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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 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 the 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.

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 true 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 it, 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 ubiquitination, glycosylation or endocytosis. Continuous Notch activation requires constant exposure to additional ligands, as NICD undergoes rapid proteasomal 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.

Keywords: Notch signaling; Liver repair; Liver cancer; Notch inhibitors.
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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, γ-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; CCl_4, carbon tetrachloride.

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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 trial for cancer treatment (reviewed in [20,21]) (see Fig. 1 and Table 1).

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 cholangiocytes). 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. With comparison 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, HNF1β, 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].

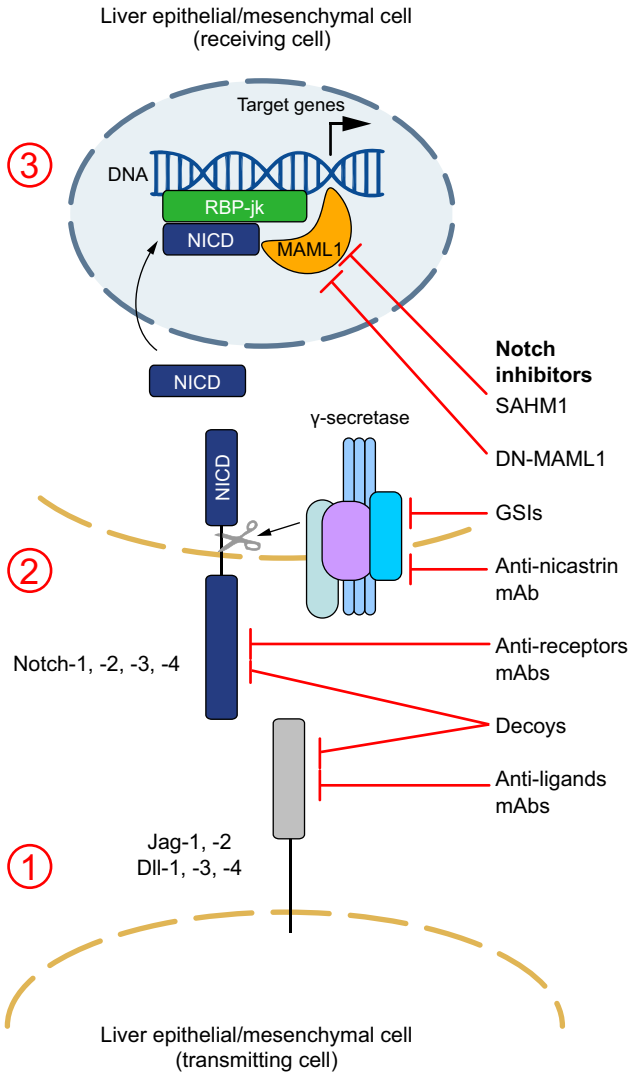

Fig. 1. Schematic representation of Notch signaling and potential inhibitory strategies. Notch pathway requires different steps to transmit the signal from the "transmitting cell" to the adjacent "receiving cell". (1) Ligand/receptor binding is the very first step that leads to signaling activation. 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).

Table 1. Available Notch-interfering agents and suggested applications for liver diseases treatment.

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 Selectively target Notch-1 or -2 in liver cancer cells
Decoys				
Soluble N1, Jag1, Dll1	*In vitro* studies Preclinical studies	• Small, soluble molecules	• Unknown pharmacokinetics • Unknown biodistribution	

Table 1 shows different classes of compounds to inhibit Notch signalling, some of which are under preclinical investigation. These agents block Notch signalling 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].

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 they 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 transdifferentiation 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 in 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 CCl_4-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 cell 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 the 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

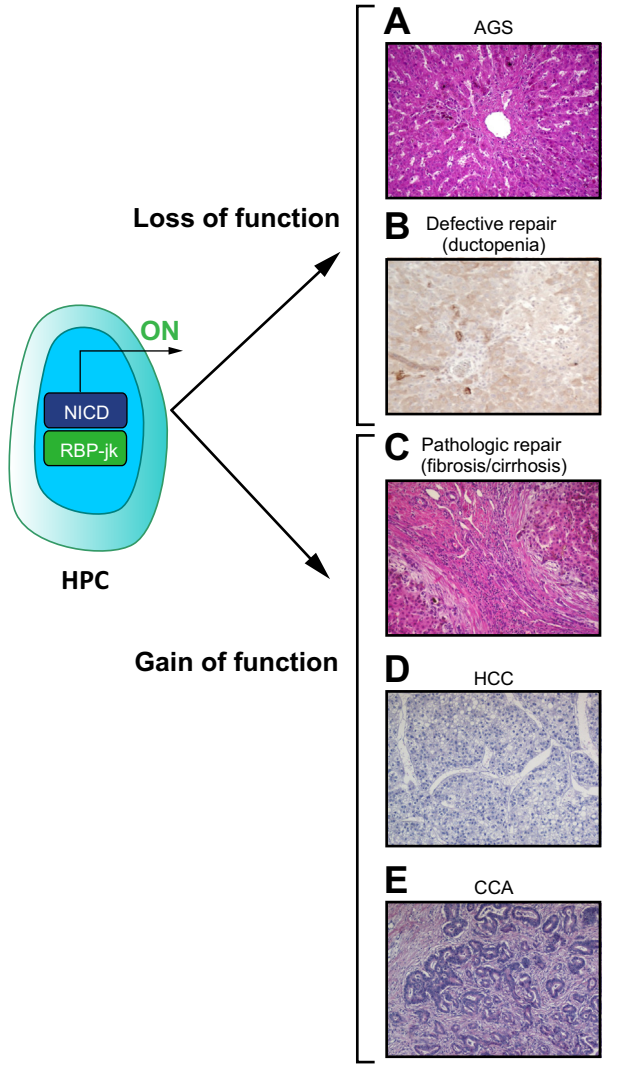

Fig. 2. Pathological conditions possibly involving Notch loss/gain of function, working hypothesis. Proper Notch activation is fundamental for HPCs biology. (A) Defective Notch signaling causes AGS, a condition characterized predominantly by ductopenia and consequent cholestasis. In this context, HPCs 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 resulting 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 H&E 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.

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 with 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 (Fig. 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 a specific cell context and the complex interplay with additional partners involved.

Key Points

- In the liver, Notch signaling is critical for biliary tree formation by committing hepatoblasts toward the cholangiocyte-fate and by orchestrating tubulogenesis. Defective Notch signaling causes Alagille Syndrome with ductopenia and cholestasis
- Notch activation has been demonstrated in response to liver damage, where it appears to be fundamental for regenerating damaged cholangiocytes and bile ductules
- Persistent activation of Notch eventually leads to pathologic liver repair and fibrosis; preliminary studies suggest Notch inhibition may reduce liver fibrosis in experimental models
- Ectopic and persistent Notch activation in the liver may be linked to hepatic cancer. Interestingly both HCC and CCA have been described in animal models overexpressing Notch in liver epithelial cells
- Understanding the pathophysiological role of Notch in liver disease may turn out to be of therapeutic relevance, in fact several therapeutic tools are being developed

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Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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