HHS Public Access

Author manuscript

*Gastroenterology*. Author manuscript; available in PMC 2016 October 01.

Published in final edited form as:

*Gastroenterology*. 2015 October ; 149(4): 876–882. doi:10.1053/j.gastro.2015.08.004.

# Liver stem cells: Experimental findings and implications for human liver disease

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## Abstract

Evidence from human histopathology and experimental studies with rodents and zebrafish has shown that hepatocytes and cholangiocytes may function as facultative stem cells for each other in conditions of impaired regeneration. The interpretation of the findings derived from these studies has generated considerable discussion and some controversies. This review examines the evidence obtained from the different experimental models and considers implications that these studies may have for human liver disease.

Few topics of liver tissue biology have attracted as much attention as the existence of liver-specific tissue stem cells. Routine liver histology reveals two types of epithelial cells, hepatocytes and cholangiocytes (also known as biliary epithelial cells). Endothelial cells line the hepatic capillaries (sinusoids), with macrophages (Kupffer cells) interspersed along the sinusoid lumen. Stellate cells exist under the sinusoids and in close proximity to hepatocytes. None of these cells appears to have functions of a fully committed tissue specific stem cell, analogous to the cells of the intestinal crypts, the basal layer of the epidermis, bone marrow stem cells, etc.

Hepatocytes and cholangiocytes can be easily identified based on their morphology and cell-specific biomarkers. Hepatocytes and cholangiocytes, however, often have mutually mixed expression of biomarkers in pathologic conditions. In patients with fulminant hepatic failure (FHF), there is rampant proliferation of cholangiocytes organized in ductular structures ("ductular reaction"[1, 2]). Many of these cholangiocytes (known as ductular hepatocytes) express biomarkers associated with hepatocytes, (HNF4, albumin, HEPPAR[3], etc.). They are seen surrounding cells ranging in size from small to typical hepatocytes, and with a gradient of expression of cholangiocyte-associated biomarkers (e.g. EpCAM) decreasing from the periphery to the center (Regenerative Clusters: see Figure 1). It is not clear in FHF whether cholangiocytes give rise to hepatocytes or vice versa. Most cells in liver tissues from patients with FHF, however, are typical cholangiocytes, so it is likely that these are the source of hepatocytes detected in the (more rarely seen) regenerative clusters.

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The term “progenitor” cells (used in tissue biology to describe the immediate progeny of stem cells) is most often used to collectively cover these proliferating cells with mixed hepatobiliary biomarkers in rats, mice, humans and fish. This may be inappropriate because it implies that such cells are generated by tissue-specific stem cells, even though such stem cells are not identifiable in the liver. Though the term “progenitor cells” does not fulfill criteria used in other tissues, it does imply a transition from one type of cell differentiation to another. Thus, the term has persisted in hepatic biology, even though it is not entirely appropriate. However, in most of the scenarios below, hepatocytes and cholangiocytes appear to function as “facultative stem cells” for each other. Thus the term “progenitor” is not entirely inappropriate. (The term “facultative stem cell”, better defined in the intestine[4], implies that normal cells function as stem cells when necessary).

We will use the term “liver progenitor cells” (LPC) to be consistent with the majority of existing literature. We should caution, however, that not all cells named LPC are necessarily the same. Cells transitioning from hepatocytes to cholangiocytes appear different than cells transitioning in the opposite direction, and the latter appear different in details from rats (oval cells) and humans (ductular hepatocytes) and zebrafish [5, 6]. The term LPC, used in a generic sense, is currently a useful term to employ until such time as the peculiarities of these cells in the different situations are better understood.

## Possible Origins of LPC

LPC might derive from preexisting hepatic cells with mixed hepatobiliary differentiation. Cholangiocytes at the end of the canals of Hering, in sites with immediate proximity to hepatocytes, have mixed expression of transcription factors[7]. LPC might also derive from pre-existing hepatic tissue-specific stem cells. As mentioned above, such cells are simply not seen under sophisticated microscopy or complete tissue dissociation. If they exist, they must be present in exceedingly small numbers. There has been no evidence provided for their existence as a standard histologic element of liver lobules.

Much discussion in the literature has focused on the possibility that LPC may derive from hepatocytes or cholangiocytes undergoing trans-differentiation. During this process, cholangiocytes and hepatocytes function as “facultative stem cells,”[4] and undergo transdifferentiation, or reprogramming, from one cell type to the other to rescue failed regeneration of hepatocytes or cholangiocytes. Several models have been designed to test these possibilities.

Cholangiocytes might become LPC to rescue hepatocytes. This model is controversial, because, until very recently[8], different results have been obtained from studies of rats and mice. In rats fed a diet containing acetylaminofluorene (AAF), which is carcinogenic in rats and causes DNA damage, levels of p21 increase, leading to hepatocyte cell cycle arrest[9]. When rats given AAF undergo partial hepatectomy, liver growth does not occur for 5–6 days. Then, rapidly proliferating cells (LPC) appear in periportal areas, resembling cholangiocytes, with markers of biliary cells and hepatocytes [10, 11]. Rats given AAF-containing diets followed by $CCl_4$-induced centrilobular necrosis have similar periportal responses [12]. A pulse of $H^3$-thymidine, administered at the time of the LPC proliferation, labeled most of the nuclei. LPC that retained sufficient $H^3$-thymidine migrated towards all

zones of the lobule, expanded in size, and became small hepatocytes. These hepatocytes, retaining the $H^3$-thymidine label of LPC, increased in size and eventually appeared throughout the lobule [10, 13, 14]. The "tagging" of the LPC with $H^3$-Thymidine did not depend on permanent genomic alterations, as in "lineage" tagging" done in mice. Critical analysis of the experimental results, however, demonstrates that the tagging was reliable and the follow-up was thorough and conclusive.

The main question in these studies was the origin of the LPC. Most of the evidence indicated that LPC derived from cholangiocytes. Prior to the emergence of LPC, expression of hepatocyte biomarkers including albumin, alpha-fetoprotein[15] and HNF4α[16] was seen in biliary ductules. Soon after their emergence as a distinct cell population, LPC expressed most biliary biomarkers (HNF1β, CK19, etc.[17]) and the above hepatocyte biomarkers. The biliary biomarkers are gradually lost as LPC expanded within the hepatic lobule and transitioned to mature hepatocytes. It should be noted that Farber et a. [18] in one publication reported that cholangiocytes did not differentiate into LPC in rats fed AAF or subjected to partial hepatectomy. However, studies from several other groups with multiple publications reported that cholangiocytes did differentiate into LPC in this model[11, 19, 20]. In other studies, administration of DAPM (a biliary-specific toxin) prior to AAF and partial hepatectomy prevented the appearance of LPC[21]. The LPC expressed EGFR, MET, FGFR1 and FGFR2.[17, 22-24]. TWEAK, a member of the tumor necrosis factor family, is a mitogen for these cells [25]. When all evidence is taken together, it appears that in rats, in response to a strong regenerative stimulus and with severely inhibited hepatocyte proliferation, some cholangiocytes express hepatocyte-associated transcription factors, proliferate as LPC, and become hepatocytes. This model satisfies the criteria for Ockham's razor (among competing hypotheses that predict equally well, the one with the fewest assumptions should be selected). There were no genetically defined lineage tags in this system; however, such an approach was pursued in the studies with mice.

Mice cannot activate AAF to carcinogenic electrophiles due to lack of a sulfotransferase and thus AAF does not generate DNA adducts that block hepatocyte proliferation[26]. AAF therefore cannot be used in such studies of mice. A porphyria-inducing diet, containing diethyl 1,4-dihydro-2,4,6-trimethyl-pyridine-3,5-dicarboxylate (DDC)[27] causes appearance of cells resembling the oval cells seen in rats. Such cells also emerge with diets causing hepatic toxicity, hepatocyte death and rapid cell turnover (choline deficient (CD) diet with ethionine (CDE), high dose galactosamine with LPS, etc.[28]). Cholangiocytes and hepatocytes can be tagged specifically and irreversibly in mice by expression of markers under control of a cell-specific promoter. Using this approach, Rodrigo-Torres et al[29] demonstrated that cells in a typical ductular reaction of human FHF expressed the cholangiocyte-specific transcription factor HNF1β. They applied this observation in mice and generated strains with hepatic cell lineages tagged based on HNF1β. In these mice there was no evidence of generation of hepatocytes from cholangiocytes, except in mice fed CDE diet, in which 1.83% of hepatocytes were "tagged". Espanol-Suner et al labeled cells using the osteopontin promoter[30] and found 2.45% of hepatocytes to derive from LPC or biliary cells. Both studies found no evidence for generation of hepatocytes from cholangiocytes (or LPC) following partial hepatectomy or $CCl_4$ injury. Schaub et al[31] tagged hepatocytes using AAV vectors that expressed Cre recombinase under the transthyretin promoter and did not

observe generation of hepatocytes from cells other than hepatocytes in mice on choline-deficient diets. Yanger et al tagged cholangiocytes by expressing a marker under the keratin 19, type I promoter, in mice placed under various conditions (including those on a DDC-containing diet); they did not observe generation of hepatocytes from tagged cholangiocytes under any of the conditions[32]. Similar results were obtained by Jors et al who tagged cholangiocytes by expressing a marker from the *Hnf1b* promoter [33].

Tarlow et al tagged SOX9-positive cells in mice, analyzed formation of organoids in culture, monitored responses of cells in mice on CDE diet or diets containing DDC, and also tracked cells transferred into *Fah*-/- mice[34]. (These mice suffer from tyrosinemia and are kept alive by administering the drug (2-[2-nitro-4-trifluoromethylbenzoyl]-1,3-cyclohexanedione (NTCB). Removal of the drug results in acute tyrosinemia, hepatocyte death and liver failure. Hepatocytes from normal, immune-compatible donors, can be transplanted and recolonize the liver of these mice. It is an excellent model to test the capacity of transplanted cells to give rise to hepatocytes.) They found less than 1% of hepatocytes derive from SOX9-positive precursors. In a subsequent study[38], these authors found a DDC-containing diet to induce generation of cholangiocytes from hepatocytes. Upon termination of the diet, these cells reverted to hepatocytes. The authors concluded that this pathway allows hepatocytes to undergo ductal metaplasia and then revert back to hepatocytes, as a mechanism of survival following severe chronic injury. The hepatocyte-derived cholangiocytes continued to express some hepatocyte-specific genes (such as HNF4, low expression of EPCAM[35]). It is not clear whether ductal cells derived from hepatocytes in this study are similar to those seen in rats with DPP4 chimeric livers[36]. Furthermore, in previous studies by the same group with *Fah*-/- mice on CDE diets, hepatocytes did not appear to be the originators of LPC, because FAH was not expressed in the LPC generated in the study[37].

In a very recent publication, Lu et al presented convincing evidence of conversion of cholangiocytes to hepatocytes when hepatocyte Mdm2 was removed[8]. $Mdm2^{flox/flox}$ mice were crossed with mice expressing AhCre and beta-napthoflavone was used to activate Cre and induce removal of Mdm2 from hepatocytes. That resulted in over-expression of p53 and p21 causing widespread hepatocyte death by apoptosis. There was a massive ductular reaction and in several months hepatic histology was restored primarily by cells that had retained Mdm2. The authors used several lineage tagging approaches as well as enhancement of the response by the LPC mitogen TWEAK to demonstrate that the source of tissue restoration was from LPC. In separate experiments they tagged cholangiocyte lineage and demonstrated that the bipotential clonal fraction of LPC derived from cholangiocytes. It is of interest that this phenomenon was finally demonstrated in mice when p21 was induced and blocked hepatocyte proliferation, similar to the rat model in which cholangiocyte conversion to LPC and hepatocytes was also seen when the AAF diet induced an upregulation of p21 in hepatocytes[9]. It is conceivable that the cholangiocyte conversion to hepatocytes via LPC is uniquely dependent on inhibition of hepatocyte proliferation due to induction of p21.

Studies with biliary organoids derived from human liver provide evidence that cholangiocytes can differentiate into hepatocytes. Huch et al isolated cholangiocytes from

human liver based on expression of EPCAM[38]. The cells were grown into organoids, induced to transdifferentiate in culture, and expressed hepatocyte-specific genes. When the human biliary organoids were transplanted into mice given retrorsine (a DNA cross-linking agent that is activated in only hepatocytes) and subjected to hepatectomy, the cholangiocyte-derived organoids colonized the livers with human hepatocytes. Cholangiocytes isolated from liver biopsies from patients with liver diseases also differentiated into hepatocytes in the organoid cultures, but still carried markers of the patients' diseases, such as globules that contain the abnormally folded ATz mutant form of α-1 antitrypsin[38]. It is important to note, however, that the transdifferentiation of cholangiocytes to hepatocytes occurred in culture—the hepatocyte phenotype detected after transplantation of the cells into mice was observed before the cells were transplanted.

There is also evidence from zebrafish studies that cholangiocytes can generate hepatocytes after the latter are severely damaged[15, 6, 39]. When hepatocytes were destroyed with metronidazole, cholangiocytes trans-differentiated to hepatocytes and rescued the liver. In one of the studies[39], similar to observations in rats[21], administration of the biliary toxin DAPM inhibited the rescue of destroyed hepatocytes by cholangiocytes.

It seems therefore fair to conclude that under most conditions of chronic toxic injury or normal liver regeneration, hepatocytes and cholangiocytes proliferate and retain their phenotype. This is strongly supported by both the rat and the mouse studies[31, 32, 34]. In extreme conditions of complete elimination of hepatocyte replication (AAF/Partial hepatectomy in rats[10, 11, 13-16, 19, 22-24, 40],metronidazole in zebrafish[6, 39], expression of p21 in mice[8]) the evidence that cholangiocyte-derived LPC give rise to cells which eventually become hepatocytes is also very strong. It is not clear, however, whether all cholangiocytes only a fraction do so [41]. Because of the findings with regenerative clusters from human liver with FHF (Figure 1), pathways by which cholangiocytes differentiate into LPC and hepatocytes should continue to be explored. They might lead to new approaches to treating human liver disease. FHF most commonly leads to death or liver transplantation. Some FHF cases however recover spontaneously[42]. LPC have been frequently detected in liver tissues from patients with FHF [43], but their significance in recovery was not assessed until recently [43]. There is also evidence that many of the hepatocyte nodules observed in patients with micro-nodular cirrhosis are derived from cholangiocytes, providing a new angle of research for liver cell biologists [44].

There is evidence from animal and human studies that hepatocytes can transdifferentiate to cholangiocytes. DPP4-negative rats were given retrorsine, a DNA crosslinking agent blocking hepatocyte proliferation[45]. When these rats received partial hepatectomies followed by injection of DPP4-positive hepatocytes, the transplanted hepatocytes colonized the host liver, resulting in a chimeric liver with predominance of donor DPP4-positive hepatocytes. Cholangiocytes are not affected by retrorsine, proliferate and remain DPP4-negative. Bile duct ligation generated biliary ductules of which 1%–2% expressed the donor hepatocyte marker (DPP4). However, when the cholangiocyte specific toxin DAPM was injected before bile duct ligation (to impair the capacity of cholangiocytes to undergo self-repair), approximately 50% of bile ductules expressed DPP4, indicating that when cholangiocytes were damaged and lost the capacity to proliferate and restore their lineage,

more cholangiocytes were derived from donor hepatocytes[36]. It is interesting that there was no evidence of formation of LPC. The immediate periportal hepatocytes transdifferentiated directly into ductal structures lined by ductular hepatocytes, and became biliary ductules[36]. This indicates that the immediate periportal hepatocytes have increased capacity to transdifferentiate to cholangiocytes, consistent with findings by Carpentier et al, that immediate periportal hepatocytes derive from remnants of the ductal plate [46]. In humans, expression of cholangiocyte-specific transcription factor HFN3β was widespread in patients with biliary obstruction or primary biliary cirrhosis[47]. In mice, Yanger et al showed that lineage-tagged hepatocytes from all areas of the lobule could transdifferentiate into cholangiocytes and implicated activation of *Notch* signaling[48] as part of this process. Yimlamai et al showed that acute inactivation of the Hippo pathway (and consequent elevation of nuclear YAP) induced dedifferentiation and activation of a ductal phenotype in hepatocytes[49]. In hepatic organoids in culture, only signals transmitted via the EGF receptor or MET (the receptor for HGF) activated transdifferentiation of hepatocytes into cholangiocytes; PI3K activation was an essential element in this process and induced only by activation of EGFR and MET[50]. Fan et al[51] and Sekiya et al[52] also provided evidence that cholangiocarcinomas can arise from hepatocytes. All these studies demonstrate that hepatocytes participate in repair and salvage of critically injured biliary epithelium in rodents and humans, and that Notch, Hippo/Yap, EGFR, HGF/MET and PI3K signaling (all of them also important for liver regeneration[53-57]) are involved in this process. A schematic illustrating the transdifferentiation relationships between cholangiocytes and hepatocytes is shown in Figure 2.

There have been reports that stellate cells can give rise to hepatocytes. Recent studies from Kordes et al[58] demonstrated that transplantation of lineage-tagged stellate cells in rats subjected to the AAF/partial hepatectomy protocol resulted in hepatocytes and cholangiocytes bearing the stellate lineage marker. On the other hand, Schwabe and coworkers using carefully controlled stellate cell specific fate-tracing could find no evidence whatsoever that epithelial cells of the liver could be traced back to stellate cells[59].

## Liver tissue maintenance in steady state

Strong evidence shown above indicates that during standard liver regeneration after hepatectomy or in conditions of selective hepatocyte or cholangiocyte damage, active proliferation of hepatic cells is involved in tissue restoration. Not much is understood however related to maintenance of hepatic tissue in steady state. BRDU or Thymidine labeling of hepatocytes in adult rodents is generally less than 0.1% and randomly distributed in the lobule. Detailed studies in mice using long term $H^3$-thymidine label immediately after birth showed extensive labeling of hepatocytes in all zones[60]. Loss of $H^3$-thymidine grains was slightly different in different lobule zones, with a slight edge in the perivenous region, suggesting a higher rate of proliferation. Other studies had shown that the immediate pericentral, glutamine-synthetase (GS) positive hepatocytes have a low rate of proliferation compared to hepatocytes in the other zones[61, 62]. Surprisingly, however, Wang et al in a recent study[63] demonstrated that lineage tagging of some pericentral GS-positive hepatocytes generated progeny of tagged hepatocytes that eventually encompassed on average 30% of the lobular area. The tagging was made based on Axin2, part of the

ubiquitin ligase complex associated with destruction of beta-catenin. Although Furuyama et al showed progressive migration of Sox9 lineage-tagged cells being the source for steady state replacement of most of the hepatocytes in all lobular zones[64], follow-up studies by Lemaigre et al did not confirm this finding [65], and it is believed that lineage tracing by Furuyama et al. was not sufficiently specific. Several studies using labeling of hepatocyte DNA in resting liver had appeared in the past, suggesting a continuous "streaming" of hepatocytes through the liver lobule[66, 67]. Other studies, however, looking into local progeny of single labeled cells did not support the concept[68]. More studies should be performed to resolve these controversies and it will be crucial to be able to reproduce any of the above findings using more than one type of methodology. Liver has many options to restore tissue damage, not all of which are understandable to us. Whatever the pathway chosen, Nature knows best.

## Conclusions

Studies of transdifferentiation between hepatic cell types have generated significant divergence of views and some controversies. At this stage, the potential of hepatocytes to rescue cholangiocytes is accepted by most groups in the field. Most of the controversy relates to the possibility of cholangiocytes giving rise to LPC and hepatocytes. On this issue, studies with mice suggest that this occurs very rarely or not at all. Recent studies, however, seem to indicate that this transdifferentiation is possible in mice and depends on inhibition of hepatocyte proliferation due to elevation of p21[8]. Findings with rats, humans and zebrafish provide evidence that in situations of severe hepatocyte depletion or near absolute inhibition of hepatocyte proliferation, cholangiocytes can generate LPC and hepatocytes. This should not be entirely surprising. Several studies from the 70s and 80s have shown the plasticity of the hepatic epithelial phenotypes. Hepatocytes in primary culture express biomarkers characteristic of cholangiocytes (gamma-glutamyl transpeptidase, alkaline phosphatase)[69]. Human cholangiocytes can differentiate into various endoderm-derived cell types, including intestinal mucosal cells, pancreatic acinar cells etc. [70-72]. Pancreatic ductules can give rise to hepatocytes[73-75]. The etiologies eliciting catastrophic conditions in cholangiocytes and hepatocytes are quite different. From an evolutionary perspective, the ability of hepatocytes and cholangiocytes to function as facultative stem cells for each other perhaps is more effective in preventing organ failure than the use of tissue-specific stem cells. Further studies of LPC could lead to new strategies for treatment of tissue damage in liver disease.

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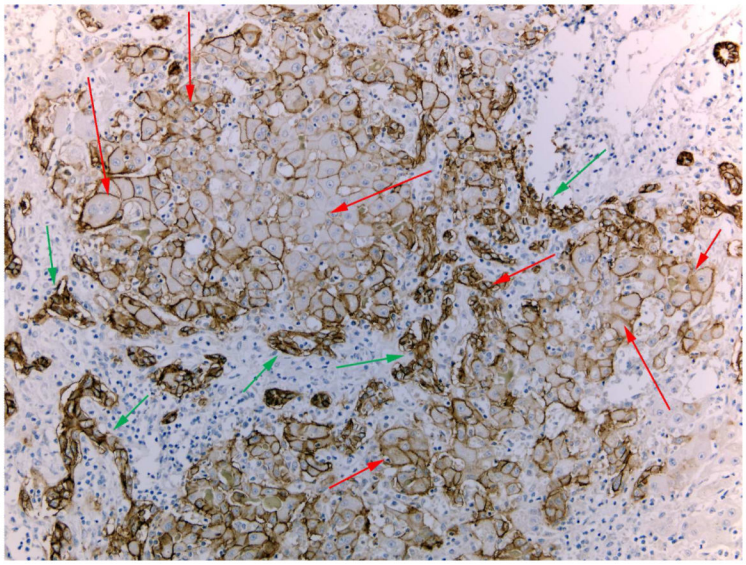

**Figure 1. Regenerative clusters of mixed phenotypes in fulminant hepatic failure**

Regenerative clusters composed of cholangiocytes (green arrows) organized in tortuous ductules surrounding and connecting with hepatocytes (red arrows). Hepatocytes express EPCAM, a marker of cholangiocytes, to various degrees. EPCAM expression decreases towards the center of the regenerative cluster. These features are frequently observed in histologic analyses of liver tissues from patients with fulminant hepatic failure with massive hepatocyte necrosis and ductular reaction.

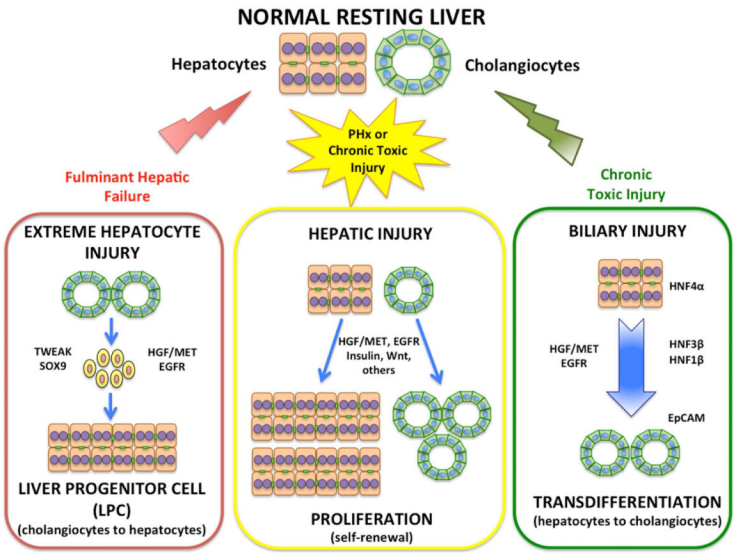


**Figure 2. Potential mechanisms of liver regeneration in relation to parenchymal cell injury**

Following partial hepatectomy (PHx), most liver cells are capable of regeneration, with hepatocytes and cholangiocytes respectively contributing to self-replication (center panel). Under extreme hepatocyte loss, such as in cases of fulminant hepatic failure or AAF exposure, cholangiocytes become progenitor cells that become hepatocytes (left panel). With biliary injury (following administration of DAPM), hepatocytes undergo transdifferentiation directly into cholangiocytes (right panel). These types of reprograming events result in phenotypic inter-conversion to replace the injured cell type, and involve a number of regenerative pathways including HGF signaling via MET, and EGFR signaling.