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Liver Progenitor Cells, Cancer Stem Cells and Hepatocellular Carcinoma

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1. Introduction

There is great interest in the biology of liver progenitor cells (LPCs) because of their stem cell-like ability to regenerate the liver when the hepatocyte pool is exhausted. Barely detectable in healthy tissue, they emerge upon chronic insult in periportal regions, proliferate and migrate to injury sites in the parenchyma and eventually differentiate into hepatocytes and cholangiocytes to restore liver mass, morphology and function. The increasing worldwide shortage of livers for orthotopic transplantation means LPCs have assumed more prominence as candidates for cell therapy as an alternative therapeutic approach for the treatment of various liver diseases. However, an LPC response is usually seen in pre-cancerous liver pathologies and their high proliferation potential makes them possible transformation targets; associations that overshadow their restorative capability. This mandates that we continue to investigate the factors that govern their activation, proliferation and especially their differentiation into mature, functional cells to effectively direct transplanted cells towards regeneration and not tumorigenicity.

2. Normal liver tissue turnover

Tissue regeneration and maintenance in healthy intestine and skin is achieved within days and weeks respectively. In contrast healthy liver has a very slow cell turnover rate and the vast majority of hepatocytes is considered to be in the quiescent, non-proliferative G_0 phase of the cell cycle. It has been estimated that at any one time only 1 in 20,000 to 40,000 hepatocytes is undergoing mitotic cell division with an average life span of 200 to 300 days (Bucher & Malt, 1971).

The mechanisms by which hepatic cells are replaced in healthy liver are controversial. An early model, the "streaming liver" hypothesis is based on the metabolic zonation and differential gene expression patterns of periportal compared to pericentral hepatocytes. Periportal cells were proposed to proliferate and migrate ("stream") towards the central area with maturation during the journey and terminal differentiation achieved when the cells reached the central zone (Zajicek *et al*., 1985; Arber *et al*., 1988; Sigal *et al*., 1992). However there is no convincing evidence for a periportal to pericentral differentiation gradient and while hepatocytes in opposing lobular areas are responsible for different

metabolic functions, cells in either location are considered to be fully differentiated. By reversing the blood flow in the liver, Thurman and Kauffman demonstrated that this lobular zonation is not dependent on hepatocyte lineage progression but rather due to metabolite-induced gene regulation (Thurman & Kauffman, 1985). Retroviral marking studies provided additional evidence against the "streaming liver" model since transplanted cells, traceable by β-galactosidase expression, remained in the original location for 15 months (Bralet *et al.*, 1994). Furthermore, experiments performed with mosaic livers of chimeric rats (Ng & Iannaccone, 1992) as well as approaches using transgenic hAAT/β-gal mice (Kennedy *et al.*, 1995) demonstrated that hepatocytes proliferate clonally during normal tissue renewal throughout the whole liver lobule. Collectively, these findings led to the conclusion that normal liver cell plates lack the existence of a main proliferative compartment and instead randomly distributed hepatocytes mediate normal liver turnover by slow clonal expansion without involvement of a liver stem cell (Ponder, 1996).

3. Liver regeneration

The liver has an enormous capacity to regenerate by (1) replication of remaining, healthy hepatocytes, (2) activation, expansion and differentiation of a stem cell compartment, or (3) by a combination of these processes. Which pathway is employed depends on the nature of the injury, its severity and duration. This is discussed in greater detail in the sections to follow.

3.1 Hepatocyte-mediated regeneration

The hepatic regenerative capacity is most clearly seen after surgical removal of liver mass. This model, referred to as partial hepatectomy (PHx), was introduced by Higgins and Anderson (Higgins & Anderson, 1931) and it is unquestionably the best studied liver regeneration model due to its simplicity of design and reproducibility. In the rat two-thirds PHx is performed, whereas in the mouse usually only the left lobe is removed due to technical difficulties in the performance of two-thirds PHx surgery in mice, with resultant high mortality (Fausto *et al.*, 2006). The removed lobes do not re-grow. Instead there is compensatory, hyperplastic growth of all residual cellular populations until the size of the organ achieves proportionality to the body size, as determined by metabolic demands of the organism (Kawasaki *et al.*, 1992; Starzl *et al.*, 1993). The different liver cell types do not divide simultaneously but show different kinetics in DNA synthesis. Periportal hepatocytes, with a presumably shorter G_1 phase than pericentral cells (Rabes, 1976), are the first to undergo a wave of mitosis but DNA synthesis progresses to eventually involve the whole lobule with the exception of a few glutamine synthetase-positive, pericentral cells (Gebhardt, 1988). The proliferating hepatocytes are thought to provide mitogenic stimuli for the other hepatic cell populations. Biliary ductular cells, Kupffer and hepatic stellate cells (HSCs) and finally sinusoidal endothelial cells enter DNA synthesis about 24 hours later (Michalopoulos & DeFrances, 1997) with synchronised proliferation of each cell type for at least the first wave of replication. The greatest increase in liver mass can be seen by 72 hours with complete mass restoration after about one week (Grisham, 1962).

Although it was known from early experiments that repeated PHx does not exhaust hepatocyte growth (Simpson & Finck, 1963), the enormous proliferative capacity of adult

hepatocytes has previously been underestimated. Rhim *et al.* showed that newborn uPA overexpressing mice with continuous hepatocytic necrosis could be rescued by transplantation of a small number of hepatocytes that required between 10 to 15 rounds of replication to generate sufficient liver mass (Rhim *et al.*, 1994; Rhim *et al.*, 1995). In addition, serial transplantation experiments performed in tyrosinemic mice caused by a deficiency for fumarylacetoacetate hydrolase (FAH) revealed that hepatocytes are capable of undergoing more than 70 cell doublings without loss of functionality (Overturf *et al.*, 1997). Conversely there is also recent evidence that hepatocytes might reach a state of "replicative senescence" under certain chronic conditions such as advanced cirrhosis, perhaps due to telomere shortening (Paradis *et al.*, 2001; Wiemann *et al.*, 2002).

3.2 Liver progenitor cell-mediated regeneration

Repeated replication of healthy hepatocytes is the most efficient way to restore liver mass and function during normal tissue renewal and repair. If this process is inhibited or blocked during chronic chemical or carcinogenic hepatocyte insult, the liver relies on stem cell-like LPCs for its restoration. These cells are also referred to as "oval cells" in rodents (Fausto & Campbell, 2003) and the "Ductular Reaction" in humans due to their rather ductular phenotype in most human chronic liver diseases (Roskams & Desmet, 1998; Theise *et al.*, 1999).

3.2.1 History, origin and features of liver progenitor cells

The appearance of oval-like cells in the livers of rats treated with the azo dye "Butter Yellow" was originally reported in 1937 (Kinosita, 1937). Two decades later, Farber introduced the term "oval cell" for this population after observing small ovoid cells with a scant basophilic cytoplasm and a high nuclear to cytoplasmic ratio following treatment of rats with carcinogenic agents (Farber, 1956a, 1956b). Shortly after, Wilson and Leduc documented the proliferation of ductular cells that gave rise to hepatocytes and possibly new interlobular bile ducts in mice fed a methionine-rich, bentonite-supplemented diet and they were the first to suggest the existence of a bipotential liver progenitor or stem cell (Wilson & Leduc, 1958). Many experimental models involving toxins and carcinogens, alone or in combination with other surgical or dietary regimes, have since been developed and these facilitated the study of these progenitor cells, which are now widely accepted to represent adult LPCs; the progeny of hepatic stem-like cells.

The precise origin of LPCs remains uncertain, even though many researchers have addressed this question. The lack of definite evidence regarding the cellular source of LPCs may reflect differences in the models used to induce them and has also been hampered by a lack of specific LPC markers. Lenzi *et al.* suggested bile ducts as the structure of origin and argued that LPCs express biliary markers such as cytokeratin (CK) 7 and CK19 and lack expression of the mesenchymal cell markers vimentin and desmin. Additionally, the degree of LPC proliferation during early ethionine-induced carcinogenesis was found to be proportional to the increase in biliary tree volume and the authors claimed that LPCs are simply part of spatially expanded cholangioles (Lenzi *et al.*, 1992).

Other investigators have proposed an extrahepatic origin for LPCs. After it became apparent that some LPCs share c-kit, CD34 and Thy-1 expression with haematopoietic stem cells

(Fujio *et al.*, 1994; Omori *et al.*, 1997; Petersen *et al.*, 1998a), Petersen *et al.* were the first to suggest that LPCs could be derived from epithelial precursors in the bone marrow (Petersen *et al.*, 1999). Bone marrow-derived cells that potentially contribute to liver regeneration would enter via the portal vasculature and locate adjacent to the ducts in the periportal region, which is why Sell extended the preceding proposition by suggesting the periductular LPC as the candidate cell for an extrahepatic, bone marrow-derived stem cell in the liver (Sell, 2001). To test the hypothesis that cells from the bone marrow contribute to the formation of LPCs and hepatocytes, several investigators performed cell transplantation studies. They generally followed the fate of male bone marrow cells or purified haematopoietic stem cells transplanted into lethally irradiated female recipients that were in most cases subjected to liver injury. It was demonstrated that very minor fractions of LPCs or hepatocytes were donor-derived in both healthy and diseased livers (Petersen *et al.*, 1999; Theise *et al.*, 2000a, 2000b; Krause *et al.*, 2001; Wang *et al.*, 2002). The responsible population in the bone marrow capable of repopulating the liver was thought to be of c-kit^{high}Thy^{low}Lin^{neg}Sca-1^{pos} phenotype (Lagasse *et al.*, 2000). Soon after, the bone marrow was found to contain another stem cell subpopulation, the multipotent adult progenitor cell (MAPC), which can be induced to express hepatocyte phenotype and functions *in vitro* (Schwartz *et al.*, 2002) and is capable of differentiating into hepatocyte-like cells when transplanted into the liver (Jiang *et al.*, 2002). When donor-derived hepatocytes were examined genotypically, it was noted that they contained both donor and host genetic markers, indicating cell fusion as the likely mechanism by which hepatocytes are generated from bone marrow and not by transdifferentiation of haematopoietic stem cells (Vassilopoulos *et al.*, 2003; Wang *et al.*, 2003b). On the other hand, haematopoietic stem cells co-cultured with injured liver tissue separated by a trans-well membrane were shown to convert to a hepatocyte phenotype without fusion due to humoral factors released from the liver tissue. When engrafted into injured liver the haematopoietic stem cells differentiated into functional hepatocytes and their plasticity was proposed to facilitate the conversion, rather than the rare cell fusion event that was only seen at later stages of the experiment (Jang *et al.*, 2004). Recently it was demonstrated by transplantation of lacZ-transgenic bone marrow into virally or steatotically challenged mice that the contribution of extrahepatic cells to LPC-generated hepatocytes is minimal (Tonkin *et al.*, 2008). Collectively, these experiments show that some bone marrow cells are capable of producing hepatocytes (with or without fusion, depending on the model and cell population used) to restore injured liver. However, it occurs at a low frequency and efficiency unless a strong selective pressure is applied (Thorgeirsson & Grisham, 2006). It is likely that the more significant role of bone marrow cells is to generate non-parenchymal cells during liver regeneration (Forbes *et al.*, 2004). The usual regeneration processes after acute and chronic liver injuries appear to rely predominantly on intrahepatic cells.

The most widely accepted view is that LPCs originate from liver-resident precursor or stem cells, which lie dormant and present in such low numbers as to be undetectable in normal liver. However, they can be activated to proliferate under certain pathological conditions (Fig. 1). Evidence from experiments showing that LPCs always emerge from periportal liver zones and the fact that selective periportal damage inhibits the LPC response (Petersen *et al.*, 1998b) have led to the conclusion that the precursor cell likely resides somewhere in the vicinity of the portal triad. Grisham and Porta found ductular proliferation in carcinogen-treated rats that they attributed to activated stem-like cells from the Canals of Hering, the anatomical boundary between terminal bile ducts and the most distal hepatocytes of the

hepatic plate (Grisham and Porta, 1964). Microscopic studies of early histological changes in rats following 2-acetylaminofluorene (2-AAF)/partial hepatectomy (PHx) treatment also show elongated ductular branches that are formed by proliferating LPCs, which originate from a stem cell compartment located in these canalicular-ductular junctions. The newly formed biliary structures represent cellular extensions of the Canals of Hering and remain connected to the terminal biliary ductules by a continuous basement membrane (Paku *et al.*, 2001). Reid and colleagues suggested epithelial cell adhesion molecule (EpCAM) as a suitable marker for isolation and study of these Canals of Hering-derived LPCs (Schmelzer *et al.*, 2007). Lineage tracing of Sry (sex determining region Y)-box 9 (Sox9)-expressing cells supports the hypothesis that LPCs derive from the epithelial lining of bile ducts (Furuyama *et al.*, 2011). Theise *et al.* conducted studies comparing normal with acetaminophen-induced necrotic liver and identified the human equivalent to the rodent Canals of Hering, a niche which is similarly thought to harbour stem-like cells that give rise to LPCs or the Ductular Reaction (Theise *et al.*, 1999).

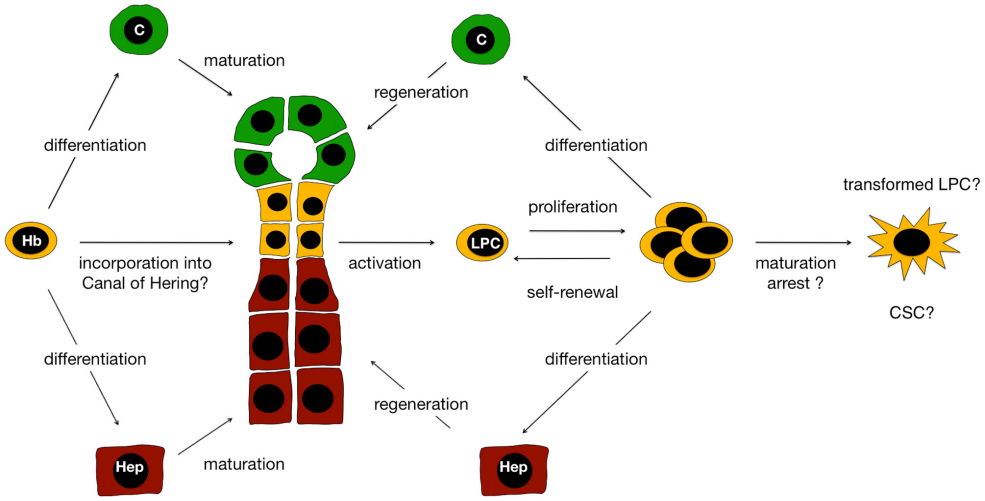

Fig. 1. LPC ontogeny. During liver development hepatoblasts (Hb) differentiate into cholangiocytes (C) and hepatocytes (Hep) and might be incorporated into the Canals of Hering to serve as a stem cell compartment during chronic liver injury. Activated liver progenitor cells (LPC) proliferate after appropriate stimuli, are capable of self-renewal and later commit towards either the cholangiocytic or hepatocytic lineage to regenerate the liver. If kept in a proliferative state, LPCs are likely candidates for transformation and might represent cancer stem cells (CSCs).

LPCs are a heterogeneous cell population and immature as well as intermediate phenotypes are observed before cells that express a differentiated phenotype are identified. Importantly, from activation to differentiation or transformation, they continuously change their morphology, phenotype and accordingly marker expression. LPCs express different combinations of phenotypic markers from both the hepatocytic and biliary lineage (Fig. 2) and also share epitopes with haematopoietic cells and cancer stem cells (CSCs; see table 1).

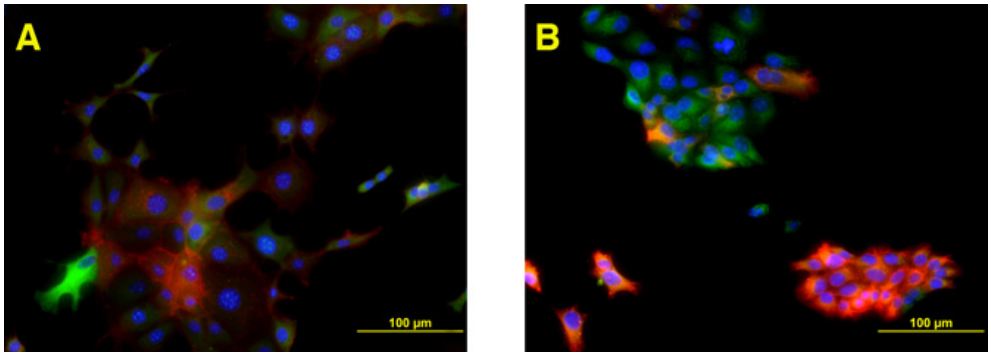

Fig. 2. Bipotentiality of LPCs. Immunofluorescent characterisation of the clonally established LPC line BMOL (Tirnitz-Parker *et al*., 2007) demonstrates the cells' bipotentiality. Immature BMOL cells co-express the hepatocytic markers muscle 2-pyruvate kinase (A, green) and transferrin (B, green) with the biliary markers A6 (A, red) and CK19 (B, red).

Marker	Hepatocytes	Cholangiocytes	LPCs	CSCs	References
A6	-	+	+	?	Engelhardt *et al.*, 1993, Tirnitz-Parker *et al.*, 2007
AFP	fetal	-	+	(+)	Sell, 1978; Evarts *et al.*, 1987; Smith *et al.*, 1996; Ishii *et al.*, 2010
Alb	+	-	+	(+)	Sell, 1978; Tian *et al.*, 1997; Yamashita *et al.*, 2010
CD24	?	(+)	+	+	Lee *et al.*, 2011; Qiu *et al.*, 2011
CD34	-	+	+	(+)	Omori *et al.*, 1997; Petersen *et al.*, 2003
CD133	?	?	+	+	Suetsugu *et al.*, 2006; Ma *et al.*, 2007; You *et al.*, 2010; Colombo *et al.*, 2011
CK7	-	+	+	?	Golding *et al.*, 1995; Clouston *et al.*, 2005
CK8	+	(+)	+	?	Sarraf *et al.*, 1994; Golding *et al.*, 1995; Sasaki *et al.*, 2008
CK14	-	(+)	(+)	(+)	Bisgaard *et al.*, 1993; Rogler, 1997; Zhang *et al.*, 2010
CK18	+	(+)	+	(+)	Golding *et al.*, 1995; Zhang *et al.*, 2010
CK19	-	+	+	?	Sarraf *et al.*, 1994; Golding *et al.*, 1995; Colombo *et al.*, 2011
c-kit	-	(+)	+	(+)	Fujio *et al.*, 1994; Fujio *et al.*, 1996; Knight *et al.*, 2008
Cx32	+	-	(+)	(+)	Zhang & Thorgeirsson, 1994; Paku *et al.*, 2004; Kawasaki *et al.*, 2011
Cx43	-	+	+	?	Zhang & Thorgeirsson, 1994; Paku *et al.*, 2004
Dlk	-	-	+	(+)	Jensen *et al.*, 2004; Yanai *et al.*, 2010
E-cad	low	high	high	?	Tirnitz-Parker *et al.*, 2007; Ueberham *et al.*, 2007; Van Hul *et al.*, 2009
EpCAM	(+)	fetal	+	+	Schmelzer *et al.*, 2007; Yamashita *et al.*, 2009; Okabe *et al.*, 2009; Yoon *et al.*, 2011
GGT IV	-	+	+	?	Petersen *et al.*, 1998a; Holic *et al.*, 2000
M_2PK	fetal	+	+	?	Tian *et al.*, 1997; Lowes *et al.*, 1999; Tirnitz-Parker *et al.*, 2007
OV-6	-	+	+	?	Dunsford *et al.*, 1989; Zhang *et al.*, 2010; Cao *et al.*, 2011
π-GST	fetal	-	+	?	Tee *et al.*, 1992; Lowes *et al.*, 1999; Oliva *et al.*, 2010
Sca-1	-	-	+	+	Petersen *et al.*, 2003; Qiu *et al.*, 2011
Thy-1	-	-	+	+	Petersen *et al.*, 1998a; Colombo *et al.*, 2011

Table 1. Marker expression by adult liver cells. A6, murine marker, epitope unknown; AFP, α-fetoprotein; Alb, albumin; CD, cluster of differentiation; CK, cytokeratin; c-kit, CD117, stem cell factor receptor; Cx, connexin; Dlk, delta-like protein; E-cad, E-cadherin; EpCAM, epithelial cell adhesion molecule; GGT IV, γ-glutamyl transpeptidase IV; M_2PK, muscle 2-pyruvate kinase ; OV-6, rat and human marker, epitope shared by CK14 and 19; π-GST, pi-glutatione-S-transferase; Sca-1, stem cell antigen 1; Thy-1, thymocyte differentiation antigen 1.

They have been shown to differentiate at least bipotentially into hepatocytes and cholangiocytes (Tirnitz-Parker *et al.*, 2007), and in some models display multipotentiality, also producing intestinal and pancreatic lineages (Tatematsu *et al.*, 1985; Yang *et al.*, 2002; Leite *et al.*, 2007). Hence it is not surprising that there is still not a single LPC-specific marker available and a combination of phenotypic markers is required for their identification or isolation.

LPCs infiltrate the parenchyma in close spatial and temporal association with hepatic stellate cells (HSCs). Following activation, HSCs differentiate from quiescent, vitamin A-rich cells into α-smooth muscle actin-positive myofibroblastic cells, which are capable of matrix degradation to generate space for cell migration as well as fibrogenesis and collagen deposition to provide chronically injured liver with architectural support. The activation, proliferation, migration and differentiation status of LPCs and HSCs, as well as their beneficial as opposed to pathological contributions, are controlled by key cytokines. LPCs and HSCs have been reported to influence each other's behaviour through paracrine signalling. LPCs produce a range of cytokines, including lymphotoxin β (LTβ), which signals via the LTβ receptor on HSCs to activate the NFkB pathway. This results in production of intercellular adhesion molecule 1 (ICAM-1) and regulated upon activation, normal T-cell expressed and secreted (RANTES), which then act as chemotactic agents for LPCs and inflammatory cells involved in the wound healing response to chronic liver injury (Ruddell *et al.*, 2009). Several other factors mediating the LPC response have been identified, including tumour necrosis factor (TNF), TNF-like weak inducer of apoptosis (TWEAK), interferon gamma (IFNγ), and transforming growth factor beta (TGFβ) among others (Knight *et al.*, 2000; Akhurst *et al.*, 2005; Knight *et al.*, 2005; Knight & Yeoh, 2005; Knight *et al.*, 2007; Tirnitz-Parker *et al.*, 2010). Abrogation of these key signalling pathways inhibits the LPC response to injury and prevents or diminishes liver fibrosis in animal models (Davies *et al.*, 2006; Lim *et al.*, 2006; Knight *et al.*, 2008). In the setting of impaired wound healing combined with chronic inflammation, the regenerative fibrotic response turns into pathological fibrogenesis, which can progress to cirrhosis and eventually hepatocellular carcinoma (HCC).

3.2.2 Rodent liver progenitor cell induction models

The majority of commonly used LPC induction models was originally developed to study the process of hepatocarcinogenesis. They generally combine an injuring mitotic stimulus, usually in the form of functional liver mass loss (chemically or otherwise-induced), with a manipulation that chronically damages hepatocytes or blocks their ability to divide and prevents them from contributing to the liver regeneration process. Described below are four examples of the most commonly used regimens.

3.2.2.1 D-galactosamine

This model is mainly used to induce liver injury in the rat. Administration of D-galactosamine inhibits RNA and protein synthesis in centrilobular hepatocytes by trapping and depleting uridine-nucleotides and UDP-glucose (Decker & Keppler, 1972), leading to acute necrosis. Hepatocyte replication is not fully blocked in this model; the response is only delayed. LPCs are resistant to the chemical as they do not metabolise D-galactosamine and are induced to proliferate within 48 hours after injury. They migrate into the parenchyma, where they generate both ductular cells and small hepatocytes (Lemire *et al.*, 1991; Dabeva & Shafritz, 1993).

3.2.2.2 Solt-Farber model and the modified 2-AAF/PHx regime

In this model, which is commonly used in rats and only rarely in mice, injection of the ethylating hepatocarcinogen diethylnitrosamine (DEN) is followed two weeks later by a two-week treatment with 2-AAF and PHx one week into 2-AAF feeding (Solt & Farber, 1976). The most commonly used regimen is a modification to the original Solt-Farber protocol, in which the "initiation" step of DEN injection is omitted and 2-AAF is administered four days before and after PHx, the 2-AAF/PHx regime (Tatematsu *et al.*, 1984). Both models induce proliferation of ductular or periductular LPCs, which accelerates when 2-AAF feeding is terminated, indicating that not only hepatocytes are growth-inhibited by 2-AAF but also LPCs, although to a lesser extent. LPCs differentiate more efficiently into hepatocytes at low doses of 2-AAF, whereas they tend to undergo apoptosis at higher dosages (Alison *et al.*, 1997). As a consequence, the rate at which LPCs differentiate into hepatocytes can easily be controlled through variation of the 2-AAF dose (Paku *et al.*, 2004).

3.2.2.3 Choline-deficient, ethionine supplemented diet (CDE diet)

A dietary deficiency of the lipotrope choline is known to induce hepatic steatosis (Lombardi *et al.*, 1966; Lombardi *et al.*, 1968). This pathology reflects an impaired release of triglycerides in the form of very low-density lipoprotein (VLDL) from hepatocytes, leading to intracytoplasmic deposition of fat vacuoles within a few hours of choline withdrawal. Choline-deficiency has also been reported to induce hepatocarcinogenesis (Ghoshal & Farber, 1984; Yokoyama *et al.*, 1985; Locker *et al.*, 1986). Similar effects were shown for another well-known carcinogen, DL-ethionine. Administered alone, ethionine is an antagonist of methionine and as such an inhibitor of *de novo* choline-biosynthesis thus induces fatty liver (Farber, 1967) and also leads to HCC (Farber, 1956a). When tested in combination with choline-deficiency, ethionine enhances the formation of liver tumours (Shinozuka *et al.*, 1978b), yet surprisingly diminishes the formation of fatty liver during choline-deficiency (Sidransky & Verney, 1969).

An interesting observation during early choline-deficient, ethionine-supplemented (CDE) diet-induced hepatocarcinogenesis studies in rats was the massive proliferation of α-fetoprotein-positive LPCs in the liver (Shinozuka *et al.*, 1978a). Numerous studies using this model to provoke an LPC response in rats were subsequently described. Due to the extensive availability of genetically engineered mouse strains, it became desirable to apply this regimen to mice. The conventional CDE diet used in rats however caused high mortality in mice and was therefore modified to a CD diet with separate administration of 0.165% DL-ethionine in the drinking water. This customised CDE diet (Akhurst *et al.*, 2001) reliably induces the proliferation of LPCs (Fig. 3, Tirnitz-Parker *et al.*, 2007; Tirnitz-Parker *et al.*, 2010) as well as inflammatory cells (Knight *et al.*, 2005) and serves as a murine model of hepatic fibrogenesis (Ruddell *et al.*, 2009; Van Hul *et al.*, 2009) and tumorigenesis following prolonged CDE diet exposure (Knight *et al.*, 2000; Knight *et al.*, 2008).

3.2.2.4 3,5-diethoxycarbonyl-1,4-dihydro-collidine diet (DDC diet)

The hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydro-collidine is also an effective inducer of LPCs as it causes extensive and prolonged liver damage while the diet is administered (Jakubowski *et al.*, 2005). However, in contrast to the CDE diet (see above), a fraction of hepatocytes continue to proliferate for the duration of diet administration (Wang *et al.*, 2003a). Thus the model is unusual in that liver regeneration is accomplished by both

hepatocytes and LPCs. It offers an alternate model to investigate mechanisms that regulate LPC proliferation and differentiation. In the context of liver cancer, the DDC model has been used extensively to demonstrate a link between LPCs and HCC. LPCs isolated from p53 null mice subjected to a DDC diet are able to generate both hepatocarcinomas and cholangiocarcinomas following transplantation into immunodeficient mice (Suzuki *et al.*, 2008). By placing a Hepatitis B Virus X transgenic mouse on a DDC diet, Wang and colleagues were able to show that LPCs overexpressing HBx were tumorigenic (Wang *et al.*, 2012). Interestingly, over the same period of seven months, DDC treatment did not induce tumours in wild type mice. In another study, the importance of the Hippo-Salvador pathway, working through inhibition of the yes-associated protein YAP, was shown by subjecting mice with liver-specific ablation of WW45 (drosophila homolog of Salvador and adaptor for the Hippo kinase) to a DDC diet. These mice displayed liver tissue overgrowth, an enhanced LPC response and they developed liver tumours with HCC as well as cholangiocarcinoma characteristics that appeared to be LPC-derived (Lee *et al.*, 2010).

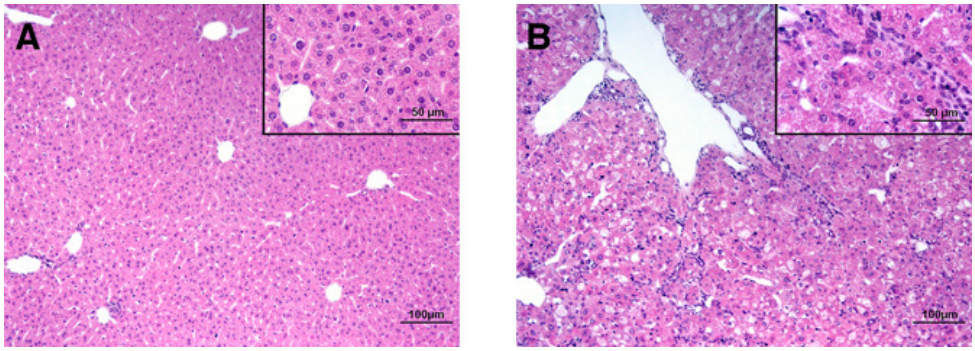

Fig. 3. Histology of normal and chronically injured liver. Adult mice on a control diet display normal liver architecture with orderly cords of hepatocytes and sinusoidal structures in-between the plates (A). On day 21 of the CDE diet, the liver architecture is highly disrupted by steatosis, scattered aggregates of infiltrated inflammatory cells and proliferating LPCs (B).

3.2.3 Liver progenitor cells in human pathologies

LPCs have been identified in a variety of human liver pathologies and are activated like their rodent counterparts to regenerate chronically injured liver (Haque *et al.*, 1996; Theise *et al.*, 1999). Like oval cells in rodents, human LPCs are usually associated with prolonged fibrosis, hepatocellular necrosis, cirrhosis and chronic inflammatory liver diseases. Hence, their proliferation is frequently seen in patients with hereditary haemochromatosis, chronic hepatitis B or C infection, alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) when hepatocytes are inhibited by DNA-damaging oxidative stress (Lowes *et al.*, 1999; Roskams *et al.*, 2003a; Clouston *et al.*, 2005). The degree of stem cell activation and the number of proliferating LPCs in these pathologies was demonstrated to correlate with the progression and severity of the underlying liver disease (Lowes *et al.*, 1999). The activation of human LPCs is characterised by the appearance of reactive ductules, also referred to as Ductular Reaction. Cirrhotic livers have been shown to contain nodules that are usually in close contact with reactive ductules and consist entirely of intermediate hepatocytes, which

strongly suggests they originate from LPCs (Roskams *et al*., 2003a; Roskams *et al*., 2003b, Falkowski *et al*., 2003). LPCs always emerge in pathologies with a predisposition to cancer and their proliferation in an environment rich in inflammatory mediators, growth factors or reactive oxygen species renders them likely targets for transformation. Furthermore, inhibition of the LPC response has been demonstrated to reduce the formation of cancerous lesions, strongly supporting a role for LPCs in hepatocarcinogenesis (Davies *et al*., 2006; Knight *et al*., 2005; Knight *et al*., 2008). Very recently LPCs have not only been discussed as cellular precursors for liver cancer but also as potential liver cancer stem cells, which could be responsible for tumour maintenance and recurrence (Marquardt *et al*., 2011; Rountree *et al*., 2012).

4. Cancer stem cells

The similarities between adult stem cells and CSC have led to confusion regarding their identity and it has often not been clear in the literature whether CSCs represent transformed progenitor or stem cells or whether both cell types are distinct cell populations that only share the expression of certain cell markers and display a similar biology. Adult tissue stem cells and CSCs are both defined by (i) highly efficient self-renewing ability through asymmetrical cell division and (ii) differentiation capacity along at least two if not more cell lineages. CSCs manifest the additional property of tumour initiation and/or maintenance. Nowadays the consensus is that the term CSC simply describes a cell's potential for self-renewal and ability to give rise to the hierarchic organisation of the heterogeneous lineages of cancer cells that constitute the tumour and does not consider the cell's origin. CSCs may arise from the differentiation arrest and transformation of a normal adult stem cell through oncogenic and/or epigenetic aberrations or the dedifferentiation of a mature cell that subsequently acquires self-renewing capacity. The CSC concept has been debated for many decades and compelling evidence of their existence has only emerged in the past decade.

4.1 Haematopoietic cancer stem cells

The existence of CSCs was first demonstrated in the haematological malignancy acute myeloid leukaemia (AML). Dick and colleagues isolated human AML cells from peripheral blood and transplanted them into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The vast majority of cells was unable to induce leukaemia, even when transplanted in larger numbers, despite the fact that they displayed a leukaemic blast phenotype such as the $CD34^+/CD38^+$ subpopulation. Only 0.01-1% of all AML cells, the $CD34^+CD38^-$ fraction, initiated AML and gave rise to a heterogeneous leukaemia tumour cell mass, classifying them as CSCs. The $CD34^+CD38^-$ cells could be serially transplanted and reliably developed AML with the same morphology and cell surface marker expression as the original tumour (Bonnet & Dick, 1997). Additional tumour–initiating AML cell populations were later identified and described to be of a $CD34^+CD19^-$ or $CD34^+CD10^-$ and $CD34^+CD4^-$ or $CD34^+CD7^-$ phenotype (Cox *et al*., 2004; Cox *et al*., 2007).

4.2 Solid tumour cancer stem cells

Using similar approaches involving cell transplantation into immunodeficient mice, CSCs have subsequently been identified in a variety of solid tumours, including breast, brain and liver cancer.

4.2.1 Breast cancer stem cells

Metastatic breast cancer was the first solid tumour in which CSCs were identified and prospectively isolated. The $CD44^+CD24^{-/low}Lineage^-$ cell population initiated tumours upon transplantation into mice with as few as 100 cells per injection. Importantly, they could be serially passaged and reliably reproduced the heterogeneous phenotype of the original breast cancer. In contrast, unsorted cells from the primary tumour or injection of a large number of alternate phenotypes, such as $CD44^+CD24^+$ cells, failed to form tumours (Al-Hajj *et al*., 2003). Furthermore, it was established that increased expression of the detoxifying enzyme aldehyde dehydrogenase (ALDH) identifies the tumorigenic breast stem cell fraction and high ALDH1 activity correlates with poorer prognosis (Ginestier *et al*., 2007).

4.2.2 Central nervous system cancer stem cells

The discovery of breast CSC was reported in the same year as the identification of tumour-initiating stem cells in the brain. Singh and colleagues identified and prospectively isolated a $CD133^+$ population of cells from a range of human brain tumours including medulloblastomas, pilocytic astrocytoma, glioblastoma and anaplastic ependymoma that *in vitro* exhibited stem cell properties and gave rise to heterogeneous cell populations with the same phenotype as the original tumour cells. Upon transplantation of as few as 100 $CD133^+$ glioma cells into the frontal lobes of NOD/SCID mice, serially transplantable tumours were initiated that mirrored the original tumour phenotype, whereas no tumours developed after injection of a much larger number of $CD133^-$ cells from the same tumour (Singh *et al*., 2003; Singh *et al*., 2004).

4.2.3 Liver cancer stem cells

Only very recently have liver CSCs been described. However the mounting evidence is compelling and ever more markers are suggested to describe the population of cells that may be responsible for liver cancer initiation, maintenance and potentially tumour recurrence after HCC resection, as described below.

4.2.3.1 Side population (Hoechst 33342 dye efflux)

The first evidence for the existence of liver CSCs came from Haraguchi and colleagues who performed Hoechst 33342 side population (SP) analyses of various human gastrointestinal cell lines and identified a subpopulation of cells with CSC properties. The SP approach is based on the finding that cells without stem cell characteristics accumulate the fluorescent nucleic acid-binding dye Hoechst 33342, whereas stem cells and CSCs do not as they are capable of effectively effluxing the dye through high activity of adenosine triphosphate (ATP)-binding cassette (ABC) transporters such as the multidrug resistance transporter 1 (MDR1) or breast cancer resistance protein (BCRP, also known as ABCG2). These ABC transporters employ ATP hydrolysis to facilitate substrate export across membranes against steep concentration gradients and thereby protect cells from cytotoxic agents and importantly from chemotherapeutic drugs such as cisplatin and doxorubicin. The authors report that the HCC lines HuH7 and Hep3B contained 0.9% to 1.8% SP cells with CSC properties, respectively, whereas no SP cells could be purified from the less aggressive hepatoma cell line HepG2 (Haraguchi *et al*., 2006). These results were confirmed shortly after by Chiba and colleagues who identified SP cells in some human liver cell lines, which

successfully induced xenograft tumours in NOD/SCID mice upon transplantation of as few as 1000 SP cells, while attempts to produce tumours with 1 x 10^6 non-SP cells failed consistently (Chiba *et al*., 2006).

4.2.3.2 CD133 (Prominin 1)

Several recent studies have used this glycoprotein initially identified as a marker for $CD34^+$ haematopoietic stem cells and later as a marker of LPCs for the isolation of liver CSCs. Suetsugu *et al*. reported that both the hepatoblastoma cell line HepG2 as well as the human fetal hepatoblast cell line Hc lacked CD133 expression, and that $CD133^+$ cells could only be demonstrated in the human HCC line Huh7. $CD133^+$ cells showed a higher proliferative potential in culture but also a greater ability to initiate tumour growth *in vivo* compared to the $CD133^-$ population (Suetsugu *et al*., 2006). Hepatic cells with a CD133 phenotype have been shown to be more resistant to chemotherapeutic drugs such as doxorubicin and 5-fluorouracil than their $CD133^-$ counterparts through preferential activation of the Akt/protein kinase B and Bcl-2 cell survival pathways. Furthermore, resistance of normal stem cells to cyclophosphamide is facilitated by the differentially expressed marker ALDH. Studies on ALDH and $CD133^+$ cells found ALDH expression only in the $CD133^+$ subpopulation and suggested a hierarchical cell organisation with regard to tumorigenicity in the order $CD133^+ALDH^+$ > $CD133^+ALDH^-$ > $CD133^-ALDH^-$, which suggests ALDH as an additional marker useful for liver CSC identification (Ma *et al*., 2007, Ma *et al*., 2008). In addition, it has been demonstrated that TGFβ signalling can induce CD133 expression in the HCC cell line Huh7 through epigenetic regulation, which results in a significant increase in tumour initiation capacity in these cells compared to $CD133^-$ Huh7 cells (You *et al*., 2010).

4.2.3.3 Epithelial cell adhesion molecule (EpCAM)

Myajima and colleagues identified EpCAM as a biliary and LPC marker, which is expressed in biliary epithelial cells and becomes upregulated in liver upon 2-AAF/PHx and DDC treatment (Okabe *et al*., 2009). Since EpCAM expression has been reported in many normal epithelial as well as in tumour cells, it is not surprising that it has been suggested as a useful CSC marker. $EpCAM^+$ cells isolated from human HCC tissues were shown to be more tumorigenic and invasive than $EpCAM^-$ cells and consistently formed invasive tumours in NOD/SCID mice, even after serial transplantation, whereas the $EpCAM^-$ population did not (Yamashita *et al*., 2009). EpCAM is a direct transcriptional target of Wnt/β-catenin signalling, which has been implicated as a CSC self-renewal pathway (Yamashita *et al*., 2007). Activation of the Wnt/β-catenin pathway increased the $EpCAM^+$ cell population, whereas knockdown of EpCAM resulted in decreased proliferation, colony formation, migration and drug resistance (Yamashita *et al*., 2009).

5. Hepatocellular carcinoma

Mortality from chronic liver disease is the most rapidly increasing cause of death in many western nations. The commonest aetiologies contributing to this escalation are chronic viral hepatitis C or B infection, alcoholic and non-alcoholic fatty liver disease. All these conditions can cause fibrosis and, subsequently, cirrhosis and HCC. Much evidence has been gathered demonstrating that HCC can arise from deregulated LPC proliferation and maturation during chronic liver injury in humans and in animal models of liver disease and carcinogenesis.

5.1 HCC: A clinically important end-stage complication of chronic liver disease

End-stage complications of chronic liver disease (cirrhosis and HCC) are the 9th commonest global cause of death and will remain so for at least the next 20 years. Of great concern is the prediction by the World Health Organisation that by 2030, deaths from HCC will for the first time exceed those from non-malignant complications of cirrhosis, such as liver failure and portal hypertension (Mathers *et al*., 2006).

Most cases of HCC in the western world arise in the setting of established cirrhosis (Bruix & Sherman, 2005; Olsen *et al.*, 2010; Sherman, 2011). The median survival of untreated HCC is in the order of 6-16 months. In view of the poor survival in the absence of therapy, strategies have been implemented to reduce the incidence of HCC through immunisation to prevent chronic HBV infection and screening of high-risk groups (i.e. those with cirrhosis). Despite these approaches, we are still faced with an escalation in the number of cases and requirement for treatment (El-Serag & Mason, 1999; Bruix & Sherman, 2005; Llovet *et al.*, 2005; Mathers & Loncar, 2006; Llovet *et al*., 2008).

Presently the treatment of choice for HCC is liver resection or orthotopic liver transplantation (OLT), either with or without adjunctive chemotherapy or non-surgical ablative therapy. Liver resection is the treatment of choice for HCC in non-cirrhotic livers, and accounts for 5% of HCC cases in western countries and 40% of cases in non-western countries. Patients with well compensated cirrhosis and who do not have portal hypertension may also be considered for resection, provided that lesions are confined to the liver and enough "functional reserve" of liver is retained to ensure survival of the patient (Bruix & Sherman, 2005; Llovet *et al*., 2005). Currently, tumour size, number and vascular invasion are still the strongest predictors of survival with up to 70% of subjects surviving five years. Tumour recurrence complicates 70% of cases at five years, reflecting either intrahepatic metastases (true recurrences) or the development of *de novo* tumours (Llovet *et al*., 2005). Based on comparative genomic hybridisation, DNA fingerprinting using loss of heterozygosity assays, or DNA microarray studies, it is estimated that just over half of recurrences correspond to intrahepatic metastases undetected by the time of resection, whereas less than half are *de novo* HCCs (Chen *et al*., 2000; Finkelstein *et al*., 2003; Ng *et al*., 2003). OLT is indicated in individuals who fulfil the "Milan criteria": patients with a single HCC of up to five centimetres in size or up to three nodules not larger than three centimetres each. Strict adherence to these criteria results in 5-year survival of up to 70% with recurrence rates usually less than 15%(Bismuth *et al*., 1993; Mazzaferro *et al*., 1996; Llovet *et al*., 2005; Mazzaferro *et al*., 2009).

5.2 Pathogenesis of HCC: The emerging role of LPCs and CSCs

The activation and proliferation of LPCs during chronic liver injury is associated with an inflammatory response that involves activation of resident and recruited inflammatory cells (Fig. 4). These inflammatory cells initiate tissue regeneration by promoting the removal of cellular debris and by stimulating LPCs to proliferate through release of mitogenic growth factors and cytokines (Lowes et al., 2003; Knight et al., 2005). Whilst LPCs play an important role in normal liver repair processes, dysregulation of their proliferation and differentiation has been linked to fibrogenesis and carcinogenesis (Lowes *et al.*, 1999; Clouston *et al*., 2005; Knight *et al*., 2008; Ruddell *et al*., 2009; Tirnitz-Parker *et al*., 2010). Clear demonstration of a role for LPCs, and possibly CSCs, in HCC development was reported by Shachaf and

colleagues (Shachaf *et al.*, 2004). Inactivation of the Myc oncogene was sufficient to induce sustained regression of invasive HCC in a murine model. Tumour cells differentiated into hepatocytes and biliary epithelial cells. This process was associated with rapid loss of expression of the tumour marker α-fetoprotein, increase in expression of liver cell markers CK8 and carcinoembryonic antigen, and in some cells the biliary LPC marker CK19. Many of the "reverted" tumour cells remained dormant as long as Myc remained inactivated; however, Myc reactivation immediately restored their neoplastic features. Using array comparative genomic hybridisation, Shachaf and coworkers confirmed that the dormant liver cells and the restored tumour retained the identical molecular signature and hence were clonally derived from the tumour cells. Thus, tumours have pluripotent capacity to differentiate into normal cellular lineages and tissue structures, while retaining their latent potential to become cancerous

Several other studies have confirmed a LPC phenotype in a substantial number of HCCs. Detailed immunophenotyping revealed that 28–50% of HCCs express markers of LPCs, such as CK7 and CK19. Histologically, these tumours consist of cells that have an intermediate phenotype between LPCs and mature hepatocytes. Furthermore, HCCs that express both hepatocyte and biliary cell markers such as albumin, CK7 and CK19, carry a significantly poorer prognosis and higher recurrence after surgical resection and liver transplantation (Roskams, 2006; Yao & Mishra, 2009). The "precursor-product" relationship between LPCs, CSCs and HCC is further strengthened by the observation that 55% of small dysplastic foci, which represent the earliest premalignant lesions, are comprised of LPCs and intermediate hepatocytes (Weinstein *et al.*, 2001). Finally, inhibition of the LPC response to liver injury using a broad range of pharmacological therapies such as interferon alpha 2b (Lim *et al.*, 2006), COX-II inhibitors (Davies *et al.*, 2006), or tyrosine kinase inhibitors (Knight *et al.*, 2008) is associated with a reduction in the severity of hepatic fibrosis and incidence of HCC. These observations provide more evidence in support of a critical role for LPCs and CSCs in the carcinogenic process. Collectively these studies suggest that anti-inflammatory agents may be useful therapeutically in reducing the incidence of liver cancer among patients with chronic liver pathologies.

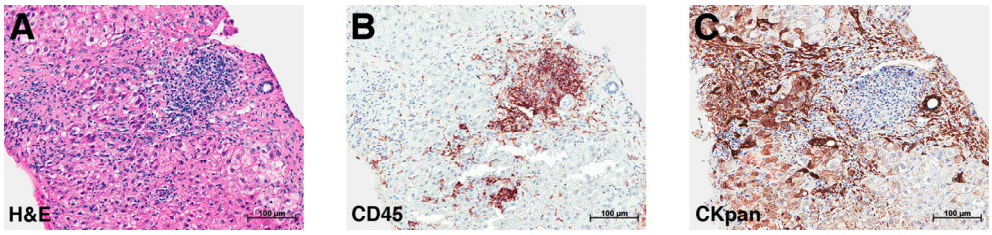

Fig. 4. Co-regulation of inflammatory response and LPC proliferation in hepatitis C patients. Haematoxylin and eosin (H&E) staining of a liver section from a hepatitis C virus-infected patient demonstrates disrupted liver architecture through infiltration and proliferation of small basophilic cells as well as steatotic changes in hepatocytes (A). Staining for the common leukocyte marker CD45 (B) and the biliary LPC marker CKpan (C) suggests co-regulation of the inflammatory response with the Ductular Reaction.

6. Conclusion

The cellular target of transformation leading to HCC is currently undefined. Potential candidates include the hepatocyte and the LPC and they need not be mutually exclusive. However, there is substantial circumstantial as well as some direct evidence implicating LPCs. This view would also be compatible with the increasingly popular theory of the stem cell basis of cancer. In the context of HCC, a variety of animal models, which induce chronic liver injury ultimately produce liver cancers and most of these pathologies display increased proliferation of LPCs. To conform to current views on carcinogenesis i.e. it is a rare event that affects a few cells and there are multiple stages in the process, it is necessary to hypothesise that a minority of LPCs are tumorigenic and that these have incurred the early genetic alterations that have initiated their progression to cancer. The challenge for future strategies to treat liver cancer is to identify these initiated LPCs and to show their direct link to HCC. This should be followed up with studies to elucidate progressive changes at the molecular level, which govern their behaviour and to exploit their vulnerability. Such knowledge will facilitate better diagnosis as well as treatment and prevention of HCC.

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