



# Biliary stem cells in health and cholangiopathies and cholangiocarcinoma

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## Purpose of review

This review discusses evidence regarding progenitor populations of the biliary tree in the tissue regeneration and homeostasis, and the pathobiology of cholangiopathies and malignancies.

## Recent findings

In embryogenesis biliary multipotent progenitor subpopulation contributes cells not only to the pancreas and gall bladder but also to the liver. Cells equipped with a constellation of markers suggestive of the primitive endodermal phenotype exist in the peribiliary glands, the bile duct glands, of the intra- and extrahepatic bile ducts. These cells are able to be isolated and cultured easily, which demonstrates the persistence of a stable phenotype during *in vitro* expansion, the ability to self-renew *in vitro*, and the ability to differentiate between hepatocyte and biliary and pancreatic islet fates.

## Summary

In normal human livers, stem/progenitors cells are mostly restricted in two distinct niches, which are the bile ductules/canals of Hering and the peribiliary glands (PBGs) present inside the wall of large intrahepatic bile ducts. The existence of a network of stem/progenitor cell niches within the liver and along the entire biliary tree inform a patho-biological-based translational approach to biliary diseases and cholangiocarcinoma since it poses the basis to understand biliary regeneration after extensive or chronic injuries and progression to fibrosis and cancer.

## Keywords

cholangiocarcinoma, cholangiocytes, peribiliary glands, primary sclerosing cholangitis, stem cells

## INTRODUCTION

Liver, biliary tree, and ventral pancreas share a common origin from an endodermal bud of the foregut [1–3]. Only recently, the precise lineage hierarchy and succession of events leading to the segregation of an endoderm progenitor compartment into hepatic, biliary and pancreatic structures have been depicted which demonstrated that a multipotent progenitor subpopulation persists in the pancreato-biliary organ rudiment, contributing cells not only to the pancreas and gall bladder but also to the liver [4]. These results are crucial to understand trajectories taken by the biliary progenitor cells along ongoing organogenesis and in cholangiopathies. On the same line, another significant and recent investigation demonstrated that intrahepatic cholangiocyte regenerate upon resumption of Jagged/Notch signaling, from multipotent progenitors originating from an Fgf-dependent extrahepatic stem cell niche in a zebrafish model of Alagille Syndrome [5]. In normal human livers, epithelial cell adhesion molecule (EpCAM) positive stem/progenitors cells are mostly restricted in two

distinct niches, which are the bile ductules or canals of Hering and the mucous glands present inside the wall of large intrahepatic bile ducts [the so-called peribiliary glands (PBGs)] [1,2]. The elegant single-cell transcriptomic atlas compiled by Aizarani *et al.* confirmed the presence of transcriptomic heterogeneity in the EpCAM+ population in human and, within this population, identified the cell fraction with the highest potential to form liver organoids

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## KEY POINTS

- In embryogenesis, a biliary multipotent progenitor subpopulation contributes cells not only to the pancreas and gall bladder but also to the liver.
- Stem/progenitor cells expressing markers of primitive endoderm exist in the peribiliary glands, the bile duct glands, of the intra- and extrahepatic bile ducts.
- The bile duct wall constitutes a well-organized barrier: the biliary barrier.
- Human biliary tree stem cells and peribiliary glands niches are involved in primary sclerosing cholangitis and intrahepatic large bile duct and perihilar cholangiocarcinoma development.

and to putatively serve as a stem cell compartment: a Mucin 6 high EpCAM+ population residing in intrahepatic large bile ducts furnished of PBGs [6]. The organoid technique was precious instrument to analyze the phenotype and functional heterogeneity of the biliary tree [intrahepatic bile ducts (IHBD) vs. extrahepatic bile ducts (EHBD)]. Taken together, results demonstrated that differences exist between IHBD and EHBD organoids [7–14]. Although, the existence of small and large cholangiocytes is a historical discovery made by Alpini and Coll. almost 30 years ago [15–17], only recently, thanks to a wise experimental approach, it has been clarified that IHBD and EHBD have distinct lineage fate and that EHBD-derived organoids provide a competent model to study bile duct diseases like cystic fibrosis [11]. As a result of an international long lasting collaboration started in 2009 [1,2,18–21], we have participated to the discovery of the presence of cells equipped with a constellation of markers suggestive of the primitive endodermal phenotype in the PBGs [1,2,18–21], the bile duct glands residing within the duct wall or even in the peribiliary tissue outside the wall, of the IHBD and EHBD. These cells are able to be isolated and cultured easily, which demonstrates the persistence of a stable phenotype during *in vitro* expansion [1,2,18–21]. These cells showed the ability to self-renew *in vitro*, a fundamental property of stem cells. They differentiated between hepatocyte and biliary and pancreatic islet fates in defined mediums. Notably, transplantation into the liver of immune-deficient (SCID) mice or into the epididymal fat of diabetic SCID mice resulted in differentiation towards the hepatic or pancreatic islet lineage and detectable levels of human c-peptide, respectively [1,2,18–21]. For this reason, we conclude that biliary glands contain cells with a differentiating capacity for mature endodermal fates. We

have called them human biliary tree stem/progenitor cells (hBTSCs) [1,2,18–21]. At anatomical level in human, a longitudinal axis exists in the biliary tree: from the hepatopancreatic ampulla, where the most primitive stem cells reside, to intrahepatic bile ducts [2,18,22]. hBTSCs (adult and fetal liver) constitute a physiologic source of hepatocytes and  $\beta$ -cells and a possible target for therapeutic strategies [23,24]. New specific markers for study and isolation are under investigation. hBTSCs from fetal liver have already been used in cell therapy of cirrhotic patients and are among the few identified sources ready for liver regenerative medicine [25]. hBTSCs are a tool for *in vitro* disease modeling in 2D and 3D [26]. Once discovered a new tissue progenitor niche, a sort of cognitive knowledge could be identified, in which the areas of interest range from the role of the stem/progenitor in development/embryogenesis, up to pathophysiology and carcinogenesis. In this review we will follow this theoretical streamline by presenting experimental and translational evidence regarding the study of progenitor populations of the biliary tree: the tissue regeneration and homeostasis, the pathobiology of diseases and malignancies, the application into the regenerative medicine.

## RADIAL (TRANSVERSAL) AXIS, AND THE BILIARY BARRIER

As largely demonstrated for the intestine, similarly, the bile duct wall constitutes a well-organized barrier capable of counteracting the toxic environment of bile through active energy expenditure [27]. The histo-morphological, molecular and functional features of the biliary barrier have not largely investigated [27]. The biliary barrier and defense systems include mechanisms shared with the gut, for example, immunoglobulins A (IgA), defensins, Toll-like receptor (TLR)-mediated immune activation, etc. Microorganisms must possess tolerance mechanisms in order to resist bile action [26,27]. Experiments in germ-free *mdr 2* knockout mice show exacerbated biochemical and histological features of sclerosing cholangitis [28]. These results demonstrate the physiological role of the commensal microbiota [28]. In human, primary sclerosing cholangitis (PSC) is characterized by an altered microbiome of the upper alimentary tract and bile ducts [29]. The biliary tree possesses a discrete vasculature: the peribiliary vascular plexus (PBVP) [30]. PBVP represents the substrate for a recirculation of biliary components in the liver. Thus, maintaining the analogy with gut-liver axis, it could be conceivable to define such a communication as bilio-hepatic axis.

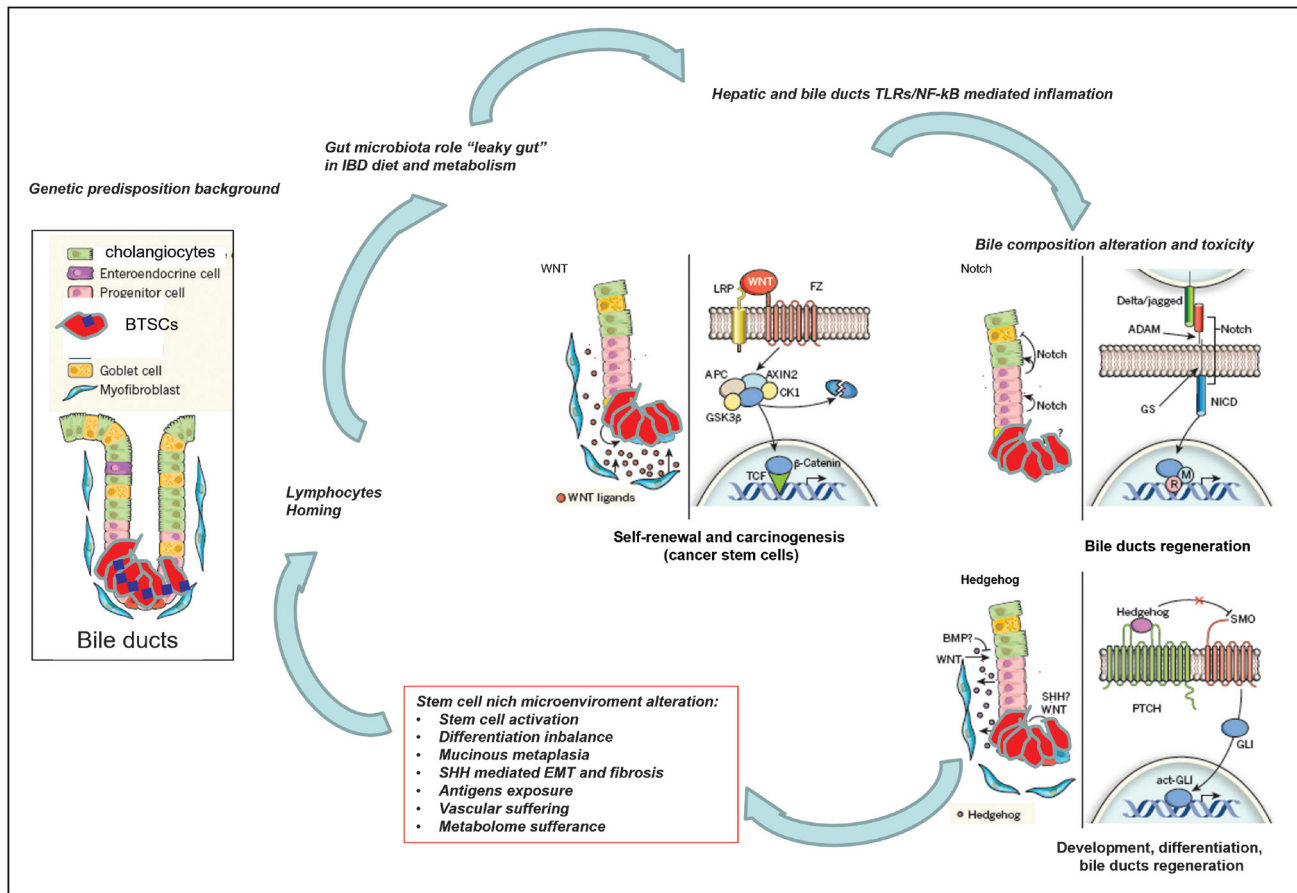
## PERIBILIARY GLAND NICHE IN HOMEOSTASIS OF BILE DUCTS

The existence of multiple cell lines within PBGs and robust proliferation of PBG cells following duct injury were confirmed in mice [31]. Moreover, using an *ex vivo* model based on precision-cut slices it was demonstrated that human biliary progenitor cells within PBGs are able to respond to bile duct epithelial loss with proliferation, differentiation, and maturation to restore epithelia [32]. In the colony formation assay, PBG cells showed significantly higher colony formation capacity than cholangiocytes lining the biliary epithelia [33]. Notably, different epithelial tissues showed a similar functional organization as demonstrated in experimental models of lineage tracing [34–39]. The ablation of lining epithelia cells reactivates the multipotency of basal cells from multiple epithelia both *in vivo* in mice and *in vitro* in organoids [34]. On this line, investigations in experimental model of large bile duct/extrahepatic bile duct injury demonstrated clearly the contribution of PBG cells in the regeneration of mature biliary epithelium in experimental models of sclerosing cholangitis, being governed in concert by Wnt and Notch pathways [40]. The differentiation of PBG cells into mature cholangiocytes implied the switch from a glycolytic to an oxidative metabolism [41,42].

## HUMAN BILIARY TREE STEM/PROGENITOR CELLS AND PERIBILIARY GLAND NICHE INVOLVEMENT IN PATHOLOGY

Following the conceptual framework of the cell/tissue of origin within defined pathological process (benign or malignant), in the last decade in a collaborative international effort the role of the two defined stem/progenitor niche of the liver (hepatic progenitors within the bile ductules/canals of Hering and BTSCs in PBGs) have been investigated to shed light into the patho-biology of cholangiopathies and cholangiocarcinoma [2,43]. Historically PSC and primary biliary cholangitis (PBC) have been coupled within the spectrum of cholangiopathies [2,43]. Both diseases are characterizing by the appearance of ductular reaction (DR) as the results of hepatic progenitor cell activation [43]. Nevertheless, progenitor cell activation differs between PSC and PBC and is characterized by a divergent fate commitment and different signaling pathway predominance [43]. In other words, it appeared that PBC is sustained by a liver parenchymal injury directed vs. interlobular cholangiocytes with a consequential biliary fate committed DR, while on the contrary, PSC-induced ascending cholestasis

damages hepatocytes with the appearance of intermediate hepatocytes in the portal space. This evidence highlights the relevance of phenotyping DR to define undetermined cholestasis. In PSC samples, progressive hyperplasia, and mucinous metaplasia of PBGs characterize fibrotic large bile ducts [44]. PBGs undergo massive hyperplasia in relation to the worsening of histology and clinical stage in PSC [44]. Hyperplasia of PBGs is determined by the expansion of BTSCs, which also contributes to biliary fibrosis through epithelial-to-mesenchymal transition and is sustained by the signaling pathway mediated by hedgehog (Hh) ligands [44]. Histologically, dysplastic lesions and CCA in PSC patients mainly arise in diseased large IHBD and EHBD [45]. Pretumoral and tumor lesions are associated with high inflammation and PBG area and further determine the increase of bile duct wall thickness [45]. These observations indicate a ‘field cancerization’ in PSC, in analogy with inflammatory bowel diseases’ carcinogenesis occurring in ulcerative colitis, in which the epithelium is preconditioned to the future development of neoplastic lesions, for example, through hyper-vascularization [45]. So, it is rational nowadays to insert PBG at the center of PSC pathogenesis [2,44,46,47]. In a condition of genetic predisposition background, gut and biliary microbial dysbiosis and an increased intestinal permeability (‘leaky gut’) characterizing inflammatory bowel disease and/or unhealthy diet/and or metabolic disorders, culminate in bile composition alteration and toxicity, which in turn induce a stem/progenitor niche microenvironment alteration with several consequences such as, stem cell activation, differentiation imbalance, mucinous metaplasia, Hh pathway mediated EMT and fibrosis, antigens exposures, vascular suffering, cell metabolism suffering, etc. This bile duct wall ongoing chronic injury may predispose to gut-primed lymphocyte homing, which in turn perpetuate the inflammatory injury of the bile duct in a virtually irreversible vicious circle leading the sclerosing duct fibrosis and CCA development (Fig. 1). Clearly, cholangiopathies, both benign and malignant, are lineage-dependent diseases, leading to a crucial question whether we should consider an advancement into the classification and nomenclature for cholangiopathies, as done recently for CCA. Biliary barriers and biliary tree stem cell niche could be a therapeutic target in cholangiopathies or constitute a conceptual framework to conceive innovative early diagnostic tools [48]. The depiction of the molecular anatomy of PBG could lead to innovative biomarkers [49,50]. Large bile duct involvement in PSC evaluated by MRI and radiomic is becoming more and more relevant for risk stratification



**FIGURE 1.** Peribiliary glands at the center of PSC pathogenesis: a working hypothesis [2,19,20,40,43,45,46]. In the situation of genetic predisposition background, gut and biliary microbial dysbiosis, intestinal permeability, or 'leaky gut,' which characterizes inflammatory bowel disease and/or unhealthy diet and metabolic disorders, result in altered and toxic bile composition, which in turn induces an alteration in the stem/progenitor niche microenvironment, including activation of stem cells, differentiation imbalance, mucinous metaplasia, fibrosis mediated by the Hh pathway, exposure to antigens, vascular suffering, impaired cell metabolism, etc. This bile duct wall ongoing chronic injury may predispose to gut-primed lymphocyte homing, which in turn perpetuate the inflammatory injury of the bile duct in a virtually irreversible vicious circle leading the sclerosing duct fibrosis and CCA development. APC, adenomatous polyposis coli; BMP, bone morphogenetic protein; CK1, Casein kinase 1; EMT, epithelial-mesenchymal transition; FZ, frizzled; GLI, glioma-associated oncogene; GS, gamma secretase; GSK3 $\beta$ , glycogen synthase kinase-3-beta; IBD, inflammatory bowel disease; LRP, low-density lipoprotein receptor-related proteins; NF- $\kappa$ B, nuclear factor-kappa-light-chain-enhancer of activated B cells; NICD, notch intracellular domain; PTCH, protein patched homolog; SHH, sonic hedgehog; SMO, smoothened; TLRs, toll-like receptors; WNT, wingless-related integration site.

[51–53,54<sup>¶</sup>]. The field cancerization appears a relevant translation finding that in our opinion should inform the surgical approach in PSC liver transplantation in order to prevent CCA recurrence [54<sup>¶</sup>,55]. PBGs are closely associated with several diseases, e.g. IgG4 associated SC, PSC, hepatolithiasis, liver flukes, CCA, etc. [56,57]. PBGs, since the discovery of hBTSCs became a very relevant pathogenic tool to understand post transplantation biliary complications, the nonanastomotic biliary strictures, largely understudied before [32,41,58–61]. Human PBGs contain biliary progenitor cells and are able to

respond to bile duct epithelial loss with proliferation, differentiation, and maturation to restore epithelial integrity [41]. PBGs have a key role in the pathophysiology of ischemia-mediated cholangiopathies after liver transplantation, implying alterations in the peribiliary vascular plexus and in nutrient and oxygen inflow [41]. Clinically, these recent data have important implications, indicating the rationale for the use of normothermic machine perfusion (NMP) in orthotopic liver transplantation (OLT) procedures [61]. Favorable bile chemistry during NMP correlates well with better-preserved biliary

microvasculature and PBGs, with a preserved capacity for biliary regeneration [61]. PBGs and BTSCs are currently under investigation to depict biliary atresia pathogenesis both in experimental models [62,63], and in children undergoing Kasay procedure [64,65]. Interestingly, even in genetic determined intrahepatic cholestasis, such as ABCB4-related LPAC syndrome associated with an ABCB4 gene variant, large and extrahepatic bile duct injury mimicking a sclerosing cholangitis has been demonstrated [66]. Thus, also the raising knowledge determined by the advent of NGS to define cholestasis and cholangiopathies suggest to re-consider the classification and nomenclature for cholestasis and cholangiopathies based on cell/tissue of origin and patho-biological determinant over the clinical appearance.

## LINEAGE-BASED CARCINOGENESIS

To further demonstrate the importance of biliary tree progenitor niche, its role in neoplastic development must be considered [67]. It has been suggested that intraductal papillary neoplasm of bile duct (IPNB) derived from PBGs are preneoplastic lesions of mucin-producing CCAs that morphologically resemble PBGs [68]. Interestingly, interleukin (IL)-33-mediated biliary epithelial injury-induced regenerative response accelerates the development of extrahepatic CCA from peribiliary glands, as demonstrated by lineage tracing models [69,70]. The role of PBGs in the genesis of ampullary tumors has been demonstrated in lineage tracing models [71]. Based on the existence of multiple cells of origin, the histology of CCA has been recently reclassified in the ICD-O 3.2 (5th edition of the WHO classification) [72–74]. This classification is based on the anatomical organization of the intrahepatic biliary tree and recapitulates the level or size of the displayed bile duct. Different intrahepatic cholangiocarcinoma (iCCA) histological subtypes can be identified, including large bile duct type [which histologically resembles perihilar cholangiocarcinoma (pCCA) and distal cholangiocarcinoma (dCCA)] and small bile duct type, which comprises also the so called cholangiolocarcinoma [72–75]. The molecular alterations of CCA ranges from SNVs, insertions and deletions, to copy number alterations and chromosome rearrangements/functions [76–82,83<sup>■</sup>,84,85]. This leads to a very complex pathway through analysis in routine clinical practice [76–82,83<sup>■</sup>,84,85]. There is a wide heterogeneity of the molecular alterations based on anatomical classification [76–82,83<sup>■</sup>,84,85]. From the point of view of markers for target therapies, intrahepatic CCA is among the tumors with the greatest actionable

mutations, in particular IDH1/2, FGFR2, whereas p/dCCA present a very low percentage of cases with actionable mutations, mainly in ERBB2 [75–82,83<sup>■</sup>,84,85]. Notably, etiology of chronic biliary or hepatic inflammation impact strongly on the molecular pathogenesis [79]. In other words, defined etiologies co-segregate with defined molecular alterations, for example, liver flukes and TP53 and/or ARID1A, PSC and KRAS [81,82]. Notably, a recent meta-analysis of  $n = 1481$  iCCA (52.1% surgical specimens), where pair-wise co-occurrences or mutual exclusivities of seven recurrent genetic driver mutations have been evaluated, showed an interesting clinico-pathological and molecular clustering [83<sup>■</sup>]. Namely, cluster 1 [KRAS (17%), TP53 (22%), and/or SMAD4 (6%)] resulted constituted by large bile duct iCCAs, and cluster 2 [FGFR2-fus (7%), IDH (15%), or BAP1 (12%)] by small bile duct iCCAs. Cluster 1 demonstrated the worst outcome in term of overall survival (OS) or recurrence-free survival (RFS) [81]. Importantly, there is a significant difference when it comes to treatment choice; the small duct type is known to harbor isocitrate dehydrogenase (IDH)-1 and -2 mutations and fibroblast growth factor receptor (FGFR) fusions which are treatable with currently available targeted therapies. On the other hand, the large duct type often presents with KRAS, and SMAD4 mutations, also observed in the perihilar and distal CCA [76–82,83<sup>■</sup>,84,85]. These data indicate the utility of subtyping iCCA in terms of clinical outcome and treatment choice [83<sup>■</sup>]. The inter-tumor heterogeneity of CCA might be due to the interplay of distinct tissues/cells of origin, the underlying disease, and the associated molecular clustering based on driver mutations which shape the pathobiological features of the different CCA subtypes [84]. Multiparametric and holistic approach to the patho-biological characterization of the CCA patients, is useful in a prognostic-therapeutic sense and also for defining patients at risk for screening/early diagnosis purposes. Studies that depict the correlates of homogenous clusters of subjects based on a tissue-based patho-biological classification could lead to have effective noninvasive tools (liquid biopsy) for precision medicine in CCA.

## CONCLUSION

A systemic rational approach based on the concept of keeping the fidelity through the multiple domains (clinical and high throughput-based) depicting overall each patient with her/his disease, as per the network medicine precepts, will bring us into future tools for an effective precision medicine. The existence of a network of stem/progenitor cell

niches within the liver and along the entire biliary tree should inform a patho-biological-based translational approach to biliary diseases and CCA [2,86].

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## Conflicts of interest

There are no conflicts of interest.

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- of special interest
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