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R-spondin 1 and noggin facilitate expansion of resident stem cells from non-damaged gallbladders

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Abstract

Pioneering studies within the last few years have allowed the *in vitro* expansion of tissue-specific adult stem cells from a variety of endoderm-derived organs, including the stomach, small intestine, and colon. Expansion of these cells requires activation of the receptor Lgr5 by its ligand R-spondin 1 and is likely facilitated by the fact that in healthy adults the stem cells in these organs are highly proliferative. In many other adult organs, such as the liver, proliferating cells are normally not abundant in adulthood. However, upon injury, the liver has a strong regenerative potential that is accompanied by the emergence of Lgr5-positive stem cells; these cells can be isolated and expanded *in vitro* as organoids. In an effort to isolate stem cells from non-regenerating mouse livers, we discovered that healthy gallbladders are a rich source of stem/progenitor cells that can be propagated in culture as organoids for more than a year. Growth of these organoids was stimulated by R-spondin 1 and noggin, whereas in the absence of these growth factors, the organoids differentiated partially toward the hepatocyte fate. When transplanted under the liver capsule, gallbladder-derived organoids maintained their architecture for 2 weeks. Furthermore, single cells prepared from dissociated organoids and injected into the mesenteric vein populated the liver parenchyma of carbon tetrachloride-treated mice. Human gallbladders were also a source of organoid-forming stem cells. Thus, under specific growth conditions, stem cells can be isolated from healthy gallbladders, expanded almost indefinitely *in vitro,* and induced to differentiate toward the hepatocyte lineage.

Keywords gallbladder; noggin; organoids; R-spondin 1; stem cells
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Introduction

The identification and characterization of tissue-specific adult stem cells is an important goal, not only in terms of understanding normal tissue biology and homeostasis, but also as a possible means toward the development of novel therapies. Among the many organs for which an enhanced understanding of stem cells could have clinical benefit, the liver ranks high, as there are many causes of impaired liver function and they are often associated with severe morbidity [1].

During embryogenesis, the liver originates from the foregut, which also gives rise to the stomach, small intestine, pancreas, and gallbladder [2]. The stem cells of the small intestine have been characterized quite well. By lineage tracing experiments, these cells have been shown to reside at the base of the intestinal crypts and to express a number of stem cell markers, including the G protein-coupled receptor Lgr5 [3]. Lgr5, a receptor for R-spondin 1, potentiates Wnt signaling, which in turn stimulates cell division and helps maintain the stem cell phenotype. The small intestine stem cells can be isolated as Lgr5-positive cells and, in the presence of R-spondin 1, be propagated indefinitely in tissue culture, as organoids bearing crypt-like structures [4,5]. It is likely that the high proliferative rate of Lgr5-positive small intestine stem cells *in vivo* contributes to the ability of these cells to establish organoids *in vitro*.

Unlike the small intestine, proliferating cells are not abundant in adult healthy livers [6]. However, when damaged, the liver exhibits regenerative capacity [7,8]. Partial hepatectomy leads to robust proliferation of hepatocytes and cholangiocytes, which are highly differentiated cells. However, if hepatocyte proliferation is impaired, for example, by chemical insult, then liver repair can be facilitated by the emergence of so-called oval cells resident in the canals of Hering. Oval cells are bipotent and can differentiate into cholangiocytes (also known as biliary epithelial cells) or hepatocytes [6,9–12].

In mice, the contribution of oval cells and, more broadly, liver stem cells to acutely induced liver regeneration is limited [13,14]. Nevertheless, chemical damage induces the emergence of Lgr5-positive

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cells in mouse livers, and in the presence of R-spondin 1, these cells can be expanded *in vitro* as organoids that can differentiate along the cholangiocyte and hepatocyte lineages [15]. Intrahepatic biliary duct (IHBD) cells from adult human livers can also be expanded as organoids in the presence of R-spondin 1 [16]. Similar to mouse, these organoids express stem cell markers, such as Lgr5 and Prom1 (prominin-1/CD133).

From a clinical perspective, obtaining an expandable liver cell population for transplantation would benefit patients with chronic liver diseases or with genetic defects. However, access to healthy livers, as a potential source of stem cells, is limited. Here, we propose an alternative, since we observed that stem/progenitor cells could be isolated easily from mouse gallbladders. These cells could be propagated for more than a year in tissue culture as organoids without the need for feeder layers and could be induced to partially differentiate toward the hepatocyte fate. Using a similar protocol, stem cells could also be isolated from human gallbladders.

Results

Establishment of liver and gallbladder 3D cell cultures

In an effort to isolate liver stem cells, non-damaged livers together with their extrahepatic biliary ducts (EHBDs) and gallbladder were harvested from 2-month-old mice, minced, and incubated with PBS/EDTA for 2 h to generate small cell clusters. These cells were then embedded in Matrigel® and cultured in serum-free media containing nicotinamide and a cocktail of growth factors (epidermal growth factor, EGF; fibroblast growth factor 10, FGF10; hepatocyte growth factor, HGF; R-spondin 1; and noggin). Within a day, numerous spheroids (organoids) formed. These organoids could be propagated, by weekly passaging, for more than a year, suggesting that they contained stem cells (Fig EV1). Single cells isolated from these organoids were also capable of establishing organoids that, again, could be propagated for prolonged periods of time in tissue culture (data not shown).

The ability to obtain organoids from non-damaged livers with such high efficiency was surprising to us, given that in previous studies, optimization of stem cell isolation was achieved either by carbon tetrachloride (CCl_4)-induced damage to the liver or by flow sorting for cells expressing specific stem cell markers [15]. We, therefore, decided to perform a more careful dissection of the liver and associated biliary tree. Liver lobes were processed separately from the EHBDs and from the gallbladder. No spheroids were obtained from tissue fragments derived from the liver lobes, whereas the gallbladder was a rich source of spheroids. Some organoids were also derived from the EHBDs, although their number was much lower than the number of organoids obtained from the gallbladder (Fig 1A). This experiment was repeated several times using mice, whose age ranged from 7 days to 1 year, with identical results (Fig 1B). Thus, the organoids obtained in our initial experiments most likely originated from the gallbladder and EHBDs, rather than from the liver parenchyma itself. Overall, these data suggest that non-damaged gallbladders are a source of stem/progenitor cells that can be propagated for extended time periods *ex vivo*.

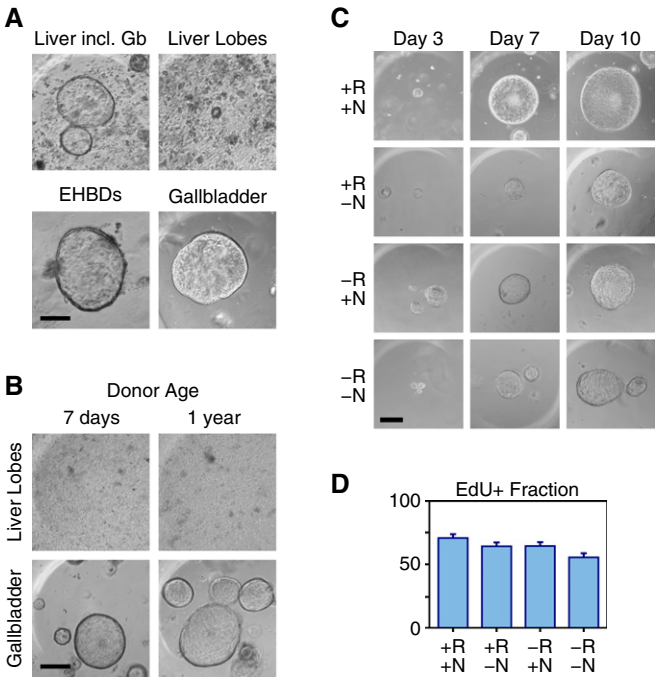

Figure 1. Derivation of organoids from mouse gallbladder tissue.

A Liver with the extrahepatic biliary ducts (EHBDs) and gallbladder (Liver incl. Gb), liver lobes, EHBDs, or gallbladder tissues were minced and cultured in Matrigel® in the presence of R-spondin 1 and noggin. Phase-contrast microscopy images were acquired 1 week later. Scale bar: 200 μm.

B Liver lobes or gallbladders harvested from 7-day- or 1-year-old mice were minced and cultured in Matrigel® in the presence of R-spondin 1 and noggin. Phase-contrast microscopy images were acquired 1 week later. Scale bar: 200 μm.

C Phase-contrast images of gallbladder organoids cultured for 3, 7, or 10 days in the presence (+) or absence (−) of R-spondin 1 (R) and/or noggin (N), as indicated. The same organoid was photographed at each time point. Scale bar: 200 μm.

D Fraction of EdU-positive (EdU+) cells in organoids cultured for 5 days in media containing R-spondin 1 and/or noggin, as indicated. The results, presented as means and standard errors, are derived from three independent experiments, each examining five organoids per condition. Statistically significant differences: +R+N vs. −R−N ($P < 0.001$); +R−N vs. −R−N ($P < 0.05$); −R+N vs. −R−N ($P < 0.05$).

R-spondin 1 and noggin facilitate growth of gallbladder organoids

The growth and survival of small intestine-derived organoids is absolutely dependent on R-spondin 1 and noggin [4,5]. Organoids derived from CCl_4-damaged livers also require R-spondin 1 for survival beyond 1–2 weeks [15]. To examine the dependency of gallbladder organoids on R-spondin 1 and noggin, freshly isolated gallbladder cells were placed in Matrigel® with full media or full media lacking R-spondin 1 or noggin or both. Organoids formed and could be propagated in all conditions tested. However, the organoids grew larger when both R-spondin 1 and noggin were present (Fig 1C). The differences in organoid size were paralleled by differences in the fraction of cells in S phase, as determined by incorporation of the thymidine analog EdU (Figs 1D and EV2A). Furthermore, consistent with noggin being an inhibitor of TGFβ signaling [17,18], a TGFβ receptor kinase inhibitor stimulated the

formation of very large organoids in the absence of noggin, but not R-spondin 1 (Fig EV2B).

In conclusion, these results show a dependency of gallbladder-derived organoids on R-spondin 1 and noggin. However, this dependency was much lower than for small intestine organoids, since the latter do not survive in tissue culture even for a few days in the absence of R-spondin 1 [4].

Gene expression patterns in gallbladder organoids

As a first step toward characterizing the gallbladder-derived organoids, we determined the expression levels of 21,258 annotated transcripts using gene expression arrays. For comparison, we also analyzed organoids derived from livers including the EHBDs and gallbladder (hereafter, liver organoids), organoids derived from the small intestine and freshly harvested gallbladder, liver, and small intestine tissue. For each organoid/tissue, two independent biological replicates were examined and, in all cases, the concordance between the replicates was very high (R^2 between 0.93 and 0.99; Fig EV3A and B). The quality of the array expression data was further examined by selecting 114 genes implicated in stem cell or gallbladder and liver biology and determining their expression levels in all our samples (organoids and tissues) using Nanostring®, a method that relies on single molecule counting. The concordance between the array and Nanostring® data was also very high ($R^2 = 0.77$) with discrepancies observed primarily in the context of genes expressed at very low levels, reflecting the different sensitivities of the two assays (Fig EV3C; Dataset EV1).

To probe the relation between the gallbladder, liver, and small intestine organoids, we first compared the expression of all 21,258 transcripts in these samples. The gallbladder and liver organoids had very similar gene expression patterns ($R^2 = 0.95$), while the small intestine organoids had more divergent patterns ($R^2 = 0.82$; Fig EV3D). The high similarity between gallbladder and liver organoids is consistent with the liver organoids originating primarily from the gallbladder and EHBDs and reflects our inability to obtain organoids from the lobes of healthy livers (Fig 1B).

To further explore whether the organoids from the entire liver were primarily of gallbladder origin, we focused on expression of tissue-specific genes. For the purposes of this study, tissue-specific genes were defined as the genes, whose expression was at least twofold higher in one tissue, as compared to each of the other two tissues being analyzed. This criterion identified 413 gallbladder-specific, 190 liver-specific, and 572 small intestine-specific genes (Fig 2A; Dataset EV2). Interestingly, the gallbladder and liver organoids expressed the gallbladder tissue-specific, but not the liver- or small intestine tissue-specific genes, whereas the small intestine organoids expressed the small intestine-specific genes (Fig 2A). These results suggest that the organoids maintained the gene expression patterns of the tissue from which they were derived and are consistent with the entire liver organoids being primarily of gallbladder and EHBD origin. Interestingly, the list of gallbladder tissue-specific genes included the stem cell marker *Prom1* and the transcription factor *Sox17* [19] (Figs 2A and EV4).

As part of this analysis, we also identified genes that were expressed in the gallbladder and liver tissues, but not in the small intestine, again applying twofold differences in expression as the threshold. A total of 250 genes fit these criteria; of these, 115 were also expressed in the gallbladder organoids at levels comparable to those in gallbladder tissue (Fig 2B and D), whereas the remaining 135 genes were expressed at lower levels in the gallbladder organoids (Fig 2B; Dataset EV3).

We concluded the gene expression array analysis by identifying organoid-specific genes. These were defined as the genes, whose expression in the gallbladder and small intestine organoids differed by more than twofold (higher or lower) from their expression in the tissues. Twenty-nine genes were expressed at higher levels in the organoids, including the stem cell markers *Lgr5*, *Msh2*, *Sox4,* and *Sox9* and two genes, *Phgdh* and *Psat1*, that function in the metabolic pathway that converts 3-phosphoglycerate to phosphoserine [20,21] (Fig 2C and D; Dataset EV4). The list of genes whose expression was lower in the organoids, included *Lrp1*, a gene that inhibits canonical Wnt signaling [22] (Figs 2C and EV4). However, most of the other genes in this category were genes expressed by connective tissue cells, simply reflecting the fact that the organoids, but not the tissues, consist entirely of epithelial cells.

Expression of stem and biliary cell markers in gallbladder-derived organoids

The gallbladder has a common developmental origin with EHBDs. Indeed, several genes in the gallbladder-specific cluster, such as *Cd44*, *Prom1,* and *Sox17* (Fig 2A), are also expressed in extrahepatic biliary epithelial cells [23,24]. Thus, to further characterize the gallbladder-derived organoids, we examined by immunofluorescence the expression of selected biliary cell markers using liver tissue as control. Prom1 (prominin-1/CD133), Cldn3 (claudin-3), Epcam (epithelial cell adhesion molecule), and Itga6 (integrin A6) were all highly expressed in the gallbladder organoids, whereas their expression in the liver was restricted to the bile ducts (Cldn3 and Itga6) or was not observed (Fig 3).

As expected, gallbladder and EHBD tissues also stained positive for biliary cell markers, including Krt19, Sox17, and A6 (Fig 4A). Interestingly, most A6-positive cells of the gallbladder and EHBDs also expressed Hnf4α, which is considered to be a hepatocyte marker (Fig 4B). Co-expression of biliary and hepatocyte markers is characteristic of embryonic hepatoblasts [25] and is also observed in periportal hepatocytes of regenerating livers [26], but generally not in healthy livers (Fig 4B). Apparently, in the gallbladder tissue and organoids, there is expression of both biliary cell markers and markers considered to be hepatocyte specific (Figs 2B, 3, 4 and EV4).

Finally, we also examined in gallbladder organoids, by immunofluorescence, the intracellular localization of Yap1, a downstream target of the Hippo pathway. Yap1 exhibited nuclear localization in the organoids, but was cytoplasmic in the liver (Fig 3), consistent with the intracellular localization of Yap1 being a key determinant of its pro-proliferative activity [27].

Differentiation of gallbladder organoids *in vitro*

Stem cell cultures have the potential to serve as a replenishable source of differentiated cells. Given the common origin of the gallbladder and liver during embryonic development and the expression of hepatocyte markers in gallbladder epithelial cells (Figs 2B, 4 and EV4), we examined whether the gallbladder organoids could be induced to differentiate along a hepatocyte fate.

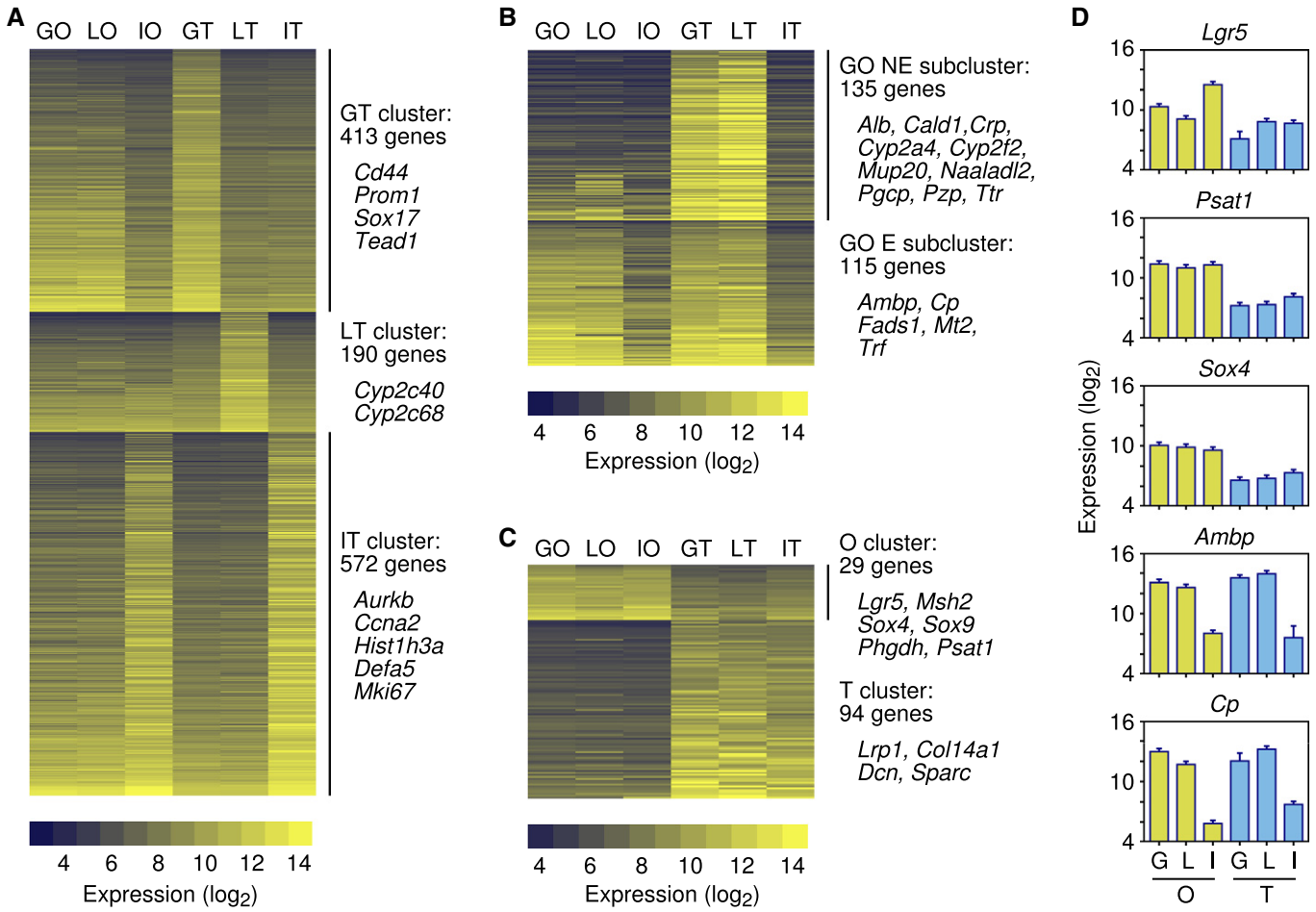

Figure 2. Gene expression profiles of gallbladder-derived organoids.

A Clusters of gallbladder tissue (GT)-specific, liver tissue (LT)-specific, and small intestine tissue (IT)-specific genes (Dataset EV2). The number of genes and the names of selected genes in each cluster are indicated. Gene expression levels (log_2 scale) are shown for gallbladder organoids (GO), liver organoids (LO), intestinal organoids (IO) and gallbladder (GT), liver (LT), and intestinal (IT) tissues.

B Genes expressed in gallbladder and liver tissues, but not in the intestine, divided into two subclusters, depending on whether the genes were expressed or not in gallbladder organoids (Dataset EV3). The number of genes and the names of selected genes in each subcluster are indicated.

C Clusters of organoid (O)-specific and tissue (T)-specific genes (Dataset EV4). The number of genes and the names of selected genes in each cluster are indicated.

D Expression levels of selected genes from the clusters shown in (B) and (C). G, gallbladder; L, liver; I, intestine; O, organoid; T, tissue. Error bars are square root of MSE from ANOVA 2F (see Materials and Methods) ($n = 2$).

We tried a number of protocols to induce the differentiation of gallbladder organoids and in the end converged on simply removing R-spondin 1, noggin, and nicotinamide from the media for a period of 2 weeks. At the end of this time period, we determined the expression levels of the same 21,258 annotated transcripts, as cited above, using gene expression arrays. Two independent biological replicates were examined, yielding highly reproducible results ($R^2 = 0.99$). The gene expression levels in the differentiated organoids were then compared to the levels in the non-differentiated organoids and in gallbladder and liver tissues. Only a small number of genes showed changes in gene expression greater than twofold, indicating incomplete differentiation (Dataset EV5). Sixty-seven genes were upregulated upon differentiation (Fig 5A); these included two liver-specific genes, *Cyp2c40* and *Cyp2c68* (Figs 2A and 5A), and many liver-/gallbladder-specific genes that were not expressed in the undifferentiated organoids, including *Cald1*, *Crp*, *Cyp2a4*, *Cyp2f2*, *Mup20*, *Naaladl2*, *Pgcp*, *Pzp*, and *Ttr* (Figs 2B and 5A). The level of induction for many of these genes was remarkable (Fig 5B). Of the genes downregulated upon differentiation, *Lgr5* is notable (Fig 5A and B).

As a second method to monitor differentiation, we prepared gallbladder organoids from mice in which a tamoxifen-dependent Cre recombinase was inserted in the 3′ untranslated region of the *Alb* gene; these mice also contained a gene encoding a red fluorescent protein (RFP) that only becomes expressed after the induction of Cre recombinase activity [28,29]. Organoids grown in the presence of R-spondin 1, noggin, and nicotinamide did not express RFP, whereas when these three factors were removed from the media, RFP was expressed (Fig 5C), suggesting that the *Alb* promoter had been activated.

Finally, we examined whether differentiation led to the accumulation of intracellular glycogen, which can be monitored by PAS staining. Indeed, a fraction of the cells in the differentiated

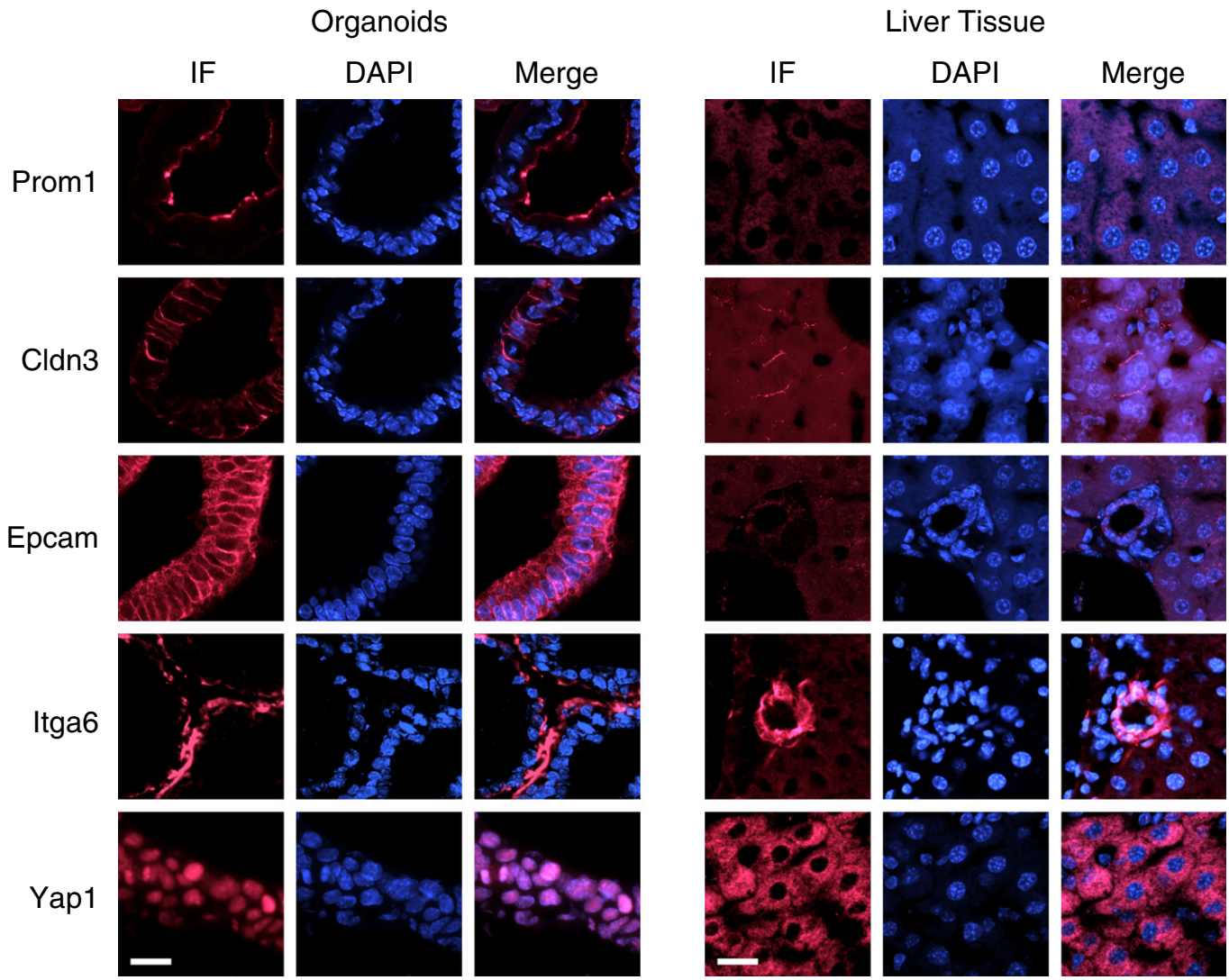

Figure 3. Expression of biliary cell markers in gallbladder organoids.
Expression of Prom1, Cldn3, Epcam, Itga6, and Yap1 in gallbladder-derived organoids and liver tissue, as monitored by immunofluorescence (IF). The cell nuclei were counterstained with DAPI. Scale bars: 25 μm.

gallbladder organoids were PAS positive, indicating differentiation toward a hepatocyte fate (Fig 5D).

Engraftment of gallbladder organoids and organoid cells *in vivo*

Having identified conditions for establishing gallbladder organoid cultures *in vitro*, we next ascertained their potential for engraftment. For this purpose, gallbladder organoids were established from mice harboring a *GFP* transgene under the control of the human ubiquitin C promoter. Organoids prepared from these mice expressed GFP (Fig EV5A), thereby allowing the engrafted cells to be distinguished from the host cells.

GFP-positive gallbladder-derived organoids suspended in Matrigel® were injected into the hepatic subcapsular space of immunocompromised B6.Rag2xγc mice and engraftment was monitored 2 weeks later by immunohistochemistry. In five out of six injected mice, organoids were successfully engrafted. These organoids maintained the same shape and tissue architecture, as observed *in vitro*, and continued to express biliary cell markers, such as Krt19, Cd44, and Cldn3 (Fig 6A and B). Interestingly, almost no cell in the engrafted organoids expressed Ki67, indicating that these cells were no longer actively proliferating (Fig 6B). We next examined whether the organoids persisted for longer periods of time in the engrafted mice; however, we could not detect any organoids 4 weeks after engraftment in a total of eight mice.

As a second approach to study engraftment, organoids were dissociated into single cell suspensions and injected into the mesenteric vein of B6.Rag2xγc mice for homing to the liver. Two days before cell injection, CCl_4 had been administered intraperitoneally to the mice to induce liver damage, thereby possibly providing a niche that would allow incorporation of the injected stem cells into the liver parenchyma. Engraftment was monitored by immunohistochemistry 1 week after cell injection (Fig EV5B). Interestingly, small clusters of GFP-positive cells were observed that expressed also

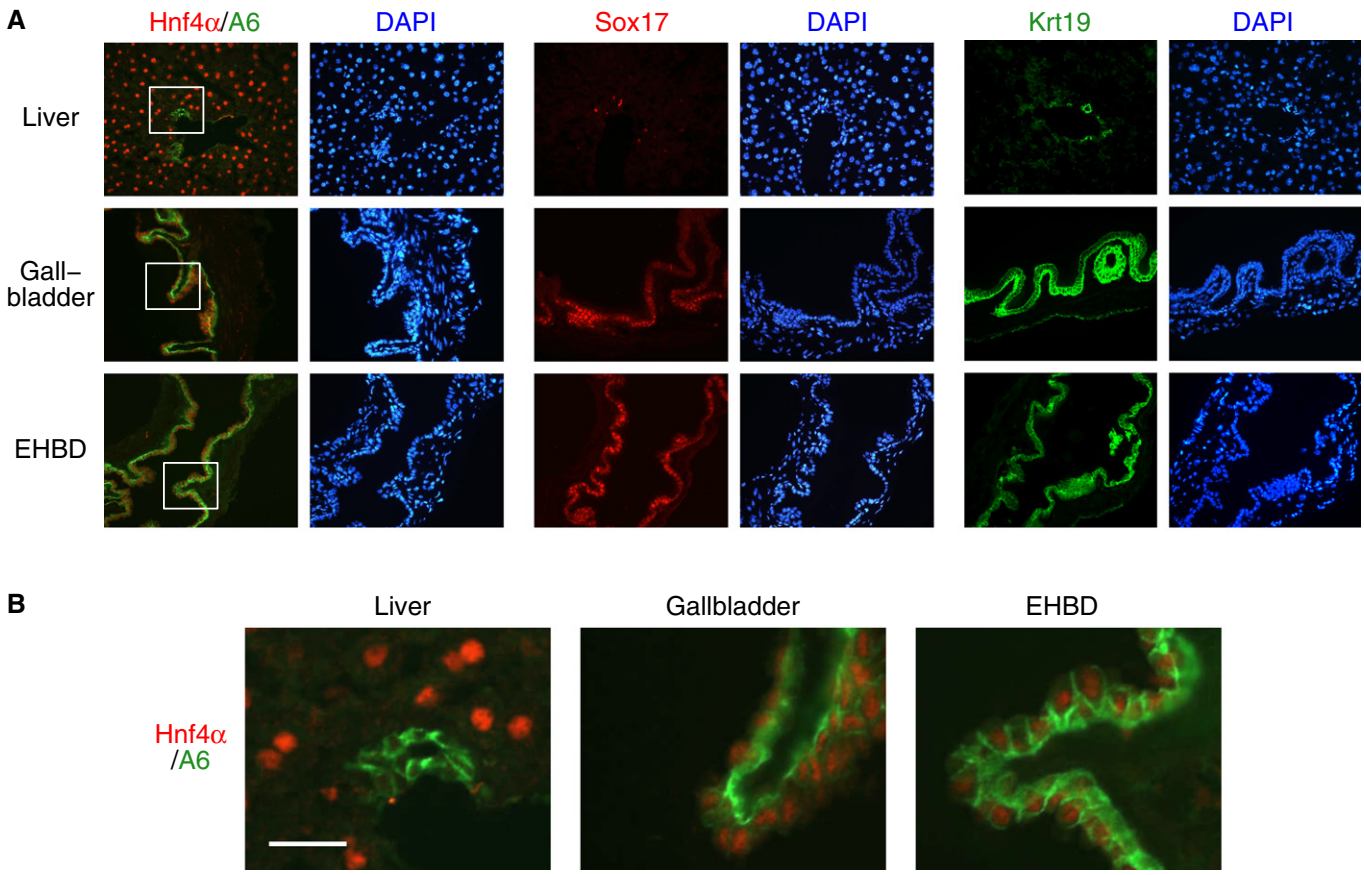

Figure 4. Expression of biliary cell and hepatocyte markers in gallbladder tissue, EHBDs, and liver lobes.

A Immunofluorescence analysis of expression of Hnf4α, A6, Sox17, and Krt19. The nuclei were counterstained with DAPI. EHBD, extrahepatic biliary duct.

B Higher magnification of the regions demarcated by the white rectangles in (A). Scale bar: 25 μm.

Prom1, Sox9 (stem cell markers), and Afp (alpha-fetoprotein; a hepatoblast marker). However, a similar analysis performed 4 weeks after cell injection failed to identify any GFP-positive cells.

Establishment of human gallbladder organoids

To determine whether the conditions allowing the establishment and propagation of mouse gallbladder organoids were also suitable for humans, we obtained samples from patients undergoing removal of their gallbladder. We followed essentially the same protocol as the one described above for mice, except that human recombinant growth factors (EGF, HGF, and FGF10) were used. We obtained organoids that morphologically were indistinguishable from the mouse gallbladder organoids (Fig EV6A). The human organoids could be propagated in tissue culture for at least 16 weeks and expressed biliary cell markers, similar to the mouse organoids (Fig EV6B).

Discussion

The liver has long been recognized as unique among organs for its ability to regenerate [30]. Regeneration is mediated by the proliferation of differentiated hepatocytes, as well as by precursor cells. In rats, the precursor cells express markers of both hepatocytes and biliary cells and were named oval cells [9]. In mice and humans, the contribution of bipotent precursor cells, like oval cells, to liver regeneration appears to be more limited [13,14]. However, a recent study has identified in injured mouse livers periportal hepatocytes that express both biliary and hepatocyte markers; these hybrid hepatocytes can regenerate the liver parenchyma, suggesting that precursor cells with oval cell-like properties are not limited to rats [26]. Precursor cells probably represent a transit cell compartment derived from intrahepatic stem cells [31,32]. The precise niche of these stem cells has not been fully elucidated, but significant evidence points to the canals of Hering within the liver lobules, where the bile ducts originate [33–35].

Efforts to identify hepatic stem/precursor cells have relied heavily on models of liver damage. One property of stem cells is their capacity to self-regenerate, and a recent study has indeed described the isolation of intrahepatic stem cells that retain high proliferative capacity for more than one year [15]. Briefly, following hepatocyte injury with CCl_4, putative stem cells expressing Lgr5 can be identified in close proximity to the IHBDs. These cells were isolated by FACS and could be expanded almost indefinitely in Matrigel® in the presence of R-spondin 1, EGF, HGF, and FGF10. Furthermore, these

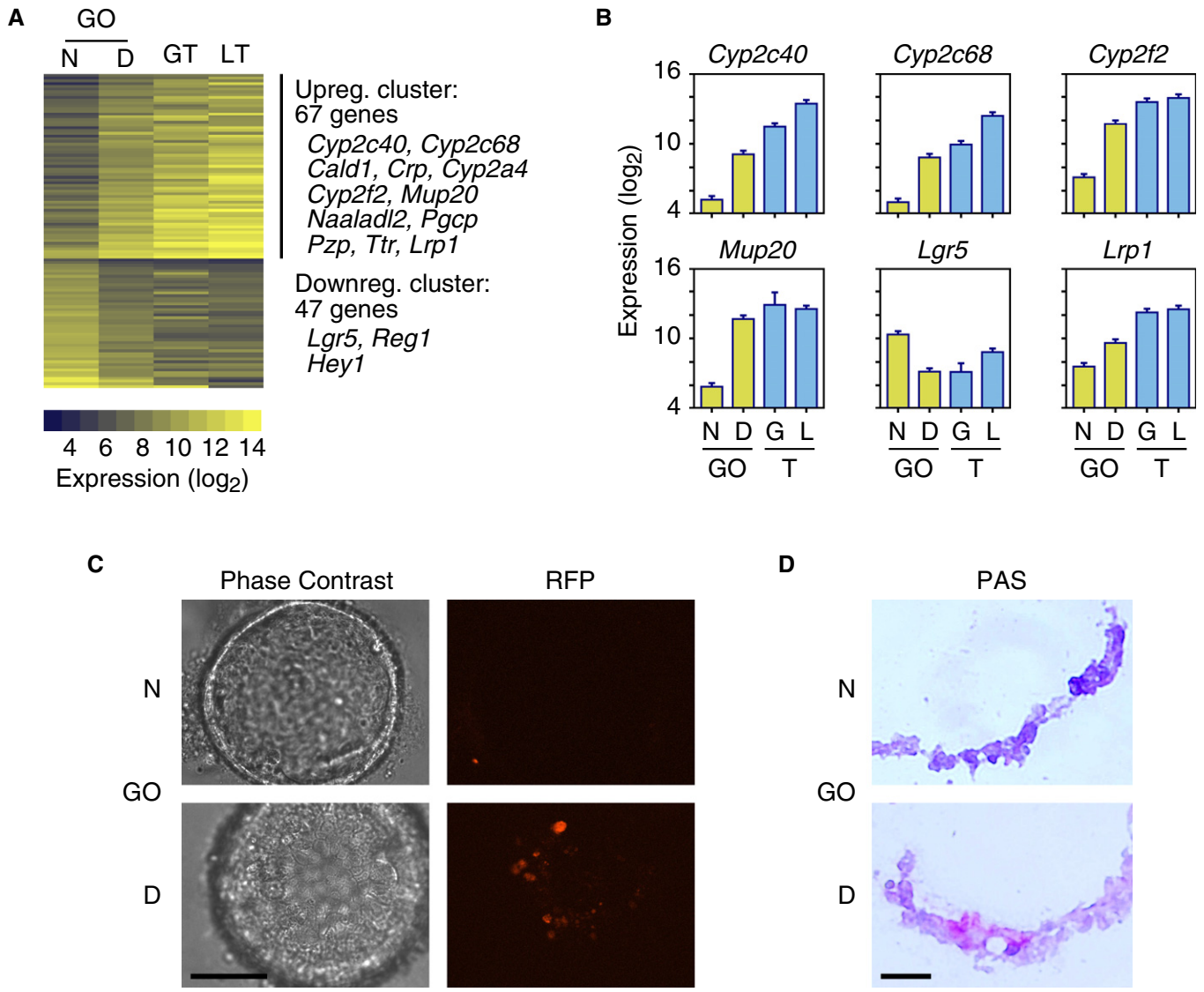

Figure 5. Partial differentiation of gallbladder organoids toward the hepatocyte fate.

A Clusters of genes, whose expression changes toward the hepatocyte fate, upon the removal of R-spondin 1 and noggin from the organoid culture media (Dataset EV5). The number of genes and the names of selected genes in each cluster are indicated. Gene expression levels (log_2 scale) are shown for non-differentiated (N) and differentiated (D) gallbladder-derived organoids (GO), gallbladder tissue (GT), and liver tissue (LT). Upreg, upregulated; Downreg, downregulated.

B Expression levels of selected genes from the clusters of panel A. GO, gallbladder organoid; N, non-differentiated; D, differentiated; G, gallbladder; L, liver; T, tissue. Error bars are square root of MSE from ANOVA 2F (see Materials and Methods) ($n = 2$).

C Removal of R-spondin 1 and noggin from the media induces the expression of red fluorescent protein (RFP) in a subset of gallbladder organoid cells. A transgene expressing tamoxifen-inducible Cre is driven by the endogenous *Alb* promoter; a second transgene expresses RFP after Cre-mediated rearrangement. Scale bar: 200 μm.

D Glycogen accumulation upon organoid differentiation, as revealed by periodic acid–Schiff (PAS) staining (red). Scale bar: 25 μm.

putative liver stem cells could be differentiated *in vitro* to acquire hepatocyte functions and, following such differentiation, they could be transplanted into the livers of $Fah^{-/-}$ mice [15]. Thus, Lgr5-positive liver cells represent a source of stem cells with therapeutic potential [36]. The only drawback relates to the method of preparing these cells, which involves either inducing liver damage or, alternatively, isolating IHBDs.

The EHBDs and gallbladder have also emerged as a source of stem/progenitor cells [37–39]. In fact, stem cells can be isolated from these tissues without inducing tissue injury. In one study, stem cells residing in the mouse gallbladder were isolated by sorting $Epcam^{+}/Cd49f(Itga6)^{++}$ cells; these cells could self-renew on a feeder layer for more than 20 passages and could be engrafted into the subcutaneous neck region space [39]. Stem cells were also isolated from human gallbladders and again cultured on feeder layers [40,41]. The mouse and human gallbladder-derived stem cells had gene expression profiles that distinguished them from stem cells derived from IHBDs; nevertheless, both the IHBD and gallbladder-derived stem cells could be differentiated into hepatocytes *in vitro* [42].

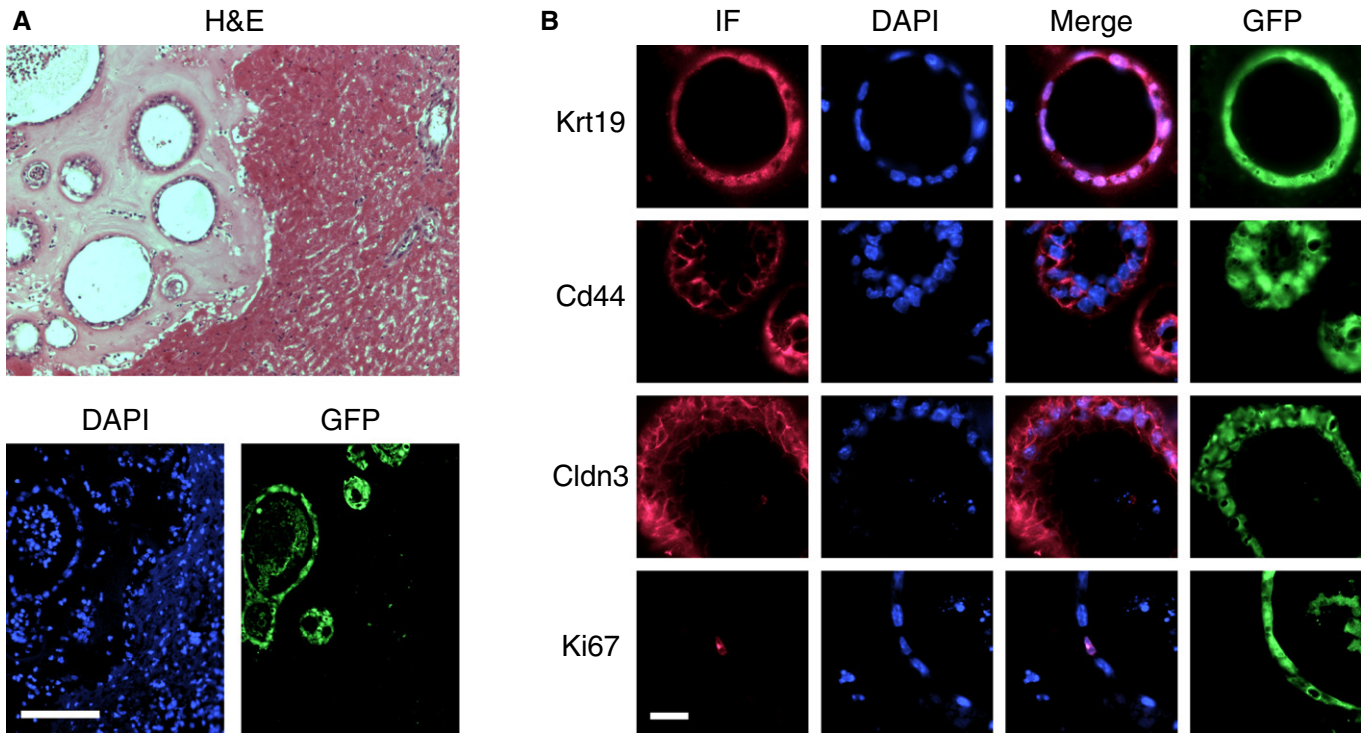

Figure 6. Engraftment of gallbladder organoids in the hepatic subcapsular space.

A (Top) Low-magnification view showing organoids embedded in Matrigel® (left) next to the liver parenchyma (right) 2 weeks after engraftment. H&E, hematoxylin & eosin staining. (Bottom) Immunofluorescence staining for GFP expression to mark the engrafted organoids and DAPI counterstaining of the cell nuclei. The liver parenchyma corresponds to the right half of the images. Scale bar: 200 μm.

B Characterization of engrafted organoids by immunofluorescence (IF). Almost no organoid cell expressed Ki67 (the one positive cell shown in the image was an exception), in contrast to the high levels of cell proliferation and Ki67 expression observed in organoids cultured *in vitro*. Scale bar: 25 μm.

Our study provides further evidence for the presence of stem/progenitor cells in the gallbladder. In our case, though, the method of isolating these cells was quite simple and did not involve a cell sorting step. Instead, non-injured gallbladders were minced and the cells plated directly on Matrigel®. The gallbladder-derived cells could be propagated for more than one year in culture and expressed stem cell markers, such as Lgr5, Prom1, Sox4, and Msh2 (Figs 2 and EV4). On the basis of expression of the markers cited above and the capacity to propagate these cells as organoids for more than one year in tissue culture, one may consider that the cells that we have isolated are indeed stem cells. Nevertheless, we have not demonstrated that these cells function as stem cells *in vivo* and, therefore, pending further characterization, we cannot formally rule out that we have isolated progenitor cells.

An important question is why the gallbladder is a more efficient source of stem/progenitor cells than the liver itself. Perhaps, the answer relates to the fact that the gallbladder epithelial cells express both biliary cell and hepatocyte markers and, in this way, resemble hepatoblasts and precursor cells of regenerating livers. Like the precursor cells in regenerating livers, hepatoblasts are bipotent cells and give rise to both hepatocytes and biliary epithelial cells during embryonic development [25]. Hepatoblasts can be readily detected in E10.5 to E15.5 mouse livers, but their number decreases greatly at later-stage embryos and they are apparently eliminated during postnatal life. Our study raises the possibility that, during development, cells segregated to the gallbladder epithelium maintain stem/progenitor-like features by escaping differentiation toward the hepatocyte or biliary epithelial lineages.

The relationship of the stem/progenitor cells identified here with those obtained in other studies remains to be determined. It is likely that the IHBD-derived stem cells are distinct from the gallbladder-derived stem cells, since the former are much more dependent on R-spondin 1 for growth and have higher levels of Lgr5 expression than the cells that we isolated [15]. In contrast, the stem cells obtained from mouse and fetal human gallbladders by others may be similar to the ones that we isolated, simply because the tissue of origin is the same. However, this needs to be experimentally determined, because in the other studies the stem cells were cultured on feeder cell layers, whereas we cultured the cells in Matrigel® in the presence of R-spondin 1 and noggin [38–41]. Even if the cells are similar or identical, the ability to bypass the need for feeder layers and the apparently higher capacity to expand organoids are clear advantages of the method that we described.

Stem cells isolated from the gallbladder could be differentiated toward a hepatocyte fate without the addition of chemicals or lentiviral transfection, by simply removing R-spondin 1, noggin, and nicotinamide from the media. Furthermore, gallbladder-derived stem cells survived well when engrafted into mice, at least in the short term. However, our experience matches that of others in that the differentiation protocol and engraftment into mice were

inefficient [15]. Thus, better methods are needed to induce efficient differentiation of gallbladder organoids into hepatocytes and for engraftment of these cells into patients.

In conclusion, the use of gallbladder-derived stem/progenitor cells could have important implications for the study of liver and biliary tree diseases. Moreover, it is possible to foresee that in the future gallbladder-derived stem cells could be differentiated into hepatocytes and transplanted into patients, allowing new therapies to be developed for liver-related genetic diseases and chronic liver insufficiency [43]. Along these lines, the gallbladder is not an essential organ and its removal is a routine medical procedure.

Materials and Methods

Mice

C57BL/6J and C57BL/6-Tg(*UBC-GFP*)30Scha/J mice were purchased from Jackson Labs; C57BL/6JRccHsd mice from Harlan Laboratories; and B6.Rag2 × common gamma chain (γc)-double-knockout mice from Taconic Biosciences. *Alb-Cre-ERT2* mice [28] were obtained from Pierre Chambon, and Gt(ROSA)26Sor^{tm1Hjf} mice [29] from Ivan Rodriguez. The latter two strains were crossed to generate mice that contain both the *Cre-ERT2* and *td-RFP* alleles in homozygous form.

Organoid preparation and culture

Entire livers (including the EHBDs and gallbladder) were isolated from C57BL/6J mice. The tissues were cut into 2- to 3-mm-diameter pieces, washed in cold PBS, and incubated in PBS–EDTA overnight at 4°C. The next day, the cells were filtered through 70-μm cell strainers (BD Bioscience). After centrifugation, the cell pellets were mixed with cold Matrigel® (Corning) and plated in 24-well plates. After the Matrigel® formed a gel, tissue culture media were added. The tissue culture media were based on AdDMEM/F12 (Life Technologies) supplemented with B27, N2 (Life Technologies), and 1.25 μM N-acetylcysteine (Sigma-Aldrich). The following growth factors were also added: 50 ng/ml murine recombinant EGF (Life Technologies), 50 ng/ml murine recombinant FGF10 (R&D Systems), 10 mM nicotinamide (Sigma-Aldrich), 50 ng/ml murine recombinant HGF (Peprotech), and R-spondin 1 and noggin home-made conditioned media (prepared as in [4]). TGFβ receptor kinase inhibitor (TRKI) (SB-431542; Calbiochem) was added to the media at 2 mM final concentration for the experiment described in Fig EV2. Medium was changed every 3 days, and organoids were split every 4–5 days by mechanical dissociation. For EHBD- and gallbladder-derived organoid cultures, EHBDs or gallbladder, respectively, were isolated from C57BL/6J mice, and the tissues were cut into pieces and processed, as described above, except that the initial incubation with PBS–EDTA was for 2 h, instead of overnight.

Immunohistochemistry and immunofluorescence

Liver tissues were embedded in OCT (Tissue-Tek), and slices of 10 μm thickness were cut using a cryostat (Leica CM 1850). Organoids were removed from Matrigel® using Cell Recovery Solution (Corning) and then embedded in OCT and sliced, as described above. Tissue and organoid sections were fixed for 1 h in 4% paraformaldehyde at room temperature and stained using standard immunofluorescence techniques and commercially available antibodies (Yap1, Cell Signaling; Cldn3, GeneTex; Epcam, AbCAM; Prom1, E-Bioscience; integrin A6, Millipore; Krt19, Dako; Hnf4α, Santa Cruz; Sox17, R&D; A6, gift from Valentina Factor, NIH). Nuclei were counterstained with DAPI, and images were acquired using a Zeiss 700 confocal microscope. Cell proliferation was assessed with the EdU Click-it Kit (Life Technologies), and images were acquired with the organoids still embedded in Matrigel® using the confocal microscope. To assess glycogen storage, organoids were examined by the periodic acid–Schiff staining method (PAS, Sigma-Aldrich).

Gene expression array and Nanostring® analysis

RNA was extracted from liver, gallbladder, or small intestine tissues and liver, gallbladder, or small intestine organoids using the RNeasy Mini RNA Extraction Kit (Qiagen). Transcript expression levels were determined using GeneChip® Mouse Gene 1.0 ST Arrays (Affymetrix) for 21,258 transcripts. Expression levels of 114 selected genes (Dataset EV4) were additionally determined by the Nanostring® method (Nanostring® Technologies), to validate the array data.

After scaling, the array and Nanostring® data were converted into log2 values. The data from all samples and replicates were processed together using a two-factor (gene vs. sample type) ANOVA with multiple replicates, thus allowing a uniform mean variance (mean square of the error, MSE) to be calculated for all comparisons, except for specific comparisons, for which a Cochran's test indicated the need to use individual variances. The gene expression array data have been deposited at the GEO database with accession number GSE77461; the Nanostring® data are shown in Dataset EV1.

Organoid differentiation

To induce differentiation, we cultured the organoids for 2 weeks in the presence of the media described above, except for not adding R-spondin 1, noggin, and nicotinamide. To monitor differentiation by RFP expression, organoids were prepared from mice containing homozygous *Cre-ERT2* and *td-RFP* alleles and then switched to media lacking R-spondin 1, noggin, and nicotinamide for 2 weeks. Then, 4-hydroxytamoxifen (1.3 μM, Sigma-Aldrich) was added, and 2 days later, the organoids, while still embedded in Matrigel®, were scored for fluorescence under a confocal microscope.

Engraftment assays

Gallbladder organoids were prepared from mice harboring a *GFP* transgene under the control of the human ubiquitin C promoter (C57BL/6-Tg(UBC-GFP)30Scha/J mice).

Liver subcapsular whole organoid injection

Dense organoid cultures were gently disrupted with a pipette to break the Matrigel® into small fragments, while preserving the organoids as whole spheres. The organoids were then gently centrifuged, resulting in intact organoids being collected at the bottom of the tube; most of the media and Matrigel® were then removed with a

pipette. The organoid pellets were chilled on ice and resuspended in ice-cold Matrigel®. The organoid and Matrigel® suspension was then loaded into cold 300-μl syringes with 30-G needles and stored on ice until use to prevent Matrigel® polymerization. B6.Rag2xγc mice were anaesthetized with isoflurane, and the abdominal cavity was opened by a short transverse laparotomy (~1 cm) 3–5 mm below the xyphoid/costal arch. The left liver lobe was lifted and fixed with the help of a sterile cotton swab, and then 50 μl of the organoid suspension was injected into the subcapsular space. After controlling hemorrhage, the abdominal wall was closed by suturing. The mice were euthanized 2 weeks later, and the site of organoid injection was examined by histology.

Mesenteric vein organoid cell injection

For mesenteric vein delivery, gallbladder organoid cultures were gently disrupted with a pipette and incubated with Dispase (Corning). The resultant cell suspension was washed three times in PBS and resuspended in PBS at a concentration of 3×10^6 cells/ml. Two days before the cell injection, B6.Rag2xγc mice were injected i.p. with CCl_4 (1 ml/kg, Sigma-Aldrich) dissolved in olive oil. Animals were anaesthetized with isoflurane; a 2-cm midline laparotomy was performed, the mesentery was exposed and immobilized on a moist swab, and 50 μl of the cell suspension, containing approximately 1.5×10^5 cells, was injected into the mesenteric vein. The injection site was compressed to control hemorrhage and suturing closed the laparotomy.

Human organoid preparation

Gallbladder specimens were obtained from patients undergoing surgery at the Inselspital Bern. Informed consent was obtained prior to surgery in compliance with the local ethical committee. Immediately following the removal of the gallbladder, a small piece was transferred to the laboratory in cold PBS. The tissue was further washed in cold PBS and its luminal part was separated and used for organoid isolation (approximately 4 cm^2 for each isolation). The tissue was minced, incubated in PBS containing EDTA at 4°C for 2 h, and then repeatedly passed up and down through a 5-ml pipette in order to release the cells. The cell preparation was then filtered through a 70-μm sterile mesh, centrifuged, the supernatant removed, and the pellet chilled on ice for 5 min. Depending on the size of the pellet, cells were resuspended in 200–600 μl of Matrigel® and plated. After the Matrigel® solidified by incubation at 37°C, it was covered with culture media, which had the same composition as the media used for mouse organoids, except that the mouse recombinant growth factors (FGF10, HGF, EGF) were substituted with human factors.

Expanded View for this article is available online.

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Author contributions

NL, IK, AK, TM, and MES performed experiments. OS and TDH performed the bioinformatic analysis. NL, IK, AK, IT, DC, DS, and TDH conceived the project, analyzed the data, interpreted results, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Nussler A, Konig S, Ott M, Sokal E, Christ B, Thasler W, Brulport M, Gabelein G, Schormann W, Schulze M *et al* (2006) Present status and perspectives of cell-based therapies for liver diseases. *J Hepatol* 45: 144 – 159
2. Lemaigre FP (2009) Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. *Gastroenterology* 137: 62 – 79
3. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ *et al* (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449: 1003 – 1007
4. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ *et al* (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459: 262 – 265
5. Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, Barker N, Shroyer NF, van de Wetering M, Clevers H (2011) Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* 469: 415 – 418
6. Duncan AW, Dorrell C, Grompe M (2009) Stem cells and liver regeneration. *Gastroenterology* 137: 466 – 481
7. Michalopoulos GK (2007) Liver regeneration. *J Cell Physiol* 213: 286 – 300
8. Michalopoulos GK (2010) Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am J Pathol* 176: 2 – 13
9. Evarts RP, Nagy P, Marsden E, Thorgeirsson SS (1987) A precursor-product relationship exists between oval cells and hepatocytes in rat liver. *Carcinogenesis* 8: 1737 – 1740
10. Fausto N (2004) Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. *Hepatology* 39: 1477 – 1487
11. Dipaola F, Shivakumar P, Pfister J, Walters S, Sabla G, Bezerra JA (2013) Identification of intramural epithelial networks linked to peribiliary glands that express progenitor cell markers and proliferate after injury in mice. *Hepatology* 58: 1486 – 1496
12. Michalopoulos GK (2014) The liver is a peculiar organ when it comes to stem cells. *Am J Pathol* 184: 1263 – 1267
13. Schaub JR, Malato Y, Gormond C, Willenbring H (2014) Evidence against a stem cell origin of new hepatocytes in a common mouse model of chronic liver injury. *Cell Rep* 8: 933 – 939
14. Yanger K, Knigin D, Zong Y, Maggs L, Gu G, Akiyama H, Pikarsky E, Stanger BZ (2014) Adult hepatocytes are generated by self-duplication rather than stem cell differentiation. *Cell Stem Cell* 15: 340 – 349
15. Huch M, Dorrell C, Boj SF, van Es JH, Li VS, van de Wetering M, Sato T, Hamer K, Sasaki N, Finegold MJ *et al* (2013) In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature* 494: 247 – 250
16. Huch M, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MM, Ellis E, van Wenum M, Fuchs SA, de Ligt J *et al* (2015) Long-term culture

of genome-stable bipotent stem cells from adult human liver. *Cell* 160: 299 – 312

17. Smith WC (1999) TGF beta inhibitors. New and unexpected requirements in vertebrate development. *Trends Genet* 15: 3 – 5
18. Massague J (2012) TGFbeta signalling in context. *Nat Rev Mol Cell Biol* 13: 616 – 630
19. Uemura M, Hara K, Shitara H, Ishii R, Tsunekawa N, Miura Y, Kurohmaru M, Taya C, Yonekawa H, Kanai-Azuma M *et al* (2010) Expression and function of mouse Sox17 gene in the specification of gallbladder/bile-duct progenitors during early foregut morphogenesis. *Biochem Biophys Res Commun* 391: 357 – 363
20. Zilberberg A, Yaniv A, Gazit A (2004) The low density lipoprotein receptor-1, LRP1, interacts with the human frizzled-1 (HFz1) and down-regulates the canonical Wnt signaling pathway. *J Biol Chem* 279: 17535 – 17542
21. Jaeken J, Detheux M, Van Maldergem L, Foulon M, Carchon H, Van Schaftingen E (1996) 3-Phosphoglycerate dehydrogenase deficiency: an inborn error of serine biosynthesis. *Arch Dis Child* 74: 542 – 545
22. Hart CE, Race V, Achouri Y, Wiame E, Sharrard M, Olpin SE, Watkinson J, Bonham JR, Jaeken J, Matthijs G *et al* (2007) Phosphoserine aminotransferase deficiency: a novel disorder of the serine biosynthesis pathway. *Am J Hum Genet* 80: 931 – 937
23. Carpino G, Cardinale V, Onori P, Franchitto A, Berloco PB, Rossi M, Wang Y, Semeraro R, Anceschi M, Brunelli R *et al* (2012) Biliary tree stem/progenitor cells in glands of extrahepatic and intrahepatic bile ducts: an anatomical in situ study yielding evidence of maturational lineages. *J Anat* 220: 186 – 199
24. Cardinale V, Wang Y, Carpino G, Mendel G, Alpini G, Gaudio E, Reid LM, Alvaro D (2012) The biliary tree–a reservoir of multipotent stem cells. *Nat Rev Gastroenterol Hepatol* 9: 231 – 240
25. Si-Tayeb K, Lemaigre FP, Duncan SA (2010) Organogenesis and development of the liver. *Dev Cell* 18: 175 – 189
26. Font-Burgada J, Shalapour S, Ramaswamy S, Hsueh B, Rossell D, Umemura A, Taniguchi K, Nakagawa H, Valasek MA, Ye L *et al* (2015) Hybrid periportal hepatocytes regenerate the injured liver without giving rise to cancer. *Cell* 162: 766 – 779
27. Piccolo S, Dupont S, Cordenonsi M (2014) The biology of YAP/TAZ: hippo signaling and beyond. *Physiol Rev* 94: 1287 – 1312
28. Schuler M, Dierich A, Chambon P, Metzger D (2004) Efficient temporally controlled targeted somatic mutagenesis in hepatocytes of the mouse. *Genesis* 39: 167 – 172
29. Luche H, Weber O, Nageswara Rao T, Blum C, Fehling HJ (2007) Faithful activation of an extra-bright red fluorescent protein in "knock-in" Cre-reporter mice ideally suited for lineage tracing studies. *Eur J Immunol* 37: 43 – 53
30. Higgins GM, Anderson RM (1931) Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch Pathol* 12: 186 – 202
31. Kamiya A, Inagaki Y (2015) Stem and progenitor cell systems in liver development and regeneration. *Hepatol Res* 45: 29 – 37
32. Fausto N, Campbell JS (2003) The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mech Dev* 120: 117 – 130
33. Wilson JW, Leduc EH (1958) Role of cholangioles in restoration of the liver of the mouse after dietary injury. *J Pathol Bacteriol* 76: 441 – 449
34. Engelhardt NV, Factor VM, Medvinsky AL, Baranov VN, Lazareva MN, Poltoranina VS (1993) Common antigen of oval and biliary epithelial cells (A6) is a differentiation marker of epithelial and erythroid cell lineages in early development of the mouse. *Differentiation* 55: 19 – 26
35. De Alwis N, Hudson G, Burt AD, Day CP, Chinnery PF (2009) Human liver stem cells originate from the canals of Hering. *Hepatology* 50: 992 – 993
36. Huch M, Boj SF, Clevers H (2013) Lgr5(+) liver stem cells, hepatic organoids and regenerative medicine. *Regen Med* 8: 385 – 387
37. Kuver R, Savard CE, Lee SK, Haigh WG, Lee SP (2007) Murine gallbladder epithelial cells can differentiate into hepatocyte-like cells in vitro. *Am J Physiol Gastrointest Liver Physiol* 293: G944 – G955
38. Lee SP, Savard CE, Kuver R (2009) Gallbladder epithelial cells that engraft in mouse liver can differentiate into hepatocyte-like cells. *Am J Pathol* 174: 842 – 853
39. Manohar R, Komori J, Guzik L, Stolz DB, Chandran UR, LaFramboise WA, Lagasse E (2011) Identification and expansion of a unique stem cell population from adult mouse gallbladder. *Hepatology* 54: 1830 – 1841
40. Carpino G, Cardinale V, Gentile R, Onori P, Semeraro R, Franchitto A, Wang Y, Bosco D, Iossa A, Napoletano C *et al* (2014) Evidence for multipotent endodermal stem/progenitor cell populations in human gallbladder. *J Hepatol* 60: 1194 – 1202
41. Manohar R, Li Y, Fohrer H, Guzik L, Stolz DB, Chandran UR, LaFramboise WA, Lagasse E (2015) Identification of a candidate stem cell in human gallbladder. *Stem Cell Res* 14: 258 – 269
42. Cardinale V, Wang Y, Carpino G, Cui CB, Gatto M, Rossi M, Berloco PB, Cantafora A, Wauthier E, Furth ME *et al* (2011) Multipotent stem/progenitor cells in human biliary tree give rise to hepatocytes, cholangiocytes, and pancreatic islets. *Hepatology* 54: 2159 – 2172
43. Kadyk LC, Collins LR, Littman NJ, Millan MT (2015) Proceedings: moving toward cell-based therapies for liver disease. *Stem Cells Transl Med* 4: 207 – 210