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Transcription Factors GATA4 and GATA6 in Pediatric Liver Disease

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ACADEMIC DISSERTATION

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To my boys

ABSTRACT

GATA transcription factors are evolutionary highly conserved zinc-finger proteins. They are essential for the normal differentiation of various tissues, and their aberrant expression has been connected to several diseases including malignancies. GATA4 and GATA6 are indispensable for early development of the mammalian liver, but their expression is low in normal postnatal hepatocytes.

Hepatoblastoma (HB) is a rare liver malignancy of small children, with a largely unknown etiology and molecular pathology. Histologically HBs resemble fetal or embryonal liver tissue synthesizing alphafetoprotein, used as a diagnostic marker. Biliary atresia (BA) is a neonatal cholestatic condition caused by fibroinflammatory obstruction of the extrahepatic bile ducts, with subsequent histological changes in liver, including fibrosis and expansion of the intrahepatic bile ductules.

In this thesis work, we demonstrate that GATA4 is frequently overexpressed in childhood HB unlike in normal liver hepatocytes or adulthood liver tumors. The effects of GATA4 in HB cell malignancy were studied *in vitro* utilizing an established human HB cell line HUH6. GATA4 expression was modified in these cells by siRNA, adenoviral vectors, or plasmid constructs. The changes in cell function and gene expression were subsequently analyzed. We found that GATA4 1) protects HB cells from the commonly used cytostatic drug doxorubicin by regulating the BCL2 protein family balance in the intrinsic apoptotic pathway, and 2) shifts the transcriptomic profile of HB cells to more mesenchymal-type and enhances their migration, thus promoting cancer cell survival and metastasis.

A second focus of this thesis is the role of GATA6 transcription factor in BA pathogenesis. We found that GATA6, normally restricted to biliary epithelium in postnatal liver, is highly expressed in the hepatocytes of BA patients, i.e. in regions of ductal metaplasia known to lead to pathological changes in BA livers. GATA6 expression levels correlate to known prognostic markers, including bile ductule expansion and age at portoenterostomy. The expression of GATA6 decreases significantly after successful portoenterostomy and resolution of cholestasis. Analogously to its increased expression in BA, GATA6 is also upregulated in the hepatocytes of two mouse models with biliary obstruction. Furthermore, forced expression of GATA6 in human primary hepatocytes and human hepatoma cell line HepG2 drives their gene expression towards cholangiocyte lineage.

Taken together, transcription factors GATA4 and GATA6 are essential for the initial differentiation of hepatoblasts and for liver organogenesis. Expression of GATA4 and GATA6 is low in normal postnatal hepatocytes, but in these two pediatric diseases of the liver, HB and BA, their expression is upregulated respectively. Overexpression of

these GATA factors affects the gene expression of hepatocytes, and thus are putative drivers of hepatocyte metaplasia and neoplasia. This thesis work clarifies the mechanisms by which GATA4 promotes the malignant phenotype of HB cells and demonstrates GATA6 as a marker and driver of hepatocyte ductal metaplasia in biliary atresia.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Soini T*, Haveri H*, Elo J, Kauppinen M, Kyrönlahti A, Salo MK, Lohi J, Andersson LC, Wilson DB, and Heikinheimo M. Transcription Factor GATA-4 Is Abundantly Expressed in Childhood but not in Adult Liver Tumors. *Journal of Pediatric Gastroenterology and Nutrition*, 2012;54:101-8.

II Soini T, Pihlajoki M, Kyrönlahti A, Andersson LC, Wilson DB, and Heikinheimo M. Downregulation of Transcription Factor GATA4 Sensitizes Human Hepatoblastoma Cells to Doxorubicin-induced Apoptosis. *Tumor Biology*, 2017;39:1010428317695016

III Soini T*, Eloranta K*, Pihlajoki M*, Kyrönlahti A, Akinrinade O, Andersson N, Lohi J, Pakarinen MPP, Wilson DB, and Heikinheimo M. Transcription Factor GATA4 Associates with Mesenchymal-like Gene Expression in Human Hepatoblastoma Cells. *Tumor Biology*, 2018; in press.

IV Soini T, Pihlajoki M, Andersson N, Lohi J, Huppert KA, Rudnick DA, Huppert SS, Wilson DB, Pakarinen MP, and Heikinheimo M. Transcription Factor GATA6: A Novel Marker and Putative Inducer of Ductal Metaplasia in Biliary Atresia. *American Journal of Physiology – Gastrointestinal and Liver Physiology*, 2018;1.

*The authors contributed equally to the study.

The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

ADD3 = adducin 3
AFP = alpha-fetoprotein
ALB = albumin
ALT = alanine amino transferase
BA = biliary atresia
BCL2 = B-cell lymphoma 2
BDE = bile ductule expansion
BDL = bile duct ligation
BMP = bone morphogenic protein
CFC1= cryptic family 1
COL4α1 = collagen 4 alpha 1
CK = cytokeratin
DKK1 = dickkopf-related protein 1
DLK1 = delta-like non-canonical Notch ligand 1
DOCK8 = dedicator of cytokinesis 8
Dox = doxorubicin
DR = ductular reaction
E = embryonal day
E-cad = epithelial cadherin = CDH1
ECM = extra-cellular matrix
EMT = epitehlial-mesenchymal transition
EpCAM = epithelial adhesion molecule
EPO = erythropoietin
FABPL = fatty acid-binding protein, liver type
FAP = familial adenomatous polyposis coli
FGF = fibroblast growth factor
FFPE = formalin-fixed paraffin embedded
FN1 = fibronectin 1
FOG = friend of GATA
GPC1 = glypican 1
GW = gestational week
HB = hepatoblastoma
HCC = hepatocellular carcinoma
HGF = hepatocyte growth factor
HNF = hepatocyte nuclear factor
HSC = hepatic stellate cell
HTT-I = hereditary tyrosinemia type 1
IGFBP1 = insulin-like growth factor binding protein 1
INVS = inversin
LSEC = liver sinusoidal endothelial cell

MMP1 = matrix metalloproteinase 1
N-cad = neural cadherin = CDH2
NFE2L2 = Nuclear factor (erythroid-derived 2)-like 2
P = postnatal day
PE = portoenterostomy
PFIC = progressive familial intrahepatic cholestasis
PROM1 = prominin 1
RHO = Ras homolog family member
siRNA = small interfering RNA
SOX9 = SRY-box 9
SYTL2 = synaptotagmin-like 2
TGF = transforming growth factor
TIMP2 = tissue inhibitor of metalloproteinases 2
TRAIL = TNF-related apoptosis-inducing ligand

REVIEW OF THE LITERATURE

1 LIVER

1.1 Development

The liver organogenesis consists of seven stepwise phases. They include a priming phase, a phase of increasing specification, expansion of the liver bud, migration of hepatoblasts into the transverse septum mesenchyme, a phase of liver vascularisation, an organ growth phase, and the terminal differentiation of the hepatocyte and cholangiocyte lineages [reviewed in (1)].

Three germ layers are formed during gastrulation: ectoderm, mesoderm, and endoderm. Liver is derived from the ventral foregut formed from the definitive endoderm. To start the programming towards hepatoblasts, the cells of the foregut endoderm require inductive signals, including fibroblast growth factors (FGF) and bone morphogenic proteins (BMP), from the adjacent cardiogenic mesoderm (2) and septum transversum mesenchyme (3), respectively. In response to these signals, a primary liver bud is formed from the progenitor cells in the ventral wall of the foregut. In a mouse embryo, this formation of the liver bud occurs by embryonic day (E) 8.5-9.0 (4). Among several other transcription factors, GATA4 and GATA6 are crucial for the expansion of the liver bud and the early hepatic development (5). After the invasion of the hepatoblasts of the liver bud to septum transversum, they start to organize to form the specific architecture of the mature liver. This requires the differentiation of the various liver cell types. Simultaneously, the liver volume grows rapidly, requiring extensive proliferation of the hepatocyte cell population [reviewed in (4)].

Hepatoblasts are the bipotential precursors of both epithelial cell types of the liver, hepatocytes and cholangiocytes. The process of cell fate determination of fetal hepatoblasts is not completely clear, but several signaling pathways and transcription factors are implicated as inductors of both cell lineages. The biliary differentiation is dependent on Notch and Wnt/β-catenin signaling pathway components, and hepatocyte nuclear factors (HNF) 1β and HNF6 [reviewed in (6)], whereas hepatocyte differentiation is driven by hepatocyte growth factor (HGF), HNF3, HNF4α, and HNF1α [reviewed in (7)] (Figure 1).

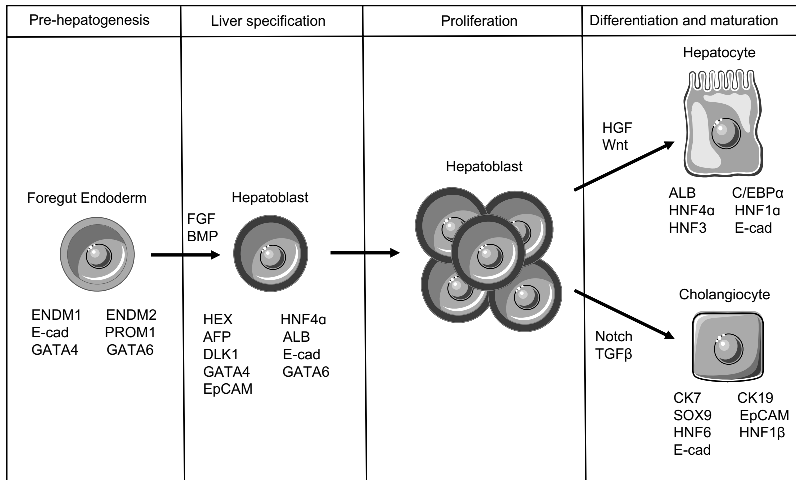

Figure 1. Differentiation of hepatocytes and cholangiocytes from common progenitor cells. *Expression of distinct cellular markers and inducing factors/pathways at different stages of liver development. ENDM=endoderm-specific surface marker, E-cad=E-cadherin, PROM1=prominin 1, HEX= Hematopoietically Expressed Homeobox, HNF=hepatic nuclear factor, AFP=alfafetoprotein, ALB=albumin, DLK1=Delta Like Non-Canonical Notch Ligand 1, EpCAM=epithelial cell adhesion molecule, FGF=fibroblast growth factor, BMP=bone morphogenic protein, HGF=hepatocyte growth factor, C/EBPα=CCAAT/enhancer binding protein α, CK=cytokeratin, SOX9=SRY-box 9, TGFβ=transforming growth factor β. Modified from (8). Created using image vectors from Servier Medical Art (http://smart.servier.com/), licensed under the Creative Commons Attribution 3.0 Unported License.*

1.2 Gross anatomy

Liver is the largest intestinal organ of the body, accounting approximately 2-3% of body weight in adults, and up to 5-6% in children. The liver is enclosed in the capsule of Glisson and located in the upper right quadrant of the abdominal cavity. It consists of left and right lobes, separated by the falciform ligament, attaching the liver to anterior abdominal wall (Figure 2). The liver is further divided into eight segments based on its blood supply, and each segment is supplied by a portal triad (portal vein, bile duct, and hepatic artery) (9). The liver has a dual blood supply, from common hepatic artery (30%) originating from the caeliac trunk, and from the portal vein (70%), bringing blood from the other intestinal organs. Three major hepatic veins drain the blood from the liver to the inferior vena cava (Figure 2) (9).

Bile flow starts from the intrahepatic bile canaliculi and moves on to larger interlobular ducts. These ducts combine to form left and right hepatic duct and further

a common hepatic duct outside the liver. The gallbladder is connected to this duct to form a common bile duct carrying bile to the small intestine (Figure 2) (10).

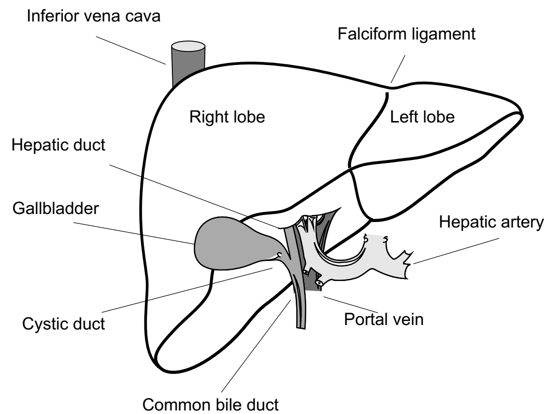

Figure 2. Anatomy of the liver and extrahepatic bile ducts.
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1.3 Physiology and function

Liver has several vital functions [reviewed in (11)]. In the fetal period, erythropoiesis takes place in the liver (12). Postnatally, liver controls the metabolism of carbohydrates, proteins, and lipids. Cholesterol and other lipids, as well as several important serum proteins, like coagulation factors and albumin are produced by the liver. Furthermore, liver serves as a storage of many essential vitamins and minerals, glycogen and fatty acids, and it maintains the blood homeostasis of glucose. Bile, consisting of water, bile salts, cholesterol, and bilirubin, is necessary for lipid digestion and secreted from the liver to small intestine. Several hormones are produced in the liver. Liver has an important detoxification function; it metabolizes drugs, alcohol, and other toxic agents. All extrinsic molecules absorbed in the intestine are passed through portal vein to liver before entering the systemic circulation. Liver macrophages, Kupffer cells, are an important part of the immune system, and they digest microbes from the blood flowing through the liver.

1.4 Histology and cell types

1.4.1 Cellular anatomy

The liver consists of three functional systems: 1) hepatocyte system, 2) biliary system, and 3) blood circulatory system [reviewed in (13)]. Hepatocytes are arranged in hexa- or pentagonal structural units, called hepatic lobules. Central vein is in the middle of each lobule, surrounded by radiating colums of hepatocytes, and 4-6 portal triads (consisting of a branch of portal vein, hepatic artery, and bile ductule). Hepatocytes of the lobule are divided into three zones based on their location, and thereafter their nutrient and oxygen saturation, metabolic and regeneration activity, and pronity to toxins. This functional unit is called a hepatic acinus (Figure 3).

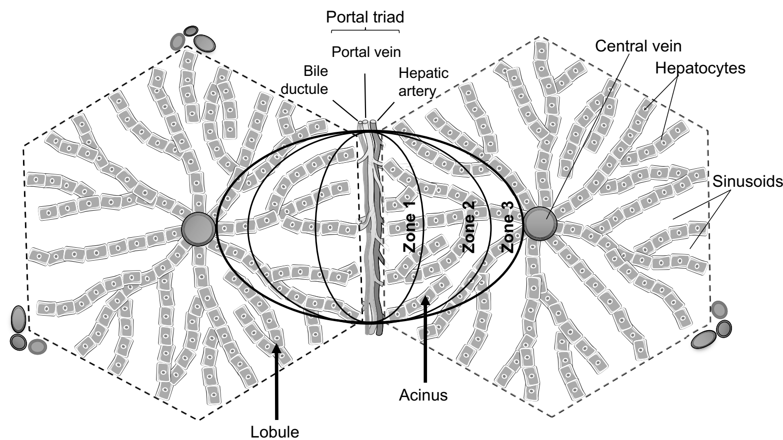

Figure 3. Structural and functional architecture of the liver: lobule and acinus.
Lobules are hexagonal structural units of the liver, consisting of hepatocyte columns with a central vein in the middle, and surrounded by portal triads. Acini are the functional units of the liver, consisting of three zones of hepatocytes according to their location to portal vein and central vein. Created using image vectors from Servier Medical Art (http://smart.servier.com/), licensed under the Creative Commons Attribution 3.0 Unported License.

The blood entering the lobule from portal vein, flows through spaces between the hepatocytes called sinusoids, and drains to the central vein. The area between the sinusoidal endothelial cells and the hepatocyte columns is called the space of Disse. Between adjacent hepatocyte columns is a tubular bile canaliculi structure, where the bile flows to opposite direction with blood, ending up in bile ducts on the periphery of the lobule (Figure 4).

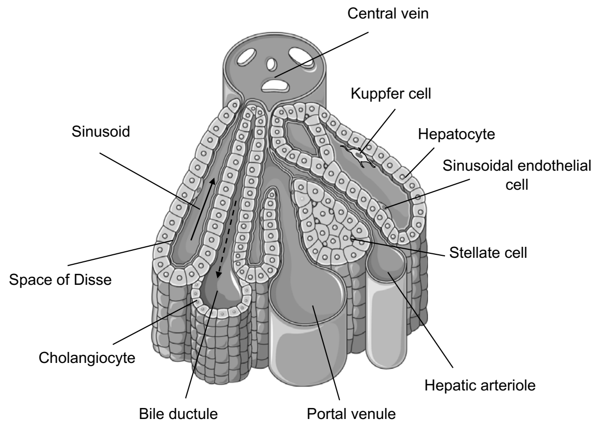

Figure 4. Structure and cell types of the liver lobule.
Bile flows in bile ductules (arrow with dashed line) to opposite direction with blood in sinusoids (arrow with solid line). Created using image vectors from Servier Medical Art (http://smart.servier.com/), licensed under the Creative Commons Attribution 3.0 Unported License.

1.4.2 Hepatocytes

Hepatocytes are the parenchymal cells of the liver that comprise almost 80% of the liver volume and 60% of the cell population. Hepatocytes are derived from bipotential stem cells of the liver, hepatoblasts, by gradually acquiring liver-enriched transcription factors. These factors regulate gene expression of the developing hepatocytes to gain the several vital functions of mature hepatocyte [reviewed in (7)]. Hepatocytes are the functional units of the liver. They are cuboidal, structurally and functionally polarized epithelial cells that have endocrine and exocrine properties (4). Adjacent hepatocytes are connected with intercellular junctional complexes. Hepatocytes have a great regeneration ability after injury. The regenerative hepatocytes derive from mitotic activity of mature hepatocytes, cholangiocytes and endothelial cells, but when the proliferative capacity of the mature hepatocytes is impaired, hepatic progenitor cells i.e. oval cells, can participate to the regenerative process by differentiating to hepatocytes (14).

1.4.3 Cholangiocytes

Cholangiocytes are epithelial cells lining the intrahepatic and extrahepatic bile ducts. They are highly polarized and joined together near the apical surface by tight junctions. The primary bile secreted by hepatocytes is transported into bile canaliculi, where it is alkalinized and fluidized by the cholangiocytes (15). Cholangiocytes are functionally and morphologically heterogenous along the biliary tree. The cholangiocytes lining small bile ducts have a more reactive phenotype and are able to proliferate and re-differentiate in case of hepatic injury or pathological stimuli (16). Like hepatocytes, the intrahepatic cholangiocytes are derived from hepatoblasts. In

contrast, cholangiocytes lining the extrahepatic bile ducts share a common developmental origin with the pancreas and duodenum (17). When a hepatoblast has committed to differentiate to cholangiocyte, it starts to express cholangiocyte markers, like cytokeratins (CK) 7 and 19.

1.4.4 Other cell types of the liver

In addition to cholangiocytes; sinusoidal endothelial cells, hepatic stellate cells (HSCs), hepatic progenitor cells (HPCs), Kupffer cells, and pit cells (liver-specific natural killer cells) represent the majority of non-hepatocyte cell types in the liver.

Liver sinusoidal endothelial cells (LSEC) are fenestrated, highly specialized endothelial cells, located between the hepatocytes and HSCs. They control the tone of the hepatic vasculature and keep the portal pressure stable. LSECs also control the activation of HSCs, and thus contribute to the onset of liver fibrosis in disease state [reviewed in (18)].

HSCs, also know as Ito cells, are located in the space of Disse, and in normal quiescent state they function as storage of vitamin A. HSCs are activated under liver damage, when they differentiate into proliferating portal myofibroblasts, producing ECM components, and thus have an important role in development of liver fibrosis. HCSs also participate in inflammation and immune regulation of the liver (19).

HPCs are bipotential cells residing in the canals of Hering in the normal liver, able to participate in hepatocyte or cholangiocyte renewal. Their proliferation is evident in primary liver cancer and reactive conditions of the liver [reviewed in (20)].

Kupffer cells are liver macrophages and a part of the reticuloendothelial system. They phagocytose microbes and aged erythrocytes. Kupffer cells are localized inside the liver sinusoids, and in a healthy liver they do not response to every immunologic stimulus derived from the intestinal blood flow. In chronic inflammatory diseases, Kupffer cells are activated and lose the tolerogenic phenotype, thus start sending signals through the endothelial cells for the hepatocytes and stellate cells, enhancing the pathologic fibrogenesis and liver injury [reviewed in (21)].

The different cell types of the liver constantly interact with one other; this intercellular signaling is essential for normal functions of the liver, but it is also likely to significantly contribute to the pathological processes occurring during liver disease and injury (22, 23).

2 PEDIATRIC LIVER PATHOLOGY

Liver pathology in children is divided into neonatal conditions and diseases occurring in older children. The most frequent causes of liver pathology in these two groups are listed in table 1.

Table 1. Frequent causes of pediatric liver disease according to age.
Modified from (24).

<table>
<tr><th>Neonates and infants</th><th>Older children and adolescents</th></tr>
<tr><td rowspan="5">Cholestatic diseases
• Biliary atresia
• Paucity of the intrahepatic bile ducts
• Cholelithiasis
• Progressive familial intrahepatic cholestasis (PFIC)
• Benign recurrent intrahepatic cholestasis</td><td>Hepatitis</td></tr>
<tr><td>Sclerosing colangitis</td></tr>
<tr><td>Parasitic infections</td></tr>
<tr><td>Toxins and pharmacologic remedies</td></tr>
<tr><td>Fatty liver of obesity</td></tr>
<tr><td>Idiopathic neonatal hepatitis</td><td>Occlusion of the hepatic veins</td></tr>
<tr><td>Viral hepatitis and other infectious diseases in the neonate</td><td>Wilson disease</td></tr>
<tr><td>Methabolic disease</td><td>Hypotension/ischemia/cardiac failure</td></tr>
<tr><td>Toxic or pharmacologic injury</td><td>Fatty liver of pregnancy</td></tr>
<tr><td>Tumors
• Malignant: hepatoblastoma, hepatocellular carcinoma, embryonal rhabdomyosarcoma
• Benign: hemangioendothelioma, mesenchymal hamartoma</td><td>Malignant tumors
• Hepatocellular carcinoma, undifferentiated embryonal sarcoma</td></tr>
</table>

2.1 Hepatoblastoma

2.1.1 Incidence and etiology

Hepatoblastoma (HB) is a pediatric liver tumor with an incidence of 1.7 per million children per year (25). Although rare, it accounts for nearly 70% of all malignant tumors of the liver in children (26). Most HB cases occur before the age of four, the median age of diagnosis being 1.5 years (27). They are 1.5-times more common

among boys than girls for unknown reason, and in nearly all cases there is no family history of HB (25).

The embryonal/fetal histology and the young age of the patients imply that genetic and developmental factors play a role in HB pathobiology. There are rare cases of HB in school-aged children and adolescents, but they commonly have features of both HB and hepatocellular carcinoma (HCC) and are thus called translational liver tumors (27). HCC is the most common liver cancer in adulthood, with an etiology based on the predisposing hepatic injury or cirrhosis by exposure to viruses or toxic agents (26).

Most HB cases are sporadic, but HB occurrence is also connected to congenital syndromes, like Beckwith-Wiedemann and Sotos overgrowth syndromes (28, 29) and familial adenomatous polyposis coli (FAP) (30). There are several studies about the perinatal risk factors for HB and their results vary, as HB is connected to prematurity (25, 31), maternal factors during pregnancy (32), and congenital anomalies (31, 33, 34) i.a. Only low birth weight has been implicated as a risk factor in several studies (25, 32, 33, 35, 36).

2.1.2 Histological subtypes

HBs are very heterogenous tumors and one tumor can consist of several histologically distinct areas. HBs can be divided into two main groups: pure epithelial (56%) or mixed epithelial/mesenchymal (44%) tumors (Table 2) (37, 38).

The epithelial component can either resemble different developmental stages of embryonal or fetal liver or can be small cell undifferentiated (SCUD) or macrotrabecular subtype. Embryonal and fetal subtypes present hepatocyte lineage cells with immature, blastic features. Extramedullary hematopoiesis is present in these subtypes (38). Pure fetal subtype has the most favorable prognosis (39), and can be treated surgically without chemotherapy (40). SCUD cell subtype consists of highly proliferative stem cell-like cells with low AFP production, and its presence in tumor predicts a poor prognosis (41). Macrotrabecular HB, often presenting HCC or translational tumor-like features, is named after the growth pattern of the epithelial cells (38).

The mixed epithelial-mesenchymal tumors include neoplastic areas of stromal components, mainly immature osteoid, fibroblastoid, or myofibroblastoid loci. Less frequently, cartilagous or rhabdomyoplastic tissue may be present (37).

Table 2. HB histological classification according to SIOPEL liver tumor group. Modified from (38).

HB histological subtypes
Epithelial HB
Pure fetal
Mixed embryonal/fetal
Small cell undifferentiated
Macrotrabecular
Mixed epithelial/mesenchymal HB
With teratoid features
Without teratoid features
Unspecified HB

2.1.3 Genetics and cytogenetics of hepatoblastoma

Although HB pathobiology is still relatively unknown, new molecular mechanisms are constantly emerging as the new sequencing methods have become more accessible and revolutionised the area of cancer genomics and molecular biology. The most common known genetic mutations, altered pathways, and karyotypic changes in HBs are listed in Table 3.

The fetal origin of HBs is evident in the gene expression profile of hepatoblasts. Variety of growth factor signaling pathways and developmental pathways are connected to HB pathogenesis. HB cells produce AFP, a glycoprotein normally synthesized by the fetal yolk sac and liver. Majority of HB tumors express hepatic progenitor cell markers, including stem cell epithelial adhesion molecule EpCAM, hepatoblast and cholangiocyte marker CK19, and EGF-like protein DLK-1, which activates the Notch pathway in fetal liver cells (42). Downregulation of genes important in typical liver functions, like fatty acid and xenobiotic metabolics, are frequent in HB tumors (43). *NFE2L2* gene, encoding a transcription factor that activates the cellular antioxidant response to fight xenobiotics and oxidative stress, is mutated in approximately 10% of of HBs (44).

The hallmark molecular event in HB pathogenesis is mutation in the canonical Wnt/β-catenin signaling pathway. A mutation of the *CTNNB1* gene (encoding β-catenin) is present in approximately 80% of HB tumors (43, 45), but mutations in other components of the pathway, including *APC*, *AXIN,* and *LEF1* are also common (38, 43, 46, 47). Mutations of β-catenin prevent its normal degradation in the cytoplasm, and β-catenin is transferred to nucleus where it activates the transcription of the Wnt

target genes, preventing apoptosis and differentiation (48). One of these genes is the *C-MYC* proto-oncogene (*MYC*), which plays a role in cell cycle progression, apoptosis and cellular transformation (49). Activation of MYC signaling has been connected to poorly differentiated and highly proliferative HB subclass (50).

Processes involved in cell cycle regulation and proliferation are shown to play important role in HB pathogenesis. Mutations and downregulation of oncogenes *PLK1* and *P53* controlling the cell cycle have been reported (47, 52). Furthermore, hypermethylation of *SOCS1* gene, an inhibitor of the JAK/STAT signaling pathway affecting cell proliferation, apoptosis, and differentiation, is common in these tumors (53). The expression of pro-apoptotic members of BCL2 family is frequently downregulated in HBs, whereas the anti-apoptotic, prosurvival members are upregulated (54, 55).

Abnormalities in karyotype are common in HBs. The cytogenetic changes are usually acquisition (chromosomes 2, 8, and 20) or loss (chromosome 18) of whole chromosomes, often occurring in conjunction with structural translocations (27, 56). The exact mechanism by which the cytogenetic abnormalities contribute to tumorigenesis or their prognostic significance is still relatively unknown (27).

Table 3. Major known mutations, differentially expressed pathways and karyotypic changes in HB tumors. Modified from (51).

HB genetics and cytogenetics
Wnt pathway mutations
CTNNB1: 67.5%
APC: 2%
AXIN1: 5%
Other genes and pathways
Antioxydant response: *NFE2L2*: 11.7%
Transcription regulators
Chromatin modifiers
Ubiquitin pathway
Genomic alterations
Gains: chr 2, 1q, 8, 6, 12, 17, 20
Losses: chr 4q, 11p

2.1.4 Diagnosis

The first symptoms of HB are a palpaple mass, abdominal distension, and pain. Generalized fatigue, jaundice, and growth retardation are often present. Serum AFP is commonly elevated, being the most important marker for diagnosis and follow-up. However, 5-10% of HB patients have normal or very low AFP, which is connected to poor prognosis (57). Other serum markers, like DLK1 and Glypican3 have been suggested for HB, but are not currently in clinical use. Imaging of the tumor size, localization, and distribution is performed with ultrasonography (US), computer tomography (CT) or magnetic resonance imaging (MRI). US-guided core needle biopsy from the tumor is histologically assessed to confirm the diagnosis and subtype of the tumor [reviewed in (58)].

2.1.5 Staging and prognosis

HB stage and risk level is preoperatively evaluated based on radiological tumor extent (PRETEXT) staging system, developed by the Childhood Liver Tumor Study Group of the International Society of Pediatric Oncology (SIOPEL) (59). The liver is divided into four anatomical segments by hepatic veins and the portal vein; left lateral (segments 2 and 3), left medial (segments 4a and 4b), right anterior (segments 5 and 8) and right posterior (segments 6 and 7). PRETEXT 1-3 are localized tumors with high AFP and categorized as the standard-risk tumors. Tumors involving all four hepatic sections (PRETEXT 4), and patients with <100ng/mL serum AFP (reflecting the SCUD histological subtype), belong to the high-risk group. Very high-risk group patients have distant metastases of HB, usually in the lungs, at diagnosis (60). PRETEXT is based on the tumor distribution, but also the assessment of histological subtype (61), and resectability of the tumor (58) are important factors in the final prognosis. The overall 3-year survival rate for standard-risk HB is 91% and for high-risk HB 53% (59).

2.1.6 Treatment

The outcome of HB has dramatically improved during the past decades due to effective cisplatin and doxorubicin (Dox) -based neoadjuvant chemotherapy (62). Cisplatin monotherapy is used for standard-risk patients (63) and combination of cisplatin and Dox (PLADO) is used for high-risk patients (64) [reviewed in (65)]. After tumor shrinkage with chemotherapy, it is surgically resected. If resection is not anatomically possible, yet no extrahepatic metastases exist, a liver transplantation is performed (58).

2.2 Biliary atresia

2.2.1 Insidence and etiology

Biliary atresia (BA) is a rare idiopathic disorder of the neonates with an obstructive fibroinflammatory process obliterating the extrahepatic bile ducts, resulting in cholestasis and progressive fibrosis. If untreated, BA leads to cirrhosis and death before the age of 2 years. The incidence varies geographically, being highest in East Asia (1.5 per 10 000), and 0.5-0.8 per 10 000 in Europe (66, 67). The incidence in Finland is 0.54 per 10 000 (68).

About 20% of BA cases are associated with congenital malformations, most commonly splenic malformations, vascular malformations, or malrotation (69, 70). They are often referred to as “embryonic” form of BA, since they are hypothesized to arise during development of laterality during early gestation (71). This theory has not, however, been scientifically proven, and there most likely exist several etiopathogenetic explanations also in this group of BA (72).

The pathogenetic process of isolated BA without other malformations is thought to initiate closer to birth. The exact molecular mechanisms and perinatal events that trigger the sclerosing inflammatory process, are widely unknown. Majority of BA cases are sporadic. It has also been suggested that viral infections, immune-mediated injury, genetic causes, exposure to toxins, or defects in prenatal circulation might cause this disease (71, 73).

BA is grouped into three main types according to the extent of fibrosis in the extrahepatic bile ducts. Type I atresia involves the common bile duct, type II extends up to the common hepatic duct, and type III atresia involves also the porta hepatic and thus encompasses all extrahepatic bile ducts. Type III is the most common subclass and accounts for over 90% of all BA cases (74).

2.2.2 Pathobiology

2.2.2.1 Genetic mutations and alterations in gene expression

Polymorphism or mutations of genes regulating bile duct development and morphogenesis are suggested to predispose to BA. Notch signaling is crucial for the initiation of the biliary lineage determination of hepatoblasts and duct morphogenesis (75). Abnormal Notch signaling, especially alterations in *JAGGED1* and *HES1* expression, is connected to pathogenesis of BA (76-78). Hepatic nuclear factors HNF1β and HNF6 are required for early development of the biliary tree, and mice bearing mutations in these genes have anomalies and atresia of extrahepatic bile ducts (79-81).

Aberrant hepatic Hedgehog signaling has been indicated in BA, and suggested to induce the epithelial-mesenchymal transition and expansion of cholangiocytes (82). Mutations in or reduced expression of *Glypican-1* (*GPC1*) gene, regulator of Hedgehog signaling and inflammation, are demonstrated in some BA patients, and a zebrafish model reinforces the role of *GPC1* in normal development and function of biliary tracts (83).

Abnormal expression of genes associated with left-right patterning, e.g. *Inversin* (*INVS*) and *Cryptic Family 1* (*CFC1*), are also connected to BA in animal models (84, 85). This theory is consistent with the observation that the embryonal/fetal form of BA is often related to laterality defects causing also accompanying developmental anomalies in other organs.

2.2.2.2 Histological changes

Ductular reaction (DR) is a dynamic system of expanding bile duct-like cells, accompanied by mesenchymal and inflammatory cells, driving the fibrogenesis of BA liver (86). The origin of the epithelial cell type of the DR is controversial. The majority of these cells derive from proliferating cholangiocytes, but cholestasis also triggers metaplasia of periportal hepatocytes towards a cholangiocyte-like phenotype, as an adaptive injury escape mechanism (87-89). Furthermore, hepatic progenitor cells (HPCs), located in the canals of Hering, are capable of differentiating to either cholangiocytes or hepatocytes, and participate in DR by producing reactive intermediate hepatocyte- or duct-like cells [reviewed in (90)]. The cells in DR area have paracrine communications with myofibroblasts, inflammatory cells and endothelial cells, leading to an epithelial-mesenchymal transition and subsequent fibrosis [reviewed in (91)].

The progressive fibrosis, starting from periportal areas, leads to portal hypertension and liver cirrhosis in BA. The fibrosis typically forms bridges within and adjacent to DR areas of the BA liver. The cellular origin of the fibrosis and extracellular matrix production is not clear and it is likely that many cell types of the liver participate in this process. Both hepatocytes and cholangiocytes are shown to contribute to the fibrosis by epithelial-mesenchymal transition (EMT) (82, 92). Furthermore, transdifferentiation of hepatic stellate cells to myofibroblasts has also been demonstrated (93). Additionally, HPCs are likely to participate in the process of ECM deposition, but their role in the development of fibrosis remains unclear (94). Mavila et al. demonstrated an expansion of a cell population that expresses stem cell marker prominin-1. The proliferation of these collagen-producing cells was associated with activation of fibroblast growth factor (FGF) and transforming growth factor-beta (TGFβ) signaling pathway, which in turn are inducers of EMT and fibrosis (95). All in all, a complex crosstalk among cholangiocytes, hepatocytes, progenitor cells, myofibroblasts, endothelial, and inflammatory cells is required for the onset of DR and the subsequent periportal fibrosis in BA.

2.2.3 Diagnosis

Typical symptoms at the time of diagnosis are jaundice, acholic stools, dark urine, and sometimes hepatomegaly in an otherwise healthy and thriving newborn. Coexistence of other malformations can cause additional symptoms. BA patients typically have conjugated bilirubinemia, as well as elevated gamma-glutamyl transferase, alkaline phosphatase, and total serum bile acids, as a result of cholestasis. A common serum marker of hepatocellular injury, alanine aminotransferase, is usually only moderately elevated at diagnosis.

Radiological examinations are essential for BA diagnostics. In abdominal ultrasonography, gallbladder does not visualize in 4/5 of cases and a “triangular cord sign” of the hilar structures is often present. Gamma biligraphy shows the accumulation of the radioactive label in the liver and very little or no access to intestine. Percutaneous needle biopsy presents typical histological changes, including cholestasis, ductular reaction (DR), portal inflammation, and fibrosis. The definitive diagnosis is established by intraoperative cholangiography which also reveals the level of obstruction (71) .

2.2.4 Treatment and follow-up

BA is surgically managed using the Kasai portoenterostomy (PE) operation, named by a Japanese surgeon Morio Kasai, who developed the operation method in the 1950s. In the operation, the gallbladder and biliary remnants are removed, and the porta hepatis is anastomosed to the bowel in a Roux-en-Y fashion. An early diagnosis and undelayed treatment are predictive for a better prognosis (96). In addition, postoperative adjuvant therapy including steroids, ursodeoxycholic acid, and prophylactic antibiotics seems to improve the clearance of jaundice and native liver survival rates (97-99).

In Finland, the diagnostic protocol, surgical care, postoperative treatment, and follow-up of BA patients were standardized in 2005 when BA management was centralized to Helsinki University Hospital by a governmental decision (68). All patients are currently administered steroids, ursodeoxyxholic acid, and antibiotics postoperatively. Abdominal ultrasonography and liver biochemistry are monitored at regular follow-up visits, and upper GI endoscopies are performed annually to screen for the development of esophageal varices. In addition, routine follow-up liver biopsies are taken 1, 5, and 10 years after PE (68).

If the PE operation is not successful (bilirubin remains elevated) or when the slowly progressing liver damage either progresses to end-stage liver disease or causes other life-threating complications, the treatment is liver transplantation. BA is the most frequent indication for liver transplantation among children. Compared to other pediatric surgeries, the PE operation relatively often fails in its objective regardless of the operator and other circumstances. A recent meta-analysis of five large studies (US, France, Japan) reports that over 30% of BA patients that underwent PE do not achieve any improvement in jaundice and over half the patients are listed for transplantation

within a year of the operation (100). In the long term, only ~20% of BA patients survive to adulthood without a liver transplantation (101). The reason for the ongoing liver injury after successful clearance of jaundice by PE remains largely unknown and is an area of active investigation. Immunological and inflammatory factors have been suggested to play a role in this process (102-104).

2.2.5 Prognosis

In a large French study with 472 BA patients treated with PE and/or liver transplantation, the overall five- and 10-year survival rates were 70% and 68%, respectively. Better prognosis was related with early age at PE, the favorable anatomical patterns of BA, the absence of other malformations, and the experience of the treatment center (105). After the centralization of BA treatment in Finland, 5-year native liver survival increased from 38% to 70%, and 5-year overall survival increased from 68% to 94% (106).

3 GATA TRANSCRIPTION FACTORS

GATA transcription factors are zinc-finger proteins, which bind to a specific (A/T) GATA (A/G) DNA motif and can either activate or repress transcription depending on the context (107). There are approximately 7 million GATA motifs in the human genome (108). In addition to this consensus GATA binding site, these factors can bind to large variety of other DNA motifs. The ability to recognize the alternative motifs varies between different GATA factors and enables the differential gene regulation in cells expressing several GATA factors (109). Furthermore, post-translational modifications and varying interactions with cofactors cause functional diversity among these transcription factors.

The C-terminal domain is required for the DNA binding, whereas the N-terminal domain can bind to other parts of DNA or cofactor proteins and may also stabilize the DNA binding (110). GATA factors are evolutionary highly conserved and found in eukaryotic organisms ranging from mold to vertebrates substantiating their fundamental roles in development and gene regulation (111).

There are six members in the vertebrate family of GATA factors, named in the order of their discovery. They are divided into two subfamilies; GATA1/2/3 are mainly expressed in the hematopoietic tissues [reviewed in (112)], whereas GATA4/5/6 are expressed in endoderm-derived tissues; including heart, lung, gonad, and gastrointestinal tract [reviewed in (113)], (Figure 5, Table 4). GATA factors play pivotal roles in cellular differentiation and cell-fate specification, cell proliferation and movement. Their expression is required for the normal development of various organs during gestation. Nevertheless, mutations or altered tissue expression of different GATA factors are also connected to many human diseases and neoplasias (Table 4).

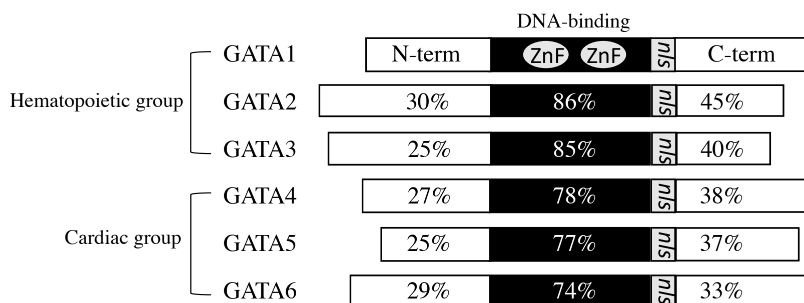

Figure 5. Structure and homology of the GATA proteins. *All GATA factors have two zinc fingers (ZnF) –containing DNA-binding domain. Transactivation domains are located in N- and C-terminus. The percentage indicates the homology between the domains of different GATA factors (in mouse). nls=nuclear localization signal. Modified from (107).*

Table 4. GATA transcription factors in mice and humans.

	Postnatal expression	Phenotype of knockout mice	Human mutations
GATA1	hematopoietic lineage	lethal (E11.5-12.5), erythroid cell maturation defect	hereditary thrombocytopenia and anemia, DS-AMKL
GATA2	hematopoietic lineage	lethal (E12.5), anemia	AML and CML, MDS
GATA3	CNS, kidney, T-lymphocytes	lethal (E11-E12), CNS deformities, internal bleeding	HDR-syndrome, breast cancer, ALL
GATA4	heart, lung, gonads, digestive organs	lethal (E7.0-E9.0), cardiac defect	CHD, pancreatic agenesis, diaphragmatic hernia, defects in testicular development
GATA5	heart, digestive organs, genitourinary tract	viable, female genitourinary defects	CHD
GATA6	heart, gonads, digestive organs, lung, adrenal gland	lethal (E6.5-E7.5), gastrulation defect	CHD, pancreatic agenesis, biliary tract anomalies, diaphragmatic hernia

E=embryonic day, DS-AMKL= down syndrome - acute megakaryoplastic leukemia, CNS=central nervous system, acute myeloid leukemia, CML=chronic myeloid leukemia, HDR=hypoparathyreidism, deafness, and renal dysplasia, ALL=acute lymphoblastic leukemia, CDH=congenital heart defect, MDS=myelodysplastic syndrome. References: (5, 107, 113-119)

3.1 GATA factors in cancer

Considering their role as regulators of cell survival and maturation, it is not surprising that altered expression or activity of GATA factors is associated with carcinogenesis. They are connected to variety of human cancers including leukemia and solid tumors of many tissues. They affect the metabolism, proliferation, cell death signaling and invasiveness of tumor cells. The activity of GATA factors in tissues can be altered by mutation, loss of expression mostly by promoter methylation, overexpression, or functional interference by interacting proteins [reviewed in (107, 120)]. The function of one GATA factor depends on both the cell type and the context of the promoters and may thus be even opposite in different cancers.

GATA1/2/3 are strongly connected to different types of leukemia (121), and GATA3 plays a role in breast cancer progression (122). GATA4/5/6 are associated e.g. with tumors of the gastrointestinal tract, lungs, and ovaries. The expression of GATA4 and GATA6 is altered in several endocrine-related tumors including ovarian granulosa cell tumors, adrenocortical tumors, and pancreatic tumors [reviewed in (118)].
In gastrointestinal malignancies, *GATA4* gene is amplified in a substantial part of esophageal adenocarcinomas (123). *GATA6* is frequently elevated and suggested to

act as oncogene in pancreatobiliary neoplasms (124, 125). GATA6 is shown to promote invasion and metastasis of colon cancer (126, 127). There are no previous reports about the expression or function of GATA factors in HBs.

In line with their pivotal role in organogenesis, GATA factors affect important developmental signaling pathways, like TGFβ and WNT/β-catenin pathways (128, 129), also connected to oncogenesis in many organs. For instance, GATA4 regulates components of TGFβ pathway in adrenocortical tumors (128, 130). Furthermore, GATA factors are important regulators of cell growth, proliferation and survival during gestation. Accordingly, GATA4 is shown to inhibit apoptotic pathways and cell cycle regulation in ovarian granulosa cell tumors (131, 132), where high level of GATA4 expression is connected to clinically poor prognosis (133).

3.2 Expression and function of GATA4 and GATA6

GATA4 and GATA6 are master regulators of endoderm formation demonstrated with mouse embryonic stems cells which differentiate into extra-embryonic endoderm when either of these factors is overexpressed (134).

GATA4 gene is located in the short arm of chromosome 8. *Gata4* was first cloned and characterized in mouse in 1993 by Arceci *et al.* (135), and it is expressed in the heart (136, 137), proximal parts of the gastrointestinal tract (138), lungs (139), and gonads (140, 141). Mice null for *Gata4* die during embryogenesis at E7.0-E9.0 for heart defects and point mutations in *GATA4* cause congenital cardiac septal defects in humans (136, 142). GATA4 is required for the migration and folding morphogenesis of the precardiogenic mesodermal cells, and for regulation of carciac angiogenesis (136, 143). GATA4 has an important role in the regulation of gonadal gene expression and sex development (144, 145).

In addition to binding DNA, GATA factors interact with cofactors and other transcription factors. GATA4 interacts with a transcriptional modifying protein, Friend of GATA 2 (FOG2), predominantly expressed in heart, gonads, and brain (146). GATA4 binds to FOG2 with its N-terminal zinc-finger (147). Presence of FOG2 is essential for the function of GATA4 in heart morphogenesis, and *Fog2*$^{-/-}$ mouse embryos die in midgestation due to cardiac defects (148).

GATA6, cloned in human in 1996 by Suzuki *et al*. (149), maps in the long arm of chromosome 18 and has an overlapping expression profile with GATA4. GATA6 is expressed in gastrointestinal tract (138), heart (150), lungs (151), gonads (140, 141), and adrenal gland (152). The cells of murine *Gata6*$^{-/-}$ embryonic ectoderm undergo apoptosis shortly after the *Gata6* onset in the wild type embryos, and thus *Gata6*-null mice die at E6.5-E7.5 (153). This is partly due to downregulation of transcription factor *Hnf4*, a direct downstream target of GATA6 in visceral edoderm, required for the normal gastrulation (153, 154). GATA6 is required for the vascular development and expressed in the vascular smooth muscle cells during embryogenesis and

postnatally (150). In the developing gonads, GATA4 and GATA6 have overlapping but distinct expression patterns, suggesting some functional redundancy (140, 141).

3.2.1 GATA4 and GATA6 in the liver

GATA4 and GATA6 are expressed in murine foregut endoderm and are both essential for the normal development of the liver (155). GATA4 and GATA6 belong to the pioneer factors that determine which cells of the endoderm become hepatoblasts by modulating the chromatin structure (156, 157). GATA4 potents the hepatic gene expression in the primary liver bud. It binds to *Albumin* (*Alb*) enhancer before the onset of *Alb* expression in the developing liver (157). The crucial role for *Gata4* and *Gata6* is shown also in early zebrafish liver development, where these factors are shown to have functional redundancy in the onset of hepatic development, but independent roles in regulating liver organ development (158).

Tetraploid embryo complementation studies (providing *Gata4*$^{-/-}$ *or Gata6*$^{-/-}$ embryos with *Gata4*$^{+/+}$ or *Gata6*$^{+/+}$ extraembryonic endoderm) have enabled the generation of post-gastrulation stage embryos of homozygote knockout mice (5, 116). These studies demonstrate abnormal formation of the liver bud and expression of normal hepatocyte markers at E9.5 in both *Gata4*$^{-/-}$ and *Gata6*$^{-/-}$ embryos. As GATA4 expression is diminished in the liver diverticulum already at this stage, the defect in liver development is likely caused by lack of *Gata4*-mediated signaling from adjacent septum transversum. GATA6 in contrary, is present in hepatoblasts at E14.5 (155) and is suggested to have a more cell-autonomous role in liver bud expansion (5).
In human liver, GATA4 is expressed in hepatocytes at gestational week 8, whereas in normal postnatal liver, GATA4 is expressed mainly in sinusoidal endothelial cells and Kupffer cells (159). There are contradictory reports about the normal expression of GATA4 and GATA6 in postnatal murine hepatocytes (160-162). There are no comprehensive studies about GATA6 expression in normal human liver.

AIMS OF THE STUDY

This doctoral thesis focuses on the role of GATA transcription factors in HB and BA pathogenesis.

The specific aims of the presented research are:

1. To assess the expression and tissue localization of GATA4 and GATA6 in normal liver versus HB and BA

2. To clarify the effect of *GATA4* gene silencing on the function and gene expression of a human HB cell line, focusing on apoptosis and epithelial-mesenchymal transition

3. To explore the role of GATA6 in ductal metaplasia of hepatocytes in BA

MATERIALS AND METHODS

1 Patients and clinical data (I, III, IV)

HB and BA patients included in this study were treated in Children's Hospital, Helsinki University Hospital during years 1986-2015. Clinical data was obtained from patient medical records.

2 Tissue samples (I, III, IV)

The use of the patient samples and medical records in this study was approved by the Ethical Committee of Helsinki University Hospital (approval number: 98/13/03/03/2013) and by the Finnish National Authority of Medicolegal Affairs and Health (approval number: THL/1239/5.05.01/2015). All animal experiments were approved by the Institutional Animal Care and Use Committee in accordance with the National Research Council's (NRC) Guide for Care and Use of Laboratory Animals.

2.1 Human tissue (I, III, IV)

Paraffinized human tissue samples were obtained from Helsinki University Hospital's archives of pathology. Postnatal control samples were obtained from healthy organ donors and fetal control samples were obtained from obductions. Fresh frozen tissue samples from BA, and control livers was also utilized.

2.2 Mouse tissue (IV)

Mouse tissue samples were obtained from collaboration laboratory in Cincinnati Children's Hospital Medical Center, USA. Two mouse models affecting the biliary tree development or function, mimicking cholestatic liver disease were used; surgical bile duct ligation model and a genetic model for induced ductular reaction (a liver-specific double knockout for *Hnf6* and *Rbpj* genes). These animal models have been previously described (163-165).

3 Cell culture (I, II, III, IV)

Two immortalized, adherent, human liver cancer cell lines were utilized. Human HB cell line HUH6 clone 5 was obtained from Health Science Research Bank (Osaka, JP). HUH6 is derived from a mixed epithelial/mesenchymal HB of an Asian 12-month-old

male (166). Human cell line HepG2 is derived from a hepatic tumor of a 15-year old Caucasian male. This cell line has originally been falsely described as hepatocellular carcinoma (167), and later re-characterized as epithelial HB in literature (168). Both cell lines were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Basel, CH) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin sulfate. The cell lines were regularily tested for mycoplasma contamination (Promokine PCR Mycoplasma test kit, Promocell, Germany).

Commercially available primary human hepatocytes were derived from 51-year old male (III) and 62-year old female (IV) with a non-hepatic cause of death (Lonza, Basel, CH), and cultured in hepatocyte-specific supplemented medium according to manufacturer's instructions (Lonza).

4 Modification of gene expression in cell cultures

Three methods were used for transient transfections of HUH6, HepG2, and primary human hepatocyte cells. Cells were plated 24 h prior to transfections at a density to reach 60-70% confluency.

4.1 Adenoviral transfections (I)

In HUH6 cells, GATA4 overexpression was mediated with a construct expressing rat wild-type GATA4 (169). Dominant negative GATA4 adenovirus, producing a GATA4 and repressor domain fusion protein, was utilized to disturb the function of endogenous GATA4 (170).

4.2 Liposome-mediated siRNA transfections (II, III)

Small-interfering RNA (siRNA) was used to disturb the transcription and subsequent knockdown *GATA4* expression. A pool of four siRNAs targeting *GATA4* or a non-targeting (NT) control siRNA were subjected to cells using RNA iMAX Lipofectamine reagent in Opti-MEM medium (both from Life Technologies, Carlsbad, NY) in the absence of antibiotics. The final siRNA concentration was 0.1μM.

4.3 Liposome-mediated plasmid transfections (III, IV)

Cells were transfected with either pMT2-GATA4 expression plasmid (135), pcDNA3-GATA6 expression plasmid (150), or corresponding control plasmid. Optimem

medium and jetPEI™Hepatocyte DNA transfection reagent (Polyplus Transfection, Illkirch, FR) for human primary hepatocytes or Lipofectamine LTX with Plus™ Reagent (Thermo Fischer Scientific, MA, USA) for HUH6 and HepG2 cells was used to perform the transfections.

5 RNA expression

5.1 RNA extraction and Real-time quantitative PCR (II, III, IV)

RNA was isolated [Nucleospin RNA/protein kit, Machrey-Nagel, Düren, DE (cell line samples) or RNA Easy Mini Kit, Qiagen, Hilden, DE (tissue samples)] and reverse transcribed (Reverse transcriptase Core kit, Eurogentec, Liege, BE). qRT-PCR was performed using SYBR GREEN (qPCR MasterMix Plus, Eurogentec) and expression of gene of interest was normalized to housekeeping genes *GAPDH* or *PPIG* (encoding cyclophilin G). Primer pairs used are listed in Table 5.

5.2 RNA microarray hybridization (III)

After 72h of transfection with *GATA4* siRNA or non-targeting control siRNA, RNA was extracted from two sample sets of HUH6 cells (n=3 in each sample set) and purified (NucleoSpin RNA Clean-Up XS kit, Machrey-Nagel). RNA quality was assessed with Bioanalyzer (Agilent, CA, USA). All 12 samples were subjected to RNA microarray hybridization, performed by the Functional Genomics Unit at the University of Helsinki using an Illumina Human HT-12 v4 oligonucleotide expression BeadChip (Illumina, CA, USA). Background correction was done on raw array data using BeadStudio software (Illumina, San Diego, CA) before quantile normalization and log2 transformation were performed using the BeadArray Bioconductor package (171). Linear models for microarray data (LIMMA) (172) with Benjamini-Hochberg correction were used to identify differentially expressed genes. Expression level changes with a false discovery rate (FDR) below 5% were considered as significantly differentially expressed. Microarray data was subjected to average linkage clustering with uncentered correlation using Cluster (173), and heatmap was generated with R (174). GOstats Bioconductor package was used to perform gene set enrichment analysis of the differentially expressed genes (175).

Table 5. qRT-PCR primers.

Gene	Primer sequence 5′→ 3′	Gene	Primer sequence 5′→ 3′
ADD3	F: GGCTCTGCGGCGCTTA R: CTTGTTATCTCGCAGCGCGT	*GATA4*	F: CTCCTCTGCACATTGCTGTT R: GTGTGGGGAGGCGTAGTAT
AFP	F: GAGGGAGCGGCTGACATTAT R: CATGGCCTCCTGTTGGCATA	*GATA6*	F: GTGCCCAGACCACTTGCTAT R: TGGAATTATTGCTATTACCAGAGC
AHNAK	F: CTCGTCGCCGCCAGTAG R: TCTCGGTCACAACCTGAGGG	*Gata6 (m)*	F: ATTCACCAGCAGCGACTACG R: TTGATTCCTCGAGCGATGTG
ALB	F: GCTGTCATCTCTTGTGGGCTGT R: AAACTCATGGGAGCTGCTGGTT	*HNF1β*	F: GGCCTACGACCGGCAAAAGA R: GGGAGACCCCTCGTTGCAAA
BCLXL	F: TCCCCATGGCAGCAGTAAAG R: TCCACAAAAGTATCCTGTTCAAAGC	*HNF4α*	F: TGTCCCGACAGATCACCTC R: CACTCAACGAGAACCAGCAG
BCL2	F: GGATAACGGAGGCTGGGATGC R: GCAGAGTCTTCAGAGACAGCC	*HNF6*	F: TGTGGAAGTGGCTGCAGGA R: TGTGAAGACAACCTGGGCT
BID	F: GTCGCCACTGGGACACTG R: GGAACCGTTGTTGACCTCAC	*IGFBP1*	F: TTTAGCCAAGGCACAGGAGA R: ATGGATGTCTCACACTGTCTGC
BAD	F: TGTGGACTCCTTTAAGAAGGGAC R: CACCAGGACTGGAAGACTCG	*JAG1*	F: TGCCCTCCAGGACATAGTGG R: ACTCTCCCCATGGTGATGCA
BAX	F: TCCCCATGGCAGCAGTAAAG R: CAAACAGGCTGGTGGCAATC	*MMP1*	F: AGTCCAGAAATACCTGGAAAAATA R: TTTTTCAACCACTGGGCCAC
BAK	F: TCATCGGGGACGACATCAAC R: CAAACAGGCTGGTGGCAATC	*MSF*	F: CCCATCCAGTGGAATGCACC R: GGTGGGATACTCACAGGTCT
BCLW	F: CTTTGTCTTTGGGGCTGCAC R: CTGTGAACTCCGCCCAGC	*NOTCH2*	F: GGCATTAATCGCTACAGTTGTGTC R: GGAGGCACACTCATCAATGTC
BMP4	F: CTGCGGGACTTCGAGGCGACACTTCT R: TCTTCCTCCTCCTCCTCCCCAGACTG	*PPIG*	F: CAATGGCCAACAGAGGGAAG R: CCAAAAACAACATGATGCCCA
CDH1	F: CACCACGGGCTTGGATTTTG R: TGGGGGCTTCATTCACATCC	*RHOB*	F: GTGTGTCTGTTCGACTCCCC R: AGGGATATCAAGCTCCCGCT
CDH2	F: GCGTCTGTAGAGGCTTCTGG R: GCAGTTGCTAAACTTCACATTGAG	*RHOU*	F: TGCCGGACAGGATGAATTTGA R: TGGGACAGTGGCATCGAATC
CK19	F: CTGCGGGACAAGATTCTTGGT R: CCAGACGGGCATTGTCGAT	*SRC*	F: CAGATTGTCAACAACACAGAGGG R: CACGTAGTTGCTGGGGATGT
COL4A2	F: GGATGGCTATCAAGGGCCTG R: CTGGCACCTTTTGCTAGGGA	*SMAD5*	F: CTATGTTGGAGAGGTGTATG R: CAGCACGTGGTGGGATGAAA
CTNNB1	F: ATTGAAGCTGAGGGAGCCAC R: TGCATATGTCGCCACACCTT	*SOX9*	F: GTACCCGCACTTGCACAAC R: TCTCGCTCTCGTTCAGAAGTC
DKK1	F: TCACACCAAAGGACAAGAAGG R: ATCTTGGACCAGAAGTGTCTAGC	*SYTL2*	F: AGTGAAGGCTCGCAACGC R: CACCTACCTCCGAGTCGCT
DOCK8	F: GACCCTAGAAGCCACCGAAC R: TCCGCTGAAGAATACCTGTTGA	*TIMP2*	F: CAGATGTAGTGATCAGGGCCAA R: CCTTCTCAGGCCCTTTGAACA
GAPDH	F: GGTCATCCATGACAACTTTGG R: CCATCCACAGTCTTCTGGGT	*TAT*	F: GGGGACCCTACTGTGTTTGG R: ACTGGATAGGAAGCCGATGG

5.3 RNA *in situ* hybridization (III, IV)

Formalin-fixed paraffin embedded (FFPE) tissue sections (thickness 5 μm) were subjected to RNA *in situ* hybridization using RNAscope 2.5 HD detection kit-RED (#322350, ACDBio, Milano, IT) for chromogenic target mRNA detection and RNAscope Multiplex Fluorescent Reagent Kit Version 2 (#323100, ACDBio) for fluorescent target detection. After baking for 1 h at 60°C, tissue sections were deparaffinized and treated with hydrogen peroxide for 10 min at RT. Target retrieval was performed for 15 min at 97°C, followed by protease treatment for 15 min at 40°C. The probes were hybridized for 2 h at 40°C followed by signal amplification. For chromogenic detection, the samples were incubated for 45 min with AMP 5–RED reagent. The samples were then treated with fast red for 10 min at room temperature followed by counterstaining with 50% hematoxylin.

For fluorescent detection, Signal amplification and development for HRP channels were performed according to the manual. TSA Plus Cyanine 3 fluorophore (NEL744001KT, Perkin Elmer), and TSA Plus Cyanine 5 fluorophore (NEL745001KT) were used used at 1:1500 dilutions for different probes. The sections were counterstained with DAPI. Probes used for ISH are listed in Table 6.

Table 6. RNA *in situ* hybridization probes.

Gene	Probe ID#	Article
JAG1	546181	IV
HNF6	490081	IV
HNF1β	490071	IV
GATA6	603131	IV
GATA4	579821	III
CK7	550151	IV
CFTR	603291	IV

6 Protein expression

6.1 Protein extraction and western blotting (I, II, IV)

Protein was isolated from cell culture lysates and tissue samples using Nucleospin RNA/Protein kit (Machrey-Nagel), separated by weight with 7.5% SDS-PAGE, and transferred onto a PVDF membrane (Invitrogen, CA, USA). Nonfat 5% milk in 0.1% Tween-TBS was used to block unspecific binding. Primary antibodies were incubated o/n at 4°C and secondary antibodies were incubated for 1h at RT. Proteins were visualised with Enhanced Chemiluminescence Prime kit (G&E healthcare, IL, USA). β-Actin or GAPDH were used as loading controls. The primary and secondary antibodies used are listed in Table 7.

6.2 Immunoperoxidase and immunofluorescence stainings (I, III, IV)

FFPE liver sections were deparaffinized, hydrated, and treated with 10 mM citric acid in 97°C for 20 min or Target retrieval solution for 35 min (Dako, Glostrup, DK). Endogenous peroxidase activity was blocked with 3% H2O2, and nonspecific binding was prevented by using 1.5% normal serum. Immunoperoxidase staining was performed using a polymerized reporter enzyme staining system (ImmPRESS reagent kit; Vector Laboratories, Burlingame, CA or Novolink Polymer Detection System; Leica Biosystems, Newcastle, GB) to visualize the bound antibody. Primary

antibodies, listed in Table 7, were incubated overnight at 4°C. In control experiments, nonimmune serum replaced the primary antibody. Sections were counterstained with hematoxylin.

For immunocytochemistry, HUH6 cells were cultured in Matrigel coated Nunc LabTek 2-well chamber slide systems (Thermo Scientific, Waltham, MA). Cells were fixed and permeabilized in 100% methanol. Unspecific binding was blocked with UltraVision Protein Block (Thermo Scientific). Cells were incubated in primary antibody for 1h at RT (listed in Table 7).

Images were collected using 3DHISTECH Panoramic 250 FLASH II digital slide scanner at Genome Biology Unit (Research Programs Unit, Faculty of Medicine, University of Helsinki, Biocenter Finland).

Table 7. Primary antibodies used in this study.

Antigen	Species	Reference#	Method (dilution)	Article
β-actin	goat	SC-1616	WB (1:10 000)	I,II
BCL2	mouse	MO887	WB (1:500)	II
BCLXL	rabbit	2762S	WB (1:500)	II
BID	mouse	SC-373939	WB (1:500)	
Cytokeratin 7	rabbit	SP52	IHC (1:1000)	IV
E-cadherin	mouse		IF (1:200)	III
Erythropoietin	rabbit	SC-7956	IHC (1:100)	I
GATA4 (human)	mouse	AF2606	IHC (1:6000), ICC (1:200), IF (1:150)	I,III
GATA4 (human/mouse)	goat	SC-1237	IHC (1:200), WB (1:1000)	I, II
GATA6 (human)	rabbit	SC-9055	IHC (1:1200), ICC (1:200)	I,IV
GATA6 (human/mouse)	goat	AF1700	IHC (1:1200), WB (1:1000)	IV
Ki-67	mouse	SC-15402	IHC (1:200)	I
N-cadherin	mouse		IF (1:50)	III

7 Apoptosis, proliferation, and cell viability assays (II)

Apoptosis was analysed from HUH6 cells with Caspace-Glo 3/7 luminescent Assay (Promega, Madison, WI, USA), measuring the activity of apoptosis effector caspaces 3 and 7. Luminometer (Lab systems Luminoskan RS, Helsinki, Finland) was used to detect the luminescence levels. Cell proliferation was assessed by BrdU Assay (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The assay measures incorporation of a thymidine analog, into the newly synthesised DNA of proliferating cells. Cell viability was measured with WST-1 assay (Roche, Basel, CH), based on the level of mitochondrial enzymes present in viable cells. Colorimetric

BrdU and WST-1 assays were analyzed with a spectrophotometer (Multiskan EX, Thermo Scientific, MA, USA).

8 Migration assays (III)

For wound healing assay, HUH6 cells were cultured for 24h on a collagen I–coated plates. A scratch was created in the confluent cell monolayer and images were captured in the beginning (0h) and after 24h. Remainder of migrating cell area from the two timepoints was calculated.

For transwell migration assay, a suspension of $6x10^4$ HUH6 cells was applied to 8µm-pore inserts, with the bottom of the insert coated with collagen I. Serum-free media was applied in the upper chamber, and normal growth media in the lower chamber. Cells were allowed to migrate for 40 hours, after which the cells were fixed with 4% PFA and stained with crystal violet. The number of migrated cells was manually counted under light microscope from 5 fields per well.

RESULTS AND DISCUSSION

1 GATA4 IN HEPATOBLASTOMA PATHOGENESIS (I, II, III)

1.1 GATA4 expression in normal liver and hepatoblastomas (I, III)

Transcription factor GATA4 is expressed in early human fetal liver at gestational week (GW) 8 (159). We demonstrated that by GW12 it is mostly restricted to the non-hepatocyte cell population of the liver (I). Contradictory reports about the expression and function of GATA4 in postnatal hepatocytes have been published (159, 160, 162, 176). We therefore studied the expression levels of GATA4 in livers of healthy organ transplant donors with a normal liver histology (n=18). Low or negligible levels of GATA4 mRNA and protein were expressed in normal hepatocytes (Figure 6A-B). GATA4 protein expression varied to some extent among the liver samples and through different areas of the samples, whereas *GATA4* mRNA exression remained more consistent. Contrary to the findings in hepatocytes, high GATA4 expression was found in vena endothelium, sinusoidal endothelial cells, and Kupffer cells in all the samples (Figure 6B).

In HB tissue, GATA4 was highly expressed compared to hepatocytes of normal liver samples and to the liver parenchyma adjacent to the tumor cell areas in HB samples. Especially tumor areas with a distinct embryonal, undifferentiated histology were highly positive for GATA4. This high expression of GATA4 was reflected in both mRNA and protein levels (Figure 6C-D).

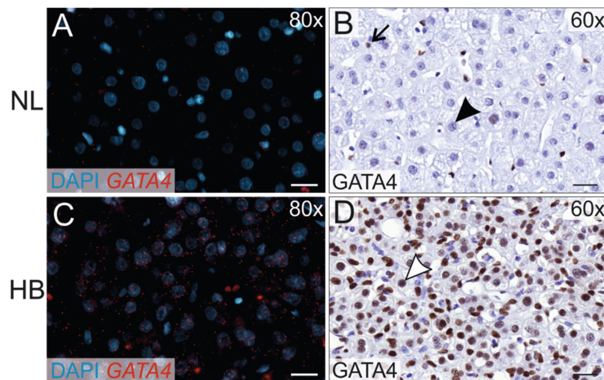

Figure 6. GATA4 expression is absent in normal hepatocytes and high in HB tumor cells.
A-B: In situ hybridization (A) and immunohistochemistry (B) of GATA4 in normal adult liver (NL) demonstrate very low or absent expression of GATA4 in hepatocytes (black arrowhead), and high expression in Kupffer cells (arrow).
C-D: In situ hybridization (C) and immunohistochemistry of GATA4 in an embryonal hepatoblastoma (HB) demonstrate abundant GATA4 expression in tumor cells (white arrowhead). Red indicates positive signal of GATA4 mRNA and blue indicates positive signal of DAPI (A and C). Brown indicates positive signal for GATA4 (B and D).

1.1.1 GATA4 in HUH6 cell line

In this study, a human HB cell line HUH6 was used as an *in vitro* model. We found a high endogenous GATA4 expression at both mRNA and protein level in this cell line, and HUH6 cells were thus well suited for our purpose to study the role and function of GATA4 in HBs. An indisputable weakness of this study is the usage of only one cell line to model the actions of GATA4 in HBs. This is due to the fact that there are only few other HB cell lines established, and some of them are indefinitive as to the character or diagnosis of the originating tumor (177). For instance, HepG2 cell line, derived from a 15-year-old child with liver tumor, has been commonly referred to as hepatocellular carcinoma but recently re-characterized as epithelial HB (168). Consistent with this, we found high levels of *GATA4* mRNA in HepG2 cells (data not shown), and in the planned future experiments, also this cell line will be utilized beside HUH6.

The disadvantages of cell lines in general are lacking of the tumor microenvironment and loss of their original characteristics during years or growing on plate. An interesting and more reliable model to test tumor behavior would be transplantation-based or genetically engineered (GEMM) mouse models [reviewed in (178)]. Patient-derived xenograft model where the tumor cells of a patient are transplanted into an immunodeficient mouse, are able to recapitulate the original nature and gene expression of the tumor cells, and even to develop pulmonary metastasis (179, 180).

GEMM mouse models for HB include c-Myc or mutant β-catenin expressing transgenic mice [reviewed in (180)].

1.1.2 GATA4 expression in hereditary tyrosinemia type 1 and childhood HCCs

To find out if the high GATA4 levels were specific to HBs, we also assessed GATA4 expression profile in hepatocellular carcinomas (HCC). We found that in contrast to HBs, 9/10 of the adult HCCs were negative for GATA4 similar to normal postnatal hepatocytes (I). In children, HCC is more uncommon than HB and its incidence increases with age. Only 0.5-1% of all childhood liver malignancies are HCCs (26, 181). Hereditary tyrosinemia type 1 (HTT-I) is a metabolic disorder enriched in Finland leading to abnormal liver function and associates with HCC tumorigenesis (182). HTT-I is caused by deficiency of the enzyme fumaryl acetoacetate hydrolase (183). It ultimately causes liver cirrhosis and increased regeneration of liver tissue; the regeneration nodules are preneoplastic and have a high risk of transforming into HCC if early liver transplantation is not performed. Interestingly, majority of childhood HCCs (chHCC) and HTT-I regeneration nodules, predisposing to HCC tumors, both abundantly expressed GATA4 (Figure 7). This reflects the fact that although theoretically different cellular origins and etiologies of these tumors, there is a thin red line between pediatric HCC and HB, and the histological diagnosis is not always clear.

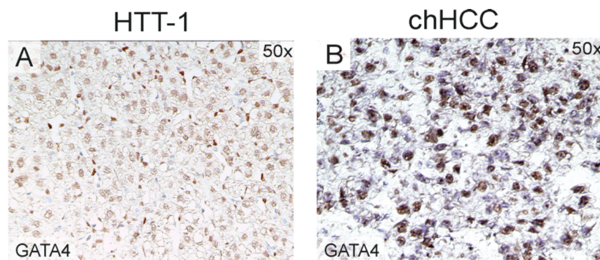

Figure 7. GATA4 expression is high in HTT-I and chHCC.
Immunohistochemical stainings demonstrate abundant expression of GATA4 in A: HTT-I regeneration nodulus and B: childhood HCC tumor. Brown indicates positive signal for GATA4 in nuclei.

1.2 Modification of GATA4 level in HUH6 cells (I, II, III)

We hypothesized, that GATA4 is a driver of a malignant phenotype of HB, thus providing these neoplastic cells an advantage in growth, survival, or metastasis. For assessing the significance of GATA4 in HB cells, modification of GATA4 expression

levels in HUH6 cell line was performed, and thereafter the subsequent changes in function and gene expression were measured with several methods.

GATA4 was overexpressed in HUH6 cells with a GATA4 wild type (WT) adenovirus vector and LacZ vector was used as a control (Figure 8A). However, since HUH6 cells readily had a high endogenous expression of GATA4, the primary aim was to downregulate it to find significant differences in cell behavior in the absence of GATA4. GATA4 function was disturbed by a GATA4 DN adenovirus (I). A flag subunit was attached to the GATA4 mutant construct, and as shown in Figure 8B, the GATA4 DN was strongly expressed in HUH6 cells. Actual knockdown of *GATA4* down to 20% from the original expression level was reached with siRNA constructs (II, III). The silencing of *GATA4* was demonstrated at mRNA (Figure 8C) and protein (Figure 8D-E) levels. An even more specific and efficient way of gene manipulating that could be used in our future studies is the CRISPR Cas9 technology, in which a site-specific double strand break in the DNA is created with Cas9 endonuclease to silence the gene of interest (184).

GATA4 and GATA6 are shown to have functional redundancy in many tissues (158, 185). As GATA6 is expressed in untreated HUH6 cells and also in many HB tumors, we measured the expression of *GATA6* mRNA 72 hours after knockdown of GATA4 and found that *GATA6* levels remained unaltered. It is, however, possible that GATA6 can take up some functions of GATA4, even though its expression remained constant.

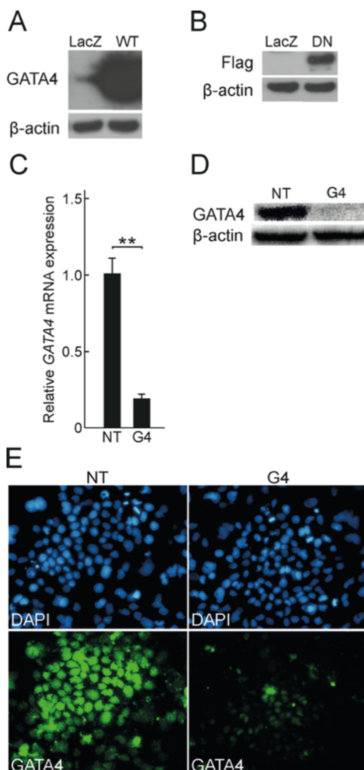

Figure 8. Modification of GATA4 expression in HUH6 cell line.
A: Western blot (WB) of GATA4 overexpression with WT virus compared to LacZ control virus. β-actin serves as loading control in WBs.
B: Flag protein is detected with WB in the cells infected with DN, unlike LacZ control.
*C: Relative expression of GATA4 mRNA after treatment with nontarget (NT) siRNA and GATA4 (G4) siRNA. **=p<0.01.*
D: WB of GATA4 after siRNA treatment.
E: Immunofluorescence staining demonstrates reduced GATA4 expression after G4 siRNA compared to control NT siRNA. Blue indicates positive signal of DAPI, green indicates positive signal of GATA4.

1.3 Relation of GATA4 to HB cell apoptosis (II)

Apoptosis is a process of programmed cell death, normally activated by a DNA damage which is beyond repair. Apoptosis plays an important role in development and tissue homeostasis, and its dysregulation leads to many diseases, including cancer (186). One hallmark of tumor development is the ability of the cancer cell to escape apoptosis. Two apoptotic pathways exist; extrinsic (death receptor pathway) is activated when a ligand binds to the death receptor on the cell surface, whereas intracellular stimuli can induce apoptosis by releasing mitochondrial cytochrome-c. Both pathways trigger the caspace cascade which eventually executes the programmed cell death [reviewed in (187)].

The balance of the anti- and proapoptotic B-cell lymphoma-2 (BCL2) protein family members determines the threshold of the intrinsic apoptotic pathway [reviewed in (188)]. The antiapoptotic members include BCL-2, BCL-X_L, BCL-X_S, BCL-w, and BAG; and the proapoptotic members include BCL-10, BAK, BAX, BID, BIM, BIK, and BLK (189). The expression of BCL2 proapoptotic genes is frequently downregulated in HBs, whereas the anti-apoptotic, prosurvival genes are upregulated (54, 55).

GATA4 regulates the cell survival signaling in other cell types. More specifically, it is shown to have an anti-apoptotic role in cardiomyocytes, ovarian granulosa cells, lung vascular smooth muscle, and childhood acute lymphoblastic leukemia (140, 190-192).

1.3.1 Effects of GATA4 modulation on HB cell viability

Cell viability can be altered by changes in cell proliferation or cell death. In addition to apoptosis, programmed cell death can occur through three other pathways: necrosis, autophagy, or pyroptosis [reviewed in (193)]. Necrosis and pyroptosis occur mostly with presence of pathogens and result to an inflammatory response, autophagic cell death has characteristics of both necrosis and apoptosis and is often accompanied by other cell death pathways. In this study, we focused on HB cell proliferation and apoptosis.

First, the effect of modifying GATA4 expression on baseline viability of HUH6 cells was assessed. Although there was a trend towards a reduced viability and increased apoptosis (measured by activity of executioner caspases -3 and -7) with siRNA-mediated silencing of *GATA4* (Figure 9A-B) this result did not reach a statistical significance. *GATA4* overexpression by WT adenovirus did not affect baseline proliferation or apoptosis (Figure 9C-D).

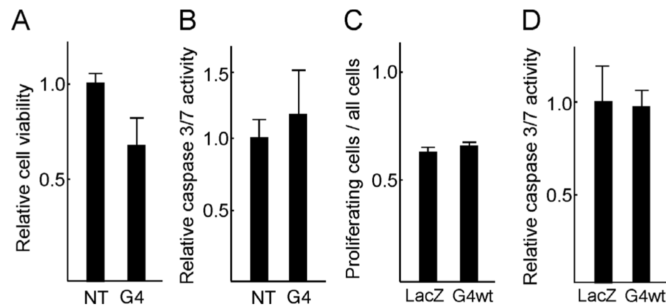

Figure 9. Cell viability, proliferation, and apoptosis in HUH6 cells after GATA4 modification.
A: Relative cell viability of HUH6 cells after NT siRNA or G4 siRNA measured by WST-1 assay.
B: Relative apoptosis rate of HUH6 cells after NT siRNA or G4 siRNA measured by caspase 3/7 assay.
C: Ratio of proliferating cells to all cells after LacZ or WT adenovirus measured by BrdU assay.
D: Relative apoptosis rate of HUH6 cells after LacZ or WT adenovirus measured by caspase 3/7 assay. $p>0.05$ compared to NT or LacZ in all experiments.

Since there was no significant difference in the viability of HUH6 cells after silencing *GATA4*, we presumed that high expression of GATA4 benefits HB cells in some other way. In ovarian granulosa cell tumors, GATA4 is shown to protect the cancer cells from the extrinsic apoptotic pathway induced by TRAIL (TNF-related apoptosis-inducing ligand) and also to regulate the components of intrinsic apoptotic pathway (131, 132). Next, we assessed whether GATA4 has a similar protective role from extrinsic inducers of cell death also in HB cells.

1.3.2 Effects of Doxorubicin on HUH6 cells

Doxorubicin (Dox) is an anthracycline drug commonly used in the treatment of high-risk HB patients. The main mechanisms of its action are disruption of topoisomerase-II-mediated DNA repair and generation of free radicals damaging cell components [reviewed in (194)]. A well-known side effect of Dox therapy is cardiomyopathy (195). Downregulation of GATA4, abundantly expressed in normal cardiomyocytes and essential for their function, has been implicated in pathogenesis of Dox-induced cardiomyopathy. Dox is shown to suppress GATA4 expression and its DNA-binding activity in cardiomyocytes (196-201). Furthermore, silencing *GATA4* in these cells enhances the Dox-induced autophagy and apoptosis by affecting BCL2 (B-cell lymphoma-2) mediated intrinsic pathway (198, 202) (Figure 13).

The expression of BCL2 proapoptotic genes is frequently downregulated in HBs, whereas the anti-apoptotic, prosurvival genes are upregulated (54, 55). Since Dox is effective for treatment of HB, we hypothesized it may act partially through GATA4 mediated apoptotic mechanisms also in HB cells. Increasing concentrations of Dox

were administered to HUH6 cells and a significant apoptotic response was reached on after 48 hours of Dox treatment with a concentration of 100ng/ml (II).

1.3.3 DOX inhibits GATA4 expression and alters the balance of BCL2 protein family

Similar to its actions in cardiomyocytes (198), Dox markedly decreased GATA4 mRNA and protein levels in HUH6 cells with concentration of 50ng/ml (Figure 10). We also measured the effect of Dox on the expression of several BCL2 family members (anti-apoptotic *BCL2, BCLXL, BCL-W,* and pro-apoptotic *BAK, BID, BAD, BAX*) and found a statistically significant decrease in *BCL2* and an increase in *BAK* with concentration of 100ng/ml (Figure 10).

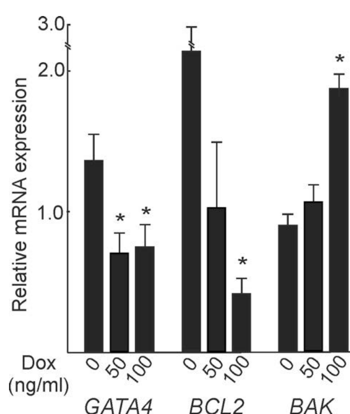

Figure 10. Effects of Dox on GATA4, BCL2, and BAK in HUH6 cells.
*Dox treatment for 48 hours causes downregulation of GATA4 and antiapoptotic BCL2, and an upregulation of proapoptotic BAK. *=p<0.05 compared to Dox 0 ng/ml.*

1.3.4 DOX-induced apoptosis is enhanced with GATA4 silencing

To investigate whether the reduction of GATA4 after Dox treatment was causing the altered expression of the BCL2 protein family members, we measured their expression after siRNA-mediated *GATA4* silencing. Two members of the BCL2 family were altered, antiapoptotic BCL2 and proapoptotic BID, demonstrated at both mRNA (Figure 11A) and protein (Figure 11B) levels. Like the expression changes of BCL2 and BAK after Dox treatment (1.3.3), also these changes shifted the balance of the intrinsic apoptotic pathway towards a proapoptotic direction.

Finally, we measured the cell viability and apoptosis of HUH6 cells after combination of *GATA4* siRNA (72h) and Dox (48h) treatment. We found a significant difference

in both cell viability and apoptosis at concentration 100ng/ml compared to control cells treated with NT siRNA and Dox (II and Figure 11C).

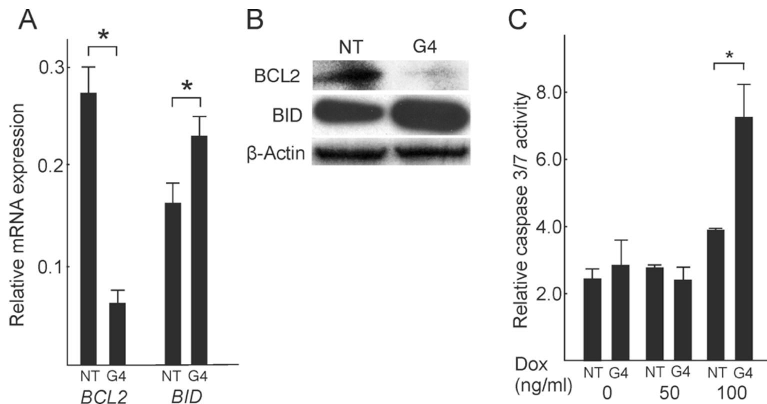

Figure 11. GATA4 silencing affects the expression of BCL2 and BID, and increases Dox-induced apoptosis in HUH6 cells.
A-B: Relative expression of BCL2 is reduced and BID is increased with after GATA4 silencing (G4) compared to control (NT), as shown with qPCR (A) and western blotting (B). β-actin serves as loading control.
*C: Relative apoptosis rate after Dox treatment is higher with GATA4 siRNA (G4) compared to control siRNA (NT), measured by caspace 3/7 assay. *=p<0.05 compared to NT.*

It is not known, whether GATA4 is a direct regulator of *BCL2* and *BID* in HUH6 cells, or what other mechanisms and pathways are mediating this effect. Chromatin immunoprecipitation (ChIP) studies are needed in the future to clarify the interaction of GATA4 and promoters of these genes in HUH6 cells. In cardiomyocytes, GATA4 is demonstrated to bind GATA site of the *BCL2* promoter and positively regulate this gene (203). The mode of action of Dox and GATA4 silencing in cardiomyocytes is demonstrated in Figure 12A. The present results demonstrate that silencing GATA4 sensitizes HUH6 cells to Dox-induced apoptosis (Figure 12B). Based on these findings, we hypothesize that GATA4 may protect HB cells from Dox also *in vivo* and thus enhance the drug resistance of these tumors.

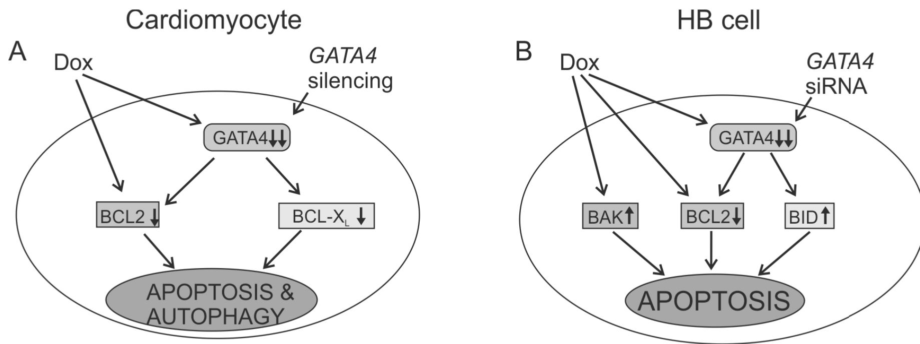

Figure 12. Schematic figure of sensitizing HUH6 cells to Dox-induced cell death in cardiomyocytes vs. HB cells by silencing GATA4.

A: In cardiomyocytes, Dox decreases GATA4 expression, subsequently leading to downregulation of antiapoptotic BCL2 and BCL-X$_L$ These changes promote cardiomyocyte autophagy and apoptosis (202, 204).

B: In HB cells, Dox affects the expression of BCL2 and BAK and downregulates GATA4. GATA4 downregulation with siRNA alters the expression of BCL2 and BID. When these two are combined, the balance of the BCL2 proteins shifts towards pro-apoptotic and Dox-induced apoptosis is enhanced (II). Dox=doxorubicin.

1.4 The significance of GATA4 in hepatoblastoma migration and epithelial-mesenchymal transition (III)

1.4.1 RNA microarray hybridization of GATA4 silenced HUH6 cells

To unravel the role of GATA4 in HB pathophysiology, a more unbiased approach was needed in search for putative target genes and pathways. For this purpose, we performed an mRNA microarray hybridization on GATA4 silenced and control HUH6 cells. We used two sets of samples (n= 3 NT + 3 G4 in each set) and listed the genes that were differentially expressed in both sample sets. Purpose of this was to find the set of genes that were constantly altered by GATA4 silencing, since HUH6 cells tend to differentiate in culture with time. In line with this notion, there was a statistically significant difference between the gene expression of the two sample sets of control HUH6 cells collected within 14 days of time. The list of overlapping differentially expressed genes included 106 genes, from which 79 were upregulated and 34 were downregulated (Figure 13).

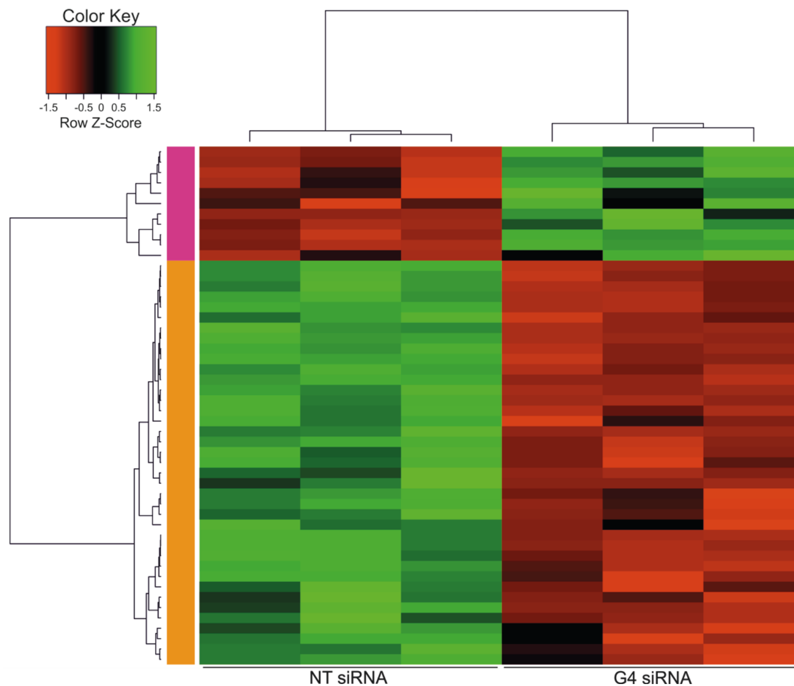

Figure 13. A heatmap of top 50 differentially expressed genes in HUH6 cells after GATA4 silencing.
Heatmap and two-dimensional clustering of gene expression changes (by adjusted p-values) in RNA microarray hybridization of GATA4 silenced (G4) compared to control (NT) HUH6 cells.

Gene Ontology (GO) analysis (205) was performed on the differentially expressed genes to identify biological processes associated with GATA4 inhibition. A substantial proportion of the altered genes and biological processes were connected to epithelial-mesenchymal transition (EMT) (Table 8). EMT is a gradual process, in which the polarized epithelial cell detaches from the basement membrane and adjacent cells, obtains enhanced migratory capacity, and produces increased amounts of extracellular matrix components. The end point of EMT process is change in cell phenotype to a mesenchymal cell, capable of degrading and invasing the basement membrane, and migrating from its original site [reviewed in (206)]. Tumor cells frequently harness developmental processes and pathways. Accordingly, EMT is crucial for normal embryogenesis and organ development, but also a hallmark molecular phenomenon in cancer metastasis [reviewed in (207)]. EMT has been recognized as an important factor also in hepatocarcinogenesis (208).

Table 8. Gene set enrichment analysis of GATA4 silenced HUH6 microarray data.

Biological Process	GO ID	p-value
Wound healing	GO:0042060	0.0003
Establishment or maintenance of monopolar cell polarity	GO:0061245	0.003
Tissue morphogenesis	GO:0048729	0.004
Epithelial cell differentiation	GO:0030855	0.009
Positive regulation of substrate-dependent cell migration	GO:1904237	0.012
Establishment or maintenance of bipolar cell polarity	GO:0061245	0.018
Establishment of apical/basal cell polarity	GO:0035089	0.023
Calcium-independent cell-matrix adhesion	GO:0007161	0.023
Protein localization to adherens junction	GO:0071896	0.023
Regulation of endothelial cell migration	GO:0010594	0.028
Planar cell polarity pathway involved in axis elongation	GO:0003402	0.035
Positive regulation of cell-cell adhesion	GO:2000049	0.041
Establishment of tissue polarity	GO:0007164	0.044

The changes in EMT-related processes were not completely surprising, since previous work in Drosophila and MDCK (Madin-Darby canine kidney) cells suggests GATA4 and GATA6 as conserved repressors of cell epithelial characteristics and as promoters of migration and mesenchymal gene expression (209). Another transcription factor, HNF4α, promoting hepatocyte lineage differentiation from hepatoblasts, is also connected to EMT in HCCs. Loss of HNF4α expression has been demonstrated to cause loss of cell polarity, and cell-cell and cell-ECM adhesion in mouse HCC xenografts (210). Of interest, HNF4α has been connected to GATA-factors in endoderm development and in HepG2 cell culture experiments (153, 211).

1.4.2 Altered expression of EMT genes in HUH6 cells after GATA4 silencing

For further analyses, we selected genes related to epithelial-mesenchymal-balance, adhesion, migration, and invasion from the list of differentially expressed genes (DEGs) in HUH6 RNA microarray and validated the results with qPCR. As the microarray sensitivity is relatively low, we also selected some key genes that were not significantly altered in the array, but important for EMT or connected to GATA4 in other tissues, for qPCR analysis. The altered genes were categorized into three groups according to their function: 1) organization of the actin cytoskeleton and migration, 2) cell-to-cell adhesion and cadherin switch, 3) remodelling of the extracellular matrix (ECM) (Table 9).

Table 9. Altered gene expression of HUH6 cells after GATA4 silencing.

Gene	RNA microarray Ratio G4/NT	RNA microarray p-value	qRT-PCR Ratio G4/NT	qRT-PCR p-value
1) Cytoskeleton reorganization and migration				
ADD3	0,75	0.0057**	0,73	0.043*
AHNAK	0,78	0.0007**	0,61	0.015*
DOCK8	0,97	0.62	0,71	0.033*
RHOU	0,76	9.61E-05**	0,69	0.002**
SYTL2	0,66	8.18E-07**	0,48	0.008**
SRC	0,99	0.45	0,77	0.033*
RHOB	1,28	0.0023**	1,46	0.034*
2) Cadherin switch				
CDH1	1,02	0.074	1,47	0.001**
CDH2	0,95	0.12	0,71	0.045*
3) ECM remodelling				
MSF	0,64	6.88E-06**	0,57	0.005**
IGFBP1	0,76	0.0006**	0,51	0.007**
TIMP2	0,93	0.08	0,88	0.048*
MMP1	0.99	0.13	0,46	0.030*
COL4A1	1,32	0.0031**	1,46	0.048*

*ADD3=adducin 3, AHNAK=ahnak nucleoprotein, DOCK8=dedicator of cytokinesis 8, RHOU=Ras homolog family member U, SYTL2=synaptotagmin like 2, SRC=SRC proto-oncogene, RHOB=Ras homolog family member B, CDH1=cadherin 1 (encoding E-cadherin), CDH2=cadherin 2 (encoding N-cadherin), FN1-7.3=fibronectin 1 splice variant 7.3 (encoding FN-EDB), IGFBP1=IGF binding protein 1, TIMP2=tissue inhibitor of metalloproteinase 2, MMP1=matrix metalloproteinase 1, COL4A1=collagen type 4 alpha 1 chain. *$P<0.05$, **$P<0.01$.*

1.4.3 GATA4 promotes reorganization of actin cytoskeleton and cadherin switch in HB cells

After siRNA mediated silencing of GATA4, a change in the organization of the filamentous actin (F-actin) fibers was evident with phalloidin immunostaining. In control cells with abundant GATA4 expression, F-actin formed stress fiber bundles in cytoplasm enabling cell contraction and movement (Figure 14A). In GATA4 silenced cells, F-actin was re-localized mainly in the peripheral parts of the cell, adjacent to cell membrane and stress fiber formation was reduced (Figure 14B).

Cadherin switch is a hallmark molecular phenomenon in the EMT process. Epithelial E-cadherin is downregulated, and mesenchymal N-cadherin is upregulated, loosening the attachment of the cell to adjacent epithelial cells and increasing its migratory

capacity (212). Cadherin switch is reported to occur in metastatic HCCs and is connected to poor prognosis (213). We demonstrated a reverse cadherin switch when GATA4 was downregulated in HUH6 cells, i.e expression of E-cadherin was increased after G4 siRNA (Figure 14D) compared to NT siRNA (Figure 14C), whereas N-cadherin was decreased after G4 siRNA (Figure 14F) compared to NT siRNA (Figure 14E). Of note, GATA4 regulates E-cadherin and N-cadherin expression also in other tissues, including MDCK cells and ovarian granulosa cells (209, 214)

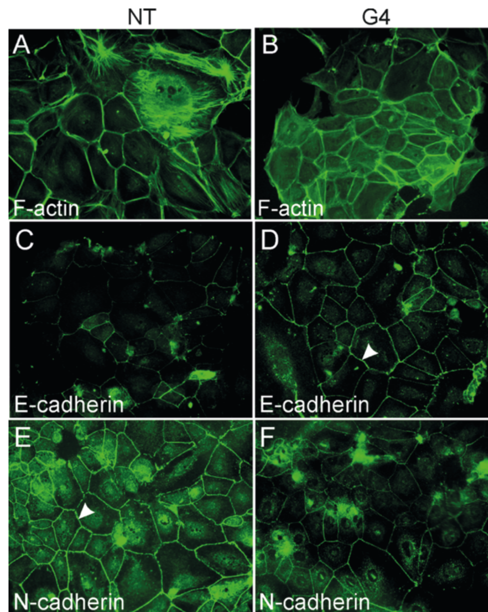

Figure 14. Changes in F-actin cytoskeleton organization and cadherin localization after GATA4 silencing.
Immunofluorescence stainings of
A-B: In control HUH6 cells (A), F-actin filaments form stress fibers that enable migration, after silencing GATA4 (B), F-actin is located mostly on the cell surface and stress fibers are scarce.
C-D: epithelial-type E-cadherin is increased on cell surface after GATA4 silencing (D) compared to control (C).
E-F: mesenchymal-type N-cadherin is increased on cell surface after GATA4 silencing (F) compared to control (E). NT=nontarget siRNA, G4=GATA4 siRNA

1.4.4 GATA4 promotes migration of HUH6 cells

Although EMT is a recognized hallmark of cancer progression, there are only few studies about EMT in HBs. In a recent report, Pei *et al.* demonstrated that GATA4 promotes the migration and invasion of HUH6 cells (215). We measured the migration

of HUH6 cells after GATA4 silencing with two parallel methods and confirmed that cells with less GATA4 migrate significantly slower than control cells (Figure 15). This result suggests, that GATA4 is a key factor required for maintaining the migratory capacity of HB cells, in the same manner as previously shown in MDCK cells (209). Presumably, the specific alterations in gene expression demonstrated above account for this change in HUH6 cell function.

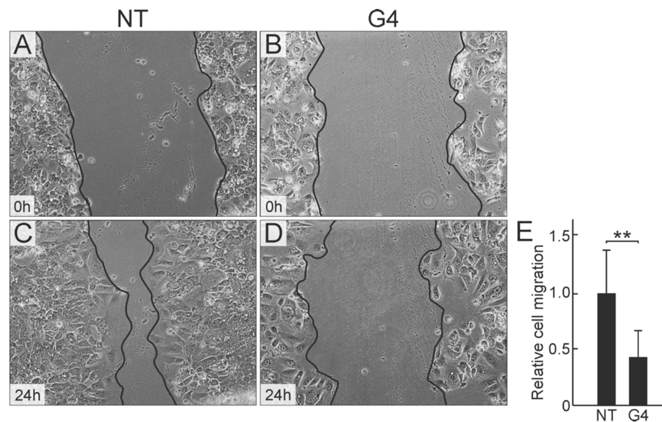

Figure 15. Changes in HUH6 migration after GATA4 silencing.
*A scrach migration assay demonstrates that the area of migrating cells from timepoint 0h to 24h is 2.6 times (E) larger in NT control cells (A and C) compared to GATA4 silenced (G4) cells (B and D). **=p<0.01 compared to NT. The assay was performed in triplicate.*

1.4.5 Enforced expression of GATA4 alters gene expression in human primary hepatocytes

As GATA4 maintains the migratory and mesenchymal-like gene expression in HB cells, the next interesting question is whether GATA4 overexpression is able to initiate this gene expression profile in normal hepatocytes. To test this hypothesis, we forced the overexpression of *GATA4* by plasmid-mediated transfections in primary human hepatocyte culture. The baseline expression of *GATA4* was very low in primary hepatocytes, and the GATA4 expression plasmid led to a 229-fold increase in its expression level. The subsequent changes in gene expression in terms of the EMT-associated genes altered in *GATA4* silenced HUH6 cells was assessed with qPCR. The majority (10/14) of these same genes were significantly altered in primary hepatocytes after GATA4 overexpression (Figure 16). Out of the altered genes, 8/10 were altered in opposite direction with GATA4 silenced HUH6 cells, implying that GATA4 can change the gene expression profile of hepatocytes to a mesenchymal direction, and thus have a role also in cancer initiation.

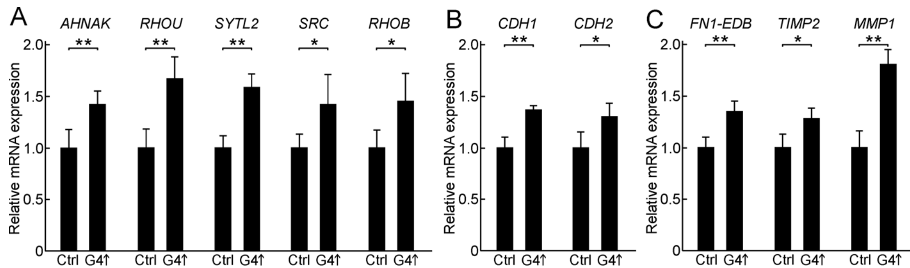

Figure 16. Changes in gene expression of primary human hepatocytes after forced GATA4 overexpression.
Differentially expressed genes were involved in
A: migration or cytoskeleton reorganization, B: cadherin switch, C: ECM remodulation.
**P<0.05, **P<0.01, compared to control cells.*

1.5 Summary of the findings in HB (I, II, III)

Our findings of GATA4 acting as an oncogene in HBs contradicts a recent report where GATA4 was demonstrated as a tumor suppressor, frequently silenced in HCCs (176). GATA4 is a tumor suppressor in many types of cancer, including colorectal, esophageal, and lung cancer, where the *GATA4* promoter is frequently methylated (216-218). On the other hand, high GATA4 expression is associated with poor prognosis and malignant behavior of ovarian granulosa cell tumors (131-133). It is possible that GATA4 has variant or even opposite roles in different tissues, or even in the same tissue during different phases of development or cellular differentiation. HCCs are typically tumors of adults or older children, derived from mature hepatocytes, with a different etiology than HBs. It is likely that a complex network of molecules and pathways is required for the HB tumorigenesis, GATA4 being but a small part of the entity.

All in all, the present results demonstrate that transcription factor GATA4 is frequently overexpressed in HB tumors compared to normal liver. However, it is not known whether GATA4 is an "innocent bystander" or plays an actual role in the function, growth, or survival of HB cells. In a HB cell line, silencing GATA4 promotes Dox-induced apoptosis, decreases migration, and shifts their gene expression towards a more epithelial type. On the contrary, overexpression of GATA4 in normal hepatocytes increases the expression of genes promoting mesenchymal phenotype. These results cannot be directly extrapolated to HB tumors *in vivo*, as immortalised cell lines are in many aspects not the ideal disease models. In the future experiments of GATA4 in HB pathobiology, other models like patient derived xenografts and genetically engineered mouse models might give more reliable answers to these questions.

2 GATA6 IN BILIARY ATRESIA PATHOGENESIS (IV)

2.1 GATA6 expression in normal liver and biliary atresia

GATA6 is required for the early murine liver development, and later in gestation it colocalizes in cells expressing HNF1α (155, 160) . GATA6 expression is reported also in adult mouse and rat hepatocytes, where it activates the fatty acid binding protein gene *Fabpl* in cooperation with GATA4, GATA5 and HNF1α (160, 162). In normal postnatal human liver, GATA6 protein expression is shown to be restricted to non-hepatocyte cell population (127).

To clarify the expression profile of GATA6 in normal human fetal and postnatal liver, we performed mRNA *in situ* hybridization and immunohistochemistry. At gestational week (GW) 13, all cells of the liver abundantly expressed GATA6 (Figure 17A). By GW 32, GATA6 expression was found in bile duct epithelium, vena endothelium, and inflammatory cells of the liver. In adult human liver, the expression pattern was similar to the late stage fetal liver, where hepatocytes showed negative or weak signal (Figure 17B-C) and cells of bile duct epithelium, cholangiocytes, were strongly positive for GATA6 mRNA (Figure 17D) and protein (Figure 17C).

The expression of GATA6 in BA differed from that of normal liver. GATA6 mRNA and protein were highly expressed in the hepatocytes of BA livers, especially around the ductal reactive areas (Figure 17E-F). The expression was strongest in the differentiated bile duct epithelium, similar to normal liver. A descendent gradient in GATA6 immunoreactivity could be seen in hepatocytes from the portal areas towards the central vein.

Double *in situ* hybridization of GATA6 and cytokeratin 7 (CK7) demonstrates that GATA6 expression is not restricted to reactive ductular cells or hepatocytes undergoing ductal metaplasia but expressed in vast majority of hepatocytes in BA livers (Figure 17F). A similar expression pattern is reported with another transcription factor essential for normal cholangiocyte differentiation, SOX9, suggested to play a rolein the development of DR. Expression of SOX9 in newly formed ductal reactive cells and adjacent hepatocytes correlates to blood markers of inflammation and liver injury, and furthermore with patient age at PE (219).

2.1.2 GATA6 expression correlates to established prognostic factors of BA

We correlated GATA6 protein expression in BA patients to several histological [bile ductule expansion (BDE), fibrosis, periportal inflammation] and clinical (survival with native liver, syndromic/nonsyndromic BA, age at PE, blood laboratory values) variables. We found a significant correlation to bile ductule expansion rate (visualized

by CK7 staining), age at PE, and plasma alanine aminotransferase (p-ALT), a marker for hepatocyte injury (IV). Histologically limited BDE and PE at early age are both known indicators of positive prognosis for BA (96, 220).

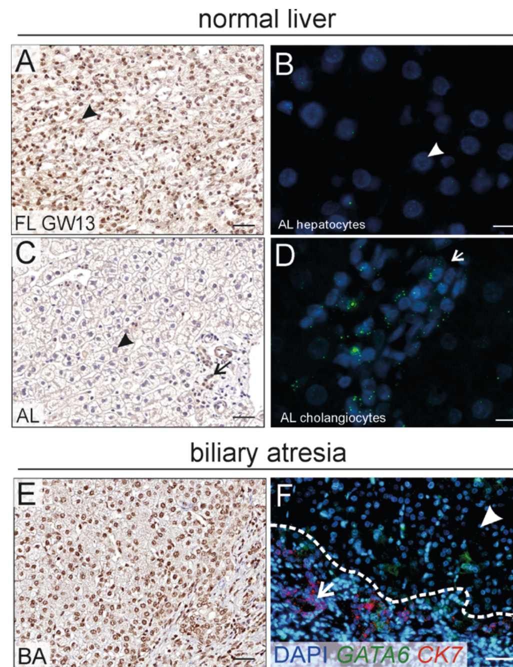

Figure 17. GATA6 is differentially expressed in normal liver and biliary atresia.
A: GATA6 protein is expressed in fetal liver hepatoblasts at gestational week 13.
B-C: GATA6 in situ hybridization (B and D) and immunohistochemistry (C) of normal adult liver demonstrates positive signal in cholangiocytes and negative hepatocytes.
E and F: GATA6 protein (E) and mRNA (F) are abundantly expressed in BA hepatocytes in addition to its normal expression in cholangiocytes. Dashed line demonstrates the ductal reaction area.
Arrowhead=hepatocyte, arrow=cholangiocyte. Brown indicates positive signal for GATA6 protein in nuclei. Green indicates positive signal for GATA6 mRNA, red indicates positive signal for CK7 mRNA, and blue indicates DAPI staining in the nuclei. Scale bars: 20µm (B and D) or 200µm (others).

2.2 GATA6 expression in mouse models for BA

Several animal models for BA have been developed to study the etiology and treatment of this disease [reviewed in (221)]. In this study, we used two mouse models to study GATA6 expression which both develop severe cholestasis and DR. 14 days after surgical bile duct ligation (BDL) (222), GATA6 was highly expressed in DR areas and hepatocytes (Figure 18B). The sham-operated mice had very low GATA6 immunoreactivity in hepatocytes, but high GATA6 expression in bile duct epithelium, in keeping with our findings in normal human postnatal liver (Figure 18A). Another

model for DR was the *Alb*-Cre;$Rbpj^{flox/flox}$;$Hnf6^{flox/flox}$ mouse [liver-specific double knockout (DKO)] (164), which failed to develop the intrahepatic small branches of biliary tree at postnatal day (P) 30, and thereafter had a subsequent prominent DR. The bile flow is normalized by P120, when the intrahepatic ductules regenerate. The control mice (*Alb*-Cre negative $Rbpj^{flox/flox}$;$Hnf6^{flox/flox}$ mice) demonstrated very low or negative GATA6 mRNA and protein expression at P30 and P120 (Figure 18C and E), whereas DKO mice hepatocytes where strongly positive for GATA6 (Figure 18D). By P120, when the liver histology was normalized, GATA6 expression in DKO hepatocytes had abolished (Figure 18F). Other animal models of BA could be used in future studies to confirm these findings; the closest of them to mimic the human disorder is the rhesus rotavirus A-induced extrahepatic biliary obstruction (223).

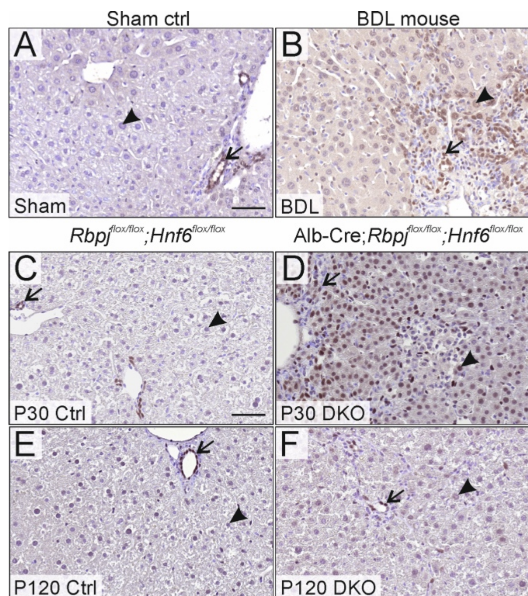

Figure 18. GATA6 protein expression in two mouse models for BA.
GATA6 immunohistochemistry in A: sham control mouse liver, B: BDL mouse liver (14 post-operative days), C: P30 control mouse liver, D: P30 DKO mouse liver, E: P120 control mouse liver, F: P120 DKO mouse liver. Arrowhead=hepatocyte, Arrow=cholangiocyte. Brown indicates positive staining of GATA6 in nuclei. Scale bar 50µm.

2.3 GATA6 expression decreases after portoenterostomy operation

PE is a palliative operation in which the bile flow is re-established and cholestasis alleviated. A successful PE (i.e. normalized serum bilirubin after operation) leads to improvement of the patient clinical parameters, the cholestatic laboratory values, and reduction of portal inflammation in liver (224, 225).

We measured GATA6 expression in BA follow-up biopsies (BA-post-PE) taken 302-6887 days after PE. There was a significant reduction in hepatocyte GATA6 expression after succesful PE. qPCR from whole liver biopsies demonstrates high *GATA6* expression levels in BA samples compared to normal late fetal liver (FL), adult liver (AL), BA-post-PE samples, and other cholestatic neonatal conditions (DC) (Figure 19A). To assure that this reduction is not due to decreased amount of DR and bile ducts, we localized *GATA6* mRNA with *in situ* hybridization in BA (Figure 19B) and BA-post-PE (Figure 19C) samples. We confirmed that in BA samples GATA6 expression was localized in hepatocytes and was decreased after PE. The downregulation of GATA6 after PE was also evident in protein level as shown by WB (Figure 19D).

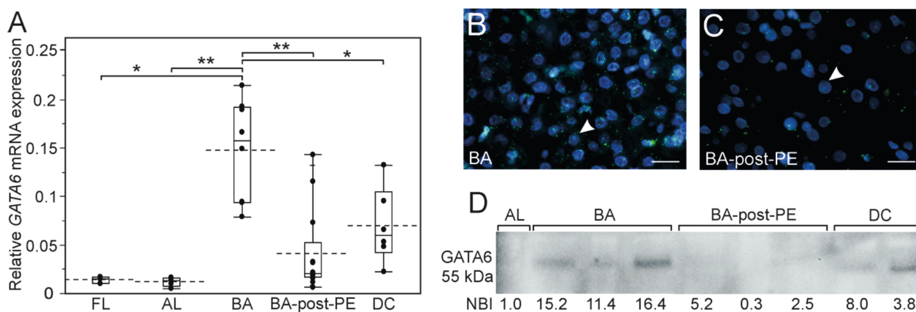

Figure 19. GATA6 expression is diminished after successful PE.
*A: A boxplot of relative GATA6 mRNA expression, as determined by qPCR, in different liver sample groups. *=p<0.05, **=p<0.01 as compared to BA group. B: In situ hybridization of GATA6 in BA liver. C: In situ hybridization of GATA6 in BA-post-PE liver. Green indicates positive signal of GATA6 mRNA, blue indicates positive signal for DAPI in nuclei. Scale bar 150 µm. D: WB detection of GATA6 protein in different liver sample groups. NBI=normalised band intensity.*

2.4 Forced expression of GATA6 in primary human hepatocytes promotes hepatocyte-cholangiocyte transdifferentiation

GATA6 is highly expressed in normal cholangiocytes, and *GATA6* mutations are shown to cause bile duct anomalies, including gallbladder agenesis and biliary atresia (226, 227). Therefore, we hypothesized that the elevated GATA6 in BA is related to a compensation mechanism of the cholestatic liver to produce new bile ducts. Major proportion of the neoductules in BA livers arise from proliferating cholangiocytes, but also ductal metaplasia of hepatocytes is shown to contribute to BDE (87, 89). GATA6 correlated to the level of BDE in BA patients and is particularly high in hepatocytes around the DR areas.

To test our hypothesis of GATA6 as a driver of hepatocyte ductal metaplasia, we used two hepatocyte cell models, primary human hepatocytes and HepG2 cell line.

Enforced overexpression of *GATA6* (mRNA levels approximately 1500-fold) was reached with plasmid transfections. Subsequent changes in gene expression were analysed with qPCR. In both cell types, we found significant upregulation of cholangiocyte lineage markers *HNF1β, HNF6*, and *JAGGED1* (*JAG1*) (Figure 20). This result suggests, that GATA6 promotes the expression of genes required for early differentiation of cholangiocytes from hepatoblasts. In primary human hepatocytes, also *DKK1* and *HNF4α* were upregulated (Figure 20). DKK1 is an antagonist of the Wnt signaling pathway, inhibiting the hepatocyte differentiation of hepatoblasts during development and disease, thus driving their lineage determination towards cholangiocytes (228, 229). Interestingly, one hepatocyte lineage marker, *HNF4α,* was also significantly upregulated with GATA6 overexpression. GATA6 is known to directly activate this gene also in embryonal visceral endoderm (153).

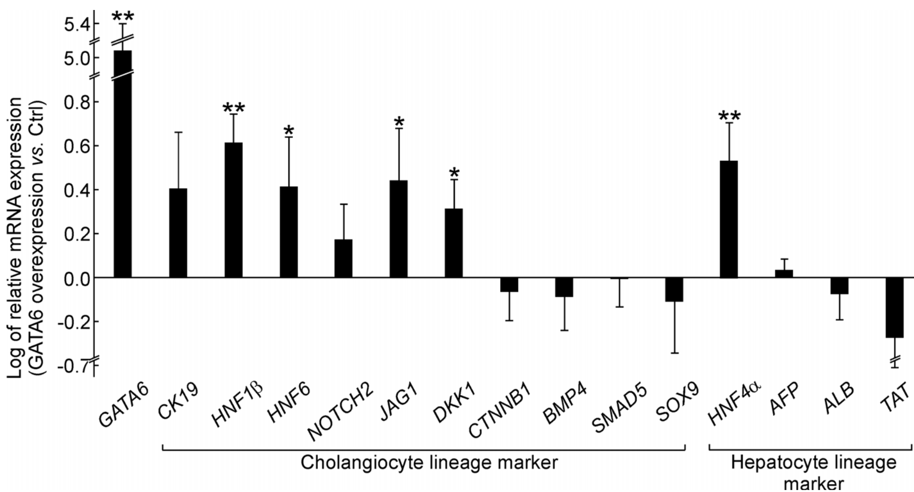

Figure 20. Altered gene expression after enforced expression of GATA6 in primary human hepatocytes.
Ratio of relative mRNA expression (Log) in GATA6 overexpression vs. control primary human hepatocytes. Value <0 indicates downregulation and value >0 indicates upregulation of a gene after GATA6 overexpression. Cholangiocyte lineage markers on the left side and hepatocyte lineage markers on the right side.

2.5 Expression of *HNF1β, HNF6,* and *JAG1* is elevated in biliary atresia

Finally, we compared the mRNA expression profiles of the three cholangiocyte lineage markers altered in both hepatocyte cell models after GATA6 overexpression (*HNF1β, HNF6*, and *JAG1*) to that of *GATA6*. We found that, like *GATA6*, these genes are upregulated in BA samples compared to normal liver, and their expression is significantly downregulated after PE (Figure 21A-C). *HNF1β, HNF6*, and Notch signaling, are shown to be dysregulated in BA and other biliary diseases (76-80).

Mutations in *JAG1* are shown to cause Alagille syndrome, characterized by severe paucity of the intrahepatic bile ducts (230). GATA4 and GATA6 are shown to modulate Notch signaling also in the developing intestinal epithelium (231).

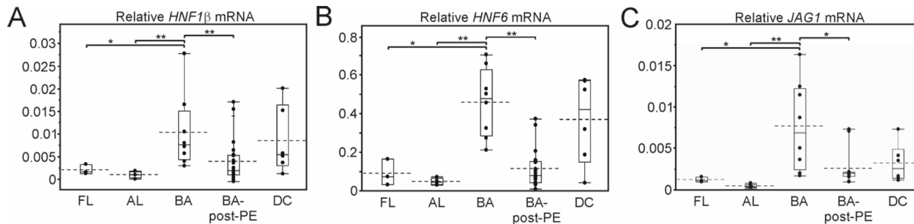

Figure 21. Expression of HNF1β, HNF6, and JAG1 in different liver sample types.
*Boxplots of relative HNF1β (A), HNF6 (B), or JAG1 (C) mRNA expression, as assessed by qPCR, in different liver sample groups. *=p<0.05, **=p<0.01 as compared to BA group.*

We assessed the localization of the *HNF1β, HNF6*, and *JAG1* mRNA by *in situ* hybridization in liver samples. In BA samples, mRNA of these genes was expressed in both cholangiocytes and hepatocytes, and thus their downregulation after PE is not caused solely by reduction of BDE. Their expression in periportal hepatocytes was diminished, similar to *GATA6* expression. Furthermore, we correlated the mRNA expression levels (qPCR) of *HNF1β, HNF6*, and *JAG1* to that of *GATA6* in BA and BA-post-PE samples, and found a linear correlation between *GATA6* and all three genes in BA-post-BE samples (IV). This result may indicate that GATA6 regulates these genes also *in vivo*, and thus promotes the pathologic hepatocyte-cholangiocyte metaplasia in BA livers.

Taken together, GATA6 is overexpressed in BA hepatocytes, compared to normal liver and other neonatal cholestatic conditions. Human *GATA6* mutations are connected to biliary malformations, suggesting a crucial role for this factor in the normal development and function of the biliary tree (226, 227). We hypothesize that the elevated GATA6 in BA hepatocytes promotes their ductal metaplasia and thus enhances the development of the DR and subsequent liver damage. When the cause of the cholestasis is eliminated (successful PE), GATA6 expression together with the pathologic changes in histology are reduced. With time, however, vast majority of the operated patients develop liver cirrhosis and end up requiring a liver transplantation (101). The causes of this ongoing fibrosis, as well as the role of GATA6 in BA progression in long term after PE, remain pivotal future questions. The follow-up biopsy samples collected from BA patients in Helsinki University Hospital are invaluable in unravelling this issue, and they will be utilized in ongoing studies of the GATA6-related and other pathways more globally in BA.

CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis work, we have investigated the pathogenesis of two severe diseases of pediatric liver; HB and BA. These two conditions have very different pathophysiologies and manifestations, yet both affecting infants and small children. These studies focused on the differentiation and gene expression changes of hepatocytes and its precursors driven by the two transcription factors crucial for normal liver development, GATA4 and GATA6. Figure 22 demonstrates a simplified cartoon of the expression and role of GATA4 and GATA6 in transdifferentiation of different liver cell types from normal liver to HB and BA.

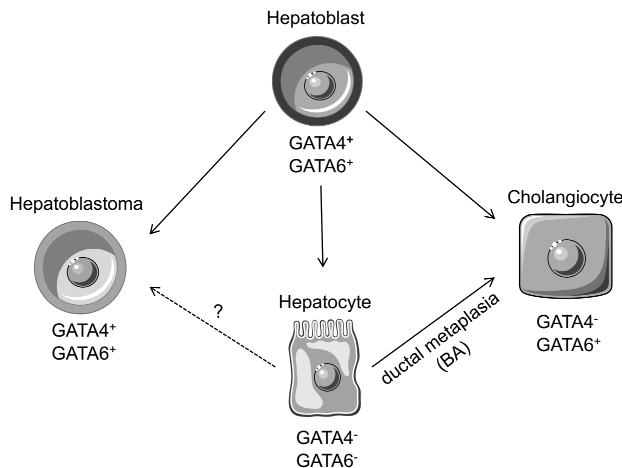

Figure 22. Schematic figure of GATA4 and GATA6 in different cell types of normal liver, HB, and BA. *Image created using image vectors from Servier Medical Art: (http://smart.servier.com/), licensed under the Creative Commons Attribution 3.0 Unported License.*

Point-by-point conclusions and future directions of the study:

1) <u>GATA4 regulates apoptosis and increases drug resistance in HB cells.</u>

GATA4 is highly expressed in HBs, whereas its expression in normal postnatal hepatocytes is low or absent. In HUH6 human HB cell line, GATA4 regulates the balance of the BCL2 protein family members and drives them towards an anti-apoptotic and pro-survival direction. Silencing GATA4 in these cells sensitizes them to the apoptotic effects of doxorubicin, a commonly used cytotoxic drug in the treatment of HB.

2) GATA4 promotes the malignant mesenchymal phenotype of HB cells.

GATA4 silencing alters the gene expression of HUH6 HB cells towards an epithelial phenotype and reduces the migratory capacity in these cells. Altered expression of EMT-related genes affecting 1) cytoskeleton reorganization, 2) cadherin swich, and 3) ECM remodulation was demonstrated after GATA4 silencing in HB cells. Opposite changes occurred in cultured primary hepatocytes with GATA4 overexpression, suggesting that GATA4 not only maintains this phenotype in HB cells, but may also be capable of inducing a mesenchymal-type gene expression when overexpressed in normal liver.

Future aspects: More specific knowledge about the gene regulation by GATA4 in HBs is required. Chromatin immunoprecipitation studies would identify the direct target genes of GATA4 and will be performed on HUH6 cells in the future. On the other hand, it is not known which factors upstream regulate the expression of GATA4. Recently, microRNA-based therapy targeting the Wnt/β-catenin pathway has been proposed for HB treatment (232). Efforts to downregulate GATA4 or disturb its function *in vivo*, would be helpful for the management of this childhood malignancy. Of interest, this has previously been successfully done with another GATA factor, GATA2, in an *in vitro* model for prostate cancer (233). In addition to the cell line studies, more sophisticated and reliable models with an *in vivo* tumor microenvironment, are required to assess the role of GATA4 in HBs. Comparison of patients' clinical outcome to GATA4 levels in HB tumors in a larger international study group may eventually enable the use of this factor as a prognostic marker in practice.

3) GATA6 is overexpressed in BA and potentially drives the ductal metaplasia of hepatocytes

GATA6, normally expressed in bile duct epithelium, is overexpressed in the hepatocytes of BA livers. This expression is significantly reduced after a successful alleviation of the cholestasis with PE operation. GATA6 correlates with pathological expansion of the bile ductules, patient age at PE, and liver injury marker ALT. In primary hepatocytes, GATA6 enhances the expression of genes involved in cholangiocyte differentiation and BA pathogenesis. Furthermore, GATA6 expression correlates to and colocalizes with these genes in BA livers.

Future aspects: Co-culture experiments with cholangiocytes are required to explore the effect of intercellular signaling in GATA6 driven hepatocyte ductal metalplasia. Furthermore, a mouse model of BA combined with inducible hepatocyte-specific *GATA6* knockout would shed light onto the role of GATA6 in development of DR and fibrosis. The final step would be unraveling the factors regulating GATA6 itself in BA (components of bile, cytokines and other signals of inflammation or cellular stress, transcription factors, growth factors etc.), thus enabling modifying these factors also *in vivo* to reduce the pathological processes in BA livers.

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Helsinki, June 2018

Tea Soini

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