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STEM CELLS, CELL TRANSPLANTATION AND LIVER REPOPULATION

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Abstract

Liver transplantation is currently the only therapeutic option for patients with end-stage chronic liver disease and for severe acute liver failure. Because of limited donor availability, attention has been focused on the possibility to restore liver mass and function through cell transplantation. Stem cells are a promising source for liver repopulation after cell transplantation, but whether or not the adult mammalian liver contains hepatic stem cells is highly controversial. Part of the problem is that proliferation of mature adult hepatocytes is sufficient to regenerate the liver after two-thirds partial hepatectomy or acute toxic liver injury and participation of stem cells is not required. However, under conditions in which hepatocyte proliferation is blocked, undifferentiated epithelial cells in the periportal areas, called “oval cells”, proliferate, differentiate into hepatocytes and restore liver mass. These cells are referred to as facultative liver stem cells, but they do not repopulate the normal liver after their transplantation. In contrast, epithelial cells isolated from the early fetal liver can effectively repopulate the normal liver, but they are already traversing the hepatic lineage and may not be true stem cells. Mesenchymal stem cells and embryonic stem cells can be induced to differentiate along the hepatic lineage in culture, but at present these cells are inefficient in repopulating the liver. This review will characterize these various cell types and compare the properties of these cells and the conditions under which they do or do not repopulate the liver following their transplantation.

Keywords

stem/progenitor cells; bipotency; oval cells; cell transplantation; liver repopulation

The origin and properties of stem cells

Stem cells originate from the inner cell mass during development of the blastocyst. They are pluripotent and are referred to as embryonic stem (ES) cells [1]. These cells give rise to somatic stem cells that differentiate further into multipotent tissue-specific stem cells [1–3]. These multipotent tissue-specific stem cells then give rise to progenitor cells that subsequently proliferate and differentiate into mature somatic phenotypes that comprise organ mass (Figure 1). Stem cells are generally considered to exhibit four major properties: (1) capacity for self-renewal or self-maintenance (generally slowly cycling); (2)

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multipotency (capable of producing progeny in at least two lineages); (3) functional, long-term tissue reconstitution; and (4) serial transplantability. For stem cells to maintain themselves in adult tissue and at the same time provide cells to maintain the differentiated function(s) of that tissue, some of the cells must undergo division without differentiation or asymmetric division, such that one of the progeny remains undifferentiated, while the other proliferates and differentiates to generate new tissue mass [4–6]. Concerning the liver, some studies on asymmetric cell division have been conducted *in vitro* with hepatic-derived cell lines [7], but this property has not been identified *in vivo*. An alternative method to study self-maintenance *in vivo* is to identify "label retaining cells" and follow their fate after inducing their proliferation and differentiation. Such studies have also been conducted in skin epithelia [8] but not yet in the liver.

Self-renewal is a property unique to stem cells, whereas progenitor cells that are the progeny of stem cells also proliferate and differentiate into somatic populations but do not maintain themselves. They may have single or multi-lineage potential, but are capable of only short-term tissue reconstitution. Progenitor cells have also been well-studied in skin epithelia and the intestinal tract, where they have also been termed "transit amplifying cells" [4]. Activated "oval cells" exhibit many features of "transit amplifying cells" and thus may represent the liver counterpart to these latter cells in an organ where tissue mass turns over very slowly.

Liver regeneration

In the normal adult liver, hepatocytes are in a quiescent state and turn over very slowly (1–2 times/year). However, following two-thirds partial hepatectomy (PH) or acute toxic liver injury in rodents, the liver regenerates very quickly (within 1–2 weeks). A similar process occurs in larger animals and in humans, but at a somewhat slower rate (~1 month). The final size of the liver is proportional to total body weight (~3.0–3.5% in rodents); however, the precise mechanism that regulates hepatic mass has not been determined.

In the 1960s, it was shown in rats that during liver regeneration, hepatocytes throughout the liver parenchyma are actively engaged in DNA synthesis, and it was estimated that 70–90% of hepatocytes undergo at least one round of cell division during this process [9]. However, after two-thirds PH, only one or two divisions of each remaining hepatocyte is required to restore liver mass, so that the proliferative response is rather small. Under normal conditions, liver regeneration is achieved through proliferation of differentiated hepatocytes (including tetraploid cells) and does not require the participation of stem cells [10,11]. However, whether stem cells are involved in normal liver homeostasis or in maintenance of hepatic mass or function during chronic liver injury has not been determined.

Hepatocytes as liver stem cells?

In the last decade, landmark studies have demonstrated that hepatocytes, under specialized circumstances, have virtually unlimited proliferative potential. In urokinase plasminogen activator (uPA) transgenic mice, in which host hepatocytes are continually being destroyed [12], transplanted normal (wt) hepatocytes undergo more than 12 cell divisions on average and replace most of the host liver [13]. In fumarylacetoacetate hydrolase (FAH) knockout mice, a model of Hereditary Tyrosinemia Type 1, in which there is also extensive and continuous liver injury, the metabolic disorder can be corrected by transplanting wt hepatocytes, with full restoration of normal lobular structure and function [14,15]. Using this cell transplantation model, Grompe and coworkers demonstrated further that normal adult hepatocytes can be serially transplanted through seven generations of FAH null mice with each transplanted cell undergoing an average of 69 or more divisions [15]. Therefore, under selected circumstances, the proliferative capacity of mature hepatocytes is virtually infinite.

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In other rodent models of liver repopulation by transplanted cells, host hepatocytes have been rendered incapable of proliferation by DNA damage through treatment with DNA alkylating agents, retrorsine [16] or monocrotaline [17], or by x-irradiation, using a Phillips orthovoltage irradiator [18,19]. Other studies have used genetically modified p27 null mouse hepatocytes exhibiting increased proliferative activity [20] or Bcl-2 transgenic mouse hepatocytes that are resistant to apoptosis [21], in conjunction with repeated host liver injury by carbon tetrachloride (CCl_4) administration or anti-Fas antibody administration to stimulate liver regeneration. All of these models exhibit two critical features: 1) extensive and continuous or repeated liver injury and 2) a selective advantage for transplanted cells to proliferate and/or survive compared to host hepatocytes. It has also been shown recently in rats that hepatocytes can differentiate into cholangiocytes and form mature bile ducts, when there is massive bile duct injury [22]. Thus, hepatocytes fulfill all the criteria for stem cells. However, transplanted hepatocytes do not significantly repopulate the normal (or near normal) adult liver [23,24] and therefore do not behave as stem cells under normal circumstances.

Hepatic stem cells in the adult liver?

Studying liver regeneration in mice after severe nutritional injury in the late 1950's, Wilson and Leduc observed that non-parenchymal cells of the distal cholangioles proliferate and, after cessation of injury, differentiate into hepatocytes and possibly into new interlobular bile ducts and they proposed that there may be stem cells in the adult liver [25]. During the same period, it was established that hepatocytes and bile duct epithelial cells are of common embryonic origin and it was proposed that hepatocytes are derived from epithelial precursors in the distal cholangioles [26,27]. However, specific identification of these cells has not been possible, because unique liver stem cell markers are not currently available. Nonetheless, numerous studies during the last decade have identified cells both within and outside the liver that exhibit properties of hepatic stem cells and can differentiate into hepatocytes and/or bile duct epithelial cells both in culture and after their transplantation (see below).

"Oval cells" as facultative stem cells

The term "oval cells" was first introduced by Farber [28], who found non-parenchymal cells with a characteristic morphologic appearance after treatment of rats with carcinogenic agents, ethionine, 2-acetylaminofluorine (2-AAF) and 3-methyl-4-dimethyl aminobenzene. He described these cells as "small oval cells with scant lightly basophilic cytoplasm and pale blue-staining nuclei" [28]. Thorgeirsson and coworkers subsequently showed that "oval cells", induced to proliferate in the periportal area by treatment of rats with 2-AAF/PH, take up [^{3}H] thymidine and the [^{3}H] thymidine labeled cells subsequently accumulate in the mid parenchyma as clusters of basophilic hepatocytes [29]. The expression of bile duct markers (CK-7, CK-19 and OV-6) and hepatocytic markers (AFP and Alb) in [^{3}H] thymidine labeled "oval cells" over time also suggested a precursor/product relationship between "oval cells" and hepatocytes [29,30]. Moreover, the temporal pattern of liver-enriched transcription factor expression (HNFs and C/EBPs) during "oval cell" activation in rats by 2-AAF/PH also mirrored their expression pattern during liver development [31]. Finally, studies showing activation of stem cell genes, c-kit [32], CD34 [33], flt 3 receptor [34] and LIF [35] during "oval cell" proliferation, suggested that they exhibit stem cell-like properties.

Liver "oval cells" have been referred to as facultative stem cells or as a reserve stem cell compartment [36,10,11]. Clearly, the rat liver regenerates after 2-AAF/PH administration (conditions under which hepatocyte proliferation is blocked) and "oval cells" that are induced in this model differentiate into hepatocytes; however, the percentage of

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repopulation that is derived from “oval cells” has not been determined. The finding that “oval cells” also express hematopoietic stem cell (HSC) genes (such as c-kit, CD34, Sca-1 and Thy-1) has led to the suggestion that they originate from HSC [37–40]. However, several recent studies in mice and rats have shown that transdifferentiation of HCS into “oval cells” is a very rare event, which probably does not have physiological significance [41–43].

Other models of “oval cell” activation

Cells with the morphologic appearance of “oval cells” are also induced to proliferate in rodents by a number of other regimens: administration of a choline-deficient (CD) diet supplemented with ethionine in mice [25], treatment of mice with other DNA alkylating agents; 1,4-bis[N,N′-di(ethylene)-phosphamide]piperazine (Dipin) [44] or 12,18-dihydroxy-senecionan-11-16-dione (retrorsine) in combination with PH in rats [45], feeding 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) in mice [46], phenobarbital/cocaine induced liver injury in mice [47], acute allyl alcohol toxicity in rats [48] or D-galactosamine administration in rats, which temporarily blocks hepatocyte proliferation [49,50].

Transplantation of “oval cells”

The most important property that would establish “oval cells” as liver stem cells would be restoration of liver mass after their transplantation. Isolated “oval cells” and “oval cell” lines have been transplanted into the liver of normal, carcinogen-fed or metabolically defective rats or mice. More than 15 years ago, it was reported that “oval cells” isolated from the liver of rats fed a CD/2-AAF diet produced colonies, i.e. clusters of cells with a phenotype resembling hepatocytes, in the livers of rats fed the same diet but not in recipients receiving a normal diet [51]. However, the level of liver repopulation by these “colonies” was not determined. An epithelial cell line originating from normal rat liver (WB-344), that exhibits stem cell properties *in vitro* [52], also showed very little capacity to repopulate the liver when transplanted into syngeneic rats [53]. “Oval cells” isolated from the liver of D-galactosamine treated rats also engraft and undergo 5–7 rounds of cell division, as opposed to adult hepatocytes that undergo no more than 2–3 cell divisions under the same conditions [54]. This augmented proliferation, however, was not sufficient for “oval cells” to significantly repopulate the normal adult liver.

“Oval cells” isolated from the liver of Long-Evans Cinnamon (LEC) rats and transplanted into LEC or Nagase analbuminemic rats, also displayed an hepatocytic phenotype and produced albumin (Alb) [55], but, once again, liver repopulation was low. However, “oval cells”, isolated from the livers of DDC-fed mice and transplanted into FAH null mice, repopulate the metabolically compromised liver very well, i.e. at least as efficiently as mature hepatocytes [41]. “Oval cells” from GFP transgenic mice, maintained on a DDC diet, were isolated by selection on immunomagnetic beads (MACS), using the surface marker Sca-1 [56]. These “oval cells” were transduced with human α_1-antitrypsin (α_1-AT) and transplanted into monocrotaline-treated C57BL/6 mice in conjunction with PH. In this model, there was 40–50% liver repopulation by GFP expressing cells, of which 5–10% also expressed human α_1-AT [56]. These latter studies show that substantial liver repopulation can be achieved with “oval cells”, but, as with hepatocytes, this requires extensive modification of the host liver.

Thy-1$^+$ cells

In 1998, Petersen et al [37] identified expression of Thy-1 by “oval cells” in the rat 2-AAF/CCl_4 model of liver injury/regeneration. Since Thy-1 is normally expressed by hematopoietic stem and progenitor cells, they subsequently transplanted bone marrow (BM)

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cells into rats and mice, preconditioned by BM ablation, and showed the presence of donor BM derived hepatocyte-like cells in the recipient liver [40]. Avital et al [57] subsequently isolated β_2-microglobulin$^-$/Thy-1$^+$ cells from the BM of rats that had undergone bile duct ligation and showed hepatocytic gene expression of these cells in culture and differentiation into hepatocytes after their transplantation. In more recent studies, it has been reported that Thy-1$^+$ cells isolated from the rat BM repopulate the liver under conditions of severe hepatocyte growth inhibition, together with a liver regenerative stimulus (monocrotaline/BM transplantation/2-AAF/PH), but once again the levels of liver repopulation were still low [58]. Thy-1$^+$ cells have also been identified in the developing rodent and human fetal liver [59,60]. Thy-1$^+$ cells isolated from rat fetal liver showed proliferative activity and express liver specific genes in culture (CK-18, AFP and Alb) [61]. However, these cells repopulate the liver only under severe endogenous hepatocyte growth suppression (retrorsine/PH) [62].

Studies with “oval cell” lines

Stable “oval cell” lines have been established from normal liver of Fischer (F)-344 rats [63] and from the liver of rats fed a CD diet supplemented with DL-ethionine [63,64] or allyl alcohol treatment [48], from livers of LEC rats, an animal model of Wilson’s disease [55], from livers of transgenic rats expressing the Ras oncogene [65], from p53 knockout, normal or TAT-GRE lacZ mice fed a CD, ethionine-supplemented diet [66,64] and from human liver [67]. The common feature of these “oval cell” lines is their ability to differentiate into cells expressing hepatocytic or biliary epithelial cell genes in cell culture. This suggests that they are bipotent, but *in vivo* transplantation studies with these cell lines are again very limited.

Hepatic stem cells in the fetal liver

In the mouse, endodermal stem cells begin to proliferate when the ventral wall of the endoderm becomes positioned next to the developing heart on embryonic day (ED) 8.0 [26,68–70]. Specification toward the hepatic epithelial lineages occurs at ED8.5 and requires fibroblast growth factor (FGF) signaling from the cardiogenic mesoderm [71] and bone morphogenic protein (BMP) signaling from the septum transversum mesenchyme [72]. By ED9.0–9.5, these cells begin to express GATA4 and liver-enriched, nuclear factor HNF4α, as well as liver-specific genes, AFP followed by Alb [70,73]. These events occur one day later in the rat. The hepatic-specified cells are now referred to as hepatoblasts and proliferate massively. Cords of hepatoblasts invade the septum transversum mesenchyme that contains stellate cells and sinusoidal endothelial cells. These cells secrete a variety of cytokines and growth factors, such as EGF, FGF, HGF, TGFβ, TNFα and IL-6, that are known to be involved in liver development, as well as in the hepatocyte proliferative response during liver regeneration [70,11].

At ED11 in the rat, HSC invade the liver bud to form a visible liver structure that is primarily an hematopoietic organ. Hepatoblasts continue to expand rapidly and begin to express placental alkaline phosphatase, intermediate filament proteins CKs-14, 8 and 18 and γ-glutamyl-transpeptidase (GGT), and later α_1-AT, glutathione-S-transferase (GST)-P and fetal isoforms of aldolase, lactic dehydrogenase and muscle pyruvate kinase (M2-PK) [74–77]. Just prior to ED16, hepatoblasts diverge along two lineages, hepatocytes and cholangiocytes [73,78,79]. Differentiation along the cholangiocytic lineage is promoted by Notch signaling and is antagonized by HGF, which in conjunction with oncostatin M promotes hepatocytic differentiation [80]. After ED16, there is a massive change in the gene expression profile of rat fetal liver epithelial cells to a more differentiated phenotype [81] and the percentage of bipotent cells, i.e., those expressing genes in both the hepatocytic and cholangiocytic lineages (e.g., AFP or Alb and CK-19, respectively), is markedly reduced

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[78,79,82,24]. At this point, most of the cells are unipotent and irreversibly committed to either the hepatocytic or cholangiocytic lineage [79,24]. As organogenesis proceeds, intrahepatic bile ducts are formed in the vicinity of large portal vein branches, beginning on ~ED17 [83]. The basic lobular structure is now completed, although the parenchymal cords do not become fully mature until several weeks after birth.

Bipotential cells and cell lines generated from rodent fetal liver

Single cells that are $RT1A^{1-}/OX18^{low}/ICAM\text{-}1^{+}$ have been isolated from rat ED13 fetal liver by Kubota and Reid [84]. These cells are clonogenic in culture and exhibit bipotential differentiation, producing epithelial colonies expressing CK-19, Alb or both proteins. Suzuki et al. [85] isolated highly proliferative cells from mouse liver ($Ter119^{-}/CD45^{-}/c\text{-}kit^{-}/CD29^{+}/CD49f^{+}$), termed hepatic colony-forming-units (H-CFU-C), that showed hepatocytic and biliary lineage markers in cell culture. They subsequently isolated H-CFU-C as single cells by flow cytometry and these cells exhibited bipotential differentiation *in vivo* [86]. These cells also exhibited pancreatic, gastric and intestinal epithelial differentiation *in vitro* and *in vivo* and were regarded as endodermal stem cells, although their ability to repopulate the liver was very low. Specific surface markers for mouse hepatoblasts have also been identified: Liv2 [87], E-cadherin [88] and Dlk-1 [89], and it has been shown that all of these markers identify a population of $AFP^{+}/Alb^{+}/Pan\text{-}CK^{+}/Sca\text{-}1^{+}/c\text{-}kit^{-}/CD34^{-}/CK\text{-}19^{-}$ hepatoblasts at ED12.5 [90]. Both E-cadherin and Dlk-1 can be used to purify fetal hepatoblasts by immunomagnetic beads or flow cytometry [88–90]. Most recently, Neighbor of PuncE11 (Nope), a cell surface gene similar to neogenin, that is expressed during transition of cells from an undifferentiated to a differentiated cell phenotype [91], has been identified in mouse fetal liver epithelial cells and may serve as a specific marker for these cells [92].

Cell lines have been established from mouse embryonic foregut at ED9.5 [93]. One of these lines, HBC-3, grows well at low cell density and exhibits bipotential differentiation properties. This cell line also identified the importance of the Wnt signaling pathway during hepatocytic differentiation [94]. After introduction into mouse blastocysts, HBC-3 cells are incorporated into most tissues of the body and maintain their hepatic epithelial phenotype [95]. Using transgenic mice expressing a constitutively active truncated human Met receptor (c-met), met murine hepatocyte (MMH) cell lines have been established from mouse ED14.5 fetal livers and neonatal liver [96]. These MMH cell lines were subsequently subcloned, producing cells with an epithelial morphology expressing liver-enriched transcription factors ($LETF^{+}$) (HNF1α and HNF4), as well as E-cadherin and Zo-1, but not expressing Alb, TTR or β-fibrinogen. Cells with a fibroblastic appearance, termed palmate cells, were essentially negative for all hepatic epithelial markers but surprisingly gave rise to epithelial clusters or bile duct-like structures when cultured under selective differentiation conditions [97].

Bipotential cell lines have also been established from non-transgenic mouse embryonic and adult liver (BMEL and BMAL, respectively) that exhibit bipotential capacity *in vitro* and participate in liver regeneration in uPA/SCID mice *in vivo* [98,99]. These cell lines exhibit some properties of stem cells including: 1) clonality, 2) bipotential differentiation, 3) high proliferative capacity in culture, and 4) ability to differentiate into mature hepatocytes and bile duct cells *in vivo*. However, despite the fact that these cells grow very well in culture and can be readily passaged, they produce significant liver repopulation only under conditions of massive liver injury and preferential selection of transplanted cells, the same conditions that allow liver repopulation by adult hepatocytes [13,14]. It is thought that this may reflect a low engraftment efficiency. However, in the early 1990s, Grisham and colleagues reported in rats that the host liver exerts an anti-proliferative and anti-tumor

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effect on transplanted, chemically transformed WB-344 cells and that this effect decreases with age of the host recipient [100].

Epithelial-to-mesenchymal transition (EMT)

The palmate cells, originally described in mice by Weiss and colleagues [97], probably represent an epithelial-to-mesenchymal transition (EMT), a process that is now well-known in cultured cells. Cultured fetal rat hepatocytes have been shown to undergo EMT when treated with TGFβ or fetal bovine serum [101]. These cells express high levels of vimentin and Snail and lose expression of CK-18 and E-cadherin. Both hepatic stellate cells and cultured human hepatic epithelial progenitor cells undergo EMT in culture [102]. Most recently, primary adult hepatocytes were shown to undergo EMT, also when cultured with TGF-α1 [103]. Co-expression of hepatic epithelial and hematopoietic stem and progenitor cell markers by adult rat or human BM-derived cells in culture, such as c-kit, CD34 and Thy-1, may also represent EMTs [104,105]. The reverse process, i.e. mesenchymal-to-epithelial transition (MET), has also been reported with cells derived from human islets that first undergo EMT when cultured in serum containing medium to produce fibroblast-like cells, which then proliferate very well [106]. By simply switching to serum-free medium, these cells can be reconverted from fibroblast-like cells into proinsulin and proglucagon producing islet-like cells (MET).

EMT is also known to occur *in vivo* during remodeling of tissues in early embryogenesis [107,108] and recent studies in nematodes and zebrafish suggest that endoderm and some portion of the mesoderm may be derived from a bipotential layer of cells called mesendoderm [109]. EMT has been reported in fetal liver in stromal cells that express both mesenchymal and epithelial markers [102]. What is most interesting is that EMT of hepatocytes to fibroblasts has been clearly demonstrated *in vivo* by lineage tracing studies in lacZ transgenic mice chronically treated with CCl_4 (103). In these studies, almost 50% of fibroblasts observed in CCl_4-induced hepatic fibrosis were derived from hepatocytes by EMT, although it is possible that the cells undergoing EMT were hepatocyte progenitor cells. These findings have significant implications in terms of the pathogenesis of chronic liver disease.

Stem cell properties of primary rat fetal liver epithelial cells

As indicated previously, the ultimate test for a putative stem cell is to demonstrate its ability to self-renew *in vivo* and to functionally repopulate a tissue or organ, long-term (properties that have been shown repeatedly with hematopoietic stem cells). Sandhu et al. [24] reported 5–10% repopulation of $DPPIV^-$ mutant F344 rat liver by transplanting wt ED14 fetal liver epithelial cells in conjunction with two-thirds PH. Liver repopulation by transplanted cells increased progressively over six months, and the bulk of repopulating clusters contained both hepatocytes and mature bile ducts. The transplanted cells were integrated into the host parenchyma and formed hybrid bile canaliculi with host hepatocytes. Thus, transplanted rat ED14 fetal liver epithelial cells exhibited three major properties of liver stem cells, 1) extensive proliferation, 2) bipotency and 3) long-term repopulation *in vivo* [24]. Liver repopulation by transplanted rat fetal liver cells was achieved in a non-selective host liver environment but required PH to initiate the process. Using greater numbers of unfractionated rat ED14 fetal liver cells, Oertel et al. [110] achieved 23.5% replacement of liver mass at 6 months under the same non-selective conditions. Repopulation continues to increase for up to one year, reaching an average of ~30% for the total liver (Figure 2; Oertel et al, unpublished data). This represents greater than 1,000-fold amplification of transplanted fetal liver epithelial cells in the host organ. The mechanism for liver repopulation by rat fetal liver stem/progenitor cells is cell competition (110), a process originally described in *Drosophila*

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during wing development [111,112]. However, self-renewal or serial transplantability has not yet been demonstrated with these cells, and thus they have been termed “fetal liver stem/progenitor cells” (FLSPC) [110]. These cells have been crypreserved with full ability to repopulate the normal adult liver after thawing [113] and most recently, rat FLSPC have been enriched to 95% purity by immunoselection [114].

Liver repopulation by extrahepatic and embryonic stem cells

Various studies have reported that cells from the BM are released into the circulation, migrate to the liver and differentiate into hepatocytes. However, the extent to which this occurs and the mechanism(s) involved remain highly controversial [for reviews, see 115–117]. Estimates of liver repopulation by hematopoietic cells vary widely, ranging from < 0.01% to 40% [40,118–128]. Originally, Petersen and coworkers reported that BM stem cells from $DPPIV^+$ F344 rats transplanted into sublethally irradiated $DPPIV^-$ F344 rats repopulate the BM and then migrate to the liver and “transdifferentiate” into hepatocytes by entering the liver “oval cell” progenitor pathway [40]. This mechanism was generally accepted until the studies of Wang et al [41] using lacZ marking, showed that BM cells did not enter the “oval cell” pool in wt mice treated with DDC or contribute to liver repopulation by “oval cells” in secondary $FAH^{-/-}$mouse recipients, and Dabeva and coworkers [42] showed in rats that $DPPIV^+$ BM cells transplanted into $DPPIV^-$ rats contributed less than 1% to “oval cells” expanded by 3 different methods: 1) 2-AAF/PH, 2) retrorsine/PH or 3) D-galactosamine-induced liver injury.

In $FAH^{-/-}$ mice and other model systems, it has been shown that cell fusion, rather than transdifferenitation, represents the mechanism whereby hematopoietic cells acquire an hepatocytic phenotype. Initial studies in cell culture revealed that BM and neuronal cells can fuse with ES cells [129,130]. Wang et al [131] and Vassilopoulos et al [132] subsequently showed that hematopoietic stem cells fuse with hepatocytes in FAH null mice to produce cells expressing the deficient enzyme, which then expand massively to restore liver mass and function [131,132]. Fusion also occurs between hematopoietic cells and neurons or muscle cells [133,134] and it has been shown that myelomonocytic cells can fuse with hepatocytes [135,136] or muscle cells [137] to produce somatic hybrids expressing genes from both parental cell types.

However, in other studies reported during the same period, fusion did not appear to be required for BM-derived cells to differentiate into hepatocytes [138–140]. In two of these studies, transplanted BM-derived cells are scattered sparsely throughout the hepatic parenchyma, repopulation clusters are very small and total liver repopulation is low. However, in the third study, Jang et al. [140] reported much higher levels and rapid liver repopulation using a specific subpopulation of HSC that were pre-homed to the BM, reisolated and then transplanted into the liver. When BM pre-homed HSC were placed in a culture chamber over minced tissue from CCl_4-injured liver, separated by a 0.4 μm pore size membrane in a trans-well apparatus, these cells exhibited many hepatocytic gene expression characteristics [140]. After transplantation of BM pre-homed HSC into mice pretreated with CCl_4 to induce liver regeneration, there was 7.6% liver repopulation in one week. This finding was most surprising, as it required nearly 100% engraftment efficiency of pre-homed cells, approximately 7 divisions of transplanted cells and no loss of transplanted cells or their progeny during the regenerative process.

Other studies have transplanted unfractionated or $CD34^+$ enriched cells from human cord blood [141–144], multipotent adult progenitor cells (MAPC) [145,146] or mesenchymal stem cells [147–149] into the liver of immunodeficient mice that express a differentiated hepatocytic phenotype [141–152]. However, liver repopulation by these cells is also very

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low. Several recent studies report that mesenchymal stem cells, isolated from adipose tissue and differentiated in culture along the hepatocytic lineage, can also engraft in the liver parenchyma and contribute to liver regeneration [153,154]. In one of these studies [154], large repopulation clusters were obtained with hepatocyte-differentiated mesenchymal stem cells, but this required retrorsine treatment. These studies are promising, but the ability of mesenchymal stem cells to repopulate the adult liver under more normal, clinically viable circumstances will need to be established. Similarly, it has been reported that ES cells can be induced along the endodermal and hepatocytic lineages in culture [155–162], and then transplanted into the liver with differentiation into both mature hepatocytes [161,162] and bile duct epithelial cells [162]. The levels of liver repopulation obtained with hepatocyte-differentiated ES cells are also low, but somewhat higher when transplanted into MUP-uPA/SCID mice [162]. However, it is hoped that conditions will be developed in the future in which lineage-specified ES cells will be therapeutically effective.

In GFP transgenic mice transplanted with a wt liver, it has been shown that extrahepatic cells, expressing stem cell markers c-kit and Sca-1, enter the liver and continue to express these genes, as well as liver progenitor cell markers, A6 and human specific antigen [163]. Overall, GFP^+ cells comprised 9% of cells in periportal areas at 28 days after transplantation of an undersized, but not a normal size graft. However, at present, it is unclear what % of GFP^+ cells were hepatocytic. These experiments are reminiscent of cross gender studies in humans in which males with end stage liver disease received transplants of female livers [119,120]. The presence of extrahepatic-derived male cells in the female liver (presumably of hematopoietic origin) was documented by Y-chromosome *in situ* hybridization in mice and humans and it was reported that the cells containing Y-chromosomes were hepatocytes [118–121,123]. However, fluorescence *in situ* hybridization (FISH) is very difficult technologically, and it is difficult to quantify this process and determine the specific phenotype(s) of extrahepatic-derived Y-chromosome positive cells in the liver.

Other roles for BM and mesenchymal cells in liver regeneration

Recent studies have discovered that BM stem cells may play a major role in either generation or resolution of hepatic fibrosis, as well as in liver regeneration following acute or chronic liver injury. Forbes and colleagues observed that a substantial proportion (6.8–22%) of myofibroblasts in human liver scar tissue are of extrahepatic (BM) origin [164]. Subsequently, studies in mice showed that the contribution of BM cells to parenchymal regeneration is minor (0.6%), whereas the BM contributed extensively to the hepatic stellate (68%) and myofibroblast (70%) cell populations [165]. Moreover, in mice myofibroblasts in the liver originate largely from mesenchymal stem cells and macrophages derived from the BM, and these myofibroblasts coordinate both production of hepatic fibrosis and liver injury and resolution of fibrosis during recovery from inflammatory liver injury [166].

Other studies have reported that injections of BM-derived stem cells can stimulate liver regeneration and restore liver function during chronic liver injury by enhancing the degradation of liver fibrosis in mice [167,168]. Such events are associated with induction of metalloproteinases, especially MMPs 2, 9, and 13 [169]. Most recently, it has been reported that BM-derived endothelial progenitor cells (EPCs), injected into the spleen during liver injury, engraft in the liver, form new blood vessels and secrete growth factors, such as HGF, TGFα, EGF and VEGF, that stimulate liver regeneration and improve survival of animals with massive liver injury [170]. Thus, the role of BM stem cells in liver regeneration may be supportive in generating new parenchymal mass and, under some circumstances, in ameliorating hepatic fibrosis. Similar mechanisms have been reported in mice to be responsible for improved cardiac function following transplantation of BM cells [171].

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Human “oval cells” and stem cells

A human counterpart to “oval cell” activation has been described in liver tissue obtained from patients with extensive chronic liver injury or submassive hepatic necrosis, i.e. the so-called “ductular reaction” (for a detailed description, see ref. 172). In very simple terms, “ductular reactions” are comprised of collections of cells in ductular arrays with the morphologic appearance and immunohistochemical markers comparable to those found in rodent “oval cells”. They are present primarily in the portal tracts and extend into the parenchyma expressing both hepatocytic and bile ductular markers, as well as certain neuroendocrine genes [172–175]. Using simultaneous double and triple immunohistochemistry, Zhou et al [176] have most recently shown that “ductular reactions” are bipolar structures with cells at one pole exhibiting hepataocytic morphology and gene expression (HepPar1 or HepPar1/NCAM) and cells at the other pole exhibiting biliary morphology and gene expression (CK-19 or CK-19/NCAM), with undifferentiated epithelial cells in the center expressing only NCAM. Cells with similar morphologic and immunohistochemical properties have also been identified in the human fetal liver beginning at 4 weeks gestation [177].

A number of investigators have isolated, cultured and/or passaged human fetal liver epithelial cells with bipotent properties, and several of these studies have demonstrated their differentiation into hepatocytes after transplantation into SCID or nude mice [178–180]. Schmelzer, et al [181] have most recently identified two populations of hepatic progenitor cells from human fetal, neonatal and pediatric liver that exhibit stem cell properties. One population is thought to represent an hepatic stem cell (AFP^{-}/Alb^{+}) and the other a slightly more differentiated hepatoblast (AFP^{+}/Alb^{+}). In a most recent study, Schmelzer et al. [182] report data suggesting that these cells may reside in the Canals of Hering and in culture, they can differentiate into hepatoblasts, possibly requiring cues from companion cells that copurify with them during immunoselection. One curious question, which remains unanswered, is that the presumed hepatic-specified stem cells in human neonatal, pediatric and adult liver are Alb positive but AFP negative, which is precisely opposite to what one might expect based on expression of AFP before Alb during liver development [73]. Nonetheless, these studies suggest that a human liver somatic stem cell might exist and hopefully future studies will demonstrate *in vivo* self-renewal and long-term repopulation of the liver by these cells, proving that they are indeed stem cells.

In summary, considerable advances have been made in identifying cells in the fetal, neonatal and adult liver with stem-cell properties. Cell lines have also been established, including ES cells, fetal liver cells, and oval (progenitor) cells that also exhibit stem cell properties and differentiate into hepatocytes and/or bile ducts *in vitro* and *in vivo* (Figure 3). However, all of these cells and cell lines have shown only limited repopulation of the normal liver at the current state-of-the-art, except for rat fetal liver stem/progenitor cell that produce substantial long-term replacement and function. In order to further advance the field of liver cell therapy, it will be necessary to find conditions under which cells and cell lines derived from ES, fetal liver or adult liver, can be expanded in culture and successfully repopulate the liver under conditions that will be clinically acceptable. Such cell therapy can be readily visualized for treatment of inborn metabolic disorders, as well as chronic liver diseases of various etiologies.

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List of abbreviations

ES	embryonic stem
PH	partial hepatectomy
uPA	urokinase plasminogen activator
wt	wild-type
FAH	fumarylacetoacetate hydrolase
CCl_4	carbon tetrachloride
2-AAF	2-acetylaminofluorine
CK	cytokeratin
AFP	α-fetoprotein
Alb	albumin
HNF	human nuclear factor
C/EBP	CCAAT/enhancer-binding protein
flt	fms-like tyrosine kinase
LIF	leukemia inhibitory factor
HSC	hematopoietic stem cell
CD	choline-deficient
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
LEC	Long-Evans Cinnamon
GFP	green fluorescent protein
FACS	fluorescence activated cell sorting
α_1-AT	alpha1-antitrypsin
BM	bone marrow
F	Fischer
ED	embryonic day
FGF	fibroblast growth factor
BMP	bone morphogenic protein
GATA	GATA binding protein (globin transcription factor)
EGF	epidermal growth factor
HGF	hepatocyte growth factor
TGF	transforming growth factor
TNF	tumor necrosis factor
IL	interleukin
GGT	gamma-glutamyltranspeptidase
GST	glutathione-S-transferase
M2-PK	muscle pyruvate kinase

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ICAM	intracellular adhesion molecule
H-CFU-C	hepatic colony-forming-unit
Dlk	delta-like protein
HBC	hepatoblast cell line
MMH	met murine hepatocyte
TTR	transthyretin
SCID	severe combined immunodeficiency
EMT	epithelial-to-mescenchymal transition
MET	mesenchymal-to-epithelial transition
DPPIV	dipetidyl peptidase IV
VEGF	vascular endothelial growth factor
HepPar	hepatocyte paraffin
NCAM	neural cell adhesion molecule

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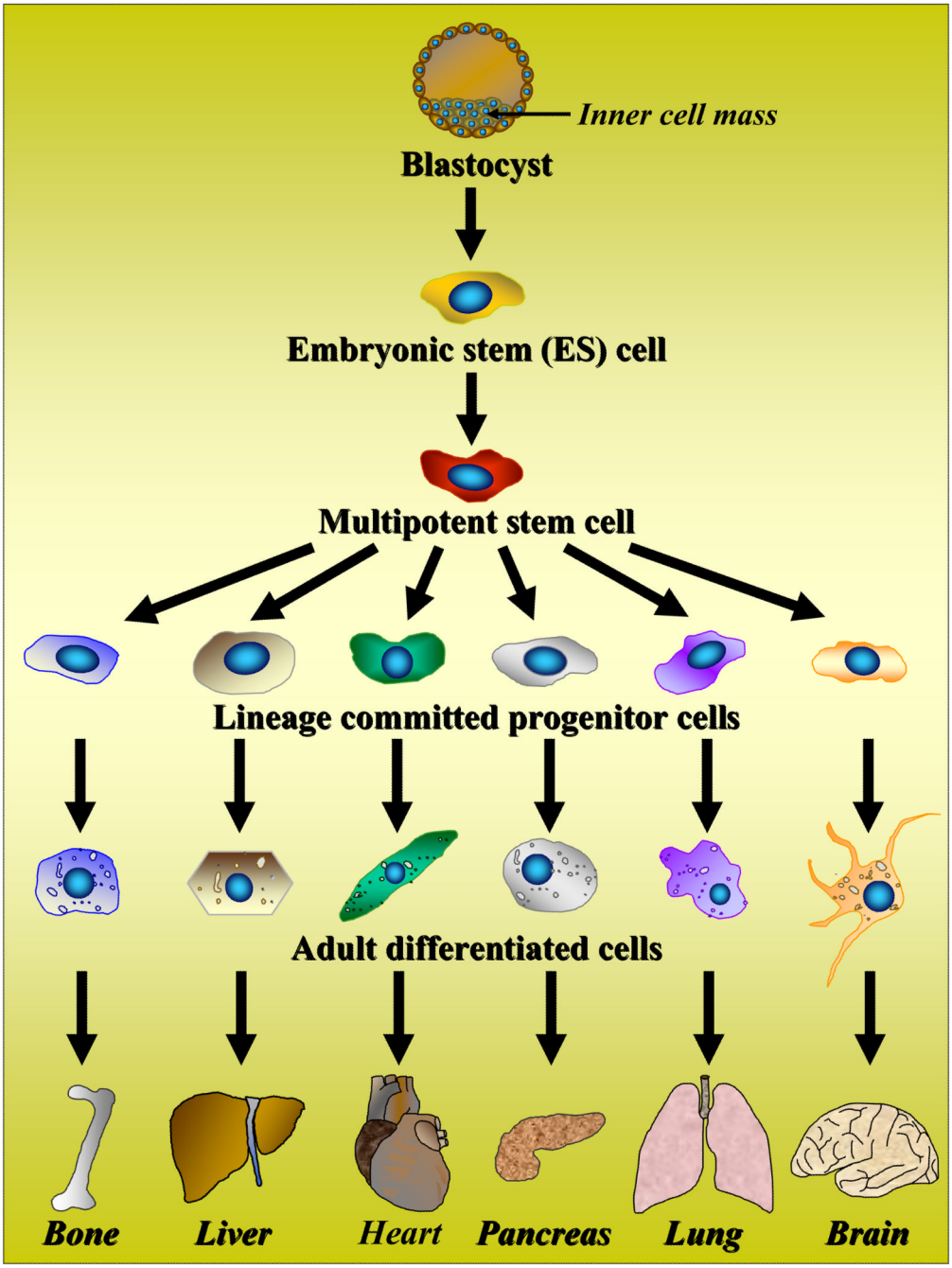

Figure 1.
Schematic diagram showing the lineage progression of stem cells in the mammalian blastocyst to adult somatic cells in various tissues.

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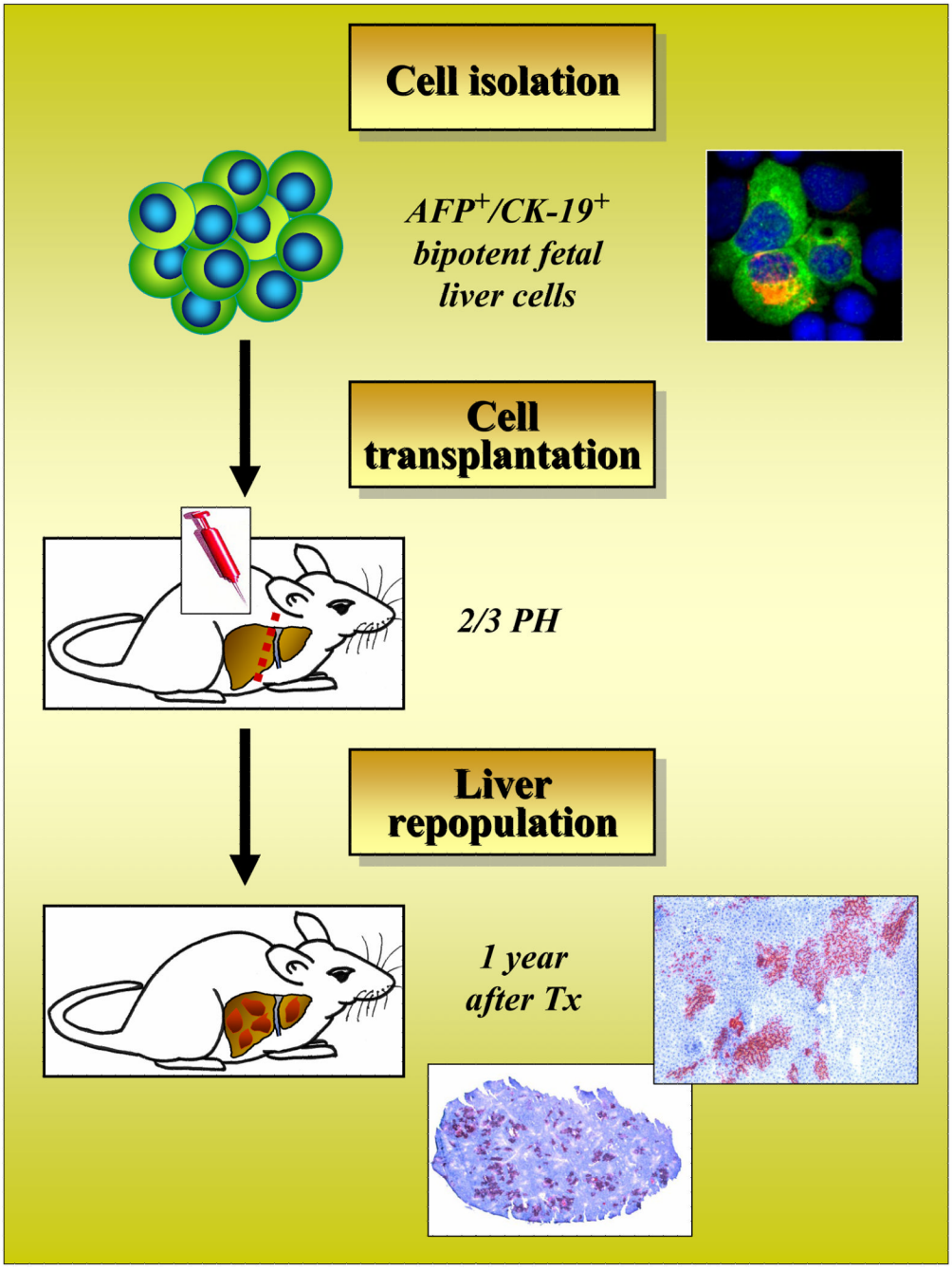

Figure 2.
Experimental design used to show repopulation of the rat liver by fetal liver stem/progenitor cells. Cells isolated from wt ($DPPIV^+$) ED14 F344 rat liver are transplanted into the liver of adult $DPPIV^-$ mutant F344 rats immediately after two-thirds PH and repopulation is followed over time by enzyme histochemistry for DPPIV.

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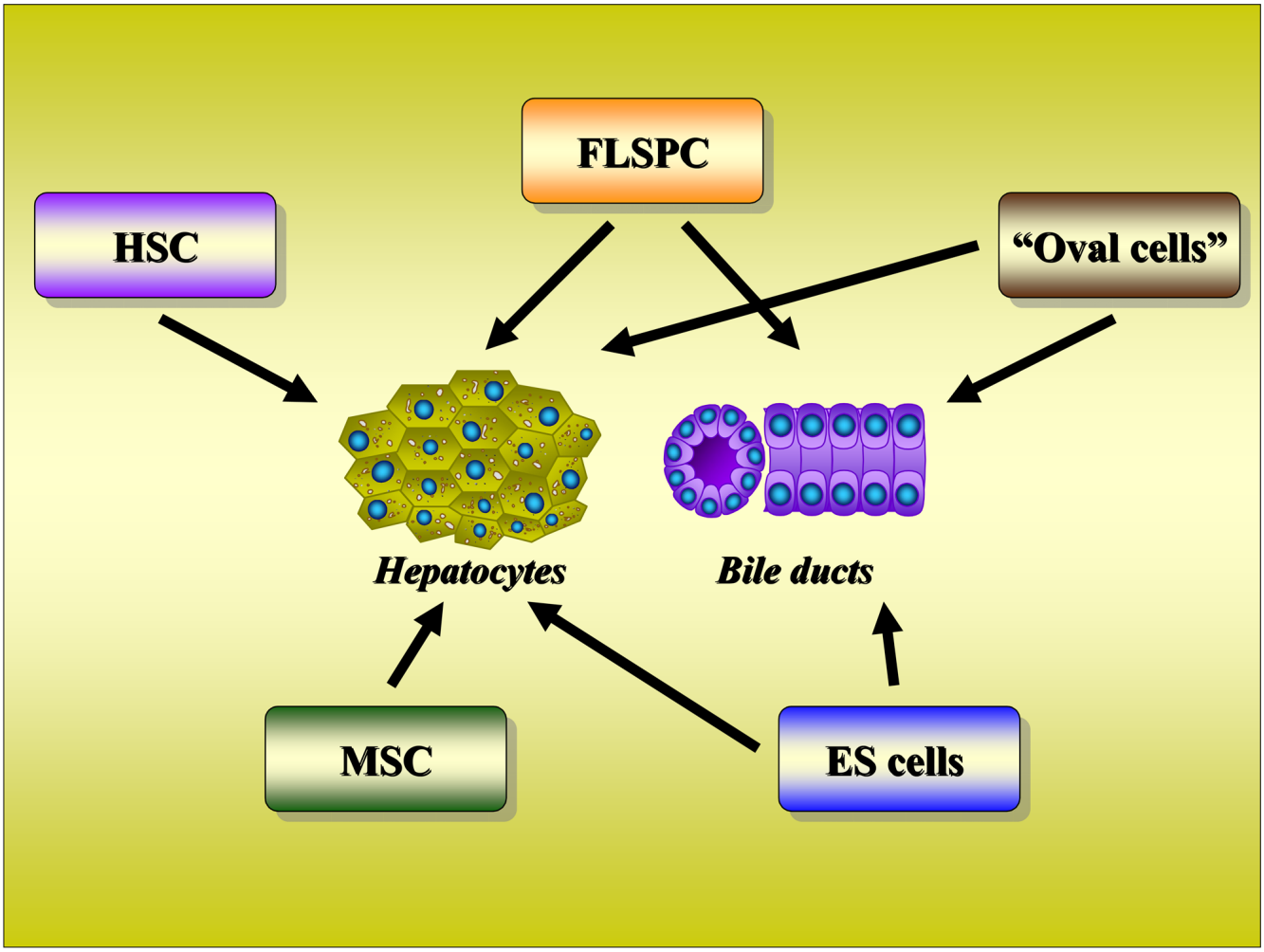

Figure 3.
Schematic diagram summarizing the various stem or progenitor-like cells that have been transplanted into rats or mice, producing progeny exhibiting either an hepatocytic or bile ductular phenotype. (FLSPC = fetal liver stem/progenitor cells, HSC = hematopoietic stem cells, MSC = mesenchymal stem cells, ES = embryonic stem)