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Review article

Evaluating the regenerative potential and functionality of human liver cells in mice

Antson Kiat Yee Tan[a,1], Kyle M. Loh[b,1], Lay Teng Ang[a,*]

[a] Stem Cell & Developmental Biology Group, Genome Institute of Singapore, A*STAR, Singapore 138672, Singapore
[b] Stanford Institute for Stem Cell Biology and Regenerative Medicine and the Stanford—UC Berkeley Siebel Stem Cell Institute, Stanford University School of Medicine, Stanford, CA 94305, USA

ABSTRACT

Liver diseases afflict millions of patients worldwide. Currently, the only long-term treatment for liver failure is the transplantation of a new liver. However, intravenously transplanting a suspension of human hepatocytes might be a less-invasive approach to partially reconstitute lost liver functions in human patients as evinced by promising outcomes in clinical trials. The purpose of this essay is to emphasize outstanding questions that continue to surround hepatocyte transplantation. While adult primary human hepatocytes are the gold standard for transplantation, hepatocytes are heterogeneous. Whether all hepatocytes engraft equally and what specifically defines an "engraftable" hepatocyte capable of long-term liver reconstitution remains unclear. To this end, mouse models of liver injury enable the evaluation of human hepatocytes and their behavior upon transplantation into a complex injured liver environment. While mouse models may not be fully representative of the injured human liver and human hepatocytes tend to engraft mice less efficiently than mouse hepatocytes, valuable lessons have nonetheless been learned from transplanting human hepatocytes into mouse models. With an eye to the future, it will be crucial to eventually detail the optimal biological source (whether *in vivo*- or *in vitro*-derived) and presumptive heterogeneity of human hepatocytes and to understand the mechanisms through which they engraft and regenerate liver tissue *in vivo*.

1. Introduction

The liver is essential to life: it discharges a wide range of crucial roles, including bodily metabolism and neutralization of toxins, amongst other functions (Stanger, 2015; Jungermann, 1995; Jungermann and Kietzmann, 1996; Gebhardt, 1992). Despite its ability to regenerate upon two-thirds physical resection (Nagasue et al., 1987), the liver cannot sustain injuries beyond a certain threshold. After extensive damage, liver functions (including detoxification) fail, leading to the accumulation of toxins and eventually, coma and death (Karl et al., 1953; Bernal and Wendon, 2013). Liver disease is one of the 12 leading causes of adult death in the United States (U.S.) (Asrani et al., 2013) and it is estimated that annually > 1 million patients die worldwide due to liver failure (Mokdad et al., 2014).

Therefore there is a pressing need for new therapeutic interventions to restore liver functions after injury. Currently, the only effective long-term treatment for liver failure is to transplant a new liver derived from another human being (Goss et al., 1998; Jain et al., 2000; Buescher et al., 2016). However, liver transplantation is critically limited by the shortage of livers available from organ donors (Buescher et al., 2016; Hansel et al., 2014; Ibars, 2016). Moreover, it is a surgically-invasive and expensive procedure (Hansel et al., 2014), by some estimates costing ~$145,000-$182,000 in the U.S. per patient (Haddad et al., 2017). These estimates are coupled with additional costs incurred while waiting for an organ transplant and post-transplantation medications and services (Brand et al., 2004).

Various clinical trials have explored a less-invasive alternative, namely to transplant a single-cell suspension of hepatocytes into the portal vein of patients with specific types of liver disease. The intention of such "hepatocyte transplantation" is to temporarily restore liver functions and bridge patients until they can receive a liver transplant (Dhawan, 2016). In one of the best cases of hepatocyte transplantation, infusion of human hepatocytes (constituting ~2–5% of original liver mass) into a pediatric patient with Crigler-Najjar syndrome Type 1 partially corrected the disorder and sustained the patient for 4 years before she received a whole-organ liver transplant (Fox et al., 1998; Fisher and Strom, 2006).

* Corresponding author.
E-mail address: anglt1@gis.a-star.edu.sg (L.T. Ang).
[1] Co-first author.

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It is important to emphasize that hepatocyte transplantation has thus far only been used to temporarily bridge patients until they can receive a liver transplant; moreover, hepatocyte transplantation has yet to see widespread clinical application for a number of reasons. First, primary human hepatocytes show considerable batch-to-batch variability and lack consistency given their provenance from different donors. The lack of a renewable source of transplantable hepatocytes has impeded the large-scale application of hepatocyte transplantation. Second, patients transplanted with primary human adult hepatocytes sometimes lose their graft after several months or years (Jorns et al., 2012; Hughes et al., 2005). It remains unclear whether this can be attributed to the dissatisfactory proliferative ability of transplanted hepatocytes and/or to immune rejection (for immune-mismatched hepatocyte transplants). Third, hepatocyte transplantation has enjoyed the greatest success in treating monogenic, inherited metabolic diseases in human patients (Fisher and Strom, 2006). Whether it will also enjoy similar success in treating more complex liver failure syndromes such as cirrhosis—where disarrayed liver architecture and fibrosis might hamper the distribution and vascularization of transplanted hepatocytes—remains a mystery. These limitations underscore the need to understand the basic mechanisms through which hepatocytes incipiently engraft in, and eventually repopulate, a recipient liver if we wish to eventually achieve long-term liver reconstitution in patients (Jorns et al., 2012; Hughes et al., 2005).

This essay explores the plausibility of hepatocyte transplantation and focuses on the as-of-yet-unanswered question of what defining properties enable hepatocyte engraftment and regeneration *in vivo*. To this end, mouse xenograft models enable tests of human hepatocyte engraftment, repopulation and function *in vivo*, with the proviso that the mouse liver might not be fully representative of the human liver. This issue is further complicated by the fact that hepatocytes are a heterogeneous population of cell-types (Halpern et al., 2017), and current clinical hepatocyte transplantation protocols engraft bulk, unpurified populations. Here we discuss 1) the various subpopulations of hepatocytes present in the liver, 2) new potential sources of transplantable hepatocytes besides human donors, 3) various genetically- and chemically-induced mouse models of liver injury that enable the assessment of transplanted human hepatocytes and 4) challenges surrounding the engraftment of human hepatocytes in the mouse liver. First, we begin with the issue that there are multiple hepatocyte subtypes, each of whose regenerative potential and "transplantability" has yet to be fully explored.

2. Hepatocytes are heterogeneous and comprise multiple subtypes distributed throughout the liver lobule

The liver is a tapestry of multiple cell-types—including several subtypes of hepatocytes—interwoven in a spatially-stereotyped way (Si-Tayeb et al., 2010). The liver is subdivided into several lobes and each lobe is composed of smaller functional units known as liver lobules (Si-Tayeb et al., 2010; Miyajima et al., 2014). Each liver lobule is a roughly polygonal unit comprising several subtypes of hepatocytes distributed between central vein and portal triad (constituting portal vein, hepatic artery, and bile duct), amongst other substructures (Si-Tayeb et al., 2010). Amongst these lineages, hepatocytes constitute the major secretory and metabolic workhorse of the liver and collectively execute a wide range of distinct functions including the metabolism of carbohydrates, amino acids, fatty acids and ammonia (Gebhardt, 1992).

However it is clear that there is no singular type of "hepatocyte"; instead, there are at least three subtypes of hepatocyte (periportal, midlobular and pericentral), which have non-overlapping metabolic functions and express distinctive, subtype-specific markers. These distinct hepatocyte subtypes are located at spatially distinct, stereotyped locations within each lobule (Fig. 1). Periportal hepatocytes encircle the portal vein, whereas midlobular hepatocytes span between the portal and central veins and pericentral hepatocytes circumscribe the central vein (Jungermann, 1995; Jungermann and Kietzmann, 1996; Colnot and Perret, 2011). Periportal hepatocytes express the carbohydrate-metabolizing enzyme Phosphoenolpyruvate Carboxykinase 1 (Pepck1) (Benhamouche et al., 2006) and predominantly perform gluconeogenesis, ureagenesis and cholesterol biosynthesis. Conversely, pericentral hepatocytes express the ammonia-metabolizing enzyme Glutamine Synthetase (Gs) (Gebhardt, 1992) and largely participate in glycolysis, glutamine synthesis and bile acid biosynthesis (Jungermann, 1995; Jungermann and Kietzmann, 1996; Colnot and Perret, 2011). A wealth of additional hepatocyte subtypes likely exist, as suggested by a recent single-cell RNA-Seq profiling survey which revealed that the classification of hepatocytes as either periportal, midlobular or pericentral is likely an oversimplification (Halpern et al., 2017). Moreover, this transcriptional survey revealed that half of liver-expressed genes examined showed clear gradients in expression, either biased to pericentral or periportal hepatocytes (Halpern et al., 2017). Finally, aside from gene expression, hepatocytes spanning the liver lobule also differ in their genomic content (ploidy): while two-thirds of adult mouse hepatocytes are polyploid, pericentral hepatocytes are preferentially diploid (Wang et al., 2015).

In sum, there is no single hepatocyte that discharges all liver metabolic functions – these functions are subdivided amongst subsets of complementary hepatocytes. The spatial distribution of different hepatocyte subsets and functions across distinct parts of the liver lobule is referred to as "zonation". The evolutionary imperative underlying the zonation of metabolic functions across multiple hepatocytes subsets remains unclear (Halpern et al., 2017). Nonetheless, if hepatocytes are heterogeneous with respect to gene expression, metabolic function, and ploidy, this logically raises the question of whether hepatocytes are likewise heterogeneous with respect to regenerative activity.

3. Which hepatocyte populations regenerate the liver during homeostasis and regeneration?

In accord with the aforementioned molecular heterogeneity of hepatocytes, it has been proposed that certain hepatocyte subset(s) are more regenerative and proliferative than others—if so, this would nominate them for therapeutic use, for instance for transplantation into patients. To that end, at least two complementary methods have been used to test the regenerative potential of hepatocytes: transplantation or lineage tracing. In the former approach, hepatocytes are transplanted into a recipient liver, whereupon they proliferate and ultimately repopulate the liver. Lineage tracing (Kretzschmar and Watt, 2012) entails first genetically labeling hepatocytes *in vivo* (typically based on their expression of a specific marker) and then tracing whether they subsequently give rise to progeny in either the uninjured or injured liver, without recourse to transplantation. To date, studies have focused on the regenerative abilities of at least two hepatocyte subsets: pericentral and periportal hepatocytes. First, we focus on pericentral hepatocytes.

Pericentral hepatocytes encircle and directly abut the endothelium of the central vein and have been proposed to be uniquely regenerative. Central vein endothelial cells produce extracellular Wnt signaling pathway agonists and co-agonists (Wnt and R-spondin proteins, respectively (Wang et al., 2015; Rocha et al., 2015)), which activate the Wnt pathway in adjacent pericentral hepatocytes. In normal circumstances, Wnt activation is largely confined to pericentral hepatocytes (Stanger, 2015; Yang et al., 2014) and is a dominant driver of the pericentral hepatocyte state. Indeed supernumerary activation of the Wnt pathway across the liver lobule (for instance, by deleting the Wnt pathway antagonist *Apc* (Benhamouche et al., 2006) or by treating mice with soluble Wnt agonists (Janda et al., 2017)) leads to expression of pericentral-specific hepatocyte markers (*e.g., Gs, Axin2* , *RhBg*, *Glt1* and *Lect2*) (Benhamouche et al., 2006) by the majority of hepatocytes

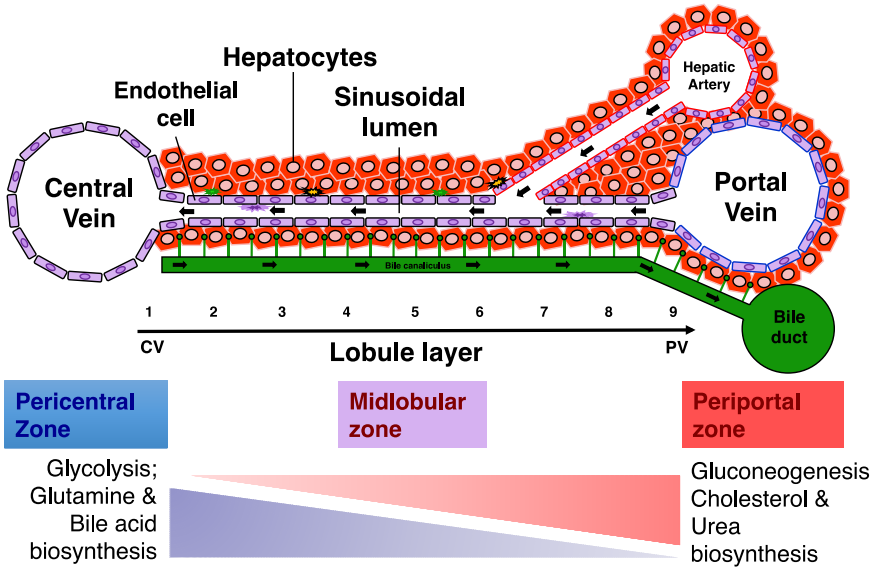

Fig. 1. A liver lobule consisting of at least 3 broad zones: periportal (near portal vein), midlobular region and pericentral (encircling central vein) zones. Zones of hepatocytes were further divided into 9 zones; hepatocytes at each zone express different levels of hepatic genes (Halpern et al., 2017).

across the liver lobule. Conversely, conditional deletion of the Wnt transcriptional activator *β-catenin* extinguishes the pericentral expression of *Gs* (Sekine et al., 2006).

Wnt-responding pericentral hepatocytes have immense proliferative and regenerative ability. In the uninjured liver, pericentral hepatocytes are preferentially the most proliferative amongst all hepatocyte subsets, dividing once every ~2 weeks (Wang et al., 2015). Indeed given that pericentral hepatocytes are enriched for diploid hepatocytes (Wang et al., 2015), these cells may be endowed with a replicative advantage by contrast to polyploid hepatocytes located elsewhere in the liver lobule. Striking, genetic lineage tracing of pericentral hepatocytes (expressing the Wnt target gene *Axin2*) has demonstrated that labeled pericentral hepatocytes proliferate long-term in the uninjured liver to maintain constant hepatocyte numbers during tissue renewal (Wang et al., 2015), thus offsetting the natural attrition of hepatocytes. Over the course of 1 year, pericentral hepatocytes can replace up to one-third of the total hepatocytes in the liver (including periportal hepatocytes) (Wang et al., 2015), exemplifying that they are engines of hepatocyte proliferation. Indeed, reducing Wnt signaling in the uninjured liver (by simultaneously deleting the Wnt coreceptors *Lgr4* and *Lgr5*) reduces hepatocyte proliferation, and consequently, overall liver weight (Planas-Paz et al., 2016). Finally, aside from their role in maintaining normal homeostasis, pericentral hepatocytes seem to be drivers of liver regeneration. Combined loss of *Lgr4* and *Lgr5* impairs liver regeneration (Planas-Paz et al., 2016) and after partial hepatectomy of the neonatal mouse liver, pericentral hepatocytes preferentially expand (Tsai et al., 2017). In summary, pericentral hepatocytes are capable of giving rise to more of themselves (self-renewal) in addition to periportal hepatocytes; hence they might be particularly apt for regenerative applications.

While they are typically less proliferative in the uninjured liver, periportal hepatocytes have also been postulated as an additional contributor to liver regeneration upon injury. In the uninjured liver, genetic lineage tracing has shown Mfsd2a^{+} periportal hepatocytes are less proliferative (Pu et al., 2016). In fact, their numbers gradually wane over time as they undergo attrition over time without renewing themselves (Pu et al., 2016). Nonetheless, upon injury (partial hepatectomy), Mfsd2a^{+} periportal hepatocytes expand their numbers by ~1.5-fold, giving rise to more of themselves in addition to reconstituting pericentral hepatocytes (Pu et al., 2016). Lineage tracing has also suggested that Sox9^{+} periportal hepatocytes can also expand after several different types of injury (Font-Burgada et al., 2015). Hence while periportal hepatocytes do not undergo significant expansion in the uninjured liver, upon injury they may be facultatively called to action—in addition to pericentral hepatocytes, they might serve as a second hepatocyte subpopulation with regenerative abilities. Understanding the mechanism(s) through which proliferative abilities are reignited in, or conferred upon, periportal hepatocytes might be of general interest to understand the molecular basis of endogenous liver regeneration.

Finally, bile duct cells (known as cholangiocytes) have also been reported to contribute to hepatocytes (Raven et al., 2017), although this remains an area of debate (Alison and Lin, 2016). Isolated human or mouse cholangiocytes (identified by a variety of markers) can form 3-dimensional "organoids" in culture; these expanded liver organoids were then capable of engrafting the *Fah*$^{-/-}$ mouse model (described below) of liver injury and regenerating hepatocytes (Huch et al., 2014; Li et al., 2017; Huch et al., 2013). However, this required intervening expansion and manipulation in culture. By contrast, direct genetic lineage tracing of *Krt19*$^{+}$ cholangiocytes suggests that in typical circumstances, cholangiocytes rarely if at all transdifferentiate into hepatocytes *in vivo* (Yanger et al., 2014), though this conclusion has recently been challenged (Raven et al., 2017). Other non-hepatocyte lineages (*e.g.,* bipotent hepatobiliary progenitors [oval cells] (Theise et al., 1999)) have also been proposed to regenerate hepatocytes (Miyajima et al., 2014); however an extensive treatment of this area is beyond the scope of the current review.

While lineage tracing has been used to non-invasively assess the regenerative potential of hepatocyte subtypes, there is a pressing need to prospectively isolate distinct hepatocyte subsets and test their regenerative ability upon *transplantation* into animal models of liver injury. Current clinical hepatocyte transplantation protocols typically transplant all hepatocytes *en masse* despite their marked heterogeneity and might be refined by transplanting purified hepatocyte subsets. Such an effort might rely on identifying distinctive surface markers of hepatocyte subsets to enable their prospective isolation, as classically applied to blood tissues (Weissman and Shizuru, 2008). While surface markers for mouse embryonic liver progenitors (Tanaka et al., 2009; Suzuki et al., 2008; Suzuki et al., 2000) and human fetal hepatocytes (Xiong et al., 2008) have been defined, surface markers that can parse different adult hepatocyte subsets remain to be discovered. In sum, it will be imperative to determine whether different hepatocyte subsets are all "engraftable" and whether the liver reconstitution activity

Table 1
Hepatocytes reprogrammed from fibroblasts.

Transcription factors	Species	Sources of cells	Cell type generated	Engraftability	Reports
HNF1A, HNF4A, HNF6, ATF5, PROX1, and *CEBPA*	Human	Embryonic fibroblasts	Hepatocytes	Engraft up to 30% of livers of Tet-uPA mice	(Du et al., 2014)
FOXA3, *HNF1A,* and *HNF4A*	Human	Fetal or adult fibroblasts	Immortalized hepatocytes	Engraft in *Fah*-deficient mice and improve survival	(Huang et al., 2014)
Hnf1β and *Foxa3*	Mouse	Embryonic fibroblasts	Bipotent hepatic stem cells	Engraft FRG mice and ameliorate liver injury	(Yu et al., 2013)
Foxa3, *Gata4*, *Hnf1a,* and *Hnf4a*	Mouse	Hepatic myofibroblasts	Hepatocytes	Attenuate liver injury induced by CCl_4	(Song et al., 2016)
Hnf4a with *Foxa1, Foxa2* or *Foxa3*	Mouse	Fibroblasts	Hepatocytes	Engraft in FRG mice	(Sekiya and Suzuki, 2011)

observed in bulk hepatocyte transplants can be ascribed to specific purified hepatocyte subset(s). A side-by-side comparison of the engraftment potential of purified pericentral, midlobular and periportal hepatocytes in various mouse models of liver injury could shed light on which subtype(s) is/are more apropos for hepatocyte transplantation therapies.

4. Sources of transplantable liver cells: primary hepatocytes, but what developmental stage?

Broadly speaking, one could construe two general sources of hepatocytes for transplantation—either derived from primary patient liver tissue (*in vivo*-derived) or generated/expanded *in vitro*. The current standard for hepatocyte transplantation in clinical trials is to transplant primary adult human hepatocytes (Dhawan et al., 2010; Fox and Chowdhury, 2004; Dhawan et al., 2006). First, we consider primary liver tissue as a source of transplantable hepatocytes.

In considering a suitable source of hepatocytes for transplantation, another question is whether developmentally "younger" or "aged" hepatocytes harbor differences in regenerative capacity. One might intuit that *adult* hepatocytes might be the most compatible for transplantation into the injured *adult* liver, as these cells would be chronologically-matched with their recipient environment. Indeed when transplanted into the injured adult mouse liver (injured by expression of *urokinase-type plasminogen activator* [*uPA*]; described below), adult mouse hepatocytes engraft and repopulate better, by comparison to mouse hepatocytes derived from 11.5 or 13.5 days of embryonic development (E11.5 and E13.5, respectively) (Haridass et al., 2009). Similarly, human adult hepatocytes and fetal hepatocytes respectively repopulate ~46.6% and ~12.1% of the *uPA*-injured adult mouse liver respectively (Haridass et al., 2009). Taken together, this implies that developmentally more differentiated hepatocytes engraft or repopulate better in the adult liver environment than early liver progenitors/hepatocytes. One hypothesis is that the adult hepatocytes are more compatible with adult mouse liver environment, as they might express compatible surface antigens and receptors to enable their initial engraftment and response to adult regenerative signals, respectively.

By contrast, other data imply that more immature hepatocytes have an advantage in liver engraftment and reconstitution. For instance, neonatal hepatocytes engraft and repopulate to a larger extent than adult hepatocytes upon transplantation into Gunn rats (Tolosa et al., 2015a). A uniquely regenerative phenotype of "younger" hepatocytes has also been implied by findings that younger (neonatal) mouse livers regenerate without fibrosis, by contrast to adult mouse livers (Tsai et al., 2017).

However, one proviso to take into account in comparison of hepatocytes of various developmental stages is that for each developmental stage, heterogeneous bulk cell populations were transplanted. Resolving the contribution of individual hepatocyte subsets to regeneration upon transplantation (at any developmental stage) remains an outstanding issue.

5. Another source of transplantable liver cells: derived *in vitro via* reprogramming or stem cell differentiation?

While currently primary adult hepatocytes are the cell-of-choice for clinical hepatocyte transplantation, *in vitro*-produced hepatocytes might constitute a second alternate source in the future. To this end, at least two approaches have emerged to generate hepatocytes *in vitro*: to transdifferentiate them from unrelated cell-types by enforced transcription factor overexpression ("reprogramming") or to differentiate them from pluripotent stem cells.

Current efforts have successfully transdifferentiated fibroblasts into hepatocyte-like cells through two general approaches. Lentiviral overexpression of two sets of hepatic transcription factors—either *FOXA3*, *HNF1A* and *HNF4A* or alternatively, *HNF1A*, *HNF4A*, *HNF6*, *ATF5*, *PROX1,* and *CEBPA*—can "directly" transdifferentiate human fibroblasts into hepatocytes (Du et al., 2014; Huang et al., 2014) (Table 1). (Similar approaches have been applied to transdifferentiate mouse fibroblasts into hepatocytes (Sekiya and Suzuki, 2011; Song et al., 2016; Yu et al., 2013)). The resultant transdifferentiated hepatocytes were capable of engrafting mouse models of liver injury to some extent. However, these approaches required lentiviral transcription factor overexpression, and in one approach, overexpression of SV40 large T antigen was used to help immortalize the reprogrammed fibroblasts (Huang et al., 2014); in the other approach, *MYC* and a *p53*-targeting siRNA was introduced to enhance reprogramming (Du et al., 2014). Thus while human fibroblasts can be successfully transdifferentiated into hepatocyte-like cells, the preclinical safety of these cells might benefit from safer, non-lentiviral means to accomplish transdifferentiation.

A parallel approach has been to "indirectly" transdifferentiate human fibroblasts into hepatocytes by first converting them into a quasi-pluripotent state. Specifically, human fibroblasts were converted into a quasi-pluripotent state *via* overexpression of *OCT4*, *SOX2* and *KLF4*, followed by replating these cells into endoderm- and liver-inducing differentiation conditions. The resultant hepatocytes derived *via* indirect transdifferentiation improved the survival of FRG mice (described further below) with chronic liver injury (Zhu et al., 2014). However, reprogramming efficiencies were quite low (Zhu et al., 2014).

Another approach to generating human hepatocyte-like cells is *via* the differentiation of pluripotent stem cells. Human pluripotent stem cells (hPSCs), including human embryonic (Thomson et al., 1998) and induced pluripotent stem cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007), have the ability to differentiate into all the hundreds of diverse cell-types in the body ("pluripotency") and they can be indefinitely expanded in culture (Thomson et al., 1998; Levenstein et al., 2006), dividing once every ~18 h. Thus, it should be theoretically possible to generate very large numbers of hepatocyte-like cells from hPSCs for transplantation. However, it is not currently possible to generate a pure population of mature hepatocyte-like cells from hPSCs. While current approaches to differentiate hPSCs towards hepatocyte-like cells have demonstrated partial engraftment in mouse models of chemically-induced liver injury (where CCl_4, acetaminophen,

or ganciclovir were used to induce liver damage (Takebe et al., 2013; Tolosa et al., 2015b; Nagamoto et al., 2016)), significant challenges lay ahead.

Two major challenges impeding the use of hPSC-derived hepatocyte-like cells are the (1) presence of unwanted, non-liver cell-types and (2) limited functional maturity of the resultant hepatocyte-like cells. We discuss both of these challenges in sequence.

hPSCs can differentiate into hundreds of cell-types through a sequence of branching lineage choices; however due to incomplete "lineage guidance" most prevailing differentiation protocols end up generating a mixture of liver and non-liver lineages from hPSCs. This is likely due to incomplete knowledge of the extracellular signals that specify cell-fate at each developmental juncture. Due to these uncertainties, it has been challenging to direct stem cells to differentiate into a pure population of the desired cell-type at each lineage choice, as there tends to be a subset of stem cells differentiating towards the unwanted (*i.e.,* non-liver) lineage at certain lineage branch points. To provide additional context, hPSCs first differentiate into a cell-type known as the primitive streak before generating a key lineage known as definitive endoderm, which is the common progenitor to epithelial cells in multiple internal organs including the lung, liver, pancreas and intestines (Tam and Beddington, 1987; Lawson et al., 1991). However, the extracellular signals BMP and FGF have been reported to specify both liver and intestines from endoderm (Rossi et al., 2001; Jung et al., 1999; Shin et al., 2007; Chung et al., 2008). Hence, while prevailing protocols treat hPSC-derived endoderm with both BMP and FGF to generate liver, it is likely that they also concurrently induce other non-liver fates. Attaining a detailed understanding of the signals (Loh et al., 2014) that control each lineage choice between liver and non-liver fates during hPSC differentiation will be pivotal to overcome this hurdle. Speaking to the danger of transplanting heterogeneous cellular populations containing liver and non-liver cells, early efforts to transplant impure hPSC-derived hepatocyte-containing populations led to teratoma formation (Haridass et al., 2009; Cai et al., 2007). This emphasizes the need to generate purer hepatocyte populations or to purify them and eliminate the risk of teratoma/tumor formation.

The next roadblock is the functional immaturity of hPSC-derived hepatocyte-like cells. While recent studies have reported derivation of hepatocyte-like cells with increased maturity in 3-dimensional culture systems (Ogawa et al., 2013; Ma et al., 2013), it is generally accepted that hPSC-derived hepatocyte-like cells generally correspond to fetal-like hepatocytes rather than that of primary adult hepatocytes (Camp et al., 2017). Indeed, after immature hepatocytes are formed in the fetal liver *in vivo*, they upregulate the expression of key metabolic enzymes over the course of protracted embryonic and postnatal development (Blake et al., 2005; Rich and Boobis, 1997). Precisely how hepatocytes mature *in vivo* remains a black box, and understanding the nuts and bolts of this maturation process will be key to generate more functional hepatocyte-like cells from hPSCs. In determining the functionality of hPSC-derived hepatocyte-like cells (or in fact, derived from any origin), *in vivo* transplantation has emerged as one of the most compelling assays.

6. Genetic models of mouse liver injury to test human hepatocyte transplantability and function

In evaluating the quality of human hepatocytes—whether obtained from human donors or *in vitro* sources—the gold standard has been to transplant them into rodent models of liver injury to test if they could restore liver functions *in vivo* (Goldring et al., 2011). Immunodeficient mouse models of liver injury are generally considered a proof-of-principle to test the safety and functional efficacy of human hepatocytes in the injured liver before proceeding to clinical hepatocyte transplantation. That notwithstanding, an important qualification for the discussion that follows is that no rodent model of liver injury is likely to fully mimic human liver failure. Bearing that in mind, 3 rodent models of liver injury have been traditionally employed by the field: physical resection of the liver (hepatectomy) or chemically- or genetically-induced liver damage. Of these models, chemically- or genetically-induced liver damage is probably the most germane to human patients and we confine our discussion to these two points. What is important to appreciate is that each liver injury model is somewhat unique and tests the abilities of transplanted human hepatocytes in a different way; therefore there is no single "generic" liver injury model.

Various inborn metabolic disorders in human patients are caused by loss-of-function mutations in single genes encoding liver metabolic enzymes; knockout mice that lack the same enzymes are therefore considered representative models of such human inborn metabolic disorders (Jorns et al., 2012). For instance, Type I Tyrosinemia is caused in human patients by disruptions in the *FUMARYLACETOACETATE HYDROLASE* (*FAH*) gene, which encodes an enzyme that usually degrades a toxic byproduct of tyrosine metabolism (fumarylacetoacetate) (St-Louis and Tanguay, 1997). $Fah^{-/-}$ mice serve as a representative model of Type I Tyrosinemia (Azuma et al., 2007; Overturf et al., 1996): in both mouse and humans, loss of *Fah* leads to liver damage and mortality as toxic fumarylacetoacetate accumulates. Liver damage can be temporarily forestalled by treating mice with a drug, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), that prevents the synthesis of fumarylacetoacetate at an upstream biochemical step (Grompe et al., 1995). However, to make $Fah^{-/-}$ mice amenable to the engraftment of human hepatocytes, they must be rendered immunodeficient (for instance, by breeding them with $Rag2^{-/-}Il2g^{-/-}$ mice). The resultant $Fah^{-/-}Rag2^{-/-}Il2g^{-/-}$ (FRG) mice can be readily engrafted by human hepatocytes and have been increasingly used to test the functionality of human hepatocytes in the injured mouse liver (Wilson et al., 2014; Bissig et al., 2007).

FRG mice exemplify a genetic mouse model of chronic liver injury in several ways. First, liver damage is confined to the recipient mouse hepatocytes, as only the $Fah^{-/-}$ recipient hepatocytes become progressively damaged. This spares any transplanted wild-type hepatocytes and allows any engrafted wild-type hepatocytes to progressively repopulate the liver. Second, liver damage can be temporally controlled: mice are maintained on the drug NTBC to forestall liver damage prior to transplantation (Overturf et al., 1996), yet after hepatocyte transplantation the reduction, or cyclic withdrawal, of NTBC induces periodic bouts of mouse liver injury (Azuma et al., 2007), providing a chance for transplanted human hepatocytes to gradually proliferate and reconstitute over time. Third, if left untreated, FRG mice typically perish after a few months of cyclical NTBC withdrawal (Wilson et al., 2014), providing a long window for human hepatocytes to engraft and take root in this chronic model of liver injury, by contrast to acute liver injury models where engrafted hepatocytes have to provide an immediate therapeutic benefit in hours or days.

However, the FRG model is not without its shortcomings. For instance transplantation of primary human adult hepatocytes rescue only ~40% of FRG mice (Azuma et al., 2007), by contrast to how transplanted primary wild-type mouse hepatocytes successfully rescues virtually all $Fah^{-/-}$ mice. The incomplete rescue of FRG mice by human hepatocytes may be attributed to a partial incompatibility of human hepatocytes with the mouse liver environment (discussed further below) or the inconsistent quality or heterogeneity of primary human hepatocyte populations.

Other genetic mouse models that mirror inborn metabolic disorders of urea and bilirubin metabolism in humans could also be used as receptacles for transplanted human hepatocytes to test their authenticity. First, $Arg1^{-/-}$ mice lack the urea metabolic enzyme *Arginase 1*, and consequently develop hyperargininemia similar to that seen in human patients (Sin et al., 2013), eventually leading to neurological impairment, growth retardation and hyperammonemia (Grody et al., 1992; Iyer et al., 1998). Second, the most common cause of urea cycle

disorders in human patients is mutations in the *OTC* gene (*ORNITHINE TRANSCARBAMYLASE*), an enzyme that converts ornithine to citrulline in the urea cycle. Likewise, *Otc*$^{-/-}$ (otherwise known as *Spf-ash*) mutant mice model congenital ornithine transcarbamylase deficiency (Ohtake et al., 1986; Briand et al., 1982). Third, Crigler-Najjar syndrome Type 1 patients suffer from jaundice due to an accumulation of bilirubin, caused by mutations in the *UGT1A1* (*URIDINE DIPHOSPHATE GLUCURONOSYLTRANSFERASE*, otherwise known as *UDPGT*) enzyme that naturally functions to clear bilirubin (Ciotti et al., 1997; Gantla et al., 1998; Strauss et al., 2006). Crigler-Najjar syndrome Type 1 can be modeled in Gunn rats or mice lacking the same gene (Nguyen et al., 2008), which also develop dysfunctional bile salt export or bile conjugation functions. These mice have also been used as rodent models to test for the ability of human hepatocytes to conjugate bilirubin *in vivo* (Tolosa et al., 2015a; Chen et al., 2015; Guha et al., 2002). Other genetic mouse models of liver injury have been described elsewhere (Jorns et al., 2012). In sum, for inborn human metabolic disorders caused by mutations in a single gene, mouse models genetically lacking the same gene might serve as a useful test bed for the functional efficacy of transplanted human hepatocytes (Jorns et al., 2012; Hughes et al., 2005) As long as the transplanted human hepatocytes (whether *in vivo*- or *in vitro*-derived) express the missing gene (and are capable of initial engraftment and proliferation), this might be enough to provide a functional benefit in these monogenic inherited metabolic disorders.

Separately, other genetic mouse models of liver injury employ transgenes to ablate hepatocytes in a fashion that is not reminiscent of observed causes of liver disease in human patients. While these models are useful in that they damage the mouse liver to provide "space" for transplanted hepatocytes, it is unclear whether they are clinically relevant to human liver injury conditions. They are nonetheless useful to gauge the activities of human hepatocytes in certain contexts.

One such popular model is the enforced expression of the protease *urokinase-type plasminogen activator* (*uPA*), which induces damage of endogenous hepatocytes and proteolysis of extracellular matrices (Sandgren et al., 1991). Transgenic *SCID* mice expressing a *uPA* transgene (Dandri et al., 2001) under the control of the *albumin* enhancer or promoter suffer liver injury and can be repopulated by human hepatocytes (Meuleman et al., 2005; Mercer et al., 2001). One benefit of uPA-mediated injury is that uPA cleaves an inactive single-chain form of the cytokine hepatocyte growth factor (HGF) into an active two-chain form, thus enhancing signaling through its cognate receptor tyrosine kinase (MET) (Shanmukhappa et al., 2009) and enhancing hepatocyte proliferation and regeneration (Borowiak et al., 2004). However, it is unclear if there is a parallel for such a trigger of liver injury in human patients. One disadvantage of this model is that liver injury in this model is constitutive and is not temporally controllable (as is the NTBC-tunable *Fah*$^{-/-}$ chronic mouse liver injury model, described above). This disadvantage has been overcome with the advent of *uPA*-expressing adenovirus, which can be delivered to wild-type mice at any time (Azuma et al., 2007).

A second transgenic mouse model of liver damage is the *TK-NOG* (NOD-SCID *Il2r*$^{-/-}$ *Tg[Alb-TK]*) mouse wherein hepatocytes are destroyed by drug-induced activation of a *TK* (simplex virus type 1 thymidine kinase) transgene. These *TK-NOG* mice positively select for transplanted human hepatocytes as endogenous albumin^{+} hepatocytes expressing *thymidine kinase* (*TK*) are conditionally destroyed upon exposure to a drug known as ganciclovir, providing space for exogenously transplanted human hepatocytes to engraft (Hasegawa et al., 2011). Such *TK-NOG* mice can be repopulated by primary human adult hepatocytes with up to 90% of the liver becoming humanized (Xu et al., 2014).

In short, these various genetic mouse models of liver failure have served as excellent test beds for primary human adult hepatocytes to engraft and propagate *in vivo*, as reviewed elsewhere (Grompe and Strom, 2013). Impressively, the activities of all three phases (Azuma et al., 2007) of human cytochrome p450 family enzymes (spanning from phase 1 metabolism to phase 2 conjugation (Zhu et al., 2014)) and finally, phase 3 transport excretion (Azuma et al., 2007; Strom et al., 2010) and high human serum ALBUMIN levels (Azuma et al., 2007; Wilson et al., 2014) have been observed in humanized mice, underscoring that once they have taken root *in vivo*, human hepatocytes can exercise their metabolic and secretory functions. That notwithstanding, these results were observed by transplanting bulk primary adult human hepatocytes. Whether particular hepatocyte subset(s) are particularly capable of engrafting and proliferating *in vivo* remains to be determined.

7. Chemical models of mouse liver injury to test human hepatocyte transplantability and function

Aside from genetic models of liver injury, other mouse models of liver injury with even more widespread clinical relevance are those involving the use of chemical agents. After all, ingestion of chemical agents (*e.g.,* drugs or alcohol) is a prevalent driver of liver failure in human patients. Yet, when treating animal models with such hepatotoxic chemicals, at least three important considerations come to mind. First, is the mechanism of liver injury similar between humans and mice? Second, is the degree of induced liver damage similar between humans and mice? This issue is admittedly more elusive to tackle because the dose and frequency of administration are of paramount importance in both humans and mice. Third (and related to the second point), different chemically-induced models of liver failure have distinct rates of injury, each mandating a different rate for hepatocytes to engraft and to restore liver function, with some being more acute or more chronic.

Although mouse models sometimes mirror chemical-induced injury in humans, there are certain differences in the respective injury patterns. While chronic and excessive alcohol consumption causes liver injury in both humans and mice, only mild fibrosis or cirrhosis was detected in alcoholic mice whereas significant fibrosis is observed in the livers of alcoholic human patients (Keegan et al., 1995; Brandon-Warner et al., 2011; Best et al., 1949). In retrospect, this is not too surprising, as cirrhosis and liver fibrosis entail reciprocal interactions between multiple cell-types (hepatocytes, fibroblasts, immune cells and perhaps other lineages) and any differences in any of these cell-types between human and mouse might account for these differences. Conversely, other hepatotoxins such as acetaminophen (a common over-the-counter analgesic) induce hepatotoxicity in both human and mouse (Fontana, 2008) through similar putative mechanisms. In particular, pericentral and midlobular hepatocytes are thought to react with excessive levels of acetaminophen to form reactive intermediates that precipitate in mitochondrial membrane damage, impairment in ATP synthesis and nuclear DNA fragmentation (McGill et al., 2012; Hinson et al., 2009).

One emergent feature of chemically-induced liver injury models is that different chemicals damage distinct parts of the liver lobule (see Table 2), perhaps offering a way to test whether transplanted hepatocytes (Tolosa et al., 2015b; Nagamoto et al., 2016) can replace—or compensate for the loss of—specific zonated hepatocyte subsets. For example, acetaminophen, CCl_4, and alcohol preferentially destroy pericentral hepatocytes whereas allyl alcohol or a choline-deficient/ethionine-supplemented diet instead selectively induces periportal liver damage (Table 2). Here we emphasize CCl_4-mediated injury in particular, due to its prevalence in the literature. While a single dose of CCl_4 leads to targeted necrosis of pericentral hepatocytes and zonated steatosis in mice (Shi et al., 2010), chronic delivery can lead to liver fibrosis, occasionally cirrhosis (Domenicali et al., 2009) and eventually hepatocellular carcinoma (Pérez Tamayo, 1983). CCl_4 is metabolized into the reactive free radical CCl_3, which has been purported to deplete glutathione and increase oxidative stress, ulti-

Table 2
Chemicals that preferentially damage specific hepatocyte subtypes.

Chemical/diet	Rodent	Hepatocytes damaged
CCl_4	Rats, mice	Centrilobular necrosis (Shi et al., 2010; Pérez Tamayo, 1983; Rubin et al., 1963), fibrosis or cirrhosis (Domenicali et al., 2009)
Acetaminophen	Rats, mice	Centrilobular necrosis (McGill et al., 2012; Davis et al., 1974; Mitchell et al., 1973; Walker et al., 1983)
Ethanol	Rats	Pericentral (significant), periportal (García-Ruiz et al., 1994)
Choline-deficient and ethionine-supplemented diet (CDE)	Mice	Periportal injury and fibrosis (Köhn-Gaone et al., 2016; Akhurst, 2001)
Methionine-choline-deficient diet (MCD)	Mice	Fatty liver (hepatic steatosis), lobular/periportal inflammation and perisinusoidal fibrosis (Akhurst, 2001; Machado et al., 2015)
3,5 Diethoxycarbonyl-1,4-dihydrocollidine (DDC)	Mice	Ductular proliferation (Preisegger et al., 1999)

mately damaging mitochondria and lysosomes (Hinson et al., 2009), amongst various purported mechanisms.

8. Is the injured mouse liver a representative environment conducive to engraftment by transplanted human hepatocytes?

So, how useful are mouse liver injury models when assaying the transplantability and functionality of human hepatocyte populations? Of course, there are clear differences between the human and mouse livers. Human hepatocytes can engraft both the FRG (*Fah*$^{-/-}$) and *uPA*-expressing mouse models of liver injury (Haridass et al., 2009)—albeit to a lesser extent than mouse hepatocytes. While an individual mouse can be highly repopulated by human hepatocytes, there is typically wide mouse-to-mouse variability in the extent of liver chimerism (Azuma et al., 2007). This implies that there is a partial species-related incompatibility between the injected human hepatocytes and the environment of the surrounding recipient mouse liver.

Interspecific compatibility between transplanted human hepatocytes and the recipient mouse liver may be due in part to incompatibility between mouse growth factors and their cognate receptors on human cells. For example, despite > 90% amino acid sequence homology between human and rodent HGF, mouse HGF does not efficiently bind to the human HGF receptor (known as MET) (Bussolino et al., 1992; Fiaschi-Taesch et al., 2008). Given the key role of HGF-MET signaling in spurring hepatocyte proliferation, in retrospect, it is surprising that human hepatocytes can even repopulate the mouse liver as well as they have thus far. To that end, one intriguing study has shown that reactivating the MET receptor on transplanted human hepatocytes can enhance their engraftment in mouse models (Ohashi et al., 2000). Specifically, if human hepatocytes were transplanted under the renal capsule of mice and then treated with a human-specific MET agonist antibody, this potently enhanced their engraftment by supplying this otherwise-missing hepatocyte proliferation cue (Ohashi et al., 2000). Ultimately this suggests that a "humanized" signaling environment may be adequate to support the long-term survival of human hepatocytes *in vivo*. Indeed, in the parallel case of human hematopoietic stem cell transplantation into mice, their subsequent differentiation into various hematopoietic lineages can be vastly enhanced by simultaneously expressing the human-specific homologs of various hematopoietic growth factors *in vivo* (Rongvaux et al., 2014). One could envisage an analogous effort to express humanized hepatocyte proliferation factors *in vivo* to boost human hepatocyte reconstitution of the mouse liver.

However, this issue of potential growth factor-receptor incompatibility illustrates a broader point—namely, that we understand little about the initial molecular mechanisms that allow hepatocytes to engraft the injured liver and proliferate. Presumably, defects in any such mechanism(s) could attribute to the partially-reduced engraftment of human hepatocytes in mouse models, and perhaps even stymie the clinical pursuit of hepatocyte transplantation into injured patients. There is clearly a need to define the surface molecules on human hepatocytes that mediate successful homing to, and transversal of, mouse liver endothelial cells, and to define the receptors that subsequently enable human hepatocytes to respond to proliferative (or anti-proliferative) signals emanating from the injured mouse environment. Eventually, the discovery of such receptors might serve as useful biomarkers for batches of hepatocytes meant for clinical transplantation to prospectively estimate the efficiency with which these hepatocytes might initially engraft and seed the recipient liver. Alternately, enforced expression of such receptors/surface molecules on hepatocytes prior to transplantation might augment their engraftment or proliferation.

Finally, besides a hepatocyte-centric view of hepatocyte transplantation, another overlooked regulator of hepatocyte regeneration and injury is the immune system. Of course, studies of human hepatocyte transplantation into mice entail immunodeficient mice, lest the mouse immune system reject the transplanted xenogenic cells. Yet, while immunodeficient mice are tolerant to human hepatocytes, they lack certain factors that compound liver disease progression, including the inflammatory response to hepatocyte injury and fibrosis (Pellicoro et al., 2014). For example, mice lacking B cells demonstrate attenuated liver fibrosis in response to injury (Novobrantseva et al., 2005); it follows that immunodeficient mice (used as recipients for human hepatocyte transplants) would therefore show idiosyncratic liver injury responses that may not be representative of the situation found in wild-type animals. One bold idea is to concurrently humanize mice with human hematopoietic stem cells and hepatocytes derived from the same donor (Wilson et al., 2014), which might enable study of how human immune cells interact with human hepatocytes.

9. Conclusion

This essay has sought to highlight outstanding questions that continue to surround hepatocyte transplantation. Indeed, despite its value in specific clinical situations (Fisher and Strom, 2006; Dhawan et al., 2010; Dhawan et al., 2006; Strom et al., 1997), hepatocyte transplantation has yet to be pervasively deployed. This is due in part to (1) the need to demonstrate long term engraftment, functional efficacy and safety in more complex liver failure syndromes (beyond monogenic inherited metabolic disorders) and also due to (2) the shortage of reliable human hepatocytes suitable for transplantation (Ibars et al., 2016; Puppi et al., 2012). Speaking to the latter point, moving ahead it will be critical to be able to predict the clinical activity of a given hepatocyte population—whether derived *in vivo* from primary tissue or artificially generated *in vitro*—prior to transplantation. Indeed it is unsurprising that different batches of primary adult human hepatocytes derived from different donors of distinct genetic backgrounds and ages might show considerable variability. A robust *in vitro* source of human hepatocytes (derived from reprogramming, differentiating pluripotent stem cells or even other means) could potentially fulfill the need for a consistent and reliable source of hepatocytes for transplantation. However, these *in vitro*-derived hepatocyte-like cells warrant closer preclinical scrutiny for safety and efficacy.

With an eye to the future, it will be critical to determine the heterogeneity of human hepatocytes and to determine which subtype(s), if any, preferentially engraft *in vivo*. One could imagine that certain subtypes of hepatocytes might be innately more regenerative—for instance, the aforementioned adult pericentral and/or periportal hepatocyte subsets (Halpern et al., 2017). Alternately, transplantable hepatocyte subtype(s) might be distinguished by expression of transmembrane receptors that allow them to properly home to the injured liver upon portal vein infusion, or that allow them to respond to regenerative cues *in vivo*. In any case, surface markers that identify transplantable hepatocyte subset(s) would be useful as biomarkers to prospectively evaluate the "quality" of different primary human hepatocyte batches and would enable purification of these subset(s) prior to clinical transplantation.

Moreover, there is the issue of what is the "best" animal model of liver injury to test the functionality of transplanted human hepatocytes. The most parsimonious case is that of monogenic inherited human metabolic disorders (Puppi et al., 2012). These can be modeled in mice by genetically deleting the corresponding mouse gene and should in theory be rescued by transplanting wild-type human hepatocytes that express the functional version of that gene (Puppi et al., 2012). This strategy is exemplified by the successful rescue of $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mice by wild-type human hepatocytes, which can effectively treat Type 1 Tyrosinemia in this preclinical model.

Yet for more complex types of liver failure, it is unclear whether the injured mouse liver will be representative of the environment of the injured human liver—as exemplified by the differential response of human and rodent livers to drugs (Muruganandan and Sinal, 2008). A point that cannot be understated is that hepatocytes exhibit hundreds of metabolic functions; hence it is unlikely that a singular animal model can generically test all such functions. In particular, at advanced stages of human liver injury induced by alcohol, there is significant fibrosis, which might impede the engraftment of transplanted hepatocytes (Fisher and Strom, 2006; Dhawan et al., 2010). In the cirrhotic liver, it is unclear whether the underlying fibrosis has to be ameliorated in the recipient liver to permit successful donor hepatocyte engraftment, or alternatively, whether wholesale transplantation of a new liver might be the most effective strategy.

Last but not least, even though certain mouse models enable a high degree of hepatocyte repopulation (*e.g.,* FRG mice), > 60% of transplanted mice are not engrafted by human hepatocytes (Azuma et al., 2007). Hence, there is also the added complication that human hepatocytes tend to engraft the mouse liver less efficiently than mouse hepatocytes (Haridass et al., 2009), perhaps due to interspecific incompatibility. Although the mouse liver can be extensively humanized by transplanted hepatocytes, typically engraftment is less consistent than if mouse hepatocytes were transplanted in their stead (Haridass et al., 2009). Enforced expression of human homologs of hepatocyte proliferation proteins *in vivo* might enhance the ability of human hepatocytes to engraft mice (Ohashi et al., 2000), akin to what has been done to enhance human hematopoietic engraftment in mice. Taken in collective this warrants investments on multiple fronts to advance hepatocyte transplantation—investigating the most suitable, consistent source of human hepatocytes for transplantation; identifying which subsets are transplantable and purifying them; learning about the mechanisms of hepatocyte homing and engraftment; and finally, developing more accurate simulacra of liver failure in mouse models.

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