# **REVIEW**

# Liver cancer: the role of stem cells

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Abstract. Studies of aggregation chimaeras and X-linked polmorphisms strongly suggest that liver tumours are derived from single cells (monoclonal), but the important question is, which cell? Stem cell biology and cancer are inextricably linked. In continually renewing tissues such as the gut mucosa and epidermis, where a steady flux of cells occurs from the stem cell zone to the terminally differentiated cells that are imminently to be lost, it is widely accepted that cancer is a disease of stem cells, since these are the only cells that persist in the tissue for a sufficient length of time to acquire the requisite number of genetic changes for neoplastic development. In the liver the identity of the founder cells for the two major primary tumours, hepatocellular carcinoma and cholangiocarcinoma, is more problematic. The reason for this is that no such obvious unidirectional flux occurs in the liver, although it is held that the centrilobular hepatocytes may be more differentiated (polyploid) and closer to cell senescence than those cells closest to the portal areas. Moreover, the existence of bipotential hepatic progenitor cells, along with hepatocytes endowed with longevity and long-term repopulating potential suggests there may be more than one type of carcinogen target cell. Cell proliferation at the time of carcinogen exposure is pivotal for 'fixing' any genotoxic injury into a heritable form, thus any proliferative cell in the liver can be susceptible to neoplastic transformation. Hepatocytes are implicated in many instances of hepatocellular carcinoma, direct injury to the biliary epithelium implicates cholangiocytes in some cases of cholangiocarcinoma, while hepatic progenitor cell/ oval cell activation accompanies many instances of liver damage irrespective of aetiology, making such cells very likely carcinogen targets. Of course, we must qualify this assertion by stating that many carcinogens are both cytotoxic and cytostatic, and that hepatic progenitor cell proliferation may be merely a bystander effect of this toxicity. An in-depth discussion of causes of cancer in the liver is beyond the scope of this review, but infectious agents (e.g. hepatitis B and C viruses) play a major role, not just in transactivating or otherwise disrupting cellular proto-oncogenes (hepatitis B virus), but also in causing chronic inflammation (hepatitis C and B viruses). Sustained epithelial proliferation in a milieu rich in inflammatory cells, growth factors and DNA-damaging agents (reactive oxygen and nitrogen species – produced to fight

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infection), will lead to permanent genetic changes in proliferating cells. Up-regulation of the transcription factor NF- $\kappa$ B in transformed hepatocytes, through the paracrine action of TNF- $\alpha$  from neighbouring endothelia and inflammatory cells, may be critical for tumour progression given the mitogenic and antiapoptotic properties of proteins encoded by many of NF- $\kappa$ B's target genes.

#### INTRODUCTION

This review focuses on the cellular origins of the two major primary cancers of the liver, hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). HCC is the fifth most common cancer worldwide and the third most common cause of cancer death. HCC is defined by the World Health Organization as a malignant tumour composed of cells resembling hepatocytes but abnormal in appearance; a plate-like organization around sinusoids is common and is nearly always present somewhere in a tumour. Most HCCs (80%) arise in a cirrhotic liver, i.e. a situation where there has been long-standing hepatocyte damage and chronic inflammation leading to fibrosis. There are huge geographical variations in the incidence of HCC, with the highest incidence in areas such as eastern Asia and sub-Saharan Africa where chronic hepatitis B virus (HBV) infection is a major risk factor (Brechot 2004). In Europe and USA, the incidence of HCC is low but slowly increasing, probably as a result of the rise in people infected with hepatitis C virus (HCV). Apart from hepatotropic viruses, the other major risk factors for HCC are other conditions leading to cirrhosis such as alcohol abuse and metabolic liver disease, and mutagens such as aflatoxins, toxic metabolites of the food mould *Aspergillus* sp.

Cholangiocarcinomas are believed to arise from biliary epithelium that is either within the liver (intrahepatic) or the extrahepatic. The tumour is much less common than HCC, but its incidence and associated mortality has been increasing steadily over the past two to three decades, with most tumours arising in persons over 50 years of age, suggesting carcinogenesis is a protracted and (possibly) multi-step process (Sirica 2005). Injury to the biliary epithelium in chronic inflammation, together with impedance of bile flow, are common factors in high risk conditions for CC such as primary sclerosing cholangitis, hepatolithiasis (gall stones) and liver fluke infestation by *Opistorchis viverrini* and *Clonorchis sinensis*. Thus, chronic inflammation features prominently in the histogenesis of both HCC and CC, where the coordinated actions of enzymes such as iNOS and COX-2 will lead to oxidative DNA damage, cell proliferation and a suppression of apoptosis (Pikarsky *et al.* 2004).

## LIVER STEM CELLS: HEPATOCYTES

The fetal liver is clearly a source of bipotential progenitor cells (hepatoblasts) as seen by their ability to colonize the diseased livers of rats after transplantation (Alison *et al.* 2004). In postnatal animals, hepatocytes are highly differentiated cells with multiple synthetic and metabolic functions; they are also the functional stem cells in the liver under most circumstances. In health, individual hepatocytes have a life expectancy of over a year. Therefore, in the normal adult liver, there is little cell proliferation. However, in response to parenchymal cell loss, the hepatocytes restore the liver mass by self-replication. This is a very efficient system, and in rodents, when two-thirds of the liver is resected by partial hepatectomy (PH), the remaining remnant can re-

408

grow to the original liver size in approximately 10 days. This model has been intensively studied and has provided much data on the mechanisms controlling liver regeneration (Michalopoulos & DeFrances 1997; Fausto 2004). In response to this stimulus, the normally quiescent hepatocytes leave G<sub>0</sub> to enter the cell cycle under the influence of many growth factors. Hepatocyte proliferation begins in the periportal region of the liver and spreads to the centrilobular region. This regeneration requires each hepatocyte to undergo, on average, only 1.4 rounds of replication to restore the liver to its original size. This does not, however, mean that hepatocytes have a limited replication potential. Hepatocyte transplantation models in mice have shown that hepatocytes are capable of significant clonal expansion within the diseased livers of experimental animals. In the fumarylacetoacetate hydrolase (FAH)-deficient mouse, a model of hereditary type 1 typosinaemia, there is strong positive selection pressure exerted on transplanted wild-type hepatocytes as host hepatocytes readily undergo cell death because of the cytoplasmic accumulation of fumarylacetoacetate (FAA). Without transplantation, the FAH null genotype is lethal unless the mice are protected by 2-(2-nitro-4-trifluoro-methylbenzoyl)-1.3-cyclohexanedione (NTBC), a compound that prevents the accumulation of cytotoxic FAA. When 10<sup>4</sup> normal hepatocytes from congenic male wild-type mice are intrasplenically injected into mutant female mice and the NTBC treatment is withdrawn, these cells colonize the mutant liver efficiently (Overturf et al. 1997). Moreover, serial transplantations from such colonized livers to other mutant livers have indicated that at least 69 hepatocyte doublings can occur, thereby confirming the clonogenic potential of hepatocytes and fulfilling one of the crucial properties that define stem cells.

Many studies have examined the transplantation potential of adult hepatocytes, and provided a selection pressure has been applied to encourage the growth of the transplanted cells, they have largely confirmed that many hepatocytes are capable of significant long-term repopulation activity and clonal expansion. In particular, if hepatocytes are injected into recently hepatectomized syngeneic dipeptidyl peptidase (DPPIV)-deficient F344 rats that have been pretreated with the DNA-binding pyrrolizidine alkaloid retrorsine (to block indigenous liver regeneration) then the almost complete replacement of the recipient liver by donor hepatocytes can occur (Laconi et al. 1998). On the other hand, if rats are just administered retrorsine prior to being subjected to a two-thirds PH, regeneration is accomplished by the activation, expansion and differentiation of so-called small hepatocyte-like progenitors (SHPCs). These cells have shown phenotypic traits of fetal hepatoblasts, oval cells and fully differentiated hepatocytes, but they are morphologically and phenotypically distinct from all three (Gordon et al. 2000). Cytochrome (CYP) P450 enzymes have a pivotal role in hepatocyte function, but these cell clusters lacked CYP enzymes that are readily induced by retrorsine, and this probably accounted for their resistance to its antiproliferative effects. When such cells were established in short-term culture and then transplanted into syngeneic rats, they gave rise to differentiated hepatocytes (Gordon et al. 2002). There are data suggesting that these SHPCs are clonally derived, seemingly at random from amongst all mature hepatocytes (Avril et al. 2004); equally well they may have their origins in relatively undifferentiated progenitor cells.

The clonogenic ability of human hepatocytes in chronic hepatitis can be indirectly estimated. Using mathematical modelling of viral infection kinetics, it has been estimated that in chronic HBV infection, between 0.3% and 3% of all hepatocytes are killed daily and therefore replaced to maintain a stable liver cell mass (this approximates to  $10^9$  of the liver's  $2 \times 10^{11}$  hepatocytes) (Nowak *et al.* 1996). This accords with hepatocyte proliferation levels in chronic hepatitis B and C, where proliferating cell nuclear antigen (PCNA) indices of 0.1–3.6% have been found, and Ki-67 labelling indices in hepatitis C of 1–14% (Donato *et al.* 2002; Freeman *et al.* 2003). In chronic hepatitis, the parenchymal mass can therefore be maintained through prolonged hepatocyte self-replication and such cells could be the targets for DNA-damaging agents and thus initiation events.

Hepatocyte proliferation rate increases in hepatitis C with increasing histological damage until cirrhosis is reached, at which point the proliferation rate falls (Falkowski *et al.* 2003). The reason for this fall has not been fully resolved. It may represent the hepatocytes coming to the end of their division potential and undergoing replicative senescence (Marshall *et al.* 2005), although other factors such as distortion of blood flow through the liver are also likely to play a part. Whatever the reason, the reduction in hepatocyte proliferation indices in chronic hepatitis occurs concurrently with the activation of a potential stem cell compartment located within the smallest branches of the intrahepatic biliary tree. This so-called *ductular reaction* in human liver is equivalent to the *oval cell reaction* seen in many rodent models of hepatocarcinogenesis. The development of an oval cell reaction in response to hepatocyte replicative senescence has been demonstrated in a transgenic mouse model of fatty liver and DNA damage. In this model, mice developed fatty livers and a large number of senescent hepatocytes. A striking oval cell response occurred in these mice, which related to the number of senescent mature hepatocytes (Yang *et al.* 2004).

# LIVER STEM CELLS: OVAL CELLS (HEPATIC PROGENITOR CELLS)

#### Rodents

After very extensive liver damage or in situations where hepatocyte regeneration after damage is compromised, a potential stem cell compartment located within the smallest branches of the intrahepatic biliary tree is activated. This oval cell response or ductular reaction amplifies a biliary population of transit amplifying cells that are at least bipotential, capable of differentiating into either hepatocytes or cholangiocytes. Most rodent models of oval cell activation have employed potential carcinogens to inhibit hepatocyte replication in the face of a regenerative stimulus. For example in the rat, protocols have included administering 2-acetylaminofluorene (2-AAF) to inhibit hepatocyte proliferation before creating a demand for hepatocyte proliferation by partial hepatectomy or a necrogenic dose of carbon tetrachloride (Alison et al. 1996). The need to maintain parenchymal cell mass results in the development of an oval cell response in the liver that spreads from the edge of the portal tract to deep into the parenchyma (Fig. 1a). Oval cells are small cells with a large nuclear to cytoplasmic ratio, in which the nucleus has a distinctive ovoid shape. A wide range of markers has been used to identify oval cells including gamma-glutamyl transpeptidase (GGT) and glutathione-S-transferase (GST) activity (Alison 2003), along with a host of monoclonal antibodies raised against cytoskeletal proteins and surface antigens (see Table 1). Because oval cells express some of the antigens traditionally associated with haematopoietic cells (c-kit, flt-3, Thy-1 and CD34), there has been speculation that hepatic oval cells were directly derived from bone marrow precursor cells. However, most studies now concur that the location of a stem cell niche for oval cells is in the canals of Hering, which is a transitional zone between the periportal hepatocytes and the biliary cells lining the smallest terminal bile ducts.

#### Humans

The human counterparts to the oval cells described in rodents are often referred to as hepatic progenitor cells (HPC). These have been observed after severe hepatocellular necrosis, chronic viral hepatitis, alcoholic liver disease and non-alcoholic fatty liver disease. Activation of this potential stem cell compartment leads to the formation of reactive ductules, anastomosing cords of immature biliary cells with an oval nucleus and a small rim of cytoplasm (Fig. 1b).



Figure 1. (a) An oval cell reaction in the rat liver. Oval cells, highlighted by cytokeratin 19 immunoexpression, branch out from the portal tract (PT). (b) An extensive ductular reaction in a human liver in response to parenchymal necrosis. Both hepatocytes and ductular cells express cytokeratin 18, but the strongest expression is in the ductular cells.

Table 1. Markers that have aided in the identification of oval cells in mammalian liver

OV-6 OC.2, OC.3, OC.4, OC.5, OC.10 BDS7 Thy-1 c-kit CD34 ABCG2/BCRP1 (breast cancer resistance protein) Connexin 43 CK7, CK19, CK14 AFP GGT (gamma-glutamyl transpeptidase) GST-P (placental form of glutathione-S-transferase) flt-3 ligand/flt-3 DMBT1 (deleted in malignant brain tumour 1) NCAM-1/CD56 (neural cell adhesion molecule 1) Chromogranin A PTHrP (parathyroid hormone-related peptide)



Figure 2. Ductular reaction in a case of massive hepatic necrosis due to acetaminophen toxicity, immunohistochemically stained for CK7. Note many intermediate cells still with a bilary-type staining pattern (CK7 +), but with a morphology midway between cholangiocytes and hepatocytes (courtesy of Professor Tania Roskams and with permission from John Wiley Publishers).

Differentiation towards the hepatocyte lineage occurs via intermediate hepatocytes, these are polygonal cells with a size and a phenotype intermediate between progenitor cells and hepatocytes (Roskams et al. 2003). After a submassive liver cell necrosis, reactive ductules, in continuity with intermediate hepatocytes, can be seen (Fig. 2), suggesting gradual differentiation into hepatocytes, analogous with what is seen in rat models of chemical injury associated with impaired hepatocyte replication. Elegant 3D reconstructions of serial sections of human liver immunostained for cytokeratin-19 have shown that the smallest biliary ducts, the canals of Hering, normally extend into the proximate third of the lobule (unlike those in rodents), and it is envisaged that these canals react to massive liver damage (like a trip-wire), proliferating and then differentiating into hepatocytes (Theise et al. 1999). Oval cell numbers in human liver rise with increasing severity of liver disease (Lowes et al. 1999). Some commentators have argued that this ductular reaction could be a ductular metaplasia of damaged hepatocytes rather than a stem cell response, but Falkowski and colleagues have clarified this issue in human liver. Using serial sections and 3D reconstructions of human liver cirrhosis, it was noted that intraseptal hepatocyte buds (small nodules of hepatocytes) were invariably connected to areas of ductular reaction (Falkowski et al. 2003), implying a stem cell origin of the buds. Furthermore 'cholestatic' hepatocytes were very largely not associated with a ductular reaction, clearly supporting the notion that ductular reactions are not metaplastic hepatocytes.

### LIVER STEM CELLS: BONE MARROW CELLS

Petersen *et al.* (1999) were the first to report that occasional oval cells/hepatocytes could be derived from circulating bone marrow cells, using the now familiar lethally irradiated and bone

marrow sex-mismatched transplanted animals (in their case, rats), subsequently inflicted with liver damage. Similar findings came from mice and ultimately humans, although the proportions of bone marrow-derived hepatocytes in human liver have ranged from nonexistent in some studies of long-term liver allografts, to over 40%. One suspects that this variation is partly due to differences in the severity of parenchymal damage, but equally well may be a reflection of the ability (or lack of it) of the intrahepatic stem/progenitor cells to mount an effective regenerative response to damage.

The 'proof of principle' demonstration that bone marrow could cure mice with a potentially fatal metabolic liver disease, hereditary tyrosinemia type 1, was a landmark publication (Lagasse et al. 2000). In the setting of liver failure, wild-type bone marrow could apparently switch lineage determination and differentiate into hepatocytes expressing the enzyme 'fumarylacetoacetate hydrolase' (fah), the component of the tyrosine catabolic pathway absent in tyrosinaemic (fah-/-) animals. Such lineage switching has been called transdifferentiation or plasticity. However, it is now clear that the new functioning liver cells in the fah-/- mouse result from cell fusion between donor bone marrow-derived macrophages and fah-/- hepatocytes and their subsequent proliferation (Willenbring et al. 2004). From an oncology point of view, the interest in such heterokaryons is that many of these cells appeared to be genomically unstable, seemingly shedding chromosomes at random to become aneuploid (Wang et al. 2003). While the exact significance of bone marrow-derived cells to liver disease is far from fully elucidated, the fact that damaged hepatocytes can alter the lineage commitment of haematopoietic stem cells (HSCs) toward that of hepatocytes without cell fusion occurring (Jang et al. 2004) and that a number of studies now report on the ability of human cord blood mononuclear cells to give rise to hepatocytes in the livers of non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice (reviewed in Alison et al. 2004), the possibility that primary liver tumours could be initiated in bone marrow-derived cells can not be discounted.

The bone marrow may also indirectly influence liver stem cell responses and hepatocarcinogenesis. As discussed, the more or less permanent deposition of abnormal amounts of connective tissue (cirrhosis) may contribute to hepatocyte senescence and oval cell activation – the fibrogenic cells may be bone marrow-derived. In the mouse liver, transplanted bone marrow cells can acquire the phenotype of quiescent stellate cells and after liver injury become activated into alpha smooth muscle actin ( $\alpha$ SMA)-expressing cells (Baba *et al.* 2004), while in human cirrhosis, by examining sex-mismatched allografts, our group has shown that up to 40% of myofibroblasts are of bone marrow origin (Forbes *et al.* 2004). Moreover, we have shown that the desmoplastic (fibrotic) response that surrounds many carcinomas emanates from bone marrow-derived cells (Direkze *et al.* 2004) and, in HCC and hepatoblastoma, HGF produced by tumour-associated myofibroblasts undoubtedly promotes invasion and tumour growth (Neaud *et al.* 1997; van Schweinitz *et al.* 2000).

#### STEM CELLS AND CANCER IN THE LIVER

Assessment of DNA alteration in tumour cells allows a precise determination of their clonality. Where HBV is involved, there is no doubt that the integration of HBV-DNA into the hepatic genome is a significant event in hepatocarcinogenesis (Shafritz & Kew 1981; Shafritz *et al.* 1981; Brechot 2004). Moreover, inspection of viral integration sites among tumour cells clearly indicates that each tumour is monoclonal, i.e. is derived from a single cell (Esumi *et al.* 1986; Govindarajan *et al.* 1988; Yamamoto *et al.* 1999; Ng *et al.* 2003). Likewise, studies of HCC clonality based on restriction fragment length polymorphisms of X-linked genes such as the

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androgen receptor gene (HUMARA) come to the same conclusion (Zhang, Cong & Wu 2004). The important question is: which cell is involved in cancer initiation? As discussed previously, in the liver there are many cells endowed with longevity and long-term repopulating potential, suggesting there may be more than one type of carcinogen target cell. Irrespective of which target cell is involved, what is clear is that cell proliferation at the time of carcinogen exposure is pivotal for 'fixation' of the genotoxic injury into a heritable form.

#### Animal models

Many models of liver cancer utilize a brief exposure to an initiating carcinogen at a time when the liver is in a proliferative state, either during the period of postnatal growth or shortly after a PH or necrogenic insult. For example, Craddock (1975) very clearly demonstrated the carcinogenic effects of dimethylnitrosamine (DMN) on the rat liver when it was administered 1 day after a PH (when some 30–40% of hepatocytes would be in S-phase), whereas the same compound, at the same dose, was not carcinogenic to normal adult rats.

Taking this view, Sell has opined that in models of experimental hepatocarcinogenesis, there may be at least four distinct cell lineages susceptible to neoplastic transformation (Sell 2002, 2003, 2004) (Fig. 3). This belief is based on the fact that there is considerable heterogeneity in the proliferative responses that ensue after injury in the many different models of hepatocarcinogenesis. Thus, hepatocytes are implicated in some models of HCC, direct injury to the biliary



Figure 3. Schematic diagram of the various lineages that respond to specific cell damaging insults and therefore are likely founder cells for the tumours that subsequently develop. (1) The cells that normally respond to hepatocyte loss are the hepatocytes themselves; (2) potential stem cells may reside in the canal of Hering and they or their progeny (oval cells/hepatic progenitor cells) may give rise to most HCCs; (3) the interlobular bile duct epithelia may give rise to CCs associated with fluke infection; and (4) periductular cells are associated with experimental hepatocarcinogenesis when animals are fed ethionine in a choline-deficient diet. Largely based on an idea by Stewart Sell.

epithelium implicates essentially unipotent cholangiocytes in some models of CC, while HPC/ oval cell activation accompanies very many instances of liver damage irrespective of aetiology, making such cells very likely carcinogen targets. A fourth cell type that might be susceptible to neoplastic transformation is the so-called nondescript periductular cell that responds to periportal injury; the suggestion that such a cell maybe of bone marrow origin would be experimentally verifiable in the context of a sex-mismatched bone marrow transplantation (see above) and in the appropriate carcinogenic regimen.

Reviewing the experimental hepatocarcinogenesis literature, Sell finds, for example, that after diethylnitrosamine (DEN) exposure, there is little oval cell proliferation but the emergence of alpha-fetoprotein (AFP)-positive hepatocytes, followed by AFP<sup>+</sup> foci and eventually AFP<sup>+</sup> HCC, inferring that HCC develops from hepatocytes in this model. On the other hand, direct injury to the bile ducts induced by furan leads to bile duct hyperplasia and intestinal metaplasia, and prolonged furan exposure results in CCs with a smaller number of HCCs, observations consistent with a bile duct cell origin of these tumours and a developmental origin of bile ducts and hepatocytes from the foregut. Likewise, fluke infection can lead to marked bile duct hyperplasia and subsequent exposure to DMN leads to the rapid development of CC. Many models of hepatocarcinogenesis are characterized by a striking proliferation of oval cells, in particular the so-called 'Solt-Farber' model. Here a single exposure to DEN is followed by a course of 2-AAF designed to block the regenerative ability of normal hepatocytes, thus when a PH is performed only those cells 'initiated' and therefore resistant to the antiproliferative effects of 2-AAF can respond - hence the 'resistant hepatocyte' model of carcinogenesis (reviewed in Alison et al. 1998). Despite the name of the model, Sell concludes that the sequence of hepatocyte foci, to nodules of increasing size to HCC is most likely to have its origins in bipotential oval cells. In animal models of metabolic liver disease such as Wilson's disease, hepatocyte destruction and inflammation is accompanied by a marked presence of oval cells (Fig. 4), and, perhaps not surprisingly, a high incidence of both HCC and CC. A fourth type of cell that may be involved in carcinogenesis is the periductular oval cell (Novikoff & Yam 1998) that proliferates and expresses AFP in response to carcinogens such as ethionine in animals fed on a choline-deficient diet (CDE diet).



Figure 4. An oval cell reaction in a Long Evans Cinnamon (LEC) rat. These animals develop HCC and CC with a high frequency.

The direct involvement of hepatocytes in hepatocarcinogenesis has been clearly established in rats. Gournay *et al.* (2002) found that some preneoplastic foci (expressing gamma-glutamyl transpeptidase and the placental form of glutathione-S-transferase) were directly descended from hepatocytes. This was achieved by stably labelling hepatocytes at 1 day after a 2/3 PH with  $\beta$ -galactosidase using a recombinant retroviral vector containing the  $\beta$ -galactosidase gene; subsequent feeding with 2-AAF leads to foci, some of which were composed of  $\beta$ -galactosidaseexpressing cells. Using the same labelling protocol, Bralet *et al.* (2002) observed that 18% of hepatocytes expressed  $\beta$ -galactosidase at the completion of regeneration after a 2/3 PH; subsequent chronic treatment with DEN resulted in many HCCs of which 17.7% of them expressed  $\beta$ -galactosidase, leading to the conclusion that a random clonal origin of HCC from mature hepatocytes was operative in the model.

It is widely believed that the extensive polyploidy is associated with terminal differentiation and cell senescence, and in many models of chronic injury it precedes overt oval cell development (reviewed in Gupta 2000). Moreover, the carcinogenic process in animals and man is associated with the presence of more diploid cells - consistent with an expansion of oval/HPCs during ongoing liver injury, cells that eventually give rise to so-called 'small cell dysplasia', a likely precursor lesion for HCC (see below). If tumours do arise from oval/HPCs, then this would suggest a block in oval cell differentiation, a process called 'stem cell maturation arrest' by Sell (Sell 1993a,b; Sell & Pierce 1994). The mechanisms behind such a block are unclear, but one candidate tumour suppressor gene, Tg737, thought to control oval cell differentiation and mutated in some rat and human HCCs (Isfort et al. 1997), has not been found to be mutated in another series of human HCC (Bonura et al. 1999). If HCCs are derived from differentiation-arrested oval cells, one would predict a range of neoplastic phenotypes recapitulating stages in normal development, a prediction supported experimentally (Hixson et al. 2000). Using a range of monoclonal antibodies OC.4, OC.5 and OC.10 generated from a mouse immunized with an oval cell line, it was found that HCCs induced by a CDE diet did express many of these oval/bile duct cell markers, suggesting a transitional state between a bipotent oval cell and a hepatocyte. In fact the transition can go in the opposite direction: murine HCC produced by Myc over-expression can revert to apparent normality after Myc is switched off, with both normal hepatocytes and biliary epithelia appearing in a tumour graft (Shachaf et al. 2004). In normal rat liver, hepatocyte to biliary cell transdifferentiation can be seen after severe biliary injury (Michalopoulos, Barua & Bowen 2005). Direct evidence for the involvement of oval cells in the histogenesis of HCC was obtained by Dumble et al. (2002) who isolated oval cells from p53 null mice; when these cells were transplanted into athymic nude mice they produced HCCs. The possibility that bone marrow-derived cells could be involved in hepatocarcinogenesis has been explored: female mice were transplanted with male bone marrow from  $\beta$ -galactosidase transgenic mice, and then HCCs were induced by DEN; however, none of the tumours were male or X-gal-positive (Ishikawa et al. 2004). However, more extensive studies would be needed to rule out such a possibility, and indeed an origin of murine gastric adenocarcinoma from bone marrow has been claimed (Houghton et al. 2004), although here chronic gastritis first ablated the indigenous stem cell population.

#### Human studies

As in rodents, HCC appears to evolve from focal precursor lesions that reflect the stages of multi-step carcinogenesis. Usually in a setting of chronic inflammation with liver cell damage and concurrent regeneration, activation of HPCs invariably follows (Lowes *et al.* 1999; Falkowski *et al.* 2003; Roskams *et al.* 2003; Xiao *et al.* 2003), and the first lesions are thought to be either foci of small cell dysplasia or low grade dysplastic nodules (Libbrecht, Desmet & Roskams 2005). Further rounds of mutation and clonal expansion eventually lead to HCC (Fig. 5). It



Figure 5. (a) A multistep model for the progression to HCC in human liver. Chronic inflammation may not only initiate the carcinogenic process, but may also be important for subsequent progression via inflammatory cytokines such as TNF $\alpha$ , that through NF- $\kappa$ B signalling cause (1) more oxidative cell damage (iNOS), (2) promoting cell growth (COX-2) and (3) suppressing apoptosis (Bcl-X<sub>L</sub> and IAPs) – see Pikarsky *et al.* (2004). HPCs may be involved in the histogenesis of many HCCs and give rise to foci of small cell dysplasia. Further rounds of mutation and clonal expansion may give rise to other precancerous lesions such as 'low grade dysplastic nodules' (LGDN) and 'high grade dysplastic nodules' (HGDN) before HCC develops. (b) Cartoon of the morphological correlates of this process.

seems likely that mature polyploid hepatocytes are not the cells of origin of most HCCs, but rather that most HCCs have their origin in HPCs; the fact that oval cells/HPCs can be infected with HBV is also consistent with a possible histogenesis of HCC from such cells (Hsia *et al.* 1994). Ploidy studies also indicate diploid cells in precursor lesions and early HCCs, but generally an

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Figure 6. HPCs (arrowheads) in a hepatic adenoma immunoreactive for CK19 (a), OV-6 (b) and chromogranin-A (c). Intermediate hepatocyte-like cells (arrows) positive for OV-6 and chromogranin-A surround the HPCs (courtesy of Professor Tania Roskams).

upward shift in ploidy with chronic hepatitis (Marshall *et al.* 2005; Toyoda *et al.* 2005), probably resulting in so-called large cell dysplasia (sometimes called large cell change), thought to be a reflection of cellular senescence rather than a precursor lesion for HCC.

An origin of HCC from HPCs is often inferred from the fact that many tumours contain an admixture of mature cells and cells phenotypically similar to HPCs (Fig. 6). This would include small oval-shaped cells expressing OV-6, CK7 and 19, and chromogranin-A, along with cells with a phenotype intermediate between HPCs and the more mature malignant hepatocytes (Libbrecht & Roskams 2002). Likewise, foci of small cell dysplasia (probable precursor lesions) have an HPC phenotype, while foci of large cell dysplasia do not have an HPC phenotype (Libbrecht *et al.* 2000). Cells resembling HPCs (e.g. OV.1<sup>+</sup> or OV-6<sup>+</sup>) have also been noted in hepatoblastoma (Ruck *et al.* 1996, 1997; Xiao *et al.* 2003; Fiegel *et al.* 2004); this tumour, the most common liver tumour in childhood, is widely believed to be stem cell-derived given there can be both epithelial and mesenchymal tissue components. These tumours can even have structures mimicking intrahepatic bile duct formation with the formation ductal plate-like structures (Libbrecht, Desmet & Roskams 2003; Gornicka *et al.* 2001). Hepatoblastoma has also been recorded in a case of biliary atresia where there are high numbers of HPCs (Taat, Bosman & Aronson 2004).

Cells with an HPC phenotype have also been noted in a relatively rare subset of hepatic malignancies where there are clearly two major components, an HCC component and a cholangiocarcinoma component, again suggestive of an origin from a bipotential progenitor (Theise *et al.* 2003), while there has been a case of a tumour with combined HCC, CC and squamous components (Tsuneyama *et al.* 2003). In many human tumours it is becoming apparent that only tumour stem cells are capable of transferring the disease to NOD/SCID mice (see articles in this issue), so it would be not surprising if HPCs in HCCs are the only cells capable of propagating the tumour in immunodeficient mice.

### CONCLUSIONS

In this review we have tried to summarize the role of the several cell lineages that might be involved in liver regeneration in the virally infected and/or chronically damaged liver. The ability of hepatocytes, the unipotent cholangiocytes and the bipotent oval/HPCs to contribute to liver regeneration is not in doubt, although the identity of the cells within the parenchymal mass and biliary tree deserving of the appellation of 'stem cells' is still unclear. The role of extrahepatic cells in liver regeneration, in particular HSCs, is also uncertain with claims for and against a participation in the regenerative process. However, HSCs do contribute to the scarring, which is an inevitable feature of the chronically damaged liver, and the evidence proffered for an origin of many HCCs from oval/HPCs is becoming increasingly persuasive.

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