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## Cholangiocyte Biology as Relevant to Cystic Liver Diseases

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### CONTENTS

INTRODUCTION  
MORPHOLOGY AND SECRETORY  
FUNCTIONS OF THE NORMAL BILIARY  
EPITHELIUM  
CHOLANGIOCYTE REACTION TO DAMAGE  
MECHANISMS OF CYSTIC LIVER  
DISEASES: CILIA AND BEYOND  
CELLULAR MECHANISMS OF LIVER CYST  
FORMATION AND GROWTH  
NEW THERAPEUTIC STRATEGIES IN  
PREVENTING CYST GROWTH  
REFERENCES

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### *Summary*

Polycystic liver diseases are hereditary disorders that affect the biliary epithelium, often in conjunction with the renal tubule epithelium. Characterized by the progressive formation of cysts

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throughout the liver and kidney, they can often lead to severe life-threatening complications. Polycystins and fibrocystin, the defective proteins in the dominant and in the recessive form of the disease, respectively, are mainly expressed in the primary (nonmotile) cilia of cholangiocytes, the epithelial cells that line the intrahepatic biliary tree. Important clues for understanding the pathogenesis of cystic diseases come from understanding the biology and pathobiology of cholangiocytes. In this chapter, cholangiocyte function and morphology is first briefly described, with particular emphasis on the regulation of their secretory properties and the complex intercellular signaling. Then, we discuss a number of possible mechanisms leading to cyst formation and progressive growth of the cysts. In both autosomal dominant and recessive forms, liver cysts arise from an aberrant development of intrahepatic bile duct epithelium. During cyst expansion, different factors, including excessive fluid secretion, extracellular matrix remodeling, increased proliferation of the epithelial cells lining the cyst, and aberrant hypervascularization around the cyst wall, variably take part in promoting progressive cyst growth. Many of these factors act via autocrine mechanisms. Each of them represents a possible target for therapies aimed at reducing the growth of liver cysts.

**Key Words:** Cholangiocytes, ADPKD, ARPKD, Polycystin-1, Polycystin-2, Fibrocystin, VEGF, Cilium, Ductal plate malformation

## INTRODUCTION

Polycystic liver diseases are genetic disorders that affect mainly the bile duct and the renal tubule epithelia. Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited diseases, occurring in 1:400 to 1:1,000 individuals; it is characterized by the formation of multiple cysts in the kidney, liver, and pancreas. Although synthetic liver function is usually preserved in ADPKD, severe cyst complications (mass effect, hemorrhage, infection, or rupture) may develop and thus require urgent liver transplantation. Autosomal recessive polycystic kidney disease (ARPKD) and its liver-related phenotypes Caroli disease (CD) and congenital hepatic fibrosis (CHF) are, by contrast, rare disorders with an estimated prevalence of 1:20,000 live births. CD and CHF are characterized by recurrent acute cholangitis and severe portal hypertension due to an excessive peribiliary fibrosis that can be further complicated by the development of biliary malignancies.

The even rarer “isolated” polycystic liver disease (PCLD) is phenotypically similar to ADPKD, except that the kidney is not affected. In all cases of cystic liver disease, the pathologic condition targets the biliary epithelium, justifying the inclusion of these forms among the genetic cholangiopathies [1].

ADPKD is caused by mutations in one of two genes, *PKD1* (polycystic kidney disease 1) (85–90% of the cases) or *PKD2* (10–15%) encoding for polycystin-1 (PC1) and polycystin-2 (PC2), respectively. Polycystins act as mechanoreceptors, chemoreceptors, and calcium ( $\text{Ca}^{2+}$ ) channels, able to sense changes in apical flow. ARPKD/CD and CHF are caused by mutations in the *PKHD1* (polycystic kidney and hepatic disease 1) gene, encoding for fibrocystin, a protein whose functions remain largely unknown. Polycystins and fibrocystin are expressed in the primary cilia of cholangiocytes. In secretory epithelia, primary nonmotile cilia are involved in the regulation of multiple epithelial functions including secretion, proliferation, differentiation, and interactions with cell matrix. In the liver, cilia are preferentially expressed by cholangiocytes. Although the impact of ciliary dysfunction on cholangiocyte physiology is unknown, animal models with defects in ciliary proteins, such as polycystins, fibrocystins, and polaris, show different degrees of biliary dysgenesis. In the liver, both ADPKD and ARPKD/CHF/CD are morphologically characterized by aberrant development of the biliary epithelium that retains an immature, ductal plate-like architecture with the formation of multiple biliary microhamartomas that progressively dilate to macroscopic cysts, scattered throughout the liver parenchyma.

The isolated polycystic liver disease (PCLD) on the other hand is caused by mutations in *PRKCSH*, a gene coding for protein kinase C substrate 80 K-H, also called hepatocystin, or in the *SEC63* gene. *SEC63* encodes for a component of the molecular machinery regulating translocation and folding of newly synthesized membrane glycoproteins. Hepatocystin and *SEC63* are not expressed in cilia, but in the endoplasmic reticulum, thus cystic diseases of the liver can also be caused by defects in proteins that are not expressed in cilia.

Diseases of the biliary epithelium caused by single-gene defects that alter a critical physiologic process provide an invaluable clue for understanding epithelial function and pathophysiology. As a consequence, in the last few years, interest for polycystic liver diseases has consistently grown. In this chapter we will review the aspects of cholangiocyte biology that more closely relate to the pathogenesis and treatment of cystic diseases of the liver.

## MORPHOLOGY AND SECRETORY FUNCTIONS OF THE NORMAL BILIARY EPITHELIUM

The biliary epithelium forms a branching system of conduits within the liver where bile flows from the hepatocytes to the gallbladder and intestine. The biliary tree is organized in a complex tridimensional network that starts at the canals of Hering, located at the limiting plate with hepatocytes, and forms tubules ( $<15\text{ }\mu\text{m}$  in diameter) which gradually converge to create ducts of progressively larger size (up to  $300\text{--}800\text{ }\mu\text{m}$ ): interlobular, septal, major ducts, and hepatic ducts embedded into the portal space [2]. The biliary tree is lined by cholangiocytes. These are epithelial cells with absorptive and secretory properties that actively contribute to bile formation, regulating its volume, pH, and composition according to physiological needs. Ductular secretion may account for about 40% of bile flow in humans, a percentage that can be rapidly increased during the digestive phase.

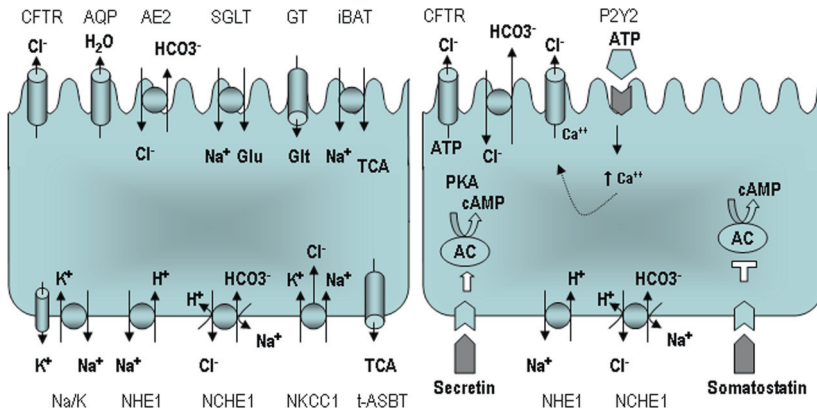
The morphology of cholangiocytes, as well as their function, varies along the biliary tree: cholangiocytes in the small interlobular bile ducts are cuboidal epithelial cells, but become columnar and mucus-secreting in larger ducts approaching the extrahepatic portion [3]. This morphological heterogeneity also corresponds to a functional regional specialization: cholangiocytes lining the large interlobular and major ducts are mostly involved in secretory functions. Conversely, cholangiocytes in the smaller bile duct branches, cholangioles and ducts of Hering, perform other important biological properties such as the ability to proliferate in response to liver damage, participate in the inflammatory response, and undergo limited phenotypic changes. Furthermore, liver progenitor cells are believed to arise from subpopulations of cholangiocytes residing in the canal of Hering [4]. This functional specificity is substantiated by the fact that most cholangiopathies show a site-restricted bile duct injury. For instance, primary biliary cirrhosis (PBC) targets specifically the interlobular bile ducts, whereas primary sclerosing cholangitis (PSC) affects the larger intrahepatic and extrahepatic ducts. Interestingly, the “small duct” variant of PSC, where damage is restricted to the finest branches of the biliary tree, has distinct clinical manifestations.

The biliary tree runs along the portal spaces between the hepatic lobules, in close vicinity to a branch of the portal vein and to one or two branches of the hepatic artery. While portal blood perfuses hepatocytes in the hepatic lobules, the cholangiocyte blood supply is provided by the hepatic artery. Branches of the hepatic artery, at the periphery of the liver lobule, create a peribiliary vascular plexus (PBP), a network of

capillaries which nourishes the cholangiocytes and eventually merges into the hepatic sinusoids.

Bile formation starts at the hepatocyte canalicular membrane, with the secretion of bile acids, other organic and inorganic solutes, electrolytes, and water. As the primary bile flows through the bile ducts on its route toward the duodenum, its composition is regulated by the intrahepatic bile duct epithelium that reabsorbs fluids, amino acids, glucose, and bile acids, while secreting water, electrolytes, and immunoglobulin A (IgA) [5, 6]. Fifteen years of investigation have partly unveiled the complexity of the transport function of cholangiocytes and of its regulation (see Fig. 2.1). Here we will limit our discussion to the aspects that may be relevant for understanding cystogenesis in the liver.

Ultimately, secretion and alkalization in the bile ducts is mainly associated with a net flux of chloride ( $\text{Cl}^-$ ) and bicarbonate ( $\text{HCO}_3^-$ ) into the lumen which induces the secretion of water and regulates bile pH. In contrast with hepatocytes, where the major driving force for bile production is the active secretion of bile acids by adenosine triphosphate (ATP)-driven transporters, cholangiocytes secrete fluid and electrolytes in response to paracrine or endocrine stimuli. A number of different ion channels and transporters have been identified and shown to be specifically located at the basolateral or apical membrane. As in all mammalian cells, the driving force for facilitated membrane transport in cholangiocytes is provided by the  $\text{Na}^+/\text{K}^+$  ATPase, which actively extrudes sodium ( $\text{Na}^+$ ) from the cell and, together with potassium ( $\text{K}^+$ ) channels, maintains the transmembrane potential. At the basolateral side, the  $\text{Na}^+$  gradient regulates the  $\text{Na}^+/\text{H}^+$  exchanger isoform 1 (NHE1) and the  $\text{Na}^+:\text{HCO}_3^-$  symporter (or  $\text{Na}^+$ -dependent  $\text{Cl}^-/\text{HCO}_3^-$  exchanger in humans, NCHE) which mediate the reabsorption of  $\text{HCO}_3^-$  necessary for acid extrusion, while the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  cotransporter (NKCC1), a major determinant of fluid secretion, mediates the chloride uptake into the cell. On the apical side of the cell,  $\text{Cl}^-$  efflux is mainly mediated by a cyclic adenosine monophosphate (cAMP) activated, slow conductance,  $\text{Cl}^-$  channel encoded by the cystic fibrosis transmembrane conductance regulator (CFTR). The opening of chloride channels (CFTR) in the apical membrane leads to an efflux of  $\text{Cl}^-$  and the generation of an osmotic gradient which induces the release of water into the lumen through aquaporins (AQP-1 and AQP-4). The  $\text{Cl}^-$  gradient regulates the  $\text{Na}^+$ -independent  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (AE2) which extrudes bicarbonate into the bile providing biliary alkalization, in accordance with intracellular pH. Other carriers such as the  $\text{Na}^+$ -dependent glucose transporter (SGLT1), the glutamate transporter, and the ileal bile acid transporter (iBAT) expressed on the apical membrane of cholangiocytes mediate the reabsorption of



**Fig. 2.1.** Secretory function of cholangiocytes and its regulation. *Left:* secretion and alkalization in bile ducts is ultimately associated with a net flux of  $\text{Cl}^-$  (which induces fluidification) and  $\text{HCO}_3^-$  (alkalinization) into the lumen mediated by specific transporters localized to the apical or basolateral membrane of cholangiocytes. The  $\text{Na}^+/\text{K}^+$  pump creates the membrane potential necessary for cell homeostasis and maintains the  $\text{Na}^+$  gradient across the membrane necessary for facilitated transports. At the basolateral side, the  $\text{Na}^+/\text{H}^+$  exchanger NHE1 and the  $\text{Na}:\text{HCO}_3^-$  symporter NCHE1 mediate the reabsorption of  $\text{HCO}_3^-$  into the cell and the acid extrusion, while chloride uptake occurs through the  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  cotransporter NKCC1. On the apical side, the  $\text{Cl}^-$  is released into the bile by cystic fibrosis transmembrane conductance regulator (CFTR) inducing a parallel osmotic movement of  $\text{H}_2\text{O}$  through aquaporines. The  $\text{Cl}^-/\text{HCO}_3^-$  exchanger AE2 (located at both the basolateral and the apical membrane) mediates carbonate release into the bile. Specific  $\text{Na}^+$ -dependent apical carriers, GT and iBAT, mediate the reabsorption of glutamate and taurocholate, respectively. Biliary acids are then secreted in the peribiliary plexus via t-ASBT. *Right:* Choleretic hormone secretin stimulates cAMP production by adenylate cyclase with consequent activation of CFTR via PKA mediated phosphorylation.  $\text{Cl}^-$  released by CFTR promotes  $\text{HCO}_3^-$  secretion by AE2 and bile alkalization. Similarly luminal purinergic nucleotides can activate a  $\text{Ca}^{++}$ -dependent  $\text{Cl}^-$  channel and stimulate secretion. In contrast, somatostatin decreases bile secretion and alkalization by adenylate cyclases inhibition.

biliary constituents, such as glucose and glutathione breakdown products and conjugated bile acids. This is particularly important because bile acids can stimulate proliferation of biliary epithelial cells. Biliary bile acids are then secreted in the peribiliary plexus via t-ASBT, a truncated isoform of the apical sodium-dependent bile acid transporter (ASBT), or via MRP3 (multidrug-resistant protein 3), a p-glycoprotein. This cholehepatic circulation of bile acids is also important in the overall regulation of bile secretion.

The secretory function of the bile ducts is finely regulated by rapid hormone-mediated signaling. The net amount of fluid and secreted  $\text{HCO}_3^-$  is determined by the integration of different pro-secretory (secretin [5], glucagon [7], VIP [8], acetylcholine [9], bombesin [10]) and anti-secretory (somatostatin [11], endothelin-1 [12]) stimuli. All these hormone signals ultimately act on the adenylyl cyclases (ACs), the transmembrane enzymes that regulate the intracellular level of the second messenger cAMP, converting ATP to cAMP. Secretin, the main choleretic hormone, increases cAMP/PKA (protein kinase A). This activates CFTR, and consequently stimulates  $\text{Cl}^-$  and  $\text{HCO}_3^-$  efflux and inhibits the  $\text{Na}^+/\text{H}^+$  exchanger (NHE)-dependent  $\text{Na}^+$  absorption [13, 14]. Cholinergic agonists,  $\beta$ -adrenergic agonists, and  $\text{HCO}_3^-$ -mediated signals also regulate bile secretion through the cAMP and PKA pathway. ACs may thus represent an important means of integration of multiple secretory signals. So far nine different isoforms of AC have been identified (AC1-9), each displaying tissue-specific expression and regulation. Interestingly the AC6 isoform was found to be located in cholangiocyte cilia, thus further suggesting a correlation between ductal bile secretion and ciliary function [15].

The secretory functions of the biliary epithelium are also regulated by molecules (such as bile salts, glutathione, and purinergic nucleotides) secreted by hepatocytes into the canalicular bile and delivered to receptors and transporters located in the apical membrane of cholangiocytes [5]. For instance, ATP, which is released into the bile by hepatocytes or by cholangiocytes themselves, can bind to apical P2Y2 purinergic receptors and stimulate apical  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels and basolateral  $\text{Na}^+/\text{H}^+$  exchanger (NHE-1), thus promoting  $\text{Cl}^-$  efflux into the bile and basolateral  $\text{HCO}_3^-$  influx [16]. Certain bile acids may also stimulate cholangiocyte secretion of  $\text{HCO}_3^-$  by inducing ATP dependent  $\text{Cl}^-$  secretion by CFTR and purinergic activation of apical  $\text{Ca}^{++}$ -activated or volume-activated  $\text{Cl}^-$  channels [17].

## CHOLANGIOCYTE REACTION TO DAMAGE

Cholangiocytes possess receptors for a number of cytokines, chemokines, and growth factors and angiogenic factors that enable an extensive cross talk with other liver cell types, including hepatocytes, stellate cells, and endothelial cells [6]. This property becomes particularly relevant when the liver or the biliary tree is damaged. In fact, the cholangiocyte compartment can significantly expand in response to liver injury. Cholangiocyte proliferation occurs in most pathologic conditions, including cholestasis, viral hepatitis, and hepatic necrosis, and represents a key mechanism of regeneration and repair,



which ensures the integrity of the biliary tree following liver damage. These “reactive” or “activated” cholangiocytes are believed to arise from a progenitor cell compartment located in close contact with the smallest radicals of the biliary tree, the terminal cholangioles at the canals of Hering. Reactive cholangiocytes show a less differentiated secretory phenotype, but acquire the capability to secrete a number of proinflammatory and chemotactic cytokines and growth factors. They can recruit inflammatory and mesenchymal cells and induce them to proliferate and to produce extracellular matrix (ECM) components [18]. There are, in fact, intimate contacts and exchange of signals between mesenchymal cells and reactive cholangiocytes. While mesenchymal cells are considered the effectors of fibrosis, reactive cholangiocytes are considered the “pacemaker of liver fibrosis” [19]. The list of cytokines, chemokines, inflammatory factors and growth factors, and receptors that mediate the epithelial/mesenchymal cross talk in the liver is continuously increasing. It includes interleukin-6 (IL-6), IL-8, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interferon- $\gamma$  (IFN $\gamma$ ), monocyte chemotactic protein-1 (MCP-1), cytokine-induced neutrophil chemoattractant (CINC), and nitric oxide, which regulate the immune activity of lymphocytes and polymorphonuclear cells. Reactive cholangiocytes also produce growth factors such as vascular endothelial growth factor (VEGF), endothelin-1 (ET-1), platelet-derived growth factor-BB (PDGF-BB), transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2), and connective tissue growth factor (CTGF).

In addition to establishing paracrine communications with mesenchymal cells, cholangiocytes may also participate in the generation of liver fibrosis through a process of epithelial to mesenchymal transition (EMT). EMT is a process of cellular reprogramming whereby epithelial cells acquire some of the phenotypic and functional characteristics of mesenchymal cells, such as the expression of fibroblast-specific markers (FSP-1, vimentin), the ability to migrate by locally dismantling the basement membrane upon which the epithelial sheet resides, and the ability to generate different connective tissue components (fibronectin, collagen, elastin, tenascin). EMT may thus contribute to the accumulation of activated fibroblasts in association with the loss of bile ducts. This biological process has also been described in the pathogenesis of organ fibrosis in the kidney [20] and lung [21]. Recent studies suggest that EMT may also be involved in liver fibrosis [22, 23].

In response to liver injury, reactive cholangiocytes also acquire a neuroendocrine-like phenotype and express receptors that enable them to respond to regulation by neural terminations. In fact, reactive cholangiocytes express  $\beta$ 1 and  $\beta$ 2 adrenergic receptors, the M3 acetylcholine receptor [24], serotonin 1A and 1B receptors [25]. During cholestasis,



cholangiocytes can also directly secrete serotonin, thus further limiting the growth of bile ducts by an additional autocrine inhibitory loop. Furthermore, in experimental cholestasis, cholangiocytes secrete neuropeptides, such as nerve growth factor (NGF), that can stimulate cholangiocyte proliferation [26]. A large number of other regulatory neurotransmitters and neuropeptides are expressed by reactive cholangiocytes, but will not be mentioned here. The role of neuroendocrine signaling in cyst growth in the polycystic liver diseases has not been experimentally addressed yet.

## MECHANISMS OF CYSTIC LIVER DISEASES: CILIA AND BEYOND

A novel concept in biliary physiology is that primary cilia are involved in the regulation of fundamental biological activities including cell differentiation, proliferation, and secretion [27, 28]. The presence of primary cilia at the apical domain of cholangiocytes has long been known; however, their role in biliary physiology remained undefined. The discovery that mutations in several proteins relevant to ciliary function are associated with cystic diseases in several organs, including kidney, liver, and pancreas, strongly revived interest in the function of this organelle [29].

Primary cilia of cholangiocytes, as opposed to cilia of lung epithelial cells, are nonmotile, but can be bent in response to changes in luminal fluid flow, thereby transducing a mechanical force into an intracellular calcium signal [30]. Masuyk et al. investigated ciliary function in the biliary epithelium using microperfused rat intrahepatic bile duct units (IBDU) and showed that changes in luminal flow increased  $[Ca^{2+}]_i$  and inhibited forskolin-stimulated cAMP production [15]. These changes were significantly inhibited by removal of cilia with chloral hydrate or by silencing of ciliary protein (such as PC1 or PC2) or of adenylyl cyclases 6 (AC6) [15, 31]. PC2 is believed to function as a nonselective  $Ca^{2+}$  channel, activated by PC1 through its C-terminus, while AC6 is a  $Ca^{2+}$ -inhibitable AC expressed in cilia, which interacts with PC2. Ciliary dysfunction in ADPKD would thus reduce intracellular calcium levels, thereby increasing AC6 activity and the levels of cAMP. It is well known that cAMP stimulates cholangiocyte secretion through the Src/Ras/MEK/ERK1-2 pathway and thus can promote cyst formation. Additionally, PC1 may have a direct transcriptional effect mediated by the proteolytic cleavage and nuclear translocation of its carboxy-terminal tail to the nucleus [32]. In kidney cells, PC1 was also shown to regulate the signal transducer mTOR [33, 34]. The

constitutive activation of both these pathways results in progressive cyst growth [33].

Much of the attention has been focused on ciliary function; however, it should be noted that morphologic alterations of cilia have not been consistently reported in cystic liver diseases. Recent electron microscopy studies on human ADPKD liver described heterogeneous abnormalities on the apical surface of cyst epithelium, depending on cyst size [35]. The epithelial cells lining small cysts (1 cm) showed a relatively normal apical surface with cilia and microvilli represented in the expected number and size. On the other hand, the apical surface of medium-sized cysts (2–3 cm) showed areas free of microvilli with rare and shortened cilia, while large hepatic cysts (3 cm) totally lack microvilli and primary cilia. These progressive morphological abnormalities of primary cilia and microvilli may represent a mechanic effect of enhanced endoluminal pressure during cyst growth, rather than a primary consequence of defective ciliary proteins.

Most “ciliary proteins” are not exclusively expressed in cilia. For example, PC2 is also strongly expressed in the endoplasmic reticulum (ER), where it interacts with ryanodine or InsP3 receptors to regulate ER and cytoplasmic calcium levels. Furthermore, the proteins encoded by the *PRKCSH* and *SEC63* genes are not expressed in cilia, but in the ER. Defects of other enzymatic activities associated with the ER, such as xylosyltransferase 2, an initiator of heparin sulfate and chondroitin sulfate biosynthesis, have been linked to the development of renal and liver cysts [36].

It is interesting that cyst formation is not a common reaction of the biliary epithelium to liver damage. In obstructive cholestasis the biliary epithelium reacts by forming multiple branching tubules, while in inflammatory biliary disease the epithelium forms a ramified mesh of reactive cells that for the most part lack a lumen. On the other hand, conditional PC1 or PC2 mice in which the genetic deletion is induced after birth develop multiple cysts in the liver, indicating that polycystins remain key determinants of biliary architecture during adult life.

An important property of epithelial sheets is planar cell polarity – the capacity to orient the axis of cell division in such a way that the growth of the epithelial sheet is “polarized” within the plane of the cell sheet. This means, for example, that the epithelial cells of the kidney align their mitotic spindle along the tubule axis so that the daughter cell will be inserted in a way that elongates the tubule rather than increases the size of its lumen. Interestingly, in rodent models with low *Pkhd1* expression, the orientation of the mitotic spindle is distorted [37]. *Pkhd1* is also localized to the basal body [29, 38–40], a sub-cellular organelle that originates from the mother centriole in the

centrosome and is responsible for the assembly of the cilium. Centrioles organize the mitotic spindle and serve as microtubule organizing center (MTOC). An anomalous cell division would result in tubule enlargement rather than tubule elongation. Direct experimental evidence for this model in cholangiocytes has not yet been produced.

## CELLULAR MECHANISMS OF LIVER CYST FORMATION AND GROWTH

Mechanisms leading to the progressive growth of liver and kidney cysts are being actively investigated. In both autosomal dominant and recessive forms, liver cysts arise from an aberrant development of intrahepatic bile duct epithelium. During cyst expansion different factors, including excessive fluid secretion, extracellular matrix remodeling, increased proliferation of the epithelial cells lining the cyst, and the pericystic vasculature, variably take part to promoting the progressive cyst growth.

### *Altered Biliary Developmental Program (Ductal Plate Malformation)*

The developmental role of polycystins is evident from studies in genetically modified mice and was clear to early pathologists that recognized a morphology suggestive of a blockage in ductal plate maturation. They classified cystic liver disease as a malformative condition.

The ontogenesis of the intrahepatic biliary tree begins around the 8th week of gestation and proceeds centrifugally from the ileum to the periphery of the liver. Still immature at birth, the biliary tree completes its development during the first year of life. Its formation starts when the periportal hepatoblasts surrounding branches of the portal vein undergo a phenotypic switch and assemble into a sheath of small flat epithelial cells, called “primordial ductal plate.” Over the following weeks, some segments of the ductal plate perimeter are duplicated by a second layer of cells (double layered ductal plate), while the remaining single layer portions are deleted by apoptosis. The double layered ductal plate then dilates and starts to form a tubular structure which is incorporated into the mesenchyme of the developing portal space (migratory stage) and later undergoes a branching process to form the biliary tree [41]. Ductal plate remodeling during fetal and postnatal development is thus a fine balance between proliferative and apoptotic processes. A failure in ductal plate remodeling causes a number of developmental cholangiopathies, hence classified as ductal plate malformations (DPM) of which PKD is one [42].

However, in humans, cysts appear to develop throughout adult life. Mice with conditional knockout of *Pkd1* or *Pkd2* show a progressive formation of liver and renal cysts reminiscent of human diseases even when the induction is performed weeks after birth [43–45]. This indicates that there is also a role for polycystin in maintaining a normal biliary architecture during adult life. It is interesting to note that there are fundamental structural differences between ADPKD and ARPKD liver cysts. In ADPKD, the nascent cysts detach from the original duct and form autonomous structures that no longer communicate with the duct; in ARPKD, cysts mostly remain open. This fundamental difference helps to explain the different clinical manifestations between ARPKD/CHF/Caroli and ADPKD.

### ***Altered Epithelial Fluid Secretion***

Studies performed by Everson et al. on ADPKD patients have shown that hepatic cysts are able to generate ion secretion under basal conditions and when stimulated with secretin [46]. The increased intraluminal pressure may contribute to cyst expansion as secretion into the closed cyst would stretch the lining epithelial cells and induce proliferation. In cell culture models of epithelial cysts, increasing intraluminal pressure increased the rate of cell proliferation [47, 48]. Stretch may activate apical secretion of purinergic agonists [49], major players in the regulation of cholangiocyte secretion and proliferation [16, 50]. Studies in kidney cyst cells from both ARPKD and ADPKD have shown that the cyst epithelium releases substantial amounts of ATP in culture [51] and expresses P2X and P2Y purinergic receptors, along with  $\text{Ca}^{2+}$ -stimulated  $\text{Cl}^-$  channel activation [52], leading to cystic fluid accumulation. It is presently unclear if these findings apply to cholangiocytes as well and if purinergic signaling is actually different from the normal epithelium. Cystic cholangiocytes appear to have an altered intracellular  $\text{Ca}^{2+}$  homeostasis, so without direct experimental evidence, it is difficult to predict the overall role of purinergic activation.

On the other hand, there is important cross talk between the cAMP and the  $\text{Ca}^{2+}$ -dependent pathways. Increased cellular cAMP content due to the reduced  $\text{Ca}^{2+}$ -dependent inhibition of AC6 would favor CFTR-dependent secretory events. It is interesting to note that the severity of ADPKD was milder in two cases in which the diseases coexisted with cystic fibrosis [53], a disease that impairs CFTR-dependent  $\text{Cl}^-$  secretion [54]. Consistent with these reports and the role of CFTR-dependent secretion, small molecule CFTR inhibitors slowed cyst growth in experimental polycystic kidney disease [55, 56].

In spite of the evidence of active fluid secretion in cystic kidney and liver disease, there is no definitive proof that unregulated fluid secretion is actually the major pathophysiologic mechanism leading to cyst growth in the liver. Furthermore 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 [57].

### *Cholangiocyte Proliferation*

Most observations indicate that increased proliferative activity of the cystic epithelium may be the major determinant of cyst growth. As discussed earlier, cholangiocytes lining liver cysts present functional similarities with the reactive ductules, and express a vastly similar array of growth factors, growth factor mediators, cytokines, and chemokines. While in reactive ducts this property facilitates progenitor cell-mediated liver repair, their expression in cystic cholangiocytes likely represents the phenotypic and functional signature of a relative loss of differentiation. For example, we have shown that the pattern of angiogenic factors expression by cystic cholangiocytes in ADPKD is similar to that of the ductal plates.

Increased epithelial levels of cAMP is one of the factors determining the increased proliferative activity of cystic cholangiocytes and fluid secretion [58–60]. The increased cAMP level in cystic epithelia may be related to changes in the intracellular  $\text{Ca}^{2+}$  homeostasis and the cross talk with the  $\text{Ca}^{2+}$ -inhibitable adenylate cyclase 6. The relevance of cAMP in promoting cholangiocyte growth is demonstrated by the fact that in vivo treatment of normal rats with the adenylate cyclase (AC) stimulator forskolin induces cholangiocyte proliferation in association with increased activity of protein kinase A (PKA) and the activation of the cAMP/PKA/Src/ERK1/2 cascade [61]. Inhibition of cAMP production has been exploited as a therapeutic strategy in ARPKD. In particular somatostatin and its analogs, such as octreotide, were used to inhibit the secretin-induced increases in cAMP levels observed in cholangiocytes from bile duct-ligated (BDL) rats [62]. Somatostatin represses AC function through its receptor SSTR2, which is expressed in the liver only by cholangiocytes [63]. Masyuk et al. showed that 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 epithelia [60].

The cystic fluid from patients with ADPKD also contains elevated levels of cytokines and growth factors that could potentially promote

cell proliferation and cyst expansion [64–66]. These include high levels of IL-8, IL-6, EGF, and VEGF [65, 67, 68]. Histological studies have shown a marked over-expression of estrogen receptors, insulin-like growth factor (IGF), IGF-receptor, growth hormone receptor, and pAKT [35, 68, 69]. Estrogens and IGF-1 are major factors able to induce proliferation of cyst epithelium, given their capability to activate specific proliferative and/or survival pathways. Like cAMP, estrogens [70], IGF-1 [71] and VEGF [71] may promote cholangiocyte growth via the ERK pathway, which is the main pathway of regulation of cholangiocytes proliferation. However, the strong immunoreactivity for IGF1, IGFR-1, and pAKT in liver cysts from ADPKD patients also indicate activation of the PI3-kinase pathway. Through this pathway IGF1 can activate mTOR (mammalian target of rapamycin) and thus promote cell proliferation via cyclins. In fact, phospho-mTOR is over-expressed in the liver cystic epithelium of ADPKD patients and mouse models. It is interesting to note that mTOR can also stimulate HIF1 $\alpha$ -dependent VEGF secretion.

Cystic cholangiocytes over-express VEGF, angiopoietin-1, and their cognate receptors VEGF receptors 1, 2 and Tie-2 [68]. Thus, VEGF and angiopoietin-1 may exert an important autocrine proliferative effect and promote the growth of liver cysts.

### *Autocrine and Paracrine VEGF Signaling*

Ductal plate malformations are frequently associated with an abnormal vasculature ramification: a morphologic feature known as “pollard willow pattern” which derives from an alteration in the normal relationship between bile ducts and portal vascular structures. The close anatomic relationship between intrahepatic bile ducts and hepatic arterial vascularization is already evident during the developmental stages and it appears to be crucial for the maintenance of the integrity and function of the biliary epithelium [72]. In immunohistochemical studies, Fabris et al. investigated the expression of angiogenic growth factors (VEGF and angiopoietins) and their cognate receptors during biliary and arterial development in humans and showed that, during development, VEGF released by cholangiocytes promotes the angiogenesis of the PBP in close vicinity to the maturing bile ducts [73]. Likewise, the PBP support is also fundamental during ductular reaction in response to liver damage or disease. Indeed, in many forms of liver injury, cholangiocyte proliferation is accompanied by an increase in number of the surrounding vascular structures [74]. In rat models of cholangiocyte proliferation

(common bile duct ligation), Gaudio et al. observed that a marked proliferation of the PBP became apparent only after the extension of the bile duct system occurred, underscoring the role of proliferating cholangiocytes in directly promoting angiogenesis [75]. Indeed they showed a significantly higher expression of VEGF in cholangiocytes isolated from BDL rats compared to normal rats [71].

VEGF is one of the most potent angiogenic factors and its role in vascular proliferation associated with tumor growth or wound healing has been widely documented in different organs. Also in human diseases related to developmental ductal plate malformation (i.e., PKD) the dysmorphic bile ducts are surrounded by hyperplastic vascular structures. Fabris et al. showed that in the cystic biliary epithelium of fibropolycystic liver diseases (ADPKD and Caroli disease), VEGF and angiopoietin-1 are markedly up-regulated, together with their receptors VEGFR2 and Tie2, and their enhanced expression is closely related to the microvascular density around biliary cysts [68]. In polycystic diseases, the biliary epithelium retains thus an immature phenotype characterized by up-regulation of VEGF and angiopoietins. The growing cysts stimulate angiogenesis to meet their need of vascular supply for metabolic support.

VEGF production is controlled in most tissues by hypoxia-inducible factor 1 (HIF-1), one of the key regulators of oxygen homeostasis. HIF-1 is a transcription factor active in hypoxic conditions, and the loss of microvillar structure and decreased microvillar density in ADPKD liver cyst epithelium are also features consistent with an ischemic damage [35]. However, isolated cystic cholangiocytes also overproduce VEGF, indicating that this is the direct result of the loss of PC1 and PC2 function, rather than the consequence of the cystic epithelium becoming hypoxic (C. Spirli et al., submitted). Mice deficient in PC2, in particular, show a severe liver phenotype with higher proliferation rate of the cystic epithelium and higher expression of VEGF and its receptor VEGFR-2, pERK1/2 and HIF1 $\alpha$ , suggesting that PC2 acts as repressors of the Raf/MEK/ERK cascade in physiological conditions and the lack of its function leads to activation of this pathway and the consequent increase in proliferation (C. Spirli et al., submitted).

The significance of this effect is even more relevant in light of the fact that many of the growth factors that stimulate cholangiocytes appear to act through this pathway. The list of HIF-1-regulated genes is ample and includes genes coding for proteins involved in angiogenesis, energy metabolism, erythropoiesis, cell proliferation and viability, vascular remodeling, and vasomotor responses. Interestingly, HIF-1 $\alpha$



transcription can also be stimulated in normoxic conditions by a number of growth factors, cytokines, and extracellular mediators (IL-1, IL-6, EGF, HGF, TGF $\beta$ , 17- $\beta$ -estradiol, IGF-1) which can stabilize or phosphorylate HIF-1 $\alpha$  via PI3K/AKT/tuberin/mTOR or Raf/MEK/ERK or STAT3.

### *Extracellular Matrix Remodeling*

In the normal liver ECM is scarce, and it is essentially composed of elastin, fibronectin, collagen type I, and collagen types III, IV, V, and VI in low quantity [76]. However, progressive accumulation of ECM components with an altered composition in the portal tract is a common feature in ADPKD, where a remodeling of the ECM is a prerequisite to allow the expansion of the cyst wall [76, 77]. These ECM abnormalities have also been found in the renal interstitium. The progressive establishment of fibrosis within the renal interstitium as well as within the hepatic portal space is particularly abundant in ARPKD, where dense fibrotic tissue is formed in close vicinity to aberrant bile ducts. In congenital hepatic fibrosis, Ozaki et al. recently showed that connective tissue growth factor (CTGF) is diffusely retained in the heparan sulfate proteoglycan web in the portal tract where it can be responsible for non-resolving fibrosis due to persistent activation of many mast cells and portal myofibroblasts [78]. Not surprisingly, the growth of liver cysts requires the enhanced degradation of ECM induced by an altered interplay between matrix metalloproteinases (MMPs) and their specific tissue inhibitors (TIMP). MMPs are typically involved in the breakdown of extracellular matrix in embryonic development as well as in tissue repair and remodeling. IL-8, a cytokine that has been shown to be up-regulated in the liver cyst epithelium of animal models of ARPKD, stimulates MMP2 and MMP9 production by endothelial cells and portal myofibroblasts [79, 80].

Studies in PKD rodent models [81, 82] and in cultured mouse renal tubular cells [83] showed that MMP2 was consistently increased in cystic epithelial cells: in the PKD mouse model (C57BL/6 *J-cpk*), Rankin et al. observed an increased kidney cystic cell content of MMP-2 both in vivo [81] and in vitro [83]. Once isolated, cultured cells were able to migrate through collagen gels, further indicating they possess proteolytic activity. In addition, high MMP2, MMP9, MMP3, TIMP1, and TIMP2 levels were found in the culture medium [83]. Thus, increased expression of MMPs contributes to the overall reorganization and restructuring of the ECM necessary for cyst expansion.

## NEW THERAPEUTIC STRATEGIES IN PREVENTING CYST GROWTH

In this chapter, we reviewed the aspects of cholangiocyte physiology and pathophysiology that are relevant for polycystic liver diseases. In particular, we have reviewed the anatomy and the function of the biliary tree and the mechanisms of cholangiocyte secretion and its regulation. We have discussed the role of some of the chemokines, cytokines, and growth factors expressed by cholangiocytes in pathologic conditions and in polycystic liver diseases. Many of these factors play a role in liver cysts growth. We have also discussed the signaling pathways that mediate cholangiocyte function. Each pathway represents a potential target for therapies aimed at reducing the growth of liver cysts. For example, the VEGF and HIF-1 $\alpha$  signaling pathways could be targeted by inhibiting VEGFR2 or using blocking antibodies to VEGF receptors. We have shown that the treatment of ADPKD mice models with VEGFR-2 inhibitors significantly reduced liver cysts area and decreased the VEGF-induced pERK-1/2 activation providing a proof of concept for the potential use of anti-angiogenic therapy in polycystic liver diseases (C. Spirli et al., submitted).

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