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POLYCYSTIC LIVER DISEASES: CONGENITAL DISORDERS OF CHOLANGIOCYTE SIGNALING

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SUMMARY

Polycystic liver diseases (PLD) are inherited disorders of the biliary epithelium, caused by genetic defects in proteins associated with intracellular organelles, mainly the endoplasmic reticulum and the cilium. PLD are characterized by the formation and progressive enlargement of multiple cysts scattered throughout the liver parenchyma, and include different entities, classified based on their pathology, inheritance pattern, involvement of the kidney and clinical features. PLD should be considered as congenital diseases of cholangiocyte signaling. Here, we will review the changes in signaling pathways involved in liver cyst formation and progression, and their impact on cholangiocyte physiology. Each pathway represents a potential target for therapies aimed at reducing disease progression.

Keywords

Liver cysts; cholangiopathies; gene defects; biliary epithelium; liver transplantation; ADPKD; ARPKD; PCLD

Introduction

Polycystic liver diseases (PLD) are a genetically and clinically heterogeneous group of inherited cholangiopathies^{1–3}. **Autosomal Dominant Polycystic Kidney Disease (ADPKD)** occurs in 1:500-1,000 individuals; it is characterized by the formation of multiple cysts in the kidney, liver and pancreas, and by a variety of vascular abnormalities. Despite extensive cyst substitution of the hepatic parenchyma, liver function is well preserved, portal hypertension is rare, and the patient is asymptomatic, unless complications develop (including compression, cyst infections or bleeding). The “isolated” **Polycystic Liver**

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Disease (PCLD) is a far less common disease that shares many clinical features of ADPKD, except for the renal involvement. Fibropolycystic diseases, i.e., **Congenital Hepatic Fibrosis (CHF)**, **Caroli disease (CD)**, and **Autosomal Recessive Polycystic Kidney Disease (ARPKD)** are also rare diseases, with a prevalence of 1:20,000 live births. CD and CHF are characterized by recurrent acute cholangitis (CD) and severe portal hypertension, due to excessive peribiliary fibrosis (CHF). CD patients have an increased risk of cholangiocarcinoma. Other, rarer disorders associated with PLD, have been reviewed elsewhere⁴. The clinical approach to PLD has been recently reviewed⁵. Here we will discuss the pathogenic aspects that are specific to the liver.

Genetics of PLD

ADPKD is associated with mutations in two genes: *PKD1*, and *PKD2*, which encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively. **PC1**, a 460 kDa transmembrane protein, is localized in the primary cilium, plasma membrane and *adherens junctions*. PC-1 functions as a mechanoreceptor that, sensing changes in apical flow, stimulates PC2-mediated Ca^{2+} signals¹. In addition, cleaved fragments of the PC1 cytoplasmic tail, translocate to the nucleus and bind to β -catenin, preventing its transcriptional activity (fragment p200)⁶, or interact with STAT6/P100 (fragment 112)⁷, or decrease the ERK-dependent phosphorylation of tuberousclerosis-2-complex⁸. **PC2** (TRPP2) is a 110-kDa transmembrane protein with both the N- and C-terminus located intracellularly. PC1 and PC2 colocalize in the cilium and physically interact through their C-terminus, however, the largest pool of PC2 is located in the ER^{9,10}. PC2 is a member of the transient receptor potential channels (TRP) family and functions as a non-selective Ca^{2+} channel. TRP channels have the ability to multimerize with other proteins that determine their function^{11–13}. Thus, PC2 responds to mechanical stimuli (bending of the cilium) or to biliary osmolarity or participates in receptor-operated Ca^{2+} -signaling, depending on its interactions with PC1, TRVP4, or IP3R^{9,10}. PC2 also interacts with the two major intracellular Ca^{2+} channels (i.e. ryanodine receptors–RyR- and inositol,1,4,5-trisphosphate receptors–IP3R) and regulates cytoplasmic and ER- Ca^{2+} homeostasis^{9,10}.

PRKCSH and **SEC63**, the genes responsible for PCLD^{14,15}, encode for proteins expressed in the ER that are associated with the processing of glycoproteins in the endoplasmic reticulum (ER). **PRKCSH**, encodes for the protein kinase-C-substrate 80K-H (or hepatocystin), a protein that regulates the correct localization in the ER, of enzymes involved in the quality control of newly synthesized glycoproteins¹⁵. The **SEC63** gene encodes for a component of the molecular machinery regulating translocation and folding of newly synthesized membrane glycoproteins¹⁴.

Mutations in the **PKHD1** gene cause ARPKD, CD and CHF¹. **PKHD1** encodes for fibrocystin, a large protein with a single transmembrane domain, and sequence homologies with plexins and the HGF receptor (C-Met). Fibrocystin is localized in primary cilia and basal bodies of several epithelia. Fibrocystin complexes with PC2, and is involved in cell Ca^{2+} homeostasis; fibrocystin can also signal via the proteolytic cleavage of its C-terminus^{16,17}. A variety of functions have been proposed for fibrocystin, from proliferation to secretion, terminal differentiation, tubulogenesis¹⁸. Transcription of **PKHD1** is regulated by HNF-1 β a transcription factor expressed in cholangiocyte¹⁹.

Cellular mechanisms of liver cysts formation and growth

In ADPKD and PCLD, liver cysts are scattered throughout the parenchyma, without connection to the biliary tree, that appears anatomically intact and without accompanying fibrosis. By contrast, in fibropolycystic diseases, liver cysts are connected to the biliary tree, which appear distorted and embedded in abundant fibrotic tissue. Cyst formation is not a

common reaction of the biliary epithelium to liver damage. In response to obstructive cholestasis, the biliary epithelium generates multiple branching tubules, whereas in inflammatory processes, reactive cholangiocytes form a ramified mesh with cellular chords that do not encircle a lumen. Mice with conditional PC knock-out show a progressive formation of liver cysts, even when the induction is performed weeks after birth^{20,21}. This indicates that polycystins are necessary to maintain a normal biliary architecture during adult life. It is not clear whether the lack of polycystin expression induces pathologic changes throughout the biliary tree, or only in specialized portions that maintain “developmental” capabilities. The pathogenic sequence leading to liver cyst formation and progressive growth is the subject of extensive investigation.

Altered biliary developmental program (Ductal plate malformation)

The biliary tree is a branching system of tubules that begins at the canals of Hering, and then converges to create ducts of progressively larger size. Biliary ontogenesis begins when periportal hepatoblasts surrounding branches of the portal vein, undergo a phenotypic switch and assemble into a layer of epithelial cells, called “ductal plate”^{3,22}. Over the following weeks, specific segments of the ductal plate form a second layer of cells, which eventually arranges into tubular structures that mature and become incorporated into the mesenchyme of the developing portal space. Non-duplicated ductal plates are then reabsorbed by apoptosis (ductal plate remodeling). PLD are characterized by macroscopic cysts and by the presence of multiple biliary microhamartomas, scattered throughout the liver parenchyma. Both structures resemble ductal plates that failed to remodel, justifying the inclusion of PLD among the ductal plate malformations (DPM^{3,22}).

Cilia and beyond

Primary cilia are involved in the regulation of fundamental biological activities of epithelial cells, including differentiation, proliferation and secretion. Several proteins encoded by genes associated with PLD are expressed in primary cilia; DPM is consistently observed in mice defective for ciliary proteins. Cholangiocyte primary cilia are non-motile, but can bend in response to changes in luminal fluid flow and transduce a mechanical force into an intracellular calcium signal. Changes in luminal flow increased $[Ca^{2+}]_i$ and cAMP production, an effect inhibited by silencing PC1, PC2, or adenylyl-cyclases-6 (AC6)^{2,23}. By contrast proteins associated with PLD, are not exclusively expressed in cilia. For example, PC2 is also highly expressed in the ER, where it regulates calcium levels^{9,13}. Furthermore, hepatocystin and *SEC63* (PCLD) are expressed in the ER, and yet, the clinical presentation is similar¹⁴.

Altered epithelial fluid secretion

Cholangiocytes possess secretory properties and actively contribute to bile formation, thereby regulating its volume, pH and composition²⁴. The net amount of fluid secreted is the end-result of several pro-secretory and anti-secretory stimuli. Autocrine, paracrine and endocrine stimuli are integrated at the level of adenylyl cyclases (ACs)²⁵, the enzymes that synthesize the second messenger cAMP. CFTR, a cAMP/PKA-activated Cl^- channel, is the main regulator of bile secretion. CFTR stimulates Cl^- and HCO_3^- efflux, inhibits NHE_3^- dependent Na^+ absorption and facilitates apical release of ATP and purinergic receptors activation²⁴. Of note, the severity of ADPKD was milder in two cases in which ADPKD coexisted with cystic fibrosis, a disease that impairs CFTR-dependent Cl^- secretion²⁶. However, fluid secretion is not the major pathophysiologic mechanisms leading to cyst growth in the liver. To account for the very slow growth rates of the cysts, the net difference between absorption and secretion should be very subtle and constant in the face of increasing intraluminal pressure. However, the increased intraluminal pressure may stretch

the lining epithelial cells and induce cell proliferation, via apical secretion of purinergic agonists, and their interaction with P2X and P2Y purinergic receptors.

Increased cholangiocyte proliferation

The cystic epithelium has a very high mitotic index (as judged by PCNA and Ki67 expression)^{20,21}, suggesting that increased proliferation of cystic cholangiocytes is the major determinant of cyst growth. Markers of proliferation and apoptosis are both present in liver cysts, however, the size of the liver progressively increases (about 2% of the liver volume per year), indicating that apoptosis does not match proliferation.

During cell division, the epithelial cells of a tubular structure must align their mitotic spindle in such a way that the daughter cells will grow along the longitudinal, rather than the horizontal, axis. The ability to organize cell division and the growth of the epithelial layer along a specific plane ("planar cell polarity") is an important property of epithelia, regulated by the non-canonical Wnt pathway; its malfunction may result in tubule enlargement rather than tubule elongation. In rodent models with fibrocystin (Pkd1) deficiency, the orientation of the mitotic spindle of kidney cells is distorted²⁷. Direct experimental evidence for this model in ADPKD and in cholangiocytes is yet to be produced.

Cystic fluid and conditioned medium of cultured cystic cholangiocytes contain elevated levels of cytokines and growth factors, which are able to promote cell proliferation and cyst expansion. Among them, IL-6, EGF, VEGF and IGF-1^{28,29} utilize autocrine loops to stimulate the proliferation of cystic cholangiocytes. Their expression likely represents the phenotypic and functional signature of a relative loss of cell differentiation. For example, the pattern of angiogenic factor expression by cystic cholangiocytes in ADPKD is similar to that of the ductal plates³⁰.

Estrogens³¹ and VEGF^{21,28} promote cyst growth via ERK1/2, which is the main pathway of regulation of cholangiocyte proliferation, whereas the PI3-kinase/AKT/mTOR pathway is activated by IGF1 in ADPKD liver cysts²⁰. In cholangiocytes, cAMP/PKA activates Ras and ERK1/2 and cell proliferation, an effect that is significantly increased in cystic cholangiocytes. This is a major difference with kidney cells in which cAMP inhibits replication in normal condition, but stimulates replication if polycystin are defective. The reason for these differences may lay in the differential sensibility to cAMP of cells-specific B-Ras and C-Ras isoforms.

Autocrine and paracrine angiogenic signaling

Ductal plate malformations are frequently associated with an abnormal vascular morphology, thereby resulting from increased angiogenic signaling between bile ducts and liver vascular structures. The anatomic relationship between intrahepatic bile ducts and hepatic arterial vascularisation begins during the developmental stages and is mediated by VEGF released by maturing bile ducts³⁰. Cystic cholangiocytes overexpress VEGF, angiopoietin-1 and their cognate receptors VEGFR2 and Tie-2. VEGF and angiopoietin-1 stimulate cholangiocyte proliferation and the formation of vessels around the growing cysts²⁸. VEGF production is controlled by Hypoxia-Inducible Factor-1 α (HIF-1). Cystic cholangiocytes express more HIF-1 α than normal cholangiocytes, when cultured under the same conditions, indicating expression of VEGF/HIF-1 α is caused by the loss of PC1 and PC2 function, rather than by hypoxia of the cystic epithelium²¹. HIF-1 α levels can be modulated in normoxic conditions by a number of factors, (including IL-1, IL-6, EGF, HGF, TGF β , 17- β -estradiol, IGF-1)²⁹, which can stabilize or phosphorylate HIF-1 α via PI3K/AKT/tuberin/mTOR or Raf/MEK/ERK, or STAT3. Inhibition of VEGF production or blockage of VEGF-R2 *in vivo* in PC2 defective mice inhibits cyst growth²¹.

Extracellular matrix (ECM) remodeling

Remodeling of the ECM is necessary for the expansion of the cyst wall. Matrix metalloproteinases (MMPs) are involved in the breakdown of extracellular matrix in embryonic development as well as in tissue repair and remodeling. IL-8, a cytokine known to be up-regulated in the liver cyst epithelium, stimulates MMP2 and MMP9 production by endothelial cells and portal myofibroblasts. MMP2 is increased in polycystic kidneys and participate to the ECM remodeling necessary to accommodate cyst expansion²⁹. On the contrary, a major feature of ARPKD/CHF/CD, is the progressive establishment of fibrosis in the hepatic portal space in close vicinity to aberrant bile ducts. The mechanisms of fibrogenesis in fibropolycystic diseases are not clear.

Future directions: PLD as disorders of cholangiocytes signaling

There is convincing evidence that PLD are disorders of intracellular signaling; altered Ca^{2+} homeostasis, and increased production of cAMP being the main defects. Lower intracellular Ca^{2+} and higher cAMP levels have been consistently reported in cystic epithelia from ADPKD and ARPKD models^{10, 23}. The mechanistic relationship between defective polycystins/fibrocystin and the disorder of these second messengers is unclear. Here, we will provide a reasonable, but yet speculative, working model based on experimental evidence generated in ADPKD models (Fig1). It is important to emphasize that the fine molecular tuning of these interactions remains largely unknown.

Several studies, including our own observations in cystic cholangiocytes, indicate that intracellular Ca^{2+} levels are lower in the cystic epithelium (below 100 nM)^{10,23}. PC2 functions as a membrane Ca^{2+} channel, in response to mechanical stimulations (in partnership with PC1) or to variations in osmolarity (in partnership with TRPV4)³². PC-2 also interacts both physically and functionally with IP_3R and RyR, thereby regulating Ca^{2+} homeostasis in the ER^{9,10}, the largest controllable intracellular Ca^{2+} store in non-excitabile cells. Thus, a defect in PC-2 may affect resting $[\text{Ca}^{2+}]$ by reducing extracellular Ca^{2+} entry and by altering ER Ca^{2+} homeostasis.

The amount of cAMP produced by a given cell is the result of the activity of several adenylyl cyclase (AC) isoforms, which respond to different stimuli and second messengers. At least seven of them are expressed in cholangiocytes²⁵. Among them, AC8 is regulated by Ca^{2+} /calmodulin, while AC5 and AC6 are inhibited at physiological Ca^{2+} concentrations (100 to 200 nM), but can be activated at the Ca^{2+} concentrations measured in polycystin-defective cells. Thus, at the $[\text{Ca}^{2+}]$ measured in cystic cholangiocytes, AC6 becomes more prone to activation than AC8. Of note, AC6 gene silencing abolishes shear stress-induced signaling in polarized cholangiocytes²³.

Thus, the increased cAMP level in cystic epithelia may be related to the activation of AC6, facilitated by the changes in intracellular Ca^{2+} homeostasis. Increased epithelial levels of cAMP stimulate fluid secretion and also the proliferative activity of cystic cholangiocytes. Alpini and coworkers have shown that cAMP stimulates proliferation in normal cholangiocytes via the PKA/Src/Raf/MEK/ERK1/2 cascade³³. In cystic cholangiocytes, the proliferative response and ERK1/2 activation in response to cAMP is significantly higher, suggesting that cAMP levels are not the only determinant of ERK1/2 activation. In fact, polycystin may have additional effects directly mediated by the proteolytic cleavage and nuclear translocation of its carboxy-terminal tail to the nucleus.

Activation ERK1/2 has a number of effects, including stimulation of the mTOR pathway²⁰. Both ERK1/2 and mTOR converge in stimulating cyclins and HIF1 α . The list of HIF-1 α -regulated genes is large, and includes genes coding for proteins involved in energy

metabolism, erythropoiesis, and cell proliferation, in addition to VEGF. Mice deficient in PC2 show a severe liver phenotype, high proliferation rate of the cystic epithelium and high expression of VEGF and its receptor VEGFR-2. Expression of pERK1/2, p-mTOR and HIF1 α , are also increased, suggesting that wild type PC2 acts as repressors of the cAMP/PKA/Raf/MEK/ERK/mTOR cascade^{20, 21}. The observation that changes in Ca²⁺/cAMP signalling similar to the one described in PC-2 defective cells are common to cystic diseases characterized by different genetic defects and phenotypes suggests that PC2 is central to the pathogenesis of all PLD forms.

The pathophysiological relevance of this model is demonstrated by the reduction of cyst growth *in vivo*, after administration of SU5418 (inhibition of VEGFR2 signalling), or rapamycin (inhibition of mTOR and of VEGF production)^{20,21}. Inhibition of cAMP production was also exploited as a therapeutic strategy. Somatostatin represses AC function through its receptor SSTR2, which is expressed in the liver only by cholangiocyte. Octreotide, given *in vivo* to PCK rats, reduced liver and kidney weight, hepatic and renal cyst volume and fibrosis, and diminished the rate of cell proliferation in hepatic and renal epithelia³⁴. A recent randomized controlled double blind clinical trial revealed a 5% reduction in liver cyst volume in patients with symptomatic ADPKD/PLCD treated with the long-acting somatostatin analogue lanreotide³⁵.

Several open questions remain. The mechanism leading to reduced cellular levels of Ca²⁺ are still obscure, and the impact of the lack of polycystins on ER Ca²⁺ homeostasis, and the role of AC6 need better understanding. In wild type animals, the result of increased cAMP-dependent proliferation is an expansion of the bile duct mass, rather than cystic transformation. Thus, the above working model addresses the mechanisms of progression of the established cysts, but does not explain how polycystins and fibrocystins actually interfere with the morphogenetic mechanisms in the biliary tree.

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Abbreviations

ADPKD	autosomal dominant polycystic kidney disease
AKT	v-akt murine thymoma viral oncogene homolog 1
ARPKD	autosomal recessive polycystic kidney disease
CD	Caroli disease
CFTR	cystic fibrosis transmembrane conductance regulator
CHF	congenital hepatic fibrosis
DMP	ductal plate malformation
ECM	extracellular matrix
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERK1/2	Extracellular-Regulated Kinase-1 and -2

HGF	hepatocyte growth factor
HNF-1β	hepatocyte nuclear factor 1 homeobox B
IGF-1	Insulin-like growth factor 1
IL	interleukin
IP3R	inositol 1,4,5-trisphosphate receptor
MEK	Mitogen-activated protein kinase kinase
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
NHE3	sodium-hydrogen exchanger 3
PC1	polycystin-1;
PC2	polycystin-2
PCLD	“isolated” polycystic liver disease
PKD	polycystin kidney disease gene
PLD	polycystic liver disease
PRKCSH	protein kinase C substrate, 80 Kda protein
Raf	proto-oncogene serine/threonine-protein kinase
RyR	Ryanodine receptor
SEC63	SEC63 homolog (<i>S. cerevisiae</i>)
SSTR2	Somatostatin receptor 2
STAT3	Signal transducer and activator of transcription 3
STAT6	signal transducer and activator of transcription 6, interleukin-4 induced
TGFβ	Transforming growth factor beta
Tie-2	TEK tyrosine kinase
TRPP2	transient receptor potential polycystic 2
TRVP4	transient receptor potential vanilloid 4
VEGF	vascular endothelial growth factor
VEGF-R	vascular endothelial growth factor receptor

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fig. 1.
Alterations of intracellular signaling in PLD. See text for details.