

Invited Review

# Stem cells in liver regeneration, fibrosis and cancer: the good, the bad and the ugly

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No conflicts of interest were declared.

## Abstract

The worldwide shortage of donor livers to transplant end stage liver disease patients has prompted the search for alternative cell therapies for intractable liver diseases, such as acute liver failure, cirrhosis and hepatocellular carcinoma (HCC). Under normal circumstances the liver undergoes a low rate of hepatocyte 'wear and tear' renewal, but can mount a brisk regenerative response to the acute loss of two-thirds or more of the parenchymal mass. A body of evidence favours placement of a stem cell niche in the periportal regions, although the identity of such stem cells in rodents and man is far from clear. In animal models of liver disease, adopting strategies to provide a selective advantage for transplanted hepatocytes has proved highly effective in repopulating recipient livers, but the poor success of today's hepatocyte transplants can be attributed to the lack of a clinically applicable procedure to force a similar repopulation of the human liver. The activation of bipotential hepatic progenitor cells (HPCs) is clearly vital for survival in many cases of acute liver failure, and the signals that promote such reactions are being elucidated. Bone marrow cells (BMCs) make, at best, a trivial contribution to hepatocyte replacement after damage, but other BMCs contribute to the hepatic collagen-producing cell population, resulting in fibrotic disease; paradoxically, BMC transplantation may help alleviate established fibrotic disease. HCC may have its origins in either hepatocytes or HPCs, and HCCs, like other solid tumours appear to be sustained by a minority population of cancer stem cells.

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**Keywords:** bone marrow cells; cholangiocytes; cell senescence; chronic inflammation; cirrhosis; fibrosis; hepatic progenitor cells; hepatocytes; oval cells; stem cells

## Introduction

Normally the liver exhibits a very low level of cell turnover, but when an abnormal hepatocyte loss occurs, a rapid regenerative response is elicited from all cell types in the liver to restore the organ to its pristine state [1]. More severe liver injury, particularly longstanding iterative injury (eg chronic viral hepatitis) or when replicative senescence ensues (eg steatohepatitis), activates a facultative stem cell compartment located within the intrahepatic biliary tree, giving rise to cords of bipotential transit amplifying cells (named oval cells in rodents and hepatic progenitor cells in man) that can ultimately differentiate into hepatocytes and biliary epithelial cells [2]. A third population of stem cells with hepatic potential resides in the bone marrow; these stem cells usually make little contribution to regeneration but, after fusing with metabolically defective hepatocytes, can be reprogrammed to contribute in a major way to restoring liver function [3–5]. Mesenchymal cells from bone

marrow and other locations, particularly adipose tissue, appear to be the most suitable extra-hepatic candidate cells for hepatic differentiation. This review summarizes recent advances in our knowledge of these indigenous cell types and their possible therapeutic application.

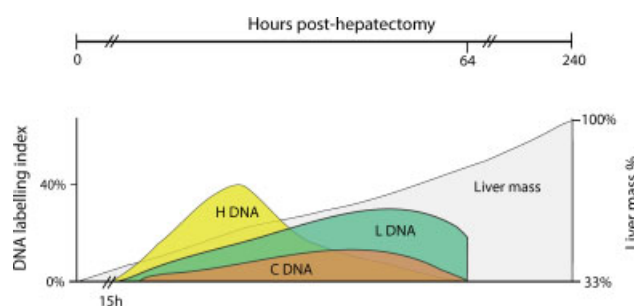
In the context of disease, the bone marrow may also harbour cells with fibrogenic potential, contributing significantly to end-stage liver fibrosis [6,7]; conversely, autologous bone marrow cell therapies may ameliorate this condition and a small number of clinical trials are providing some evidence of this. In common with other tissues, there is persuasive evidence that in the liver, stem cells can be the founder cells of primary hepatic malignancies, such as hepatocellular carcinoma (HCC). HCC is also likely to possess so-called cancer stem cells, otherwise known as tumour-initiating cells, although their identity is far from clear, with the side population (SP), CD133 and Thy-1 (CD90) all being proposed as markers for these cells.

## Liver regeneration: more than one mechanism

### Hepatocytes

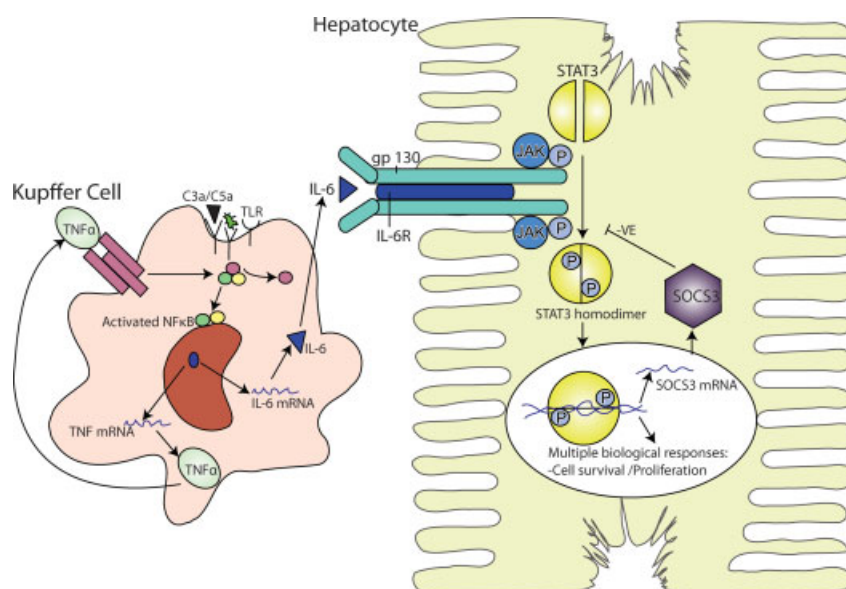
In postnatal animals and humans, hepatocytes are highly differentiated cells with multiple synthetic and metabolic functions. Notwithstanding this, hepatocytes are the cells that normally shoulder the burden of regenerative growth after liver damage, so they can be considered as the functional stem cells under most circumstances. In health, individual hepatocytes have a life expectancy of over a year, thus there is only minimal 'wear-and-tear' renewal. The regenerative capacity of the liver is spectacularly demonstrated when two-thirds of the rat liver is resected by a procedure called a partial hepatectomy (PH), because the remaining remnant can re-grow to the original liver mass in about 10 days (Figure 1). This model has been intensively studied and has provided many data on the mechanisms controlling liver regeneration [1]. In response to this stimulus all the normally proliferatively quiescent hepatocytes leave  $G_0$  to enter the cell cycle under the influence of many growth factors (Figures 2, 3). Hepatocyte proliferation is initiated first in the periportal region of the liver and spreads to the centrilobular region. This regenerative response requires each hepatocyte to undergo, on average, only 1.4 rounds of replication to restore normal liver size. However, this does not mean that hepatocytes have limited replication potential. Hepatocyte transplantation experiments in mice have shown that hepatocytes are capable of large-scale clonal expansion within a diseased liver (see below). Age has a bearing on the response, as only in young rats (2–3 months old) do all hepatocytes enter the cell cycle at least once after a two-thirds PH, but in aged rats (>2 years old) a significant number of hepatocytes do not respond and so appear to have become reproductively senescent [8]. After a two-thirds PH there is a lag period of about 15 h before the first periportal hepatocytes enter DNA synthesis, and at the peak of the response at 24 h the hepatocyte DNA labelling index reaches 40% [9]. After 4 days this hyperplastic response is effectively curtailed and further increases in liver mass are achieved through hepatocyte hypertrophy. To retain correct cellular relationships, the non-parenchymal cells also proliferate, although the kinetics of cell cycle entry lag a few hours behind that of hepatocytes (Figure 1).

Numerous growth factors and cytokines have been implicated in the initiation and control of this regenerative response [1,10–16], with the activation of seemingly critical transcription factors, such as AP-1, NF $\kappa$ B and STAT-3. Liver injury can be associated with a defective intestinal barrier, leading to exposure to bacterial products such as lipopolysaccharides and components of the innate immune system (complement fragments), and their activation of the NF $\kappa$ B pathway in Kupffer cells, leading to the production and secretion

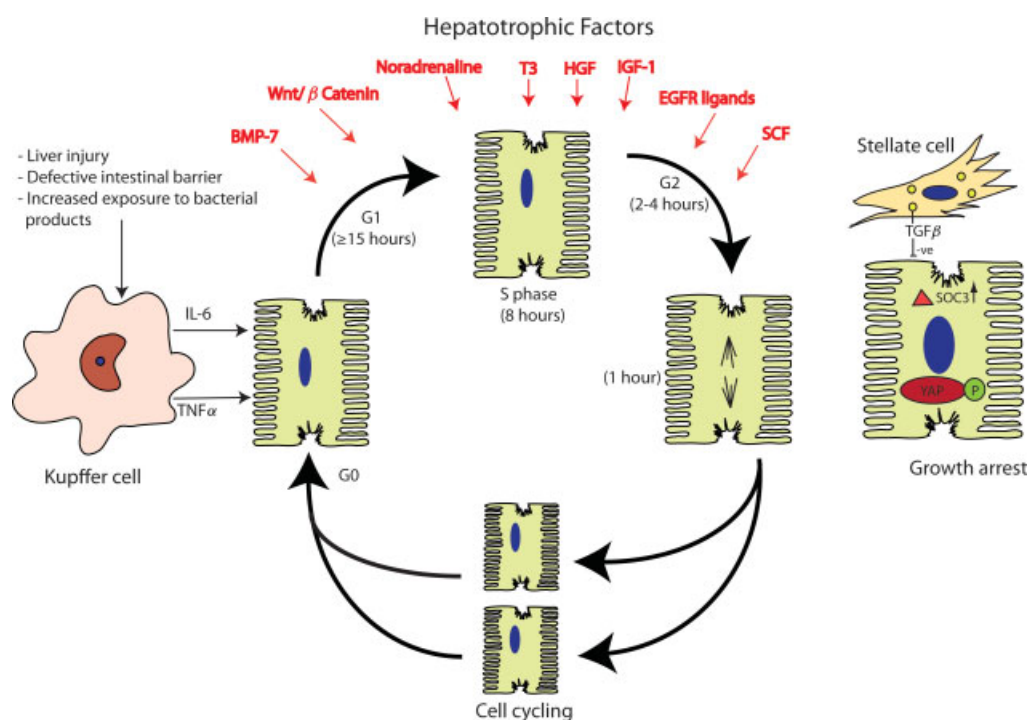


**Figure 1.** The kinetics of liver regeneration in the rat after a two-thirds PH. The hepatocyte DNA labelling index (H DNA) rises after 15 h, peaking at 24 h with a labelling index of ~40%. The entry of littoral (sinusoid-lining) cells (L DNA) and cholangiocytes (C DNA) into DNA synthesis lags behind that of hepatocytes. The hyperplastic response is essentially complete by 96 h, but continued hepatocyte hypertrophy restores the pre-operative liver weight within 10 days

of IL-6, appears to be a significant event leading to the initiation of DNA synthesis in hepatocytes (Figure 2). Having said this, it is worth noting that liver regeneration can still be achieved by livers depleted of Kupffer cells, and no single factor amongst the plethora of so-called hepatotrophic factors, eg IL-6, TNF $\alpha$ , HGF, amphiregulin, SCF, IGF-1, T3, BMP-7 and Wnt/ $\beta$ -catenin, appears absolutely critical for the process to occur (Figure 3). Equally important is what actually governs the magnitude of the regenerative response; TGF $\beta$  produced by the stellate cells certainly inhibits hepatocyte replication, and the IL-6 response mediated by STAT3 homodimers (Figure 2) is negatively regulated through transcriptional up-regulation of Socs3 (suppressor of cytokine signalling 3), a molecule that prevents further interaction of JAKs with STAT3, thus blocking further JAK-mediated phosphorylation (activation) of STAT3. Nevertheless, SOCS is not essential for termination of the regenerative response; Socs3 knock-out mice do have higher levels of hepatocyte proliferation after PH than their wild-type counterparts and do restore their liver weight 2 days earlier, but proliferation still ceases after 4 days and there is no inexorable rise in liver weight [15]. On the other hand, over-expression of the Yes-associated protein (YAP) in a conditional YAP transgenic mouse led to a phenomenal liver weight increase through hyperplasia, reaching 25% of body weight — normal liver weight is ~5% of body weight [16]. YAP transcriptionally activates cell cycle proteins such as Ki-67 and c-Myc and also inhibitors of apoptosis, and its phosphorylation by way of activation of the 'Hippo' pathway blocks its ability to shuttle to the nucleus [17]. The Hippo pathway is considered to be a 'size checkpoint' operating at the level of the organ's total mass, perhaps reminiscent of the 'chalone' hypothesis that was fashionable in the 1970s, which held that the size of each organ was controlled by a tissue-specific chalone, produced by differentiated cells, that negatively regulated the progenitor compartment [18,19].



**Figure 2.** Stimulated by components of the innate immune system, Kupffer cells produce and secrete IL-6 to kick-start the regenerative response. IL-6 helps stimulate hepatocyte proliferation via STAT3 activation; in turn, this response is negatively regulated by SOCS3



**Figure 3.** A wide range of cytokines and growth factors have been implicated in mediating the liver's regenerative response (see text) and, while most hepatocytes will traverse the cell cycle only once after a two-thirds PH, a few will cycle a second or even a third time. Factors that control hepatocyte decycling include  $TGF\beta$ , SOCS3 and activation of the Hippo pathway, resulting in YAP phosphorylation

### Are there parenchymal stem cells and where do they live?

By definition, stem cells should be self-renewing and clonogenic, and models of hepatocyte transplantation have indeed shown that transplanted hepatocytes are capable of significant clonal expansion in the diseased liver [20,21]. In the fumarylacetoacetate hydrolase (Fah)-deficient mouse, a model of

hereditary type 1 tyrosinaemia, a strong positive selection pressure is exerted on any transplanted wild-type hepatocytes because host hepatocytes readily undergo cell death due to 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  $10^4$

normal hepatocytes from congenic male wild-type mice were intrasplenically injected into mutant female mice and the NTBC treatment withdrawn, these hepatocytes colonized the mutant liver very effectively [21]. Moreover, serial transplantations of wild-type cells from colonized livers to other Fah null mice indicated that at least 69 doublings would have been necessary from the original hepatocytes for six rounds of liver repopulation. This estimate is likely to be a minimal figure, since it assumes that all injected hepatocytes migrate to the liver from the spleen and take part equally in the cycles of regeneration. In fact, probably at best only 15% of intrasplenically transplanted hepatocytes migrate to the liver, and if all these participated equally in repopulation, a minimum of 86 doublings would be required for six serial transplants. This figure might be even higher if not all the cells that migrated to the liver actually took part in repopulation, and the authors suggested that there might be a sub-population of hepatocyte stem cells designated as 'regenerative transplantable hepatocytes' (RTHs). We could speculate that perhaps these RTHs are analogous to the small hepatocyte-like progenitors (SHPCs) described by Gordon [22]? If rats are pretreated with retrorsine, a pyrrolizidine alkaloid metabolized by the hepatocyte's cytochrome P450 system to metabolites that form DNA adducts, then after a two-thirds PH, regeneration is accomplished by the activation, expansion and differentiation of these so-called SHPCs. These cells showed phenotypic traits of fetal hepatoblasts, oval cells and fully differentiated hepatocytes, but they were morphologically and phenotypically distinct from all three. Cytochrome (CYP) P450 enzymes have a pivotal role in hepatocyte biology, but typically these cell clusters lacked CYP enzymes that are usually readily induced by retrorsine, and this probably accounted for their resistance to the anti-proliferative effects of retrorsine. When such cells (H4-positive) were isolated, established in short-term culture and then transplanted into syngeneic rats, they gave rise to differentiated hepatocytes, as shown by expression of albumin and transferrin but lack of  $\alpha$ -fetoprotein [23]. Cell foci resembling SHPCs have also been observed in retrorsine-treated mice with chronic liver injury [24]. From retroviral lineage tracing studies in rats, the origin of these cells is thought to be hepatocytes [25] but their location is unclear, seeming to be located at random in the parenchyma rather than confined to a particular niche.

In the mouse there has been some evidence for a parenchymal stem cell niche close to the portal area. By labelling cells with BrdU after a necrogenic dose of acetaminophen, and then administering another dose 2 weeks later to induce several divisions of previously labelled cells, so-called label-retaining cells (LRCs), considered to be slowly dividing stem cells, were found as both cholangiocytes of interlobular ducts and peribiliary hepatocytes and so-called null cells [26]. It is not clear if the latter cells correspond to the peribiliary Sca-1<sup>+</sup> cells, also found in the murine liver

[27]. In human liver as well, rare putative stem cells that strongly express STAT3 and the embryonic stem cell-associated pluripotency-associated factors Oct4 and nanog are apparently also located near portal tracts [28].

A niche close to portal tracts would be compatible with the 'streaming liver' hypothesis. Advocated by Gershom Zajicek and colleagues [29,30], it has been proposed that the liver, and other very slowly turning-over glandular cell populations, such as kidney and salivary glands, are in fact organized like the intestine with a unidirectional flux of cells. In the case of liver, cells would be born at one end of the flux, the portal area, and migrate down a path leading to the hepatic (central) vein. This hypothesis was put forward based upon some very simple observations; intact adult rats were injected with tritiated thymidine and from those killed 1 h later, labelled hepatocytes were on average some 70  $\mu$ m away from the portal rim. The remaining rats were killed at intervals of up to 4 weeks later, and the average distance of labelled cells from the portal rim gradually increased to 140  $\mu$ m, so leading to the conclusion that hepatocytes migrated at a velocity of over 2  $\mu$ m/day from the portal rim to the central vein. However, other observations do not fit with this theory; Bralet and colleagues [31] labelled proliferating hepatocytes at 24 h after a two-thirds PH through retroviral-mediated gene transfer of the *Escherichia coli*  $\beta$ -galactosidase gene, and then studied the fate of the labelled cells over the next 15 months. Over time, not only did each labelled cell develop into a small cluster of labelled cells, but their pattern of distribution over the three zones did not alter in 15 months: if the livers 'streamed', one might have expected a significant shift of the labelled clusters out of the periportal zone towards the hepatic veins.

## Cell therapies using hepatocytes

Successful cell therapy depends on either the innate clonogenicity of the administered cells or that a situation can be engineered in which the transplanted cells have a selective growth advantage over the indigenous population. In the diseased human liver there may not be the substantial selective growth advantage for transplanted cells that exists in many of the rodent models, so is it possible to enrich for cells that would continue to expand in the recipient liver in the absence of a major growth stimulus — these might simply be fetal cells or a subpopulation of antigenically distinct adult cells. An array of markers have been used to select for clonogenic cells from both human fetal and adult liver (Table 1), and these have had varying degrees of success in treating animal models of liver disease.

Many liver diseases appear to be amenable to hepatocyte transplantation therapy. These include genetic diseases that produce liver disease, such as Wilson's

**Table 1.** Cell surface markers used to enrich for clonogenic cells isolated from human hepatic parenchyma

Source	Surface markers	Comments	Reference
Human fetal and adult	EpCAM <sup>+</sup> , AFP <sup>-</sup> , CK19 <sup>+</sup> , CD44h <sup>+</sup> , CD133 <sup>+</sup> , N-CAM <sup>+</sup> , claudin 3 <sup>+</sup> , Albumin <sup>+/+</sup>	150 doublings <i>in vitro</i> . Produce mature hepatocytes after Tx in NOD/SCID	37,38
Human fetal	EpCAM <sup>+</sup> , AFP <sup>+</sup> , CK 18 <sup>+</sup> , 19 <sup>+</sup> , CD44h <sup>+</sup> , CD34 <sup>+</sup> , CD90 <sup>+</sup> , c-kit <sup>+</sup> , c-Met <sup>+</sup> , SSEA-4 <sup>+</sup> , Albumin <sup>-</sup>	>100 doublings <i>in vitro</i> . Hepatic and cholangiocyte differentiation and others (pluripotent)	39
Human fetal	CD117 <sup>+</sup> , CD34 <sup>+</sup> , Lin <sup>-</sup>	Contributed to 5% of liver after Tx to nude mice with D-galactosamine injury	40
Human adult	CD29 <sup>+</sup> , CD44 <sup>+</sup> , CD73 <sup>+</sup> , CD90 <sup>+</sup> , AFP <sup>+</sup> , albumin <sup>+</sup> , CD34 <sup>-</sup> , CD45 <sup>-</sup> , CK19 <sup>-</sup> , CD117 <sup>-</sup> , CD133 <sup>-</sup>	Hepatic differentiation plus others <i>in vitro</i> —pluripotential. Contributed to hepatocytes in N-acetyl-paraaminophen damaged SCID mice	41
Human adult	Non-parenchymal cell fraction. CK8/18 <sup>+</sup> , CD105 <sup>-</sup> , CD90 <sup>-</sup> , c-Kit <sup>-</sup> , CD34 <sup>-</sup> , OV-6 <sup>-</sup> , CD45 <sup>-</sup>	20–25 doublings <i>in vitro</i> . Hepatic differentiation <i>in vitro</i> .	42

AFP,  $\alpha$ -fetoprotein; CK, cytokeratin; EpCAM, epithelial cell adhesion molecule; h, human; Lin, lineage; N-CAM, neural cell adhesion molecule; NOD/SCID, non-obese diabetic/severe combined immunodeficiency; SSEA, stage-specific embryonic antigen; Tx, transplantation.

disease (copper accumulation), Crigler–Najjar syndrome (lack of bilirubin conjugation activity) and tyrosinaemia, and cases where there is extrahepatic expression of the disease, eg Factor IX deficiency. Transplantation of adult rat hepatocytes has been effective in normalizing bilirubin levels and improving bilirubin conjugation activity in Gunn rats (a model of Crigler–Najjar syndrome) [32,33]. These cells were reversibly immortalized and transduced with the bilirubin-uridine 5'-diphosphoglucuronate glucuronosyltransferase gene (*ugt1a1*) and engraftment was improved by prior irradiation and partial hepatectomy of the recipient rats. Many strategies have been employed to improve the proliferation of transplanted cells within the recipient liver. Preconditioning with irradiation and ischaemia reperfusion injury (IRI) markedly improved the repopulation of dipeptidyl peptidase IV (DPPIV)-negative livers by syngeneic F344 wild-type hepatocytes [34]; likewise, transient IRI performed on LDL receptor-deficient Watanabe rabbits improved the therapeutic benefit (ie lowering of LDL cholesterol) of multiple intraportal infusions of allogeneic hepatocytes [35]. In the retrorsine pretreated and partially hepatectomized DPPIV-deficient rat, superior repopulation by wild-type hepatocytes was achieved by selecting donor cells with low rather than high asialoglycoprotein receptor expression [36].

Hepatocyte transplantation in humans has met with varied success. An infusion of isolated hepatocytes through the portal vein equivalent to 5% of the parenchymal mass to a patient with Crigler–Najjar syndrome achieved a medium-term reduction in serum bilirubin and increased bilirubin conjugate levels in the bile [43]. Hepatocyte transplantation has also been successful in the treatment of human glycogen storage disease type 1a [44], but not in the treatment of severe ornithine transcarbamylase deficiency, where rejection of the transplanted cells was thought to be the reason for only temporary (11 days) relief [45]. Also, a temporary, but impressive, reduction in the requirement for exogenous recombinant factor VII was achieved by transplantation of adult hepatocytes to two children with inherited severe factor VII deficiency,

although orthotopic liver transplants were required by both recipients after 6 months [46]. A better success was seen in a 3 year-old patient with a urea cycle disorder, arginosuccinate lyase deficiency, where a heroic course of 11 hepatocyte transplants achieved a peak of 19% donor hepatocytes at 8 months [47].

Exploiting natural clonogenicity while also providing a selective growth advantage for the transplanted cells is clearly a desirable objective. Shafritz and colleagues [48] have shown that fetal liver epithelial progenitors (FLEPs) from ED14 rats are more clonogenic than normal adult hepatocytes, for when wild-type FLEPs were injected into recently hepatectomized syngeneic DPPIV-deficient F344 rats they proliferated for at least 6 months and constituted 7% of the recipient liver at this time, compared to colonization of only 0.06% of the liver by wild-type adult hepatocytes. However, much greater colonization of the mutant liver was observed when the recipient rats were given prior administration of retrorsine (a compound whose metabolites form DNA adducts in hepatocytes, preventing them from undergoing DNA replication); at 6 months after transplantation, 60–80% of the recipient liver was occupied by DPPIV<sup>+</sup> hepatocytes [49]. Important from a human standpoint, such cells can be cryopreserved for up to 20 months with no loss of repopulating activity [50]. Many studies have also examined the transplantation potential of adult hepatocytes in the DPPIV<sup>-</sup> mutant rat, combining retrorsine treatment with a mitogenic stimulus, such as partial hepatectomy or triiodothyronine (T3), leading to the rapid replacement of DPPIV<sup>-</sup> cells by DPPIV<sup>+</sup> donor cells [51,52]; even in the absence of a mitogenic stimulus, near-total replacement by donor cells occurs within 12 months [53].

Bipotential progenitors have been isolated from fetal mouse liver in a number of studies. Tanimizu *et al* [54] have selected such cells on the basis of expression of Dlk, a type I membrane protein that has six EGF-like repeats in its extracellular domain, and Dlk combined with immunopositivity for AFP, albumin, E-cadherin and Liv2 (a molecule only expressed at ED 9.5–12.5) has selected for a

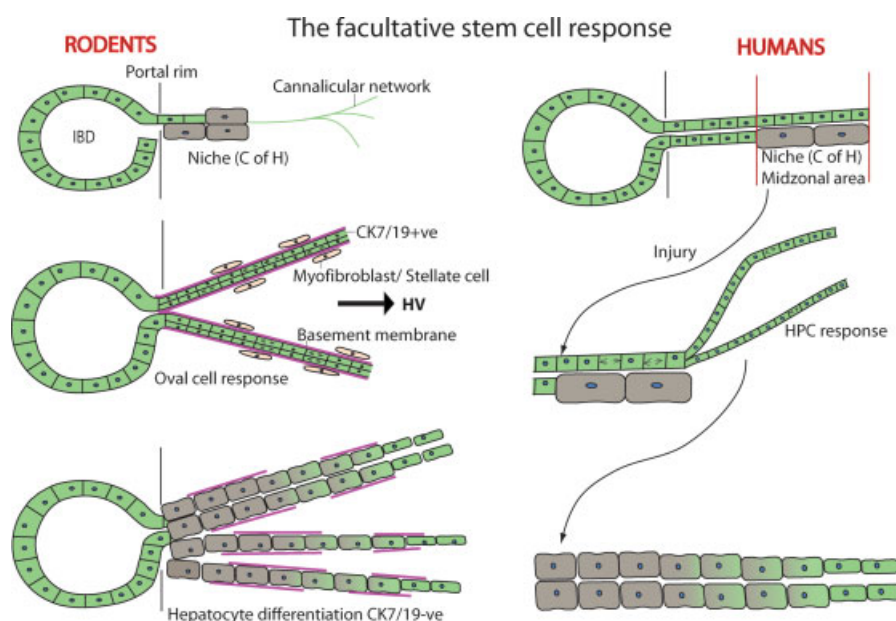


population capable of an 80% replacement of the DPPIV-deficient mouse liver [55]. The key to the success of the transplantation was again that the mice had been pre-treated with retrorsine (blocking indigenous hepatocyte replication) and given courses of hepatocyte-destroying  $\text{CCl}_4$ , both before and after cell transplantation. Clonogenic cells have also been isolated by Suzuki *et al* [56] from fetal mouse liver (ED13.5), on the basis of expressing the integrins  $\alpha 6$  (CD49f) and  $\beta 1$  (CD29) but not c-kit, CD45 or Ter119 (erythroid precursor antigen). Designated 'hepatic colony-forming units in culture' (H-CFU-C), this sorting achieved a 35-fold enrichment of H-CFU-C over total fetal liver cells. Further selection based on c-Met-positivity enriched for H-CFU-C and these cells produced both hepatocytes (albumin-positive) and biliary cells (cytokeratin-19-positive) in culture [57]; EGFP-marked cells from these clonally-derived H-CFU-C also produced hepatocytes and biliary cells when injected into mice and, more surprisingly, were found to apparently differentiate into pancreatic ducts and acini and duodenal mucosal cells when injected directly into these organs. The expression of the  $\alpha 6$  integrin, in combination with CK19 and A6 (oval cell marker), also selects for clonogenic cells from adult mice, cells capable of forming hepatocytes and biliary cells in albumin-uPA/SCID mice [58].

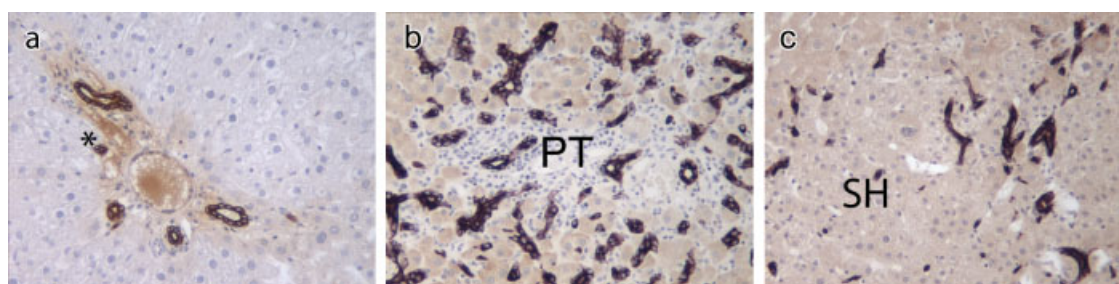
### The facultative stem cell response: oval/hepatic progenitor cells

Overwhelming liver injury, chronic liver injury or large-scale hepatocyte senescence results in a *potential*

stem cell compartment being activated from within the smallest branches of the intrahepatic biliary tree. For example, hepatocyte proliferation rates increase in hepatitis C with increasing histological damage until cirrhosis is reached, when the proliferation rate falls [59]. This fall probably reflects replicative senescence [60], although the diversion of blood flow through the liver probably plays a part. This reduction in hepatocyte proliferation in chronic hepatitis occurs concurrently with the activation of this potential stem cell compartment. The development of an oval cell reaction in response to hepatocyte replicative senescence has also been demonstrated in a transgenic mouse model of fatty liver and DNA damage [61]. In both humans [62] and mice [63], the extent of this reaction is dependent on the severity of the damage. This so-called 'oval cell' or 'ductular reaction' amplifies a cholangiocyte-derived (biliary) population before these cells differentiate into either hepatocytes or cholangiocytes [2,64–67]. Oval cells are derived from the canal of Hering, and in rodents this canal barely extends beyond the limiting plate (Figures 4, 5); in contrast, in human liver the organization of the biliary tree is different, with the canal of Hering extending to the proximate third of the lobule [68] and so apparently requiring a name change from oval cells to 'hepatic progenitor cells' (HPCs) [69]. An enormous range of markers has been used to identify oval cells (Table 2) [70–83] and some, such as Dlk, may signal imminent hepatocyte differentiation [71]. A popular experimental procedure to elicit an oval cell response in rats is to pre-treat the animals with 2-acetylaminofluorene (2-AAF) before performing a two-thirds PH (the 2-AAF/PH protocol). The 2-AAF is metabolized by the



**Figure 4.** The facultative stem cell response involves biliary epithelial cells in both rodents and man. In mice and rats (LH side), the stem cell niche is the canal of Hering (C of H) located close to the portal space, and once activated an 'oval cell' response ensues, sending cords of cells outwards towards the hepatic veins. Subsequently these biliary-derived cells can differentiate to hepatocytes, losing their biliary (CK7/19<sup>+</sup>) phenotype. In man (RH side), the same course of events occurs, but here the C of H is located some distance from the portal area [68]



**Figure 5.** Oval cell behaviour in the rat liver treated by the AAF/PH protocol. Corresponding to Figure 4: (a) CK19 immunoreactivity (brown) in the uninjured liver confined to interlobular bile ducts and possible canals of Hering (\*); (b) the oval cell response can be visualized by CK19 immunostaining (brown-purple), with cords of cells emanating from the portal tract (PT) — note the prominent inflammatory response that has been implicated in partly mediating this reaction [83]; (c) at later times the cords of oval cells differentiate into small immature hepatocytes (SH) with a notable lack of CYP2E1 immunoreactivity (brown staining). Note occasional residual oval cell ductules expressing CK19 (purple staining) at the outer margins

**Table 2.** Markers used in the identification of oval cells/HPCs in the damaged mammalian liver

A6 antigen (mouse marker)
ABCG2/BCRP1 (breast cancer resistance protein)
AFP ( $\alpha$ -fetoprotein)
Cadherin 22
CD 34, CD24, CD44, CD133
Chromogranin A
CK7, CK19, CK14
c-Kit (CD117)
Claudin7
Connexin 43
Dlk ( $\delta$ -like protein)
DMBT1 (deleted in malignant brain tumour 1)
E-cadherin
flt-3 ligand/flt-3
Fn14 (TWEAK receptor)
GGT ( $\gamma$ -glutamyltranspeptidase)
GST-P (placental form of glutathione-S-transferase)
M2-PK (muscle type pyruvate kinase)
MUC1
NCAM-1/CD56 (neural cell adhesion molecule-1)
OV-1, OV-6, OC.2, OC.3, OC.4, OC.5, OC.10
PTHrP (parathyroid hormone related peptide)
Thy-1 (CD90)*

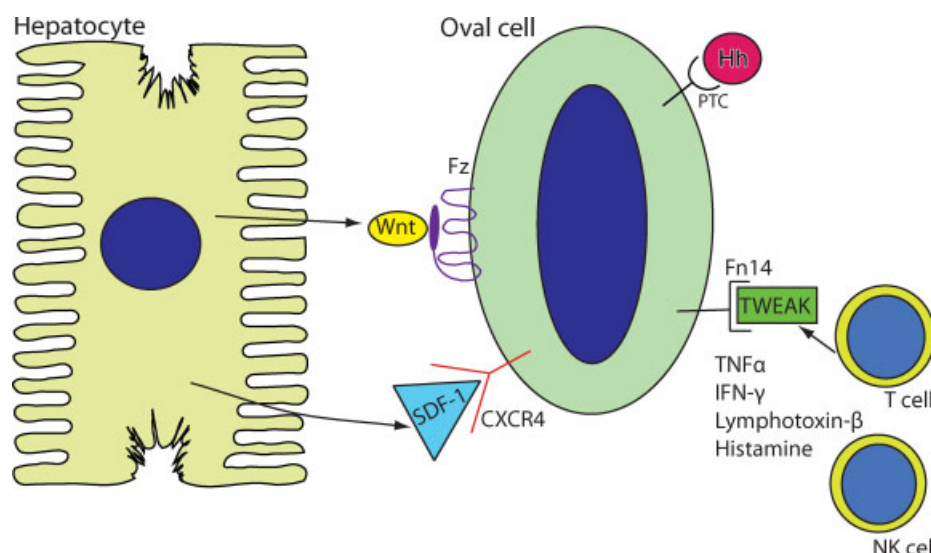
Many of these markers are also expressed on normal biliary epithelial cells. \* Controversial.

hepatocyte's cytochrome P450 monooxygenase system, yielding metabolites that form DNA adducts, resulting in the inability of hepatocytes to enter the cell cycle in response to the subsequent PH. Thus, the burden of regeneration is shifted to the biliary cell compartment that is resistant to the growth-inhibitory effects of 2-AAF [64]; whether there are specific stem cells is unclear, but certainly it is the small rather than the larger bile ducts from where the response emanates. The regulation of HPC maturation is likely to involve paracrine signals from Thy1<sup>+</sup> mesenchymal cells that are in intimate contact with the expanding ductules [84], although differentiation of rat oval cells to hepatocytes can be hastened by outside influences such as T3 [85].

Some antigens traditionally associated with haematopoietic cells (c-kit, flt-3, CD34) can also be expressed by oval cells/HPCs, leading to the notion that at least

some hepatic oval cells are directly derived from a precursor of bone marrow origin. Most studies suggest that this is unlikely, eg oval cell reactions were elicited by three different protocols in DPPIV-deficient mutant rats that had been previously lethally irradiated and given a wild-type bone marrow, but no oval cells were found to be of bone marrow origin [86]. On the other hand, careful manipulation of the protocol for eliciting oval cells in the female DPPIV-deficient rat can result in some (~20%) oval cells apparently being of bone marrow origin [87] — as usual the devil is in the detail. Prior exposure to monocrotaline, another pyrrolizidine alkaloid, was used to inhibit hepatocyte regeneration; thus, an oval cell reaction would be stimulated by liver damage. However, monocrotaline also inhibits bone marrow cell activation, and so only if monocrotaline is administered *before* a bone marrow transplant will bone marrow contribute to the oval cell reaction. The possible bone marrow origin of oval cells has also been tested in another model of activation, namely one in which rats are fed a ethionine-supplemented, choline-deficient (CDE) diet [88]; indeed, 3–4% of oval cells expressed the same marker as the transplanted bone marrow — GFP. However, these same cells also had the recipient trait, the Y chromosome, so in fact had been created by cell fusion.

Surprisingly, oval cells/HPCs have attracted little attention in terms of therapeutic potential, perhaps because isolated human cells bearing appropriate markers, eg CD34 or c-kit, only differentiate *in vitro* into biliary cells [89]. The hepatocyte potential of transplanted oval cells has been clearly demonstrated in animal models: oval cells isolated from LEC rats were transplanted into LEC/Nagase albuminaemic double mutant rats, where they differentiated into albumin-expressing hepatocytes [90]. Oval cells can also act as transplantable hepatocyte progenitors in the Fah knockout mouse; furthermore, the fact that oval cells generated in bone marrow transplanted wild-type mice can repopulate Fah knockouts, but lack markers of the original bone marrow donor, strongly suggests that oval cells *do not* originate from



**Figure 6.** Cartoon indicating some possible interactions between oval cells/HPCs and hepatocytes and inflammatory cells that influence the facultative stem cell response

bone marrow in this particular model [91]. Transplanted oval cells can make a modest ( $\sim 2\%$  after 1 month, falling to  $0.4\%$  at 6 months) contribution to the hepatocyte population in the  $\text{CCl}_4$ -damaged mouse liver [92]. The progenitor response can be manipulated *in vivo*; in the 2-AAF/PH model the oval cell reaction can be significantly boosted by G-CSF [93], and a small study of patients with alcoholic steatohepatitis found that 5 days of G-CSF treatment resulted in a four-fold increase in proliferating HPCs [94].

The regulation of the oval cell response appears multifactorial (Figure 6). Hatch *et al* [95] have highlighted the significance of the up-regulation of the chemokine SDF-1 by hepatocytes in the  $\text{CCl}_4$ -damaged liver for the activation of CXCR4-expressing oval cells; importantly, SDF-1 was only up-regulated in the face of such damage when hepatocyte DNA synthesis was concurrently blocked by 2-AAF. Autocrine and paracrine Wnt signalling is also clearly involved in the oval cell response in mice [96], rats [97] and humans [98], and is likely responsible for the expression of EpCAM [99]. Hedgehog (Hh) signalling acting through the receptor Patched (PTC) on oval cells/HPCs is required for their survival [76]. Perhaps most significantly, inflammatory cells produce a range of cytokines and chemokines that initiate the response [82,83]; SDF-1 attracts CXCR4<sup>+</sup> T cells, and these cells express TNF-like weak inducer of apoptosis (TWEAK), which stimulates oval cell proliferation by engaging its receptor Fn14 [80]. Other elements of the inflammatory response that may stimulate oval cells include lymphotoxin- $\beta$ , IFN $\gamma$ , TNF $\alpha$  and even histamine [81]. A resistance to the growth inhibitory effects of TGF $\beta$  has been credited with allowing oval cells to proliferate under conditions inhibitory to hepatocytes [100].

## Bone marrow

Considerable excitement and not a little controversy has been generated since the original 'proof-of-concept' demonstration of the potential therapeutic utility of bone marrow to cure mice with the potentially fatal metabolic liver disease, hereditary tyrosinaemia type 1 [3]. The salient point to arise from this powerful demonstration of the therapeutic potential of bone marrow cells was that, although the initial engraftment was low (approximately one bone marrow cell for every million indigenous hepatocytes), the strong selection pressure exerted thereafter on the engrafted bone marrow cells resulted in their clonal expansion to eventually occupy almost half the liver. This positive selection was achieved by cycles of withdrawal of NTBC, the compound that blocks the breakdown of tyrosine to hepatotoxic fumarylacetoacetate (FAA) and maleylacetoacetate in the *Fah*<sup>-/-</sup> mice, so protecting against liver failure. In the absence of NTBC, FAA accumulates and destroys the hepatocytes; thus, the ensuing regenerative stimulus promotes the growth of the engrafted cells. Of course, we now know that the new healthy liver cells in the transplanted *Fah*<sup>-/-</sup> mouse contain chromosomes from both recipient and donor cells, with the donor haematopoietic cell nuclei being reprogrammed when they fused with the unhealthy *Fah*<sup>-/-</sup> hepatocyte nuclei, creating functional hepatocytes [4].

Since publication of the 'curing' of the *Fah*<sup>-/-</sup> mouse by a wild-type bone marrow transplant, considerable effort has been expended into clarifying the significance of the bone marrow–liver axis (reviewed in [5]), and it is indeed difficult to argue with a review of the literature that concludes that 'haematopoietic cells contribute little to hepatocyte formation under either physiological or pathological conditions' [101]. Nevertheless, bone marrow cell therapies in animal



models and patients have produced some interesting results. For example, while bone marrow fails to contribute to liver development in neonatal mice [102], impressive numbers of hepatocytes appear to be human cord blood (CD34<sup>+</sup>lin<sup>-</sup>) -derived when the cord blood is transplanted in to either pre-immune fetal sheep [103] or goats [104]. In animal models, the most promising cells for therapy appear to be mesenchymal stem cells (MSCs). If human MSCs are xenografted to the allyl alcohol-damaged livers of immunosuppressed rats, large groups of human albumin-positive hepatocytes are generated without cell fusion being involved [105]. Transplanted human MSCs also contribute to the hepatocyte mass, predominantly periportal, in the partially hepatectomized livers of immunodeficient mice when regeneration is impeded by a  $\beta$ -receptor antagonist [106]. Human MSCs from adipose tissue, selected on the basis of CD105 (endoglin) expression, also generate hepatocytes when injected into nude mice at 24 h after CCl<sub>4</sub> injury [107]. Rat MSCs can also differentiate into hepatocytes *in vitro* and these cells continue to function after transplantation into DPPIV-negative rats, again predominantly in the periportal area [108]. As expected, a number of transplantation studies report fusion between donor and recipient cells, including human cord blood with sublethally irradiated mouse hepatocytes [109] and mouse bone marrow with mouse hepatocytes after CCl<sub>4</sub> injury [110]: the benefits of cell fusion in the context of hepatocyte injury are not immediately obvious.

Autologous CD133<sup>+</sup> bone marrow cells have been used to great effect to boost regeneration in human liver [111]: three patients with major liver tumours were subjected to portal vein embolization of the tumour-bearing lobe (induces atrophy), thereby inducing contralateral lobe hypertrophy and thus increasing the size of the future remnant liver volume before an extensive partial hepatectomy. By CT criteria, the bone marrow-treated lobes enlarged by at least 2.5-fold more than the non-bone marrow cell-treated controls.

## Liver fibrosis

Chronic liver injury is invariably accompanied by progressive fibrosis [112]. In the progression to chronic liver injury/inflammation, hepatic stellate cells (HpSCs) become activated, proliferate and synthesize and secrete interstitial collagens. HpSCs express  $\alpha$  smooth muscle actin ( $\alpha$ -SMA), the histological hallmark of myofibroblasts, and are thought to be the pivotal to the pathogenesis of liver fibrosis. As in other responses, Kupffer cells seem to be key cells, activating HpSCs by secretion of TGF $\beta$ , and bacterial products such as lipopolysaccharide (LPS) engage toll-like receptors on stellate cells, down-regulating the inhibitory TGF $\beta$  pseudoreceptor Bambi, allowing unrestricted activation of HpSCs by the Kupffer cells [113]. Apart from activated stellate cells, portal fibroblasts and hepatocytes/cholangiocytes undergoing epithelial–mesenchymal transition have been

implicated in the fibrogenic response [112]. Furthermore, a growing body of evidence indicates that bone marrow cells can contribute to the fibrogenic population (Table 3a). For example, female mice, having had a male bone marrow transplant, were chronically poisoned with CCl<sub>4</sub> and, up to 70% of HpSCs and myofibroblasts associated with septal scars were bone marrow-derived, most likely from MSCs [7]. A bone marrow contribution to hepatic fibrogenesis has also been shown in the bile duct-ligated mouse, a model of cholestatic liver disease [116].

Paradoxically, the intravenous injection of bone marrow cells, particularly MSCs, appears to be therapeutically useful to animals with ongoing hepatic damage (Table 3b), and some Phase I trials involving the injection of autologous bone marrow cells to cirrhotic patients have reported modest improvements in clinical scores (reviewed in [126,127]). The fate of the cells in the human liver is unknown, neither is the mechanism that underlies any improvement clear; one animal study [121] claims that bone marrow cells differentiate to hepatocytes and secrete MMPs that degrade the bands of collagen.

## Stem cells and liver cancer

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer death. Most HCCs (80%) arise in a cirrhotic liver, ie in 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 [128]. In Europe and the USA, the incidence of HCC is low but slowly increasing, probably due to the rise in people infected with HCV. Apart from hepatotropic viruses, the other major risk factors for HCC are other factors leading to cirrhosis, such as alcohol abuse and metabolic liver disease, and mutagens such as aflatoxins, toxic metabolites of the food mould *Aspergillus* spp.

Cholangiocarcinomas (CC) are believed to arise from biliary epithelium that is either within the liver (intrahepatic) or 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, suggesting that carcinogenesis is a protracted and (possibly) multi-step process [129]. Injury to the biliary epithelium with 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 *Opisthorchis viverrini* and *Clonorchis sinensis*.

**Table 3a.** Bone marrow cell involvement in hepatic fibrosis

Reference	Species	Method	Injury	Evidence	Comments
[6]	Human	Sex-mismatch liver allografts, one female had male BMTx	Cirrhosis	6–22% of SMA <sup>+</sup> cells in female donor liver were Y <sup>+</sup> .	12.4% of SMA <sup>+</sup> cells were Y <sup>+</sup> in female recipient of male BMTx—bone marrow origin Very rare MSC-derived hepatocytes
[114]	Human to mouse	hMSC Tx to NOD/SCID	CCl <sub>4</sub>	1.6% of liver nuclei were human with myofibroblast morphology	
[115]	Mouse	GFP <sup>+</sup> BMTx	CCl <sub>4</sub>	GFP <sup>+</sup> cells in liver express GFAP and desmin, after injury express SMA	
[116]	Mouse	Col I <sup>+</sup> GFP <sup>+</sup> BMTx	BDL 2 weeks after adoptive transfer	GFP <sup>+</sup> cells were SMA <sup>+</sup> but expressed CD45 and Col $\alpha$ 1 (I)	Injury recruits BM-derived fibrocytes
[7]	Mouse	Male BMTx to female	CCl <sub>4</sub>	70% of SMA <sup>+</sup> myofibroblasts were BM-derived	No cell fusion, Y <sup>+</sup> cells express mRNA for procollagen $\alpha$ 1 (I)
[117]	Mouse	GFP <sup>+</sup> HSCTx (clonally-derived from lin <sup>+</sup> Sca-1 <sup>+</sup> c-Kit <sup>+</sup> CD34 <sup>+</sup> cells)	CCl <sub>4</sub>	50–60% of GFP <sup>+</sup> cells were GFAP <sup>+</sup> and $\alpha$ -SMA <sup>+</sup>	Male-to-male Tx, no double Y <sup>+</sup> cells—no fusion
[118]	Mouse	GFP <sup>+</sup> BMTx	BDL at 4 weeks after Tx	After 7 days, 25% of $\alpha$ -SMA <sup>+</sup> cells were GFP <sup>+</sup>	

BDL, bile duct ligation; BMTx, bone marrow transplant; CCl<sub>4</sub>, carbon tetrachloride; Col I, collagen I; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein, SMA, smooth muscle actin.

**Table 3b.** Experimental bone marrow cell transplantation therapies that have reduced hepatic fibrosis

Reference	Species	Method	Injury	Evidence	Comments
[119]	Mouse	Flk1 <sup>+</sup> MSCTx immediately after first CCl <sub>4</sub> injection	DMN or CCl <sub>4</sub>	Reduced fibrosis, limited differentiation to albumin <sup>+</sup> epithelial cells	MSCTx after 1 week of CCl <sub>4</sub> has no effect
[120]	Rat	MSCTx during liver injury regime	CCl <sub>4</sub>	Reduced fibrosis	Animal survival rates improved
[121–123]	Mouse	GFP <sup>+</sup> BMTx midway through 8 week CCl <sub>4</sub> regime	CCl <sub>4</sub>	Reduced fibrosis linked to MMP-9 expression	Engrafted cells claimed to be hepatocytes
[124]	Rat	MSCTx at start of CCl <sub>4</sub> treatment	CCl <sub>4</sub>	Reduced fibrosis: Restoration of normal albumin levels	If MSCs not exposed to HGF before Tx, then no effect
[125]	Mouse	EGFP <sup>+</sup> BMTx	CCl <sub>4</sub>	MMP-9 and -13 expressed by GFP <sup>+</sup> and GFP <sup>-</sup> cells	G-CSF enhanced engraftment and accelerated fibrosis regression

DMN, dimethylnitrosamine; G-CSF, granulocyte-colony stimulating factor; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase; MSCTx, mesenchymal stem cell transplantation; see Table 3a for other abbreviations.

## What are the founder cells of HCC and CC?

Stem cells and cancer are inextricably linked. In continually renewing tissues such as the intestinal 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, HCC and CC, is more problematic. The reason for this is the existence of HPCs, along with hepatocytes 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. Taking this view, Sell has opined that in models of experimental hepatocarcinogenesis as a whole, there may be at least four distinct cell lineages susceptible to neoplastic transformation [130]. This 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 epithelium implicates 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 'non-descript 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-mismatch bone marrow transplantation and the appropriate carcinogenic regimen. In the mouse, an origin of HCC from bone marrow has been discounted in a model of chemical hepatocarcinogenesis [131].

Hepatocytes are implicated in many instances of HCC, eg in mouse models of HCC, oncogenic transgenes are driven by albumin promoters. Many models of liver cancer utilize a brief exposure to a genotoxic 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 [132] demonstrated very clearly that the carcinogenic effects of dimethylnitrosamine (DMN) on the rat liver were only apparent if the compound was administered shortly after a PH, particularly after 1 day, when some 30–40% of hepatocytes would be in S phase (see Figure 1), whereas the same compound, at the same dose, was not carcinogenic to normal adult rats. Thus, hepatocytes appear to be the origin of cancer here. Likewise, in rats following 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 also develops from hepatocytes in this model [133].

The direct involvement of hepatocytes in hepatocarcinogenesis has been clearly established in rats. Gournay *et al* [134] found that some pre-neoplastic foci (expressing  $\gamma$ -glutamyl transpeptidase and the placental form of glutathione-S-transferase) were directly descended from hepatocytes. This was established by stably labelling hepatocytes at 1 day after a two-thirds PH with  $\beta$ -galactosidase, using a recombinant retroviral vector containing the  $\beta$ -galactosidase gene; subsequent feeding with 2-acetylaminofluorene led to foci, some of which were composed of  $\beta$ -galactosidase-expressing cells. Using the same labelling protocol, Bralet *et al* [135] observed that 18% of hepatocytes expressed  $\beta$ -galactosidase at the completion of regeneration after a two-thirds PH; subsequent chronic treatment with diethylnitrosamine (DEN) resulted in many HCCs, of which 17.7% of the tumours expressed  $\beta$ -galactosidase, leading to the conclusion that a random clonal origin of HCC from mature hepatocytes was operative in the model.

As discussed above, there is now compelling evidence that oval cells/HPCs are at the very least bipotent, capable of giving rise to both hepatocytes and cholangiocytes. The fact that oval cell activation (ductular cell reaction) precedes the development of HCC in almost all models of hepatocarcinogenesis and invariably accompanies chronic liver damage in humans makes it almost certain that the mature hepatocyte is not the cell of origin of all HCCs; indeed, perhaps only a small minority of HCCs are derived from the mature hepatocyte. The fact that oval cells/HPCs can be infected with HBV is also consistent with a possible histogenesis of HCC from such cells [136]. 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. 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 [137]. 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 [138]. Cells resembling HPCs (eg OV.1<sup>+</sup> or OV-6<sup>+</sup>; see Table 2) have also been noted in hepatoblastoma [139–142], the most common liver tumour in childhood, likely to be stem cell-derived given there can be both epithelial and mesenchymal tissue components. Hepatoblastomas can even have structures mimicking embryonic intrahepatic bile duct formation, with the formation ductal plate-like structures [143,144].

Direct evidence of a role for oval cells in the histogenesis of HCC can be obtained experimentally; Dumble *et al* [145] isolated oval cells from p53-null mice

and when the cells were transplanted into athymic nude mice they produced HCCs. A probable origin of HCC from oval cells is suggested by the fact that if oval cell expansion is blocked in the CDE diet mouse model by targeting c-Kit with imatinib mesylate, then tumour formation is reduced [146]. If tumours do arise from oval/HPCs, then this would suggest a block in oval cell differentiation, a process termed 'stem cell maturation arrest' [147,148]. This hypothesis is supported by the fact that HCCs induced by a CDE diet exhibit a range of neoplastic phenotypes recapitulating stages in normal development, suggesting transitional states between bipotent oval cells and hepatocytes [149]. Along these lines of thought in humans, four prognostic subtypes of HCC have been identified, corresponding to a hierarchy of liver cell lineages [150]. Those with the poorest prognosis possessed a sizeable proportion of either EpCAM<sup>+</sup>AFP<sup>+</sup> cells (hepatoblast-like) or EpCAM<sup>-</sup>AFP<sup>+</sup> cells (HPC-like), whereas those with EpCAM<sup>-</sup>AFP<sup>-</sup> cells (mature hepatocyte-like) or EpCAM<sup>+</sup>AFP<sup>-</sup> cells (cholangiocyte-like) had a more favourable outcome. Moreover, gene expression profiling has identified a subset of HCCs with a poor prognosis that have a profile consistent with an origin from HPCs [151], and simple enumeration of CK19-positive cells in HCC can identify a patient group who have a shorter time to recurrence [152].

### Cancer stem cells in HCC

There is a growing realization that many, if not all, cancers contain a minority population of self-renewing stem cells, the *cancer stem cells* (CSCs) which are entirely responsible for sustaining the tumour as well as giving rise to proliferating but progressively differentiating cells that are responsible for much of the cellular heterogeneity that is so familiar to histopathologists [153]. We have suggested that many liver tumours probably have their origins in normal liver stem cells, particularly HPCs, but do liver tumours

have CSCs? With the development of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice for the xenografting of human tumours came the first good *in vivo* evidence for the existence of CSCs in leukaemia, breast and brain tumours. This assay has been criticized as simply reflecting the ability of human cells to grow in a foreign, inappropriate murine microenvironment, and as such the cells should be called 'tumour-initiating cells' (TICs), rather than CSCs. Whatever the limitations of the assay, several markers have been advocated with which to prospectively isolate human HCC CSCs (Table 4).

Chiba and colleagues [154] have reported that two of the four HCC cell lines that they studied had SP cells comprising 0.25% and 0.8% of the cell population. These cells were highly proliferative and relatively resistant to apoptosis *in vitro*. Microarray analysis indicated that several genes implicated in 'stemness', eg *WNT* pathway genes, were substantially up-regulated in the SP cells in comparison to non-SP cells. Using the 'gold-standard' NOD/SCID mouse assay for CSCs, they found that transplanting 10<sup>3</sup> liver SP cells consistently yielded tumours, whereas transplantation of 10<sup>6</sup> non-SP cells failed to give rise to tumours — so are SP cells in HCC the CSCs? They could be in some cases? Prominin-1 (CD133) a pentaspan membrane protein, whose function is as yet unclear, has been suggested as a CSC marker in many different tumours, including colon, pancreas, brain and prostate [153,162,163], although its utility has recently been called into question [164]. Using the CD133/1 antibody, a number of studies have suggested that the CD133-positive fraction enriches for HCC CSCs (Table 4). As might be expected of CSCs, they appear as a minority (<2%) population in primary tumours, although continued passaging has resulted in considerable enrichment of CD133<sup>+</sup> cells in some HCC cell lines, up to 90% [157]. HCCs with higher than the median number (1.32%)

**Table 4.** Markers used to isolate putative cancer stem cells from human HCC, tested by tumorigenicity in immunodeficient mice (tumour-initiating cells)

Marker	Frequency (%)	Tumorigenicity*	Comments	Reference
SP	<1% in 2/4 HCC cell lines	1 × 10 <sup>3</sup>		[154]
SP	1–28% in four HCC cell lines	2 × 10 <sup>3</sup> for two lines 2 × 10 <sup>4</sup> for two lines	Frequency of SP related to metastatic potential of primary	[155]
Oct4 <sup>+</sup> TGFβRII <sup>-</sup> ELF <sup>-</sup>	Very rare	Not tested	Elf <sup>+</sup> / <sup>-</sup> mice develop HCC	[28]
CD133	0.05–47% in three HCC cell lines	1 × 10 <sup>7</sup>	1 × 10 <sup>7</sup> CD133 <sup>-</sup> cells were not tumourigenic	[156]
CD133	~2% in primary tumours	5 × 10 <sup>4</sup>	1 × 10 <sup>4</sup> CD133 <sup>+</sup> cells were not tumourigenic	[157]
CD133/ALDH double positive	65% in Hu7 cell line	500 vs. 1000 for CD133 <sup>+</sup> -only cells	Not all CD133 <sup>+</sup> cells were ALDH <sup>+</sup>	[158]
CD133	0.1–1% in primary tumours	500		[159]
CD90 <sup>+</sup> /CD45 <sup>-</sup>	0–1.85% in seven cell lines. 2.5% in HCC	4 × 10 <sup>3</sup>	CD44 blockade prevents tumour formation	[160,161]
OV6	0.2–3% in five HCC cell lines	5 × 10 <sup>3</sup>	Most OV6 <sup>+</sup> cells were CD133 <sup>+</sup>	[98]

\* Cell number injected that formed tumours in majority of recipients, not necessarily the minimum number.

ALDH, aldehyde dehydrogenase; SP, side population.



of CD133-positive cells are correlated with shorter survival, higher recurrence rates and higher tumour grade [165], and CD133<sup>+</sup> cells appear highly resistant to conventional therapeutic drugs, such as 5-FU and doxorubicin [166]. Mishra and colleagues [28] have suggested that HCC CSCs are descendants of normal parenchymal stem cells that have lost sensitivity to the inhibitory growth effects of TGF $\beta$ , while cell selection based on Thy-1 (CD90), a disputed marker of oval cells (see Table 2), in combination with CD44, has also produced cells with aggressive tumorigenic potential [160,161]. It remains to be seen how much overlap there is between these various markers, or whether there is a 'one-fits-all' marker for CSCs in HCC and indeed in other tumour types as well.

## Conclusions

This review has summarized our current knowledge of how the liver regenerates itself after both acute and more chronic iterative damage. Under normal circumstances the differentiated parenchymal cells (hepatocytes) are the functional stem cells, but in more extreme circumstances a 'potential' stem cell compartment can be recruited into action, providing HPCs from the intrahepatic biliary system that can differentiate into hepatocytes. Most cases of HCC arise within a cirrhotic setting, a scenario associated with hepatocyte replicative senescence and HPC activation. Observation and experimental evidence points to a possible origin of HCC from either HPCs or hepatocytes, and HCC itself appears to have a minority population of CSCs. Some of the fibrogenic cells in cirrhosis may be bone marrow-derived, although conversely autologous bone marrow cell therapy may lessen the fibrosis by way of the fibrolytic actions of secreted MMPs.

## Teaching Materials

Power Point slides of the figures from this Review may be found in the supporting information.

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