The role of liver progenitor cells during liver regeneration, fibrogenesis, and carcinogenesis

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Köhn-Gaone J, Gogoi-Tiwari J, Ramm GA, Olynyk JK, Tirnitz-Parker JE. The role of liver progenitor cells during liver regeneration, fibrogenesis, and carcinogenesis. Am J Physiol Gastrointest Liver Physiol 310: G143–G154, 2016. First published November 25, 2015; doi:10.1152/ajpgi.00215.2015.—The growing worldwide challenge of cirrhosis and hepatocellular carcinoma due to increasing prevalence of excessive alcohol consumption, viral hepatitis, obesity, and the metabolic syndrome has sparked interest in stem cell-like liver progenitor cells (LPCs) as potential candidates for cell therapy and tissue engineering, as an alternative approach to whole organ transplantation. However, LPCs always proliferate in chronic liver diseases with a predisposition to cancer; they have been suggested to play major roles in driving fibrosis, disease progression, and may even represent tumor-initiating cells. Hence, a greater understanding of the factors that govern their activation, communication with other hepatic cell types, and bipotential differentiation as opposed to their potential transformation is needed before their therapeutic potential can be harnessed.

The liver is known for its remarkable capacity to regenerate after acute injury, such as loss of liver mass, as observed following partial hepatectomy (PHx), or acetaminophen poisoning via compensatory hyperplasia, mediated by remaining healthy hepatocytes and only very limited progenitor cell involvement. However, chronic hepatic damage due to constant viral, toxic, or carcinogenic injury compromises the regenerative capacity of hepatocytes through induction of replicative arrest and regeneration relies on activation and differentiation of liver progenitor cells (LPCs). This review discusses the ontology of LPCs, models for isolation and characterization, and their role in chronic liver injury-associated processes involved with hepatic regeneration, fibrogenesis, and carcinogenesis.

LPCs: Where Do They Come From and Where Do They Go?

LPCs were originally identified by Kinosita (52) in rats treated with the azo dye and former food additive “Butter Yellow” and later introduced by Farber as “ovoid cells,” describing their cytologic appearance in rodents following treatment with carcinogenic agents (28). Since then, a variety of different terminologies have been used to describe this extremely heterogeneous cell population that is hardly detectable in healthy liver but is progressively activated under chronic liver injury conditions, including hepatic stem-like/progenitor cells, oval cells (mainly used in rodent models), transit-amplifying ductular cells, and they are described as a component of so-called ductular reactions (mainly used in human pathologies, detailed further below). They are defined as small cells (7–10 μm in diameter) featuring a high nuclear-to-cytoplasmic ratio, a small ovoid nucleus, and a basophilic character, and variably express both biliary and hepatocytic, as well as hematopoietic markers (see Table 1).

Their origin remains highly controversial. Since their proliferation is always first seen in periportal hepatic regions, the general view has been that they are the progeny of a yet to be identified liver-resident stem cell, residing in the Canals of Hering. These represent the biliary-hepatocytic interphase at the anatomical boundary between the terminal bile ductules of the biliary tree and the hepatocyte canalicular system. Evidence for their role as progenitor cell origin was provided by three-dimensional reconstructions in healthy adult tissue and following massive hepatic necrosis secondary to acetaminophen toxicity, demonstrating LPC proliferation located topographically in the Canals of Hering structures (123). Label...
Table 1. Adult LPC marker expression in mouse, rat, and human hepatic cell lineages

<table>
<thead>
<tr>
<th>Mouse/Rat Marker</th>
<th>LPC</th>
<th>Hepatocyte</th>
<th>Cholangiocyte</th>
<th>Model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>2-AAF/PHx, DDC, CDE, DDB1 deletion</td>
<td>23, 89, 108, 118, 128, 130, 133</td>
</tr>
<tr>
<td>AFP</td>
<td>+</td>
<td>fetal</td>
<td>–</td>
<td>2-AAF/PHx, DEN, CDE</td>
<td>25, 48, 109, 114, 128</td>
</tr>
<tr>
<td>Alb</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>CDE, 2-AAF</td>
<td>109, 125</td>
</tr>
<tr>
<td>CD13</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>2-AAF/PHx, DDC</td>
<td>90, 108, 144</td>
</tr>
<tr>
<td>CD24</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>2-AAF/PHx, DDC</td>
<td>82, 89</td>
</tr>
<tr>
<td>CD44</td>
<td>low</td>
<td>–</td>
<td>–</td>
<td>2-AAF/PHx</td>
<td>133</td>
</tr>
<tr>
<td>CD133</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>2-AAF/PHx, DDC</td>
<td>58, 101, 118</td>
</tr>
<tr>
<td>CK7</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>2-AAF/PHx</td>
<td>13, 83, 106</td>
</tr>
<tr>
<td>CK8</td>
<td>+</td>
<td>low (membranous)</td>
<td>+ (cytoplasmic)</td>
<td>2-AAF/PHx</td>
<td>36, 106, 107</td>
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<tr>
<td>CK18</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>2-AAF/PHx</td>
<td>36, 106</td>
</tr>
<tr>
<td>c-Kit</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>2-AAF/PHx, CDE, D-gal</td>
<td>33, 34, 56</td>
</tr>
<tr>
<td>CD44</td>
<td>low (intermediate state)</td>
<td>–</td>
<td>–</td>
<td>D-gal</td>
<td>58</td>
</tr>
<tr>
<td>CD133</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>2-AAF/PHx, CDE, retnorsine</td>
<td>48, 49</td>
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<tr>
<td>E-cad</td>
<td>+/high</td>
<td>–</td>
<td>+/high</td>
<td>CDE</td>
<td>128, 133, 135</td>
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<tr>
<td>EpCAM</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>2-AAF/PHx, DDB1 deletion, DDC, D-gal</td>
<td>23, 80, 108, 144</td>
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<tr>
<td>Fox1</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>DDC, CDE, BDL</td>
<td>103, 112</td>
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<tr>
<td>GGT</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>CCl4, DDC, MCDE</td>
<td>44, 88</td>
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<tr>
<td>Lgr5</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>CCl4, DDC, MCDE</td>
<td>46, 18</td>
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<tr>
<td>MIC1-1C3</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>DDC</td>
<td>17, 18</td>
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<tr>
<td>M-protein</td>
<td>+</td>
<td>fetal</td>
<td>+</td>
<td>2-AAF/PHx, CDE</td>
<td>48, 114, 122, 128</td>
</tr>
<tr>
<td>NCAM</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>DDC</td>
<td>108</td>
</tr>
<tr>
<td>OC-2</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>2-AAF, 2-AAF/CCl4, 2-AAF/PHx, CDE</td>
<td>42, 88</td>
</tr>
<tr>
<td>OPN</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>DDC, CDE</td>
<td>12, 24</td>
</tr>
<tr>
<td>OV-6</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>2-AAF, 2-AAF/CCl4, 2-AAF/PHx, CDE</td>
<td>84, 88, 142</td>
</tr>
<tr>
<td>EpCAM</td>
<td>+</td>
<td>fetal</td>
<td>–</td>
<td>CDE</td>
<td>74, 81, 114, 122</td>
</tr>
<tr>
<td>Scare</td>
<td>+</td>
<td>low</td>
<td>–</td>
<td>DDC</td>
<td>89</td>
</tr>
<tr>
<td>Sox9</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>DDC, CDE</td>
<td>12, 35</td>
</tr>
<tr>
<td>Thy1/CD90</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>2-AAF/CCl4, 2-AAF/PHx, DDC</td>
<td>88, 89</td>
</tr>
<tr>
<td>Trop2</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>DDC, CDE</td>
<td>12, 80</td>
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</tbody>
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Progenitor cell compartments in noninjured livers

<table>
<thead>
<tr>
<th>Human Marker</th>
<th>LPC</th>
<th>Hepatocyte</th>
<th>Cholangiocyte</th>
<th>Disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Acetaminophen-induced necrosis, acute necrotizing hepatitis</td>
<td>116, 123</td>
</tr>
<tr>
<td>CD90</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>HCV</td>
<td>66, 136</td>
</tr>
<tr>
<td>CD109</td>
<td>+</td>
<td>low</td>
<td>low</td>
<td>Acute necrotizing hepatitis, HBV, HCV, primary biliary cirrhosis</td>
<td>81, 116</td>
</tr>
<tr>
<td>CD133</td>
<td>+</td>
<td>–</td>
<td>(+)</td>
<td>Alcoholic hepatitis, acute necrotizing hepatitis, HCV, pNASH, primary biliary cirrhosis</td>
<td>78, 105, 116, 136</td>
</tr>
<tr>
<td>CK7</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Alcoholic hepatitis, acute necrotizing hepatitis, HCV, pNASH, primary biliary cirrhosis</td>
<td>66, 133</td>
</tr>
<tr>
<td>CD133</td>
<td>+</td>
<td>low</td>
<td>+</td>
<td>Acetaminophen-induced necrosis, alcoholic liver disease, genetic hemochromatosis, HBV, HCV</td>
<td>30, 66, 74, 123, 136</td>
</tr>
<tr>
<td>c-Kit</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Acetaminophen-induced necrosis, acute liver failure, extrahepatic biliary atresia (cirrhosis)</td>
<td>7, 66, 123</td>
</tr>
<tr>
<td>EpCAM</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Alcoholic hepatitis, HBV, HCV</td>
<td>91, 105, 136</td>
</tr>
<tr>
<td>Jagged 1</td>
<td>+</td>
<td>–</td>
<td>(+)</td>
<td>Acute necrotising hepatitis, HCV, primary biliary cirrhosis</td>
<td>116</td>
</tr>
<tr>
<td>M-protein</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Genetic hemochromatosis, alcoholic liver disease, HCV</td>
<td>74</td>
</tr>
<tr>
<td>NCAM</td>
<td>+</td>
<td>–</td>
<td>(+)</td>
<td>Acute necrotising hepatitis, HBV, HCV, primary biliary cirrhosis</td>
<td>91, 116</td>
</tr>
<tr>
<td>OV-6</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>HCV, hepatitis C virus</td>
<td>66</td>
</tr>
</tbody>
</table>

Note: +, positive; –, negative; (+), occasionally expressed; “low” denotes weakly expressed, while “high” denotes highly expressed. 2-AAF, 2-acetylaminofluorene; PHx, partial hepatectomy; CDE, choline-deficient, ethionine-supplemented; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DDB1, damaged DNA binding protein 1; DEN, diethylnitrosamine; diethylnitrosamine; D-gal, D-galactosamine; t-gal, t-galactosamine; MCDE, methionine CDE; BDL, bile duct ligation; HBV, hepatitis B virus; HCV, hepatitis C virus; HCV, hepatitis C virus.
retention assays on the basis of bromodeoxyuridine incorporation equally identified the Canals of Hering and, in addition, intraductal cholangiocytes, periductal “null cells” (lacking expression of hepatobiliary markers) and the first hepatocytes of the hepatic acinus as potential functional stem cell niche locations (61). A recent study using a hepatocyte-chimeric lineage tracing strategy by extensive RNA sequencing and ultrastructural analysis suggested that mature, fully differentiated hepatocytes can contribute to the LPC pool by undergoing reversible metaplasia to a biliary-like progenitor state during 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-induced liver injury (120). Kordes et al. (59) demonstrated through hepatic stellate cell (HSC) transplantation experiments in two chronic liver injury models that HSCs were able to contribute to the regeneration process by bipotential differentiation into hepatocytes and cholangiocytes and may, therefore, be another candidate cell contributing to the LPC population under certain experimental conditions (59). Another lineage tracing study that followed the fate and regenerative capacity of hepatocyte nuclear factor 1α-positive biliary cells demonstrated that this population did not contribute to the generation of new hepatocytes in healthy liver or after acute injury; however, they can differentiate into hepatocytes in certain chronic injury regimes, such as shown using the choline-deficient-ethionine supplemented (CDE) dietary mouse model (98), via an intermediate LPC phenotype.

LPCs, defined by their immature stem cell-like marker expression profile, have been shown to differentiate bipotentially into biliary cells and hepatocytes (32, 103, 112, 118), and in some models, they have demonstrated multipotentiality by differentiation into pancreatic and intestinal lineages (64, 121, 143). However, the actual regenerative capacity of LPCs has been highly debated. A recent genetic lineage tracing study used yellow fluorescent protein-osteopontin-marked cholangiocytes and LPCs to study their role during (J) PHx, 2) carbon tetrachloride (CCL4)-induced acute injury, or 3) chronic injury following administration of either a CDE or DDC diet (24). This study revealed that osteopontin-expressing LPCs only demonstrated a significant contribution to hepatocellular regeneration in the CDE model of chronic liver injury, highlighting injury context specificity. Further evidence that LPCs might play an important role during liver mass reconstitution comes from experiments using a reporter mouse for the LPC marker Fkh1, demonstrating that Fkh1+ LPCs and their descendants are critically required for hepatocyte generation during recovery from CDE-induced injury (111). In contrast, Tarlow et al. (119) reported conflicting results by showing that LPCs labeled via expression of SRY-related HMG box transcription factor 9 (Sox9) only rarely (<1%) produced new hepatocytes in the CDE model (119). However, it should be noted that this study used supplementation with only 0.1% ethionine in the drinking water, as opposed to the more common concentration of 0.15% also used by Shin et al. (111), highlighting the fact that observed differences might be the result of differences in the severity of induced liver injury. A very recent study defined a population of highly expandable, clonogenic cells negatively sorted for hematopoietic (cluster of differentiation, CD, Ref. 45), endothelial (CD31), and erythroid (Ter-119) markers and with the expression profile epithelial cell adhesion molecule (EPCAM)+CD24−CD133+, as capable of efficiently repopulating the liver bipotentially following repeated rounds of induced hepatocyte senescence in mice lacking functional E3 ubiquitin-protein ligase Mdm2 in hepatocytes (76). Observed discrepancies and contradicting results between studies probably stem from a lack of uniformity with regard to the animal model used, the degree of evoked liver injury, and differences in the underlying injury and repair mechanisms. However, taken together, these data suggest that the adult liver may retain a certain level of plasticity and the ability to draw on multiple distinct cellular sources for LPC generation and subsequent epithelial tissue regeneration (see Fig. 1).

Animal Models to Study LPCs

Rodent models are commonly used to study chronic liver disease and associated processes as rats and mice generally display high levels of genetic similarity to humans, while the ever-increasing availability of genetically manipulated mice makes them powerful tools for the mechanistic evaluation of disease development and progression. They are easy to handle due to their size, easy to breed in captivity, and have a relatively short gestational period and life span, allowing cost-effective analyses. Nevertheless, no model can be a complete replica of the corresponding human liver disease due to differences in the immune system and metabolic rates during tissue homeostasis, as well as the metabolic response to injury stimuli (70). Importantly, remarkable heterogeneity with respect to phenotypic disparity has been displayed by LPCs in different models, warranting careful selection and interpretation. Most of the models described below were initially developed to follow hepatocellular carcinoma development and only later adapted to study induction and subsequent biology of LPCs.

d-Galactosamine model. The noncarcinogenic agent d-galactosamine has been routinely used to induce LPCs in rats. It is metabolized by centrilobular hepatocytes, where it blocks RNA synthesis, leading to an inhibition of protein synthesis by trapping uridine nucleotides and uridine diphosphate glucose, and subsequently to the development of necrosis (29). Activation of LPC-like nonparenchymal cells occurs in the perportal area due to impaired hepatocyte proliferation within 48 h of administration of a single dose of d-galactosamine (70 mg/100 g body wt), followed by expansion into the parenchyma, forming a network of cells expressing γ-glutamyl transpeptidase (14). d-Galactosamine was shown to induce the proliferation of LPCs and small hepatocytes (only up to 16 μm in diameter) that are positive for the fetal form of α-fetoprotein (AFP), which importantly does not seem to be induced in mouse models featuring LPC proliferation (65).

Solt-Farber model and the 2-AAF/PHx regimen. This model, which is commonly used in rats and only rarely in mice, is composed of three stages of disease/LPC induction: 1) injection of the ethylating hepatocarcinogen diethylnitrosamine (DEN), which acts as a disease initiator, 2) administration of 0.02% 2-acetylaminofluorene (2-AAF) 2 wk later, and 3) partial hepatectomy (PHx), as a growth stimulus, 1 wk into 2-AAF feeding (115). This regimen is frequently modified by omission of the DEN initiation step, and 2-AAF is administered 4 days prior and after PHx. Both models induce proliferation of ductular or periductular LPCs, which accelerates when 2-AAF feeding is terminated, indicating that not only
hepatocytes but also LPCs are growth-inhibited by 2-AAF, although to a lesser extent. LPCs tend to differentiate more efficiently into hepatocytes at low 2-AAF doses, whereas they undergo apoptosis at higher dosages (3). As a consequence, the rate of hepatocytic LPC differentiation can be controlled through variation of the 2-AAF dose (84).

**DDC diet model.** DDC is a potent xenobiotic hepatotoxin that stimulates robust biliary and LPC proliferation, while inducing mitochondrial stress, hepatocyte ballooning, apoptosis, the formation of cytoplasmic aggregates termed Mallory-Denk bodies that resemble hepatocyte inclusion bodies observed in human alcoholic and nonalcoholic steatohepatitis, and hepatomegaly (94, 145). Feeding of 0.1% DDC in chow leads to a ductular reaction involving cytokeratin (CK) 19+ cells and significantly increased biliary secretion within the 1st wk, which is followed by segmental bile duct obstructions through deposition of porphyrin pigment plugs in the lumina of small bile ducts—a distinct histological feature of this cholestatic liver injury model (see Fig. 2, middle). At later disease stages, activated periductal myofibroblasts cause progressive biliary liver fibrosis with portal-portal septa, resembling human sclerosing cholangitis (31). Interestingly, this model only results in activation of LPC proliferation in mice and not in rats (48), and in liver phenotype (in particular the extent of hepatomegaly and pigment deposition induced by DDC feeding) largely depends on the genetic background of the mouse strain used in the model (41).

**CDE diet model.** Dietary deficiency of the lipotrope choline combined with 0.05 or 0.1% of the methionine-agonist and hepatocarcinogen ethionine was initially developed as a chronic liver injury model in rats, where it produces alterations in phospholipid metabolism, fatty liver, and substantial numbers of proliferating AFP+ LPCs by 3 wk (113). Because of its high murine morbidity and mortality, an alternative protocol was later developed for use in mice that involved feeding of a choline-deficient diet with separate administration of 0.15% of ethionine dissolved in the drinking water (1). The CDE diet reliably induces steatosis, an inflammatory response, proliferation of hepatocytic and biliary LPCs (see Fig. 2, left), portal fibrosis, and, eventually, hepatocellular carcinoma after 10–12 mo (56, 128).

**TAA supplementation model.** Prolonged administration of thioacetamide (TAA), either via intraperitoneal injection at concentrations of 150–200 mg/kg body wt 3 times a week, or given orally in the drinking water at 200–300 mg/l, is a well-established rodent model of hepatotoxicity and cirrhosis, akin to human cirrhosis (69). TAA is a thiono-sulfur-containing compound that is converted by microsomal flavin-adenine dinucleotide-containing monooxygenase, as well as the P-450 cytochrome enzyme CYP2E1 through reduction of dioxygen to superoxide anion and subsequent catalysis to hydrogen peroxide, which is responsible for the observed oxidative stress-induced liver injury, lipid peroxidation, and centrilobular necrosis (73). TAA administration has been used to induce chronic inflammation, LPC proliferation (see Fig. 2, right), fibrosis, cirrhosis, cholangiocarcinoma (CCA), as well as hepatocellular carcinoma (HCC) (9, 104).

**Identification and Isolation Strategies for LPCs**

Primary cell culture is of considerable value for LPC research, as these cells most closely mimic in vivo status, while facilitating the study and direct manipulation of cell proliferation, lineage commitment, and differentiation. Since LPCs are hardly detectable in healthy liver, most isolation procedures involve an induction protocol, followed by a series of standard isolation steps, including tissue perfusion, digestion and fluorescence-activated cell sorting (FACS) or cell centrifugation and fractionation for further purification.
To establish a mixed culture containing a variety of heterogeneous LPC phenotypes, centrifugal elutriation based on cell size and density can be used to separate LPC populations from other liver cell fractions, followed by growth in LPC-promoting culture conditions (128). However, the resultant LPC subpopulation, as well as cell quantity, viability, and features of LPCs maintained in culture, very much depends on the method of isolation, the surface coating of the culture dish, and the combination and concentration of growth factors in the culture medium. These factors vary considerably among laboratories, making it difficult to interpret in vitro results obtained by different groups, and it is clear that reliable, standardized protocols are urgently required.

Importantly, the term LPC primarily describes a cell’s bilineage marker expression and ability to differentiate into cholangiocytes and hepatocytes in vitro or in vivo and does not describe a specific cell origin, cell ontogenesis, or predefined lineage fate. In addition, LPCs may continuously change their phenotype, according to their transient proliferation and differentiation status. Consequently, the LPC compartment is composed of heterogeneous cell populations, including immature, intermediate, and more mature or differentiated phenotypes, and, so far, no marker is available that is specific for LPCs and able to discriminate between LPCs and other cell types sharing certain markers, such as cholangiocytes, hepatocytes, and hematopoietic stem cells (see Table 1 for published LPC expression profiles). Thus, a combination of different markers is necessary to identify and FACS-isolate specific LPCs. Several markers have been linked to populations of clonogenic bipotential LPCs, including the MIC1-1C3 antigen, CD24, CD133, EpCAM, Fox11, and neighbor of punc E11 protein (Nope) (18, 51, 76, 80, 112, 118). The transmembrane glycoprotein trophoblast antigen 2 (Trop2), which has been suggested as a regulator of EpCAM-induced cell signaling, was shown to distinguish between activated proliferative LPCs during DDC-induced chronic liver injury and normal biliary cells in healthy liver (80), indicating its potential value as a specific LPC marker. However, its exclusive expression profile has not been confirmed in other experimental models. Very recently, Huch et al. (47) demonstrated that single cells positive for leucinerich repeat-containing G protein-coupled receptor 5 (also known as Lgr5) can be expanded as epithelial three-dimensional organoids in vitro and can be hepatocyte-differentiated in vitro and form new hepatocytes upon transplantation into CCl4-retrorsine-treated mice.

**LPCs in Human Chronic Liver Disease**

In contrast to the dogma based on rodent experiments that LPCs are only activated in case of massive hepatocyte loss or hepatocyte replicative arrest, in humans, LPCs are activated in the majority of liver diseases, even in minimal degrees of liver damage. In rodents and humans, LPCs proliferate either as single cells, strings of cells, or as part of ductular reactions in the portal-parenchymal interphase. The latter are composed of a diversity of cellular components, including activated or reactive biliary epithelial cells and LPCs—providing the ductular component of the name—as well as mesenchymal, vascular, neural, hematopoietic, and inflammatory cells. These cellular changes go hand-in-hand with extracellular matrix modifications and depositions, and there is an enormous range of patterns seen clinically, depending on the etiology, severity of underlying stimuli, and the respective disease progression state (for reviews, see Refs. 26, 37, 138). The surface glycoprotein neural cell adhesion molecule (NCAM) regulates cell migration and differentiation through cell-cell and cell-matrix interactions. During liver regeneration, polysialic acid modifies NCAM expressed by ductular reactions and LPCs and weakens the interactions with other hepatic cells and the surrounding matrix. These modifications facilitate cell migration from the...
periportal niche to sites of damage, as demonstrated in experimental liver injury induced by CDE or DDC treatment (131). A study by Lunz et al. (77) in a mouse model of decompensated biliary cirrhosis demonstrated that ductular reactions and LPC proliferation are induced when mitochondria-rich hepatocytes are targeted by oxidative stress and undergo replicative arrest through upregulation of the cyclin-dependent kinase inhibitor p21. Oxidative stress plays a major role during alcoholic liver disease and nonalcoholic fatty liver disease (NAFLD), which along with chronic hepatitis represent the most prevalent risk factors for the development of hepatocellular carcinoma in the Western world. All of these conditions feature ductular reactions and LPC proliferation, and the magnitude of this response directly correlates with the progression of fibrosis and the severity of the underlying disease (13, 74, 100). In addition, continuous iron loading of hepatocytes leading to impaired hepatocyte replication in hereditary hemochromatosis has been linked to LPC proliferation and the ductular reaction, with both the presence of the ductular reaction and portal inflammation strongly associated with hepatic fibrosis progression (74, 126, 139). A recent retrospective cohort study on hepatitis C virus (HCV)-infected patients demonstrated that inflammatory, fibrogenic, and LPC-associated responses are intricately linked and coregulated during disease progression, as well as during HCV recurrence after orthotopic liver transplantation (93).

LPCs and Hepatic Fibrosis

In chronic liver disease, mortality and morbidity are the results of uncontrolled and inappropriate hepatic wound healing and regeneration, which can ultimately lead to cirrhosis and hepatocellular carcinoma. Wound healing is facilitated by the activation and transdifferentiation of HSCs and portal fibroblasts, into collagen-producing myofibroblasts. This process involves an inflammatory phase, extracellular matrix remodeling and subsequent collagen deposition by HSCs. If liver injury is halted, matrix is resorbed and normal liver architecture restored. While HSCs produce the collagen responsible for fibrosis, they are transformed from a quiescent state to an activated profibrogenic myofibroblast by growth factors and cytokines produced by other resident and infiltrating cells in response to tissue injury (for review, see Refs. 6, 96). A variety of studies have proposed that HSCs are influenced by their hepatic paracrine environment (22, 127, 138), mediated by the local production of soluble mediators, as well as by direct cell-cell contact with LPCs, inflammatory cells, and other parenchymal cells (97, 102, 129). HSC activation is generally

Fig. 3. The injury and regeneration niche in CDE- and TAA-treated mouse liver. Immunofluorescent labeling of LPCs (panCK, green), activated myofibroblasts (α-smooth muscle actin, αSMA, red) and inflammatory cells (CD45, white) in 1-wk CDE- and 6-wk TAA-treated mouse liver illustrates the close spatial relationship of these cell populations that form a niche facilitating cellular crosstalk. PV, portal vein; CV central vein.
associated with the inflammatory, as well as the LPC response in both a spatial and temporal manner (see Fig. 3). BALB/c mice lacking T helper type 1 immune signaling show significantly reduced LPC proliferation and fibrosis in response to a CDE diet, compared with wild-type C57BL/6 animals (53). Furthermore, mitotic stimulation of the CDE-induced LPC response through interferon γ supplementation equally leads to an acceleration of HSC activation and fibrosis (54). As discussed earlier, the ductular reaction has been correlated with fibrosis severity in several human liver diseases, but the underlying cellular and molecular mechanisms are poorly understood.

Patsenker et al. (86) demonstrated that integrin αβ6, a local activator of the profibrotic protein transforming growth factor β 1 (TGFβ1), is induced in CK19+ proliferating bile duct epithelial cells (and possibly LPCs) in bile duct-ligated rats and that its inhibition significantly impedes fibrosis progression. These findings were recently extended by showing that genetic or pharmacological inactivation of either integrin αβ6 or TGF-β markedly inhibits the LPC response in two mouse models of sclerosing cholangitis and, thus, diminishes liver fibrosis and tumorogenesis (87). In addition, it has been shown that connective tissue growth factor, expressed by ductular reactions and LPCs, can function together with integrin αβ6 to activate TGF-β1 during chronic liver injury and augment biliary fibrosis (90). Some studies have proposed potential HSC-derived mediators of LPC biliary fate, including Notch signaling via jagged1 (8, 10). Other reports suggest a role for direct cell-cell contact between LPCs and HSCs in regulating HSC activation, inflammation, and fibrogenesis (102, 127). Lymphotoxin-β (LTβ), is a cell surface-bound member of the tumor necrosis factor (TNF) superfAMILY involved in both autocrine and paracrine proinflammatory signaling to adjacent cells expressing the LTβ receptor (LTβR), which, once activated, initiates NFκB-mediated downstream signaling. Increased LTβ expression has been demonstrated in chronic liver injury induced by bile duct ligation (62) and the CDE diet (2, 55) in rodents, as well as in human HCV (75), with a significant correlation between hepatic LTβ mRNA and hepatic fibrosis. In CDE-treated LTβR−/− mice, there is a decrease in LPC and HSC numbers, as well as fibrosis (2, 102). Evidence has been provided supporting the potential cross-talk between LTβ+ LPCs and LTβR+ HSCs (102). In this study, LTβ induced the expression of chemotaxis-associated factors intercellular adhesion molecule 1 and regulated upon activation, normal T cell expressed and secreted (or RANTES) in HSCs, which was proposed to play a role in mediating recruitment of LPCs, HSCs, and leukocytes required for wound healing and regeneration during liver injury. Thus, LTβR signaling via cellular cross-talk may act as one of the coordinating pathways controlling fibrogenesis.

The question remains, however, which cell type is activated first and successively regulates the biology of the other? Are LPCs absolutely required for HSC activation and fibrosis, and what role do HSCs play in LPC-driven hepatic regeneration? In chronic liver injury, LPCs and a subpopulation of activated HSCs have been shown to demonstrate inducible expression of fibroblast growth factor-inducible 14 (Fn14) (129), the receptor for the TNF family member TNF-like weak inducer of apoptosis (TWEAK), suggesting that TWEAK/Fn14 signaling may regulate both LPC proliferation and fibrogenesis. While TWEAK is a recognized LPC mitogen, little is known of its effects on HSC biology. Two recent studies have investigated the role of TWEAK/Fn14 on fibrogenesis. Tirnitz-Parker et al. (129) demonstrated a potential link between TWEAK-regulated LPC and fibrogenic responses in Fn14-deficient mice subjected to a CDE diet, showing decreased LPC proliferation in addition to reduced tissue inhibitor of metalloproteinases 1 and 2 mRNA expression and collagen deposition. Kuramitsu et al. (60) subjected mice undergoing 70% PHx to the administration of recombinant TWEAK, which resulted in the expansion of A6+ duct-like structures and LPCs, as well as upregulated transcript levels of profibrogenic mediators. Conversely, pharmacological blocking of TWEAK signaling produced the opposite effect, again suggesting that the LPC compartment contributes functionally to hepatic wound healing by amplification of the profibrogenic response (60). However, the underlying mechanism remained elusive. Indeed, it is possible that fibrosis inhibition following TWEAK pathway manipulation could mean that either TWEAK acts directly on HSCs, or TWEAK-induced LPC expansion may indirectly control fibrogenesis via LPC/HSC cross-talk, i.e., via Notch or LTβ signaling (127). Evidence from other organ systems suggests that TWEAK can directly activate myofibroblast production of collagen (16, 79), although similar evidence in liver disease is still lacking.

There remains controversy as to the chronological events leading to LPC-HSC interplay in driving fibrosis. Van Hul et al. (135) demonstrated that matrix is deposited early in the CDE model, prior to evidence of a significantly increased ductular reaction, which implies HSC activation may occur at least, in part, independently of LPC expansion in early liver disease to establish the required niche for the LPC response. Others believe that LPC expansion and the ductular reaction are prerequisites and key drivers of periportal fibrosis (13). However, these opposing patterns are not mutually exclusive, and the order of events may depend on the injury source, the hepatic target area, and the induced signaling pathways. Williams et al. (138) suggested that both the cellular heterogeneity and polarity of the ductular reaction allow for the promotion of different responses with regard to LPC biology and collagen deposition. Despite this controversy of cause and effect, it is clear that interactions between LPCs and components of the extracellular matrix are critical for wound healing, fibrosis, and regeneration, and, in particular, the regulation of LPC fate. Hepatocytic differentiation of LPCs in vivo has been associated with downregulation of the laminin-receptor integrin α6 and subsequent loss of contact with this basement membrane component (84). In vitro experiments have further confirmed that exposure to laminin keeps LPCs in a proliferative and rather biliary state, whereas fibronectin promotes differentiation toward a hepatocyte phenotype (72).

**LPCs and Carcinogenesis**

Liver cancer ranks among the most common solid cancers worldwide and leads to cancer-related death in high frequencies, while its global incidence and disease burden are steadily increasing. HCC embodies its major subtype, accounting for up to 90% of all liver cancers, with major risk factors being represented by chronic HBV and HCV infection, continuous excessive alcohol consumption, NAFLD and other metabolic
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disorders (71). A study by Paradis and colleagues (85) established that HCC development in patients with metabolic syndrome as the only risk factor may develop from malignant transformation of preexisting liver cell adenomas in the absence of significant liver fibrosis and cirrhosis. Because of the increasing prevalence of obesity and Type 2 diabetes worldwide, which significantly predispose patients to the development of metabolic fatty liver disorders, NAFLD has, therefore, been of particular concern. Currently, surgical resection, radiofrequency ablation, orthotopic liver transplantation, transcatheter arterial chemoembolization, and administration of the small-molecule tyrosine kinase inhibitor sorafenib are the treatments of choice for HCC (71). The second most common primary liver cancer, CCA, accounts for 10–25% of primary hepatobiliary malignancies worldwide, with the greatest incidence being recorded in Southeast Asian regions, due to higher prevalence of risk factors, such as parasitic infections with the hepatobiliary flukes Opisthorchis viverrini and Clonorchis sinensis, as well as hepatolithiasis (gall stones). In the Western world, the most common CCA risk factor is primary sclerosing cholangitis, with other less-established risk factors, including inflammatory bowel disease, HBV and HCV infection, cirrhosis, chronic alcohol consumption, diabetes, and obesity (132). While the global CCA incidence has been rising rapidly, this malignancy remains untreatable due to its multifocal nature and chemoresistant profile, signifying a very poor prognosis and survival rate of only 5–10% at 5 yr postdiagnosis (110).

Cancer, in general, is caused by sequential gene mutations leading to either sequence alterations or changes in the epigenetic signature of genes, including oncogenes and tumor suppressor genes (39, 40). The most affected genes play key roles in cell cycle control mechanisms, cell proliferation, self-renewal, and differentiation. Both HCC and CCA develop from focal precursor lesions, reflecting the multistep process of hepatic carcinogenesis (67).

Considering cell transformation is caused by accumulated gene mutations, long-living cells, such as stem cells, and highly proliferating cells, such as transit-amplifying progenitor cells, are likely targets for transformation. In addition to LPCs, hepatocytes represent the other major target population for cell transformation in HCC, a hypothesis that is based on the dedifferentiation theory vs. the maturation arrest theory (99). According to the dedifferentiation theory, hepatocytes undergo clonal proliferation during carcinogenesis, dedifferentiate, and gain a high proliferative capacity due to a newly acquired immature phenotype (11). Fan et al. (27) demonstrated Notch-mediated reprogramming of fully differentiated hepatocytes into CCA precursors via atypical biliary cells. In addition, Dubois-Pot-Schneider et al. (19) “retrodifferentiated” hepatocyte-like cells, derived from HCV-induced HCC, into bipotential LPCs through crosstalk of TGFβ1, TNF, and interleukin 6, proposing that the proinflammatory microenvironment frequently associated with most chronic liver diseases may trigger this pathogenic mechanism.

In contrast, the maturation arrest theory is based on a combination of proliferation and blocked ontogeny in progenitor cells (92). In the case of malignant LPC transformation during carcinogenesis, terminal differentiation is suppressed, and an accumulation of maturation-arrested cells occurs due to a lack of apoptosis, making them prone to genetic alterations. Many laboratories worldwide support a progenitor-product relationship between LPCs and HCCs. Phenotypical tumor features give a hint for the cell of origin in HCCs, as most tumors still share characteristics with their precursor cell. Several studies based on immunohistochemical analysis of HCCs detected the expression of LPC markers such as CK7, CK19, oval cell marker (OV)-6 and EpCAM (43, 68), with CK19 positivity denoting a particularly poor prognosis for HCC patients (21, 99). A prospective study of 242 HCC samples including resection, as well as biopsy material, confirmed the prognostic value of CK19 in a Caucasian cohort, regardless of the underlying etiology, and revealed that HCC cells with a CK19+ LPC-like phenotype featured higher invasive or metastatic capacity and chemoresistant properties than CK19− counterparts (38). Consistent with data demonstrating that proliferating, immature LPCs are generally surrounded by the glycoprotein laminin (72), Govaere et al. (38) also showed a strong correlation of cytoplasmic laminin and CK19 expression in more aggressive HCCs, suggesting that laminin secretion by CK19+ tumor cells might represent an effective autocrine mechanism to maintain stemness. On the basis of immunohistochemical and microarray studies using several HCCs, different subtypes can be clustered according to their expression profiles (4). Yamashita et al. (140) classified HCCs based on their expression levels of EpCAM and AFP and proposed that different stages of hepatic cell lineages may be involved in the transformation process: 1) hepatic stem cell-like HCC, 2) bile duct epithelium-like HCC, 3) hepatocytic LPC-like HCC, and 4) hepatocyte-like HCC, where 1) and 3) were associated with a particularly poor prognosis. They further demonstrated that EpCAM+ HCCs downregulate hepatocyte-specific genes, while upregulating the Wnt/β-catenin pathway, which has been implicated in the maintenance of stemness and stem cell self-renewal and was demonstrated to represent a major regulator of the LPC response in rodents (5).

Immunohistochemical phenotyping of cirrhotic human livers revealed that at least half of the earliest premalignant precursor lesions or small dysplastic foci consist of immature LPC phenotypes and intermediate hepatocytes, being consistent with a progenitor cell origin (68). Furthermore, studies targeting LPC proliferation in chronically injured livers correlated LPC inhibition with reduced tumor development (15, 56, 57, 63), potentially linking LPC activation and proliferation with HCC development. Importantly, various studies have demonstrated that only a few mutations are necessary for the transformation of LPCs. For instance, the loss of the tumor suppressor gene p53 enables LPCs to immortalize in culture and to form poorly differentiated HCCs after transplantation into nude mice (20, 118). Finally, the existence of tumors of a combined hepatocellular/cholangiocarcinoma (HCC-CCA) phenotype with characteristics of both hepatocytes and cholangiocytes further suggests the bipotential LPC as a potential tumor-initiating cell (124). Several LPC markers, including CD133 and EpCAM, have been used to isolate cells with cancer stem cell features, which are thought to dictate tumor aggressiveness and to be responsible for liver tumor recurrence, metastasis, and chemoresistance (141). The Notch signaling pathway, originally described as a key parameter for biliary lineage specification during liver development and LPC-mediated regeneration, has been suggested as a cancer stem cell regulator during hepatocarcinogenesis (117). Villanueva et al. (137) demonstrated through a comparative, functional
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