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# Liver Progenitor Cells Yield Functional Hepatocytes in Response to Chronic Liver Injury in Mice

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**BACKGROUND & AIMS:** Self-renewal of mature hepatocytes promotes homeostasis and regeneration of adult liver. However, recent studies have indicated that liver progenitor cells (LPC) could give rise to hepatic epithelial cells during normal turnover of the liver and after acute injury. We investigated the capacity of LPC to differentiate into hepatocytes in vivo and contribute to liver regeneration. **METHODS:** We performed lineage tracing experiments, using mice that express tamoxifen-inducible Cre recombinase under control of *osteopontin* regulatory region crossed with yelow fluorescent protein reporter mice, to follow the fate of LPC and biliary cells. Adult mice received partial (two-thirds) hepatectomy, acute or chronic administration of carbon tetrachloride ($CCl_4$), choline-deficient diet supplemented with ethionine, or 3,5-diethoxycarbonyl-1,4-dihydrocollidine diet. **RESULTS:** LPC and/or biliary cells generated 0.78% and 2.45% of hepatocytes during and upon recovery of mice from liver injury, respectively. Repopulation efficiency by LPC and/or biliary cells increased when extracellular matrix and laminin deposition were reduced. The newly formed hepatocytes integrated into hepatic cords, formed biliary canaliculi, expressed hepato-specific enzymes, accumulated glycogen, and proliferated in response to partial hepatectomy, as neighboring native hepatocytes. By contrast, LPC did not contribute to hepatocyte regeneration during normal liver homeostasis, in response to surgical or toxic loss of liver mass, during chronic liver injury ($CCl_4$-induced), or during ductular reactions. **CONCLUSIONS: LPC or biliary cells terminally differentiate into functional hepatocytes in mice with liver injury.**

*Keywords:* Liver Disease; Differentiation; Mouse Model; Liver Regeneration.

BASIC AND TRANSLATIONAL LIVER

The liver has a remarkable capacity for regeneration.[1] Self-renewal of hepatocytes is the main mechanism responsible for liver mass homeostasis and for liver regeneration after acute (moderate) liver injury and reduction of liver mass.[2] However, in conditions of chronic liver injury or submassive liver cell loss, such capacity for self-renewal is overwhelmed, exhausted, or impaired, leading to liver failure or insufficiency. In those conditions, liver progenitor cells (LPC), which are dormant and found in periportal location in a healthy liver, actively proliferate, and yield transit-amplifying cells (or oval cells). This reaction is known as ductular reaction in human beings or oval cell proliferation in rodents.[3,4]

A highly debated question is whether LPC contribute to the maintenance of liver mass homeostasis in the healthy and damaged liver. The streaming liver hypothesis[5] recently revived by the observations by Alison's group postulates that stem/progenitor cells replenish the liver to maintain its homeostasis.[6] Moreover, Furuyama et al[7] reported that liver SRY-related HMG box transcription factor 9 $(SOX9)^+$ progenitor cells are the predominant source of hepatocytes in mouse liver homeostasis and afford near-complete turnover of hepatocyte mass within 6 months. This work also suggests that LPC contribute significantly to liver mass recovery after partial hepatectomy or acute injury induced by carbon tetrachloride. More recent lineage tracing studies from our group and others, rather show that hepatocytes are the main cell contributing to liver maintenance under normal conditions, and that participation of non-hepatocyte cells is negligible.[8–11]

Ductular reactions are encountered in virtually all human liver disorders in which there is organ-wide chronic liver damage and cell loss. They are formed by a proliferative transit-amplifying population derived from hepatobiliary progenitor cells. Phenotypically, cells in ductular reactions are immature biliary-like cells with a large nuclear to cytoplasm ratio, expressing biliary markers such as SOX9 and keratin 19 (K19)[12] forming (pseudo-) ductular structures within or around the portal mesenchyma or strings of less-differentiated cells invading the liver parenchyma. Several publications support that ductular reactions are a source of hepatocyte restoration in the chronically injured adult human liver. Those are based on identification of cytochrome c–negative nodules of regen-

*Abbreviations used in this paper:* $\alpha$-SMA, $\alpha$-smooth muscle actin; CDE, choline-deficient ethionine-supplemented; K19, keratin 19; $iCreER^{T2}$, codon-improved cyclization recombinase estrogen receptor ligand binding domain variant T2; CTGF, connective tissue growth factor; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; ECM, extracellular matrix; HNF4$\alpha$, hepatocyte nuclear factor 4$\alpha$; LPC, liver progenitor cells; OPN, osteopontin; PH, partial hepatectomy; SOX9, SRY-related HMG box transcription factor 9; TGF$\beta$1, transforming growth factor $\beta$1; YFP, yellow fluorescent protein.


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eration in cirrhotic livers[13] and on the topography and immunophenotype of epithelial cell adhesion molecule-positive hepatocyte-like cells in relation to ductular reaction in chronic viral hepatitis.[14]

Several groups isolated discrete populations of LPC or transit-amplifying cells both from healthy and injured livers.[15–19] Such studies showed the clonogenic potential of LPC or transit-amplifying cells and their dual capacity to differentiate into hepatocytes or cholangiocytes in vitro and in transplantation experiments. The occurrence of such phenomenon in vivo remains to be shown. The aims of the study thus were to examine the fate of LPC in vivo to assess the contribution of hepatocytic differentiation of transit-amplifying cells in liver cell replacement. We took advantage of restricted expression of osteopontin (OPN) in cells originating from the embryonic ductal plate, namely cholangiocytes lining the ductules or the canals of Hering,[8] from which LPC derive and generated a mouse line expressing inducible codon-improved cyclization recombinase estrogen receptor ligand binding domain variant T2 (iCreER$^{T2}$) recombinase under control of *Opn* regulatory region as a cell tracking tool for LPC and biliary cells in the adult liver. We show that hepatobiliary precursors do not contribute to liver mass homeostasis or to liver regeneration in the healthy liver. By contrast, in chronic liver injury (induced by a diet deficient in choline and supplemented with ethionine) expanded transit-amplifying cells give rise to a small proportion of hepatocytes that are well differentiated, polarized, and respond to pro-proliferative stimuli as normal native hepatocytes.

## Materials and Methods

### *Animal Models*

Experiments were performed with approval of the University Animal Welfare Committee.

In OPN-iCreER$^{T2}$ mice, iCreER$^{T2}$ was inserted in the *Opn* locus of a bacterial artificial chromosome that was injected in fertilized oocytes (Figure 1*A* and Supplementary Materials and Methods). OPN-iCreER$^{T2}$ mice were crossed with ROSA26R$^{yellow\ fluorescent\ protein(YFP)/YFP}$ reporter mice leading to OPN-iCreER$^{T2}$;ROSA26R$^{YFP}$ mice. The resulting mice have a CD1-enriched background; males and females were used in all experiments. To achieve Cre-LoxP recombination, tamoxifen (T5648; Sigma, Bornem, Belgium) dissolved in corn oil at a concentration of 30 mg/mL was injected intraperitoneally at a dose of 100 mg/kg of body weight, in 20-day-old mice, unless otherwise specified in the legends to the figures. At 4 weeks of age (or 20 g body weight), mice were fed a diet deficient in choline (MP Biomedicals, Irvine, CA) for 3 weeks supplemented with 0.15% (wt/vol) ethionine (E5139; Sigma-Aldrich, Bornem, Belgium) in drinking water (choline-deficient ethionine-supplemented [CDE]) or a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)- containing diet (137030; Sigma-Aldrich). After 3 weeks of a CDE or DDC diet, animals were either killed for liver examination or returned to a standard chow and plain water diet for 2 weeks to allow recovery from liver injury (STOP model). Controls received standard rodent chow. In all models, transgenic mice without tamoxifen injection were used in parallel as negative control for Cre recombination. Two-thirds partial hepatectomy (PH) was performed as described.[20] Livers were harvested and examined 44 hours after PH. Bromodeoxyuridine (500 $\mu$g/10 g body weight) was injected intraperitoneally 2 hours before death. Acute toxic liver injury was induced by an injection of $CCl_4$ (900 $\mu$L/kg body weight; intraperitoneally) and chronic liver injury was induced with fibrosis by repeated injections of $CCl_4$ (500 $\mu$L/kg body weight, intraperitoneally, 3 times a week for 4 weeks). Livers were harvested 48 hours after the last dose of $CCl_4$. In a separate experiment, iloprost (Ilomedine; a gift from Bayer Santé France, Bayer Healthcare Pharmaceuticals, Loos, France) was administered to tamoxifen-injected and CDE-fed OPN-iCreER$^{T2}$;ROSA26R$^{YFP}$ mice via an Alzet osmotic minipump (model D1002; DUREC corporation, Cupertino, CA) to allow delivery of 20 ng/kg/min for the last 10 days of the 21-day CDE experiment. For clarity, the details of each experimental design are recapitulated in the corresponding figure.

Analytic methods are available as Supplementary material.

## Results

### *Generation of OPN-iCreER$^{T2}$ Mice and Monitoring of OPN-iCreER$^{T2}$ Activity in Liver*

To investigate how the progenitor cells contribute to repopulate the liver, we generated a mouse model (OPN-iCreER$^{T2}$;Rosa26R$^{YFP}$) that allows us to genetically trace the fate of the progenitor cells. In this model the progenitor cells and their progeny are detected by expression of enhanced YFP: a loxP-flanked stop cassette in the Rosa26R$^{YFP}$ reporter locus[21] is removed at a specific time point and in a specific cell population by tamoxifen-inducible iCreER recombinase, thereby enabling expression of YFP. *Opn* gene regulatory regions were selected to drive iCreER$^{T2}$ expression because in the healthy adult mouse liver, OPN expression was found in cholangiocytes lining the canals of Hering, in the interlobular bile ducts, and the intralobular ductules. No other cell type was found to express OPN (Supplementary Figure 1 and not shown).

In the absence of tamoxifen, no YFP expression was detected in liver of OPN-iCreER$^{T2}$;Rosa26R$^{YFP}$ (Supplementary Figure 2). We then injected 3- to 8-week-old OPN-iCreER$^{T2}$;Rosa26R$^{YFP}$ mice with tamoxifen (100 mg/kg) twice at a 36-hour interval and the livers were analyzed for YFP expression 1 week after the second injection. OPN-iCreER$^{T2}$–induced YFP was found in OPN-expressing cells (Figure 1*B*,i–iii). These cells co-expressed SOX9 and K19 (Figure 1*B*,iv–vi), and correspond to cholangiocytes lining all segments of the intrahepatic biliary tree, including the canals of Hering (Figure 1*B*,x–xii). No other cell type than cholangiocytes was found to express OPN-iCreER$^{T2}$–induced YFP in normal liver. Hepatocytes, which express hepatocyte nuclear factor 4$\alpha$ (HNF4$\alpha$), express neither OPN nor OPN-iCreER$^{T2}$–induced YFP (Figure 1*B*,vii–ix). Also, tamoxifen induction of Cre recombinase in OPN-iCreER$^{T2}$;Rosa26R$^{YFP}$ did not alter the expression of the murine native OPN gene (Supplementary Figure 3).

The efficiency of OPN-iCreER$^{T2}$–mediated recombination of the Rosa26R$^{YFP}$ locus was calculated. Two injec-