University of Alberta

Investigating The Role Of Fibrocystin/Polyductin In Cholangiocarcinoma

by

Yasser Habeeb Abuetabh

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Master of Science

Medical Sciences – Laboratory Medicine and Pathology

Spring 2013

Edmonton, Alberta

I dedicate this thesis to my lovely wife and daughter

Abstract

Cholangiocarcinoma (CCA) represents a very devastating liver neoplasm, whose incidence is increasing worldwide. CCA is often diagnosed in its late stages, and the treatment options for this disease are restricted to surgical resection and liver transplantation.

Our group demonstrated that fibrocystin/polyductin (FPC) plays a significant role in the development of the bile duct. Furthermore, FPC expression was detected in infantile cholangiopathies as well as in adult CCA.

This thesis demonstrates the presence of differential abnormal expression and localization patterns of cell adhesion molecules in the CCA cell lines, which was not associated with FPC expression. Furthermore, this data supports that FPC is an essential growth factor for CCA cells.

Although much remains to be elucidated, the data presented in this thesis may represent a step forward for investigating the intracellular mechanisms that underline the development of CCA, hence providing insight into future therapeutic options for CCA.

Acknowledgment

First, I would like to express my gratefulness and appreciation to my supervisor, Dr. Consolato Sergi. He has been extremely supportive, patient and understanding, providing me with invaluable skills, opportunities, and knowledge. As a result, I am very proud to be one of his students.

I would like also to thank Dr. Sujata Persad, who showed extreme generosity towards me. Her constructive feedback and continuous support are very much appreciated.

My gratitude is also extended to Dr. Judith Hugh and Dr. Kim Solez for their insightful instruction, valuable critiques and continual advice.

Many thanks to Dr. Jonathan Martin for coordinating the defense, as his efforts are highly valued.

I am thankful to all of my lab colleagues, especially Redha Albahrani and Wesam Bahitham, for their support, wisdom, and friendship.

I am grateful to the Saudi Arabia Cultural Bureau for their generous financial support.

I would also extend my appreciation to my family. My parents' love, support, and encouragement always kept me optimistic. Without my parents' continuous prayers, this thesis would not have been possible.

Finally, I would like to express my profound and sincere gratitude to my wonderful wife, Fatimah, for her permanent love, support, and encouragement as well as for believing in me.

Table of Contents

List of Figures

Abbreviations

aa	Amino acids
ADPKD	Autosomal dominant polycystic kidney diseases
AJ	Adherent junctions
APC	Adenomatous polyposis coli
ARPKD	Autosomal recessive polycystic kidney diseases
BA	Biliary atresia
BCA	Bicinchoninic acid
BD	Bile duct
BECs	Biliary epithelial cells
BSA	Bovin serum albumin
CA125	Carbohydrate antigen 125
CA19-9	Carbohydrate Antigen 19-9
CCA	Cholangiocarcinoma
CEA	Carcinoembryonic antigen
CHF	Congenital hepatic fibrosis
COX-2	Cyclooxygenase-2

DP	Ductal Plate
DPM	Ductal plate malformation
Dsh	Dishevelled
E-cad	E-cadherin
ECL	Enhanced chemi-luminescence
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular-regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FPC	Fibrocystic/polyductin
GIT	Gastrointestind tract
GJ	Gap junctions
GSK-3	Glycogen synthetase kinase-3
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus

HEK293	Human Embryonic kidney 293
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth-factor receptor
HRP	Horse radish peroxidase
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IPT	Immunoglobulin-like plexin-transcription-factor
K-19	Keratin-19
MAPK	Mitogen Activated protein kinase
Mcl-1	Myeloid cell leukemia-1
MDCK	Madin-darby canine kidney epithelial cells
mTMCD-K2	mouse inner medulary collecting duct cell line
mTOR	mammalian target of rapamycin
N-cad	N-cadherin
NO	Nitric oxide
ORF	Open reading frame
PbH1	Parallel beta-helix-1

PBS	Phosphate buffer saline
PC	Proprotein convertase
PC1	Polycystin-1
PC2	Polycystin-2
Pg	Plakoglobin
PI3K	Phosphatidylinositol 3-kinase
PKD	Polycystic kidney diseases
PKHD-1	Polycystic kidney hepatic disease-1
PM	Portal mesenchyme
PSC	Primary sclerosing cholangitis
PV	Portal vein
PVDF	Polyvinylidene difluoride membrane
RBD	Remodelling bile duct
RDP	Remodelling ductal plate
RGD	Arginine-glycine-aspartate
RIP	Regulated intramembrane proteolysis
RIPA	Radio-immuno precipitation assay

RPMI-1680	Roswell park memorial institute-1680
RT-PCR	Real-time polymerase reaction
SDS-PAGE	Sodium-dodecyle-sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
STAT6	Single transducer and activator of transcription6
TALH	Thick ascending limb of henle
TCF/LEF	T-cell factor /Lymphoid enhancer factor
TGF-β	Transforming growth factor beta
TJ	Tight junctions
TNF-α	Tumour necrosis factor-α
X-cat	X-catenin
α-FP2	α-fused protein-2
β-cat	β-catenin

CHAPTER 1: INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Polycystic kidney diseases (PKD)

Polycystic kidney diseases (PKDs) are congenital inherited disorders that mainly affect the kidney and liver. PKDs entail diverse forms with various pathogeneses and clinical features. Specifically, its two main forms include autosomal recessive PKD (ARPKD) and autosomal dominant PKD (ADPKD), both of which are clinically significant and characterized by cysto-genesis in the kidney, liver and other gastrointestinal tract (GIT) organs (Wilson and Goilav, 2007).

1.1.1 Autosomal Dominant Polycystic Kidney Disease

ADPKD, commonly referred to as "adult-PKD," is the most frequent and severe form of PKD, with an incidence rate of 1:1000 live births. The symptoms of ADPKD commonly appear at early adulthood, and include hypertension, urinary tract infections, kidney stones, hematuria and polyuria. Although the major characteristic features of ADPKD involve kidney enlargement as well as numerous renal, liver and pancreatic cysts, ADPKD patients may also develop diverticulosis in addition to intracerebral and aortic aneurysms (Wilson, 2004;Wilson and Goilav, 2007). Approximately half of ADPKD patients will incur end-stage kidney failure. The main causes of ADPKD involve mutation in PKD1 (~ 85%) and PKD2 (~ 15%) genes. Polycystin-1 (PC1) and polycystin-2 (PC2) compose the protein products of PKD1 and PKD2 genes, respectively. PC1

(~460kDa) and PC2 (110kDa) are membranous proteins with short intracellular C-terminals that contain many potential phosphorylation sites (Figure 1.1) (Giamarchi,Padilla, et al, 2006;Hughes,Ward, et al, 1995). Unlike PC2, which possesses a short intracellular N-terminal domain, PC1 contains a long extracellular N-terminal domain. These characteristics suggest that PC1 functions as a plasma-membrane receptor while PC2 acts as the calcium channel (Figure 1.1) (Giamarchi,Padilla, et al, 2006;Hughes,Ward, et al, 1995). Studies have illustrated that PC1 and PC2 interact at their cytoplasmic domains and form a heterodimeric complex (Newby,Streets, et al, 2002). Both proteins have been found to be localized in primary cilia (Yoder,Hou and Guay-Woodford, 2002). The complexes of both polycystin proteins affect a wide range of signal transduction pathways, such as the canonical Wnt pathway, the mammalian target of rapamycin (mTOR) pathway, the phosphatidylinositol 3-Kinase (PI3K) pathway, the JAK/STAT pathway, and the Ras pathway (Bhunia,Piontek, et al, 2002;Boca,D'Amato, et al, 2007;Kim,Arnould, et al, 1999;Ong and Harris, 2005;Weimbs, 2006).

1.1.2Autosomal Recessive Polycystic Kidney disease

ARPKD is characterized by cystic change, abnormal formation of the bile duct system, bile duct dilation and marked kidney enlargement, all of which lead to both kidney failure and death. Although ARPKD constitutes one of the major causes of renal and hepatic-related deaths among infants and newborns, its incidence is considerably rare, occurring in 1:20,000 - 1:40,000 live births. ARPKD has been frequently found in association with congenital hepatic fibrosis

(CHF) and Caroli's syndrome. Specifically, lung hypoplasia represents the main cause of death in 30% of newborns with ARPKD, whose complications include hypertension, portal hypertension and liver fibrosis. ARPKD forms are caused by mutations in the Polycystic Kidney and Hepatic Disease-1 (PKHD1) gene. While Ward C. et al. (2002) have identified and named the protein product of PKHD1 as Fibrocystin (Ward,Hogan, et al, 2002), Onuchic L. et al. (2002) have termed this protein as Polyductin (Onuchic,Furu, et al, 2002). Hence, most recently published articles refer to this protein as Fibrocystin/Polyductin (FPC). Furthermore, Xiong H. et al. (2002) have identified this product as Tigmin (Xiong,Chen, et al, 2002). Additional information about ARPKD can be obtained in a significant body of research (Al-Bhalal and Akhtar, 2008;Dell, 2011;Menezes and Onuchic, 2006;Onori,Franchitto, et al, 2010;Wen, 2011;Wilson and Goilav, 2007;Xiong,Chen, et al, 2002)).

1.1.2.1 The PKHD1 gene and its transcriptional protein product, the FPC

The PKHD1 gene has been discovered and mapped to chromosome 6p12.2. In particular, PKHD1 is a large human gene with a genomic size of ~ 469 kb and more than 86 exons. Although PKHD1 is assumed to encode multiple alternatively-spliced transcripts, its longest open reading frame (ORF) encodes a membrane-bond protein, the FPC, which has a molecular weight of ~ 440 kDa and contains 4,074 amino acids (aa). Structurally, FPC possesses a single transmembrane domain, a long extracellular N-terminal and a short cytoplasmic C-terminal (Figure 1.1) (Onuchic,Furu, et al, 2002;Ward,Hogan, et al, 2002). Researchers have predicted the N-terminal to contain several immunoglobulin-

like fold (IPT) domains and multiple parallel beta-helix 1 repeats (PbH1); conversely, the C-terminal includes a number of potential cAMP/cGMP-dependent protein kinase phosphorylation sites. Therefore, FPC was found to share variable homologies with different proteins that play a fundamental role in cell proliferation, such as the hepatocyte growth-factor receptor (HGFR), the Ron class of tyrosine kinase receptors, D86 and plexins (Onuchic,Furu, et al, 2002;Ward,Hogan, et al, 2002). Furthermore, the literature has identified an important motif in FPC that contains an arginine-glycine-aspartate (RGD) domain, which is found in various proteins that fulfill a cell adhesion role. Accordingly, FPC is believed to participate in cell adhesion (Onuchic,Furu, et al, 2002). The expression profile of PKHD1 has been elucidated in various infant and adult organs. Specifically, PKHD1 is strongly expressed in the kidneys and slightly expressed in the pancreas and liver (Onuchic,Furu, et al, 2002;Ward,Hogan, et al, 2002). Ward et al. and Menezes et al. have generated several monoclonal and polyclonal antibodies to not only detect and identify the expression profile of FPC but also to detect potential FPC isoforms (Menezes,Cai, et al, 2004;Ward,Yuan, et al, 2003). For the most part, both research groups revealed consistent results; western blot data analysis illustrated three distinct bands with various molecular weights: > 450 kDa, 220-230 kDa and 140-150 kDa. The largest band was detected in kidney, liver and pancreas samples from normal human adults, rats and mice. Moreover, the 220-230kDa band was also identified in kidney and liver specimens. Both large bands apparently signified membrane-bond products; however, the largest band represented the expected

product of the longest ORF that encodes the FPC (Menezes,Cai, et al, 2004;Ward,Yuan, et al, 2003). Menezes et al. identified a strong 140 kDa band in soluble cellular fractions of the kidney, liver and pancreas, prompting the authors to conclude that this band comprises a soluble protein (Menezes,Cai, et al, 2004). Immunohistochemical analysis revealed that FPC intensively stained the cortical and medullar collecting ducts as well as the thick ascending limb of Henle (TALH) in the human adult kidney. In addition, researchers also showed FPC staining on biliary and pancreatic duct epithelia (Menezes,Cai, et al, 2004;Ward,Yuan, et al, 2003). The embryonic human and mouse kidney samples revealed strong staining in the branching ureteric bud and collecting ducts as well as in the intra- and extrahepatic biliary tracts and pancreatic ducts (Menezes,Cai, et al, 2004;Ward,Yuan, et al, 2003). Immunofluorescent studies using Madin-Darby canine kidney epithelial cells (MDCK) and mouse inner medullary collecting duct cell lines (mIMCD-K2) demonstrated that FPC is localized in the cytoplasm, apical plasma membrane and primary cilia. Further immunoelectron microscopy studies on normal human and mouse kidney samples demonstrated that FPC is located in the primary cilia (Menezes,Cai, et al, 2004;Ward,Yuan, et al, 2003). Several studies have highlighted the proteolytic processes involving possible FPC functions (Hiesberger,Gourley, et al, 2006;Kaimori,Nagasawa, et al, 2007). In particular, research has speculated that FPC undergoes sophisticated and regulated intramembrane proteolysis (RIP) processes that are accomplished by the cleavage and release of its cytoplasmic tail via γ-secretase. In this case, the released C-terminal fragments translocate to the nucleus. However, the cleavage

and release of the ectodomain of FPC is essential prior to C-terminal cleavage. The N-terminal domain of FPC contains a proprotein convertase (PC) site. Upon cleavage, a large N-terminal fragment is shed by a member of a disintegrin and metalloproteinase (ADAM) family. Furthermore, Hiesberger T. et al. demonstrated that the release of the C-terminal fragment is regulated by calcium (Hiesberger,Gourley, et al, 2006) and that the released C-terminal fragments translocate to and localize in nucleoli (Hiesberger,Gourley, et al, 2006). Since the FPC undergoes a regulated proteolytic processing, Kaimori J. et al speculated that FPC might function as a bidirectional signaling molecule (Kaimori,Nagasawa, et al, 2007). Moreover, these authors showed that FPC's N-terminal fragment is released from the primary cilia into the lumen, producing the hypothesis that this process may be essential for delivering cell signals to distant targets (Kaimori,Nagasawa, et al, 2007). Similar to FPC, PC1 has been found to undergo proteolytic processes, where its released C-terminal fragments translocate into the nucleus. The nuclear accumulation of PC1C-terminal fragments interacts with the signal transducer and activator of transcription 6 (STAT6) as well as the co-activator P100. These interactions stimulate STAT6-dependent gene expression (Low,Vasanth, et al, 2006). Nevertheless, FPC's location in the primary cilia is not linked to PC1, since pkd1 null/null cells demonstrated normal FPC expression patterns (Wang,Luo, et al, 2004). Mai W. et al (2005) revealed that the down-regulation of FPC mediated by short hairpin (sh) RNA in mouse IMCD cell lines revealed numerous abnormalities, including tubular malformation, abnormal cell-cell contact, aberrant cell proliferation and apoptosis as well as the dysregulation

of extracellular-regulated kinase (ERK) and focal adhesion kinase (FAK) signalling pathways (Mai,Chen, et al, 2005). Furthermore, Fischer D. et al (2009) established that the AKT/mTOR pathway is activated in ARPKD (Fischer,Jacoby, et al, 2009), and Sun L. et al (2011) demonstrated that the down-regulation of FPC in human embryonic kidney 293 (HEK293) cell lines increases PI3K/AKT activity, which ultimately causes aberrant apoptosis. Therefore, these authors concluded that FPC fulfills a crucial function in the regulation of apoptosis (Sun,Wang, et al, 2011). Our research group has investigated the expression profile and role of FPC in the development of the primitive intrahepatic biliary system as well as in perinatal and infantile cholangiopathies. Moreover, they also studied the FPC expression profile in liver carcinomas represented by hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA)(Dorn,Menezes, et al, 2009). Data provided by Dorn L. et al (2009) demonstrated that FPC fulfills a fundamental role in the development of the primitive intrahepatic biliary system, where FPC is predominantly expressed during early stages, ductal plate (DP) and remodelling ductal plate (RDP), and absent when the bile duct is remodelled (remodelled bile duct (RBD)) (Figure 1.2) (Dorn,Menezes, et al, 2009). However, FPC was re-expressed during abnormal development of the biliary system, which is also known as ductal plate malformation (DPM) (Figure 1.2) (Dorn,Menezes, et al, 2009). Moreover, FPC expression was also detected in infantile cholangiopathies including CHF, neonatal cholestasis, biliary atresia (BA), and neonatal hepatitis. While FPC expression was detected in CCA samples, it was absent in HCC samples (Dorn,Menezes, et al, 2009). Consequently, the potential

role of FPC in carcinogenesis remains obscure. This ambiguity prompted many questions: 1) Does FPC fulfill significant roles in the malignant transformation of cholangiocytes? 2) What are the consequences of impairing the expression of FPC in CCA cell lines? 3) Does FPC have potential therapeutic actions in CCA?

1.2 Bile Duct Development

During liver development, endoderm-derived bipotential progenitor cells, or hepatoblasts, surrounding the portal vein develop into biliary epithelial cells (BECs) while the parenchymal hepatoblasts become hepatocytes. For the duration of the 9^{th} to 12^{th} gestational weeks, the ductal plate (DP) forms around the portal vein, where the surrounding hepatoblasts grow a double-layered cylinder of biliary-type cells that possess decreased hepatic genes and elevated biliary-type genes such as keratin-19 (K-19). Consequently, throughout the 13^{th} to 17^{th} gestational weeks, some biliary-type cells migrate into the mesenchyme in the direction of the portal vein. This stage of development is referred to as remodeling ductal plate (RDP). From the 18^{th} to the 40^{th} gestational weeks, ductal plate regression mediated by apoptosis and mature bile duct formation are recognized; this stage is known as remodeled bile duct (RBD) (Figure 1.3) (Haruna,Saito, et al, 1996;Sergi,Adam, et al, 2000;Sergi,Adam, et al, 2000). Two or more of these developmental stages might occur simultaneously. The occurrence of multiple phases in the same liver sample is considered normal, as the development of the

intrahepatic bile duct progresses from the hilum portion to the peripheral portion of the liver (Sergi,Adam, et al, 2000). Ductal plate malformation (DPM) consists of incomplete regression or the continued appearance of the ductal plate. This condition is evidenced in many hepato-cholangiopathies, such as Meckel syndrome, Caroli's disease and CHF. Intrahepatic bile duct development abnormalities, including choledochal cysts, Caroli's syndrome and fibropolycystic diseases of the liver and kidney, have been intensively studied and linked to CCA development in young patients (Lipsett,Pitt, et al, 1994;Scott,Shousha, et al, 1980;Yamada and Alpers, 2009). Nevertheless, the carcinogenesis mechanism remains poorly understood. Many signaling pathways have been identified as fulfilling essential roles during bile duct development, including transforming growth factor (TGF)-β, Wnt/β-catenin (β-cat) and Notch (Zorn, 2008). Specifically, TGF-β and Wnt/β-cat interactions have been intensively investigated and linked to the regulation of many biological events, such as embryonic development as well as cell proliferation, migration differentiation and malignant transformation. Decaens T. et al (2008) successfully proved that the Wnt/β-cat pathway regulates the differentiation of the hepatoblast via down-regulation of the hepatic genes, which favors the remodeling of the ductal plate (Decaens,Godard, et al, 2008). Alternatively, Clotman F. et al (2005) demonstrated that a gradient expression of the Activin/TGF-β signaling pathway is compulsory for hepatoblast differentiation. Their data revealed that the high expression of Activin/TGF-β around the portal vein area is responsible for differentiating hepatoblasts into BECs. On the other hand, the lower expression of Activin/TGF-β detected in the

parenchyme is required for the differentiation of hepatoblasts into hepatocytes (Clotman,Jacquemin, et al, 2005). Indeed, our group's data on FPC has probably imperative implications for human intrahepatic bile duct development (Dorn,Menezes, et al, 2009).

1.3 Cell-Cell interaction of the adhesion molecules

Cell-cell junctions are not only important in cell adhesion but they also participate in morphogenesis and fulfill central roles in maintaining the rigidity and integrity of solid tissues. Based on the various molecular compositions and ultrastructural appearances, these junctions can be classified into gap junctions (GJ), tight junctions (TJ), adherent junctions (AJ), and desmosomes (Franke, 2009;Giepmans and van Ijzendoorn, 2009). However, this thesis focuses specifically on AJs and desmosomes. GJs are mostly described as cell-cell channels that are responsible for micro-molecule (<1KDs) exchanges. In particular, the connexin protein family constitutes an essential component of this junction type (Wei,Xu and Lo, 2004). Furthermore, the crucial building-block units of TJs include transmembranous proteins, named claudins, which are described as branched and tightened bands forming transport barriers (Martin and Jiang, 2009).

1.3.1 Desmosomes

Desmosomes represent another cell-cell junction category that provides extremely strong adherent points between adjacent cells, which protect them from

mechanical stress. The extracellular region of desmosomes consists of special membrane-bound proteins of the cadherin family, known as desmogleins and desmocollins, or the desmosomal cadherins. The cytoplasmic domains of cadherins interact with members of the armadillo family, including plakoglobin (Pg), also known as γ-catenin, and the plakophilins, which subsequently bind to desmoplakin. Ultimately, desmoplakin binds to the intermediate filaments of the cytoskeleton (Delva,Tucker and Kowalczyk, 2009;Garrod and Chidgey, 2008;Stokes, 2007). Various diseases and disorders, such as epidermal blistering and cardiomyopathy, have been associated with the disruption of desmosomal components (Brooke,Nitoiu and Kelsell, 2012;Chidgey, 2002). In general, several studies have proposed that some dysregulation of desmosomal components may be linked to the progression and invasiveness of several malignant neoplasms, including lung, prostate, gastric and colorectal carcinomas (Breuninger,Reidenbach, et al, 2010;Dusek and Attardi, 2011;Furukawa,Daigo, et al, 2005). Nonetheless, studies have documented that the re-expression of desmosomal components might possess the ability to suppress tumour invasiveness (Dusek and Attardi, 2011).

1.3.2 Adherent Junctions

AJs are generally represented by cadherin-catenin units, which create the greatest strength between adjacent cells. These units fulfill significant functions in the regulation of many crucial biological events comprising cell differentiation, motility, morphology and proliferation. Thus, the dysregulation, destruction or loss of cadherin-catenin units could feasibly trigger cell disconnection,

dissociation and abnormal morphology as well as tumor development, progression and invasiveness (Hirohashi, 1998;Pinho,Matos, et al, 2007;Schmalhofer,Brabletz and Brabletz, 2009). Cadherins encompass a large family of membranous glycoproteins, including epithelial E-cadherins and neural N-cadherins (E-cad and N-cad). On one side, the cadherin-catenin units are constructed when the cytoplasmic domains of cadherins bind to a chain of catenins, including β-catenin (β-cat), Pg, p120-catenin and α-catenin (α-cat), which eventually attach to the actin of the cytoskeleton. On the other side, adjacent cells are tethered together via the interactions between the extracellular domains of cadherins (Meng and Takeichi, 2009;Ozawa and Kemler, 1992;Pecina-Slaus, 2003;Shapiro and Weis, 2009). The dysregulation of E-cad and N-cad expressions have been strongly linked to cancer prognosis, progression, invasiveness and metastasis (Hirohashi, 1998;Kim,Islam, et al, 2000;Pinho,Matos, et al, 2007). Furthermore, aberrant E-cad and N-cad localizations and expressions have shown the ability to induce one of the cancer hallmarks: epithelial to mesenchymal transition (EMT) (Agiostratidou,Hulit, et al, 2007). The chief member of the catenin family is β-cat, a multifunctional protein that possesses numerous structural and transcriptional functions (Lin,Xia, et al, 2000). Research has established that aberrant β-cat transcriptional activity is correlated with malignant transformation and tumour progression in several types of cancers (Takayama,Shiozaki, et al, 1996). Moreover, β-cat is considered the central molecule in the Wnt canonical signalling pathway. In the unstressed cells and in the absence of Wnt protein, β-cat creates a cascade with Anxin and Adenomatous Polyposis Coli (APC) as

well as glycogen synthetase kinase-3 (GSK-3). Consequently, GSK-3 activates the phosphorylation of β-cat through key sites involving the serine/threonine residues of β-cat. Thus, phosphorylated β-cat undergoes protosomal degradation and ubiquitination. Nevertheless, the binding of Wnt ligands to the frizzled receptors activates the phosphorylation of the dishevelled family proteins (Dsh). Since Dsh dephosphates the sub-membrane complex, including ANXIN, APC and GSK3, β-cat escapes from its association with the complex. Accordingly, β-cat molecules accumulate in the cytoplasm and subsequently translocate into the nucleus, where they bind with the T-cell factor/lymphoid enhancer factor (TCF/LEF) complex (Eastman and Grosschedl, 1999) to synchronize specific gene targets. β-cat mediates cell growth and cell proliferation by targeting several genes, such as the oncogenes c-Myc and c-Jun (Anna,Iida, et al, 2003), survivin and cyclin D (Smalley and Dale, 1999). Although Pg, which shares a structural homology with β-cat, has shown the ability to translocate into the nucleus and bind with TCF/LEF, the Pg/TCF/LEF complex has no capability of binding with the DNA. Accordingly, the nuclear localization of Pg may negatively regulate the transcriptional activity of β-cat, thus indicating that Pg contains the potential for tumour suppression activities (Aktary,Chapman, et al, 2010;Rieger-Christ,Ng, et al, 2005;Simcha,Shtutman, et al, 1998;Zhurinsky,Shtutman and Ben-Ze'ev, 2000).

1.4 Bile Duct Cancer

The primary tumor of the bile duct, known as cholangiocarcinoma (CCA), has attracted significant attention as the second most common malignant liver

neoplasm due to its unique etiology, association with inflammation and increased incidence in certain geographical regions. CCA has been classified according to its areas of origin in the intra-hepatic and extra-hepatic epithelium of the bile duct. Among all patients with liver malignancies, CCA accounts for 10-20% of patient deaths. As a highly infiltrative tumour, CCA possesses significant potential for expanding and metastasizing within the liver, resulting in its frequent diagnosis at very advanced stages. Consequently, the treatment options for CCA are limited to surgical resection and liver transplantation (Blechacz and Gores, 2008;Blechacz and Gores, 2008;Gatto,Bragazzi, et al, 2010;Mosconi,Beretta, et al, 2009;Nakagohri,Kinoshita, et al, 2008;Nakeeb,Pitt, et al, 1996). The pathogenesis of CCA and the causes of its globally increasing incidence remain unknown; as a result, CCA requires extensive investigation to clarify its pathogenesis and hence to identify alternative therapeutic pathways.

1.4.1 Anatomical and histological classifications of CCA

Anatomically, CCA has been classified as extra- and intrahepatic. Extrahepatic CCA is further subdivided according to its distance from the liver hilum: perihilar or Klatskin, middle and distal (Mosconi,Beretta, et al, 2009). Furthermore, both extra- and intrahepatic CCAs are morphologically categorized into additional subtypes. Thus, intrahepatic CCA can be subdivided into mass-forming, periductal infiltrating, and intraductal growth, while subdivisions of extrahepatic CCA include sclerosing, nodular, and papillary (Chung,Kim, et al, 2009). From a histologic point of view, CCA is classified into several types, 90% of which are

adenocarcinomas. These histological types include adenosequamous carcinoma, sequamous cell carcinoma, papillomatosis, malignant fibrous histocytoma, mucinous adenocarcinoma, and small cell carcinoma (Mosconi,Beretta, et al, 2009).

1.4.2 Epidemiology of CCA

According to the International Agency for Research on Cancer (IARC), cancer-related death accounted for approximately 13% of all worldwide fatalities in 2008. Among these mortalities, hepatobiliary cancer is one of the leading cancer-causing deaths along with lung, stomach and colorectal cancers (Ferlay,Shin, et al, 2010). Next to HCC, CCA is identified as the second most common primary malignant liver neoplasm and accounts for 10% - 20% of all liver cancer deaths. Although both sexes are susceptible to CCA, more men than women are likely to incur this condition (Shaib and El-Serag, 2004). CCA is frequently diagnosed in patients in their forties and fifties and its occurrence varies worldwide, likely due to the use of diverse pathogenetic manners (Shaib and El-Serag, 2004). In particular, the highest incidence rate was ascertained in Southeast Asia, where the manifestation with the most recognizable CCA risk factor, liver flukes, such as *Opisthorchis viverrini* and *Clonorchis sinensis*, are endemic (Shaib and El-Serag, 2004;Shin,Oh, et al, 2010;Srivatanakul,Parkin, et al, 1991).

1.4.3 Risk factors and pathogenesis of CCA

Chronic inflammation of biliary epithelium, particularly those mediated by *Opisthorchis viverrini* and *Clonorchis sinensis*, demonstrates the highest association with CCA development (Mairiang,Laha, et al, 2012;Pinlaor,Ma, et al, 2004). In particular, Northwest Thailand is considered as an endemic region of *O. viverrini* infection due to the local consumption of undercooked freshwater fish that host this parasite (Kaewpitoon,Kaewpitoon and Pengsaa, 2008;Ohshima,Bandaletova, et al, 1994). In Thailand, the occurrence of *O. viverrini* has been significantly increasing over the past 100 years (Sithithaworn,Andrews, et al, 2012). In addition to *O. viverrini*, primary sclerosing cholangitis (PSC) represents another chronic inflammation that has been significantly linked to CCA development, with an incidence rate of 5-15% (Burak,Angulo, et al, 2004;Halliday,Djordjevic, et al, 2012;LaRusso,Shneider, et al, 2006). Furthermore, patients with hepatolithiasis, which is endemic in East Asia, are also in danger of developing CCA (Lesurtel,Regimbeau, et al, 2002;Mori,Sugiyama and Atomi, 2006;Tsui,Lam, et al, 2011). Although viral hepatitis infections from the hepatitis B virus (HBV) and the hepatitis C virus (HCV) have been associated with biliary carcinogenesis, their pathogenesis remains poorly understood (Lee,Lee, et al, 2008;Na,Huang, et al, 2011;Srivatanakul,Honjo, et al, 2010). Another well-established risk factor involves exposure to thorotrast, which was used as a versatile medical radiography contrast agent from the 1920s to the 1940s. Specifically, thorotrast toxicity has led to CCA, HCC, and angiosarcoma (Charles,Mill and Darley,

2003;Cohn,Gusmano and Robertson, 1967;Lee,Tharakan, et al, 1996;Lipshutz,Brennan and Warren, 2002;Silverman,Ram and Korobkin, 1983;Stern,Zamenhof and Dawson-Hughes, 1993;Zhu,Lauwers and Tanabe, 2004). As previously mentioned, patients with intrahepatic bile duct development abnormalities, such as choledochal cysts, Caroli syndrome and fibropolycystic diseases of the liver and kidney, are also at risk for CCA (Lipsett,Pitt, et al, 1994;Scott,Shousha, et al, 1980;Yamada and Alpers, 2009).

Based on these conditions, many typical biological reactions that occur during chronic inflammation might lead to the malignant transformation of cholangiocytes. For instance, responses occurring in the biliary microenvironment may include the recruitment of inflammatory cells, the release of cytokines and chemokines and the release of growth factors as well as the activation and deactivation of numerous important cell signaling pathways (Berthiaume and Wands, 2004;Fava,Marzioni, et al, 2007;Ustundag and Bayraktar, 2008). Cholangiocytes, along with inflammatory cells and hepatocytes, abundantly express cytokines during the course of chronic inflammation. These cytokines have been demonstrated to stimulate inducible nitric oxide synthase (iNOS) expression by cholangiocytes, and hence, the accumulation of nitric oxide (NO). Aberrant NO expression has a significant association with the downregulation of DNA repair proteins and up-regulation of Notch proteins in PSC and CCA (Ishimura,Bronk and Gores, 2005;Jaiswal,LaRusso, et al, 2000;Spirli,Fabris, et al, 2003;Torok,Higuchi, et al, 2002). Furthermore, in the state of chronic inflammation, BECs express abnormal levels of several molecules, including

interleukin-6 (IL-6), TGF-β, hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-α) and cytooxygenase-2 (COX-2), all of which are associated with promoting the carcinogenic mechanisms of CCA (Brown and DuBois, 2005;Isomoto,Kobayashi, et al, 2005;Lai,Radaeva, et al, 2000;Yazumi,Ko, et al, 2000;Yoon,Werneburg, et al, 2002;Zhang,Lai and Sirica, 2004). For instance, the anti-apoptotic protein, myeloid cell leukemia-1 (Mcl-1), can be up-regulated during IL-6 elevation. This protein also increases the activity of STAT3, which subsequently controls Mcl-1 expression. Therefore, the up-regulation of IL-6 promotes cell survival in CCA(Gores, 2003;Isomoto,Kobayashi, et al, 2005;Isomoto,Mott, et al, 2007;Kobayashi,Werneburg, et al, 2005;Okada,Shimizu, et al, 1994;Park,Tadlock, et al, 1999;Sugawara,Yasoshima, et al, 1998). The accumulation of bile acid during cholestasis is also considered as a malignant promoter in which bile acids promote the activity of epidermal growth factor receptor (EGFR), PI3K, mitogen-activated protein kinase (MAPK) and COX-2(Lipson,Pang, et al, 1998;Werneburg,Yoon, et al, 2003;Yoon,Higuchi, et al, 2002).

1.4.4 Laboratory Analysis and Biomarkers in CCA Diagnosis

CCA is widely recognized as a “silent killer” because the symptoms usually appear at an advanced stage of the disease. Specifically, CCA is difficult to diagnose at early stages due to the lack of specific biomarkers that could be used for public screening(Alvaro, 2009;Briggs,Neal, et al, 2009;Morris-Stiff,Bhati, et al, 2008;Patel and Singh, 2007;Schulick, 2008). In fact, serum and bile are the only biological fluids used in CCA diagnosis. The biomarker most frequently

utilized in CCA investigations is carbohydrate antigen 19-9 (CA19-9), which was significantly increased in 85% of CCA patients (Nehls,Gregor and Klump, 2004). In patients suffering from both CCA and PSC, the sensitivity and specificity of CA19-9 at a cutoff value of 129U/ml are 79% and 98% respectively, while at a cutoff value of >63U/L, the sensitivity and specificity are increased to 90% and 98% respectively (Levy,Lymp, et al, 2005). Nevertheless, CA19-9 is not specific to CCA, as it is also increased in some gastrointestinal neoplasms (Akdogan,Sasmaz, et al, 2001;Albert,Steinberg and Henry, 1988). Furthermore, CA19-9 is undetectable in patients who do not possess the Lewis antigen blood type (Narimatsu,Iwasaki, et al, 1998;Vestergaard,Hein, et al, 1999). Other CCA biomarkers include carcinoembryonic antigen (CEA) and carbohydrate antigen-125 (CA125); however, these biomarkers lack specificity to CCA because they are also increased during cholangitis and other gastrointestinal malignancies (Chen,Shiesh, et al, 2002). In addition to these biomarkers, alkaline phosphate and gamma glutamyltransferase are also elevated in CCA (Furmanczyk,Grieco and Agoff, 2005;Siqueira,Schoen, et al, 2002). Cytologically, bile duct brush and lymph node pathology are generally used (Furmanczyk,Grieco and Agoff, 2005).

1.4.5 CCA Treatment

Currently, the therapeutic options available for CCA are limited to surgical resection and liver transplantation, and in many cases, surgical resection is not attainable. The five-year survival rates for CCA treatment are still poor (Casavilla,Marsh, et al, 1997;Isaji,Kawarada, et al, 1999;Lieser,Barry, et al, 1998;Madariaga,Iwatsuki, et al, 1998;Nakeeb,Pitt, et al, 1996;Ohtsuka,Ito, et al,

2002;Valverde,Bonhomme, et al, 1999). After resection, the three-year survival rates are 40% to 60% (Jarnagin,Fong, et al, 2001). Specifically, the survival rates following negative margin resection in extrahepatic CCA are less than 50% (Nagorney and Kendrick, 2006). In CCA cases with PSC, liver transplantation represents the preferred treatment option because the surgical resection outcomes are poor with five-year survival rates of less than 10% (Han,Leng, et al, 2004;Parkin,Srivatanakul, et al, 1991) due to further CCA development and recurrent cholangitis postoperatively (Bettschart,Clayton, et al, 2002;Chalasani,Baluyut, et al, 2000;Klempnauer,Ridder, et al, 1997;Tocchi,Mazzoni, et al, 2001). Options such as chemotherapy and radiotherapy are not recommended for patients with extrahepatic CCA (McMasters,Tuttle, et al, 1997;Wiedmann,Caca, et al, 2003). Following liver transplantation, the five-year survival rates for extra- and intrahepatic CCA are 23% - 26% and up to 18%, respectively (Casavilla,Marsh, et al, 1997;Iwatsuki,Todo, et al, 1998;Meyer,Penn and James, 2000;O'Grady,Polson, et al, 1988;Pascher,Jonas and Neuhaus, 2003;Pichlmayr,Weimann, et al, 1995;Weimann,Varnholt, et al, 2000). However, noticeable advancements in liver transplantation approaches have achieved encouraging results, with five-year survival rates of 76% (De Vreede,Steers, et al, 2000;Gores,Nagorney and Rosen, 2007;Heimbach,Gores, et al, 2006;Sudan,DeRoover, et al, 2002). Molecular therapies targeting the altered signaling pathways in CCA are currently under intensive investigations. For instance, the inhibition of Mcl-1, IL-6, Notch and Cox-2 signaling pathways have demonstrated the ability to decrease tumor cell

growth(Han,Leng, et al, 2004;Ishimura,Bronk and Gores, 2005;Isomoto,Mott, et al, 2007;Mott,Kobayashi, et al, 2007;Nzeako,Guicciardi, et al, 2002;Park,Tadlock, et al, 1999;Sirica,Lai, et al, 2002;Wehbe,Henson, et al, 2006;Wu,Leng, et al, 2004;Yu,Bruzek, et al, 2005).

1.5 Rationale and thesis hypothesis

In 2012, cancer is a dramatic reality, as it has been estimated that 75,700 Canadians will die of cancer (http//www.cancer.ca). These numbers are inflated by the paucity of precise approaches for detecting and preventing cancer, thus highlighting the necessity of additional studies to comprehend the biology of this disease. Specifically, CCA is a devastating liver neoplasm that arises from the bile duct. CCA's relevance emanates from recently evolving data necessitating further study, including the facts that (1) CCA's etiology remains obscure (2) most patients with CCA are diagnosed at advanced stages, and (3) the incidence of CCA is steadily increasing.

In cooperation with national and international researchers, our group has demonstrated that FPC plays a central role in the development of the primitive intrahepatic biliary system, as FPC is expressed during the early stages and remains absent when the biliary system is remodelled (Dorn,Menezes, et al, 2009). Moreover, FPC has been proposed as an excellent biomarker for distinguishing CCA from HCC, since FPC is only expressed in CCA.

Nonetheless, the tumorigeneic roles of FPC in CCA are still unknown and require detailed investigation.

This thesis discusses the possible roles and functions of FPC in CCA. First of all, I explored and challenged the notion that FPC is associated with AJ molecules, thus hypothesizing that the restoration of FPC expression in CCA will alter the expression of AJ in CCA. Secondly, I investigated the potential biological significance of FPC in CCA, leading to the hypothesis that knockdown the PKHD1 gene will affect cell proliferation and cell growth in CCA.

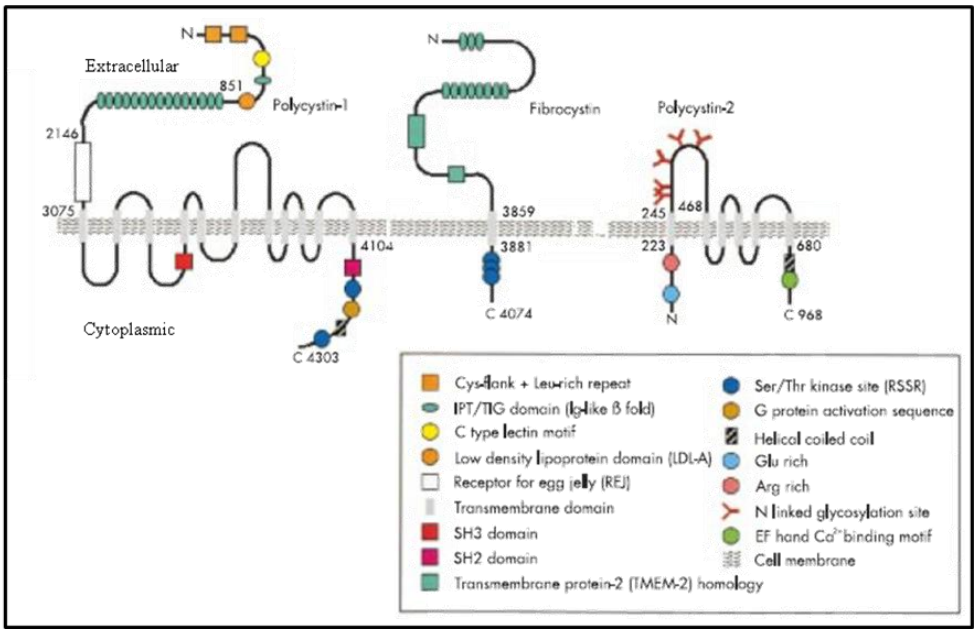

Figure 1.1: Molecular domain structure of FPC, PC1 and PC2. This diagram was modified with permission from the author (Johnson,Gissen and Sergi, 2003) .

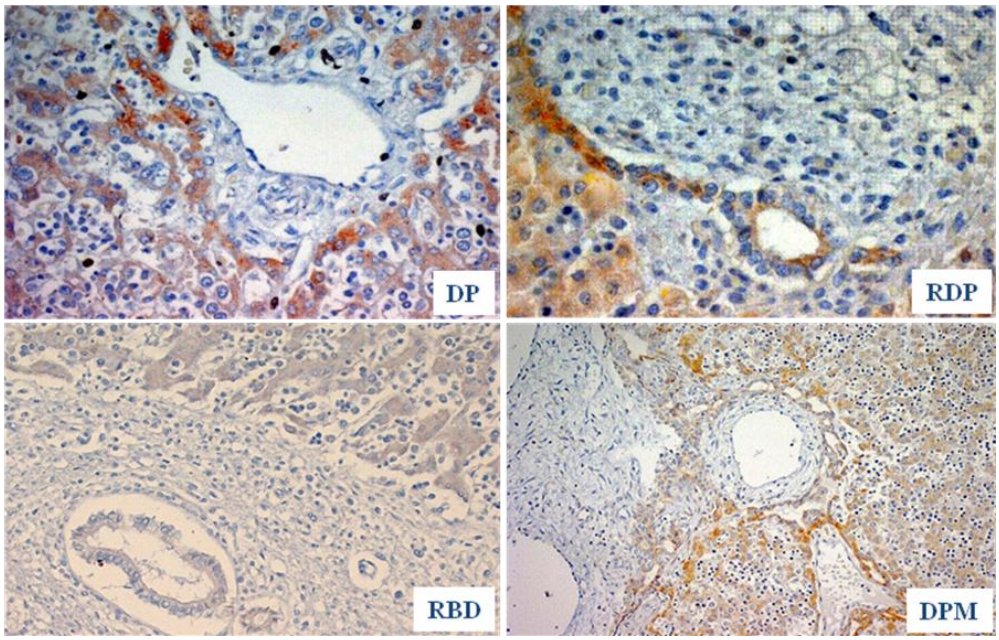

Figure 1.2: FPC expression in human bile duct development. This photograph was taken with permission from the authors (Dorn,Menezes, et al, 2009).

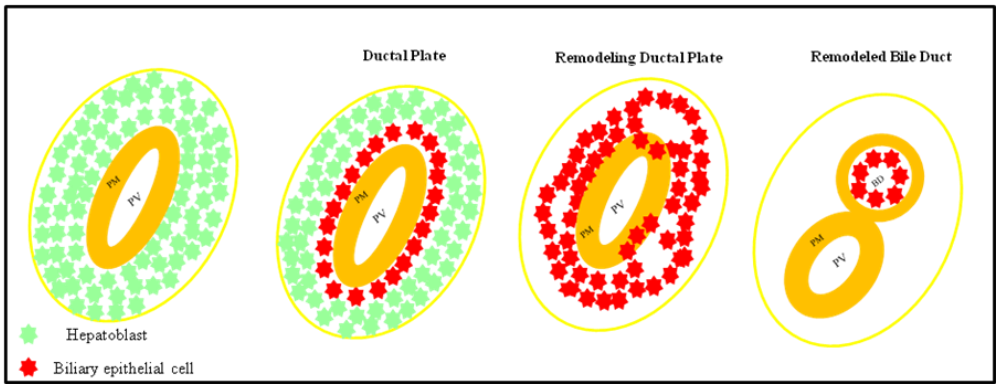

Figure 1.3: Developmental stages of the bile duct.

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines

The previously-published CCA cell lines, OZ, Huh-28 and HuCCT-1, were obtained from the cell culture bank of the Japan Health Sciences Foundation (Homma,Nagamori, et al, 1987;Kusaka,Tokiwa and Sato, 1988;Miyagiwa,Ichida, et al, 1989). All cell lines were grown as a monolayer culture in their appropriate media. Specifically, OZ was grown in William's E Medium (Invitrogen Canada Inc., Burlington, ON, Canada) and supplemented with 10% Fetal Bovine Serum (FBS) (PAA Laboratories Inc., Etobicoke, ON, Canada). Huh-28 and HuCCT-1 were cultured in Roswell Park Memorial Institute (RPMI) 1680 medium (Invitrogen Canada Inc., Burlington, ON, Canada) and supplemented with 10% FBS. Both the hepatocellular carcinoma cell line (HepG-2) and the larynx carcinoma cell line (Hep-2) were purchased from American Type Culture Collection (ATCC) and cultured in their appropriate media according to the provider's instructions. All cell lines were maintained at 37°C and 5% CO_2.

2.2 Antibodies

In most cases, all antibodies were purchased from Cell Signaling Technology Inc.

(Danvers, MA, USA). The primary antibodies that we used in western blot and immunofluorescence staining include E-cadherin, N-cadherin (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), β-catenin, plakoglobin (kindly denoted by Dr. Pasdar, M., Department of Oncology, University of Alberta, Edmonton, AB, Canada) and β-actin. The fused protein, α-FP2, is a polyclonal antibody that has been designed and established with an intracellular carboxy-terminus of FPC (Menezes,Cai, et al, 2004). This antibody has a high binding affinity to FPC (Dorn,Menezes, et al, 2009;Menezes,Cai, et al, 2004). Among the secondary antibodies, Alexa Flour 555 and 488 are used for immunofluorescence staining, while ECL anti-mouse horseradish peroxidase (HRP) and ECL anti-rabbit-HRB (GE Healthcare Inc., Baie d'Urfe, Quebec, Canada) are used for western blotting.

2.3 Total proteins extraction and western blotting

Protein extraction and western blot were performed as previously described (Abuetabh,Persad, et al, 2011). Cells were grown until they achieved 80% confluence and then lysed in Radio-Immuno Precipitation Assay (RIPA) buffer (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Protein concentrations were measured by using the bicinchoninic acid (BCA) assay (Fisher Scientific Company, Ottawa, ON, Canada). Specifically, 30-50 ug of total proteins extracted from each cell line were separated in 7% Sodium-Dodecyl-Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) gel or 4% Tricine gel. Proteins were subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Inc., Baie d'Urfe, Quebec, Canada), which was

blocked in 5% milk or 3% BSA and incubated with the appropriate antibodies. Then, PVDFs were incubated with HRP-conjugated secondary antibodies, and finally, protein bands were visualized by enhanced chemi-luminescence (ECL) system detection according to the protocol provided by the manufacturer (Perkin-Elmer Inc., Massachusetts, USA) and developed on Kodak film (Kodak Graphic Communications Company, Burnaby, BC, Canada).

2.4 Immunofluorescence analysis

Immunofluorescence staining was performed as previously discussed (Abuetabh,Persad, et al, 2011).To localize the AJ components (E-cad, N-cad, β-cat and Pg) in the CCA cell lines, a confocal laser microscopy was used. Each cell line was grown on cover slips for 24-48 hours and then fixed in 4% formaldehyde. After blocking with 1% bovin serum albumin (BSA) (Fisher Scientific Company, Ottawa, ON, Canada), cells were incubated with the applicable antibodies overnight at 4 degrees Celsius. After undergoing washing with phosphate buffer saline (PBS), cells were incubated with fluorochrome-conjugated secondary antibodies. All primary and secondary antibodies were diluted in BSA-PBS solution. The images were obtained using a confocal microscope (Zeiss LSM 510 Confocal Microscope, Carl Zeiss MicroImaging Inc., Toronto, ON, Canada).

2.5 Visualization of ultrastructures

To visualize the ultrastructures of the CCA cell lines, including cell-cell junction, cell-cell adhesion and desmosomes, the electron microscope was used. Specifically, 70-80 % confluent petri-dish plates of each CCA cell lines were washed in PBS and fixed with 2% glutaraldehyde for one hour at 4°C. Subsequently, cells were scraped from their plates and collected in eppendorf tubes. After centrifugation, osmium tetroxide was applied to cell pellets overnight at room temperature. Following graded dehydration, pellets were epon embedded and sectioned into ultra-thin fragments. Sections were mounted on metal mesh grids and contrasted with uranyl acetate and lead citrate. The grids were examined in a Hitachi H-7650 transmission electron microscope (TEM) (Hitachi High-Technologies Canada Inc., 89 Galaxy Blvd. Suite 14, M9W 6A4, Rexdale, ON Canada).

2.6 PKHD1-shRNA transfection

HuCCT1 cells were grown in 6-well plates until reaching 60-70% confluency and then transfected with either 1 ug of PKHD1-shRNA or scrambled shRNA plasmid following the manufacturer's directions (Santa Cruz, CA, USA). Cells were harvested 48-72h after transfection. The mRNA levels of PKHD1 were assessed by StepOnePlus real-time polymerase chain reaction (RT-PCR) (Applied Biosystems, CA, USA). PKHD1-shRNA is a pool of three different shRNA

plasmids prepared and provided by Santa Cruz, Ca, USA. The hairpin and corresponding siRNA sequences are as follow:

(1) Hairpin sequence:
GATCCGGATTGGTGTCCAGATTCATTCAAGAGATGAATCTGGACACCA
ATCCTTTTT
Corresponding siRNA sequences:
• Sense: GGAUUGGUGUCCAGAUUCAtt
• Antisense: UGAAUCUGGACACCAAUCCtt

(2) Hairpin sequence:
GATCCGGAAGGTGTTAGCCTGATATTCAAGAGATATCAGGCTAACACC
TTCCTTTTT
Corresponding siRNA sequences:
• Sense: GGAAGGUGUUAGCCUGAUAtt
• Antisense: UAUCAGGCUAACACCUUCCtt

(3) Hairpin sequence:
GATCCCAACTTCCTTGCAATGTAATTCAAGAGATTACATTGCAAGGAA
GTTGTTTTT
Corresponding siRNA sequences:
• Sense: CAACUUCCUUGCAAUGUAAtt
• Antisense: UUACAUUGCAAGGAAGUUGtt

All sequences are provided in 5′ → 3′ orientation. The scrambled-shRNA was purchased from Santa Cruz (Control shRNA Plasmid-A: sc-108060).

2.7 RNA isolation, cDNA synthesis and RT-PCR

Total RNA was isolated by the TRIzol® (Life Technologies, CA, USA) method according to the manufacturer's instructions. cDNAs were synthesized using the QuantiTect RES. Transcription Kit (QIAGEN Inc., ON, Canada). TaqMan® Gene

Expression Assay for PKHD1 (ID: Hs00370969) and for β-actin (ID: Hs99999903) with TaqMan® Fast Advanced Mix were used to detect the mRNA levels of PKHD1. StepOnePlus RT-PCR (Applied Biosystems, CA, USA) was used.

2.8 MTS assay

HuCCT1 cells transfected with either PKHD1-shRNA or scrambled shRNA were seeded in 96-well plates at a density of $0.5x10^4$. After periods of 24, 48 and 72 hours, 20 ul of Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay Kit was added according to the manufacturer's guidelines (Promega Corporation, USA). Wells containing only media were used as negative controls. The absorbance of the samples was normalized to that of the controls and measured by SpecraMax M3 Microplate Reader (Molecular Devices, CA, USA) at 490nm. The experiment was conducted a total of three times.

2.9 Statistical measures

The unpaired student t test and the analysis of variance (ANOVA) tests were used appropriately to determine the statistical significance, where P values of less than 0.05 were considered significant.

CHAPTER 3: RESULTS

CHAPTER 3: RESULTS

3.1 Western blot analysis of FPC expression in CCA cell lines

Western blot analysis revealed that FPC is expressed by OZ, Huh-28 and HuCCT-1 CCA cell lines but unexpressed by HepG-2 or Hep-2 (Figure 3.1a-d). These results are strongly compatible with previous data (Dorn,Menezes, et al, 2009), where, using immunohistochemical approaches, FPC expression was present in CCA tissues but absent in HCC tissues. The experiment was repeated three times for confirmation. Preimmune serum corresponding to α-FP2 (A polyclonal antibody against FPC) was used to determine the specificity of α-FP2 to detect the FPC. β-actin was used as a loading control.

3.2 Western blot analysis of AJ's molecules expressions in CCA cell lines

Western blot (WB) analysis for E-cad/β-cat/Pg expression (Figure 3.2) revealed that all three CCA cell lines express β-cat. Unlike OZ and HuCCT-1, Huh-28 does not express E-cad or Pg. Both Huh-28 and HuCCT-1 express N-cad while OZ did not express this protein (Figure 3.2). In this experiment, Hep2 and HepG2 were

used as controls since they have been previously characterized as such (Cimbora-Zovko,Ambriovic-Ristov, et al, 2007;Pan,Zhang, et al, 2010) and the resulting expression patterns confirm the data published in the literature. While Hep2 expresses β-cat and Pg, it does not express E-cad. On the other hand, HepG2 has demonstrated expression of E-cad and β-cat. Moreover, HepG2 was used to determine the presence of any diagnostic differences between CCA and HCC regarding the expression of AJ components. Finally, β-actin was used as a loading control.

3.3 Confocal and Electron microscopy analysis of AJ molecules and cell-cell junctions in CCA cell lines

This experiment further characterized AJ components in the CCA cell lines by revealing the localization patterns of those proteins through immunofluorescence staining techniques. Specifically, immunofluorescence staining showed that E-cad is mainly located in the membrane of OZ cells (Figure 3.3a), which corresponds to normal patterns. In contrast, HuCCT-1 demonstrated a diffuse cytoplasmic localization of E-cad (Figure 3.3b), which represents an abnormal localization pattern, indicating that E-cad may be functionally inactive or structurally altered. For the localization patterns of β-cat, both Huh-28 and HuCCT-1 possessed a cytoplasmic localization as detected by immunofluorescence staining (Figure 3.4b, c and d). In particular, a very faint nuclear deposit of β-cat appeared in Huh-

28 cells. On the other hand, OZ cells revealed a membranous localization of β-cat (Figure 3.4a). Although Pg is generally located in the membrane of OZ cells, it is also located in the nucleus (Figure 3.5a), thus indicating that Pg's patterns of localization can be interpreted as normal. Furthermore, HuCCT-1 also demonstrated a very faint cytoplasmic localization of Pg (Figure 3.5b). The study showed that N-cad is localized mainly in the cytoplasm of Huh-28 and HuCCT-1 cells (Figure 3.6a and b), representing an abnormal localization of N-cad in both cell lines.

Ultrastructural investigation revealed that OZ cells possess an appropriate number of desmosomes (Figure 3.7a and b) and display normal structures with strong cell-cell junctions and adhesions. However, few desmosomes were apparent in HuCCT-1 (Figure 3.8a), and no desmosomes were found in the Huh-28 cell line. The ultrastructure of cell-cell junctions and cell-cell adhesions are also illustrated in OZ (Figure 3.7c), HuCCT-1 (Figure 3.8b) and Huh-28 (Figure 3.9a and b). In particular, OZ cells displayed abundant and strong cell-cell junctions. While Huh-28 contained very few and weak cell-cell junctions, there were a moderate number of cell-cell junctions in HuCCT-1. These data are consistent with the expression of AJ component data. The number of desmosomes ($p=0.0021$), the number of cell-cell junctions ($p=0.0071$) and the length of those cell-cell junctions ($p=0.0339$), differ significantly between these CCA cell lines. In particular, six different electron microscopic fields from each cell line were compared, and the statistical significances of such comparisons were determined by an ANOVA test.

3.4 Down-regulation of PKHD1 decreases cell proliferation in HuCCT1 cell line

PKHD1-shRNA is a pool of 3 different shRNA plasmids against PKHD1 mRNA was utilized to down-regulate the gene expression in HuCCT-1 cell lines in order to explore the biological significance of FPC in CCA. Approximately 40.3% knockdown of the gene was successfully achieved, as assessed by RT-PCR (Figure 3.10). MTS data showed that in comparison to scrambled shRNA, the down-regulation of PKHD1 mRNA in HuCCT1 cell lines significantly decreases proliferation and cell growth (Figure 3.11). Experiments were performed in triplicate.

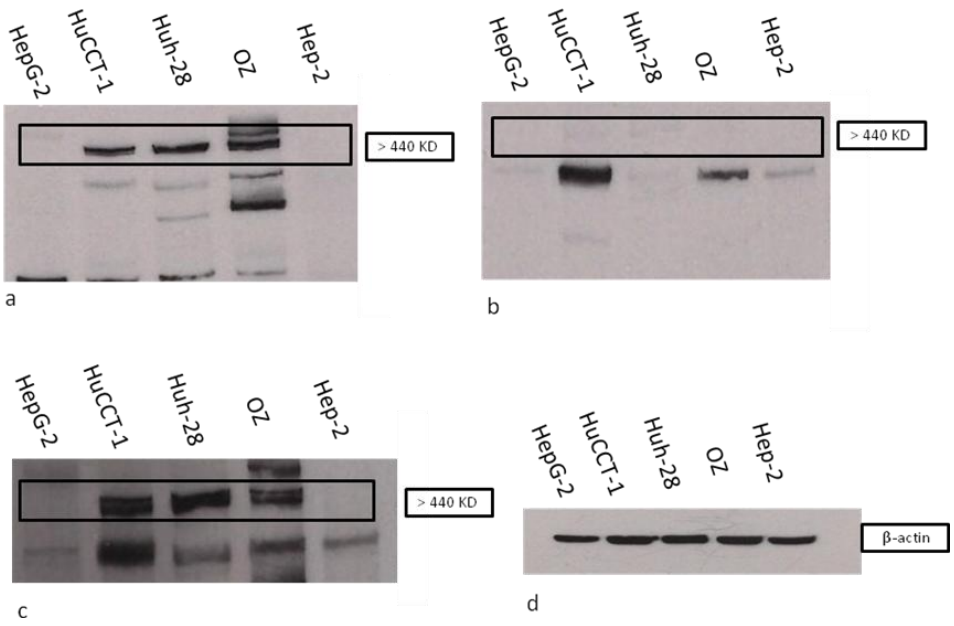

Figure 3.1: Western blotting analysis of FPC expression in CCA and HCC cell lines.

FPC bands (>440 kD) were detected by using α-FP2 polyclonal antibody (a). In (b), preimmune serum corresponding to α-FP-2 was used as a control to determine the specificity of α-FP2 polyclonal antibody to detect the FPC. The membrane in (b) was re-probed with α-FP2 (c). Protein lysates were probed with β-actin monoclonal antibody to demonstrate the loading and protein quality (d).

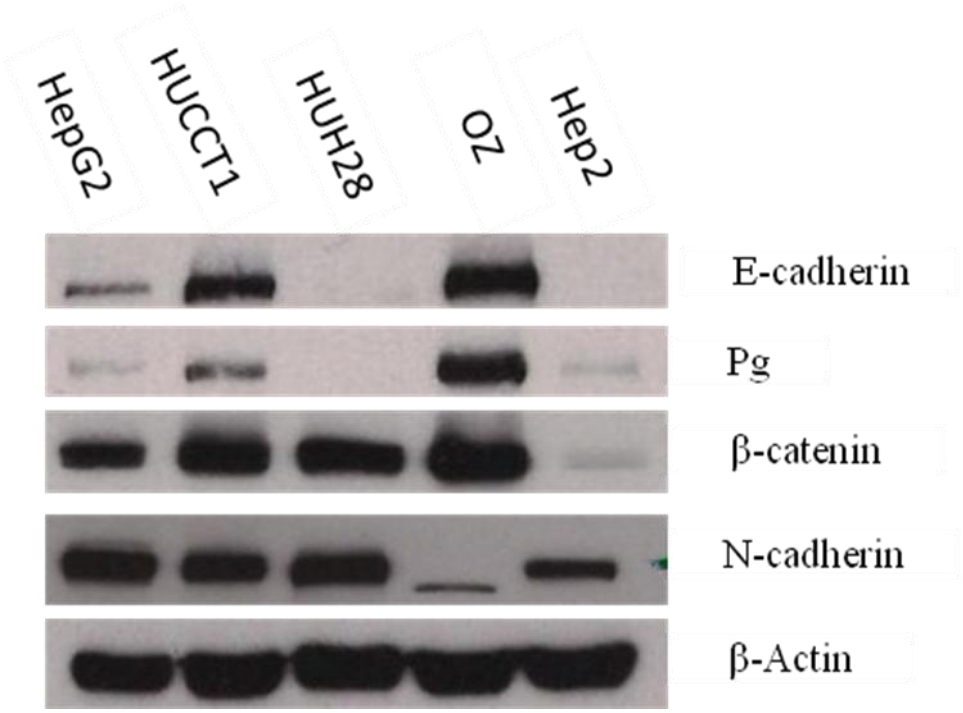

Figure 3.2: Expression of AJ components in cholangiocarcinoma cell lines and controls. Western blot analysis was performed on whole cell lysates of OZ, Huh-28 and HuCCT-1 for E-cadherin (E-cad), N-cadherin (N-cad), Plakoglobin (Pg), β-catenin (β-cat) and β-actin (control) expression. Human hepatocellular carcinoma (HepG2) cells were used as a positive control for E-cad and β-cat expression. Human larynx sequamous cell carcinoma was used as a positive control for Pg and N-cad expression and as a negative control for E-cad expression.

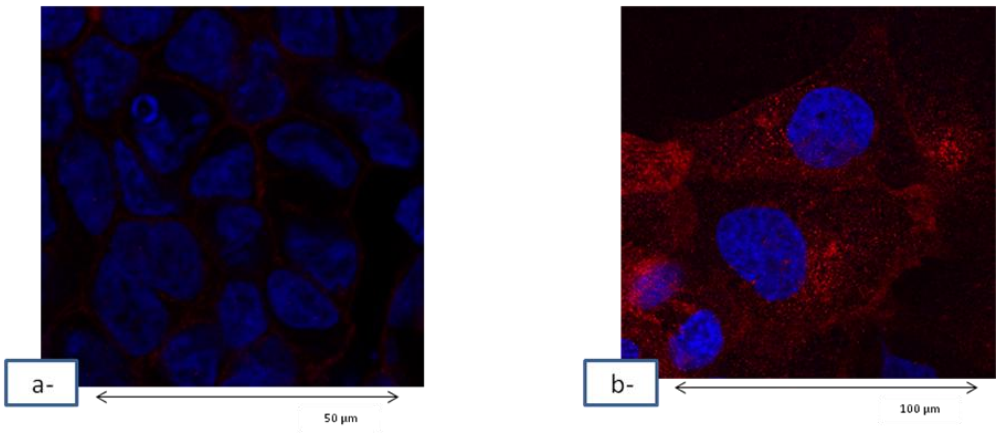

Figure 3.3: Immunolocalization of E-cadherin in OZ and HuCCT-1. OZ and HuCCT-1 cells were stained with antibodies against E-cadherin as demonstrated by confocal microscopy (a and b respectively). Red fluorescence indicates E-cad, while DAPI (blue) indicates nuclei. Scale bars are shown below the photograph.

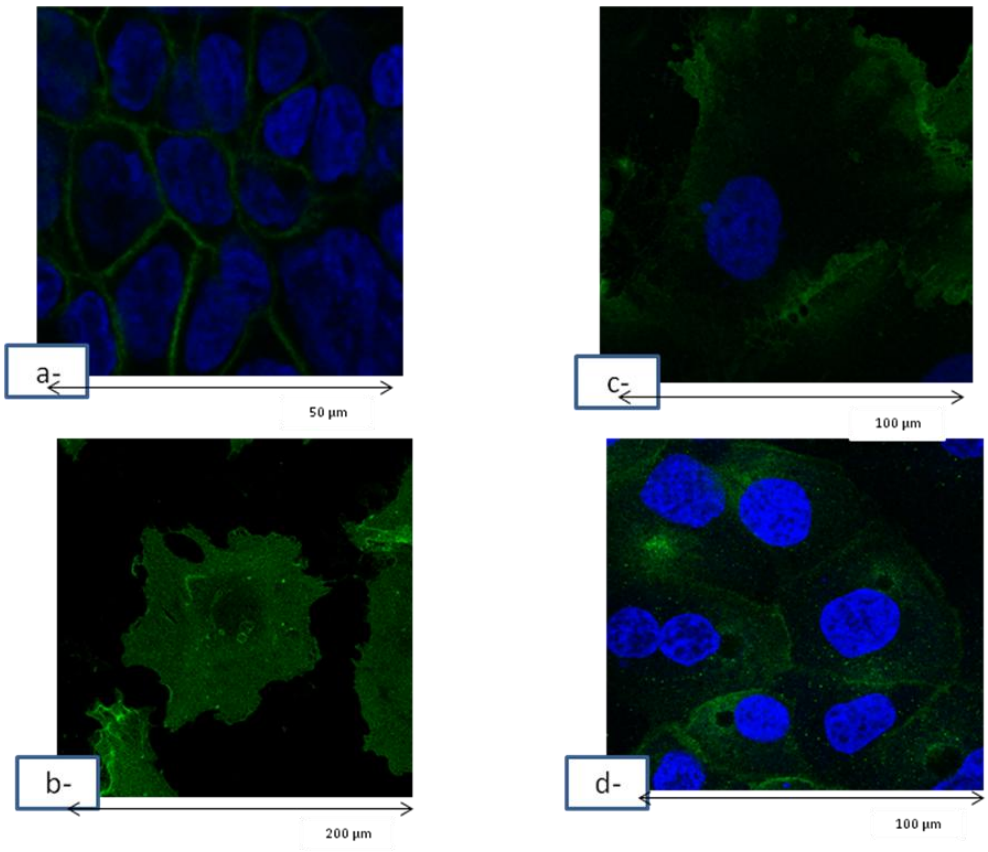

Figure 3.4: Immunolocalization of β-catenin in OZ, HuCCT-1 and Huh-28. OZ, Huh-28 and HuCCT1 cells were stained with antibodies against β-catenin as illustrated by confocal microscopy (a, (b,c) and d respectively). Green fluorescence indicates β-cat, while DAPI (blue) indicates nuclei. Scale bars are shown below the photograph.

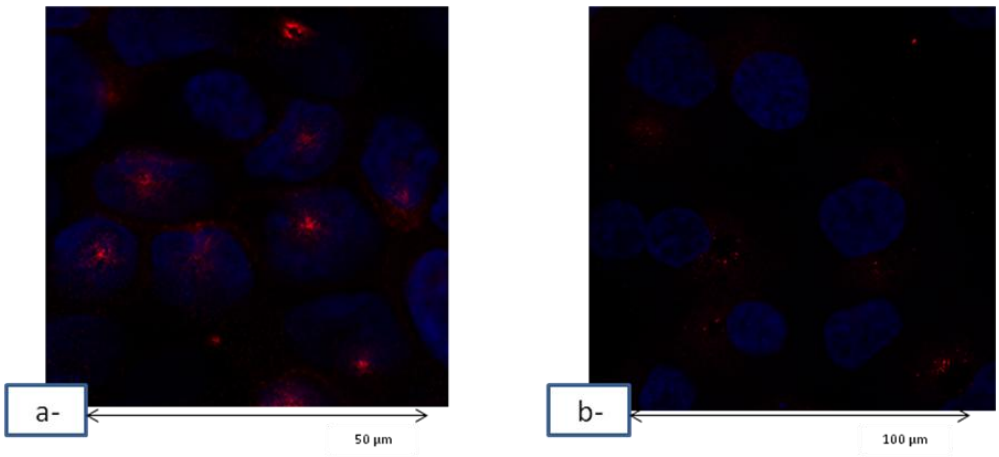

Figure 3.5: Immunolocalization of Plakoglobin in OZ and HuCCT-1. OZ and HuCCT1 cells were stained with antibodies against plakoglobin as demonstrated by confocal microscopy (a and b respectively). Red fluorescence indicates Pg, while DAPI (blue) indicates nuclei. Scale bars are shown below the photograph.

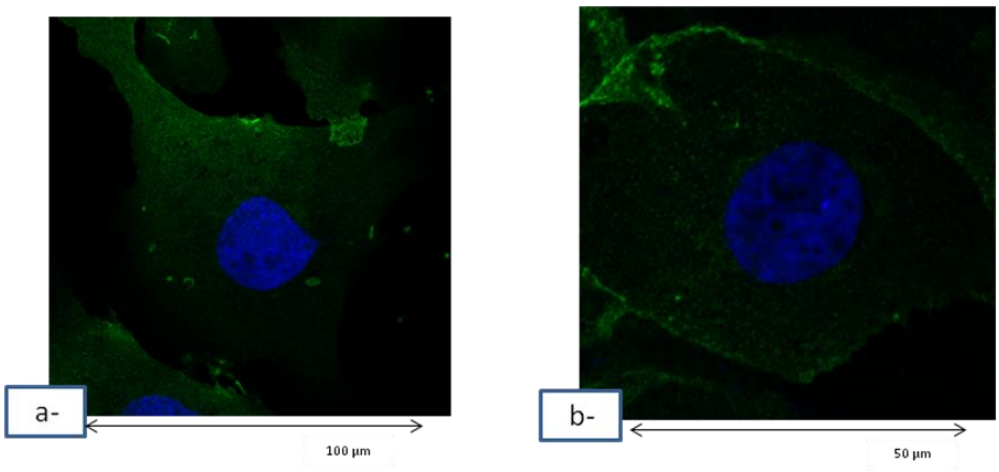

Figure 3.6: Immunolocalization of N-cadherin inHuCCT-1 and Huh-28. Huh-28 and HuCCT1 cells were stained with antibodies against N-cadherin as illustrated by confocal microscopy (a and b respectively). Green fluorescence indicates N-cad, and DAPI (blue) indicates nuclei. Scale bars are shown below the photograph.

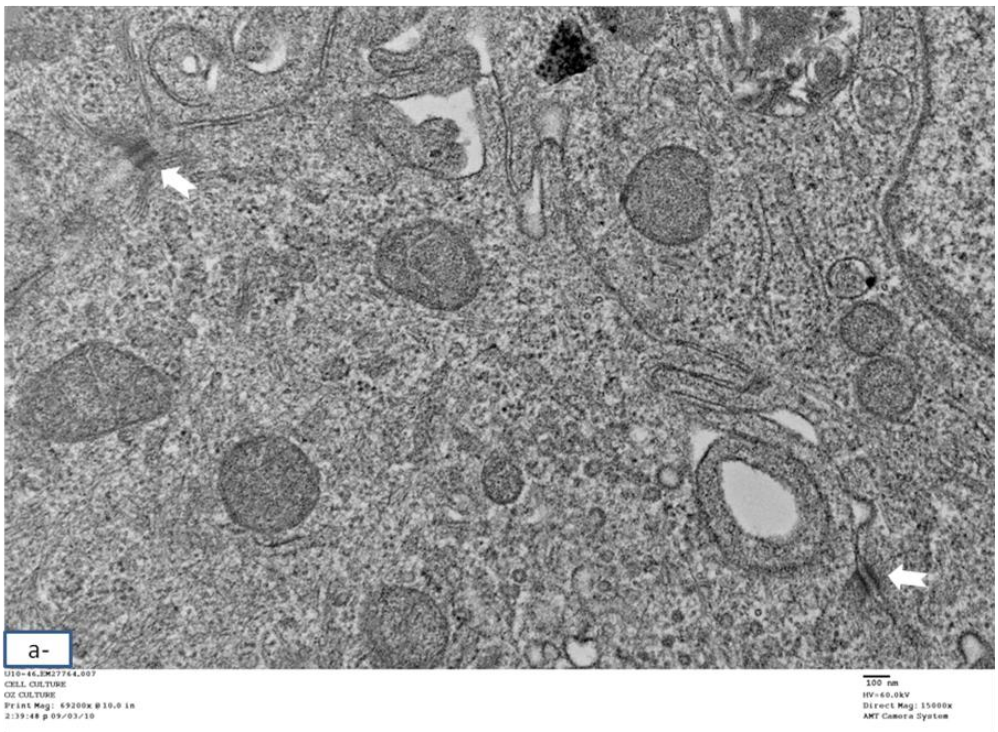

Figure 3.7a: Ultrastructure of cell-cell junctions and desmosomes in OZ. White arrows signify desmosomes. Scale bars are shown under the photograph.

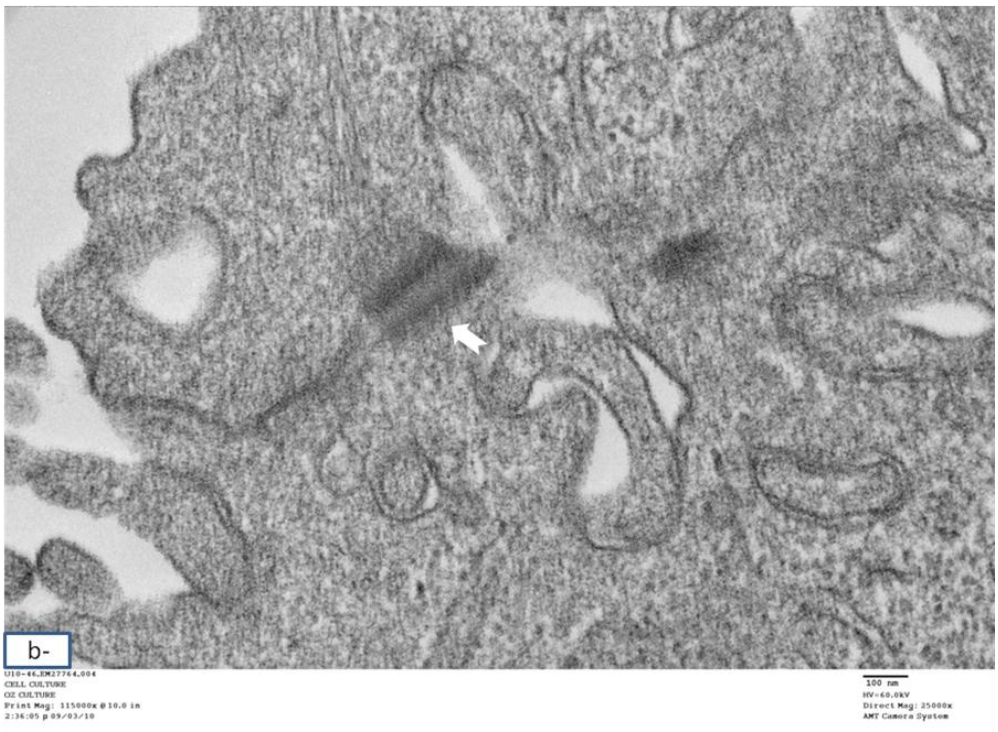

Figure 3.7b: Ultrastructure of cell-cell junctions and desmosomes in OZ. White arrows indicate desmosomes. Scale bars are shown under the photograph.

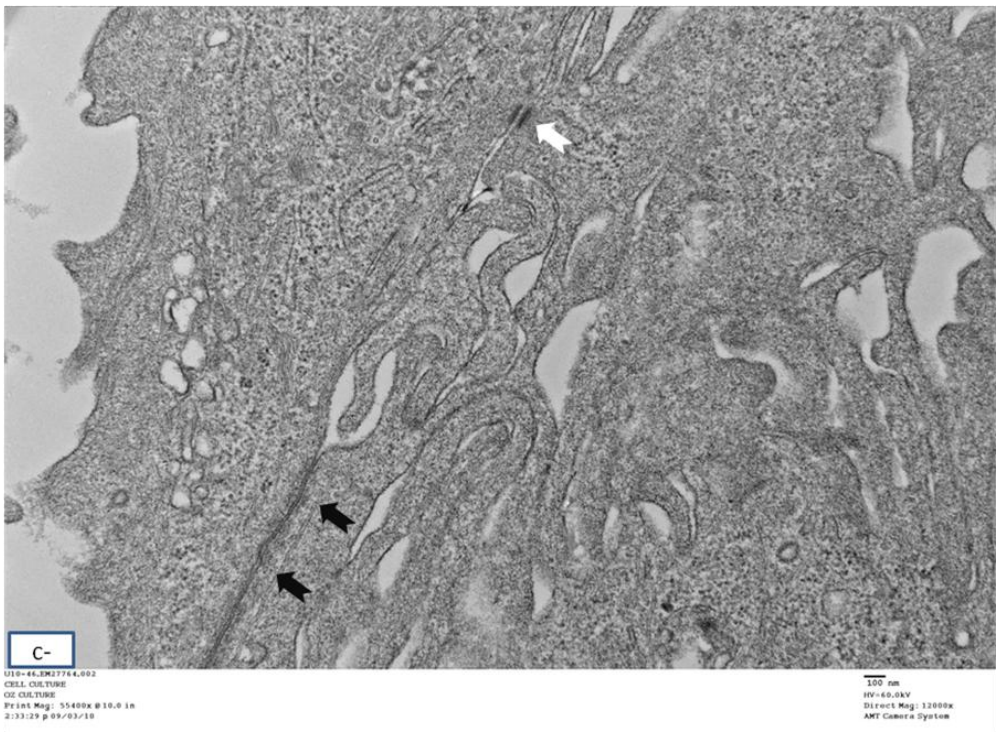

Figure 3.7c: Ultrastructure of cell-cell junctions and desmosomes in OZ. White arrows denote desmosomes, whereas black arrows signify cell-cell junctions. Scale bars are shown below the photograph.

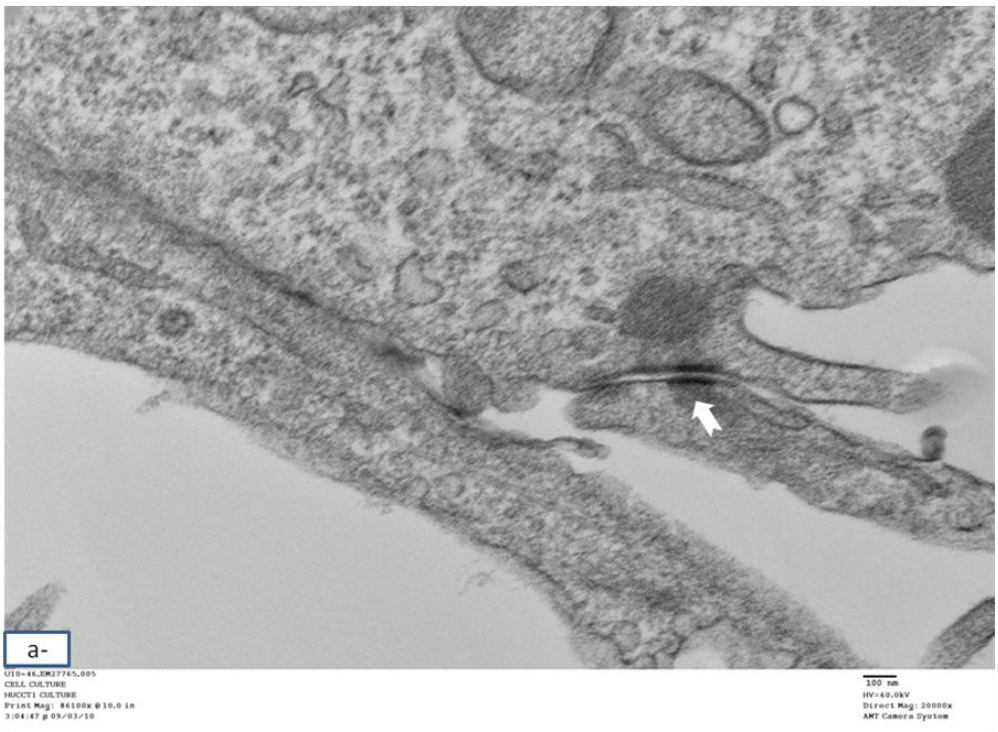

Figure 3.8a: Ultrastructure of cell-cell junctions and desmosomes in HuCCT-1. White arrows reveal the desmosomes. Scale bars are shown under the photograph.

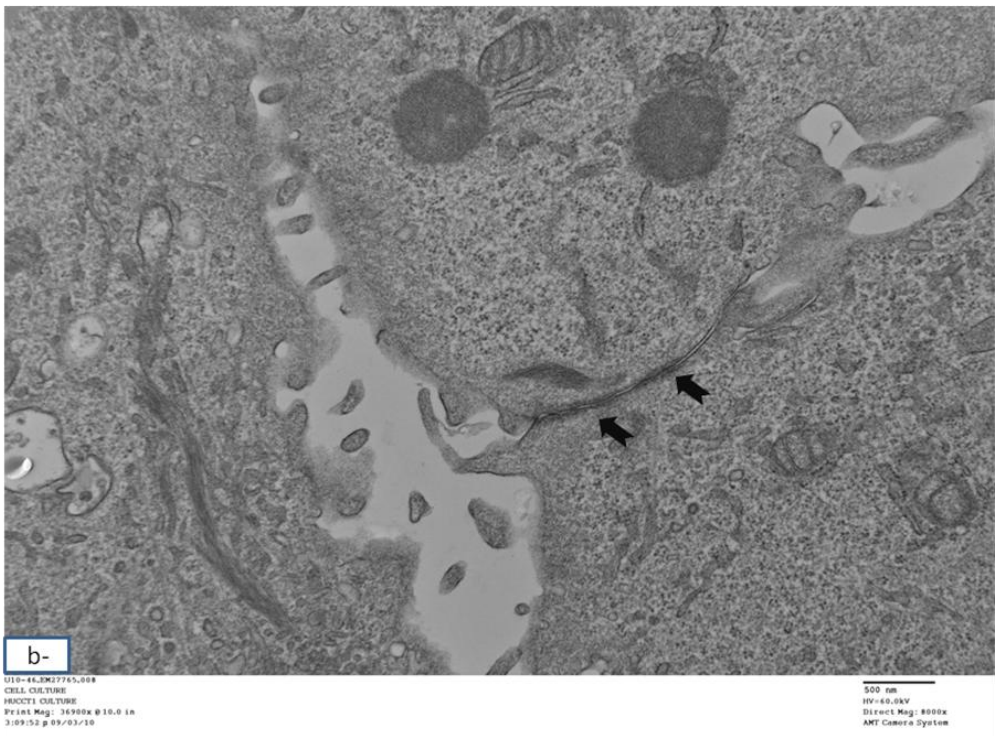

Figure 3.8b: Ultrastructure of cell-cell junctions and desmosomes in HuCCT-1. Black arrows signify cell-cell junctions. Scale bars are shown below the photograph.

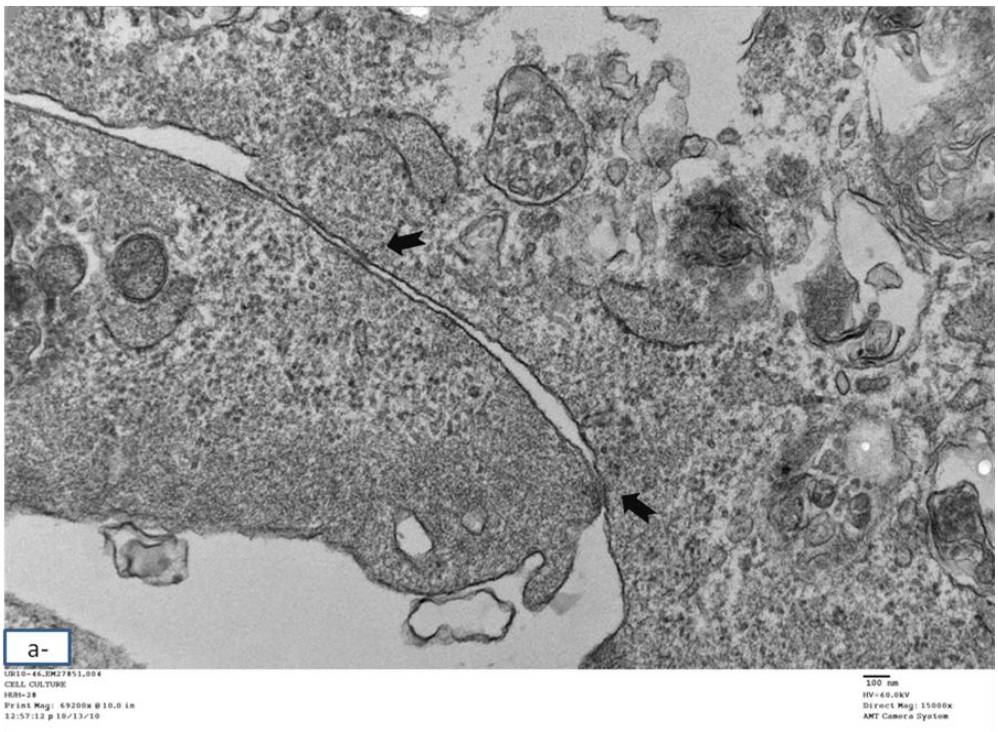

Figure 3.9a: Ultrastructure of cell-cell junctions in Huh-28. Black arrows indicate cell-cell junctions, and scale bars are shown under the photograph.

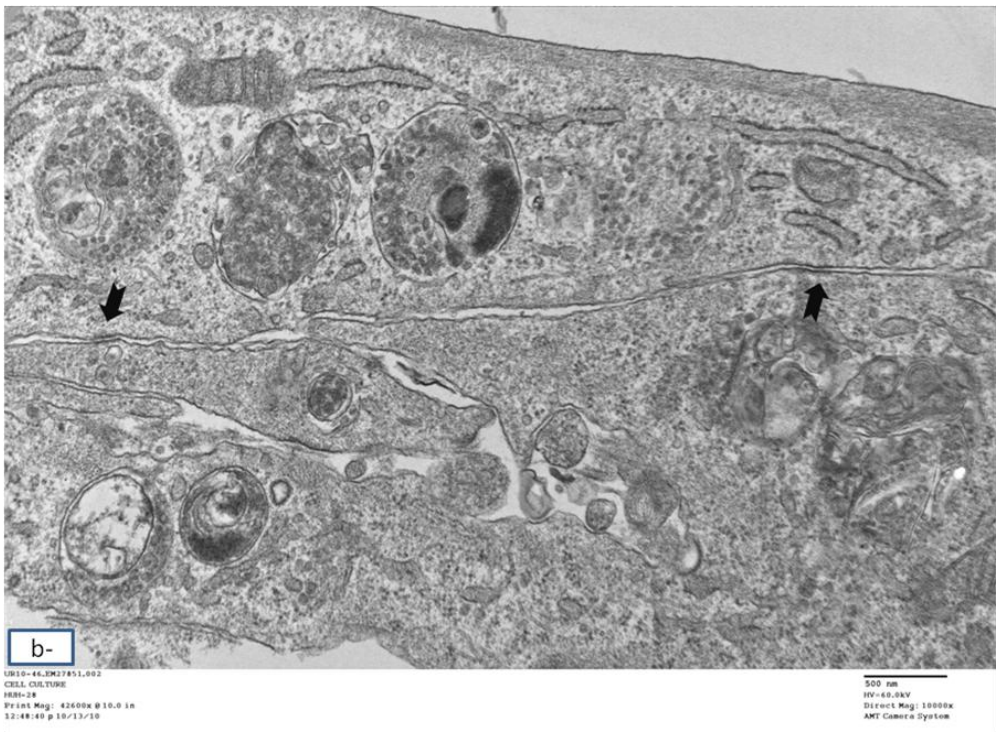

Figure 3.9b: Ultrastructure of cell-cell junctions in Huh-28. Black arrows highlight cell-cell junctions, and scale bars are shown below the photograph.

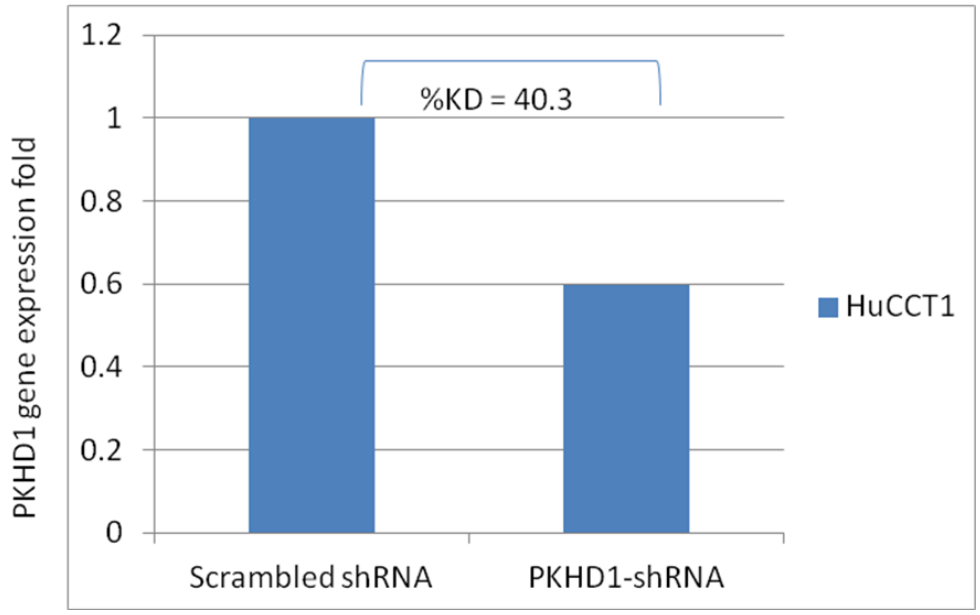

Figure 3.10: Down-regulation of PKHD1 mRNA in HuCCT1 cell line. shRNA plasmids against PKHD1 mRNA was successful to knockdown a 40.3% of the gene, as assessed by RT-PCR. %KD: Percentage Knockdown.

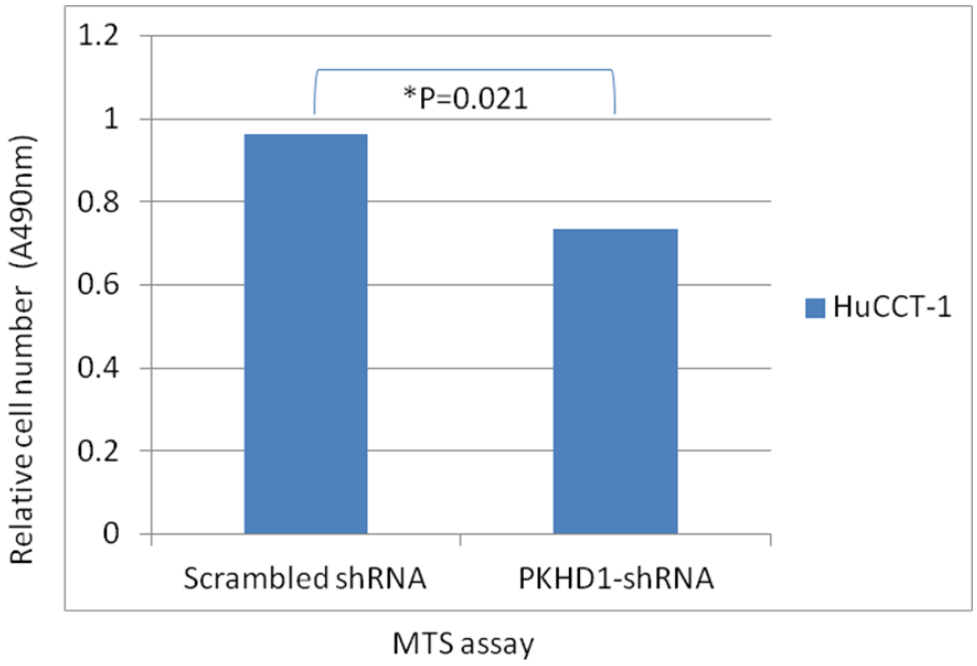

Figure 3.11: Down-regulation of PKHD1 (FPC) decreases cell proliferation in cholangiocarcinoma. MTS data shows that in comparison to scrambled shRNA, the down-regulation of PKHD1 mRNA in HuCCT1 cell lines significantly decreases proliferation and cell growth.

CHAPTER 4: DISCUSSION

CHAPTER 4: DISCUSSION

4.1 General discussion

A comprehensive and detailed investigation of cancer biology may lead to the precise prognosis, treatment and prevention of multiple malignant neoplasms. Bile duct cancer or cholangiocarcinoma (CCA) is a very devastating liver neoplasm characterized by diagnosis that often occurs at an advanced stage of the disease. The cause of CCA is obscure and its incidence is increasing worldwide. Our group demonstrated that FPC fulfills a central role in the development of the primitive intrahepatic biliary system (Dorn,Menezes, et al, 2009). Furthermore, intense immunohistochemical staining of FPC was identified in the abnormal development of the biliary system. As expected, I found that FPC is expressed in three CCA cell lines, while it was not expressed in the HCC cell line. To the best of my knowledge, there is no data currently available concerning either FPC expression or action in CCA and HCC cell lines. Since much research has investigated FPC in kidney tissue and cell lines, studies examining the potential functional roles of FPC in CCA are crucial. Accordingly, this thesis demonstrated that FPC is an important growth factor for CCA cells; these results may provide a possible pathogenic pathway of FPC as well as novel treatment options for CCA.

4.2 Differential expression and localization patterns of AJ's components in CCA cell lines

The data presented in this thesis represents an initial step toward a molecular characterization of cell-cell junctions in three CCA cell lines with the aim of improving understanding about the nature of bile duct carcinoma. I investigated the quality of cell-cell junctions in three CCA cell lines: OZ, Huh-28 and HuCCT-1. Specifically, cell-cell junctions were examined by studying mainly the expression and localization patterns of adherent junction components and the presence of desmosomal structures in the transmission electron microscope. The cytoplasmic domains of both AJ components and desmosomal proteins entail the anchorage site of the cytoskeleton (Delva,Tucker and Kowalczyk, 2009;Meng and Takeichi, 2009;Pecina-Slaus, 2003;Shapiro and Weis, 2009;Stokes, 2007). AJ components include E-cad, N-cad, β-cat and Pg. Experimental results found that while OZ and HuCCT-1 expressed E-cad/β-cat/Pg, these components displayed different localization patterns. In HuCCT-1, E-cad, β-cat and Pg were all localized in the cytoplasm, while in OZ, E-cad and β-cat were localized only in the plasma membrane. In OZ, Pg was located mainly in the nucleus, with a minute amount in the cytoplasm membrane. In contrast, Huh-28 only expressed β-cat with diffused cytoplasmic and faint nuclear localization patterns. Moreover, with the exception of OZ, both Huh-28 and HuCCT-1 expressed N-cad with cytoplasmic localization. The electron microscope findings of the three CCA cell lines showed that OZ contains an abundant number of desmosomes, while HuCCT-1 possesses

few desmosomes. In contrast, Huh-28 contains no desmosomes and displays very weak cell-cell contact.

It has been consistently demonstrated that cadherin-catenin complexes are distorted in many cancers such as breast, stomach, colon, and skin cancers. This condition increases the cell's potential to undergo uncontrolled proliferation and hence invasion and metastasis (Gat,DasGupta, et al, 1998;Guilford, 1999;Guilford,Hopkins, et al, 1999;Korinek,Barker, et al, 1997). The data presented in this thesis promotes many speculations and hypotheses. For instance, although OZ has been described as a metastasizing cell line, this cell line demonstrated typical localization patterns for E-cad and β-cat, both of which are localized in the plasma membrane. However, because studies have established that the shutting and activating mechanisms of E-cad are reversible, one can speculate that E-cad has been lost via hypermethylation activity that occurred prior to the metastasis event (Brabletz,Jung, et al, 2001). Furthermore, one might assume that OZ cells may contain mutations in the CTNNA1 gene, which encodes α-cat, since research has shown that those mutations cause cell adhesion abnormalities (Hirano,Kimoto, et al, 1992). Surprisingly, this study found that Pg possesses a nuclear and membranous localization in OZ; this result is consistent with electron microscope findings in which OZ contains abundant desmosomes, as the expression of Pg is positively correlated with desmosomal protein expressions (Rieger-Christ,Ng, et al, 2005). Moreover, the nuclear localization of Pg in OZ cells could compete with β-cat, since Pg shares a structural homology

with β-cat and has shown the ability to translocate into the nucleus and bind with TCF/LEF. However, the Pg/TCF/LEF complex has no capability of binding with the DNA. Accordingly, the nuclear localization of Pg may negatively regulate the transcriptional activity of β-cat, leading to the hypothesis that Pg has tumor suppression activities (Aktary,Chapman, et al, 2010;Rieger-Christ,Ng, et al, 2005;Simcha,Shtutman, et al, 1998;Zhurinsky,Shtutman and Ben-Ze'ev, 2000). Future studies could knockdown the expression of Pg to investigate its biological importance in OZ cells as well as its effect on β-cat expression.

Among all CCA cell lines, Huh-28 is the most intriguing cell line, as it expresses neither E-cad nor Pg while expressing β-cat and N-cad with cytoplasmic localizations. This result led to the conclusion that the cell-cell junction in Huh-28 is both scarce and altered, which was subsequently confirmed by electron microscope findings revealing that Huh-28 possesses no desmosomes and contains very few and weak cell-cell junctions. The Huh-28 cell line was established from poorly differentiated CCA; thus, these data indicate that Huh-28 cells are losing their epithelial markers and cell-cell contacts, which might result in the metastasis of these cells. Such characteristics represent one of the cancer hallmarks known as epithelial to mesenchymal transition (EMT). In particular, E-cad might be hypermethylated in Huh-28, as the hypermethylation of E-cad has been reported in other CCA cell lines (Ku,Yoon, et al, 2002).

Moreover, this investigation illustrated that E-cad, N-cad and β-cat were localised in the cytoplasm of HuCCT1 cell lines. Some researchers have demonstrated that

cytoplasmic localization of E-cad is directly associated with poor prognosis of colon cancer as well as postoperative recurrence (Guzinska-Ustymowicz,Chetnik and Kemona, 2004). Furthermore, Gao S. et. al. (2005) showed that the hypermethylation of the APC gene is significantly linked with the cytoplasmic localization of both E-cad and β-cat (Gao,Eiberg, et al, 2005). This pattern of localization may indicate that E-cad could possess a mutation in its extra-cellular domain or cytoplasmic tail. Moreover, the cytoplasmic localization of E-cad may signify the mesenchymal phenotype (Schmalhofer,Brabletz and Brabletz, 2009). Moreover, one may speculate that β-cat is not binding to E-cad. In addition, since no nuclear accumulation of β-cat was observed, one could assume that β-cat is adequately degraded and Wnt signaling is not actively functioning. Interestingly, the loss of desmosomes might be considered as an early event that ends with tumor invasion and progression (Dusek and Attardi, 2011). Indeed, this hypothesis could also apply to HuCCT-1 cell lines, as they contained very few desmosomes. Since HuCCT-1 cells were derived from a moderately differentiated CCA, our data may indicate that these cells might denote an early stage of EMT. Therefore, one can speculate that the cadherin switch, where E-cad is replaced with N-cad, has begun with the diminishing of cell-cell junctions. Finally, the number of desmosomes ($p=0.0021$), the number of cell-cell junctions ($p=0.0071$) and the length of those cell-cell junctions ($p=0.0339$) differ significantly between these CCA cell lines.

4.3 No significant correlation between FPC and AJ components has been established

The investigation results determined that OZ, Huh-28 and HuCCT-1 are FPC-expressing CCA cell lines, whereas the HCC cell line does not express FPC. These results are compatible with our previous study, which demonstrated that FPC serves as an excellent marker for distinguishing between CCA and HCC. Nevertheless, considering the structural domains of FPC and its similarity to adhesion molecules, several studies have proposed that FPC may function and associate with cell adhesion molecules. Hence, this thesis hypothesized that the re-expression of FPC will affect the expression and localization of cell adhesion molecules in CCA. However, since this study revealed different expression and localization patterns of the various adhesion molecules in the CCA cell lines as well as steady expression levels of FPC, one can assume that there is no significant association between the expression of FPC and AJ components in CCA. Nevertheless, numerous approaches, including protein fractionations and immunoprecipitation experiments, can be performed to support or contradict this assertion.

4.4 FPC is an important growth promoting factor for CCA cells

shRNA plasmids containing 3 different shRNA sequences against PKHD1 mRNA was utilized to downregulate the gene expression in HuCCT-1 cell lines for the purposes of exploring the biological significance of FPC in CCA. MTS data showed that the downregulation of PKHD1 mRNA in HuCCT1 cell lines significantly decreased the proliferation and cell growth compared to scrambled shRNA, leading to the conclusion that FPC might be an essential factor that promotes CCA cell growth. Specifically, FPC undergoes sophisticated and regulated intramembrane proteolysis (RIP) processes that are accomplished by the cleavage and release of its cytoplasmic tail via γ-secretase. Subsequently, the released C-terminal fragments translocate to the nucleus. Furthermore, Hiesberger T. et al (2006) demonstrated that the released C-terminal fragments translocate to and localize in nucleoli (Hiesberger,Gourley, et al, 2006). Therefore, one can speculate that FPC might have essential roles in the regulation of genes that have implications in proliferation and cell cycles.

4.5 Future directions

The most important risk factor for CCA development is PSC. Until recently, there has been no precise biomarker for assisting PSC patients in screening for potential CCA development. To the best of our knowledge, FPC has not been investigated in PSC, and hence, our research group can hypothesize that FPC might be detected in PSC samples and that FPC expression is correlated with CCA development. Additional support from this hypothesis derives from the fact that FPC undergoes a regulated proteolytic processing similar to that of Notch (Kaimori,Nagasawa, et al, 2007), whose expression is significantly up-regulated in PSC and CCA. The outcomes of this hypothesis might clarify the role of FPC in triggering the malignant transformation of cholangiocytes and identify a precise prognostic marker, which will subsequently aid in an earlier detection of CCA.

4.6 Summary

According to the International Agency for Research on Cancer, approximately 7.6 million worldwide deaths resulted from cancer in 2008. The most common cancer-causing deaths include hepatobiliary, stomach and colorectal cancers (Ferlay,Shin, et al, 2010). Currently, CCA patients experience a poor prognosis due to the lack of feasible therapeutic options. The increased incidence of CCA emphasizes the necessity for improved understanding of the molecular events in cholangiocarcinogenesis. FPC is the main product of polycystic kidney and

hepatic disease 1 gene. In particular, PKHD1 mutations are responsible for most autosomal recessive polycystic diseases. FPC expression was identified in several tissues, including liver, kidney, and pancreas. Accordingly, our group demonstrated that FPC fulfills a central role in the development of the primitive intrahepatic biliary system, as FPC is expressed during the early stages of system development and absent when the biliary system is remodelled. FPC is an excellent biomarker for distinguishing CCA from HCC, especially since FPC is only expressed in CCA. Consequently, this thesis discussed the possible roles of FPC in CCA. Firstly, I investigated the association between FPC and AJ molecules, as suggested in several studies. Thus, the thesis hypothesized that the restoration of FPC expression in CCA will alter the expression of AJ molecules in CCA. However, since AJ molecules demonstrated variable expression and localization patterns in the CCA cell lines and steady expression levels of FPC were present, the thesis concluded that there is no significant association between FPC and cell adhesion molecule expressions in CCA. Secondly, this project investigated the potential biological significance of FPC in CCA, hypothesizing that down-regulation of the PKHD1 gene will affect cell proliferation and cell growth in CCA. Using shRNA, the PKHD1 mRNA was down-regulated and cell proliferation was assessed using MTS assay. The data demonstrated that FPC is an important growth factor that promotes cell growth in CCA. Although there are still many gaps in understanding, the data demonstrated in this thesis might represent a step forward for investigating intracellular mechanisms that underlie

the development of CCA, hence providing insight into future therapeutic options for CCA.

References

Reference List

Abuetabh, Y., Persad, S., Nagamori, S., Huggins, J., Al-Bahrani, R. and Sergi, C. (2011). Expression of E-cadherin and beta-catenin in two cholangiocarcinoma cell lines (OZ and HuCCT1) with different degree of invasiveness of the primary tumor. Ann. Clin. Lab. Sci. *3,* 217-223.

Agiostratidou, G., Hulit, J., Phillips, G.R. and Hazan, R.B. (2007). Differential cadherin expression: potential markers for epithelial to mesenchymal transformation during tumor progression. J. Mammary Gland Biol. Neoplasia *2-3,* 127-133.

Akdogan, M., Sasmaz, N., Kayhan, B., Biyikoglu, I., Disibeyaz, S. and Sahin, B. (2001). Extraordinarily elevated CA19-9 in benign conditions: a case report and review of the literature. Tumori *5,* 337-339.

Aktary, Z., Chapman, K., Lam, L., Lo, A., Ji, C., Graham, K., Cook, L., Li, L., Mackey, J.R. and Pasdar, M. (2010). Plakoglobin interacts with and increases the protein levels of metastasis suppressor Nm23-H2 and regulates the expression of Nm23-H1. Oncogene *14,* 2118-2129.

Albert, M.B., Steinberg, W.M. and Henry, J.P. (1988). Elevated serum levels of tumor marker CA19-9 in acute cholangitis. Dig. Dis. Sci. *10,* 1223-1225.

Al-Bhalal, L. and Akhtar, M. (2008). Molecular basis of autosomal recessive polycystic kidney disease (ARPKD). Adv. Anat. Pathol. *1,* 54-58.

Alvaro, D. (2009). Serum and bile biomarkers for cholangiocarcinoma. Curr. Opin. Gastroenterol. *3,* 279-284.

Anna, C.H., Iida, M., Sills, R.C. and Devereux, T.R. (2003). Expression of potential beta-catenin targets, cyclin D1, c-Jun, c-Myc, E-cadherin, and EGFR in chemically induced hepatocellular neoplasms from B6C3F1 mice. Toxicol. Appl. Pharmacol. *2,* 135-145.

Berthiaume, E.P. and Wands, J. (2004). The molecular pathogenesis of cholangiocarcinoma. Semin. Liver Dis. *2,* 127-137.

Bettschart, V., Clayton, R.A., Parks, R.W., Garden, O.J. and Bellamy, C.O. (2002). Cholangiocarcinoma arising after biliary-enteric drainage procedures for benign disease. Gut *1,* 128-129.

Bhunia, A.K., Piontek, K., Boletta, A., Liu, L., Qian, F., Xu, P.N., Germino, F.J. and Germino, G.G. (2002). PKD1 induces p21(waf1) and regulation of the cell cycle via direct activation of the JAK-STAT signaling pathway in a process requiring PKD2. Cell *2,* 157-168.

Blechacz, B. and Gores, G.J. (2008). Cholangiocarcinoma: advances in pathogenesis, diagnosis, and treatment. Hepatology *1,* 308-321.

Blechacz, B.R. and Gores, G.J. (2008). Cholangiocarcinoma. Clin. Liver Dis. *1,* 131-50, ix.

Boca, M., D'Amato, L., Distefano, G., Polishchuk, R.S., Germino, G.G. and Boletta, A. (2007). Polycystin-1 induces cell migration by regulating phosphatidylinositol 3-kinase-dependent cytoskeletal rearrangements and GSK3beta-dependent cell cell mechanical adhesion. Mol. Biol. Cell *10,* 4050-4061.

Brabletz, T., Jung, A., Reu, S., Porzner, M., Hlubek, F., Kunz-Schughart, L.A., Knuechel, R. and Kirchner, T. (2001). Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proc. Natl. Acad. Sci. U. S. A. *18,* 10356-10361.

Breuninger, S., Reidenbach, S., Sauer, C.G., Strobel, P., Pfitzenmaier, J., Trojan, L. and Hofmann, I. (2010). Desmosomal plakophilins in the prostate and prostatic adenocarcinomas: implications for diagnosis and tumor progression. Am. J. Pathol. *5,* 2509-2519.

Briggs, C.D., Neal, C.P., Mann, C.D., Steward, W.P., Manson, M.M. and Berry, D.P. (2009). Prognostic molecular markers in cholangiocarcinoma: a systematic review. Eur. J. Cancer *1,* 33-47.

Brooke, M.A., Nitoiu, D. and Kelsell, D.P. (2012). Cell-cell connectivity: desmosomes and disease. J. Pathol. *2,* 158-171.

Brown, J.R. and DuBois, R.N. (2005). COX-2: a molecular target for colorectal cancer prevention. J. Clin. Oncol. *12,* 2840-2855.

Burak, K., Angulo, P., Pasha, T.M., Egan, K., Petz, J. and Lindor, K.D. (2004). Incidence and risk factors for cholangiocarcinoma in primary sclerosing cholangitis. Am. J. Gastroenterol. *3,* 523-526.

Casavilla, F.A., Marsh, J.W., Iwatsuki, S., Todo, S., Lee, R.G., Madariaga, J.R., Pinna, A., Dvorchik, I., Fung, J.J. and Starzl, T.E. (1997). Hepatic resection and transplantation for peripheral cholangiocarcinoma. J. Am. Coll. Surg. *5,* 429-436.

Chalasani, N., Baluyut, A., Ismail, A., Zaman, A., Sood, G., Ghalib, R., McCashland, T.M., Reddy, K.R., Zervos, X., Anbari, M.A. and Hoen, H. (2000). Cholangiocarcinoma in patients with primary sclerosing cholangitis: a multicenter case-control study. Hepatology *1,* 7-11.

Charles, M.W., Mill, A.J. and Darley, P.J. (2003). Carcinogenic risk of hot-particle exposures. J. Radiol. Prot. *1,* 5-28.

Chen, C.Y., Shiesh, S.C., Tsao, H.C. and Lin, X.Z. (2002). The assessment of biliary CA 125, CA 19-9 and CEA in diagnosing cholangiocarcinoma--the influence of sampling time and hepatolithiasis. Hepatogastroenterology *45,* 616-620.

Chidgey, M. (2002). Desmosomes and disease: an update. Histol. Histopathol. *4,* 1179-1192.

Chung, Y.E., Kim, M.J., Park, Y.N., Choi, J.Y., Pyo, J.Y., Kim, Y.C., Cho, H.J., Kim, K.A. and Choi, S.Y. (2009). Varying appearances of cholangiocarcinoma: radiologic-pathologic correlation. Radiographics *3*, 683-700.

Cimbora-Zovko, T., Ambriovic-Ristov, A., Loncarek, J. and Osmak, M. (2007). Altered cell-cell adhesion in cisplatin-resistant human carcinoma cells: a link between beta-catenin/plakoglobin ratio and cisplatin resistance. Eur. J. Pharmacol. *1-3*, 27-36.

Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C.E., Van der Smissen, P., Dietz, H.C., Courtoy, P.J., Rousseau, G.G. and Lemaigre, F.P. (2005). Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. Genes Dev. *16*, 1849-1854.

Cohn, S.H., Gusmano, E.A. and Robertson, J.S. (1967). Calculation of radiation dose from thorotrast using whole-body gamma-ray spectral data. Ann. N. Y. Acad. Sci. *3*, 608-622.

De Vreede, I., Steers, J.L., Burch, P.A., Rosen, C.B., Gunderson, L.L., Haddock, M.G., Burgart, L. and Gores, G.J. (2000). Prolonged disease-free survival after orthotopic liver transplantation plus adjuvant chemoirradiation for cholangiocarcinoma. Liver Transpl. *3*, 309-316.

Decaens, T., Godard, C., de Reynies, A., Rickman, D.S., Tronche, F., Couty, J.P., Perret, C. and Colnot, S. (2008). Stabilization of beta-catenin affects mouse embryonic liver growth and hepatoblast fate. Hepatology *1*, 247-258.

Dell, K.M. (2011). The spectrum of polycystic kidney disease in children. Adv. Chronic Kidney Dis. *5,* 339-347.

Delva, E., Tucker, D.K. and Kowalczyk, A.P. (2009). The desmosome. Cold Spring Harb Perspect. Biol. *2,* a002543.

Dorn, L., Menezes, L.F., Mikuz, G., Otto, H.F., Onuchic, L.F. and Sergi, C. (2009). Immunohistochemical detection of polyductin and co-localization with liver progenitor cell markers during normal and abnormal development of the intrahepatic biliary system and in adult hepatobiliary carcinomas. J. Cell. Mol. Med. *7,* 1279-1290.

Dusek, R.L. and Attardi, L.D. (2011). Desmosomes: new perpetrators in tumour suppression. Nat. Rev. Cancer. *5,* 317-323.

Eastman, Q. and Grosschedl, R. (1999). Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Curr. Opin. Cell Biol. *2,* 233-240.

Fava, G., Marzioni, M., Benedetti, A., Glaser, S., DeMorrow, S., Francis, H. and Alpini, G. (2007). Molecular pathology of biliary tract cancers. Cancer Lett. *2,* 155-167.

Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C. and Parkin, D.M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int. J. Cancer *12,* 2893-2917.

Fischer, D.C., Jacoby, U., Pape, L., Ward, C.J., Kuwertz-Broeking, E., Renken, C., Nizze, H., Querfeld, U., Rudolph, B., Mueller-Wiefel, D.E., Bergmann, C. and Haffner, D. (2009). Activation of the AKT/mTOR pathway in autosomal recessive polycystic kidney disease (ARPKD). Nephrol. Dial. Transplant. *6,* 1819-1827.

Franke, W.W. (2009). Discovering the molecular components of intercellular junctions--a historical view. Cold Spring Harb Perspect. Biol. *3,* a003061.

Furmanczyk, P.S., Grieco, V.S. and Agoff, S.N. (2005). Biliary brush cytology and the detection of cholangiocarcinoma in primary sclerosing cholangitis: evaluation of specific cytomorphologic features and CA19-9 levels. Am. J. Clin. Pathol. *3,* 355-360.

Furukawa, C., Daigo, Y., Ishikawa, N., Kato, T., Ito, T., Tsuchiya, E., Sone, S. and Nakamura, Y. (2005). Plakophilin 3 oncogene as prognostic marker and therapeutic target for lung cancer. Cancer Res. *16,* 7102-7110.

Gao, S., Eiberg, H., Krogdahl, A., Liu, C.J. and Sorensen, J.A. (2005). Cytoplasmic expression of E-cadherin and beta-Catenin correlated with LOH and hypermethylation of the APC gene in oral squamous cell carcinomas. J. Oral Pathol. Med. *2,* 116-119.

Garrod, D. and Chidgey, M. (2008). Desmosome structure, composition and function. Biochim. Biophys. Acta *3,* 572-587.

Gat, U., DasGupta, R., Degenstein, L. and Fuchs, E. (1998). De Novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. Cell *5*, 605-614.

Gatto, M., Bragazzi, M.C., Semeraro, R., Napoli, C., Gentile, R., Torrice, A., Gaudio, E. and Alvaro, D. (2010). Cholangiocarcinoma: update and future perspectives. Dig. Liver Dis. *4*, 253-260.

Giamarchi, A., Padilla, F., Coste, B., Raoux, M., Crest, M., Honore, E. and Delmas, P. (2006). The versatile nature of the calcium-permeable cation channel TRPP2. EMBO Rep. *8*, 787-793.

Giepmans, B.N. and van Ijzendoorn, S.C. (2009). Epithelial cell-cell junctions and plasma membrane domains. Biochim. Biophys. Acta *4*, 820-831.

Gores, G.J. (2003). Cholangiocarcinoma: current concepts and insights. Hepatology *5*, 961-969.

Gores, G.J., Nagorney, D.M. and Rosen, C.B. (2007). Cholangiocarcinoma: is transplantation an option? For whom? J. Hepatol. *4*, 455-459.

Guilford, P. (1999). E-cadherin downregulation in cancer: fuel on the fire? Mol. Med. Today *4*, 172-177.

Guilford, P.J., Hopkins, J.B., Grady, W.M., Markowitz, S.D., Willis, J., Lynch, H., Rajput, A., Wiesner, G.L., Lindor, N.M., Burgart, L.J. *et al* . (1999). E-

cadherin germline mutations define an inherited cancer syndrome dominated by diffuse gastric cancer. Hum. Mutat. *3*, 249-255.

Guzinska-Ustymowicz, K., Chetnik, A. and Kemona, A. (2004). Effects of changes at the site of E-cadherin expression as an indicator of colon cancer aggressiveness. Rocz. Akad. Med. Bialymst. 70-72.

Halliday, J.S., Djordjevic, J., Lust, M., Culver, E.L., Braden, B., Travis, S.P. and Chapman, R.W. (2012). A unique clinical phenotype of primary sclerosing cholangitis associated with Crohn's disease. J. Crohns Colitis *2*, 174-181.

Han, C., Leng, J., Demetris, A.J. and Wu, T. (2004). Cyclooxygenase-2 promotes human cholangiocarcinoma growth: evidence for cyclooxygenase-2-independent mechanism in celecoxib-mediated induction of p21waf1/cip1 and p27kip1 and cell cycle arrest. Cancer Res. *4*, 1369-1376.

Haruna, Y., Saito, K., Spaulding, S., Nalesnik, M.A. and Gerber, M.A. (1996). Identification of bipotential progenitor cells in human liver development. Hepatology *3*, 476-481.

Heimbach, J.K., Gores, G.J., Haddock, M.G., Alberts, S.R., Pedersen, R., Kremers, W., Nyberg, S.L., Ishitani, M.B. and Rosen, C.B. (2006). Predictors of disease recurrence following neoadjuvant chemoradiotherapy and liver transplantation for unresectable perihilar cholangiocarcinoma. Transplantation *12*, 1703-1707.

Hiesberger, T., Gourley, E., Erickson, A., Koulen, P., Ward, C.J., Masyuk, T.V., Larusso, N.F., Harris, P.C. and Igarashi, P. (2006). Proteolytic cleavage and nuclear translocation of fibrocystin is regulated by intracellular Ca2+ and activation of protein kinase C. J. Biol. Chem. *45,* 34357-34364.

Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S. and Takeichi, M. (1992). Identification of a neural alpha-catenin as a key regulator of cadherin function and multicellular organization. Cell *2,* 293-301.

Hirohashi, S. (1998). Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. Am. J. Pathol. *2,* 333-339.

Homma, S., Nagamori, S., Fujise, K., Yamazaki, K., Hasumura, S., Sujino, H., Matsuura, T., Shimizu, K., Kameda, H. and Takaki, K. (1987). Human bile duct carcinoma cell line producing abundant mucin in vitro. Gastroenterol. Jpn. *4,* 474-479.

Hughes, J., Ward, C.J., Peral, B., Aspinwall, R., Clark, K., San Millan, J.L., Gamble, V. and Harris, P.C. (1995). The polycystic kidney disease 1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. Nat. Genet. *2,* 151-160.

Isaji, S., Kawarada, Y., Taoka, H., Tabata, M., Suzuki, H. and Yokoi, H. (1999). Clinicopathological features and outcome of hepatic resection for intrahepatic cholangiocarcinoma in Japan. J. Hepatobiliary. Pancreat. Surg. *2,* 108-116.

Ishimura, N., Bronk, S.F. and Gores, G.J. (2005). Inducible nitric oxide synthase up-regulates Notch-1 in mouse cholangiocytes: implications for carcinogenesis. Gastroenterology *5*, 1354-1368.

Isomoto, H., Kobayashi, S., Werneburg, N.W., Bronk, S.F., Guicciardi, M.E., Frank, D.A. and Gores, G.J. (2005). Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells. Hepatology *6*, 1329-1338.

Isomoto, H., Mott, J.L., Kobayashi, S., Werneburg, N.W., Bronk, S.F., Haan, S. and Gores, G.J. (2007). Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing. Gastroenterology *1*, 384-396.

Iwatsuki, S., Todo, S., Marsh, J.W., Madariaga, J.R., Lee, R.G., Dvorchik, I., Fung, J.J. and Starzl, T.E. (1998). Treatment of hilar cholangiocarcinoma (Klatskin tumors) with hepatic resection or transplantation. J. Am. Coll. Surg. *4*, 358-364.

Jaiswal, M., LaRusso, N.F., Burgart, L.J. and Gores, G.J. (2000). Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. Cancer Res. *1*, 184-190.

Jarnagin, W.R., Fong, Y., DeMatteo, R.P., Gonen, M., Burke, E.C., Bodniewicz BS, J., Youssef BA, M., Klimstra, D. and Blumgart, L.H. (2001). Staging, resectability, and outcome in 225 patients with hilar cholangiocarcinoma. Ann. Surg. *4*, 507-17; discussion 517-9.

Johnson, C.A., Gissen, P. and Sergi, C. (2003). Molecular pathology and genetics of congenital hepatorenal fibrocystic syndromes. J. Med. Genet. *5*, 311-319.

Kaewpitoon, N., Kaewpitoon, S.J. and Pengsaa, P. (2008). Opisthorchiasis in Thailand: review and current status. World J. Gastroenterol. *15*, 2297-2302.

Kaimori, J.Y., Nagasawa, Y., Menezes, L.F., Garcia-Gonzalez, M.A., Deng, J., Imai, E., Onuchic, L.F., Guay-Woodford, L.M. and Germino, G.G. (2007). Polyductin undergoes notch-like processing and regulated release from primary cilia. Hum. Mol. Genet. *8*, 942-956.

Kim, E., Arnould, T., Sellin, L.K., Benzing, T., Fan, M.J., Gruning, W., Sokol, S.Y., Drummond, I. and Walz, G. (1999). The polycystic kidney disease 1 gene product modulates Wnt signaling. J. Biol. Chem. *8*, 4947-4953.

Kim, J.B., Islam, S., Kim, Y.J., Prudoff, R.S., Sass, K.M., Wheelock, M.J. and Johnson, K.R. (2000). N-Cadherin extracellular repeat 4 mediates epithelial to mesenchymal transition and increased motility. J. Cell Biol. *6*, 1193-1206.

Klempnauer, J., Ridder, G.J., Werner, M., Weimann, A. and Pichlmayr, R. (1997). What constitutes long-term survival after surgery for hilar cholangiocarcinoma? Cancer *1*, 26-34.

Kobayashi, S., Werneburg, N.W., Bronk, S.F., Kaufmann, S.H. and Gores, G.J. (2005). Interleukin-6 contributes to Mcl-1 up-regulation and TRAIL resistance via

an Akt-signaling pathway in cholangiocarcinoma cells. Gastroenterology *7,* 2054-2065.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B. and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science *5307,* 1784-1787.

Ku, J.L., Yoon, K.A., Kim, I.J., Kim, W.H., Jang, J.Y., Suh, K.S., Kim, S.W., Park, Y.H., Hwang, J.H., Yoon, Y.B. and Park, J.G. (2002). Establishment and characterisation of six human biliary tract cancer cell lines. Br. J. Cancer *2,* 187-193.

Kusaka, Y., Tokiwa, T. and Sato, J. (1988). Establishment and characterization of a cell line from a human cholangiocellular carcinoma. Res. Exp. Med. (Berl) *5,* 367-375.

Lai, G.H., Radaeva, S., Nakamura, T. and Sirica, A.E. (2000). Unique epithelial cell production of hepatocyte growth factor/scatter factor by putative precancerous intestinal metaplasias and associated "intestinal-type" biliary cancer chemically induced in rat liver. Hepatology *6,* 1257-1265.

LaRusso, N.F., Shneider, B.L., Black, D., Gores, G.J., James, S.P., Doo, E. and Hoofnagle, J.H. (2006). Primary sclerosing cholangitis: summary of a workshop. Hepatology *3,* 746-764.

Lee, F.I., Tharakan, J., Vasudev, K.S. and Isaacs, P.E. (1996). Malignant hepatic tumours associated with previous exposure to Thorotrast: four cases. Eur. J. Gastroenterol. Hepatol. *11*, 1121-1124.

Lee, T.Y., Lee, S.S., Jung, S.W., Jeon, S.H., Yun, S.C., Oh, H.C., Kwon, S., Lee, S.K., Seo, D.W., Kim, M.H. and Suh, D.J. (2008). Hepatitis B virus infection and intrahepatic cholangiocarcinoma in Korea: a case-control study. Am. J. Gastroenterol. *7*, 1716-1720.

Lesurtel, M., Regimbeau, J.M., Farges, O., Colombat, M., Sauvanet, A. and Belghiti, J. (2002). Intrahepatic cholangiocarcinoma and hepatolithiasis: an unusual association in Western countries. Eur. J. Gastroenterol. Hepatol. *9*, 1025-1027.

Levy, C., Lymp, J., Angulo, P., Gores, G.J., Larusso, N. and Lindor, K.D. (2005). The value of serum CA 19-9 in predicting cholangiocarcinomas in patients with primary sclerosing cholangitis. Dig. Dis. Sci. *9*, 1734-1740.

Lieser, M.J., Barry, M.K., Rowland, C., Ilstrup, D.M. and Nagorney, D.M. (1998). Surgical management of intrahepatic cholangiocarcinoma: a 31-year experience. J. Hepatobiliary. Pancreat. Surg. *1*, 41-47.

Lin, S.Y., Xia, W., Wang, J.C., Kwong, K.Y., Spohn, B., Wen, Y., Pestell, R.G. and Hung, M.C. (2000). Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. Proc. Natl. Acad. Sci. U. S. A. *8*, 4262-4266.

Lipsett, P.A., Pitt, H.A., Colombani, P.M., Boitnott, J.K. and Cameron, J.L. (1994). Choledochal cyst disease. A changing pattern of presentation. Ann. Surg. *5*, 644-652.

Lipshutz, G.S., Brennan, T.V. and Warren, R.S. (2002). Thorotrast-induced liver neoplasia: a collective review. J. Am. Coll. Surg. *5*, 713-718.

Lipson, K.E., Pang, L., Huber, L.J., Chen, H., Tsai, J.M., Hirth, P., Gazit, A., Levitzki, A. and McMahon, G. (1998). Inhibition of platelet-derived growth factor and epidermal growth factor receptor signaling events after treatment of cells with specific synthetic inhibitors of tyrosine kinase phosphorylation. J. Pharmacol. Exp. Ther. *2*, 844-852.

Low, S.H., Vasanth, S., Larson, C.H., Mukherjee, S., Sharma, N., Kinter, M.T., Kane, M.E., Obara, T. and Weimbs, T. (2006). Polycystin-1, STAT6, and P100 function in a pathway that transduces ciliary mechanosensation and is activated in polycystic kidney disease. Dev. Cell. *1*, 57-69.

Madariaga, J.R., Iwatsuki, S., Todo, S., Lee, R.G., Irish, W. and Starzl, T.E. (1998). Liver resection for hilar and peripheral cholangiocarcinomas: a study of 62 cases. Ann. Surg. *1*, 70-79.

Mai, W., Chen, D., Ding, T., Kim, I., Park, S., Cho, S.Y., Chu, J.S., Liang, D., Wang, N., Wu, D. *et al* . (2005). Inhibition of Pkhd1 impairs tubulomorphogenesis of cultured IMCD cells. Mol. Biol. Cell *9*, 4398-4409.

Mairiang, E., Laha, T., Bethony, J.M., Thinkhamrop, B., Kaewkes, S., Sithithaworn, P., Tesana, S., Loukas, A., Brindley, P.J. and Sripa, B. (2012). Ultrasonography assessment of hepatobiliary abnormalities in 3359 subjects with Opisthorchis viverrini infection in endemic areas of Thailand. Parasitol. Int. *1*, 208-211.

Martin, T.A. and Jiang, W.G. (2009). Loss of tight junction barrier function and its role in cancer metastasis. Biochim. Biophys. Acta *4*, 872-891.

McMasters, K.M., Tuttle, T.M., Leach, S.D., Rich, T., Cleary, K.R., Evans, D.B. and Curley, S.A. (1997). Neoadjuvant chemoradiation for extrahepatic cholangiocarcinoma. Am. J. Surg. *6*, 605-8; discussion 608-9.

Menezes, L.F., Cai, Y., Nagasawa, Y., Silva, A.M., Watkins, M.L., Da Silva, A.M., Somlo, S., Guay-Woodford, L.M., Germino, G.G. and Onuchic, L.F. (2004). Polyductin, the PKHD1 gene product, comprises isoforms expressed in plasma membrane, primary cilium, and cytoplasm. Kidney Int. *4*, 1345-1355.

Menezes, L.F. and Onuchic, L.F. (2006). Molecular and cellular pathogenesis of autosomal recessive polycystic kidney disease. Braz. J. Med. Biol. Res. *12*, 1537-1548.

Meng, W. and Takeichi, M. (2009). Adherens junction: molecular architecture and regulation. Cold Spring Harb Perspect. Biol. *6*, a002899.

Meyer, C.G., Penn, I. and James, L. (2000). Liver transplantation for cholangiocarcinoma: results in 207 patients. Transplantation *8,* 1633-1637.

Miyagiwa, M., Ichida, T., Tokiwa, T., Sato, J. and Sasaki, H. (1989). A new human cholangiocellular carcinoma cell line (HuCC-T1) producing carbohydrate antigen 19/9 in serum-free medium. In Vitro Cell. Dev. Biol. *6,* 503-510.

Mori, T., Sugiyama, M. and Atomi, Y. (2006). Gallstone disease: Management of intrahepatic stones. Best Pract. Res. Clin. Gastroenterol. *6,* 1117-1137.

Morris-Stiff, G., Bhati, C., Olliff, S., Hubscher, S., Gunson, B., Mayer, D., Mirza, D., Buckels, J. and Bramhall, S.R. (2008). Cholangiocarcinoma complicating primary sclerosing cholangitis: a 24-year experience. Dig. Surg. *2,* 126-132.

Mosconi, S., Beretta, G.D., Labianca, R., Zampino, M.G., Gatta, G. and Heinemann, V. (2009). Cholangiocarcinoma. Crit. Rev. Oncol. Hematol. *3,* 259-270.

Mott, J.L., Kobayashi, S., Bronk, S.F. and Gores, G.J. (2007). mir-29 regulates Mcl-1 protein expression and apoptosis. Oncogene *42*, 6133-6140.

Na, B., Huang, Z., Wang, Q., Qi, Z., Tian, Y., Lu, C.C., Yu, J., Hanes, M.A., Kakar, S., Huang, E.J. *et al* . (2011). Transgenic expression of entire hepatitis B virus in mice induces hepatocarcinogenesis independent of chronic liver injury. PLoS One *10*, e26240.

Nagorney, D.M. and Kendrick, M.L. (2006). Hepatic resection in the treatment of hilar cholangiocarcinoma. Adv. Surg. 159-171.

Nakagohri, T., Kinoshita, T., Konishi, M., Takahashi, S. and Gotohda, N. (2008). Surgical outcome and prognostic factors in intrahepatic cholangiocarcinoma. World J. Surg. *12*, 2675-2680.

Nakeeb, A., Pitt, H.A., Sohn, T.A., Coleman, J., Abrams, R.A., Piantadosi, S., Hruban, R.H., Lillemoe, K.D., Yeo, C.J. and Cameron, J.L. (1996). Cholangiocarcinoma. A spectrum of intrahepatic, perihilar, and distal tumors. Ann. Surg. *4*, 463-73; discussion 473-5.

Narimatsu, H., Iwasaki, H., Nakayama, F., Ikehara, Y., Kudo, T., Nishihara, S., Sugano, K., Okura, H., Fujita, S. and Hirohashi, S. (1998). Lewis and secretor gene dosages affect CA19-9 and DU-PAN-2 serum levels in normal individuals and colorectal cancer patients. Cancer Res. *3*, 512-518.

Nehls, O., Gregor, M. and Klump, B. (2004). Serum and bile markers for cholangiocarcinoma. Semin. Liver Dis. *2*, 139-154.

Newby, L.J., Streets, A.J., Zhao, Y., Harris, P.C., Ward, C.J. and Ong, A.C. (2002). Identification, characterization, and localization of a novel kidney polycystin-1-polycystin-2 complex. J. Biol. Chem. *23*, 20763-20773.

Nzeako, U.C., Guicciardi, M.E., Yoon, J.H., Bronk, S.F. and Gores, G.J. (2002). COX-2 inhibits Fas-mediated apoptosis in cholangiocarcinoma cells. Hepatology *3*, 552-559.

O'Grady, J.G., Polson, R.J., Rolles, K., Calne, R.Y. and Williams, R. (1988). Liver transplantation for malignant disease. Results in 93 consecutive patients. Ann. Surg. *4*, 373-379.

Ohshima, H., Bandaletova, T.Y., Brouet, I., Bartsch, H., Kirby, G., Ogunbiyi, F., Vatanasapt, V. and Pipitgool, V. (1994). Increased nitrosamine and nitrate biosynthesis mediated by nitric oxide synthase induced in hamsters infected with liver fluke (Opisthorchis viverrini). Carcinogenesis *2*, 271-275.

Ohtsuka, M., Ito, H., Kimura, F., Shimizu, H., Togawa, A., Yoshidome, H. and Miyazaki, M. (2002). Results of surgical treatment for intrahepatic cholangiocarcinoma and clinicopathological factors influencing survival. Br. J. Surg. *12*, 1525-1531.

Okada, K., Shimizu, Y., Nambu, S., Higuchi, K. and Watanabe, A. (1994). Interleukin-6 functions as an autocrine growth factor in a cholangiocarcinoma cell line. J. Gastroenterol. Hepatol. *5*, 462-467.

Ong, A.C. and Harris, P.C. (2005). Molecular pathogenesis of ADPKD: the polycystin complex gets complex. Kidney Int. *4*, 1234-1247.

Onori, P., Franchitto, A., Mancinelli, R., Carpino, G., Alvaro, D., Francis, H., Alpini, G. and Gaudio, E. (2010). Polycystic liver diseases. Dig. Liver Dis. *4*, 261-271.

Onuchic, L.F., Furu, L., Nagasawa, Y., Hou, X., Eggermann, T., Ren, Z., Bergmann, C., Senderek, J., Esquivel, E., Zeltner, R. *et al* . (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. Am. J. Hum. Genet. *5*, 1305-1317.

Ozawa, M. and Kemler, R. (1992). Molecular organization of the uvomorulin-catenin complex. J. Cell Biol. *4*, 989-996.

Pan, F.Y., Zhang, S.Z., Xu, N., Meng, F.L., Zhang, H.X., Xue, B., Han, X. and Li, C.J. (2010). Beta-catenin signaling involves HGF-enhanced HepG2 scattering through activating MMP-7 transcription. Histochem. Cell Biol. *3*, 285-295.

Park, J., Tadlock, L., Gores, G.J. and Patel, T. (1999). Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line. Hepatology *5*, 1128-1133.

Parkin, D.M., Srivatanakul, P., Khlat, M., Chenvidhya, D., Chotiwan, P., Insiripong, S., L'Abbe, K.A. and Wild, C.P. (1991). Liver cancer in Thailand. I. A case-control study of cholangiocarcinoma. Int. J. Cancer *3*, 323-328.

Pascher, A., Jonas, S. and Neuhaus, P. (2003). Intrahepatic cholangiocarcinoma: indication for transplantation. J. Hepatobiliary. Pancreat. Surg. *4,* 282-287.

Patel, T. and Singh, P. (2007). Cholangiocarcinoma: emerging approaches to a challenging cancer. Curr. Opin. Gastroenterol. *3,* 317-323.

Pecina-Slaus, N. (2003). Tumor suppressor gene E-cadherin and its role in normal and malignant cells. Cancer. Cell. Int. *1*, 17.

Pichlmayr, R., Weimann, A., Oldhafer, K.J., Schlitt, H.J., Klempnauer, J., Bornscheuer, A., Chavan, A., Schmoll, E., Lang, H. and Tusch, G. (1995). Role of liver transplantation in the treatment of unresectable liver cancer. World J. Surg. *6,* 807-813.

Pinho, S.S., Matos, A.J., Lopes, C., Marcos, N.T., Carvalheira, J., Reis, C.A. and Gartner, F. (2007). Sialyl Lewis x expression in canine malignant mammary tumours: correlation with clinicopathological features and E-Cadherin expression. BMC Cancer 124.

Pinlaor, S., Ma, N., Hiraku, Y., Yongvanit, P., Semba, R., Oikawa, S., Murata, M., Sripa, B., Sithithaworn, P. and Kawanishi, S. (2004). Repeated infection with Opisthorchis viverrini induces accumulation of 8-nitroguanine and 8-oxo-7,8-dihydro-2'-deoxyguanine in the bile duct of hamsters via inducible nitric oxide synthase. Carcinogenesis *8,* 1535-1542.

Rieger-Christ, K.M., Ng, L., Hanley, R.S., Durrani, O., Ma, H., Yee, A.S., Libertino, J.A. and Summerhayes, I.C. (2005). Restoration of plakoglobin expression in bladder carcinoma cell lines suppresses cell migration and tumorigenic potential. Br. J. Cancer *12,* 2153-2159.

Schmalhofer, O., Brabletz, S. and Brabletz, T. (2009). E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. Cancer Metastasis Rev. *1-2,* 151-166.

Schulick, R.D. (2008). Primary sclerosing cholangitis: detection of cancer in strictures. J. Gastrointest. Surg. *3,* 420-422.

Scott, J., Shousha, S., Thomas, H.C. and Sherlock, S. (1980). Bile duct carcinoma: a late complication of congenital hepatic fibrosis. Case report and review of literature. Am. J. Gastroenterol. *2,* 113-119.

Sergi, C., Adam, S., Kahl, P. and Otto, H.F. (2000). The remodeling of the primitive human biliary system. Early Hum. Dev. *3,* 167-178.

Sergi, C., Adam, S., Kahl, P. and Otto, H.F. (2000). Study of the malformation of ductal plate of the liver in Meckel syndrome and review of other syndromes presenting with this anomaly. Pediatr. Dev. Pathol. *6,* 568-583.

Shaib, Y. and El-Serag, H.B. (2004). The epidemiology of cholangiocarcinoma. Semin. Liver Dis. *2,* 115-125.

Shapiro, L. and Weis, W.I. (2009). Structure and biochemistry of cadherins and catenins. Cold Spring Harb Perspect. Biol. *3,* a003053.

Shin, H.R., Oh, J.K., Masuyer, E., Curado, M.P., Bouvard, V., Fang, Y., Wiangnon, S., Sripa, B. and Hong, S.T. (2010). Comparison of incidence of intrahepatic and extrahepatic cholangiocarcinoma--focus on East and South-Eastern Asia. Asian Pac. J. Cancer. Prev. *5,* 1159-1166.

Silverman, P.M., Ram, P.C. and Korobkin, M. (1983). CT appearance of abdominal thorotrast deposition and Thorotrast-induced angiosarcoma of the liver. J. Comput. Assist. Tomogr. *4,* 655-658.

Simcha, I., Shtutman, M., Salomon, D., Zhurinsky, J., Sadot, E., Geiger, B. and Ben-Ze'ev, A. (1998). Differential nuclear translocation and transactivation potential of beta-catenin and plakoglobin. J. Cell Biol. *6,* 1433-1448.

Siqueira, E., Schoen, R.E., Silverman, W., Martin, J., Rabinovitz, M., Weissfeld, J.L., Abu-Elmaagd, K., Madariaga, J.R. and Slivka, A. (2002). Detecting cholangiocarcinoma in patients with primary sclerosing cholangitis. Gastrointest. Endosc. *1,* 40-47.

Sirica, A.E., Lai, G.H., Endo, K., Zhang, Z. and Yoon, B.I. (2002). Cyclooxygenase-2 and ERBB-2 in cholangiocarcinoma: potential therapeutic targets. Semin. Liver Dis. *3,* 303-313.

Sithithaworn, P., Andrews, R.H., Nguyen, V.D., Wongsaroj, T., Sinuon, M., Odermatt, P., Nawa, Y., Liang, S., Brindley, P.J. and Sripa, B. (2012). The current status of opisthorchiasis and clonorchiasis in the Mekong Basin. Parasitol. Int. *1*, 10-16.

Smalley, M.J. and Dale, T.C. (1999). Wnt signalling in mammalian development and cancer. Cancer Metastasis Rev. *2*, 215-230.

Spirli, C., Fabris, L., Duner, E., Fiorotto, R., Ballardini, G., Roskams, T., Larusso, N.F., Sonzogni, A., Okolicsanyi, L. and Strazzabosco, M. (2003). Cytokine-stimulated nitric oxide production inhibits adenylyl cyclase and cAMP-dependent secretion in cholangiocytes. Gastroenterology *3*, 737-753.

Srivatanakul, P., Honjo, S., Kittiwatanachot, P., Jedpiyawongse, A., Khuhaprema, T. and Miwa, M. (2010). Hepatitis viruses and risk of cholangiocarcinoma in northeast Thailand. Asian Pac. J. Cancer. Prev. *4*, 985-988.

Srivatanakul, P., Parkin, D.M., Jiang, Y.Z., Khlat, M., Kao-Ian, U.T., Sontipong, S. and Wild, C. (1991). The role of infection by Opisthorchis viverrini, hepatitis B virus, and aflatoxin exposure in the etiology of liver cancer in Thailand. A correlation study. Cancer *11*, 2411-2417.

Stern, D., Zamenhof, R. and Dawson-Hughes, B. (1993). Spurious dual-energy X-ray absorptiometry images in a patient exposed to the contrast agent Thorotrast. Osteoporos. Int. *5*, 283-285.

Stokes, D.L. (2007). Desmosomes from a structural perspective. Curr. Opin. Cell Biol. *5*, 565-571.

Sudan, D., DeRoover, A., Chinnakotla, S., Fox, I., Shaw, B.,Jr, McCashland, T., Sorrell, M., Tempero, M. and Langnas, A. (2002). Radiochemotherapy and transplantation allow long-term survival for nonresectable hilar cholangiocarcinoma. Am. J. Transplant. *8*, 774-779.

Sugawara, H., Yasoshima, M., Katayanagi, K., Kono, N., Watanabe, Y., Harada, K. and Nakanuma, Y. (1998). Relationship between interleukin-6 and proliferation and differentiation in cholangiocarcinoma. Histopathology *2*, 145-153.

Sun, L., Wang, S., Hu, C. and Zhang, X. (2011). Down-regulation of PKHD1 induces cell apoptosis through PI3K and NF-kappaB pathways. Exp. Cell Res. *7*, 932-940.

Takayama, T., Shiozaki, H., Shibamoto, S., Oka, H., Kimura, Y., Tamura, S., Inoue, M., Monden, T., Ito, F. and Monden, M. (1996). Beta-catenin expression in human cancers. Am. J. Pathol. *1*, 39-46.

Tocchi, A., Mazzoni, G., Liotta, G., Lepre, L., Cassini, D. and Miccini, M. (2001). Late development of bile duct cancer in patients who had biliary-enteric drainage for benign disease: a follow-up study of more than 1,000 patients. Ann. Surg. *2*, 210-214.

Torok, N.J., Higuchi, H., Bronk, S. and Gores, G.J. (2002). Nitric oxide inhibits apoptosis downstream of cytochrome C release by nitrosylating caspase 9. Cancer Res. *6,* 1648-1653.

Tsui, W.M., Lam, P.W., Lee, W.K. and Chan, Y.K. (2011). Primary hepatolithiasis, recurrent pyogenic cholangitis, and oriental cholangiohepatitis: a tale of 3 countries. Adv. Anat. Pathol. *4*, 318-328.

Ustundag, Y. and Bayraktar, Y. (2008). Cholangiocarcinoma: a compact review of the literature. World J. Gastroenterol. *42,* 6458-6466.

Valverde, A., Bonhomme, N., Farges, O., Sauvanet, A., Flejou, J.F. and Belghiti, J. (1999). Resection of intrahepatic cholangiocarcinoma: a Western experience. J. Hepatobiliary. Pancreat. Surg. *2*, 122-127.

Vestergaard, E.M., Hein, H.O., Meyer, H., Grunnet, N., Jorgensen, J., Wolf, H. and Orntoft, T.F. (1999). Reference values and biological variation for tumor marker CA 19-9 in serum for different Lewis and secretor genotypes and evaluation of secretor and Lewis genotyping in a Caucasian population. Clin. Chem. *1,* 54-61.

Wang, S., Luo, Y., Wilson, P.D., Witman, G.B. and Zhou, J. (2004). The autosomal recessive polycystic kidney disease protein is localized to primary cilia, with concentration in the basal body area. J. Am. Soc. Nephrol. *3,* 592-602.

Ward, C.J., Hogan, M.C., Rossetti, S., Walker, D., Sneddon, T., Wang, X., Kubly, V., Cunningham, J.M., Bacallao, R., Ishibashi, M. *et al* . (2002). The gene mutated in autosomal recessive polycystic kidney disease encodes a large, receptor-like protein. Nat. Genet. *3,* 259-269.

Ward, C.J., Yuan, D., Masyuk, T.V., Wang, X., Punyashthiti, R., Whelan, S., Bacallao, R., Torra, R., LaRusso, N.F., Torres, V.E. and Harris, P.C. (2003). Cellular and subcellular localization of the ARPKD protein; fibrocystin is expressed on primary cilia. Hum. Mol. Genet. *20,* 2703-2710.

Wehbe, H., Henson, R., Meng, F., Mize-Berge, J. and Patel, T. (2006). Interleukin-6 contributes to growth in cholangiocarcinoma cells by aberrant promoter methylation and gene expression. Cancer Res. *21,* 10517-10524.

Wei, C.J., Xu, X. and Lo, C.W. (2004). Connexins and cell signaling in development and disease. Annu. Rev. Cell Dev. Biol. 811-838.

Weimann, A., Varnholt, H., Schlitt, H.J., Lang, H., Flemming, P., Hustedt, C., Tusch, G. and Raab, R. (2000). Retrospective analysis of prognostic factors after liver resection and transplantation for cholangiocellular carcinoma. Br. J. Surg. *9,* 1182-1187.

Weimbs, T. (2006). Regulation of mTOR by polycystin-1: is polycystic kidney disease a case of futile repair? Cell. Cycle *21,* 2425-2429.

Wen, J. (2011). Congenital hepatic fibrosis in autosomal recessive polycystic kidney disease. Clin. Transl. Sci. *6,* 460-465.

Werneburg, N.W., Yoon, J.H., Higuchi, H. and Gores, G.J. (2003). Bile acids activate EGF receptor via a TGF-alpha-dependent mechanism in human cholangiocyte cell lines. Am. J. Physiol. Gastrointest. Liver Physiol. *1,* G31-6.

Wiedmann, M., Caca, K., Berr, F., Schiefke, I., Tannapfel, A., Wittekind, C., Mossner, J., Hauss, J. and Witzigmann, H. (2003). Neoadjuvant photodynamic therapy as a new approach to treating hilar cholangiocarcinoma: a phase II pilot study. Cancer *11,* 2783-2790.

Wilson, P.D. (2004). Polycystic kidney disease. N. Engl. J. Med. *2,* 151-164.

Wilson, P.D. and Goilav, B. (2007). Cystic disease of the kidney. Annu. Rev. Pathol. 341-368.

Wu, T., Leng, J., Han, C. and Demetris, A.J. (2004). The cyclooxygenase-2 inhibitor celecoxib blocks phosphorylation of Akt and induces apoptosis in human cholangiocarcinoma cells. Mol. Cancer. Ther. *3,* 299-307.

Xiong, H., Chen, Y., Yi, Y., Tsuchiya, K., Moeckel, G., Cheung, J., Liang, D., Tham, K., Xu, X., Chen, X.Z. *et al* . (2002). A novel gene encoding a TIG multiple domain protein is a positional candidate for autosomal recessive polycystic kidney disease. Genomics *1,* 96-104.

Yamada, T. and Alpers, D.H. (2009). Atlas of gastroenterology (Oxford: Wiley-Blackwell)

Yazumi, S., Ko, K., Watanabe, N., Shinohara, H., Yoshikawa, K., Chiba, T. and Takahashi, R. (2000). Disrupted transforming growth factor-beta signaling and deregulated growth in human biliary tract cancer cells. Int. J. Cancer *6*, 782-789.

Yoder, B.K., Hou, X. and Guay-Woodford, L.M. (2002). The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia. J. Am. Soc. Nephrol. *10,* 2508-2516.

Yoon, J.H., Higuchi, H., Werneburg, N.W., Kaufmann, S.H. and Gores, G.J. (2002). Bile acids induce cyclooxygenase-2 expression via the epidermal growth factor receptor in a human cholangiocarcinoma cell line. Gastroenterology *4*, 985-993.

Yoon, J.H., Werneburg, N.W., Higuchi, H., Canbay, A.E., Kaufmann, S.H., Akgul, C., Edwards, S.W. and Gores, G.J. (2002). Bile acids inhibit Mcl-1 protein turnover via an epidermal growth factor receptor/Raf-1-dependent mechanism. Cancer Res. *22,* 6500-6505.

Yu, C., Bruzek, L.M., Meng, X.W., Gores, G.J., Carter, C.A., Kaufmann, S.H. and Adjei, A.A. (2005). The role of Mcl-1 downregulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. Oncogene *46,* 6861-6869.

Zhang, Z., Lai, G.H. and Sirica, A.E. (2004). Celecoxib-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. Hepatology *4*, 1028-1037.

Zhu, A.X., Lauwers, G.Y. and Tanabe, K.K. (2004). Cholangiocarcinoma in association with Thorotrast exposure. J. Hepatobiliary. Pancreat. Surg. *6,* 430-433.

Zhurinsky, J., Shtutman, M. and Ben-Ze'ev, A. (2000). Differential mechanisms of LEF/TCF family-dependent transcriptional activation by beta-catenin and plakoglobin. Mol. Cell. Biol. *12*, 4238-4252.

Zorn, A.M. (2008). Liver development. In StemBook, (Cambridge (MA): Aaron M. Zorn)