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Permalink

<https://escholarship.org/uc/item/3wb8f75h>

ISBN

978-1-118-67062-0

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Publication Date

2015-05-18

DOI

10.1002/9781118670613.ch14

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Basic Science of Liver Cancer Stem Cells and Hepatocarcinogenesis

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14.1 Introduction

14.1.1 Overview

The basic science of liver cancer stem cells (LCSCs) and hepatocarcinogenesis is best understood by familiarity with the increasingly complex epidemiology of hepatocellular carcinoma (HCC), with the still unknown cell origin(s) of HCC, with the anatomy and function of the liver lobule and with the historical and some novel methods for the study and identification of adult tissue stem cells and LCSCs. These background subjects are briefly discussed in this section.

14.1.2 Epidemiology of HCC

Environmental agents, including carcinogens, diet and genetic predisposition, are associated with toxicity-induced liver cell death and chronic inflammation. Both liver cell death and inflammation can contribute to development of compensatory hepatocyte proliferation and to hepatocarcinogenesis (El-Serag and Mason, 1999; El-Serag and Rudolph, 2007; El-Serag, 2011; American Cancer Society, 2013; World Health Organization, 2014).

HCC will likely cause 745 000 deaths worldwide in 2014 (World Health Organization, 2014). It is the fourth or fifth most common cancer in the world

and its incidence rate is increasing (El-Serag, 2011; National Cancer Institute, 2014a, 2014c). In 2014, in the United States alone, approximately 33 190 new cases of HCC will have been diagnosed, with 23 000 subsequent deaths (American Cancer Society, 2014). Major causes of HCC are alcoholism, in the United States and Europe; dietary carcinogens, such as aflatoxin, in Africa and East Asia; hepatitis B virus (HBV), in Africa and Asia; and hepatitis C virus (HCV), in Japan, Europe and the United States (Venook *et al.*, 2010). Chronic HBV and HCV infections are the major factors underlying HCC risk (~10–80%), a risk still higher in co-infected patients (Ikeda *et al.*, 1993; Benvegnù *et al.*, 1994; Chiaramonte *et al.*, 1999; Venook *et al.*, 2010). Cirrhosis of any aetiology, including both alcoholic and biliary cirrhosis, is also a major risk factor, and is present at autopsy in 80–90% of HCC livers (Farinati *et al.*, 1994; Fattovich *et al.*, 2004; Silveira *et al.*, 2008; Hytioglou *et al.*, 2012; Floreani and Farinati, 2013). Genetic risk factors include α_1 -antitrypsin deficiency, haemochromatosis, porphyria cutanea tarda, tyrosinemia and Wilson's disease, nonalcoholic steatohepatitis, and metabolic diseases, such as dyslipidaemia, obesity, type 2 diabetes, glycogen storage disease, as well as autoimmune hepatitis (Bugianesi *et al.*, 2002; Fattovich *et al.*, 2004; Mattner, 2011; Hytioglou *et al.*, 2012). HCC incidence is also two- to eightfold higher in males than in females (Buch *et al.*, 2008; Howlader *et al.*, 2012). With a new depth and accuracy of cellular identification and structural biopsy interpretation, fuelled also by inbred animal studies and *in vitro* culture investigations, the increasing volume of epidemiological studies provides direction for future research into the heretofore unsolved problems of HCC cell and LCSC origins, and into earlier-detection assays for these cell types.

14.1.3 Problems surrounding HCC cell origins

The cellular origins of HCC are a long-standing problem in liver research (Sell and Leffert, 1982, 2008), compounded by the complex clinical phenotypes of HCCs (van Malenstein *et al.*, 2011). The definitions of cancer stem cells (CSCs) are confusing, since CSCs can both generate and be derived from malignant tumours (Reya *et al.*, 2001; Clarke *et al.*, 2006; Rahman *et al.*, 2011; van Malenstein *et al.*, 2011; Magee *et al.*, 2012; Valent *et al.*, 2012). Generally, the definition of CSCs is a function of their experimental derivation as an isolate from tumour, and as a precursor to a secondary tumour. For the purposes of this article, the definition of a liver cancer precursor cell, or tumour-initiating cell (TIC), will be one preceding the primary tumour and requiring a chronically injured environment for progression to a primary tumour. On the other hand, an LCSC will be a liver cancer precursor, usually isolated from the primary tumour, unless otherwise noted, and fully capable of forming a secondary tumour in an untreated animal. Because the putative parent HCCs are of complex aetiology and phenotype, and of undoubtedly multiple possible origins (Figure 14.1), the biology of LCSCs isolated from them will likely be equally complex.

Figure 14.1 Lineage models of candidate HCC progenitor cells (HcPCs): hepatocytes, LSCs such as OCs and/or LCSCs. HCCs might develop from mono- (pathway [1]) or binucleated (pathways [1a] or [1b]) mature hepatocytes; from preneoplastic nodules derived from hepatocytes (pathway [1c]); from intrahepatic multipotent stem cells (MSCs) directly (pathway [2a]); or indirectly (pathway [2]) from bipotential OC or 'null' cell intermediates (pathway [2b]) formed from MSCs or other postulated MSC pathways. Alternatively, HCCs might develop from 'large' mononucleated hepatocytes derived from OCs or null cells (pathway [2c]); directly from intrahepatic stem cells (pathway [2d]); from small hepatocytes (pathway [3]) derived from intrahepatic LSCs like OCs or from OCs derived from injured HSCs (pathway [3a]) (Yang L. *et al.* 2008), AFP⁻ null cells (pathway [3b]) or mono- (pathway [3c]) or binucleated (pathway [3d]) hepatocytes; or from extrahepatic stem cells (pathways [3e], [4] or [4a–4c]). Red arrows indicate likely OC → HCC lineages (pathways [2b] and [3b]). Pathways [5] and [6] reflect putative reversible formation of LCSCs from HCCs, or of HCCs from LCSCs, and concomitant differentiation of LCSCs into mononucleated hepatocytes, respectively (arrows, → or ←, indicate the direction of the cell lineage). Koch and Leffert 2004. Reproduced with permission of Springer Science + Business Media.

Investigations of HCC origins following different injury regimens have focused particularly on hepatocytes and liver oval cells (OCs), small bipotential cells with liver stem cell (LSC)-like properties, both as possible HCC precursors. For instance, after marking proliferating rat hepatocytes with retroviral β -galactosidase (β -gal) reporter-encoding vector transduction following partial hepatectomy, the chemical hepatocarcinogen diethylnitrosamine (DEN) was administered by drinking water, and the resulting

hepatic populations and HCCs were examined for β -gal positivity (β -gal⁺), as well as for the preneoplastic, nodular and HCC marker placental glutathione-S-transferase (GSTp). The percentage of β -gal⁺ hepatocytes following compensatory growth post partial hepatectomy was the same as that of HCC cells following DEN administration (~18%); clonal growth of nodules and HCCs was indicated by the whole nodule or tumour β -gal positivity; and, whereas all HCCs expressed GSTp, although to different levels, only ~50% of surrounding β -gal⁺ hepatocytes were GSTp⁺, suggesting possible hepatocyte HCC precursor subtypes. Disadvantageously uninterpretable in such a marking–mapping system are the unmarked β -gal⁻ HCC cells (82% β -gal⁻), originated either from unmarked hepatocyte precursors or from other unmarked precursors, such as OCs (Bralet *et al.*, 2002).

Likewise, in a strategy designed to infect and mark both rat OCs and hepatocytes, and a second design to prelabel hepatocytes alone, with a carefully timed β -gal retroviral transduction during an acetylaminofluorene (AAF) hepatocarcinogen regimen known to induce massive OC proliferation and concomitant hepatocyte death, some hepatocytes, preneoplastic foci and nodules, as well as HCCs in *both* transductions, expressed β -gal and GSTp, thus again suggesting that the hepatocyte could be an HCC precursor, and one which might have transited through a less differentiated state (Gournay *et al.*, 2002). The possibility of simultaneous OC infection and proliferation in either of these two systems cannot be rigorously excluded.

Hepatocytes were also implicated as HCC precursors in an Alb-Cre recombinase hepatocyte-targeted *Ikk β* -deletion mouse system, shown to produce *Ikk β* -deleted HCC, following DEN administration (Maeda *et al.*, 2005). The patency of this interpretation rests upon the specificity of the albumin expression that drives the Cre recombinase necessary for the *Ikk β* deletion, and that can be shared by some differentiating OCs.

Subsequently, a system of transplantation has been developed whereby C57BL/6 hepatocytes, isolated from a DEN-treated mouse, are injected intrasplenically into the liver of a recipient mouse (He *et al.*, 2010). These cells will develop HCCs within the recipient liver if it has been subjected to chronic injury and to compensatory proliferation, such as the liver of a MUP/uPA mouse (Weglarz *et al.*, 2000), but will not develop HCCs in a normal liver recipient. The transplantation system has now been used to test both other animal donor cells and other recipient models, as well as to develop a more precise characterization of the HCC progenitor cells (HcPCs) (He *et al.*, 2013). Isolate separations by aggregate formation capacity and by CD44 positivity established the most effective HCC precursors. The small size of HcPCs is similar to that of cells of dysplastic foci that develop many months prior to HCC formation in the DEN-treated mouse or rat. The transcriptomes of HcPCs, OCs and HCC are very similar, but the dysplastic foci following DEN administration are in liver zone 3 near the Cyp2E1 DEN-metabolizing hepatocytes, whereas OCs are thought to be initially located portally in the Canals of Hering. Nevertheless, OCs can proliferate

and migrate throughout the liver following certain types of injury (Guest *et al.*, 2010), although they are not noticeable following DEN, as in this model.

In all of these precursor ‘fate mapping-like’ systems, the results suggest that parenchymal, possibly dedifferentiated, hepatocytes are HCC tumour progenitors. Hepatocytes are the predominant liver epithelial cell (Baratta *et al.*, 2009) and the major sites of metabolic activation of chemical carcinogens and carcinogen toxicity (Guengerich, 2000). While mature hepatocytes are not considered tissue stem cells, they appear to be able to dedifferentiate under certain circumstances. Nevertheless, many other putative intra- and extrahepatic LSCs, including hepatic OCs (Figure 14.1), were not eliminated as HCC precursors in these investigations (see Section 14.2.8). Thus, instead of supporting LSC origins, these observations suggest, but do not prove, an HCC origination model of cumulative genetic, epigenetic alteration and clonal selection from DEN-exposed hepatocytes (Nowell, 1976; Greaves and Maley, 2012; Guichard *et al.*, 2012).

It is still not known whether the cells of origin of HCC can be found within the dysplastic foci observed prior to HCC appearance and following DEN and other carcinogen administration. Whether these dysplastic foci are or are not HCC precursors, the origin of cells in the foci is also not known. Finally, in summary, it is not known whether HCCs originate from OCs, from dedifferentiated hepatocytes or from some other precursor cells.

The development of treatments to target the modulation or elimination of aggressive HCC rests upon the identification and biology of the earliest cells of origin of HCC: hepatocyte, altered hepatocyte, LSC, LCSC or other. Current therapies (National Cancer Institute, 2014b), including treatments of recurrent HCC following tumour resection, might only be providing suitable environments for drug resistance and metastasis, or LCSC proliferation, by focusing treatment on elimination of the wrong cells.

14.1.4 Anatomy and function of the liver lobule

Knowledge of lobular anatomy both in its normal state (Deleve *et al.*, 2011) and in response to different forms of acute or chronic injury provides us with information about the locations of hepatocytes, OCs and other non-parenchymal cells (NPCs); which of these cells proliferate, when, and where they migrate within the lobule; and the possible relevance of this dynamic structure to LSC homeostasis and LCSC formation, latency/proliferation and migration. An adult liver lobule is diagrammed in Figure 14.2.

Mature hepatocytes, with diameters of 20–60 μm , form bilaminar plates of differentiated cells, separated by opposing bile canalicular membranes. These membranes enclose canalicular spaces, into which biliary acids are secreted, for progression into the bile ducts.

Highly oxygenated blood from the hepatic artery comes along with nutrient-rich blood from the portal vein, originating in the intestines, and flows unidirectionally and collectively into and throughout the liver sinusoids.

Figure 14.2 Three-dimensional and cross-sectional diagrams of the cellular and structural anatomy of the adult liver lobule. The zones are numbered: 1, Portal triad (Space of Mall); 2, Midzonal – cells between zones 1 and 3; and 3, Centrilobular. Harwin, Lyons and Monsen, 1984. Reproduced with permission of Wolters Kluwer/Lippincott Williams and Wilkins.

This sinusoidal blood flows through a fenestrated endothelial cell layer into the Space of Disse, which contains migratory NPCs such as Kupffer cells, hepatic stellate cells (HSCs) and B and T lymphocytes (Crispe, 2009), and then through an extracellular matrix (ECM) adjacent to hepatocyte basal membranes. The depleted sinusoidal blood finally exits the lobule through the central vein and empties into the inferior vena cava.

Three hepatic zones are positionally defined (Figure 14.2): zone 1, the Portal Triad (Space of Mall), where portal veins, bile ductules and hepatic arterioles are enclosed within a fibrous sheath, proximal to the undifferentiated cells of the Canal of Hering, which separate bile ductules from hepatocyte plates; zone 2, the intermediate region between zones 1 and 3; and, zone 3, the centrilobular region surrounding the central vein. Zones 1–3 are functionally specialized. For example, hepatocytes synthesize and secrete albumin in all zones, 1–3, but alcohol dehydrogenase and hepatocarcinogen metabolizing Cyp450 enzymes are selectively enriched in the hepatocytes of zones 1 and 3, respectively. Bile acids secreted across apical membranes into canalicular spaces flow retrograde to blood, from zone 3 to zone 1; they collect in the bile ductules of the portal triad. Early postnatal life is a time of marked hepatocyte proliferation (from 10% to <1% of cells in S-phase), as lobular structure and zonal formation mature. During this time, α_1 -fetoprotein (AFP), a widely used

OC, LSC, TIC and HCC marker, is constitutively expressed in all zones. But as hepatocytes further mature and proliferation subsides to a steady state of ~0.005% of M-phase cells, AFP expression is extinguished directionally, from zone 3 to zone 1, and its blood levels fall to <50 ng/ml (Ramesh *et al.*, 1995).

Hepatocyte receptors are in a steady state with various soluble growth-stimulating and growth-inhibiting endocrine, paracrine, autocrine and juxtacrine factors that emanate from different organs and cells, and with associated factors that bind reversibly to the liver matrix. Depending upon exogenous or endogenous hepatotoxic insults, different stimulatory and inhibitory growth factor steady states shift, as a result of loss of liver mass, stimulating hepatocytes to proliferate in the different zones at different times in order to restore lobular mass. Alternatively, if loss of liver mass is too great for extant hepatocyte recovery, other cells, such as OCs, perhaps other LSCs or even, potentially, LCSCs, proliferate to restore, or disrupt, liver function. The nature of the response is dependent on the type of injury, its chronicity or acuteness and the milieu in which it occurs, such as the age of the animal.

The regenerative response following partial hepatectomy is a basic example of a range of defined injuries and quantifiably measurable responses. For example, regeneration after partial hepatectomy, which affects all liver zones, can be quantified as the time and rate of hepatocyte S- and M-phase entry (deHemptinne and Leffert, 1983). These rates fall with age, in parallel with decreasing telomerase length (Hoare *et al.*, 2010; Schmucker and Sanchez, 2011); regeneration occurs over a longer time frame, but restores the same mass. Quiescent rodent livers (G_0) regenerate in a 30–70% partial hepatectomy dose- and zone-dependent fashion (zone 1 → zone 2 → zone 3); within 3–7 days, hepatocytes divide one to three times to restore liver mass. Zonal effects depend partly upon circulating growth factors, because surgical reversal of bloodflow redirects hepatocyte $G_0 \rightarrow S \rightarrow G_2 \rightarrow M$ -phase entry times (zone 3 → zone 2 → zone 1). NPC proliferation is also humorally regulated, and collectively occurs after the start of hepatocyte proliferation. In general, this defined regenerative response provides the background for study of different types of liver injuries, varied by dose, duration, lobular location and recruitment of parenchymal cells and NPCs, both mature and stem cell, to deliver acute to chronic liver insult, resulting in a range of regenerative to fibrotic responses, respectively.

14.1.5 Methods of identification and properties of adult stem cells

In a classical series of experiments, using a spleen colony-forming assay (SCA), a suspension of bone marrow cells (BMCs) from a donor mouse formed discrete colonies, proportional to the injection dose, in the spleen of a lethally irradiated mouse recipient, indicating the existence of bone marrow stem cells (BMSCs) (Siminovitch *et al.*, 1963). This assay provided a way to

fate map putative stem cells, revealed key stem cell properties and served as the prototype for the identification and isolation of somatic stem cells, as well as tissue CSCs such as BMSCs (Spangrude *et al.*, 1988; Reya *et al.*, 2001; Clarke *et al.*, 2006) and CSCs from gliomas (Lathia *et al.*, 2011) and from breast (Russo *et al.*, 2010).

Six principle observations were made: (i) Consistent with clonal origin, the number of intravenously injected BMCs generated linear dose–response curves of macroscopic haematopoietic spleen colonies (Till and McCulloch, 1961). (ii) Radiation log-linear dose–response killing curves of donor BMCs suggested fewer than two hits per colony were necessary for killing (Till and McCulloch, 1961). (iii) Examination of the chromosome spreads of donor BMCs, heavily irradiated shortly after their injection into the recipient mouse in order to generate unique chromosomal markers, showed the majority of cells within the subsequent clones to have the unique colony-specific marker karyotype (Becker *et al.*, 1963). (iv) When lethally irradiated mice were used as recipients of dispersed haematopoietic tissues to determine the number of spleen colony-forming units (CFUs), these colonies were found to be of a variable number per suspension, and each colony was found to contain both undifferentiated and differentiated cells, in variable ratios. Single colonies were therefore isolated, dispersed and used as donor suspensions in a new round of irradiated recipients, at one colony per recipient; the growth of CFUs from single colonies was suggested by statistical analysis to follow a curve most similar to that of a stochastic process of stem cell self-renewal ('birth') or stem cell differentiation ('death'), with the variance between colonies exceeding the mean value (Till *et al.*, 1964). (v) Again using the SCA, physical separation studies distinguished cells with the greatest self-renewing ability as the lowest velocity-sedimenting population, indicating that the probability of self-renewal versus differentiation of a single cell was perhaps not completely random, after all (Worton *et al.*, 1969a). Fractions of bone marrow suspensions separated by both velocity sedimentation and equilibrium density-gradient sedimentation were assayed both for CFU capacity by *in vivo* SCA (CFU-S) and for CFU capacity in an *in vitro* culture assay (CFU-C). Although fractions contained CFU capacity in both CFU assays, there were peaks of CFU *in vitro* capacity that did not overlap with CFU *in vivo* capacity, and vice versa, indicating potential multiple cellular subtypes and putative complex environmental interactions regulating stem cell 'birth' and 'death' – self-renewal and differentiation – respectively (Worton *et al.*, 1969b). (vi) CFU assays showed that a single clone of radiation-karyotypically-marked donor CFU spleen cells was able to repopulate thymus, bone marrow and lymph nodes, as both erythropoietic and granulocytic lineages, an observation consistent with the pluripotency of BMSCs (Wu *et al.*, 1968). Some years later, asymmetric cell division of BMSCs was shown directly by timelapse videos (Wu *et al.*, 2007). All of these studies framed the definition of stem cells, as well as many of the questions still being asked about cell autonomy and cellular interplay with the environment or niche.

Normal stem cells are generally classified as pluripotent, capable of generating the next descendant cell types, or multipotent, generating fewer of the descendant types. Normal stem cells appear to function constitutively in the bone marrow, intestine and skin; their activation is enhanced by toxicity and disease (Potten and Loeffler, 1990; Deasy *et al.*, 2003; Martin-Belmonte and Perez-Moreno, 2012). Stem cell frequencies range from 1×10^{-13} cells/20 g mouse (e.g. the totipotent zygote) to $\leq 1 \times 10^{-4}$ cells/tissue (e.g. bone marrow: Spangrude *et al.*, 1988). Stem cells, by definition, can divide in at least two ways (Cairns, 1975, 2002; Potten *et al.*, 1978; Deasy *et al.*, 2003; Wu *et al.*, 2007): symmetrically (1 stem cell $\rightarrow$ 2 undifferentiated daughter stem cells) and asymmetrically (1 stem cell $\rightarrow$ 1 undifferentiated stem cell + 1 differentiated daughter). Generally, the stem cell is thought to replenish its pool by self-renewal, but otherwise to be relatively quiescent, while the differentiated daughter cell can expand itself exponentially, according to need or injury.

Hypotheses of the inheritance of parental DNA by nonrandom sister chromatid segregation have been developed to postulate protection from accumulation of mutation and genomic rearrangement within a stem cell population (Cairns, 1975, 2006). The immortal-strand hypothesis is difficult to validate or prove experimentally, and has putatively been shown in some tissues and not in others (Charville and Rando, 2013; Falconer and Lansdorp, 2013; Yadlapalli and Yamashita, 2013; Yennek and Tajbakhsh, 2013), but DNA label-retention analysis, used to take advantage of the functional characteristic of slow turnover by some stem cells, has provided a measure of stem cell location within some organ systems, in spite of the need to activate a quiescent population with low-grade injury in order to label it for visualization. This technique has been used to good effect to label four different stem cell populations in the mouse liver (Kuwahara *et al.*, 2008). By injection of sublethal acetaminophen (APAP) and bromodeoxyuridine (BrdU), followed 14 days later with a large chase dose of APAP, the BrdU is diluted from the differentiated daughter cells, which are presumed to be proliferating rapidly in response to the injury, but is retained in the SC daughter cell, also having incorporated BrdU during the division following the first APAP, but having returned to quiescence by the time of the second injury. The Canals of Hering harboured one population of label-retaining cells (LRCs); OCs have historically been associated with this niche. A second population of LRCs was near the OCs, adjacent to the parenchyma and to the biliary ductules at the Canals of Hering; these were called small peribiliary hepatocytes, and by serial section examination, appear possibly to have been a descendant population of OCs. Both OCs and peribiliary small hepatocytes still retained label at 8 weeks post APAP/BudR. If these *are* found to be descendants of OCs, then an asymmetric division will presumably have produced two stem cells of distinct potencies, an event clearly common throughout normal embryological development but occasioned by toxicity in this case. A third population of LRCs was not within the parenchyma, but instead consisted of occasional, labelled cholangiocytes. The fourth

population was small periductular cells with characteristics of ‘null’ cells, often considered to be a subtype of OCs that does not express AFP or other hepatobiliary markers; ‘null cells’ historically respond to allyl alcohol toxicity in the rat (Yavorkovsky *et al.*, 1995). There is as yet no definitive lineage connecting these stem cell types to one another, or to hepatocytes, tumour progenitors, TICs, HcPCs, LCSCs, HCCs or any other liver cancer.

14.2 Experimental evidence of LCSCs

14.2.1 Overview

CSC hallmarks of LCSC candidates, reported from investigations of phenotypes shared by various CSCs and nontumour cells (Sell and Leffert, 2008; Magee *et al.*, 2012; Valent *et al.*, 2012), include spheroid formation *in vitro*, side population (SP) formation, the expression of drug resistance and many CSC-associated markers, limiting dilution xenoengraftment; key evidence of asymmetric cell division is limited. There is evidence that OCs express some hallmarks of TICs, LCSCs and HCCs. Future LCSC investigations might include the hepatic properties of polyploidy, carcinogen bystander effects and definitions of niche on formation, on establishment and on migration to a new environment.

14.2.2 LCSCs and spheroids

Many CSCs form spheroids, but whether spheroid formation is a consistent property of an isolate of primary LCSCs is unclear. However, several observations of different aggregated forms have been made in cultured HCC-derived LCSC-like cell (LCSCLC) lines and strains, as well as in HcPC, and even in normal hepatocyte cultures (see Table 14.1).

A study of spheroid formation in cultures of LCSCLCs from *Ikkβ^{F/F}* (*Ikkβ* normal) mice has been reported (He *et al.*, 2010). Putative LCSCLC strains isolated from DEN-initiated HCCs in *Ikkβ^{F/F}* mice were established in culture by supplementation with phenobarbital and epidermal growth factor (EGF). Three of the DEN-induced HCC (dih) strains, di10, 11 and 12, all expressing AFP and albumin (consistent with formation from AFP⁺ HCCs), were selected and found to form spheroids under crowded culture conditions. Because it was previously found that hepatocyte-targeted deletion of *Ikkβ* enhanced di10 formation in these *Ikkβ^{ΔHEP}* mice compared to their *Ikkβ^{F/F}* siblings, an adenovirus, Adv-Cre, infection of the di10 *Ikkβ^{F/F}* strain of LCSCLC was used to delete *Ikkβ* from di10, thereby producing a deficient substrain, *IkkβΔ di10*. Under serum-free conditions in Petri plates, the cells of *IkkβΔ di10* formed two and three times more floating spheroids in 1° and 2° spheroid cultures, respectively. Spheroid formation was also enhanced in *Ikkβ^{F/F} di10* cells transduced with an IκBα super-repressor, again suggesting that IKKβ inhibited spheroid formation. For evaluation of the tumourigenicity of spheroid-forming di10 cells, *Ikkβ^{F/F}* and *IkkβΔ di10* cells

Table 14.1 Spheroid formation in normal, CSC and CSC- and LCSC-like cells.

Cell or tumour type	Cell system(s)	References
Normal rat liver	Hepatocyte cell suspensions	Jeejeebhoy <i>et al.</i> (1975)
Paediatric HCC	Single cells from tumour biopsy	Tomuleasa <i>et al.</i> (2010)
Mouse HcPCs	Aggregate-forming hepatocytes from DEN-treated BL/6; precursors to HCCs in chronically injured liver	He <i>et al.</i> (2013)
EpCAM ⁺ HCC	<i>In vitro</i> malignant cell lines	Terris <i>et al.</i> (2010)
CD133 ⁺ HCC	±BMP4-sensitive <i>in vitro</i> malignant cell lines	Zhang <i>et al.</i> (2012); Ma (2013)
Prostate	<i>In vitro</i> malignant cell lines without or with fibroblasts; intact glands	Wartenberg <i>et al.</i> (2001); Khoei <i>et al.</i> (2004); Liao <i>et al.</i> (2010)
Cervical	<i>In vitro</i> malignant cell lines	Olive <i>et al.</i> (2004)
Mammary	<i>In vitro</i> malignant cell lines	Günther <i>et al.</i> (2007); Krohn <i>et al.</i> (2009)
Colorectal	<i>In vitro</i> malignant cell lines in three-dimensional cultures	Valcárcel <i>et al.</i> (2008)
Glioblastoma	Dissociated 1° tumours	Ernst <i>et al.</i> (2009)
Melanoma	<i>In vitro</i> malignant cell lines	Na <i>et al.</i> (2009)
Neck	<i>In vitro</i> malignant cell lines	Hirschhaeuser <i>et al.</i> (2010)
Mouse	<i>In vitro</i> AFP ⁺ spheroid-forming cell strains from DEN-initiated HCCs (dih) from <i>Ikkβ^{F/F}</i> and <i>IkkβΔ</i> hepatocytes	He <i>et al.</i> (2010)

were transplanted subcutaneously into C57BL/6 mice. *IkkβΔ* cells grew faster than *Ikkβ^{F/F}* cells; after 6 weeks, *IkkβΔ* 2° HCC volumes were four times greater than those of *Ikkβ^{F/F}* HCCs, and BrdU labelling indices (LIs) were increased, as expected (Koch *et al.*, 2009; He *et al.*, 2010). Likewise, HCCs from *IkkβΔ* di12 cells had greater proliferative indices than those of *Ikkβ^{F/F}* di12 when grown in MUP/uPA mice. These experiments suggest that IKKβ suppresses both spheroid formation and tumourigenicity. Of particular interest is that these spheroid-forming di12 cells, isolated from HCCs, are able to generate secondary HCCs after subcutaneous transplantation into C57BL/6 mice, whereas the aggregate-forming HcPC tumour progenitor cells, isolated from DEN-treated liver prior to tumour formation, do not form HCC under this condition (He *et al.*, 2013), distinguishing clearly between tumour progenitor and LCSCLC.

14.2.3 LCSCs and SPs

SP formation has been investigated widely with respect to CSCs and to LCSCs (Chiba *et al.*, 2006). Following staining with Hoechst 33342 dye, cells are separated by fluorescence-activated cell sorting (FACS) into SP and non-SP, based upon active uptake and efflux of the dye by SP via ATP-dependent membrane

transporter, ABCG2, and to a lesser extent ABCB1 (Golebiewska *et al.*, 2011; Liu *et al.*, 2011). Separation of SPs is particularly effective when both SP and non-SP fractions are used for comparison to other signature marker distributions, such as the correspondence to shRNA knockdown of cMyc, or spheroid formation, or tumourigenicity (see Holczbauer *et al.*, 2013).

Notably, Hoechst dye can be cytotoxic, and some of the fractional differences in tumour initiation or transplantation, for example, may be a result of this (Sell and Leffert, 2008; Golebiewska *et al.*, 2011). Finally, other ABC transporters, such as ABCB1, also effect uptake and efflux of Hoechst 33342, although with less efficiency than ABCG2. The cellular heterogeneity of transporters within a population, as well as culture and growth state conditions, can lead to variable results, such that the SP determination is not yet an accurate measure of specific transporter activity (Smith *et al.*, 2013).

14.2.4 LCSCs and drug resistance

Enhanced HCC recurrence and reduced patient survival are characterized by overexpression of ATP-dependent membrane ABC transporters, including the multigene multidrug resistance (MDR/P-glycoprotein) family containing ABCB1, which is particularly studied in liver (Kato *et al.*, 2001). These glycoproteins, and other transporter family members, accelerate drug efflux during chemotherapy (Dean *et al.*, 2005; D'Alessandro *et al.*, 2007; Sukowati *et al.*, 2010; Golebiewska *et al.*, 2011; Liu *et al.*, 2011; Oishi and Wang, 2011). HCC procarcinogens like aflatoxin B₁ and AAF upregulate the formation of reactive oxygen species (ROS) and stimulate transporter overexpression (Kuo and Savaraj, 2006). This overexpression has been reported in CD90⁺CD133⁺ LCSC spheroid-forming 1^o HCC cultures (Tomuleasa *et al.*, 2010) and in xenoengraftable CD44⁺CD133⁺ HCC cell lines (Zhu *et al.*, 2010). As previously noted, the expression of some of the transporters to effect rapid efflux of Hoechst dyes is the basis of SP separations, which presently lack specificity of identification of the many members in the large drug-resistance gene family. Although many cells, particularly in the liver, express drug-resistance genes, knowledge of the dynamic spectrum of drug-resistance expression in the putative LCSC population may be critical for potential detection and targeting if LCSCs are found to form a drug-resistant pool of liver cancer precursors which seed recurrent tumours.

14.2.5 LCSCs and LCSC-like markers

Table 14.2 summarizes the properties of some markers considered important in the classification of LCSCs and LCSCLCs (Klonisch *et al.*, 2008; Sell and Leffert, 2008; Liu *et al.*, 2011; Yamashita and Wang, 2013). For example, poor survival and accelerated relapse are reported in human patients with HCCs expressing CD13, CD24, CD44, CD90, CD133, Keratin 19, EpCam, OV6, AFP and *SALL4*. Just as expression in individual HCCs is variable, most

Table 14.2 Markers of putative LCSCs and LCSCLCs.

Marker or marker set	Comments	References
CD13	Enhances growth of human HCC cell lines and inhibits <i>in vivo</i> relapse of CD13 ⁺ HCCs	Haraguchi <i>et al.</i> (2010)
CD24	Mouse TICs form CD24 ⁺ HCCs through STAT3-mediated NANOG regulation; sporadic expression in human tissue HCCs	Lee <i>et al.</i> (2011)
CD44	CD44 ⁺ CD133 ⁺ HCC cell lines; both markers confer aggressive xenoengraftment	Zhu <i>et al.</i> (2010)
CD90	CD90 ⁺ HCC human cell lines and tissue HCCs; CD90 ⁺ CD44 ⁺ HCC cells generate HCC nodules in liver and produce lung metastases in SCID mice	Yang Z.F. <i>et al.</i> (2008)
CD133	Most prevalent HCC CD marker, induced by HBV and HCC infections; HCC expression predicts reduced survival and confers malignant potential by regulating metalloproteinases in HCC cell lines; BMP4 induces differentiation in HCC lines and blocks their tumorigenicity and drug resistance after xenoengraftment; BMP4 can be isolated from normal mouse liver	Ma <i>et al.</i> (2007, 2013); Kohga <i>et al.</i> (2010); Rountree <i>et al.</i> (2011); Sasaki <i>et al.</i> (2010); Zhang <i>et al.</i> (2012); Ma (2013)
EpCAM	Human EpCAM ⁺ AFP ⁺ HCC tissues display properties of self-renewal and aggressive xenoengraftment	Terris <i>et al.</i> (2010)
β -catenin ^a	Human mutation and overexpression, and depletion of hepatocyte β -catenin in adult knockout mice promote LSC and HCC formation; poor survival is also seen in human patients	Wong <i>et al.</i> (2001); Wang <i>et al.</i> (2011)
Keratin 19	Poor prognosis for patients with keratin 19 ⁺ HCCs; these HCCs also express CD133 and EpCam	Kim <i>et al.</i> (2011)
Anti-Ca ²⁺ channel MnAb 1B50-1	Targets human TICs from HCC tissues; binds to HCC Ca ²⁺ channel α 2 δ 1 subunit; HCC lines also express CD13, CD133, AFP and EpCam	Zhao <i>et al.</i> (2013)
h-Dlk-1	Associated with foetal liver progenitor cells; expressed in 20% of adult human HCCs, and more in patients younger than 50 with AFP ⁺ HCC	Yanai <i>et al.</i> (2010); Nishina (2012)
CD44, CD133, EpCAM, CD90 and AFP	Unique combinations of CDs and other LCSCLC markers are expressed in HCC tissues from different patients, and in CD133 ⁺ CD90 ⁺ biopsies	Tomuleasa <i>et al.</i> (2010); van Malenstein <i>et al.</i> (2011); Wilson <i>et al.</i> (2013)

(continued overleaf)

Table 14.2 (continued)

Marker or marker set	Comments	References
<i>SALL4</i>	Oncofetal protein not found in normal hepatocytes but high in a subset of human xenoengraftable HCCs; confers poor prognosis; <i>SALL4</i> elevates PTEN expression; PTEN fusion TAT peptide reduces HCC cell line formation in SCID mice	Yong <i>et al.</i> (2013)
ALDH ^{HIGH}	Co-expression of CD133 ⁺ CD90 ⁺ is correlated with human tissue ALDH ^{HIGH} LCSCs	Lingala <i>et al.</i> (2010)
SPs	Expression of CSC and LCSCs SPs is controversial	Sell and Leffert (2008); Golebiewska <i>et al.</i> (2011)

^a Marker-related effects.

MnAb, monoclonal antibody; TAT, transactivator of transcription protein transduction domain; ALDH, aldehyde dehydrogenase

markers are also expressed variably in isolated putative TICs and LCSCs. One of the longstanding difficulties in identifying and isolating LSCs and LCSCs has been the lack of specific markers by which to distinguish these cells. Identification now rests upon the functional and structural contexts in which these cells are found, in conjunction with expression of a panel of likely markers. The current genome-transcriptome-proteome projects should increase the prospects for specific markers, or perhaps unique subsets of markers.

14.2.6 LCSCs and limiting-dilution xenoengraftment

Co-expression of CD44⁺CD133⁺ in human HCC cell lines appears to correlate with augmented xenoengraftment in severe combined immunodeficiency disease (SCID) mice (Ma *et al.*, 2007; Zhu *et al.*, 2010; Ma, 2013). Similar observations have been made in HCC lines that overexpress Bm1 (Wang *et al.*, 2008), the expression of which is also associated with reduced patient survival (Chiba *et al.*, 2007). Heightened xenoengraftment of human CD90⁺, OV6⁺ and *SALL4*⁺ LCSC tissues in immunocompromised mice has also been reported (Table 14.2) (Yang Z.F. *et al.*, 2008; Yong *et al.*, 2013). Recent attempts to xenoengraft or transplant with low doses of LCSCs have also been reported.

In one case, in order to isolate LCSCs from the human HCC line PLC/PRF/5, label-retaining cancer cells (LRCCs) were sorted and collected by FACS for high-Cy5 dUTP (Cy5) labelled DNA, following one labelling cell cycle. After this first sorting into high and lowest Cy5, the high fraction was grown for eight more cycles without Cy5; the high Cy5 (Cy5^{high}) and the lowest Cy5 (Cy5^{low}) were then again sorted and collected. After this, either 10 Cy5^{high}

cells or 10 Cy5^{low} cells were injected subcutaneously into nude/SCID mice and followed for 16 weeks. From the non-LRCC fraction, 2 of 20 mice developed tumours; from the Cy5^{high} fraction, 14 of 20 had tumours (Xin *et al.*, 2012).

In the second report, 100 cells of the CD44-positive fraction of C57BL/6 HcPCs, collected 3–5 months after DEN treatment, were transplanted intrasplenically into MUP/uPA mice. Zero of five and zero of three mice formed tumours after dosing with 100 sorted CD44-negative cells and 100 unsorted cells, respectively, whereas a dose of 100 sorted CD44-positive cells generated tumours in two of four mice. At higher doses, all three groups formed tumours in all mice. These HcPCs required the chronically injured MUP/uPA mouse as recipient, and they would not form subcutaneous tumours in BL/6 mice as the diH cells would do. The diH strains are LCSCs, having been isolated from HCCs, whereas the HcPCs are tumour-initiating or tumour progenitor cells, having been isolated before the appearance of a tumour (He *et al.*, 2010, 2013).

Finally, there is one report of xenoengraftment of LCSCs isolated from primary human HCC tumour (Muramatsu *et al.*, 2013). These cells were enriched with a nice system to mark asymmetry of division (see Section 14.2.7). Following subcutaneous injection of a range of doses into nonobese diabetic (NOD)/SCID mice, 100 of the self-renewing enriched cells produced HCC in three of six mice, while 100 of the unsorted cells produced tumours in zero of six mice. The low 26S proteasome activity utilized in the asymmetry determination for sorting these LCSC populations had previously been found to be distinctive of CSCs of human glioma and breast, and low ROS can be characteristic of haematopoietic stem cells and breast CSCs.

14.2.7 LCSCs and asymmetric cell division

In two of the systems reporting enhanced tumorigenicity in xenoengraftment studies, the asymmetry of cell division was the distinguishing characteristic by which the cells were sorted. Both studies capture live asymmetric division of putative LCSCs: from HCC cell lines in the first case (Hari *et al.*, 2011; Xin *et al.*, 2012) and from cultures established from four primary human resected HCCs, as well as from cell lines, in the second (Muramatsu *et al.*, 2013). In the first case, capture was with confocal microscopy cinematography; in the second, with 21-hour timelapse video microscopy. The first system utilized DNA labelling strategies to examine the roles of nonrandom sister chromatid segregation in asymmetric division and used label retention to examine asymmetry and/or slow cell cycling.

In order to test for nonrandom sister chromatid segregation, human HCC line PLC/PRF/5 cells were grown for one DNA cycle with microporated Cy5-dUTP, then sorted by FACS to enrich for a Cy5^{high} population. These cells were grown through the second cycle with Alexa555-dUTP, and the population was then sorted by FACS into Alexa555^{high} and into Alexa555^{high} + Cy5^{high}. A pair of cells, each with both Alexa555 and Cy5 nuclear labelling,

were daughter cells of a symmetrical division, while another pair of cells, one labelled with Alexa555 alone, and the other with both Alexa555 and Cy5, had undergone asymmetric division, presumably by one cell retaining the parental strands. That this asymmetric division had occurred was confirmed by Z-stack registration from confocal microscopy of single cells in cytokinesis, with one dual-labelled nucleus and one singly-labelled nucleus, both still in the same cytoplasm, and both still held together by a bridge of this cytoplasm. To examine label retention, the same PLC/PRF/5 cells were singly labelled for the first cycle with Cy5-dUTP, as previously, and then FACS sorted for collection of Cy5^{high}. Following eight more cycles of growth without label, consistently more cells were Cy5^{high} labelled than expected by dilution from symmetric division: 1.54–5.00%, versus 0.39% expected, suggesting that significant label retention had occurred by cell cycle slowing or asymmetric division, possibly by nonrandom chromosome segregation. It was this population of Cy5^{high} cells that was further collected for tumourigenicity studies by xenoengraftment, as described earlier (Xin *et al.*, 2012).

The second study takes advantage of the distinctive metabolic properties of low 26S proteasome and low mitochondrial superoxide, characteristic of CSCs of glioma, breast and haematopoietic systems. The four cultures from freshly isolated human HCC resections were retrovirally transduced with the ZsGreen-fused degron sequence of ODC protein (Gdeg). This is a specific substrate for 26S proteasome degradation, so that expression of the ZsGreen reporter is inversely proportional to 26S proteasome activity. In order to measure ROS, cells can be incubated with MitoSOX Red and can be sorted by FACS for either of these metabolic markers. The percentage of Gdeg^{high} cells in the primary HCC populations was low, at 0.1%. After four passages of growth to expand the transduced human primary HCC cells, they were sorted by FACS into Gdeg^{high} and Gdeg^{low} and grown in separate culture for 21-hour timelapse video microscopy (Figure 14.3). Gdeg^{high} cells divided asymmetrically into a Gdeg^{high} cell and a Gdeg^{low} cell, but Gdeg^{low} cells never divided into Gdeg^{high}, only into more Gdeg^{low}. As already discussed, the Gdeg^{high} ROS^{low} cells were significantly more tumourigenic in NOD/SCID xenoengraftment than unsorted cells (Muramatsu *et al.*, 2013).

14.2.8 OCs as LSCs

With regard to OCs, the principal question is whether LSC → OC → LCSC transitions occur. Are OCs an LSC, or a type of LSC? Do they become LCSCs? Developmental liver lineage studies indicate that embryonic endodermal and mesodermal interaction precedes the appearance of embryonic liver bud and progenitor cells, and that these in turn precede the formation of embryonic hepatocytes and bile duct epithelia (BDE) (Lemaigre and Zaret, 2004), but the exact lineage of adult LSCs with respect to these embryonic liver progenitor cells is presently unknown with completeness.

Figure 14.3 Asymmetric cell division of a single Gdeg^{high} HCC cell. Panels (A)-(D), from left to right, are single frames from time lapse videomicroscopy over a period of ~21 hrs. Single cells are ~25 μ m in diameter. (A) 0 hour, 1st cytokinesis begins. (B) 7 hour, 1st cell division ends. One green daughter (top); one unstained daughter (bottom). (C) 12 hour, unstained daughter cell divides; progeny remain unstained. Green parental cell does not divide further. (D) 21 hour, unstained daughter cells continue to divide (bottom); single green parental cell does not. See original article and supporting time lapse video; HEP_26345_sm_SuppMov.AVI. Taken from Figure 1B, p. 222, of Muramatsu S. *et al.* (2013) Visualization of stem cell features in human hepatocellular carcinoma reveals in vivo significance of tumor-host interaction and clinical course. *Hepatology*, 58(1), 218–228, © 2013 American Association for the Study of Liver Diseases; Reproduced with kind permission of John Wiley and Sons.

Liver OCs, widely considered to be rare adult LSCs, were first reported in chemical hepatocarcinogenesis studies (Price *et al.*, 1952; Farber, 1956; Abelev, 1971; Shinozuka *et al.*, 1978; reviewed in Sell and Leffert, 1982, 2008; Koch and Leffert, 2004). OCs appear, or increase greatly in number, after some types of injury, but decrease with age and tissue turnover (Guest *et al.*, 2010; Sell, 2010). They are $\leq 6 \mu$ m diameter, whereas binucleated hepatocytes are $\sim 40 \times 60 \mu$ m in size (see Sell and Leffert, 1982). OCs are rare; given the fact that they are really only detectable following extensive proliferation after injury, and yet, therefore, still assuming that they exist in the normal animal in very small numbers, their numbers have been estimated at 1×10^{-5} cells/0.8 g mouse liver (Braun and Sandgren, 2000). OCs show high nucleus to cytoplasm ratios (see Sell and Leffert, 1982) and express AFP (see Sell, 2010), CK19, CK7 and many other markers, according to growth state, species and specific toxic and carcinogenic conditions.

OCs are thought to be involved in normal hepatocellular repair when hepatocyte proliferation is blocked by carcinogens or other toxic chemicals (Trautwein *et al.*, 1999; Yovchev *et al.*, 2008). Depending upon the toxin or chemical carcinogen, this OC proliferative process can encompass injury to bile ducts, as well as to hepatocytes and NPCs in different lobular zones. Adult OCs are widely said to be bipotential: they appear to be able to form hepatocytes and BDE. When excessive hepatotoxicity occurs, OCs are activated to grow periportally, or adjacent to injury sites, where they are thought to differentiate and compensate for tissue deficits. For example, AFP⁺ OCs are induced periportally in the location of alcohol dehydrogenase (Leffert *et al.*, 1987), after zone 1-specific allyl alcohol injury (Guest *et al.*, 2010), and

are ultimately found dispersed but initially centrilobularly, in the location of Cyp2E1 and Cyp2B1/2 carcinogen and drug-metabolizing enzymes (Guenrich, 2000), after zone 3-specific CCl₄ injury and DEN toxicity. It has been suggested for some time, following very careful [3H]thymidine labelling and microscopy studies in mice treated with one dose of Dipin followed by partial hepatectomy, that a small pool of OCs within the Canal of Hering and in contact with immediately adjacent periportal hepatocytes progresses through a series of transitional cells to small hepatocytes, while apparently spreading portally to centrally through the liver acini, to replace damaged and dying hepatocytes. Following common bile duct ligation (BDL), on the other hand, bile duct proliferation occurred in the portal area, which appeared to proceed by way of BDE cells, which were larger and had a lower nucleus to cytoplasm ratio than OCs. In this model, microscopy did not support these cells proceeding to hepatocytes, but only to BDE. It was noted that a progression of OCs could indicate that there were a number of OC subtypes in various stages of growth and differentiation, or that the progression through various OC specifications was sensitive to variable microenvironment (Factor and Radaeva, 1993; Factor *et al.* 1994). It seems possible that these Canal of Hering OCs and portal BDEs represent cells from two of the Kuwahara label-retaining niches (Kuwahara *et al.*, 2008; see Section 14.1.5).

Several recent fate-mapping investigations have suggested independently that some subtypes of mouse OCs, or LSCs, generated following different conditions of adult liver injury, can be traced from cells marked during embryogenesis; or, possibly, following certain types of liver injury, from HSCs, marked during adult quiescence, prior to injury. Short descriptions of these studies follow:

1. The progeny of a *Foxl1*-Cre cross to Rosa26R lacZ mice, subjected at 10–12 weeks of age to BDL, to toxic liver injury with an OC inducer (3,5-diethoxycarbamyl-1-4-dihydrocollidine, DDC) or to a choline deficient/ethionine (CDE) carcinogen diet, and followed for 21 days, exhibited a significant immediate induction of β -gal⁺ cells in periportal bile ductular reactions, which, in time, extended, by appearance of blue hepatocyte-appearing cells, out towards the parenchyma. By 3 days post BDL, some double-positive, blue β -gal⁺ CK19⁺ cells (suggestive of BDE-type differentiation) were seen in the portal bile ductular reactions; by 5 days, some blue β -gal⁺ hepatocyte-like cells appeared around the portal biliary reactions; and by 14 days, a few double-positive blue β -gal⁺ HNF-4- α ⁺ cells (suggestive of hepatocellular differentiation) were seen as the β -gal⁺ CK19⁺ cells began to wane and a population of β -gal⁺ CK19⁻ HNF-4- α ⁻ cells was peaking. The E12.5 liver-bud primordium did not stain blue in this mouse, suggesting that *Foxl1* was not expressed in these embryonic cells; it is likely that the blue β -gal⁺ cells originated from somewhere other than the hepatic liver bud, or that expression of *Foxl1* occurs later in development. There were negligible to no blue cholangiocytes in sham animals. Both DDC injury and CDE carcinogen

diet also induced rapid and large β -gal⁺ ductular reaction populations, and, within days, subsets of these were β -gal⁺ CK19⁺, but followed by many fewer β -gal⁺ HNF-4- α ⁺ cells in comparison to the BDL animals. In all three injury models, the initially observed blue cells appeared to be in the intraductal cholangiocyte, and in the peribiliary label-retaining niches of Kuwahara (Kuwahara *et al.*, 2008; see Section 14.1.5). Because no triple-positive cells were scored and there was a β -gal⁺ CK19⁺ HNF-4- α ⁺ population peak, intermediate in time, it seems possible that either the initial Foxl1-positive population is composed of two subtypes or a single population is bipotential (Sackett *et al.*, 2009).

2. At least two *Sox9* promoter-tamoxifen inducible-Cre;*Rosa26R* mouse model systems were employed to mark *Sox9*-expressing cells and their descendants, generally with YFP or lacZ and GFP *Rosa* reporters. Because *Sox9* expression in embryogenesis is exclusive at E15.5 to ductal plate-forming cells, transient tamoxifen injection of the pregnant *Sox9*-Cre;*Rosa* YFP females at E15.5 caused Cre recombination in the *Sox9*-expressing ductal plate cells, thereby marking them and their descendants with constitutively expressed YFP. Subsequent examination of these livers 4 weeks postnatally indicated YFP⁺ cholangiocytes of the interlobular bile ducts and intralobular biliary ductules and the Canals of Hering, as well as a small number of zone 1 YFP⁺ hepatocytes. Following known OC-inducing CDE and DDC diets, given at 4 weeks postnatally, for 21 and 14 days, respectively, to these E15.5 *Sox9*⁺ ductal plate YFP-marked animals, these livers exhibited significant YFP⁺ periportal OC proliferation, suggesting that at least one significant subtype of OC had been derived from the ductal plate cells. Perhaps because these diets do not cause significant liver injury when given briefly, there was no apparent accumulation of new YFP⁺ hepatocytes greater than the limited population of control YFP⁺ periportal hepatocytes. There is no evidence as yet for a lineage from the ductal plate *Sox9* OC to the hepatocyte, the test of which will perhaps require a different or more chronic injury model than the protocols used here. Nevertheless, in this system, except for the limited periportal YFP⁺ hepatocytes, the initial population of hepatocytes did not appear to have originated from the E15.5 *Sox9*⁺ ductal plate cells, nor did these marked ductal plate cells or progeny appear to be involved in normal hepatocellular homeostasis up to 4 weeks postnatally (Carpentier *et al.*, 2011; Dorrell *et al.*, 2011). Finally, on the other hand, examination of the liver of a *Rosa26R* LacZ mouse containing a different *Sox9*-Cre construct, in which the tamoxifen induction-Cre recombinase sequence was placed within the 3' untranslated region of the native *Sox9* locus, rather than within a bacterial artificial chromosome as in the previously described system, revealed β -gal labelling of a *Sox9*-expressing biliary progenitor pool within 1 day following high-dose tamoxifen injection at 8 weeks postnatally. Within 10–30 days post injection, the hepatocyte population became β -gal labelled from the portal to the centrilobular regions, in

contrast to any progression following from the E15.5 marking in the previous *Sox9* system. Although a subtype of 8-week-postnatal hepatocytes might actually express *Sox9*, sufficiently for Cre-recombination, in which case tamoxifen-induced β -gal labelling of these hepatocytes would be indistinguishable from labelling as a descendant of any other labelling, it might also be possible that the 8-week-postnatal intrahepatic biliary cells were expressing *Sox9*, and had, in fact, become the precursors to hepatocytes. It is not really possible to compare results from tamoxifen induction at E15.5 with those from induction at postnatal 8 weeks. There is, in fact, a suggestion that there is a 'switch' from the early embryonic hepatocyte origin from *Sox9*⁻ hepatoblasts to postnatal origin from *Sox9*⁺ intrahepatic biliary cells. It is interesting to note that in this system, the postnatally marked *Sox9* biliary cells did not appear to contribute to the hepatocellular proliferative response following DDC injury or partial hepatectomy, but were a precursor to a marked hepatocyte component following a methionine/choline deficient/ethionine (MCDE) diet (Furuyama *et al.*, 2011). Clearly, these animal systems are complex, and promising.

3. The progeny of a glial fibrillary acidic protein (GFAP)-Cre X Rosa GFP mouse cross (GFAP-Cre/GFP) were used to investigate the fate of GFAP-expressing, quiescent hepatic stellate cells (Q-HSC), which downregulate GFAP, synthesize alpha smooth-muscle actin (α -SMA) and begin to proliferate as myofibroblastic hepatic stellate cells (MF-HSCs) following chronic liver injury, such as occurs in rodents with an MCDE diet. In normal, untreated 3–4-month-old GFAP-Cre/GFP mice, there were GFAP⁺GFP⁺ sinusoidal Q-HSCs, as well as unexpected GFAP⁺GFP⁺ portal bile ductular cells, and neither GFAP⁺ nor GFP⁺ hepatocytes. Following MCDE diet administration, the portal biliary ductular cells remained GFAP⁺GFP⁺ and did not proliferate significantly, whereas the Q-HSCs became GFAP⁻GFP⁺ and proliferated greatly, particularly within the centrilobular and midzonal sinusoids. Over a 6-week period, involving diet for 3 weeks and return to normal for 3 weeks, a few isolated, and then increasingly large numbers of hepatocytes became GFP⁺ (GFAP⁻), from centrilobular to midzonal to portal. Most of the proliferating GFAP⁻GFP⁺ HSCs began to express the myofibroblastic α -SMA progressively from central to portal sinusoids, followed by an occasional GFP⁺ α -SMA⁺ hepatocyte, also expressing progenitor AE1/AE3 reactive cytokeratins, in the same zones; then, as the α -SMA expression declined rapidly in HSCs, and then in hepatocytes, and the GFP⁺ HSCs disappeared, a large proliferation of albumin-expressing GFP⁺ hepatocytes, negative for AE1/AE3 cytokeratins, spread periporally, ultimately making up one-third of the hepatocellular population. It would appear that, following MCDE injury in these mice, HSCs might

give rise to mature hepatocytes by undergoing a transition through a hepatic progenitor (Yang L. *et al.*, 2008).

To date, these fate-mapping studies have raised many new questions, but have not yet mapped OCs → HCCs or OCs → LCSCs. Are early alterations proceeding from liver injury found in the proliferating population of responding OCs, or are there changes in other injured (adjacent) cells, such as hepatocytes? And which altered cells lead to HCCs or to LCSCs? It is, perhaps, instructive in this regard that mouse adult hepatic progenitor cells (HPCs), hepatoblasts (HBs) and adult hepatocytes (AHs), all transduced with H-Ras and SV40LT, contained SPs with LCSC properties. All three transduced cells of origin, following single-cell clonal expansion and transplantation of each line, generated HCC, cholangiocarcinoma (CCA) and combined hepatocellular cholangiocarcinoma (CHC), but in consistently different lineage-based ratios, thus, presumably, indicating that single cells from these transduced lines were multipotent but still retained the signature of their lineage of origin. Transcriptome comparison with the cells of origin indicated that the AHs required the greatest dysregulation of gene expression in the acquisition of tumour-initiating capacity or tumour formation, followed by HBs and HPCs requiring less (Holczbauer *et al.*, 2013). It seems quite possible that the OC, its subtypes, and various injury states will be a part of this group of cells which are LCSCs, or are progressing to LCSCs, and to liver cancers.

14.2.9 LCSCs and polyploidy

Postnatal hepatocytes are mononucleated and diploid. With maturation, more than half of hepatocytes become binucleated and polyploid (Gupta, 2000; Baratta *et al.*, 2009; Celton-Morizur *et al.*, 2010). Hepatocytes are 2N, 4N (or 2N + 2N), 8N (or 4N + 4N, or 4N + 2N + 2N, etc.) or 16N (8N + 8N, etc.) per cell, or more. Polyploidy increases with liver age (Enesco and Samborski, 1983) and after 70% partial hepatectomy (Gentric *et al.*, 2012).

It has recently been found that not only is polyploidy generated, usually by failed cytokinesis, and also probably by endomitosis and endoreplication, but it is also reduced by multipolar mitotic spindle formation and/or by chromosome missegregation, to form daughter cells of reduced ploidy or of aneuploidy (Duncan *et al.*, 2010). Of particular interest is that aneuploidy in daughter cells includes abnormal chromosomal numerical value, and can also include genetically monoallelic gene expression as a result of segregation of uniparental chromosome sets. Just as the normally 5–10% of epigenetically monoallelically expressed genes provide opportunities for adaptation with diversity (Gimelbrant *et al.*, 2007), so it is proposed that each aneuploid hepatocyte or clone newly formed by ploidy reduction could represent a unique clonally expandable possibility of viable genetic diversity that, particularly under conditions of xenobiotic or chemical stress common to the liver, represents a heritable genotypic chance of resistance (Duncan *et al.*, 2010). Clearly, resistance is potentially either deleterious or beneficial.

That an extant pool of genotypically slightly diverse cells might well facilitate either resistance to or progression to tumour initiation, or to tumour evolution, is apparent by examination of the evolutionary trees of single-cell DNA sequencing of two human breast cancers and their liver metastases (Navin *et al.*, 2011). The polygenomic tumour contains only three subpopulations, each separated from the others probably by a single major genetic event and clonal expansion of the previous subpopulation, one of which is the metastatic population. There are many ‘primary pseudodiploids’, which have deviated probably from the earliest subpopulation but have neither expanded nor metastasized. The consequences of the ploidy genotype, expansion and reduction within the normal or injured hepatocyte population on this process of tumourigenesis, and on the generation of LCSCs (see Zhang *et al.*, 2014, for polyploidy in an ovarian system), await further study.

14.2.10 LCSCs and bystander effects

Bystander effects have been defined as soluble signalling by radioactive metabolites via the microenvironment (Barcellos-Hoff and Brooks, 2001). Similar effects might apply to the induction of HCCs by chemical procarcinogens (Alison, 2005; Fukushima *et al.*, 2005). For instance, Cyp1A2⁻ OCs, stimulated to proliferate in order to renew a population of hepatocytes lost by hepatocellular death due to carcinogen toxicity (e.g. AAF), might be exposed to the activated carcinogen and to subsequent mutation by diffusion of such activated carcinogen from the extant Cyp1A2⁺ hepatocyte, acting in a paracrine manner to crossfeed undifferentiated OCs. The chronology of OC differentiation from Cyp1A2⁻ OC to ALB⁺Cyp1A2⁺ OC may well affect the outcome of such injury. Such bystander effects represent some of the complexities in perturbation, subsequent and secondary genetic or epigenetic OC alteration and respecification of OC or LSC transitions to HCC and/or LCSC.

14.2.11 LCSCs and niche

Four stem cell niches have been identified in liver by the DNA label-retaining cells found within them (Kuwahara *et al.*, 2008; see Section 14.1.5). These cells are all putative stem cells with apparently different sizes and shapes, and each responds with proliferation to different injury models. Their developmental lineages are being slowly deciphered by recent fate-mapping studies, but there are indications that some of these cells may be a step in transition to one of the others, in which case there must be interaction between their respective niches. Many of these cells, OCs in particular, are so rare as to be visible only in proliferation following injury, and often in response to concomitant hepatocellular loss, so that it seems possible that OCs exist only as injured cells. But until proven otherwise, they are considered normal stem cells. There is no definitive evidence that they are precursors to liver cancers, to LCSCs or to tumour progenitor cells, or that their niches are potential sites of origin of LCSCs or of tumour progenitor cells.

The most reliable niche in which to activate tumour progenitor cells is the chronically injured liver, but until these progenitor cells and LCSCs can be identified for visualization before isolation, it is difficult to study them in their microenvironments. On the other hand, significant progress is being made in defining the vascular niche of the liver sinusoidal endothelial cells, and the interplay between regenerative CXCR7 and fibrotic CXCR4 ‘angiocrine’ paracrine pathways, during acute and chronic liver injuries (Ding *et al.*, 2014).

14.3 Conclusions

Although populations of putative LSCs often proliferate massively following delivery of certain types of liver injury, such as carcinogen feeding, the origins and dispositions of these cells are not yet known; they are thought to replenish the hepatocyte population following injury, but there is currently no direct evidence for this. And because these cells appear in large numbers in response to liver injury, which is itself an environment conducive, if not requisite, for liver cancer formation, it is also possible that these proliferating LSCs are tumour progenitors. If so, it is important to determine their relationship to LCSCs, as well as the origins of LCSCs within the tumour which have already progressed to capability of full malignancy, but which, putatively, by definition, cycle within the tumour, or its environment, as a relatively quiescent stem cell parent, capable of giving rise asymmetrically to actively malignant progeny cells. The origins of these cells are also not known. Do they arise before or after overt malignant capability of the tumour? What is the lineal relationship of both of these populations to normal or injured LSCs? A clear understanding of the progression to malignancy of all these cell populations is necessary for the development of early detection and of treatment strategies.

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While writing this article, the authors were supported by the National Cancer Institute of the National Institutes of Health under Award Number R21CA161510 to K.S.K. and H.L.L. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We apologize to any colleagues whose work could not be cited due to space limitations.