Universita' degli Studi di Milano-Bicocca
Scuola di Dottorato in Scienze Mediche Sperimentali e Cliniche

Indirizzo: TECNOLOGIE BIOMEDICHE, Ciclo XXIII
Dipartimento di Medicina Sperimentale, Ambientale e Biotecnologie Mediche

ROLE OF EPITHELIAL TO MESENCHYMAL TRANSITION IN GENETIC CHOLANGIOPATHIES RELATED TO FIBROCYSTIN DEFICIENCY

Silvia Lecchi

Supervisors:
Prof. Mario Strazzabosco, Yale University (CT, USA)
Dott. Luca Fabris, CeLiveR Lab. (Ospedali Riuniti di Bergamo, IT)

Tutor: Prof. Marzia Galli-Kienle

A.A. 2009/2010

To my mom,
with all my admiration, profound respect and love

Table of Contents

ABSTRACT

Autosomal recessive polycystic kidney disease (ARPKD), congenital hepatic fibrosis (CHF) and Caroli disease (CD) are a group of genetically related disorders affecting bile ducts and kidney tubules. These diseases are characterized by the progressive formation of fluid-filled cysts and fibrosis in kidneys and liver. Beside the cyst's mass effect, severe life threatening complications can arise i.e. recurrent cholangitis in the liver and the hazard of the cysts becoming infected or rupture. The most severe clinical consequences however, are, actually, due to the massive portal fibrosis which causes portal hypertension, hypersplenism, and the formation of varices and ascites in the esophagus, increasing the risk for cholangiocarcinoma. ARPKD, CHF and CD are all related to mutations in the PKHD1 gene, acronym for Polycystic Kidney and Hepatic Disease 1. The gene encodes for Fibrocystin (FPC), a protein whose function is still not fully known, normally expressed mainly on the primary cilia of renal tubular and biliary epithelial cells, consistently with the sites primarily affected by the disease, however the mechanistic relationship between a FPC defect and the development of portal fibrosis are still not fully understood. Other cystic diseases are related to defects in ciliary proteins: the Adult Dominant Polycystic Kidney Disease (ADPKD) is caused by mutations in PKD1 or PKD2 encoding respectively for Polycystin 1 and Polycystin2 (PC1 and PC2) and is similarly characterized by cysts formation in kidney and liver, but generally does not present scarring liver fibrosis. The purpose of this project was to investigate the mechanisms leading to portal fibrosis in ARPKD/CHF/CD. In particular our hypothesis was that the defective fibrocystin could activate a program of epithelial mesenchymal transition (EMT) in cholangiocytes that would acquire mesenchymal characteristics, including cells motility and the ability to secrete extracellular matrix proteins, and thus contribute to the development of massive fibrosis in ARPKD. To that end we studied a transgenic mouse harboring a

deletion in the Pkhd1 gene (Pkhd1$^{del4/del4}$ (Gallagher et al., 2008), that mimics the human disease ARPKD. Mice defective in Polycystin (Pkd2KO), mmicking ADPKD, and WT mice were used as control. We examined immunophenotipic markers of EMT and compared liver samples from biopsy of healthy and diseased patients and also from the WT mice and the diseases models. We also investigated motility and invasiveness of cholangiocytes isolated from Pkhd1 mice and their profile of cytokines secretion in order to explore their capabilities in epithelial mesenchymal cross-talk.

We demonstrated that cholangiocytes in ARPKD display some phenotypic characteristics of mesenchymal cells, suggestive of a partial transition EMT and this may contribute to fibrosis, in association with a hyper-secretive profile of the biliary epithelium that may activate fibrocytes recruited in proximity of biliary cysts

INTRODUCTION

1. The Liver

1.1. Liver anatomy and function

Function

Situated in the upper right portion of the abdominal cavity, the liver is the largest solid organ in the human body. It is a vital organ playing a major role in metabolism, digestion and detoxification and there is currently no way to compensate for the absence of liver function other than transplant. Liver functions are mainly carried out by hepatocytes (main liver cells) and include glucose, protein and lipid metabolism: the liver performs gluconeogenesis, glycogenolysis, glycogenesis, insulin break down, and also protein synthesis and degradation, cholesterol synthesis and production of triglicerydes. This organ is also responsible for bile production; the bile produced in the liver is directly secreted into the duodenum for emulsifying dietary fats, or can be stored in the gall bladder for later release. Furthermore, the liver produces or breaks down several hormones: it produces IGF-1 and trombopoietin (that regulates platelets production by bone marrow), angiotensinogen (vasoconstrictor, when activated it increases blood pressure) and also albumin (major osmolar component of the blood serum). Likewise it breaks down or modifies toxic substances and drugs. On top, it is an important storage supply for Vitamins A, D and B12, iron and copper.

Anatomy

Based on surface features, traditional anatomy divides the liver in four lobes of unequal size and shape: the right and left lobes (divided by the falciform ligament, on the anterior side) and the caudate lobe and quadrate lobe on the visceral side. The whole organ is enveloped in a fibrous collagen shroud named Glisson's capsule. The liver dense parenchyma is mostly constituted by hepatocytes, organized into the hepatic lobules of roughly hexagonal shape (in section) where hepatocytes are arranged radially and in layers (Fig.1).

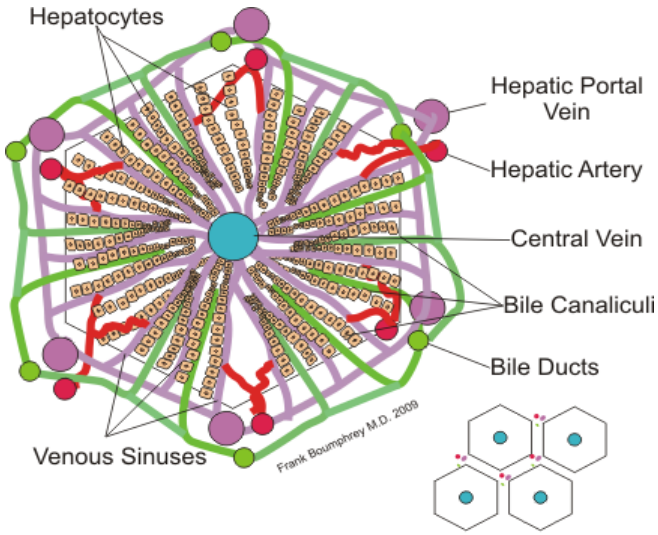

Figure 1. Basic structure of the liver lobule
Image of Boumphreyfr, 2009. Licensed under GNU Free Documentation License.
Available at http://commons.wikimedia.org/wiki/File:Hepatic_structure.png

The lobule is the basic functional unit of the liver where blood flow and bile flow run in opposite directions gathering and delivering substances involved in the hepatocytes metabolism. Branches of the main blood vessels of the liver: portal vein and hepatic artery, and the bile ducts (that together constitute the so called portal triad) together with lymphatic vessels and nerves, run, parallel, at the corner between adjacent lobules, in the portal space, lying in a network of connective tissue tightly surrounded by hepatocytes. The ring of hepatocytes abutting the connective tissue of the triad is named limiting plate.

Blood flow and bile flow

The hepatic vascular system has several unique characteristics. The blood reaches the liver through two main vessels: the hepatic artery, which supplies the oxygenated blood from the aorta, and the portal vein that carries to the liver the blood containing digested nutrients from the gastrointestinal tract and the spleen. Both blood vessels access the organ from its lower side (porta hepatis), in the liver they subdivide repeatedly, terminating in small capillaries, each one leading to a lobule where arterial and portal blood mixes and enters in sinusoids running toward the center of the lobule. Hepatocytes in the lobules are organized

in rows radiating from the central point, in close contact with the blood filled sinusoids. Thus oxygen and nutrients diffuse from the sinusoids through the capillary walls into the liver cells. The blood then leaves the liver via a central vein located in each lobule, which finally drains into the hepatic vein and then into the inferior vena cava.

Bile secretion flows parallel to the blood in the sinusoids, but in the opposite direction. The bile is produced by the hepatocytes. Its formation starts with the secretion of bile acids, other organic and inorganic solutes, electrolytes and water into dilated intercellular spaces between adjacent hepatocytes: the canaliculi. It is then delivered (centrifugally) to a system of ducts: the bile ducts, lined by specialized epithelial cells: cholangiocytes. At the limiting plate with hepatocytes, the biliary system begins in small twigs (canals of Hering) and it develops in a complex tridimensional network of tubules (<15μm in diameter) which gradually converge to create ducts of increasingly larger size (up to 300-800 μm): interlobular, septal, major ducts, and hepatic ducts embedded into the portal space (Strazzabosco & Fabris, 2008). The bile exits the liver through the common hepatic duct which joins the cystic duct from the gall bladder. The bile can then be stored in the gallbladder or delivered to the intestinal lumen where it aids in emulsifying dietary lipids and facilitates the absorption of nutritional fats and lipo-soluble vitamins (A, D, E, K) (Choi et al., 1994). The human liver can produce up to one liter of bile a day (depending on body size), this is a very efficient system and about 95% of the salts secreted in the bile are re-adsorbed in the terminal ileum and re-used. Blood from the ileum flows directly to the hepatic portal vein and returns to the liver where the hepatocytes re-adsorb the salts and return them to the bile ducts to be reused, up to three times per meal.

Hepatic Cells

Hepatocytes, performing the main liver functions, and **cholangiocytes**, the epithelial cells lining the bile ducts, are both of hepatic progenitor origin and are the main constituents of the liver parenchyma. The liver is also populated by other important populations of non parenchymal cells: 15% of resident liver cells

is represented by **hepatic stellate cells** (**HSC**) normally residing in the "space of Disse": the perisinusoidal space that divides hepatocytes and sinusoids and is filled with serum. HSC are also called Ito cells, they store retinoid (Vit. A) and lipid droplets. During injury these cells can become myofibroblasts-like and represent key effectors in the fibrotic process producing extracellular matrix proteins. (Friedman, 2000). About 10% of the total liver population is represented by **Kupffer cells** (Baffy, 2009), resident macrophages in the liver, that line the walls of the sinusoids, they are the primary effectors of the innate immune response within the liver. When activated Kupffer cells can produce a variety of chemokines and cytokines (including TNF that promotes Collagen I production by activated HSC) (Wheeler et al., 2001). Not last the liver **sinusoidal endothelial cells**. Liver sinusoids differ from the other capillaries in the body because of the presence of fenestrae or pores, lacking a diaphragm and a basal lamina underneath the endothelium. These fenestrae selectively filter fluids, solutes and particles that are exchanged between the sinusoidal lumen and the space of Disse. Liver sinusoidal endothelial cells actively uptake a wide variety of substances from the blood through a high, receptor-mediated, endocytotic capacity, and act as a "scavenger system" which clears the blood from many different macromolecular waste products.

During cirrhosis, typically, the deposition of an endothelial basement membrane together with the defenestration and capillarization of the sinusoidal endothelium results in an impaired bidirectional exchange between the sinusoidal blood and the parenchymal cells which contributes to hepatic failure (Braet & Wisse, 2002).

1.2. The cholangiocytes and the biliary system.

Cholangiocytes lining the biliary vessels are characterized by distinctive absorptive and secretory properties and their function is not limited to funneling the bile, they can actively contribute to its formation regulating its composition, volume, and pH. Actually, in humans, about 40% of bile flow is produced by

cholangiocytes (Nathanson & Boyer, 1991) and even more is secreted during the digestive phase. Cholangiocytes regulate bile secretion, fluidity and pH by actively pumping Cl^- and $HCO3^-$ into the bile ducts lumen, thus inducing the secretion of water and increasing the alkalinity of the bile. In the intestine, the bicarbonate within the bile acts balancing the acidic chime, the semi fluid mass of partly digested food, containing hydrochloric acid and with a pH of around 2, that reaches the duodenum from the stomach. The duodenum can stimulate the release of more bile from the gallbladder by producing powerful choleretic hormones: secretin and cholecystokinin (CKK).

In fact the secretory function of bile ducts is finely regulated by rapid hormone-mediated signaling and the net amount of fluid and secreted $HCO3^-$ is determined by the integration of diverse pro-secretory and anti-secretory stimuli. Secretin is the major choleretic hormone (Strazzabosco, 1997), but also glucagon (Lenzen et al., 1997), VIP (Cho & Boyer, 1999b), acetylcholine (Alvaro et al., 1997) and bombesin (Cho & Boyer, 1999a) have a pro-secretory effect. Conversely somatostatin (Gong et al., 2003) and endothelin1 (Caligiuri et al., 1998) inhibit cholangiocytes secretory function. Altough exerting different effects, for most of these hormones the downstream signal cascade eventually acts on the adenyl cyclases, the transmembrane enzymes that convert ATP to cAMP regulating the intracellular level of the second messenger cAMP. Other molecules can also regulate the secretory functions of the biliary epithelium: bile salts, glutathione and purinergic nuclotides, secreted by hepatocytes into the canalicular bile (Strazzabosco, 1997).

Cholangiocytes function (and morphology) is topologically different along the biliary tree: the larger ducts and the extrahepatic portions are lined by columnar cholangiocytes that specifically express a number of different ion channels and transporters at the basolateral or apical domain (Glaser et al., 2006) and are mostly involved in secretory functions; conversely, cholangiocytes in the small interlobular bile ducts and, even more, those in cholangioles and ducts of Hering, are cubical epithelial cells that, rather than secretory functions, present other important biological properties such as the ability to proliferate in response to

liver damage, to participate in the inflammatory response (reactivity), and undergo limited phenotypic changes (plasticity). In fact liver progenitor cells are believed to arise from subpopulations of cholangiocytes residing in the canal of Hering (Sell, 2001). This functional specificity is also substantiated by the fact that most cholangiopathies show a site restricted bile duct injury. For instance, primary biliary cirrhosis targets specifically the interlobular bile ducts, whereas primary sclerosing cholangitis affects the larger intrahepatic and extrahepatic ducts. Moreover, the "small duct" variant of primary sclerosing cholangitis, where damage is restricted to the finest branches of the biliary tree, has distinct clinical manifestations.

2. Polycystic liver diseases

2.1. Fibrosis is a key feature of Autosomal Recessive Polycystic Kidney Disease (ARPKD).

Polycystic liver diseases (PCLD) are a group of genetic disorders affecting bile ducts with the progressive formation of multiple cysts. PCLD can affect just the liver, as in the case of the rare Autosomal Dominant Polycystic Liver Disease (ADPLD, estimated having an incidence of 1:100000 births); or can occur in association with kidney polycystic diseases (PKDs) (Francis et al., 2004; Masyuk, Masyuk & LaRusso, 2009). Autosomal Dominant Polycystic Kidney Disease (ADPKD), belonging to this latter category, is one of the most common inherited diseases, occurring in 1:400 to 1:1,000 individuals; it is characterized by growing cysts in the kidney, liver and pancreas. Although physiological liver function is usually preserved in ADPKD, life threatening cyst complications (mass effect, hemorrhage, infection, or rupture) may develop and demand urgent liver transplantation. Our studies focused yet on another member of this family of genetic disorders: the Autosomal Recessive Polycystic Kidney Disease (ARPKD) and its liver-related phenotypes Caroli disease (CD) and Congenital Hepatic Fibrosis (CHF). Relatively rare disorders with an estimated prevalence of 1:20,000 live births (Zerres et al., 1998), but with a high mortality rate. Up to 30% of affected people die at birth or within the first year of life or soon develop kidney and liver disfunctions. Alongside with the severe complications related to the growing cysts, CD and CHF are characterized by severe portal hypertension and recurrent and acute cholangitis due to a massive peribiliary fibrosis that also causes hypersplenism, esophageal varices and ascites, and increased risk of biliary malignancies. Peribiliary fibrosis is a key pathogenic mechanism in ARPKD and will also be the focus of this project (Fig. 2).

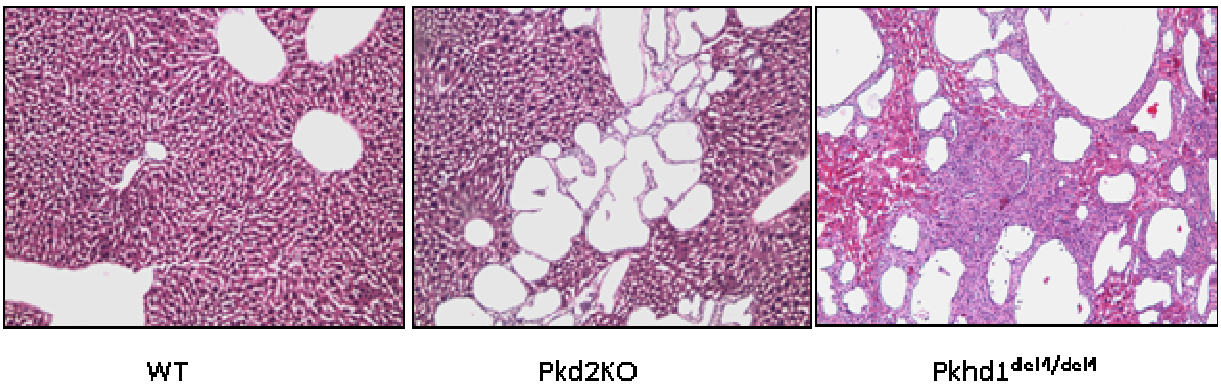

Figure 2. Fibrosis is a key feature of CHF: The comparison of micrograph of liver sections from a WT mouse (left), a mouse model for ADPKD (Pkd2KO, center), and a mouse model for ARPKD ($Pkhd1^{del4/del4}$, right), reveals the presence of biliary cysts in diseased mice. A massive periiliary fibrosis characterizes the ARPKD mouse.

2.2. Genetics and molecular pathology of polycystic liver diseases.

The isolated polycystic liver disease (PCLD) is caused by mutations in PRKCSH, a gene coding for hepatocystin (also called protein kinase C substrate 80K-H), or in the SEC63 gene which encodes for a protein residing in the endoplasmic reticulum and which is a component of the molecular machinery regulating translocation and folding of newly synthesized membrane glycoproteins. Other cystic diseases of the liver are, conversely, caused by defects in proteins that are expressed in cilia suggesting a link between ciliary dysfunction and cystogenesis. ADPKD can derive from mutations in 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.

PKD1 Mutations are more frequent and determine cysts of larges size. PC1 is a large (>460 kD) heavily glycosylated integral membrane protein (Hughes et al., 1995). It has a large N terminal extracellular portion (about 2500 amino acids long), 11 transmembrane domains and a short C-terminal cytoplasmic tail through which it interacts with PC2 forming a etherodimeric complex critical for the regulation of both proteins.

PC-2 is a smaller protein (968 amino acids) with 6 transmembrane domains and C and N termini both cytoplasmic. Based on sequence homology it likely functions as non selective Ca^{++} channel. Functional analysis of PKD1 transgenic lines suggested a direct role for PC1's extracellular domain in mediating cell-cell adhesion via homophile interactions (Streets et al., 2003; Streets et al., 2009). Furthermore PC-1 can potentially act as mechanoceptor and sense changes in apical flow (Qian et al., 2005) triggering a PC2-dependent Ca^{++} signaling response. Downstream events include activation of Rac1 heterotrimeric G protein, JAK/STAT, phosphatidylinositol 3-kinase (PI3-K)/Akt/ mTOR (mammalian target of rapamycin) and activator protein 1 (AP-1)/mitogen-activated protein kinases (MAPK) pathways (Ong & Harris, 2005).

ARPKD also derives from defects in a protein located mainly in primary cilia/basal body. The gene responsible for ARPKD is termed "polycystic kidney and hepatic disease 1" (PKHD1), and maps on human chromosome 6p21.1–p12 (Guay-Woodford et al., 1995; Zerres et al., 1994). It was identified only in very recent times, with the completion of the human genome: its long and largely dispersed structure made it difficult to localize it just by genetic linkage analyses (Bergmann et al., 2005). It is exceptionally big: the longest open reading frame comprises 67-exons, the predicted full-length protein counts 4074 amino acids and is called fibrocystin/polyductin (FPC), reflecting the hepatic and renal changes associated with this disorder. It is predicted to be an integral membrane protein with a single transmembrane domain near its carboxyl terminus and a hydrophobic signal peptide near the N-terminus. The most part of the protein is predicted to reside extracellullarly and to be heavily glycosylated. The sequence of the amino-terminal region contains multiple copies of the TIG/IPT domain (immunoglobulin-like fold) (Ward et al., 2002), a domain found in the extracellular parts of several transcription factors and receptor proteins such as the hepatocyte growth factor receptor (HGFR), the macrophage-stimulating protein receptor and plexins, proteins involved in the regulation of cell proliferation, and in cellular adhesion and repulsion.

Expression analysis suggests that PKHD1 and its murine orthologue can undergo a complex pattern of alternative splicing (Nagasawa et al., 2002; Onuchic et al., 2002) which can potentially result in different isoforms, including secreted products lacking the transmembrane domain (Ward et al., 2002). However a full sequencing of these other forms of the protein has not yet been performed. Recent studies have shown that FPC undergoes a notch-like proteolytic cleavage resulting in regulated release from the cells apical surface (Kaimori et al., 2007). Another proteolytic cleavage, Ca^{++} dependent, can also occur in the cytoplasmic region and causes the release of a fragment which harbors a nuclear localization sequence and can translocate to the nucleus and affect genes transcription (Hiesberger et al., 2006).

2.3. Fibrocystin function

Fibrocystin function is not known, although it is likely involved in maintenance of ducts architecture by regulating the planar orientation of the epithelial sheet and mediating interactions with the extracellular matrix (ECM). Sequence homology of the extracelullar portion suggest a possible function as a receptor or ligand, though there is no direct evidence for either role and the complex splicing profile could lead to combination of products with different properties.

The common location of cystic proteins on primary cilia suggests the existence of a link between ciliary dysfunction and cystogenesis. Primary non-motile cilia in secretory epithelia are involved in the regulation of multiple epithelial functions including secretion, proliferation, differentiation, and interactions with cell matrix, thus several possible mechanisms underlying cysts formation had been proposed, but it is still not clear how mutations in either gene lead to cyst formations. Common features of ARPKD and ADPKD also suggest a functional role of polycystins in development and maintenance of bile ducts architecture. Both ADPKD and ARPKD/CHF/CD represent examples of “ductal plate malformation” (DPM): characterized by the persistence of an excessive number of bile duct structures in the portal tracts, at different levels of the biliary tree, a

feature typical of the immature biliary epithelium and suggestive of a blockage in the biliary tree formation. Ontogenetically, the biliary tree starts to be apparent around the eight week of gestation and its formation proceeds centrifugally from the ileum to the periphery of the liver. During development, several periportal hepatoblasts adjacent to branches of the portal vein undergo a phenotypic change and assemble into sheaths of flat cells called primordial duct plate. Some of these primordial plates are then duplicated by a second layer of cells (double layered ductal plate) while the others are deleted by apoptosis. The double layered ductal plate progressively dilates forming the tubular structure of the biliary tree branches embedded into the portal space (Roskams & Desmet, 2008). At birth the biliary tree still displays features of immatureness, and its development completes during the first year of life. However, the persistence of a multiple ductal plate-like morphology in the adult is a characteristic of polycystic diseases, indicating a dysregulation of the proliferative and apoptotic processes that accompany the development of the biliary tree (Desmet, 1992). In polycystic diseases the multiple biliary microhamartomas progressively dilate to macroscopic cysts, scattered throughout the liver parenchyma, interestingly, in ADPKD the cysts are not connected with the biliary tree and form autonomous structures, while in ARPKD they remain as enlargements of the tree itself. This fundamental difference could perhaps help to explain the different clinical manifestations between ARPKD/CHF/Caroli and ADPKD. It is important to emphasize that in patients cysts continue to appear throughout adult life. Furthermore mice conditional knockout for Pkd1 or Pkd2 develop cysts as in the human disease even tough the gene inactivation occurs weeks after birth (Koptides & Deltas, 2000; Wu et al., 1998). Thus Polycystins seem to play a role also in maintaining a normal architecture of the biliary tree during adult life.

Studies in mouse renal tubular cells (IMCD, inner medullary collecting duct) cell lines, grown in three-dimensional cultures, showed that Pkhd1 silencing results in impaired tubule formation and aberrant organization of the actin cytoskeleton along with abnormalities in cell-cell and cell-extra cellular matrix (ECM) interactions. These features, together with the fact that fibrosis is one of the

peculiarity of ARPK compared to ADPKD, may suggest the activation of an epithelial to mesenchymal transition (EMT) program: a biological process that has recently gained much interest because of its possible involvement in the pathogenesis of organ fibrosis in the kidney, the lung and, perhaps in the liver. Cholangiocytes undergoing EMT may contribute to the accumulation of activated fibroblasts, the principal effectors of the excessive deposition of extra-cellular matrix (ECM) leading to fibrotic scar formation, this hypothesis will be further discussed below.

3. Liver fibrosis: cross talk between epithelial and mesenchymal cells and epithelial to mesenchymal transition

3.1. Fibrosis is the consequence of a persistent injury.

Hepatic fibrosis, the main pathogenic mean of ARPKD/CHF/CD, represents a wound healing response to liver injury, common in a wide variety of etiologies and consists in the excessive deposition of extracellular matrix by mesenchymal cells (myofibroblasts) in a reparative attempt. It occurs as a consequence of a chronic perturbation of the hepatic homeostasis or a prolonged unabated inflammation. Most chronic liver diseases follow this common course progressing from persistent epithelial damage and mild inflammation, to more severe inflammation, to -still reversible- fibrosis, and finally to cirrhosis. Despite that, the molecular pathogenesis linking inflammation or persistent tissue damage and fibrosis are still not fully described. Several cellular events can trigger the signals that activate the fibrotic process. Persistent liver injury can derive from cholestasis or necrosis. Hepatocytes necrosis or apoptosis can induce fibrosis. Classically believed to be just a secondary consequence of liver injury, nowadays **hepatocytes apoptosis** is rather considered as an important mediator of the fibrotic process. In fact it has been shown that the inhibition of hepatocytes apoptosis, obtained with pancaspase inhibitors, hampered fibrosis in mouse models (Witek et al., 2009). Necrosis of cells, as well as apoptosis may also constitute an inflammatory stimulus. **Chronic inflammation** is another trigger for liver fibrosis. The liver macrophages Kupffer cells (KC), but also hepatocytes, liver endothelial cells, biliary cells and HSC all express TLR4, receptor for bacterial lipopolysaccaride (LPS). LPS exposure elicits a fibrogenic response in KC -that will produce extra cellular matrix, pro-inflammatory cytokines and reactive oxigen species (ROS, potential fibrotic mediators)-, and in HSC, by down regulation of BMP (bone morphogenic protein) promoting TGF-β sensitization trough a NF-kappaB-dependent pathway (Seki et al., 2007). TLR4-activated HSC then produce pro-inflammatory cytokines further attracting (recruiting) Kupffer

cells. **Oxidative stress** is an important mediator of liver fibrosis: Reactive oxygen species (ROS) derived from lipids peroxidation can originate from hepatocytes, hepatic stellate cells, macrophages and inflammatory cells. Alcohol consumption, for example, can produce oxidative stress by activating cytochrome P450 2E1 in the hepatocytes. Similarly the NADPH oxidase in activated Kupffer cells, macrophages, and also expressed by hepatic stellate cells can produce ROS leading to fibrosis (De Minicis et al., 2008). Finally, last but not least, **TGF-β**, is a potent fibrogenic cytokine. TGFβ induces fibrosis through multiple mechanisms: it can directly activate HSCs, stimulating the production of multiple ECM proteins, increasing its own synthesis through the AP-1 site on its own gene and promoting the synthesis of tissue inhibitors of metalloproteases (TIMPs) inhibiting ECM degradation. It is also one of the main mediators of epithelial mesenchymal transition (EMT) further discussed below.

Important insights into the pathological process were provided by models of liver fibrosis developed in rats and mice and these still represent the main approach for these studies. Animal models of post-necrotic fibrosis are obtained through the administration of carbon tetrachloride (CCl_4), while bile ducts ligation provides the model for secondary biliary fibrosis. These studies yielded important information about critical signals and molecular mediators that stimulate fibrogenesis however a genetic model of ARPKD, as is the model used in this study, would supply the possibility to study the establishment of primary fibrosis and also the opportunity to focus on the downstream effect of a single genetic product.

3.2. Fibrosis derives from an integrated cellular response to tissue injury

The fibrotic response to an enduring injury ultimately consists in the excessive deposition of extracellular matrix mainly produced by activated myofibroblasts, however, the progression of chronic liver entry to fibrosis is the result of an integrate cellular response where all hepatic cells undergo specific changes and contribute to this end. Injured hepatocytes undergo apoptosis. Cholangiocytes

wage the so called “ductular reaction”, sinusoidal endothelial cells undergo a loss of fenestrae (termed acapillarization) of the sinusoids. Kupffer cells, the resident liver macrophages, activate and produce a variety of chemokines and cytokines. Lymphocytes infiltrate the injured liver and contribute to the inflammation. Finally, the quiescent stellate cells are activated, they start to proliferate and to produce extracellular matrix (ECM) proteins (Brenner, 2009).

This complex interplay between the damaged epithelial cells, inflammatory cells, and mesenchymal cells, eventually leads to the activation of myofibroblasts, the proliferative, fibrogenic, contractile cells actively responsible for extracellular matrix deposition and the fibrotic process in basically all chronic liver diseases. The cellular sources of the excessive ECM and the factors promoting their activation during liver fibrogenesis remain the focus of an intense debate and investigations.

The question recently regained new attention as new findings demonstrated that several cells potentially can accomplish this task. It was initially shown that cells activated to become myofibroblasts are mainly liver resident cell: either the **tissue fibroblast** located in the portal tract of the liver (portal fibroblasts) or the quiescent **hepatic stellate cells** (HSC) located in the Space of Disse. Most evidence substantiates the primary role of the quiescent HSC in becoming myofibroblast and then produce the fibrous scar found in chronic liver diseases (Brenner, 2009). In recent years growing evidence has shown that also bone marrow-derived **fibrocytes** or circulating mesenchymal cells can be recruited through the injured liver and become myofibroblast as shown by Kisseleva and colleagues using cross-sex bone marrow transplantation (Kisseleva et al., 2006). Furthermore, several studies of lineage tracking demonstrated that fibroblasts can also derive locally from hepatocytes, cholangiocytes, or even endothelial cells undergoing **epithelial to mesenchymal transition** (EMT) and transitioning to acquire characteristics of mesenchymal cells, eventually becoming activated myofibroblasts (Zeisberg et al., 2007). (Kruglov et al., 2006). The contribution of these cells to the fibrogenic process will be described in more detail.

Hepatic stellate cells activation.

The quiescent hepatic stellate cells (HSCs) reside in the perisinusoidal space of Disse, between sinusoidal endothelium and hepatocytes; they are Vit-A (retinoid) storing cells, often detected by desmin immunostaining. They also express neural markers (and most likely derive from neural crests). In response to fibrogenic stimuli these cells lose the retinoids and start de novo expressions of proteins such as α-smooth muscle actin (α-SMA, characteristic of activated myfibroblasts), and receptors: the platelet derived growth factor receptor (PDGFR) and transforming growth factor-β receptor (TGF-β R) above all. As a result PDGF is a very powerful mitogen and chemoactractant for HSC (Friedman, 2008a). In addition to the well-characterized A and B chains of PDGF, C and D isoforms also have been discovered more recently; in fact, PDGF-D is the most potent and physiologically relevant PDGF subunit in HSC activation described thus far (Borkham-Kamphorst et al., 2007). Other stellate cell mitogens include vascular endothelial growth factor (VEGF), thrombin and its receptor, epidermal growth factor (EGF), TGFα, keratinocyte growth factor and basic fibroblast growth factor bFGF (Friedman, 2008a). Among HSC chemoactractants: MCP1 (monocyte chemoactractant protein, also called CCL2) which can also be produced by cholangiocytes reacting to damage, and CXCR3 ligands (CXCL9, CXCL10, CXCL11). Conversely, TGFβ1 has a fibrogenic effect on HSC. HSC can also secrete TGFβ1 that can act in an autocrine or paracrine way and initiate several positive feedback pathways that further amplify the signal. It represents a potent stimulus for the production of ECM proteins, mainly collagen type I, and other matrix components including cellular fibronectin and proteoglycans that alter ECM composition from type IV collagen, heparin sulfate proteoglycan, and laminin (as in the basal lamina) to fibril-forming collagen type I and III. HSCs also contribute to the degradation of the normal extracellular matrix through the production of the metalloproteinase, MMP2, thus leading to the release of growth factors from matrix-bound reservoirs, that may further stimulate the process. ECM alterations, the disassembly of focal-adhesion and cells migration, are sensed by integrins and integrin-linked kinases transduce the signal to other

membrane receptors, to Rho and Rac (membrane-bound GTP binding proteins) which act on the actin cytoskeleton promoting migration and contraction. Integrins also sense the enhanced density of ECM and the increased matrix stiffness, a significant stimulus to HSC activation. (Friedman, 2008b).
ESC contribution to the pathology of fibrosis is not limited to ECM production and degradation, stimulated by endothelin-1 they acquire contractility and may also contribute to portal hypertension. Moreover, through the production of reactive oxidative species generated by NADPH oxidase, HSCs add to the oxidative stress. By producing chemokines and cytokines such as MCP-1, HSC exert an autocrine and also paracrine chemotactic effect on leukocytes. Finally, the HSCs are the major source of hepatocyte growth factor (HGF), the primary mytogen for hepatocytes. Their multifaceted contribution to liver fibrosis thus includes proliferation, fibrogenesis, contractility, matrix degradation, and proinflammatory signals (Brenner, 2009).

Fibrocytes: bone marrow derived collagen producing cells.

Fibrocytes are collagen producing cells of bone marrow origin. They were first identified in wound repair studies as a population of cells circulating in the blood that exhibit mixed morphological and molecular characteristics of hematopoietic stem cells, monocytes and fibroblasts (Bucala et al., 1994). Common fibroblasts intervening in wound repair generally derive from resident mesenchymal cells and are not of hematopoietic origin, conversely fibrocytes express CD45, a common lymphoid marker, and CD34, early hematopoietic marker, proof of their origin; they also express the immunological markers typical of an antigen-presenting cell and at the same time are positive for fibroblasts specific markers such as collagen I and fibronectin and can participate in wound healing and fibrogenesis (Asawa et al., 2007; Kisseleva et al., 2006; Quan et al., 2004). Moreover fibrocytes are important producers of cytokines, chemokines and growth factors: most importantly they produce platelet-derived growth factor α (PDGF-α) which promotes proliferation and migration of endothelial cells and activates HSC; they produce TGFα, which enhances extracellular matrix

expression and deposition and reduces MMPs activity, TGF-β and also mediators of the inflammatory and immune response such as macrophage-colony stimulating factor (M-CSF), macrophage-colony stimulating factor (GM-CSF), macrophage inflammatory protein-1 (MIP-1α), macrophage inflammatory protein-2 (MIP-2), Interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α). Not last vascular endothelial cell growth factor (VEGF) and bFGF, stimulating angiogenesis, matrix metalloproteinase-9 (MMP-9), Il-6, Il-8, and MCP-1 (Quan et al., 2004). Not all studies agree on the fact that these cells could be myofibroblasts precursor. Lineage tracking experiments upon bone marrow transplantation in mice demonstrated that fibrocytes contribute for about 5-10% to collagen-I producing cells in BDL mice models of fibrosis. However, in two similar studies Kisseleva et al. did not find $CD45^+/\alpha SMA^+$ cells (Kisseleva et al., 2006), conversely Asawa et al. (Asawa et al., 2007) did find αSMA positive cells derived from the transplanted bone marrow, recruited in the periportal area, however, αSMA expression was limited to 7 days after transplant and decreased thereafter, suggesting that differentiation of bone marrow derived cells into myofibroblasts could occur transitorily at the early stages of periductal fibrosis. Transplanted cells acquired a fibroblast phenotype and remained fibroblast-specific protein positive. On the other hand it has been shown that fibrocytes spontaneously gain expression of αSMA in culture and gradually lose CD34 and CD45, differentiating in myofibroblasts-like cells, especially upon exposure to TGFβ (Bucala, 2008; Kisseleva et al., 2006). Fibrocyte differentiation appears to be driven by a complex profile of cytokines: pro-fibrotic cytokines IL-4 and IL-13 promote fibrocyte differentiation from peripheral blood monocytes without inducing proliferation, whereas the anti-fibrotic cytokines IL-12 and interferon (IFN)γ inhibit fibrocyte differentiation (Shao et al., 2008): While IL-4, IL-13 and IFN-γ have a direct effect on fibrocyte differentiation IL-12 was found to have an indirect effect possibly through $CD16^+$ NK cells.

The signaling that mediates fibrocytes recruitment is still poorly understood. Fibrocytes express chemokines receptors CXCR4 and CXCR7, and the CXCR4-CXCL12 axis plays an important role in lung fibrosis in both human disease

(Mehrad et al., 2007) and mouse models (Mehrad, Burdick & Strieter, 2009), however, it seems that the mechanism can vary depending on the disease involved and on the target tissue (Keeley, Mehrad & Strieter, 2010).

Cholangiocytes: epithelial-mesenchymal cross talk and ductular reaction.

Epithelial-mesenchymal cross talk is a physiological routine. Cholangiocytes normally display receptors for a number of cytokines, chemokines and growth factors and angiogenetic factors that enable an extensive cross-talk with other liver cell types, including hepatocytes, Kupfer cells, stellate cells, fibroblasts, endothelial cells, lymphoid cells (Strazzabosco, Spirli & Okolicsanyi, 2000). This characteristic becomes particularly relevant when the liver or the biliary tree are damaged favouring an intense exchange of signals between mesenchymal cells, that can release these cytokines into the microenvironment, and cholangiocytes that react to the damage. Liver injury or biliary tree damage, in fact, triggers a reactive response (called ductular reaction) by cholangiocytes that start to actively proliferate and expand. Cholangiocytes proliferation occurs in most pathologic conditions, including cholestasis, viral hepatitis, 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 arise from a progenitor cell compartment located in close contact with the smallest twigs of the biliary tree, the terminal cholangioles at the canals of Hering. At least three types of ductular reaction have been described in animal models related to cholangiocytes proliferation in response to different types of liver damage. The typical is "type I", observed in BDL rats and in human obstructive cholestasis (Svegliati-Baroni, De Minicis & Marzioni, 2008) which result in increased number of fully functional bile ducts (hyperplasia) into the portal spaces. Type II ductular reaction is conversely, characterized by irregular proliferation and sprouting of ineffective bile ducts in all the liver parenchyma, also outside the portal area (Alvaro, Gigliozzi & Attili, 2000; Alvaro et al., 2007; Svegliati-Baroni et al., 2008), probably as a consequence of proliferation of pre-existing ductules and cells from the progenitor cells compartment. It had been

described in primary biliary cirrhosis, in primary sclerosing cholangitis (PBC, PSC) and in alchoolic liver disease. Type III also called "oval cell" proliferation, occurs in the early stages of carcinogenesis in rat liver and involves the proliferation of the formation of disorganized tubular structures with a poorly defined duct lumen which randomly spread into hepatic lobules, creating a distorted hepatic architecture (Alvaro et al., 2000), (Priester, Wise & Glaser, 2010).

Reactive cholangiocytes also lose some of their characteristic channels and transport proteins and acquire the capability to secrete de novo a number of proinflammatory and chemotactic cytokines and growth factors and can thus recruit inflammatory and mesenchymal cells (Strazzabosco, Fabris & Spirli, 2005). The secreted cytokines can trigger an autocrine and paracrine regulation of various activities in biliary epithelial cells as well. Desmet V.J. coined this expression which effectively describes this cross-talk and the potential contribution of cholangiocytes to the fibrogenic process: "while mesenchymal cells are considered the effectors of fibrosis, reactive cholangiocytes are considered the pacemaker of liver fibrosis" (Desmet, 1998). Several cytokines, have been proven to mediate the epithelial/mesenchymal cross talk in the liver, among them inflammatory mediators such as IL-6, IL-8, TNFα, IFNγ, PDGFβ (Grappone et al., 1999), and also, monocyte chemotactic protein-1 (MCP-1) (Marra et al., 1999), 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 VEGF, which promotes angiogenesis, endothelin-1 (ET-1), platelet derived growth factor-BB (PDGF-BB), transforming growth factor-β2 (TGF-β2) and connective tissue growth factor (CTGF), MCP1, IL-6, IL-8 immunomodulatory cytokines. Recently, Popov et al. have shown that in experimental and human liver fibrosis reactive cholangiocytes also express integrin αvβ6, normally expressed by biliary epithelium only during development. Integrins are family of transmembrane, heterodimeric cellular receptors mediating cell-cell and cell-ECM interactions. Expression of αvβ6 integrin by cholangiocytes appears to be a crucial step

triggering fibrogenesis: integrin αvβ6 is a transmembrane adhesion receptor for the matrix molecule fibronectin and also binds TGF-β, a major stimulator of extracellular matrix production. In fact, TGF-β1 is synthetized as inactive form, and it needs to be tethered to different cell surface receptors, including αvβ6 integrin, for its activation. Notably, integrin αvβ6 does not co-activate TGF-β2. (Popov et al., 2008), (Patsenker et al., 2008).

3.3 EMT: epithelial-mesenchymal transition can contribute to fibrosis

Classically it was believed that cholangiocytes contribution to liver fibrosis was limited to their capability to establish paracrine communications with mesenchymal cells, possibly recruiting ECM producing cells. In recent years it has been proposed that, cholangiocytes may also participate in the generation of liver fibrosis through a process of epithelial to mesenchymal transition (EMT). This idea is modifying the definition of cholangiocytes as pacemaker and not effectors of fibrosis. EMT is a process of cellular reprogramming whereby epithelial cells acquire some of the phenotypic and functional characteristics of mesenchymal cells, such as the ability to migrate by locally dismantling the basement membrane upon which the epithelial sheet resides and, most importantly, the ability to generate connective tissue components (fibronectin, collagen, elastin, tenascin). EMT may thus contribute to the accumulation of matrix producing cells, in association with the damage to bile ducts. This biological process has been described in the pathogenesis of organ fibrosis in the kidney (Zeisberg & Kalluri, 2008) and lung (Willis, duBois & Borok, 2006b), (Iwano et al., 2002; Liu, 2004; Willis et al., 2006b; Zeisberg & Kalluri, 2008). Recent studies suggest that EMT may also be involved in liver fibrosis as seen in mice treated with CCl_4, and in recurrent primary biliary cirrhosis after liver transplantation (Robertson et al., 2007), (Omenetti et al., 2008; Rygiel et al., 2007; Xia et al., 2006). Both hepatocytes and cholangiocytes showed the capability to undergo some degree of EMT in chronic liver disease (Robertson et

al., 2007; Rygiel et al., 2008; Zeisberg et al., 2007) however, strong evidence for EMT involving cholangiocytes is yet lacking.

Historically, the process of EMT was described for the first time in chicks' embryos about 40 years ago and it is now well know that during gastrulation, EMT (as well as the subsequent MET, mesenchymal to epithelial transition) is an essential step for germ layer formation (mesoderm) and cell migration in the early vertebrate embryos (Hay, 1968), for gastrulation and organogenesis. New data are now showing that EMT is not limited to the embryos but it can also occur in the adult body in pathological conditions. EMT was recognized as a mechanism important for the initial step of metastasis: the migratory behavior acquired by metastatic cells immediately prompted comparisons with cells that have undergone EMT. In fact, since the early '90s different studies have drawn a parallel between genes implicated in EMT during embryogenesis and genes that have been shown to control metastasis (Huber, Kraut & Beug, 2005; Wu & Zhou, 2008). In addition the aspect of a functional transition of cells with the acquired ability to produce extra cellular matrix components suggested EMT's possible contribution to the pathogenesis of organ fibrosis. EMT was described in the pathogenesis of organ fibrosis in kidney and lung. Kalluri and colleagues, in experiments of lineage tracking showed that in a mouse model of renal fibrosis as much as 36% of new fibroblasts were derived from local EMT (Iwano et al., 2002; Kalluri & Neilson, 2003).

A specific and detailed description of the phenomenon called for the classification of EMT in three different subtypes based on the biological context in which they occur (Kalluri & Weinberg, 2009), (EMT International Association's Meetings, Cracow, Poland 2007, and Cold Spring Harbor Laboratories 2008). Nevertheless, despite the differences and peculiarities in these described EMT processes, especially as to functional consequences, a common set of landmarks (markers) at the molecular and transcriptional level was clearly identifiable. Embryonic EMT belongs to type 1 EMT. Primary EMT events (i.e., those that occur in tissues that have never undergone a previous EMT process) take place during implantation of the embryo into the uterus, during embryogenesis (Acloque et al., 2009), and

during organ development. Mesodermal and endodermal mesenchyme generated by type 1 EMT then undergoes MET to generate secondary epithelia that, in turn, undergo further rounds of EMT/MET to form various organs (Choi et al 2009). These processes emphasizes a remarkable phenotypic plasticity of the embryonic epithelia that enables it to move back and forth between epithelial and mesenchymal states via the processes of EMT and MET (Lee et al., 2006). Type 1 EMT does not cause fibrosis, neither induces an invasive phenotype resulting in systemic spread through the circulation (Kalluri & Weinberg, 2009).

The EMT associated with wound healing, tissue regeneration, and organ fibrosis belongs to a second type. The program originates as part of a repair-associated event that normally generates fibroblasts and other related cells in order to reconstruct tissues following trauma and inflammatory injury. This response is supposed to be transitory and to cease once inflammation is attenuated however, in the setting of organ fibrosis, type 2 EMT can continue to respond to ongoing inflammation, leading eventually to organ destruction. As mentioned, tissue fibrosis is in essence an unabated form of wound healing due to persistent inflammation.

The EMT observed in neoplastic cells is yet of a different type and occurs in cells that have previously undergone genetic and epigenetic changes, specifically in genes that favor clonal outgrowth and the development of localized tumors. These changes, notably affecting oncogenes and tumor suppressor genes, act together with the EMT regulatory circuitry to produce outcomes different from those observed in the other two types of EMT. Carcinoma cells undergoing a type 3 EMT may invade and metastasize and thereby generate the final, life-threatening manifestations of cancer progression. Importantly, cancer cells may engage in EMTs to different extents, with some cells retaining many epithelial traits while acquiring some mesenchymal ones and other cells losing all vestiges of their epithelial origin and becoming fully mesenchymal (Kalluri & Weinberg, 2009).

Despite their different biological features a common set of genetic and biochemical elements underlies these externally diverse phenotypic programs

(Kalluri, 2009) a common molecular machinery involved at the transcriptional and post-transcriptional level, with specific phenotypic markers, able to univocally describe the EMT process.

Molecular mechanism of EMT.

Epithelial cells and mesenchymal cells are characterized by very distinct features; such complex transition requires the fine integration of sequential molecular signals at the transcriptional and post transcriptional level. Epithelial cells are adherent cells; they attach laterally to each other through intercellular adhesion complexes (tight junctions, adherens junctions, desmosomes, and gap junctions) forming impermeable continuous layers where cells display apico-basal polarity, and typically the epithelial sheets lay on a basal membrane that separates the epithelium from other tissues. By contrast, mesenchymal cells are non polarized spindle shaped cells and lack intercellular junctions, such that they can move as individual cells throughout the extracellular matrix. A first step in response to EMT-induction signals is the progressive destruction of intercellular adhesion complexes and the consequent loss of the apico-basal polarity of epithelial cells, cytoskeleton modifications, crucial for permitting cell motility, and basement membrane disruption so that the cells that acquired migratory and invasive properties can migrate through the extracellular matrix.

Figure 3 schematizes the main players involved in the EMT process. A major activator of EMT is TGF-β. Acting through the Smad-dependent cascade (mainly Smad-2/3) (Rhyu et al., 2005) it regulates the expression of many proteins of the EMT proteome. Among them, β-catenin is crucial, as it is involved in both cell adhesion and the transcriptional mediation of the Wnt signalling pathway. Its participation in either role is actively regulated by phosphorylation. In normal conditions, β-catenin is bound to the cadherin complex and clasps to the actin cytoskeleton contributing to the stability of adherens junctions which maintain the polarization of the epithelial sheet. The cytoplasmic and nuclear pool of ß-catenin is maintained at low level by phosphorylation, and subsequent ubiquitination and proteosomal degradation.

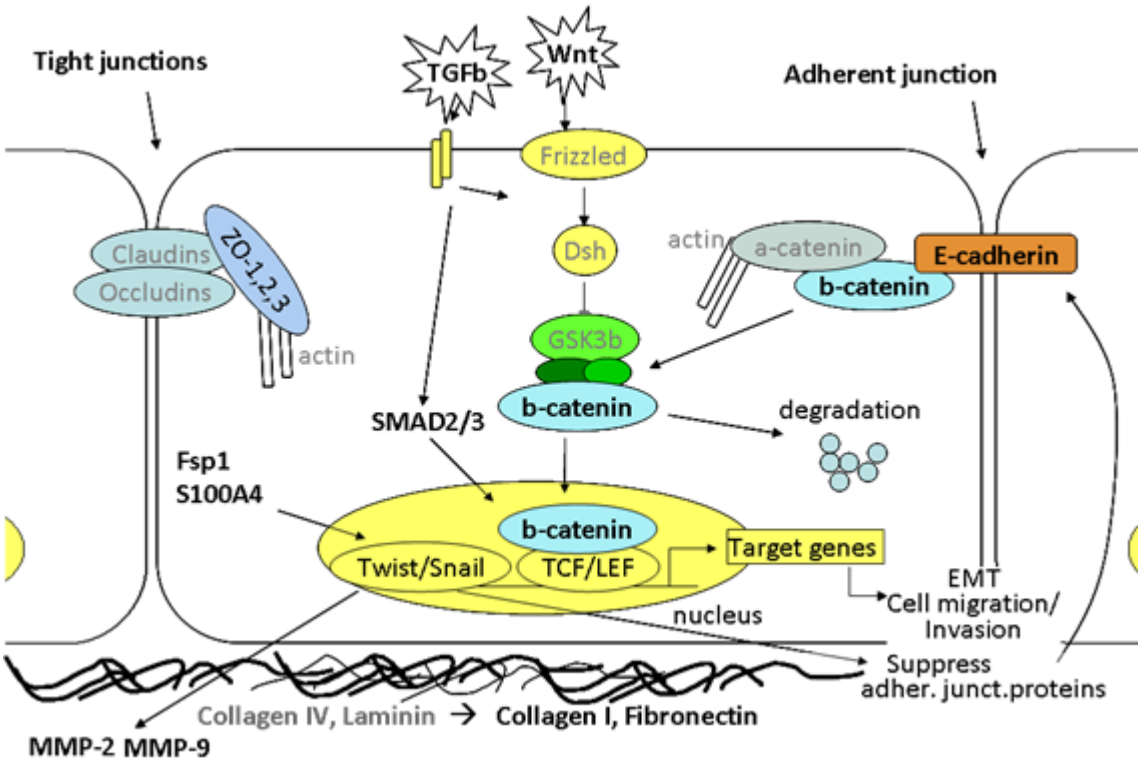

Figure 3. The EMT machinery. Diagram of the main molecules involved in the EMT process (description in the text).

This phosphorylation occurs through Casein kinase 1 alpha (CK1α) and glycogen syntase 3β (GSK3β) that phosphorylate the protein at Ser 45 and Ser 33/Ser37/Thr41 (respectively) in the NH2-terminal region, inducing its subsequent proteosomal degradation. Conversely, in response to Wnt signalling, the Wnt receptor, Frizzled (Fz) inhibit the GSK3β-dependent posphorylation of β-catenin that thus accumulates in the cytoplasm and translocates into the nucleus where it activates the transcription factors T cell factor and lymphoid enhancer factor (TCF/LEF). Among other functions, these transcription factors activate the transcription factor Snail and suppress the epithelial (E)-cadherin expression (Lee et al., 2006).

E-cadherin is an important marker of the epithelial phenotype thus its loss is considered a hallmark of EMT. E-cadherin is a cell-cell adhesion protein, important component of the adherent junctions between homotypic epithelial

cells (Junghans, Haas & Kemler, 2005). Its extracellular domain mediates a Ca^{++}-dependent (generally homophilic) cell-cell binding and the cytoplasmic tail links to the cytoskeleton establishing strong adhesions. This link is mediated by E-cadherin binding to ß-catenin, which in turn binds to α-catenin. The α-catenin bridges the cadherin–ß-catenin complex to the actin cytoskeleton either directly or indirectly via actin-binding proteins such as α-actinin or profilin (Junghans et al., 2005). E-cadherin provides the epithelial sheet with the cell-cell adhesion strength that determines cell polarity, with apical, basal and lateral compartments. Several transcription factors have been implicated in the transcriptional repression of E-cadherin. Mainly zinc finger protein Snail-1, Twist, ZEB1, SIP1 (Behrens et al., 1991; Giroldi et al., 1997). Studies on invasive breast cancer suggest that Snail1 is implicated in the initial migratory phenotype while ZEB1 and twist would be responsible for the maintenance of migratory behavior (Wu & Zhou, 2008). Snail in particular seems to be a pivotal point in the integration of EMT signals. Its expression is regulated by the Wnt/β catenin signaling (Yook et al., 2006), by Hedgehog (Katoh & Katoh, 2009), Notch, Ras, PKA, HIF1α, VEGF, ROS, PDGF, TGFβ (Omenetti et al., 2008), and it functions as transcriptional regulator for E-cadherin and for many genes involved in the epithelial differentiation (cytokeratins, N-cadherin, HNF4, HNF1β, MMP2, MMP9) (Katoh & Katoh, 2009). The EMT process is also characterized by expression of S100A4 by epithelial cells (Robertson et al., 2007). Its homologous in mouse is called FSP-1- fibroblast specific protein (Kalluri & Neilson, 2003; Lee et al., 2006). It is a cytoskeleton-associated, Ca^{++}-binding protein, normally expressed only by fibroblasts, it interacts with other cytoskeletal proteins, such as myosin-IIa, causing a cell reshaping that enables motility. Its expression in epithelial cells does not normally occur in cholangiocytes. S100A4 also up-regulates Twist and Snail1 (Venkov et al., 2007), the transcription factors just described above, that suppress adherens junction proteins, including E-cadherin.

AIMS

Given the fact that

- a defective fibrocystin expressed by cholangiocytes in ARPKD causes progressive fibrosis in the absence of inflammation;
- silencing Pkhd1 in IMCD cells alters cytoskeletal organization and impairs cell-cell and cell-matrix contact, features consistent with EMT in epithelial cells;

Pkhd1 mutations may offer a model of EMT specifically related to a single genetic defect.

We propose the hypothesis that cholangiocytes with defective fibrocystin are primed to EMT, leading to progressive accumulation of activated fibroblasts ultimately responsible for the generation of portal fibrosis. For this study we analyzed human liver biopsies from CHF/CD patients and from ADPKD patients and healthy individuals, together with mice models of both ARPKD and ADPKD

- to explore phenotypic evidence of EMT in patients with CHF/CD and in Pkhd1$^{del4/del4}$ mice
- to study if cholangiocytes isolated from Pkhd1$^{del4/del4}$ mice are functionally engaged in EMT in vitro and display increased motility and invasivity.
- to study the effect of the altered biliary phenotype on the cross talk between epithelial and mesenchymal cells in ARPKD.

METHODS

Animal models.

Pkhd1$^{del4/del4}$, the mouse model of ARPKD used in this study, was a kind gift of Prof. S. Somlo (Yale University, New Haven, CT). The mouse, of a mixed C57BL6/129Sv background, carries a inactivating deletion in the exon 4 of the Pkhd1 gene (orthologue of the human PKHD1 gene) (Gallagher et al., 2008). These mice (from now called Pkhd1) mimic the human hepatic disease, developing cysts and progressive portal fibrosis in the liver, already apparent at 1-3 month (Gallagher et al., 2008). Mice were genotyped by polymerase chain reaction (PCR) analysis of DNA extracts from tail sections. The primers used to screen for the deletions are the forward primer 5'-TTAGGGAAGAATGGCTCTC-3', and the reverse primer 5'-TTCAGAGGGAGGAAAAGCAA-3' for the WT allele, to produce a 580-bp fragment. The reverse primer 5'-GCCAGAGGCCACTTGTGTAG-3', amplifying a 171-bp product was used for the Pkhd1^{del4} allele, as described in Gallagher et al., 2008). WT and mice homozygotes for the deletion were used for this study; heterozygotes were maintained for the breeding.

Mice models for ADPKD (also a kind gift from Prof. Somlo's Laboratory): carry conditional deletion of the Pkd1 or Pkd2 gene in combination with the tamoxifen inducible pCXCreER (Guo, Yang & Lobe, 2002; Shibazaki et al., 2008), (Spirli et al., 2010). Pkd1 or Pkd2 were conditionally inactivated upon exposure to tamoxifen as tested by PCR on tail DNA extracts. These mice (Pkd1KO and Pkd2KO) mimic the human ADPKD and develop biliary cysts without generating liver fibrosis (Spirli et al., 2008). (Spirli C, Okolicsanyi S, et al, Gastroenterology 2010). Together with WT mice of the same genetic background (C57BL/6J) were used as control in our experiments.

Liver tissue from human patients.

Samples of fibropolycystic liver diseases including CHF, CD, ARPKD and ADPKD liver tissue (n = 15) formalin-fixed and paraffin-embedded were from archival tissue stored at the Department of Pathology, Ospedali Riuniti di

Bergamo. CHF, CD, ARPKD and ADPKD samples were from explants of patients undergoing liver transplantation or from hepatectomy of patients undergoing surgical resection; additional samples were obtained from needle biopsies performed for diagnostic purposes. All diagnoses are based on clinical and laboratory data and on histopathological examination of histological samples. Wedge biopsies (n = 20), taken from liver donors successfully used for liver transplantations were used as normal controls.

Liver tissue from mouse models.

Whole liver was collected from cohorts of Pkhd1 mice of different ages (3, 6, 9 and 12 months, total n = 20). Data were compared with those from WT littermates of similar age (n = 10) or those from Pkd1KO and Pkd2KO mice. Liver samples from the mice were fixed in 4% formalin for 4 hours, and then switched to 70% EtOH until tissues were embedded in paraffin and cut in 5 µm sections.

Immunohistochemical studies on human and murine liver samples.

For the staining paraffin blocks were deparaffinized in xylene, gradually rehydrated in graded ethanol and washed in phosphate buffered saline (PBS). Antigen retrieval was performed using Signet pH-All-2 (Signet Laboratories, Inc., Dedham, MA) for 25 minutes (exceptions: 10mmol/L citrate buffer, pH 6 for CD-34 antibody; proteinasi K, 20µg/ml in TE buffer, pH8 for Laminin) while steaming, then slides were cooled to room temperature and washed in PBS. Murine sections were incubated for 40 minutes in blocking buffer containing 2% bovin serum albuminin (Sigma Aldrich, USA) and then in primary antibody overnight at 4°C. Human sections were incubated with primary antibody in 5% human serum, overnight at 4°C. Table 1 lists the primary antibody used in this study.

Table 1

Antigen recognized	Clone	Antibody name	Dilution	Host	Source *
Actin, α-Smooth Muscle	1A4	αSMA, a5228	1:200	Mouse	Sigma-Aldrich
β catenin	β-Catenin-1	M3539	1:20	Mouse	Dako
CD-34	MEC14.7	Gtx28158	1:500	Rat	GeneTex
CD-45,	30-F11	550539 Leukocyte Common Antigen (LCA)	1:20	Rat	BD/Pharmingen
Collagen I	COL-1	C2456	1:100	Mouse	Sigma-Aldrich
Cytokeratin 19	Troma III	Troma III	1:100	Mouse	DSHB
E-cadherin	ECCD-2	M108	1:200	Mouse	Takara
Fibronectin	Polyclonal	A0245	1:300	Rabbit	Dako
Laminin	Polyclonal	Z0097	1:100	Rabbit	Dako
PDGFR-β	28E1	3169	1:100	Rabbit	Cell-Signaling
PDGF-D	Polyclonal	H00080310-D01	1:50	Rabbit	Abnova
PanCK	PCK-26	C1801	1:200	Mouse	Sigma-Aldrich
PanCK	Polyclonal	Z0622	1:300	Rabbit	Dako
S100A4	Polyclonal	A5114	1:100	Rabbit	Dako
Vimentin	VIM-13.2	V5255	1:50	Mouse	Sigma-Aldrich
Snail	Polyclonal	ab17732	1:	Rabbit	Abcam

*** Source legend:**

- Abcam Transduction Labs, Cambridge, MA, USA
- Abnova, Taipei City, Taiwan
- BD: Becton, Dickinson and Company, NJ, USA
- Cell Signaling technology, MA, USA
- Dako Cytomation, Denmark
- DSHB: Developmental Studies Hybridoma Bank, IA, USA
- GeneTex, CA, USA
- PharMingen, San Diego, CA, USA
- Santa Cruz Biotechnology, CA, USA
- Sigma-Aldrich, St Louis, MO, USA
- Takara Bio inc., Japan
- R&D Systems, MN, USA

Slides were then incubated with the appropriate secondary antibody for 40 minutes at room temperature and washed in PBS. Finally slides were either mounted in fluorescence in mounting media with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) or incubated with diaminobenzidine (Sigma-Aldricht) for HRP detection.
Hematoxylin-eosin staining were performed by the Yale Pathology Tissue Services, Yale School of Medicine, New Haven, CT.

Quantitation collagen-I.

Liver samples were stained with anti collagen-I antibody. Digital images of 5 random non overlapping fields for each sample were recorded with a digital camera at 200X magnification and the collagen positive area was calculated as the percentage of pixels above the threshold value versus the total pixels per field, using the Image analysis software LuciaG 5.0 (Nikon)

Quantitation of E-cadherin.

Sections from the two major mouse liver lobes for each sample were immunostained with a pancytokeratin antibody (PCK) for a correct identification of the biliary cysts structures and E-cadherin antibody. The relative number of E-cadherin negative cells among PCK positive biliary cells was then calcultated in the following way. For each pair of liver lobes, 5 random non-overlapping fields were recorded by a digital camera, at 200X magnification, to a total number of 10-fields per each mouse. Each PCK^+ /$E\text{-cadherin}^-$ or PCK^+ /$E\text{-cadherin}^+$ cell per each field was then manually counted by two investigators blinded to the sample code, using the Image-J software (NIH, Bethesda, MD). Data were expressed as the percentage of $E\text{-cadherin}^-$ cholangiocytes over pancytokeratin $^+$ cells.

Measurement of fibronectin along cysts profile.

Upon fibronectin staining 5 random non-overlapping fields for each main liver lobe were recorded with a digital camera at 200X magnification, to a total number of 10-fields per each mouse. The total cyst profile and the fraction of

cysts profile stained by fibronectin were manually measured using Image J software (NIH, Bethesda, MD).

Isolation and characterization of murine cholangiocytes.

Murine cholangiocytes and cystic epithelial cells were isolated and cultured from WT, PkdKO and Pkhd1 mice as previously described (Spirli et al., 2005). Cholangiocytes from WT mice were isolated through micro dissection of intrahepatic bile ducts, whilst, for conditional KO mice, cells were isolated directly from micro dissected cysts (as in Fabris et al., 2006). Cells were cultured on rat tail collagen in a specific medium, NMC (Vroman & LaRusso, 1996) additioned with 10% fetal bovine serum, and eventually formed a monolayer with measurable trans-epithelial resistance. Biliary phenotype was confirmed by cytokeratin-19 staining. Cells were subcultured for up to ten passages.

Cell Motility/ Invasivity Assay

The invasivity of cholangiocytes obtained from Pkhd1 or from control mice was assessed using transwell chambers coated with a thin layer of freshly prepared rat tail collagen. Upon 24 hours starvation, primary cultures of cholangiocytes were seeded on the 8 μm pore size transwell chambers (Corning Life Sciences, Ma, USA) suspended in a receiver (7.5 X 10^4 cell/cm^2). Forty-eight hours later, cells on the upper layer were gently removed with a cotton swab whilst cells migrated through the filter to the lower side of the membrane were fixed and stained using Diff-Quick Baxter® (Baxter, Deerfield, IL, USA) and counted on a light microscope.

Cytokines array.

Cells were seeded as monolayer in a transwell chamber, over a thin layer of rat tail collagen. Upon confluence (measured by testing the transepithelial resistance) cells were starved. Medium from the upper and the lower chamber were collected 24 hours later, centrifuged to remove debris, and stored at -20 °C or analyzed for the presence of a broad spectrum of cytokines (32 different

cytokines) using a quantitative multiplex bead immunoassay: a Milliplex Map Mouse Cytokine/Chemokine panel (Millipore) coupled with the Luminex® xMAP® platform, (xMAP® system Technology by Luminex) according to the manufacturers protocol.

Briefly, Luminex® is based on the use of 5.6 μm microspheres internally colored with fluorescent dyes. The combination of two different dyes in defined concentration ratios produces 100 distinct sets of color-coded beads, each of which is coated with a specific capture antibody for a defined cytokine. Beads are first exposed to the analyte, then incubated with a biotinilated detection antibody and a Streptavidin-PE conjugated reporter molecule to complete the reaction on the surface of each microsphere. During the assay, a laser excites the internal dyes (for the specific recognition of each bead), and a second laser excites the fluorescent PE on the surface. Each sphere (specific for a defined cytokine) can thus be identified and, based on the magnitude of the fluorescent reporter signal, the relative amount of cytokine bound to the sphere is quantified. The immunoassay includes a calibration set for each cytokine in order to associate the measured fluorescence with the cytokine concentration. Data were normalized to the proteins obtained from the cell lysis of each sample in the wells. Total cell proteins were prepared using a lysate buffer (50 mM Tris-HCl, 1% NP40, 0.1% SDS, 0.1% Deoxycholic acid, 0.1 mM EDTA, 0.1 mM EGTA) containing fresh protease and phosphatase inhibitor cocktails (Sigma, St Louis, CA). Protein concentration was measured using the Comassie protein assay reagent (Pierce, Rockford, IL).

RESULTS

We aimed at defining if fibrocystin deficiency promotes a process of epitelial to mesenchymal transition in the cystic epithelium in the liver and we investigated how this phenotypic change affects cholangiocytes interaction with the surrounding cells.

To this end we analyzed liver samples from biopsies of diseased or healthy individuals and also a mouse model of ARPKD: Pkhd1 (Gallagher et al., 2008). WT mice and PkdKO mice from the same background were used as control. We pursued our goal by

-exploring phenotypic evidence of EMT in patients with CHF/CD and in Pkhd1 mice, through immunohistochemistry

-testing in vitro the cell motility of cholangiocytes obtained from Pkhd1 mice: increased cell motility is a functional sign of engagement in EMT

-investigating the altered cross talk between epithelial and mesenchymal cells in ARPKD

1. Pkhd1 mouse displays biliary dysgenesis, liver fibrosis and portal hypertension, characteristics of ARPKD

For this study we bred a mouse model for ARPKD recently generated by inactivation of the Pkdh1 gene (murine hortologue of the human PKHD1) through a deletion of exon 4 (Gallagher et al., 2008). Mice were born at a close to normal Mendellian ratio (21:60:21, WT, eterozygous, null, where null were 51/240), grew and lived normally for more than 12 months. They developed cysts throughout life, displayed liver enlargement (Fig. 4a) with progressive cysts formation (Fig. 4b) and fibrosis deposition (Fig. 4c) mimicking the ARPKD phenotype. Intrahepatic bile duct lesions were readily observable already at 2 weeks post birth and extrahepatic bile duct dilatation was also common in mice 9 month old or older. More than 50% of the mice displayed splenomegaly, a sign of clinically relevant portal hypertension (Fig. 4a).

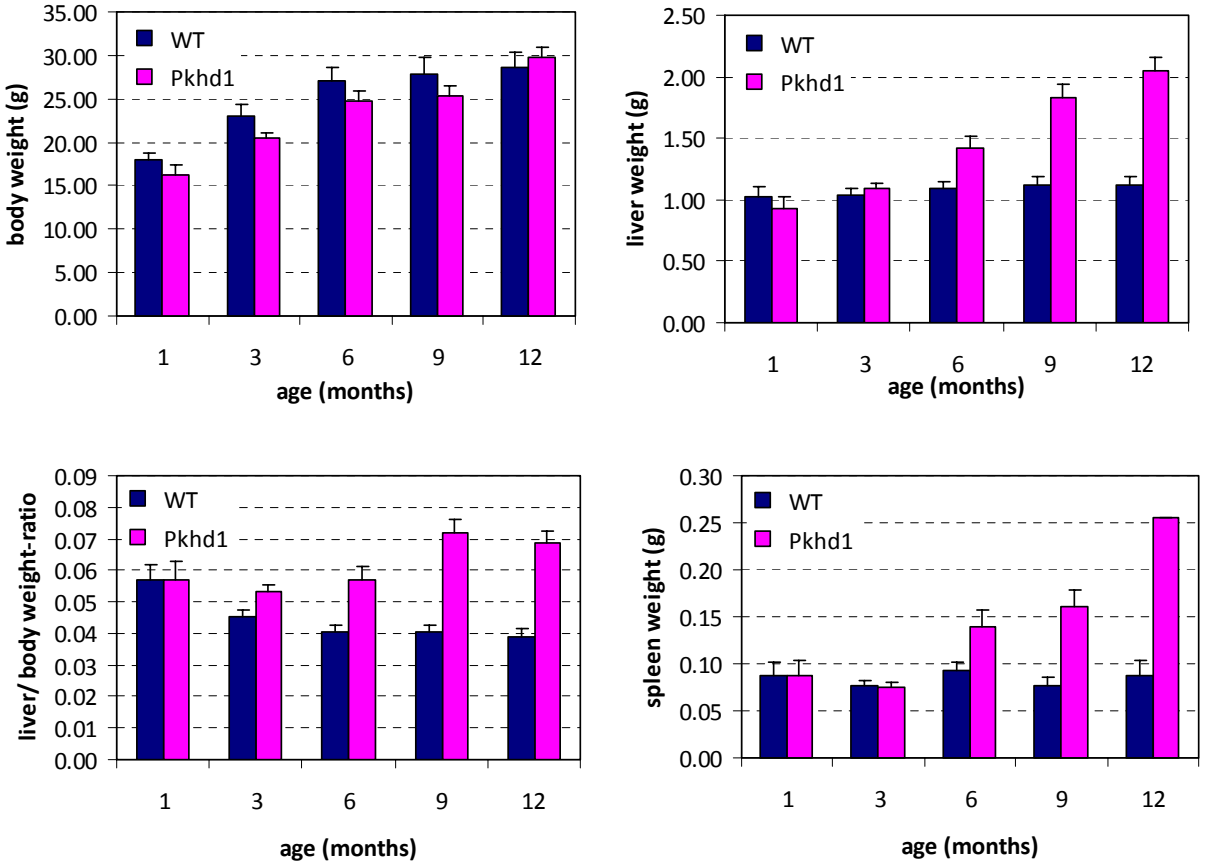

Figure 4a. Body, liver and spleen weight comparison between WT and Pkhd1 mice. Cohorts of WT and Pkhd1 mice measured at progressive age had a comparable body weight (upper panel, left). Conversely, the liver weight (upper panel right) and consequently the liver/body-weight ratio (lower panel left) were significantly bigger in Pkhd1 mice at 6 months and progressively increased with age. Pkhd1 mice displayed splenomegaly, and spleen weight was noticeably higher compared to WT starting at 6 months age (lower panel right), a sign of portal hypertension.

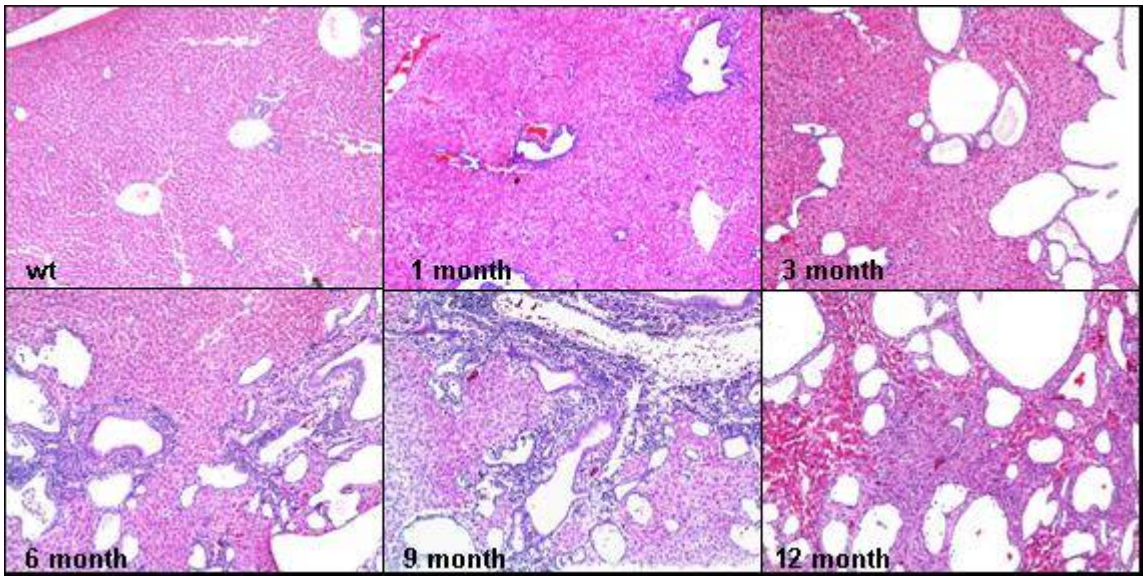

Figure 4b. Liver histology: the comparison of hematossilin-eosin staining of liver sections from WT mice (wt) and from Pkhd1 mice at 1, 3, 6, 9, 12 month of age shows the progressive bile ducts dilatation and the deposition of fibrotic tissue in portal spaces in Pkhd1 mice.

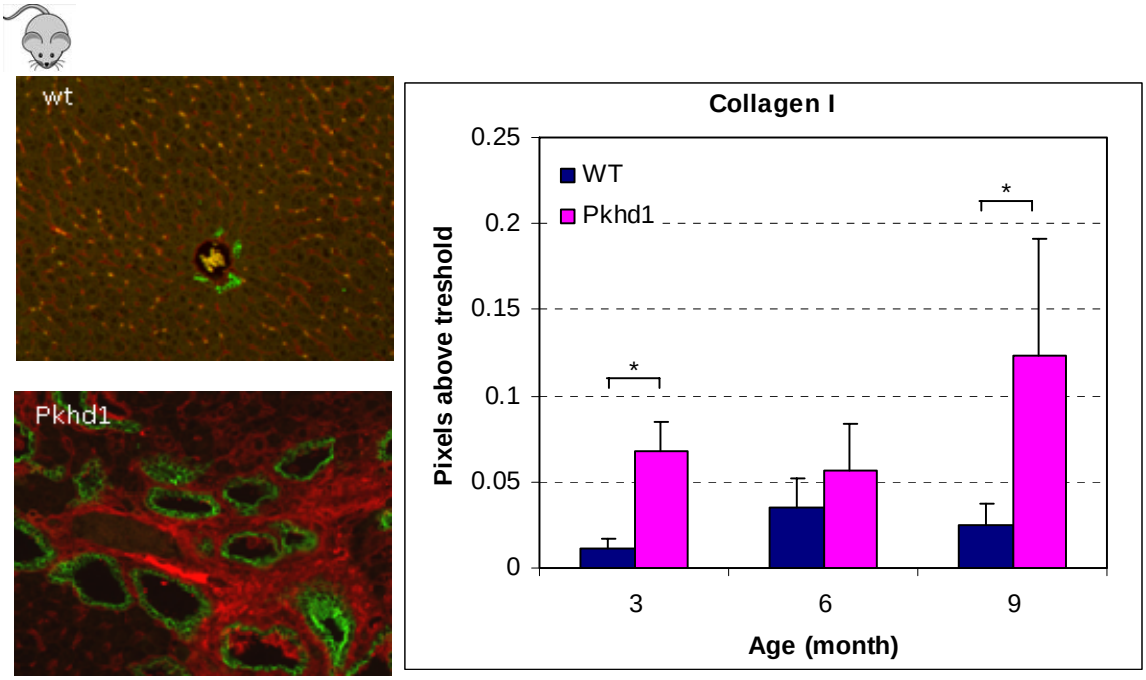

Figure 4c. Pkhd1 mice develop progressive portal fibrosis. Collagen deposition was measured in liver sections from cohorts of WT and Pkhd1 mice at age 3, 6, 9 month (2 mice for each group). Slides were stained in immunofluorescence (left panels) with anti CK19 antibody (in green) to identify bile ducts, and anti collagen-I antibody (in red) (Magnification 200X). The percentage of collagen-I positive area (in the graph) was measured as the percentage of pixels above threshold in the red channel in digital images taken from 5 random non overlapping fields at 200X magnification. (*= P<0.01)

2. Immunohistochemical evidences of EMT in human CHF/CD and in a mouse model of ARPKD:

2.1 Decreased expression of epithelial markers in ARPKD, a sign of ongoing EMT.

E-cadherin is down regulated in liver samples of ARPKD. In this study, immunohistochemical analysis showed that while E-cadherin clearly defined tight junctions in normal liver tissues, it was downregulated in liver samples from CD patients (Fig. 5a) as well as in Pkhd1 mice (Fig 5b). E-cadherin is a major component of the adherens junction complex in epithelial tissue where it determines the strength of intercellular adhesion. The reduction in the adhesion properties of the epithelial cells, and the acquisition of migratory properties is a hallmark of EMT. A reduction in E-cadherin levels is therefore an important indicator of ongoing EMT.

The quantitation of E-cadherin negative cells in Pkhd1 mice showed a significant loss of E-cadherin already at 1 and 3 months compared to WT mice of the same age (Fig. 5c).

Staining for the transcription factor **snail** was negative on normal human Fig. 6A) and murine cholangiocytes (Fig. 6 C, E) whilst it was found to be strongly up-regulated in nuclei of biliary epithelial cells in human CHF (Fig. 6B) and in Pkhd1mice (Fig. 6D, F).

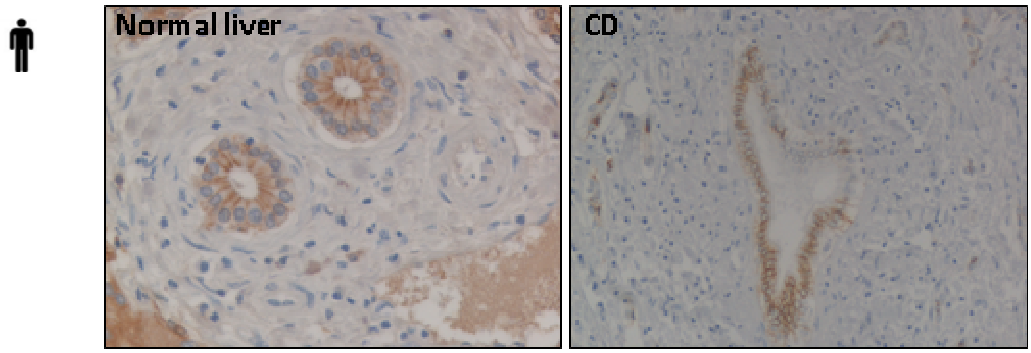

Figure 5a. E-cadherin is focally downregulated in human CD. Immunostaining revealed that E-Cadherin is downregulated and uneven in human liver samples from CD (right panel, 400X) compared to normal liver (left panel, magnification 200X).

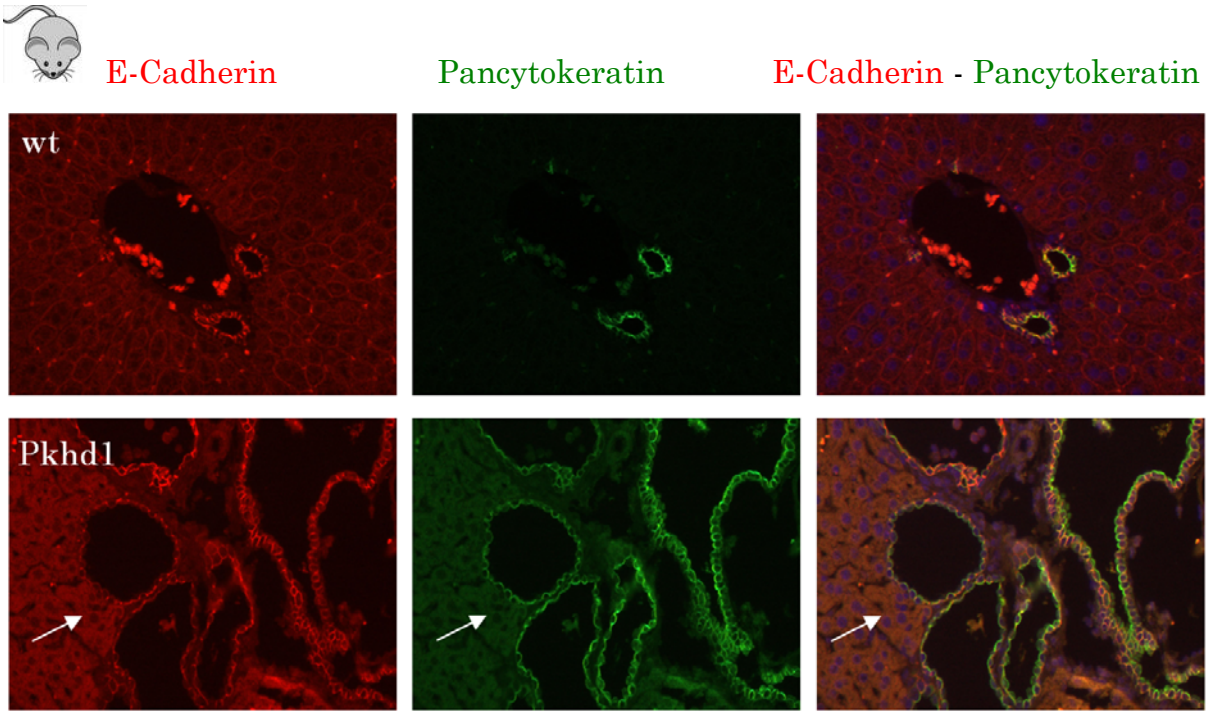

Figure 5b. E-cadherin is focally downregulated in Pkhd1 mice. Mice liver sections were immunostained with anti-pancytokeratin antibody (which in the liver reacts with cholangiocytes), for an unambiguous identification of bile ducts (in green), and with anti E-Cadherin antibody (in red). Double immunostaining revealed that while E-Cadherin clearly stains each tight junction between cholangiocytes in wt mice (upper panels), it is downregulated and patchy in liver samples from Pkhd1 mice (lower panels, arrow) (Magnification 200X).

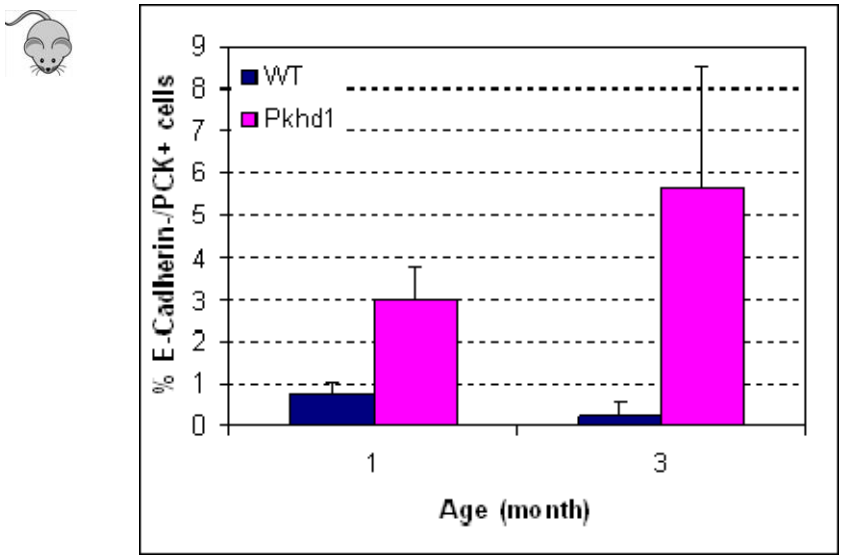

Figure 5c. Quantitation of E-cadherine negative cells showed a progressive loss of E-cadherin in Pkhd1 mice. Liver sections from both liver lobes of WT or Pkhd1 mice were immunostained for PKC and E-Cadherin. The percentage of E-cadherin negative cells among PCK positive biliary cells, calcultated over 5 random non-overlapping fields, was significantly higher in Pkhd1 mice versus WT already at 1 month and at 3 month. (n=3 for each sample)

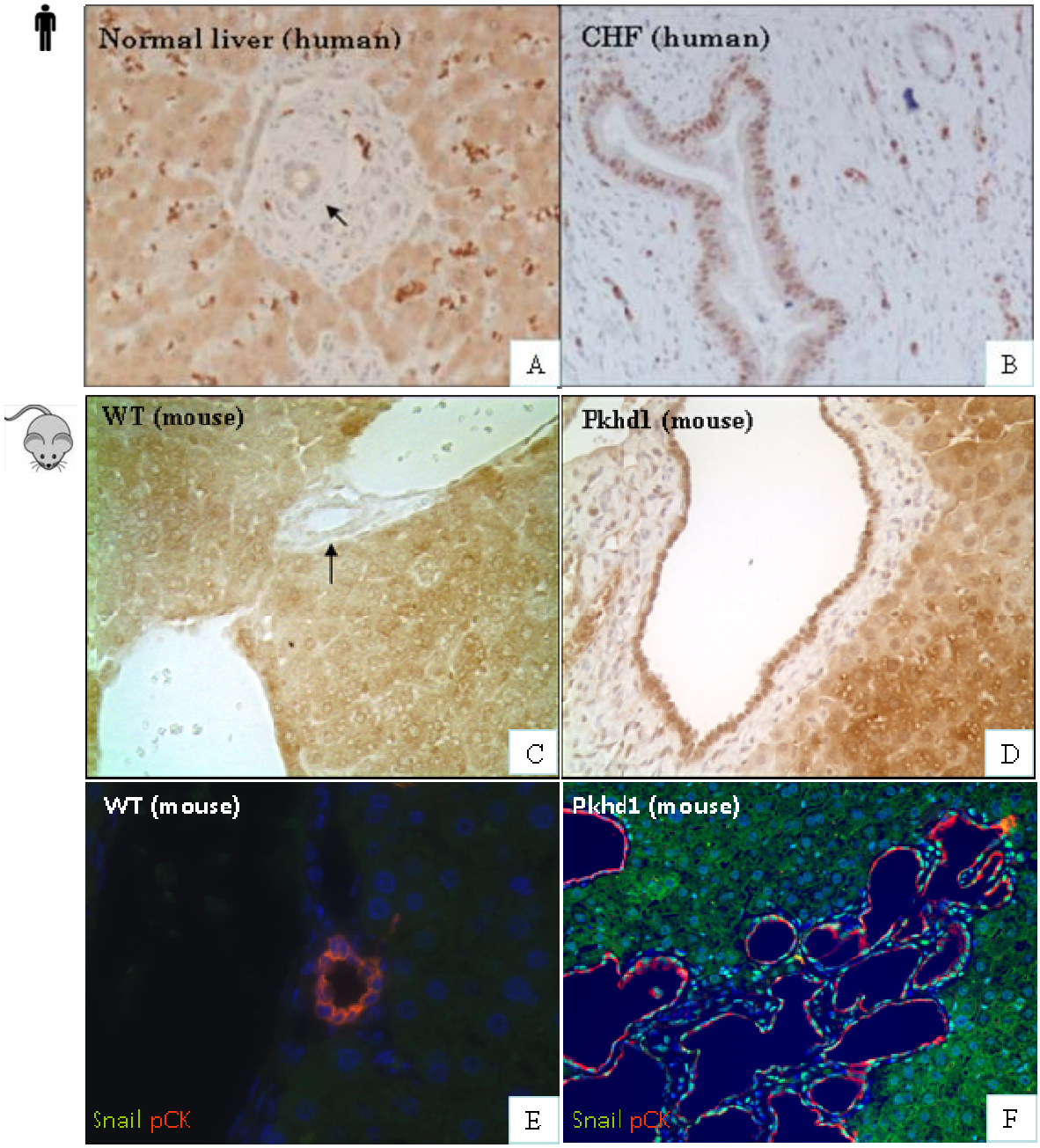

Figure 6 Nuclear staining for the transcription factor snail was negative in normal human liver (A) whilst it was clearly detectable in cholangiocytes in human CHF (B). Snail immunodetection on liver sections from WT or Pkhd1 mice was performed using HRP (C, D respectively) or immunofluorescence tecniques (E, F, the signal for pancytokeratin is in red and snail in green). Cholangiocytes in Pkhd1 mice displayed nuclear staining for snail whilst cholangiocytes in WT did not (A-E: magnification 200X, F: magnification 100X).

Snail is a transcription factor strongly implicated in EMT: it inhibits the expression of genes with conserved E-boxes in their promoter regions, including E-cadherin. Snail may directly inhibit the transcription of other epithelial markers, among which cytokeratin-19, a biliary-specific marker.

β-Catenin is mislocalized in CD and in Pkhd1 mice. Staining for β-catenin revealed a clear downregulation and mislocalization of this protein to the perinuclear region of biliary epithelial cells both in human ARPKD versus normal liver (Fig. 7A, B), and in Pkhd1 mice compared to WT and Pkd2KO mice (Fig. 7C-F).

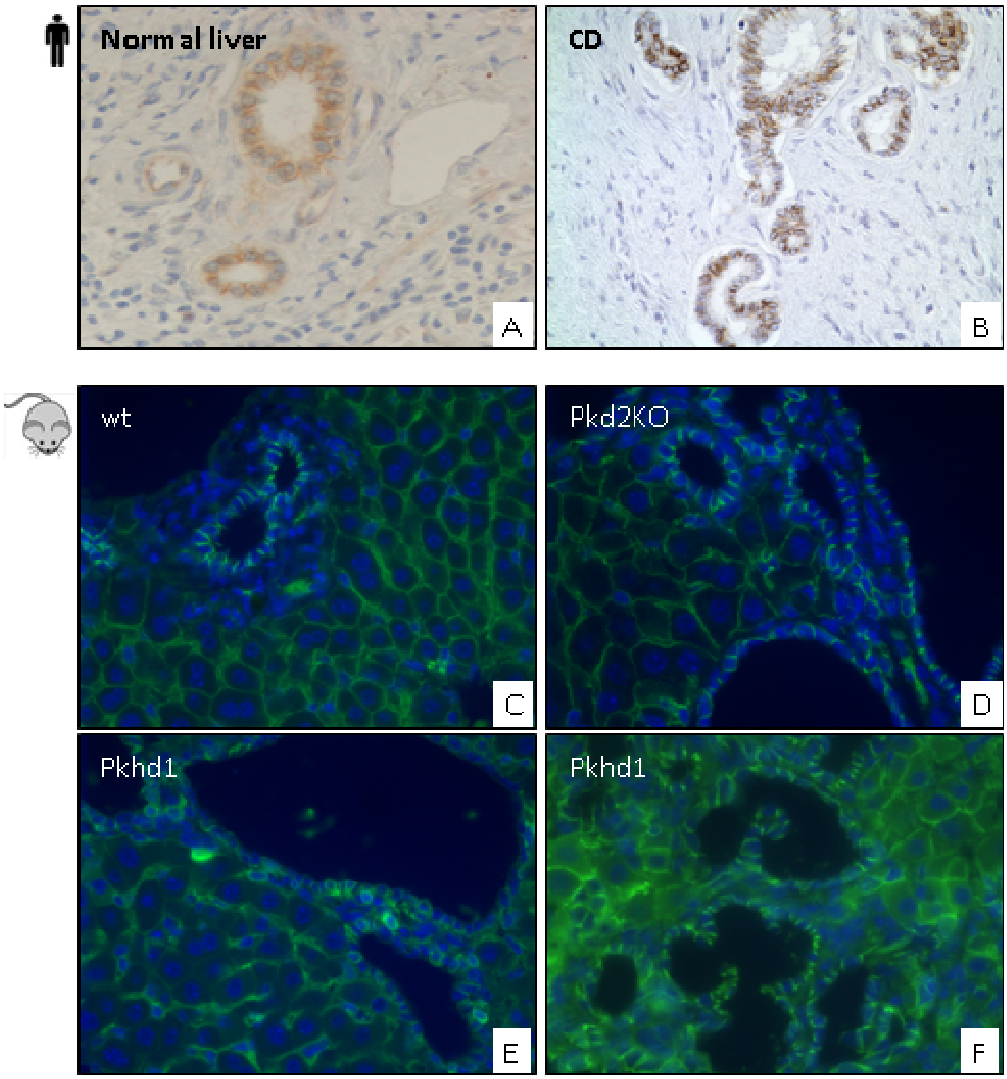

Figure 7. **β-catenin staining.** In immunohistochemistry β-catenin staining clearly defined adherens junctions in normal human liver (A), whilst it had a more diffuse pattern in Caroli disease (B). On mice liver, **β-catenin** antibody stained junctions in WT and in Pkd2KO mice (C, D), while it gave a more diffused signal in Pkhd1 mice (E, F). (A, C-F: magnification 200X, B: magnification 100X)

β-catenin is an important component of the adhesion complex formed by E-Cadherin in the normal epithelial sheet. Nevertheless it can be involved in the transcriptional regulation downstream of the Wnt signalling pathway: when detached from the adherens junctions, β cateins turnover is normally regulated by posphorylation and rapid degradation in proteasomes. If not degraded, β-catenin may translocate into the nucleus where, through the activation of the TCF/LEF transcription factors, it modulates a series of transitional events. Nuclear import of ß-catenin upon activation of the Wnt pathway is considered a key molecular step in EMT and is strictly related to the reduction of E-cadherin levels.

2.2 Epithelial basement membrane is focally dismanteled in ARPKD.

Laminin: as showed in Fig. 8 progressive laminin discontinuity was observed along bile ducts profile in Pkhd1 mice. The epithelial layer normally lies on the basal membrane, a thin sheet of fibers that act as a mechanical barrier and anchor the epithelium to the connective tissue that lies underneath. Laminin is the main component of the basement membrane and focal destruction of laminin is a pre-requisite for the migration of epithelial cells.

2.3 ECM deposition in ARPKD

Fibronectin. Immunohistochemical staining for fibronectin showed that this protein is up-regulated in Pkhd1 mice, and it is clearly visible on the basal side of cholangiocytes along the cysts perimeter. Fibronectin is an extracellular matrix glycoprotein which binds to integrins (transmembrane receptor proteins), and to extracellular matrix components such as collagen and fibrin causing a reorganization of the cell's cytoskeleton and facilitating cell movement. It is involved in embryogenesis and cell migration/adhesion allowing cells to move through the ECM. It is upregulated in EMT.

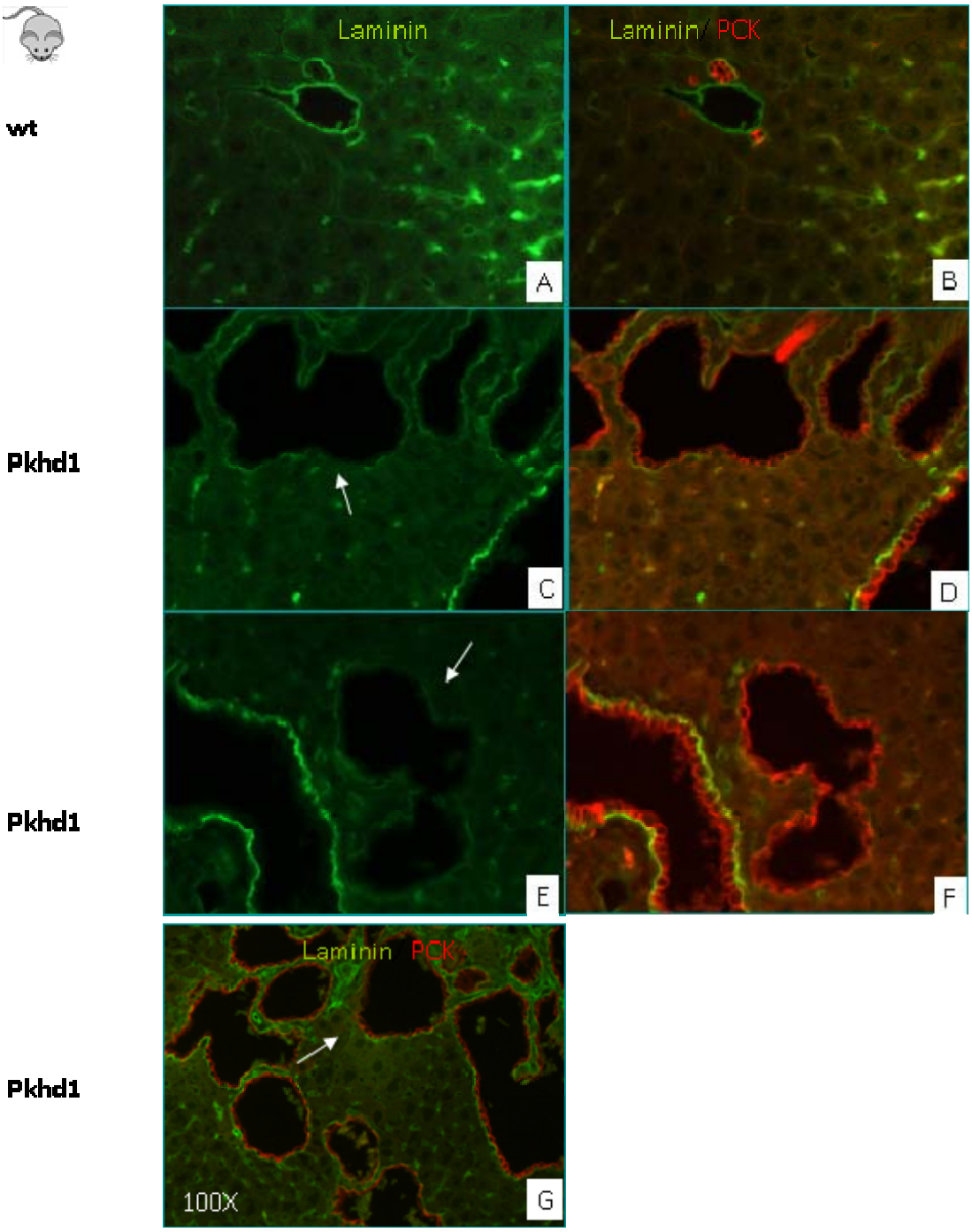

Figure 8. Discontinuity in the bile ducts laminin rim in Pkhd1 mice: wt (A, B) and Pkhd1 (C-G) liver slides were stained with anti PCK antibody to correctly identify cholangiocytes (in red), and anti laminin, a basement membrane marker (in green). Micrographs A, C and E display only the green channel, corresponding to the laminin antibody, in all other pictures red and green channels are merged. The staining reveals the dismantlement of the basal membrane around cysts in the ADPKD mouse model (arrows). Pictures were taken at 200X magnification, for a broader view picture G was taken at 100X magnification.

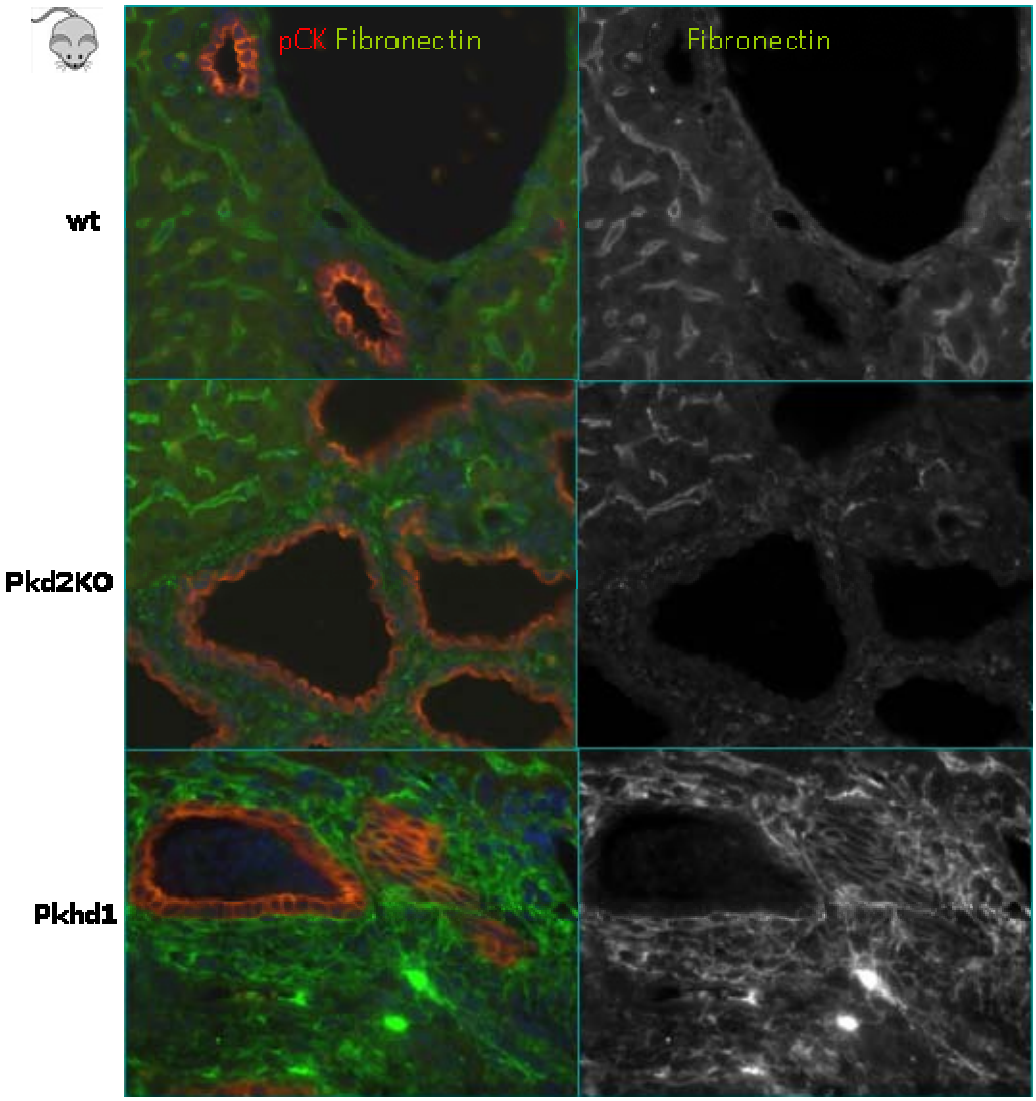

Figure 9a. Fibronectin deposition along the cysts profile in Pkhd1 mice. Double immunostain for PCK (red) and Fibronectin (green) on mice liver slides from wt (top) Pkd2KO (middle), Pkhd1 (lower panels) mice. The panels to the right show the green channel only of the picture, corresponding to the fibronectin signal. Fibronectin staining was negative around cysts in wt and Pkd2KO mice, while a strong Fibronectin positive rim was observed around the basal side of cholangiocytes in Pkhd1 mice. (Magnification 200x)

The chart (Fig. 9b) describe the progressive deposition of fibronectin around cysts profile. Liver slides from Pkhd1 mice at progressive age (3, 6, 9 month) were double stained in immunofluorescence using anti pancytokeratin antibody (in order to detect cholangiocytes) and anti-fibronectin antibody and five random pictures were taken (200X) for each sample The full length of the cysts profile (as evidenced by pCK staining) and the cholangiocytes basal profile stained by anti-fibronectin antibody were measured. Fibronectin deposition was plotted as the ratio between the length of cysts profile staining with anti-fibronectin antibody and the full profile length for pCK, determining the percentage of fibronectin-positive cysts profile. Fibronectin positive profile in PkdKO mice was never above 10 %.

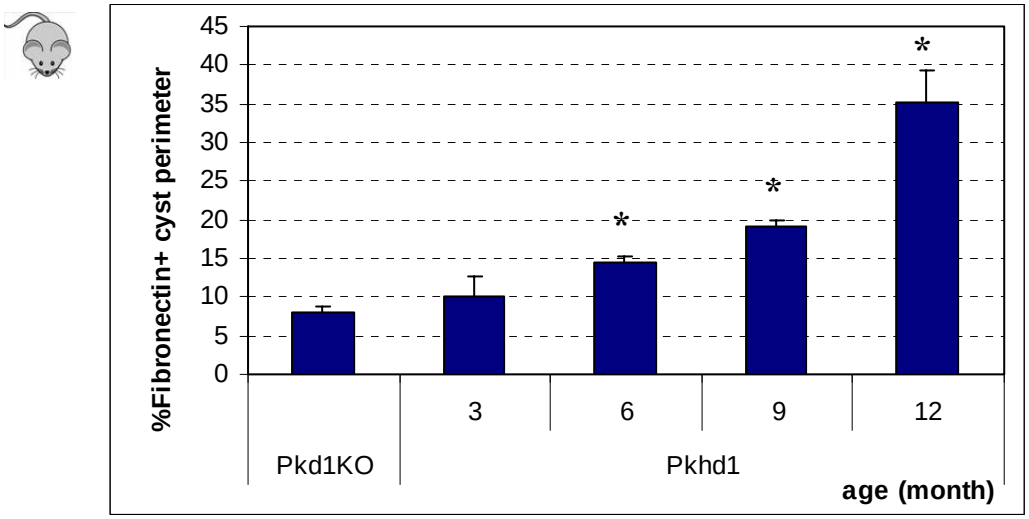

Figure 9b Measurement of fibronectin deposition along cholangiocytes basal side in Pkh1 mice compared to Pkd1KO mice (n=2 for each group). Fibronectin deposition at 6 month and beyond was significantly higher in Pkhd1 mice compared to Pkhd1KO (* P<=0.05)

2.4 Biliary epithelial cells in ARPKD do not express the mesenkymal markers S100A4 and α-SMA.

S100A4 is a cytoskeleton-associated, calcium binding protein, normally expressed by fibroblasts and not expressed by epithelial cells. By interacting with the cytoskeletal protein Myosin-IIa, S100A4 induces the cell reshaping that enables

motility. It is considered a marker of EMT, suggesting the engagement in a molecular program primed to the mesenchymal phenotype. In our study S100A4 was not readily detectable in biliary cells in either ARPKD or normal liver samples. Liver cholangiocytes were also negative for aSMA (not shown)

The results of the immunohistological analyses are summarized in Table 2.

Table 2

	EMT Markers	**Pkhd1**
Loss of cell-cell contact	E-cadherin	Focally down-regulated
	Snail	Diffusely up-regulated
	β-catenin	Focally mislocalised and down-regulated
Disruption of basement membrane	Laminin	Progressive loss along bile ducts profile as maturation proceeds
ECM	Fibronectin	Focal basal expression along cysts profile
	Collagen-I	Strong stain into the portal space along cysts
Mesenchymal markers	S100A4	Negative
	αSMA	Negative

In summary, this study generated morphological evidence that cholangiocytes in CHF/CD patients and Pkhd1 mice display mesenchymal characteristics, compatible with their engagement in biliary EMT.

3. Murine cholangiocytes from Pkhd1 mice constitutionally show aberrant migratory and invasive capabilities.

Cholangiocytes undergoing EMT would also acquire motility and the capability to migrate more typical of mesenchymal cells. The migration of epithelial cells toward a new location implies the local rupture of the basement membrane on which the epithelium rests and the degradation of the extracellullar matrix (ECM).

We intended to test the invasive potential of WT and mutated cholangiocytes. To this end, primary cultures were established, over a layer of rat tail collagen, from cholangiocytes freshly isolated from WT, Pkd2KO and Pkhd1 mice. According to our experience, cholangiocytes in culture maintain their epithelial characteristics: they grow in monolayer and develop tight junctions creating a measurable transepithelial resistance. At the ultrastructural level cells appear to be polarized: they display apical cilia and present extensive basolateral digitations (Fiorotto et al., 2007). We tested the invasive potential of Pkhd1 cholangiocytes and controls using a transwell system: a monolayer of cells was plated on a permeable support, a polycarbonate 10 μm membrane with 8 μm pores suspended in a receiver. The support was first coated with collagen-I (prepared form rat tail) mimicking the fibrillary extracellular matrix, which is produced especially during disease. Primary cultures of cholangiocytes isolated from Pkd2KO or Pkhd1 mice were starved for 24 hours, then harvested and seeded on the transwell chamber and incubated in regular NMC medium with 10% FBS. Forty-eight (48) hours later, cells on the upper layer were gently removed and cells migrated to the lower layer were fixed, stained and counted on a light microscope. Pkhd1 cells displayed enhanced migratory properties compared to WT and Pkd2KO cells, feature consistent with a process of EMT. The number of cells that migrated through the collagen barrier was 3 times higher for cholangiocytes obtained from Pkhd1 mice than in Pkd2KO (Fig.10). The capability of migrating through the collagen coating also implies the ability to erode the local extracellular matrix and basal membrane components, permitting cell migration.

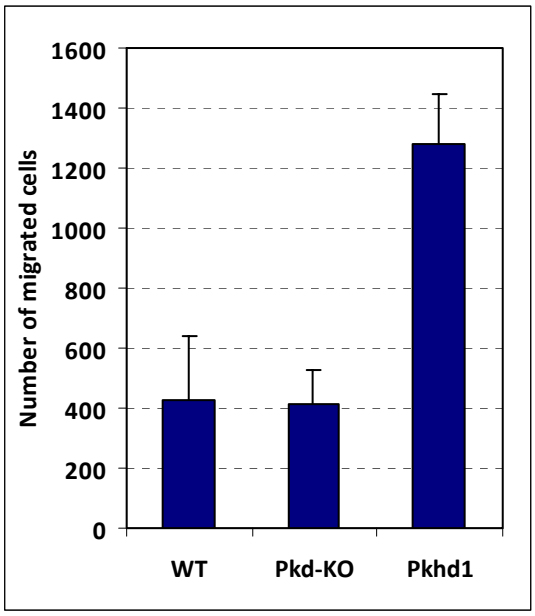

Figure 10. Cholangiocytes isolated from Pkhd1 mice display increased migratory properties compared to cholangiocytes from wt and Pkd-KO mice. Cells ($7.5x10^4$) were seeded on a porous support coated with a thin layer of collagen. After 48 hours the number of cells migrated through the collagen barrier was significantly higher for cholangiocytes isolated from Pkhd1 mice than for cholangiocytes from wt or Pkd-KO (* $P<0.01$). Mice (n=4).

4. Fibrocytes and hepatic stellate cells are recruited in proximity to cysts in ARPKD

In an extensive immunophenotypic analysis of ARPKD cholangiocytes we found proofs of a cholangiocytes engagement in a EMT process, however we did not observe a complete trans-differentiation of the epithelial cells, we never observed S100A4 nor αSMA expression on cholangiocytes, characteristic of mesenchymal cells. In order to better understand the mechanisms leading to fibrosis in ARPKD we also run an immunophenotypic characterization of mesenchymal cells that are found in the fibrotic areas, in strict proximity to the bile duct epithelium. Fig 11 shows the main findings of our analysis, outlined at the following points.

Activated stellate cells populate the fibrotic portal area in Pkhd1 mice. The resident fibroblasts and hepatic stellate (HSC) cells have been traditionally viewed as the primary cells involved in promoting liver fibrosis. In response to liver injury HSC undergo morphological and functional changes and become activates assuming a myofibroblastic phenotype and start to express alpha

smooth muscle actin (αSMA) and collagen type I. α-SMA and vimentin staining detects activated stellate cells (Fig. 11 A, B) in Pkhd1 mice, noticeably they are not strictly adjacent to the cysts and are detectable mostly in mice older than 9 month.

Peribiliary recruitment of PDGFRβ positive cells. Interestingly we observed a progressive enrichment of PDGFRβ positive cells in strict proximity to biliary microhamartomas (Fig.11C), correlated with the progressive increase of portal fibrosis through different maturation ages in Pkhd1 mutated mice.

Immunophenotypic characterization of PDGF-Rβ-positive cells. Cells expressing PDGF-Rβ were localized in close proximity to the epithelial layer lining biliary microhamartomas in Pkhd1, whilst they were absent in normal portal space from WT mice. These cells have spindle shape morphology, but are negative for α-SMA. Interestingly, they are variably decorated by the CD45 and CD34 (Fig.11D, F) antibodies, a phenotype consistent with a hematopoietic origin. They strongly co-express collagen type I (Fig. 11E), as also shown by dual immunolabeling with CD45 (Fig. 11F), thereby indicating their relevant role in ECM deposition. This immunophenotype corresponds to bone-marrow-derived circulating fibrocytes, cells that may generate fibrosis. Fibrocytes were first identified in 1994 as circulating cells that, extravasating into wounds, contributed to wound repair (Quan Bucala JBC 2004) and are characterized by their unique surface phenotype. These peripheral blood fibrocytes express the CD34 and CD45 cell surface antigens indicative of their hematopoietic origin and produce collagen and other matrix proteins.

PDGFR-β expression in human Caroli Disease. Extensive accumulation of α-SMA negative / PDGFRβ positive cells was also observed in human CD (Fig. 11 G).

PDGF-D is up-regulated in biliary cysts of CD/CHF. In human liver, normal bile ducts were consistently negative for both PDGF-B and PDGF-D isoforms (not shown). In liver from CD patients (n=2), cyst cholangiocytes were negative for PDGF-B but were strongly positive for PDGF-D, showing a cytoplasmic granular pattern compatible with a secretory behavior (Fig. 11H) suggesting that PDGF-D produced by biliary epithelium may actually exert a paracrine effect regulating α-SMA negative mesenchymal cells recruitment.

Figure 11

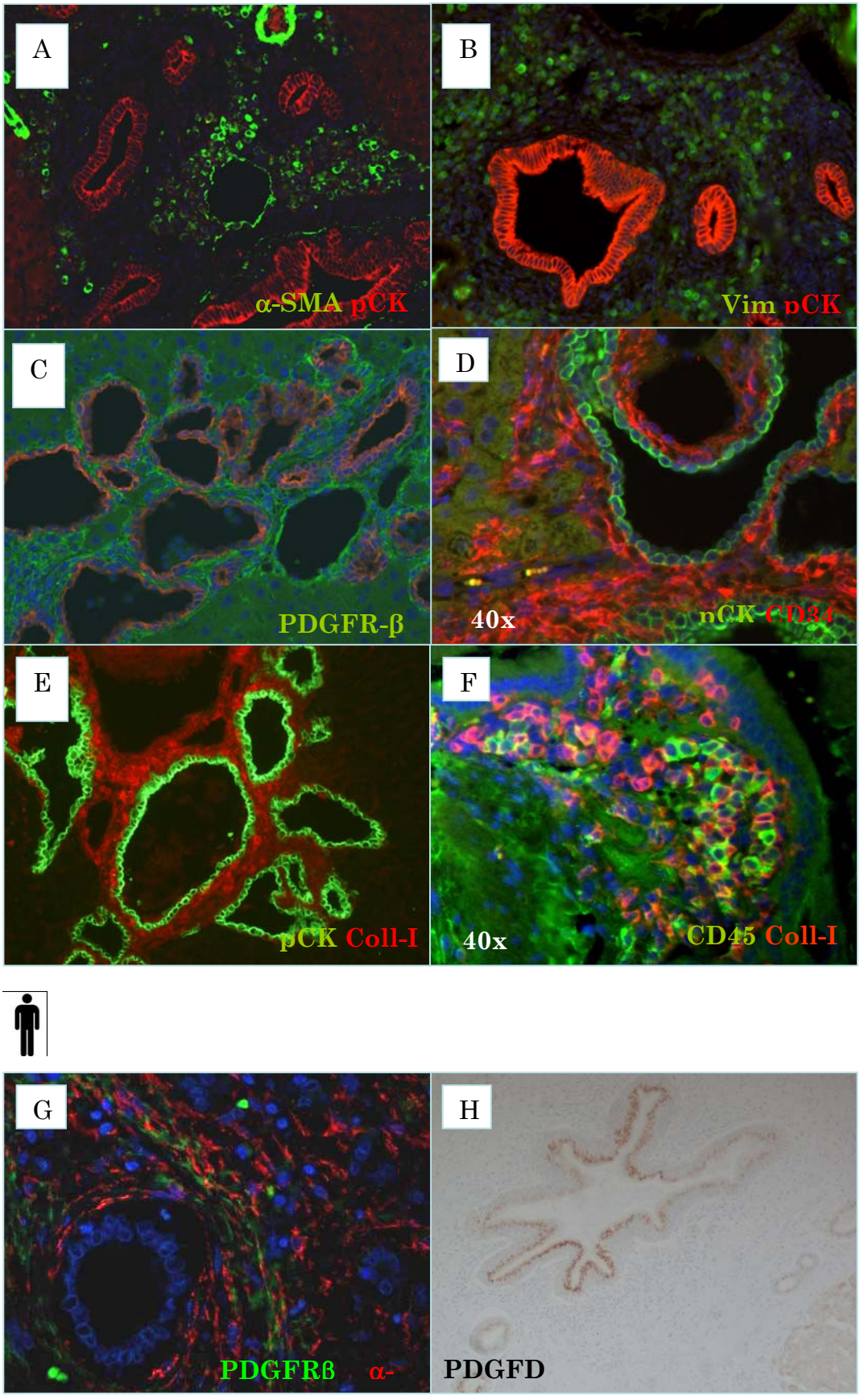
A
α-SMA pCK
B
Vim pCK
C
PDGFR-β
D
40x
pCK CD34
E
pCK Coll-I
F
40x
CD45 Coll-I
G
PDGFRß α-
H
PDGFD

Figure 11. A-F) Immunophenoptypic characterization of portal infiltrate in Pkhd1 mice. Liver slides were immuno-stained with **A)** anti αSMA (green) and anti pCK (red) antibody; **B)** Fibronectin (green) and pCK (red); **C)** PDGFRβ (green), pCK (red); **D)** pCK (green), CD34 (red), **E)** pCK (green), Collagen-I (red); **F)** CD45 (green), Collagen-I (red). **G-H) Immunostaining of human liver from CD patients**. **G)** Double immunostain for PDGFRβ (green) and αSMA (red); H) immunostain with anti PDGF-D antinody. (A, B, C, E, G, H: magnification 200x; D, F: magnification 400x).

5. Multiplex cytokines assay reveals a hyper secretion of cytokines by Pkhd1 cholangiocytes, especially on the cells basal side, compatible with an active recruitment of mesenchymal cells.

Cholangiocytes isolated from Pkhd1, Pkd1KO, or WT mice were seeded as monolayer in a transwell chamber, over a thin layer of collagen. Upon confluence (measured by testing the transepithelial resistance), cells were starved. Medium from the upper and the lower chamber were collected 24 hours later and analyzed for the presence of a broad spectrum of cytokines (32 different cytokines, listed in Table 3) using a quantitative multiplex bead immunoassay.

Table 3

Milliplex ID	Gene ID	Description
Eotaxin	CCL11	chemokine (C-C motif) ligand 11
G-CSF	CSF3	colony stimulating factor 3 (granulocyte)
GM-CSF	CSF2	colony stimulating factor 2 (granulocyte-macrophage)
IFN-g	IFNG	interferon, gamma
IL-1a	IL1A	interleukin 1, alpha
IL-1b	IL1B	interleukin 1, beta
IL-2	IL2	interleukin 2
IL-3	IL3	interleukin 3 (colony-stimulating factor, multiple)
IL-4	IL4	interleukin 4
IL-5	IL5	interleukin 5 (colony-stimulating factor, eosinophil)
IL-6	IL6	interleukin 6 (interferon, beta 2)
IL-7	IL7	interleukin 7
IL-9	IL9	interleukin 9
IL-10	IL10	interleukin 10
IL-12(p40) (29)	IL12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
IL-12(p70) (62)	IL12 (complex)	complex of interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35) and interleukin 12B

IL-13	IL13	interleukin 13
IL-15	IL15	interleukin 15
IL-17	IL17	interleukin 17°
IP-10	CXCL10	chemokine (C-X-C motif) ligand 10
KC	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
LIF	LIF	leukemia inhibitory factor (cholinergic differentiation factor)
LIX	CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
MCP-1	CCL2	chemokine (C-C motif) ligand 2
M-CSF	CSF1	colony stimulating factor 1 (macrophage)
MIG	CXCL9	chemokine (C-X-C motif) ligand 9
MIP-1a	CCL3	chemokine (C-C motif) ligand 3
MIP-1b	CCL4	chemokine (C-C motif) ligand 4
MIP-2	CXCL3	chemokine (C-X-C motif) ligand 3
RANTES	CCL5	chemokine (C-C motif) ligand 5
TNF-a	TNF	tumor necrosis factor
VEGF	VEGFA	vascular endothelial growth factor A

Figure 12 shows the average results of 3 experiments run on medium collected from the apical or basal side of WT, Pkd1KO, or Pkhd1 cells in culture. For an easier visualization, concentrations were transformed to log10 scale in order to accommodate the wide range of the concentration values. Some of the analytes measured were not detectable. Comparing Pkhd1 cholangiocytes and WT, we observed a general cytokine over-expression, more pronounced on the basal side (Fig. 12). The same analysis was run comparing Pkd1KO cells and WT (Fig. 13).

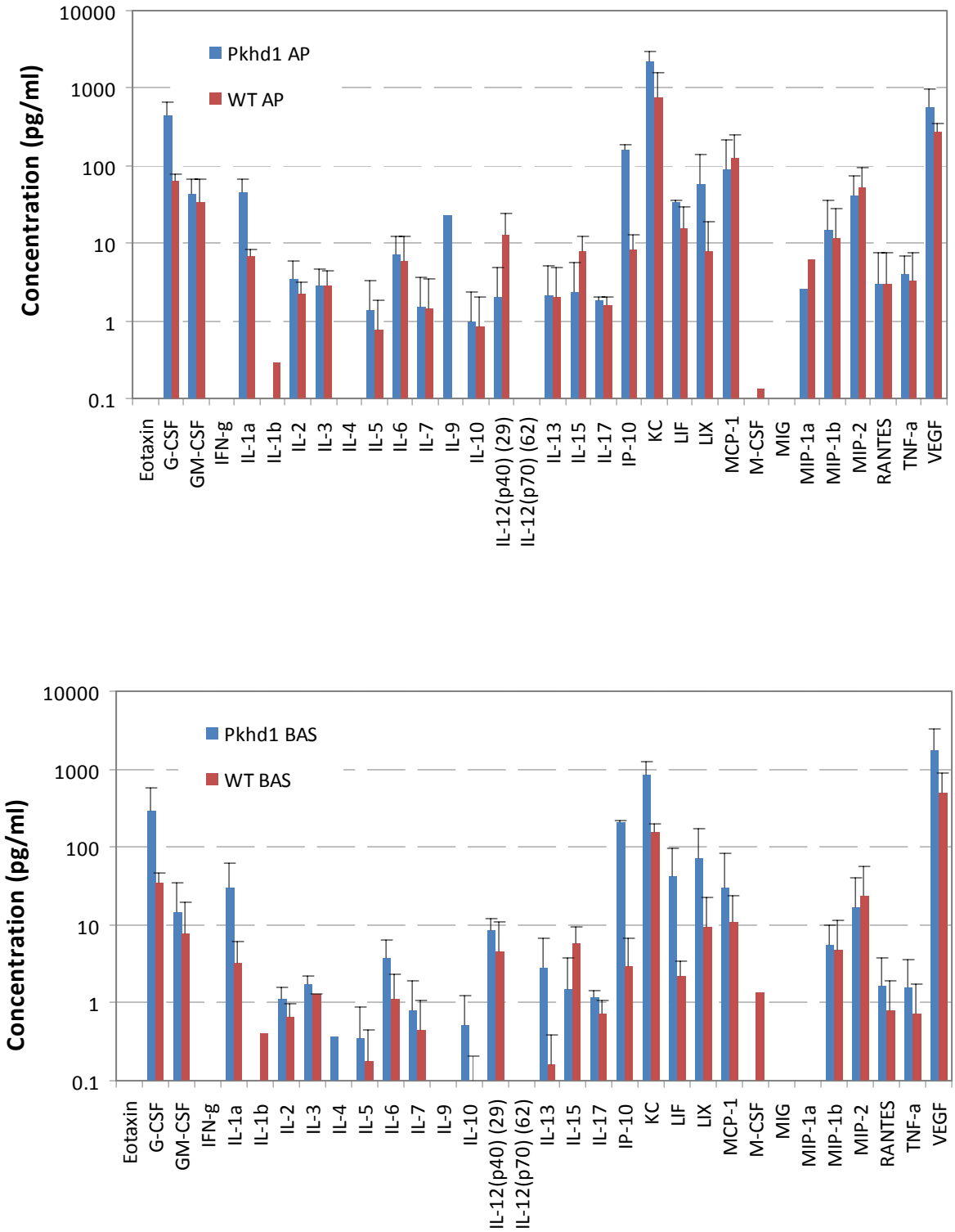

Figure 12. Cytokines concentrations measured in medium collected from the apical (upper panel) or basal (lower panel) side of cultured Pkhd1 (blue) or Wild Type (red) cholangiocytes. Concentrations are plotted on a log scale to accommodate the wide range of values, (n=3). Standard deviation is given where computable.

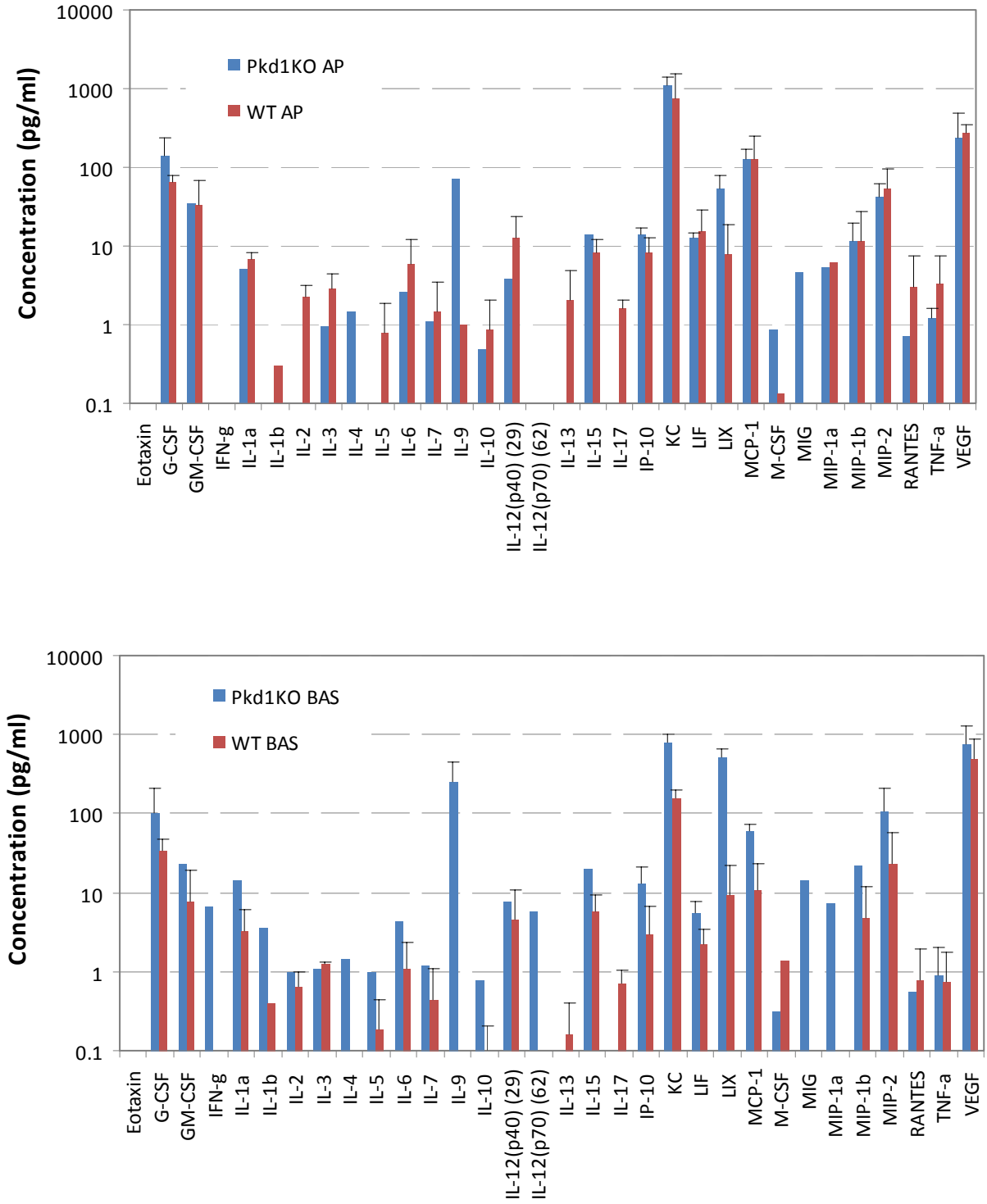

Figure 13. Cytokines concentrations measured in medium collected from the apical (upper panel) or basal (lower panel) side of cultured Pkd-KO (blue) or Wild Type (red) cholangiocytes. Concentrations are plotted on a log scale to accommodate the wide range of values, (n=3). Standard deviation is given where computable.

For further analysis, the fold variation of all cytokines comparing Pkhd1 and wild type (from either basal or apical side) were calculated. Fold-change values between 0 and 1 were expressed with the negative inverse (-1/x) (e.g. 0.5 is converted to -2). Only cytokines with at least a 2 fold variation (absolute value) were further considered (Fig. 14). All cytokines in this latter group were upregulated on the basal side of Pkhd1 cholangiocytes (with the exception if IL-15), revealing a general hyper-secretive phenotype, while the picture was less straightforward on the apical side where, for example, IL-12 was 6.4 times less secreted in Pkhd1 versus WT.

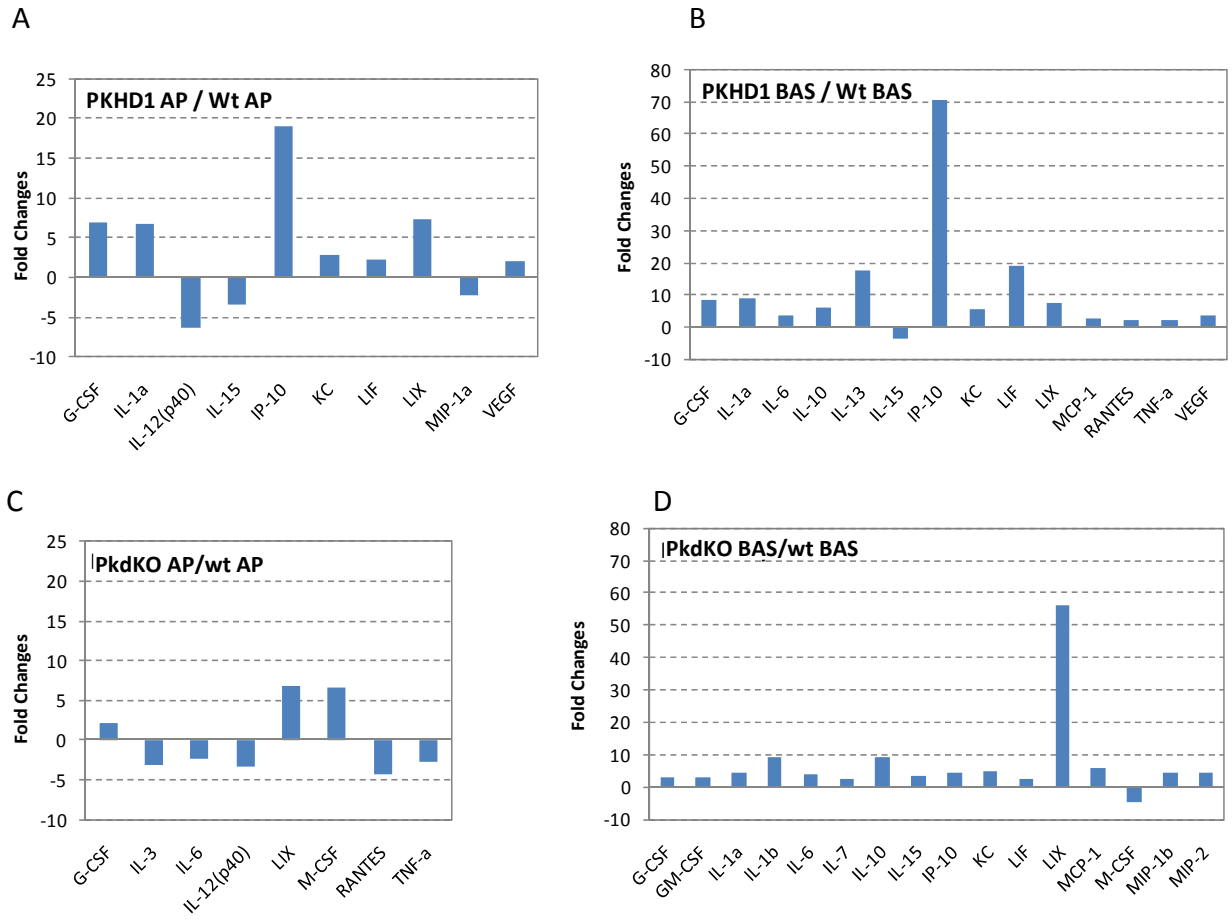

Fig. 14. The following four comparison pairs were considered: A) Pkhd1 versus WT for APICAL secretion, B) Pkhd1 versus WT for BASAL secretion, C) PkdKO versus WT for APICAL secretion, D) PkdKO versus WT for BASAL secretion.
For each one of the above pairs the fold variation of all cytokines was determined and a 2-fold variation (either positive or negative) was set as cutoff. Only cytokines with a detectable variation in concentration above or below the given cutoffs were plotted.

A Venn diagram gives a better visualization of the differences in cytokines production of Pkhd1 versus WT and Pkd1KO versus WT (Fig. 15a and 15b). In both comparisons, differentially concentrated cytokines were grouped according their specific variation in either mutant. The basal compartment in mutants (both Pkhd1 and Pkd1KO) is characterized by a general higher cytokine production (see also histograms Fig. 14).

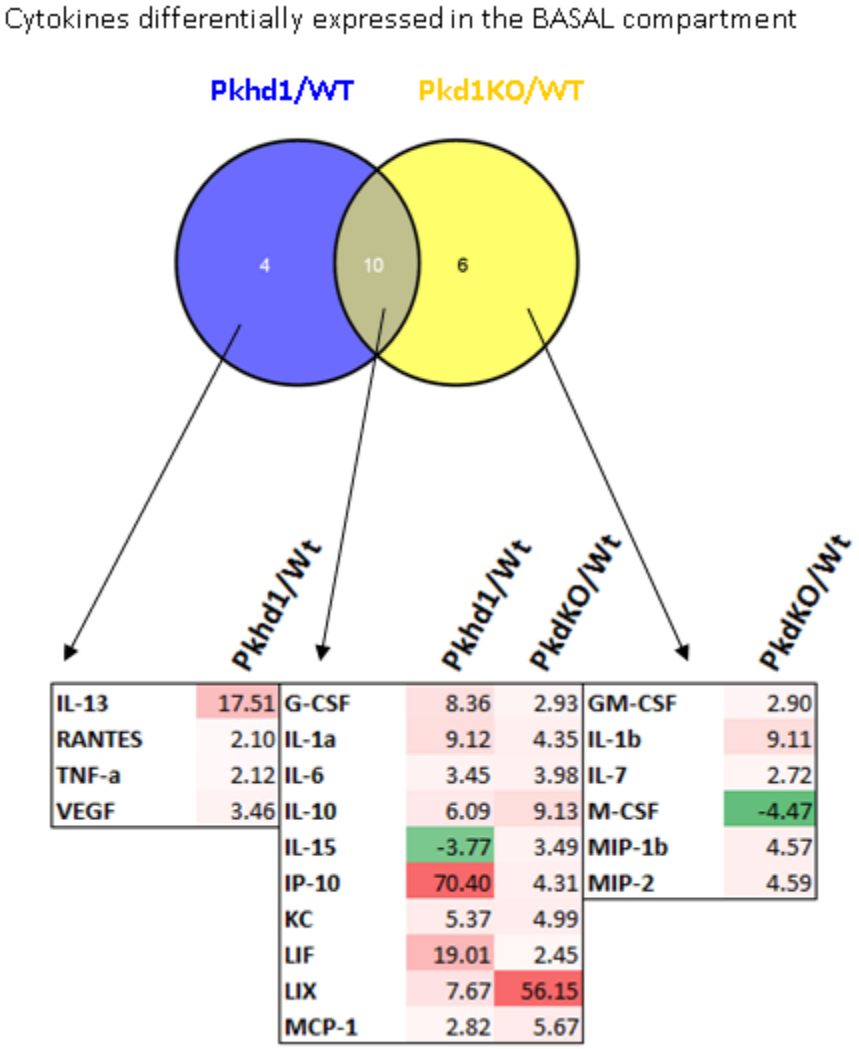

Figure 15a. Circles represent Venn diagrams depicting the number of cytokines differentially secreted on the basal side of Pkhd1 cholangiocytes versus WT (blue circles) and the number of cytokines differentially expressed by Pkd1KO cholangiocytes versus WT (yellow circles) or altered in both mutants (overlapping area). Over the 14 different cytokines altered from the basal side of Pkhd1 cholangiocytes, 10 were abnormally produced also by PkdKO mutants, wile 4 were exclusively altered in Pkhd1 cholangiocytes. Altered cytokines corresponding to each category are listed below the circles and are color coded in red for over expression and green for lower.

The apical compartment, conversely, is characterized by a lower number of variations and a more heterogeneous pattern of differential expression in mutants, with some cytokine being up-regulated and some down-regulated. As for the basal compartment, the diagram shows that IL-13, Rantes (CCL5), TNFα and VEGF were upregulated only in Pkhd1; IL-13, in particular was 17.5 folds more concentrated in supernatant from Pkhd1 basal side than in WT culture. IL-13 is a potent inducer of MMP9 (metalloproteinase) and TGFb1 expression. Together with IL-4 it is a pro-fibrotic cytokine, it promotes fibrocytes differentiation toward fibroblasts.

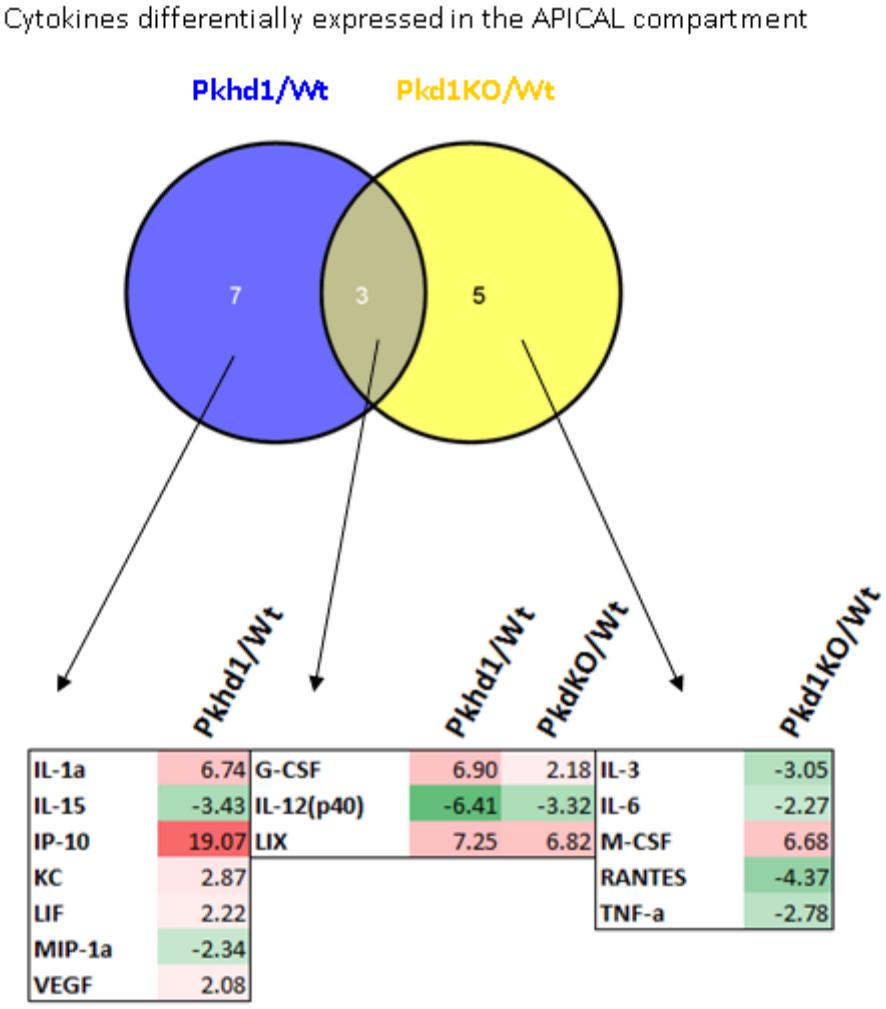

Figure 15b. Venn diagrams were drawn as in figure 15a this time analyzing the differential secretion of cytokines from the APICAL side.

Cytokines G-CSF, IL-1α, IL6, IL10, IP10, KC, LIF, LIX, MCP-1 were upregulated in both mutants, while other cytokines, IL1-b in particular, were up-regulated only from the basal side of Pkd1KO cultures.

In particular IL-1α was 9.12 fold more abundant in the medium harvested from the basal side of Pkhd1 cholangiocytes, and VEGF 3.46 fold. Il-1α (also called fibroblast-activating factor (FAF) or lymphocyte-activating factor (LAF), it promotes angiogenesis in vivo via VEGFR-2. This amplification of the VEGF signaling in fibrogenic conditions would lead to an excess or aberrant neo-angiogenesis.

All these factors may affect in turn both epithelial cells and their intense cross-talk with mesenchymal cells sustaining the fibrogenic response (reviewed in Pinzani and Rombouts, 2004, Parola, Pinzani, 2008).

SUMMARY of results

Our data generate morphological evidence that liver fibrosis in ARPKD/CHF/CD may initially be elicited by a process of epithelial–mesenchymal transition during which cholangiocytes acquire aberrant secretory capabilities enabling them to establish an intensive cross talk with surrounding mesenchymal cells. In detail:

- Pkhd1 mouse display biliary dysgenesis, liver fibrosis and portal hypertension, featuring CHF
- Immunohistochemistry revealed evidence of EMT in human CHF/CD and in a mouse model of ARPKD
- Murine cholangiocytes from Pkhd1 mice constitutionally possess aberrant migratory and invasive capabilities
- Fibrocytes are recruited in proximity to cysts in ARPKD
- Pkhd1-mutated cholangiocytes display an activated profile of cytokines and growth factors secretion on the basal side (G-CSF, IL1α, IL-13, CXCL 10, and VEGF) compatible with their ability to promote mesenchymal cells recruitment.

DISCUSSION

This study was performed to clarify the mechanisms underlying portal fibrosis in ARPKD starting from the hypothesis that cholangiocytes in ARPKD are engaged in a EMT program and able to establish a dynamic cross-talk with mesenchymal cells, actively contributing to fibrosis.
We considered liver specimens derived from human patients as well as from a mouse model of ARPKD through different maturation ages.

Cholangiocytes in ARPKD display features consistent with the activation of an EMT program

Epithelial cells undergoing EMT would lose intercellular cohesion and translocate from the epithelial compartment into the interstitium where, gaining a full mesenchymal phenotype, they can participate in the synthesis of the fibrotic matrix. We showed that cholangiocytes in ARPKD possess morphological and functional properties compatible with a partial transition towards a mesenchymal phenotype.
We observed an uneven, focal reduction in the epithelial markers, including E-cadherin, responsible for tight junction strength and intercellular adhesion in bile ducts. Its reduction implies a loosening of tight junctions, with all the deriving consequences, an increased motility of epithelial cells and a possible rearrangement in cells cytoskeleton and polarity. We also observed mislocalization of ß-catenin, another component of the epithelial adhesion complexes, which is also involved in the transcriptional regulation of the Wnt signaling pathway. When it is not bound to cells junctions, ß-catenin can translocate to the nucleus and contribute to the activation of a transcriptional machinery that leads to the upregulation of Snail and Twist, transcription factors actively promoting EMT. We observed a marked up regulation of snail, which inhibits (inter alia) E-cadherin transcription. Finally, we found that fibronectin was also up-regulated with a clear basal aspect suggesting that in Pkhd1 mice cholangiocytes likely contribute to ECM production. Fibronectin regulates cell adhesion, growth and migration by interacting with integrins (membrane-spanning receptor proteins) and with extracellular matrix components, such as collagen, fibrin and proteoglycans or fibronectin itself. Laminin discontinuity

along bile ducts profile in Pkhd1 mice indicates dismantling of the basement membrane, a fundamental prerequisite for permitting the migration of epithelial cells upon the loosening of tight junctions. In fact, cholangiocytes isolated from Pkhd1 mice also possessed enhanced migratory and invasive properties typical of an ongoing EMT process. However, in our studies we did not find α-SMA positive cholangiocytes along biliary cysts suggesting that, though EMT occurs in cholangiocytes in ARPKD, it does not lead to a full phenotypic switch.

Our results are in line with studies run in other laboratories. Recently, it was shown that in a rat model of polycystic kidney (PKC rat) cholangiocytes acquire mesenchymal features (flat shape, reduction in CK19 expression) during aging and participate in progressive hepatic fibrosis by producing extracellular matrix molecules (vimentin, fibronectin and collagen-I), however the authors did not observe a full transdifferentiation of cholangiocytes to myofibroblasts and expression of αSMA was never observed, nor in vivo and neither in vitro in cultured cholangiocytes upon administration of TGFβ1 (the primary EMT inducer) (Sato et al., 2007). In both studies cholangiocytes seem to undergo a partial EMT that enables cells to initiate a dedifferentiating program, acquire mesenchymal characteristics and motility and actively participate in progressive hepatic fibrosis by producing extracellular matrix molecules.

Proofs of EMT in the liver.

As of today proofs of EMT occurring in the liver are still somewhat controversial. There are several reasons why EMT could be underestimated or remain undetected: it appears to be a dynamic process that becomes visible on a wavelike fashion and some authors pointed out that αSMA expression (often transitory) might not be the most reliable marker for detecting EMT (Liu, 2004). All these studies depend on a transitional stage were cells co-express both epithelial and mesenchymal markers at the same time, however the duration of this transitional stage is not known. Actually, in the obstructed kidney where α-SMA expression was detected in epithelial cells, clearly proving EMT, the sudden loss of E-cadherin and immediate induction of αSMA within a short period of time

from day 3 and day 7, indicates that the transitional phase of EMT may be very short, making it difficult to identify cells in transition (Liu, 2004; Yang & Liu, 2001). The picture is further complicated by the heterogeneity observable within a cells population and by the patchy characteristics of this phenomenon.

EMT is a well recognized phenomenon in kidneys (Liu, 2010) and in the lungs fibrosis (Willis, duBois & Borok, 2006a). Since pioneering studies in kidney indicated that tubular epithelial cells could express fibroblast markers in disease states, thus postulating for the first time the possibility of a epithelial-to-mesenchymal transition (EMT) (Strutz et al., 1995), several subsequent studies were done, in vitro and in vivo, that reinforced this finding. Studies in vitro were straightforward and showed that, upon incubation with TGFβ1, tubular epithelial cells undergo a clear phenotypic switch to mesenchymal characteristics, with de novo αSMA expression. Studies in vivo were more challenging however they gave coherent results in various animal models of chronic kidney diseases including obstructive nephropaty, (Yang & Liu, 2001), (Zeisberg & Kalluri, 2008) and remnant kidney after 5/6 nephrectomy (Lan, 2003; Ng et al., 1998). In the latter model, for instance, such studies provided morphologic and phenotypic evidence for the existence of EMT by showing that tubular cell began to express (de novo) αSMA and progressively acquired a spindle shape morphology, losing their apical-basal polarity, and migrated into the peritubular interstitium through the disruption of the tubular basement membrane (Ng et al., 1998). Clinical studies on human kidney biopsies also reported tubular expression of S100A4 or Vimentin in tubular cells from various progressive chronic kidney diseases (Liu, 2010) for a rev.). Iwano et al. provided the most convincing evidence by using lineage tracking studies, dissipating concerns that were raised that these FSP-1 -positive tubular cells may represent an infiltration of interstitial myofibroblasts. With their genetic constructs they showed that in a mouse model of unilateral ureteral obstruction, up to 36% of all FSP-1 positive fibroblasts within the interstitial space originate from renal proximal tubules (Iwano et al., 2002). These studies highlighted the potential remarkable plasticity of fully differentiated epithelial cells unhinging a concept that was considered consolidated for years.

As for the liver, several authors describe the actual EMT contribution to liver fibrosis in a variety of human chronic liver diseases, including PBC and PSC and end-stage alcoholic cirrhosis. In these studies cholangiocytes lining the smallest branches of the biliary tree (reactive ductules, small- and medium-sized bile ducts), show phenotypic markers of ongoing EMT: reduction in E-cadherin, expression of S100A4, and of phosphorylated forms of Smad2/3 into the nucleus, (Robertson et al., 2007), (Rygiel et al., 2008), (Harada et al., 2009; Robertson et al., 2007; Rygiel et al., 2008; Sato et al., 2007). It has been suggested that in PBC and PSC, TGFβ, possibly presented by infiltrating T cells, may be the trigger of biliary EMT, or it can be activated by an innate immunity response (Harada et al., 2009). Features of EMT have been also observed in cholangiocarcinoma (CCA), where EMT is induced in neoplastic cholangiocytes by TGFβ1/Snail activation and is associated with an enhanced invasive growth (Sato et al., 2010). However, as pointed-out by Wells in a recent commentary, the use of morphologic and immunophenotypic studies with putative biomarkers (revealing loss of cell-cell adhesion, acquisition of mesenchymal traits, and breakdown of the basement membrane), can only provide a clue, but do not prove the existence of a process that is, by its nature, elusive. Furthermore, Wells questions the specificity of some of the EMT biomarkers: vimentin or collagen-I expression, for example, are not necessarily limited to mesenchymal cells, and the widely used FSP-1, (S100A4) considered fibroblasts-specific, is also expressed by leukocytes and macrophages (Le Hir et al., 2005). Not least, their detection just by immunostaining could be affected by the presence of infiltrating cells adjacent or overlapping the field of cysts. According to this line of thoughts only the genetic lineage experiments can provide the necessary proofs.

The first lineage tracking studies in liver were designed to track hepatocytes fate and were made in CCl_4 mice models with a β-Gal reporter gene under the control of the albumin promoter. With this approach the group of Kalluri (Zeisberg et al., 2007) reached the conclusion that some of the FSP^+ cells were of epithelial origin. However this study generated many questions also because about 30% of hepatocytes lacked the reporter gene expression and important technical pitfalls of the used techniques were highlighted. New studies tracking hepatocytes

(Taura et al., 2010) or cholangiocytes (Scholten et al., 2010) lineages in CCl_4 mice were published in 2010 and did not find clear evidence of EMT. Lineage tracking in cholangiocytes was done using a mouse with a tamoxifen inducible Cre-mediated labeling where, upon tamoxifen induction, CK19 expressing cells (cholangiocytes) express the fluorescent protein YFP. Fibrosis was induced by BDL (bile ducts ligation) or CCl_4. This study showed that cells with a history of CK19 expression did not express α-SMA, FSP-1 or desmin. According to Wells, these experiments put the question to rest establishing that EMT in liver does not occur (Wells, 2010). On the other hand it is important to remark that CCl_4 is a mouse model of induced liver fibrosis and the outcome could be different in different pathologies. Moreover, as pointed out by Schuppan and Popov (Popov & Schuppan, 2010), this technique using inducible Cre, even though it is the most suitable to detect EMT during injury because it excludes events linked to development, has some downside. It labels a highly specific population of mature cholangiocytes at the time of injury, and it has been shown that the cholangiocytes population can be rather heterogeneous, especially in response to injury and especially in the smallest branches of the biliary tree and in the canal of Herings (Priester et al., 2010). Schuppan and Popov underline that in all the elegant and well performed genetic studies run so far, using the Cre-Lox system to follow the lineage conversion, the authors never reported an efficacy exceeding 70% of Cre expressing cells and it is unknown why recombination does not occur in the remaining 30%. Actually they speculate that a specific subset of cholangiocytes could not be traced either owing to weak CK19 expression or other unknown cell phenotypes, perhaps even related to cell differentiation or plasticity (or EMT).

In consideration of all these observations, the actual impact of EMT in liver diseases remains unclear and is becoming strongly controversial. We think that the controversy on EMT and liver fibrosis is mainly a matter of definitions. Although, there is no demonstration that a "full" transdifferentiation to a mesenchymal phenotype, marked by the acquisition of the α-SMA immunoreactivity, actually occurs in ARPKD, it is clear that mutated

cholangiocytes express several morphologic and functional markers commonly associated to a mesenchymal phenotype. This is a fundamental property; as a consequence, cholangiocytes reduce the strength of their tight junctions, acquire motile properties, and are able to process and remodel the ECM actively contributing to the fibrotic process. Another important point is that, after admitting the occurrence of EMT, it remains difficult to establish how important its contribution is to fibrosis. Since many cell types can produce ECM proteins, the actual impact of EMT on fibrogenesis can be difficult to estimate. Alternatively, phenotypic changes affecting mutated cholangiocytes may also enhance their ability to interact with surrounding ECM producing cells.

Fibrocytes are recruited around biliary cysts, likely through paracrine effects exerted by Pkhd1-defective cholangiocytes.

The involvement of cholangiocytes in a fibrogenic program in ARPKD may also derive from an extensive cross talk with other mesenchymal cell types, enabled by an aberrant secretory phenotype endorsed by a partial EMT.

Different fibrogenic cell types may contribute to the deposition of extracellular matrix in ARPKD. In our studies, the immunophenotypical characterization of the fibrotic periductal area, showed a progressive enrichment of $S100A4^{+}$ cells, most likely fibroblasts, even though only few αSMA positive cells could be observed, especially in younger mice. Similarly, other published studies on patients with Caroli disease found that αSMA positive cells were negligible, suggesting that portal fibrosis in CHF is mediated by other cell types in addition to the activated myofibroblasts (Ozaki et al., 2005). Interestingly we found a progressive portal enrichment in a population of Coll-1/CD34/CD45-positive cells, likely of hematopoietic origin, in close vicinity to biliary cysts in Pkhd1 mice. This unique immunophenotype is characteristic of fibrocytes, cells that can play a major role in wound healing and fibrosis. Fibrocytes are not activated resident mesenchymal cells, but instead differentiate from circulating monocytes to be recruited to the wound sites. Upon cytokine stimulation they mature to a fibroblast-like phenotype and can start to express αSMA and to produce ECM

proteins (Abe et al., 2001). Fibrocytes contribution to fibrosis is not limited to ECM production, they also release a variety of cytokines and growth factors which are relevant for fibrogenesis (Chesney et al., 1998) as well as for angiogenesis (Metz, 2003).

Several factors can affect fibrocytes differentiation into myofibroblasts-like cells. In addition to the stimulation with TGF-β1 (which is also the main promoter of EMT), the direct contact with T lymphocytes in co-cultures (Abe et al., 2001) and the exposure to PDGF-B, a main activator of mesenchymal cells (Oh et al., 1998) have been reported (Varcoe et al., 2006). PDGF is actually one of the most relevant players in liver fibrogenesis, in several fibrotic diseases the fibroblasts population expresses PDGFR and responds to VEGF and PDGF stimuli. In fact, we found that also in Pkhd1 mutated mice and human CD, PDGFRβ was strongly expressed by the CD45-positive cells strictly adjacent to the biliary microhamartomas. PDGFRβ binds to PDGF B and PDGF D dimers with high affinity, whereas it does not bind PDGF AA nor CC or eterodimers (Fredriksson, Li & Eriksson, 2004). Interestingly in our study, biliary cysts were strongly positive for PDGF D in human CD, suggesting that PDGF-D may actually represent a paracrine factor regulating the recruitment of α-SMA negative mesenchymal cells around the cysts.

PDGF D chain (and also the C chain), was discovered more recently and is less known than PDGF A and B chains (Bergsten et al., 2001; Uutela et al., 2001). Expression studies showed that the expression pattern and level of PDGF-D in human diseases is more restricted and limited compared with that of PDGF-B, implying a more specific, spatially controlled function (Fredriksson et al., 2004), though its function is far to be fully elucidated. In vitro studies showed that PDGF-D is as effective as PDGF B in stimulating HSC proliferation and ECM production (Borkham-Kamphorst et al., 2007) and recently, PDGF-D has been indicated to behave as an important mediator of proliferation of mesangial cells, the kidney homolog of HSC in liver, in mesangioproliferative glomerulonephritis (Floege et al., 2007; Hudkins et al., 2004).

Cholangiocytes from Pkhd1 mice showed a brisk hyper-secretory profile, particularly from the basolateral side, enabling an active recruitment of mesenchymal cells.

We used a cytokine array to analyze the medium differentially harvested from the apical or from the basolateral side of murine cholangiocytes grown in a polarized monolayer. Pkhd1-mutated cholangiocytes were compared with Pkd1 and WT cholangiocytes. We found that Pkhd1 defective cholangiocytes possessed a hyper-secretory phenotype, expecially from the basolateral side. Among the different cytokines found to be strongly released in the basolateral medium of Pkhd1 mutated cholangiocytes, G-CSF, CXCL10, IL13, IL1α and were of particular interest given their potential effects on leukocyte recruitment and activation.

G-CSF in particular is one of the main hematopoietic growth factors. It is the most commonly used agent for counteracting neutropenia and its potency on mobilizing bone marrow derived hematopoietic cells is well documented (Thomas, Liu & Link, 2002). The mobilization and recruitment of bone marrow-derived cells to the wound site is a general reparative mechanism of tissue fibrosis and wound healing however the extent of this phenomenon largely depends on the tissue microenvironment (Ishii et al., 2005). In particular G-CSF exerts its main biologic effect on commited circulating myeloid cells, inducing their terminal differentiation to neutrophils and promoting their proliferation (Demetri & Griffin, 1991; Ebihara et al., 2000; Nagata & Fukunaga, 1991).

CXCL10 (IP10, IFN-inducible protein 10) is another important chemokine responsible for the recruitment and localization of inflammatory cells to sites of tissue damage. Its role in liver fibrosis has been described in several diseases and animal models (Hintermann et al.). Studies on the role of CXCL10 in a model of liver fibrosis showed that CXCL10 was upregulated in the liver of CCl_4 treated mice, and fibrosis was less severe in CXCL10 deficient mice. Most importantly the authors show that CXCL10 promotes HSC chemotaxis and lymphocytes migration to the liver. In fact HSC, dendritic cells, activated CD4T cells, and B lymphocytes, all express CXCR3, which is the CXCL10 specific receptor (Hintermann et al.). The recruitment of inflammatory cells to the wound site

results in further active release of cytokines, chemokines and growth factors targeting surrounding cells and activating a cellular response to the tissue damage.

Most interestingly IL-13 was also hypersecreted on the basal side of mutated clangiocytes. IL-13 is an important profibrotic mediator and binds to specific receptors on fibroblasts actively promoting collagen production (Murata et al., 1998), (Chiaramonte et al., 1999; Oriente et al., 2000; Saito et al., 2003). Most importantly, this cytokine is also a major promoter of fibrocytes differentiation to αSMA^+ cells without inducing proliferation (Shao et al., 2008). IL-13 produced by mutated cholangiocytes in ARPKD thus may be an important trigger that promotes fibrocytes differentiation and consequent fibrosis next to biliary cysts. IL-13 also indirectly activates TGF-β by upregulating the expression of MMPs that mediate its activation (Lanone et al., 2002; Lee et al., 2001).

Conclusions

Congenital Hepatic Fibrosis (CHF) and Caroli's Disease (CD) are genetic disorders of the liver and kidney caused by deficiency in fibrocystin, the protein encoded by the PKHD1 gene. The liver disease is characterized by dysmorphic intrahepatic bile ducts associated with the progressive establishment of portal fibrosis, ultimately leading to portal hypertension and its severe manifestations (hypersplenism, esophageal varices and ascites). The mechanistic relationship between the genetic defect and development of portal fibrosis is not known. In this study we have found that fibrocystin-defective cholangiocytes acquire some mesenchymal-like features without a full phenotypic conversion (partial EMT). This mechanism is relevant for enabling cholangiocytes to respond to liver damage and initiate the reparative response.

The reparative response requires epithelial cells to reduce their barrier function in favor of functional properties typical of mesenchymal cells, such as increased motility and migration, and the ability to erode the basal membrane. The reparative response also requires epithelial cells to secrete factors able to cross-

talk with other cell type involved in liver repair (mesenchymal, inflammatory, endothelial cells). In our model, fibrocystin-defective cholangiocytes a) possess increased motility, b) express phenotypic markers associated with EMT, c) secrete factors able to promote migration of portal leukocytes, compatible with a fibrocytes phenotype. Therefore, these results may have a broader significance, since the Pkhd1$^{del4/del4}$ mice represents a valuable model for studying the mechanistic relationships between cholangiocyte dysfunction and portal fibrosis.

Abbreviations

ADPKD: Adult Dominant Polycystic Kidney Disease

ADPLD: Autosomal Dominant Polycystic Liver Disease

ARPKD: Autosomal recessive polycystic kidney disease

CD:Caroli Disease

CHF: congenital hepatic fibrosis

ECM: extra cellular matrix

EGF: epidermal growth factor

EMT: epithelial-mesenchymal transition

FPC: Fibrocystin

HSC: hepatic stellate cells

IGF: Insulin-like growth factor

MET: mesenchymal-epithelial transition

MMP: Metalloproteinase

PBC: primary biliary cirrhosis

PC1 and PC2: Polycystin 1 and Polycystin2

PCLD: Polycystic liver disease

PDGFR: platelet derived growth factor receptor

PSC: primary sclerosing cholangitis

ROS: Reactive oxygen species

TGF-β: transforming growth factor-β

TNF: tumor necrosis factor

VEGF: vascular endothelial growth factor

Acknowledgements

I would like to thank Professor Strazzabosco whose support enabled me to develop this project and I'm also heartily thankful to Dott. Fabris, whose guidance and contagious enthusiasm sustained me during the completion of this work. I'm indebted to all my colleagues in Strazzabosco's laboratory for their collaboration and for all we shared during these years in the lab, in particular to Max and Luigi for their active contribution to this project. I would like to thank Professor Galli-Kienle for her helpful advises. I'm deeply grateful to Professor Slayman for her inspiring teachings, and to Ken, exceptional component of the "dynamic duo". My most heart felted gratitude goes to Riccardo, for his precious help and unconditional love.

REFERENCES

Abe, R., Donnelly, S. C., Peng, T., Bucala, R. & Metz, C. N. (2001). Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol* 166(12), 7556-62.

Acloque, H., Adams, M. S., Fishwick, K., Bronner-Fraser, M. & Nieto, M. A. (2009). Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest* 119(6), 1438-49.

Alvaro, D., Alpini, G., Jezequel, A. M., Bassotti, C., Francia, C., Fraioli, F., Romeo, R., Marucci, L., Le Sage, G., Glaser, S. S. & Benedetti, A. (1997). Role and mechanisms of action of acetylcholine in the regulation of rat cholangiocyte secretory functions. *The Journal of clinical investigation* 100(6), 1349-62.

Alvaro, D., Gigliozzi, A. & Attili, A. F. (2000). Regulation and deregulation of cholangiocyte proliferation. *Journal of hepatology* 33(2), 333-40.

Alvaro, D., Mancino, M. G., Glaser, S., Gaudio, E., Marzioni, M., Francis, H. & Alpini, G. (2007). Proliferating cholangiocytes: a neuroendocrine compartment in the diseased liver. *Gastroenterology* 132(1), 415-31.

Asawa, S., Saito, T., Satoh, A., Ohtake, K., Tsuchiya, T., Okada, H., Neilson, E. G. & Gotoh, M. (2007). Participation of bone marrow cells in biliary fibrosis after bile duct ligation. *Journal of gastroenterology and hepatology* 22(11), 2001-8.

Baffy, G. (2009). Kupffer cells in non-alcoholic fatty liver disease: the emerging view. *Journal of hepatology* 51(1), 212-23.

Behrens, J., Lowrick, O., Klein-Hitpass, L. & Birchmeier, W. (1991). The E-cadherin promoter: functional analysis of a G.C-rich region and an epithelial cell-specific palindromic regulatory element. *Proc Natl Acad Sci U S A* 88(24), 11495-9.

Bergmann, C., Senderek, J., Windelen, E., Kupper, F., Middeldorf, I., Schneider, F., Dornia, C., Rudnik-Schoneborn, S., Konrad, M., Schmitt, C. P., Seeman, T., Neuhaus, T. J., Vester, U., Kirfel, J., Buttner, R. & Zerres, K. (2005). Clinical consequences of PKHD1 mutations in 164 patients with autosomal-recessive polycystic kidney disease (ARPKD). *Kidney international* 67(3), 829-48.

Bergsten, E., Uutela, M., Li, X., Pietras, K., Ostman, A., Heldin, C. H., Alitalo, K. & Eriksson, U. (2001). PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor. *Nat Cell Biol* 3(5), 512-6.

Borkham-Kamphorst, E., van Roeyen, C. R. C., Ostendorf, T., Floege, J., Gressner, A. M. & Weiskirchen, R. (2007). Pro-fibrogenic potential of PDGF-D in liver fibrosis. *Journal of hepatology* 46(6), 1064-74.

Braet, F. & Wisse, E. (2002). Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. *Comparative Hepatology* 1(1), 1.

Brenner, D. A. (2009). Molecular pathogenesis of liver fibrosis. *Trans Am Clin Climatol Assoc* 120, 361-8.

Bucala, R. (2008). Circulating fibrocytes: cellular basis for NSF. *J Am Coll Radiol* 5(1), 36-9.

Bucala, R., Spiegel, L. A., Chesney, J., Hogan, M. & Cerami, A. (1994). Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med* 1(1), 71-81.

Caligiuri, A., Glaser, S., Rodgers, R. E., Phinizy, J. L., Robertson, W., Papa, E., Pinzani, M. & Alpini, G. (1998). Endothelin-1 inhibits secretin-stimulated ductal secretion by interacting with ETA receptors on large cholangiocytes. *The American journal of physiology* 275(4 Pt 1), G835-46.

Chesney, J., Metz, C., Stavitsky, A. B., Bacher, M. & Bucala, R. (1998). Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes. *J Immunol* 160(1), 419-25.

Chiaramonte, M. G., Donaldson, D. D., Cheever, A. W. & Wynn, T. A. (1999). An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J Clin Invest* 104(6), 777-85.

Cho, W. K. & Boyer, J. L. (1999a). Characterization of ion transport mechanisms involved in bombesin-stimulated biliary secretion in rat cholangiocytes. *Journal of hepatology* 30(6), 1045-51.

Cho, W. K. & Boyer, J. L. (1999b). Vasoactive intestinal polypeptide is a potent regulator of bile secretion from rat cholangiocytes. *Gastroenterology* 117(2), 420-8.

Choi, I., Kang, H. S., Yang, Y. & Pyun, K. H. (1994). IL-6 induces hepatic inflammation and collagen synthesis in vivo. *Clinical and experimental immunology* 95(3), 530-5.

De Minicis, S., Seki, E., Oesterreicher, C., Schnabl, B., Schwabe, R. F. & Brenner, D. A. (2008). Reduced nicotinamide adenine dinucleotide phosphate oxidase mediates fibrotic and inflammatory effects of leptin on hepatic stellate cells. *Hepatology* 48(6), 2016-26.

Demetri, G. D. & Griffin, J. D. (1991). Granulocyte colony-stimulating factor and its receptor. *Blood* 78(11), 2791-808.

Desmet, V. J. (1992). Congenital diseases of intrahepatic bile ducts: variations on the theme "ductal plate malformation". *Hepatology* 16(4), 1069-83.

Desmet, V. J. (1998). HISTOPATHOLOGY OF CHRONIC CHOLESTASIS AND ADULT DUCTOPENIC SYNDROME. *Clinics in Liver Disease* 2(2), 249-64.

Ebihara, Y., Xu, M.-j., Manabe, A., Kikuchi, A., Tanaka, R., Kato, S., Nakahata, T. & Tsuji, K. (2000). Exclusive expression of G-CSF receptor on myeloid progenitors in bone marrow CD34+ cells. *British Journal of Haematology* 109(1), 153-61.

Fabris, L., Cadamuro, M., Fiorotto, R., Roskams, T., Spirli, C., Melero, S., Sonzogni, A., Joplin, R. E., Okolicsanyi, L. & Strazzabosco, M. (2006). Effects of angiogenic factor overexpression by human and rodent cholangiocytes in polycystic liver diseases. *Hepatology* 43(5), 1001-12.

Fiorotto, R., Spirli, C., Fabris, L., Cadamuro, M., Okolicsanyi, L. & Strazzabosco, M. (2007). Ursodeoxycholic acid stimulates cholangiocyte fluid secretion in mice via CFTR-dependent ATP secretion. *Gastroenterology* 133(5), 1603-13.

Floege, J., van Roeyen, C., Boor, P. & Ostendorf, T. (2007). The role of PDGF-D in mesangioproliferative glomerulonephritis. *Contrib Nephrol* 157, 153-8.

Francis, H., Glaser, S., Ueno, Y., LeSage, G., Marucci, L., Benedetti, A., Taffetani, S., Marzioni, M., Alvaro, D., Venter, J., Reichenbach, R., Fava, G., Lynne Phinizy, J. & Alpini, G. (2004). cAMP stimulates the secretory and proliferative capacity of the rat intrahepatic biliary epithelium through changes in the PKA/Src/MEK/ERK1/2 pathway. *Journal of hepatology* 41(4), 528-37.

Fredriksson, L., Li, H. & Eriksson, U. (2004). The PDGF family: four gene products form five dimeric isoforms. *Cytokine Growth Factor Rev* 15(4), 197-204.

Friedman, S. L. (2000). Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *The Journal of biological chemistry* 275(4), 2247-50.

Friedman, S. L. (2008a). Hepatic fibrosis -- overview. *Toxicology* 254(3), 120-9.

Friedman, S. L. (2008b). Mechanisms of hepatic fibrogenesis. *Gastroenterology* 134(6), 1655-69.

Gallagher, A. R., Esquivel, E. L., Briere, T. S., Tian, X., Mitobe, M., Menezes, L. F., Markowitz, G. S., Jain, D., Onuchic, L. F. & Somlo, S. (2008). Biliary and pancreatic dysgenesis in mice harboring a mutation in Pkhd1. *Am J Pathol* 172(2), 417-29.

Giroldi, L. A., Bringuier, P. P., de Weijert, M., Jansen, C., van Bokhoven, A. & Schalken, J. A. (1997). Role of E boxes in the repression of E-cadherin expression. *Biochem Biophys Res Commun* 241(2), 453-8.

Glaser, S., Francis, H., Demorrow, S., Lesage, G., Fava, G., Marzioni, M., Venter, J. & Alpini, G. (2006). Heterogeneity of the intrahepatic biliary epithelium. *World J Gastroenterol* 12(22), 3523-36.

Gong, A. Y., Tietz, P. S., Muff, M. A., Splinter, P. L., Huebert, R. C., Strowski, M. Z., Chen, X. M. & LaRusso, N. F. (2003). Somatostatin stimulates ductal bile absorption and inhibits ductal bile secretion in mice via SSTR2 on cholangiocytes. *American journal of physiology* 284(5), C1205-14.

Grappone, C., Pinzani, M., Parola, M., Pellegrini, G., Caligiuri, A., DeFranco, R., Marra, F., Herbst, H., Alpini, G. & Milani, S. (1999). Expression of platelet-derived growth factor in newly formed cholangiocytes during experimental biliary fibrosis in rats. *Journal of hepatology* 31(1), 100-9.

Guay-Woodford, L. M., Muecher, G., Hopkins, S. D., Avner, E. D., Germino, G. G., Guillot, A. P., Herrin, J., Holleman, R., Irons, D. A., Primack, W. & et al. (1995). The severe perinatal form of autosomal recessive polycystic kidney disease maps to chromosome 6p21.1-p12: implications for genetic counseling. *American journal of human genetics* 56(5), 1101-7.

Guo, C., Yang, W. & Lobe, C. G. (2002). A Cre recombinase transgene with mosaic, widespread tamoxifen-inducible action. *Genesis* 32(1), 8-18.

Harada, K., Sato, Y., Ikeda, H., Isse, K., Ozaki, S., Enomae, M., Ohama, K., Katayanagi, K., Kurumaya, H., Matsui, A. & Nakanuma, Y. (2009). Epithelial-mesenchymal transition induced by biliary innate immunity contributes to the sclerosing cholangiopathy of biliary atresia. *The Journal of pathology* 217(5), 654-64.

Hay, E. D. (1968). Organization and fine structure of epithelium and mesenchyme in the developing chick embryo. In Epithelial-mesenchymal interactions. In Fleischmajer, R. & Billingham, R.E. (Eds.), (pp. 31-55). Baltimore, Mariland, USA: Williams and Wilkins.

Hiesberger, T., Gourley, E., Erickson, A., Koulen, P., Ward, C. J., Masyuk, T. V., Larusso, N. F., Harris, P. C. & Igarashi, P. (2006). Proteolytic cleavage and nuclear translocation of fibrocystin is regulated by intracellular Ca2+ and activation of protein kinase C. *The Journal of biological chemistry* 281(45), 34357-64.

Hintermann, E., Bayer, M., Pfeilschifter, J. M., Luster, A. D. & Christen, U. CXCL10 promotes liver fibrosis by prevention of NK cell mediated hepatic stellate cell inactivation. *J Autoimmun* 35(4), 424-35.

Huber, M. A., Kraut, N. & Beug, H. (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Current opinion in cell biology* 17(5), 548-58.

Hudkins, K. L., Gilbertson, D. G., Carling, M., Taneda, S., Hughes, S. D., Holdren, M. S., Palmer, T. E., Topouzis, S., Haran, A. C., Feldhaus, A. L. & Alpers, C. E. (2004). Exogenous PDGF-D Is a Potent Mesangial Cell Mitogen and Causes a Severe Mesangial Proliferative Glomerulopathy. *Journal of the American Society of Nephrology* 15(2), 286-98.

Hughes, J., Ward, C. J., Peral, B., Aspinwall, R., Clark, K., San Millan, J. L., Gamble, V. & Harris, P. C. (1995). The polycystic kidney disease 1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. *Nature genetics* 10(2), 151-60.

Ishii, G., Sangai, T., Sugiyama, K., Ito, T., Hasebe, T., Endoh, Y., Magae, J. & Ochiai, A. (2005). In vivo characterization of bone marrow-derived fibroblasts recruited into fibrotic lesions. *Stem Cells* 23(5), 699-706.

Iwano, M., Plieth, D., Danoff, T. M., Xue, C., Okada, H. & Neilson, E. G. (2002). Evidence that fibroblasts derive from epithelium during tissue fibrosis. *The Journal of clinical investigation* 110(3), 341-50.

Junghans, D., Haas, I. G. & Kemler, R. (2005). Mammalian cadherins and protocadherins: about cell death, synapses and processing. *Current opinion in cell biology* 17(5), 446-52.

Kaimori, J. Y., Nagasawa, Y., Menezes, L. F., Garcia-Gonzalez, M. A., Deng, J., Imai, E., Onuchic, L. F., Guay-Woodford, L. M. & Germino, G. G. (2007). Polyductin undergoes notch-like processing and regulated release from primary cilia. *Human molecular genetics* 16(8), 942-56.

Kalluri, R. (2009). EMT: when epithelial cells decide to become mesenchymal-like cells. *J Clin Invest* 119(6), 1417-9.

Kalluri, R. & Neilson, E. G. (2003). Epithelial-mesenchymal transition and its implications for fibrosis. *The Journal of clinical investigation* 112(12), 1776-84.

Kalluri, R. & Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *J Clin Invest* 119(6), 1420-8.

Katoh, Y. & Katoh, M. (2009). Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. *Curr Mol Med* 9(7), 873-86.

Keeley, E. C., Mehrad, B. & Strieter, R. M. (2010). Fibrocytes: bringing new insights into mechanisms of inflammation and fibrosis. *Int J Biochem Cell Biol* 42(4), 535-42.

Kisseleva, T., Uchinami, H., Feirt, N., Quintana-Bustamante, O., Segovia, J. C., Schwabe, R. F. & Brenner, D. A. (2006). Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis. *Journal of hepatology* 45(3), 429-38.

Koptides, M. & Deltas, C. C. (2000). Autosomal dominant polycystic kidney disease: molecular genetics and molecular pathogenesis. *Human genetics* 107(2), 115-26.

Kruglov, E. A., Nathanson, R. A., Nguyen, T. & Dranoff, J. A. (2006). Secretion of MCP-1/CCL2 by bile duct epithelia induces myofibroblastic transdifferentiation of portal fibroblasts. *Am J Physiol Gastrointest Liver Physiol* 290(4), G765-71.

Lan, H. Y. (2003). Tubular epithelial-myofibroblast transdifferentiation mechanisms in proximal tubule cells. *Curr Opin Nephrol Hypertens* 12(1), 25-9.

Lanone, S., Zheng, T., Zhu, Z., Liu, W., Lee, C. G., Ma, B., Chen, Q., Homer, R. J., Wang, J., Rabach, L. A., Rabach, M. E., Shipley, J. M., Shapiro, S. D., Senior, R. M. & Elias, J. A. (2002). Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling. *J Clin Invest* 110(4), 463-74.

Le Hir, M., Hegyi, I., Cueni-Loffing, D., Loffing, J. & Kaissling, B. (2005). Characterization of renal interstitial fibroblast-specific protein 1/S100A4-positive cells in healthy and inflamed rodent kidneys. *Histochem Cell Biol* 123(4-5), 335-46.

Lee, C. G., Homer, R. J., Zhu, Z., Lanone, S., Wang, X., Koteliansky, V., Shipley, J. M., Gotwals, P., Noble, P., Chen, Q., Senior, R. M. & Elias, J. A. (2001).

Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med* 194(6), 809-21.

Lee, J. M., Dedhar, S., Kalluri, R. & Thompson, E. W. (2006). The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *The Journal of cell biology* 172(7), 973-81.

Lenzen, R., Elster, J., Behrend, C., Hampel, K. E., Bechstein, W. O. & Neuhaus, P. (1997). Bile acid-independent bile flow is differently regulated by glucagon and secretin in humans after orthotopic liver transplantation. *Hepatology (Baltimore, Md* 26(5), 1272-81.

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

Liu, Y. (2010). New insights into epithelial-mesenchymal transition in kidney fibrosis. *J Am Soc Nephrol* 21(2), 212-22.

Marra, F., DeFranco, R., Grappone, C., Parola, M., Milani, S., Leonarduzzi, G., Pastacaldi, S., Wenzel, U. O., Pinzani, M., Dianzani, M. U., Laffi, G. & Gentilini, P. (1999). Expression of monocyte chemotactic protein-1 precedes monocyte recruitment in a rat model of acute liver injury, and is modulated by vitamin E. *J Investig Med* 47(1), 66-75.

Masyuk, T., Masyuk, A. & LaRusso, N. (2009). Cholangiociliopathies: genetics, molecular mechanisms and potential therapies. *Current opinion in gastroenterology* 25(3), 265-71.

Mehrad, B., Burdick, M. D. & Strieter, R. M. (2009). Fibrocyte CXCR4 regulation as a therapeutic target in pulmonary fibrosis. *Int J Biochem Cell Biol* 41(8-9), 1708-18.

Mehrad, B., Burdick, M. D., Zisman, D. A., Keane, M. P., Belperio, J. A. & Strieter, R. M. (2007). Circulating peripheral blood fibrocytes in human fibrotic interstitial lung disease. *Biochem Biophys Res Commun* 353(1), 104-8.

Metz, C. N. (2003). Fibrocytes: a unique cell population implicated in wound healing. *Cell Mol Life Sci* 60(7), 1342-50.

Murata, T., Husain, S. R., Mohri, H. & Puri, R. K. (1998). Two different IL-13 receptor chains are expressed in normal human skin fibroblasts, and IL-4 and IL-13 mediate signal transduction through a common pathway. *Int Immunol* 10(8), 1103-10.

Nagasawa, Y., Matthiesen, S., Onuchic, L. F., Hou, X., Bergmann, C., Esquivel, E., Senderek, J., Ren, Z., Zeltner, R., Furu, L., Avner, E., Moser, M., Somlo, S., Guay-Woodford, L., Buttner, R., Zerres, K. & Germino, G. G. (2002). Identification and characterization of Pkhd1, the mouse orthologue of the human ARPKD gene. *J Am Soc Nephrol* 13(9), 2246-58.

Nagata, S. & Fukunaga, R. (1991). Granulocyte colony-stimulating factor and its receptor. *Prog Growth Factor Res* 3(2), 131-41.

Nathanson, M. H. & Boyer, J. L. (1991). Mechanisms and regulation of bile secretion. *Hepatology* 14(3), 551-66.

Ng, Y. Y., Huang, T. P., Yang, W. C., Chen, Z. P., Yang, A. H., Mu, W., Nikolic-Paterson, D. J., Atkins, R. C. & Lan, H. Y. (1998). Tubular epithelial-myofibroblast transdifferentiation in progressive tubulointerstitial fibrosis in 5/6 nephrectomized rats. *Kidney international* 54(3), 864-76.

Oh, S. J., Kurz, H., Christ, B. & Wilting, J. (1998). Platelet-derived growth factor-B induces transformation of fibrocytes into spindle-shaped myofibroblasts in vivo. *Histochemistry and Cell Biology* 109(4), 349-57.

Omenetti, A., Porrello, A., Jung, Y., Yang, L., Popov, Y., Choi, S. S., Witek, R. P., Alpini, G., Venter, J., Vandongen, H. M., Syn, W. K., Baroni, G. S., Benedetti, A., Schuppan, D. & Diehl, A. M. (2008). Hedgehog signaling regulates epithelial-mesenchymal transition during biliary fibrosis in rodents and humans. *J Clin Invest* 118(10), 3331-42.

Ong, A. C. & Harris, P. C. (2005). Molecular pathogenesis of ADPKD: the polycystin complex gets complex. *Kidney international* 67(4), 1234-47.

Onuchic, L. F., Furu, L., Nagasawa, Y., Hou, X., Eggermann, T., Ren, Z., Bergmann, C., Senderek, J., Esquivel, E., Zeltner, R., Rudnik-Schoneborn, S., Mrug, M., Sweeney, W., Avner, E. D., Zerres, K., Guay-Woodford, L. M., Somlo, S.

& Germino, G. G. (2002). PKHD1, the polycystic kidney and hepatic disease 1 gene, encodes a novel large protein containing multiple immunoglobulin-like plexin-transcription-factor domains and parallel beta-helix 1 repeats. *American journal of human genetics* 70(5), 1305-17.

Oriente, A., Fedarko, N. S., Pacocha, S. E., Huang, S. K., Lichtenstein, L. M. & Essayan, D. M. (2000). Interleukin-13 modulates collagen homeostasis in human skin and keloid fibroblasts. *J Pharmacol Exp Ther* 292(3), 988-94.

Ozaki, S., Sato, Y., Yasoshima, M., Harada, K. & Nakanuma, Y. (2005). Diffuse expression of heparan sulfate proteoglycan and connective tissue growth factor in fibrous septa with many mast cells relate to unresolving hepatic fibrosis of congenital hepatic fibrosis. *Liver Int* 25(4), 817-28.

Patsenker, E., Popov, Y., Stickel, F., Jonczyk, A., Goodman, S. L. & Schuppan, D. (2008). Inhibition of integrin alphavbeta6 on cholangiocytes blocks transforming growth factor-beta activation and retards biliary fibrosis progression. *Gastroenterology* 135(2), 660-70.

Popov, Y., Patsenker, E., Stickel, F., Zaks, J., Bhaskar, K. R., Niedobitek, G., Kolb, A., Friess, H. & Schuppan, D. (2008). Integrin alphavbeta6 is a marker of the progression of biliary and portal liver fibrosis and a novel target for antifibrotic therapies. *Journal of hepatology* 48(3), 453-64.

Popov, Y. & Schuppan, D. M. D. (2010). Epithelial-to-Mesenchymal Transition in Liver Fibrosis: Dead or Alive? *Gastroenterology* 139(3), 722-5.

Priester, S., Wise, C. & Glaser, S. (2010). Involvement of cholangiocyte proliferation in biliary fibrosis. *World J Gastrointest Pathophysiol* 1(2), 30-7.

Qian, F., Wei, W., Germino, G. & Oberhauser, A. (2005). The nanomechanics of polycystin-1 extracellular region. *The Journal of biological chemistry* 280(49), 40723-30.

Quan, T. E., Cowper, S., Wu, S. P., Bockenstedt, L. K. & Bucala, R. (2004). Circulating fibrocytes: collagen-secreting cells of the peripheral blood. *Int J Biochem Cell Biol* 36(4), 598-606.

Rhyu, D. Y., Yang, Y., Ha, H., Lee, G. T., Song, J. S., Uh, S. T. & Lee, H. B. (2005). Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *J Am Soc Nephrol* 16(3), 667-75.

Robertson, H., Kirby, J. A., Yip, W. W., Jones, D. E. & Burt, A. D. (2007). Biliary epithelial-mesenchymal transition in posttransplantation recurrence of primary biliary cirrhosis. *Hepatology (Baltimore, Md* 45(4), 977-81.

Roskams, T. & Desmet, V. (2008). Embryology of Extra- and Intrahepatic Bile Ducts, the Ductal plate. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology* 291(6), 628-35.

Rygiel, K. A., Robertson, H., Marshall, H. L., Pekalski, M., Zhao, L., Booth, T. A., Jones, D. E., Burt, A. D. & Kirby, J. A. (2008). Epithelial-mesenchymal transition contributes to portal tract fibrogenesis during human chronic liver disease. *Laboratory investigation; a journal of technical methods and pathology* 88(2), 112-23.

Rygiel, K. A., Robertson, H., Marshall, H. L., Pekalski, M., Zhao, L., Booth, T. A., Jones, D. E. J., Burt, A. D. & Kirby, J. A. (2007). Epithelial-mesenchymal transition contributes to portal tract fibrogenesis during human chronic liver disease. *Lab Invest* 88(2), 112-23.

Saito, A., Okazaki, H., Sugawara, I., Yamamoto, K. & Takizawa, H. (2003). Potential action of IL-4 and IL-13 as fibrogenic factors on lung fibroblasts in vitro. *Int Arch Allergy Immunol* 132(2), 168-76.

Sato, Y., Harada, K., Itatsu, K., Ikeda, H., Kakuda, Y., Shimomura, S., Shan Ren, X., Yoneda, N., Sasaki, M. & Nakanuma, Y. (2010). Epithelial-mesenchymal transition induced by transforming growth factor-{beta}1/Snail activation aggravates invasive growth of cholangiocarcinoma. *Am J Pathol* 177(1), 141-52.

Sato, Y., Harada, K., Ozaki, S., Furubo, S., Kizawa, K., Sanzen, T., Yasoshima, M., Ikeda, H., Sasaki, M. & Nakanuma, Y. (2007). Cholangiocytes with mesenchymal features contribute to progressive hepatic fibrosis of the polycystic kidney rat. *Am J Pathol* 171(6), 1859-71.

Scholten, D., Osterreicher, C. H., Scholten, A., Iwaisako, K., Gu, G., Brenner, D. A. & Kisseleva, T. (2010). Genetic labeling does not detect epithelial-to-mesenchymal transition of cholangiocytes in liver fibrosis in mice. *Gastroenterology* 139(3), 987-98.

Seki, E., De Minicis, S., Osterreicher, C. H., Kluwe, J., Osawa, Y., Brenner, D. A. & Schwabe, R. F. (2007). TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 13(11), 1324-32.

Sell, S. (2001). Heterogeneity and plasticity of hepatocyte lineage cells. *Hepatology (Baltimore, Md* 33(3), 738-50.

Shao, D. D., Suresh, R., Vakil, V., Gomer, R. H. & Pilling, D. (2008). Pivotal Advance: Th-1 cytokines inhibit, and Th-2 cytokines promote fibrocyte differentiation. *J Leukoc Biol* 83(6), 1323-33.

Shibazaki, S., Yu, Z., Nishio, S., Tian, X., Thomson, R. B., Mitobe, M., Louvi, A., Velazquez, H., Ishibe, S., Cantley, L. G., Igarashi, P. & Somlo, S. (2008). Cyst formation and activation of the extracellular regulated kinase pathway after kidney specific inactivation of Pkd1. *Human molecular genetics* 17(11), 1505-16.

Spirli, C., Fiorotto, R., Song, L., Santos-Sacchi, J., Okolicsanyi, L., Masier, S., Rocchi, L., Vairetti, M. P., De Bernard, M., Melero, S., Pozzan, T. & Strazzabosco, M. (2005). Glibenclamide stimulates fluid secretion in rodent cholangiocytes through a cystic fibrosis transmembrane conductance regulator-independent mechanism. *Gastroenterology* 129(1), 220-33.

Spirli, C., Okolicsanyi, S., Fiorotto, R., Fabris, L., Cadamuro, M., Lecchi, S., Tian, X., Somlo, S. & Strazzabosco, M. (2008). Endothelial Growth Factor (VEGF) signaling reduces ERK1/2 activation and cyst growth in polycysti-2 defective mice. *Hepatology* 48.

Spirli, C., Okolicsanyi, S., Fiorotto, R., Fabris, L., Cadamuro, M., Lecchi, S., Tian, X., Somlo, S. & Strazzabosco, M. (2010). ERK1/2-dependent vascular endothelial growth factor signaling sustains cyst growth in polycystin-2 defective mice. *Gastroenterology* 138(1), 360-71 e7.

Strazzabosco, M. (1997). New insights into cholangiocyte physiology. *Journal of hepatology* 27(5), 945-52.

Strazzabosco, M. & Fabris, L. (2008). Functional anatomy of normal bile ducts. *Anat Rec (Hoboken)* 291(6), 653-60.

Strazzabosco, M., Fabris, L. & Spirli, C. (2005). Pathophysiology of cholangiopathies. *Journal of clinical gastroenterology* 39(4 Suppl 2), S90-S102.

Strazzabosco, M., Spirli, C. & Okolicsanyi, L. (2000). Pathophysiology of the intrahepatic biliary epithelium. *Journal of gastroenterology and hepatology* 15(3), 244-53.

Streets, A. J., Newby, L. J., O'Hare, M. J., Bukanov, N. O., Ibraghimov-Beskrovnaya, O. & Ong, A. C. (2003). Functional analysis of PKD1 transgenic lines reveals a direct role for polycystin-1 in mediating cell-cell adhesion. *J Am Soc Nephrol* 14(7), 1804-15.

Streets, A. J., Wagner, B. E., Harris, P. C., Ward, C. J. & Ong, A. C. (2009). Homophilic and heterophilic polycystin 1 interactions regulate E-cadherin recruitment and junction assembly in MDCK cells. *Journal of cell science* 122(Pt 9), 1410-7.

Strutz, F., Okada, H., Lo, C. W., Danoff, T., Carone, R. L., Tomaszewski, J. E. & Neilson, E. G. (1995). Identification and characterization of a fibroblast marker: FSP1. *The Journal of cell biology* 130(2), 393-405.

Svegliati-Baroni, G., De Minicis, S. & Marzioni, M. (2008). Hepatic fibrogenesis in response to chronic liver injury: novel insights on the role of cell-to-cell interaction and transition. *Liver Int* 28(8), 1052-64.

Taura, K., Miura, K., Iwaisako, K., Osterreicher, C. H., Kodama, Y., Penz-Osterreicher, M. & Brenner, D. A. (2010). Hepatocytes do not undergo epithelial-mesenchymal transition in liver fibrosis in mice. *Hepatology* 51(3), 1027-36.

Thomas, J., Liu, F. & Link, D. C. (2002). Mechanisms of mobilization of hematopoietic progenitors with granulocyte colony-stimulating factor. *Curr Opin Hematol* 9(3), 183-9.

Uutela, M., Lauren, J., Bergsten, E., Li, X., Horelli-Kuitunen, N., Eriksson, U. & Alitalo, K. (2001). Chromosomal location, exon structure, and vascular expression patterns of the human PDGFC and PDGFD genes. *Circulation* 103(18), 2242-7.

Varcoe, R. L., Mikhail, M., Guiffre, A. K., Pennings, G., Vicaretti, M., Hawthorne, W. J., Fletcher, J. P. & Medbury, H. J. (2006). The role of the fibrocyte in intimal hyperplasia. *J Thromb Haemost* 4(5), 1125-33.

Venkov, C. D., Link, A. J., Jennings, J. L., Plieth, D., Inoue, T., Nagai, K., Xu, C., Dimitrova, Y. N., Rauscher, F. J. & Neilson, E. G. (2007). A proximal activator of transcription in epithelial-mesenchymal transition. *The Journal of clinical investigation* 117(2), 482-91.

Vroman, B. & LaRusso, N. F. (1996). Development and characterization of polarized primary cultures of rat intrahepatic bile duct epithelial cells. *Laboratory investigation; a journal of technical methods and pathology* 74(1), 303-13.

Ward, C. J., Hogan, M. C., Rossetti, S., Walker, D., Sneddon, T., Wang, X., Kubly, V., Cunningham, J. M., Bacallao, R., Ishibashi, M., Milliner, D. S., Torres, V. E. & Harris, P. C. (2002). The gene mutated in autosomal recessive polycystic kidney disease encodes a large, receptor-like protein. *Nature genetics* 30(3), 259-69.

Wells, R. G. (2010). The epithelial-to-mesenchymal transition in liver fibrosis: here today, gone tomorrow? *Hepatology* 51(3), 737-40.

Wheeler, M. D., Kono, H., Yin, M., Nakagami, M., Uesugi, T., Arteel, G. E., Gabele, E., Rusyn, I., Yamashina, S., Froh, M., Adachi, Y., Iimuro, Y., Bradford, B. U., Smutney, O. M., Connor, H. D., Mason, R. P., Goyert, S. M., Peters, J. M., Gonzalez, F. J., Samulski, R. J. & Thurman, R. G. (2001). The role of Kupffer cell oxidant production in early ethanol-induced liver disease. *Free Radic Biol Med* 31(12), 1544-9.

Willis, B. C., duBois, R. M. & Borok, Z. (2006a). Epithelial origin of myofibroblasts during fibrosis in the lung. *Proceedings of the American Thoracic Society* 3(4), 377-82.

Willis, B. C., duBois, R. M. & Borok, Z. (2006b). Epithelial Origin of Myofibroblasts during Fibrosis in the Lung. *Proc Am Thorac Soc* 3(4), 377-82.

Witek, R. P., Stone, W. C., Karaca, F. G., Syn, W. K., Pereira, T. A., Agboola, K. M., Omenetti, A., Jung, Y., Teaberry, V., Choi, S. S., Guy, C. D., Pollard, J., Charlton, P. & Diehl, A. M. (2009). Pan-caspase inhibitor VX-166 reduces fibrosis in an animal model of nonalcoholic steatohepatitis. *Hepatology* 50(5), 1421-30.

Wu, G., D'Agati, V., Cai, Y., Markowitz, G., Park, J. H., Reynolds, D. M., Maeda, Y., Le, T. C., Hou, H., Jr., Kucherlapati, R., Edelmann, W. & Somlo, S. (1998). Somatic inactivation of Pkd2 results in polycystic kidney disease. *Cell* 93(2), 177-88.

Wu, Y. & Zhou, B. P. (2008). New insights of epithelial-mesenchymal transition in cancer metastasis. *Acta Biochim Biophys Sin (Shanghai)* 40(7), 643-50.

Xia, J. L., Dai, C., Michalopoulos, G. K. & Liu, Y. (2006). Hepatocyte growth factor attenuates liver fibrosis induced by bile duct ligation. *The American journal of pathology* 168(5), 1500-12.

Yang, J. & Liu, Y. (2001). Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. *Am J Pathol* 159(4), 1465-75.

Yook, J. I., Li, X. Y., Ota, I., Hu, C., Kim, H. S., Kim, N. H., Cha, S. Y., Ryu, J. K., Choi, Y. J., Kim, J., Fearon, E. R. & Weiss, S. J. (2006). A Wnt-Axin2-GSK3beta cascade regulates Snail1 activity in breast cancer cells. *Nat Cell Biol* 8(12), 1398-406.

Zeisberg, M. & Kalluri, R. (2008). Fibroblasts emerge via epithelial-mesenchymal transition in chronic kidney fibrosis. *Front Biosci* 13, 6991-8.

Zeisberg, M., Yang, C., Martino, M., Duncan, M. B., Rieder, F., Tanjore, H. & Kalluri, R. (2007). Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *The Journal of biological chemistry* 282(32), 23337-47.

Zerres, K., Mucher, G., Bachner, L., Deschennes, G., Eggermann, T., Kaariainen, H., Knapp, M., Lennert, T., Misselwitz, J., von Muhlendahl, K. E. & et al. (1994).

Mapping of the gene for autosomal recessive polycystic kidney disease (ARPKD) to chromosome 6p21-cen. *Nature genetics* 7(3), 429-32.

Zerres, K., Rudnik-Schoneborn, S., Steinkamm, C., Becker, J. & Mucher, G. (1998). Autosomal recessive polycystic kidney disease. *J Mol Med* 76(5), 303-9.