WNT5A on ADPKD cyst cells, we hypothesized that knockdown of *WNT5A* in these cells may have an inhibitory effect on their proliferation. To test this, we used shRNA lentiviruses targeted against *WNT5A* in ADPKD cells to assess the effect on cell growth, compared to non-specific shRNA treatment, over a 7-day time period.

To first identify the lentiviral constructs that promote the most efficient *WNT5A* knockdown, we co-transfected 8 different WNT5A pGIPZ shRNA plasmids into HEK293T cells separately, each along with a *WNT5A* expression vector. Western blot for WNT5A identified three shRNA constructs that yielded greater than 75% knockdown efficiency, one of which, shRNA-3256, generated a 94% knockdown relative to control (Figure 4.3A). The GFP-expressing lentiviruses carrying the three shRNA constructs, pGIPZ-3256, pGIPZ-449 and pGIPZ-479, as well as a non-silencing (NS) shRNA (negative control), were then generated to test their knockdown efficiency in human ADPKD cyst cells. In these experiments, cell were first infected with each respective lentivirus separately, and then sorted for the GFP-expressing cells using flow cytometry. Knockdown of *WNT5A* in the sorted selected cells was assessed using qRT-PCR since the WNT5A antibody lacked the sensitivity to detect protein with the small number of cells available to test. Each of the three *WNT5A* targeting shRNAs produced a knockdown of 90% or greater compared to the NS control (Figure 4.3B).

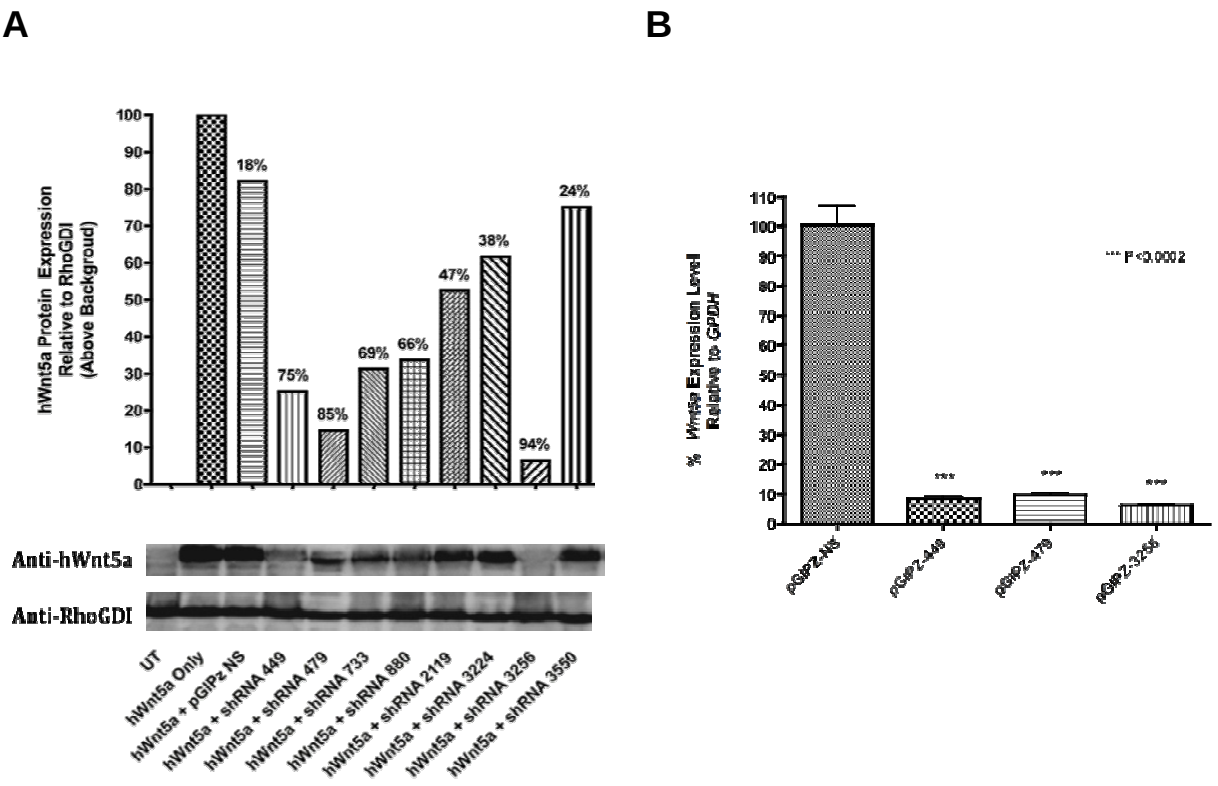


Figure 4.3: WNT5A-specific shRNA plasmid knockdown of *WNT5A in vitro.* A: Eight WNT5A pGIPZ shRNA plasmids were co-transfected into HEK293T cells separately, along with a WNT5A expression vector for each. Western blot for WNT5A identified three shRNA constructs that yielded greater than 75% knockdown efficiency compared to a non-silencing control plasmid. B: The GFP-expressing lentiviruses carrying the three shRNA constructs, pGIPZ-3256, pGIPZ-449 and pGIPZ-479, as well as a non-silencing (NS) shRNA (negative control), were infected into human ADPKD cyst cells. qRT-PCR showing the knockdown of endogenous *WNT5A* using *WNT5A* specific lentiviruses demonstrated over 90% knockout compared to NS control in human ADPKD cyst cells for each (n=3).

WNT5A knockdown cells generated using the above mentioned lentiviruses were selected and grown *in vitro* to assess the effects of *WNT5A* depletion on ADPKD cell proliferation after 0, 1, 3 and 5 days after seeding (corresponding to days 2, 3, 5 and 7 post-infection, respectively). Surprisingly, not only did we observe complete inhibition of growth, but also a reduction in the overall number of *WNT5A*-depleted cells compared to NS control cells, which began to recover slightly by the 7th day post-infection (Figure 4.4). Similar results were obtained using other lentivurses as well (data not shown). This

suggests that there may be a basic requirement for WNT5A in promoting survival of these cells.

It may be that a basal level of WNT5A is required for survival, while elevated levels of this protein may promote proliferation. Future experiments using lentiviruses with varying degrees of *WNT5A* knockdown will be tested and used on ADPKD cyst cells co-cultured with macrophages to determine the specific contribution of elevated *WNT5A* expression to macrophage-induced ADPKD cyst cell proliferation. Nonetheless, these data collectively suggest a role for WNT5A in ADPKD cyst cell proliferation.

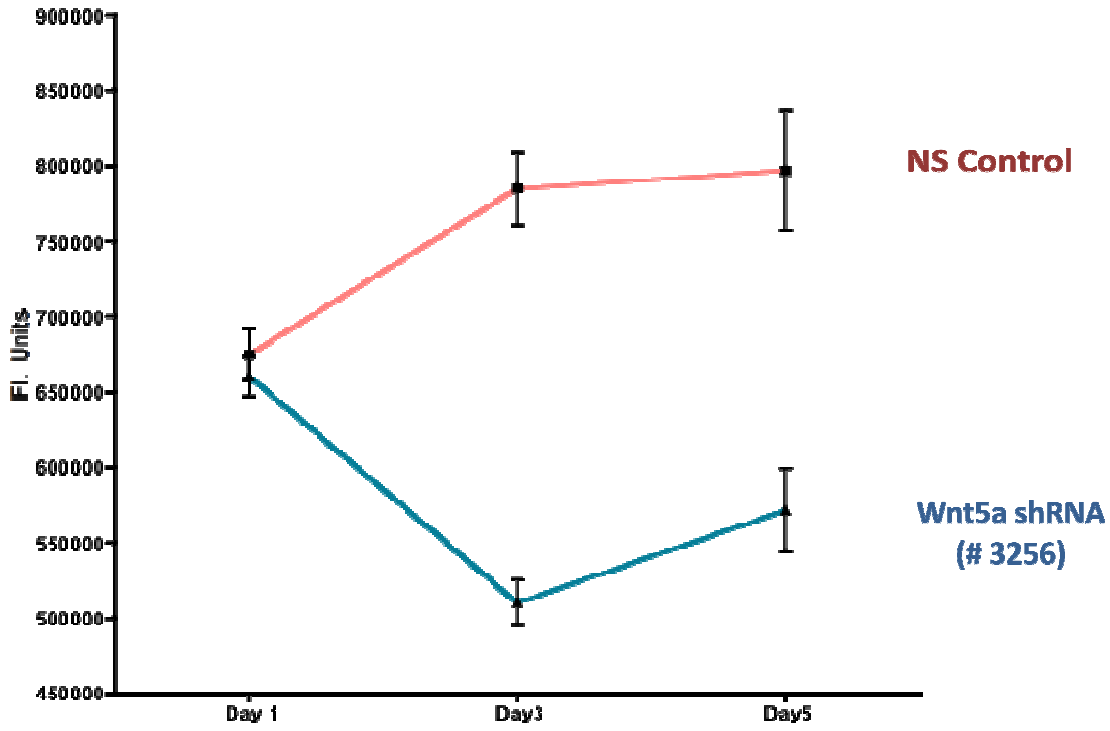


Figure 4.4: WNT5A-specific shRNA lentivirus knockdown of *WNT5A in vitro.* ADPKD cyst cells infected with pGIPZ-3256 or NS-control were sorted for GFP and seeded in for a proliferation assay. Cells were collected and proliferation measured after 0, 1, 3 and 5 days post-sorting (corresponding to days 2, 3, 5 and 7 post-infection, respectively). Knockdown of *WNT5A* in ADPKD cyst cells using the pGIPZ-3256 lentivirus as compared to a non-silencing control lentivirus resulted in decreased ADPKD cyst cell growth.

Discussion

In this study, we sought to uncover the role that WNT5A, which primarily activates non-canonical Wnt signaling, may contribute to the progression of ADPKD. ADPKD is a chronic kidney disease that shares many common features with kidneys responding to ischemic or obstructive injury. The elevated levels of *WNT5A* expression in ADPKD cyst cells compared to non-cystic tubule epithelial cells, demonstrated in these studies, may arise from the same initiating events that promote the upregulation of *WNT5A* in the kidney following acute ischemic injury[137]. In those studies, the transcript levels for *WNT5A*, as well as for several other members of the Wnt family were found to be elevated in whole kidneys at five days post-ischemic injury[137], a time during which large numbers of infiltrating macrophages are present[195]. Whether the presence of these macrophages contributed to the elevated expression of *WNT5A* in these injured kidneys was not determined. However, this seems to be a possibility, given the upregulation of *WNT5A* in cyst-derived tubule epithelial with macrophage co-culture (Figure 4.1).

Exogenous WNT5A treatment in our studies was found to promote both ADPKD cyst cell proliferation and microcyst expansion compared to non-WNT5A treated cells. This proliferative effect in 2D culture was demonstrated to be specific for WNT5A since the effect was eliminated after depletion of WNT5A with a specific antibody. This pro-proliferative effect appears to conflict with the traditional classification of WNT5A as a tumor-suppressor. WNT5A is best known as an antagonizer of the canonical Wnt signaling pathway, promoting the degradation of β-catenin and, as a result, has been shown to act as a tumor suppressor[245]. It was shown to be downregulated in a number

of different cancers including colorectal cancer[246, 247], neuroblastoma[248], ductal breast cancer[249, 250] and leukaemias[251-253]. Furthermore, exogenous expression of WNT5A in colorectal-cancer or thyroid-cancer cell lines has been shown to decrease their invasion, migration, and proliferation[246, 254]. However, there is accumulating evidence for the oncogenic effects of WNT5A in tumors from other tissues such as melanoma[255], breast cancer[256], gastric cancer[257], pancreatic cancer[258], lung cancer[259] and prostate cancer[260]. WNT5A also demonstrates a pro-proliferative ability in the embryo by regulating proliferation of certain progenitor cells[261]. Therefore, given the proliferative capacity of WNT5A and its demonstrated upregulation in the presence of macrophages in this study, combined with its overexpression post-injury, it is possible that WNT5A contributes to regeneration of cells post-injury, although this has yet to be examined.

We were unable to delineate the specific contribution of WNT5A to macrophage-induced ADPKD cell proliferation in this study since knockdown of *WNT5A* using shRNA lentiviruses not only halted proliferation of ADPKD cyst cells, but also compromised the survival of these cells. This suggests a basic requirement for *WNT5A* in epithelial cell survival. Use of lower levels of *WNT5A* knockdown to establish partial, non-deleterious knockdown in these cells will be necessary to assess WNT5A contribution, if any, on macrophage-induced ADPKD cyst cell proliferation.

Nonetheless, this study supports the role of WNT5A in the proliferation of ADPKD cyst cell and microcyst growth *in vitro*. Experiments are underway to determine the effect of WNT5A depletion in an orthologous mouse model of ADPKD. If proven effective in ameliorating disease progression, WNT5A itself, or components of relevant

downstream signaling pathways that are yet to be identified, are likely to provide new targets for therapy.

Chapter Five

Summary, conclusions and future directions

Polycystic kidney disease (PKD) represents a family of genetic disorders characterized by renal cystic growth and progression to kidney failure. No treatment is currently available for individuals with PKD. However, since the discovery of the genes that cause PKD onset, namely *PKD1* and *PKD2*, research in the field has naturally focused on the cell autonomous, intrinsic aberrations found in the cyst cells. Our research proposes a unique approach by addressing an extrinsic factor, macrophages, to further enhance current research on PKD progression. A role for macrophages in the progression of PKD was established by the observations that they were capable of promoting ADPKD cyst cell proliferation *in vitro* and that their chemical depletion in animal models of PKD was able to attenuate cyst growth and expansion *in vivo*[144, 145]. This is not dissimilar to the scenario that occurs during acute kidney injury (AKI), where depletion of wound-healing macrophages during the recovery phase post-injury resulted in reduced tubular cell proliferation and repair[140]. In order to leverage this finding for the development of specific PKD therapies, a more detailed understanding of the mechanisms by which macrophages accomplish this is required. Consequently, with this work, I set out to elucidate molecular mechanisms by which macrophages promote disease progression.

We first sought to identify any major macrophage-recruitment factors produced by PKD cells, since their inhibition is likely to prevent attraction of macrophages to the injured cells, reproducing similar results to that of macrophage depletion. MCP-1 is a

major chemokine involved in the attraction of monocytes from the blood stream across the vascular endothelium in response to inflammation and injury as well as for routine immunological surveillance of tissues[160, 161]. Both MCP-1 and its receptor CCR2 have been demonstrated to be upregulated and involved in various diseases including multiple sclerosis[262], rheumatoid arthritis[263], atherosclerosis[264], and diabetes[265] and have been shown to be a potential target of therapy for these diseases. Although monocytes and macrophages are typically the major source of MCP-1[266, 267], it is also produced by a variety of other cell types including endothelial, fibroblasts, smooth muscle and epithelial cells[268, 269]. Our data is the first to demonstrate the upregulation of MCP-1 in the epithelial tubular cells lining cysts, which had been suggested by previous studies[162, 163]. To our surprise, MCP-1 was also upregulated in surrounding non-cystic tubules. Whether or not this is a result of the intrinsic loss of PKD causing genes in these cells or due to environmental factors, secondary to disease progression, has yet to be determined.

Since MCP-1 had been found to be upregulated in cyst-fluid and urine[162] from PKD patients, and in this current study in renal epithelial cells of PKD patients, we decided to investigate the presence of this chemokine in CM media obtained from human ADPKD cyst epithelial cells and mouse PKD cells in this study. We were able to determine using neutralizing antibodies that not only is this factor present, but also it is responsible for the majority of monocyte chemoattractant activity produced by ADPKD cells. Interestingly, CM obtained from NHK cells was also found to attract monocytes, at the same level as CM from PKD cells, predominantly via MCP-1 (data not shown). It is not clear why this may be the case, particularly since we found that kidney sections

from NHK patients only expressed very low levels of MCP-1, but it is likely NHK cells that have been processed for isolation from explanted kidneys have also undergone stress-inducing conditions that may be interpreted as “injury” by these cells, causing them to secrete injury-response factors, including MCP-1[162].

MCP-1 has been linked to several diseases and its overexpression or deficiency *in vivo* has been shown to increase or decrease disease severity, respectively. For example, overexpression of MCP-1 using a transgenic mouse model system has been shown to contribute to insulin-resistance in diabetic patients as a result of increased macrophage recruitment[270]. In a rodent model for multiple sclerosis, it was shown that MCP-1 or CCR2-deficient mice had reduced severity of disease as a result of decreased recruitment of monocytes into the CNS[271]. Testing the effect of loss of MCP-1 and, subsequently, monocytes/macrophages on disease progression in PKD in this study, we found that MCP-1 deficiency increased survival in our *cpk* mouse model of PKD. Although this did not correlate with a significant improvement in kidney function as measured by BUN at PN 13, experiments are currently underway assessing kidney function at earlier time-points when a measurable difference is likely to be found. Furthermore, the correlation between loss of MCP-1 and decreased macrophage populations in the kidney, as well as proliferation will also be analyzed. Nevertheless, this study is the first to demonstrate a significant improvement in a mouse model of PKD resulting from MCP-1 deficiency, warranting further studies into its potential as an intervention point for the treatment of this disease.

Once monocytes are recruited to the site of inflammation or injury, they are stimulated by their environment to differentiate into distinct types of macrophages. This

is due to the capacity of macrophages to be “programmed” to express different markers as well as secrete distinct factors, depending on the various stimuli present within this microenvironment. In the case of injury, the initial environment is conducive of a pro-inflammatory and pro-injurious type of macrophage that mainly expresses markers of *in vitro*-defined M1 macrophages. After several days, these macrophages are converted to a more wound-healing, M2-like phenotype based on signals in their environment. As such, these macrophages begin secreting pro-proliferative and regenerative factors that promote the replenishment of cells lost during the injurious event[128, 129]. Tumor associated macrophages (TAMs) have been demonstrated to be mainly of this latter phenotype, demonstrating little cytotoxicity for tumor cells but rather promoting tumor-cell proliferation[272]. Similarly, our previous studies have demonstrated that PKD-associated macrophages are predominantly of the M2-like phenotype *in vivo*, with the capacity to be programmed to this phenotype in the presence of renal epithelial cells *in vitro.* Consistent with this identity, the programmed macrophages have an enhanced proliferative capacity compared to unprogrammed macrophages (see Chapter three). It is important to note, however, that a large portion of the macrophage-induced proliferative effect can still be produced by “unprogrammed” macrophages. It is not clear what the precise phenotypic state of these macrophages is, but it is likely that they may share similar properties of M2-like macrophages, even prior to programming with ADPKD CM. Consistently in our previous studies, naïve RAW macrophages treated with media alone expressed the prototypic M2 macrophage marker, *Mrc1,* but not the *Arg1* M2-marker, which was only expressed upon programming with ADPKD cyst cells[144]. This suggests that naïve macrophages *in vitro* are capable of expressing M2-like

markers and are, as such, likely still able to promote a pro-proliferative response in ADPKD cyst cells. Nonetheless, programming with ADPKD cyst cells ensures the generation of M2-like macrophages that more closely mimic those present in the *in vivo* environment that demonstrate an enhanced pro-proliferative capacity exceeding that of naïve macrophages.

In this study, we were able to identify and characterize factors secreted by these ADPKD CM programmed M2-like macrophages that contribute to the pro-proliferative response in ADPKD cyst cells. We showed that one or more proteins is likely responsible for the activity (Figure 3.4). The secretion of one factor, CXCL1 (GRO-α), was found to be significantly increased (~10 fold) in macrophages after programming with ADPKD cyst cell CM (Figure 3.4), as was its message (Table 3.2). Inhibition of the Gro-α signaling response, using an antagonist to its CXCR2 receptor, was effective in reducing the macrophage-induced pro-proliferative response in ADPKD cyst cells.

CXCR2 agonists, including GRO-α, IL-8 and epithelial neutrophil-activating peptide (ENA-78), have been shown to induce proliferation of epithelial cells[196]. Interestingly, these factors were detected by ELISA in kidney cyst fluid from ADPKD patients; albeit at a much lower level than in fluid obtained from human liver cysts. Treatment with cyst fluid from human liver or kidneys was able to induce proliferation of epithelial cells, which was reduced in liver cyst fluid experiments (but not kidney cyst fluid) with pretreatment with the same CXCR2 antagonist (SB225002) used in our study. Furthermore, the proliferative effect was attributed to IL-8 stimulation in that study, based on its higher expression levels in liver cyst fluid compared to GRO-α and ENA-78 and its ability to directly induce proliferation of the cells. However, the effect of

exogenous treatment with GRO-α and ENA-78 on epithelial cell proliferation was not assessed[273]. Of the CXCR2 agonists mentioned in that study, only GRO-α, which has previously been demonstrated to promote tumor survival, angiogenesis and metastasis[215, 216], has been found to be upregulated in programmed macrophages based on our cytokine array and RNA sequencing data. Neither ENA-78 nor IL-8 was upregulated in our cytokine array experiment. Furthermore, neither of these cytokines were upregulated in our RNA sequencing data. These variations are likely due to differences in the source of CXCR2 agonists produced, since macrophages and cyst epithelial cells, from which IL-8 production was attributed to in the mentioned study[273], are likely to produce distinct cytokine profiles. Nonetheless, it remains that inhibition of signaling of the common receptor, CXCR2, using SB225002 promotes the reduction of epithelial cell proliferation in both studies. The direct contribution of GRO-α in this response is under current investigation in our lab, as well as the effects, if any, of other potential factors identified in this study, in the macrophage-induced ADPKD cyst cell pro-proliferative response. Of particular interest, is the role of ENA-78 in this effect since, as with GRO-α[218], this gene can be expressed in response to IL-1β[217], the latter of which was found to be upregulated in our array data.

While identification of signaling ligands secreted by programmed macrophages has proven promising, characterization of downstream signaling pathways involved in the pro-proliferative effect on ADPKD cyst cells is a complementary approach that may also reveal targets for therapy. Pathways known to contribute to ADPKD cell proliferation, including the mTOR, Wnt/B-catenin and cAMP/PKA/MAPK/ERK pathways, were investigated in this study. Of these, only the downstream components of the cAMP

cascade, namely ERK and CREB, were found to be activated upon stimulation with conditioned media from programmed macrophages. Furthermore, inhibition with a PKA-selective inhibitor resulted in the inhibition of activation for these downstream targets, which correlated with a reduction in macrophage-induced proliferation of ADPKD cyst cells. Whether this is a result of direct stimulation of cAMP has yet to be established. Nonetheless, our data support the targeting of cAMP downstream signaling components, particularly PKA and/or ERK, in an effort to hinder disease progression, while implicating macrophages as an alternate, external source for their activation. Future experiments will focus on confirming the direct contribution of cAMP stimulation and its downstream signaling components to the pTCM-induced proliferative effect, and the connection, if any, between this pathway and CXCR2 signaling in ADPKD cysts cells.

Consistent with ADPKD as a model of chronic injury, another portion of our study has focused on assessing the role that WNT5A, the expression of which is induced in models of renal acute injury[137], plays in ADPKD cyst cell proliferation. Not only was WNT5A upregulated in ADPKD cyst epithelial cells compared to NHK cells, but this expression was further enhanced in the presence of macrophages. How macrophages contribute to this upregulation was not addressed in this study, but a potential target of future investigation would likely include analysis of WNT7B. After acute renal injury in mice, Wnt7b is upregulated in whole kidneys and in infiltrating, wound healing renal macrophages[137]. In mice where *Wnt7b* was genetically knocked out specifically in macrophages, regeneration of renal epithelial cells following kidney injury was impaired. However, the direct effect of Wnt7b on these epithelial cells was not shown.

Interestingly, *Wnt5a* was also upregulated in whole kidneys following acute injury, although its potential effects on tubule epithelial cell regeneration were not assessed[137]. Because *Wnt5a* expression can be regulated by *Wnt7b* during early embryonic mouse kidney development[243], it is plausible to hypothesize that, similarly, *Wnt7b* expressed in M2-like macrophages, can induce expression of *Wnt5a* in ADPKD cyst cells post-injury. It would be important to first assess the effects of WNT7B stimulation on *WNT5A* expression in ADPKD cyst cells. If WNT7B is capable of inducing *WNT5A* expression in these cells, the effects of *WNT7B*-depleted macrophages on the induction of ADPKD WNT5A following co-culture would be assessed. Although WNT7B was not upregulated in programmed macrophages based on our RNA sequencing data (unavailable on cytokine array), it may be a common factor produced by both programmed and naïve macrophages, which as previously mentioned, may still exhibit M2-like characteristics.

Another potential stimulator of WNT5A expression in ADPKD cyst cells are activators of STAT3. Conserved binding sites for this transcription factor are found within the WNT5A gene and certain stimuli that promote STAT3 activation have also been shown to induce *WNT5A* in some cells, including cardiac myocytes and embryonic stem-cells[245]. Thus, macrophage factors that can promote STAT3 activation in ADPKD cells are potential candidates for promoting WNT5A induction and, consequently, proliferation. One such promoter of STAT3 activation is IL-10[274], a cytokine that has been previously found to be expressed in our programmed mouse macrophages[144] and human macrophages (not shown). However, treatment of ADPKD cells with exogenous IL-10 was not found to stimulate ADPKD cyst cell proliferation in our experiments (data not shown). Furthermore, co-culture of ADPKD cyst cells with

macrophages did not stimulate further activation of STAT3 in the ADPKD cyst cells (data not shown). Regardless of the stimulating agent, our studies, utilizing both overexpression and knockout experiments, are the first to implicate WNT5A as a growth promoting factor for ADPKD cyst cells *in vitro.* Ongoing studies assessing the *in vivo* role of conditional and global knockout of Wnt5a in a mouse model of PKD will likely confirm the significance of this gene in disease progression and identify it as a likely candidate of targeted therapy against PKD. Identifying downstream effectors of WNT5A-induced proliferation is outside the scope of this study; however, this approach could also uncover further therapeutic targets.

Our study highlights the pivotal role macrophages play in PKD progression. This is consistent with the characterization of PKD as a neoplastic disease that also manifests as a chronic injury condition. Based on this study, we are able to conclude that, as in the case with acute injury or in certain cancers, injured or damaged cyst epithelial cells in PKD kidneys secrete monocyte attractant factors, predominantly MCP-1, in an attempt to recruit macrophages and repair damaged cells or replace those lost to injury. This enhanced regenerative and pro-proliferative capacity is also a result of programming signals secreted by renal epithelial cells. Although the primary purpose of macrophage infiltration is to provide an opportunity to improve organ function via restoration of damaged cells, a simultaneous, albeit inadvertent, side-effect is the promotion of cyst epithelial cell growth and hence cyst expansion. This is because macrophages are incapable of spatial-targeting their growth-stimulating effects. Subsequent to further cyst expansion, compressive injury is also further enhanced, likely resulting in recruitment of even more macrophages in a perpetual loop of a failed

reparative response. Although the absence of macrophages in this case is not likely to prevent cyst initiation or completely hinder cyst growth, it may possibly slow down disease progression considerably by eliminating the additional growth-stimulating effects induced by macrophages. This is supported by our previous studies, which demonstrated reduced cyst expansion after chemical depletion of macrophages in a mouse model of PKD[144]. This current study uncovers several potential candidates that are directly involved in the various processes of macrophage contribution to PKD progression, beginning with macrophage recruitment, secretion of specific pro-proliferative factors, as well as downstream macrophage-induced signaling components contributing to PKD cyst cell growth (Figure 5.1). Any combination of one or more of these factors is likely to provide a promising novel alternative target for therapy to slow ADPKD progression.

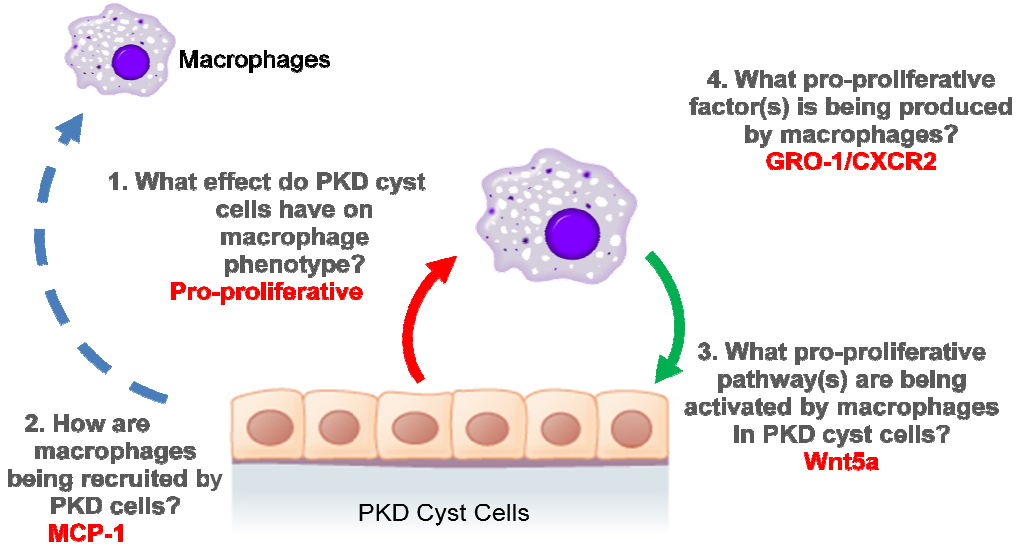


Figure 5.1: Schematic summarizing presented data.

References

1. Igarashi P, Somlo S. Genetics and pathogenesis of polycystic kidney disease. *J Am Soc Nephrol* 2002; **13:** 2384-2398.

2. Grantham JJ. Lillian Jean Kaplan International Prize for advancement in the understanding of polycystic kidney disease. Understanding polycystic kidney disease: a systems biology approach. *Kidney Int* 2003; **64:** 1157-1162.

3. Wilson PD, Falkenstein D. The pathology of human renal cystic disease. *Curr Top Pathol* 1995; **88:** 1-50.

4. Avner ED, Piesco NP, Sweeney WE, Jr.*, et al.* Hydrocortisone-induced cystic metanephric maldevelopment in serum-free organ culture. *Lab Invest* 1984; **50:** 208-218.

5. Hildebrandt F, Zhou W. Nephronophthisis-associated ciliopathies. *J Am Soc Nephrol* 2007; **18:** 1855-1871.

6. Hildebrandt F, Otto E. Molecular genetics of nephronophthisis and medullary cystic kidney disease. *J Am Soc Nephrol* 2000; **11:** 1753-1761.

7. Martinez JR, Grantham JJ. Polycystic kidney disease: Etiology, pathogenesis, and treatment. *Disease-a-month : DM* 1995; **41:** 693-765.

8. Wilson PD. Polycystic kidney disease. *N Engl J Med* 2004; **350:** 151-164.

9. Calvet JP, Grantham JJ. The genetics and physiology of polycystic kidney disease. *Semin Nephrol* 2001; **21:** 107-123.

10. Gabow PA. Autosomal dominant polycystic kidney disease. *N Engl J Med* 1993; **329:** 332-342.

11. Torres VE. New insights into polycystic kidney disease and its treatment. *Curr Opin Nephrol Hypertens* 1998; **7:** 159-169.

12. Ibraghimov-Beskrovnaya O, Bukanov N. Polycystic kidney diseases: from molecular discoveries to targeted therapeutic strategies. *Cell Mol Life Sci* 2008; **65:** 605-619.

13. Perrone RD. Extrarenal manifestations of ADPKD. *Kidney Int* 1997; **51:** 2022-2036.

14. Consortium: TIPKD. Polycystic kidney disease: the complete structure of the PKD1 gene and its protein. The International Polycystic Kidney Disease Consortium. *Cell* 1995; **81:** 289-298.

15. Mochizuki T, Wu G, Hayashi T*, et al.* PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. *Science* 1996; **272:** 1339-1342.

16. Torres VE, Harris PC, Pirson Y. Autosomal dominant polycystic kidney disease. *Lancet* 2007; **369:** 1287-1301.

17. Gallagher AR, Germino GG, Somlo S. Molecular advances in autosomal dominant polycystic kidney disease. *Adv Chronic Kidney Dis* 2010; **17:** 118-130.

18. Harris PC, Torres VE. Polycystic kidney disease. *Annu Rev Med* 2009; **60:** 321-337.

19. Nadella R, Blumer JB, Jia G*, et al.* Activator of G protein signaling 3 promotes epithelial cell proliferation in PKD. *J Am Soc Nephrol* 2010; **21:** 1275-1280.

20. Grantham JJ, Mulamalla S, Swenson-Fields KI. Why kidneys fail in autosomal dominant polycystic kidney disease. *Nat Rev Nephrol* 2011; **7:** 556-566.

21. Grantham JJ, Geiser JL, Evan AP. Cyst formation and growth in autosomal dominant polycystic kidney disease. *Kidney Int* 1987; **31:** 1145-1152.

22. Malhas AN, Abuknesha RA, Price RG. Interaction of the leucine-rich repeats of polycystin-1 with extracellular matrix proteins: possible role in cell proliferation. *J Am Soc Nephrol* 2002; **13:** 19-26.

23. Geng L, Segal Y, Pavlova A*, et al.* Distribution and developmentally regulated expression of murine polycystin. *Am J Physiol* 1997; **272:** F451-459.

24. Zhou J. Polycystins and primary cilia: primers for cell cycle progression. *Annu Rev Physiol* 2009; **71:** 83-113.

25. Torres VE, Harris PC. Autosomal dominant polycystic kidney disease: the last 3 years. *Kidney Int* 2009; **76:** 149-168.

26. Hogan MC, Manganelli L, Woollard JR*, et al.* Characterization of PKD protein-positive exosome-like vesicles. *J Am Soc Nephrol* 2009; **20:** 278-288.

27. Cai Y, Maeda Y, Cedzich A*, et al.* Identification and characterization of polycystin-2, the PKD2 gene product. *J Biol Chem* 1999; **274:** 28557-28565.

28. Hanaoka K, Qian F, Boletta A*, et al.* Co-assembly of polycystin-1 and -2 produces unique cation-permeable currents. *Nature* 2000; **408:** 990-994.

29. Pelucchi B, Aguiari G, Pignatelli A*, et al.* Nonspecific cation current associated with native polycystin-2 in HEK-293 cells. *J Am Soc Nephrol* 2006; **17:** 388-397.

30. Yu Y, Ulbrich MH, Li MH*, et al.* Structural and molecular basis of the assembly of the TRPP2/PKD1 complex. *Proc Natl Acad Sci U S A* 2009; **106:** 11558-11563.

31. Yoder BK, Hou X, Guay-Woodford LM. The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia. *J Am Soc Nephrol* 2002; **13:** 2508-2516.

32. Nauli SM, Alenghat FJ, Luo Y*, et al.* Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat Genet* 2003; **33:** 129-137.

33. Patel V, Chowdhury R, Igarashi P. Advances in the pathogenesis and treatment of polycystic kidney disease. *Curr Opin Nephrol Hypertens* 2009; **18:** 99-106.

34. Tobin JL, Beales PL. The nonmotile ciliopathies. *Genet Med* 2009; **11:** 386-402.

35. Hildebrandt F, Otto E. Cilia and centrosomes: a unifying pathogenic concept for cystic kidney disease? *Nat Rev Genet* 2005; **6:** 928-940.

36. Moyer JH, Lee-Tischler MJ, Kwon HY*, et al.* Candidate gene associated with a mutation causing recessive polycystic kidney disease in mice. *Science* 1994; **264:** 1329-1333.

37. Pazour GJ, Dickert BL, Vucica Y*, et al.* Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene tg737, are required for assembly of cilia and flagella. *J Cell Biol* 2000; **151:** 709-718.

38. Qian F, Boletta A, Bhunia AK*, et al.* Cleavage of polycystin-1 requires the receptor for egg jelly domain and is disrupted by human autosomal-dominant polycystic kidney disease 1-associated mutations. *Proc Natl Acad Sci U S A* 2002; **99:** 16981-16986.

39. Chauvet V, Tian X, Husson H*, et al.* Mechanical stimuli induce cleavage and nuclear translocation of the polycystin-1 C terminus. *J Clin Invest* 2004; **114:** 1433-1443.

40. Low SH, Vasanth S, Larson CH*, et al.* Polycystin-1, STAT6, and P100 function in a pathway that transduces ciliary mechanosensation and is activated in polycystic kidney disease. *Dev Cell* 2006; **10:** 57-69.

41. Wei W, Hackmann K, Xu H*, et al.* Characterization of cis-autoproteolysis of polycystin-1, the product of human polycystic kidney disease 1 gene. *J Biol Chem* 2007; **282:** 21729-21737.

42. Xu C, Rossetti S, Jiang L*, et al.* Human ADPKD primary cyst epithelial cells with a novel, single codon deletion in the PKD1 gene exhibit defective ciliary polycystin localization and loss of flow-induced Ca2+ signaling. *Am J Physiol Renal Physiol* 2007; **292:** F930-945.

43. Yu S, Hackmann K, Gao J*, et al.* Essential role of cleavage of Polycystin-1 at G protein-coupled receptor proteolytic site for kidney tubular structure. *Proc Natl Acad Sci U S A* 2007; **104:** 18688-18693.

44. Wilson PD. Polycystin: new aspects of structure, function, and regulation. *J Am Soc Nephrol* 2001; **12:** 834-845.

45. Parnell SC, Magenheimer BS, Maser RL*, et al.* The polycystic kidney disease-1 protein, polycystin-1, binds and activates heterotrimeric G-proteins in vitro. *Biochem Biophys Res Commun* 1998; **251:** 625-631.

46. Vassilev PM, Guo L, Chen XZ*, et al.* Polycystin-2 is a novel cation channel implicated in defective intracellular Ca(2+) homeostasis in polycystic kidney disease. *Biochem Biophys Res Commun* 2001; **282:** 341-350.

47. Koulen P, Cai Y, Geng L*, et al.* Polycystin-2 is an intracellular calcium release channel. *Nat Cell Biol* 2002; **4:** 191-197.

48. Anyatonwu GI, Estrada M, Tian X*, et al.* Regulation of ryanodine receptor-dependent calcium signaling by polycystin-2. *Proc Natl Acad Sci U S A* 2007; **104:** 6454-6459.

49. Li Y, Santoso NG, Yu S*, et al.* Polycystin-1 interacts with inositol 1,4,5-trisphosphate receptor to modulate intracellular Ca2+ signaling with implications for polycystic kidney disease. *J Biol Chem* 2009; **284:** 36431-36441.

50. Yamaguchi T, Hempson SJ, Reif GA*, et al.* Calcium restores a normal proliferation phenotype in human polycystic kidney disease epithelial cells. *J Am Soc Nephrol* 2006; **17:** 178-187.

51. Vainio S, Lin Y. Coordinating early kidney development: lessons from gene targeting. *Nat Rev Genet* 2002; **3:** 533-543.

52. Little M, Georgas K, Pennisi D*, et al.* Kidney development: two tales of tubulogenesis. *Curr Top Dev Biol* 2010; **90:** 193-229.

53. Van Adelsberg J, Chamberlain S, D'Agati V. Polycystin expression is temporally and spatially regulated during renal development. *Am J Physiol* 1997; **272:** F602-609.

54. Ward CJ, Turley H, Ong AC*, et al.* Polycystin, the polycystic kidney disease 1 protein, is expressed by epithelial cells in fetal, adult, and polycystic kidney. *Proc Natl Acad Sci U S A* 1996; **93:** 1524-1528.

55. Lu W, Peissel B, Babakhanlou H*, et al.* Perinatal lethality with kidney and pancreas defects in mice with a targetted Pkd1 mutation. *Nat Genet* 1997; **17:** 179-181.

56. Wu G, D'Agati V, Cai Y*, et al.* Somatic inactivation of Pkd2 results in polycystic kidney disease. *Cell* 1998; **93:** 177-188.

57. Sedman A, Bell P, Manco-Johnson M*, et al.* Autosomal dominant polycystic kidney disease in childhood: a longitudinal study. *Kidney Int* 1987; **31:** 1000-1005.

58. Belibi FA, Reif G, Wallace DP*, et al.* Cyclic AMP promotes growth and secretion in human polycystic kidney epithelial cells. *Kidney Int* 2004; **66:** 964-973.

59. Yamaguchi T, Pelling JC, Ramaswamy NT*, et al.* cAMP stimulates the in vitro proliferation of renal cyst epithelial cells by activating the extracellular signal-regulated kinase pathway. *Kidney Int* 2000; **57:** 1460-1471.

60. Yamaguchi T, Nagao S, Wallace DP*, et al.* Cyclic AMP activates B-Raf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys. *Kidney Int* 2003; **63:** 1983-1994.

61. Yamaguchi T, Wallace DP, Magenheimer BS*, et al.* Calcium restriction allows cAMP activation of the B-Raf/ERK pathway, switching cells to a cAMP-dependent growth-stimulated phenotype. *J Biol Chem* 2004; **279:** 40419-40430.

62. Calvet JP. Strategies to inhibit cyst formation in ADPKD. *Clin J Am Soc Nephrol* 2008; **3:** 1205-1211.

63. Wallace DP. Cyclic AMP-mediated cyst expansion. *Biochim Biophys Acta* 2011; **1812:** 1291-1300.

64. Wang X, Wu Y, Ward CJ*, et al.* Vasopressin directly regulates cyst growth in polycystic kidney disease. *J Am Soc Nephrol* 2008; **19:** 102-108.

65. Hanaoka K, Devuyst O, Schwiebert EM*, et al.* A role for CFTR in human autosomal dominant polycystic kidney disease. *Am J Physiol* 1996; **270:** C389-399.

66. Davidow CJ, Maser RL, Rome LA*, et al.* The cystic fibrosis transmembrane conductance regulator mediates transepithelial fluid secretion by human autosomal dominant polycystic kidney disease epithelium in vitro. *Kidney Int* 1996; **50:** 208-218.

67. Li H, Findlay IA, Sheppard DN. The relationship between cell proliferation, Cl- secretion, and renal cyst growth: a study using CFTR inhibitors. *Kidney Int* 2004; **66:** 1926-1938.

68. Magenheimer BS, St John PL, Isom KS*, et al.* Early embryonic renal tubules of wild-type and polycystic kidney disease kidneys respond to cAMP stimulation with cystic fibrosis transmembrane conductance regulator/Na(+),K(+),2Cl(-) Co-transporter-dependent cystic dilation. *J Am Soc Nephrol* 2006; **17:** 3424-3437.

69. Somlo S, Torres V, Caplan M. Autosomal dominant polycystic kidney disease and inherited cystic diseases. *The kidney* 2008; **2:** 2283-2314.

70. Shillingford JM, Murcia NS, Larson CH*, et al.* The mTOR pathway is regulated by polycystin-1, and its inhibition reverses renal cystogenesis in polycystic kidney disease. *Proc Natl Acad Sci U S A* 2006; **103:** 5466-5471.

71. Distefano G, Boca M, Rowe I*, et al.* Polycystin-1 regulates extracellular signal-regulated kinase-dependent phosphorylation of tuberin to control cell size through mTOR and its downstream effectors S6K and 4EBP1. *Mol Cell Biol* 2009; **29:** 2359-2371.

72. Dere R, Wilson PD, Sandford RN*, et al.* Carboxy terminal tail of polycystin-1 regulates localization of TSC2 to repress mTOR. *PLoS One* 2010; **5:** e9239.

73. Myrvang H. Polycystic kidney disease: modified rapamycin targets PKD. *Nat Rev Nephrol* 2012; **8:** 554.

74. Ibraghimov-Beskrovnaya O, Natoli TA. mTOR signaling in polycystic kidney disease. *Trends Mol Med* 2011; **17:** 625-633.

75. Grantham JJ, Bennett WM, Perrone RD. mTOR inhibitors and autosomal dominant polycystic kidney disease. *N Engl J Med* 2011; **364:** 286-287; author reply 287-289.

76. Bhunia AK, Piontek K, Boletta A*, et al.* PKD1 induces p21(waf1) and regulation of the cell cycle via direct activation of the JAK-STAT signaling pathway in a process requiring PKD2. *Cell* 2002; **109:** 157-168.

77. Li X, Luo Y, Starremans PG*, et al.* Polycystin-1 and polycystin-2 regulate the cell cycle through the helix-loop-helix inhibitor Id2. *Nat Cell Biol* 2005; **7:** 1202-1212.

78. Liang G, Yang J, Wang Z*, et al.* Polycystin-2 down-regulates cell proliferation via promoting PERK-dependent phosphorylation of eIF2α. *Hum Mol Genet* 2008; **17:** 3254-3262.

79. Parnell SC, Magenheimer BS, Maser RL*, et al.* Polycystin-1 activation of c-Jun N-terminal kinase and AP-1 is mediated by heterotrimeric G proteins. *J Biol Chem* 2002; **277:** 19566-19572.

80. Yu W, Kong T, Beaudry S*, et al.* Polycystin-1 protein level determines activity of the Galpha12/JNK apoptosis pathway. *J Biol Chem* 2010; **285:** 10243-10251.

81. Yu W, Ritchie BJ, Su X*, et al.* Identification of polycystin-1 and Galpha12 binding regions necessary for regulation of apoptosis. *Cell Signal* 2011; **23:** 213-221.

82. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol* 2002; **4:** E131-136.

83. Arnould T, Kim E, Tsiokas L*, et al.* The polycystic kidney disease 1 gene product mediates protein kinase C alpha-dependent and c-Jun N-terminal kinase-dependent activation of the transcription factor AP-1. *J Biol Chem* 1998; **273:** 6013-6018.

84. Le NH, van der Wal A, van der Bent P*, et al.* Increased activity of activator protein-1 transcription factor components ATF2, c-Jun, and c-Fos in human and mouse autosomal dominant polycystic kidney disease. *J Am Soc Nephrol* 2005; **16:** 2724-2731.

85. Puri S, Magenheimer BS, Maser RL*, et al.* Polycystin-1 activates the calcineurin/NFAT (nuclear factor of activated T-cells) signaling pathway. *J Biol Chem* 2004; **279:** 55455-55464.

86. Horsley V, Pavlath GK. NFAT: ubiquitous regulator of cell differentiation and adaptation. *J Cell Biol* 2002; **156:** 771-774.

87. He X, Semenov M, Tamai K*, et al.* LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development* 2004; **131:** 1663-1677.

88. Komiya Y, Habas R. Wnt signal transduction pathways. *Organogenesis* 2008; **4:** 68-75.

89. Niehrs C. The complex world of WNT receptor signalling. *Nat Rev Mol Cell Biol* 2012; **13:** 767-779.

90. Kim E, Arnould T, Sellin LK*, et al.* The polycystic kidney disease 1 gene product modulates Wnt signaling. *J Biol Chem* 1999; **274:** 4947-4953.

91. Saadi-Kheddouci S, Berrebi D, Romagnolo B*, et al.* Early development of polycystic kidney disease in transgenic mice expressing an activated mutant of the beta-catenin gene. *Oncogene* 2001; **20:** 5972-5981.

92. Trudel M, D'Agati V, Costantini F. C-myc as an inducer of polycystic kidney disease in transgenic mice. *Kidney Int* 1991; **39:** 665-671.

93. Husson H, Manavalan P, Akmaev VR*, et al.* New insights into ADPKD molecular pathways using combination of SAGE and microarray technologies. *Genomics* 2004; **84:** 497-510.

94. Lanoix J, D'Agati V, Szabolcs M*, et al.* Dysregulation of cellular proliferation and apoptosis mediates human autosomal dominant polycystic kidney disease (ADPKD). *Oncogene* 1996; **13:** 1153-1160.

95. Cowley BD, Jr., Smardo FL, Jr., Grantham JJ*, et al.* Elevated c-myc protooncogene expression in autosomal recessive polycystic kidney disease. *Proc Natl Acad Sci U S A* 1987; **84:** 8394-8398.

96. Harding MA, Gattone VH, 2nd, Grantham JJ*, et al.* Localization of overexpressed c-myc mRNA in polycystic kidneys of the cpk mouse. *Kidney Int* 1992; **41:** 317-325.

97. Lal M, Song X, Pluznick JL*, et al.* Polycystin-1 C-terminal tail associates with beta-catenin and inhibits canonical Wnt signaling. *Hum Mol Genet* 2008; **17:** 3105-3117.

98. Miller MM, Iglesias DM, Zhang Z*, et al.* T-cell factor/beta-catenin activity is suppressed in two different models of autosomal dominant polycystic kidney disease. *Kidney Int* 2011; **80:** 146-153.

99. Sugiyama N, Tsukiyama T, Yamaguchi TP*, et al.* The canonical Wnt signaling pathway is not involved in renal cyst development in the kidneys of inv mutant mice. *Kidney Int* 2011; **79:** 957-965.

100. Kim I, Ding T, Fu Y*, et al.* Conditional mutation of Pkd2 causes cystogenesis and upregulates beta-catenin. *J Am Soc Nephrol* 2009; **20:** 2556-2569.

101. Fischer E, Legue E, Doyen A*, et al.* Defective planar cell polarity in polycystic kidney disease. *Nat Genet* 2006; **38:** 21-23.

102. Nishio S, Tian X, Gallagher AR*, et al.* Loss of oriented cell division does not initiate cyst formation. *J Am Soc Nephrol* 2010; **21:** 295-302.

103. Qian F, Watnick TJ, Onuchic LF*, et al.* The molecular basis of focal cyst formation in human autosomal dominant polycystic kidney disease type I. *Cell* 1996; **87:** 979-987.

104. Brasier JL, Henske EP. Loss of the polycystic kidney disease (PKD1) region of chromosome 16p13 in renal cyst cells supports a loss-of-function model for cyst pathogenesis. *J Clin Invest* 1997; **99:** 194-199.

105. Vujic M, Heyer CM, Ars E*, et al.* Incompletely penetrant PKD1 alleles mimic the renal manifestations of ARPKD. *J Am Soc Nephrol* 2010; **21:** 1097-1102.

106. Rossetti S, Kubly VJ, Consugar MB*, et al.* Incompletely penetrant PKD1 alleles suggest a role for gene dosage in cyst initiation in polycystic kidney disease. *Kidney Int* 2009; **75:** 848-855.

107. Sampson JR, Maheshwar MM, Aspinwall R*, et al.* Renal cystic disease in tuberous sclerosis: role of the polycystic kidney disease 1 gene. *Am J Hum Genet* 1997; **61:** 843-851.

108. Piontek K, Menezes LF, Garcia-Gonzalez MA*, et al.* A critical developmental switch defines the kinetics of kidney cyst formation after loss of Pkd1. *Nat Med* 2007; **13:** 1490-1495.

109. Davenport JR, Watts AJ, Roper VC*, et al.* Disruption of intraflagellar transport in adult mice leads to obesity and slow-onset cystic kidney disease. *Curr Biol* 2007; **17:** 1586-1594.

110. Patel V, Li L, Cobo-Stark P*, et al.* Acute kidney injury and aberrant planar cell polarity induce cyst formation in mice lacking renal cilia. *Hum Mol Genet* 2008; **17:** 1578-1590.

111. Takakura A, Contrino L, Zhou X*, et al.* Renal injury is a third hit promoting rapid development of adult polycystic kidney disease. *Hum Mol Genet* 2009; **18:** 2523-2531.

112. Happe H, Leonhard WN, van der Wal A*, et al.* Toxic tubular injury in kidneys from Pkd1-deletion mice accelerates cystogenesis accompanied by dysregulated planar cell polarity and canonical Wnt signaling pathways. *Hum Mol Genet* 2009; **18:** 2532-2542.

113. Bastos AP, Piontek K, Silva AM*, et al.* Pkd1 haploinsufficiency increases renal damage and induces microcyst formation following ischemia/reperfusion. *J Am Soc Nephrol* 2009; **20:** 2389-2402.

114. Prasad S, McDaid JP, Tam FW*, et al.* Pkd2 dosage influences cellular repair responses following ischemia-reperfusion injury. *Am J Pathol* 2009; **175:** 1493-1503.

115. Bell PD, Fitzgibbon W, Sas K*, et al.* Loss of primary cilia upregulates renal hypertrophic signaling and promotes cystogenesis. *J Am Soc Nephrol* 2011; **22:** 839-848.

116. Lantinga-van Leeuwen IS, Leonhard WN, van der Wal A*, et al.* Kidney-specific inactivation of the Pkd1 gene induces rapid cyst formation in developing kidneys and a slow onset of disease in adult mice. *Hum Mol Genet* 2007; **16:** 3188-3196.

117. Chapin HC, Caplan MJ. The cell biology of polycystic kidney disease. *J Cell Biol* 2010; **191:** 701-710.

118. Weimbs T. Third-hit signaling in renal cyst formation. *J Am Soc Nephrol* 2011; **22:** 793-795.

119. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature* 2013; **496:** 445-455.

120. Nelson PJ, Rees AJ, Griffin MD*, et al.* The renal mononuclear phagocytic system. *J Am Soc Nephrol* 2012; **23:** 194-203.

121. Hoeffel G, Wang Y, Greter M*, et al.* Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med* 2012; **209:** 1167-1181.

122. Van Ginderachter JA, Movahedi K, Hassanzadeh Ghassabeh G*, et al.* Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. *Immunobiology* 2006; **211:** 487-501.

123. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* 2009; **27:** 451-483.

124. Wang Y, Wang YP, Zheng G*, et al.* Ex vivo programmed macrophages ameliorate experimental chronic inflammatory renal disease. *Kidney Int* 2007; **72:** 290-299.

125. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol* 2004; **4:** 583-594.

126. Mantovani A, Sica A, Sozzani S*, et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 2004; **25:** 677-686.

127. Hao NB, Lu MH, Fan YH*, et al.* Macrophages in tumor microenvironments and the progression of tumors. *Clin Dev Immunol* 2012; **2012:** 948098.

128. Sean Eardley K, Cockwell P. Macrophages and progressive tubulointerstitial disease. *Kidney Int* 2005; **68:** 437-455.

129. Duffield JS. Macrophages and immunologic inflammation of the kidney. *Semin Nephrol* 2010; **30:** 234-254.

130. Solez K, Morel-Maroger L, Sraer JD. The morphology of "acute tubular necrosis" in man: analysis of 57 renal biopsies and a comparison with the glycerol model. *Medicine (Baltimore)* 1979; **58:** 362-376.

131. Bonventre JV. Pathophysiology of acute kidney injury: roles of potential inhibitors of inflammation. *Contrib Nephrol* 2007; **156:** 39-46.

132. Beck-Schimmer B, Oertli B, Pasch T*, et al.* Hyaluronan induces monocyte chemoattractant protein-1 expression in renal tubular epithelial cells. *J Am Soc Nephrol* 1998; **9:** 2283-2290.

133. Dong X, Swaminathan S, Bachman LA*, et al.* Resident dendritic cells are the predominant TNF-secreting cell in early renal ischemia-reperfusion injury. *Kidney Int* 2007; **71:** 619-628.

134. Li L, Huang L, Sung SS*, et al.* NKT cell activation mediates neutrophil IFN-gamma production and renal ischemia-reperfusion injury. *J Immunol* 2007; **178:** 5899-5911.

135. Jo SK, Sung SA, Cho WY*, et al.* Macrophages contribute to the initiation of ischaemic acute renal failure in rats. *Nephrol Dial Transplant* 2006; **21:** 1231-1239.

136. Day YJ, Huang L, Ye H*, et al.* Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages. *Am J Physiol Renal Physiol* 2005; **288:** F722-731.

137. Lin SL, Li B, Rao S*, et al.* Macrophage Wnt7b is critical for kidney repair and regeneration. *Proc Natl Acad Sci U S A* 2010; **107:** 4194-4199.

138. Menke J, Iwata Y, Rabacal WA*, et al.* CSF-1 signals directly to renal tubular epithelial cells to mediate repair in mice. *J Clin Invest* 2009; **119:** 2330-2342.

139. Kim MG, Boo CS, Ko YS*, et al.* Depletion of kidney CD11c+ F4/80+ cells impairs the recovery process in ischaemia/reperfusion-induced acute kidney injury. *Nephrol Dial Transplant* 2010; **25:** 2908-2921.

140. Lee S, Huen S, Nishio H*, et al.* Distinct macrophage phenotypes contribute to kidney injury and repair. *J Am Soc Nephrol* 2011; **22:** 317-326.

141. Ibrahim S. Increased apoptosis and proliferative capacity are early events in cyst formation in autosomal-dominant, polycystic kidney disease. *ScientificWorldJournal* 2007; **7:** 1757-1767.

142. Ashizawa M, Miyazaki M, Furusu A*, et al.* Nephronophthisis in two siblings. *Clin Exp Nephrol* 2005; **9:** 320-325.

143. Anders HJ, Ryu M. Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. *Kidney Int* 2011; **80:** 915-925.

144. Swenson-Fields KI, Vivian CJ, Salah SM*, et al.* Macrophages promote polycystic kidney disease progression. *Kidney Int* 2013; **83:** 855-864.

145. Karihaloo A, Koraishy F, Huen SC*, et al.* Macrophages promote cyst growth in polycystic kidney disease. *J Am Soc Nephrol* 2011; **22:** 1809-1814.

146. Park EY, Seo MJ, Park JH. Effects of specific genes activating RAGE on polycystic kidney disease. *Am J Nephrol* 2010; **32:** 169-178.

147. Zhou J, Ouyang X, Cui X*, et al.* Renal CD14 expression correlates with the progression of cystic kidney disease. *Kidney Int* 2010; **78:** 550-560.

148. Mrug M, Zhou J, Woo Y*, et al.* Overexpression of innate immune response genes in a model of recessive polycystic kidney disease. *Kidney Int* 2008; **73:** 63-76.

149. Gardner KD, Jr., Evan AP, Reed WP. Accelerated renal cyst development in deconditioned germ-free rats. *Kidney Int* 1986; **29:** 1116-1123.

150. Gardner KD, Jr., Reed WP, Evan AP*, et al.* Endotoxin provocation of experimental renal cystic disease. *Kidney Int* 1987; **32:** 329-334.

151. Werder AA, Amos MA, Nielsen AH*, et al.* Comparative effects of germfree and ambient environments on the development of cystic kidney disease in CFWwd mice. *J Lab Clin Med* 1984; **103:** 399-407.

152. Cowley BD, Jr., Gudapaty S, Kraybill AL*, et al.* Autosomal-dominant polycystic kidney disease in the rat. *Kidney Int* 1993; **43:** 522-534.

153. Takahashi H, Calvet JP, Dittemore-Hoover D*, et al.* A hereditary model of slowly progressive polycystic kidney disease in the mouse. *J Am Soc Nephrol* 1991; **1:** 980-989.

154. Phillips JK, Hopwood D, Loxley RA*, et al.* Temporal relationship between renal cyst development, hypertension and cardiac hypertrophy in a new rat model of autosomal recessive polycystic kidney disease. *Kidney Blood Press Res* 2007; **30:** 129-144.

155. Schmidt-Weber CB, Rittig M, Buchner E*, et al.* Apoptotic cell death in activated monocytes following incorporation of clodronate-liposomes. *J Leukoc Biol* 1996; **60:** 230-244.

156. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 1994; **174:** 83-93.

157. van Rooijen N, Sanders A, van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. *J Immunol Methods* 1996; **193:** 93-99.

158. Hughes J, Ward CJ, Peral B*, et al.* The polycystic kidney disease 1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. *Nat Genet* 1995; **10:** 151-160.

159. Ward CJ, Hogan MC, Rossetti S*, et al.* The gene mutated in autosomal recessive polycystic kidney disease encodes a large, receptor-like protein. *Nat Genet* 2002; **30:** 259-269.

160. Deshmane SL, Kremlev S, Amini S*, et al.* Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res* 2009; **29:** 313-326.

161. Yadav A, Saini V, Arora S. MCP-1: chemoattractant with a role beyond immunity: a review. *Clin Chim Acta* 2010; **411:** 1570-1579.

162. Zheng D, Wolfe M, Cowley BD, Jr.*, et al.* Urinary excretion of monocyte chemoattractant protein-1 in autosomal dominant polycystic kidney disease. *J Am Soc Nephrol* 2003; **14:** 2588-2595.

163. Cowley BD, Jr., Ricardo SD, Nagao S*, et al.* Increased renal expression of monocyte chemoattractant protein-1 and osteopontin in ADPKD in rats. *Kidney Int* 2001; **60:** 2087-2096.

164. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25:** 402-408.

165. Fry JL, Jr., Koch WE, Jennette JC*, et al.* A genetically determined murine model of infantile polycystic kidney disease. *J Urol* 1985; **134:** 828-833.

166. Hou X, Mrug M, Yoder BK*, et al.* Cystin, a novel cilia-associated protein, is disrupted in the cpk mouse model of polycystic kidney disease. *J Clin Invest* 2002; **109:** 533-540.

167. Preminger GM, Koch WE, Fried FA*, et al.* Murine congenital polycystic kidney disease: a model for studying development of cystic disease. *J Urol* 1982; **127:** 556-560.

168. Segerer S, Nelson PJ, Schlondorff D. Chemokines, chemokine receptors, and renal disease: from basic science to pathophysiologic and therapeutic studies. *J Am Soc Nephrol* 2000; **11:** 152-176.

169. Noris M, Bernasconi S, Casiraghi F*, et al.* Monocyte chemoattractant protein-1 is excreted in excessive amounts in the urine of patients with lupus nephritis. *Lab Invest* 1995; **73:** 804-809.

170. Rovin BH, Doe N, Tan LC. Monocyte chemoattractant protein-1 levels in patients with glomerular disease. *Am J Kidney Dis* 1996; **27:** 640-646.

171. Grandaliano G, Gesualdo L, Bartoli F*, et al.* MCP-1 and EGF renal expression and urine excretion in human congenital obstructive nephropathy. *Kidney Int* 2000; **58:** 182-192.

172. Giunti S, Barutta F, Perin PC*, et al.* Targeting the MCP-1/CCR2 System in diabetic kidney disease. *Curr Vasc Pharmacol* 2010; **8:** 849-860.

173. Wada T, Yokoyama H, Furuichi K*, et al.* Intervention of crescentic glomerulonephritis by antibodies to monocyte chemotactic and activating factor (MCAF/MCP-1). *FASEB J* 1996; **10:** 1418-1425.

174. Ojeda JL. Abnormal tenascin expression in murine autosomal recessive polycystic kidneys. *Nephron* 1999; **82:** 261-269.

175. Gattone VH, 2nd, Calvet JP, Cowley BD, Jr.*, et al.* Autosomal recessive polycystic kidney disease in a murine model. A gross and microscopic description. *Lab Invest* 1988; **59:** 231-238.

176. Gattone VH, 2nd, MacNaughton KA, Kraybill AL. Murine autosomal recessive polycystic kidney disease with multiorgan involvement induced by the cpk gene. *Anat Rec* 1996; **245:** 488-499.

177. Shyy JY, Lin MC, Han J*, et al.* The cis-acting phorbol ester "12-O-tetradecanoylphorbol 13-acetate"-responsive element is involved in shear stress-induced monocyte chemotactic protein 1 gene expression. *Proc Natl Acad Sci U S A* 1995; **92:** 8069-8073.

178. Shyy YJ, Hsieh HJ, Usami S*, et al.* Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium. *Proc Natl Acad Sci U S A* 1994; **91:** 4678-4682.

179. Flores D, Battini L, Gusella GL*, et al.* Fluid shear stress induces renal epithelial gene expression through polycystin-2-dependent trafficking of extracellular regulated kinase. *Nephron Physiol* 2011; **117:** p27-36.

180. Lu B, Rutledge BJ, Gu L*, et al.* Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med* 1998; **187:** 601-608.

181. Taub DD, Ortaldo JR, Turcovski-Corrales SM*, et al.* Beta chemokines costimulate lymphocyte cytolysis, proliferation, and lymphokine production. *J Leukoc Biol* 1996; **59:** 81-89.

182. Gu L, Tseng S, Horner RM*, et al.* Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* 2000; **404:** 407-411.

183. Denger S, Jahn L, Wende P*, et al.* Expression of monocyte chemoattractant protein-1 cDNA in vascular smooth muscle cells: induction of the synthetic phenotype: a possible clue to SMC differentiation in the process of atherogenesis. *Atherosclerosis* 1999; **144:** 15-23.

184. Reape TJ, Groot PH. Chemokines and atherosclerosis. *Atherosclerosis* 1999; **147:** 213-225.

185. Werle M, Schmal U, Hanna K*, et al.* MCP-1 induces activation of MAP-kinases ERK, JNK and p38 MAPK in human endothelial cells. *Cardiovasc Res* 2002; **56:** 284-292.

186. Viedt C, Dechend R, Fei J*, et al.* MCP-1 induces inflammatory activation of human tubular epithelial cells: involvement of the transcription factors, nuclear factor-kappaB and activating protein-1. *J Am Soc Nephrol* 2002; **13:** 1534-1547.

187. Rollins BJ. Monocyte chemoattractant protein 1: a potential regulator of monocyte recruitment in inflammatory disease. *Mol Med Today* 1996; **2:** 198-204.

188. Kunkel SL. Through the looking glass: the diverse in vivo activities of chemokines. *J Clin Invest* 1999; **104:** 1333-1334.

189. Gerard C, Rollins BJ. Chemokines and disease. *Nat Immunol* 2001; **2:** 108-115.

190. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* 2006; **354:** 610-621.

191. Power CA, Meyer A, Nemeth K*, et al.* Molecular cloning and functional expression of a novel CC chemokine receptor cDNA from a human basophilic cell line. *J Biol Chem* 1995; **270:** 19495-19500.

192. Kim MS, Magno CL, Day CJ*, et al.* Induction of chemokines and chemokine receptors CCR2b and CCR4 in authentic human osteoclasts differentiated with RANKL and osteoclast like cells differentiated by MCP-1 and RANTES. *J Cell Biochem* 2006; **97:** 512-518.

193. Boring L, Gosling J, Chensue SW*, et al.* Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest* 1997; **100:** 2552-2561.

194. Daly C, Rollins BJ. Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies. *Microcirculation* 2003; **10:** 247-257.

195. Lin SL, Castano AP, Nowlin BT*, et al.* Bone marrow Ly6Chigh monocytes are selectively recruited to injured kidney and differentiate into functionally distinct populations. *J Immunol* 2009; **183:** 6733-6743.

196. Wang Y, Yang J, Gao Y*, et al.* Regulatory effect of e2, IL-6 and IL-8 on the growth of epithelial ovarian cancer cells. *Cell Mol Immunol* 2005; **2:** 365-372.

197. Li X, Magenheimer BS, Xia S*, et al.* A tumor necrosis factor-alpha-mediated pathway promoting autosomal dominant polycystic kidney disease. *Nat Med* 2008; **14:** 863-868.

198. Wood LD, Richmond A. Constitutive and cytokine-induced expression of the melanoma growth stimulatory activity/GRO alpha gene requires both NF-kappa B and novel constitutive factors. *J Biol Chem* 1995; **270:** 30619-30626.

199. Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* 1999; **68:** 821-861.

200. Ishizaki T, Uehata M, Tamechika I*, et al.* Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol Pharmacol* 2000; **57:** 976-983.

201. Chen RH, Abate C, Blenis J. Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. *Proc Natl Acad Sci U S A* 1993; **90:** 10952-10956.

202. Xing J, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* 1996; **273:** 959-963.

203. Gupta P, Prywes R. ATF1 phosphorylation by the ERK MAPK pathway is required for epidermal growth factor-induced c-jun expression. *J Biol Chem* 2002; **277:** 50550-50556.

204. Wiggin GR, Soloaga A, Foster JM*, et al.* MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. *Mol Cell Biol* 2002; **22:** 2871-2881.

205. Mantovani A, Sozzani S, Locati M*, et al.* Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002; **23:** 549-555.

206. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* 2004; **4:** 71-78.

207. An T, Sood U, Pietruk T*, et al.* In situ quantitation of inflammatory mononuclear cells in ductal infiltrating breast carcinoma. Relation to prognostic parameters. *Am J Pathol* 1987; **128:** 52-60.

208. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol* 2002; **196:** 254-265.

209. Mantovani A, Allavena P, Sica A. Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. *Eur J Cancer* 2004; **40:** 1660-1667.

210. Tsutsui S, Yasuda K, Suzuki K*, et al.* Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. *Oncol Rep* 2005; **14:** 425-431.

211. Grantham JJ. Time to treat polycystic kidney diseases like the neoplastic disorders that they are. *Kidney Int* 2000; **57:** 339-340.

212. Dhawan P, Richmond A. Role of CXCL1 in tumorigenesis of melanoma. *J Leukoc Biol* 2002; **72:** 9-18.

213. Richmond A, Balentien E, Thomas HG*, et al.* Molecular characterization and chromosomal mapping of melanoma growth stimulatory activity, a growth factor structurally related to beta-thromboglobulin. *EMBO J* 1988; **7:** 2025-2033.

214. Anisowicz A, Bardwell L, Sager R. Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. *Proc Natl Acad Sci U S A* 1987; **84:** 7188-7192.

215. Nannuru KC, Sharma B, Varney ML*, et al.* Role of chemokine receptor CXCR2 expression in mammary tumor growth, angiogenesis and metastasis. *J Carcinog* 2011; **10:** 40.

216. Horton LW, Yu Y, Zaja-Milatovic S*, et al.* Opposing roles of murine duffy antigen receptor for chemokine and murine CXC chemokine receptor-2 receptors in murine melanoma tumor growth. *Cancer Res* 2007; **67:** 9791-9799.

217. Schmouder RL, Streiter RM, Walz A*, et al.* Epithelial-derived neutrophil-activating factor-78 production in human renal tubule epithelial cells and in renal allograft rejection. *Transplantation* 1995; **59:** 118-124.

218. Omari KM, John G, Lango R*, et al.* Role for CXCR2 and CXCL1 on glia in multiple sclerosis. *Glia* 2006; **53:** 24-31.

219. Siu YT, Jin DY. CREB--a real culprit in oncogenesis. *FEBS J* 2007; **274:** 3224-3232.

220. Bolitho C, Hahn MA, Baxter RC*, et al.* The chemokine CXCL1 induces proliferation in epithelial ovarian cancer cells by transactivation of the epidermal growth factor receptor. *Endocr Relat Cancer* 2010; **17:** 929-940.

221. Puma C, Danik M, Quirion R*, et al.* The chemokine interleukin-8 acutely reduces Ca(2+) currents in identified cholinergic septal neurons expressing CXCR1 and CXCR2 receptor mRNAs. *J Neurochem* 2001; **78:** 960-971.

222. Shibata F, Konishi K, Nakagawa H. Chemokine receptor CXCR2 activates distinct pathways for chemotaxis and calcium mobilization. *Biol Pharm Bull* 2002; **25:** 1217-1219.

223. Pinto CS, Reif GA, Nivens E*, et al.* Calmodulin-sensitive adenylyl cyclases mediate AVP-dependent cAMP production and Cl- secretion by human autosomal dominant polycystic kidney cells. *Am J Physiol Renal Physiol* 2012; **303:** F1412-1424.

224. Arnaout MA. Molecular genetics and pathogenesis of autosomal dominant polycystic kidney disease. *Annu Rev Med* 2001; **52:** 93-123.

225. Bonventre JV. Dedifferentiation and proliferation of surviving epithelial cells in acute renal failure. *J Am Soc Nephrol* 2003; **14 Suppl 1:** S55-61.

226. Devarajan P. Update on mechanisms of ischemic acute kidney injury. *J Am Soc Nephrol* 2006; **17:** 1503-1520.

227. Forbes JM, Hewitson TD, Becker GJ*, et al.* Ischemic acute renal failure: long-term histology of cell and matrix changes in the rat. *Kidney Int* 2000; **57:** 2375-2385.

228. Liu Y. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. *J Am Soc Nephrol* 2004; **15:** 1-12.

229. Wu MJ, Wen MC, Chiu YT*, et al.* Rapamycin attenuates unilateral ureteral obstruction-induced renal fibrosis. *Kidney Int* 2006; **69:** 2029-2036.

230. Rodriguez-Iturbe B, Pons H, Herrera-Acosta J*, et al.* Role of immunocompetent cells in nonimmune renal diseases. *Kidney Int* 2001; **59:** 1626-1640.

231. Zeier M, Fehrenbach P, Geberth S*, et al.* Renal histology in polycystic kidney disease with incipient and advanced renal failure. *Kidney Int* 1992; **42:** 1259-1265.

232. Pagtalunan ME, Olson JL, Tilney NL*, et al.* Late consequences of acute ischemic injury to a solitary kidney. *J Am Soc Nephrol* 1999; **10:** 366-373.

233. Burne-Taney MJ, Yokota N, Rabb H. Persistent renal and extrarenal immune changes after severe ischemic injury. *Kidney Int* 2005; **67:** 1002-1009.

234. He W, Dai C, Li Y*, et al.* Wnt/beta-catenin signaling promotes renal interstitial fibrosis. *J Am Soc Nephrol* 2009; **20:** 765-776.

235. Terada Y, Tanaka H, Okado T*, et al.* Expression and function of the developmental gene Wnt-4 during experimental acute renal failure in rats. *J Am Soc Nephrol* 2003; **14:** 1223-1233.

236. Behrens MI, Lendon C, Roe CM. A common biological mechanism in cancer and Alzheimer's disease? *Curr Alzheimer Res* 2009; **6:** 196-204.

237. Jin T. The WNT signalling pathway and diabetes mellitus. *Diabetologia* 2008; **51:** 1771-1780.

238. Pulkkinen K, Murugan S, Vainio S. Wnt signaling in kidney development and disease. *Organogenesis* 2008; **4:** 55-59.

239. Kohn AD, Moon RT. Wnt and calcium signaling: beta-catenin-independent pathways. *Cell Calcium* 2005; **38:** 439-446.

240. Carroll TJ, Das A. Planar cell polarity in kidney development and disease. *Organogenesis* 2011; **7:** 180-190.

241. Zhou D, Li Y, Lin L*, et al.* Tubule-specific ablation of endogenous beta-catenin aggravates acute kidney injury in mice. *Kidney Int* 2012; **82:** 537-547.

242. Oishi I, Suzuki H, Onishi N*, et al.* The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. *Genes Cells* 2003; **8:** 645-654.

243. Yu J, Carroll TJ, Rajagopal J*, et al.* A Wnt7b-dependent pathway regulates the orientation of epithelial cell division and establishes the cortico-medullary axis of the mammalian kidney. *Development* 2009; **136:** 161-171.

244. Yun K, Ajima R, Sharma N*, et al.* Non-canonical Wnt5a/Ror2 signaling regulates kidney morphogenesis by controlling intermediate mesoderm extension. *Hum Mol Genet* 2014.

245. Katoh M, Katoh M. STAT3-induced WNT5A signaling loop in embryonic stem cells, adult normal tissues, chronic persistent inflammation, rheumatoid arthritis and cancer (Review). *Int J Mol Med* 2007; **19:** 273-278.

246. Dejmek J, Dejmek A, Safholm A*, et al.* Wnt-5a protein expression in primary dukes B colon cancers identifies a subgroup of patients with good prognosis. *Cancer Res* 2005; **65:** 9142-9146.

247. Ying J, Li H, Yu J*, et al.* WNT5A exhibits tumor-suppressive activity through antagonizing the Wnt/beta-catenin signaling, and is frequently methylated in colorectal cancer. *Clin Cancer Res* 2008; **14:** 55-61.

248. Blanc E, Roux GL, Benard J*, et al.* Low expression of Wnt-5a gene is associated with high-risk neuroblastoma. *Oncogene* 2005; **24:** 1277-1283.

249. Jonsson M, Dejmek J, Bendahl PO*, et al.* Loss of Wnt-5a protein is associated with early relapse in invasive ductal breast carcinomas. *Cancer Res* 2002; **62:** 409-416.

250. Dejmek J, Leandersson K, Manjer J*, et al.* Expression and signaling activity of Wnt-5a/discoidin domain receptor-1 and Syk plays distinct but decisive roles in breast cancer patient survival. *Clin Cancer Res* 2005; **11:** 520-528.

251. Liang H, Chen Q, Coles AH*, et al.* Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue. *Cancer Cell* 2003; **4:** 349-360.

252. Roman-Gomez J, Jimenez-Velasco A, Cordeu L*, et al.* WNT5A, a putative tumour suppressor of lymphoid malignancies, is inactivated by aberrant methylation in acute lymphoblastic leukaemia. *Eur J Cancer* 2007; **43:** 2736-2746.

253. Ying J, Li H, Chen YW*, et al.* WNT5A is epigenetically silenced in hematologic malignancies and inhibits leukemia cell growth as a tumor suppressor. *Blood* 2007; **110:** 4130-4132.

254. Kremenevskaja N, von Wasielewski R, Rao AS*, et al.* Wnt-5a has tumor suppressor activity in thyroid carcinoma. *Oncogene* 2005; **24:** 2144-2154.

255. Da Forno PD, Pringle JH, Hutchinson P*, et al.* WNT5A expression increases during melanoma progression and correlates with outcome. *Clin Cancer Res* 2008; **14:** 5825-5832.

256. Fernandez-Cobo M, Zammarchi F, Mandeli J*, et al.* Expression of Wnt5A and Wnt10B in non-immortalized breast cancer cells. *Oncol Rep* 2007; **17:** 903-907.

257. Kurayoshi M, Oue N, Yamamoto H*, et al.* Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion. *Cancer Res* 2006; **66:** 10439-10448.

258. Ripka S, Konig A, Buchholz M*, et al.* WNT5A--target of CUTL1 and potent modulator of tumor cell migration and invasion in pancreatic cancer. *Carcinogenesis* 2007; **28:** 1178-1187.

259. Huang CL, Liu D, Nakano J*, et al.* Wnt5a expression is associated with the tumor proliferation and the stromal vascular endothelial growth factor--an expression in non-small-cell lung cancer. *J Clin Oncol* 2005; **23:** 8765-8773.

260. Wang Q, Williamson M, Bott S*, et al.* Hypomethylation of WNT5A, CRIP1 and S100P in prostate cancer. *Oncogene* 2007; **26:** 6560-6565.

261. Yamaguchi TP, Bradley A, McMahon AP*, et al.* A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* 1999; **126:** 1211-1223.

262. Sorensen TL, Ransohoff RM, Strieter RM*, et al.* Chemokine CCL2 and chemokine receptor CCR2 in early active multiple sclerosis. *Eur J Neurol* 2004; **11:** 445-449.

263. Hayashida K, Nanki T, Girschick H*, et al.* Synovial stromal cells from rheumatoid arthritis patients attract monocytes by producing MCP-1 and IL-8. *Arthritis Res* 2001; **3:** 118-126.

264. Kusano KF, Nakamura K, Kusano H*, et al.* Significance of the level of monocyte chemoattractant protein-1 in human atherosclerosis. *Circ J* 2004; **68:** 671-676.

265. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A* 2003; **100:** 7265-7270.

266. Yoshimura T, Robinson EA, Tanaka S*, et al.* Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutinin-stimulated human blood mononuclear leukocytes. *J Immunol* 1989; **142:** 1956-1962.

267. Yoshimura T, Yuhki N, Moore SK*, et al.* Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. *FEBS Lett* 1989; **244:** 487-493.

268. Cushing SD, Berliner JA, Valente AJ*, et al.* Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci U S A* 1990; **87:** 5134-5138.

269. Standiford TJ, Kunkel SL, Phan SH*, et al.* Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. *J Biol Chem* 1991; **266:** 9912-9918.

270. Kamei N, Tobe K, Suzuki R*, et al.* Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J Biol Chem* 2006; **281:** 26602-26614.

271. Izikson L, Klein RS, Charo IF*, et al.* Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2. *J Exp Med* 2000; **192:** 1075-1080.

272. Dinapoli MR, Calderon CL, Lopez DM. The altered tumoricidal capacity of macrophages isolated from tumor-bearing mice is related to reduce expression of the inducible nitric oxide synthase gene. *J Exp Med* 1996; **183:** 1323-1329.

273. Amura CR, Brodsky KS, Gitomer B*, et al.* CXCR2 agonists in ADPKD liver cyst fluids promote cell proliferation. *Am J Physiol Cell Physiol* 2008; **294:** C786-796.

274. Wehinger J, Gouilleux F, Groner B*, et al.* IL-10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected genes. *FEBS Lett* 1996; **394:** 365-370.