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Stem and progenitor cells for liver repopulation: can we standardise the process from bench to bedside?

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ABSTRACT

There has been recent progress in the isolation and characterisation of stem/progenitor cells that may differentiate towards the hepatic lineage. This has raised expectations that therapy of genetic or acquired liver disease might be possible by transplanting stem/progenitor cells or their liver-committed progeny. However, it is currently impossible to determine from the many documented studies which of the stem/progenitor cell populations are the best for therapy of a given disease. This is largely because of the great variability in methods used to characterise cells and their differentiation ability, variability in transplantation models and inconsistent methods to determine the effect of cell grafting in vivo. This manuscript represents a first proposal, created by a group of investigators ranging from basic biologists to clinical hepatologists. It aims to define standardised methods to assess stem/progenitor cells or their hepatic lineage-committed progeny that could be used for cell therapy in liver disease. Furthermore standardisation is suggested both for preclinical animal models to evaluate the ability of such cells to repopulate the liver functionally, and for the ongoing clinical trials using mature hepatocytes. Only when these measures have been put in place will the promise of stem/progenitor-derived hepatocyte-based therapies become reality.

Cell therapy can be defined as "the use of living cells to restore, maintain or enhance tissue and organ function".¹ Such a strategy has emerged as an achievable therapeutic approach in the past decades due to progress in methods of isolation and culture of cells derived from several organs and tissues. Bone marrow (BM) transplantation was the first example of successful cell therapy, and this therapy is no longer experimental.²⁻⁵ Examples of more recent approaches to substitute or improve the function of organs include, among others, islet transplantation to treat type I diabetes,⁴ fetal midbrain transplantation in Parkinson disease⁵ or transurethral injection of muscle-derived stem cells and fibroblasts in urinary incontinence.⁶ However, even if proof of principle on the clinical efficacy of cell therapy has been obtained in several of these clinical trials, many unanswered questions remain.

CURRENT CLINICAL CELL THERAPY FOR LIVER DISEASE

Hepatocyte transplantation has been performed for >10 years,⁷ chiefly in the setting of inherited

disorders of liver metabolism (Supplementary table S1; because of space constraints, many of the papers referred to have been entered in supplementary tables, which can be found as supplementary information online. These studies provide "proof of principle" that hepatocyte transplantation may cure or alleviate congenital metabolic diseases of the liver. A general problem for all cell-based therapies is the limited repopulation capacity of grafted cells. For at least some of the genetic liver ailments, however, replacement of 2–5% of the liver parenchyma with normal hepatocytes may be sufficient to improve liver function significantly.⁸ The clinical hepatocyte transplantation experience includes initial sporadic cases and a few small series of patients (table S1). In 1998, Fox *et al* reported on a 10-year-old Crigler–Najjar patient with bilirubin-induced brain damage after infusion of 7.5×10^9 hepatocytes, leading to a 60% reduction in bilirubin levels and reduced need for phototherapy.⁹ Since then, several patients have received hepatocytes for a number of indications. In most instances, clinical improvement for up to 12 months was observed (table S1).

In patients with acquired acute and acute-on-chronic liver failure, hepatocyte-based therapies have included the use of human and porcine hepatocytes in bioartificial liver devices, where they are combined with artificial scaffolds that interface with the patient's blood,¹⁰ or direct transplantation of human hepatocytes. Although most studies employing bioartificial devices failed to demonstrate a beneficial effect on patient survival, a subgroup of patients with fulminant liver failure may have benefited from this therapy (table S1). Results from transplantation of hepatocytes in patients with acute liver failure have been described as encouraging (table S1). However, the interpretation of these studies is complicated by the heterogeneity of the disorders treated, the limited number of patients per series and the lack of true control groups. Both transplantation of hepatocytes in the setting of acute liver failure and the use of bioartificial liver devices can currently only be viewed as a method to improve the metabolic condition of extremely ill patients prior to transplantation.

One major impediment to evaluate the efficacy of hepatocyte transplantation and bioartificial liver devices further is the shortage of human hepatocytes.

In contrast to orthotopic liver transplantation, which is a well-established, life-saving procedure, hepatocyte transplantation and bioartificial liver devices are experimental. Therefore, it is ethically difficult to assign cadaveric livers to experimental approaches while many patients still die on the waiting list. Although one could establish banks of cryopreserved hepatocytes, with which some clinical success has been reported,^{11 12} this may not solve the problems of cell shortage because human hepatocytes are easily damaged during the freezing–thawing procedure. Hence there is need for another source of cells, such as stem or progenitor cells that can differentiate into hepatocytes.

The use of hepatocytes derived from stem/progenitor cells may have several advantages over transplantation of mature hepatocytes. First, stem/progenitor cells, especially if from an extrahepatic source, capable of generating hepatocyte-like cells would circumvent the shortage of hepatocytes, as they can be expanded *ex vivo*. Secondly, grafting stem/progenitor cells may in theory yield better long-term repopulation and persistent metabolic activity due to the constant generation of newly formed hepatocytes. However, a multitude of questions remains to be addressed before stem/progenitor cell-derived hepatocytes will be clinically usable.

In the next sections we will describe what is currently known regarding potential sources of stem/progenitor cells for hepatic repopulation (fig 1 and table S2), methods to induce their *in vitro* differentiation and maturation, and the utility of various animal models to demonstrate functional liver repopulation by these cells or their progeny *in vivo*. We will then formulate the multiple

questions remaining to be addressed and suggest possible ways to start addressing these.

STEM/PROGENITOR CELL-DERIVED HEPATOCYTES: WHAT ARE THE CRITERIA TO ASSESS THEM BY?

Several hundred studies have been published demonstrating that stem/progenitor cells can be differentiated towards “hepatocyte-like cells”, a term extensively used in the field for cells generated *in vitro* that show some characteristics of mature hepatocytes but are not fully mature and/or characterised. However, the criteria to identify a differentiated cell as a hepatocyte have not been standardised and differ greatly among different studies. Moreover, due to differences in the starting cell population, differentiation protocols, assays performed to assess differentiation and endpoints of preclinical transplantation studies, it is currently impossible to determine which is the most suitable stem/progenitor population to repopulate the liver.

In table S2 we summarise selected publications and the experiments that led to the conclusion that a given stem/progenitor cell type differentiates into a hepatocyte-like cell, keeping in mind the caveat that the conclusion that a given cell population has generated hepatocytes is only as good as the criteria that are used to define a functioning mature hepatocyte both *in vitro* and *in vivo*. (The manuscripts listed in the table are referred to in the text as S2-X, etc.)

STEM/PROGENITOR CELLS WITH REPORTED HEPATOCYTE DIFFERENTIATION ABILITY

Intrahepatic stem/progenitor cells

Perhaps the most logical source of stem/progenitor cells for liver repopulation would be liver-intrinsic stem/progenitor cells. However, regeneration of the liver parenchyma is mostly the result of proliferation of mature hepatocytes. Nevertheless, (facultative) hepatocyte progenitors are present in the bile ductules and the canals of Hering, which are lined by small biliary cells and hepatocytes.¹³ These progenitor cells (in rodents called oval cells) are activated following liver injury especially when replication of mature hepatocytes is inhibited.^{14 15} These hepatic progenitors differentiate to hepatocytes via intermediate hepatocytes,^{15 16} which co-express markers of biliary cells (keratin 7 and 19) and immature hepatocytes (α -fetoprotein (AFP)).¹⁵ While the mechanisms controlling progenitor cell activation are partially understood in rodent models, very little is known in humans.

Hepatic progenitor cells have been isolated from rodent livers following liver damage, cultured and differentiated towards hepatocytes and cholangiocytes (table S2-11). However, the phenotype of such rodent hepatic progenitors remains controversial, and the relevance of hepatic progenitors obtained after liver damage as a source of transplantable cells is not clear. A more clinically relevant population of hepatic stem/progenitor cells has been isolated from non-damaged fetal

Figure 1 Possible stem/progenitor cells for liver repopulation. A number of different cell populations have been suggested to be capable of liver regeneration. Hepatic stem/progenitor cells present near the canals of Hering can proliferate and differentiate via a hepatoblast stage to hepatocytes in clinical settings where hepatocyte proliferation is significantly diminished. Although the phenotype is not fully known, they are a potential cell source for hepatic regeneration. Stem/progenitor cells from extrahepatic origin can be divided into pluripotent cells, such as embryonic stem cells (ES cells) and the more recently described induced pluripotency stem cells (iPS cells), and perhaps other cells derived from adult tissues with increased potency. These cells can be induced down an endoderm, hepatic endoderm, hepatoblast and hepatocyte differentiation path *in vitro* under specific culture conditions. There are also a number of studies that demonstrate a possible lineage switch from a haematopoietic stem cell (HSC) in either the bone marrow (BM) or umbilical cord blood (UCB), or mesenchymal stem cells (MSC) present in BM, to cells with hepatic features, by transplantation in the liver and to a lesser extent following *in vitro* culture-mediated differentiation.

(table S2-6,9) and adult rodent or human liver (table S2-1-6,8,9). Again, the exact phenotype of the hepatic progenitor cells is unclear, and their suitability for hepatic repopulation in the clinical setting still needs to be ascertained. Identification of these intrahepatic progenitor cells opens up the possibility that, once signals that govern their proliferation and differentiation to mature hepatocytes are better understood, clinically feasible methods to induce liver repopulation from these endogenous progenitor/stem cells could be developed. In addition, such insights may be of significant importance for developing methods to allow maturation of non-hepatic stem/progenitor cells to hepatocytes *in vivo*.

Embryonic stem cells (ESCs)

At the other end of the spectrum of cells that may be used for hepatic regeneration are pluripotent ESCs. ESCs differentiate spontaneously towards the hepatic lineage by simple removal of factors that prevent their differentiation (table S2-13,15,16), or can be guided towards a hepatocyte-like fate with the appropriate cytokines (table S2-12,14,17-20). ESC-derived hepatocyte-like cells engraft the liver of several animal models of liver disease, although in general at levels <2% (table S2-14,18-20). In one study, albumin-expressing cells, reselected from teratomas generated in the liver following transplantation of ESCs, integrated and functioned as mature hepatocytes when grafted into secondary animals, suggesting that they might have adapted to an adult liver environment in the initially transplanted animal (table S2-19). Obviously ESCs are allogeneic in nature, and transplantation of hepatocyte-like cells generated from ESCs will require immunosuppression, and ethical concerns may constitute limitations for their use. The newly described induced pluripotent stem cells (iPS cells),^{17 18 19} generated by introduction of Oct4, Sox2, cMyc and Klf4 or OCT3/4, SOX2, NANOG and LIN28 in terminally differentiated cells, displaying many if not all features of ESCs might circumvent the problems due to human leucocyte antigen (HLA) mismatching invariably associated with ESC-based therapies.²⁰

"Pluripotent" adult stem cells

Between 2002 and 2008, different cell types with extended potency have been isolated from multiple tissues, including BM, umbilical cord and umbilical cord blood (UCB), amniotic fluid and fetal liver, among others (table S2-21-28). These cells have in common that they can be expanded extensively without loss of potency and have an unexpectedly broad differentiation ability, as they appear to generate differentiated progeny of all three germ layers. All these cell populations may differentiate into cells with some or many features of hepatocytes *in vitro*, and, for at least some of them, some degree of liver repopulation *in vivo* has been shown (Table S2-23,25). Whether these cells naturally exist or have—like iPS cells—acquired greater

differentiation ability due to the culture conditions applied to isolate them from the different tissues remains unclear.

Haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs)

The first suggestion that non-hepatic and non-pluripotent cells may generate hepatocytes *in vivo* came from the identification of Y chromosome-positive hepatocytes in female patients receiving a male BM graft (table S2-29), which was subsequently also demonstrated in mice and rats (table S2-30-32). However, several subsequent studies demonstrated that this apparent plasticity is at least in part due to fusion between transplanted cells and recipient hepatocytes (table S2-33,34). Adult BM mainly comprises two populations of precursor cells, HSCs and MSCs. HSCs are also present in circulating blood and UCB, and can sustain production of all blood cells throughout life.²¹ MSCs can be isolated from several other tissues, including adipose tissue, placenta, amniotic fluid, UCB and fetal tissues, and can differentiate into osteocytes, adipocytes, chondrocytes, smooth muscle cells and haematopoietic supportive stroma.

Apparent transdifferentiation of HSCs (in many instances this is relatively crude BM) to hepatocyte-like cells has been described, chiefly in animals that underwent BM/HSC transplantations followed by induction of liver damage (table S2-35-42). Lagasse *et al* demonstrated that highly purified HSCs repopulated not only the haematopoietic system, but also the liver of animals with hereditary tyrosinaemia, rescuing the animals from liver failure (table S2-40). Subsequent studies demonstrated that liver repopulation was not HSC-autonomous, but required fusion between donor monocytes and host hepatocytes (table S2-42). Nevertheless, other studies have suggested that BM/HSC can differentiate *in vitro* into hepatocyte-like cells (table S2-37-39) or may generate low percentages of the liver parenchyma without fusion (table S2-36,39). Similar results have been reported with UCB (table S2-44-48). Likewise, a number of studies have suggested that MSCs may differentiate into hepatocyte-like cells *in vitro* or *in vivo* (table S2-48-52). As for HSCs, transdifferentiation of MSCs to hepatocyte-like cells *in vivo* may in part be due to fusion, but some reports suggest cell-autonomous transdifferentiation (table S2-48,52). All these studies have in common that they have not analysed hepatic differentiation from BM cells at the clonal level, precluding the definite conclusion that an HSC or MSC has the capability to generate hepatocytes aside from their haematopoietic or mesenchymal differentiation potential, respectively.

METHODS USED FOR IN VITRO DIFFERENTIATION OF STEM/PROGENITOR CELLS TOWARDS THE HEPATIC LINEAGE

Whilst some studies suggest that mature adult hepatocytes are the better candidates for liver repopulation, other studies suggest that fetal liver

cells might be superior as they have a greater proliferation potential and exhibit less apoptosis following transplantation.²² Hence, whether the progenitor cells should be differentiated to a fetal or adult hepatocyte phenotype is currently an open question. Compared with mature hepatocytes, ED14 fetal liver cells have the advantage of being (1) bipotential, giving rise to both hepatocytes and cholangiocytes; (2) highly proliferative; (3) less immunogenic; and (4) small in size, allowing easier and safer intraportal injection and dispersion.²³

Spontaneous differentiation towards hepatocyte-like cells is seen when ESCs are allowed to develop to embryoid bodies (EBs) (table S2-13–17,20). To increase the percentage of hepatocyte-like cells, specific cytokines, known to play a role in liver differentiation, have been added to differentiating EBs (table S2-14,17,20). As ESC generates not only definitive endoderm but also primitive endoderm, differentiating cells should be evaluated for the expression of a small set of proteins expressed only in definitive endoderm (box 1). Subsequent differentiation into hepatic endoderm, hepatoblasts and mature hepatocytes can then be followed by assessing expression of a panel of genes expressed in subsequent steps of differentiation. We believe that monitoring the expression of developmental markers specific for certain developmental stages during the course of the *in vitro* differentiation process is essential to demonstrate that bona fide hepatocyte-like cells have been generated. This is important during differentiation of not only ESCs but also other stem/progenitor cells.

For non-ESC stem/progenitor cells, “directed” differentiation has been obtained by cultivating cells in a one- (table S2-21,22,24,28,37,38,45,48,51) or two-step protocol (table S2-26,27,49,50). Few if any of these studies have assessed whether differentiation of extrahepatic stem/progenitor cells follows classical developmental rules, as has been shown for ESCs.

The final progeny in differentiation assays is often a mixed population of differentiated hepatocyte-like cells, other non-hepatic differentiated cells and persisting undifferentiated cells, which in the case of ESC-derived grafts will create teratomas. As many proteins found in hepatocytes are also expressed in other cells, it is imperative to demonstrate that multiple proteins found in mature hepatocytes are indeed co-expressed in some of the progeny. Only this approach proves that mature hepatocytes but not immature hepatoblasts, or unrelated cells, have been generated. To purify the differentiated hepatocyte-like cells further, current methods employ transgenic cells expressing fluorochromes (table S2-14–20) or an antibiotic resistance gene under the control of liver-specific genes. However, clinically relevant enrichment methods are still needed. Another, commonly omitted, method to verify generation of hepatocyte-like cells is the demonstration that the stem/progenitor cells have differentiated into cells with typical hepatocyte ultrastructural features.

Although each morphological characteristic is again not exclusive of mature hepatocytes, hepatocyte-like cells should show several characteristics of mature hepatocytes (box 1).

Ultimate *in vitro* proof that mature hepatocyte-like cells have been generated requires demonstration of functional hepatocyte characteristics, such as nutrient processing, detoxification and plasma protein synthesis (box 1). It is important to note that most metabolic enzymes are expressed in the perinatal period, but become functional after birth.^{24–25} It is thus not sufficient to demonstrate expression of enzymes, but their functional activity should be measured to determine the degree of maturation attained. As is true for phenotype features, no single test is sufficient to prove the generation of functional mature hepatocyte-like cells, as most functions are not hepatocyte specific.

Most of the studies listed in table S2 only partially characterised hepatocyte-like cells generated from the different stem/progenitor populations. Hence, in many instances, it cannot be determined whether indeed bona fide hepatocyte-like cells were generated, what stage of differentiation was achieved and what the efficiency of the differentiation system was.

IN VIVO REPOPULATION OF THE LIVER PARENCHYME WITH STEM/PROGENITOR CELLS

Although *in vitro* tests may suggest that a given cell population can generate hepatocyte-like cells, the ultimate proof that differentiation has been achieved is to demonstrate that a cell or its progeny can repopulate the liver and restore liver function in an animal model of liver disease. Whether an undifferentiated stem or progenitor cell, except for intrahepatic progenitors, can be induced to commit in a cell-autonomous manner to a hepatocyte by simple intrahepatic transplantation is not clear (table S2-25,36,39–41,44–47). However, cues provided by the, often injured, hepatic microenvironment may induce further maturation of partially differentiated cells.

Stem/progenitor cells may fuse with host cells, giving the false impression that the cells themselves differentiated into hepatocytes (table S2-33,34,42,44,47). Most studies demonstrating cell fusion have been performed with adult stem cells. Few studies wherein ESC progeny are grafted have specifically investigated whether engraftment is due to cell fusion with parenchymal cells even though *in vitro* ESC fusion has been reported.^{26–27} Several techniques, when used in combination, can exclude that fusion underlies apparent hepatic differentiation of stem/progenitor cells or their progeny *in vivo*. These comprise the identification of the Y chromosome in sex-mismatched transplants (table S2-30,33,34,39,42–44), ALU sequences in the setting of xenogeneic transplants (table S2-27,36,48), exclusion of the presence of donor- and host cell-specific transcripts and proteins in a single cell (table S2-33,36,44,46–48) and the use of cre-Lox approaches, with Cre being expressed in either the

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Box 1 Recommendations for the characterisation of stem/progenitor-derived hepatocyte-like cells

As should be clear from the description in the text, no single test is sufficient to determine if hepatocyte differentiation has been obtained from stem or progenitor cells. In fact, one could state that the more assays are done that indicate that the final cells are confirmed hepatocytes, the better. We would recommend the following sequence of tests to investigate whether stem/progenitor cells have generated hepatocytes:

(1) qRT-PCR (quantitative reverse transcription-PCR) analysis of transcripts in hepatocyte-like cells generated in vitro (this depends on the degree of maturity of cells that are used to initiate differentiation cultures)

- (a) For primitive cells (ESCs, "pluripotent" adult stem cells): demonstration of commitment/specification to definitive endoderm via
 - (i) an epiblast-like phase (brachyury, Nodal, Fgf8, Cripto; not primitive endoderm—Sox7)
 - (ii) a mesendoderm phase (Eomes, brachyury, Gsc, E-cadherin and Foxa2)
 - (iii) a definitive endoderm phase (Hex, Mixl1, Cxcr4, Tm4sf2, Gsc, Sox17, E-cadherin; not Sox7)
- (b) For less primitive cells and cells from ESCs/"pluripotent" adult stem cell cultures that have reached the definitive endoderm stage
 - (i) early hepatoblast genes (Afp, Ttr, Krt19, albumin) and
 - (ii) late fetal, postnatal hepatocyte genes:
 - 1. initial screen to include: albumin, Krt8, Krt18, α -1At, Tat, G6P;
 - 2. further extended evaluation: Adh1C, ApoF, Hnf1 α , Mrp2, C/EBP α , CPSase I, Pepck, Cx32, HepPar1, To, Hnf4 α , Cyp2B6, Cyp3A4, Cyp7A1, Bsep, glycogen synthase
 - (iii) expression of liver-specific transcription factors Hnf4 α , Hnf1 α , Hnf6, Prox1, Hex, C/EBP α , C/EBP β),

(2) Evaluation of protein expression in hepatocyte-like cells generated in vitro

- (a) immunohistochemistry for Sox17 and Foxa2 (early), Ttr, Afp, albumin (mid), Hnf4 α , α -1At, Mrp2, Cx32, Bsep (late)
- (b) western blot for the same proteins

(3) Ultrastructural evaluation of hepatocyte-like cells generated in vitro

- (a) large polygonal polarised cells, large cytoplasm to nuclear ratio, canaliculus formation (apical pole), presence of gap and tight junctions (lateral pole)
- (b) presence of glycogen granules, high number of mitochondria, well organised rough endoplasmic reticulum

(4) Functional analysis of hepatocyte-like cells generated in vitro (in order of complexity)

- (a) albumin secretion
- (b) glycogen storage
- (c) ureagenesis
- (d) bilirubin metabolism, with generation of bilirubin monoglucuronides, and bilirubin diglucuronide
- (e) steroid metabolism
- (f) CYP function using, for instance, the 7-pentoxoresorufin-*O*-dealkylation (PROD) assay
- (g) lidocaine clearance
- (h) ability to secrete "bile" (after formation of 3D clusters) to assess functional polarisation using fluorescein isothiocyanate (FITC)—glycocholate as substrate

(5) Engraftment, differentiation and functional repopulation in vivo

- (a) demonstration of cell-autonomous differentiation of stem cell-derived progeny (ideally a combination of the following)
 - (i) sex-mismatched transplantations, evaluation of donor and host Y vs X chromosome, combined with deconvolution confocal microscopy to ensure that the nucleus is part of the evaluated cell
 - (ii) xenogeneic transplantations: evaluation of donor and host ALU sequences, combined with deconvolution confocal microscopy, evaluation of donor and host Y vs X chromosome, combined with deconvolution confocal microscopy
 - (iii) xenogeneic transplantations: demonstration of donor-specific hepatocyte protein, if 100% species-specific antibody exists. Expression of liver-specific proteins without proteins characteristic of donor cell type grafted
 - (iv) cre-Lox system, wherein grafted cells ideally contain a construct encoding a first fluorochrome, followed by a floxed STOP cassette and a second fluorochrome, to be grafted in animals wherein a universal or hepatocyte-specific promoter drives Cre (or vice versa)
- (b) demonstration of cell differentiation to hepatocytes (ideally a combination of the following)
 - (i) presence of mature hepatocyte-specific proteins, including albumin, α -1AT, Cx32, BSEP in donor cell
 - (ii) grafting donor cells containing a transgene wherein a liver promoter gene drives β -galactosidase or a fluorochrome
 - (iii) ultrastructural evaluation of labelled donor cells
- (c) demonstration of functional properties of stem cell-derived hepatocytes
 - (i) transplantation in animal models with liver dysfunction, such as APO-E-deficient animals, haemophilic animals, hypoalbuminaemic animals, where blood can be evaluated for production of the specific proteins
 - (ii) transplantation in animal models of liver or metabolic failure such as FAH mice, uPA mice, Gunn rats or Nagase rats where levels of bilirubin, albumin, etc. can provide an indication of liver rescue, and ultimately survival of animals can be used as a readout for liver rescue
 - (iii) morphological analysis using species-specific antibodies in combination with cell lineage-specific antibodies can provide evidence of functional integration: if a bile canaliculus starts between mouse hepatocytes and continues between human hepatocytes and further again between mouse hepatocytes, a functional integration of transplanted cells has been shown
 - (iv) another feature of transplanted cells that can be characterised is the use of a biologically plausible time course, and sampling at more than one time point can provide additional information—this adds weight to the functional characterisation of the cells including their stability in vivo

host or donor cells (table S2-30) and the Lox-p-flanked marker such as green fluorescent protein or β -galactosidase in the donor or host cell, respectively.

Even though hepatocyte-like cells generated from stem/progenitor cells may yield higher levels of engraftment than undifferentiated cells, these levels fall far short of those seen when mature or fetal hepatocytes are engrafted.^{23 28} The reasons underlying the poor performance of stem/progenitor-derived cells compared with primary liver-derived cells when engrafted into the liver are unknown.

For the engraftment of mature hepatocytes to occur, translocation from the sinusoids into liver plates is required, with disruption of the sinusoidal endothelium, allowing cells to home and engraft between parenchymal cells.^{29 30} Subsequently, transplanted cells must proliferate at the expense of autologous hepatocytes. This requires that (1) "space is made", by for instance partial hepatectomy or hepatocyte necrosis and/or apoptosis, and that (2) the transplanted cells have a growth advantage over endogenous hepatocytes.³¹ The rules governing parenchymal repopulation by hepatocytes are likely to be similar for hepatocyte-like cells generated from stem/progenitor cells. However, a number of differences between mature hepatocytes and stem/progenitor-derived hepatocyte-like cells may explain the differential degree of liver repopulation seen. Compared with mature hepatic cells that are retained in liver sinusoids because of their relatively large size,^{32 33} the smaller progeny from stem/progenitor cells may require specific signals to be retained within the liver, allowing subsequent homing to the liver parenchyma and engraftment. Signals that govern this process when stem/progenitor cell-derived hepatocytes are grafted remain to be determined. It is also not known whether stem/progenitor cell-derived hepatocytes may have a proliferative disadvantage over mature hepatocytes that have extensive proliferation potential,²⁸ and/or may undergo significantly more apoptosis when grafted.

AVAILABLE ANIMAL MODELS TO MIMIC HUMAN DISEASE

Prior to the commencement of phase I clinical trials, animal models should be designed to provide "proof of principle" that a given stem/progenitor cell population can repopulate the liver parenchyma. The different animal models and their suitability for testing the efficacy of cells to repopulate the liver with functioning hepatocytes are summarized in box 2. As most of these have not been standardised and the predictive value of the animal models for the efficacy with which hepatocytes will repopulate human liver is unknown, we will only briefly go over the different models.

Several genetic disorders involving hepatocytes have been modelled in rodents. In addition, a number of toxin-induced models exist. Some of the genetic models provide neither space nor a proliferative advantage of grafted over host cells, and hence are the most difficult models to demonstrate hepatic engraftment (box 2). Nevertheless, such

models mimic clinical situations where life-threatening genetic defects may not be associated with significant liver damage. Other genetic or toxin-induced models are associated with loss of mature hepatocytes, creating space. However, as the remaining hepatocytes still have extensive proliferative potential, there is no competitive advantage for the donor cells.²⁸ Similar genetic defects in humans or acute drug-mediated liver toxicity are clinical situations akin to these models, and may be the most likely clinical situation in the foreseeable future where transplants of stem/progenitor-derived hepatocyte-like cells will be used. A certain set of genetic disorders, such as the fumarylacetoacetate hydrolase (FAH) model³⁴ or induction of toxicity by a combination of toxins that cause hepatocyte death as well as inhibition of hepatocyte proliferation should yield the most robust regeneration from transplanted cells, even though this situation is seldom seen in the clinic. Nevertheless, it may be possible to create such conditions by, for instance, combining hepatic irradiation and partial hepatectomy or an ischaemia/reperfusion injury. Aside from models for acute liver failure, a number of models are also available to recreate chronic liver failure (box 2).

The same models crossed with immunodeficient animal models such as the NOD-SCID mouse, the Rag2^{-/-} mouse, or NOD-SCID or Rag2^{-/-} mice also deficient in the IL2R γ c receptor, are available to evaluate the ability of human hepatocytes or stem/progenitor-derived hepatocyte-like cells to engraft *in vivo*.³⁵ An alternative model is the preimmune fetal sheep model wherein human cells are tolerated and, owing to the fetal environment, may allow robust human-derived cell repopulation.³⁶ However, the latter model is impractical for many groups around the world and the costs of such animal studies are also likely to be prohibitive. Obviously, repopulation of the liver in humans by hepatocyte-like cells represents a challenge that is orders of magnitude greater than repopulation of mouse or rat livers. Therefore, non-human primates, dogs or swine can be used to test hepatocyte engraftment. An advantage of such larger animal models is that they allow clinically translatable surgical interventions.

It should be kept in mind that none of the existing transplantation models is entirely representative of the human situation. Moreover, no studies have addressed which animal model has the greatest predictive value for subsequent clinical human transplantations. Such standardised studies will be needed to move hepatocyte or hepatocyte-like cell transplantation to a clinically useful therapy.

Haematopoietic cell transplantation is an excellent example of the close relationship between preclinical and clinical investigation. Many preclinical studies, followed by clinical and then again by preclinical studies done over the last 40 years, have led to the now standard practice of haematopoietic cell grafts. After the initial successes in the late 1960s in small numbers of patients, extensive standardisation of the type and number

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Box 2 Recommendations for the use of specific experimental animal models**(1) Proof that a given cell population can be used to ameliorate liver disease****(a) Animal models with no space and no selective advantage of grafted cells**

- ▶ Gunn rat (model for Crigler–Najjar type I)^{41 42}
- ▶ spf-ash mouse (model of ornithine transcarbamylase deficiency)⁴³
- ▶ Factor VIII-deficient mice (haemophilia)⁴⁴

(b) Animal models with space generation but no selective advantage of grafted cells

- ▶ Toxic milk mouse (Wilson disease)⁴⁵
- ▶ Long Evans cinnamon rat (model of Wilson disease)⁴⁶
- ▶ mdr2 mouse (model of progressive familial intrahepatic cholestasis type 3)⁴⁷
- ▶ Fas ligand-mediated damage^{48 49}
- ▶ acetaminophen injury⁵⁰
- ▶ carbon tetrachloride (CCl₄) administration⁵¹
- ▶ partial hepatectomy

(c) Animal models with space generation and selective advantage of grafted cells

- ▶ FAH mouse (model of tyrosinaemia type I)³⁴
- ▶ uPA model: hepatocyte-specific expression of the uroplasinogen activator under the control of the albumin promoter^{52 53}

(d) Liver progenitor cell proliferation**Combination of acute liver failure and**

- ▶ choline-deficient ethionine-supplemented diet⁵⁴
- ▶ carefully dosed acetaminophen toxicity⁵⁵
- ▶ 3,5-diethoxycarbonyl-bonyl-1,4-dihydrocollidine
- ▶ D-galactosamine⁵⁶
- ▶ use of hepatocyte injury combined with retrorsine to inhibit mature hepatocyte replication⁵⁷

(e) Chronic liver failure

- ▶ bile duct ligation^{58 59}
- ▶ chronic hepatitis by administration of toxins^{60–62}
- ▶ diphtheria toxin receptors under the control of an albumin promoter⁶³
- ▶ hepatitis B surface antigen mouse⁵⁷
- ▶ HSV-tk transgenic mouse⁶⁴

(2) Proof that a given human cell population can differentiate into a parenchymal liver cell in vivo

- ▶ NOD/SCID or the Rag2^{−/−} and/or IL2Rγ^{−/−} mice^{20 65 66}
- ▶ FAH^{−/−} Rag2^{−/−} IL2Rγ^{−/−} mice³⁵
- ▶ uPA-Rag2^{−/−} IL2Rγ^{−/−} mice³⁵

(3) If animal models require procedures which cannot be reproduced in patients

Develop translational models allowing cell engraftment and differentiation in a clinically compatible setting; such models may include syngeneic, allogeneic and/or xenogeneic transplantation⁶⁷

(4) When size matters

- ▶ swine⁶⁸
- ▶ hyperuricaemic Dalmatian dog⁶⁹
- ▶ Watanabe rabbit: hypercholesterolaemia caused by a low-density lipoprotein receptor defect^{70–72}
- ▶ preimmune fetal sheep³⁶

(5) Tumourigenesis and side effects, such as colonisation of unwanted sites
Suitable small or large animal models

of cells to be transplanted in standardised mouse, and to a lesser extent dog, models occurred. This moved the field of haematopoietic cell transplantation from the experimental to a well developed, clinically standardised therapy. From these studies it is also obvious that testing engraftment of human cells in a xenogeneic setting cannot take into account the specific nature of the human cells

and the stem cell niche, and the species-specific nature of factors governing human cell proliferation and differentiation *in vivo*. However, histopathological studies of human liver diseases have already yielded important new insights into the factors that govern human hepatocyte progenitor activation and differentiation.¹⁴ These and future insights should therefore be applied to the different animal models described above to generate models that are as close as possible to the human situation.

DEALING WITH GRAFT REJECTION

One of the major hurdles to successful hepatic repopulation by stem/progenitor cells or their progeny is the likely allogeneic nature of the cells, necessitating life-long immunosuppression. Although one may be able to model some of the questions in rodents, lessons learned from, for instance, clinical haematopoietic cell transplantation indicate that results obtained in rodent models may not necessarily be translatable to humans.^{13 37} Alternatives are transplantation studies in larger animal models, such as dogs, a process proven useful in evaluating BM transplantation.³⁸ Another possible model to study hepatic repopulation is to develop mouse models where a fully established human immune system is available including antigen-presenting cells.³⁹ This approach may have clinical ramifications, as an approach that has been proposed for ESCs and other stem cell-based therapies is first to render the recipient a haematopoietic chimera, and subsequently transplant cells from the same donor stem cell population. Although such an approach would not be useful in the setting of acute liver failure, it could be considered in individuals with genetic liver disorders, in whom liver failure can be prevented temporarily using drugs. Alternatively, progress in genetic modification, including site-specific genetic correction, may make it possible to correct genetically intrahepatic stem/progenitor cells or pluripotent cells created from the patient, hence allowing one to generate donor-specific hepatocyte-like cells that can be engrafted without the need for long-term immunosuppression. One interesting side-note regarding MSCs, is that these cells may also modify the immunological response to allogeneic transplantation, and thus help overcome immune rejection.⁴⁰

CONCLUSIONS

It should be clear from this review that many outstanding questions will need answers before the promise of stem/progenitor cells capable of generating hepatocyte-like cells can be translated into successful clinical therapies (fig 2 and boxes 1 and 2). These include:

- ▶ What is the optimal differentiation stage of hepatocyte stem/progenitor cells to achieve the highest level of engraftment and function? What are the criteria that should be used to define the nature of the hepatocyte committed progeny?

Figure 2 Standardising the process from bench to bedside. Standardisation of the process from basic research to the development of clinical trials will be essential to ensure successful cell-based therapies for liver diseases. (1) Obviously, basic research characterising cells and developing differentiation protocols cannot be standardised as this is per definition innovative research. (2) However, once putative hepatic stem/progenitor cells or their differentiated progeny are being considered for clinical application, standardised methodologies should be used to characterise the cells to be grafted, and grafting should occur in standardised well-validated animal models. Characterisation of the cell population to be grafted should be done using a standard set of phenotypic markers, as well as a standard set of in vitro functional studies. In addition, the cryopreservation protocols should be standardised, and thawed cells should again be evaluated using standard assays for phenotype and function. (3) Although many animal models are currently being used to evaluate the ability of hepatocytes, hepatic stem/progenitor cells or their differentiated progeny to repopulate the liver functionally, these animal models have not been validated. Hence, validation could be obtained by transplanting hepatocytes not only in current ongoing clinical protocols but also in animals, much like studies in the haematopoietic cell transplantation field. (4) Finally, it will be paramount that ongoing clinical trials using hepatocytes, and planned clinical trials using hepatic stem/progenitor cells or their differentiated progeny also use as many as possible standardised protocols for standardised procedures to define the nature of cells grafted, the patients enrolled, the transplantation procedure and pretreatment of the liver to create space and/or a proliferative advantage of the grafted cells, as well as standard data collection regarding efficacy, and possible side effects; with information going back and forth between clinicians and the scientists performing the preclinical studies to optimise further over time both the preclinical and clinical aspects of the transplantation therapies.

- How does one ensure that hepatocyte stem/precursor cells or their hepatocyte-like progeny home to and are retained in the liver parenchyma?
- Is there a need for the creation of space?
- What are the signals needed to induce fully the mature function of hepatocytes derived from stem/progenitor cells following repopulation of the recipient liver?
- In which disorders should transplantation of hepatocytes derived from stem/progenitors be tested?

- Is it possible to obtain and use autologous stem/progenitor cells, or is it necessary to use allogeneic material with the inevitable drawbacks of immunosuppressive therapy?
- What are the possible side effects of transplantation of hepatocytes derived from stem/progenitor cells?

We believe that answers on these questions will only be forthcoming via an intense collaborative effort from basic cell biologists familiar with developmental biology of the liver, as well as stem cell characterisation and manipulation; translational scientists familiar with functional characterisation of hepatocytes in vitro as well as in vivo in animal models of liver disease; and clinicians familiar with treating the diseases that are probably clinical targets. This manuscript was therefore conceived and written by a group of scientists spanning from basic biologist, translational scientists and clinicians dealing with patients with liver failure on a daily basis in the hope that all aspects of this very complex clinical problem could be highlighted. The suggestions below should not be considered binding, but we hope that they will form the basis for further modification and refinement by investigators worldwide. We hope that within the foreseeable future, most basic studies, translational studies and clinical trials will therefore generate information that is comparable between laboratories and clinical centres—providing the necessary insights in this complex area such that successful clinical therapies for liver failure/liver disease can become a reality.

Our suggestions are:

- To standardise the characterisation and testing of stem/progenitor cells and their differentiated progeny.

Extensive comparative preclinical testing of the different stem/progenitor cells that have been proposed as suitable for liver repopulation is needed. This should be done using standard criteria for phenotypic differentiation and functional capabilities. These criteria are outlined in box 1. The underlined text is what we believe are the minimal criteria to be able to compare studies between labs and clinical centres, and should also be considered as the minimal criteria prior to moving a given stem/progenitor cell to a “first in man” clinical study.

- To standardise the animal models for testing the repopulation ability of hepatocytes and hepatocyte-like cells in the liver.

Many animal models are currently being used to evaluate the repopulation ability of hepatocytes, hepatic stem/progenitor cells and their differentiated progeny. However, none of these models have been truly validated for clinical predictability. Thus, for animal models to be useful, two areas of standardisation will be needed: clinical hepatocyte grafts should be transplanted not only in human recipients but also in the most likely useful animal

models, and the efficacy of repopulation compared across the two species. In addition, standardised procedures are needed between laboratories using animal models regarding the transplantation method, methods for generating space, for providing proliferative advantage of the engrafted cells and for evaluation of phenotypic and functional engraftment.

► Clinical studies.

Finally, it will be paramount that ongoing clinical trials using hepatocytes, and planned clinical trials using hepatic stem/progenitor cells or their differentiated progeny, are as standardised as is practical. This could be modelled from the field of haematopoietic cell transplantation where standardisation at the preclinical end has been mirrored by standardisation of clinical protocols, including careful characterisation of the grafted cell population, patient population, transplantation procedures and data collection to measure efficacy and toxicity.

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