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Biliary tree stem/progenitor cells in glands of extrahepatic and intraheptic bile ducts: an anatomical *in situ* study yielding evidence of maturational lineages

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SUMMARY

Stem/progenitors have been identified intrahepatically in canals of Hering and extrahepatically in glands of the biliary tree. Glands of the biliary tree (peribiliary glands: PBGs) are tubulo-alveolar glands with mucinous and serous acini, located deep within intrahepatic and extrahepatic bile ducts. We have shown that biliary tree stem/progenitors (BTSCs) are multipotent, giving rise *in vitro* and *in vivo* to hepatocytes, cholangiocytes or pancreatic islets. Cells with the phenotype of BTSCs are located at the bottom of the PBGs near the fibromuscular layer. They are phenotypically heterogeneous expressing transcription factors and surface and cytoplasmic markers for stem/progenitors of liver (e.g. SOX9/17), pancreas (e.g. PDX1) and endoderm (e.g.

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Y.W. and V.C. are co-equal authors in all the original studies identifying the stem cells in the PBGs. Also, Y.W. also provided the initial evidence of ongoing organogenesis for liver and biliary tree;
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SOX17, EpCAM, NCAM, CXCR4, Lgr5, OCT4), but not for mature markers (e.g. albumin, secretin receptor, or insulin). Subpopulations co-expressing liver and pancreatic markers (e.g. PDX1+/SOX17+), are EpCAM±, and are assumed to be the most primitive of the BTSC subpopulations. They give rise to descendents undergoing a maturational lineage process from the interior to the surface of ducts and varying in mature cells generated: pancreatic cells in hepato-pancreatic ducts; liver cells in large intrahepatic bile ducts; and bile duct cells along most of the biliary tree. We hypothesize that there is ongoing organogenesis throughout life with BTSCs giving rise to hepatic stem cells in the canals of Hering and to committed progenitors within the pancreas. The BTSCs are likely to be central to normal tissue turnover and injury repair and to be key elements in the pathophysiology of liver, pancreas and biliary tree diseases including oncogenesis.

Keywords

multipotent stem cells; endoderm; peribiliary glands; biliary tree; pancreas; liver; Secretin Receptor; insulin; albumin

INTRODUCTION

The biliary tree is a complex 3-dimensional network of interconnected ducts of increasing diameter from liver to intestine (Roskams et al., 2004). This network can be subdivided into two portions: the intrahepatic bile ducts (IHBDs) and the extrahepatic bile ducts (EHBDs). The EHBDs consist of the left and right hepatic ducts, the common hepatic duct, the gallbladder with the cystic duct, the bile duct (choledochus) and the hepatopancreatic ampulla. The hepatopancreatic ampulla drains into the duodenum via the Papilla of Vater. IHBDs start at the ductular-canalicular junction with the canals of Hering and continue with bile ductules, interlobular, septal, area and segmental ducts. Area and segmental ducts are considered large intrahepatic bile ducts, while septal ducts represent an intermediate link between the large and interlobular biliary system (Nakanuma et al., 1997). Large and septal IHBDs have several morphological/histological aspects and a embryological origin in common with EHBDs (Nakanuma et al., 1997).

From a histological point of view, a unique feature that joins the large intrahepatic bile ducts and the extrahepatic biliary system is the presence of glands in the duct walls. In the scientific literature, these glands are currently named peribiliary glands (PBGs) (Nakanuma et al., 1997) but, in the latest version of nomina anatomica, the term "*glands of bile duct*" is adopted. Here, we will refer to them as (peri-)biliary glands (or biliary tree glands) and the abbreviation "PBGs" will be used. PBGs are tubulo-alveolar glands with mucinous and serous glandular acini located in the deeper tissue of the bile duct walls and communicating with the duct lumen (Nakanuma et al., 1997).

From an embryological point of view, the biliary system shares a common origin with ventral pancreas (Nakanuma, 2010). A common stem/progenitor for liver, bile duct system, and pancreas exists at earlier stages of development, when the anterior definitive endoderm is forming the foregut (Lemaigre, 2009, Si-Tayeb et al., 2010, Wandzioch and Zaret, 2009, Zong and Stanger, 2011). The extrahepatic biliary tract originates directly from a portion of the ventral endoderm deriving from a pancreatobiliary stem/progenitor expressing PDX1 and SOX17 (Roskams and Desmet, 2008, Spence et al., 2009). The segregation of pancreatic and biliary precursors depends on SOX17; the primitive multipotent precursors are SOX17+/PDX1+ and give rise to SOX17+/PDX1− extrahepatic biliary cells and SOX17−/PDX1+ pancreatic cells (Spence et al., 2009). The endodermal proliferation occurring at the porta hepatis is actually considered the common phenomena giving rise to

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large (area and segmental) intrahepatic bile ducts. By contrast, the interlobular bile duct formation during the early phase of embryologic development derives from the differentiation of hepatic stem cells within the ductal plates, and their descendents, hepatoblasts, located closely to the forming portal tract and is driven by the appearance of SOX 9 expression (Zhang et al., 2008, Antoniou et al., 2009, Furuyama et al., 2010).

The canals of Hering have long been proposed to be a stem cell niche in postnatal livers (Alison et al., 1996, Schmelzer et al., 2007, Zhang et al., 2008, Gaudio et al., 2009, Okabe et al., 2009, Spee et al., 2010). They have been shown recently to derive from the ductal plates of fetal and neonatal livers (Zhang et al., 2008). In these ductal plates or canals of Hering are multipotent hepatic stem cells (HpSCs) that give rise to hepatoblasts, that are, in turn, bi-potent stem cells able to differentiate *in vivo* and *in vitro* to mature hepatocytes and cholangiocytes (Schmelzer et al., 2006, Schmelzer et al., 2007, Zhang et al., 2008, Wang et al., 2010, Turner et al., 2011).

The cell lineages within and along the biliary tree have not been investigated. So, it is unknown how many there are or their orientation. Nor has it been clarified whether there are alternative stem cell niches furnishing the biliary lineage of the bile ducts distal to the interlobular ones. It was recently shown (Furuyama et al., 2010) that adult intestinal cells, hepatocytes and pancreatic acinar cells are derived from SOX9-expressing stem/progenitors located throughout the biliary and pancreatic ductal epithelia suggesting interdependence between the structure and homeostasis of endodermal organs and with SOX9 expression being linked to stem/progenitor cell status. However, a more recent study (Carpentier et al., 2011) indicates that some of these findings are suspect, because the method of marking the cells for lineage tracing using Cre-Lox technologies was found to induce SOX9 expression; therefore, the findings using these technologies could have resulted in artifacts.

Recently, we have described the possibility to isolate multipotent biliary tree stem/progenitor cells (BTSCs) from human fetal and adult extrahepatic bile ducts (Cardinale et al., 2011). Those cells are located *in situ* in glands of biliary tree and are able, *in vitro* and *in vivo*, to differentiate into hepatocytes, cholangiocytes and pancreatic endocrine cells. We have not yet to assess whether they can also give rise to pancreatic acinar cells.

Accordingly, the aim of the present paper is to furnish a detailed characterization of the presence of progenitor-like cells (biliary tree stem/progenitor cells: BTSCs) with a endoderm-like phenotype located in glands (PBGs) along extrahepatic and intrahepatic bile duct system of adult human organs, to confirm their origin from foetal structures and to ascertain orientation and other facets of maturational lineages of cells derived from the BTSCs.

MATERIALS AND METHODS

Fetal Human Samples

Fetal livers and biliary tree tissue were provided by the Department of Gynecology of Sapienza University of Rome from fetuses of 18–22 week gestational age, and that were obtained from elective terminations of pregnancy. Informed consent was obtained from pregnant woman for use of the tissues for research purposes, protocols received Institutional Review Board approval, and processing was compliant with Good Manufacturing Practice. Extra-hepatic bile ducts (EHBD) were dissected from liver parenchyma. In particular, we included in this study: fetal gallbladder (N=3) and fetal common hepatic duct (N=3).

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Adult Human Samples

Human biliary, liver and pancreatic tissues were obtained from cadaveric donors. Livers were obtained when rejected for transplantation because of steatosis, whereas most of the biliary tree and pancreatic tissues were obtained because they were not used for transplantation. All tissues were obtained from the surgical department of Sapienza University of Rome, Italy. Informed consent was obtained from next of kin for use of the tissues for research purposes, protocols received Institutional Review Board approval, and processing was compliant with Good Manufacturing Practice. All of the samples derived from adults, ages 19–73. We included in this study the following tissues: adult liver fragments (N=10); adult gallbladder (N=10); adult cystic duct (N=10); adult common hepatic duct at hepatic hilum (N=5); adult common bile duct (N=5); and adult hepato-pancreatic ampulla (N=10).

Light Microscopy (LM), Immunohistochemistry (IHC) and Immunofluorescence (IF)

Specimens were fixed in 10% buffered formalin for 2–4 hours, embedded in low-temperature-fusion paraffin (55–57°C), and 3–4 μm sections were stained with hematoxylin-eosin. For IHC, sections were mounted on glass slides coated with 0.1% poly-L-lysine. Sections were hydrated in graded alcohol and rinsed in phosphate-buffered saline (PBS, pH 7.4). Endogenous peroxidase activity was blocked by a 30 min incubation in methanolic hydrogen peroxide (2.5%). The endogen biotin was then blocked by the Biotin Blocking System (Dako, code X0590, Glostrup, Denmark) according to the instructions supplied by the vendor. Antigens were retrieved by applying Proteinase K as suggested by the vendor, (Dako, code S3020) for 10 min at room temperature. Sections were then incubated overnight at 4°C with primary antibodies. A complete list of primary antibodies, sources, dilutions and their role as stem or progenitor marker is reported in Table 1. Samples were rinsed twice with PBS for 5 min, incubated for 20 min at room temperature with secondary biotinylated antibody (LSAB+ System-HRP, Dako, code K0690) and then with Streptavidin-HRP (LSAB+ System-HRP, Dako, code K0690). Diaminobenzidine (Dako) was used as substrate, and sections were counterstained with hematoxylin or PAS staining.

For Immunofluorescence, non-specific protein binding was blocked by 5% normal goat serum. Sections were incubated with primary antibodies at room temperature for 1 hour. All primary antibodies were diluted (1:50) in 1% BSA in PBS-Tween 20 (PBS-T). Then, sections were washed twice with PBS-T and incubated for 1 h with labeled isotype-specific secondary antibodies. These included anti-mouse AlexaFluor-488, -546, anti-rabbit Alexafluor-488. -536, anti-goat AlexaFluor-488, -546, anti-guinea pig-488 (Alexa fluor®, Invitrogen Ltd, Paisley, UK). They were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for visualization of cell nuclei. For all immunoreactions, negative controls consisted of the primary antibody being replaced with pre-immune serum.

Sections were examined in a coded fashion using the Leica Microsystems DM 4500 B Light and Fluorescence Microscopy (Weltzlar, Germany) equipped with a Jenoptik Prog Res C10 Plus Videocam (Jena, Germany). LM, IHC and IF observations were processed with an Image Analysis System (IAS - Delta Sistemi, Roma- Italy) and were performed independently by two pathologists in a blind fashion. The number of positive cells was counted in a random, blinded fashion in six non-overlapping fields (magnification × 20) for each slide. The data are expressed as % positive cells.

In the present paper, several markers of stem, progenitor and mature cells has been used; in particular, the markers have been considered as following (see also Table 1): i) OCT4, FOXa2, CXCR4: markers of definitive endoderm (Zaret and Grompe, 2008); ii) PDX1/ SOX17 (co-expression): markers of bilio-pancreatic progenitors iii) PDX1 (alone): marker

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of pancreatic progenitors; iv) SOX17 (alone): marker of biliary progenitors (Spence et al., 2009); v) Lgr5: marker of intestinal stem cells (Schepers et al., 2011); vi) SOX9, EpCAM, NCAM are wider markers which are expressed by definitive endoderm, bilio-pancreatic progenitor, hepatic stem cells; vii) insulin, albumin, HepPar-1, Secretin Receptor, CK19, CK7 are markers of mature cells (β-pancreatic cells, hepatocytes and cholangiocytes) (Turner et al., 2011).

For morphometric analysis, the surface occupied by PBGs was obtained in six non-overlapping fields (magnification ×10) for each slide and measured as volume occupied by glandular acini (μm^2). In Table 2, the average of mass occupied by endoderm-like/progenitor-like cells (in brackets) has been calculated as *PBG mass (average) * % of PDX1+/SOX19+ cells (average)*. In order to help the reader, data are summarized by a semi-quantitative score as previously elsewhere (Glaser et al., 2009b): − < 1%; +/− = 1–5%; + = 5–30%; ++ = 30–50%; +++ > 50%.

RESULTS

Extrahepatic and intrahepatic bile ducts in fetal tissues (Figure 1)

We examined gallbladders and common hepatic ducts from foetuses of 18–22 weeks gestational age (N=3). In common hepatic ducts, the bud of PBGs is observed as a small evagination of the surface epithelium (Figure 1A). At this stage, mucin-producing cells are not present (not shown). Biliary epithelial cells and PBG cells are diffusely positive for endodermal stem/progenitor markers, located primarily at the bottom of the developing glands (Figure 1A).

We examined livers (N=3) from fetuses of 18–22 week gestational age. At the hilum, large connective tracts could be observed containing segmental and septal bile ducts with well-formed ducts consisting of cells co-expressing EpCAM, PDX1, and SOX17 (Figure 1B). These ducts were continuous with ductal plates located at the interface of liver parenchyma (arrowheads in B). At this gestational age, no bud of PBGs could be observed at the level of the intrahepatic biliary tree.

Extrahepatic bile ducts in adults

Phenotype of cells within glands of EHBDs (Figures 2, 3 and 4)—We examined adult liver fragments (N=10); adult gallbladder (N=10); adult cystic duct (N=10); adult common hepatic duct at hepatic hilum (N=5); adult common bile duct (N=5); and adult hepatopancreatic ampulla (N=10).

In hepatopancreatic ampulla, bile duct, common hepatic duct and cystic duct, PBGs were present in duct walls and were heterogeneous in cellular composition. The distribution of (peri-) biliary glands along EHBD was not homogeneous: the highest glandular mass could be observed at hepatopancreatic ampulla where PBGs occupied an average volume of 31056.67 ± 1673.12 μm^2 for 10X-field. In common hepatic duct (at hepatic hilum) and in cystic duct, the PBG mass occupied an average volume of 6251.22 ± 691.15 μm^2 and 6867.47 ± 175.54 μm^2 respectively ($p< 0.01$ versus hepatopancreatic ampulla). Finally the lowest values have been calculated along bile duct (3115.53 ± 543.69 μm^2, $p< 0.01$ versus other EHBDs).

Extrahepatic bile ducts were composed mostly of cholangiocytes and mucin-producing cells which together represented more than 60% of the cells of the PBGs. The immunohistochemical analyses revealed that ≈12% of cells within PBGs expressed surface markers (EpCAM, NCAM, CXCR4, LGR5) and/or transcription factors (FOXa2, SOX9, OCT4) typical of endodermal stem cells and progenitors (Figure 2). Immunofluorescence

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protocols revealed that endoderm-like/progenitor-like cells [i.e. PDX1+/SOX17+ (or FOXa2+] accounted for 11 ± 3.81% of cells within PBGs (Figures 3A and 4, Table 2). These cells predominantly showed EpCAM expression: EpCAM+/PDX1+/SOX17+ cells represented 8 ± 2.55% of the cells whereas EpCAM−/PDX1+/SOX17+ cells accounted for 2 ± 1% (Figure 3A). However, the most interior of the cells were EpCAM− and transitioned to EpCAM+ cells with progression towards to the lumen. This suggests that the EpCAM− cells are giving rise to the EpCAM+ cells, an hypothesis supported also by findings in culture (Cardinale et al., 2011).

At the level of hepatopancreatic ampulla, the investigation of mature phenotypic markers indicated that cells in PBGs express markers of goblet cells (PAS+: 37 ± 5.70%), mature cholangiocytes (secretin receptor+: 27 ± 5.70%), β-islet cells (insulin+: 9.8 ± 3.56%), and, rarely, of hepatocytes (albumin+, HepPar1+: less than 5%). Moreover, dual immunostainings with EpCAM and mature cell markers showed that there was a large and heterogeneous population of intermediate cells (EpCAM+/PAS+, EpCAM+/SR+, EpCAM+/Insulin+, EpCAM+/Albumin+). These constituted 26 ± 6.52% of cells within PBGs (Figure 3B). Intermediate cells could be interpreted as newly derived cells originating from endoderm-like/progenitor-like cells (Yoon et al., 2011). Observations from hepatic duct and cystic duct showed similar results (Figure 4) with the exception of i) the percentage of insulin+ cells, that was found to be rare (less than 5%, $p<0.01$ vs hepatopancreatic ampulla; Table 2), ii) the percentage of HepPar1+ cells (12.2 ± 5.4%), that was higher in comparison with hepatopancreatic ampulla (less than 1%, $p<0.01$, Table 2); iii) the presence of pancreatic progenitor-like cells (i.e. PDX1+/SOX17− cells), which accounted for 13.2 ± 4.44% at the level of hepatopancreatic ampulla while could be rarely individuated in hepatic ducts (less than 5%, $p<0.01$).

Gallbladder is devoid of PBGs. However, cells expressing EpCAM, NCAM, CD133, CXCR4, LGR5, PDX1, and SOX17 were present within the surface epithelium (not shown).

Organization of stem cell niche in PBGs of EHBDs (Figure 5)

Progenitor-like and mature cells showed a typical organization in PBGs (Figure 5). Glandular acini located deep within duct wall (near the fibromuscular layer) were mostly composed of cells expressing stem/progenitor cell markers (e.g. EpCAM and PDX1: Figure 5C and F). In parallel, acini located near the surface epithelium were mostly constituted by cells negative for stem/progenitor cell markers and positive for markers of goblet cells (PAS +, Figure 5B). Finally, cells with a committed phenotype (e,g, goblet cells or mature cholangiocytes) were mostly present in acini located in the intermediate portion of glands of biliary tree (EpCAM+/PAS+: Figure 5A).

The expression of PCNA, a marker of cell proliferation, followed the distribution of stem cell markers (Figure 3 G–I). PCNA positive cells constitute 14.6 ± 3.50% of cells within PBGs and are located mostly at the bottom of PBGs. The number of PCNA positive cells progressively decreases as one moves towards the surface epithelium. At the surface, the PCNA+ cells are rare (less than 5%).

Intrahepatic bile ducts in adults (Figures 6 and 7)

We examined separately large (area and segmental), medium (septal), interlobular bile ducts and bile ductules within adult liver parenchyma (N=10). PBGs were found predominantly at the level of large bile ducts. In these ducts, two types of glands were observed: *intramural* and *extramural* PBGs. The intramural PBGs were positioned inside the duct wall and appear as shallow evaginations of the bile duct epithelium. Extramural PBGs were located outside the wall and are composed of PAS-negative cells (Figures 6, 7). At the level of septal bile

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ducts, PBGs can be observed occasionally as small evaginations of the bile duct epithelium. Finally, interlobular bile ducts are devoid of glands.

The morphometric analysis showed that (peri-)biliary glands of IHBDs accounted for a restricted area of liver parenchyma mostly located near the hilum (0.72% ± 0.38 of examined parenchyma). Moreover, the glandular mass occupied an average volume of 2629.21 ± 638.38 μm^2 for 10X-field which was significant lower when compared with hepatopancreatic ampulla, common hepatic ducts and cystic ducts ($p<0.01$).

In segmental, area and septal bile ducts, PBGs and biliary epithelia contained cells expressing EpCAM, PDX1, SOX17, NCAM, CD133, CXCR4, and LGR5. Observations on serial sections (Figure 7) indicated that in intra- and extramural PBGs, PDX1+/SOX17+ (endoderm-like/progenitor-like cells) cells were present accounting respectively for the 8.44 ± 2.96% and for 38 ± 10.46% of cells. As regards EpCAM expression, intramural (peri-)biliary glands were mostly composed of positive cells whereas extramural glandular cells displayed a faint positivity (Figure 6). Accordingly, extramural PBGs were mostly composed by endoderm-like/progenitor-like cells in comparison with intramural glands which, in turn, express higher level of EpCAM (intermediate phenotype).

Moreover, similar to that in the common hepatic duct, intrahepatic PBGs showed (Figures 4 and 7, Table 2): i) a rare percentage of insulin+ cells (less than 1%, $p<0.01$; Table 2), ii) a percentage of HepPar1+ cells (14.4 ± 3.78%), that was higher in comparison with hepatopancreatic ampulla; iii) the amount of pancreatic progenitor-like cells (i.e. PDX1+/SOX17− cells) which could identified was rare and less than 5%.

Finally, the epithelia of interlobular bile ducts do not show any cells co-expressing EpCAM, PDX1 and SOX17 (Table 2). Rarely, EpCAM positive cholangiocytes have been found in interlobular bile ducts.

DISCUSSION

In the present paper, we demonstrated that, in human, glands of the biliary tree (PBGs) represent niches of cells with classic phenotypic traits of stem/progenitor cells of endodermal origin with respect to transcription factors, surface and cytoplasmic markers, and with evidence for proliferation.

Since it is not correct that a cell is defined as a “stem cell” just because of the expression of certain markers without demonstrating a long term persistence, self-renewal and without a lineage tracing study, we refer to such cells as “progenitor-like”, or “endoderm-like”. However, our observations in human tissues should integrated with in vitro demonstration that (Pdx1+/Sox17+/EpCAM+) cells isolated from biliary tree have long term (in vitro) persistence, self-renewal and are able to give rise to a more restricted progeny (Cardinale et al., 2011). Moreover, in the paper by Spence et al., a lineage tracing study has been performed demonstrating that SOX17/PDX1 positive cells are the bilio-pancreatic precursors (Spence et al., 2009).

The study of the organization of cells within glands of biliary tree showed that: i) the progenitor-like cells and transit-amplifying cells were located at the bottom of the glands; ii) the cells with an intermediate phenotype between progenitor-like cells and mature cells were found in the middle of the glands; and iii) the fully differentiated cells were in continuum with the surface epithelium. Cells producing low levels of insulin are found within PBGs, especially in the hepatopancreatic common ducts, whereas those producing albumin are found in portions of the biliary tree near to liver. Endoderm-like/progenitor-like cells are

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also found in PBGs intrahepatically within large bile ducts, representing a candidate niche for the renewal of the intrahepatic cells.

Glands of biliary tree (PBGs) are tubular-alveolar glands composed of serous and mucinous acini located along extrahepatic and large intrahepatic bile ducts (Nakanuma et al., 1997). Glandular epithelial cells are resorptive with relevance to the mechanisms of concentration and resorption of bile constituents from the duct lumina to the surrounding vessels, including the lymphatics. These glands show some secretory activities (secretion of mucinous substances) and are also positive for pancreatic digestive enzymes (Terada et al., 1994).

Recently, we isolated multipotent stem/progenitor cells from fetal and adult extrahepatic biliary tree tissue. These cells were able *in vitro* and *in vivo* to generate mature hepatocytes, cholangiocytes and β-pancreatic cells (Cardinale et al., 2011). We have yet to test whether they can give rise to pancreatic acinar cells. In the present paper, we provide a more detailed description of the anatomical location of the BTSCs, the cellular phenotypes present and evidence for a maturational lineage process from the interior of the ducts to the luminal surface.

To assess our aims, several markers of stem, progenitor and mature cells has been evaluated; OCT4, FOXa2, CXCR4 are typical markers of definitive endoderm (Zaret and Grompe, 2008); biliopancreatic progenitor cells are SOX17+/PDX1+ and give rise to SOX17+/PDX1− biliary progenitors and SOX17−/PDX1+ pancreatic progenitors (Spence et al., 2009). Finally, Lgr5 is a recognized marker for intestinal stem cells (Schepers et al., 2011) while SOX9, EpCAM, NCAM are more diffusely distributed markers which are expressed by definitive endoderm, bilio-pancreatic progenitor and hepatic stem cells (Turner et al., 2011).

In PBGs, approximately 10% of the cells co-expressed PDX1, SOX17 and FOXa2, a phenotype indicative of the early stem cell subpopulation (endoderm-like cells). Moreover, we showed that glands of biliary tree were also composed by populations of cells with less primitive phenotypes which were characterized by the loss of FOXa2 and PDX1 expression (biliary progenitor-like cells) or, alternatively, by the loss of FOXa2 and SOX17 (pancreatic progenitor-like cells). Both the cell populations maintained the expression of EpCAM which is a wider expressed marker. Several lines of evidence indicate that EpCAM expression is tightly regulated and only occurs in case of a temporary need for proliferation and is immediately downregulated upon terminal differentiation (Trzpis et al., 2007, Maetzel et al., 2009).

Biliary Tree Stem Cells could represent the remnant in adults of the common stem/progenitor (PDX1+/SOX17+) for liver, bile duct system and pancreas which exists at earlier stages of development (Spence et al., 2009) and which is considered to derive from ventral endoderm (Wandzioch and Zaret, 2009, Zong and Stanger, 2011).

Within the body of the PBGs, we identified the presence of a committed intermediate (transit-amplifying) compartment in which were present cells co-expressing both stem cell markers and markers of mature cells: secretin receptor (cholangiocytes), mucins (goblet cells), insulin (endocrine pancreas) and, rarely, albumin (hepatocytes). The relative proportion of cells expressing mature cell markers indicative of liver versus bile duct versus pancreas correlated with the location along the length of the biliary tree. This portion of the compartment could be descendent of the endoderm-like/progenitor-like cells located at the base of the glands (Furuyama et al., 2010, Yoon et al., 2011).

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In accordance, PBGs present an organization along the duct wall: cells with an undifferentiated phenotype (EpCAM± /PDX1+/SOX17+/LGR5+) are situated mostly in the gland base near the fibromuscular layer. The transit-amplifying progenitor population (EpCAM+ /PDX1±/SOX17±/LGR5−) are concentrated in the body of the glands (corresponding to the middle of the duct wall). Finally, mature cells (e.g. cholangiocytes, goblet cells, hepatocytes, pancreatic cells) are located at the neck of PBGs in strict contact with the surface epithelium; which mature cell type is found depends on whether the portion of the biliary tree is near the liver (hepatocytes); in the middle of the biliary tree (cholangiocytes); or near the duodenum (pancreatic cells and goblet cells). The distribution of proliferating cells followed a similar gradient; in fact, the number of PCNA-positive cells was higher at the bottom of glands and decreased with progression towards the lumen. Interestingly, the cells of the surface epithelium were mostly PCNA negative. Taken together, these data indicate that PBGs contain progenitor-like cells which normally proliferate and are responsible for the renewal of the surface epithelium generating mature cells such as cholangiocytes and goblet cells (in the middle of the biliary tree) or hepatocytes (near the liver) or islet cells (near the pancreas).

In summary, the endoderm-like/progenitor-like cell compartment within PBGs resembles the organization of the stem cell niche within intestines. In the intestine, proliferation takes place at the base of intestinal crypts in which are located LGR5+ stem cells interspersed by Paneth cells (Pech and Artandi, 2011, Schepers et al., 2011). They give rise to progenitors produced from stem cells in the crypts (transit-amplifying population) migrate upwards and fully differentiate towards mature cells. The phenotype of the mature cell (e.g. whether stomach, duodenum, small intestine or large intestine) depends on the position of the villus in the anterior to posterior axis along the intestine (Shaker and Rubin, 2010, Sancho et al., 2003).

In PBGs, particularly those near the hepatopancreatic ampulla, there are individual cells able to produce insulin. The co-expression of EpCAM by insulin-producing cells seems to indicate that these elements represent intermediate cells that have partially differentiated. In mouse, the presence of pancreatic β-cells exhibiting a glucose-stimulated insulin secretion has already been shown in extrahepatic bile ducts (Dutton et al., 2007, Nagaya et al., 2009). Moreover, studies in genetically manipulated animals have indicated that the biliary tract is able to demonstrate pancreatic differentiation (Nakanuma, 2010, Eberhard et al., 2008). In $Hes1^{-/-}$ mice, biliary epithelia differentiate into endocrine and exocrine cells and form acini and islet-like structures in the mutant bile ducts (Sumazaki et al., 2004, Fukuda et al., 2006). In humans, the presence of insulin-producing cells within extrahepatic biliary tract was unknown until our studies (Cardinale et al., 2011). Clearly future studies must devote detailed characterization of the biliary tree, especially the hepatopancreatic ampulla, as the native reservoir of stem cells for pancreatic organogenesis in adults.

In adult human livers, a well-described stem cell niche is located within canals of Hering (Schmelzer et al., 2007, Zhang et al., 2008, Gaudio et al., 2009, Spee et al., 2010, Turner et al., 2011). The adult hepatic stem cell niche is derived from the ductal plates of fetal and neonatal livers (Zhang et al., 2008) and is composed by stem/progenitor cells named recently human hepatic stem cells (hHpSCs) (Schmelzer et al., 2007, Roskams and Desmet, 2008, Furuyama et al., 2010, Turner et al., 2011).

In normal conditions, hHpSCs are mostly quiescent taking no or minimal part in the renewal of liver parenchyma which is sustained by the replication of adult hepatocytes within the lobules and of cholangiocytes in interlobular bile ducts (Fausto, 2004, Gaudio et al., 2009). However, a recent study by Furuyama et al (Furuyama et al., 2010) showed by a Cre-based

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lineage tracing that hepatocytes are supplied physiologically from Sox9-expressing progenitors located within Canals of Hering.

In a variety of chronic and acute liver diseases, the proliferation of mature parenchymal liver cells is impaired by a variety of insults leading to the activation of a secondary proliferative pathway of hHpSCs and the appearance of reactive ductules (ductular reaction) which represent tortuous structures with no discernable lumen and composed of progenitor cells, with highly variable marker profiles (Spee et al., 2010). A number of recent evidence demonstrated how ductular reaction represents the morphological expression of activation of resident stem/progenitor cell pool aimed to repair liver damage either through the replacement of death cells or by driving fundamental repair processes including fibrosis and angiogenesis (Glaser et al., 2009a, Omenetti et al., 2008). In particular, in the setting of fibrosis following chronic hepatic injuries, hHpSCs has been demonstrated to be able to underlie an Epithelial to Mesenchynal Transition (EMT)(Omenetti et al., 2008, Svegliati-Baroni et al.); however, the exact role of EMT and its contribution in the hepatic fibrogenesis have not been fully clarified and are still under debate (Wells, 2010).

Beside the well-known niche at the level of Canals of Hering, our results indicate an additional set, indeed, a large number of cells with the phenotype of endodemal stem/progenitor cells within PBGs of the large intrahepatic bile ducts both in foetal and adult life implicating a newly identified reservoir of stem cells for the renewal of the large bile duct epithelium.

Our observations suggest that these niches are closely related to the ductal plate which has been recently demonstrated to generate the liver parenchyma during embryogenesis (Carpentier et al., 2011). This relationship is supported by our observation in fetal tissue in which progenitors in large bile ducts were continuous with ductal plates which transition into the canals of Hering (Zhang et al., 2008). Moreover, our observations are in touch with the hilum to periphery model of bile duct morphogenesis recently proposed by Lemaigre et associates (Antoniou et al., 2009).

Segmental, area and septal intrahepatic bile ducts share several common features with EHBDs such as the presence of PBGs (Nakanuma et al., 1997, Terada et al., 1994) and, interestingly, the presence of endoderm-like/progenitor-like cells that are EpCAM±/PDX1+/SOX17+/LGR5+. These observations are in accordance with the possibility of isolating multipotent stem cells from fetal and adult livers of rodents and other mammals (Suzuki et al., 2002, Yang et al., 2002).

Our observations could also have profound pathophysiologic implications since the PBGs, as stem cell niches, could play a relevant role in the pathogenesis of inflammatory (primary sclerosing cholangitis) and neoplastic (cholangiocarcinoma) diseases of the biliary tree. With respect to this point, the PBGs have morphology and phenotypic traits strongly similar to intestinal stem cells described by Cleaver and associates (Sancho et al., 2003) and to the pancreatic duct glands characterized by Bonner-Weir and associates or by Thayer and associates (Strobel et al., 2010). Thayer and her associates have determined that the pancreatic duct glands are comprised of progenitors and are sites responding to injuries and diseases. The morphology and phenotypic traits of the cells of the pancreatic duct glands are strikingly similar to those of the biliary tree glands. Further study is needed to clarify if the biliary tree glands and pancreatic duct glands are, in fact, variations on the same entities that share in the organogenesis process leading to both liver and to pancreas.

A number of recent studies have focused on the role of stem cells in the origins of cancer (Reya et al., 2001). A large body of current literature deals with the involvement of stem/progenitors in oncogenesis of the liver (Komuta et al., 2008, Yamashita et al., 2009, Ji et al.,

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2009, Marquardt et al., 2011, Woo et al., 2011). Human hepatic stem cells (hHpSCs) are actually considered cells of origin of some hepatocarcinoma and intrahepatic cholangiocarcinoma (Komuta et al., 2008, Yamashita et al., 2009, Ji et al., 2009). They have demonstrated how stem cell activators such as Wnt/beta-catenin, TGF-beta, Notch and Hedgehog signaling pathways also expedite liver tumorigenesis (Marquardt et al., 2011, Woo et al., 2011). Primary liver cancers arise in the context of hepato-biliary diseases of different aetiologies (Welzel et al., 2007, Cardinale et al., 2010, Tyson and El-Serag, 2011). As for cells of the pancreatic duct glands (Strobel et al., 2010), those of the PBGs are sites for increased vulnerability to diseases especially in response to the death of the injured mature cells or to continued stimuli to achieve a “restitutio ad integrum”, represents the initial step of the oncogenic process. Similarly cell proliferation and PBG hyperplasia represent common features associated with the pathologies that affect the large intrahepatic and the extrahepatic bile ducts, which are currently indicated as risk factors associated to the development of the cholangiocarcinoma (Cardinale et al., 2010). Our histological observation of stem cell niches within biliary tree suggests the possibility that they could be involved in cholangiocarcinoma development. In particular, our results suggest that endoderm-like stem cells within PBGs could represent the cells of origin of mucin-producing intrahepatic cholangiocarcinomas; this histological subtype of cholangiocarcinoma is characterized by mucin-producing cells and typically arises at the hepatic hilum in the context of intrahepatic segmental bile ducts (Blechacz et al., 2011); our data demonstrated how PBGs contain mucin-producing cells and their location overlaps with the sites in which the mucinous cholangiocarcinoma typically occurs; moreover, cholangiocarcinomas express several markers that are in common with PBG cells such as EpCAM, OCT4, CD133 (Komuta et al., 2008); finally, hilar cholangiocarcinoma has been correlated with definite risk factors (such as primary slerosing cholangitis, liver fluke infection) which determinate the proliferation of PBGs (Nakanuma et al., 2011, Tyson and El-Serag, 2011). The cancer cell of origin has great importance in tumor cell fate and pathology; the activation of the same genetic/epigenetic mutation in different cellular compartment of a given organ may have profound implication in malignant potential. The identification of the cells of origin of cancers has as prerequisite the characterization of the normal cellular hierarchy within a given tissue and the study of stem cells niches. The most primitive cells, stem cells, are candidates for targets of transformation because of their self-renewal and longevity, which would allow the sequential accumulation of genetic or epigenetic mutations required for oncogenesis (Visvader, 2011). Further studies on cholangiocarcinoma are needed to test the possibility that this tumour or its histological subtypes could derive from cells within PBGs.

In summary, the main and original findings of the present study indicate that in humans: i) glands of extrahepatic bile ducts (peribiliary glands: PBGs) represent the niche of endoderm-like and progenitor-like cells (biliary tree stem/progenitor cells: BTSC); ii) this niche follows the model of intestinal stem cell niche: proliferating cells and cells with a more primitive phenotype are located at the bottom of the gland, cells with an intermediate phenotype are placed in the middle of the gland and fully differentiated cells are in continuum with the surface epithelium; iii) insulin producing cells are located within PBGs of human adult extrahepatic bile ducts; iv) in intrahepatic biliary tree, endoderm-like and bilio-pancratic progenitor-like cells are present and located in PBGs of large bile ducts representing a new niche for the renewal of biliary epithelium; v) the endodermal stem/progenitor-like cells in the adult seem to be the remnant of endoderm and bilio-pancratic progenitor cells that in fetal life determinate the formation of biliary tree. Our data have implications for the knowledge on biliary tree development, physio-pathology and carcinogenesis and open novel scenarios in regenerative medicine, since the extrahepatic biliary tree could be a reservoir of stem cells useful in cell therapy programs for liver, biliary tree, and pancreas (e.g. diabetes).

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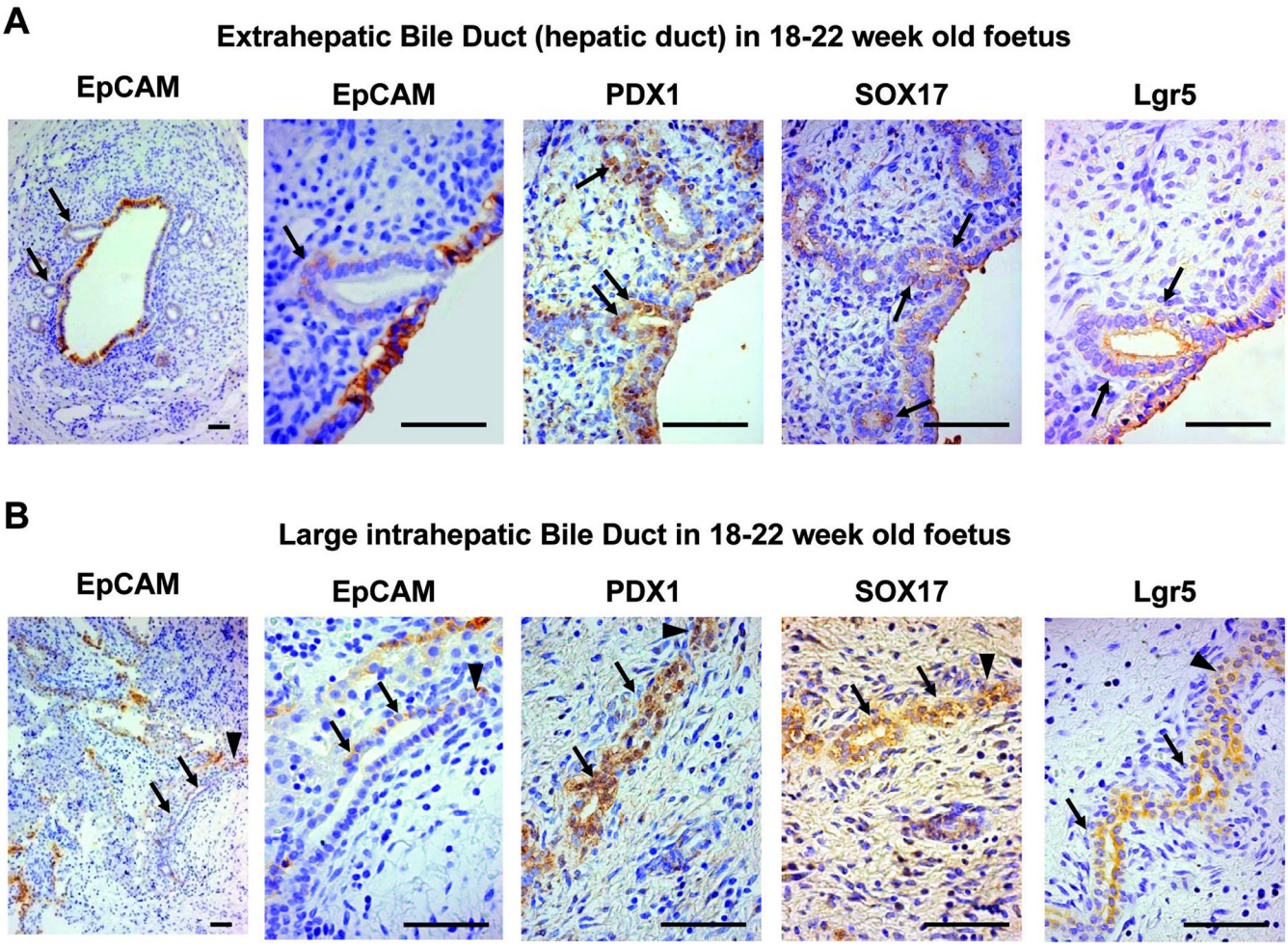

Figure 1.

A) 18–22 weeks old, fetal common hepatic duct (serial sections). The epithelia of fetal common hepatic duct are comprised of hepato-pancreatic progenitors (EpCAM ±, PDX1+, SOX17+, Lgr5+). Small evaginations of the epithelium, representing the bud of (peri-)biliary glands, were observed (arrows). The bud of PBGs are comprised of endodermal progenitors (arrows) that are located mostly at the bottom of the glands (see the arrow in the magnified image of cells stained with EpCAM). B) In 18–22 weeks old fetuses, the large intrahepatic bile duct can be found at the hilum. These ducts are composed mostly of endodermal (EpCAM±/PDX1+/SOX17+/Lgr5+) stem/progenitor cells (arrows). Scale Bar= 50μm.

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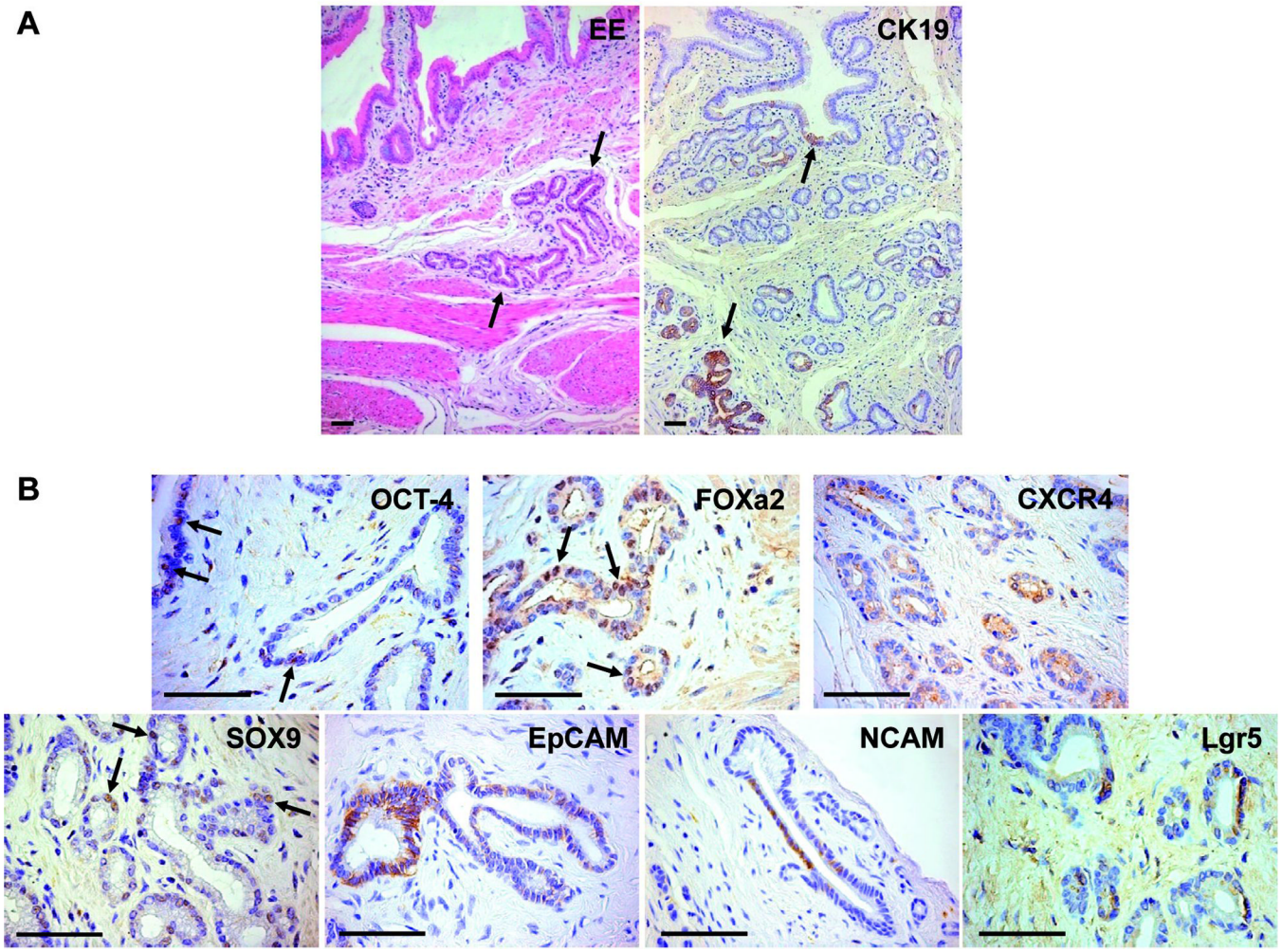

Figure 2.

Phenotype of glands within extrahepatic bile ducts. **A)** In haematoxylin-eosin stains, (peri-)biliary glands are tubulo-alveolar glands located in the lamina propria of duct walls (arrows). Immunohistochemistry showed the presence of Cytokeratin-19 (CK 19)+ cells in the surface epithelium (cholangiocytes) and in the PBGs (arrows). **B)** Immunohistochemistry for nuclear transcription factors and surface markers characterizing endodermal stem cells. Upper panels: cells within PBGs express markers typical of endodermal stem cells such as FOXa2, CXCR4, OCT4 (arrows). Lower panels: PBGs are composed of cells expressing typical markers of intestinal stem cell (LGR5) and hepatic stem cell such as EpCAM, NCAM, SOX9. Scale Bar= 50μm.

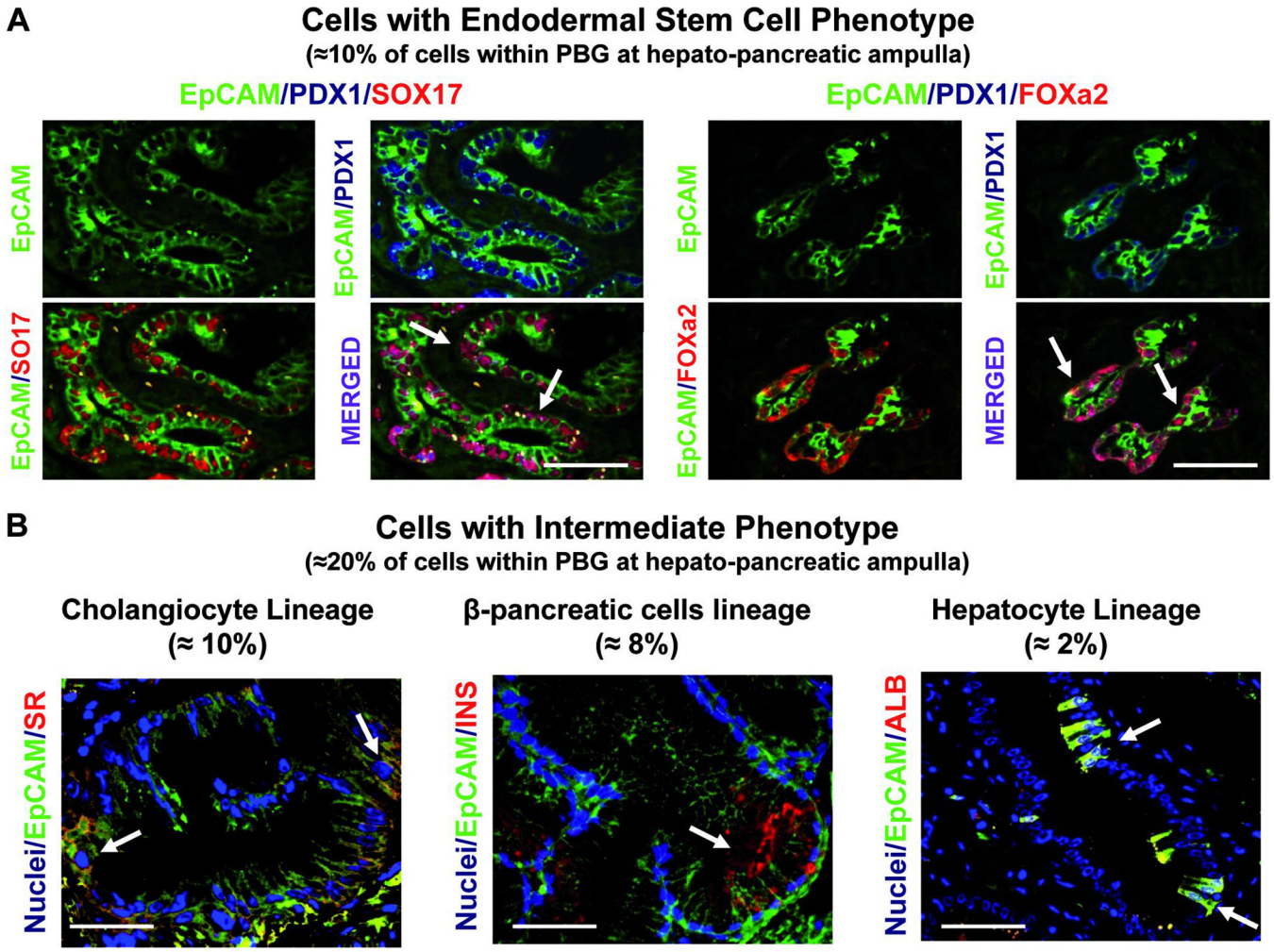

Figure 3.

Triple/double immunofluorescence staining demonstrating the presence of endoderm-like and progenitor-like cells (BTSCs) and cells with an intermediate phenotype within PBGs. A) EpCAM ± /PDX1+/SOX17+(or FOXa2+) cells were present in PBGs (arrows) representing ≈10% of the PBG cells. B) Double immunofluorescence staining indicated the presence of a population of EpCAM+ cells which co-express markers of mature cells such as secretin receptor (cholangiocyte lineage), insulin (β-pancreatic cells) and, rarely, albumin (hepatocyte lineage). This intermediate compartment represents ≈ 20% of cells of PBGs at hepato-pancreatic ampulla. Scale Bar= 50μm.

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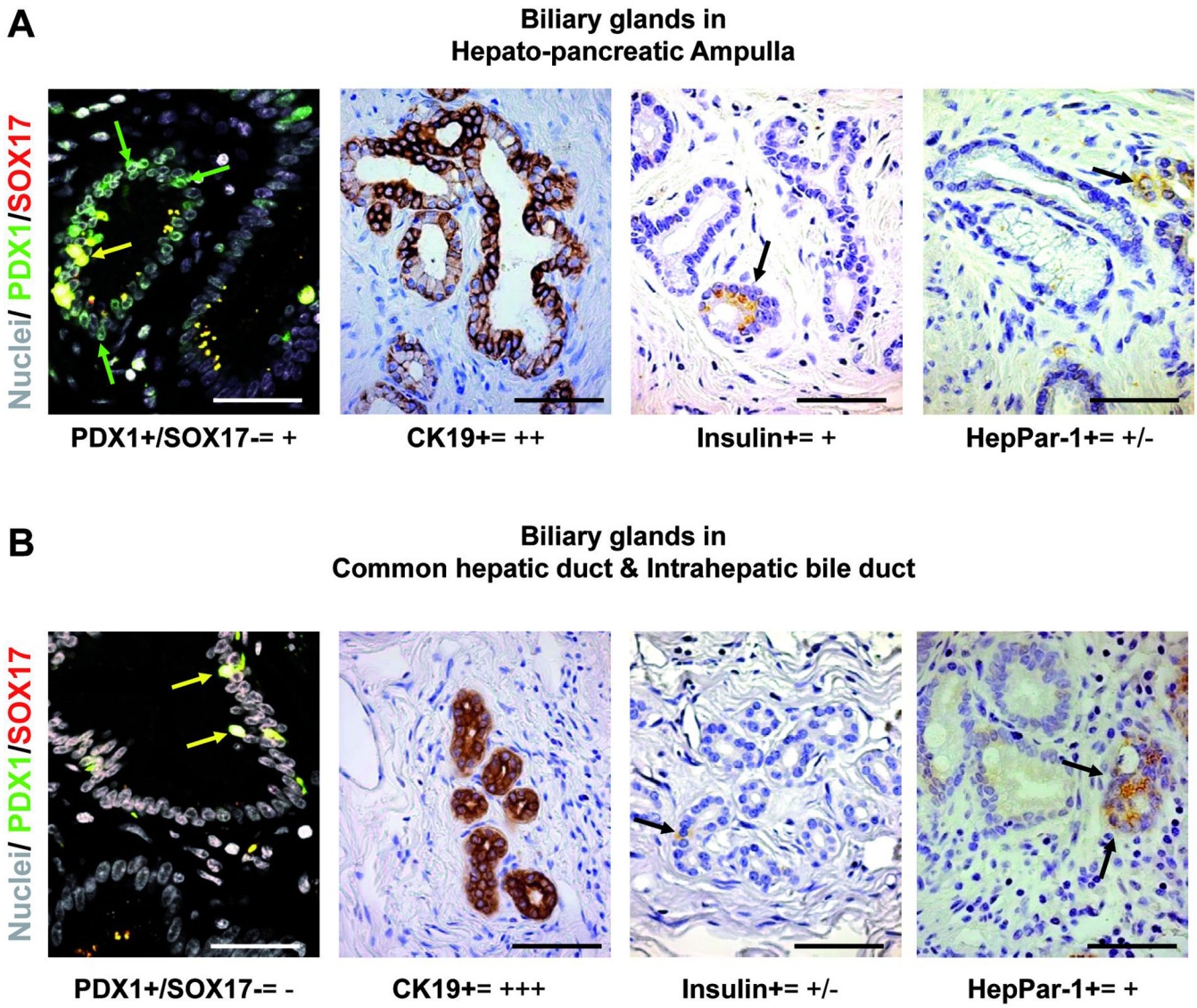

Figure 4.

Demonstration of the distinction in mature marker fate as one progresses along the biliary tree. A–B) Double immunofluorescence for PDX1 and SOX19 and immunohistochemistry for Cytokeratin 19, HepPar-1 and Insulin at the level of hepatopancreatic ampulla (A) and common hepatic/large intrahepatic bile ducts (B). Scale Bar= 50μm.

Several differences could be noted: the presence of pancreatic progenitor-like cells (i.e. PDX1+/SOX17− cells, green arrows) and insulin+ cells was higher near the hepatopancreatic ampulla when compared with hepatic/large intrahepatic bile ducts; by the contrast, the presence of HepPar+ cells was higher in common hepatic/large intrahepatic bile ducts in comparison with hepato-pancreatic ampulla (see also Table 2).

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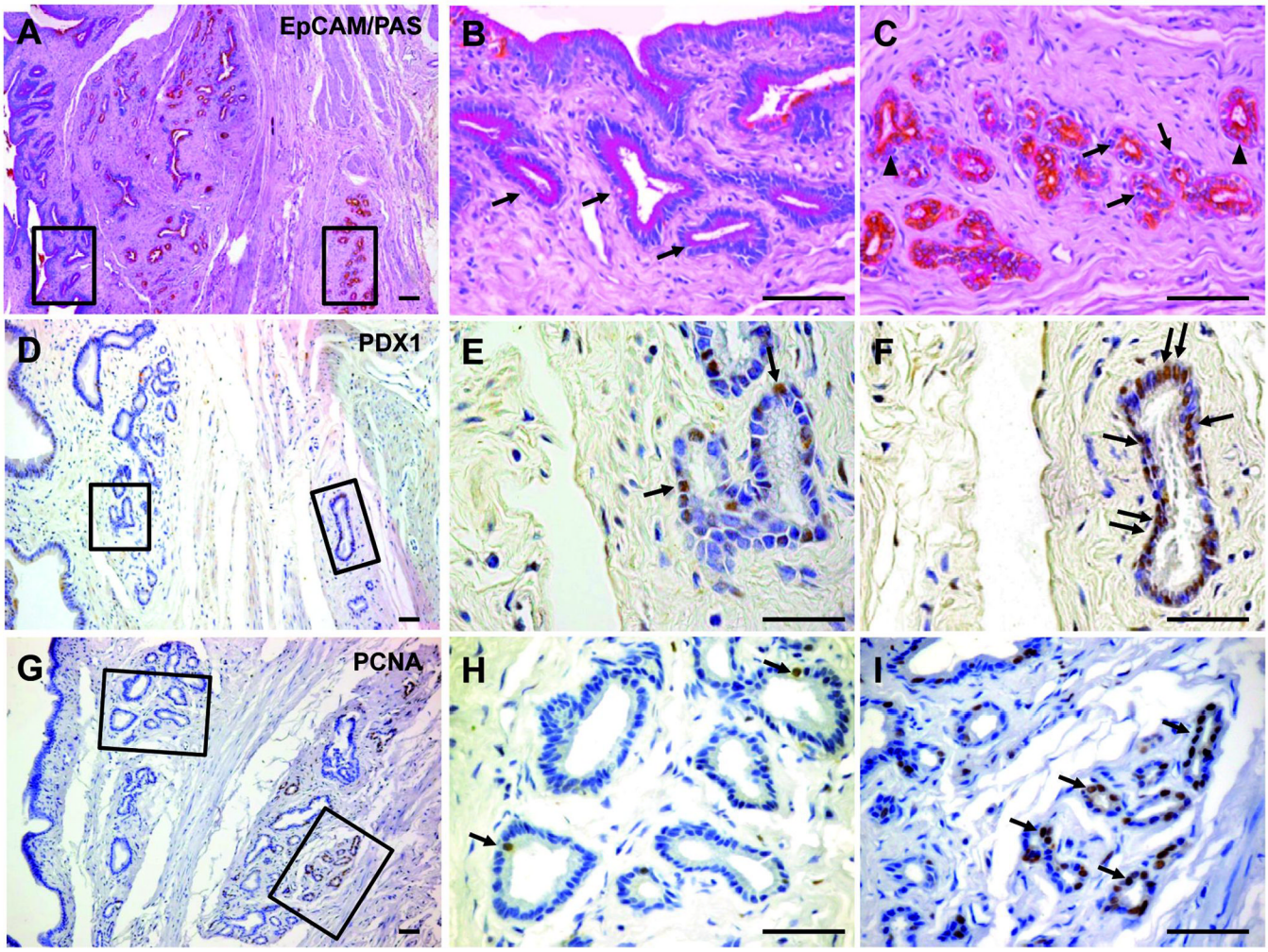

Figure 5.

Gradient in the expression of stem cell markers and of the proliferation of PBGs cells. A–C) Immunohistochemistry for EpCAM counterstained with PAS. Glandular element just beneath the surface epithelium (see magnification in B) are mostly EpCAM negative and PAS positive (goblet cells); by contrast, acini deeply located near the fibromuscular layer (see magnification in C) are composed of cells that are mostly EpCAM+ and PAS negative (arrows) or by EpCAM+/PAS+ cells (arrowhead). D–F) Immunohistochemistry for PDX1. PDX1+ cells are mostly situated deeply within duct walls (see magnification in F: arrows). PBGs near the surface epithelium are occasionally PDX1+ (arrow in E). G–I) Immunohistochemistry for PCNA. Proliferating cells are mostly present in glandular elements located near the fibromuscular layer (I: arrows). Few cells are positive in more superficial acini. Notably, surface epithelial cells are mostly negative for PCNA. Scale Bar= 50μm.

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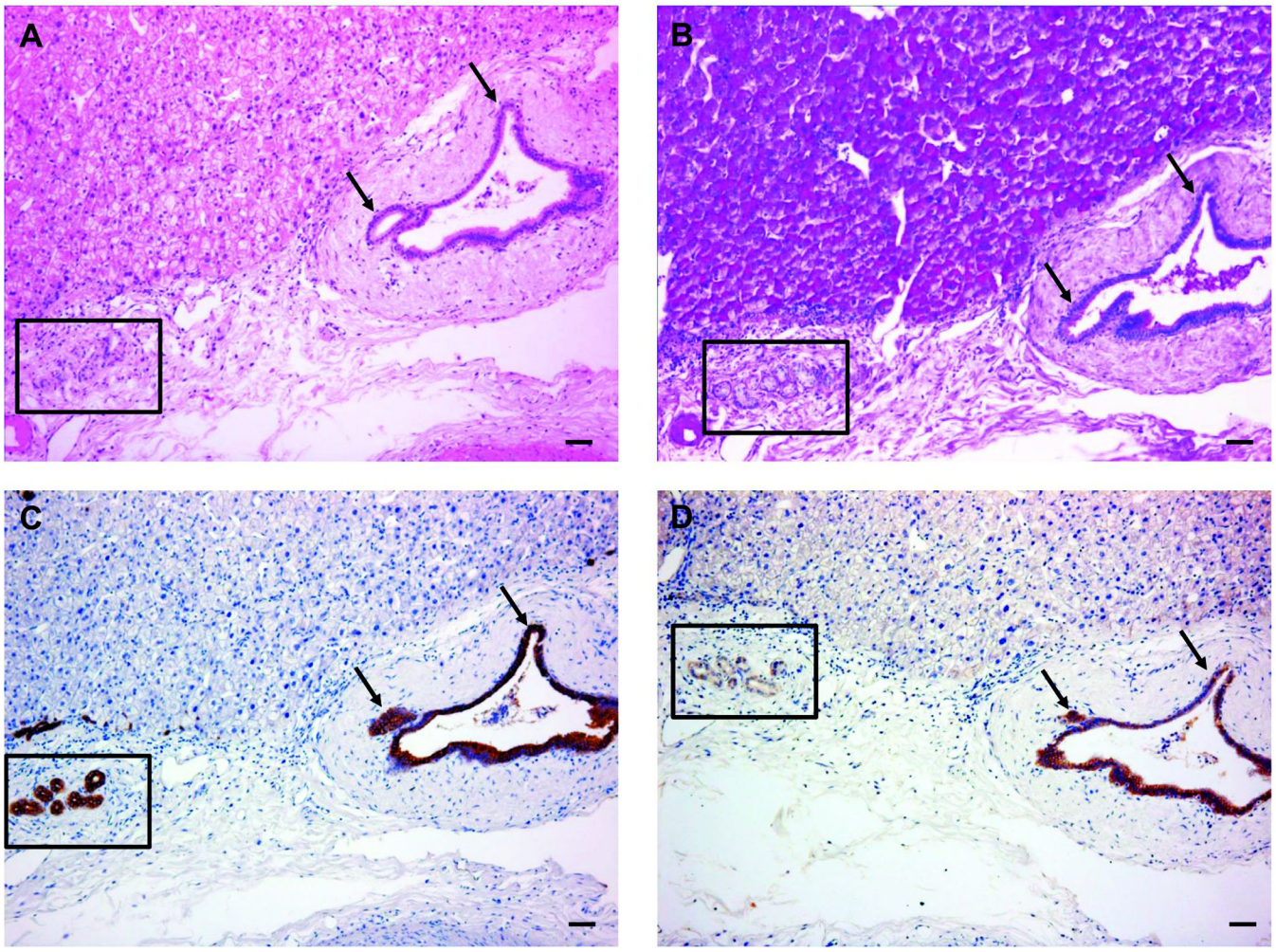

Figure 6.

Morphological aspects of glands within intrahepatic bile ducts. A–B) Haematoxylin-eosin and PAS stains. PBGs were predominantly found at the level of large bile ducts; in these ducts, two types of (peri-)biliary glands could be observed: *intramural* PBGs (arrows) were positioned inside the duct wall and appeared as shallow evaginations of the bile duct epithelium; *extramural* PBGs (box) were located outside the wall and were composed of PAS negative cells. C–D) Immunohistochemistry for CK19 and EpCAM showed the presence of positive cells in the surface epithelium, intra- (arrows) and extramural (box) PBGs. Scale Bar= 50μm.

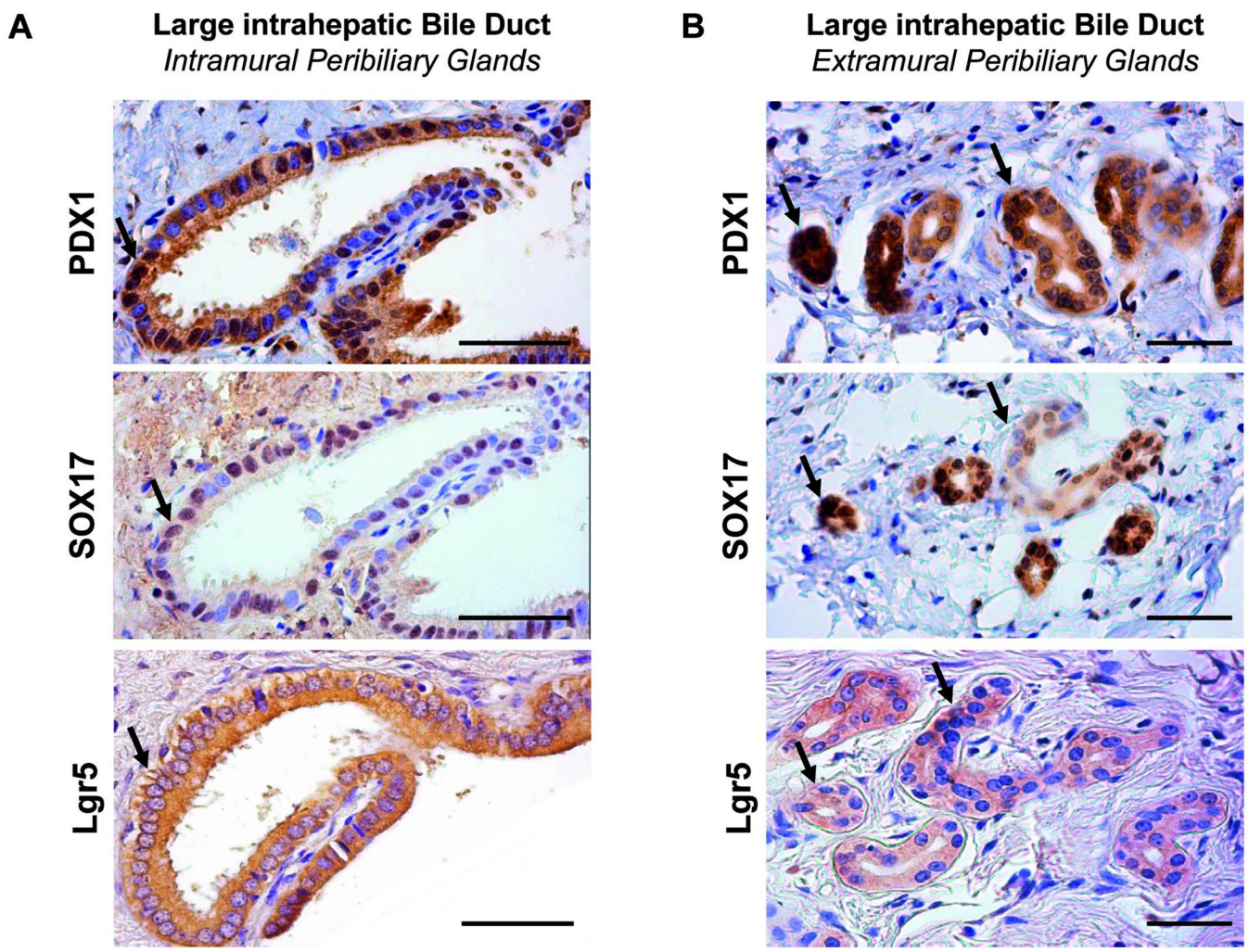

Figure 7.

Immunohistochemistry for PDX1, SOX17 and Lgr5 in intrahepatic bile ducts. **A)** In large intrahepatic bile ducts, the surface epithelium and the intramural PBGs contain endoderm-like and progenitor-like cells (PDX1+/SOX+/Lgr5+ cells) as indicated in seriated sections (arrows). **B)** Extramural (peri-)biliary glands are composed mostly of endoderm-like and progenitor-like cells which co-express specific markers (PDX1+/SOX+/Lgr5+). Notably, PDX1+/SOX19− cells were rarely found. Scale Bar= 50μm.

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Table 1

List of used antibodies

Name	Source	Catalog Number	Dilution	Role as marker for stem and progenitor cells
OCT4	Abcam	Ab19857	1:500	Embryonic stem cell
FOXa2	Abcam	Ab40874	1:500	Definitive endoderm
CXCR4	Abcam		1:100	Definitive endoderm Bilio-pancreatic progenitors
PDX1	Santa Cruz	SC-25403	1:50	Pancreatic progenitors
SOX17	Santa Cruz	Sc-17355	1:50	Biliary progenitors
SOX9	Chemicon	Ab 5535	1:50	Definitive endoderm Bilio-pancreatic progenitors Hepatic stem cell
Lgr5	Santa Cruz		1:50	Intestinal stem cell
CD326 / EpCAM	Santa Cruz	sc-59782	1:50	Definitive endoderm Bilio-pancreatic progenitors Hepatic stem cell
CD56 / NCAM	Santa Cruz	SC-7326	1:50	Bilio-pancreatic progenitors Hepatic Stem Cells
Insulin	DAKO	IS002	1:100	β-pancreatic cells
HepPar-1	DAKO	M7158	1:50	Mature Hepatocyte
Albumin	DAKO	F0117	1:50	Mature Hepatocyte
CK19 (cytokeratin 19)	DAKO	M0888	1:100	Biliary Cytokeratin
CK7 (cytokeratin 7)	DAKO	M7018	1:100	Biliary Cytokeratin
Secretin receptor	Santa Cruz	SC-26633	1:50	Mature cholangiocytes

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Table 2

Summary of distribution and phenotype of cells within glands of biliary tree

	PBG mass (μm^2)	PDX1+/SOX17+ (Biliary Tree Stem Cells) [BTSC mass]	PDX1+/SOX17− (Pancreatic progenitors)	HepPar-1 (hepatocyte fate) [albumin]	Insulin (β-pancreatic fate)
Hepatopancreatic Ampulla	31056.7 ± 1673.1§	+ [≈ 3416]	+ §	− [+/−]§	+§
Common Hepatic Duct	6251.2 ± 691.2	+ [≈ 687]	+/−	+ [+]	+/−
Cystic Duct	6867.5 ± 175.5	+ [≈ 707]	+/−	+ [+]	+/−
Large intrahepatic Bile Duct	2629.2 ± 638.4$^{\#}$	+ [≈ 499]	-	+ [+]	-
Interlobular Bile Duct	None	N/A	N/A	N/A	N/A

The table is focused on the proportion of a given (peri-)biliary gland positive for the indicated markers along with the mass occupied by glands in a given region of biliary tree. Scoring system as percentage of positive cells: − < 1%; +/− = 1–5%; + = 5–30%; ++ = 30–50%; +++ > 50% (Glaser et al., 2009b).

§= p< 0.01 versus other groups;

$^{\#}$= p<0.01 versus common hepatic and cystic ducts.

Notably, the percentage of cells with a phenotype of Biliary Tree Stem/Progenitor Cells did not vary in the different regions of biliary tree; however, an estimation of the mass occupied by these cells has been indicated in brackets to underscore that, taking in account the high differences in PBG mass along the biliary tree, the absolute amount of cells with a phenotype of Biliary Tree Stem/Progenitor Cells is highly variable.

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