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Cell therapy for liver disease

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Cell therapy for liver disease: From liver transplantation to cell factory

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Summary

Work over several decades has laid solid foundations for the advancement of liver cell therapy. To date liver cell therapy in people has taken the form of hepatocyte transplantation for metabolic disorders with a hepatic basis, and for acute or chronic liver failure. Although clinical trials using various types of autologous cells have been implemented to promote liver regeneration or reduce liver fibrosis, clear evidence of therapeutic benefits have so far been lacking. Cell types that have shown efficacy in preclinical models include hepatocytes, liver sinusoidal endothelial cells, mesenchymal stem cells, endothelial progenitor cells, and macrophages. However, positive results in animal models have not always translated through to successful clinical therapies and more realistic preclinical models need to be developed. Studies defining the optimal repopulation by transplanted cells, including routes of cell transplantation, superior engraftment and proliferation of transplanted cells, as well as optimal immunosuppression regimens are required. Tissue engineering approaches to transplant cells in extrahepatic locations have also been proposed. The derivation of hepatocytes from pluripotent or reprogramed cells raises hope that donor organ and cell shortages could be overcome in the future. Critical hurdles to be overcome include the production of hepatocytes from pluripotent cells with equal functional capacity to primary hepatocytes and long-term phenotypic stability *in vivo*.
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Introduction

Orthotopic liver transplantation (OLT) is the standard of care for people with end-stage liver disease and for certain liver-based metabolic defects [1]. However, successful replacement of deficient liver functions by transplantation of healthy hepatocytes, e.g., in animal models and people with Crigler-Najjar syndrome due to UGT1 enzyme deficiency, familial hypercholesterolemia due to low-density lipoprotein receptor (LDLR) deficiency, or acute and chronic liver failure indicated that OLT could possibly be avoided [2–6]. This general concept has been emphasized by similar successes with auxiliary partial orthotopic liver transplantation (APOLT) for enzymatic deficiency states or acute liver failure [7]. In the latter case, discontinuation of immunosuppression when the native liver regenerates after APOLT may lead to spontaneous rejection and atrophy of the allogeneic liver graft [8,9] The clinical experience with APOLT gives credence to the hypothesis that the relevant functional unit of the liver – "the hepatocyte" could be used to correct discrete enzyme defects and support metabolic functions for the failing liver after injury whilst it regenerates. Similarly, successful correction of haemophilia by OLT, indicated that consideration of cell therapy will be appropriate for other classes of diseases. In principle, cell transplantation is far simpler than either OLT or APOLT, because 1) cells from a donor liver may be transplanted into multiple recipients; 2) cell transplantation is simpler using cell administration via intravascular catheters rather than complex surgery; 3) if cryopreserved cells are used, therapies could be undertaken in a prospective non-emergency setting; 4) cells may even be transplanted repeatedly, the procedure can be considered "reversible" since the native liver is not removed; and 5) the costs of transplanting cells should be considerably less than that of organ transplantation.

Subsequent to the early demonstrations of whether transplanted cells may engraft and function in the liver and in a variety of extrahepatic sites [10], a large body of work in many small and large animal models supported studies of the potential of hepatocyte transplantation [11,12]. More recently, the therapeutic value of other liver cell types was elucidated. For instance, transplantation of liver sinusoidal endothelial cells (LSECs) cured haemophilia A in mice after LSECs were found to be the major

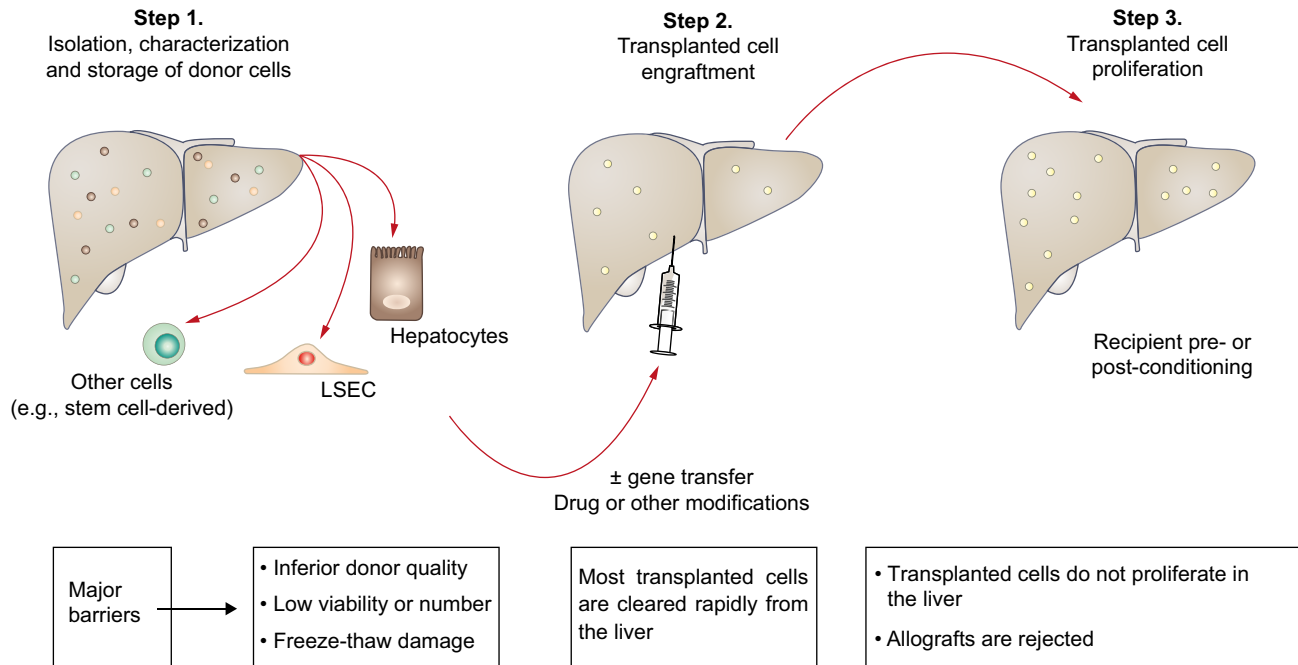**Critical components of liver cell therapy and current barriers**

Fig. 1. **Depiction of critical components in liver cell therapy and barriers in various steps.** The first step in cell therapy requires isolation, characterization and storage of suitable donor cells. These steps are restricted by donor organ shortages or their inferior quality, procedural limitations in isolating cells of high viability and large numbers, as well as difficulties in cryopreservation of cells. The second critical step concerns engraftment of transplanted cells in the liver (or extrahepatic sites), which requires overcoming of early transplanted cell clearances. Cells may be modified by gene transfer vectors, drugs or other ways for improving cell viability, engraftment and proliferation. In the third and final step, transplanted cells must survive over the long-term and also proliferate to the necessary extents for imparting therapeutic benefits, which may require conditioning of recipients either before or after cell transplantation, as well as development of suitable regimens for controlling allograft rejection.

source of FVIII [13]. Applications of LSECs may extend to liver repair since these cells have been shown to be critical for liver regeneration in mice [14]. Pathophysiological processes that could be altered during chronic liver injury and fibrosis by the cell transplantation approach have also gained interest [15]. In some people with acute liver failure, cell transplantation has been successful for bridging to OLT, whereas in other instances, people with liver failure or enzymatic deficiency states had to be treated with OLT because cell therapy proved unsuccessful [6]. In part, this difficulty in achieving superior outcomes of cell therapy has been related to immunosuppression following allogeneic cell transplants, since optimal regimens for inducing tolerance to transplanted liver cells are to be established.

In the setting of metabolic liver disease and hepatic injury, e.g., hereditary tyrosinemia type-1 or Wilson's disease, animal studies established that disease correction can be achieved because even modest numbers of healthy transplanted hepatocytes can proliferate and repopulate the liver [16,17]. This process of liver repopulation has been shown in rodents to be accelerated by recipient organ preconditioning [18]. By contrast, in the setting of metabolic diseases where the native liver is unaffected and remains totally healthy, as in Crigler-Najjar syndrome or familial hypercholesterolemia, transplanted hepatocytes engraft but do not proliferate in the liver because this is not physiologically required. Therefore, in achieving therapeutic levels of repopulation further manipulation is required by either: a) preconditioning of the recipient's liver using techniques such as DNA-adduct forming chemicals, radiation, oxidative stress or by b) modification of

donor cells by altering liver growth or cell cycle controls, such that transplanted cells receive survival and/or proliferation advantages over native cells [18,19]. In this way, the concept of "liver transplantation to cell factory" may be gained if one considers that successive generations of daughter cells may emanate in the recipient liver from transplanted hepatocytes, as was elegantly established using serial hepatocyte transplants in the fumarylacetoacetate-hydrolase-deficient ($FAH^{-/-}$) mouse model [20]. If these concepts regarding liver repopulation are reduced to drug-based approaches then barriers in transplanted cell engraftment and proliferation will be overcome for more effective clinical trials.

For many reasons, the clinical application of liver cell therapy has proceeded at a gradual pace in people compared to the successes in preclinical animal studies. Some of the obstacles concern limited availability of donor livers, difficulties in isolating good-quality cells from often suboptimal donor livers, mechanistic restrictions in cryopreserving human liver cells without losing viability, low levels of engraftment and proliferation in transplanted liver cells, as well as the general lack of therapeutic benefits over the long-term due to allograft rejection (Fig. 1). Another important point is that the animal models used often translate poorly to the clinic. Liver damage may have accumulated over decades in patients with severe distortion of liver architecture and impairment of function. The models of liver injury developed in mice and rats typically occur over days or weeks and are often milder than the human diseases they seek to model. An important message is that more realistic models of these liver injuries are required.

Key Points

- Liver cell therapy has extensive value for paediatric and adult populations with many enzyme deficiency states, metabolic diseases, coagulation disorders, as well as liver failure

- Much of the clinical literature is based upon case reports although some controlled studies have also been conducted

- Widespread applications of liver cell therapy have been constrained by donor organ shortages and limitations in transplanted cell engraftment and proliferation

- Utilization of stem cells offers hope for producing suitable liver cells of clinical utility although barriers include the production of cells with the necessary levels of differentiated function and karyotypic stability

- Approaches to regenerate liver or reducing liver fibrosis or promoting endogenous repair and regeneration are being developed and tested

Table 1. Potential clinical indications for liver cell therapy.*

A. Congenital disorders

- Alpha-1 antitrypsin deficiency*
- Crigler-Najjar syndrome type 1*
- Familial hypercholesterolemia*
- Congenital coagulation factor VII deficiency*
- Hemophilia A
- Glycogen storage disease type I*
- Infantile Refsum disease
- Maple syrup urine disease
- Neonatal hemochromatosis
- Progressive familial intrahepatic cholestasis type 2 (PFIC2)
- Urea cycle defects - ornithine transcarbamylase (OTC) deficiency; arginosuccinate lyase deficiency; carbamoylphosphate synthase type 1 deficiency; citrullinemia*
- Wilson's disease

B. Acquired disorders

- Acute liver failure (multiple etiologies)*
- Fatty liver of pregnancy*
- Acute-on-chronic liver failure (multiple etiologies)*

*Indicates conditions treated by cell transplantation in people.

Major clinical indications and roles of cell therapy

The premise for early studies in people was based on transplanting healthy cells to replace deficient functions in acquired or inborn errors of metabolism besides supporting the failing liver (Table 1). Liver-based metabolic defects are usually secondary to a missing enzyme with consequences secondary either to the lack of a normally functioning protein or the upstream accumulation of toxic substances due to impaired metabolism of a protein. These diseases could be further classified into those with no effects on the liver and those leading to liver injury and fibrosis/cirrhosis. The rationale for hepatocyte transplantation in people with these conditions came from animal studies showing that healthy hepatocytes transplanted into hepatic or extrahepatic sites possessed properties as follows (Table 2): 1) engraftment and proliferation in the liver or other sites; 2) enzymatic activity for detoxification, e.g., bilirubin glucuronidation followed by the biliary excretion of conjugated bilirubin in Gunn rats modelling Crigler-Najjar syndrome; 3) release of secreted proteins, e.g., albumin synthesis and release in Nagase analbuminemic rats (NAR); 4) receptor-mediated ligand uptake, e.g., LDLR-dependent clearance of cholesterol in Watanabe heritable hyperlipidemic cholesterolemic rabbits (WHHL); 5) ammonia-fixation in rats with portacaval shunts; and 6) improvements in mortality in rats and rabbits with induced acute liver failure [21–24]. These demonstrations were better understood after the important discovery in rodents that hepatocytes transplanted into liver sinusoids enter liver parenchyma and integrate with adjacent native hepatocytes along with the reconstitution of plasma membrane structures permitting restoration of cell polarity and transport or exchange functions (Fig. 2) [25–27].

Moreover, studies in rat showed that transplanted cells retained position-specific and physiologically-regulated patterns of gene expression, and exhibited lifetime survival with normal liver growth controls in the absence of allograft rejection

Table 2. Selected examples of animal models used in cell transplantation studies.

Mechanisms in cell engraftment and proliferation
Dpp4-deficient F344 rats and Dpp4 knockout mice
Transgenic donor mice (HBV, human alpha-1 antitrypsin, beta-galactosidase, alb-uPA, etc.)
Fumarylacetoacetate hydroxylase (FAH) knockout mice

Defects in hepatic detoxification

- FAH knockout mice (hereditary tyrosinemia, type-1)
- Gunn rat and MRP-2 knockout mice (Crigler-Najjar syndrome, type-1)
- Histidinemia mice (histidinemia)
- MSUD knockout mouse (Maple syrup urine disease)
- Spf-ash mice (OTC deficiency)

Secretory protein deficiency

- Nagase analbuminaemic rat (hypoalbuminaemia)
- Hemophilia A mice

Diseases of receptor function

- Watanabe heritable hyperlipidemic rabbit (familial hypercholesterolaemia)
- Apolipoprotein E knockout mouse (hypercholesterolemia)

Transport defects

- TR- rat and Mdr2 knockout mouse (PFIC1)
- BSEP knockout mouse (PFIC2)
- Long-Evans Cinnamon rat and Atp7b knockout mouse (Wilson's disease)

Acquired disorders

- Induced acute liver failure (hepatectomy, chemicals, drugs, viruses, physical methods)
- Chronic liver disease (CCl_4, thiocetamide, acetyl-aminofluorene, etc.)
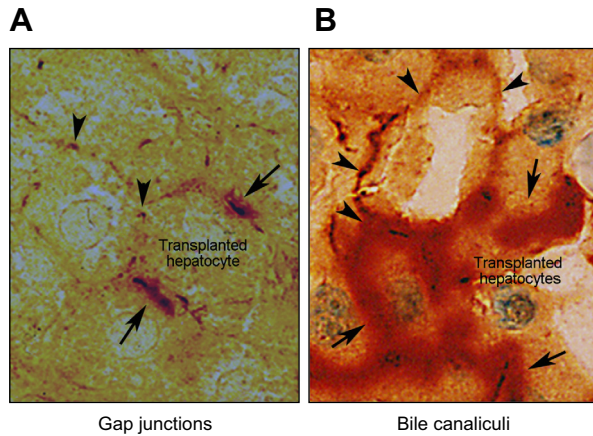
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Fig. 2. Integration of dipeptidyl peptidase IV (Dpp4)-positive adult hepatocytes in liver parenchyma of healthy Dpp4-negative rats. (A) Combined staining for Dpp4 (red color, arrows) and hepatic connexin 32 (brown color, arrowheads) showing sharing of gap junctions by adjacent transplanted and native hepatocytes. (B) Reconstitution of bile canalicular networks in transplanted hepatocytes (Dpp4 staining, red color, arrows) and native hepatocytes (ATPase staining, brown color, arrowheads). Modified from Gupta *et al.* Proc Natl Acad Sci 1995.

[28,29]. These findings prompted numerous studies of liver cell biology and stimulated clinical hepatocyte transplantation (listed in Table 2). Although hepatocyte transplantation in animal studies has been shown to correct enzymatic, receptor, proteolytic or transport defects in the setting of no liver injury and early in disease processes, in conditions associated with progressive liver injury, e.g., alpha-1 antitrypsin deficiency, Wilson's disease, biliary phospholipid excretion (Mdr2/3 deficiency) or progressive familial intrahepatic cholestasis (BSEP deficiency) [18,30], realizing such gains in people with these conditions awaits further advances as discussed below.

In an early human trial, autologous hepatocytes isolated from resected liver were returned via intrasplenic injection in several cirrhotic individuals but without clear clinical benefits [31]. The unique vascular and extracellular matrix architecture of the spleen permits survival and proliferation of transplanted hepatocytes although studies in rat show that the majority ($\approx$90%) of transplanted cells enter instantaneously into the portal vein and then liver sinusoids [32]. Similarly, after acute injury, the liver can be seeded by transplanting cells into the portal vein. On the other hand, attachment of hepatocytes to extracellular matrix scaffolds can help in their engraftment in extrahepatic sites, e.g., subcutaneous fat pads or peritoneal cavity as demonstrated in the rat [24]. Therefore, it was possible to examine major principles for supporting the failing liver, i.e., to understand the role of reseeding the liver with healthy cells *vs.* provision of metabolic support and paracrine factors from transplanted cells in liver regeneration. Whilst cell transplantation improved outcomes in animals with induced liver failure, it was also found in rat that besides intact cells, fragmented cells, culture medium supernatant from cells, and transplantation of even allogeneic or xenogeneic hepatocytes that were rapidly cleared, were also capable of rescue in acute liver failure [33]. This raised the possibility that paracrine factors could be important for cell therapy in liver failure, which recently gained renewed attention.

The experience in single cases or uncontrolled trials of hepatocyte transplantation via spleen, portal vein or intraperitoneal administration in several adults and children with acute liver failure has been mixed but with some notable successes, as defined by spontaneous recovery or bridging to OLT [6]. Of course, injection of cells into the portal venous system is made difficult in these patients by coagulopathy and associated portal hypertension, which presents the risk of systemic embolization or portal vein thrombosis. Moreover, liver inflammation and injury could be aggravated by ischemic injury produced by the arrival of transplanted cells in liver sinusoids and activation of ischemic events. Immunosuppression after the transplantation of allogeneic cells also adds the risk of sepsis in this vulnerable population.

In preclinical rat studies, transplantation of healthy hepatocytes in the liver after acute toxic injury established that the transplanted cells required several days before they could proliferate and liver repopulation was very limited [34]. Therefore, it was of interest when investigators used the peritoneal cavity for transplanting freshly isolated, allogeneic fetal human hepatocytes in people with acute liver failure [35,36]. Of eight persons with acute liver failure and hepatocyte transplantation in this manner, four people recovered. In these studies hepatocytes were transplanted without matrix support or anchorage, which would have led to the rapid loss of transplanted cells, raising a question about the mechanisms underlying the rescue. More recently, animal studies established ATM (ataxia telangiectasia mutant) gene network-related molecular lesions in hepatocytes after drug-induced acute liver failure, as another explanation of the failure of liver regeneration [37]. These studies established that rescue did not require reseeding of the liver with transplanted cells since hepatocytes transplanted into the peritoneal cavity provided metabolic support along with paracrine factors to advance liver regeneration. Therefore, transplantation of hepatocytes with matrix support offers a viable alternative approach for rescue in acute liver failure as shown in mice [38]. This should be simplified by the possibility of cryopreserving immobilized hepatocytes [39]. In ongoing studies, human hepatocytes encapsulated in alginate beads (to avoid exchange of immunoglobulins and immunocytes driving alloresponses but not of desirable proteins) were intraperitoneal administered to several children with acute liver failure. *In vitro* experiments showed that albumin and factor VII were produced for at least 2 weeks or longer when microbeads were cultured in ascites fluid obtained from children in the post liver-transplant period [40]. Of seven children with acute liver failure, three avoided OLT after intraperitoneal transplantation of alginate-encapsulated hepatocytes (Dhawan A, personal communication unpublished observations). These results offer mechanisms for further cell therapy approaches for acute liver failure, including studies with additional cells of therapeutic interest.

For cell therapy in the setting of advanced chronic liver disease or cirrhosis due to hepatitis, alcohol or unspecified injury, one must take into account the depletion of parenchymal cells, architectural distortions, vascular reorganization, inflammation and excessive extracellular matrix deposition, and myofibroblast activation and proliferation. This distorted liver microenvironment is naturally challenging for the engraftment and proliferation of transplanted cells. In preclinical studies in rats using carbon tetrachloride-induced cirrhosis, transplanted hepatocytes engrafted but did not proliferate significantly in fibrotic liver after withdrawal of the injurious agent and no benefits were noted in

outcomes related to hepatic function or liver fibrosis [41]. By contrast, transplantation of hepatocytes into the spleen of rats with end-stage liver failure was found to prolong survival [42]. There have been a number of clinical studies seeking to transplant adult hepatocytes into patients with liver cirrhosis [15]. These studies have attempted various ways to transplant hepatocytes, including directly into the liver or via spleen as well as in the peritoneal cavity. Transplantation of cells into the liver in this situation is obviously difficult because of coagulopathy and portosystemic collaterals with risks of serious cardiopulmonary complications. However, despite the possibility that selected parameters may have improved in some cases, in general these studies have not met with great success. In the absence of disease processes that may be modified by transplanted hepatocytes, e.g., mobilization of copper in Wilson's disease, removal of toxins in progressive familial intrahepatic cholestasis, or replenishment of disease-resistant cells in chronic hepatitis, underlying issues of portal hypertension, excess extracellular matrix deposition and vascular distortions may not improve. An alternative approach recently suggested, following positive results in mice, the seeding of hepatic cells into the lymphatics [43]. Whether this interesting approach is translatable in the setting of human cirrhosis remains to be seen.

An alternative cell therapy approach to liver cirrhosis is the transplantation of cells that may stimulate endogenous regeneration or decrease fibrosis (see Table 4 for different types of potential cell therapy depending upon disease scenario). For instance, Thomas *et al.* studied the intraportal injection of bone marrow-derived macrophages for liver fibrosis and found significant reduction in liver scarring and improvements in regeneration and metabolic function in mice [44]. Macrophages directly activate the liver's ductular response via Tweak/FN14 signalling [45] and stimulate such cells towards an hepatocyte phenotype via Wnt signalling [46]. Injected macrophages have strong paracrine and chemotactic effects which amplify their benefit but only survive for a short time in the liver, which may limit efficacy without repeated administration. Nakamura *et al.* used endothelial progenitor cells (EPCs) in rat models of liver fibrosis and found them to be anti-fibrotic and also capable of stimulating liver regeneration [47]. Similarly, DeLeve and colleagues established that bone marrow-derived liver sinusoidal endothelial progenitor cells could be targeted to the liver with improvement in liver inflammation and hepatic injury in rats [48].

Terai *et al.* utilized autologous mononuclear cells from patients with advanced liver disease by bone marrow aspiration [49]. The cells were purified prior to re-infusion via the peripheral vein. A pilot study showed encouraging preliminary results where liver function and histological parameters of liver regeneration improved following cell therapy. The peripheral administration of autologous mesenchymal stem cells (MSCs) have been tested in a randomized, placebo-controlled trial in cirrhosis but no beneficial effect was seen, indeed 3 out of 15 patients that received the MSCs died in the first 5 months following cell administration compared to zero from 12 in the control group [50]. The clinical studies of various autologous cells for liver disease have been systematically reviewed by Moore *et al.* and to date no convincing benefit has been noted in adequately powered randomized controlled studies [51]. However it is worth noting that due to the early state of the "therapies" tested many studies were small uncontrolled studies that did not allow a measure of efficacy.

Main criteria
- Fresh or cryopreserved hepatocytes
- Cell viability should be >60%
- ABO blood group compatible
- Up to 10^9 cells per infusion
- Portal pressure monitoring
- Repeated up to 5-10% liver mass
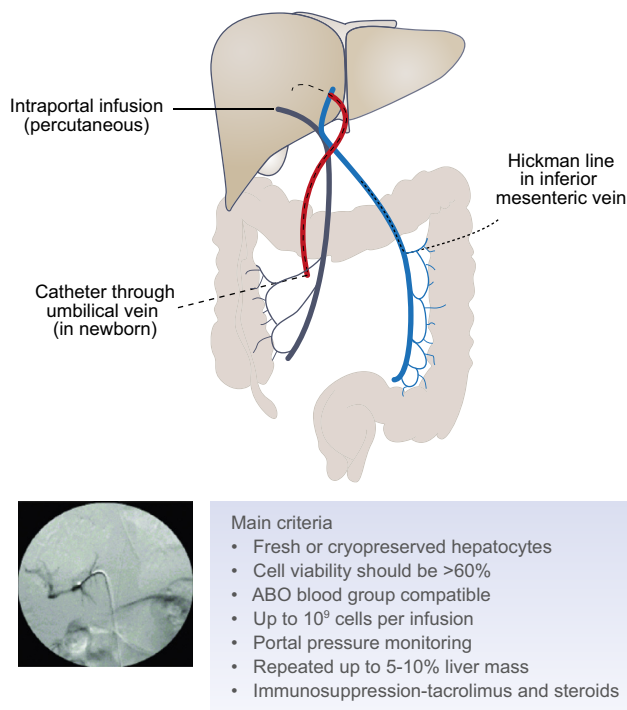- Immunosuppression-tacrolimus and steroids

Fig. 3. Routes for delivering cells into the portal venous system and major criteria for donor cell usage in the clinical setting. The radiographic image in the box indicates hepatocytes being infused via the umbilical vein into the liver of a new-born with urea cycle deficiency disorder. From Dhawan, Transplantation. Used with permission.

The issues of cell delivery

The relatively superficial location of the liver and access through a variety of modalities, including percutaneous, intravascular through either portal vein or hepatic artery, as well as vessels upstream of the portal vein offer multiple ways to deliver cells (Fig. 3). However, studies in rat have established that for transplanted cells to integrate in the liver parenchyma, the most effective approach is to deliver cells into hepatic sinusoids [52]. Injecting cells directly into the liver parenchyma has the risk of inadvertent entry into hepatic venous outflow tract and entry of cells into pulmonary capillaries with embolic consequences. Injection of cells into the hepatic or splenic artery is technically convenient but this may lead to organ infarcts, again due to embolic processes [53]. Under high-flow conditions of the arterial circulation transplanted cells may be destroyed rapidly by shear forces, which is equally applicable to the pulmonary, hepatic and splenic arterial beds. By contrast, transplantation of hepatocytes under low-flow conditions coupled with access to additional cell attachment factors and extracellular matrix components, as in the liver or spleen sinusoids, has been most effective for hepatocyte delivery, persistence and engraftment. In the case of the spleen in rat, most (90%) of the cells instantaneously migrate into the portal vein followed by redistribution equally to all liver lobes in accordance with blood flow and liver volumes [32]. The number of cells that may be safely accommodated in the sinusoids is substantial and approaches 5–10% of the total number of parenchymal cells in the healthy rat liver [54]. Moreover, studies in rodents have shown that cells may be transplanted repeatedly

without deleterious consequences on hepatic vasculature or liver sinusoids [55].

The initial transplanted cell distributions has been examined in rodents using radiolabeled or genetically-marked reporter liver cells, which provide additional methods for non-invasive tracking of the initial fate of transplanted cells [27,32]. A clinically relevant finding is that cell transplantation results in transient portal hypertension, which is due to transplanted cell size-dependent occlusions in the periportal vascular complex, followed by the restoration of blood flow through the opening of alternative vascular channels as well as by the entry of transplanted cells into the liver parenchymal structure over several hours [56]. However, the transplantation of cells in the presence of pre-existing portal hypertension and chronic liver disease may lead to excessive translocation of cells into lungs through portosystemic collaterals or channels with cardiovascular complications. These may be related to the dosages of cells administered. Therefore, clinical trial design needs to incorporate mechanisms for the successful initial delivery of cells to liver sinusoids.

Engraftment of transplanted cells including tracking and monitoring of the delivered cells

After delivery of transplanted cells to liver sinusoids, several steps follow before cells are fully integrated in the liver parenchyma (hepatocytes) or in appropriate niches of the liver structure (LSEC and other cell types) [13,56,57]. Studies in rats have shown that the initial series of events are driven by the mechanical process by which cells enter vascular spaces beyond the portal areas, which is dependent upon the size of cells and the diameter of hepatic sinusoids [54]. The larger the size of cells, the more proximal will these be to portal areas, and the smaller the size of cells, the more distal in the liver lobule will these be in relationship to portal areas. This essential size-structure relationship drives the nature of cell-cell adhesions, as well as the extent of vaso-occlusive processes that may be initiated by cell transplantation. Rodent studies have shown that subsequent cell-cell signalling events are important. The ischemic injury rapidly activates neutrophils, Kupffer cells, LSECs and hepatic stellate cells [58,59]. The initial engraftment processes where vascular responses involve release of locally-acting vasoactive molecules, such as NO and prostacyclins, complement, platelet-related thrombogenic substances, endothelin, cyclooxygenases, cytokines/chemokines/receptors, are largely deleterious. However, release of some substances, such as vascular endothelial growth factor (VEGF) from native hepatocytes, Kupffer cells, monocytes and hepatic stellate cells helps in permeabilization of endothelial cells. Coupled with the activation of LSEC, transplanted cells are able to penetrate the endothelial barrier and then integrate themselves into the liver parenchyma [56]. This part of the process requires 16–20 h from cell delivery. However, cumulatively, nearly 80–90% of all transplanted hepatocytes are destroyed due to sinusoidal events, including lack of entry into sinusoids, inadequate or no adhesion to sinusoidal endothelium, oxidative stress, cytokine-mediated toxicity, etc. Therefore, considerable efforts have been devoted to understanding how these deleterious processes could be harnessed and thus yield drug-based approaches for improving cell therapy outcomes.

The next process in transplanted cell engraftment concerns integration of transplanted cells in the liver parenchyma. This requires reconstitution over 1–5 days of plasma membrane structures and physical joining together of transplanted and native hepatocytes [56]. Evidence of this process in rats include re-associated gap junctions and bile canalicular networks with components from both native and transplanted hepatocytes [25], as noted above. Despite delays in the integration of transplanted hepatocytes, it should be noteworthy that transplanted cells continue to express the normal repertoire of genes without interruptions. However, it has been shown in rodents that hepatic gene expression in transplanted cells is better supported in the hepatic microenvironment compared with that in the spleen, which in turn is superior to that in the peritoneal cavity [60]. The process of transplanted cell integration in liver is aided by the activation of stellate cells, which release matrix metalloproteinases and tissue inhibitors of metalloproteinases to coordinate disruptions and restitutions of the extracellular matrix during the entry and resettling of transplanted cells [61]. After transplanted cells become integrated in the liver parenchyma, gene expression profiles are driven by their position in the liver lobule, which is similar to adjacent native hepatocytes, and is under position-specific regulation [29]. Moreover, transplanted cells exhibit normal patterns of proliferative activity, i.e., no proliferation in the healthy liver, and graded proliferation in response to injury in the liver lobule that spares them, which is similar to the responses in adjacent healthy cells. In these approaches, transplanted cells are noted to life-long survival in rodents [28].

In recent years, novel targets have been defined to improve engraftment of transplanted cells at these stages and several drug-based strategies have been developed in preclinical models that are potentially clinically relevant for enhancing cell engraftment in the liver (Table 3). The major concepts have concerned use of drugs to treat subjects prior to cell transplantation, such that vascular or inflammatory changes induced by cell transplantation are abolished or minimized, the endothelial barrier interposed between liver sinusoids and parenchyma is disrupted, or hepatic stellate cells are induced to release beneficial substances, e.g., VEGF [58,59,62,63]. Similarly, novel concepts have been developed in rodents where donor cells may be modified prior to transplantation, e.g., by addition of extracellular matrix components for better endothelial adhesion or incubation with drugs to block endothelin (ET)-1 receptors, which otherwise may transduce deleterious intracellular signals to activate NF-κB-mediated cell death [63,64]. Of note, prior treatment of recipients with anti-inflammatory drugs, e.g., the TNF-α blocker, etanercept, had remarkable effects in rat by preventing deleterious cytokine/chemokine/receptor responses, leading to improvements in transplanted cell survival and engraftment in the liver [65]. The possibility of combining these approaches has also been and is being examined for further beneficial effects. To this effect, the initial losses of transplanted cells may be mitigated by relatively straightforward approaches using available drugs, which should aid future cell therapy applications.

The *in vivo* monitoring of delivered cells in patients is surprisingly challenging. Whilst cells can be genetically labelled with fluorescent or other probes in animal models, cell tracking in humans requires alternative methods. For short-term tracking of transplanted cells, radiolabeling methods have been effective, e.g., Indium-111- or 99m-technetium-labelled cells, in animals as well as in people [32,66–68]. These methods are particularly suitable for tracking the initial distribution and redistribution of transplanted cells in various vascular beds. An example of this

Table 3. Drug-based approaches for improving transplanted cell engraftment and proliferation.

A. Pre-treatment of recipients prior to cell transplantation
Alteration of hepatic vascular responses
• ET-1 receptor blockade (bosentan, darusentan)
• Nitroglycerine
• Prostacyclin
Blockade of inflammatory cytokines/chemokines/receptors
• Etanercept
• Thalidomide
Disruption of hepatic endothelial barrier
• Cyclophosphamide
• Doxorubicin
• Rifampicin/phenytoin
Activation of hepatic stellate cells
• Naproxen
• Celecoxib
B. Pre-treatment of donor cells prior to transplantation
• Extracellular matrix coating with adhesion factors or engineered molecules
• Dual ET-1 A and B receptor blockade (bosentan)
C. Combined preconditioning of recipients and of donor cells prior to or after cell transplantation
• Various combinations of drug regimens
• Thyroid hormone treatments
• Inhibitors of fumarylacetoacetate hydroxylase activity
• Other drugs promoting liver regeneration or cell survival under study

approach is the ability to determine and quantitate the risks of intrapulmonary shunting of transplanted cells via portosystemic collaterals. Moreover, transplanted cells may be localized in extra-hepatic sites by imaging of hepatic receptor function, e.g., asialo-glycoprotein receptor as has been shown in mice and rats [69]. For longer-term tracking of transplanted cells, molecular imaging methods have also been developed. One such approach developed in rodents and non-human primates used genetic constructs (herpes simplex virus thymidine kinase) for metabolizing ganciclovir substrates for PET imaging [70]. Another approach tested in rats utilised the imaging of natural ligands, e.g., radiocopper probes, to demonstrate restoration of biliary copper excretion after transplantation of healthy hepatocytes [71]. Furthermore, genetic reporters capable of identifying unique alleles, including HLA-specific alleles short tandem repeats, have been proposed as additional molecular methods to establish organ chimerism with transplanted cells in mice [72]. Nonetheless, further clinically applicable methods are needed for tracking transplanted cells and monitoring their function in humans.

Inducing proliferation in transplanted cells for liver repopulation

Despite the integration and indefinite persistence of transplanted cells in the absence of allograft rejection, more needs to be learned about ways to induce proliferation in transplanted cells. Although repeated transplantation of cells in large numbers coupled with ways to decrease losses of transplanted cells could substantially increase delivery of cells to the liver [65], replacing large amounts of the liver parenchyma with transplanted cells is of enormous interest for many conditions. The fundamental principle driving this area was gained from studies in animals

Table 4. Different types of potential cell therapy depending upon disease scenario.

| Cell type	Potential indication	Clinical use?	Advantages	Disadvantages	Reference
Hepatocytes	• Metabolic liver disease • Paediatric liver failure	Yes	• In routine clinical use • Key metabolic and synthetic cell	• Shortage of supply • Engraftment in damaged liver problematic • Susceptible to infection with hepatitis viruses	
MSCs	• Liver cirrhosis • Liver failure • Immune mediated liver disease	Some clinical reports and small number of randomised trials	• Easy to isolate and expand • Generally immune tolerising • May be used with other cell types to reduce inflammation	• Some clinical studies have been negative • Poorly defined cell type	[50, 111]
EPCs	• Liver cirrhosis	No	• Appear anti-fibrotic and pro-regenerative	• Isolation and clinical use unclear	[47]
Macrophages	• Liver cirrhosis	No	• Multiple effects on fibrosis and regeneration • Stimulate host macrophages to amplify the response	• Transient liver engraftment • May be pro-fibrotic in certain circumstances	[44, 45]
Embryonic stem cells (ESCs)	• Metabolic liver disease • Liver failure	No	• May be infinitely expandable population of cells • Available from GMP compatible sources	• Ethics of use has been questioned • Long-term stability unclear	[93]
Induced pluripotent stem cells (IPSCs)	• Metabolic liver disease • Liver failure	No	• May be infinitely expandable population of cells • Autologous use is possible, this would require gene modification for genetic diseases	• Question over completeness of functionality • Long-term stability as yet unproven	[102]
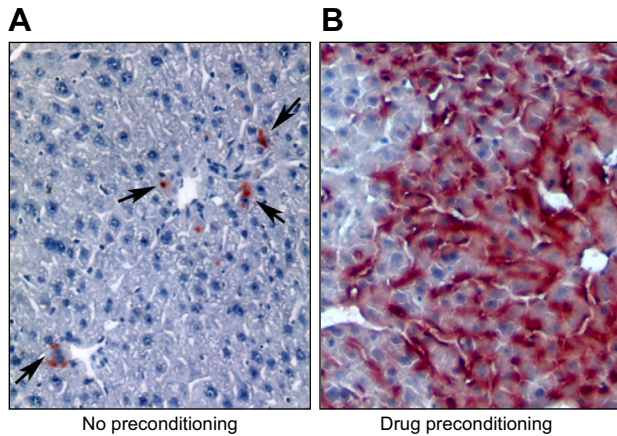
Fig. 4. Proliferation of transplanted cells in the liver of healthy Dpp4 knockout mice. (A) Control animal subjected to no hepatic preconditioning showing occasional transplanted cells with Dpp4 activity (red color, arrows) after 2 months. (B) Preconditioning of animals with rifampicin, phenytoin and monocrotaline followed by hepatocyte transplantation showed extensive liver repopulation to approximately 50% after 2 months.

with progressive liver injury that spared healthy transplanted hepatocytes. For instance, in alb-uPA transgenic mice, transplantation of healthy hepatocytes resulted in progressive liver repopulation [18]. Similarly, in *FAH*$^{-/-}$ mice with progressive liver injury, transplanted healthy hepatocytes or hepatocytes derived as fusion products with components from extrahepatic sources, such as donor bone marrow-derived mononuclear cells, proliferated with progressive liver repopulation [73]. In LEC rats modelling Wilson's disease, extensive liver repopulation was observed when healthy hepatocytes were transplanted [17].

Furthermore, induced injuries that constituted hepatic preconditioning were successful in promoting proliferation of transplanted cells in rodents [18]. For instance, DNA-adduct forming pyrollizidine alkaloids, retrorsine and monocrotaline, were found to be effective in combination with additional injuries, such as partial hepatectomy, carbon tetrachloride, or other drugs, for inducing liver repopulation by transplanted cells. Preconditioning of the liver with radiation plus partial hepatectomy, ischemia-reperfusion or toxic bile salts has also been effective for liver repopulation. This included replacement of mouse liver with transplanted hepatocytes as well as LSECs [13,74]. However, these manipulations are not well-suited to clinical applications and more conceptual development is necessary, especially in regards to suitable drugs. Some progress has been made in this area and further progress is anticipated (Fig. 4).

An alternative approach is to alter the proliferation of transplanted cells by inactivation of cell cycle suppressors, which accelerated liver repopulation kinetics with transplanted hepatocytes in *FAH*$^{-/-}$ mice [19]. However, this approach is not without cancer-risk. More recently, regulation of liver growth control by the Hippo signalling pathway has come to attention [75] which may provide additional targets to promote transplanted cell proliferation.

It may be that hepatic preconditioning to replace LSECs will be simpler than the replacement of hepatocytes because suitable endothelial injury could possibly be achieved by ischemia-reperfusion injury or other available drugs. Although this needs further study this would be of benefit for haemophilia A targeted cell therapy.

Obtaining sufficient hepatocytes in the clinic therapy

Human adult hepatocytes

Human hepatocytes are isolated from donor organ livers that are either unused surplus or are rejected for transplantation in people. Cells are typically isolated by using collagenase perfusion techniques under clinical Good Manufacturing Practice (cGMP) conditions [76]. The shortage of good-quality donor organs has led to the use of marginal donors or segment IV/caudate lobes when the donor liver is split for transplantation into more than one recipient [77,78]. Progress is being made in understanding molecular mechanisms underpinning the difficulty in isolating viable hepatocytes from non-heart-beating cadaveric donors [79]. Progress is also being made to improve the viability and metabolic function of hepatocytes isolated from steatotic livers, e.g., by the addition of N-acetylcysteine as an antioxidant to the perfusion solution [80]. Given the increasing rates of obesity and thus the frequency of fatty organs that are offered for transplantation this area of research is of major importance for the future. Isolated human hepatocytes may be transplanted freshly after isolation or after cryopreservation even several years later if stored appropriately, depending on regulatory practices [81]. However, the cell yield after thawing is low and the freezing process also has a detrimental effect on the metabolic function and cell attachment properties [82]. Therefore, an optimised protocol may provide significant improvements in cryopreserved cells but further refinements in the processes of cryopreservation and mechanisms capable of preserving or enhancing cell viability are required [83].

Alternative sources of hepatocyte-like cells

Fetal hepatocytes

Clinical studies using fetal hepatocytes have suggested they are a potentially useful source of cells for clinical therapy [35,36]. Understanding their behaviour in the host liver will help to define conditions for their optimal usage. Interestingly, in the rat, fetal hepatocytes have been shown to proliferate within the host liver, even in the absence of the usual mitogenic stimulus required for transplanted adult hepatocytes [84]. An interesting feature of this was that the transplanted cells could induce apoptosis in the recipient's nearby host cells and thereby have a form of selective advantage over the recipient liver cells. Although the proliferative capacity of the rat fetal cells is encouraging, other studies utilizing human fetal cells have found different results. Haridass *et al.* compared human fetal hepatocytes to adult hepatocytes in an immunodeficient mouse transplantation model. In this system the fetal cells had less repopulation capacity on a cell per cell basis than the adult hepatocytes [85]. Intriguingly, a recent study has suggested that rat fetal hepatocytes may have anti-fibrotic properties when transplanted into damaged livers, which would have the dual benefit of supporting parenchymal regeneration whilst targeting the scarring component of chronic liver disease [86]. Whether this finding is a general phenomenon, seen using human fetal hepatocytes, particularly in the setting of chronic liver injury where there is significant scarring is unknown. Whether fetal cells could be less immunogenic compared with adult hepatocytes is also unresolved at present. One issue in

transferring these interesting preclinical studies findings into clinical therapy is the issue of ethically sourcing sufficient human fetal hepatocytes for therapy in people. This aspect will likely prevent fetal hepatocytes becoming a widely used clinical resource, however the positive features of the cells may be used to guide and influence the production of stem cell derived hepatocytes in the future.

Hepatic progenitor cells (HPCs)

Whilst the normal human liver can regenerate efficiently through hepatocyte division during severe or chronic liver disease the regenerative capacity of hepatocytes is compromised. Under these circumstances it has been thought that there are endogenous HPCs that are activated and due to their bipotential nature are able to regenerate both biliary epithelia and hepatocytes [87]. Recently this understanding has been challenged by studies in mouse which suggest hepatocytes supply all the regenerative capacity of the parenchyma, and the ductular reactions seen in chronic injury do not regenerate parenchyma. Furthermore some of the ductular reactions may arise from the de-differentiation of mature hepatocytes during injury [88,89]. However recent data using developmental ablation of foxl1 marked HPCs in mice suggested that HPCs were a significant source of parenchymal regeneration and ablation of these foxl1 HPCs had a detrimental effect upon the recovery from liver injury [90]. Further studies are awaited in this area with conditional ablation of such cell types to further delineate their regenerative role of such HPCs.

An important unresolved question is whether such studies in mice are directly translatable to human liver disease. The injury models used are often relatively mild and short lived compared to the severe liver injury that occurs sometimes over decades in humans and results in the significant impairment of hepatocyte proliferation. This controversial area is beyond the scope of this review but is relevant for the question of whether an expandable source of cells with hepatocyte or biliary potential could be derived for cell therapy [91]. Lgr5 has been shown in mouse to identify cells with a HPC characteristic. These Lgr5+ cells could be grown into organoids with a high clonogenic capacity [46]. When such organoids were used in the $FAH^{-/-}$ mouse they could engraft within the parenchyma and provide nodules of functioning parenchyma. Furthermore, organoids have been derived from human livers using EpCAM selection of bipotential hepatic epithelial cells that originated in the ductal areas of the liver [92]. Importantly these cells retained chromosomal stability during prolonged culture. In order to translate these interesting findings into a clinical therapy a GMP compatible method of organoid culture will be required; furthermore the engraftment and repopulation characteristics of the organoids or cells derived from these organoids will need to be defined.

Pluripotent sources of hepatocytes: Embryonic stem (ES) cells and induced pluripotent stem cells (iPSCs)

ES cells are pluripotent stem cells derived from the inner cell mass of a blastocyst. In humans this stage is reached at 4–5 days post fertilization and consists of approximately 50–150 cells. Because the derivation of ES cells requires destruction of the blastocyst there is an inescapable moral dimension to their use. ES

cells have been studied for a considerable time and human ES cells have been derived at GMP level which would be required for clinical use. Protocols have been developed to differentiate ES cells sequentially into a hepatocyte-like cell (HLC) phenotype. These have largely been based upon the signals that arise during development including Activin A and Wnt 3A to encourage endoderm differentiation [93,94], bone morphogenic protein (BMP) and fibroblast growth factor (FGF) to aid hepatocyte differentiation and then oncostatin M and dexamethasone to promote maturity [95]. Further attempts have been made to enhance their phenotype and stability using synthetic and natural cell matrices [96], however despite this the pluripotent (ES cells and iPSCs-see later) derived HLCs have a phenotype more in keeping with a fetal than an adult hepatocyte phenotype [97]. However, *in vivo* it is possible that more mature cells may develop. A recent study demonstrated that pluripotent derived human HLCs can support HCV infection and replication *in vivo* [98]. If the signals and factors present *in vivo* can be recapitulated *in vitro* it is possible that more mature HLCs may be produced. 3D culture systems have been tested and aid the increased maturity of the HLCs [99]. Because of the source and derivation of ES cells it is unlikely that ES cell-derived HLCs would be routinely fully immunologically matched to the recipient (blood antigen, tissue type and HLA). Therefore, like whole liver transplantation, focusing upon blood antigen matching would be one solution with the resulting need for some immunosuppression.

iPSCs are pluripotent cells that can be reprogrammed from adult cells using so-called pluripotency factors [100]. They have the ethical advantage of not requiring embryonic material and have the potential clinical advantage that they can be developed from autologous starting cells, thereby obviating the requirement for immunosuppression. Obviously if iPSCs were to be used for the derivation of HLCs for the treatment of a genetic liver disease then the autologous source would mean some form of "gene surgery" would be required prior to use. Such an approach has been adopted in a preclinical model of alpha-1-antitrypsin deficiency [101].

Human HLCs can be efficiently derived from iPSCs [102], however using standard differentiation methods they are currently more fetal in their phenotype than adult primary hepatocytes [97]. The 3D culture of iPSCs has recently been shown to increase their maturity closer to that of mature hepatocytes emphasizing the need for an appropriate developmental niche for the cells [103]. An exciting development was the demonstration that when human iPSCs were cultured with endothelial and mesenchymal cells they self-formed *in vitro* into small liver organoids that could be transplanted and had metabolic and synthetic function [104]. For clinical translation of these findings a way to grow scalable organoids with appropriate 3D structure, vascularity and ideally immune cell function would be required. Alternative approaches have been taken to increase the maturity of human iPSC derived hepatocytes. Kondo *et al.* used Activin A, dimethyl sulfoxide, hepatocyte growth factor, oncostatin M, and dexamethasone to induce hepatocyte maturity including drug metabolism activity [105].

The use of pluripotent stem cell derived and directly reprogrammed HLCs have several factors that would influence their potential clinical use. Cells would need to be phenotypically stable over a long period if they were to be transplanted into the liver. This is particularly obvious in the setting of paediatric cell therapy where decades of safety would be required from

the transplanted cell. The HLC would need to be homeostatic and respond to growth and renewal requirements in an appropriate manner. Furthermore this capacity for appropriate proliferation would be tested in the setting of chronic liver injury where there are multiple and chronic signals acting to perturb the HLCs phenotype and physiological function. A particular concern when contemplating the use of pluripotent cells as a source of HLCs is the development of tumours although safety measures can be envisaged such as physical encapsulation of the cells or the use of an inbuilt suicide gene that could be activated in the situation of unwanted proliferation of the transplanted cells.

Direct reprogrammed cells

A recent technological advance is the direct reprogramming of human fibroblasts into so-called induced hepatocytes (iHeps). Sekiya and Suzuki showed that two transcription factors-Hnf4alpha plus Foxa1, Foxa2 or Foxa3 could convert mouse adult fibroblasts into iHeps *in vitro* and could rescue the $FAH^{-/-}$ mouse [106]. In a similar approach fibroblasts were converted to iHeps by the transduction of Gata4, Hnf1alpha and Foxa3 and the inactivation of p19. Furthermore, the iHeps showed good phenotypic qualities and could repopulate the livers of $Fah^{-/-}Rag2^{-/-}$ mice, rescuing a proportion of recipients [107].

Human induced hepatocytes (hiHeps) have been developed by similar techniques to that seen in mouse. Huang *et al.* found that the expression of HNF4, HNF1A, and FOXA3 in fibroblasts allows the production of hiHeps at a conversion rate approaching 20% [108]. The paper by Du *et al.* used a more comprehensive set of factors: C-MYC, HNF1A, HNF4A, HNF6, ATF5, PROX1, CEBPA, and p53 shRNA to efficiently produce hiHeps [109]. Both sets of hiHeps had similar gene expression profiles to mature human hepatocytes but by no means identical. Encouragingly the hiHeps also showed good *in vivo* functionality in a number of mouse models of liver injury including the FRG ($Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$) mouse.

A further refinement of this technique has been employed in mice whereby fibroblasts are first differentiated into induced multipotent progenitor cells (iMPCs) [110]. These iMPCs could be significantly passaged and expanded then differentiated through an endoderm stage to differentiated HLCs (so-called iMPC-Heps). These iMPC-Heps could partially repopulate the $FAH^{-/-}$ mouse liver although again the resulting hepatocytic function was short of that seen with primary hepatocytes.

Overall the use of pluripotent stem cells and directly reprogrammed hepatocytes is in its infancy and whilst we must applaud the advances made, we must also acknowledge the limitations and problems associated with these cells. To date the pluripotent derived cells are lacking sufficient maturity for widespread clinical use. Alongside this lack of maturity goes the concern that the cells may be phenotypically unstable *in vivo* over a prolonged period and when exposed to the "adverse niche" of the damaged liver. Understanding and where possible recapitulating the conditions of the developing human liver are likely to bring advances in the maturity and stability of these pluripotent derived cells. However, it is likely that understanding deeply the complex epigenetic factors and the regulation of gene expression that evolve in forming hepatocytes will also be required to produce hepatocytes that are "good enough" for clinical, and even, routine clinical use – surely the eventual goal of this push in the basic science area.

Conclusions and further perspectives

There is a clear requirement for an unlimited source of human HLCs for transplantation with good function, phenotypic stability and a near zero risk of tumorgenicity. Whilst easily stated this is a demanding goal. Cells with a stem cell like potential that are therefore able to give rise in a clonogenic manner to large numbers of progeny do carry a potential risk of unwanted and unregulated cell growth. Such risks can be minimized by screening potential candidate cells in appropriate long-term studies in animal models and by engineering in "safety devices" such as suicide genes which would allow the killing of a cell upon administration of a drug or small molecule. All risks of therapy are relative to the disease in question and the transplantation of a cell into an elderly person with a potentially fatal fulminant liver disease is an entirely different risk profile to the use of in a baby where the transplanted cell may need to perform for decades without undergoing oncogenic change.

Stem cells have frequently been invoked as the future cell to allow an almost limitless source of cells for therapy. However if hepatocytes are the facultative stem cells of the liver then perhaps it is time to re-examine the potential clonogenic capabilities of the humble hepatocyte. Adult hepatocyte exhibits almost unlimited clonogenicity *in vivo* yet *in vitro* has been very difficult to expand without the cells undergoing de-differentiation. Understanding the *in vivo* cues which allow the homoeostatic regulation of hepatocytes has progressed greatly. Hepatocytes divide readily when required yet remain quiescent at other times in the healthy liver. This *in vivo* understanding still needs to be applied *in vitro* to human adult hepatocytes and allow division without de-differentiation. Such knowledge would lead to the ideal scenario of an infinitely expandable cell source for clinical cell therapy.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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