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Liver repair mechanisms in non alcoholic steatohepatitis (NASH): defining the role of hepatic progenitor cells, ductular reaction and Notch signaling

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Chapter 1

Introduction

1. Liver repair and regeneration mechanisms

The liver is composed of many different cell types. The epithelial component is the most prominent, which comprises hepatocytes and cholangiocytes; these cells possess a unique regenerative potential that allows cellular renewal in homeostasis and, with limitations, in disease. Other liver resident cell types include macrophages (Kupffer cells), sinusoidal endothelial cells, lymphocytes, dendritic cells and hepatic stellate cells (HSC). Hepatic resident cells are highly organized along a matrix framework that allows their interaction in physiological and pathological processes. The liver is indeed exposed to many acute and chronic damages, and it has developed several mechanisms to repair and regenerate after injuries.

1.1 – Hepatic progenitor cells and ductular reaction

Hepatic regeneration mechanisms after injury vary depending on the type of damage. In case of mild acute injury, hepatocytes and cholangiocytes proliferate to restore the cellular loss; similar scenario has been reported in experimental models of partial hepatectomy, after which liver parenchyma is restored by hepatocytes replication[1]. Otherwise, if the liver is severely or chronically damaged, and the replicative ability of mature hepatocytes/cholangiocytes is compromised, liver regeneration relies upon the activation of the hepatic progenitor cells (HPC) compartment[2-5].

HPC constitute a bipotential amplifying compartment that gives rise to both cholangiocytes and hepatocytes. HPC are the resident liver stem cells pool, which niche is likely localized in the Canals of Hering (figure 1.1). Canals of Hering are the smallest branches of the intrahepatic biliary tree and constitute the interface

between ductules and the canalicular network, and they are therefore lined in part by cholangiocytes and in part by hepatocytes[6]. Histologically, HPC are described as small cells, with an ovoid nucleus and a high nucleus to cytoplasm ratio[6]. Several markers have been proposed to identify these liver stem cells, such as c-Kit, neural cell adhesion molecule (NCAM), epithelial cell adhesion molecule (EpCAM), cytokeratin 19 (CK19), CK7 or OV-6[2, 6-8], although none of these unequivocally identify HPC.

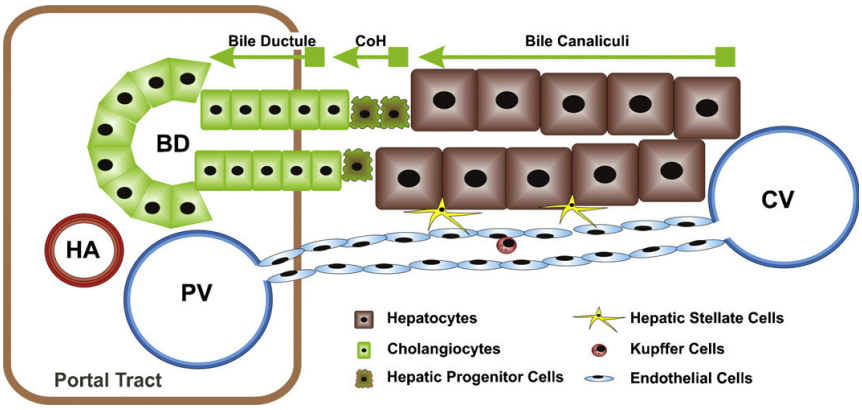

Figure 1.1. Location of hepatic progenitor cells niche within the liver. Progenitor cells are located in the canals of Hering which represent the connection between bile canaliculi and bile duct systems. BD, bile duct; CoH, canals of Hering; CV, central vein; HA, hepatic artery; PV, portal vein. (Adapted from *Gaudio et al., Digestive Liver Disease, 2009[9]*).

HPC are virtually absent in the normal liver, whereas in damaged livers they spread from the periportal niche to the pericentral lobular zone as disease progresses. Although at the beginning of the injury HPC can be found as single cells or small clumps, chronic HPC activation leads to a histological phenomenon described as “ductular reaction” (DR). DR is a reactive process involving epithelial, inflammatory and mesenchymal cells, occurring in virtually all forms of liver disease[2, 10-13]. Indeed, DR is a key step

in the repair mechanisms aimed to compensate the anatomical and functional loss of damaged livers. Depending on the type of cells mainly damaged, HPC commit toward the hepatocytic or cholangiocytic fate. Proliferating HPC differentiate in hepatocytes via intermediate hepato-biliary cells (IHBC), whereas commitment toward the biliary lineage occurs through the generation of reactive ductular cells (RDC), as shown in figure 1.2. HPC, IHBC and RDC constitute the “hepatic reparative complex”, which can be histologically recognized by the expression of CK7 and/or CK19, cytoskeletal proteins peculiar of cells of the biliary lineage. More specifically, IHBC are characterized by an intermediate phenotype between cholangiocytes and hepatocytes, lacking CK19 expression but positive for CK7, which is usually absent in mature hepatocytes[5, 14, 15].

Given the cellular heterogeneity of DR, the origin of the epithelial components has not been clearly defined yet. It is believed that DR arises mostly from activation and differentiation of HPC from the stem cell niche, or else, from the proliferation of existing bile ductules[11, 15]. Moreover, transdifferentiation or “ductular metaplasia” of hepatocytes appears to be an important mechanism of DR generation under certain circumstances[16-18]. It has also been proposed that bone marrow-derived circulating stem cells can partially contribute to DR generation[19]. Diversity of cellular origin potentially contribute to the heterogeneity in cellular elements characterizing different DRs; however, the epithelial components of the hepatic reparative complex always co-exist in DR, with one component being more prominent depending on the nature of the damage.

DR expansion occurs along with the recruitment of inflammatory mediators and the production of fibrovascular stroma.

Indeed, DR releases a vast array of paracrine signals, to establish an intensive cross-talk with inflammatory and mesenchymal cells, which is fundamental to orchestrate the intense remodelling activity characteristic of liver repair. However, although HPC activation is aimed to regenerate the damaged liver, chronic insults turn this process into a pathologic DR-driven repair, which eventually sustains disease progression and fibrotic evolution of the original injury[2, 4, 5, 10, 11, 13, 15]. Indeed, independently from the etiology, DR and liver fibrosis are strictly associated events, and their reciprocal influence eventually worsens liver damage. It is not clear yet whether DR promotes excessive fibrotic scar or on the opposite, fibrotic tissue deposition is necessary for HPC-driven hepatic regeneration. The mechanistic relationship between these two processes has not been clearly defined yet. Of relevance, DRs show different phenotypes depending on the nature of the injury[2, 13].

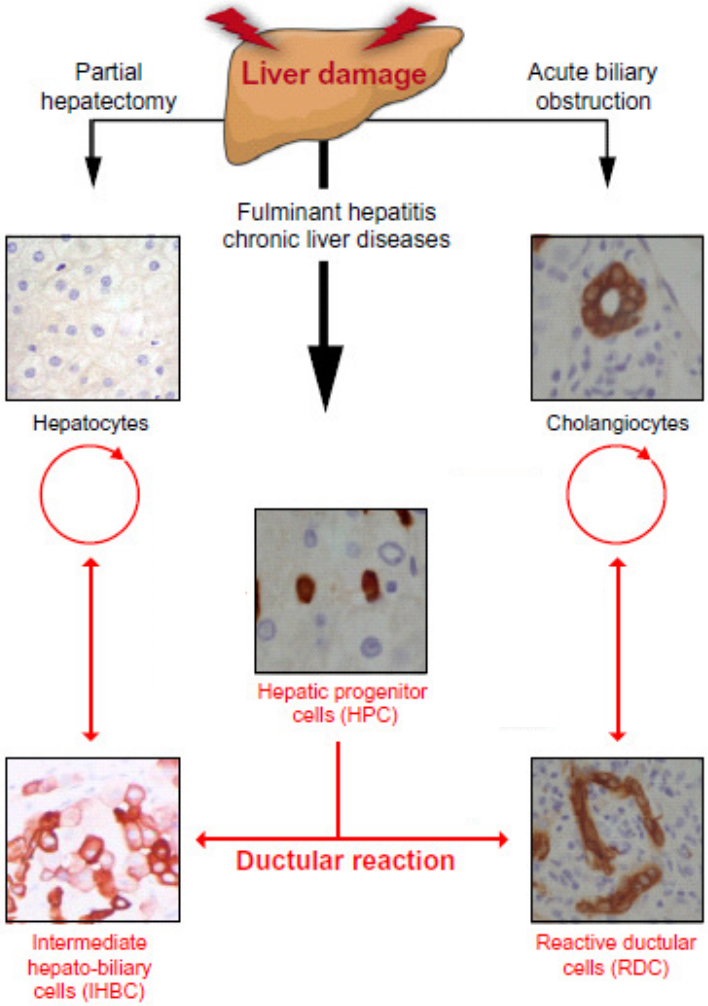

Figure 1.2. Epithelial phenotypes involved in liver repair driven by the activation of hepatic progenitor cells (''Hepatic Reparative Complex''). Hepatocyte and cholangiocyte proliferation can be directly stimulated without exploiting HPC activation in experimental models, such as partial hepatectomy and acute biliary obstruction, respectively. In massive and chronic liver diseases, liver repair is driven by the activation of HPC. HPC behave as a bipotent, transit amplifying compartment. Differentiation of HPC towards hepatocytes occurs via intermediate hepatobiliary cells (IHBC), while differentiation towards the biliary lineage leads to the formation of reactive ductular cells (RDC). HPC, IHBC and RDC constitute the ''hepatic reparative complex'', and can be distinguished by morphology and pattern of CK7 expression. (Modified from *Strazzabosco and Fabris., J Hepatol 2012[15]*).

1.2 – Ductular reaction in biliary diseases

Liver diseases in which the biliary epithelium is the primary target of the pathogenic sequence are referred to as cholangiopathies. Cholangiopathies can result from altered immunity (primary biliary cirrhosis, graft versus host disease, primary sclerosing cholangitis), infectious agents (cytomegalovirus or cryptosporidium), ischemia (arterial stenosis, post-transplant and chronic rejection), or toxic compounds. Moreover, genetic defects impairing bile duct biology can induce cholangiopathies, such as in Alagille Syndrome (AGS), Cystic Fibrosis, Autosomal Dominant and Recessive Kidney Diseases (ADPKD and ARPKD), Caroli Disease, Biliary Atresia. Although during the initial stages of disease the biliary epithelium is differentially targeted depending on the nature of the damage, its eventual chronic progression leads to cirrhosis and liver failure. Independently from the etiology, cholangiopathies are characterized by chronic inflammation around the damaged bile ducts, associated to various degrees of portal fibrosis, ductopenia and proliferation of cholangiocytes/HPC (resulting in DR formation); these processes eventually culminate in the distortion of the biliary tree architecture and cholestasis. Chronic inflammation can eventually lead to malignant transformation of cholangiocytes[3, 5, 11, 15].

DR is particularly evident in chronic cholestatic disease. After biliary damage, ductular mass expansion is aimed to build new branching biliary structures. In cholestatic disorders, there is a significant proliferation of RDC, which are initially organized in clusters of tubeless structures; as tissue remodelling progresses, RDC organize in well defined tubular network to regenerate the biliary system[3, 5, 11, 20]. During this process, fibrotic tissue deposition around proliferating DR foci is needed to re-establish proper bile

ducts architecture. Continuous activation of this mechanism further sustains DR/fibrosis, promoting disease worsening[11, 12, 15].

1.3 – Ductular reaction in hepatocellular damages

Hepatocytes can be the preferential target of toxins-induced acute fulminant hepatitis (as is the case of massive hepatic necrosis from acetaminophen toxicity) as well as in chronic liver diseases (CLD) in which hepatocellular damage can be genetically determined (such as α1-antitrypsin deficiency and genetic hemocromatosis), virus-induced (as in hepatitis C [HCV] or hepatitis B [HBV] infection) or derive from metabolic alterations (such as non-alcoholic and alcoholic steatohepatitis [NASH and ASH, respectively]). Disease progression is very variable and strictly depends on the etiology that can promote to various degrees hepatocytes senescence, apoptosis or necrosis, affecting disease outcome. Prolonged cell death in CLD eventually induces liver fibrosis, cirrhosis and end stage liver disease, factors that may drive hepatocellular carcinoma development[21, 22].

It is now clear that DR is a key event also in several human liver diseases of hepatocellular damage, when DR is generally balanced toward HPC/IHBC phenotype. The pattern of appearance and spread of DR is more variable depending on the initial injury[2, 13]. For example, dramatic DR associated with fibrosis and inflammation occurs rapidly after fulminant hepatic failure[23]. On the other hand, in chronic viral hepatitis DR is mostly found at the stromal-parenchymal interface in late stages of liver disease; in this setting, IHBC are diffused in liver parenchyma in close contact with hepatocytes and surrounded by dense fibrotic tissue[2, 24]. As discussed in the following paragraphs ("*2.2 – Ductular reaction in NASH*"), a similar scenario has been reported for non-alcoholic fatty liver disease: progression of simple steatosis to steatohepatitis is

characterized by the expansion of DR from portal areas in close contact with steatotic hepatocytes[2, 25, 26]. In general, HPC activation arises during hepatocellular CLD progression, as a result of impaired hepatocytes replication and increased cell death. As for cholangiopathies-associated DR, hepatocellular damage-induced DR is commonly associated to fibrotic tissue deposition, suggesting that DR appearance exacerbates disease progression.

2. Non Alcoholic Steatohepatitis

2.1 – Non Alcoholic Steatohepatitis (NASH)

Non-Alcoholic Steatohepatitis (NASH) is a growing worldwide health problem because of the increased incidence of risk factors such as obesity and diabetes among adults and children. NASH is considered to be a severe form of a spectrum of lesions called Non Alcoholic Fatty Liver Disease (NAFLD) (see figure 2.1). NAFLD is characterized by macrovescicular hepatic steatosis occurring in the absence of alcohol consumption, and it is believed to be the hepatic manifestation of the metabolic syndrome characterized by obesity, type II diabetes and hypertension. NAFLD ranges from simple steatosis (when more than 50% of hepatocytes contain fat droplets) to steatohepatitis with or without fibrotic scarring. NAFLD incidence reaches about 20% in the general population, which further increases up to 60% in patients with metabolic syndrome. In about 20-30% of NAFLD patients, simple steatosis progresses to inflammation and fibrosis (NASH). NASH is considered a risk factor for cirrhosis, liver failure and hepatocellular carcinoma development, since a subset of NASH patients eventually suffers from end-stage liver disease[27-31].

NASH is characterized by hepatocellular injury, inflammation and, in most cases, fibrosis. To date, liver biopsy is still the diagnostic gold standard that allows discrimination between NAFLD and NASH[28, 29, 32]. At the histological level, NAFLD and NASH are identified by triglycerides deposition in hepatocytes. Hepatocellular injury is also characterized by the presence of ballooned hepatocytes. “Ballooning” indicates enlarged hepatocytes, with swollen and rarefied cytoplasm, often with a large nucleus and a prominent nucleolus. Moreover, the pattern of expression of CK8 and CK18, typical hepatocellular cytoskeletal proteins, is altered in ballooned

hepatocytes, where they appear dispersed to the periphery. Eosinophilic intracytoplasmic inclusions of misfolded intermediate filaments (mainly CK8 and CK18), defined as Mallory Denk Bodies (MDB) are commonly seen close to the nucleus of ballooned hepatocytes. Other hepatocellular lesions that may be seen in NASH are megamitochondria, glycogenated and vacuolated nuclei, acidophil apoptotic bodies and iron deposition[28, 29, 32].

NASH is also defined by lobular inflammation; this consists of a mixed inflammatory cells infiltrate, mainly lymphocytes, along with eosinophil, few neutrophils and activated Kupffer cells. Commonly, Kupffer cells and eosinophils aggregate around lipid droplets in sinusoids in structures defined lipogranulomas[28, 32]. Moreover, NASH is characterized by portal inflammation, where lymphocytes, macrophages and neutrophils are seen in the matrix of portal tracts[25, 33].

NASH related damage can further progress to liver fibrosis. Perisinusoidal and pericellular fibrotic tissue deposition is initially recognized in centrilobular spaces (zone 3) with a characteristic “chicken wire” pattern. Progressive extracellular matrix deposition eventually remodels liver architecture: as detailed below (“*2.2 – Ductular reaction in NASH*”), portal fibrosis develops along with HPC activation, and further exacerbates distortion of liver anatomy. Dense septal fibrosis invades and disrupts liver parenchyma, eventually promoting cirrhosis, which is strongly associated with an increased risk of hepatocellular carcinoma development[26, 28, 32].

NAFLD pathogenesis and the mechanisms of progression to NASH are not completely understood. A multi-hit hypothesis has been proposed as a pathogenic mechanism[34]: the first hit is responsible of chronic hepatic fat accumulation, while the second hit (i.e. oxidative stress) prompts steatosis progression to NASH.

Different mechanisms trigger hepatic steatosis: fat accumulation in the liver can result from increased fatty acid delivery to hepatocytes (mostly deriving from diet and adipose tissue lipolysis) and *de novo* lipogenesis, along with decreased fatty acid oxidation and decreased VLDL-mediated export. Thus hepatic steatosis derives from an imbalance of fatty acids import and removal. Fatty acids accumulated in the liver are continuously metabolized by mitochondria via β-oxidation, microsomes (Cyp450) or peroxisomes (peroxisomal β-oxidation), thus generating high levels of reactive oxygen species (ROS). ROS, in turn, lead to increased lipid peroxidation, DNA and protein damage, resulting in hepatocytes apoptosis, eventually inducing a chronic inflammatory response[29, 35, 36].

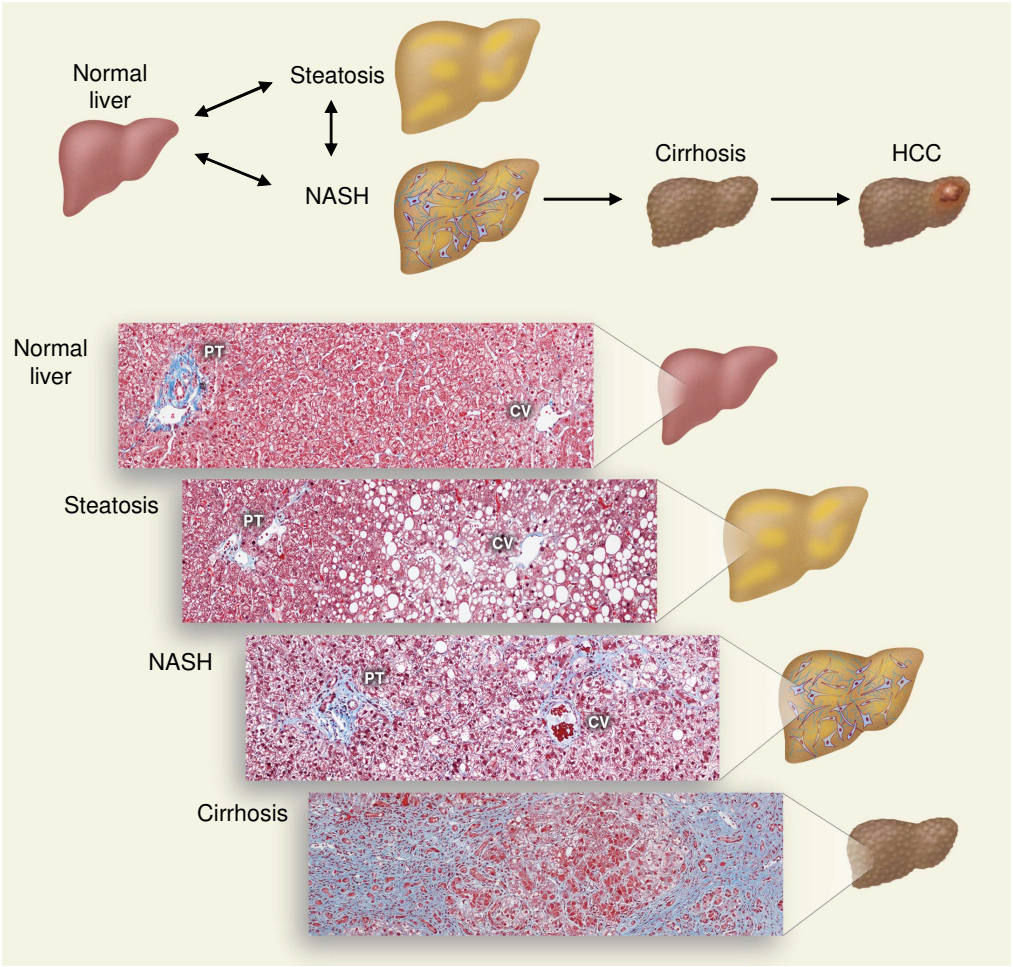

Figure 2.1. The disease spectrum of nonalcoholic fatty liver disease. (Top) Schematic progression of NAFLD. The accumulation of TG within lipid droplets in hepatocytes causes steatosis. Steatosis associated with inflammation, cell death, and fibrosis is referred to as NASH, which can progress to cirrhosis. Individuals with cirrhosis have an increased risk of hepatocellular carcinoma. (Bottom) Histological sections illustrating normal liver, steatosis, NASH, and cirrhosis. Collagen fibers are stained blue with Masson's trichrome stain. The portal triad (PT), which consists of the hepatic artery, portal vein, and bile duct, and the central vein (CV) are shown. (Modified from *Cohen et al., Science 2011[29]*).

2.2 – Ductular reaction in NASH

In NASH, HPC activation has been linked to increased risk of disease worsening[2, 24-26]. Indeed, in case of simple hepatic steatosis, DR is not present in liver parenchyma, although a slight HPC proliferation occurs in portal areas. On the other hand, increasingly evident DR appears in NASH during the progression of the damage and spreads throughout the lobule. DR is triggered in NASH as a consequence of continuous hepatocytes apoptosis and replicative arrest induced by oxidative stress. When activated, HPC proliferate and migrate in the injured parenchyma, where they establish an intimate contact with fat laden hepatocytes (figure 2.2)[2, 26]. A significant number of cells with intermediate size between hepatocytes and HPC and positive for CK7 has been reported in pediatric NASH livers, suggesting that HPC differentiation toward the hepatocytic lineage occurs in this pathologic setting[37]. However, it has to be considered that IHBC could potentially originate from transdifferentiating hepatocytes, as well. NASH-induced HPC-driven hepatic regeneration has been associated with progressive fibrosis[38]. Besides collagen deposition in sinusoidal fibrous septa, a portal fibrosis pattern has been recognized in NASH livers and periportal DR could potentially exacerbate fibrotic progression[16, 26]. This hypothesis is further supported by fibrosis pattern observed in pediatric patients: in this setting, perisinusoidal fibrosis is uncommon, whereas portal fibrosis is predominant[26, 28, 37]. Moreover, in portal areas DR extent is associated with the presence of inflammatory infiltrate: epithelial and inflammatory cells cross-talk to sustain liver damage in this pathologic setting promoting progressive fibrogenesis[25]. Although it is still unclear whether fibrosis precedes or is a consequence of DR, it is increasingly evident that DR plays a pathogenic role in NASH evolution.

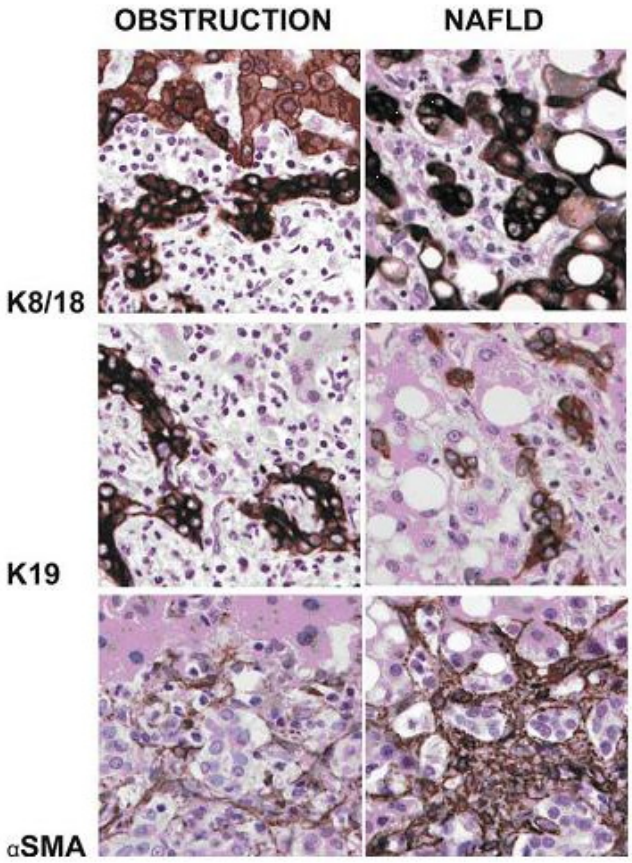

Figure 2.2. Immunohistochemical characterization of DR occurring in (left) chronic biliary obstruction, and (right) severe NAFLD (NASH). Both DRs comprise a diversity of cell types including: HPC/RDC that may be specifically highlighted with stain for keratin 19 (K19) or in combination with hepatocytes when stained for K8/18; stellate cells highlighted by stain for alpha-smooth muscle actin (αSMA) (which therefore also stains some of the matrix proteins already deposited by the stellate cells). Note that all components are present in both DRs, but their relative proportions and the cell:cell and cell:matrix relationships may differ significantly from one disease to another. HPC activation after biliary obstruction leads to HPC/RDC organization in disorganized tubeless structures in portal areas, with matrix deposition around DR, sparing liver parenchyma. In NASH livers, K19 positive cells are dispersed in liver parenchyma in small clusters or single cells and are in close contact with fatty hepatocytes. Moreover, αSMA positive cells are arranged around steatotic hepatocytes. (Modified from *Gouw et al., Hepatology, 2011[2]*).

The mechanisms involved in the pathogenesis of NAFLD/NASH are still undefined and far from being fully understood. Mechanisms driving the evolution from a condition of benign hepatic steatosis to NASH in a subgroup of patients have not been elucidated yet; moreover, there is the need to determine which risk factors are related to promotion of cirrhosis and eventually liver cancer. Since portal inflammation and portal fibrosis are recognized markers for NASH worsening[28], the pathologic expansion of the HPC compartment and the related fibrogenetic process is likely to represent a key mechanism in the progression of NASH to cirrhosis.

3. Notch signaling and liver patho-physiology

3.1 – The Notch Pathway

The Notch pathway is an evolutionarily conserved signaling crucial in regulating several biological processes, such as cellular proliferation, apoptosis, survival and lineage commitment[39, 40]. Notch is involved in developmental processes, as well as in tissue homeostasis in adult organs. Moreover, increasing evidence indicates that the reactivation of Notch occurs in injured organs, along with the activation of other morphogenic pathways, to regulate tissue repair; this phenomenon has also been linked to carcinogenic evolution.

In mammals, Notch signaling comprises four Notch receptors (Notch-1 through -4) and five ligands belonging to the Jagged (Jag1 and -2) or to the Delta-like (Dll1, -3 and -4) family. Notch receptors, as well as their ligands, are type I transmembrane proteins. Thus, ligand-dependent signal activation can only occur between neighboring cells. Cell-cell contact indeed allows the “signal sending cell” to activate Notch receptors on the “signal receiving cell”. Upon ligand binding, Notch receptors undergo sequential proteolytic cleavages that lead to the release of the Notch Extracellular Domain (mediated by the ADAM secretase) and the generation of the Notch Intracellular Domain (NICD), which is mediated by the γ-secretase complex. Thus, NICD translocates to the nucleus, where it binds to the DNA binding partner CSL (CBF1/Drosophila Su(H)/C.Elegans Lag1, also called recombination signal binding protein immunoglobulin kappa J [RBP-Jκ]): by displacing transcriptional repressors present at the DNA level, NICD/RBP-Jκ further binds to the co-activator MAML1 (Mastermind-like1). This cascade (summarized in figure 3.1) culminates with the transcription of canonical Notch target genes, among which the Hairy and Enhancer of Split homologs (Hes and Hey) transcription

factors[39-42]. In the liver, Notch activation promotes the expression of crucial determinants of biliary fate specifications, such as hepatocyte nuclear factors (HNF) and the SRY related HGM box transcription factor 9 (Sox9)[43-45].

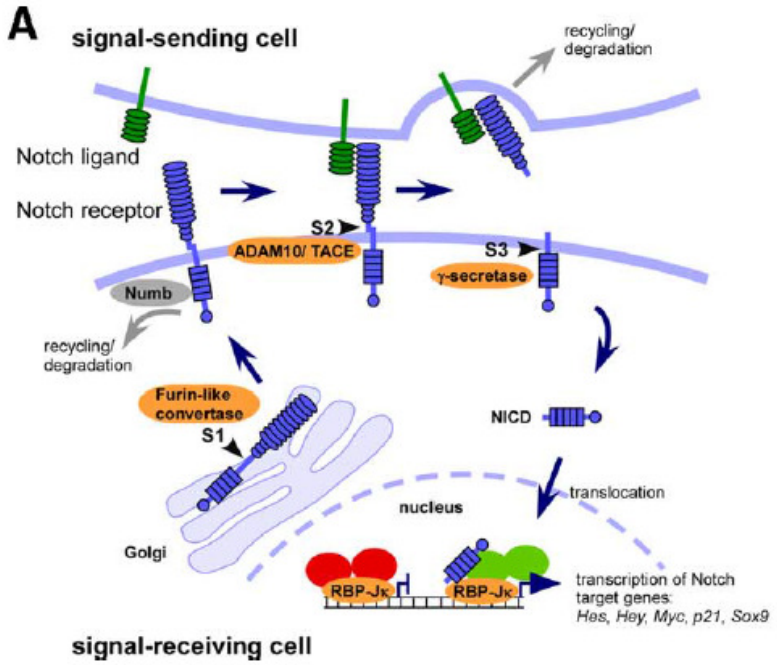

Figure 3.1. Core canonical Notch signaling pathway. (A) After synthesis, Notch receptors are cleaved by a Furin-like convertase in the trans-Golgi (S1 cleavage) to produce heterodimeric receptors containing an intra- and extracellular domain. Notch receptors are exocytosed to the cell surface of the signal-receiving cell, where Notch signaling is initiated upon binding to Notch ligands belonging to the Delta or Jagged family expressed on neighboring signal-sending cells. Steady-state levels of non-ligand-bound Notch receptors are controlled by E3 ligases and several proteins, such as Numb and a-Adaptin that control Notch turnover by recycling or lysosomal degradation. Notch ligand-receptor binding enables proteolytic cleavage of the Notch extracellular domain by ADAM10/ TACE metalloprotease (S2 cleavage) that remains bound to its ligand and is further subjected to lysosomal degradation in the signal-sending cell. The remnant receptor is then cleaved by the γ-secretase complex within its transmembrane domain at site 3 (S3 cleavage) to allow release and nuclear translocation of the NICD, where it associates with RBP-Jκ, a DNA-binding adaptor protein mediating the corpus of canonical Notch signals. In the absence of NICD, RBP- Jκ forms a corepressor complex (red) with many proteins, including histone deacetylases and SHARP. NICD binding to RBP- Jκ enables

displacement of the corepressor complex and binding of the adaptor protein, Mastermind-like (MAML) that recruits other proteins to form a coactivator complex (green), resulting in the transcriptional activation of Notch target genes. (Modified from *Geisler and Strazzabosco, Hepatology 2014[48]*).

Notch activation is finely tuned in a tissue- and time-specific manner. To modulate the signal, Notch receptors and ligands undergo several post-translational modifications that alter their expression on cell surface. Notch receptors are tightly controlled by endogenous repressors such as Numb that promotes their ubiquitylation and endocytosis[46] or by glycosylation at different sites inducing either receptors inhibition (mediated by Fringe) or activation under the influence of the O-fucosyl transferase enzyme[39, 40, 47]. Notch activation is also controlled at the NICD level by rapid proteosomal degradation induced by Numb-mediated cytosolic ubiquitylation[46] or by NICD phosphorylation in the nucleus. Therefore, continuous Notch activation requires additional ligands inducing receptor proteolysis[39, 40, 47]. Depending on the different receptors/ligands that transduce the signal and/or on the cell types in which this signal is activated, Notch promotes a variety of different, even opposing, effects. Notch activation may stimulate a phenotypical switch through lateral induction, as well as inhibit differentiation through lateral inhibition; these coordinated events allow for the spacing and timing organization of different cell types within tissues, along with maintaining the stem cells pool[39, 40, 47].

Mutations affecting the Notch pathway contribute to the pathogenesis of several human hereditary diseases (reviewed in[49] and[50]). Notch deficiencies have been linked to Alagille syndrome (AGS, OMIM 118450 and OMIM 610205[51]), spondylocostal dysostosis (SCD, OMIM 277300[52, 53]), CADASIL (cerebral autosomal dominant arteriopathy with subcotrical infarcts and

leukoencephalopathy, OMIM 125310[54]) and Hajdu-Cheney syndrome (OMIM 102500[55, 56]). AGS is caused by haploinsufficiency of Jag1[57, 58] as well as Notch-2 mutations[59]. AGS is a rare (1:70,000 live births) autosomal dominant disorder, primarly characterized by neonatal jaundice, cholestasis, and severe ductopenia. A wide range of extrahepatic manifestations also characterize AGS patients, such as characteristic facies along with abnormalities of heart, eye, skeleton, vasculature, kidney and pancreas[60]. Although disease manifestations are very heterogeneous, the hepatic phenotype (ductopenia) is present in about 90% of patients, and it is more severe at the periphery of the liver[61]; interestingly, progression to liver cirrhosis is rare. However, 15% of patients requires liver transplantation because of failure to thrive, pruritus and hypercholesterolemia[62]. As discussed in the following sections ("*3.3 – Notch and liver injuries*"), it is still unclear whether ductopenia is caused by developmental lack of bile ducts or by progressive loss of biliary structures coupled with alterations in liver repair.

3.2 – Notch and liver development

During liver development, both hepatocytes and cholangiocytes differentiate from a common bipotential progenitor, the hepatoblast, depending on their spatial organization. More specifically, hepatoblasts localized in the liver parenchyma differentiate into hepatocytes. On the other hand, hepatoblasts located in the portal mesenchyma will differentiate into cholangiocytes during bile duct morphogenesis through the remodelling of peculiar structures called "ductal plates", allowing coordinated development of both the biliary and the portal vein systems. Ductal plates are initially organized in a monolayered ring of biliary cells in proximity of portal

areas; discreet portions of these structures are then remodelled into bi-layered asymmetrical structures, lined by cholangiocyte-like cells on the portal side and cells with hepatoblast characteristics on the parenchymal side. At this point lumens dilate initiating the process of tubulogenesis, during which the immature ducts are progressively incorporated into the mesenchyma of the nascent portal space. As the portal space develops, biliary tubules are remodelled into individual bile ducts entirely lined by cholangiocytes, which proliferate and expand until complete maturation of the biliary tree[15, 63-66].

The whole process of bile duct morphogenesis (summarized in figure 3.2, panel A) starts from Notch-induced commitment of the hepatoblasts to the biliary lineage: Notch activation drives biliary specification by upregulating the cholangiocytes specific transcription factors HNF6, HNF1β, OPN and Sox9[43, 67, 68]. Sox9 is the most specific and earliest marker of biliary cells in the developing liver that controls the timing and maturation of primitive ductal structures[45, 69]. On the other hand, NICD inhibits hepatocellular differentiation by downregulating the expression of hepatocytes-specific transcription factors, such as HNF4α[45, 65]. Notch-1 and -2 receptors play an essential and redundant role in hepatic development, as a broad deficiency of these receptors is embryonically lethal[70], whereas normal embryogenesis occurs in mouse models lacking Notch-3 and -4[71, 72]. More specifically, Notch-2 is the most relevant receptor in orchestrating biliary specification of hepatoblasts[73-75] by interacting with Jag1 expressed on mesenchymal cells in the nascent portal space[45, 68, 76]. Notch-2 is also involved in bile ducts morphogenesis by regulating sequential differentiation of adjacent layers of precursor cells[45, 76]. Jag1/Notch-2 interaction is essential for portal space formation and organization, since animal models with inactivation of Jag1 in portal vein mesenchymal cells display

alterations in both biliary and portal space morphogenesis[77]. Moreover, intrahepatic bile duct morphogenesis is altered in experimental models lacking Notch effectors (i.e. Notch-1 or Notch-2[73, 78, 79], RBPjκ[45, 74, 75], Jag1[77, 80], Hes1[44]) or Notch related biliary targets (Sox9[69] and HNF1β[43]). Accordingly, hepatoblasts of mutant mice over-expressing Notch-2 intracellular domain (N2ICD) undergo a more rapid biliary specification, inducing ectopic formation of tubular and cystic structures, reminiscent of early malignant biliary lesions[74]. Although animal models lacking Notch signaling suffer from altered intrahepatic bile duct development, bile duct morphogenesis is never completely inhibited, suggesting that other developmental regulators are involved in this process. However, Notch is an essential driver of biliary morphogenesis, since so far it is the only pathway linked to a human biliary disease characterized by intrahepatic bile duct paucity[64, 65].

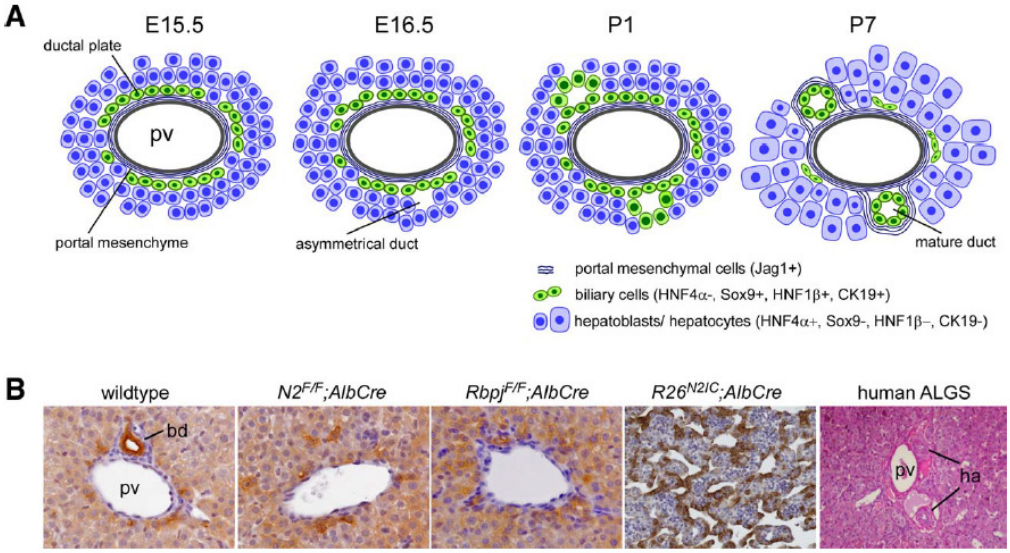

Figure 3.2. Normal embryonic development of the intrahepatic biliary tree and consequences of alterations in Notch signaling. (A) Embryonic development of the intrahepatic biliary tree starts with differentiation of a subset of Notch2+ periportal hepatoblasts expressing biliary lineage-defining proteins, such as Sox9, HNF1β, OPN, or CK19. These cells, committed to the biliary fate, encircle the Jag1+ portal mesenchyme in a monolayered ring, called the DP (for review, see previous works[65, 76]. In a second step, lumina arise at distinct sites of the DP that are lined asymmetrically by biliary cells of the first DP layer (portal side) and hepatoblasts (parenchymal side). Subsequently, during development, this asymmetry of immature ducts resolves when the duct-lining hepatoblasts maturate to biliary cells (second DP layer) by loosing HNF4α and gradually expressing Hes1, OPN, SOX9, HNF1β, and CK19. This two-step specification process in IHBD tubulogenesis is observed in mice and humans and starts at around E15.5 and W12, respectively. Because IHBD development proceeds from the hilum to the periphery, mature symmetric ducts are first observed at the hilar region, when more peripheral IHBDs may still display asymmetric tubules beyond birth before completing their remodeling program by around P2[45]. Thus, differentiation of hepatoblasts to biliary cells represents the main mechanism for intrahepatic biliary morphogenesis in the embryonic and early postnatal period. (B) Bile duct development is disturbed in Notch mutants. At P10, pan-CK+ mature bile ducts are well incorporated in the mesenchyme of medium-size portal tracts in wild-type mice. In contrast, P10 mice with hepatoblast-specific conditional deletion of Notch2 (N2F/F;AlbCre) or RBP-Jκ (RbpJκF/F;AlbCre) lack mature bile ducts as a result of impaired perinatal IHBD morphogenesis/elongation. Conversely, conditional expression of the intracellular domain of Notch2 in embryonic hepatoblasts (R26N2IC;AlbCre) directs commitment of embryonic hepatoblasts to the biliary lineage with ectopic formation of biliary-like tubular-cystic structures expressing biliary-specific Dolichos biflorus

agglutinin at P0. In human ALGS, portal tracts typically lack mature bile ducts. pv, portal vein; bd, bile duct; ha, hepatic artery. (Modified from *Geisler and Strazzabosco, Hepatology 2014[48]*).

3.3 – Notch and liver repair

Developmental pathways such as Notch, Hedgehog and Wnt are often re-activated in adult tissues when progenitor-cells dependent regeneration occurs in response to cellular damages. Despite the well documented role of Notch in liver development, Notch activation has not been investigated in depth in the diseased adult liver until recently. Increasing evidence demonstrates that Notch pathway plays a relevant role in regulating DR-driven hepatic repair processes and promotes hepatic cancer development[48].

Notch components in the adult liver are differentially regulated after damage. Cholangiocytes and HPC strongly up-regulate Notch-1 and -2 receptors upon injury[81-83]; Notch-3 and -4 are not involved in epithelial cells patho-biology, as they are mainly expressed by mesenchymal and endothelial cells[84]. Of note, once activated, hepatic stellate cells (HSC) increase Notch-3 expression while down-regulating Notch-1 receptor[85-87]. Jag1 ligand is instead broadly expressed by epithelial and mesenchymal cells and it is up-regulated in both compartments when damage occurs[81, 83, 85-88]. Strong Jag1 induction in diseased livers suggests that Jag1 is the actual ligand involved in activating Notch receptors in pathologic settings, since Dll4 expression is mainly restricted to physio/patho-biology of endothelial cells[86] and the hepatic expression of the other Notch ligands is negligible.

Notch signaling influences the reparative mechanisms by driving the HPC/DR response. Notch activation in HPC and RDC has been reported in human cholangiopathies[81, 83]. Accordingly, AGS is characterized by a peculiar DR that differs from other cholestatic

liver disorders in extent and quality[89]. In AGS patients there is an imbalance in the cellular elements involved in the reparative response, characterized by the near absence of RDCs and HPCs along with a significant increase of IHBCs[89]. The massive presence of CK7-positive IHBC suggests that when Notch is defective either HPC are forced toward the hepatocellular fate, or that hepatocytes attempt to promote liver regeneration by transdifferentiating toward the biliary lineage, but in Notch absence they are unable to proceed further and remain stuck at the intermediate phenotype. Of note, IHBC lack the expression of the biliary-specific transcription factor HNF1β (figure 3.3).

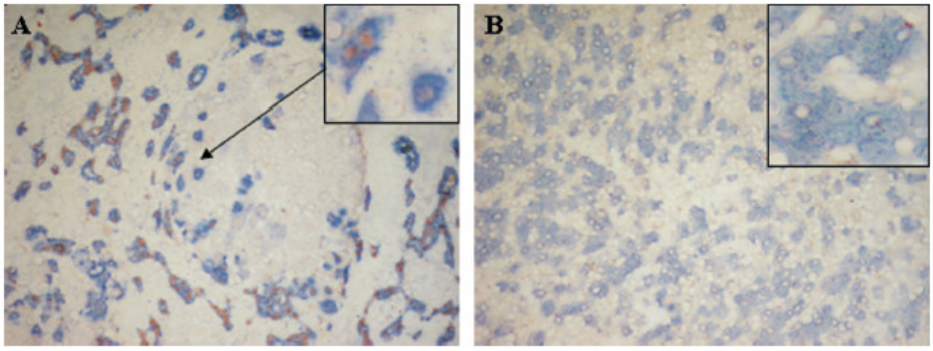

Figure 3.3. Immunophenotype of IHBC is different in AGS compared with BA. A shows the expression of the HNF1β transcription factor (a transcription factor regulated by Jagged1/Notch signaling and involved in biliary differentiation) by IHBCs, RDCs, and HPCs in a representative case of biliary atresia. **B** shows instead the lack of expression of the HNF1β transcription factor by IHBCs in a representative sample of AGS. Double immunostaining for CK7 (TrueBlue) and HNF1β (horseradish peroxidase). Magnification: X200 in all micrographs, X600 in insets. (Modified from *Fabris et al., Am Journal of Pathology, 2007[89]*).

Similarly to AGS, a complete inhibition of Notch signaling in experimental models of cholestatic damages impairs DR generation and blocks the commitment of HPC toward the biliary lineage[81, 82]. When Notch pathway is only partially defective, as in liver-specific conditional Notch-2 KO mice, DR develops after cholestatic challenges and HPC commit toward the biliary lineage. However, in this setting, biliary-committed HPC are unable to organize in tubular structures and the biliary tree cannot be regenerated (as detailed in Chapter 2), resulting in parenchymal necrosis or vanishing bile duct syndrome[82]. Conversely, overexpression of Notch-2 intracellular domain (N2ICD) specifically in the HPC/biliary compartment induces spontaneous appearance of DR[74].

Notch signaling could also be primarily involved in DR-associated fibrogenesis, by regulating epithelial-mesenchymal cross-talk. This hypothesis is supported by evidence that in AGS DR occurs in the absence of liver fibrosis[89], as opposed to what observed in most cholangiopathies. In AGS patients, collagen deposition in liver parenchyma is limited, consistent with their slow progression to cirrhosis[90]. Therefore, Notch could also play a relevant role in activating HSC and portal fibroblasts, thereby promoting collagen deposition, sustaining the fibrotic process. Accordingly, Notch signaling activation along with Notch-3 up-regulation has been reported in an experimental rat model of carbon tetrachloride (CCl_4)-induced liver fibrosis[85]. Notably, γ-secretase inhibitor treatment significantly reduced liver fibrosis extent in this setting. Moreover, up-regulation of Notch occurring in activated HSC/myofibroblasts cooperates with Hedgehog signaling, favouring fibrotic progression[87].

As mentioned above, Notch defects hamper HPC-biliary specification. Cells participating to the DR are able to commit to the

biliary lineage only if they are (at least partially) Notch responsive[82, 89]. Accordingly, to allow fully differentiation of HPC into cholangiocytes, cells surrounding the DR must be capable of inducing Notch activation in HPC. In fact in AGS patients, defect of Jag1 causes accumulation of CK7-positive IHBC that do not differentiate into cholangiocytes[89]. As in development Jag1 positive portal mesenchymal cells communicate with Notch-2-expressing hepatoblasts, the epithelial and the mesenchymal compartments are likely to interact in diseased livers as well. It has been proposed that the type of damage influences the microenvironment associated to DR. Accordingly, cholestatic damages are mainly characterized by DRs developing in portal spaces, where Jag1-expressing portal myofibroblasts accumulate, thus inducing Notch-dependent biliary commitment in HPC[81, 82]. On the other hand, the niche surrounding DRs after hepatocellular damage is mainly characterized by macrophages accumulation, which has been shown to favour hepatocellular regeneration[81, 91]. Macrophages indeed release Wnt3a, which increases Numb expression in HPC thus blunting Notch activation[81]. Therefore, hepatocytic or biliary specification of HPC is the result of a balanced Notch/Wnt signaling action (figure 3.4)[81, 92, 93].

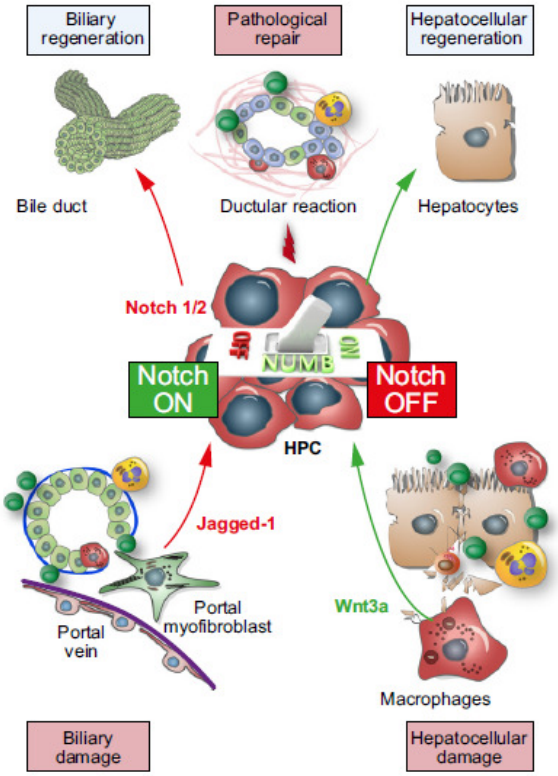

Figure 3.4. Epithelial-stromal interactions behave as critical regulators of liver repair mechanisms in response to biliary or hepatocellular damage. By alternatively activating Notch and Wnt signaling, the stroma microenvironment orchestrates the divergent specification of HPC towards the biliary or hepatocellular lineage,respectively. In biliary regeneration, the interaction of HPC with myofibroblasts expressing Jagged1 results in the activation of Notch2 in HPC, leading to their biliary specification (red left-sided section). In contrast, during hepatocellular regeneration, HPC are activated by Wnt3a released by macrophages in response to phagocytosis of debris from apoptotic hepatocytes. Wnt3a then stimulates the nuclear translocation of β-catenin, which promotes HPC differentiation towards the hepatocellular lineage (green right-sided section). In this bimodal crosstalk between HPC and adjacent inflammatory stromal cells, Numb plays a key role as ‘‘switcher’’. HPC differentiation into mature cholangiocytes or hepatocytes leads to resolution. When these mechanisms fail, reactive cholangiocytes are activated in conjunction with persistent inflammation, mesenchymal cell activation, and portal fibrosis, ultimately leading to progression of liver disease (pathologic repair, center-sided section). (Adapted from *Strazzabosco and Fabris, J Hepatol, 2013[93]*).

However, the histogenesis of HPC is not completely understood. Several papers argue on the cellular origins of HPC/DR and on their actual contribution to hepatocytes and cholangiocytes regeneration in injured livers. Some investigators provided evidence that HPC are the source of new cholangiocytes and hepatocytes in normal and disease conditions. Fate tracing studies revealed that Sox9 positive labelled progenitors could repopulate the liver during homeostatic and regenerative processes, with more than 90% of hepatocytes derived from this source[94]. Another study showed that hepatocytes were originated from osteopontin expressing-HPC, although with a low contribution[95]. Moreover, a recent paper proposed that massive loss of hepatocytes in zebrafish stimulated liver regeneration via biliary cells lineage conversion: in this setting, cholangiocytes acquired hepatocytic phenotype and morphology through an intermediate HPC-like step[96]. On the other hand, clonal fate tracing of Sox9 positive cells failed to demonstrate that HPC generate new hepatocytes in physiological conditions, whereas Sox9 positive HPC gave rise to hepatocytes with a very low frequency in experimental-induced liver damage[97]. Moreover, chimeric models (induced by hepatocytes transplantation) demonstrated that hepatocytes themselves are the predominant source of parenchymal cells in injured livers[97]. Accordingly, other groups reported that HPC do not sustain hepatocytes renewal in liver homeostasis[98-102], and that HPC contribution to hepatocytes regeneration is minimal, if not negligible in experimental models of parenchymal injury (CCl4, partial hepatectomy and acetaminophen intoxication)[100, 102] as well as in those of biliary damage (3,5-diethoxycarbonyl-1,4-dihydrocollidine, [DDC]) or in experimental protocols that induce a massive HPC activation (choline-deficient, ethionine supplemented diet [CDE])[100-102]. On the contrary, independently from the nature of the damage,

hepatocytes sustain parenchymal regeneration by self-replication[102]. These contrasting results may derive from the choice of the experimental damage that influences and affects HPC/DR activation. Of note, none of the described injury models efficiently inhibit hepatocytes proliferation, and it is believed that HPC do not generate hepatocytes when their proliferative ability is preserved[95, 101]. Moreover, most lineage tracing experiments rely on promoter-specific Cre-mediated labelling of selected cells, that in genetic models can also leak and recombinate in the opposite cell compartment, thus ectopically expressing the reporter gene and affecting the results.

Also the origin of HPC is a matter of debate. Although it is believed that HPC have a ductular origin[98-100], increasing evidence shows that hepatocytes lineage conversion might contribute to HPC/DR generation. Hepatocytes reprogramming was reported in damage models that induce HPC/DR response[18, 102, 103] and lineage tracing experiments proposed that hepatocytes are the predominant source of epithelial DR cells in injured livers[103]. Moreover, after cholestatic challenge with DDC, DR was populated by byphenotypic cells (Sox9+ve, EpCam-ve, HNF4α+ve) with hepatocyte-like morphology, suggestive of hepatocytes undergoing biliary transdifferentiation[104]. Lineage conversion of hepatocytes has been described as a slow step-wise process during which hepatocytes undergo phenotype changes (i.e. expression of biliary markers such as Sox9 or HNF1β) that progressively correlate with a smaller morphology, similar to that of HPC. CK19 expression is the last step of this process, since CK19-positive hepatocytes have been found only after prolonged exposure to damage[105]. Recent reports suggest that HPC/DR may actually derive from Notch-induced hepatocytes reprogramming[74, 105]. Indeed, continuous supply of

NICD in hepatocytes results in rapid biliary conversion[67, 74, 105]. Altogether these studies suggest that Notch may promote dedifferentiation in mature liver cells, which is consistent with Notch implication in liver cancer development.

3.4 – Notch and liver carcinogenesis

Primary liver malignancies comprise both hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). Liver cirrhosis is a risk factor for liver cancer development, since it is a common feature in HCC (85-90%) as well as in 20-25% of intrahepatic CCA[106, 107]. Cirrhosis is characterized by necroinflammation, fibrosis and HPC/DR proliferation; this pathologic environment may sustain the acquisition of a malignant phenotype in HPC, promoting their differentiation to cancer stem cells (CSC)[108, 109]. The presence of CSC would explain why a subset of tumors, identified as cholangiocellular-carcinomas, displays combined features of both HCC and CCA; interestingly, some of these cases are characterized by a Notch signature[109, 110]. Contrasting reports have been published regarding the role of Notch in liver cancer progression. Notch activation has been reported to reduce HCC cells proliferation cooperating with p53[111] or antagonizing retinoblastoma activation[112]. Other studies showed instead that HCC progression and aggressiveness was linked to Notch receptors up-regulation, which activation was promoted by the inflammatory environment or by the association with other pathways[113-115]. Of note, AGS-related liver disease rarely induces hepatic cancer[90]. Since to date Notch mutations have not been reported in solid tumors, Notch effects depend on the cellular context and on the interplay with other pathways within the tumor environment. Constitutive activation of the Notch-1 receptor consequent to chromosomal translocation[116] or

gain-of-function mutations[117] is responsible for malignant progression in T cell acute lymphoblastic leukaemia. Although there is no strong evidence of the oncogenic role of Notch in other tissues, a wide range of tumors has been linked to Notch activation[118].

However, increasing evidence strongly supports a pro-carcinogenic effect of Notch activation in the liver. Notch-2 expression has been reported in about 30% of HCC patients, with nuclear localization in 50% of cases; moreover, Notch-2 activation correlated with the expression of Jag1 and EpCam[119]. Accordingly, genomic profiling revealed that 30% of HCC cases harboured a Notch signature which correlated with cellular proliferation and Sox9 expression[120]. Notch receptors up-regulation was also reported in CCA patients[121, 122], where Notch activation was linked to increased cellular proliferation through cyclin-E induction[122]. Sox9[120] as well as EpCam[119] expression reported in subsets of human HCC suggests that HCCs with Notch signature could actually originate from CSC (figure 3.5). It could be speculated that Notch induces proliferation of HPC and/or confers progenitor-like features to mature differentiated cells during malignant progression; this would indicate a more aggressive and undifferentiated phenotype of liver tumors with active Notch signaling. Sox9 which was found increasingly expressed in these HCC subtypes[120] could potentially be used as a marker to identify a specific pluripotent population in this context.

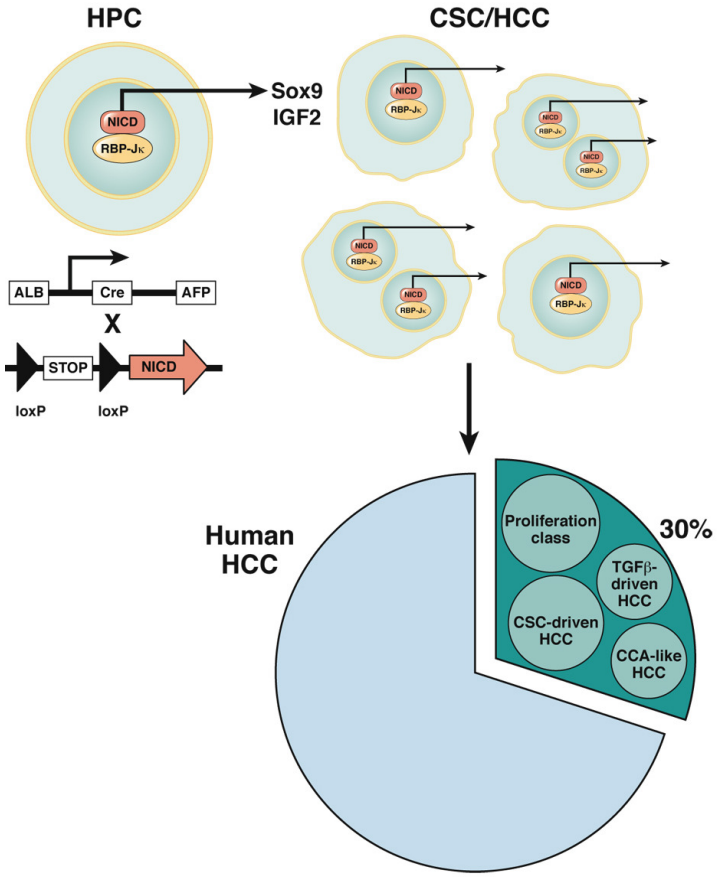

Figure 3.5. Effects of constitutively active Notch in hepatic progenitor cells. In experimental conditions, constitutional activation of Notch generated by inducing the AFP-Cre–mediated overexpression of NICD1 in embryonic hepatic progenitor cells, leads to the generation of HCC showing some features of CSC activation. Sox9 and IGF2 are critical modulators of NICD1-induced liver carcinogenesis. Signatures of Notch activation can be found in about 30% of patients with HCC. In these patients, the Notch signature co-clustered with specific molecular subgroups of HCC, mainly including the "proliferation class" and a "CSC-driven" HCC. In addition, a "CCA-like" HCC and a "TGFβ-driven" HCC are also associated with the Notch signature. CCA, cholangiocarcinoma; CSC, cancer stem cell; DLL, delta-like; HCC, hepatocellular carcinoma; HPC, hepatic progenitor cell; JAG, Jagged; NICD, Notch intracellular domain. (Modified from *Strazzabosco and Fabris, Gastroenterology, 2012[109]*).

Experimental models of hepatocarcinogenesis provide further insight on Notch in driving malignant promotion and progression. Hepatic expression of associated AKT/Ras oncogenes promoted the development of rapid pre-neoplastic lesions, giving rise to tumors with a high morphologic variability, ranging from α-fetoprotein (AFP)-positive HCC-like to EpCam-positive CCA-like tumors as well as tumors with mixed phenotypic features[119]. Notch activation occurred in this model, particularly in CCA-like lesions. Regardless of cancer phenotype, blunting Notch-2, as well as Jag1 with highly specific blocking antibodies dramatically reduced tumor progression; moreover Notch-2 and Jag1 inhibitions were also successful in preventing tumor formation. The fact that Notch-3 inhibition had no effects on tumor growth is suggestive of a prominent oncogenic role of Notch-2 receptor. Indeed, although blockade of Notch-1 efficiently reduced the formation of HCC-like tumors, it also led to a shift in tumor phenotype to CCA-like lesions, along with increasing Notch-2 activation[119]. Accordingly, constitutive expression of Notch-2 intracellular domain (N2ICD) in liver epithelial cells was sufficient to induce HCC and biliary hyperplasia. Moreover, N2ICD also accelerated diethylnitrosamine (DEN)-induced carcinogenesis, favouring the formation of both CCA and of Sox9-positive dedifferentiated HCC[123]. On the same line, liver carcinogenesis may also result from constitutive activation of Notch-1 intracellular domain (N1ICD). When expressed under the control of AFP promoter, N1ICD was able to rapidly (12 months) induce HCC-like tumors with 100% penetrance; most of the lesions showed Sox9 expression, frequently displaying progenitor-like features[120], supporting the hypothesis that Sox9 could potentially be a prognostic marker of HCC aggressiveness. Moreover, many studies suggested that continuous activation of N1ICD either alone or in association with

other oncogenes or cancerogenous stimuli induced conversion of mature hepatocytes toward the biliary fate, thus giving rise to CCA[122, 124, 125]. Notch-mediated hepatocytes reprogramming in HPC/cholangiocytes-like cells adds further credit to the involvement of Notch in more de-differentiated and aggressive forms of liver cancer.

Altogether these reports clearly show that ectopic or continuous activation of Notch eventually leads to hepatic carcinogenesis, thus establishing a pro-oncogenic role for this pathway.

Aim of the thesis

This PhD projects aims to investigate the role of Notch signaling in regulating liver repair mechanisms, in different pathologic settings. The first part of the study focuses on the role of Notch in hepatic progenitor cells (HPC) expansion and related ductular reaction, occurring in cholestatic cholangiopathies. In the second part of the study we investigated the influence of Notch on HPC response in a model of hepatocellular damage. Moreover, since the relevance of Notch in liver patho-physiology has gained much attention recently, we also reviewed the latest literature on the topic. These studies are divided into different chapters, as outlined below.

CHAPTER 2: Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. (J Hepatol. 2013 Jul;59(1):124-30. doi: 10.1016/j.jhep.2013.02.025. Epub 2013 Mar 7.)

In response to biliary damage, the expansion of the epithelial compartment in the liver is a key step of repair mechanisms activated to compensate the anatomical and functional loss of injured ducts. When the biliary tree is chronically challenged, cholangiocytes proliferation is not sufficient to sustain the repair process and the HPC compartment is activated. During this process, “reactive” cholangiocytes and HPC expand the ductular mass to form new branching tubular structures. Since Notch signaling is fundamental for the biliary differentiation of the hepatoblasts, we reasoned that in the adult liver Notch would be required to regulate biliary specification of HPC after cholestatic damages. Here we show evidence that Notch

signaling not only confers fate-instructions to HPC, but it also plays an essential role in biliary tubulogenesis.

CHAPTER 3: Notch Signaling Beyond Liver Development: Emerging Concepts in Liver Repair and Oncogenesis. (Clin Res Hepatol Gastroenterol. 2013 Nov;37(5):447-54. doi: 10.1016/j.clinre.2013.05.008. Epub 2013 Jun 24. Review.)

The unique plasticity of liver epithelial cells allows them to proliferate and promote liver restoration after injury, along with the activation of the progenitor compartment. Increasing evidence indicate that Notch is reactivated in the adult liver during tissue regeneration after injury, where it induces HPC activation, biliary differentiation and tubulogenesis. However, Notch activation needs to be finely regulated to avoid “pathologic” liver repair or aberrant cellular differentiation and proliferation that may promote hepatic cancer.

CHAPTER 4: Notch signaling and new therapeutic options in liver disease. (J Hepatol. 2014 Apr;60(4):885-90. doi: 10.1016/j.jhep.2013.11.028. Epub 2013 Dec 3.)

Altered Notch signaling activation in the adult liver has been linked to pathologic situations such as liver fibrosis and cancer. These recent findings suggest that interfering with the aberrant activation of Notch pathway could be an effective targeted therapy. Indeed, several Notch inhibitors have been developed to treat several malignancies. These compounds may be efficient in treating several forms of liver disease.

CHAPTER 5: Notch-mediated epithelial/mesenchymal interaction influences the hepatic reparative response in non-alcoholic steatohepatitis (NASH). (Submitted)

Our previous paper demonstrated that Notch signaling regulates ductular reaction occurring after cholestatic challenges. We have hypothesized that activation of Notch signaling may be also responsible for the vigorous ductular reaction characteristic of non-alcoholic steatohepatitis (NASH). In NASH, HPC expansion occurs throughout liver parenchyma and correlates with the extent of liver fibrosis. In this paper we show that in NASH, Notch signaling is aberrantly activated in subpopulations of hepatocytes, which undergo de-differentiation toward a HPC-like phenotype. Continuous Notch activation in these cells is most likely promoted by activated hepatic stellate cells over-expressing Jag1 ligand. Taken together our results suggest that aberrant Notch activation is responsible for hepatocytes reprogramming and transdifferentiation into HPC-like cells, process stimulated by the fibrotic environment. These findings indicate Jag-1 as a crucial player in NASH-activated liver repair mechanisms that drives cirrhotic evolution and eventually promotes malignant progression.

References

1. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology (Baltimore, Md 2006 Feb;43(2 Suppl 1):S45-53.

2. Gouw AS, Clouston AD, Theise ND. Ductular reactions in human liver: diversity at the interface. Hepatology (Baltimore, Md 2011 Nov;54(5):1853-1863.

3. Lazaridis KN, Strazzabosco M, Larusso NF. The cholangiopathies: disorders of biliary epithelia. Gastroenterology 2004 Nov;127(5):1565-1577.

4. Roskams TA, Libbrecht L, Desmet VJ. Progenitor cells in diseased human liver. Seminars in liver disease 2003 Nov;23(4):385-396.

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

6. Roskams TA, Theise ND, Balabaud C, Bhagat G, Bhathal PS, Bioulac-Sage P, et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology (Baltimore, Md 2004 Jun;39(6):1739-1745.

7. Crosby HA, Kelly DA, Strain AJ. Human hepatic stem-like cells isolated using c-kit or CD34 can differentiate into biliary epithelium. Gastroenterology 2001 Feb;120(2):534-544.

8. Crosby HA, Nijjar SS, de Goyet Jde V, Kelly DA, Strain AJ. Progenitor cells of the biliary epithelial cell lineage. Seminars in cell & developmental biology 2002 Dec;13(6):397-403.

9. Gaudio E, Carpino G, Cardinale V, Franchitto A, Onori P, Alvaro D. New insights into liver stem cells. Dig Liver Dis 2009 Jul;41(7):455-462.

10. Desmet V, Roskams T, Van Eyken P. Ductular reaction in the liver. Pathology, research and practice 1995 Jul;191(6):513-524.

11. Fabris L, Strazzabosco M. Epithelial-mesenchymal interactions in biliary diseases. Seminars in liver disease 2011 Feb;31(1):11-32.

12. Morell CM, Fabris L, Strazzabosco M. Vascular biology of the biliary epithelium. Journal of gastroenterology and hepatology 2013 Aug;28 Suppl 1:26-32.

13. Williams MJ, Clouston AD, Forbes SJ. Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion. Gastroenterology 2014 Feb;146(2):349-356.

14. Desmet VJ. Ductal plates in hepatic ductular reactions. Hypothesis and implications. III. Implications for liver pathology. Virchows Arch 2011 Mar;458(3):271-279.

15. Strazzabosco M, Fabris L. Development of the bile ducts: essentials for the clinical hepatologist. Journal of hepatology 2012 May;56(5):1159-1170.

16. Clouston AD, Powell EE, Walsh MJ, Richardson MM, Demetris AJ, Jonsson JR. Fibrosis correlates with a ductular reaction in hepatitis C: roles of impaired replication, progenitor cells and steatosis. Hepatology (Baltimore, Md 2005 Apr;41(4):809-818.

17. Michalopoulos GK. Liver regeneration: alternative epithelial pathways. The international journal of biochemistry & cell biology 2011 Feb;43(2):173-179.

18. Michalopoulos GK, Barua L, Bowen WC. Transdifferentiation of rat hepatocytes into biliary cells after bile duct ligation and toxic biliary injury. Hepatology (Baltimore, Md 2005 Mar;41(3):535-544.

19. Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, et al. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology (Baltimore, Md 2000 Jan;31(1):235-240.

20. Fausto N. Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology (Baltimore, Md 2004 Jun;39(6):1477-1487.

21. Luedde T, Kaplowitz N, Schwabe RF. Cell death and cell death responses in liver disease: mechanisms and clinical relevance. Gastroenterology 2014 Oct;147(4):765-783 e764.

22. Pinzani M, Rombouts K. Liver fibrosis: from the bench to clinical targets. Dig Liver Dis 2004 Apr;36(4):231-242.

23. Theise ND, Saxena R, Portmann BC, Thung SN, Yee H, Chiriboga L, et al. The canals of Hering and hepatic stem cells in humans. Hepatology (Baltimore, Md 1999 Dec;30(6):1425-1433.

24. Svegliati-Baroni G, Faraci G, Fabris L, Saccomanno S, Cadamuro M, Pierantonelli I, et al. Insulin resistance and necroinflammation drives ductular reaction and epithelial-mesenchymal transition in chronic hepatitis C. Gut 2011 Jan;60(1):108-115.

25. Gadd VL, Skoien R, Powell EE, Fagan KJ, Winterford C, Horsfall L, et al. The portal inflammatory infiltrate and ductular reaction in human nonalcoholic fatty liver disease. Hepatology (Baltimore, Md 2014 Apr;59(4):1393-1405.

26. Richardson MM, Jonsson JR, Powell EE, Brunt EM, Neuschwander-Tetri BA, Bhathal PS, et al. Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction. Gastroenterology 2007 Jul;133(1):80-90.

27. Bataller R, Rombouts K, Altamirano J, Marra F. Fibrosis in alcoholic and nonalcoholic steatohepatitis. Best practice & research 2011 Apr;25(2):231-244.

28. Brunt EM. Pathology of nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 2010 Apr;7(4):195-203.

29. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. Science (New York, NY 2011 Jun 24;332(6037):1519-1523.

30. Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. Seminars in liver disease 2008 Nov;28(4):339-350.

31. Schuppan D, Schattenberg JM. Non-alcoholic steatohepatitis: pathogenesis and novel therapeutic approaches. Journal of gastroenterology and hepatology 2013 Aug;28 Suppl 1:68-76.

32. Brunt EM, Tiniakos DG. Histopathology of nonalcoholic fatty liver disease. World J Gastroenterol 2010 Nov 14;16(42):5286-5296.

33. Brunt EM, Kleiner DE, Wilson LA, Unalp A, Behling CE, Lavine JE, et al. Portal chronic inflammation in nonalcoholic fatty liver disease (NAFLD): a histologic marker of advanced NAFLD-Clinicopathologic correlations from the nonalcoholic steatohepatitis clinical research network. Hepatology (Baltimore, Md 2009 Mar;49(3):809-820.

34. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998 Apr;114(4):842-845.

35. Larter CZ, Yeh MM, Van Rooyen DM, Teoh NC, Brooling J, Hou JY, et al. Roles of adipose restriction and metabolic factors in progression of steatosis to steatohepatitis in obese, diabetic mice. Journal of gastroenterology and hepatology 2009 Oct;24(10):1658-1668.

36. Malhi H, Gores GJ. Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. Seminars in liver disease 2008 Nov;28(4):360-369.

37. Nobili V, Carpino G, Alisi A, Franchitto A, Alpini G, De Vito R, et al. Hepatic progenitor cells activation, fibrosis, and adipokines production in pediatric nonalcoholic fatty liver disease. Hepatology (Baltimore, Md 2012 Dec;56(6):2142-2153.

38. Roskams T, Yang SQ, Koteish A, Durnez A, DeVos R, Huang X, et al. Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. The American journal of pathology 2003 Oct;163(4):1301-1311.

39. Bray SJ. Notch signalling: a simple pathway becomes complex. Nature reviews 2006 Sep;7(9):678-689.

40. Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. Journal of cell science 2013 May 15;126(Pt 10):2135-2140.

41. Fortini ME. Notch signaling: the core pathway and its posttranslational regulation. Developmental cell 2009 May;16(5):633-647.

42. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 2009 Apr 17;137(2):216-233.

43. Coffinier C, Gresh L, Fiette L, Tronche F, Schutz G, Babinet C, et al. Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development (Cambridge, England) 2002 Apr;129(8):1829-1838.

44. Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 2004 Dec;127(6):1775-1786.

45. Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, et al. Notch signaling controls liver development by regulating biliary differentiation. Development (Cambridge, England) 2009 May;136(10):1727-1739.

46. McGill MA, McGlade CJ. Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. The Journal of biological chemistry 2003 Jun 20;278(25):23196-23203.

47. Andersson ER, Sandberg R, Lendahl U. Notch signaling: simplicity in design, versatility in function. Development (Cambridge, England) 2011 Sep;138(17):3593-3612.

48. Geisler F, Strazzabosco M. Emerging roles of Notch signaling in liver disease. Hepatology (Baltimore, Md 2014 Jun 16.

49. Gridley T. Notch signaling and inherited disease syndromes. Human molecular genetics 2003 Apr 1;12 Spec No 1:R9-13.

50. Louvi A, Artavanis-Tsakonas S. Notch and disease: a growing field. Seminars in cell & developmental biology 2012 Jun;23(4):473-480.

51. Alagille D, Estrada A, Hadchouel M, Gautier M, Odievre M, Dommergues JP. Syndromic paucity of interlobular bile ducts (Alagille syndrome or arteriohepatic dysplasia): review of 80 cases. The Journal of pediatrics 1987 Feb;110(2):195-200.

52. Bulman MP, Kusumi K, Frayling TM, McKeown C, Garrett C, Lander ES, et al. Mutations in the human delta homologue, DLL3, cause axial skeletal defects in spondylocostal dysostosis. Nature genetics 2000 Apr;24(4):438-441.

53. Turnpenny PD, Whittock N, Duncan J, Dunwoodie S, Kusumi K, Ellard S. Novel mutations in DLL3, a somitogenesis gene encoding a ligand for the Notch signalling pathway, cause a consistent pattern of abnormal vertebral segmentation in spondylocostal dysostosis. Journal of medical genetics 2003 May;40(5):333-339.

54. Joutel A, Corpechot C, Ducros A, Vahedi K, Chabriat H, Mouton P, et al. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature 1996 Oct 24;383(6602):707-710.

55. Cheney WD. Acro-Osteolysis. The American journal of roentgenology, radium therapy, and nuclear medicine 1965 Jul;94:595-607.

56. Hajdu N, Kauntze R. Cranio-skeletal dysplasia. The British journal of radiology 1948 Jan;21(241):42-48.

57. Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, et al. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nature genetics 1997 Jul;16(3):243-251.

58. Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, et al. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nature genetics 1997 Jul;16(3):235-242.

59. McDaniell R, Warthen DM, Sanchez-Lara PA, Pai A, Krantz ID, Piccoli DA, et al. NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. American journal of human genetics 2006 Jul;79(1):169-173.

60. Krantz ID, Piccoli DA, Spinner NB. Clinical and molecular genetics of Alagille syndrome. Current opinion in pediatrics 1999 Dec;11(6):558-564.

61. Lykavieris P, Hadchouel M, Chardot C, Bernard O. Outcome of liver disease in children with Alagille syndrome: a study of 163 patients. Gut 2001 Sep;49(3):431-435.

62. Colombo C, Okolicsanyi L, Strazzabosco M. Advances in familial and congenital cholestatic diseases. Clinical and diagnostic implications. Dig Liver Dis 2000 Mar;32(2):152-159.

63. Lemaigre FP. Development of the biliary tract. Mechanisms of development 2003 Jan;120(1):81-87.

64. Lemaigre FP. Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. Gastroenterology 2009 Jul;137(1):62-79.

65. Raynaud P, Carpentier R, Antoniou A, Lemaigre FP. Biliary differentiation and bile duct morphogenesis in development and disease. The international journal of biochemistry & cell biology 2011 Feb;43(2):245-256.

66. Roskams T, Desmet V. Embryology of extra- and intrahepatic bile ducts, the ductal plate. Anat Rec (Hoboken) 2008 Jun;291(6):628-635.

67. Nishikawa Y, Doi Y, Watanabe H, Tokairin T, Omori Y, Su M, et al. Transdifferentiation of mature rat hepatocytes into bile duct-like cells in vitro. The American journal of pathology 2005 Apr;166(4):1077-1088.

68. Tanimizu N, Miyajima A. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. Journal of cell science 2004 Jul 1;117(Pt 15):3165-3174.

69. Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger BZ, et al. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 2009 Jun;136(7):2325-2333.

70. Harper JA, Yuan JS, Tan JB, Visan I, Guidos CJ. Notch signaling in development and disease. Clinical genetics 2003 Dec;64(6):461-472.

71. Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes & development 2004 Oct 15;18(20):2469-2473.

72. Krebs LT, Xue Y, Norton CR, Sundberg JP, Beatus P, Lendahl U, et al. Characterization of Notch3-deficient mice: normal embryonic

development and absence of genetic interactions with a Notch1 mutation. Genesis 2003 Nov;37(3):139-143.

73. Geisler F, Nagl F, Mazur PK, Lee M, Zimber-Strobl U, Strobl LJ, et al. Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology (Baltimore, Md 2008 Aug;48(2):607-616.

74. Jeliazkova P, Jors S, Lee M, Zimber-Strobl U, Ferrer J, Schmid RM, et al. Canonical Notch2 signaling determines biliary cell fates of embryonic hepatoblasts and adult hepatocytes independent of Hes1. Hepatology (Baltimore, Md 2013 Jan 12.

75. Sparks EE, Huppert KA, Brown MA, Washington MK, Huppert SS. Notch signaling regulates formation of the three-dimensional architecture of intrahepatic bile ducts in mice. Hepatology (Baltimore, Md 2010 Apr;51(4):1391-1400.

76. Zong Y, Stanger BZ. Molecular mechanisms of liver and bile duct development. Wiley interdisciplinary reviews 2012 Sep-Oct;1(5):643-655.

77. Hofmann JJ, Zovein AC, Koh H, Radtke F, Weinmaster G, Iruela-Arispe ML. Jagged1 in the portal vein mesenchyme regulates intrahepatic bile duct development: insights into Alagille syndrome. Development (Cambridge, England) 2011 Dec;137(23):4061-4072.

78. Lozier J, McCright B, Gridley T. Notch signaling regulates bile duct morphogenesis in mice. PloS one 2008;3(3):e1851.

79. McCright B, Lozier J, Gridley T. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. Development (Cambridge, England) 2002 Feb;129(4):1075-1082.

80. Loomes KM, Russo P, Ryan M, Nelson A, Underkoffler L, Glover C, et al. Bile duct proliferation in liver-specific Jag1 conditional knockout mice: effects of gene dosage. Hepatology (Baltimore, Md 2007 Feb;45(2):323-330.

81. Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, et al. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nature medicine 2012 Apr;18(4):572-579.

82. Fiorotto R, Raizner A, Morell CM, Torsello B, Scirpo R, Fabris L, et al. Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. Journal of hepatology 2013 Jul;59(1):124-130.

83. Spee B, Carpino G, Schotanus BA, Katoonizadeh A, Vander Borght S, Gaudio E, et al. Characterisation of the liver progenitor cell niche in liver diseases: potential involvement of Wnt and Notch signalling. Gut 2010 Feb;59(2):247-257.

84. Nijjar SS, Crosby HA, Wallace L, Hubscher SG, Strain AJ. Notch receptor expression in adult human liver: a possible role in bile duct formation and hepatic neovascularization. Hepatology (Baltimore, Md 2001 Dec;34(6):1184-1192.

85. Chen Y, Zheng S, Qi D, Zheng S, Guo J, Zhang S, et al. Inhibition of Notch signaling by a gamma-secretase inhibitor attenuates hepatic fibrosis in rats. PloS one 2012;7(10):e46512.

86. Sawitza I, Kordes C, Reister S, Haussinger D. The niche of stellate cells within rat liver. Hepatology (Baltimore, Md 2009 Nov;50(5):1617-1624.

87. Xie G, Karaca G, Swiderska-Syn M, Michelotti GA, Kruger L, Chen Y, et al. Cross-talk between Notch and Hedgehog regulates hepatic stellate cell fate in mice. Hepatology (Baltimore, Md 2013 May 23.

88. Nijjar SS, Wallace L, Crosby HA, Hubscher SG, Strain AJ. Altered Notch ligand expression in human liver disease: further evidence for a role of the Notch signaling pathway in hepatic neovascularization and biliary ductular defects. The American journal of pathology 2002 May;160(5):1695-1703.

89. Fabris L, Cadamuro M, Guido M, Spirli C, Fiorotto R, Colledan M, et al. Analysis of liver repair mechanisms in Alagille syndrome and biliary atresia reveals a role for notch signaling. The American journal of pathology 2007 Aug;171(2):641-653.

90. Tsai S, Gurakar A, Anders R, Lam-Himlin D, Boitnott J, Pawlik TM. Management of large hepatocellular carcinoma in adult patients with Alagille syndrome: a case report and review of literature. Digestive diseases and sciences 2010 Nov;55(11):3052-3058.

91. Van Hul N, Lanthier N, Espanol Suner R, Abarca Quinones J, van Rooijen N, Leclercq I. Kupffer cells influence parenchymal invasion and phenotypic orientation, but not the proliferation, of liver progenitor cells in a murine model of liver injury. The American journal of pathology 2011 Oct;179(4):1839-1850.

92. Huang M, Chang A, Choi M, Zhou D, Anania FA, Shin CH. Antagonistic interaction between Wnt and Notch activity modulates the regenerative capacity of a zebrafish fibrotic liver model. Hepatology (Baltimore, Md 2014 Nov;60(5):1753-1766.

93. Strazzabosco M, Fabris L. The balance between Notch/Wnt signaling regulates progenitor cells' commitment during liver repair: mystery solved? Journal of hepatology 2013 Jan;58(1):181-183.

94. Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, et al. Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. Nature genetics 2011 Jan;43(1):34-41.

95. Espanol-Suner R, Carpentier R, Van Hul N, Legry V, Achouri Y, Cordi S, et al. Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. Gastroenterology 2012 Dec;143(6):1564-1575 e1567.

96. He J, Lu H, Zou Q, Luo L. Regeneration of liver after extreme hepatocyte loss occurs mainly via biliary transdifferentiation in zebrafish. Gastroenterology 2014 Mar;146(3):789-800 e788.

97. Tarlow BD, Finegold MJ, Grompe M. Clonal tracing of Sox9+ liver progenitors in mouse oval cell injury. Hepatology (Baltimore, Md 2014 Jul;60(1):278-289.

98. Carpentier R, Suner RE, van Hul N, Kopp JL, Beaudry JB, Cordi S, et al. Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. Gastroenterology 2011 Oct;141(4):1432-1438, 1438 e1431-1434.

99. Malato Y, Naqvi S, Schurmann N, Ng R, Wang B, Zape J, et al. Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. The Journal of clinical investigation 2011 Dec;121(12):4850-4860.

100. Rodrigo-Torres D, Affo S, Coll M, Morales-Ibanez O, Millan C, Blaya D, et al. The biliary epithelium gives rise to liver progenitor cells. Hepatology (Baltimore, Md 2014 Oct;60(4):1367-1377.

101. Schaub JR, Malato Y, Gormond C, Willenbring H. Evidence against a stem cell origin of new hepatocytes in a common mouse model of chronic liver injury. Cell reports 2014 Aug 21;8(4):933-939.

102. Yanger K, Knigin D, Zong Y, Maggs L, Gu G, Akiyama H, et al. Adult hepatocytes are generated by self-duplication rather than stem cell differentiation. Cell stem cell 2014 Sep 4;15(3):340-349.

103. Sekiya S, Suzuki A. Hepatocytes, rather than cholangiocytes, can be the major source of primitive ductules in the chronically injured mouse liver. The American journal of pathology 2014 May;184(5):1468-1478.

104. Tanimizu N, Nishikawa Y, Ichinohe N, Akiyama H, Mitaka T. Sry HMG box protein 9-positive (Sox9+) epithelial cell adhesion molecule-negative (EpCAM-) biphenotypic cells derived from hepatocytes are involved in mouse liver regeneration. The Journal of biological chemistry 2014 Mar 14;289(11):7589-7598.

105. Yanger K, Zong Y, Maggs LR, Shapira SN, Maddipati R, Aiello NM, et al. Robust cellular reprogramming occurs spontaneously during liver regeneration. Genes & development 2013 Apr 1;27(7):719-724.

106. Alvaro D, Bragazzi MC, Benedetti A, Fabris L, Fava G, Invernizzi P, et al. Cholangiocarcinoma in Italy: A national survey on clinical characteristics, diagnostic modalities and treatment. Results from the "Cholangiocarcinoma" committee of the Italian Association for the Study of Liver disease. Dig Liver Dis 2011 Jan;43(1):60-65.

107. Palmer WC, Patel T. Are common factors involved in the pathogenesis of primary liver cancers? A meta-analysis of risk factors for intrahepatic cholangiocarcinoma. Journal of hepatology 2012 Jul;57(1):69-76.

108. Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nature medicine 2006 Apr;12(4):410-416.

109. Strazzabosco M, Fabris L. Notch signaling in hepatocellular carcinoma: guilty in association! Gastroenterology 2012 Dec;143(6):1430-1434.

110. Komuta M, Govaere O, Vandecaveye V, Akiba J, Van Steenbergen W, Verslype C, et al. Histological diversity in cholangiocellular carcinoma reflects the different cholangiocyte phenotypes. Hepatology (Baltimore, Md 2012 Jun;55(6):1876-1888.

111. Qi R, An H, Yu Y, Zhang M, Liu S, Xu H, et al. Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. Cancer research 2003 Dec 1;63(23):8323-8329.

112. Viatour P, Ehmer U, Saddic LA, Dorrell C, Andersen JB, Lin C, et al. Notch signaling inhibits hepatocellular carcinoma following inactivation of the RB pathway. The Journal of experimental medicine 2011 Sep 26;208(10):1963-1976.

113. Giovannini C, Gramantieri L, Chieco P, Minguzzi M, Lago F, Pianetti S, et al. Selective ablation of Notch3 in HCC enhances doxorubicin's death promoting effect by a p53 dependent mechanism. Journal of hepatology 2009 May;50(5):969-979.

114. Lim SO, Park YM, Kim HS, Quan X, Yoo JE, Park YN, et al. Notch1 differentially regulates oncogenesis by wildtype p53 overexpression and p53 mutation in grade III hepatocellular carcinoma. Hepatology (Baltimore, Md 2011 Apr;53(4):1352-1362.

115. Liu M, Lee DF, Chen CT, Yen CJ, Li LY, Lee HJ, et al. IKKalpha activation of NOTCH links tumorigenesis via FOXA2 suppression. Molecular cell 2012 Jan 27;45(2):171-184.

116. Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 1991 Aug 23;66(4):649-661.

117. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science (New York, NY 2004 Oct 8;306(5694):269-271.

118. Ranganathan P, Weaver KL, Capobianco AJ. Notch signalling in solid tumours: a little bit of everything but not all the time. Nature reviews 2011 May;11(5):338-351.

119. Huntzicker EG, Hotzel K, Choy L, Che L, Ross J, Pau G, et al. Differential effects of targeting Notch receptors in a mouse model of liver cancer. Hepatology (Baltimore, Md 2014 Oct 13.

120. Villanueva A, Alsinet C, Yanger K, Hoshida Y, Zong Y, Toffanin S, et al. Notch signaling is activated in human hepatocellular carcinoma and induces tumor formation in mice. Gastroenterology 2012 Dec;143(6):1660-1669 e1667.

121. Guest RV, Boulter L, Kendall TJ, Minnis-Lyons SE, Walker R, Wigmore SJ, et al. Cell lineage tracing reveals a biliary origin of intrahepatic cholangiocarcinoma. Cancer research 2013 Feb 15;74(4):1005-1010.

122. Zender S, Nickeleit I, Wuestefeld T, Sorensen I, Dauch D, Bozko P, et al. A critical role for notch signaling in the formation of cholangiocellular carcinomas. Cancer cell 2013 Jun 10;23(6):784-795.

123. Dill MT, Tornillo L, Fritzius T, Terracciano L, Semela D, Bettler B, et al. Constitutive Notch2 signaling induces hepatic tumors in mice. Hepatology (Baltimore, Md 2012 Nov 23.

124. Fan B, Malato Y, Calvisi DF, Naqvi S, Razumilava N, Ribback S, et al. Cholangiocarcinomas can originate from hepatocytes in mice. The Journal of clinical investigation 2012 Aug 1;122(8):2911-2915.

125. Sekiya S, Suzuki A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. The Journal of clinical investigation 2012 Nov 1;122(11):3914-3918.

Chapter 2

Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice

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ABSTRACT

BACKGROUND and AIMS: Repair from biliary damages requires the biliary specification of hepatic progenitor cells and the remodeling of ductular reactive structures into branching biliary tubules. We hypothesized that the morphogenetic role of Notch signaling is maintained during the repair process and have addressed this hypothesis using pharmacologic and genetic models of defective Notch signaling. ***METHODS:*** Treatment with DDC (3,5-diethoxycarbonyl1,4-dihydrocollidine) or ANIT (alpha-naphthyl-isothiocyanate) was used to induce biliary damage in wild-type mice and in mice with a liver-specific defect in the Notch-2 receptor (Notch-2-cko) or in RPB-Jk. Hepatic progenitor cells, ductular reaction and mature ductules were quantified using K19 and SOX-9. ***RESULTS:*** In DDC-treated wild-type mice, pharmacologic Notch inhibition with dibenzazepine decreased the number of both ductular reaction and hepatic progenitor cells. Notch-2-cko mice treated with DDC or ANIT accumulated hepatic progenitor cells that failed to progress into mature ducts. In RBP-Jκ-cko mice, mature ducts and hepatic progenitor cells were both significantly reduced with respect to similarly treated wild-type mice. The mouse progenitor cell line BMOL cultured on Matrigel, formed a tubular network allowing the study of tubule formation *in vitro;* γ-secretase inhibitor treatment and siRNAs silencing of Notch-1, Notch2 or Jagged1 significantly reduced both the length and the number of tubular branches. ***CONCLUSIONS*****:** These data demonstrate that Notch signaling plays an essential role in biliary repair**.** Lack of Notch-2 prevents biliary tubule formation, both *in vivo* and *in vitro.* Lack of RBP-Jk inhibits the generation of biliary-committed precursors and tubule formation.

Abbreviations: **DDC**, 3,5-diethoxycarbonyl1,4-dihydrocollidine; **ANIT**, alpha-naphthyl-isothiocyanate; **RPB-Jk**, recombination signal binding protein for immunoglobulin

kappa J region; **K19**, cytokeratin 19; **SOX-9**, SRY (sex determining region Y)-box 9; **BMOL**, bipotential mouse oval cell line; **AGS**, Alagille syndrome; **HPC**, hepatic progenitor cell; **RDC**, reactive ductular cell; **DBZ**, dibenzazepine; **GSI**, γ-secretase inhibitor; **DR**, ductular reaction; **Alb**, albumin; **Cyp**, cytochrome P450; **DAPT**, LY-374973 N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; **P**, postnatal day; **ALT**, alanine transaminase; **NICD**, Notch intracellular domain; **VEGF**, vascular endothelial growth factor; **+ve**, positive.

INTRODUCTION

Liver disease is the result of the interaction between ongoing liver cell damage and reparative mechanisms. Liver repair in the context of biliary diseases requires the replacement of lost or damaged cells, generation of new branching tubules and production of a fibrovascular stroma that sustains the new tissue. When these highly integrated processes fail, liver repair becomes “pathologic” and results in architectural distortion and deposition of fibrous tissue into the portal spaces.

The hallmark of ongoing biliary repair is the presence of a “ductular reaction”. “Reactive cholangiocytes”, i.e. the epithelial component of the ductular reaction, are organized in clusters that do not encircle a lumen. However, they are in contiguity with the biliary tree and extensively participate in tissue remodeling, ultimately reorganizing into tubular structures. The consequent increase in “ductal mass” is an important adaptive mechanism in cholestasis that prevents the development of extensive parenchymal necrosis.

Notch signaling, a strongly conserved developmental pathway involved in cell fate determination and stem cell biology, plays an important role in biliary development when Notch receptors expressed on ductal plate cells are stimulated by ligands expressed by the periportal mesenchyme. Mutations in the Notch signaling factors Jagged-1 or Notch-2 cause Alagille Syndrome (AGS), a cholangiopathy characterized by paucity of intrahepatic bile ducts, severe cholestasis, and extrahepatic manifestations. We have

documented that patients with AGS present a distinct pattern of liver repair responses. Recent studies indicate that Notch signaling is activated in hepatic progenitors cells (HPCs) in human cholangiopathies. Furthermore, by activating Numb, Wnt/ß-catenin signaling inhibits the biliary specification of HPCs in favor of their hepatocytic specification. In contrast to other morphogenetic pathways, like Wnt and Hedgehog, Notch signals through direct cell-cell interaction.

These observations, suggest that Notch may be involved in several steps of biliary repair, in adult life. We have used genetic models of Notch loss of function (Notch-2 and RBP-Jκ liver-conditional knock-out mice) to study the role of Notch signaling in the regulation of ductular morphogenesis during liver repair from biliary injuries.

MATERIALS AND METHODS

For reagents, immunohistochemistry, Western blotting, *see supplementary material and methods.*

Computer-assisted morphometry. K19 and SOX9 antibodies were used to quantify the ductular reaction. Reactive ductular cells are defined as K19 or SOX9 positive cells with a biliary phenotype arranged in irregularly shaped structures without a well-formed lumen, and HPCs are defined as small, oval or spindle-shaped cells positive for K19 or SOX9 with scant cytoplasm and oval nuclei, alone or in small clusters, localized in the parenchyma or at the portal interface *(see supplementary material and methods).*

Cell Culture. Bipotential Mouse Oval Cell Line (BMOL) was kindly provided by Dr. Yeoh, University of Western Australia *(see supplementary material and methods).*

Silencing of Notch-1, **Notch-2 and Jagged-1**. Gene silencing was performed using commercially available siRNAs against Notch-1, Notch-2 and Jagged-1 (Santa Cruz Biotechnology, Inc). Scramble RNAs were used to control for non-specific silencing effects. BMOL cells were transfected using the Lipofectamine 2000^{TM} transfection reagent (Invitrogen) according to the manufacturer's protocol (*see supplementary material and methods).*

Animals and experimental protocol. All experiments were performed according to protocols approved by the Yale University Institutional Animal Care and Use Committee. To produce a liver-specific deletion of Notch-2, mice Notch2$^{flox/flox}$ were crossed with mice doubly heterozygous for the Alb1-Cre transgene and the Notch2^{del2} alleles on a C57BL/6J background (all from Dr. T. Gridley, Jackson Laboratory). Offspring with the genotypes Alb1-Cre/+; Notch2$^{del2/}$ Notch2^{flox} are referred to as Notch-2-cko. Liver specific deletion of RBP-Jκ, was obtained by crossing heterozygous Alb1-Cre*; RBP-JK^{flox}/+ mice* on a CD-1 outbred background (a kind gift from Dr. T. Honjo, Kyoto University and S. Huppert, Vanderbilt University. Offspring with the genotypes Alb1-Cre;*RBP-JK$^{flox/flox}$* are referred as RBP-Jκ-cko. Experimental and wild type mice were exposed to 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) at P30 or P75 (when indicated) and alpha-naphtylisothiocyanate (ANIT) at P30 to induce biliary damage. DDC treatment, used to mimic intrahepatic obstructive cholestasis, was added to the diet at a concentration of 0.1% (BioServ, Inc. Frenchtown, NJ) for 10 days. ANIT treatment, used to induce biliary injury characterized by hyperplasia of the terminal branches of the biliary tree, was administered to the mice in a high dose (80mg/Kg) by i.p followed by a bi-weekly maintainance dose (50 mg/Kg) for 4 weeks. Dibenzazepine (DBZ) (Syncom BV), a γ-secretase inhibitor (GSI), was used to pharmacologic inhibit Notch

signaling *in vivo* at the time of biliary damage induction. DBZ was administered to wild-type mice daily for 10 days via i.p injection at a concentration of 5µmol/Kg, in combination with or without DDC treatment. At the end of each treatment the mice were sacrificed, liver tissues were explanted and the two large lobes were fixed with formalin and embedded in paraffin. The small lobes were snap frozen in liquid nitrogen.

Tubule formation *in vitro assay.* BMOL cells were plated on a thick layer of matrigel at the density of 50.000/cm^2 in growth medium. In this condition, 3 hours after plating, the cells begin to organize a network. Twenty-four hours later this network forms visible interconnecting, mesh-like structures. A second layer of matrigel applied to the 24-hour culture promotes the formation of complete tubular structures. Confocal microscopy analysis was used to assess the 3D-tubular structure, after staining with Cellmask^{TM} Orange. Images were obtained using a Zeiss LSM 510 confocal microscope using a ×63, 1.4 NA-objective lens with excitation at 555 nm and emission at 567 nm. Serial optical sections were collected for 3D-reconstruction. To inhibit Notch signaling, BMOL cells were treated with InSolution^{TM} γ-Secretase Inhibitor IX (DAPT, 10µM) (Calbiochem) at the time of plating on matrigel. The length of tubular structures and number of branches were measured using ImageJ software in 5 random, non-overlapping fields in each condition. The images were captured with a contrast-phase microscope Olympus CK40 (Micro-Tech Optical (NE), Inc., Bloomfield, CT, USA) connected to a camera (Q-color 5 RTV, Qimaging, Canada).

Statistical analysis. Results are shown as mean ± SD. Statistical comparisons were made using one-way analysis of variance or the Wilcoxon–Mann–Whitney 2-sample rank sum test, where appropriate. In the latter, the *P* value was obtained from the exact permutation null

distribution. The statistical analysis was performed using SAS software (SAS Institute Inc, Cary, NC). *P* values <.05 were considered significant.

RESULTS

Pharmacologic inhibition of Notch signaling reduces ductular reaction and HPC in mice exposed to DDC. *See supplementary results.* To investigate whether Notch signaling participates in biliary repair, we induced biliary damage in WT mice by administering DDC in the presence or the absence of DBZ, to inhibit Notch signaling. As shown in suppl-figure 1, DBZ significantly inhibited the increase in HPCs and ductular reaction (DR) induced by DDC. These are consistent with a role of Notch in biliary specification of HPCs, as suggested by Fabris and Boulter. However, because of the non-specific effects of DBZ, the demonstration of the role of Notch in biliary repair requires the use of genetic models.

Liver repair is altered in ANIT and DDC treated mice with conditional deletion of Notch-2 or RBP-Jk. Among the four Notch receptors, Notch-1 and 2 are expressed to significant extent in cholangiocytes and HPC (see also suppl-figure 2). We studied two liver-specific conditional knockout mice (Notch-2-cKO and RBP-Jκ-cKO) in which deletion of Notch-2 or RBP-Jk, respectively, is under the control of the albumin promoter. Alb-Cre mediated recombination is specific for hepatoblasts, biliary cells and hepatocytes . RBP-Jk is an essential component of Notch signalling; thus, in RBP-Jk-cKO mice both Notch1 and Notch2 signaling are defective; The phenotype of both mice has been previously described and results in paucity of bile ducts. We characterized the mice phenotypes by staining with K19 and SOX-9 during the first 8 postnatal weeks. The SOX-9

transcription factor is the earliest marker expressed by biliary precursors. At P30 and P60, the livers of Notch-2-cKO mice showed the same level of paucity of bile ducts at the periphery of the lobules and a significant increase in the number of K19, as well as SOX-9+ve cells (suppl-figures 3, 4 and 5) that extend out of the portal space; these cells are negative for Notch-2 (suppl-figure 6 and 7). The livers of RBP-Jκ cKO mice showed ductopenia, and areas of parenchymal necrosis and no increase in the number of K19/SOX-9+ve structures (Supplementary figures 3, 4 and 7). When analyzed at P30 we found an extensive expression of SOX-9 in hepatocellular-like cells. Interestingly, these SOX-9+ve cells were actually negative for the biliary/progenitor marker K19 and had a clear hepatocellular shape. These cells completely disappeared by P60, but the ductopenia did not improve by P75, suggesting that these cells could be hepatocytes making an attempt to regenerate the biliary tree (suppl- figures 8, 9 and 10).

We exposed Notch-defective mice to two protocols of biliary damage (ANIT or DDC) starting at P30. After treatment with ANIT or DDC the liver histology (figure 1 and supplementary figure 11) and the liver enzymes (suppl-table 1) were different between Notch-2-cKO, RBP-Jκ-cKO mice and WT littermates. In WT mice, both damage protocols stimulated a DR, with increased HPCs, dysmorphic bile ducts and mature bile ducts. In Notch-2-cKO mice, ANIT treatment caused an increase in dysmorphic K19+ve epithelial cells organized in clusters but lacking a tubular structure. After DDC treatment, the K19+ve cells were arranged around irregular dilatations, lined by irregularly shaped epithelial cells with nuclear pleyomorphism. Liver damage in RBP-Jκ-cKO mice was more severe: the near absence of ductular reaction was accompanied by the presence of areas of parenchymal necrosis. To exclude the

possible interference of a post-natal liver injury we repeated the DDC protocol in RBP-Jκ-cKO mice starting at P75 and we confirmed the absence of ductular reaction (see supll-figure 8). Albeit more severe, the histological picture of RBP-Jκ-cKO mice was similar to the one seen in livers of WT mice after DDC+DBZ treatment (in which GSI treatment blocks signals from both Notch receptors). To exclude confounding effects due to possible alterations in xenobiotic metabolism, as previously described in other knockout models, we analyzed the expression of Cyp1A1, 1A2, 2E1 and 3A1 and we found them similar among the mouse lines (data not shown). These observations are consistent with the concept that Notch signaling plays a major role in liver repair.

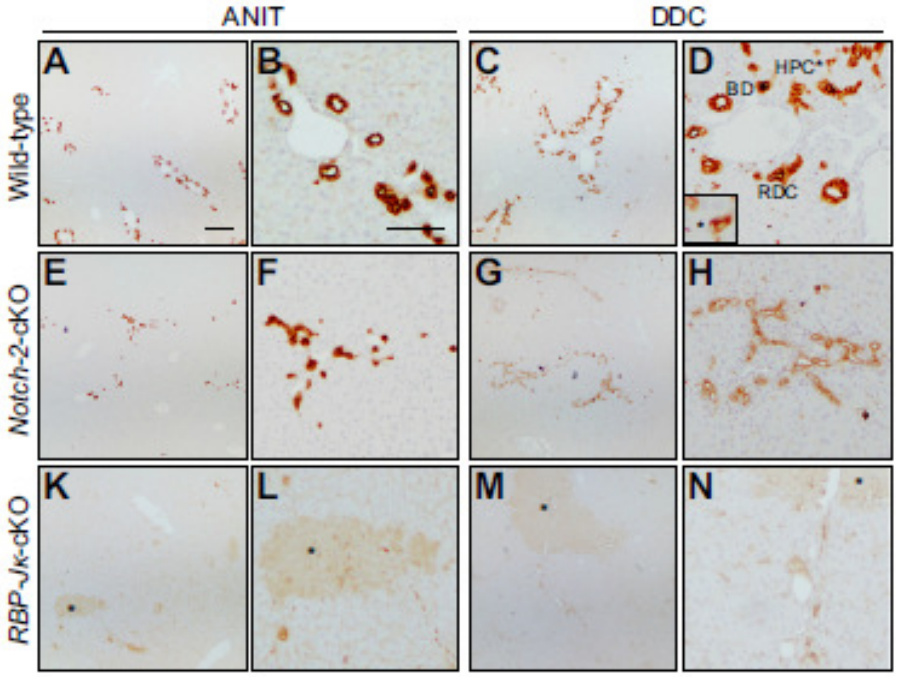

Figure 1. K19 staining in Notch-2-cKO and RBP-Jκ-cKO show a defect in liver repair after ANIT and DDC-induced damage. WT (A-D), Notch-2-cKO (E-H) and RBP-Jκ-cKO (K-N) mice were treated with ANIT and DDC at P30 (see text for details). At the end of the treatment the mice were sacrificed and liver tissues were stained with K19 antibody. After treatment with ANIT and DDC, WT mice developed the expected ductular reaction while Notch-2-cKO showed an increase in

cholangiocyte-like structures with dysmorphic epithelial cells, faintly positive for K19 and missing a tubular structure. RBP-Jκ-cKO showed no ductular reaction and presented parenchymal necrosis (*). The different components of ductular reaction are indicated in panel D: BD (bile ducts), RDC (reactive ductular cells) and HPC (hepatic progenitor cells). Scale bar is 200 µm in panels A, E, K, C, G, M and 100 µm in panels B, F, L, D, H, N.

RBP-Jk deletion, but not Notch-2 deletion affects biliary committment of HPC during biliary repair. An essential step during liver repair is the activation of HPCs. This bipotential compartment is able to differentiate into cells committed toward the hepatocellular or biliary lineage (RDCs), in order to increase the number of bile ducts at the periphery of portal spaces and rebuild the damaged biliary tree. Thus, we quantified the number of biliary progenitor cells using SOX-9 immunostaining (*supplemental material and methods*) after ANIT treatment or K19 immunostaining after DDC treatment.

The number of SOX9+ve and K19+ve HPCs at baseline was significantly higher in Notch-2-cKO but not in RBP-Jk-cKO mice compared to their WT (figure 2A-D and suppl-table 2). After ANIT treatment, the number of HPCs significantly increased in both WT mice, and more so in Notch-2-cKO mice. On the contrary, in RBP-Jκ-cKO, the number of HPCs was significantly lower than in WT treated with ANIT (figure 2B,D and suppl-table 2). Similar results were confirmed after DDC treatment by quantification of K19+ve HPCs (figure 3 A,B).

Ductular structures are decreased in Notch-2 and RBP-Jk cKO mice during liver repair. The activation of HPCs and the subsequent increase in the number of RDCs during liver repair are aimed not only at replacing damaged cells, but also at restoring the tubular structures lost during liver injury. Therefore, we quantified the number of

ductular structures (*see supplemental material and methods*) in mice treated with ANIT and DDC.

As shown in figure 2 E-F and suppl-table 3, after ANIT treatment, the number of ductular structures was significantly lower in Notch-2-cKO and RBP-Jκ-cKO mice, than in WT mice. Similar results were obtained after DDC treatment (figure 3 C, D and suppl-table 3). The increased number of HPC and the decreased number of ductular structures observed in Notch-2-cKO mice again suggests that Notch-2 is not necessary for the generation of biliary-committed cells, but rather for the ability of biliary precursor cells to organize into tubular structures. Conversely, in RBP-Jκ-cKO mice, both HPCs and ductular structures are significantly reduced, resulting in more severe impairment of biliary repair.

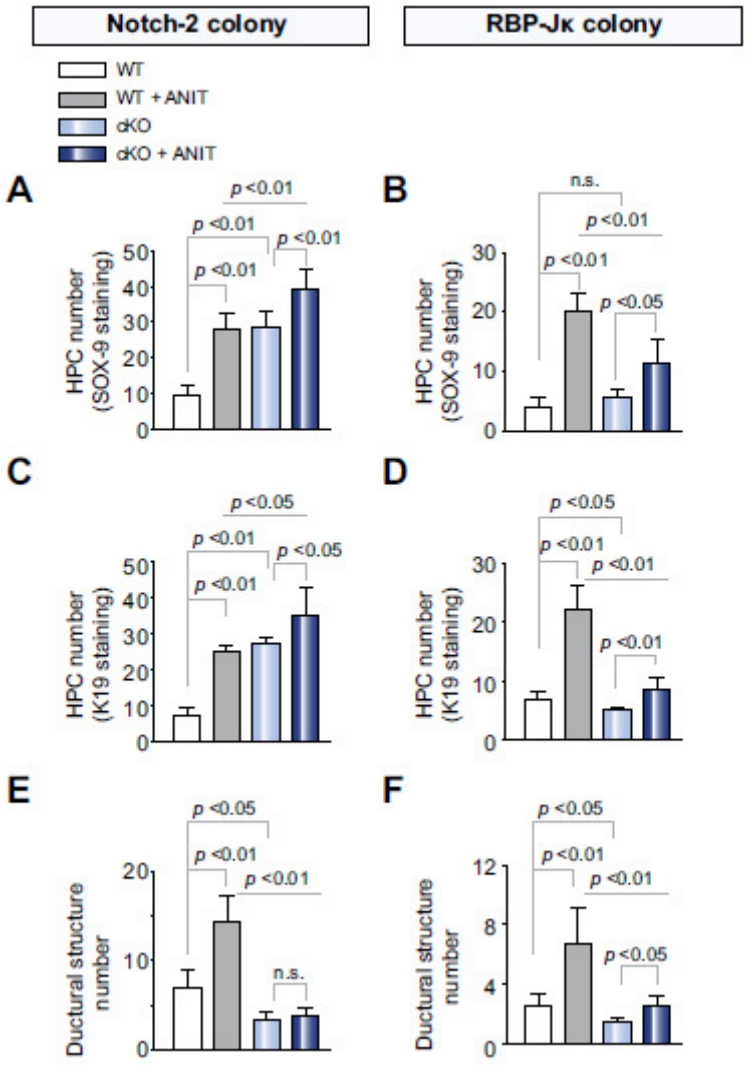

Figure 2. Biliary progenitor cells and ductular structure quantification in WT, Notch-2-cKO and RBP-Jκ-cKO mice after treatment with ANIT. Paraffin sections from Notch-2-cKO, RBP-Jκ-cKO mice and their respective WT littermates untreated or treated with ANIT were stained for SOX-9 (A-B) or K19 (C-F). See supplementary material for quantification criteria. As shown in the bar graph, (A, C) Notch-2-cKO mice showed a significant increase of HPC number (both SOX-9 or K19+ve) after ANIT damage. (B, D) Conversely, RBP-Jκ-cKO mice exhibited a significant reduction in the number of HPCs after treatment with ANIT. Variation in the baseline number of HPCs between Notch-2-WT (A) and RBP-Jκ-WT (B) are due to differences of mice strain. On the other hand, both (E) Notch-2-cKO mice and (F) RBP-Jκ-cKO mice showed a significant decrease in the number of K19+ve ductular structures after ANIT damage. Data represent average ± SD. (Data represent average ± SD, n=4-8 mice per group; p values are reported).

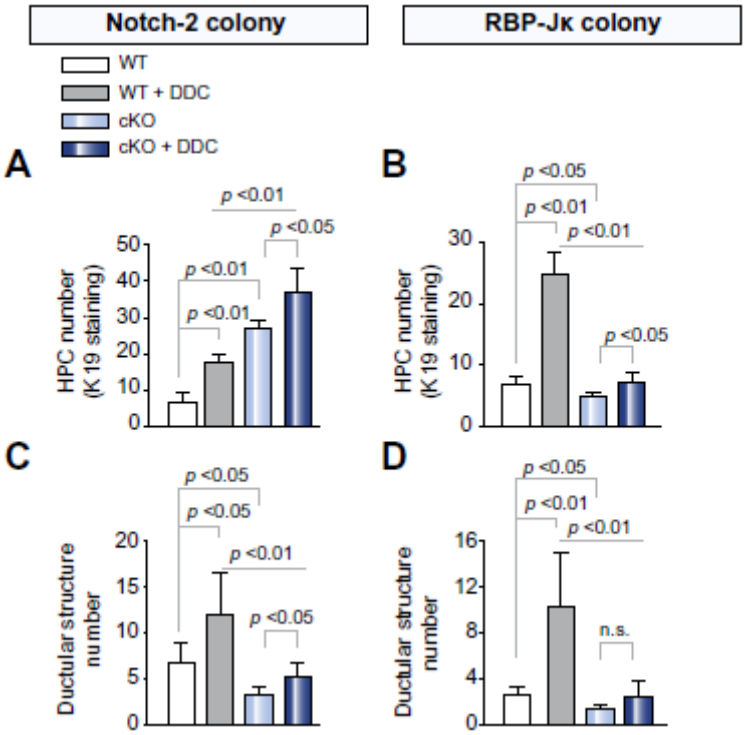

Figure 3. Biliary progenitor cells and ductular structure quantification in WT, Notch-2-cKO and RBP-Jκ-cKO mice after treatment with DDC. Paraffin sections from Notch-2-cKO, RBP-Jκ-cKO mice and their respective WT littermates untreated or treated with ANIT were stained for K19. As shown in the bar graph, (A) Notch-2-cKO mice showed a significant increase of HPC number after DDC damage. (B) Conversely, RBP-Jκ-cKO mice exhibited a significant reduction in the number of HPCs after treatment with DDC. On the other hand, both (C) Notch-2-cKO mice and (D) RBP-Jκ-cKO mice showed a significant decrease in the number of K19+ve ductular structures after DDC damage. (Data represent average ± SD, n=4-8 mice per group; p values are reported).

GSI treatment and siRNA silencing of Notch-2 and Jagged-1 inhibit tubule formation *in vitro.* The process of tubulogenesis begins during embryonic life and continues after birth until the architecture of the biliary tree is completely developed. Tubulogenesis also takes place in response to biliary injury and HPC activation. The mechanisms that regulate the initial phases of tubulogenesis during the reparative process are still unclear. To determine whether Notch plays a role in this process, we studied tubule formation *in vitro* using

a well-characterized mouse liver progenitor cell line BMOL. When plated on a thick layer of matrigel matrix BMOL cells migrated and organized into a mesh-like network of cells within 24 hours from the time of plating (figure 4A). After a second layer of Matrigel is overlayed, tubular structures are formed, as shown in figure 4B, in which the 3D confocal imaging reconstruction reveals the presence of a luminal space inside the structure.

Using this *in vitro* tubulogenesis model, we studied the effects of pharmacologic and genetic inhibition of Notch pathway factors. BMOL cells were cultured in presence of the GSI compound DAPT (10μM). The length of the tubules and the number of branches were measured 24 hours after plating using the Image-J software in 5 random, non-overlapping fields, captured with a contrast phase microscope. As shown in figure 4C, treatment with DAPT significantly decreased the length of the tubular sections between the branches.

To confirm the specific involvement of Notch signaling, we repeated the experiments using BMOL cells after gene silencing of Notch-1, Notch-2 and Jagged-1. As shown in figure 4D, tubule length and number of branches were significantly reduced 24 hours after gene silencing and this was particulary evident in the case of Notch-2 and Jagged-1 silencing. Gene expression analysis of Notch receptors (1-4) from the above experiments (see suppl-figure 2). showed no compensatory up-regulation of Notch3 and 4 in our silenced cells. These results confirm our hypothesis that inhibition of Notch signaling and specifically Notch-2 impairs the ability of progenitor cells to arrange into a tubular-like structure.

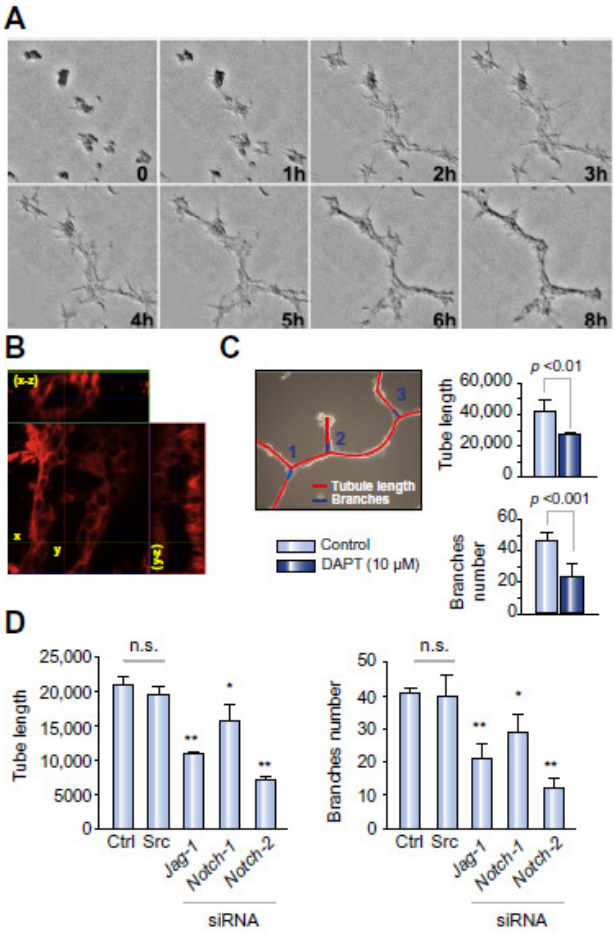

Figure 4. Inhibition of Notch signaling affects tubule formation in BMOL cultured on matrigel, a model to study biliary tubulogenesis. (A) BMOL plated on a thick layer of matrigel were imaged for 8 hours and as showed in the stack series (0-8h) they migrated to organize a network of tubule structures (8h). (B) Twenty-four hours after plating in matrigel the cell preparation was incubated with CellmaskTM Orange, a vital dye that stains cell plasma membranes and processed by confocal microscopy analysis. Serial optical sections were collected for 3D- reconstruction. The confocal microscopy analysis (see x-z projection) revealed the presence of a lumen inside the structure. (C) BMOL cells cultured on Matrigel were treated with DAPT at the time of plating. The length of tubules and the number of branches were measured 24 hours after plating in matrigel using ImageJ software in 5 random, non-overlapping fields, as described. Treatment with DAPT significantly decreased the final lenght of the tubule network and the number of branches. (D) To confirm the involvement of Notch in tubulogenesis, tubule formation was quantified in BMOL cultured on matrigel 48 hours after silencing Notch-1, Notch-2 or Jagged-1 genes. As shown in the bar graph, the tube length and number of branches were significantly reduced after silencing of Notch2 and Jagged 1, and to a lesser extent Notch1. (Average ± SD, n=3 independent experiments; *p<0.05, **p<0.01, n.s: not statistically significant).

DISCUSSION

In response to liver damage, specialized portions of the biliary tree lining the terminal cholangioles and the canals of Hering give rise to hepatic progenitor cells (HPC). These cells can eventually replace lost hepatocytes, and/or generate “ductular reactive” cells able to communicate with mesenchymal, endothelial and inflammatory cells. To successfully repair the damage to the biliary epithelium, progenitor cells and reactive cholangiocytes must have the ability to proliferate and expand the ductular mass forming branching tubular structure. Thus, “reactive” cholangiocytes, organized in tubeless structures, abandon their progenitor cell-like phenotype, characterized by motile properties and active production of growth factors and cytokines, to differentiate into cilindric ion-secreting cells arranged around tubular structures. If this process does not occur, bile may leak into the lobules causing necrosis.

Liver repair is a highly integrated process in which a number of morphogenetic pathways, including Wnt/β-catenin and Hedeghog are involved. We have previously shown that in AGS, ductular reaction is altered with reduced ability to form biliary progenitor cells. Increased expression of Notch-1, Notch-2, Jagged-1/2 and Hes-1 in reactive ductules from patients with primary biliary cirrhosis and primary sclerosing cholangitis were recently reported.

In this study we present strong evidence to support the idea that Notch signaling plays a fundamental role in the process of biliary repair. Using pharmacologic and genetic models of Notch loss of function, we showed that Notch 1 and 2 differentially regulate the biliary commitment of liver progenitor cells and the three dimensional architecture of the biliary tree.

Consistent with Boulter et al. pharmacologic inhibition of Notch in WT mice with GSI reduced the number of HPC and reactive ducts.

GSIs, are broad inhibitors of Notch signaling that prevent the cleavage of NICD from Notch receptors. Although widely used to study Notch signaling, GSI can also inhibit other proteolytic cleavage-dependent signaling pathways thus reducing the specificity of the effects. Thus, to gain further insight into the role of Notch signaling, we used two liver specific conditional KO mouse models (bearing a deletion of the Notch-2 receptor or of RBP-Jκ, a transcription factor common to all Notch receptors). Only Notch-1 and Notch-2 are expressed to a significant extent by HPCs (suppl-fig. 2) and by cholangiocytes (not shown). Thus, RBP-Jk-cKO cholangiocytes bear a defect of both Notch-1 and Notch-2, whereas in Notch-2-cKO mice, Notch1 signaling remains functional.

Consistent with previous studies, the liver phenotype of untreated conditional-KO mice shows a delay in bile duct maturation and a reduction in the number of bile ductules at the periphery of the biliary tree. Mice were viable and were not jaundiced. On the other hand, compared to wild type littermates, significant changes in biliary repair were found after treatment with DDC or with ANIT, particularly in the generation of HPC and the formation of biliary ducts.

SOX-9 is expressed by biliary-committed cells of the inner leaflet of the ductal plate cells at the duplication stage and subsequently by mature cholangiocytes and biliary-committed HPCs. In WT mice the number of SOX-9+ve biliary progenitor cells increased by 3-4 fold after ANIT damage, along with the number of bile ducts. In Notch-2-cKO mice treated with ANIT, SOX-9+ve HPC further increased. On the contrary, the number of tubular structures significantly decreased, suggesting that Notch-1 can substitute for Notch-2 in the biliary specification of HPCs but not in tubule formation. It has been reported that over-expression of Notch1-specific or Notch-2 NICD, promotes ectopic biliary differentiation in

the hepatic lobule and hyperplasia of the terminal branches of the biliary system, whereas Notch1 deletion does not alter liver development. When both Notch-2 and Notch-1 signaling are blocked, as in RBP-Jκ cKO mice, both the number of progenitor cells and the number of biliary ductules are significantly reduced. In RBP- Jκ-cKO mouse livers at post-natal day 30 (P30), we noticed an extensive expression of SOX-9 in cells with hepatocellular morphology that were negative for the biliary marker K19 and completely regresses by P60. We speculate that this phenomenon represents a transient expansion of hepatocytes making an attempt to become biliary cells, possibly under the control of other signaling pathways such as Hedgehog. We previously described a similar phenomenon in patients with Alagille Syndrome where we noticed an abundance of hepatobiliary cells positive for K7 (membranous staining), but negative for HNF-1β.

In Notch-2-cKO mice, ANIT treatment did not stimulate the expansion of ductal mass, while DDC treatment resulted in the development of irregular dilatations without a well-developed tubular structure. In RBP-Jκ-cKO mice, ductular reaction was nearly absent, wherein ANIT-induced liver damage was more severe; areas of parenchymal necrosis were present, instead of the irregular dilatations seen in Notch-2-cKO mice. Our data also show that the number of ductular structures was significantly decreased in both Notch-2-cKO and RBP-Jκ-cKO mice after damage, compared to WT injured mice. We speculate that Notch-2 is necessary to effectively form tubules. In fact, in the absence of Notch-2, the liver is still able to generate biliary progenitor cells in response to biliary injuries, but these cells cannot rebuild a tubular structure. On the other hand, in RBP-Jκ-cKO mice, lacking both Notch-1 and Notch-2 signaling, liver repair from a biliary damage is more profoundly affected and both the

ability to generate HPCs and the process of tubulogenesis are impaired.

To repair biliary damage and prevent the development of extensive liver necrosis due to bile leakage into the parenchyma progenitor cells and reactive cholangiocytes must have the ability to form new branching ductular structures. While the mechanisms of tubulogenesis taking place during development has been extensively studied, during liver repair have been neglected. Using a bipotential mouse adult liver progenitor cell line that possesses an intermediate hepato-biliary/phenotype we could model the initial phases of tubulogenesis *in vitro* (figure 4). We were able to show that pharmacologic Notch inhibition and genetic silencing of the Notch-2 receptor and Jagged-1 ligand, significantly reduced tubule length and the number of lateral branches in our system, further reinforcing the role of Notch2 signaling in promoting proper tubule formation. This *in vitro* model will be useful for future studies that will delve more deeply into different pathway components involved in biliary tubulogenesis.

In summary, our studies show that, after biliary damage, Notch-2 and RBP-JK play a fundamental role in the activation of HPC and in ductal morphogenesis (See cartoon in Figure 5). This is consistent with our earlier observation that in AGS patients, HPC are unable to differentiate along the biliary lineage and with recent reports showing stimulation/inhibition of Notch in liver progenitor cells undergoing biliary vs hepatocytic differentiation. Furthermore, our data show that proper biliary repair requires the coordinated and differential action of both Notch-1 and Notch-2. When Notch-2 is defective, biliary specification of HPC is not affected, however tubular morphogenesis is impaired both *in vivo and in vitro.* The downstream mechanisms by which Notch-2 promotes tubulogenesis are at present unknown. A cross-talk between VEGF and Notch signaling plays a

key role in vascular branching morphogenesis; our lab is currently investigating its relevance in biliary repair.

In conclusion, the demonstration of the role of Notch signaling on biliary specification of HPC and ductal morphogenesis add a further important piece to the puzzle of biliary repair and paves the way for future investigations of the mechanism of bile duct formation and indicates that it may be possible to modulate liver repair by targeting HPCs and Notch. Understanding the mechanisms of liver repair is a fundamental step for preserving organ function and prolonging the survival of patients with liver disease.

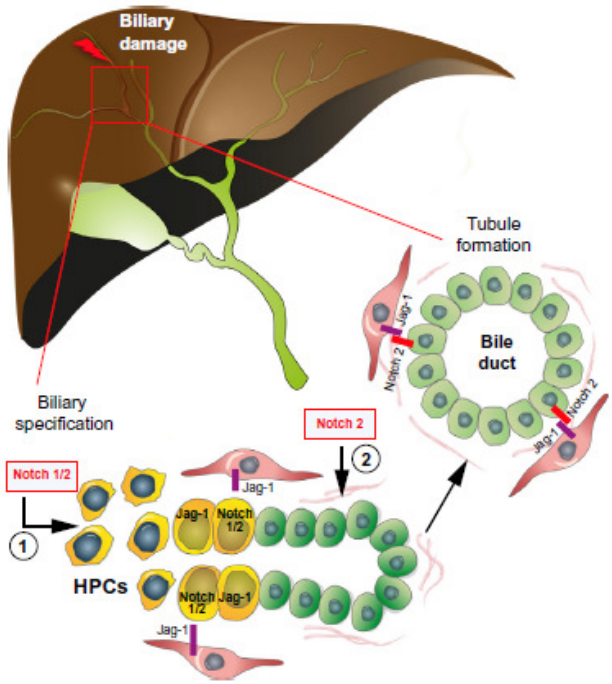

Figure 5. Notch signaling is involved in biliary repair. After biliary damage, HPCs and reactive cholangiocytes proliferate and expand the ductular mass forming branching tubular structures. Biliary specification of HPCs, requires a functional Notch signaling; Notch-2 signaling (2) is essential for tubule formation, likely responding to notch ligands expressed by the surrounding mesenchyma.

Conflict of interest: *the authors have nothing to disclose.*

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REFERENCES

[1] Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology 2006;43: S45-53.

[2] Roskams T, Desmet V. Ductular reaction and its diagnostic significance. Semin Diagn Pathol 1998;15(4): 259-269.

[3] Strazzabosco M, Fabris L. Development of the bile ducts: essentials for the clinical hepatologist. Journal of hepatology 2012;56(5): 1159-1170.

[4] Lai EC. Notch signaling: control of cell communication and cell fate. Development 2004;131(5): 965-973.

[5] Hofmann JJ, Zovein AC, Koh H, Radtke F, Weinmaster G, Iruela-Arispe ML. Jagged1 in the portal vein mesenchyme regulates intrahepatic bile duct development: insights into Alagille syndrome. Development (Cambridge, England) 2011;137(23): 4061-4072.

[6] Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, et al. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet 1997;16(3): 235-242.

[7] McDaniell R, Warthen DM, Sanchez-Lara PA, Pai A, Krantz ID, Piccoli DA, et al. NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. Am J Hum Genet 2006;79(1): 169-173.

[8] Gridley T. Notch signaling and inherited disease syndromes. Hum Mol Genet 2003;12 Spec No 1: R9-13.

[9] Fabris L, Cadamuro M, Guido M, Spirli C, Fiorotto R, Colledan M, et al. Analysis of liver repair mechanisms in Alagille syndrome and biliary atresia reveals a role for notch signaling. Am J Pathol 2007;171(2): 641-653.

[10] Spee B, Carpino G, Schotanus BA, Katoonizadeh A, Vander Borght S, Gaudio E, et al. Characterisation of the liver progenitor cell niche in liver diseases: potential involvement of Wnt and Notch signalling. Gut 2010;59(2): 247-257.

[11] Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, et al. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nat Med 2012.

[12] Tan X, Behari J, Cieply B, Michalopoulos GK, Monga SP. Conditional deletion of beta-catenin reveals its role in liver growth and regeneration. Gastroenterology 2006;131(5): 1561-1572.

[13] Omenetti A, Porrello A, Jung Y, Yang L, Popov Y, Choi SS, et al. Hedgehog signaling regulates epithelial-mesenchymal transition during biliary fibrosis in rodents and humans. J Clin Invest 2008;118(10): 3331-3342.

[14] Roskams TA, Theise ND, Balabaud C, Bhagat G, Bhathal PS, Bioulac-Sage P, et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology 2004;39(6): 1739-1745.

[15] Tirnitz-Parker JE, Tonkin JN, Knight B, Olynyk JK, Yeoh GC. Isolation, culture and immortalisation of hepatic oval cells from adult mice fed a choline-deficient, ethionine-supplemented diet. Int J Biochem Cell Biol 2007;39(12): 2226-2239.

[16] Postic C, Magnuson MA. DNA excision in liver by an albumin-Cre transgene occurs progressively with age. Genesis 2000;26(2): 149-150.

[17] Lozier J, McCright B, Gridley T. Notch signaling regulates bile duct morphogenesis in mice. PLoS One 2008;3(3): e1851.

[18] Han H, Tanigaki K, Yamamoto N, Kuroda K, Yoshimoto M, Nakahata T, et al. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. Int Immunol 2002;14(6): 637-645.

[19] Sparks EE, Huppert KA, Brown MA, Washington MK, Huppert SS. Notch signaling regulates formation of the three-dimensional architecture of intrahepatic bile ducts in mice. Hepatology 2010;51(4): 1391-1400.

[20] Preisegger KH, Factor VM, Fuchsbichler A, Stumptner C, Denk H, Thorgeirsson SS. Atypical ductular proliferation and its inhibition by transforming growth factor beta1 in the 3,5-diethoxycarbonyl-1,4-dihydrocollidine mouse model for chronic alcoholic liver disease. Lab Invest 1999;79(2): 103-109.

[21] Masyuk TV, Ritman EL, LaRusso NF. Quantitative assessment of the rat intrahepatic biliary system by three-dimensional reconstruction. Am J Pathol 2001;158(6): 2079-2088.

[22] van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 2005;435(7044): 959-963.

[23] Wolfe MS. gamma-Secretase as a target for Alzheimer's disease. Adv Pharmacol 2012;64: 127-153.

[24] Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, et al. Notch signaling controls liver development by regulating biliary differentiation. Development 2009;136(10): 1727-1739.

[25] Geisler F, Nagl F, Mazur PK, Lee M, Zimber-Strobl U, Strobl LJ, et al. Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology 2008;48(2): 607-616.

[26] Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger BZ, et al. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 2009;136(7): 2325-2333.

[27] Carpentier R, Suner RE, van Hul N, Kopp JL, Beaudry JB, Cordi S, et al. Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. Gastroenterology 2011;141(4): 1432-1438.

[28] Golbar HM, Izawa T, Yano R, Ichikawa C, Sawamoto O, Kuwamura M, et al. Immunohistochemical characterization of macrophages and myofibroblasts in alpha-

Naphthylisothiocyanate (ANIT)--induced bile duct injury and subsequent fibrogenesis in rats. Toxicol Pathol 2011;39(5): 795-808.

[29] Teachey DT, Seif AE, Brown VI, Bruno M, Bunte RM, Chang YJ, et al. Targeting Notch signaling in autoimmune and lymphoproliferative disease. Blood 2008;111(2): 705-714.

[30] Loomes KM, Russo P, Ryan M, Nelson A, Underkoffler L, Glover C, et al. Bile duct proliferation in liver-specific Jag1 conditional knockout mice: effects of gene dosage. Hepatology 2007;45(2): 323-330.

[31] Dill MT, Tornillo L, Fritzius T, Terracciano L, Semela D, Bettler B, et al. Constitutive Notch2 signaling induces hepatic tumors in mice. Hepatology 2012.

[32] Jeliazkova P, Jors S, Lee M, Zimber-Strobl U, Ferrer J, Schmid RM, et al. Canonical Notch2 signaling determines biliary cell fates of embryonic hepatoblasts and adult hepatocytes independent of Hes1. Hepatology 2013.

[33] Park J, Zhang JJ, Moro A, Kushida M, Wegner M, Kim PC. Regulation of Sox9 by Sonic Hedgehog (Shh) is essential for patterning and formation of tracheal cartilage. Dev Dyn 2010;239(2): 514-526.

[34] Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 2004;127(6): 1775-1786.

[35] Hellstrom M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 2007;445(7129): 776-780.

[36] Fabris L, Strazzabosco M. Epithelial-mesenchymal interactions in biliary diseases. Semin Liver Dis 2011;31(1): 11-32.

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY MATERIAL AND METHODS

Materials and reagents

EGF, dexamethazone, triiodothironin, EDTA, collagenase IV, forskolin and insulin were purchased by Sigma-Aldrich (St. Louis, MO). DBZ, a γ-Secretase Inhibitor, was synthesized and purchased by Syncom BV (Groningen, Netherlands). InSolution™ γ-Secretase Inhibitor IX (DAPT) was purchased from Calbiochem (La Jolla, CA). Culture media alpha-minimum essential medium, Dulbecco's minimal essential medium, HAM's F12, William's E medium, fetal bovine serum, minimum essential medium nonessential amino acids solution, minimum essential medium vitamin solutions, glyceryl monostearate, chemically defined lipid concentrate, soybean trypsin inhibitor, penicillin/streptomycin, gentamycin, trypsin/EDTA, insulin and glutamine were purchased from Invitrogen (Carlsbad, CA). IGF2 was obtained from R&D Systems (Minneapolis, MN). ANIT was purchased from Sigma Aldrich (St. Louis, MO). DDC diet was purchased from BioServ Inc. (Frenchtown, NJ). Rabbit anti-pancytokeratin was purchased from Dako (Carpinteria, CA); rat anti-

mouse K19 antibody (TROMA-III), developed by Kemler R. and rat anti-human Notch-2 intracellular domain antibody (C651.6DbHN), developed by Artavanis-Tsakonas S. were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Rabbit-anti-Sox-9 was purchased from Millipore (Temecula, CA). Notch-1, Notch-2 and Jagged-1 siRNAs and antibody anti-Notch-2 (C-terminal) and anti-calnexin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies anti-CYP1A1, 1A2, 2E1, 3A1 were purchased from Abcam (Cambridge, MA). Cellmask^{TM} Orange was obtained from Molecular Probes, Invitrogen (Carlsband, CA) and Matrigel from Becton Dickinson (Franklin Lakes, NJ).

Immunohistochemistry and immunofluorescence

Briefly, after deparaffination, sections were hydrated in alcohol and endogenous peroxidase activity was blocked with methanolic hydrogen peroxide (10%). According to the instructions supplied by the vendor, a proper retrieval was used to unmask the antigen recognized by the primary antibody. The samples were then rinsed in 0.05% Tween 20 in phosphate-buffered saline (PBS, pH 7.4) and blocked with Ultra V Block (LabVision, Fremont, CA) before applying the primary antibody. Sections were incubated overnight at 4°C with the primary antibodies K19 (TROMA-III, DSHB, 1:100) and Sox-9 (Millipore, 1:500) and Notch2 intracellular domain (C651.6DbHN, DSHB, 1:50) or Notch2 C-terminal (SantaCruz Biotechnologies, 1:100). Following incubation with the selected antibody, liver sections were rinsed with 0.05% Tween 20 in PBS and incubated with appropriated secondary antibody. After HRP secondary antibodies the sections were developed with 3-3′-diaminobenzidine.

Computer-assisted morphometry

K19 antibody was used to quantify the extention of ductular reaction. K19 HRP-immunolabeling was analyzed with a Nikon Eclipse TE2000U microscope (Nikon) supplied with a motorized stage system (Rockland). Images were collected at 4X magnification using a photometric cool snap HQ digital camera (Roper Scientific) and analyzed by Metamorph software (Molecular devices). Ductular reaction was calculated as the percentage of K19 positive pixels above the threshold value with respect to the total pixels per lobule area. The number of HPC was also determined using 10 different fields for each mouse liver.

To quantify the number of HPCs (defined as SOX-9+ve or K19+ve cells that were not incorporated into mature or dysmorphic ductular structures) and the number of ductular structures (defined as a K19+ve group of cells lining a visible and well-structured lumen that is

localized around a portal area), 5 random, non-overlapping fields for each main lobe (10 fields per mouse) were recorded on a digital camera at 100X magnification. Numbers of SOX-9+ve or K19+ve cells and ductular structures were manually counted using ImageJ software by three investigators (R.F, C.M, R.S) blinded to the treatment code. K19 staining was used in DDC samples because the different antigen retrieval used was clearing the porfirin deposits otherwise interfering with the quantification of SOX-9 staining.

Gene expression assessment by Real-Time PCR. Total RNA was isolated BMOL in matrigel after tubule formation using TRIzol Reagent (Invitrogen, Carlsband, CA) following manifacture instructions. Briefly, 800 ng RNA were coverted into PCR template using the TaqMan Reverse Transcription Kit (Applied Biosystem) and then used as the template for the real time PCR analysis using specific primers in combination with the Power SYBR Green PCR Master mix on the Applied Biosystems 7500 Real-Time PCR System. PCR was performed with specific primer pairs for mouse:

NOTCH-1 (Fw: 5'-TGGACA TCCGTGGCTCCATTGTCT-3'; Rev: 5'-TCCGCTTCTTCTTGCTGGCCTCTG-3');

NOTCH-2 (Fw: 5'-TCCTGTGTGGACCATGTGAATACC-3'; Rev: 5'-CTGCACTCATTGATATCATGGAGGC-3');

NOTCH-3 (Fw: 5'-GTGACATAGATGAGTGCCGATCTGG-3'; Rev: 5'-ATTGACACCGTCTACACACGTTCC-3');

NOTCH-4 (Fw: 5'-GCGCCCGATGTGAGAAAGACATGG-3'; Rev: 5'-TGCAGGAGCCACCATTGAGACAGG-3');

HRPT (Fw: 5'-CAGTACAGCCCCAAAATGGT-3'; Rev: 5'-CAAGGGCATATCCAACAACA-3').

Cell Culture

Bipotential Mouse Oval Cell Line (BMOL) was kindly provided by Dr. Yeoh, University of Western Australia. BMOL were isolated from the liver of wild-type mice subject to a choline-deficient, ethionine-supplemented diet as described[15]. This well characterized cell line is clonally derived and shows the immature phenotype and the bipotential characteristic of adult liver progenitor or oval cells as confirmed by their co-expression of biliary and hepatocellular lineage markers. Cells were cultured as described[15].

Western blotting

Total BMOL cell lysate was extracted using a lysate buffer (50mM Tris-HCl, 1% NP40, 0.1% SDS, 0.1% Deoxycholic acid, 0.1mM EDTA, and 0.1mM EGTA) containing fresh protease and phosphatase inhibitor cocktails (Sigma). Membrane-enriched fractions from wild type and experimental mouse liver tissues were prepared as previously described for CYP enzyme expression assessment. Equal amounts of total proteins or subcellular fractions

were loaded into a 4-12% gradient SDS polyacrilamide gel (NuPAGE® Novex Bis-Tris gel, Invitrogen) and separated at a constant voltage of 200mV with 30 mA as previously described. Calnexin was used to normalize for the amount of endosomal fractions.

Gene silencing to inhibit Notch-1, Notch-2 and Jagged-1

Gene silencing was performed using commercially available siRNAs against Notch-1, Notch-2 and Jagged-1 (Santa Cruz Biotechnology, Inc). Scramble RNAs were used to control for non-specific silencing effects. BMOL cells were transfected using the Lipofectamine 2000^{TM} transfection reagent (Invitrogen) 24 hours after plating according to the manufacturer's protocol. Cells were harvested and processed for isolation of total proteins 48 hours after transfection in order to verify the efficiency of gene silencing (not shown). In the tubule formation assay experiment, cells were plated in matrigel 24 hours after transfection and tubule formation was assessed and quantified 24 hours after plating in matrigel (48 hours after transfection).

SUPPLEMENTARY RESULTS

Pharmacologic inhibition of Notch signaling reduces ductular reaction and HPC in mice exposed to DDC. To investigate whether Notch signaling participates in biliary repair, we induced cholestatic damage in WT mice by administering DDC (0.1% in the diet) in the presence or the absence of DBZ. As shown in Supplementary figure 1, four groups of mice were studied: untreated controls, DDC alone, DBZ alone and DDC+DBZ. After 10 days, mice were sacrificed and liver specimens stained with K19 antibody to determine the extension of ductular reaction. DDC treatment caused a significant increase in HPCs and ductular reaction (DR); both HPCs and DR were significantly reduced in mice treated with DBZ and DDC, while treatment with DBZ alone had no effect compared to untreated mice. These results confirm the possible role of Notch in biliary specification of HPCs.

SUPPLEMENTARY TABLES

Supplementary table 1. Liver function tests in treated and untreated mice

	Notch-2 colony					
	WT untreated	**WT + DDC**	**WT+ ANIT**	**cKO untreated**	**cKO + DDC**	**cKO+ ANIT**
ALP (U/L)	77±13	736±107	38±5	65±13	965±209	154±50
ALT (U/L)	16±2	397±138	6±3	13±2	616±176	14±4
	RBP-Jk colony					
	WT untreated	**WT + DDC**	**WT+ ANIT**	**cKO untreated**	**cKO + DDC**	**cKO+ ANIT**
ALP (U/L)	77±5	162±7	48±9	60±10	257±47	170±74
ALT (U/L)	7±2	154±34	5±0.8	8±1	306±91	41±19

Data are expressed as means±SE

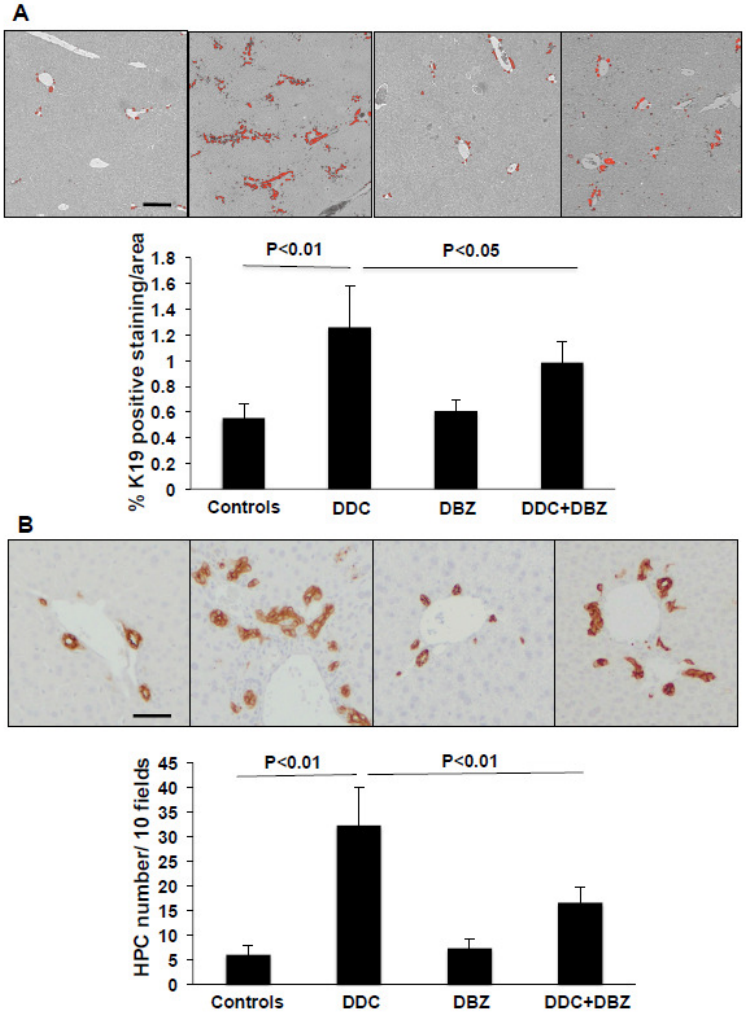
Supplementary table 2. Quantification of biliary progenitor cells in Notch-2- and RBP-Jκ-cKO mice after ANIT and DDC treatment at P60

Notch-2 colony	**SOX-9+ve cells**	**P value**	**RBP-Jκ colony**	**SOX-9+ve cells**	**P value**
Notch-2 WT (n=4)	9,70 ± 2,79		**RBP-Jκ WT (n=4)**	4,10 ± 1,43	
Notch-2 WT + ANIT (n=4)	28,18 ± 4,10	<0,001vs WT	**RBP-Jκ WT + ANIT (n=6)**	20,25± 2,82	<0,001vs WT
Notch-2-cKO (n=4)	28,55 ± 4,43	<0,001vs WT	**RBP-Jκ-cKO (n=4)**	5,60 ± 1,30	0,08 vs WT
Notch-2-cKO + ANIT (n=5)	39,1 ± 5,45	<0,01vs cKO;<0,01vs WT+ANIT	**RBP-Jκ-cKO + ANIT (n=7)**	11,37 ± 4,17	<0,05vs cKO;<0,001 vsWT+ANIT
Notch-2 colony	**K19+ve cells**	**P value**	**RBP-Jκ colony**	**K19+ve cells**	**P value**
Notch-2 WT (n=4)	7±2.4		**RBP-Jκ WT (n=4)**	6.8±1.47	
Notch-2 WT + DDC (n=8)	17.71±2.12	<0,001vs WT	**RBP-Jκ WT + DDC (n=6)**	24.7±3.63	<0,001vs WT
Notch-2-cKO (n=4)	27.32±1.68	<0,001vs WT	**RBP-Jκ-cKO (n=4)**	5.12±0.5	<0,05vs WT
Notch-2-cKO + DDC (n=6)	37.05±6.65	<0,05vs cKO;<0,01 vs WT+DDC	**RBP-Jκ-cKO + DDC (n=8)**	7.15±1.61	<0,05vs cKO;<0,001vs WT+DDC

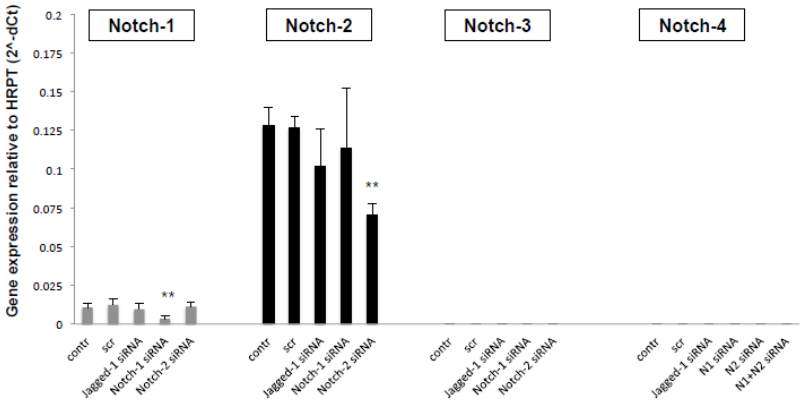
Supplementary table 3. Quantification of ductular structures in Notch-2- and RBP-Jκ-cKO mice after ANIT and DDC treatment at P60

Notch-2-cKO	Ductular structures	P value	RBP-Jκ-cKO	Ductular structures	P value
Notch-2 WT (n=4)	6,82 ± 2,11		**RBPJκ WT (n=4)**	2,58 ± 0,75	
Notch-2 WT + ANIT (n=4)	14,38 ± 2,98	<0,01 vs WT	**RBPJκ WT + ANIT (n=6)**	6,67 ± 2,48	<0,01vs WT
Notch-2 WT + DDC (n=6)	12,10 ± 4,55	<0,05 vs WT	**RBPJκ WT + DDC (n=6)**	10,38 ± 4,65	<0,01vs WT
Notch-2-cKO (n=4)	3,33 ± 0,82	<0,05 vs WT	**RBPJκ-cKO (n=4)**	1,50 ± 0,28	<0,05vs WT
Notch-2-cKO + ANIT (n=5)	3,86 ± 0,88	0,19vscKO; <0,001vs WT+ANIT	**RBPJκ-cKO + ANIT (n=7)**	2,51 ± 0,67	<0,05vs cKO;<0,001 vsWT+ANIT
Notch-2-cKO + DDC (n=6)	5,23 ± 1,56	<0,05vscKO;<0,01 vs WT+DDC	**RBPJκ-cKO + DDC (n=8)**	2,49 ± 1,32	0,08vs cKO; 0,001 vs T+DDC

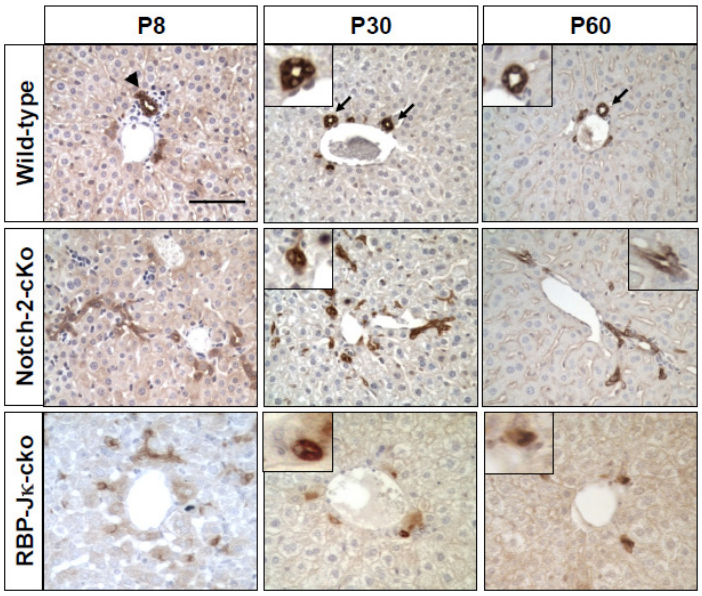
SUPPLEMENTARY FIGURES



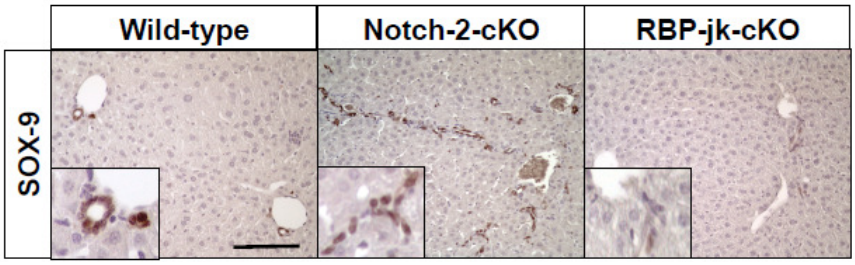
Supplementary Figure 1. Notch inhibition by DBZ significantly reduces ductular reaction and HPC proliferation in mice exposed to DDC. Liver damage was induced in wild type mice by feeding with 0.1% DDC supplemented diet for 10 days in the absence or presence of DBZ (5µmol/Kg/day) administered i.p daily. Ductular reaction was quantified by K19 staining followed by morphometric analysis as described. (A) As shown in the bar graph, ductular reaction was significantly reduced in mice treated with DDC+DBZ compared to DDC only treated mice. (B) The presence of DBZ during DDC treatment also inhibited the activation of the progenitor cell compartment as measured from the number of HPCs counted in 10 different fields for each liver lobe. Data represent average ± SD of n=6 mice for each group. Bar scale is 200 µm in panel A and 50 µm in panel B.



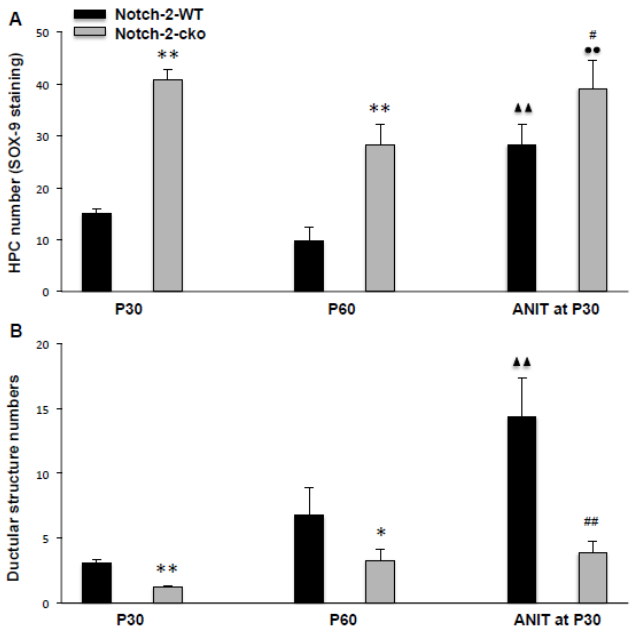
Supplementary Figure 2. Gene expression of Notch receptors in BMOL. The expression of Notch-1, Notch-2, Notch-3 and Notch-4 was evaluated by RT-qPCR in BMOL after tubule formation and after silencing of Notch-1, Notch-2 and Jagged-1. Data are normalized to HRPT and expressed as 2^-dCt. Data represent average ± SD of n=3 experiments for each group. (Scr: scramble, **p<0.01 vs scr).



Supplementary Figure 3. Phenotype of Notch-defective mice. K19 staining in Notch-2cKO and RBP-Jκ-cKO shows a defect in bile duct maturation. In WT mice biliary epithelium development was almost complete at 8 days after birth (P8) (arrowhead) with some ductal plate remnants in the final reabsorbing phase. Mature bile ducts were visible at P30 and P60 (inset and black arrows). A delay in the maturation of the biliary epithelium was visible at P8 whereas at P30 and P60 a mild defect in branching morphogenesis was present. RBP-Jκ-cKO liver phenotype was qualitatively similar to Notch-2cKO mice but more advanced bile duct paucity. Bar scale is 100 μm.

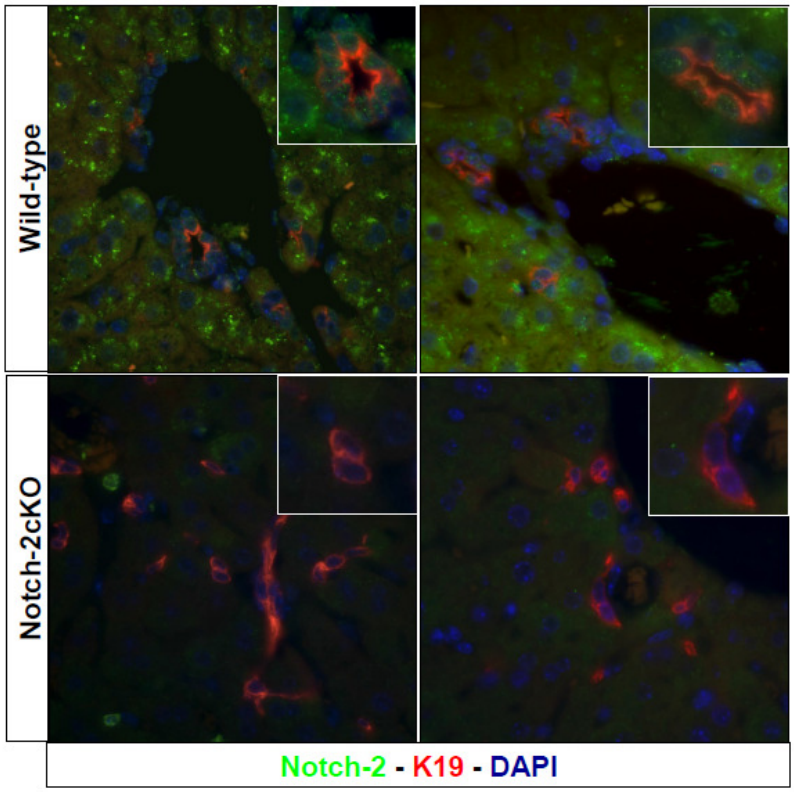


Supplementary Figure 4. Sox-9 staining shows an increase in the numbers of biliary progenitor cells in Notch-2cKO, but not in RBP-JκcKo mice. Paraffin sections from WT, Notch-2-cKO and RBP-Jκ-cKO mice at P60, were immunolabeled with SOX-9 antibody, a specific early marker of biliary cells. In WT liver, SOX-9 was and mainly expressed by mature bile ducts while in Notch-2cKO liver it was expressed by most of the reactive cells present in the portal space and the surrounding parenchyma (dysmorphic ductular structures and HPC). In RBP-Jκ Immunoreactivity for SOX-9 was detected larger ducts at the level of liver hilum and a few reactive cells. Bar scale is 100 µm.

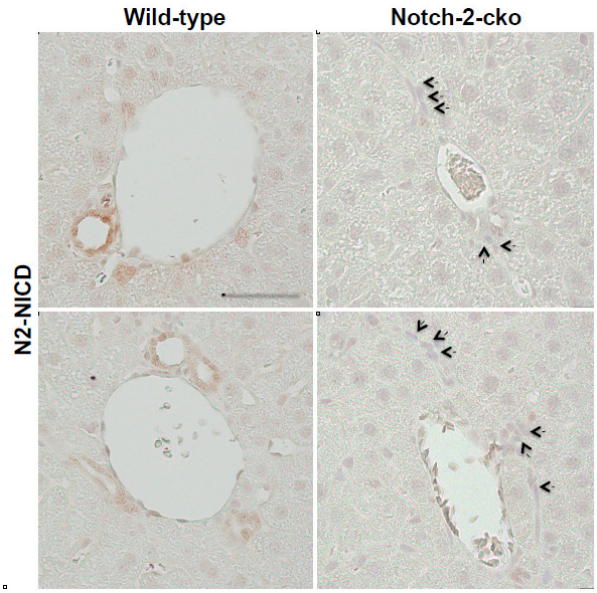


Supplementary Figure 5. Quantification of biliary progenitor cells and ductular structures in WT and Notch-2-cKO at P30, P60 and after treatment with ANIT. Paraffin sections from Notch-2-cKO mice and their respective WT littermates at P30, P60 and after treatment with ANIT by P30 were stained for SOX-9 (A) and K19 (B). As shown in the bar graph, Notch-2 cKO mice showed a significant increase in SOX-9+ve cells (A) and a decrease in bile ducts (B) compared to WT independently from the age stage. After ANIT treatment the number of SOX-9+ve cells are further increased while there is a lack of ductular structures compared to WT. (Data

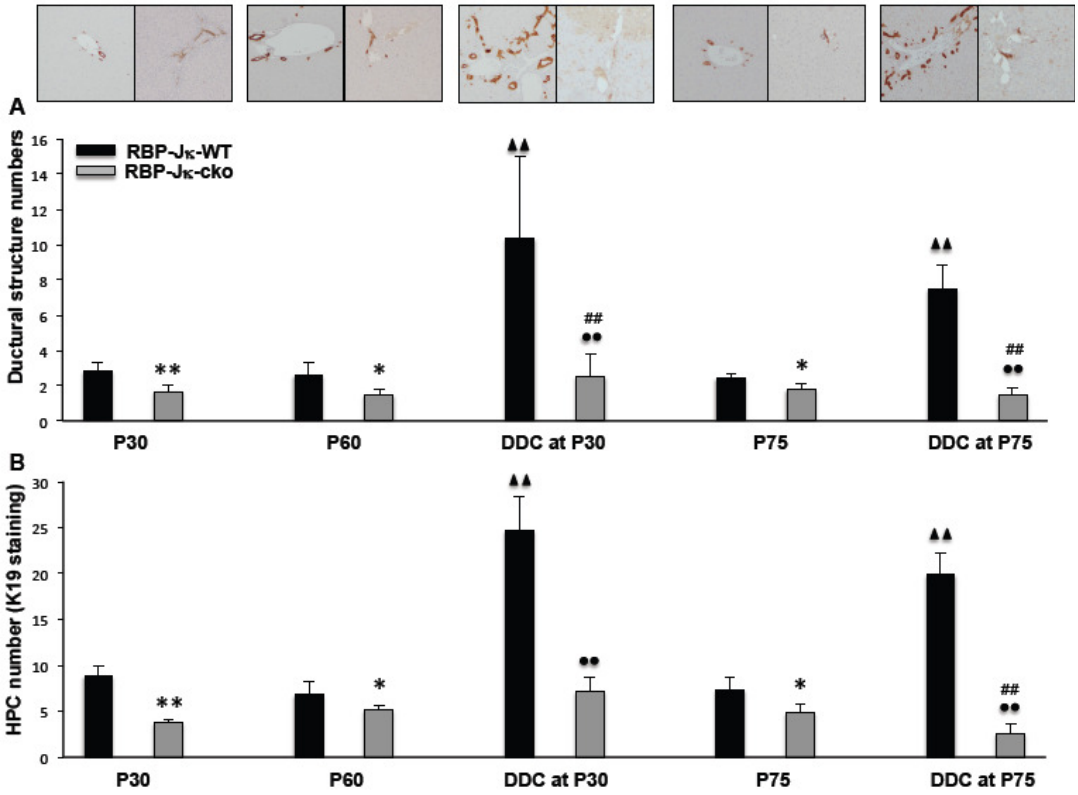
represent average ± SD, n=4-8 mice per group; *p<0.05 and **p<0.01 vs WT, ▲▲ p< 0.01 vs WT P30, ●● p< 0.01 vs cKO P30, # p<0.05 and ## p<0.01 vs WT+ANIT).



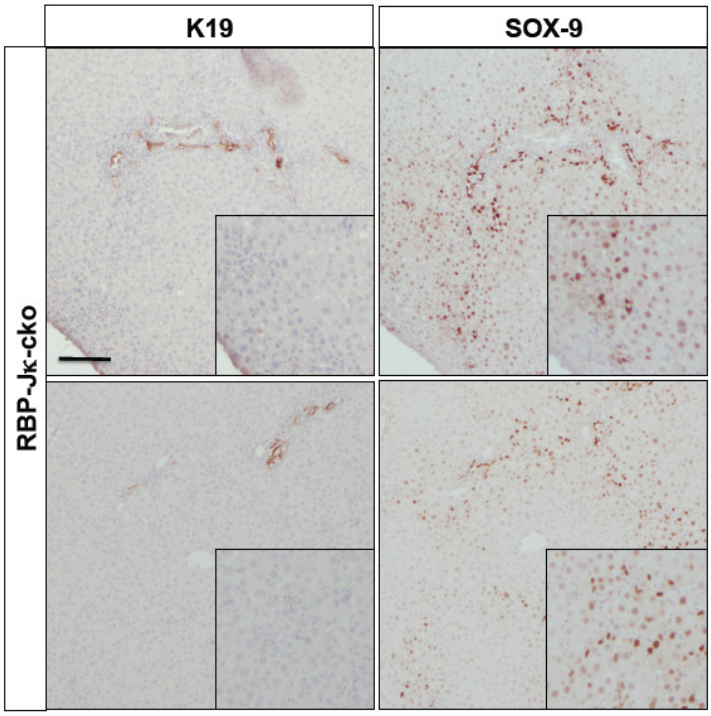
Supplementary Figure 6. Notch-2-cKO mice do not express Notch-2 in biliary progenitor cells. Notch-2-cKO and their WT littermate were stained for K19 (red), Notch-2 (green) and DAPI (blue). Bile ducts in WT mice show the double expression of K19 and Notch-2 while progenitor cells in Notch-2-cKO are positive for K19 but negative for Notch-2.



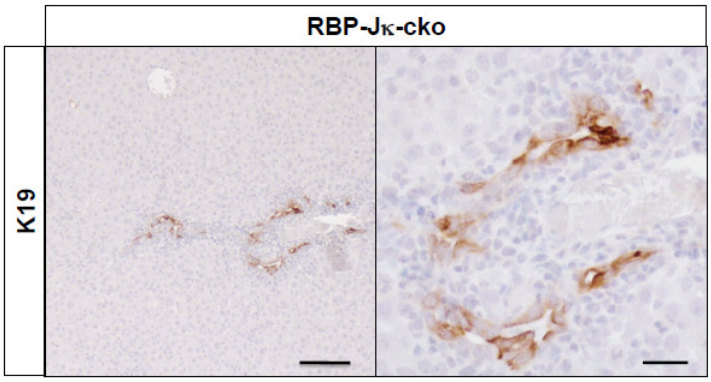
Supplementary Figure 7. Notch-2 intracellular domain is express in the nuclei in the biliary epithelium of WT but not Notch-2cKO. Notch-2-cKO and their WT littermate were stained for with an antibody against Notch-2 intracellular domain, the fragment that after gamma-secretase cleavage, translocate to the nucleus to activate the signaling. Bile ducts in WT mice show the expression of Notch-2 intracellular domain in the nuclei while Notch-2-cKO are negative. Black arrows indicate negative cells in the Notch-2-cKO with progenitor cell morphology. Bar scale is 20 μm.



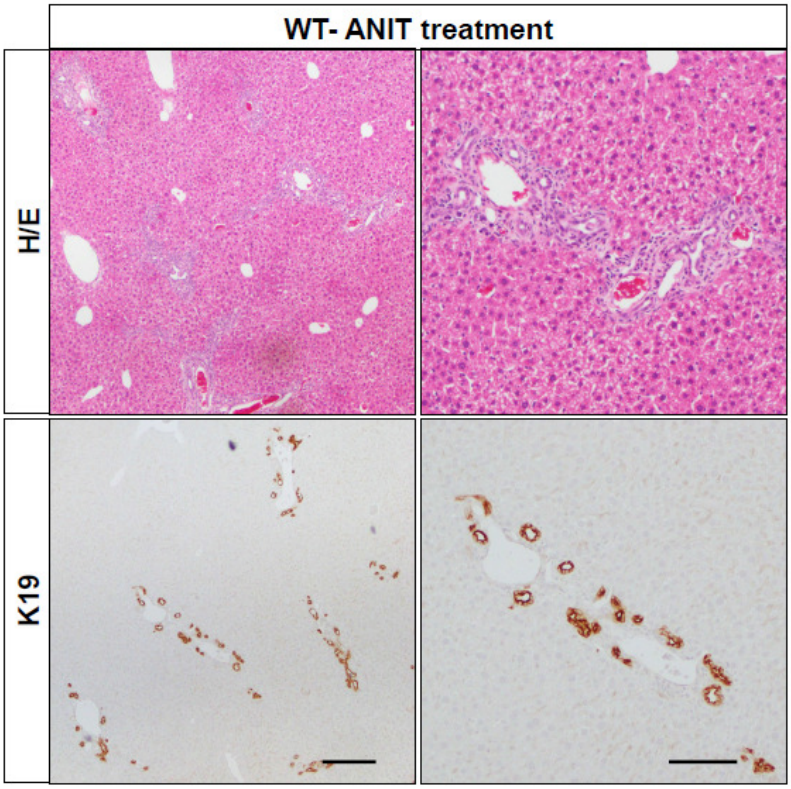
Supplementary Figure 8. Quantification of biliary progenitor cells and ductular structure in WT and RBP-Jκ -cKO at P30, P60, P75 and after treatment with DDC. Paraffin sections from RBP-Jκ-cKO mice and their respective WT littermates at P30, P60, P75 and after treatment with DDC by P30 and P75 were stained for K19. As shown in the bar graph, RBP-Jκ-cKO mice showed a significant decrease in ductular structures (A) and biliary progenitor cells (B) compared to WT at any age stage. After treatment with DDC by P30 and by P75 the number of ductular structures (A) and biliary progenitor cells (B) is significantly decreased compared to WT. (Data represent average ± SD, n=4-8 mice per group; *p<0.05 and **p<0.01 vs WT, ▲▲ p< 0.01 vs WT P30, ●● p< 0.01 vs cKO P30, # p<0.05 and ## p<0.01 vs WT+DDC).



Supplementary Figure 9. Hepatocyte like-cells expressing SOX-9 in RBP-Jκ-cKO mice at P30. Serial paraffin sections from RBP-Jκ-cKO mice at P30 were stained for K19 or SOX-9. An extensive expression of Sox-9 in cells with hepatocyte morphology was evident. These SOX-9+ve hepatocellular cells were negative for the biliary/progenitor marker K19. Bar scale is 200 µm.



Supplementary Figure 10. Biliary dilatation K19+ve rather than normal bile ducts in RBP-Jκ-cKO mice at P30. Paraffin sections from RBP-Jκ-cKO mice at P30 were stained for K19. K19 stained rare dysmorphic microcystic structures lined by epithelial cells with irregular shape. Bar scales are 200 µm and 50 µm.



Supplementary Figure 11. Characterization of chronic ANIT model in WT mice. Paraffin sections from WT mice at the end of 4 weeks treatment with ANIT were stained for H/E or for K19. ANIT treatment is characterized by hyperplasia of the terminal branches of the biliary tree and the presence of the different components of ductular reaction. Bar scales are 200 μm and 100 μm

Chapter 3

Notch Signalling Beyond Liver Development: Emerging Concepts in Liver Repair and Oncogenesis

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Summary

Notch signalling is an evolutionarily conserved intercellular pathway involved in many aspects of development and tissue renewal in several organs. The importance of Notch signalling in liver development and morphogenesis is well established. However, the post-natal role of Notch in liver repair/regeneration is only now beginning to be unveiled. Despite the simplicity of the pathway activation, a fine spatial-temporal regulation of Notch signalling is required to avoid pathologic effects. This review highlights recent advances in the field indicating that Notch signalling is involved in the reparative morphogenesis of the biliary tree and in liver carcinogenesis. Defective Notch signalling leads to impaired ability of the liver to repair liver damage, while excessive activation may be involved in liver cancer. Even though much remains to be understood about these mechanisms, including the cross-talk between Notch signalling and other liver morphogens, current evidence suggests that the modulation of the Notch pathway may represent a therapeutic target in chronic liver disease.

LIST OF ABBREVIATIONS: AGS, Alagille syndrome; CCA, cholangiocarcinoma; CSC, cancer stem cell; HCC, hepatocellular carcinoma; HNF, Hepatocyte nuclear factor; HPC, hepatic progenitor cell; IGF2, Insulin-like-growth factor 2; IHBD, Intrahepatic bile duct; Jag-1, Jagged-1; N1ICD, Notch-1 intracellular domain; N2ICD, Notch-2 intracellular domain; NICD, Notch intracellular domain; NOS2, Inducible nitric oxide synthase; RBP-Jκ, Recombination Signal Binding Protein Immunoglobulin Kappa J; Sox-9, Sex determining region Y-box 9; T-ALL, T-cell acute lymphoblastic leukaemia.

Introduction

Notch signalling is an evolutionarily conserved pathway that regulates a variety of fundamental cellular processes, including cell fate and differentiation[1, 2]. The Notch system encompasses four transmembrane Notch receptors (Notch-1, -2, -3, -4), and two types

of ligands, Serrate/Jagged (Jag-1, -2) or Delta-like (Dll-1, -3, -4). The peculiar structure of both receptors and ligands requires a cell-cell interaction between the cell expressing Notch ligands or “transmitting” cell able to activate Notch receptors on the “receiving” cell. Upon ligand binding, the Notch receptor is then cleaved by the γ-secretase complex, leading to the cytoplasmic release of the Notch intracellular domain (NICD). NICD translocates into the nucleus where it binds to the DNA binding protein CBF1/Drosophila Su(H)/ C.Elegans LAG-1 (CSL), also called Recombination Signal Binding Protein Immunoglobulin Kappa J (RBP-Jκ). Following interaction with NICD, the RBP-Jκ associated co-repressors can be displaced, thereby allowing the transcription of the Notch-target genes. Among them, the Hairy Enhancer of Split homologs transcription factors (Hes and Hey) regulate the expression of crucial determinants of the cell fate specification, as the family of the hepatocyte nuclear factors (HNF) and the Sex determining region Y-box 9 (Sox-9) in the liver[1-6]. Indeed, the activation of the Notch pathway is finely delimited in a tissue- and time-dependent fashion, driving organ morphogenesis during development. NICD activity is tightly regulated by phosphorylation and rapid proteosomal degradation. Thus, for continuous Notch activation, additional ligands inducing receptor proteolysis are required. Notch action can be also modulated by post-translational modifications: the receptor can be ubiquitylated and endocytosed under the effects of repressors, such as Numb, or alternatively, glycosylated at different sites, favouring (as is the case of the enzyme O-fucosyl transferase) or limiting (under Fringe influence) Notch activation. Moreover, cleaved or secreted ligands may antagonise Notch signalling[3].

Several papers have addressed the role of Notch signalling in liver development[5-11]. Activation of Notch signalling in hepatoblasts

stimulates their commitment towards the biliary lineage. Notch ligands, such as Jag-1, expressed by mesenchymal cells at the parenchymal/portal interface of the nascent portal space up-regulate the expression of biliary-specific markers, such as Sox-9 and HNF1β in adjacent hepatoblasts[5, 6]. Several studies based on a number of mouse models defective in either Notch effectors (i.e. Notch-1 or Notch-2 knock-out (KO)[7-9], RBP-Jκ KO[5, 10], J1 KO[12, 13], Hes-1 KO[6]) or Notch-related biliary targets (Sox-9 KO[14], HNF1β KO[15]), have demonstrated that Notch is involved in intrahepatic bile duct (IHBD) morphogenesis during foetal development and the early postnatal life (Figure1).

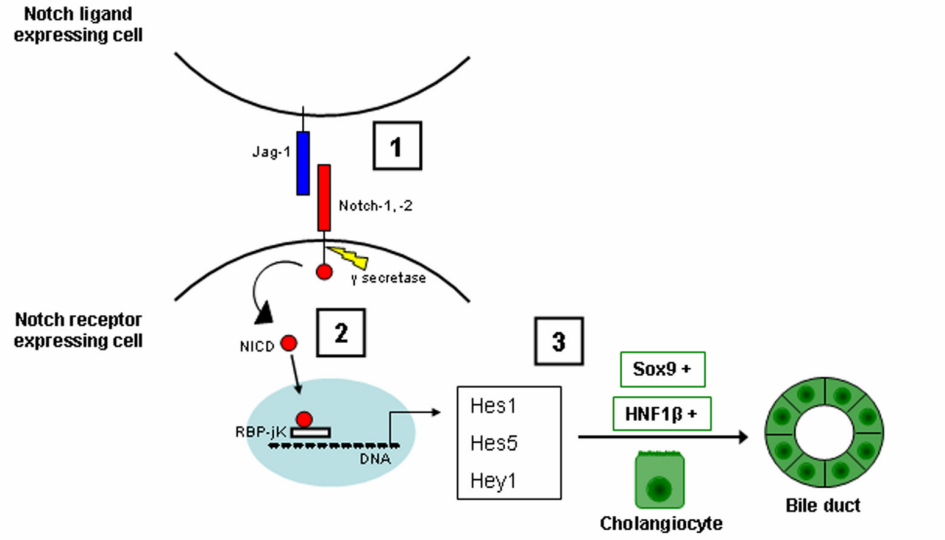

Figure 1 – Notch signalling in the liver. 1) In the liver, a Notch ligand (Jag-1) expressing cell activates Notch-1 or Notch-2 on the surface of the Notch receptor expressing cell. **2)** Upon ligand binding, Notch receptor is activated and cleaved by the action of a γ-secretase, leading to the cytosolic release of the NICD. NICD migrates into the nucleus where it binds to the transcription factor RBP-jκ, **3)** driving the expression of Notch target genes (Hes1, Hes5, Hey1) and Notch-related biliary targets (Sox-9, HNF1β). This cascade of events determines the commitment of the signal receiving cell to the biliary lineage, allowing bile duct formation. (NICD, Notch intracellular domain).

The link between Notch signalling and IHBD morphogenesis is consistent with the causative association of Jag-1[16, 17] (and less frequently, Notch-2[18]) mutations with Alagille syndrome (AGS), an autosomal dominant multisystem disorder characterised in the liver by ductopenia, mostly affecting the periphery of the liver, and cholestasis[19].

However, it is still unclear whether in AGS, ductopenia is due to a developmental failure of IHBD morphogenesis or to a progressive loss of IHBD throughout adult life resulting from the inability of the Notch-defective liver to restore the biliary tree in response to liver damage. Our group has shown that in AGS, reactive ducts are markedly reduced with respect to other paediatric cholestatic liver diseases, against an accumulation of intermediate cytokeratin-7-positive hepatocytes not expressing the biliary-specific transcription factor HNF1β, as if hepatocytes were trying, unsuccessfully, to replace the missing biliary cells[20]. These observations raised the concept that Notch pathway is involved in maintaining tissue homeostasis in the post-natal life and in the reparative reaction to biliary damage. Recently, a number of evidence has suggested that in the liver, Notch is indeed involved in liver repair[20-23], as well as in oncogenesis[24-29]. Herein, we will summarise the emerging concepts addressing the contribution of Notch to the above mechanisms (Figure 2).

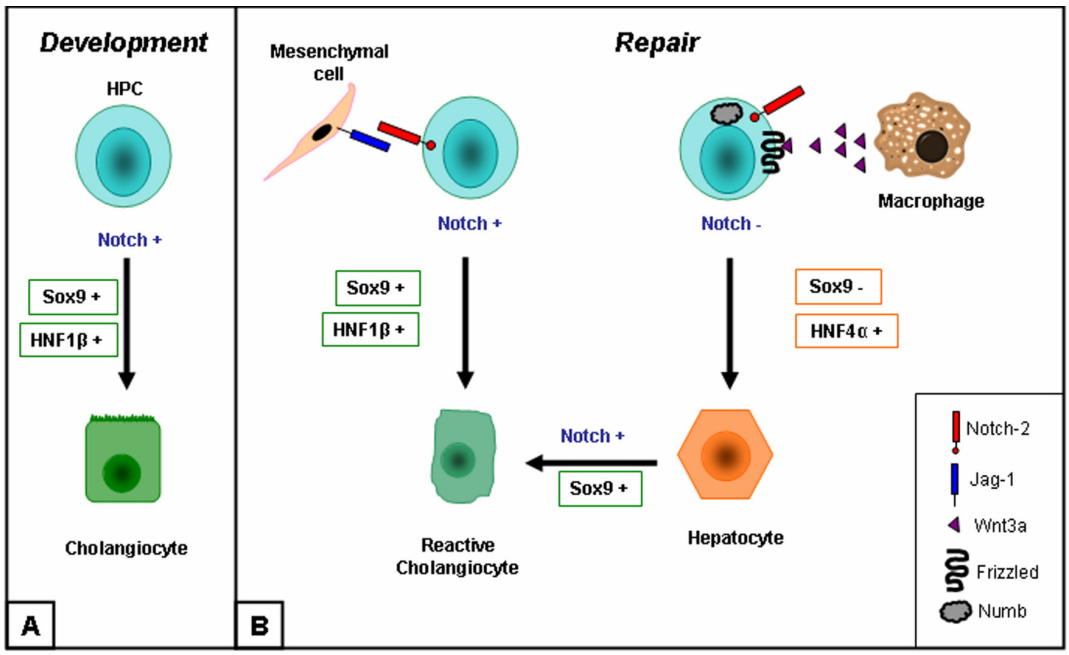

Figure 2 – Role of Notch in liver pathophysiology. A) During liver development, Notch signalling confers biliary fate instructions (Sox-9 and HNF1β) to the hepatoblast while directing bile ducts morphogenesis. **B)** Mesenchymal cells activate Notch signalling in HPCs during liver repair to generate Sox-9/HNF1β-positive reactive cholangiocytes in the presence of a biliary damage. On the other hand, hepatocellular damage results in macrophage-dependent Wnt-mediated Notch inhibition promoting HPCs commitment towards the hepatocyte lineage (blunted Sox-9, increased HNF4α expression) [21]. Recent studies also suggest that Notch-induced Sox-9 aberrant expression in hepatocytes could promote their transdifferentiation to biliary cells [46]. (HPC, hepatic progenitor cell).

Role of Notch in liver repair

Hepatocytes and cholangiocytes are quiescent cells able to proliferate in response to hepatic injury, in order to restore the liver parenchymal mass. However, when massive or chronic liver injury occurs and the proliferative ability of hepatocyte and/or cholangiocyte is compromised, the hepatic progenitor cell (HPC) compartment is activated. HPCs are likely generated in the hepatic stem cell niche located in the canals of Hering and in the terminal ductules of the biliary tree, even though transdifferentiation of hepatocytes cannot be excluded[30-33]. HPCs can give rise to hepatocytes or

cholangiocytes. The fate decision between hepatocellular or biliary commitment is dependent upon the type of inflammatory reaction and the balanced activation of Wnt or Notch signalling, respectively[11, 21, 22, 34].

Conditions of chronic damage and repair result in the generation of “ductular reactive cells”[30, 32, 33]. These cells have an intermediate phenotype between progenitor cells and biliary cells that are not yet organized into tubular structures[35, 36]. Ductular reactive cells are able to release in the microenvironment a range of cytokines and chemokines, through which they establish an intense cross-talk with multiple inflammatory cell types along with mesenchymal and vascular cells (ductular reaction)[36-39]. Thus, the “dark side” of the ductular reaction-driven tissue remodelling is the progressive deposition of a fibro-vascular stroma, ultimately responsible for the architectural distortion that is the hallmark of evolving liver diseases.

Reactivation of pathways reminiscent of embryonic development, as Wnt, Hedgehog and Notch signalling, plays a major role in these mechanisms. In the adult liver, Notch components are widely expressed in the epithelial and the mesenchymal compartment, and are differentially regulated in case of injury. All four Notch receptors are expressed in the liver[40]. Notch-1 and -2 are predominantly expressed by cholangiocytes and HPCs where they are strongly upregulated during biliary damage[21, 22, 41]; Notch-3 and -4 are more involved in mesenchymal cell biology and weakly present in the epithelial compartment. Indeed, during liver damage a strong upregulation of Notch receptors occurs in endothelial cells lining portal tract vessels (mainly Notch-2 and -3) and sinusoids, which is most likely responsible for the abnormal neovascularisation[40]. Intriguingly, Notch-1 is expressed by quiescent hepatic stellate cells[42], but downregulated during their

progression to an active myofibroblast-like state and compensated by a functional Notch-3 *de-novo* expression[43]. Jag-1 and Dll-4 are the only Notch ligands expressed in the liver[44]. Normally, Jag-1 is expressed on HPCs and biliary cells[12, 41, 44], and on smooth muscle cells in the portal vein mesenchyme[12]. Jag-1 is strongly upregulated in injured livers, becoming the most important ligand driving hepatic Notch activation. Jag-1 has been detected on proliferating bile ductules[21, 41, 44] and on hepatocytes of fibrotic livers [43], as well as on activated hepatic stellate cells[42]. Dll-4 exerts its main action on liver endothelial cells, regulating physiologic and pathologic hepatic vascularisation[44].

Notch signalling activation in HPCs and reactive ductular cells has been reported in human cholangiopathies[21, 41]. Notch-1 and Notch-2 receptors are the main Notch receptors expressed in biliary cells, reactive cells and HPCs[21, 22]. In the setting of biliary damage, a complete inhibition of Notch signalling blocks the generation of biliary-committed progenitor and reactive cells[21, 22]. While, when the Notch pathway is only partially defective (as in the case of liver-specific Notch-2 KO mice), the generation of biliary-committed HPCs is still possible (likely a compensatory effect of Notch1); however, this compensatory response is not sufficient to restore the bile duct mass, and tubule formation is inhibited[22]. In keeping with these observations, in AGS patients there is an evident imbalance in the epithelial components of the hepatic reparative complex, whereby cells with an intermediate hepatocyte-biliary phenotype accumulate in spite of the lack of biliary precursors and of the presence of ductopenia[20]. Notably, on the contrary to biliary atresia, in AGS the reduced number of reactive biliary cells is associated with limited deposition of fibrotic tissue in liver parenchyma, consistent with the slow progression to cirrhosis seen in

AGS patients[45]. As in ductal plate maturation mesenchymal cells coordinate Notch-expressing hepatoblasts through Jag-1[5, 6], it could be speculated that in the adult injured liver an epithelial-mesenchymal cross-talk depending upon Notch occurs as well. Based on this concept, accumulating portal myofibroblasts may interact with HPCs to activate Notch[21]. Noteworthy, Notch activation has been described in an experimental rat model of carbon tetrachloride-induced liver fibrosis, where Notch-3-expressing hepatic stellate cells, activated by Jag-1-positive hepatocytes, were responsible for excessive matrix deposition. In this model, pharmacological Notch inhibition was shown to be an effective strategy to reduce the extent of liver fibrosis[43]. Taking all these observations together, it is tempting to speculate that Notch signalling may play a relevant role also in epithelial–mesenchymal cross-talk and ductular reaction-associated fibrogenesis.

Constitutive activation of the Notch-2 intracellular domain (N2ICD) in the hepatoblasts during development leads to an architectural distortion of the liver lobule, often lethal at birth, characterised by the ectopic formation of tubular and cystic structures, resembling early malignant biliary lesions[46, 47]. In the adult mouse liver, continuous supply of N2ICD stimulates the proliferation of the biliary/HPC compartment. Interestingly, aberrant N2ICD activation in the adult hepatocytes induces their rapid conversion towards a biliary phenotype, where the hepatocyte specific markers HNF4α and albumin are down-regulated and in turn, the biliary Sox-9 and HNF1β markers are *de novo* expressed. These transdifferentiated cells grow as microcystic biliary structures scattered in the liver parenchyma[46]. The pathophysiological significance of ductular metaplasia of hepatocytes is uncertain, but it may represent an alternative process to generate biliary-commmitted

progenitors[20]. Notably, constitutive expression of N2ICD leads also to the expansion of the biliary/HPC compartment in adult mice[46, 47]. In addition, Notch-driven biliary conversion of hepatocytes has been reported in the setting of liver cancer[25, 26], as it will be discussed below.

Recent evidence suggests that HPCs commitment towards the biliary or the hepatocellular fate is driven by the inflammatory microenvironment localized around the stem cell niche, whereby the quality of the cellular infiltrate (macrophage, myofibroblast) is strictly dependent upon the nature of the injury[21, 48]. Biliary specification of HPCs is determined by a direct cell-cell interaction between Notch-expressing HPCs and Jag-1-expressing portal fibroblasts. However, when hepatocellular damage is prevalent, infiltrating macrophages, in response to cellular debris, secrete Wnt3a and switch off the default specification pathway for HPCs by activating the Wnt/β-catenin/Numb pathway, which turns HPCs differentiation towards a hepatocyte phenotype[21]. These findings indicate that, depending upon the type of liver damage, Notch acts in concert with other morphogens, such as Wnt or Hedgehog[49, 50], to restore liver architecture and function.

Notch signalling and liver cancer

The most common primary liver malignancies are hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA). Most cases of HCC (85-90%) and an increasing proportion of intrahepatic CCA (20-25%) arise on a background of liver cirrhosis[51, 52]. In cirrhotic livers, necroinflammation coexists with fibrosis and activation of the HPC compartment. In some cases, HPCs may acquire a malignant phenotype (cancer stem cell, CSC) and behave as cancer initiating cells[53]. A subset of tumours that exhibit both CCA and HCC characteristics, such as cholangiocellular

CCA, are thought to arise from the HPC compartment[54]. Thus it is not surprising if molecular mechanisms involved in the regulation of the HPC compartment, including Notch, Hedgehog[55, 56] and β-catenin[57, 58], are emerging as potential oncogenetic pathways.

The first evidence that Notch signalling can be involved in oncogenesis comes from studies in T-cell acute lymphoblastic leukaemia (T-ALL). Notch-1 is essential for T-cell progenitor biology, regulating their commitment to a mature T-cell fate. T-ALL development is associated with chromosomal translocation[59] or gain-of-function mutations[60] of Notch-1 that lead to the constitutive activation of the receptor and consequent neoplastic transformation.

On the other hand, the role of Notch signalling in solid tumours is still unclear. Mutations of Notch receptors have not been documented yet in solid tumours, but there is evidence for an aberrant and inappropriate Notch pathway activation[61]. To further complicate the scenario, Notch can behave as a tumour suppressor or an oncogene, depending on the cellular context. Of note, AGS liver disease rarely progresses to liver cancer[45].

Earlier studies involving Notch loss/gain of function in experimental models of liver cancer indicated that Notch might behave as a tumour suppressor[62-65]. However, more recent studies suggest that Notch activation is associated to HCC development and progression. Increased nuclear expression of Notch-1 and Notch-3 was observed in neoplastic hepatocytes when compared to the non-tumoral cirrhotic nodules[66]. Notch-3 inhibition enhanced the sensitivity to doxorubicin-induced apoptosis in a p53-dependent way. Furthermore, in hepatoma cells overexpressing wild type p53, Notch-1 activation cooperated with Snail to promote dedifferentiation and invasiveness of neoplastic hepatocytes[67]. Pro-inflammatory cytokines are also critical regulators of Notch

expression, in fact TNFα, via IKKα and the transcription factor FOXA2, may induce Notch activation by suppressing Numb[68].

More recently, a reproducible occurrence of HCC in adult mice was reported after constitutive activation of Notch-1 intracellular domain (N1ICD) in hepatoblasts[28]; after one year 100% of mice developed HCC. The neoplastic liver lesions reproduced different stages of human HCC progression and were associated with proliferation of infiltrating cytokeratin-19-positive cells and progenitor-like cells (most likely representing CSC). Genomic profiling revealed that the oncogenic role of N1ICD requires its close cooperation with molecular partners, identified in insulin-like-growth factor 2 (IGF2) and Sox-9. Interestingly, the Notch activation signature reported in N1ICD mice was found in a subset of patients with HCC; this signature is associated with genes related to cellular proliferation and with Sox-9 expression[28]. Sox-9 expression is present in less differentiated HCC and correlates with poor prognosis[69].Constitutive activation of the N2ICD in HPCs promoted the spontaneous development of aggressive and dedifferentiated HCC in the adult mouse liver[24]. Also in this case, Notch-induced malignant hepatocyte transformation was associated with the expression of Sox-9 and down-regulation of the hepatocyte-related genes. These findings suggest that Notch signalling is involved in liver oncogenesis by activating a subset of Sox-9 positive progenitors, in line with the concept that tissue resident stem cells may become tumour-initiating cells. Alternatively, persistent and combined Notch-1/Notch-2 activation in mature hepatocytes may induce the dedifferentiation of malignant cells towards a progenitor cell-like phenotype. Overexpression of N2ICD in association with cancerogenous stimuli, results in the formation of HCC in association to biliary dysplasia and eventually to CCA, with up-regulation of HNF4α expression in HNF1β-positive cells, as if

cholangiocytes were dedifferentiating into a less mature progenitor phenotype[24]. Recent investigations on the pathogenesis of the intrahepatic CCA suggest that Notch-driven transdifferentiation of hepatocytes into cholangiocytes may be a mechanism not only for liver repair but also for oncogenesis. Upregulation of N1ICD by mediators released by the inflammatory microenvironment, including the inducible nitric oxide synthase (NOS2) was originally reported in human CCA specimens[70]. More recently, Notch-1 overexpression was found to cooperate with AKT signalling to induce a more rapid and aggressive CCAs, from dedifferentiation of hepatocytes or from HPCs[25]. Along with this line, thioacetamide treatment of mice constitutively expressing activated Notch1 induced a rapid onset of CCA[26]. In this model, fate-tracing approaches documented that cytokeratin-19-malignant cells originated from the phenotypic conversion of ectopically N1ICD over-expressing hepatocytes. This finding is consistent with the emerging trend of intrahepatic CCA in patients with cirrhosis of parenchymal origin, such as in viral hepatitis[71].

Taken together, these data indicate that in specific molecular subsets of HCC and CCA, persistent activation of Notch signalling plays an oncogenic role (Figure 3). However, Notch effects are closely related to the inflammatory molecular environment, whereby critical partners (p53, Snail, TNFα/IKKα/FOXA2/Numb, Sox9, IGF2, HNF4α) may favor the pro-oncogenic functions of Notch.

Conclusions

In the last few years, Notch signalling has been recognized as a major player in liver biology and pathophysiology. Beside its role in liver development, and in AGS, a growing body of data suggests that in adult life, Notch plays a range of additional functions, from liver

repair to carcinogenesis. Indeed, Notch is required to commit HPCs to the biliary fate and to orchestrate the biliary remodelling. Notch-2 is critical for biliary tubulogenesis, which is halted when Notch-2 receptor is defective[22]. The possible role of Notch in the cross-talk between HPCs and mesenchymal cells during pathological repair and fibrosis still remains to be addressed. Emerging data suggest that Notch plays a significant role also in the pathogenesis of liver cancer. Continuous activation of the Notch pathway has been linked to both HCC and CCA. Based on these observations, Notch emerges as a potential therapeutic target, however, because of the toxicity of current Notch inhibitors[72-74], more efforts are needed to understand the molecular mechanisms regulating Notch activation in specific cell context and the complex interplay with additional partners involved.

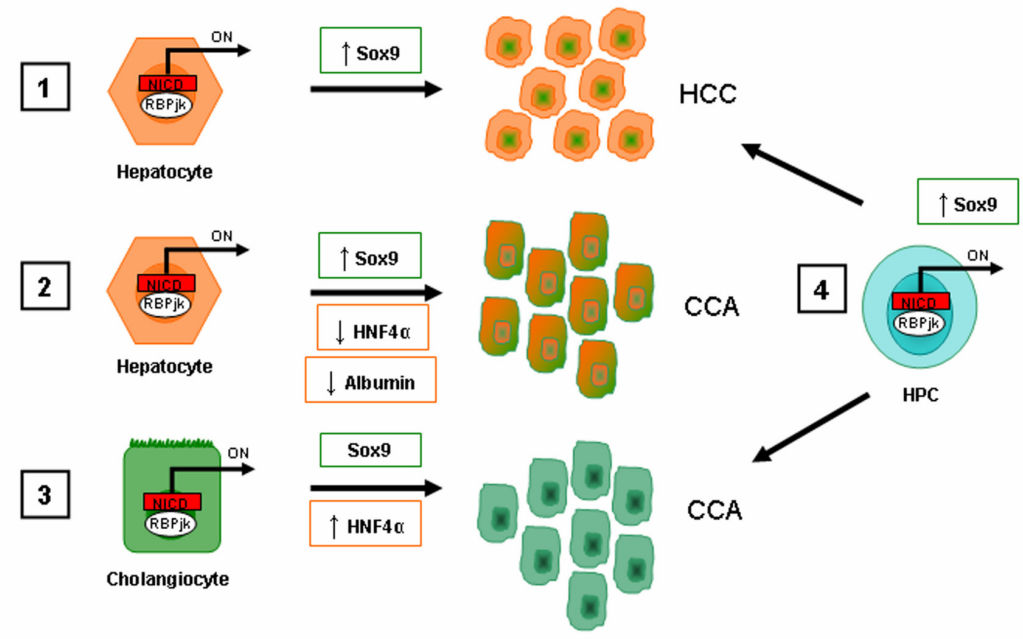

Figure 3 – Notch determines malignant phenotype in hepatocytes and cholangiocytes. Liver cancer can derive from hepatocyte or cholangiocyte or HPC. **1)** Prolonged abnormal Notch activation in hepatocytes causes aggressive and dedifferentiated HCC, in which cancer cells express the Sox-9 marker [28]. **2)** Notch

altered expression in hepatocytes is also responsible for their transdifferentiation to Sox-9 positive/HNF4α low/albumin low biliary-like cells from which intrahepatic CCA may originate [25, 26]. **3)** Aberrant activation of Notch in cholangiocytes may induce their dedifferentiation to progenitor-like cells with the re-expression of HNF4α in Sox-9 positive cells, giving rise to CCA [24]. **4)** A subpopulation of Sox-9 positive progenitors could be induced by Notch to acquire a malignant phenotype and behave as cancer initiating cells, generating either HCC or CCA. (HPC, hepatic progenitor cell; HCC, hepatocellular carcinoma; CCA, cholangiocarcinoma).

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References

1. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999 Apr 30;284(5415):770-776.

2. Lai EC. Notch signaling: control of cell communication and cell fate. Development 2004 Mar;131(5):965-973.

3. Bray SJ. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 2006 Sep;7(9):678-689.

4. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 2009 Apr 17;137(2):216-233.

5. Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, et al. Notch signaling controls liver development by regulating biliary differentiation. Development 2009 May;136(10):1727-1739.

6. Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 2004 Dec;127(6):1775-1786.

7. Geisler F, Nagl F, Mazur PK, Lee M, Zimber-Strobl U, Strobl LJ, et al. Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology (Baltimore, Md 2008 Aug;48(2):607-616.

8. Lozier J, McCright B, Gridley T. Notch signaling regulates bile duct morphogenesis in mice. PLoS One 2008;3(3):e1851.

9. McCright B, Lozier J, Gridley T. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. Development 2002 Feb;129(4):1075-1082.

10. Sparks EE, Huppert KA, Brown MA, Washington MK, Huppert SS. Notch signaling regulates formation of the three-dimensional architecture of intrahepatic bile ducts in mice. Hepatology (Baltimore, Md 2010 Apr;51(4):1391-1400.

11. Tanimizu N, Miyajima A. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. J Cell Sci 2004 Jul 1;117(Pt 15):3165-3174.

12. Hofmann JJ, Zovein AC, Koh H, Radtke F, Weinmaster G, Iruela-Arispe ML. Jagged1 in the portal vein mesenchyme regulates intrahepatic bile duct development: insights into Alagille syndrome. Development 2010 Dec;137(23):4061-4072.

13. Loomes KM, Russo P, Ryan M, Nelson A, Underkoffler L, Glover C, et al. Bile duct proliferation in liver-specific Jag1 conditional knockout mice: effects of gene dosage. Hepatology (Baltimore, Md 2007 Feb;45(2):323-330.

14. Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger BZ, et al. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 2009 Jun;136(7):2325-2333.

15. Coffinier C, Gresh L, Fiette L, Tronche F, Schutz G, Babinet C, et al. Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development 2002 Apr;129(8):1829-1838.

16. Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, et al. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet 1997 Jul;16(3):243-251.

17. Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, et al. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet 1997 Jul;16(3):235-242.

18. McDaniell R, Warthen DM, Sanchez-Lara PA, Pai A, Krantz ID, Piccoli DA, et al. NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. Am J Hum Genet 2006 Jul;79(1):169-173.

19. Lykavieris P, Hadchouel M, Chardot C, Bernard O. Outcome of liver disease in children with Alagille syndrome: a study of 163 patients. Gut 2001 Sep;49(3):431-435.

20. Fabris L, Cadamuro M, Guido M, Spirli C, Fiorotto R, Colledan M, et al. Analysis of liver repair mechanisms in Alagille syndrome and biliary atresia reveals a role for notch signaling. The American journal of pathology 2007 Aug;171(2):641-653.

21. Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, et al. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nat Med 2012 Apr;18(4):572-579.

22. Fiorotto R, Raizner A, Morell CM, Torsello B, Scirpo R, Fabris L, et al. Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. J Hepatol In press.

23. Strazzabosco M, Fabris L. The balance between Notch/Wnt signaling regulates progenitor cells' commitment during liver repair: mystery solved? J Hepatol 2012 Jan;58(1):181-183.

24. Dill MT, Tornillo L, Fritzius T, Terracciano L, Semela D, Bettler B, et al. Constitutive Notch2 signaling induces hepatic tumors in mice. Hepatology (Baltimore, Md 2012 Nov 23.

25. Fan B, Malato Y, Calvisi DF, Naqvi S, Razumilava N, Ribback S, et al. Cholangiocarcinomas can originate from hepatocytes in mice. J Clin Invest 2012 Aug 1;122(8):2911-2915.

26. Sekiya S, Suzuki A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. J Clin Invest 2012 Nov 1;122(11):3914-3918.

27. Strazzabosco M, Fabris L. Notch signaling in hepatocellular carcinoma: guilty in association! Gastroenterology 2012 Dec;143(6):1430-1434.

28. Villanueva A, Alsinet C, Yanger K, Hoshida Y, Zong Y, Toffanin S, et al. Notch signaling is activated in human hepatocellular carcinoma and induces tumor formation in mice. Gastroenterology 2012 Dec;143(6):1660-1669 e1667.

29. Razumilava N, Gores GJ. Notch driven carcinogenesis: The merging of hepatocellular cancer and cholangiocarcinoma into a common molecular liver cancer subtype. J Hepatol 2013 Jan 22.

30. Fausto N, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 2003 Jan;120(1):117-130.

31. Michalopoulos GK. Liver regeneration: alternative epithelial pathways. Int J Biochem Cell Biol 2011 Feb;43(2):173-179.

32. Roskams TA, Libbrecht L, Desmet VJ. Progenitor cells in diseased human liver. Semin Liver Dis 2003 Nov;23(4):385-396.

33. Roskams TA, Theise ND, Balabaud C, Bhagat G, Bhathal PS, Bioulac-Sage P, et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology (Baltimore, Md 2004 Jun;39(6):1739-1745.

34. Burke ZD, Reed KR, Phesse TJ, Sansom OJ, Clarke AR, Tosh D. Liver zonation occurs through a beta-catenin-dependent, c-Myc-independent mechanism. Gastroenterology 2009 Jun;136(7):2316-2324 e2311-2313.

35. Roskams T, Desmet V. Ductular reaction and its diagnostic significance. Semin Diagn Pathol 1998 Nov;15(4):259-269.

36. Strazzabosco M, Fabris L. Development of the bile ducts: essentials for the clinical hepatologist. J Hepatol 2012 May;56(5):1159-1170.

37. Fabris L, Strazzabosco M. Epithelial-mesenchymal interactions in biliary diseases. Semin Liver Dis 2011 Feb;31(1):11-32.

38. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology (Baltimore, Md 2006 Feb;43(2 Suppl 1):S45-53.

39. Strazzabosco M, Fabris L, Spirli C. Pathophysiology of cholangiopathies. J Clin Gastroenterol 2005 Apr;39(4 Suppl 2):S90-S102.

40. Nijjar SS, Crosby HA, Wallace L, Hubscher SG, Strain AJ. Notch receptor expression in adult human liver: a possible role in bile duct formation and hepatic neovascularization. Hepatology (Baltimore, Md 2001 Dec;34(6):1184-1192.

41. Spee B, Carpino G, Schotanus BA, Katoonizadeh A, Vander Borght S, Gaudio E, et al. Characterisation of the liver progenitor cell niche in liver diseases: potential involvement of Wnt and Notch signalling. Gut 2010 Feb;59(2):247-257.

42. Sawitza I, Kordes C, Reister S, Haussinger D. The niche of stellate cells within rat liver. Hepatology (Baltimore, Md 2009 Nov;50(5):1617-1624.

43. Chen Y, Zheng S, Qi D, Zheng S, Guo J, Zhang S, et al. Inhibition of Notch signaling by a gamma-secretase inhibitor attenuates hepatic fibrosis in rats. PLoS One 2012;7(10):e46512.

44. Nijjar SS, Wallace L, Crosby HA, Hubscher SG, Strain AJ. Altered Notch ligand expression in human liver disease: further evidence for a role of the Notch signaling pathway in hepatic neovascularization and biliary ductular defects. The American journal of pathology 2002 May;160(5):1695-1703.

45. Tsai S, Gurakar A, Anders R, Lam-Himlin D, Boitnott J, Pawlik TM. Management of large hepatocellular carcinoma in adult patients with Alagille syndrome: a case report and review of literature. Dig Dis Sci 2010 Nov;55(11):3052-3058.

46. Jeliazkova P, Jors S, Lee M, Zimber-Strobl U, Ferrer J, Schmid RM, et al. Canonical Notch2 signaling determines biliary cell fates of embryonic hepatoblasts and adult hepatocytes independent of Hes1. Hepatology (Baltimore, Md 2013 Jan 12.

47. Tchorz JS, Kinter J, Muller M, Tornillo L, Heim MH, Bettler B. Notch2 signaling promotes biliary epithelial cell fate specification and tubulogenesis during bile duct development in mice. Hepatology (Baltimore, Md 2009 Sep;50(3):871-879.

48. Espanol-Suner R, Carpentier R, Van Hul N, Legry V, Achouri Y, Cordi S, et al. Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. Gastroenterology 2012 Dec;143(6):1564-1575 e1567.

49. Diehl AM. Neighborhood watch orchestrates liver regeneration. Nat Med 2012 Apr;18(4):497-499.

50. Omenetti A, Choi S, Michelotti G, Diehl AM. Hedgehog signaling in the liver. J Hepatol 2011 Feb;54(2):366-373.

51. Alvaro D, Bragazzi MC, Benedetti A, Fabris L, Fava G, Invernizzi P, et al. Cholangiocarcinoma in Italy: A national survey on clinical characteristics, diagnostic modalities and treatment. Results from the "Cholangiocarcinoma" committee of the Italian Association for the Study of Liver disease. Dig Liver Dis 2011 Jan;43(1):60-65.

52. Palmer WC, Patel T. Are common factors involved in the pathogenesis of primary liver cancers? A meta-analysis of risk factors for intrahepatic cholangiocarcinoma. J Hepatol 2012 Jul;57(1):69-76.

53. Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med 2006 Apr;12(4):410-416.

54. Komuta M, Govaere O, Vandecaveye V, Akiba J, Van Steenbergen W, Verslype C, et al. Histological diversity in cholangiocellular carcinoma reflects the different cholangiocyte phenotypes. Hepatology (Baltimore, Md 2012 Jun;55(6):1876-1888.

55. Chan IS, Guy CD, Chen Y, Lu J, Swiderska-Syn M, Michelotti GA, et al. Paracrine Hedgehog signaling drives metabolic changes in hepatocellular carcinoma. Cancer Res 2012 Dec 15;72(24):6344-6350.

56. Philips GM, Chan IS, Swiderska M, Schroder VT, Guy C, Karaca GF, et al. Hedgehog signaling antagonist promotes regression of both liver fibrosis and hepatocellular carcinoma in a murine model of primary liver cancer. PLoS One 2011;6(9):e23943.

57. Anson M, Crain-Denoyelle AM, Baud V, Chereau F, Gougelet A, Terris B, et al. Oncogenic beta-catenin triggers an inflammatory response that determines the aggressiveness of hepatocellular carcinoma in mice. J Clin Invest 2012 Feb 1;122(2):586-599.

58. Zulehner G, Mikula M, Schneller D, van Zijl F, Huber H, Sieghart W, et al. Nuclear beta-catenin induces an early liver progenitor phenotype in hepatocellular carcinoma and promotes tumor recurrence. The American journal of pathology 2010 Jan;176(1):472-481.

59. Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 1991 Aug 23;66(4):649-661.

60. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004 Oct 8;306(5694):269-271.

61. Ranganathan P, Weaver KL, Capobianco AJ. Notch signalling in solid tumours: a little bit of everything but not all the time. Nat Rev Cancer 2011 May;11(5):338-351.

62. Croquelois A, Blindenbacher A, Terracciano L, Wang X, Langer I, Radtke F, et al. Inducible inactivation of Notch1 causes nodular regenerative hyperplasia in mice. Hepatology (Baltimore, Md 2005 Mar;41(3):487-496.

63. Qi R, An H, Yu Y, Zhang M, Liu S, Xu H, et al. Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. Cancer Res 2003 Dec 1;63(23):8323-8329.

64. Viatour P, Ehmer U, Saddic LA, Dorrell C, Andersen JB, Lin C, et al. Notch signaling inhibits hepatocellular carcinoma following inactivation of the RB pathway. J Exp Med 2011 Sep 26;208(10):1963-1976.

65. Wang C, Qi R, Li N, Wang Z, An H, Zhang Q, et al. Notch1 signaling sensitizes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human hepatocellular carcinoma cells by inhibiting Akt/Hdm2-mediated p53

degradation and up-regulating p53-dependent DR5 expression. J Biol Chem 2009 Jun 12;284(24):16183-16190.

66. Giovannini C, Gramantieri L, Chieco P, Minguzzi M, Lago F, Pianetti S, et al. Selective ablation of Notch3 in HCC enhances doxorubicin's death promoting effect by a p53 dependent mechanism. J Hepatol 2009 May;50(5):969-979.

67. Lim SO, Park YM, Kim HS, Quan X, Yoo JE, Park YN, et al. Notch1 differentially regulates oncogenesis by wildtype p53 overexpression and p53 mutation in grade III hepatocellular carcinoma. Hepatology (Baltimore, Md 2011 Apr;53(4):1352-1362.

68. Liu M, Lee DF, Chen CT, Yen CJ, Li LY, Lee HJ, et al. IKKalpha activation of NOTCH links tumorigenesis via FOXA2 suppression. Mol Cell 2012 Jan 27;45(2):171-184.

69. Guo X, Xiong L, Sun T, Peng R, Zou L, Zhu H, et al. Expression features of SOX9 associate with tumor progression and poor prognosis of hepatocellular carcinoma. Diagn Pathol 2012;7:44.

70. Ishimura N, Bronk SF, Gores GJ. Inducible nitric oxide synthase up-regulates Notch-1 in mouse cholangiocytes: implications for carcinogenesis. Gastroenterology 2005 May;128(5):1354-1368.

71. Sempoux C, Jibara G, Ward SC, Fan C, Qin L, Roayaie S, et al. Intrahepatic cholangiocarcinoma: new insights in pathology. Semin Liver Dis 2011 Feb;31(1):49-60.

72. Dill MT, Rothweiler S, Djonov V, Hlushchuk R, Tornillo L, Terracciano L, et al. Disruption of Notch1 induces vascular remodeling, intussusceptive angiogenesis, and angiosarcomas in livers of mice. Gastroenterology 2011 Apr;142(4):967-977 e962.

73. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 2005 Jun 16;435(7044):959-963.

74. Yan M, Callahan CA, Beyer JC, Allamneni KP, Zhang G, Ridgway JB, et al. Chronic DLL4 blockade induces vascular neoplasms. Nature 2010 Feb 11;463(7282):E6-7.

Chapter 4

Notch signaling and new therapeutic options in liver disease

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Summary

Notch signaling is a crucial determinant of cell fate decision during development and disease in several organs. Notch effects are strictly dependent on the cellular context in which it is activated. In the liver, Notch signaling is involved in biliary tree development and tubulogenesis. Recent advances have shed the light on Notch as a critical player in liver regeneration and repair, as well as in liver metabolism and inflammation and cancer. Notch signaling is finely regulated at several levels. The complexity of the pathway provides several possible targets for development of therapeutic agents able to inhibit Notch. Recent reports have shown that persistent activation of Notch signaling is associated with liver malignancies, particularly hepatocellular with stem cell features and cholangiocarcinoma. These novel findings suggest that interfering with the aberrant activation of Notch pathway may have therapeutic relevance. However, further studies are needed to clarify the mechanisms regulating physiologic and pathologic Notch activation in the adult liver, to better understand the mechanistic role(s) of Notch in liver diseases and to develop safe and specific therapeutic agents.

LIST OF ABBREVIATIONS: Jag-1, Jagged-1; **Dll**, Delta-like; **NICD**, Notch intracellular domain; **RBP-Jκ**, Recombination Signal Binding Protein Immunoglobulin Kappa J; **MAML1**, Mastermind-like 1; **Hes1**, Hairy enhancer of split-1; **Hey1**, Hairy enhancer of split-related with YRPW motif1; **Sox9**, Sex determining region Y-box 9; **HNF1β**, Hepatocyte nuclear factor 1β; **IHBD**, intrahepatic bile duct; **AGS**, Alagille syndrome; **GSI**, gamma-secretase inhibitor; **mAbs**, monoclonal antibodies; **HPC**, hepatic progenitor cell; **K7**, cytokeratin-7; **HSC**, hepatic stellate cell; **CCA**, cholangiocarcinoma; **HCC**, hepatocellular carcinoma; **CSC**, cancer stem cell; **T-ALL**, T-cell acute lymphoblastic leukaemia; **K19**, cytokeratin-19; **N1ICD**, Notch-1 intracellular domain; **N2ICD**, Notch-2 intracellular domain; **CCL4**, carbon tetrachloride.

Notch signaling is a developmental pathway that regulates several fundamental cellular processes including cell fate and

differentiation. Four transmembrane Notch receptors (Notch-1, -2, -3, -4) and two types of ligands, Serrate/Jagged (Jag-1, -2) or Delta-like (Dll-1, -3, -4) constitute the Notch system, along with several other components that transduce and regulate the signal. Activation of Notch signaling requires a direct contact between cells expressing Notch ligands and cells expressing Notch receptors; often both the "transmitting" and the "receiving" cells are modified by their interaction. Initially cells express both Notch receptor and ligands, but as the interaction continues, one cell upregulates the ligands and down regulates the receptor, becoming a "transmitting cell", whereas the opposite holds for the receiving cell[1]. Ligand-activated Notch receptors are cleaved by the γ-secretase complex, leading to the release of the Notch intracellular domain (NICD). NICD translocates into the nucleus where while binding the RBP-Jκ transcription factor, displaces the associated co-repressors and recruits associated co-activators (i.e. MAML1)[2-5]. The signal culminates with the expression of Notch target genes, such as the family of Hes and Hey related transcription factors. Regarding the liver, Notch partly controls also the expression of Sox9 and HNF1β, key players in hepatic lineage commitment[6-8].

As expected from a signaling mechanism involved in organ morphogenesis, Notch is finely tuned in a tissue- and time-dependent fashion, and it is also controlled through post-translational modifications such as ubiquitinylation, glycosylation or endocytosis. Continuous Notch activation requires constant exposure to additional ligands, as NICD undergoes rapid proteosomal degradation[2-5]. Furthermore, the effects of Notch signaling depend upon the cell types involved and the presence of signals from other pathways, including Wnt and Hedgehog.

Studies based on rodent models of Notch loss or gain of function have demonstrated that Notch is involved in several stages of intrahepatic bile duct (IHBD) morphogenesis[9]. Jag-1-positive mesenchymal cells at the parenchymal/portal interface of the nascent portal space induce the expression of cholangiocytes-specific markers in adjacent hepatoblasts, committing them to the biliary lineage. Furthermore, by regulating Sox9 and HNF1β, Notch plays an essential role in the formation of the inner leaflet of the duplicating ductal plate and also in biliary tubule formation[6-8, 10-16]. These data are consistent with the association of Alagille syndrome (AGS) (an autosomal dominant disorder characterized by ductopenia and cholestasis) with Jag-1[17, 18] (in some cases Notch-2[19]) mutations. Beyond development, other important roles of Notch are emerging that significantly impact on liver physiology and diseases. As will be discussed below, several studies indicate that the Notch pathway plays a key role in maintaining liver tissue homeostasis in the post-natal life and is involved in the reparative reaction to biliary damage, as well as in liver carcinogenesis, metabolism and inflammatory responses. This review will focus on the involvement of Notch in liver repair and carcinogenesis and the possible therapeutic implications.

Better understanding of the Notch pathway and of its relevance in pathophysiological processes prompted the development of a broad spectrum of molecules able to interfere with its signaling by 1) blocking the activation of Notch receptors (γ-secretase inhibitors or GSIs), 2) blocking the binding of the ligand (monoclonal antibodies [mAbs], decoys) or 3) blocking the transcriptional activity of NICD (blocking peptides). Some of these molecules are in a preclinical phase or in an advanced phase I clinical trials for cancer treatment (reviewed in[20, 21]) (see figure 1 and table 1).

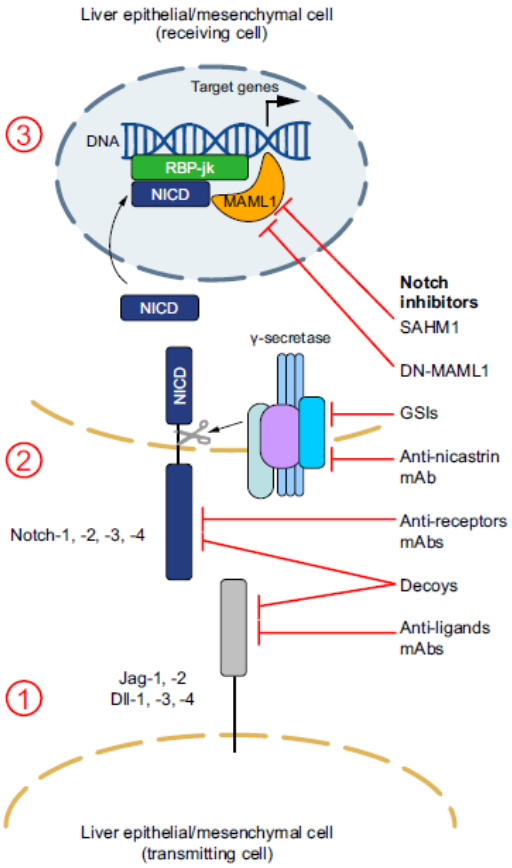

Figure 1: Schematic representation of Notch signaling and potential inhibitory strategies. Notch pathway requires different steps to transmit the signal from the "transmitting cell" to the adjacent "receiving cell". **1)** Ligand/receptor binding is the very first step that leads to signaling activation. Notch inhibition can be achieved by interfering with this step. Recent Notch **neutralizing antibodies** proved to be highly specific for the target isoforms of receptors/ligands. They target the Notch regulatory region (NRR) on the extracellular portion of the receptors and can selectively recognize Notch1 (NRR1 mAb[56, 57]), Notch2 (NRR2 mAb[57]) or Notch3 (NRR3 mAb[58]) receptors. Other mAbs compete with endogenous ligand at the ligand-binding domain level[56]. Immunostrategies directed against ligands (i.e. mAbs recognizing Jag1, Dll4, Dll1) showed inhibited tumor growth and angiogenesis[59]. These antibodies are now in phase I trials investigation (OMP-59R5 [anti-Notch2-3] and OMP-21M18 [anti-Dll4]). The high specificity of mAbs decreases the toxicity that can derive from pan-Notch inhibition. mAbs can target the desired Notch molecule that is aberrantly upregulated, sparing the other isoforms. Soluble proteins mimicking Notch receptors or ligands but lacking the transmembrane portion necessary for signal activation can be used as **Decoys** to compete with endogenous Notch1[60] Jag1[61] and Dll1[62]. **2)** The next fundamental step relies upon γ-secretase dependent receptor-proteolysis. **GSIs** are the most investigated Notch-inhibiting compounds, since they have already been tested in clinical trials to treat Alzheimer's disease[63]. GSIs are potent non-selective Notch inhibitors that target the activating proteolysis of Notch intracellular domain operated by the γ-secretase enzyme and

thus inhibit non-specifically all four Notch receptors isoforms. GSIs are being tested in phase I clinical trials for T-all leukaemia, breast cancer and other solid tumors, either alone or in combination with standard of care treatment. Although appealing, GSIs based therapy suffers from some drawbacks. GSI might have off target effects on other γ-secretase dependent pathways, and long term GSI treatment leads to intestinal toxicity as a result of combined Notch-1 and -2 inhibition. Therefore, alternative strategies have been designed, such as immunotherapy for the extracellular domain of nicastrin (i.e. one of the subunit of the γ-secretase complex). This antibody recognizes nicastrin in the active enzymatic complex, thus acting as pan-Notch inhibitor[64]. **3)** Receptor cleavage allows the release of the NICD, which translocates to the nucleus where it binds the DNA-binding partner RBP-jk and recruits the co-activator MAML1 necessary for Notch target gene transcription. Also, cell permeable **blocking peptides** (dominant negative [DN]-MAML1, stapled peptide SAHM1) can be used to interfere with the formation of the nuclear complex NICD/CSL and inhibit the transcriptional activity of NICD. These newly designed molecules reviewed in[20] are promising but need further investigation. *(mAbs, monoclonal antibodies; GSIs, γ-secretase inhibitors; NICD, notch intracellular domain).*

Blocking strategies	Status*	Advantages	Disavantages	Potential liver applications
GSIs				
γ-secretase inhibitors	Preclinical studies Phase I trials	• Potent non-selective pan-Notch inhibitors	• Off-target effects • Intestinal toxicity • Dose-limiting complications	Reduce pathologic liver repair and fibrogenic process
Blocking peptides				
DN–MAML1 SAHM1	*In vitro* studies Preclinical studies	• Small size • Structural compatibility with target protein	• Unknown pharmacokinetics • Unknown biodistribution	Blockage of Notch signaling in liver cancer
mAbs				
Anti-NRR1, NRR2, NRR3 Anti-N1, N2, N3 Anti-Jag1, Dll1, Dll4 Anti-nicastrin	*In vitro* studies Preclinical studies Phase I trials	• Decreased toxicity • High specificity • Possibility to target a specific isoform	• Limited biodistribution • Unknown pharmacokinetics	Target Jag-1 positive mesenchymal cells Target Notch-1, -2 to reduce HPC-driven ductular reaction
Decoys				
Soluble N1, Jag1, Dll1	*In vitro* studies Preclinical studies	• Small, soluble molecules	• Unknown pharmacokinetics • Unknown biodistribution	Selectively target Notch-1 or -2 in liver cancer cells

Table 1 – Available Notch-interfering agents and suggested applications for liver diseases treatment. Table 1 shows different classes of compounds to inhibit Notch signaling, some of which are under preclinical investigation. These agents block Notch signaling at different points of the pathway cascade, and each have advantages/disadvantages. Potential applications in liver disease are suggested, albeit fully speculative. * To our knowledge no phase I clinical study is being done in liver diseases. For a full discussion see refs[20,21].

Notch signaling and liver repair

In chronic liver diseases, liver repair requires the concerted action of epithelial, mesenchymal and inflammatory cells. Central to the cross talk between these cell types are hepatic progenitor cells

(HPCs or reactive cholangiocyes). This cell population, nearly absent in normal livers, expands significantly following liver injury and expresses an array of inflammatory mediators, cytokines and receptors that help establish the cellular crosstalk needed for epithelial healing. Unfortunately, continuous expansion of this reactive cell population is associated with persistent inflammation, mesenchymal cell activation, and portal fibrosis[22-24], leading to the deposition of the fibro-vascular stroma that is ultimately responsible for the architectural distortion of progressive liver diseases.

Several liver morphogenetic pathways are reactivated in HPCs during liver repair; for example, Notch acts in concert with Wnt[25]or Hedgehog[26], to restore liver architecture and function. In AGS, paucity of bile ducts is associated with impaired biliary differentiation of HPCs, consistent with the hypothesis that Notch is a default inducer of biliary specification. As respect to other cholestatic diseases, in AGS, HPCs are decreased, while intermediate cytokeratin 7 (K7)-positive hepatocytes accumulate, suggesting that HPCs are forced towards the hepatocellular fate, or that transdifferentiation of hepatocytes into HPCs is blocked[22]. Of note, HNF-1β, a transcription factor critical for biliary specification, is down-regulated in the accumulating K7-positive intermediate hepatocytes. Conversely, a reciprocal relationship between Hes1 and the transcription factor PDX-1 has been described[27].

In the adult liver, Notch components are expressed in both the epithelial and mesenchymal compartments, and are differentially regulated in case of injury. Notch-1 and -2 are expressed in epithelial liver cells and during biliary damage are upregulated in cholangiocytes and HPCs[25, 28, 29], whereas Notch-3 and -4 are expressed in mesenchymal and endothelial cells[30]. Notch-1 and Notch-3, both expressed by quiescent hepatic stellate cells (HSC)[26,

31, 32], are respectively downregulated and upregulated during HSC trasdifferentiation into myofibroblasts. The ligand Jag-1, has been detected on proliferating bile ductules[25, 29, 33] on hepatocytes[31], as well as on activated HSC[26, 31, 32] and is strongly upregulated in injured livers. It is worth noting that in patients with AGS there is limited deposition of fibrotic tissue, consistent with the slow progression to cirrhosis seen in AGS patients[34]. Thus, Jag-1, the protein defective in AGS may signal to portal myofibroblasts and induce collagen production or proliferation. Indeed, Notch has been recently associated with HSC transdifferentiation to myofibroblasts. Jag-1 and Notch-2 seem to play a role in facilitating hedgehog signaling in fibrosis[26]. Notch activation and upregulation of Notch-3 in myofibroblasts has been described in an experimental rat model of carbon tetrachloride(CCl4)-induced liver fibrosis. In this model, pharmacological Notch inhibition reduced the extent of liver fibrosis[31].

Mice with liver conditional defect in Notch receptors or in the common transcription factor RBP-Jκ are unable to mount an effective HPCs response after liver damage[28]. In addition, Notch-2 is essential for biliary tubular morphogenesis, as in liver-specific Notch-2 KO mice the generation of biliary-committed HPCs is still possible, but tubule formation is impaired[28]. Tubule formation is a fundamental aspect of biliary repair, to restore the branching architecture of the ductal system. If a proper branching structure is not regenerated, the final result will be parenchymal necrosis or vanishing bile duct syndrome and fibrosis, i.e. the final stage of several cholangiopathies.

During liver repair, cell-cell interactions between Notch-expressing HPCs and Jag-1-expressing portal fibroblasts regulate biliary specification of HPCs. The decision between the hepatocellular

and the biliary commitment depends upon the type of inflammatory reaction and the balanced activation of Wnt or Notch signaling, respectively[25, 28, 35, 36]. The histogenesis of HPCs is however not completely understood. A recent paper by Yanger et al[37], adds further credit to the hypothesis that, depending on the type of liver injury, reactive cholangiocytes may actually be generated by a Notch-dependent reprogramming of hepatocytes. This is consistent with reports showing that intrahepatic cholangiocarcinoma (CCA) may also derive from hepatocytes[38, 39] (see below).

Notch signaling and liver cancer

Liver cirrhosis is a common feature of hepatocellular carcinoma (HCC) (85-90%) and in an increasing proportion of intrahepatic CCA (85-90%)[40, 41]. The key features of cirrhosis, necroinflammation, fibrosis and HPCs-driven hepatic reparative process are permissive to the reprogramming of HPCs into cancer initiating cells (cancer stem cells, CSC)[42]. Consistent with this concept, a subset of tumours that exhibit characteristics of both CCA and HCC, are thought to arise from the HPCs compartment[43]; some show gene expression signatures of Notch activation.

On the contrary to T-cell acute lymphoblastic leukaemia (T-ALL)[44] gain-of-function mutations of Notch receptors have not been reported yet in solid tumours, but there is increasing evidence that inappropriate Notch pathway activation occurs in several cancers[20, 45], including liver cancers. Pro-mitogenic functions of Notch in hepatocytes have been shown in experimental models of partial hepatectomy[46, 47]; the pro-oncogenic role of Notch is further supported by genome wide analysis on HCC samples reporting that among others, the Notch coactivator MAML2 is a target of genetic alterations[48]. Recent studies suggest that Notch signaling is

involved in liver oncogenesis by activating a subset of Sox9 and K19-positive progenitors, (see ref[9, 49] for a discussion of the role of Notch in liver cancer). Mice with liver-specific constitutive activation of Notch-1 intracellular domain (N1ICD[50]) develop HCC once they reach adult age. The histology of liver lesions in these mice showed features similar to human HCC and the presence of proliferating K19-positive cells (most likely CSC). Genomic profiling revealed that the Notch-specific gene expression signature reported in mice overexpressing N1ICD was present in a cluster of patients with HCC and was associated with genes related to cellular proliferation and Sox9 expression[50]. Accordingly, spontaneous development of dedifferentiated HCC occurred in experimental models with constitutive Notch-2 intracellular domain (N2ICD) activation in HPCs[51]; again, Notch-induced malignant hepatocyte transformation was associated with the expression of Sox9 and down-regulation of the hepatocyte-related genes. Interestingly, when overexpression of N2ICD was associated with the administration of oncogenic stimuli, foci of CCA developed in the liver. Thioacetamide treatment in mice overexpressing hepatic N1ICD resulted to a rapid onset of CCA; fate-tracing studies proved that CCA cells derived from hepatocytes conversion to a biliary, K19 positive phenotype[39] as a consequence of ectopic Notch activation. Accordingly, N1ICD cooperation with AKT signaling in hepatocytes stimulated their malignant dedifferentiation leading to CCA development[38]. These findings are consistent with increasing incidence of intrahepatic CCAs in patients with cirrhosis of parenchymal origin[52]. Moreover, inflammatory mediators (i.e. inducible nitric oxide synthase)-stimulated N1ICD expression was reported in human CCA samples[53], further supporting a malignant role of ectopically expressed Notch in the liver.

Taken together, these data indicate that persistent activation of Notch signaling may play an oncogenic role depending on modifier factors, such as the inflammatory field or the presence of other carcinogenetic conditions, potentially giving rise to either HCC with stem cell features or to CCA.

Potential Notch-based therapeutic strategies.

The findings discussed above provide an intriguing rationale for Notch-based therapies in patients with liver diseases. Ductular reactive cells and HPCs express Notch-1 and -2 receptors, which can be activated by neighbouring cells (including Jag-1 positive mesenchymal cells) thereby regulating liver repair and regenerative processes[25, 26, 28]. GSIs efficiently inhibited Notch signaling in mouse models of cholestatic liver disease[28] and reduced fibrosis in CCL4-treated rodents[31]. It is interesting to speculate that GSIs may be used to inhibit ductular reaction and HSC activation, thereby reducing the extent of liver fibrosis and architectural distortion. Unfortunately, acting on all signaling pathways requiring proteolytic cleavage of the receptor, GSIs are not cell selective, neither system-specific. In addition, GSIs have a considerable toxicity profile, mainly affecting gut functionality, as a result of combined Notch-1 and -2 inhibition disrupting intestinal stem cells biology[54]. Thus, the use of the more selective monoclonal antibodies against Notch-1 and -2 or Jag-1 may provide a considerable advantage.

As discussed above, both HCC and CCA may arise when Notch is aberrantly and/or ectopically activated in experimental models[38, 39, 50, 51, 55]. Identification of a Notch signature, a fundamental step to design a targeted treatment, was reported in a subset of HCC patients[50], and Notch receptors were found overexpressed in human CCAs[55]. These subsets of liver cancers

may be good candidates for Notch-inhibition strategies. Silencing Notch pathway could potentially abrogate Notch-driven tumor progression and also interfere with tumor aggressiveness, given that Notch activation has been associated to a more malignant phenotype[39, 50, 51]. The presence of a reliable tissue-specific biomarker of Notch inhibition would be critical to apply Notch-directed therapy. Interestingly, the hepatic Notch target gene Sox9[8], has been associated to a worse prognosis in liver cancers[50]. Therefore, the role of Sox9 as a potential biomarker of Notch involvement and indication for Notch-targeted treatment should be explored.

Conclusions

Notch is being increasingly recognized as a major signaling mechanism in liver biology and in multiple pathophysiological conditions, from liver repair to carcinogenesis (figure 2). Indeed, Notch is necessary to regulate HPCs specification toward the biliary lineage and to orchestrate the reparative remodelling of the biliary tree[28]. However, the functional role of Notch in regulating HPCs/mesenchymal cross-talk during fibrogenic pathologic repair remains to be fully unveiled. Similarly, the oncogenetic action of \\Notch in liver malignancies requires further investigation, since persistent activation of this signaling has been associated to both, HCC and CCA.

Notch emerges as a potential therapeutic target, however, the chances of success of Notch-targeted strategies depend on a variety of factors. First of all, Notch activation has different effects depending on cellular and tissue context, in both physiologic and pathologic states. Second, Notch is strictly connected with other signaling mechanisms, indicating that combination therapies targeting also other signaling (for example Hedgehog) may be more effective to

target pathologic liver repair and carcinogenesis. Thus, more efforts are needed to understand the molecular mechanisms regulating Notch activation in specific cell context and the complex interplay with additional partners involved.

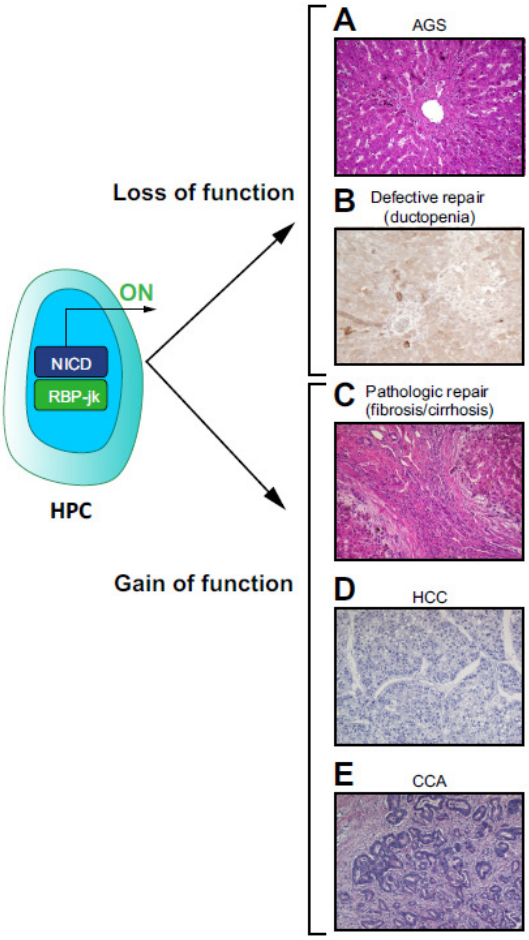

Figure 2: Pathological conditions possibly involving Notch loss/gain of function, working hypothesis. Proper Notch activation is fundamental for HPCs biology. *A)* Defective Notch signaling causes **AGS**, a condition characterized predominantly by ductopenia and consequent cholestasis. In this context, HPCs are unable to commit toward the biliary lineage and to re-organize into bile ducts. *B)* Similarly, experimental models of cholestatic liver disease[28] have demonstrated that Notch inhibition results in **altered liver repair** process and failure to regenerate bile ductules. On the contrary, *C)* continuous Notch stimulation after damage may cause **pathologic repair** that results in excessive fibrotic tissue deposition and architectural liver distortion. Moreover, aberrant and persistent Notch stimulation in HPCs may induce their malignant transformation leading to *D)* **HCC** and/or *E)* **CCA**. Micrographs represent eosin/hematoxylin staining of human samples of *A)* AGS, *C)* biliary atresia, *D)* HCC, *E)* CCA; *B)* represents K19 staining of ductular reactive/HPCs cells during liver repair in a Notch-defective mouse. (*AGS, Alagille syndrome; HPC, hepatic progenitor cells; HCC, hepatocellular carcinoma; CCA, cholangiocarcinoma; K19, cytokeratin-19).*

Conflict of interest: *the authors have nothing to disclose.*

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References

1. Fortini ME. Notch signaling: the core pathway and its posttranslational regulation. Dev Cell 2009 May;16(5):633-647.

2. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999 Apr 30;284(5415):770-776.

3. Bray SJ. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 2006 Sep;7(9):678-689.

4. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 2009 Apr 17;137(2):216-233.

5. Lai EC. Notch signaling: control of cell communication and cell fate. Development 2004 Mar;131(5):965-973.

6. Coffinier C, Gresh L, Fiette L, Tronche F, Schutz G, Babinet C, et al. Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development 2002 Apr;129(8):1829-1838.

7. Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 2004 Dec;127(6):1775-1786.

8. Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, et al. Notch signaling controls liver development by regulating biliary differentiation. Development 2009 May;136(10):1727-1739.

9. Morell CM, Fiorotto R, Fabris L, Strazzabosco M. Notch signalling beyond liver development: Emerging concepts in liver repair and oncogenesis. Clin Res Hepatol Gastroenterol 2013 Jun 24.

10. Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger BZ, et al. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 2009 Jun;136(7):2325-2333.

11. Geisler F, Nagl F, Mazur PK, Lee M, Zimber-Strobl U, Strobl LJ, et al. Liver-specific inactivation of Notch2, but not Notch1, compromises intrahepatic bile duct development in mice. Hepatology 2008 Aug;48(2):607-616.

12. Hofmann JJ, Zovein AC, Koh H, Radtke F, Weinmaster G, Iruela-Arispe ML. Jagged1 in the portal vein mesenchyme regulates intrahepatic bile duct development: insights into Alagille syndrome. Development 2010 Dec;137(23):4061-4072.

13. Loomes KM, Russo P, Ryan M, Nelson A, Underkoffler L, Glover C, et al. Bile duct proliferation in liver-specific Jag1 conditional knockout mice: effects of gene dosage. Hepatology 2007 Feb;45(2):323-330.

14. Lozier J, McCright B, Gridley T. Notch signaling regulates bile duct morphogenesis in mice. PLoS One 2008;3(3):e1851.

15. McCright B, Lozier J, Gridley T. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. Development 2002 Feb;129(4):1075-1082.

16. Sparks EE, Huppert KA, Brown MA, Washington MK, Huppert SS. Notch signaling regulates formation of the three-dimensional architecture of intrahepatic bile ducts in mice. Hepatology 2010 Apr;51(4):1391-1400.

17. Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, et al. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet 1997 Jul;16(3):243-251.

18. Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, et al. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet 1997 Jul;16(3):235-242.

19. McDaniell R, Warthen DM, Sanchez-Lara PA, Pai A, Krantz ID, Piccoli DA, et al. NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. Am J Hum Genet 2006 Jul;79(1):169-173.

20. Espinoza I, Miele L. Notch inhibitors for cancer treatment. Pharmacol Ther 2013 Aug;139(2):95-110.

21. Groth C, Fortini ME. Therapeutic approaches to modulating Notch signaling: current challenges and future prospects. Semin Cell Dev Biol 2012 Jun;23(4):465-472.

22. Fabris L, Cadamuro M, Guido M, Spirli C, Fiorotto R, Colledan M, et al. Analysis of liver repair mechanisms in Alagille syndrome and biliary atresia reveals a role for notch signaling. Am J Pathol 2007 Aug;171(2):641-653.

23. Lazaridis KN, Strazzabosco M, Larusso NF. The cholangiopathies: disorders of biliary epithelia. Gastroenterology 2004 Nov;127(5):1565-1577.

24. Strazzabosco M, Fabris L, Spirli C. Pathophysiology of cholangiopathies. J Clin Gastroenterol 2005 Apr;39(4 Suppl 2):S90-S102.

25. Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, et al. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nat Med 2012 Apr;18(4):572-579.

26. Xie G, Karaca G, Swiderska-Syn M, Michelotti GA, Kruger L, Chen Y, et al. Cross-talk between Notch and Hedgehog regulates hepatic stellate cell fate in mice. Hepatology 2013 May 23.

27. Marzioni M, Saccomanno S, Agostinelli L, Rychlicki C, De Minicis S, Pierantonelli I, et al. PDX-1/Hes-1 interactions determine cholangiocyte proliferative response to injury in rodents: possible implications for sclerosing cholangitis. J Hepatol 2013 Apr;58(4):750-756.

28. Fiorotto R, Raizner A, Morell CM, Torsello B, Scirpo R, Fabris L, et al. Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. J Hepatol 2013 Jul;59(1):124-130.

29. Spee B, Carpino G, Schotanus BA, Katoonizadeh A, Vander Borght S, Gaudio E, et al. Characterisation of the liver progenitor cell niche in liver diseases: potential involvement of Wnt and Notch signalling. Gut 2010 Feb;59(2):247-257.

30. Nijjar SS, Crosby HA, Wallace L, Hubscher SG, Strain AJ. Notch receptor expression in adult human liver: a possible role in bile duct formation and hepatic neovascularization. Hepatology 2001 Dec;34(6):1184-1192.

31. Chen Y, Zheng S, Qi D, Zheng S, Guo J, Zhang S, et al. Inhibition of Notch signaling by a gamma-secretase inhibitor attenuates hepatic fibrosis in rats. PLoS One 2012;7(10):e46512.

32. Sawitza I, Kordes C, Reister S, Haussinger D. The niche of stellate cells within rat liver. Hepatology 2009 Nov;50(5):1617-1624.

33. Nijjar SS, Wallace L, Crosby HA, Hubscher SG, Strain AJ. Altered Notch ligand expression in human liver disease: further evidence for a role of the Notch signaling pathway in hepatic neovascularization and biliary ductular defects. Am J Pathol 2002 May;160(5):1695-1703.

34. Tsai S, Gurakar A, Anders R, Lam-Himlin D, Boitnott J, Pawlik TM. Management of large hepatocellular carcinoma in adult patients with Alagille syndrome: a case report and review of literature. Dig Dis Sci 2010 Nov;55(11):3052-3058.

35. Burke ZD, Reed KR, Phesse TJ, Sansom OJ, Clarke AR, Tosh D. Liver zonation occurs through a beta-catenin-dependent, c-Myc-independent mechanism. Gastroenterology 2009 Jun;136(7):2316-2324 e2311-2313.

36. Tanimizu N, Miyajima A. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors. J Cell Sci 2004 Jul 1;117(Pt 15):3165-3174.

37. Yanger K, Zong Y, Maggs LR, Shapira SN, Maddipati R, Aiello NM, et al. Robust cellular reprogramming occurs spontaneously during liver regeneration. Genes Dev 2013 Apr 1;27(7):719-724.

38. Fan B, Malato Y, Calvisi DF, Naqvi S, Razumilava N, Ribback S, et al. Cholangiocarcinomas can originate from hepatocytes in mice. J Clin Invest 2012 Aug 1;122(8):2911-2915.

39. Sekiya S, Suzuki A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. J Clin Invest 2012 Nov 1;122(11):3914-3918.

40. Alvaro D, Bragazzi MC, Benedetti A, Fabris L, Fava G, Invernizzi P, et al. Cholangiocarcinoma in Italy: A national survey on clinical characteristics, diagnostic modalities and treatment. Results from the "Cholangiocarcinoma" committee of the Italian Association for the Study of Liver disease. Dig Liver Dis 2011 Jan;43(1):60-65.

41. Palmer WC, Patel T. Are common factors involved in the pathogenesis of primary liver cancers? A meta-analysis of risk factors for intrahepatic cholangiocarcinoma. J Hepatol 2012 Jul;57(1):69-76.

42. Lee JS, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, et al. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. Nat Med 2006 Apr;12(4):410-416.

43. Komuta M, Govaere O, Vandecaveye V, Akiba J, Van Steenbergen W, Verslype C, et al. Histological diversity in cholangiocellular carcinoma reflects the different cholangiocyte phenotypes. Hepatology 2012 Jun;55(6):1876-1888.

44. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004 Oct 8;306(5694):269-271.

45. Ranganathan P, Weaver KL, Capobianco AJ. Notch signalling in solid tumours: a little bit of everything but not all the time. Nat Rev Cancer 2011 May;11(5):338-351.

46. Kohler C, Bell AW, Bowen WC, Monga SP, Fleig W, Michalopoulos GK. Expression of Notch-1 and its ligand Jagged-1 in rat liver during liver regeneration. Hepatology 2004 Apr;39(4):1056-1065.

47. Croquelois A, Blindenbacher A, Terracciano L, Wang X, Langer I, Radtke F, et al. Inducible inactivation of Notch1 causes nodular regenerative hyperplasia in mice. Hepatology 2005 Mar;41(3):487-496.

48. Nalesnik MA, Tseng G, Ding Y, Xiang GS, Zheng ZL, Yu Y, et al. Gene deletions and amplifications in human hepatocellular carcinomas: correlation with hepatocyte growth regulation. Am J Pathol 2012 Apr;180(4):1495-1508.

49. Strazzabosco M, Fabris L. Notch signaling in hepatocellular carcinoma: guilty in association! Gastroenterology 2012 Dec;143(6):1430-1434.

50. Villanueva A, Alsinet C, Yanger K, Hoshida Y, Zong Y, Toffanin S, et al. Notch signaling is activated in human hepatocellular carcinoma and induces tumor formation in mice. Gastroenterology 2012 Dec;143(6):1660-1669 e1667.

51. Dill MT, Tornillo L, Fritzius T, Terracciano L, Semela D, Bettler B, et al. Constitutive Notch2 signaling induces hepatic tumors in mice. Hepatology 2012 Nov 23.

52. Sempoux C, Jibara G, Ward SC, Fan C, Qin L, Roayaie S, et al. Intrahepatic cholangiocarcinoma: new insights in pathology. Semin Liver Dis 2011 Feb;31(1):49-60.

\53. Ishimura N, Bronk SF, Gores GJ. Inducible nitric oxide synthase up-regulates Notch-1 in mouse cholangiocytes: implications for carcinogenesis. Gastroenterology 2005 May;128(5):1354-1368.

54. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 2005 Jun 16;435(7044):959-963.

55. Zender S, Nickeleit I, Wuestefeld T, Sorensen I, Dauch D, Bozko P, et al. A critical role for notch signaling in the formation of cholangiocellular carcinomas. Cancer Cell 2013 Jun 10;23(6):784-795.

56. Aste-Amezaga M, Zhang N, Lineberger JE, Arnold BA, Toner TJ, Gu M, et al. Characterization of Notch1 antibodies that inhibit signaling of both normal and mutated Notch1 receptors. PLoS One 2010;5(2):e9094.

57. Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y, et al. Therapeutic antibody targeting of individual Notch receptors. Nature 2010 Apr 15;464(7291):1052-1057.

58. Li K, Li Y, Wu W, Gordon WR, Chang DW, Lu M, et al. Modulation of Notch signaling by antibodies specific for the extracellular negative regulatory region of NOTCH3. J Biol Chem 2008 Mar 21;283(12):8046-8054.

59. Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature 2006 Dec 21;444(7122):1083-1087.

60. Funahashi Y, Hernandez SL, Das I, Ahn A, Huang J, Vorontchikhina M, et al. A notch1 ectodomain construct inhibits endothelial notch signaling, tumor growth, and angiogenesis. Cancer Res 2008 Jun 15;68(12):4727-4735.

61. Varnum-Finney B, Wu L, Yu M, Brashem-Stein C, Staats S, Flowers D, et al. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. J Cell Sci 2000 Dec;113 Pt 23:4313-4318.

62. Small D, Kovalenko D, Kacer D, Liaw L, Landriscina M, Di Serio C, et al. Soluble Jagged 1 represses the function of its transmembrane form to induce the formation of the Src-dependent chord-like phenotype. J Biol Chem 2001 Aug 24;276(34):32022-32030.

63. Siemers ER, Quinn JF, Kaye J, Farlow MR, Porsteinsson A, Tariot P, et al. Effects of a gamma-secretase inhibitor in a randomized study of patients with Alzheimer disease. Neurology 2006 Feb 28;66(4):602-604.

64. Hayashi I, Takatori S, Urano Y, Miyake Y, Takagi J, Sakata-Yanagimoto M, et al. Neutralization of the gamma-secretase activity by monoclonal antibody against extracellular domain of nicastrin. Oncogene 2012 Feb 9;31(6):787-798.

Chapter 5

Notch-mediated epithelial/mesenchymal interaction influences the hepatic reparative response in non-alcoholic steatohepatitis (NASH)

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ABSTRACT

BACKGROUND and AIMS: Persistent hepatic progenitor cells (HPC) activation resulting in ductular reaction (DR) is responsible for pathologic liver repair. HPC/DR expansion correlates with fibrosis in several chronic liver diseases, including non-alcoholic steatohepatitis (NASH). Increasing evidence indicates Notch signaling as one of the key regulators of HPC/DR response in several hepatic injuries. Therefore, we aimed to clarify the role of Notch in regulating HPC/DR activation in a mouse model of NASH. **METHODS:** Steatohepatitis was generated using a methionine-choline deficient (MCD) diet. For hepatocyte lineage tracing, R26R-YFP mice were infected with AAV8-TBG-Cre. HPC/DR were identified by cytokeratin19 (CK19) and Sox9. **RESULTS:** MCD diet promoted a strong HPC response that progressively diffused in the lobule, in parallel with fibrosis development. Notch was not modulated in laser-capture microdissected CK19+ve cells, whereas Notch receptors were downregulated in hepatocytes (CK19-ve). However discrete hepatocyte-like cells showed Notch-1 activation and were positive for the Notch-dependent genes Hey2 and Sox9. *In vivo* lineage tracing identified hepatocyte-derived YFP+ve cells expressing Sox9 and CK19. Treatment of a hepatocyte-cell line with immobilized Jag1 induced Sox9 expression and downregulation of albumin and the bile acid transporter (BSEP) *in vitro*. In MCD diet-fed mice, αSMA+ve HSC localized around Sox9+ve hepatocytes, suggesting that Notch activation in hepatocytes was likely promoted by hepatic stellate cells (HSC), stimulated by TGF-β1. **CONCLUSIONS:** in steatohepatitis, a hepatocytes subpopulation, exposed to myofibroblasts, contributes to pathologic DR by undergoing Notch-mediated conversion towards an

HPC-like phenotype. Our study suggests mesenchymal-Jag1 as a mediator of pathologic liver repair and a possible therapeutic target.

Abbreviations: *CK19: cytokeratin 19; DR: Ductular reaction; HPC: Hepatic progenitor cells; HSC: hepatic stellate cells; MCD: Methionine/choline deficient diet; NASH: Non alcoholic steatohepatitis; Sox9: SRY-related HMG box transcription, factor 9.*

INTRODUCTION

The liver possesses a unique regenerative potential, which is differentially regulated according to the type of damage. In chronically damaged livers, when hepatocytes and cholangiocytes replication is impaired, functional repair relies upon the contribution of the hepatic progenitor cells (HPC) compartment. HPC expansion occurs along with the recruitment of inflammatory mediators and the production of a fibrovascular stroma that sustains the newly formed tissue, giving rise to “ductular reaction” (DR). With the persistence of the damage, DR becomes the hallmark of disease progression and fibrotic evolution[1-3].

HPC/DR expansion has been associated to liver fibrosis in several chronic liver diseases, including Non-Alcoholic Steatohepatitis (NASH). Because of the increased prevalence of its risk factors (namely obesity, diabetes and metabolic syndrome) and its association with cirrhosis, liver failure and hepatocellular carcinoma (HCC), NASH is a growing worldwide health problem[4]. A strong expansion of HPC/DR, which correlates with fibrosis and with the risk of disease progression, has been described in NASH[5-7]. Because of the possibility that HPC/DR promotes disease progression and potentially cirrhotic evolution, it is fundamental to unravel the mechanisms driving HPC activation in NASH.

Signaling pathways fundamental for liver morphogenesis are also key players in liver regenerative/reparative processes. Among them, Notch signaling is well suited to finely orchestrate the cross-talk among HPC and stromal cells; in fact the signal is activated by direct homotypic or heterotypic cell-cell contacts. The ligands (Jag-1,-2, Dll-1, -3, -4) expressed on the “transmitting” cell activate Notch receptors (Notch-1 to -4) on the adjacent signal “receiving” cell; ligand/receptor interaction determines the cleavage of the Notch intracellular domain (NICD) by a γ-secretase. NICD associates with CBF1/RBPjK in the nucleus, allowing the transcription of several genes including the transcription factors Hes and Hey-related family. Notch activation is carefully tuned by the specific ubiquitynase Numb, an endogenous inhibitor that targets NICD to the proteasome[8-10].

Notch is involved in liver development, physiology and pathophysiology[9, 10]. During development, Notch modulates the expression of liver-enriched transcription factors, with Sox9 recognized as the earliest Notch-regulated marker expressed by biliary committed hepatoblasts[11]. In liver regeneration, HPC express Notch-1 and -2 receptors which may bind to Jag1 positive neighboring mesenchymal cells[12-14]. In mice lacking hepatic Notch-2 or RPB-jK undergoing a biliary damage, HPC activation is severely impaired, suggesting that Notch is involved in HPC-driven liver repair and that Notch-2 is essential for tubulogenesis[13]. Furthermore, patients with Alagille Syndrome (AGS), a cholestatic cholangiopathy linked to Jag1 or Notch-2 deficiency, show an altered reparative response, with massive accumulation of cells with an intermediate phenotype[15]. These intermediate cells may derive from activated HPC unable to further differentiate or from hepatocytes undergoing a phenotypic switch; the histogenesis of HPC is not completely clear and there is evidence in favour of both hypotheses.

Altered hepatic Notch signaling has been recently linked to liver carcinogenesis. Experimental models of constitutive activation of Notch receptors proved that Notch, in cooperation with other oncogenic pathways, leads to HCC and cholangiocarcinoma (CCA) development when aberrantly or ectopically expressed[16-20]. Accordingly, overexpression of Notch receptors has been described in human CCA[20] and a Notch signature was identified in a subset of HCC patients, along with hepatocytic Sox9 expression[19]. Interestingly, Notch activation in mature hepatocytes strongly correlates with a more malignant phenotype in hepatic cancer[16, 18, 19]. Notably, inhibition of Notch signaling with specific antibodies proved successful in decreasing tumor burden in a mouse model of liver cancer[21].

In this study we aimed to understand the role of Notch in regulating HPC/DR occurring in a mouse model of steatohepatitis. Our results show that HPC activation is linked to disease progression in terms of inflammation and fibrosis. Notch signaling (Notch-1 and Sox9) appears to be activated in a subset of hepatocytes and hepatocyte-derived HPC-like cells. These cells establish a Notch-mediated cross-talk with mesenchymal cells that could be responsible for disease progression and eventually pave the way to liver cancer development in chronically damaged livers.

MATERIALS AND METHODS

For details on immunohistochemistry, gene expression analysis and cell culture, *please see supplementary material and methods.*

Animals and experimental protocols. 8-weeks old C57BL/6 male mice were purchased from Harlan–Nossan. To induce NASH, mice were fed either methionine-choline deficient (MCD) (Laboratorio

Dottori Piccioni, Milano, Italy) or control diet for 4 up to 8 weeks. Body weight was recorded weekly throughout the experiment. For hepatocyte lineage tracing experiments, R26R-YFP reporter mice were purchased from Jacksons Laboratories. To selectively induce reporter gene (YFP) expression only in mature hepatocytes, mice were infected with the hepatocyte-specific adeno-associated virus AAV8 that carries Cre recombinase under the control of the hepatocyte-specific promoter for thyroid-binding globulin (AAV8-TBG-Cre). Viral infection with AAV8-TBG-Cre (2.5X10*11 viral particles in sterile 1X PBS) was performed via retro-orbital injection as previously described[22]. One week after injection mice were fed with MCD diet as indicated. Mice were anaesthetized with sevofluorane and blood was collected by cardiac puncture. Livers were rapidly removed, tissues were harvested and some aliquots were snap frozen in liquid nitrogen whereas portions of each liver lobe were fixed in formalin and embedded in paraffin. All animal experiments were performed according to protocols approved by the Italian Ministry of Health, by the University Commission for Animal Care following the criteria of the Italian National Research Council, and by the Yale University Institutional Animal Care and Use Committee.

Laser Capture Microdissection (LCM). LCM studies were performed to evaluate differential changes in gene expression specifically in CK19+ve cells (mainly HPC/DR) or in CK19-ve parenchymal areas (mainly hepatocytes). Briefly, 10µm thick frozen liver sections were quickly stained with CK19 to specifically label HPC/DR, and microdissected under the MMI CellCut Plus laser capture microscope (MMI, Hasslett, MI). After collecting CK19+ve cells/structures on a MMI isolation cap, CK19-ve areas from the same sample were selected in a different tube. Total RNA was extracted using RNeasy Plus Micro Kit (Qiagen) according to the

manufacturer's instructions, and reverse-transcribed with the High capacity cDNA reverse transcription Kit (Life Technologies, Carlsbad, CA). mRNA expression was quantified by real-time PCR (see supplementary section) following cDNA pre-amplification (TaqMan PreAmp Master mix, Life Technologies, Carlsbad, CA). Target genes-specific TaqMan probes were used for both amplifications.

Statistical analysis. Results are shown as means ± SD. Statistical comparisons were made using Student t-test or one-way analysis of variance (ANOVA) test with Tukey's correction for multiple comparisons where appropriate. Correlation studies were performed using Pearson's coefficient with two-tailed distribution. Statistical analysis was performed using SPSS 13.0 (Statistical Package for Social Science 2013); p values <0.05 were considered significant.

RESULTS

MCD diet induces HPC/DR expansion throughout the parenchyma along with inflammation and fibrosis. HPC/DR expansion in the liver of mice with steatohepatitis was assessed using cytokeratin-19 (CK19), a well-established marker. As shown in figure 1A and 1B, the extension of HPC/DR calculated as the percentage of the total area covered by CK19+ve cells, increased steadily in MCD diet-fed mice reaching approximately a 4-folds increase in mice treated for 8 weeks. Moreover, the pattern of CK19+ve cell distribution changed with the progression of hepatic damage, as, at the 4^{th} week of treatment, HPC/DR cells were mainly localized around portal areas, while, by the 8^{th} week they appeared to be spread into the liver lobule where CK19+ve cells established intimate contacts with fat-laden hepatocytes (figure 1A). Differential quantification of CK19 positivity in portal versus lobular areas showed that portal DR

increased by 2-folds within 8 weeks of diet, whereas lobular CK19 positivity expanded by more than 10 times (figure 1C).

In the context of liver diseases, DR is generally associated with inflammation. By investigating the expression profile of inflammatory markers we observed that gene expression of leucocyte activation markers CD11b and TNFα were both induced by approximately 4 to 7-folds after MCD diet-feeding for respectively 4 and 8 weeks (supplementary figure 1A) and correlated with CK19 positivity (supplementary figure 1B).

In parallel with the expansion of DR we observed a significant increase in hepatic fibrosis, as evaluated by collagen staining with Sirius Red (figures 2A and 2B). A strongly positive correlation was also evident between Sirius Red and the percentage of CK19+ve cells (figure 2B). DR/HPC distribution histologically paralleled collagen deposition pattern, further indicating a functional link between CK19+ve cells and collagen-producing cells. Consistent with this hypothesis, the fibrotic markers TGF-β1 and procollagen-1 (COL1A1) were significantly induced by MCD diet (figures 2C and 2D, upper panels) and positively correlated with the presence of CK19+ve cells (figures 2C and 2D, lower panels), again supporting the association between DR and liver fibrosis.

The correlation of both the inflammatory and the fibrotic processes with CK19 positivity indicates a possible link between the presence of CK19+ve cells and the worsening of MCD diet-induced liver injury.

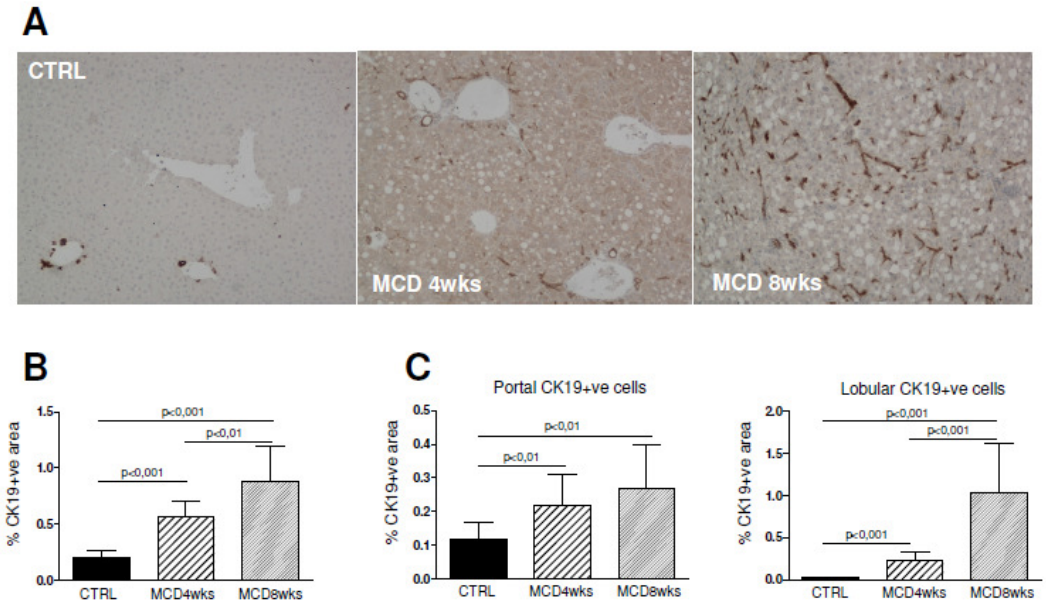

Figure 1: HPC response is activated after MCD diet. (A) CK19 staining shows progressive HPC expansion occurring in mice treated with MCD diet, as confirmed by **(B)** the quantification of the percentage of the total area covered by CK19+ve cells. As diet progressed, CK19+ve cells spread from portal areas into the lobule: as shown in panel **C**, CK19 positivity in portal areas increased after 4 weeks of MCD diet and persisted throughout the treatment, whereas in the lobule the percentage of CK19+ve cells was significantly higher by the 8th week of diet as compared to CTRL and MCD4wks groups. Data represent average±sd of n=10-11 mice per group; p values are reported. (100X magnification; CTRL=control, MCD4wks=MCD diet for 4 weeks; MCD8wks=MCD diet for 8 weeks).

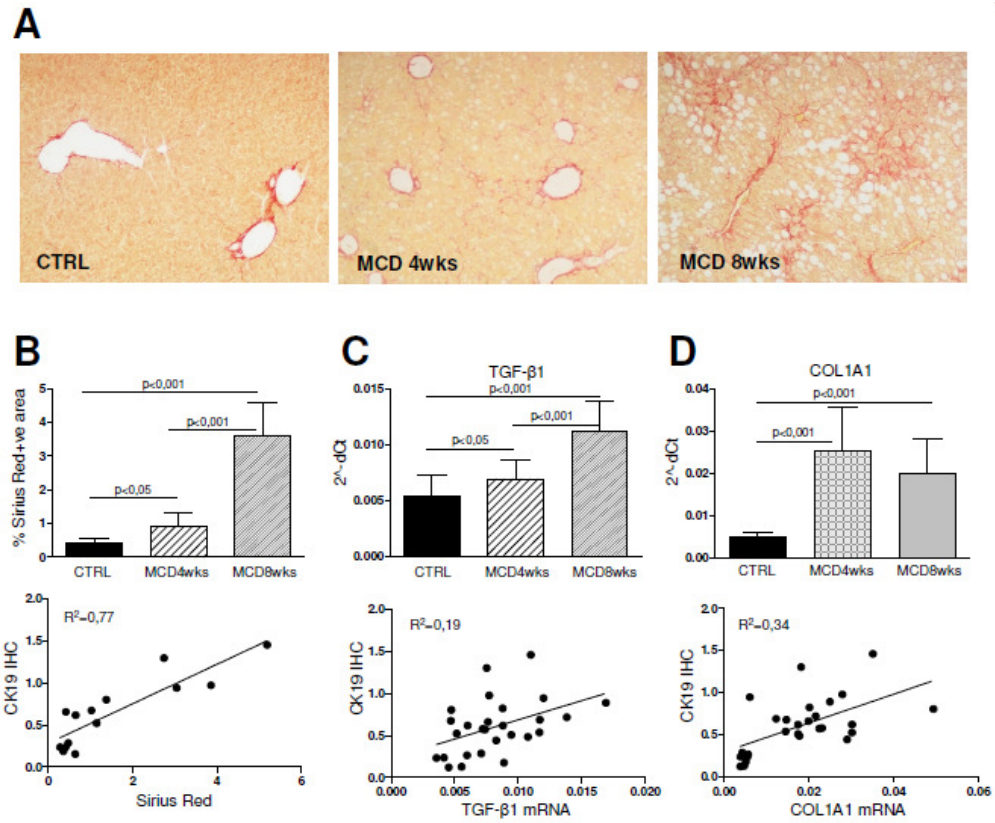

Figure 2: Fibrosis increases in MCD diet-induced steatohepatitis and correlates with HPC activation. (A) Histologic analysis on Sirius Red stained livers revealed the presence of collagen in MCD diet-treated mice, which deposition progressively increased from the 4th to the 8th week of treatment, as confirmed by (**B**, upper panel) morphometric quantification. The increase in collagen deposition strongly correlated with CK19 positivity (**B**, lower panel). MCD diet also stimulated the mRNA expression of profibrogenic mediators **(C)** TGF-β1 and **(D)** COL1A1, both of which correlated with increased HPC response (**C** and **D**, lower panels). Data represent average±sd of n=6-11 mice per group; p values are reported. (100X magnification).

Notch signaling is modulated in MCD diet-induced steatohepatitis. Notch-1 and Notch-2 mediated signaling play a primary role in regulating DR/HPC-driven repair in chronic biliary injuries[12, 13]. To understand whether Notch signaling could be involved in HPC/DR repair in the MCD model as well, we first performed gene expression analysis on total liver lysates (supplementary figure 2). We found that the expression of Notch

receptors Notch-1 and -2 (and of Dll4 ligand) significantly decreased with worsening of the disease, whereas the expression of the Jag1 ligand remained constant. The Notch target gene Hes1 was not induced whereas Hey1 showed a slight increase.

This reduction in the Notch pathway was most likely related to hepatocytes that make up the overwhelming cell population in whole liver lysates. Thus, to better define the cell type-specific expression of Notch factors, we used laser capture microdissection (LCM) to select CK19+ve areas and CK19-ve areas. In CK19+ve cells the expression of Notch related factors did not significantly change during MCD diet treatment (supplementary figure 3). On the other hand, the expression of Notch receptors and Jag1 ligand in the CK19-ve population (mainly hepatocytes) was significantly reduced (figure 3). As recent reports indicate that Notch activation supports the generation of hepatic steatosis[23], we speculated that the consistent down-regulation of Notch receptors in our mouse model could represents a defence mechanism to counteract fat accumulation occurring in hepatocytes. Numb expression was significantly reduced in the LCM isolated CK19-ve population (figure 3). Because Numb interferes with canonical Notch activation, inducing a rapid NICD degradation[12] it is likely that endogenous inhibition by Numb counterbalances the decreased expression of Notch receptors; indeed, in spite of the strong down-regulation of receptors in these cells the expression of the target genes Hes1 and Hey remained substantially unchanged.

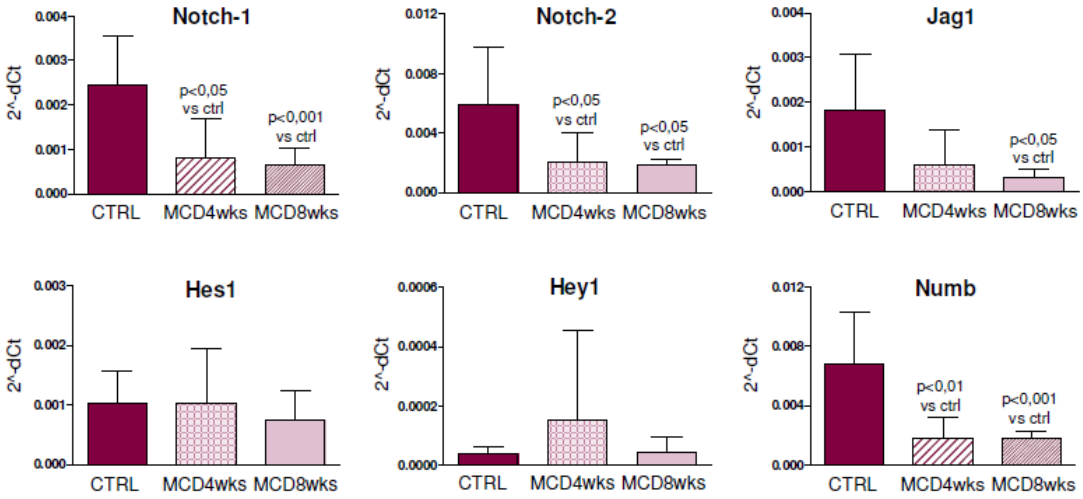

Figure 3: Notch signaling is modulated in laser-microdissected CK19-ve population. Analysis of Notch components on CK19-ve microdissected samples confirmed a significantly lower expression of Notch receptors (-1 and -2) and Jag1 ligand in hepatocytes; on the contrary, the expression of Notch target genes was not altered. Interestingly, Notch endogenous inhibitor Numb was strongly downregulated in hepatocytes by MCD diet. Data are expressed as 2^{-dCt} and represent average±sd of n=5 mice per group; p values are reported.

Notch signaling is activated in a subpopulation of hepatocytes. Thus, to better understand the pattern of Notch activation in hepatocytes, we analyzed by immunohistochemistry the expression of the Notch-regulated genes Sox9 and Hey2 and of the Notch-1 and Notch-2 intracellular domain (N1ICD and N2ICD). We found that after 8 weeks of MCD diet, approximately 3% of total hepatocytes were positive for Sox9 (figure 4) and that the number of Hey2+ve cells was also increased (figure 5). On the contrary to normal livers in which Hey2 positivity was confined to bile duct cells, in livers with steatohepatitis Hey2+ve hepatocytes appeared in the liver parenchyma, resembling Sox9 expression pattern. Moreover, in MCD diet-fed mice only, discrete hepatocyte-like cells showed a nuclear positivity for N1ICD (figure 5). On the other hand, N2ICD was expressed only in bile duct cells both in control and treated animals (figure 5). Based on these data, we hypothesized that HPC infiltrating

the lobule and adjacent to steatotic hepatocytes may actually derive from the Notch-mediated reprogramming of hepatocytes exposed to the inflammatory environment of NASH.

To study if hepatocytes can transdifferentiate into HPC during MCD diet-induced liver damage, we performed fate tracing experiments[22]. Hepatocytes were labelled *in vivo* by infecting R26R-YFP reporter mice with AAV8-TBG-Cre before inducing liver injury. The high affinity of AAV8 for hepatocytes together with the expression of Cre recombinase under the liver specific TBG promoter allows a highly efficient and selective expression of Cre recombinase in hepatocytes only. Indeed, viral infection induced Cre-mediated YFP expression in more than 99% of hepatocytes, and YFP positivity was never detected in cholangiocytes lining bile ducts ([22] and *not shown*). Using this approach, we found that in livers with steatohepatitis, approximately 10% of YFP+ve cells were also Sox9 positive, consistent with their hepatocellular origin (figure 6A). Furthermore, more than 2% of CK19+ve cells were YFP+ve, consistent with the hypothesis that a subpopulation of Sox9 positive hepatocytes may eventually transdifferentiate into HPC/DR (figure 6B) as also indicated by the HPC-like morphology of CK19+ve/YFP+ve cells. Of note, CK19+ve/YFP+ve and Sox9+ve/YFP+ve cells were strictly localized in lobular areas. These results strongly indicate that CK19+ve cells infiltrating the liver lobule in steatohepatitis partially derive from hepatocytes reprogramming, occurring after Notch-induced Sox9 expression.

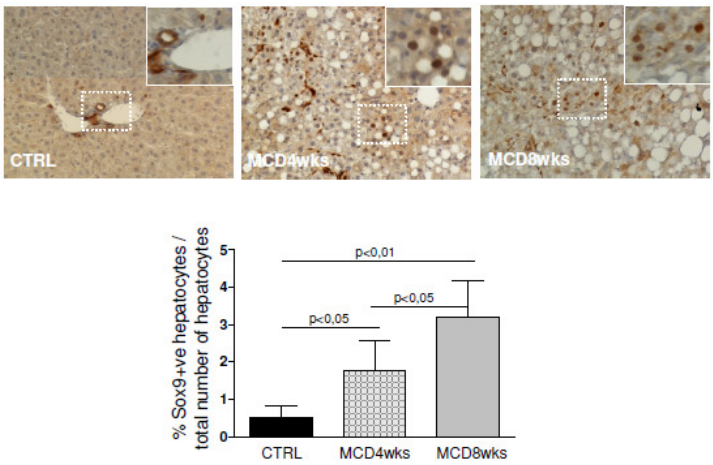

Figure 4: Sox9 expression occurs in hepatocytes after MCD diet. In control livers Sox9 is expressed by bile ducts cells; after MCD diet, Sox9+ve hepatocytes (insets) appeared in the lobule; as shown by the bar graph, the percentage of Sox9+ve cells with hepatocytic morphology increased with diet progression. Data represent average±sd of n=4 mice per group; p values are shown. (Original magnification 200X, insets 400X).

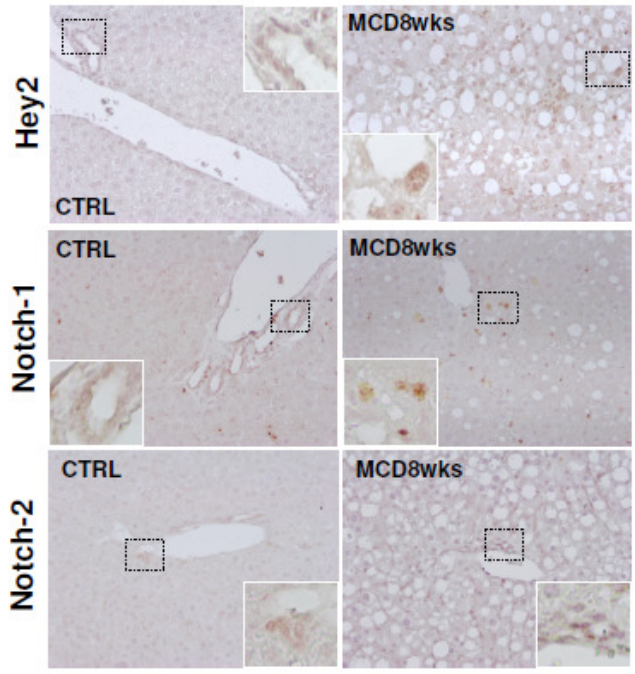

Figure 5: Notch is active in a hepatocyte subpopulation in MCD diet-injured livers. The Notch target gene Hey2 was expressed by cholangiocytes only in normal liver, whereas after MCD diet-treatment, hepatocytes showed prominent Hey2

positivity (upper right panel). Moreover, in MCD diet-treated mice, parenchymal hepatocytes also showed nuclear positivity for the intracellular domain of Notch1 (middle right panel), which expression is confined to bile ducts in controls. The expression of Notch-2 receptor is limited to bile duct cells in both control and MCD diet-treated groups. Since these antibodies recognize the intracellular portion of Notch receptors, these results indicate that Notch-1, but not Notch-2, is active in hepatocytes and is involved in MCD diet-induced hepatocytes conversion. (Original magnification 200X, insets 400X).

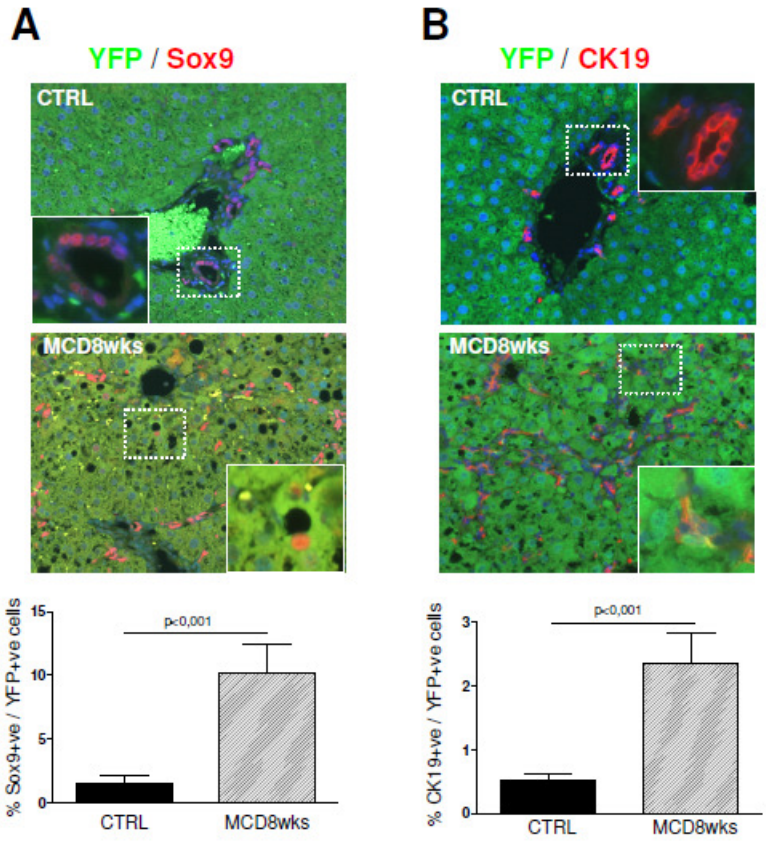

Figure 6: Lineage tracing shows hepatocytes conversion to HPC-like cells promoted by MCD diet-treatment. To confirm hepatocytes conversion, fate tracing experiments were performed in R26R-YFP mice infected with AAV8-TBG-Cre virus. After MCD treatment **(A)** approximately 10% of hepatocytes (YFP, green) expressed Sox9 (red), and **(B)** about 2% of YFP+ve/CK19+ve appeared in liver lobule. Data represent average±sd of n=4-8 mice per group; p values are shown. (Original magnification 200X, insets 400X).

Notch induces phenotypic changes in the hepatocytic cell line AML-12. To further establish the role of Notch in cellular reprogramming toward the HPC-like phenotype, we tested the effects of Notch activation in hepatocytes *in vitro*. To mimic Notch activation we cultured AML-12 cells onto immobilized Jag1, to reproduce the nature of cell-cell contact. As shown in supplementary figure 4A, continuous Notch stimulus in AML-12 cells strongly reduced the expression of albumin and the expression of bsep/abcb11, a bile acid transporter unique to hepatocytes, consistent with the overall down-regulation of hepatocytic markers induced by Notch activation. Conversely, AML-12 cells started to express biliary markers (supplementary figure 4B), including the early and persistent Sox9 positivity. The other relevant biliary transcription factor, HNF1β, significantly increased after 1week of treatment, suggesting that HNF1β is a late marker, expressed after Sox9 induction. Notch target genes were significantly induced after Jag1 treatment in AML-12 cells (supplementary figure 4C), confirming Notch activation.

Activated hepatic stellate cells (HSC) express high levels of Jag1 which may contribute to Notch-activation in hepatocytes. Notch-induced hepatocytes reprogramming occurring in MCD diet-treated mice is most likely a ligand-dependent process, as confirmed by Notch-activation assays *in vitro*. Therefore we sought to investigate which cell type was responsible of providing persistent Jag1-driven stimulus to hepatocytes. In MCD diet-fed mice, αSMA immunoreactive cells were localized around Sox9+ve hepatocytes (figure 7A) as well as in close contact with CK19+ve cells (figure 7B), suggesting that HSC/myofibroblasts could stimulate continuous Notch activation in hepatocytes. As shown in figure 7C and supplementary figure 5, in response to increasing doses of TGF-β1, HSC up-

regulated Jag1 expression in a dose-dependent manner, consistent with the hypothesis that HSC are probably the cells transmitting the Notch signal to hepatocytes during NASH. Consistent with the above hypothesis, hepatic gene expression of the ligand Jag1 and of the Notch target gene Hey1 positively correlated with COL1A1 expression (figure 7D). Consistent with this interpretation, fibrosis increased linearly with the increase in HPC/DR area (CK19+ve), and the HPC/DR area positively correlated with TGF-β1 and COL1A1 (figure 2).

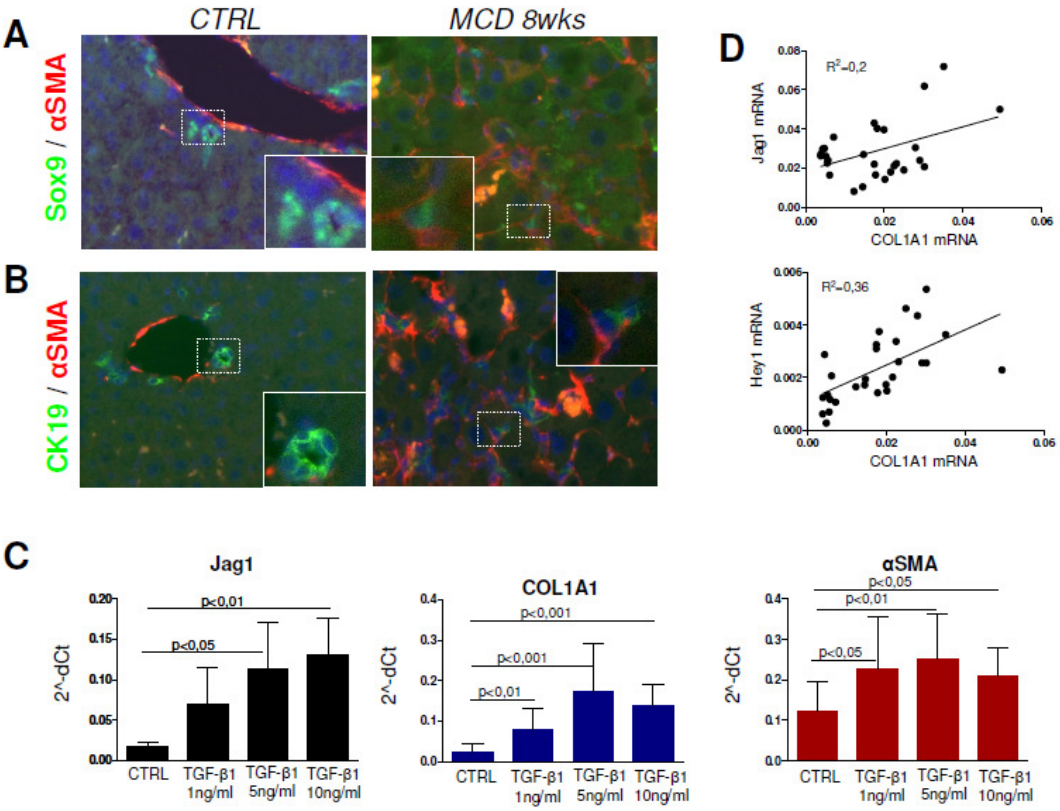

Figure 7: Jag1 promotes HSC/hepatocytes cross-talk in MCD diet-damaged livers. (A) In mice treated with MCD diet for 8 weeks Sox9+ve hepatocyte-like cells (green) established close contact with αSMA+ve cells (red); accordingly, **(B)** αSMA+ve cells (red) localized in proximity of CK19+ve cells (green). **(C)** Jag1 was strongly upregulated in primary mouse HSC activated with increasing doses of TGF-β1 (1-5-10ng/mL) for 4 days; COL1A1 and αSMA expression confirmed HSC activation. Furthermore, **(D)** Jag1 and Hey1 mRNA positively correlated with COL1A1

expression, suggesting that HSC were involved in persistent stimulation of Notch receptors on hepatocytes. Data represent average±sd of n=3 experiments; p values are reported. (Original magnification 200X, insets 400X).

DISCUSSION

Increasing evidence indicates that HPC activation actively contributes to disease progression in NASH[5-7], a condition which growing epidemics will soon present a major challenge. Understanding HPC pathobiology is therefore fundamental to unravel the mechanisms leading to cirrhosis and liver cancer in NASH. Prior work has shown that HPC are under the control of a number of signaling mechanisms, including Notch signaling, which is necessary to modulate morphogenesis during repair from biliary damages[13]. As shown in figures 1 and 2, and supplementary figure 1, in the MCD diet-induced model of steatohepatitis HPC activation correlates with inflammation and fibrosis, and HPC are spread into liver parenchyma in close contact with fat-laden hepatocytes and activated myofibroblasts.

HPC are believed to arise from the hepatic stem cell niche located in the canals of Hering and from the small ductules of the biliary tree[24, 25], but their origin has not been fully elucidated. In fact, recent reports indicate that under certain conditions, HPC may also be generated from the biliary transdifferentiation of hepatocytes (biliary metaplasia)[22, 26-28]. Our data are consistent with this interpretation and provide evidence that a subset of CK19+ve cells present in the lobule is derived from hepatocytes that underwent Notch-dependent phenotypic changes.

Given its relevance in regulating cell fate in general and HPC response in liver disease[12, 13], it is not surprising that Notch signaling promotes HPC expansion in the MCD diet-induced

steatohepatitis. Our data show that in this condition Notch activation is cell specific. Using whole liver lysates and tissue from laser capture microscope, we found that gene expression of Notch signaling factors was not modulated in CK19+ve cells, but was significantly down-regulated in CK19-ve cells. This observation is at odds with prior work from Pajani et al who reported activation of Notch signaling in the liver of mice with obesity and fatty liver[23, 29]. In Pajvani's model fatty liver was reproduced by feeding mice a high-fat diet, where steatosis (not steatohepatitis) is associated to weight gain and insulin resistance, but lacks appreciable inflammation/fibrosis. On the contrary, the MCD model (as shown in figure 2 and supplementary figure 1) is characterized by steatohepatitis and fibrosis, in the absence of obesity and insulin resistance[30]. It has been recently reported that Notch is involved in insulin resistance development and glucose metabolism[29], providing a rationale for a higher activation of the pathway for metabolic reasons. These crucial differences in experimental conditions may explain the differences in hepatocellular Notch signaling. On the other hand, down-regulation of hepatocellular Notch receptors/ligands during chronic liver damage may have a protective effect against the known carcinogenetic effects of continuative Notch signaling.

In our conditions, we observed a down regulation of the receptors and ligands in hepatocytes (CK19-ve fraction), but the Notch signal was maintained. In fact, although the expression of Notch components significantly decreased in the CK19–ve areas, the lower expression of Numb (an endogenous inhibitor of Notch) found in this population would, on the other hand, make hepatocytes more prone to Notch activation. Furthermore, a subset of parenchymal hepatocyte-like cells became positive for Sox9, a Notch target gene involved in biliary differentiation and liver carcinogenesis[11, 19].

These data indicate that in a subpopulation of hepatocytes Notch signaling is actually upregulated. Consistent with this interpretation, *in vivo* hepatocyte lineage tracing experiments showed that 8 weeks of MCD diet induced phenotype conversion in a subpopulation of mature hepatocytes. In fact we observed that 10% of YFP+ve cells with morphological features of hepatocytes expressed Sox9, indicating active Notch signaling. We also detected a percentage of YFP+ve/CK19+ve cells as well as CK19 expression in YFP+ve cells with a morphology resembling smaller HPC-like cells. These results suggest that in mice with steatohepatitis, a slow step-wise process induces hepatocytes conversion to HPC-like cells, contributing to DR as disease progresses. Of note, we have previously shown that in AGS, caused by defective Jag1/Notch-2, there is a massive accumulation of hepato-biliary intermediate cells that are not able to completely commit into functional cholangiocytes[15], supporting the idea that hepatocytes might undergo phenotypic conversion to sustain HPC-driven repair, and this requires Notch signaling for a full differentiation[22].

Sox9 expression in hepatocytes could therefore represent the starting point of Notch-mediated reprogramming occurring in disease conditions. Intriguingly, Notch-1 and the target gene Hey2 were virtually absent in the hepatic parenchyma of untreated mice, in which their expression was restricted to bile duct cells, but after MCD diet-damage, Notch-1 NICD and Hey2 were expressed in lobular hepatocytes, consistent with Notch activation. Notch-2 NICD instead was limited to bile ducts in healthy and diseased livers, further suggesting that Notch-1 is the aberrantly stimulated receptor isoform in MCD diet-induced liver damage.

In vitro studies with a hepatocytes cell line provided further evidence that Notch activation in hepatocytes leads to cellular

reprogramming driving the expression of biliary markers along with decreasing hepatocytic features. Interestingly, Sox9 was rapidly induced by Jag1-dependent Notch activation, whereas the expression of the other biliary marker HNF1β occurred only after persistent (one week) stimulus. Altogether these results highlight the relevance of Sox9 appearance in hepatocytes in order to initiate phenotype conversion. Sox9 expression has been reported in a subset of HCC and linked to a more malignant and aggressive phenotype[19]. Since HCC is a known complication of NASH, we speculated that Sox9 expression could represent an early step in NASH-related hepatocarcinogenesis. Of note, Villanueva and colleagues[19] reported that experimental persistent Notch activation in the hepatic compartment led to HCC after 12 months with 100% penetrance, suggesting that aberrant and continuous Notch activation in hepatocytes drives HCC development.

We next sought to determine which cell type could provide continuous Notch activation in hepatocytes in steatohepatitis, given that in hepatocytes Jag1 expression is actually downregulated. We reasoned that the likely source of Notch ligands could be myofibroblasts, that are known as the main contributors to liver fibrosis in MCD diet[31]. Given their intimate contact with hepatocytes and HPC, myofibroblasts could also explain why Notch would be significantly upregulated only in a subset of hepatocytes. Moreover, our data showed that hepatic expression of Jag1 and Hey1 correlated with that of collagen. Consistent with this hypothesis, we showed that treatment with the pro-fibrogenic factor TGF-β1 strongly induced a dose-dependent expression of Jag1 in primary isolated HSC and in a human HSC cell line, indicating HSC as a likely source of Jag1 in fibrotic settings. We also showed that indeed in MCD diet-fed mice, activated-HSC spread throughout liver parenchyma in close contact

with both fat-laden hepatocytes and CK19+ve cells (figure 7B). Activated-HSC localized around Sox9+ve hepatocyte-like cells (figure 7A), supporting the idea that HSC could potentially be responsible of persistent Notch stimulus in hepatocytes.

In conclusion our results are consistent with the following working model involving Notch-dependent cross-talk between hepatocytes and mesenchymal cells and hepatocellular differentiation in HPC/DR. Myofibroblasts recruited to the site of injury by the release of inflammatory mediators, and stimulated by TGF-β1, upregulate Jag1 that stimulates Notch-1 in adjacent hepatocytes. Notch1-dependent Sox9 expression initiates a series of events that eventually leads to hepatocytes reprogramming into HPC, generation of pathological DR and fibrosis. This mechanism is consistent with work by Richardson et al[5] who observed that in NASH fibrosis initiates with a pericellular distribution, becoming than periportal, which leads to cirrhosis. Our data also link fibrotic/cirrhotic evolution to increased risk of HCC development. Further knowledge of the mechanisms of Notch involvement in NASH progression may lead to therapeutic applications.

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Conflict of interests
The authors have no competing interests on the matter concerning the present manuscript.

REFERENCES

1. Fabris L, Strazzabosco M. Epithelial-mesenchymal interactions in biliary diseases. Semin Liver Dis 2011 Feb;31(1):11-32.

2. Roskams T, Desmet V. Ductular reaction and its diagnostic significance. Semin Diagn Pathol 1998 Nov;15(4):259-269.

3. Strazzabosco M, Fabris L. Development of the bile ducts: essentials for the clinical hepatologist. J Hepatol 2012 May;56(5):1159-1170.

4. Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. Semin Liver Dis 2008 Nov;28(4):339-350.

5. Richardson MM, Jonsson JR, Powell EE, Brunt EM, Neuschwander-Tetri BA, Bhathal PS, et al. Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction. Gastroenterology 2007 Jul;133(1):80-90.

6. Roskams T, Yang SQ, Koteish A, Durnez A, DeVos R, Huang X, et al. Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. Am J Pathol 2003 Oct;163(4):1301-1311.

7. Svegliati-Baroni G, Faraci G, Fabris L, Saccomanno S, Cadamuro M, Pierantonelli I, et al. Insulin resistance and necroinflammation drives ductular reaction and epithelial-mesenchymal transition in chronic hepatitis C. Gut 2011 Jan;60(1):108-115.

8. Bray SJ. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 2006 Sep;7(9):678-689.

9. Geisler F, Strazzabosco M. Emerging roles of Notch signaling in liver disease. Hepatology 2014 Jun 16.

10. Morell CM, Strazzabosco M. Notch signaling and new therapeutic options in liver disease. J Hepatol 2014 Apr;60(4):885-890.

11. Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, et al. Notch signaling controls liver development by regulating biliary differentiation. Development 2009 May;136(10):1727-1739.

12. Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, et al. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nat Med 2012 Apr;18(4):572-579.

13. Fiorotto R, Raizner A, Morell CM, Torsello B, Scirpo R, Fabris L, et al. Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. J Hepatol 2013 Jul;59(1):124-130.

14. Xie G, Karaca G, Swiderska-Syn M, Michelotti GA, Kruger L, Chen Y, et al. Cross-talk between Notch and Hedgehog regulates hepatic stellate cell fate in mice. Hepatology 2013 May 23.

15. Fabris L, Cadamuro M, Guido M, Spirli C, Fiorotto R, Colledan M, et al. Analysis of liver repair mechanisms in Alagille syndrome and biliary atresia reveals a role for notch signaling. Am J Pathol 2007 Aug;171(2):641-653.

16. Dill MT, Tornillo L, Fritzius T, Terracciano L, Semela D, Bettler B, et al. Constitutive Notch2 signaling induces hepatic tumors in mice. Hepatology 2012 Nov 23.

17. Fan B, Malato Y, Calvisi DF, Naqvi S, Razumilava N, Ribback S, et al. Cholangiocarcinomas can originate from hepatocytes in mice. J Clin Invest 2012 Aug 1;122(8):2911-2915.

18. Sekiya S, Suzuki A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. J Clin Invest 2012 Nov 1;122(11):3914-3918.

19. Villanueva A, Alsinet C, Yanger K, Hoshida Y, Zong Y, Toffanin S, et al. Notch signaling is activated in human hepatocellular carcinoma and induces tumor formation in mice. Gastroenterology 2012 Dec;143(6):1660-1669 e1667.

20. Zender S, Nickeleit I, Wuestefeld T, Sorensen I, Dauch D, Bozko P, et al. A critical role for notch signaling in the formation of cholangiocellular carcinomas. Cancer Cell 2013 Jun 10;23(6):784-795.

21. Huntzicker EG, Hotzel K, Choy L, Che L, Ross J, Pau G, et al. Differential effects of targeting Notch receptors in a mouse model of liver cancer. Hepatology 2014 Oct 13.

22. Yanger K, Zong Y, Maggs LR, Shapira SN, Maddipati R, Aiello NM, et al. Robust cellular reprogramming occurs spontaneously during liver regeneration. Genes Dev 2013 Apr 1;27(7):719-724.

23. Pajvani UB, Qiang L, Kangsamaksin T, Kitajewski J, Ginsberg HN, Accili D. Inhibition of Notch uncouples Akt activation from hepatic lipid accumulation by decreasing mTorc1 stability. Nat Med 2013 Aug;19(8):1054-1060.

24. Carpentier R, Suner RE, van Hul N, Kopp JL, Beaudry JB, Cordi S, et al. Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. Gastroenterology 2011 Oct;141(4):1432-1438, 1438 e1431-1434.

25. Rodrigo-Torres D, Affo S, Coll M, Morales-Ibanez O, Millan C, Blaya D, et al. The biliary epithelium gives rise to liver progenitor cells. Hepatology 2014 Oct;60(4):1367-1377.

26. Michalopoulos GK, Barua L, Bowen WC. Transdifferentiation of rat hepatocytes into biliary cells after bile duct ligation and toxic biliary injury. Hepatology 2005 Mar;41(3):535-544.

27. Sekiya S, Suzuki A. Hepatocytes, rather than cholangiocytes, can be the major source of primitive ductules in the chronically injured mouse liver. Am J Pathol 2014 May;184(5):1468-1478.

28. Tanimizu N, Nishikawa Y, Ichinohe N, Akiyama H, Mitaka T. Sry HMG box protein 9-positive (Sox9+) epithelial cell adhesion molecule-negative (EpCAM-) biphenotypic cells derived from hepatocytes are involved in mouse liver regeneration. J Biol Chem 2014 Mar 14;289(11):7589-7598.

29. Pajvani UB, Shawber CJ, Samuel VT, Birkenfeld AL, Shulman GI, Kitajewski J, et al. Inhibition of Notch signaling ameliorates insulin resistance in a FoxO1-dependent manner. Nat Med 2011 Aug;17(8):961-967.

30. Larter CZ, Yeh MM. Animal models of NASH: getting both pathology and metabolic context right. J Gastroenterol Hepatol 2008 Nov;23(11):1635-1648.

31. Mederacke I, Hsu CC, Troeger JS, Huebener P, Mu X, Dapito DH, et al. Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. Nat Commun 2013;4:2823.

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY MATHERIAL AND METHODS

Histology and immunohistochemistry. Tissue sections were stained by Hematoxylin and Eosin (H&E) to assess liver pathology. The severity of steatosis and lobular inflammation was scored by an experienced pathologist according to Kleiner (not shown)[S1]. Liver fibrosis was revealed by Sirius red histochemistry. Fibrosis extent was quantified with ImageJ software in 10 random micrographs (magnification 200x) by calculating the Sirius red positive area as the percentage of pixels above the threshold value with respect to the total pixels per area.

Paraffin embedded livers were analyzed for the following cellular markers: cytokeratin-19 (CK19, HPC/DR); Sox9 (biliary specific marker), αSMA (activated hepatic stellate cells [HSC] marker). Briefly, de-paraffinated sections were re-hydrated in alcohol and endogenous peroxidase activity was blocked with methanol/10% hydrogen peroxide. After unmasking with the proper antigen retrieval and treating with proper blocking solutions, the slides were incubated overnight at 4°C with the following primary antibodies against: CK19 (Troma III clone, DSHB; 1:100), Sox9 (Millipore; 1:100); αSMA (DAKO; 1:100), GFP (AbCam; 1:500); Notch1 intracellular domain (bTAN 20 clone, DSHB; 1:50); Notch2 intracellular domain (c651.6DbHN clone, DSHB; 1:50); Hey2 (Thermo Scientific; 1:50). After rinsing, slides were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody and developed with 3-3-diaminobenzidine (DAB). Alternatively, for immunofluorescence studies, slides were incubated with the proper fluorescent secondary antibody (Alexa Fluor 488, Life Technologies; 1:500; Alexa Fluor 594, Life Technologies; 1:500) and mounted with DAPI. The slides were analyzed with the Nikon Eclipse E800 microscope (Nikon) connected to a Nikon Sight DS-5Mc digital camera (Nikon).

CK19 quantification was performed in 5 random non-overlapping fields per slide (100x magnification) by calculating the CK19+ve area with the software ImageJ as percentage of pixels above the threshold value with respect to the total pixels per area. Similarly, to differentially quantify the distribution of CK19+ve cells in lobular versus portal/perportal areas, 10 random pictures (200X magnification) were taken and portal/periportal positivity was subtracted to total positivity to obtain lobular distribution. To quantify hepatocellular Sox9, the number of Sox9+ve hepatocytes (determined by cell morphology) was manually counted in 10 random non overlapping fields (200x magnification); results are expressed as

Sox9+ve hepatocytes respect to the total number of hepatocytes per field.

For hepatocyte tracing experiments, the number of cells single positive for Sox9, CK19 and double positive for Sox9/YFP and CK19/YFP was evaluated in 20 fields for each control mouse (200X magnification) and in 10 fields for each treated mouse (200X magnification). A minimum of 490 or 830 of cells positive for the selected marker was examined for control and treated mice respectively. Results are represented as the percentage of Sox9 or CK19 positive cells co-expressing the YFP label.

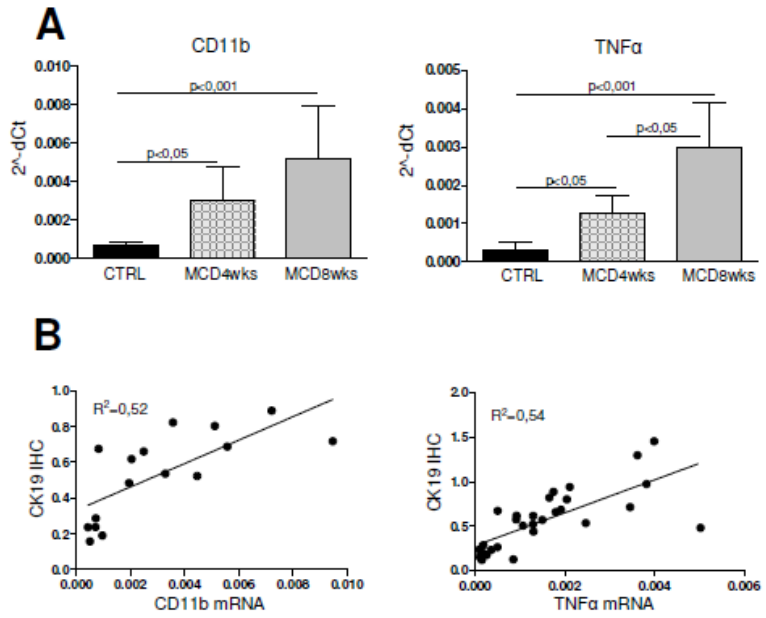
In vitro treatments. We performed *in vitro* studies using the hepatocyte cell line AML-12 (ATCC). To evaluate the effects of Notch persistent activation in hepatocytes, AML-12 cells were stimulated with immobilized Jag1 (5µg/ml; R&D System), to reproduce cell-cell contact required by Notch signaling; Notch activation was carried out for 24, 48 hours and 1 week. In other experiments, primary cultures of mouse HSCs and LX2 (a human HSC line) were treated with increasing doses of TGF-β1 (1, 5 and 10ng/mL) for 4 days or 72hrs, respectively.

Gene expression analyses by quantitative real-time RT-PCR. RNA was extracted either from cell cultures or from total liver lysates with Trizol reagent (Applied Biosystems) according to the manufacturer's instructions. 1 *µ*g of total RNA was retro-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Realtime PCR was performed in a ABI 7900 thermocycler (Life Technologies, Carlsbad, CA), using TaqMan Gene Expression Master Mix and TaqMan probes for mouse Notch-related factors (Notch1 and -2, Jag1, Dll4, Hes1, Hey1, Numb), TNF*α*, CD11b, TGF-β1, Procollagen α1(I) (COL1(A1)), Sox9, Albumin, Bsep/Abcb11, HNF1β. Experiments on mesenchymal cells (expression of Jag1, COL1(A1), α-SMA) were performed with the SYBR green technique. Data were normalized to the Gapdh and/or Hprt gene expression. The results are expressed as $2^{\wedge -dCt}$ or fold increase as indicated in bar graphs.

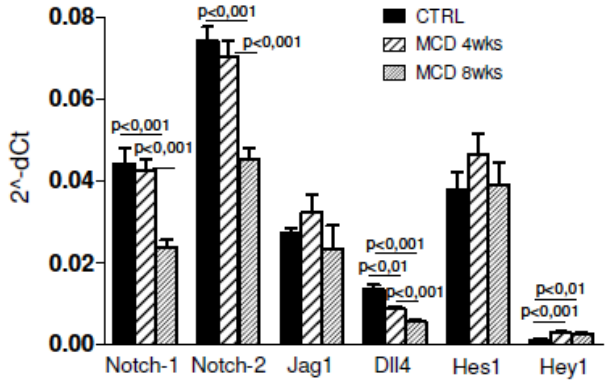
SUPPLEMENTARY REFERENCES

S1. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005 Jun;41(6):1313-1321.

SUPPLEMENTARY FIGURES

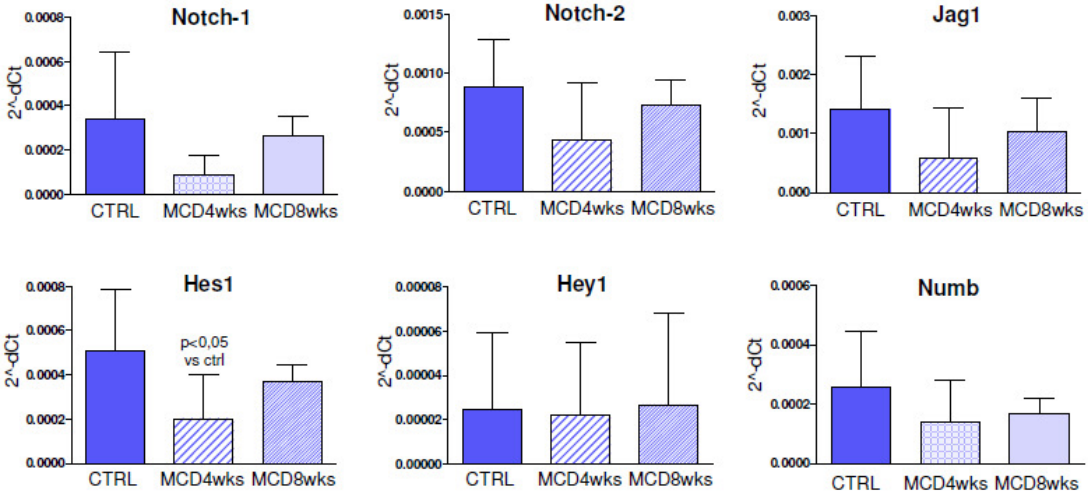


Supplementary figure 1: Inflammation increases in MCD diet-induced steatohepatitis and correlates with HPC activation. (A) MCD diet stimulated the inflammatory process, as confirmed by the mRNA expression of CD11b and TNFα, which **(B)** positively correlated with the increase of CK19+ve cells. Data represent average±sd of n=6-11 mice per group; p values are reported.

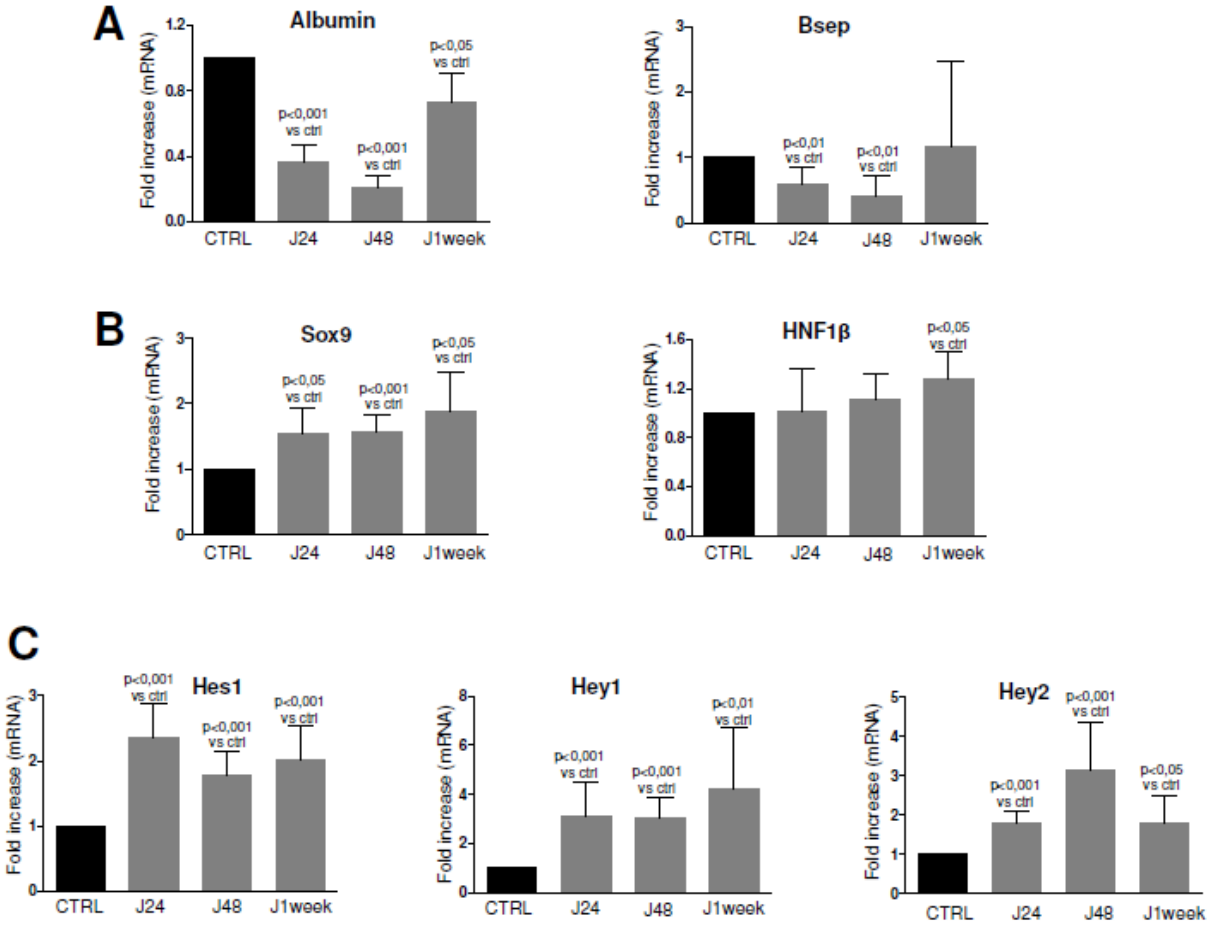


Supplementary figure 2: Gene expression analysis on total liver lysates of Notch signaling components. Real-time PCR analysis of Notch signaling components performed on total liver lysates revealed an overall downregulation of the expression of Notch receptors (Notch1 and -2) and the ligand Dll4, without reducing the expression of the Notch ligand Jag1 or of the Notch-targets Hes1 and

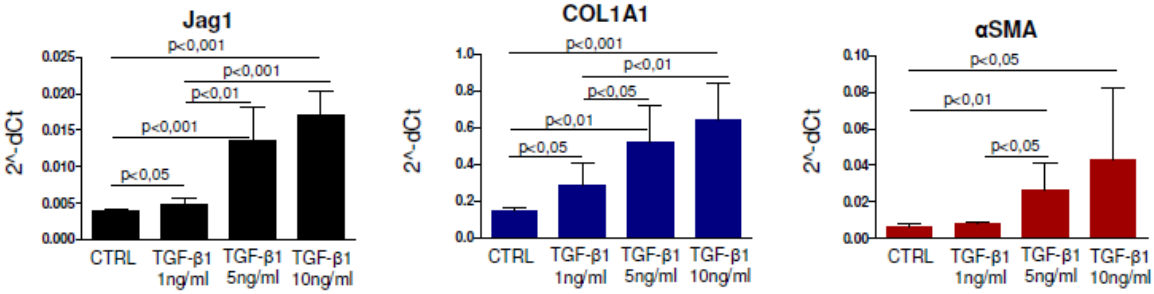
Hey1. Data are expressed as 2^{-dCt} and represent average±sd of n=10 mice per group; p values are reported.



Supplementary figure 3: Notch signaling is not modulated in the CK19+ve population. The expression of Notch-related factors was evaluated on laser-capture CK19+ve microdissected samples, which comprise HPC/DR and biliary cells. As shown in bar graphs, the Notch pathway was not significantly influenced by MCD diet in the CK19+ve population overall. Of note, Numb expression was not altered in these cells. Data are expressed as 2^{-dCt} and represent average±sd of n=5 mice per group; p values are reported.



Supplementary figure 4: Notch induces phenotypic changes in the hepatocytic cell line AML-12. AML-12 cells were treated with Jag1 to induce Notch activation for 24hours, 48hours and 1week. Notch activation assay reduced the expression of **(A)** markers of hepatocytes (albumin and Bsep), while **(B)** rapidly increasing the expression of the biliary marker Sox9; after a persistent stimulus, also Hnf1β was upregulated. **(C)** The increased expression of Notch target genes Hes1, Hey1 and Hey2 in Jag1 treated cells confirmed that this process was Notch dependent. Data are expressed as fold increase/decrease respect to control ±sd of n=4-6 experiments; p values are shown.



Supplementary figure 5: Jag1 promotes LX2 activation *in vitro*. Increasing doses of TGFβ1 (1-5-10ng/mL, for 72hrs) significantly induced Jag1 expression in the human LX2 cell line, along with increasing the expression of COL1A1 and αSMA, thus confirming LX2 activation. Data represent average±sd of n=5-15 experiments; p values are shown.

Chapter 6

Conclusions and Future Perspectives

This PhD project focused on the emerging role of Notch signaling as a regulator of liver repair mechanisms. The general approach was to study the role of Notch in two paradigmatic conditions in which liver repair is involved, i.e. biliary damage (cholangiopathies) and lobular damage (non-alcoholic steatohepatitis [NASH]). In both settings, chronic injury ultimately evolves to liver fibrosis, which progression is actively sustained by the hepatic reparative process. Liver repair in chronic disease conditions requires the activation of the hepatic progenitor cells (HPC) compartment, sustaining ductular reaction (DR)-driven reparative response[1-5]. Notch involvement in repair from liver damage has not been extensively documented; only recently Notch activation has been implicated in liver damage as well as in hepatic cancer (reviewed in[6-8]), providing insight into the patho-physiologic role of Notch in adult liver. Better understanding of this process is of fundamental relevance in order to target Notch signaling to interfere with liver diseases.

The effects of Notch activation are highly affected by the cellular environment in which it is stimulated. The studies presented here embrace different aspects of DR-driven liver repair, investigating Notch activation in various pathologic settings.

Notch-driven ductular reaction in chronic liver diseases

On one side, we assessed the relevance of Notch signaling in DR arising after cholestatic challenges ([9], *"Chapter 2"*). Biliary damage induced by DDC (3,5-diethoxycarbonyl1,4-dihydrocollidine) or ANIT (alpha-naphthyl-isothiocyanate) stimulated DR and proliferation of bile ducts, as expected. Indeed, the remodeling of ductular reactive structures into branching biliary tubules is a key step in biliary regeneration. However, complete Notch blockade achieved pharmacologically (with γ-secretase inhibitors [GSI]) or genetically

(liver conditional RPB-Jk KO mice) significantly reduced the number of both DR and HPCs. Of note, when Notch was only partially defective (Notch-2 conditional KO mice), HPC accumulated after biliary damage, but failed to progress into mature ducts. These data demonstrate that Notch signaling plays an essential role in DR-driven liver repair. More specifically, lack of Notch-2 prevented biliary tubule formation, both *in vivo* and *in vitro,* whereas lack of RBP-Jk (which blunts both Notch-1 and Notch-2 signaling) inhibited biliary morphogenesis while affecting the generation of biliary-committed precursors.

Besides coordinating the cross-talk among epithelial/inflammatory/mesenchymal cells, DR also stimulates the progressive production of fibrovascular stroma, eventually responsible for fibrotic evolution. Few recent reports[7, 10, 11] defined the role of Notch in tissue remodelling after injury. These data suggest that interfering with Notch might be successful to treat fibrotic liver diseases by hampering ductular reaction to reduce the extent of accompanying liver fibrosis and consequent hepatic architecture distortion, although very challenging.

Notch-induced hepatocellular reprogramming in chronic liver diseases

In the second part of the study (*"Chapter 5"*) we used NASH as a model of lobular damage. We reported that as NASH developed in a highly fibrogenic context, a robust HPC response occurred spreading from portal areas throughout liver parenchyma, as disease worsened. In this setting, hepatic stellate cells (HSC)/myofibroblasts provided Jag1-mediated persistent Notch activation in hepatocytes. When endogenous regulation of Notch was defective (specifically, Numb downregulation), hepatocytes underwent phenotypic

conversion to HPC-like cells. Lineage tracing experiments allowed us to document and describe this step-wise process that requires initial Sox9 expression, further progressing to morphological changes, reducing hepatocytes size and giving rise to HPC-like cells expressing CK19. These hepatocytes-derived CK19+ve cells distributed in lobular areas, therefore contributing to parenchymal DR.

Growing evidence supports the hypothesis that hepatocytes conversion contributes to DR in certain liver diseases[12-15]. Notch plays a pivotal role in this process, driving phenotypic conversion in a more rapid fashion if persistently active[16, 17]. Our study suggests that, by inducing dedifferentiation, Notch-mediated hepatocytes reprogramming represents a key event for eventual HCC generation. Indeed, recent reports demonstrated that persistent and/or ectopic Notch activation sustains HCC and CCA development[18-22], confirming Notch as a potent oncogenic stimulus in the liver. Of note, Notch has been associated to a more malignant phenotype[18, 20, 21], and Sox9, a gene downstream Notch activation in the liver[23], has been indicated as a marker of worse prognosis[21]. Therefore, interfering with Notch could potentially hamper Notch-driven tumor progression and aggressiveness.

Conclusion and translational application in medicine

Since altered Notch signaling occurs in several malignancies[24, 25], a broad spectrum of molecules able to interfere with this pathway has been developed. Therapeutic approaches either target the activation of the receptors (namely, GSIs), or the ligand/receptor binding (monoclonal antibodies [mAbs], decoys) or the transcriptional activity of Notch intracellular domain (blocking peptides) (reviewed in[8, 24, 26]). Many of these therapies are under preclinical investigation or advanced phase I clinical studies for

cancer treatment (reviewed in[24, 26]), although none of these clinical studies involve liver cancer patients.

Current therapies for advanced stage chronic liver diseases mainly rely on liver transplantation or resection. Recent advances of aberrant Notch pathway in hepatic fibrosis and liver cancer progression provide a strong rationale for introducing Notch modulating therapies to treat liver diseases. Pathologic hepatic regeneration could be therapeutically approached with GSIs or the more selective mAbs. Indeed, GSIs have been proven efficient in altering liver repair process in mouse models of cholestatic liver disease[9]. Moreover, DR/HPC crosstalk with mesenchymal cells could be blunted by the use of mAbs against Notch-1 and -2 (targeting epithelial cells) or Jag1 (targeting HSC/myofibroblasts) thus inhibiting fibrogenesis. Notch therapy could also be applied to liver carcinogenesis, since a Notch signature has been identified in a subset of highly aggressive HCC[21] and Notch receptors were found up-regulated in CCA patients[22, 27]. Of note, selective mAbs against Notch-2 and Jag1 have been proven successful in reducing tumor progression in experimental models of liver cancer with mixed HCC/CCA phenotype[28]. However, further studies are required to understand whether Notch-based therapies could be successful in treating liver diseases/malignancies. Although promising, more efforts are needed to fully understand the patho-physiological activation of Notch in the liver.

References

1. Fabris L, Strazzabosco M. Epithelial-mesenchymal interactions in biliary diseases. Seminars in liver disease 2011 Feb;31(1):11-32.

2. Gouw AS, Clouston AD, Theise ND. Ductular reactions in human liver: diversity at the interface. Hepatology (Baltimore, Md 2011 Nov;54(5):1853-1863.

3. Morell CM, Fabris L, Strazzabosco M. Vascular biology of the biliary epithelium. Journal of gastroenterology and hepatology 2013 Aug;28 Suppl 1:26-32.

4. Strazzabosco M, Fabris L. Development of the bile ducts: essentials for the clinical hepatologist. Journal of hepatology 2012 May;56(5):1159-1170.

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

6. Geisler F, Strazzabosco M. Emerging roles of Notch signaling in liver disease. Hepatology (Baltimore, Md 2014 Jun 16.

7. Morell CM, Fiorotto R, Fabris L, Strazzabosco M. Notch signalling beyond liver development: Emerging concepts in liver repair and oncogenesis. Clinics and research in hepatology and gastroenterology 2013 Jun 24.

8. Morell CM, Strazzabosco M. Notch signaling and new therapeutic options in liver disease. Journal of hepatology 2014 Apr;60(4):885-890.

9. Fiorotto R, Raizner A, Morell CM, Torsello B, Scirpo R, Fabris L, et al. Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. Journal of hepatology 2013 Jul;59(1):124-130.

10. Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, et al. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nature medicine 2012 Apr;18(4):572-579.

11. Xie G, Karaca G, Swiderska-Syn M, Michelotti GA, Kruger L, Chen Y, et al. Cross-talk between Notch and Hedgehog regulates hepatic stellate cell fate in mice. Hepatology (Baltimore, Md 2013 May 23.

12. Fabris L, Cadamuro M, Guido M, Spirli C, Fiorotto R, Colledan M, et al. Analysis of liver repair mechanisms in Alagille syndrome and biliary atresia reveals a role for notch signaling. The American journal of pathology 2007 Aug;171(2):641-653.

13. Michalopoulos GK, Barua L, Bowen WC. Transdifferentiation of rat hepatocytes into biliary cells after bile duct ligation and toxic biliary injury. Hepatology (Baltimore, Md 2005 Mar;41(3):535-544.

14. Sekiya S, Suzuki A. Hepatocytes, rather than cholangiocytes, can be the major source of primitive ductules in the chronically injured mouse liver. The American journal of pathology 2014 May;184(5):1468-1478.

15. Tanimizu N, Nishikawa Y, Ichinohe N, Akiyama H, Mitaka T. Sry HMG box protein 9-positive (Sox9+) epithelial cell adhesion molecule-negative (EpCAM-) biphenotypic cells derived from hepatocytes are involved in mouse liver regeneration. The Journal of biological chemistry 2014 Mar 14;289(11):7589-7598.

16. Jeliazkova P, Jors S, Lee M, Zimber-Strobl U, Ferrer J, Schmid RM, et al. Canonical Notch2 signaling determines biliary cell fates of embryonic hepatoblasts and adult hepatocytes independent of Hes1. Hepatology (Baltimore, Md 2013 Jan 12.

17. Yanger K, Zong Y, Maggs LR, Shapira SN, Maddipati R, Aiello NM, et al. Robust cellular reprogramming occurs spontaneously during liver regeneration. Genes & development 2013 Apr 1;27(7):719-724.

18. Dill MT, Tornillo L, Fritzius T, Terracciano L, Semela D, Bettler B, et al. Constitutive Notch2 signaling induces hepatic tumors in mice. Hepatology (Baltimore, Md 2012 Nov 23.

19. Fan B, Malato Y, Calvisi DF, Naqvi S, Razumilava N, Ribback S, et al. Cholangiocarcinomas can originate from hepatocytes in mice. The Journal of clinical investigation 2012 Aug 1;122(8):2911-2915.

20. Sekiya S, Suzuki A. Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. The Journal of clinical investigation 2012 Nov 1;122(11):3914-3918.

21. Villanueva A, Alsinet C, Yanger K, Hoshida Y, Zong Y, Toffanin S, et al. Notch signaling is activated in human hepatocellular carcinoma and induces tumor formation in mice. Gastroenterology 2012 Dec;143(6):1660-1669 e1667.

22. Zender S, Nickeleit I, Wuestefeld T, Sorensen I, Dauch D, Bozko P, et al. A critical role for notch signaling in the formation of cholangiocellular carcinomas. Cancer cell 2013 Jun 10;23(6):784-795.

23. Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, et al. Notch signaling controls liver development by regulating biliary differentiation. Development (Cambridge, England) 2009 May;136(10):1727-1739.

24. Espinoza I, Miele L. Notch inhibitors for cancer treatment. Pharmacology & therapeutics 2013 Aug;139(2):95-110.

25. Yin L, Velazquez OC, Liu ZJ. Notch signaling: emerging molecular targets for cancer therapy. Biochemical pharmacology 2010 Sep 1;80(5):690-701.

26. Groth C, Fortini ME. Therapeutic approaches to modulating Notch signaling: current challenges and future prospects. Seminars in cell & developmental biology 2012 Jun;23(4):465-472.

27. Guest RV, Boulter L, Kendall TJ, Minnis-Lyons SE, Walker R, Wigmore SJ, et al. Cell lineage tracing reveals a biliary origin of intrahepatic cholangiocarcinoma. Cancer research 2013 Feb 15;74(4):1005-1010.

28. Huntzicker EG, Hotzel K, Choy L, Che L, Ross J, Pau G, et al. Differential effects of targeting Notch receptors in a mouse model of liver cancer. Hepatology (Baltimore, Md 2014 Oct 13.

Contribution to international publications

Research articles

- Spirli C, Locatelli L, Morell CM, Fiorotto R, Morton SD, Cadamuro M, Fabris L, Strazzabosco M. *"Protein kinase A-dependent pSer675 -β-catenin, a novel signaling defect in a mouse model of congenital hepatic fibrosis".* Hepatology. 2013 Nov;58(5):1713-23.

- Fiorotto R, Raizner A, Morell CM, Torsello B, Scirpo R, Fabris L, Spirli C, Strazzabosco M. *"Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice".* J Hepatol. 2013 Jul;59(1):124-30.

- Spirli C, Morell CM, Locatelli L, Okolicsanyi S, Ferrero C, Kim AK, Fabris L, Fiorotto R, Strazzabosco M. *"Cyclic AMP/PKA-dependent paradoxical activation of Raf/MEK/ERK signaling in polycystin-2 defective mice treated with Sorafenib".* Hepatology. 2012 Dec;56(6):2363-74.

- Spirli C, Locatelli L, Fiorotto R, Morell CM, Fabris L, Pozzan T, Strazzabosco M. *"Altered store operated calcium entry increases cyclic 3',5'-adenosine monophosphate production and extracellular signal-regulated kinases 1 and 2 phosphorylation in polycystin-2-defective cholangiocytes".* Hepatology. 2012 Mar;55(3):856-68.

Reviews

- Morell CM and Strazzabosco M. *"Notch signaling and new therapeutic options in liver disease".* J Hepatol. 2014 Apr;60(4):885-90

- Morell CM, Fiorotto R, Fabris L, Strazzabosco M. *"Notch signalling beyond liver development: Emerging concepts in liver repair and oncogenesis".* Clin Res Hepatol Gastroenterol. 2013 Nov;37(5):447-54.

- Morell CM, Fabris L, Strazzabosco M. *"Vascular Biology of the Biliary Epithelium".* J Gastroenterol Hepatol. 2013 Aug;28 Suppl 1:26-32.