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The Biliary Epithelium Gives Rise to Liver Progenitor Cells

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Abstract

Severe liver diseases are characterized by expansion of liver progenitor cells (LPC), which correlates with disease severity. However, the origin and role of LPC in liver physiology and in hepatic injury remains a contentious topic. We found that ductular reaction cells in human cirrhotic livers express hepatocyte nuclear factor 1 homeobox B (HNF1β). However, HNF1β expression was not present in newly generated epithelial cell adhesion molecule (EpCAM)-positive hepatocytes. In order to investigate the role of HNF1β- expressing cells we used a tamoxifen-inducible Hnf1βCreER/R26R$^{Yfp/LacZ}$ mouse to lineage-trace Hnf1β^{+} biliary duct cells and to assess their contribution to LPC expansion and hepatocyte generation. Lineage tracing demonstrated no contribution of HNF1β^{+} cells to hepatocytes during liver homeostasis in healthy mice or after loss of liver mass. After acute acetaminophen or carbon tetrachloride injury no contribution of HNF1β^{+} cells to hepatocyte was detected. We next assessed the contribution of Hnf1β^{+} -derived cells following two liver injury models with LPC expansion, a

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website.

diethoxycarbonyl-1,4-dihydrocollidin (DDC)-diet and a choline-deficient ethionine-supplemented (CDE)-diet. The contribution of Hnf1β$^+$ cells to liver regeneration was dependent on the liver injury model. While no contribution was observed after DDC-diet treatment, mice fed with a CDE-diet showed a small population of hepatocytes derived from Hnf1β$^+$ cells that were expanded to 1.86% of total hepatocytes after injury recovery. Genome-wide expression profile of Hnf1β$^+$ -derived cells from the DDC and CDE models indicated that no contribution of LPC to hepatocytes was associated with LPC expression of genes related to telomere maintenance, inflammation, and chemokine signaling pathways.

Conclusion—HNF1β$^+$ biliary duct cells are the origin of LPC. HNF1β$^+$ cells do not contribute to hepatocyte turnover in the healthy liver, but after certain liver injury, they can differentiate to hepatocytes contributing to liver regeneration.

Liver injury from any etiology induces mature liver cells to proliferate in order to replace the damaged tissue, allowing the recovery of the parenchymal function. In most situations, this process takes place without a clear involvement of liver progenitor cells (LPCs).[1,2] LPC expansion has been described in several liver diseases, and correlates with the degree of liver injury.[3,4] We have recently shown that in alcoholic hepatitis LPC markers correlate with liver injury and predict short-term mortality.[3] This observation raises the question whether LPC expansion is a marker of liver injury or an incomplete attempt to regenerate the damaged liver. Moreover, it highlights the need for identifying the pattern of liver injury that favors LPC contribution to liver regeneration.

Ductular reaction constitutes a heterogeneous population of proliferating cells ranging from cells expressing stem cell markers with an immature phenotype, to more committed cells with an intermediate hepatobiliary phenotype.[5–8] One of the most widely investigated markers is epithelial cell adhesion molecule (EpCAM), which is expressed in ductular reaction cells but also in newly generated hepatocytes, suggesting that EpCAM-positive hepatocytes may derive from progenitor cells.[2,9,10] Several studies have shown the capacity of LPC to differentiate *in vitro* to hepatocyte-like and cholangiocyte-like cells.[10–13] However, the role of LPC in liver diseases is not well understood and whether LPCs derive from the biliary compartment and how they contribute to liver homeostasis and repair is still controversial. Moreover, it is largely unknown how the environment within the injured liver influences LPC differentiation.[3,14,15]

Genetic lineage-tracing has become a gold standard to evaluate the contribution of any given cell type to cells that arise during organ development, tissue homeostasis or disease. Recent studies aimed at evaluating the contribution of LPC to liver regeneration using this strategy have yielded disparate results. Using a sex-determining region Y-box 9 (SOX9) lineage-tracing model, Furuyama et al.[16] showed an important contribution of SOX9 progeny to hepatocyte regeneration, supporting a model of liver homeostasis and regeneration based on a permanent supply of liver cells from LPC. By contrast, other recent studies showed that SOX9-positive embryonic ductal epithelium cells and osteopontin-labeled adult liver cells have the potential to give rise to transit-amplifying progenitor cells and mature hepatocytes, although to a much lesser extent.[17,18] Moreover, lineage-tracing studies of markers not expressed in intact liver but in ductular reaction cells have shown the potential of LPC to

differentiate to hepatocytes and cholangiocytes.[13,19,20] In summary, there are conflicting evidences concerning the possible contribution of biliary duct cells and LPC to hepatocyte regeneration in response to liver injury.

Hepatocyte nuclear factor (HNF)1β is a homeobox transcription factor that plays a pivotal role during organogenesis and regulates gene expression in the adult liver and other epithelial organs.[21–23] In liver development, HNF1β is involved in the hepatobiliary specification of hepatoblasts to cholangiocytes, and it is strongly expressed throughout the embryonic and adult biliary epithelium.[21–24] However, little is known about the expression of HNF1β during liver injury and regeneration, and particularly its expression in LPC.

In this study we traced HNF1β$^+$ cells to assess the contribution of the biliary epithelium to LPC and hepatocytes during healthy liver homeostasis, liver regeneration, and in animal models of acute and repeated liver injury. We show that under physiological conditions, hepatocytes do not derive from HNF1β$^+$ cells. Only after liver injury do adult HNF1β$^+$ cells give rise to the expansion of cells with an LPC phenotype and to periportal hepatocytes. However, the population of HNF1β$^+$ cell-derived hepatocytes is small under these experimental conditions, suggesting that LPC do not substantially contribute to liver parenchymal regeneration under most liver injury insults.

Materials and Methods

Human Biopsies and Samples

Liver tissue samples were obtained from fragments of normal tissue surrounding colon metastasis collected at the moment of liver resection or from explants from liver transplantation due to alcohol-induced liver cirrhosis. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona and all patients included in this study gave written informed consent.

Animal Protocols

Hnf1bCreER transgenic mice were generated and genotyped as previously described.[25] Mice were crossed with mice bearing Cre-inducible Rosa26R reporters *LacZ* (β-galactosidase [β-GAL] or yellow fluorescent protein [YFP]). To induce Cre-recombination, 12 to 24-week-old mice were treated with three tamoxifen (Sigma-Aldrich, St. Louis, MO) doses (20 mg, 20 mg, and 10 mg) by gavage over 1 week. Tamoxifen was dissolved at 100 mg/mL in 0.9% NaCl and 10% ethanol absolute in order to facilitate sonication. All animal models of liver injury were started 1 week after the last tamoxifen treatment. Two control groups were used for each experimental setting: mice treated with tamoxifen without liver injury, and mice with liver injury but without tamoxifen treatment.

The animal models of liver injury used in this study were: acute acetaminophen (APAP) (Sigma-Aldrich); acute carbon tetrachloride injection (CCl_4, Sigma-Aldrich); two-thirds partial hepatectomy (PH); 0.1% 3,5-diethoxycarbonyl-1,4-dihydro-collidin (DDC) diet[26] (Sigma-Aldrich); choline-deficient (MP Biomedicals, Santa Ana, CA) ethionine-supplemented (0.15% in water, Sigma-Aldrich) (CDE) diet,[27] and chronic CCl_4 administration. All experimental models are described in the Supporting Material.

All animal experiments were approved by the Ethics Committee of Animal Experimentation of the University of Barcelona and were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Histochemical Procedures and Immunostaining

The staining procedure is described in the Supporting Material.

Isolation of YFP$^+$ Cells

YFP$^+$ cells were obtained by liver perfusion method followed by flow cytometry cell sorting from uninjured mice (n = 3), and from mice treated with a DDC (n = 3) or CDE diet (n = 3) for 3 weeks. A detailed procedure of isolation and following RNA extraction and gene expression analysis are described in the Supporting Material.

Statistical Analysis

Continuous variables were described as means (± standard error) and were compared using the Student *t* test. All statistical analyses were performed using SPSS v. 14.0 for Windows (Chicago, IL).

Results

Expression of HNF1β in Normal and Cirrhotic Human Livers

We first investigated the expression pattern of HNF1β in human liver tissue. HNF1β expression was restricted to biliary duct cells in healthy livers (Fig. 1A). Immunostaining of liver samples from patients with advanced alcoholic liver disease showed that ductular reaction cells were positive for HNF1β (Fig. 1B), whereas HNF1β was not expressed in mature hepatocytes as assessed by double staining of HEP PAR-1 and HNF1β (Fig. 1C,D). To further determine HNF1β expression in immature hepatocytes, we performed a double staining of HNF1β and EpCAM in healthy and alcohol-induced cirrhotic livers. Double-positive HNF1β/EpCAM cells were detected in the biliary epithelia from healthy tissue and in ductular reaction cells from cirrhotic tissue (Fig. 1E,F). Importantly, EpCAM-positive hepatocytes did not show HNF1β expression (Fig. 1F) suggesting that immature hepatocytes lost the expression of biliary markers such as HNF1β, while still retaining the LPC marker EpCAM.

Tamoxifen Induction of Cre-Recombinase in Hnf1βCreER/R26R Mice

In order to assess the specificity of Cre expression in HNF1β$^+$ biliary cells, Hnf1βCreER/R26R$^{Yfp/LacZ}$ mice were treated with tamoxifen and analyzed for Cre expression. The expression of Cre was assessed 16 hours posttamoxifen administration since Cre is transiently located in the nucleus for only 6–36 hours (Supporting Fig. 1A). Immunostaining analysis showed Cre expression restricted to the bile duct cells and colocalized with YFP expression (Supporting Fig. 1B). Importantly, Cre recombinase expression was restricted to HNF1β$^+$ cells, with an 88.47 ± 4.5% of HNF1β$^+$ cells also positive for Cre (Supporting Fig. 1C). Moreover, a 98.35 ± 0.4% of HNF1β$^+$ cells also showed expression of SOX9 (Supporting Fig. 1D). Cre expression colocalized with KRT19 and A6[26] (Supporting Fig.

1E,F). An overdose of tamoxifen is known to generate liver damage that could induce ectopic expression of HNF1β and the reporter. Tamoxifen treatment induced a mild increase in aspartate transaminase levels (AST) 42 ± 5.2 U/L versus 95.4 ± 30.2 U/L and alanine transaminase levels (ALT) 36 ± 16.9 U/L versus 76.4 ± 28.8 U/L. However, immunostaining results demonstrate that tamoxifen did not induce the expression of HNF1β or CRE in HNF4α$^+$ hepatocytes (Supporting Fig. 1G,H). Induction of reporter gene YFP or β-GAL showed a labeling efficiency of 28.7 ± 10% as assessed by KRT19/YFP or KRT19/β-GAL. These results indicate that the Hnf1βCreER/R26R$^{Yfp/LacZ}$ model can be used to trace the fate of HNF1β$^+$ biliary duct cells.

Contribution of HNF1β$^+$ Cells to Hepatocyte Turnover During Normal Liver Homeostasis

To evaluate the contribution of HNF1β$^+$ cells in a situation of normal liver homeostasis, mice were treated with tamoxifen and sacrificed 2 months later. We assessed the phenotype of the cellular progeny of HNF1β$^+$ cells by performing double staining with the reporter gene β-GAL and cell lineage markers HNF4α and KRT19. As shown in Supporting Fig. 2, we failed to find any hepatocytes that stained with the reporter marker, indicating that HNF1β$^+$ cells give rise to biliary cells but do not contribute to physiological hepatocyte turnover.

Contribution of HNF1β$^+$ Cells to Liver Regeneration

To trace the fate of HNF1β$^+$ cells in a model of liver regeneration, we performed a two-thirds PH in Hnf1βCreER^{Yfp} mice (Fig. 2A). Before PH, expression of YFP was restricted to the biliary epithelia as shown by KRT19/YFP and HNF4α/YFP staining (Fig. 2B). At 7 days after surgery mice showed a ductular reaction with HNF1β$^+$ cells restricted to bile ducts (Fig. 2C). Sporadic single hepatocytes located in periportal areas were positive for HNF4α and the reporter gene YFP (Fig. 2C). We also evaluated the contribution of HNF1β$^+$ cells in mice at 28 days after surgery to allow the complete regeneration of the liver (Fig. 2D). No contribution of HNF1β$^+$ cells to hepatocytes was detected at 28 days after surgery, demonstrating that the biliary epithelium does not make a significant contribution to hepatocyte regeneration after PH.

Gene expression of *Krt7*, *EpCAM*, *CD133*, *Trop2*, and *Krt19*[9,28] was also evaluated in mice with PH at time 0 (excised liver), 7 days, and 28 days after surgery. As shown in Fig. 2E, gene expression of LPC markers was increased at 7 days compared to time 0 and returned to basal levels at 28 days. These results suggest that resolution of the liver regenerative process is accompanied with a reduction of cells with an LPC phenotype.

Fate of HNF1β$^+$ Cells in Acute Liver Injury

In order to evaluate the proliferation and contribution of HNF1β$^+$ cells after acute liver injury, we used two well-known models of acute damage, acute APAP and acute CCl_4 administration (Fig. 3A). As shown in Fig. 3B–D,F–H, dual staining of KRT19/YFP, SOX9/YFP, and A6/YFP showed a mild induction of LPC expansion. Moreover, no duct-derived hepatocyte generation was observed based on dual staining of HNF4α/YFP (Fig. 3E,I).

Contribution of Hnf1β$^+$ Cells to Chronically Damaged Livers

LPC expansion is associated with liver damage compromising hepatocyte proliferative capacity. To determine the contribution of HNF1β$^+$ cells to LPC expansion and hepatocyte regeneration, we performed three different types of chronic liver injury: a DDC diet, which induces cholangiocytic injury, as well as chronic CCl_4 treatment and a CDE diet, both known for their hepatocyte toxic effect.

We first investigated the progeny of HNF1β$^+$ cells in a DDC diet model, which typically stimulates progenitor cell proliferation (Fig. 4A). As expected, after 4 weeks of DDC diet an important ductular reaction was observed accompanied by a significant increase in *Krt7*, *CD133*, and *Trop2* messenger RNA (mRNA) expression, but not in *EpCAM* and *Krt19* (Fig. 4B). Double immunostaining with LPC marker A6 or biliary markers SOX9 or KRT19 together with the reporter gene β-GAL, and also EpCAM/HNF1β staining demonstrated that the ductular reaction was derived from HNF1β$^+$ cells (Fig. 4C–G). However, no contribution of HNF1β$^+$ cell progeny to hepatocytes was observed (Fig. 4H). We then explored if a DDC injury-recovery model enhanced the contribution of HNF1β$^+$ cells to new hepatocytes. The replacement of the DDC diet (4 weeks) for standard chow (2 weeks) (DDC 4+2w) reduced the expression of LPC genes significantly (Fig. 4B), and double staining with HNF4α and β-GAL did not show any contribution of HNF1β$^+$ cells to hepatocyte generation (Supporting Fig. 3A–C).

Next, we assessed the contribution of Hnf1β$^+$ cells in mice treated chronically with CCl_4, which induces hepatocyte damage and liver fibrosis upon chronic treatment. After 8 weeks of CCl_4 treatment, mice presented bridging fibrosis with marked deposition of extracellular matrix and areas of hepatocyte necrosis and inflammation (Supporting Fig. 3D–G). Although some expansion of LPC was noted as assessed by KRT19 staining, only sporadic single cells with double staining for HNF4α and β-GAL were observed (Supporting Fig. 3E,F and data not shown). Although CCl_4 induces hepatocyte damage, it did not compromise hepatocyte replication as shown by Ki-67 staining (Supporting Fig. 4). Thus, we next investigated the contribution of HNF1β$^+$ cells to hepatocytes in a CDE model which presents hepatocyte damage, decreased hepatocyte proliferation, and LPC expansion (Fig. 5A; Supporting Fig. 4). This LPC expansion was confirmed by an increase of LPC markers *Krt7*, *EpCAM*, *CD133*, and *Krt19* after 3 weeks of CDE treatment, as shown in Fig. 5B. Moreover, there was a clear expansion of LPC derived from HNF1β$^+$ cells as shown by A6 (not shown), SOX9, and KRT19 double staining with YFP and EpCAM/HNF1β costaining (Fig. 5C). A 38.96% of KRT19$^+$ cells coexpressed YFP. In addition, in this model we were able to identify a population of YFP$^+$ hepatocytes in the periportal area as assessed by HNF4α/YFP staining (Fig. 5C). However, the number of HNF4α/YFP$^+$ hepatocytes in this model was low (0.22% of total hepatocytes). We next explored if a CDE injury-recovery model enhanced the number of HNF1β-derived hepatocytes. Mice treated for 3 weeks with a CDE diet followed by 2 extra weeks with standard chow (CDE 3+2w), showed a strong ductular reaction, with cells coexpressing SOX9/YFP, EpCAM/HNF1β, and 53.84% KRT19/YFP double-positive cells (Fig. 5D). Moreover, this animal model had a reduced hepatocyte proliferation as assessed by Ki-67 staining and p21 expression (Supporting Fig. 4). Importantly, we identified a double-positive population of HNF4α/YFP hepatocytes

representing 1.86% of the total hepatocyte population. Moreover, YFP^+ hepatocytes were negative for HNF1β (Fig. 5D). These results indicate that at least a fraction of $HNF1\beta^+$ cells are bipotential LPC with the capacity to give rise to cholangiocytes and hepatocytes.

Expression of LPC Markers in YFP^+ Cells

In order to determine if $HNF1\beta^+$ cells express LPC markers, YFP^+ cells were isolated from uninjured mice and mice exposed to DDC and CDE diet. Genome-wide analysis showed that YFP^+ cells from healthy and damaged livers expressed well-described LPC markers (Table 1). Moreover, they showed a mixed phenotype expressing genes typically expressed in hepatocytes such as *HNF4*α, *Krt18*, *Onecut1*, and *Onecut2*, *Foxa2*, and in cholangiocytes, *Hnf1β*, *Muc1*, and *Krt19* (Table 1). Real-time polymerase chain reaction (PCR) of *CD133*, *EpCAM*, *Trop2*, *CD24 Hnf1β*, *Sox9*, and *Krt19* was performed to validate the gene expression profile in DDC-YFP^+ cells (data not shown). In order to investigate differences in gene expression profile in YFP^+ cells from DDC and CDE-treated animals that could explain the differences in their contribution to hepatocyte generation, gene expression profiles were compared to YFP^+ cells obtained from uninjured livers. As shown in Fig. 6, a Venn diagram showed that 280 genes were differentially expressed in CDE-derived YFP^+ cells and 673 were found deregulated in DDC-derived YFP^+ cells as compared with YFP^+ cells from uninjured mice. We next performed a functional analysis of those genes differentially expressed in DDC and CDE-derived YFP^+ cells (Fig. 6; Supporting Fig. 5 and Supporting Tables 4 and 5). Canonical pathways overrepresented in both DDC and CDE-derived YFP^+ cells showed an enrichment of up-regulated genes related to cell cycle, proliferation, p53 pathway, and cycle checkpoints. Interestingly, YFP^+ cells from CDE and DDC diets showed important differences in terms of pathways overrepresented. On the one hand, as shown in Supporting Table 4, CDE treatment enhanced the expression of transcription factors and genes related to apoptosis and down-regulate genes involved in cell junction, cell-cell communication, Notch1, GSK3, or Wnt signaling pathway. On the other hand, gene set enrichment analysis of DDC YFP^+ cells showed the up-regulation of genes related to telomere maintenance, inflammatory pathways, stress, and integrin pathways, and the down-regulation of genes related to lipid metabolism and synthesis, insulin-like growth factor or peroxisome proliferator activated receptor (PPAR) signaling (Supporting Fig. 5 and Supporting Tables 4 and 5). These results confirmed that HNF1β-derived cell population is enriched with LPC and provides information regarding which pathways may be responsible for the differences in contribution of HNF1β-derived cell to hepatocyte generation.

Discussion

The present study investigated the contribution of $HNF1\beta^+$ cells from the biliary epithelium to liver regeneration. Importantly, we provide evidence that Hnf1β is expressed in cells forming the ductular reaction and expanded LPC in human diseases and in animal models of liver injury. We demonstrate that the $HNF1\beta^+$ cell population express markers of LPC but does not contribute to normal parenchymal homeostasis; however, under liver injury, $HNF1\beta^+$ cells expand and can give rise to hepatocytes. Nevertheless, our results suggest that the contribution of $HNF1\beta^+$ cells to newly generated hepatocytes is low under most liver injury models.

The main drawback of lineage tracing studies is the specificity of induction of the driving promoter. Regarding this point, the Hnf1βCreER$^{YFP/LacZ}$ mouse is an excellent tool to lineage trace ductular cells due to the specific expression of Hnf1β-Cre in the biliary tree cells and a complete absence of expression in other cell types.[25] We carefully analyzed the expression of HNF1β, Cre-recombinase and the reporter genes YFP and β-GAL in intact livers and also after tamoxifen induction in order to show that recombination was taking place exclusively in biliary cells and not in periportal hepatocytes. This issue is particularly important in experimental settings where a low number of newly generated hepatocytes is expected.

Little is known about the role of HNF1β in liver injury and resolution.[29] Recent studies showed that HNF1β participates in biliary tree formation, and HNF1β human deficiency is associated with ciliary defects in cholangiocytes.[23,24,29,30] Here we demonstrate that HNF1β is expressed in progenitor cells, suggesting that it may also play a role in the maintenance of the hepatobiliary cell phenotype. Interestingly, HNF1β is expressed in human ductular reaction cells showing EpCAM expression but not in EpCAM-positive hepatocytes, suggesting that HNF1β expression in LPC may be lost upon differentiation. In this study we attempted to confirm this hypothesis by lineage tracing HNF1β^{+} cells.

The origin of LPC and its contribution to normal liver homeostasis and repair is still debated. Histological analyses have shown them in the vicinity of bile ducts[6,31]; thus, it has been suggested that LPC derive from the biliary compartment and participate in the generation of new cholangiocytes and hepatocytes. This hypothesis has been explored in recent studies of lineage tracing with SOX9 transcription factor and osteopontin. However, these important studies have yielded opposite results, partially due to reexpression of SOX9 after tamoxifen induction[16,17] and the use of nonendogenous artificial promoters.[18] Moreover, osteopontin is shown to be expressed in cell types other than progenitor cells (e.g., hepatic stellate cells[32] and inflammatory cells[33]). This could be an important limitation of this study, since recent studies suggest that hepatic stellate cells can act as progenitor cells in the injured liver.[34,35] By performing a lineage tracing based on HNF1β, a specific marker of cells from the biliary compartment, our study clearly demonstrates that during liver injury HNF1β^{+} biliary duct cells give rise to LPC, which have the potential to generate hepatocytes. However, hepatocyte generation may only occur under specific injury conditions and not as a default mechanism during liver homeostasis.

It is difficult to estimate the net contribution of HNF1β-derived hepatocytes to liver regeneration since the number of newly generated hepatocytes varies depending on the specific liver injury model or regenerative stimulus. Moreover, the labeling of HNF1β cells is not complete, so we may be underestimating the number of generated hepatocytes. Our results do not support the continuous nature of hepatocyte supply from the biliary compartment to liver parenchyma homeostasis and regeneration and is in line with the studies showing a modest contribution of LPC to hepatocyte regeneration only after specific liver damage.[17,18] However, it is plausible that repeated cycles of injury-recovery or prolonged animal models of liver injury may show an increased significant level of contribution of LPC to liver regeneration. In this context, advanced chronic human diseases, which develop after prolonged periods of time (i.e., years), are characterized by a strong

hepatocyte arrest accompanied with a continuous and prolonged liver injury and inflammation.[36] Differences in the type, duration, and severity of liver injury between experimental models and human diseases may also suggest that the contribution of LPC to liver regeneration in human chronic diseases may exceed the extent observed in animal models.

An important finding of our study is that gene expression levels of LPC markers does not correlate with LPC contribution to liver regeneration. The gene expression profile of total liver and HNF1β-derived YFP^+ cells showed an enrichment of hepatobiliary and LPC markers, demonstrating that all animal models analyzed do induce LPC expansion. However, HNF1β-derived hepatocytes were observed only in the CDE animal model, and especially in the CDE injury-recovery model, suggesting that the liver injury milieu may influence the LPC differentiation capacity. Moreover, our study provides the first transcriptomic profile of mouse LPC comparing isolated cells from the two more widely used models of LPC expansion (i.e., DDC and CDE models). This analysis yielded important mechanistic data, since functional genome-wide analysis of YFP^+ cells isolated from both animal models showed important differences in a number of pathways that may influence LPC differentiation. In that sense, YFP^+ cells from DDC mice show an enrichment of genes related to inflammatory response, response to stress, telomerase maintenance, or cell cycle checkpoints that may be preventing $HNF1\beta^+$ cells from differentiating. Further studies will need to evaluate which may be the involvement of these pathways in LPC expansion and differentiation.

LPC expansion and contribution to liver regeneration may have an important impact on liver disease since the degree of LPC activation correlates with survival in severe human liver disease such as alcoholic hepatitis.[3] This observation suggests that in chronic liver diseases LPC expansion may be an uncontrolled response to injury that fails to effectively yield mature functioning hepatocytes. The data reported here provide important basic information regarding the adult biliary compartment as the origin of LPC. Although the lineage-tracing results shown here demonstrate that the biliary compartment may not play a role during normal liver homeostasis, it is clear that biliary cells give rise to an expanding LPC population with the potential to generate hepatocytes. Understanding the LPC contribution to hepatocyte generation is essential to better understand the mechanisms of liver regeneration and may have important implications for the development of new therapeutic strategies directed to enhance LPC expansion and differentiation aiming at favoring liver injury resolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APAP	acetaminophen
β-GAL	beta galactosidase
CCl4	carbon tetrachloride
CD133 (PROM1)	prominin-1
CDE	choline-deficient, ethionine-supplemented
DAPI	4',6-diamidino-2-phenylindole
DDC	3,5-diethoxycarbonyl-1,4-dihydro-collidin
EpCAM	epithelial cell adhesion molecule
HEP PAR-1	hepatocyte paraffin-1
HNF1β	hepatocyte nuclear factor 1 homeobox B
HNF4α	hepatocyte nuclear factor 4 alpha
Krt	keratin
LPC	liver progenitor cells
PH	partial hepatectomy
SOX9	sex determining region Y-box 9
TROP2	tumor-associated calcium signal transducer 2
YFP	yellow fluorescent protein

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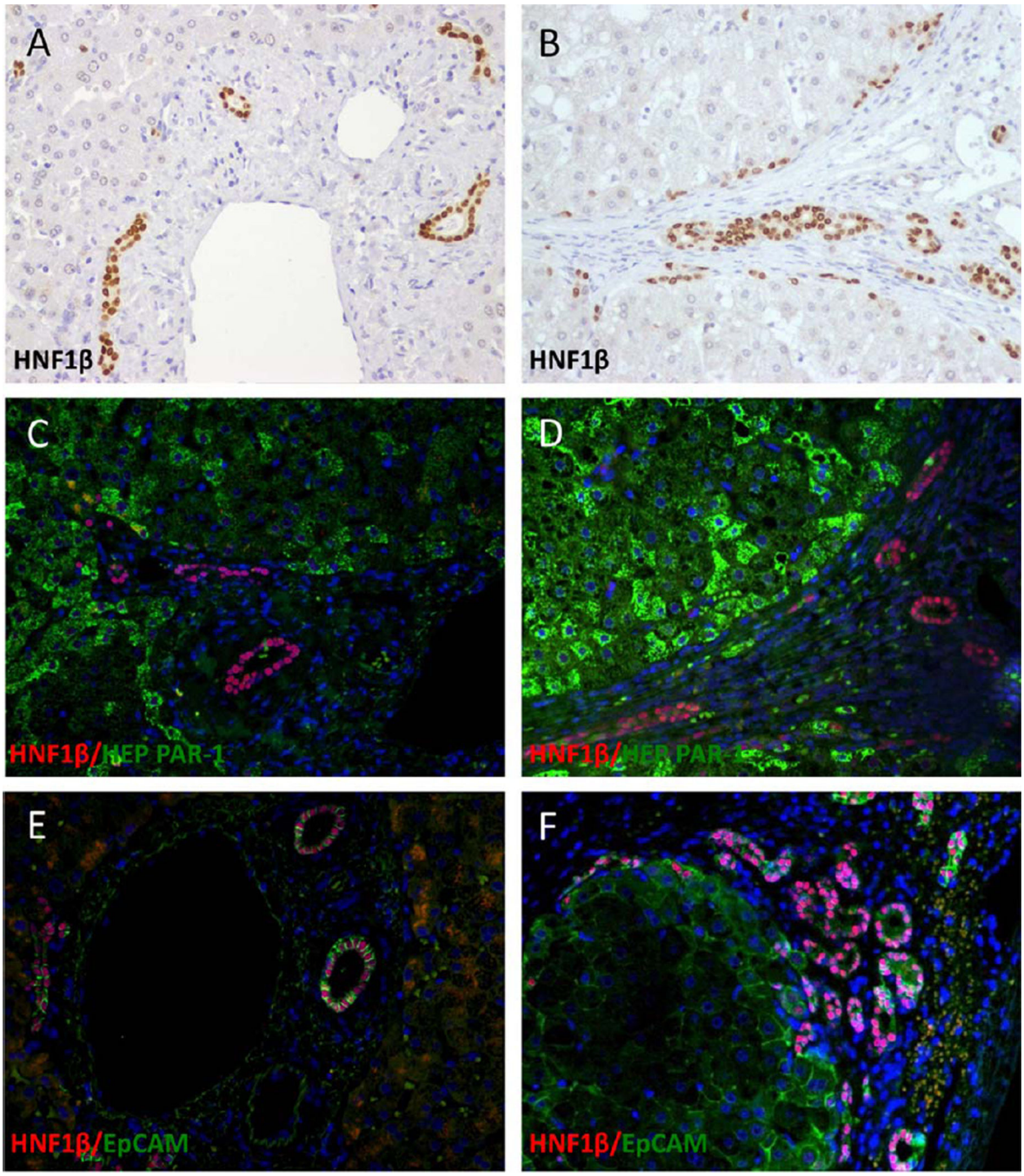

Fig. 1.

HNF1β is expressed in biliary cells and in ductular reaction cells in healthy and diseased human liver. Representative images in healthy liver of (A) HNF1β immunostaining; (C) double immunostaining for HNF1β and hepatocyte marker HEP PAR-1; and (E) double immuno-staining for HNF1β and EpCAM. Representative pictures in cirrhotic liver of (B) HNF1β immunostaining; (D) double immunostaining for HNF1β and HEP PAR-1; and (F) double immunostaining for HNF1β and EpCAM. Nuclei counterstaining was performed with DAPI (blue) (magnification ×200 and ×400).

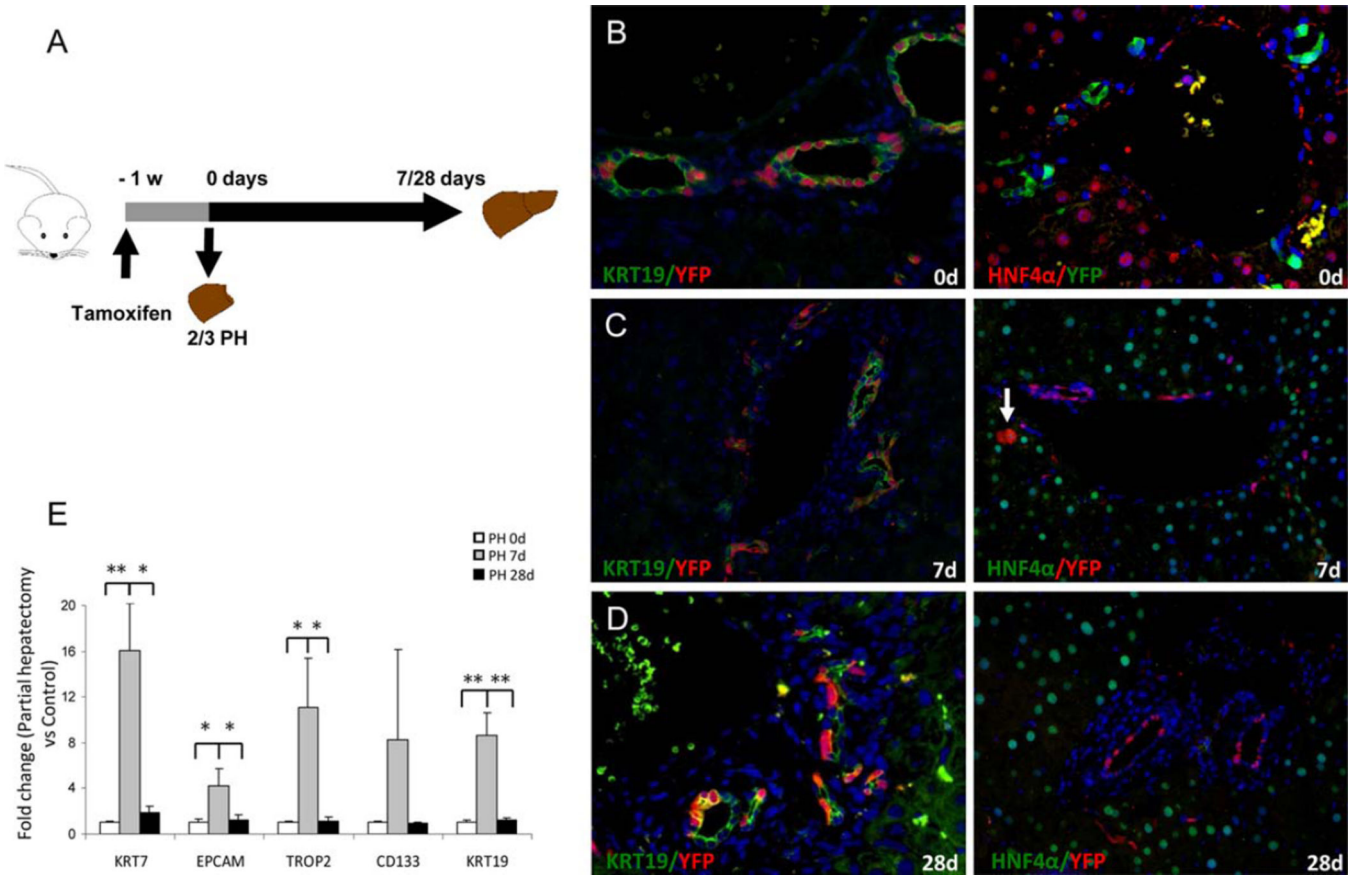

Fig. 2.

HNF1β^{+} cells do not contribute to liver regeneration after partial hepatectomy. (A) Scheme of experimental design. Double immuno-staining with YFP and KRT19 (magnification ×200) or HNF4α (magnification ×400) were performed at different timepoints: (B) Liver excised during PH (0d); (C) 7 days after PH (7d); and (D) 28 days after PH (28d). Few hepatocytes were double stained with HNF4α/YFP (white arrow). (E) *Krt7*, *EpCAM*, *Trop2*, *CD133* and *Krt19* gene expression in mice with partial hepatectomy (PH) at 7 days (PH 7d) (n = 4) or 28 days after surgery (PH 28d) (n = 4) compared to control liver (PH 0d) (n = 4) (*$P < 0.05$; **$P < 0.005$). Nuclei counterstaining was performed with DAPI (blue) (magnification ×200 and ×400). d; days; w, weeks before or after tamoxifen administration; PH, partial hepatectomy.

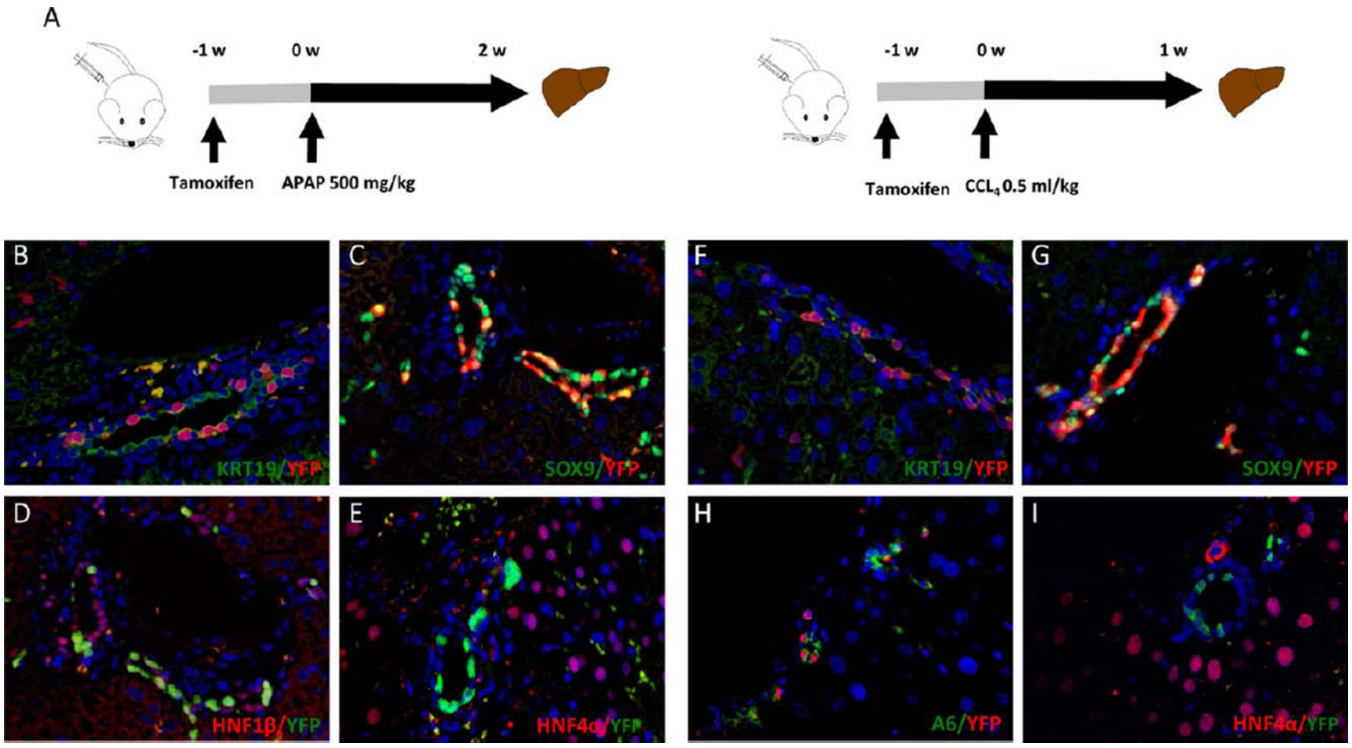

Fig. 3.

HNF1β$^+$ cells do not contribute to hepatocyte regeneration in acute injury models. (A) Scheme of APAP and CCl_4 single injection experimental design. Representative pictures of double staining with YFP and (B,F) KRT19, (C,G) SOX9, (D) HNF1β, (E,I) HNF4α, or (H) A6. HNF1β$^+$ cells did not contribute to hepatocyte population after injury damage induced by (B–E) APAP administration or (F–I) CCl_4 single injection. Nuclei counterstaining was performed with DAPI (blue) (magnification × 400). w, weeks before or after tamoxifen administration.

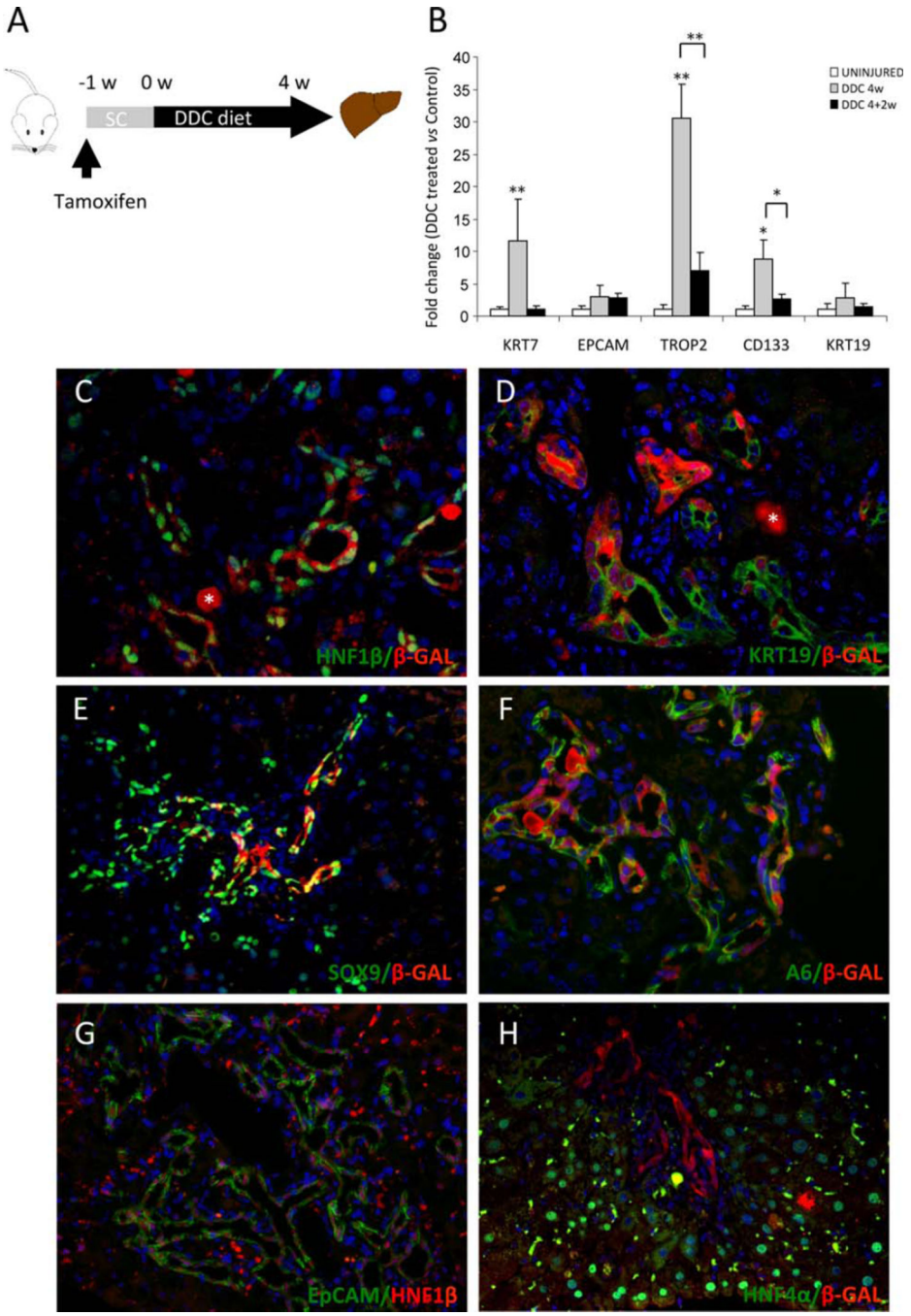

Fig. 4.

HNF1β^{+} cells generate the ductular reaction but not hepatocytes in mice treated with a DDC diet. (A) Scheme of DDC diet experimental design. (B) *Krt7*, *EpCAM*, *Trop2*, *CD133*, and *Krt19* gene expression in mice treated with a DDC diet for 4 weeks (n = 5) (DDC 4w) and DDC diet for 4 weeks plus standard chow for 2 weeks (DDC 4+2w) (n = 5) compared to uninjured mice liver (n = 4) (**P* <0.05, ***P*<0.005). Representative images of double staining of β-GAL and (C) HNF1β, (D) KRT19, (E) SOX9, (F) A6, and (H) HNF4α, and (G) EpCAM/HNF1β in mice treated for 4 weeks with a DDC diet. Asterisks mark bile

deposits. w, weeks before or after DDC feeding; SC, standard chow; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-collidin. Nuclei counterstaining was performed with DAPI (blue) (magnification ×200 and ×400).

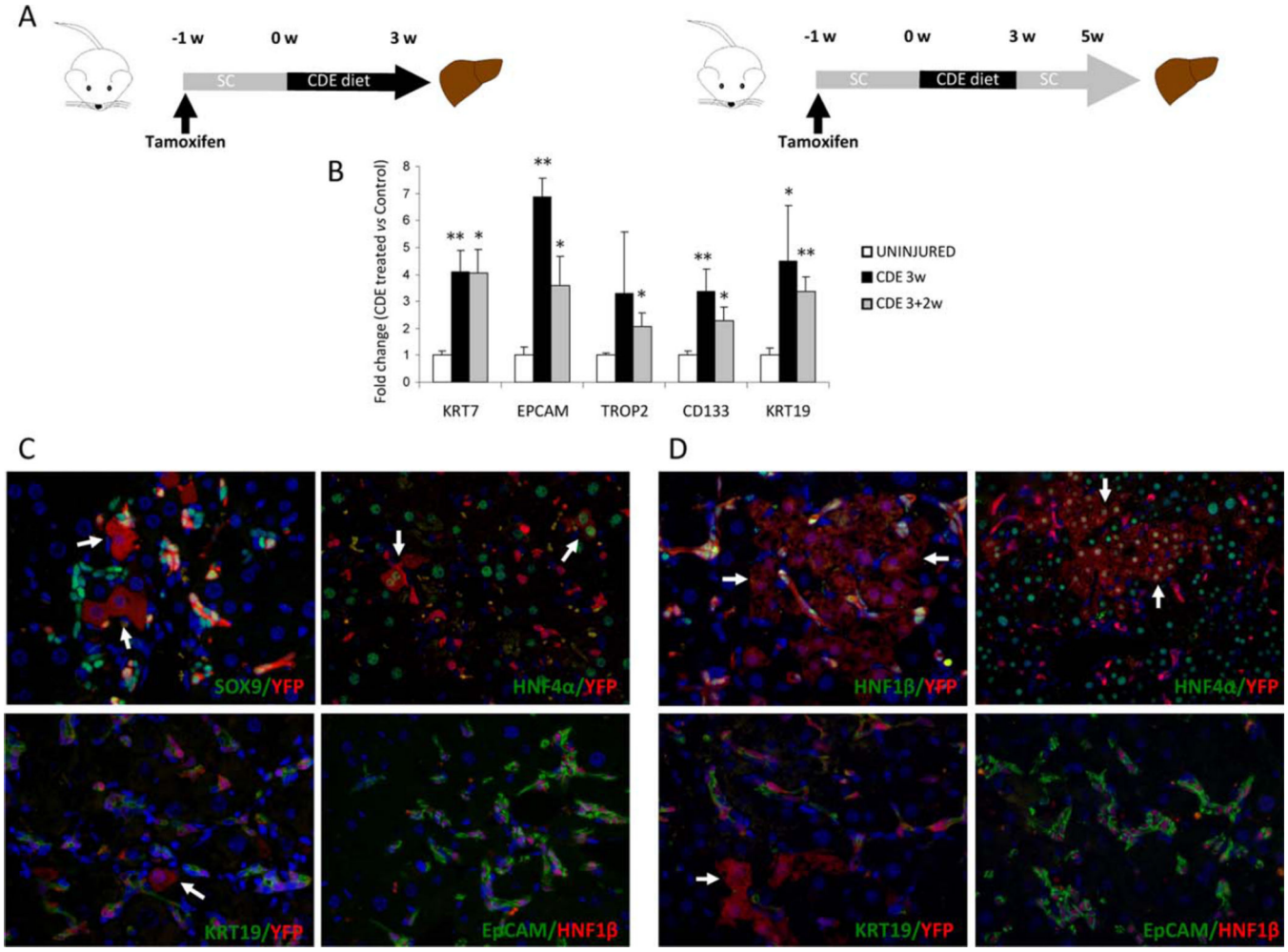

Fig. 5.

HNF1β^{+} cells contribute to ductular reaction and hepatocyte regeneration in mice treated with a CDE diet. (A) Scheme of CDE diet experimental designs. (B) *Krt7*, *EpCAM*, *Trop2*, *CD133*, and *Krt19* gene expression in mice treated with a CDE diet for 3 weeks (CDE 3w) (n = 3) and CDE injury-recovery model (CDE 3+2w) (n = 5) compared to uninjured mice liver (n = 4); (**P* < 0.05; ***P* < 0.005). (C) Representative images of double staining of YFP and SOX9, HNF4α, KRT19, and EpCAM/HNF1β in mice treated for 3 weeks with a CDE diet. HNF1β-derived hepatocytes are indicated with white arrows. (D) Representative images of double staining of YFP and SOX9, HNF4α, KRT19, and EpCAM in mice treated for 3 weeks with a CDE diet and 2 weeks with standard chow (CDE 3+2w). Nuclei counterstaining was performed with DAPI (blue) (magnification ×200 and ×400). w, weeks after or before CDE feeding; SC, standard chow; CDE, choline-deficient ethionine-supplemented.

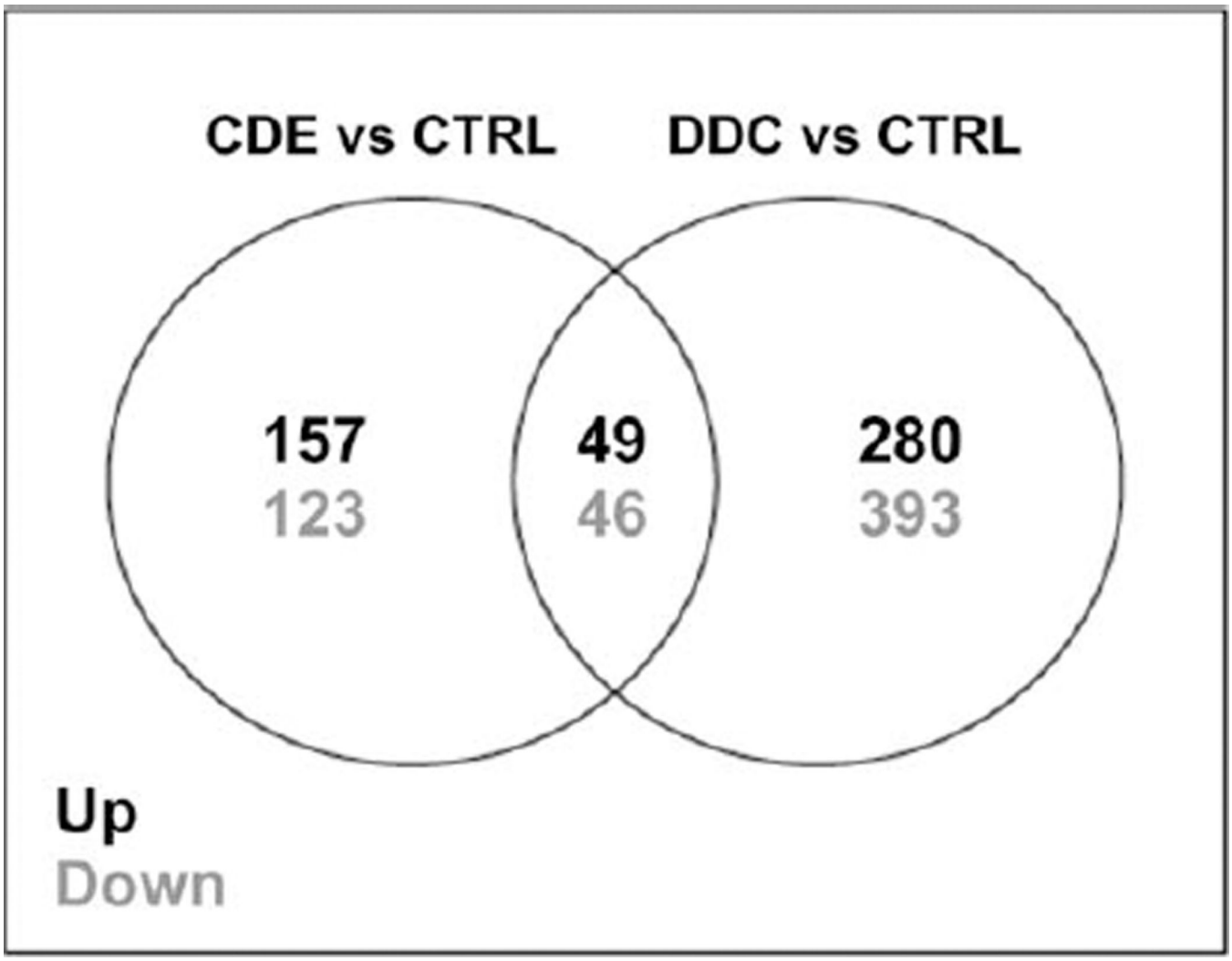

Fig. 6.

Venn diagram showing overlap of significantly regulated genes considering YFP^+ cells isolated from CDE- and DDC-treated mice versus uninjured mice (CTRL). Genes were found regulated (fold-change <1.5 and >1.5 and moderated $P < 0.05$) after assessing significance using the LIMMA package.

Table 1

Expression of Progenitor Markers in YFP$^+$ Cells

		Fold Change		
Symbol	**Gene Name**	**CDE**	**DDC**	**CTRL**
Hnf1b	HNF1 homeobox B	42.9	41.8	46.0
Sox9	SRY-box containing gene 9	37.5	36.4	38.0
Cldn7	claudin 7	28.6	27.1	29.5
Muc1	mucin 1, transmembrane	16.7	26.9	26.5
Krt19	keratin 19	21.0	24.5	26.0
Prom1	prominin 1	16.5	16.9	21.3
Krt8	keratin 8	19.2	25.2	20.8
Spp1	secreted phosphoprotein 1	21.1	22.2	20.1
Cldn3	claudin 3	17.2	20.5	19.5
Krt18	keratin 18	19.1	21.6	19.2
Onecut1	one cut domain, family member 1	18.0	18.0	17.0
Cdh1	cadherin 1	14.1	14.4	15.8
Onecut2	one cut domain, family member 2	17.9	16.5	15.8
Sox4	SRY-box containing gene 4	12.6	15.9	14.8
Krt7	keratin 7	11.4	17.4	14.6
Epcam	epithelial cell adhesion molecule	14.6	15.1	13.5
Foxa1	forkhead box A1	14.1	11.7	12.0
Foxa3	forkhead box A3	9.3	5.6	7.9
Aldh1a1	aldehyde dehydrogenase family 1, subfamily A1	9.9	7.1	7.6
Cd24a	CD24a antigen	7.2	8.0	6.9
Foxa2	forkhead box A2	5.2	5.1	5.5
Hnf4a	hepatic nuclear factor 4, alpha	3.8	4.6	5.5
Tacstd2	tumor-associated calcium signal transducer 2	4.1	10.0	4.8
Ncam1	neural cell adhesion molecule 1	1.5	2.3	4.6

CDE, choline-deficient, ethionine-supplemented treated mice; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-collidin treated mice; CTRL, uninjured mice.