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HEPATOCYTE TRANSPLANTATION: *QUO VADIS?*

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Abstract

Orthotopic liver transplantation (OLT) has been effective in managing end-stage liver disease since the advent of Cyclosporine immunosuppression therapy in 1980. The major limitations of OLT are organ supply, monetary cost and burden of lifelong immunosuppression. Hepatocyte transplantation (HT), as a substitute for OLT, has been an exciting topic of investigation for several decades. HT is potentially minimally invasive, and can also serve as a vehicle for delivery of personalized medicine through autologous cell transplant after modification *ex vivo*. However, three major hurdles have prevented large-scale clinical application: (a) availability of transplantable cells; (b) safe and efficient *ex vivo* gene therapy methods, and (c) engraftment and repopulation efficiency. This review will discuss new sources for transplantable liver cells obtained by lineage-reprogramming, clinically acceptable methods of genetic manipulation, and the development of hepatic irradiation (HIR)-based preparative regimens for enhancing engraftment

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Authors' contributions

MB planned and wrote the manuscript, prepared tables, and designed figures. PA and RK wrote portions of the manuscript and tables and revised the manuscript. NRC wrote portions of the manuscript and revised the manuscript. MK reviewed the manuscript and provided financial support. JRC wrote portions of the manuscript and revised the manuscript. CG planned the manuscript, tables, and figures and provided financial support.

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and repopulation of transplanted hepatocytes. We will also review the results of first three patients with genetic liver disorders who have undergone preparative HIR prior to HT.

INTRODUCTION

Orthotopic liver transplantation (OLT) has been highly effective in managing end stage liver disease since the advent of cyclosporine immunosuppression therapy in 1980. Between 30–40 million Americans are afflicted with liver disease [1], and the number of fatalities is climbing. Over 1.75 million people died of cirrhosis in 2010 [2], and in 2008, death due to inherited and acquired liver diseases was projected to become the 12th leading cause of mortality by 2020 [3]. Inherited metabolic liver diseases can be devastating and can lead to a significant decline in the quality of life [4]. Many patients with end stage liver disease have benefited from whole-organ liver transplantation, but to a majority of these patients, OLT remains unavailable because of the shortage of donor organs and the cost of the procedure. Currently there are 14,450 patients on the liver transplant waiting list in the US, and about 12,000 new additions to the list per year, but only about 7,000 transplants occurring per year [5].

To treat more patients and to lessen their burden, alternative therapies are being vigorously explored. Hepatocyte transplantation (HT), as a substitute for OLT, has been an exciting topic of investigation for several decades [6]. Like OLT, HT could become a viable approach to regain hepatic function. Additionally, HT could bridge a patient with acute liver injury to OLT, until a suitable donor liver becomes available [7]. HT is minimally invasive, and can also serve as a vehicle for ex vivo gene therapy for the treatment of inherited metabolic diseases after ex vivo genetic manipulation of autologous hepatocytes. However, three major hurdles have prevented large-scale clinical application of HT: (a) availability of transplantable hepatocytes; (b) safe and efficient tools for *ex vivo* gene therapy; (c) engraftment and repopulation efficiency. In this review we will discuss new sources for transplantable cells obtained through lineage-reprogramming technologies, clinically acceptable methods of gene manipulation, and the enhancement of regimens that permit effective hepatic repopulation, such as preparative hepatic irradiation (HIR) in combination with mitogenic stimuli. Efficacy in preclinical trials, safety, and clinical feasibility of HT as an indication for HIR are discussed, and the results of numerous case reports and clinical trials of HT are summarized.

Preparative regimens of HT

Effective therapy of hepatocyte-based inherited metabolic diseases requires a sufficient mass of engrafted functional hepatocytes. However, donor cell engraftment following hepatocyte transplantation (HT) is inefficient [8] and the number of donor cells that can be transplanted in a single procedure is limited by the portal hypertension and potential pulmonary embolism following transplantation [9]. Therefore, improved hepatocyte engraftment and subsequent proliferation of the engrafted cells are imperative for generating an effective mass of the therapeutic cells.

To achieve proliferation of transplanted liver cells, some investigators have injected EpCam-positive human hepatic stem cells (hHpSC) directly into the liver [10]. As these cells show very poor engraftment capacity when injected in suspension, they were injected into a liver lobe of immunodeficient nude mice embedded in a mix of soluble signals and extracellular matrix components, e.g. hyaluronan, collagen type III and laminin that are found in the putative stem cell niche of the liver. This procedure retained the injected cells within the injected lobe for at least 7 days. This approach may be potentially helpful in transplanting hepatocyte-like cells, such as iHeps, that exhibit low engraftment efficiency. However, long-term differentiation of these cells and their integration into the liver chords was not studied. Therefore, potential utility of this method in treating inherited metabolic disorders or acute liver failure is uncertain. Nevertheless, investigators have evaluated the infusion of EpCam-positive hepatic progenitor cells derived from fetal human liver into patients with liver cirrhosis [11], with apparent improvement of liver function and prevention of hepatic encephalopathy. It should be noted, however, that engraftment and survival of adult hepatocytes has not been found after infusion into hepatic artery of rodents.

Improving the initial engraftment of adult hepatocytes:

Hepatocytes infused into the portal venous system, either by injection into a portal vein tributary or by intrasplenic injection, flow from the portal venules at the periportal region through the liver sinusoids toward the central vein. From the liver sinusoids, the transplanted cells must traverse the sinusoidal endothelial barrier to enter the liver parenchyma. This process may take several hours to several days, during which time a large fraction of the hepatocytes die, the cell death and removal being partly mediated by interaction with Kupffer cells. Based on these concepts, efforts have been made to manipulate the native liver structure to improve reception of the transplanted cells. Several approaches have been described in a recent review [12]. Vasodilation using nitroglycerine injection has been used in an attempt to permit the transplanted cells to travel further through liver sinusoids, allowing improved cell entry into the liver chords. Reduction of Kupffer cell activity reduces the cell death of the hepatocytes that are not yet integrated into the liver tissue, thereby keeping the entry and engraftment window open longer. The hepatic sinusoidal endothelium is a strong barrier to the entrance of the transplanted cells from the sinusoidal space into the hepatic parenchyma. Disruption of the sinusoidal endothelium using cyclophosphamide has been reported to improve engraftment in mice. Our group has shown that hepatic irradiation, even at a low dose, can reversibly injure the liver sinusoidal endothelial cells, thereby enhancing the initial engraftment of transplanted hepatocytes [13]. A summary of the key cellular mechanisms that occur following hepatic irradiation in the context of cell transplant is described in in-text box 1 and Figure 1.

Promoting proliferation of the engrafted hepatocytes:

Although quiescent hepatocytes undergo mitosis infrequently, they retain the capacity for proliferation under appropriate stimuli. Therefore, transplanted hepatocytes have the potential to proliferate extensively in the host liver, as evidenced by transplantation studies in Fah-deficient mice or uPA-transgenic mice, where the longevity of the host hepatocytes is greatly shortened. The loss of liver mass stimulates the proliferation of the engrafted cells. However, in a majority of inherited metabolic disorders, longevity of the host hepatocytes is

not significantly compromised. In most of these diseases the host hepatocytes are capable of responding to mitotic stimuli and, therefore, the transplanted cells have no proliferative advantage. One important exception is α 1-antitrypsin deficiency, which is characterized by increased apoptosis and reduced proliferation of affected cells. In this model strong repopulation is observed without liver pretreatment [14]. To achieve preferential proliferation of the transplanted cells, the mitotic capacity of the host hepatocytes may be reduced by chemical or physical manipulations. As a proof of this principle, investigators have used retrorsine, a DNA intercalating pyrrolizidine alkaloid which interferes with DNA replication, specifically in the liver cells [15]. Following pretreatment of the host with retrorsine, the transplanted hepatocytes proliferate preferentially in response to a proliferative stimulus, such as partial hepatectomy [8]. However, systemic administration of genotoxic alkaloids is too risky for potential clinical application.

Based on the above considerations, extensive hepatic repopulation requires a combination of mitotic stimulation of the transplanted cells and growth retardation of the host hepatocytes. This naturally occurs in the developing wing of *Drosophila* during cell-cell competition and has been hypothesized to occur in the liver following transplantation of fetal hepatocytes [16,17]. Surgical options for providing mitotic stimulation include occlusion of a portal vein branch [18], which can be permanent [19,20] or reversible [21], and partial hepatectomy [8,22–28]. To circumvent the limitation of these invasive procedures, pharmacological approaches have been explored as an alternative. In experimental animals, expression of Fas-ligand [29] or administration of an activating anti-Fas antibody [30] has been used to partially ablate the host hepatocyte mass, which initiates an array of growth factors, hormones and cytokines that mediate compensatory proliferation of the remaining hepatocytes. Alternatively, hepatocyte growth factor (HGF) has been expressed using adenoviral vectors [30]. Similarly, administration of triiodothyronine (T_3 , Thyroid hormone) has been used to provide mitotic stimulus to the transplanted hepatocytes [31]. Recently, GC-1, a thyroid hormone receptor-beta ($TR\beta$) has been shown to stimulate hepatocyte proliferation, without the cardiac side effects of T_3 [32].

Preparative regimen of HIR for HT:

Our group has designed preparative protocols based on HIR to augment the initial engraftment of hepatocytes, as well as to promote subsequent preferential proliferation of the engrafted hepatocytes. Preparative HIR enhances engraftment by transient disruption of the hepatic sinusoidal endothelium and functional inhibition of Kupffer cells [13]. In addition, this method also induces a carefully targeted injury to the host hepatocytes that reduces their ability to divide, thereby imparting a relative proliferative advantage to the non-irradiated transplanted cells in the presence of a mitotic stimulus. A relative mitotic advantage of donor cells over host liver cells under this condition provides a growth advantage to the donor cells via cell-cell competition for a robust liver repopulation. Preparative HIR is more suitable for clinical application compared with pyrrolizidine alkaloids because it can be delivered in a spatially conformal manner to a desired part of the liver for a regio-specific repopulation, leaving the remaining liver unperturbed [33].

Irradiation leads to DNA damage so it is logical that either cell death or senescence will follow. We showed that HIR causes initial stress signaling and DNA damage in the liver, and expression of proteins associated with cell-cycle arrest, reduction of the phagocytic function of Kupffer cells, and damage of the sinusoidal endothelial cells (SEC) [13]. To develop a mechanistic understanding, we examined relevant signaling pathways and observed the induction of autophagy and apoptosis related genes in the liver following HIR such as ATG4D, ATG4C, Beclin, TRAIL, Merlin, p21, and p27. Koenig et al evaluated γ -H2AX staining (marker of double strand breaks) and observed increased staining shortly after hepatic irradiation (1–3hrs) followed by a decline to background levels at 6hrs [34]. We have also observed positive γ -H2AX staining specifically in irradiated liver tissue 2hrs following CT-guided hepatic irradiation. Koenig et al use a number of apoptosis measures including apoptosis-specific DNA gel patterns, low P53 expression and immunohistochemistry against caspase 9 to argue against irradiation-induced apoptosis as the major mechanism leading to proliferative advantage of transplanted cells. Induction of P21 and Cyclin D1 points to G1/S block and the observed enlarged hepatocytes (megakaryocytes) suggest a secondary mechanism of blocked G2/M characterized by replication but no cell division [34]. Others have shown similar data (notably upregulation of CyclinD1, P21, IL-6, and IL-1 β) pointing towards senescence rather than apoptosis [35].

Sultan et al [36] studied the mechanism of hepatic response to irradiation by evaluating the presence of reactive oxygen species (ROS) and the expression of Lipocalin-2 following hepatic irradiation. ROS are shown to increase gradually up to 24hrs. Concurrently, LCN2 gene and protein expression is increased significantly starting at 1hr and peaking at 12–24hrs. Irradiated hepatocytes are even more responsive with LCN upregulation when treated with IL-1 β , a cytokine upregulated concurrently following irradiation. This suggests a mechanism linking radiation-induced cellular stress activation of an acute stress response through generation of free radicals and activation of cytokines which upregulate Lipocalin 2, which is involved in the acute phase response and may be involved in apoptosis. Ischemia reperfusion also induces release of ROS and cytokines and may potentially be used to elicit a similar effect [37].

We have demonstrated that preparative HIR delivered during laparotomy combined with the expression of hepatic growth factor in the liver resulted in massive repopulation of the liver with transplanted hepatocytes (Table A) [13,19,28,33,38]. We first examined the potential of hepatocyte transplantation in ameliorating radiation induced liver disease (RILD) in F344 rats subjected to partial hepatectomy and whole liver radiation therapy. Intrasplenic or intraportal transplantation of adult primary hepatocytes, four days after high-dose liver RT, ameliorated liver function and improved survival of these animals (38). Importantly, the transplanted hepatocytes extensively repopulated and selectively replaced the irradiated host hepatocytes to maintain normal physiological function of the heavily irradiated rat liver. The studies of hepatocyte transplantation in RILD paved the way for investigators to develop a preparative regimen of liver irradiation for enhancing engraftment and selective repopulation of donor hepatocytes after hepatocyte transplantation [23,24,34,35,39,40]. Since then, HT after a preparative regimen of hepatic irradiation in combination with a hepatotropic growth stimuli, has been successfully used to ameliorate liver function in a variety of animal models

of inherited metabolic diseases, such as, Crigler-Najjar syndrome [28,41] and primary hyperoxaluria [38,42].

Clinical feasibility and safety of preparative hepatic irradiation:

Whole liver radiation (35 Gy) can cause potentially lethal radiation-induced liver disease (RILD) [43]. With advanced treatment planning very high doses (up to 90–100 Gy) can be administered if the radiation volume is small enough (~1/3 of the total liver volume) [44], and this concept is routinely used in clinical practice for the treatment of liver tumors using stereotactic body radiation therapy (SBRT). Treatment planning minimizing radiation dose to the normal liver volume is used to prevent RILD [43,45]. A study in nonhuman primates showed that whole liver irradiation above 40 Gy in 3–5 fractions produced lethal RILD [46]. In the treatment of metabolic diseases partial reconstitution of hepatic function may be sufficient to meet therapeutic goals. For example, irradiation and repopulation of 20–30% of the liver mass effectively reduces serum bilirubin down to normal levels in a model of Crigler-Najjar type I using isolated rat hepatocytes [33] as well as human iHeps [47]. We are currently developing the use of non-invasive, CT-guided regio-specific external beam radiation, which requires no abdominal surgery and is administered as fractionated, dynamic beams to minimize off target toxicity in small animals. Isolated instances using external beam irradiation for HT have been reported [23,24,40] with effective repopulation (up to ~20%, Table A) [23], although this group used antero-posterior/postero-anterior irradiation and used surgical intervention such as partial hepatectomy or portal venous occlusion to provide the mitotic stimulus. Utilizing external-beam hepatic irradiation in combination with hepatocyte-specific, pharmacological mitotic stimulants could potentially be a treatment protocol well suited for clinical applications. Encouraged by our preclinical results, clinical trials have been initiated in the University of Pittsburgh by Ira J. Fox for the treatment of phenylketonuria (NCT01465100) and other inherited liver-based metabolic diseases (NCT01345578).

CLINICAL STUDIES:

Several dozen case reports have been published since the breakthrough first example of the use of HT for the treatment of Crigler-Najjar syndrome type 1 was reported in 1998 [48]. Since the writing of our last review [9], a number of case studies have been reported describing hepatocyte transplantation in patients with metabolic diseases, and in one instance after acute liver failure following *Amanita phalloides* ingestion. For clinical application it is helpful to consider *how much engraftment and repopulation is required for therapeutic benefit*. In Table B we describe these clinical cases and categorize them according to the required level of repopulation. Children treated for a number of metabolic diseases showed reduction of abnormal metabolites and reduced psychomotor and developmental disability. These improvements persisted between 2–8 months generally, but in one instance Refsum disease did not relapse during the 18-month observation period after transplant [49]. The 62 year-old patient treated with HT for ingestion of toxic mushrooms had normal liver architecture and normal blood flow 8 weeks after transplant, and experienced a full recovery without relapse. Recently two patients with acute on chronic liver failure were treated with HT. Both patients showed markedly improved neurological

function and reduced serum ammonia levels. One patient survived after receiving a suitable liver, while the other patient died within 10 days while waiting for a suitable donor liver [7].

Eventual relapse seen in most reported cases suggest the lack of permanent repopulation. Therefore these patients must still be treated with OLT as a long-term solution. Even though the potential for bridging therapy with HT is within reach with current transplantation approaches, preparative methods and post-transplantation measures to enhance repopulation of the liver are crucial for long-term success of HT. Experimentation in Cynomolgus monkeys demonstrated that engraftment of Porcine hepatocytes could be enhanced significantly when even a small dose of HIR was used in combination with portal vein embolization (PVE), but not with HIR or PVE alone [13].

In 2011, several clinical trials were initiated in the U.S utilizing preparative HIR for HT for metabolic disorders, as well as acute liver failure (NCT01345565, NCT01345565, NCT01465100). The results of the first three patients treated with preparative hepatic irradiation prior to hepatocyte transplantation have been recently reported [50]. Two patients with urea cycle defects ages 4 and 7 months received preparative hepatic irradiation of 5 Gy and 7.5 Gy in 1 fraction, respectively, to 30–37% of the total liver volume. These two patients unfortunately had early graft loss following hepatocyte transplantation. Retrospective analysis of immune reactivity in these two patients indicated that the loss of graft function likely was secondary to rejection, and that failure to provide adequate immunosuppression during the initial donor hepatocyte infusion may have led to graft loss. A third patient, a 27 year old female with classical phenylketonuria, received 10 Gy in a single fraction of preparative hepatic irradiation. Based on the results of the initial two patients, additional immunosuppression using induction with an anti-lymphocyte antibody and serial immune monitoring was performed. There was a 36% reduction in mean peripheral blood phenylalanine levels and post-transplant liver biopsies demonstrated multiple small clusters of transplanted cells, multiple mitoses, and Ki67+hepatocytes, findings not seen routinely on liver biopsies from animals or patients undergoing hepatocyte transplant without a pre-transplant conditioning regimen. Phenylalanine levels remained at reduced levels during supervised follow-up while anti-donor activity, measured by recipient cytotoxic memory T cells, demonstrated low risk of rejection. However, graft loss occurred after follow-up became inconsistent. The results from the initial three patients using preparative hepatic irradiation demonstrated that radiation preconditioning of the liver was safe and resulted in no pathological changes to the liver, even when used in young infants [50].

TRANSPLANTABLE CELL SOURCES

Clinical HT has used primary hepatocytes isolated from cadaveric livers or explants. To avoid life-long immunosuppression, a stable and abundant source of autologous hepatocytes for transplantation is one of the main challenges for large-scale clinical applications of HT-based therapies. A variety of approaches based on isolation of mature hepatocytes or progenitor cells from mature livers have been utilized experimentally and are discussed in several previous reviews [9,51,52]. Table C contains a summary of these approaches.

Autologous cells, allogeneic cells, and xenografts have been explored as possible cell sources. Chimeric animals including mice, pigs and rats repopulated with human hepatocytes, creating animals with “humanized livers” have been developed. These can potentially be used as living bioreactors to produce large quantities of highly functional human hepatocytes [53,54]. Other exciting modern approaches have investigated the differentiation of pluripotent cells or even somatic cells into hepatocyte-like cells (HLCs).

HLCs have been made from induced pluripotent stem cells (iPSC) [27,41,55–58]. Common conversion protocols are summarized in Table D. Most rely on step-wise incubation with cytokines or forced expression of transcription factors. Very interesting work has revealed how epigenetic characteristics can be manipulated to enhance conversion [59–65]. Another interesting approach for making HLCs is through trans-differentiation (also called direct differentiation) through overexpression of transcription factors in somatic cells [27,66–70]. These cells have been validated for HT in-vivo to cause liver engraftment and rescue liver failure models and inherited metabolic disorders [47].

Optimal cell maturity for repopulation and long-term safety of HLCs is currently unknown. For instance, cells expressing more progenitor-like qualities may repopulate more effectively, but may have higher risk of tumorigenicity. Despite these caveats, the future of HT in clinical application relies on human hepatocyte substitutes such as hepatocytes isolated from humanized animal livers or HLCs, although the need for continued development and safety assessment in this field cannot be overstated.

CLINICALLY APPLICABLE REPROGRAMMING VECTORS

Cellular reprogramming techniques such as generation of iPSC and trans-differentiation of somatic cells to hepatocytes (tHeps) are fully or partially based on expression of transcription factors (TFs). In order for the resulting cells to be used in clinical applications, safety of the method of expression is critically important. Most techniques have relied on integrative viruses for the expression of various transcription factors (TFs). However, the possibility of the formation of replication competent viruses from recombination with endogenous viral sequence elements, viral integration mediated gene disruption and/or gene activation make viral mediated approaches unsuitable for clinical applications. Non-integrating, cytosolic located DNA-based protein expression systems, consisting of self-replicating episomal plasmids have been used successfully. However, all DNA based systems carry potential small risk of genomic integration, which could lead to a multitude of unwanted secondary effects due to genomic disruption as discussed above for retroviral expression systems.

To circumvent these safety concerns, mRNA-based, non-integrative protein expression systems were recently developed and applied with great success. These systems have been reviewed elsewhere [71], but the most notable advances for safe clinical use will be discussed here.

Sendai virus (SeV) is a single-stranded, negative sense RNA-only virus that does not go through a DNA stage in its life cycle. Recombinant SeV vectors are located exclusively in

the cytoplasm and are therefore considered a footprint free (no genomic integration) system for protein expression. Therefore, it poses none of the risks associated with DNA based systems [72,73]. Producing SeV vectors requires either a multi-step process involving packaging of fusion-deficient replicons into fusion-competent virions or the use of thermosensitive but otherwise competent viruses that could potentially revert to a more virulent form.

RNA can be used as a stand-alone expression vector and in vitro transcribed, biosynthetic mRNA has been used for footprint free iPSC generation [74] as well as hepatocyte trans-differentiation [70]. The mRNA is modified to reduce cellular interferon response that is otherwise activated by single stranded RNA molecules as a defense against viruses. Although efficient and safe, mRNA mediated reprogramming and trans-differentiation are expensive and time consuming, requiring careful RNase free storage and handling. The short half-life of the transfected mRNAs necessitate daily transfections, which is harsh on target cells. When using a cocktail of mono-cistronic mRNAs expressing one reprogramming factor each, even minor differences in handling could lead to differential expression of reprogramming factors in the cell and therefore lower reprogramming efficiency. Also of note, in contrast to iPSC generation, hepatocyte trans-differentiation was markedly less effective when mRNA vectors were used [70] compared with retroviral vector-based methods [66,68].

An RNA based system based on non-infectious self-replicating mRNAs derived from the Venezuelan Equine Encephalitis (VEE) virus exists exclusively in the cytoplasm of mammalian cells as a self-replicating mRNA complete with a 5' CAP and a poly A tail. It is therefore completely free of genomic footprint. This system has been used for iPSC generation [75] as well as for HIV vaccine development [76] and a simple version called Simpicon is commercially available. The presence of the construct in the cell is easily controlled. Therefore recent advances in the field have given us tools for potentially clinically applicable cell reprogramming. These approaches are also summarized in Table E.

GENE THERAPY TO CORRECT METABOLIC DISEASE PHENOTYPES

Many metabolic diseases result from a mutation leading to the loss of function of a single enzyme produced in the liver. HT can be used potentially to treat metabolic diseases using autologous cells after ex-vivo genetic editing (Figure 2). Previously, genetic modifications were limited to non-targeted approaches such as retroviral-mediated random genomic insertion [77,78], but new techniques of enhanced homologous recombination, such as Zinc-finger nucleases (ZFN), Transcription activator-like effector nucleases (TALEN), and the CRISPR-Cas9 system now make clinical application possible through highly specific genetic editing. These methods are based on generating a single-stranded or double stranded DNA break at a specific genomic site of interest.

This recruits gene repair proteins at that site, which facilitates homologous recombination of a segment of DNA present in the cell. Presence of the DNA break has been shown to greatly enhance the frequency of homologous recombination near the site of the break, permitting creation or repair of a specific genetic lesion, or insertion of a gene or interest at a genomic

“safe haven” which does not contain functional genes, but remains in euchromatin state, so that expression of the gene of interest is assured.

The most recent technology for genomic editing is based on the CRISPR system, which has been reported to outperform TALENs for the same functionality [79]. Great strides have been made in the last 5 years with the CRISPR/Cas9 system, demonstrating an unprecedented speed and ease of genomic editing. In-vivo editing of the CEBP/α gene has been performed by adenovirus-based delivery of the CRISPR/Cas9 system [80]. There are a number of studies on the use of CRISPR in-vivo using hydrodynamic injection. For example, it was used to correct fumarylacetoacetate hydrolase (Fah) deficiency in mice [81]. The donor DNA, and a plasmid expressing the guide RNA and the Cas9 nuclease were delivered by hydrodynamic injection into Fah-deficient mice, resulting in correction of the Fah deficiency in some hepatocytes, which proliferate in response to death of the host hepatocytes resulting from the metabolic abnormality [81]. While these are interesting proof of principle studies the method of delivery limits their potential for clinical translation. CRISPR-Cas9 can easily be used for robust in-vitro gene editing. We are anticipating future works demonstrating correction of metabolic diseases through *ex-vivo* genetic editing of hepatocytes or their precursors followed by cell transplantation (Figure 3), an approach with more realistic clinical applicability due to highly controlled genetic editing and testing of target cells.

Summary

The use of preparative HIR is undergoing further optimization in the clinic. Unlike many studies in experimental animals, the HIR protocol used for the initial patients undergoing HT did not include administration or expression of a mitogenic agent. We anticipate that future clinical studies will address important existing issues regarding preparative HIR, including patient safety, longevity of transplanted cells, and whether enhanced engraftment occurs preferentially in the irradiated parenchyma.

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Figure 1. Summary of mechanisms associated with preparative hepatic irradiation. (1) Increases LSEC apoptosis and sloughing, which increases porosity of sinusoids and increases migration of transplanted cells into liver parenchyma. (2) Enhancement of hepatocyte engraftment and integration in liver cords. (3) Reduction of Kupffer cell activity, which reduces phagocytic clearance of transplanted cells

Role of HIR as a preparative regimen for HT

1. Increases LSEC apoptosis and sloughing, which increases porosity of sinusoids and increases migration of transplanted cells into liver parenchyma
2. Enhancement of hepatocyte engraftment and integration in liver cords
3. Reduction of Kupffer cell activity, which reduces phagocytic clearance of transplanted cells
4. Inhibition of host hepatocyte proliferation and induction of hepatocyte death and senescence
5. In combination with hepatic growth factors, HIR provides a competitive growth advantage to donor cells
6. HIR is the only regimen that promotes regiospecific liver repopulation by donor cells

In-text box 1:

Summary of mechanisms by which preparative hepatic irradiation improves cell engraftment and repopulation

Figure 2.

The process of correction of a metabolic disease through ex-vivo gene therapy of autologous somatic cells such as fibroblasts. Fibroblasts are corrected ex-vivo and then reprogrammed and differentiated to hepatocyte-like-cells (HLCs). After transplantation, these hepatocytes integrate in the patient liver, where they produce deficient factors or enzymes.

Table A

Liver repopulation following preparative hepatic irradiation

Repopulation in nonirradiated control	Repopulation with HIR	Notable findings	Reference
Isolated cells scattered in liver parenchyma	60–80%	Bilirubin in Gunn rats reduced ~95% in PH+HIR+HT, while only 20–30% reduction was seen in control groups.	Guha et al. 2002 [28]
Not significant	70–80% within 20 weeks for fas-agonist; 90–95% for ad-HGF;	Reduction of oxalate urinary excretion in primary hyperoxaluria in HIR + adHGF	Guha et al. 2005 [38]
1–2 heps in 20–30% of periportal areas	"...frequent clustering in 70–80% of periportal regions at 50Gy" Improvement of "fivefold to 70-fold" based on counting cells.	In irradiated livers, cells formed clusters while they remained individual in non-irradiated livers. Enhancement of repopulation seen in a dose-dependent manner.	Yamanouchi et al. 2009 [13]
Only occasional cells in nonirradiated lobes.	In DPPIV-/- mice: 30–60% after 3 months; In UGT1A1-/- mice: 2030% depending on whether the median or both the median and the left lobe were irradiated.		Zhou et al. 2012 [33]
No relevant engraftment	20%		Christiansen et al. 2006 [23]
4–5%	9–20%, depending on duration of ischemia duration and reperfusion interval prior to transplantation	Highest repopulation with longest (90 min) ischemia, and shortest (1 hr) reperfusion duration.	Koenig et al. 2011 [40]
Not described	2–11%	Dose-response shows increase in repopulation based on normalized total dose. Fractioned doses used to reduce risk of radiation induced liver disease.	Krause et al. 2011 [24]

Table B

Categorized examples of clinical case reports and trials of hepatocyte transplantation

Patients	Disease	Procedure	Cells	Outcome	Reference
<i>Small number of engrafted hepatocytes lead to sufficient repopulation due to hepatocytic injury</i>					
Female 64 yo	Acute liver failure due to ingestion of amanita phalloides mushrooms	Portal vein injection	8 billion cryopreserved cells. Viability ~62% measured with Trypan blue.	Ammonia, bilirubin decreased gradually. Eight weeks after transplant, normal liver architecture and normal blood flow was seen. Physicians concluded full recovery.	Schneider et al. 2006 [82]
<i>Small number of engrafted hepatocytes needed to provide therapeutic benefit</i>					
Male	Factor VII deficiency	Inferior mesenteric vein injection	1.09 billion cryopreserved	Requirement of exogenous protein diminished to 20% dose previous to transplant. Remission began at 6 mos. Received OLT.	Dhawan et al. 2004 [83]
Male	Factor VII deficiency	Inferior mesenteric vein injection	2.18 billion fresh and cryopreserved	Requirement of exogenous protein diminished to 20% dose previous to transplant. Remission began at 6 mos. Received OLT.	
Female 9 yo	Crigler-Najjar		7.5 billion cells (5% liver mass)	50–65% reduction, remission at 3mo. Possible rejection.	Ambrosino et al. 2005 [84]
Female 9 yo	Crigler-Najjar	Venous injection	18 infusions over 5mo, total 4% of liver mass (6.1 billion cells)	Bilirubin reduced from 17.5 to 13.6, remission at 6mo	
Female 1 yo	Crigler-Najjar	Venous injection	14 infusions over 15d, total of 8.6% of liver mass (2.6 billion)	Bilirubin reduced from 17.6 to 13.3, remission at 4mo	Lysy et al. 2008 [42]
Male 6 yo	Phenylketonurea (PKU)		2.5 billion cells over 2 infusions	Phenylalanine decreased from 11.1 to 3.5	Smets et al. 2011 [85]
Female 4 mos	Carbamoyl-phosphate synthetase 1 (CPS1) deficiency	Portal vein by recanalization of the umbilical vein	689 million viable cells over 3 infusions within 26hrs of 5GY HIR	Continued to experience episodes of high ammonia	Soltys et al. 2017 [50]
Male 7 mos	OTC		2 billion cells over 4 infusions within 16hrs of 7.5GY HIR	Tolerated protein from day 20 to day 84, when ammonia acutely elevated	
Female 27 yo	PKU		5 billion cells over 3 infusions after 10GY HIR	PHE reduced by 36% for more than a year	
Male 14 mos	Urea cycle disease (Ornithine transcarbamylase (OTC) deficiency)	Portal vein injection	2.4 billion (6%)	Reduction in ammonia for 2mos. Psychomotor development improved. Eventually underwent OLT.	Stephene et al. 2005 [86]
Female 3.5 yo	Argininosuccinate Lyase deficiency	Portal vein injection	3.7 billion (9%)	Psychomotor advances acquiring fecal and urinary continence, could dress alone, and perform difficult puzzle exercises and ammonia decreased 50% and remained low for	Stephene et al. 2006 [87]

Patients	Disease	Procedure	Cells	Outcome	Reference
<i>Massive repopulation is required because toxic metabolite is generated in host hepatocytes</i>					
Female 4 yo	Infantile Refsum disease, peroxisomal biogenesis disease	spleno-mesenteric confluent injection (venous injection)	2 billion cells (5%). Half fresh half cryopreserved.	Resolution of cholestasis, metabolite decreased by 40%. Engraftment seen on biopsy. Reduction in abnormal metabolites maintained until end of measurement at 18 mos.	Sokal et al. 2003 [49]

Table C

Approaches to derive transplantable hepatocytes for the treatment of genetic diseases

Approach	Strengths	Limitations	Reference
Primary hepatocyte + <i>ex vivo</i> gene therapy	Lifelong immunosuppression not required	Isolation of cells is challenging. Expansion requires unsafe immortalization.	Fox et al. 1995 [88]
Somatic cell → iPSC + gene correction → iHep	Simple, non-invasive isolation of somatic cells. Lifelong immunosuppression not required. Easily expandable cell population.	Possible teratoma formation. Maturity profile currently uncertain.	Si-Tayeb et al. 2010 [58] Tomizawa et al. 2013 [41] Ma et al. 2013 [57] Takayama et al. 2012 [56]
Somatic cells + gene correction → tHep	Low tumorigenic potential. Potential for rapid, efficient, scalable production.	Maturity profile currently uncertain. More work needed to confirm feasibility for hepatocyte application.	Sekiya et al. 2011 [69] Huang et al. 2011 [67] Du et al. 2014 [66] Huang et al. 2014 [68] Simeonov et al. 2014 [70]

Table D

Transcription factors and culture conditions used for hepatocyte differentiation

Transcription factors	Growth factors	Use of serum	Additional factors	Induced factors	Reference
Sox 17, HEX, HNF4 α	Activin A, BMP4, HGF	Yes	Oncostatin M, Dexamethasone, ITS	GATA6, Sox17, HNF4 α	Takayama et al. [56]
FoxA2, GATA4, Hex, C/EBP α	EGF, BMP, bFGF, EGF, NGF, TGF β , HGF	No	Oncostatin M, Retinoic acid, dexamethasone, ITS (Insulin, transferrin, selenium)		Tomizawa et al. [51]
N/A	BMP, Activin A, FGF2, HGF	No	Oncostatin		Si-tayeb et al. [58]
N/A	Activin A, FGF-4, HGF, BMP2, BMP4,	Yes	Oncostatin, dexamethasone		Ma et al. [57] Duan et al [89]

Table E

Reprogramming vectors

Approach	Strengths	Limitations
Integrative virus	Easy to make. Viral transduction efficiency.	Random integration can lead to gene disruption and tumorigenesis. Recombination can result in infectious virus. Mosaic protein expression.
DNA-based vectors (e.g. episomes)	Cytoplasmic location reduces probability of integration	Any DNA-based vector can possibly lead to random integration and tumorigenesis
Non-integrating RNA virus (e.g. Sendai virus)	No genomic integration can occur. Viral transduction efficiency.	Making a virus with controllable infection is difficult and the technique has not been proven to be 100% effective. Mosaic protein expression
Self replicating mRNA	No genomic integration can occur. No infectious particles are generated. Footprint free. Equimolar expression of many proteins possible. Presence of the vector can be easily controlled with B18R.	Transfection of mRNA is less efficient than transduction. Interferon response must be mitigated.