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Assessing the Therapeutic Potential of Lab-Made Hepatocytes

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Summary

Hepatocyte transplantation has potential as a bridge or even alternative to whole organ liver transplantation. Because donor livers are scarce, realizing this potential requires the development of alternative cell sources. To be therapeutically effective, surrogate hepatocytes must replicate the complex function and ability to proliferate of primary human hepatocytes. Ideally, they are also autologous to eliminate the need for immune suppression, which can have severe side effects and may not be sufficient to prevent rejection long term. In the past decade, several methods have been developed to generate hepatocytes from other readily and safely accessible somatic cells. These lab-made hepatocytes show promise in animal models of liver diseases, supporting the feasibility of autologous liver cell therapies. Here we review recent preclinical studies exemplifying different types of lab-made hepatocytes that can potentially be used in autologous liver cell therapies. To define the therapeutic efficacy of current lab-made hepatocytes, we compare them to primary human hepatocytes, focusing on engraftment efficiency and post-transplant proliferation and function. In addition to summarizing published results, we discuss animal models and assays effective in assessing therapeutic efficacy. This analysis underscores the therapeutic potential of current lab-made hepatocytes but also highlights deficiencies and uncertainties that need to be addressed in future studies aimed at developing liver cell therapies with lab-made hepatocytes.

Introduction

Liver cell therapy using allogeneic hepatocytes is in principle clinically effective,¹ but limited availability of high-quality donor hepatocytes and immune rejection have hampered broad clinical use.² These problems could be eliminated by using autologous surrogate

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hepatocytes and methods to produce such cells now exist. The most common approach for generating human hepatocytes is to reprogram readily and safely accessible somatic cells like skin fibroblasts into induced pluripotent stem cells (iPSCs), followed by directed differentiation into hepatocytes (iPSC-Heps). Protocols for differentiating iPSCs into hepatocytes were originally developed using embryonic stem cells (ESCs) and use growth factors and cytokines known to induce hepatocyte differentiation during liver development.^{3–7} A similar approach entails partial reprogramming to pluripotency followed by directed hepatocyte differentiation, leading to induced multipotent progenitor cell-derived hepatocytes (iMPC-Heps).⁸ Directed hepatocyte differentiation without the need for prior reprogramming has been reported for mesenchymal stem cells (MSCs) like adipose-derived stem cells (ADSCs).⁹ Alternatively, fibroblasts or ADSCs can be directly reprogrammed into induced hepatocytes (iHeps) by forced expression of hepatic transcription factors.^{10, 11}

Several reviews have recently been published that provide an overview of research related to liver cell therapy, including challenges associated with clinical translation of hepatocytes generated from pluripotent stem cells or by reprogramming, here referred to as lab-made hepatocytes.^{12–15} Therefore, in this review we focus on defining the therapeutic efficacy of current lab-made hepatocytes by comparing them to primary human hepatocytes, focusing on engraftment, proliferation and function after transplantation (Fig. 1). In addition, we highlight animal models and assays that accurately assess therapeutic efficacy and can thereby guide the development of liver cell therapies with lab-made hepatocytes.

Therapeutic efficacy of current lab-made hepatocytes

To assess the therapeutic efficacy of lab-made hepatocytes, several immune-deficient animal models have been used (Supplementary Table 1). Most studies tested the function of lab-made hepatocytes and whether they can proliferate in animals with types of liver injury that provide a growth advantage for transplanted hepatocytes (Supplementary Table 1). Hepatocytes are one of a few differentiated cell types in the body that have the ability to proliferate. Replicating this feature in lab-made hepatocytes is critical for clinical translation because the human liver contains hundreds of billions of hepatocytes of which—depending on the disease—1–15% must be replaced to achieve and sustain a therapeutic effect.¹⁶ Although primary human hepatocytes (pHeps) can almost completely repopulate the liver in animal models of liver injury,^{17–25} lab-made hepatocytes are generally less effective,^{8–11, 26, 27} suggesting impaired proliferation or engraftment (Fig. 2). Liver repopulation is typically assessed by measuring human serum albumin (HSA) levels in the recipient animals and by quantifying contribution of the transplanted cells to the liver, mostly by immunostaining, but flow cytometry or PCR-based assays have also been used (Fig. 1 and Supplementary Table 1). Discrepancies between HSA and liver repopulation levels—as identified by comparison to mice repopulated with pHeps (Fig. 2)—suggest that some types of lab-made hepatocytes are not fully functional.

To define the therapeutic efficacy of current lab-made hepatocytes, in the following section we summarize the results of recently published studies of cells that could in principle be used in autologous liver cell therapies.

iMPC-Heps

One of the first reports of significant liver repopulation of an animal model with lab-made hepatocytes used iMPC-Heps.⁸ In this study pluripotency-inducing factors were used to generate iMPC-Heps from fibroblasts, but instead of establishing stable iPSC lines the cells were immediately subjected to directed hepatocyte differentiation. Transplantation of 1 million iMPC-Heps resulted in HSA levels of 100 µg/mL and 2% liver repopulation as quantified by immunostaining (Fig. 2). The recipients were mice with liver injury due to fumarylacetoacetate hydrolase (FAH) deficiency, which provides a selective growth advantage for wildtype hepatocytes. These mice die from liver failure unless they are treated with the drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) or cell or gene therapy. The mice were also immune deficient because of recombination-activating gene 2 (RAG2) and interleukin 2 receptor γ (IL2RG) deficiency, leading to lack of B and T lymphocytes and natural killer (NK) cells.¹⁸ Importantly, this study showed that iMPC-Heps resembled fetal hepatocytes in vitro but matured after transplantation until they were virtually indistinguishable from adult hepatocytes as assessed by global gene expression profiling and analysis of cytochrome P450 (CYP) enzymes by immunostaining of liver sections and in vivo drug metabolism. In addition, iMPC-Hep transplantation improved survival of FRG mice off NTBC.

iPSC-Heps

Recently, significant liver repopulation has also been achieved with iPSC-Heps (Fig. 2). Carpentier et al reported HSA levels of up to 6.415 mg/mL and up to 20% liver repopulation as quantified by immunostaining after transplantation of 4 million iPSC-Heps generated using a protocol originally developed with ESCs.^{26, 28} The recipients were severe combined immune deficiency (SCID)/Beige (Bg) mice that lacked B and T lymphocytes and functional NK cells and had liver injury due to urokinase type plasminogen activator (uPA) expressed from a major urinary protein (MUP) promoter. These mice were transplanted when they were 5–8 months old because that age afforded the highest level of liver repopulation in a previous study using pHeps.²³ In many mice HSA levels peaked 10 days after transplantation and then declined rapidly. However, several mice showed steadily increasing HSA levels over the observation period of 100 days. Serum α -fetoprotein (AFP) was undetectable in ELISA and iPSC-Heps were negative for AFP and positive for CYP in immunostainings, suggesting post-transplant maturation.

Subsequently, Chen et al reported that iPSC-Heps generated with the same protocol could repopulate livers of Gunn Rats, a model of Crigler-Najjar syndrome, in which bilirubin metabolism is defective.²⁷ The transplanted iPSC-Heps were given a selective growth advantage by preconditioning the recipients with localized liver irradiation and inducing liver regeneration with an adenovirus expressing hepatocyte growth factor (HGF). After transplantation of 2 million iPSC-Heps this resulted in HSA levels of up to 73 µg/mL and up to 7.5% repopulation in the irradiated median liver lobe as quantified by immunostaining and PCR (Fig. 2). Immune rejection of the transplanted cells was prevented with the T cell-inhibiting drug tacrolimus. Importantly, the activity of the bilirubin-metabolizing enzyme missing in Gunn rats was restored, resulting in up to 60% reduction of serum bilirubin levels.

SCi-Heps

Building on previous studies suggesting that MSCs have hepatocyte differentiation potential, Xu et al reported HSA levels of more than 1.2 mg/mL, which they estimated to correspond to 20% liver repopulation, after transplantation of 5 million ADSC-derived iHeps (Fig. 2).⁹ These so-called spherical culture iHeps (SCi-Heps) were generated by aggregating ADSCs and subjecting them to directed differentiation using hepatogenic growth factors and cytokines. The recipients were SCID mice that were also IL2RG deficient and on the non-obese diabetic (NOD) background, which has a polymorphism in signal-regulatory protein α (SIRP α) that increases its binding to the human version of the “self marker” CD47 and thereby inhibits phagocytosis of human cells by mouse macrophages.²⁹ Liver injury could be induced in these mice with the drug ganciclovir because of a herpes simplex virus thymidine kinase (TK) transgene expressed from an albumin (ALB) promoter. The metabolic function of transplanted SCi-Heps was not assessed.

iHeps

An alternative method that does not require generating or using stem/progenitor cells is direct reprogramming of somatic cells by forced expression of lineage-determining transcription factors. This strategy proved effective in generating iHeps from fibroblasts and ADSCs.^{10, 11} Huang et al generated iHeps using three hepatic transcription factors and the simian virus 40 large T antigen to overcome reprogramming-induced growth arrest. They reported HSA levels of up to 0.68 μ g/mL and up to 4.2% liver repopulation as quantified by immunostaining after transplantation of 10 million cells (Fig. 2). The recipients were mice deficient in FAH and RAG2 in which NK cells were depleted with anti-asialo-GM1 antibody and that also received tacrolimus. The metabolic function of the transplanted iHeps was not assessed, but they were as effective as pHeps in improving survival from liver injury caused by NTBC withdrawal or concanavalin A intoxication.

Du et al used six hepatic transcription factors in combination with forced MYC expression and TP53 knockdown to generate iHeps capable of proliferation. They reported HSA levels of up to 300 μ g/mL and up to 30% liver repopulation as quantified by flow cytometry after transplantation of 2 million cells (Fig. 2). The recipients were mice with liver injury caused by tetracycline (Tet)-inducible uPA expression and immune deficiency from RAG2 and IL2RG deficiency. The metabolic function of the transplanted iHeps was assessed by CYP immunostaining.

Assessment of therapeutic efficacy of lab-made human hepatocytes

The efficacy of liver cell therapy is determined by efficiency of engraftment, ability to proliferate and repopulate the liver, and complete and authentic function of the transplanted cells. In the following section we discuss animal models and assays effective in assessing the therapeutic efficacy of lab-made hepatocytes (Fig. 1). A discussion of safety, a prerequisite for clinical translation, is beyond the scope of this review.

Engraftment

Engraftment is defined as survival and integration of transplanted cells in a target organ. Hepatocytes do not home naturally, but can be targeted to the liver by injection into the portal vein, which drains into the sinusoids, leading to distribution of the transplanted cells throughout the parenchyma. Because portal vein injection is an invasive and technically challenging technique, particularly in mice, most researchers deliver hepatocytes by injection into the spleen, which connects to the portal vein via the splenic vein. To enter the liver parenchyma, transplanted hepatocytes must traverse the sinusoidal endothelium, which involves obstruction of the sinusoids and mechanical stress. Therefore, it is not surprising that only 10% of transplanted pHeps can be detected in the liver parenchyma in FRG mice after intrasplenic injection.¹⁸ The engraftment efficiency of lab-made hepatocytes has not been systematically investigated but appears to be lower than that of pHeps.⁸ Studies using immune-matched rodent hepatocytes and recipients have suggested ways to overcome barriers to initial engraftment, including drugs that break down the sinusoidal endothelium or protect hepatocytes from vasoactive or inflammatory responses.¹⁴ In accord, by causing endothelial cell injury and inhibiting phagocytic function of macrophages,³⁰ localized liver irradiation likely improved engraftment of iPSC-Heps in Gunn rats.²⁷ Along these lines, liver repopulation with pHeps is accelerated in FRG mice on the NOD background (FRGN mice) and in ALB-uPA/RG mice if mouse CD47 is expressed in the pHeps.^{24, 31}

Alternative methods of hepatocyte transplantation exist. Direct intrahepatic injection has been used to engraft SCi-Heps.⁹ iHeps contained in semi-permeable microcapsules have been transiently placed in the peritoneum.¹⁰ Organoids composed of iPSC-Heps and a vasculature formed by primary human endothelial cells and MSCs have been transplanted into well-vascularized extrahepatic sites including onto the mesentery, under the kidney capsule and onto the brain (Fig. 2 and Supplementary Table 1).³² By connecting to the recipient animal's vasculature the organoids could persist for 60 days. Life-long persistence is in principle afforded by transplantation into a lymph node, as demonstrated using primary mouse hepatocytes.³³ Transplantation into ectopic sites may be the only effective method for hepatocyte engraftment in liver cirrhosis,¹² but lack of a functional biliary system and thus release of bile from hepatocytes into the blood may require substantial residual function of the recipient's liver.

General methods available to assess engraftment of transplanted hepatocytes include immunostaining,⁸ flow cytometry,¹¹ PCR,¹⁰ and bioimaging²⁸. In addition, measuring HSA levels in live animals allows estimating engraftment and monitoring expansion of hepatocytes and will be discussed in detail below. Of note, the large fraction of hepatocytes that die in the process of transplantation release ALB, which confounds measurements of HSA levels in the first week after transplantation.³⁴

Proliferation

Clinical trials of pHep transplantation show that up to 5% of the hepatocyte mass can be safely transplanted into a patient.¹² However, because only about 10% of transplanted pHeps engraft, establishing and maintaining a therapeutically effective hepatocyte mass requires post-transplant proliferation in most liver diseases. Therefore, studies investigating the

therapeutic potential of lab-made hepatocytes typically include analyses of whether they replicate the ability of pHeps to proliferate extensively.¹⁸ For this, most researchers use immune-deficient animal models of liver injury in which transplanted hepatocytes have a growth advantage over the recipient's hepatocytes, leading to liver repopulation. Most of these animal models express a toxic transgene in hepatocytes like uPA and TK described above. MUP-uPA mice express uPA later than ALB-uPA mice, which prevents neonatal bleeding and facilitates breeding of homozygotes.^{23, 35} In both MUP-uPA and ALB-uPA mice, homozygosity limits unwanted liver repopulation with endogenous hepatocytes in which the transgene is inactivated by rearrangement.^{35, 36} In Tet-uPA and ALB-TK mice hepatotoxicity is regulatable, which provides flexibility in timing of transplantation.^{9, 11} Similarly, in FAH-deficient mice the onset, duration and extent of liver injury are regulatable because it can be suppressed with NTBC.^{8, 18}

Several nongenetic animal models of liver repopulation exist, one of which, localized liver irradiation, has been adopted as a method of preconditioning in clinical trials of pHep transplantation.²⁷ In addition to improving engraftment, liver irradiation impairs proliferation of the recipient's hepatocytes, which provides a selective growth advantage for transplanted hepatocytes if combined with stimulation of liver regeneration, e.g., using HGF.³⁰ Although restricted to the irradiated lobe, the extent of liver repopulation may be sufficient for therapy of many liver diseases.²⁷

Repeated measurement of HSA levels allows monitoring the expansion of lab-made hepatocytes in live animals. Numerous studies indicate HSA levels are correlated with percent liver repopulation—mainly assessed by immunostaining—in animals transplanted with pHeps (Fig. 2). Differences in HSA levels between these studies may be due to the quality and number of transplanted pHeps, the method of delivery and the animal model, i.e., type of liver injury, degree of immune deficiency, age and gender (Supplementary Table 1). In addition, the method of HSA analysis may affect the results, e.g., ALB levels are up to two-fold higher in serum than in whole blood. Moreover, most researchers use an ELISA from Bethyl, which appears to be affected by sample and antibody dilution and incubation time.²² Nevertheless, the results of most studies using pHeps congregate within a fairly narrow range and therefore provide a framework for interpreting results made with lab-made hepatocytes (Fig. 2). Importantly, because the secretory function of lab-made hepatocytes may differ from that of pHeps, the extent of liver repopulation suggested by HSA levels must be confirmed with an independent method, e.g., immunostaining of liver sections or flow cytometry.

Function

Because current lab-made hepatocytes are incompletely differentiated *in vitro*,^{8–11, 26, 27, 32} they must undergo post-transplant maturation to acquire complete hepatocyte function. This is well illustrated by the study by Zhu et al that showed the global gene expression profile of iMPC-Heps was very different from pHeps *in vitro* but became virtually identical after expansion in FRG mice.⁸ For this, the researchers isolated iMPC-Heps by laser-capture microdissection nine months after transplantation and analyzed them with microarrays. *In vivo* maturation was confirmed using CellNet, an open-access online tool that assesses the

authenticity of differentiation of lab-made cells based on gene regulatory networks identified in their primary counterparts.³⁷ Such comprehensive analysis of post-transplant maturation is currently not available for other types of lab-made hepatocytes but would be helpful to show complete and authentic hepatocyte differentiation or identify deficiencies.

To assess *in vivo* function of lab-made hepatocytes, most researchers analyze liver sections by immunostaining for markers of secretory and metabolic function like ALB and CYP enzymes, respectively.^{8, 11, 26} Some also exclude persistence of fetal liver markers like AFP.^{8, 26, 32} Because immunostainings are not sufficiently quantitative, additional functional analyses are used, particularly of CYP enzymes that are typically weakly expressed in lab-made hepatocytes *in vitro*. For example, Zhu et al took advantage of the specific metabolism of the drug debrisoquine by human CYP2D6 to show similar *in vivo* activity levels in iMPC-Heps and pHeps.⁸ As shown in mice repopulated with pHeps, species-specific differences in drug metabolism allow *in vivo* analysis of several other human CYP and downstream enzymes, but potential contributions from mouse orthologues must be considered.³⁸

Finally, researchers have begun to investigate the therapeutic efficacy of lab-made hepatocytes. Takebe et al showed that liver buds containing iPSC-Heps improved survival in mouse models of acute and subacute liver failure—based on diphtheria toxin receptor or TK expression in hepatocytes, respectively.³² Zhu et al and Huang et al showed that iMPC-Heps and iHeps improved survival of FAH-deficient mice, a model of the human disease tyrosinemia type I that develops subacute liver failure when taken off the drug NTBC.^{8, 10} In all of these mice liver injury was induced after transplantation and therefore did not model the clinical scenario of acute liver failure. To generate such a model, Huang et al treated wildtype mice with the fast-acting toxin concanavalin A and transplanted them with iHeps eight hours later, which was effective in prolonging survival.¹⁰ Chen et al provided the first example of specific disease-modifying activity of human lab-made hepatocytes by reversing hyperbilirubinemia in the Gunn rat model of human Crigler-Najjar syndrome.²⁷

Conclusions

Much progress has been made in generating hepatocytes from other readily and accessible somatic cells, including assessment of their therapeutic potential in animal models. The studies discussed in this review support the feasibility of autologous liver cell therapies with lab-made hepatocytes but also highlight areas in need of further development.

A prerequisite for successful liver cell therapy is establishing a hepatocyte mass of sufficient size, which requires efficient engraftment and proliferation of the transplanted cells. Several animal models have been developed that—because of severe immune deficiency and a selective growth advantage for transplanted hepatocytes—can be near-completely repopulated with pHeps. Similar levels of post-transplant expansion have not been achieved with lab-made hepatocytes and may not be needed for therapy of liver diseases like Crigler-Najjar syndrome²⁷. However, clinical translation will require distinguishing limited ability to proliferate from low engraftment efficiency as the reason for inferior liver repopulation by lab-made hepatocytes. To address this question, future studies should include analyses of

engraftment efficiency and use methods to quantify liver repopulation that allow direct comparison to previous studies.

Because lab-made hepatocytes are typically immature in vitro, knowing whether they acquire complete and authentic hepatocyte differentiation after transplantation is important for both therapeutic efficacy and safety. As a starting point, global gene expression profiling of re-isolated cells can be used. Determining in vivo function requires human-specific assays in partially repopulated animals. Ideally, animals repopulated with pHeps are used as controls. Fig. 2 shows that most lab-made hepatocytes secrete normal amounts of ALB in vivo. Whether other hepatocyte functions, e.g., drug metabolism, are fully developed in vivo is not yet known for most lab-made hepatocytes. Along these lines, more evidence for specific disease-modifying activity of lab-made hepatocytes is needed. Finally, clinical translation will require adjusting current methods for generating lab-made hepatocytes so that they are compatible with human application and in-depth safety studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADSCs	adipose-derived stem cells
AFP	α -fetoprotein
ALB	albumin
Bg	Beige
CYP	cytochrome P450
ESCs	embryonic stem cells
FAH	fumarylacetoacetate hydrolase
FRG	FAH/RAG2/IL2RG
FRGN	FAH/RAG2/IL2RG/NOD
HGF	hepatocyte growth factor
HSA	human serum albumin

iHeps	induced hepatocytes
IL2RG	interleukin 2 receptor γ
iMPCs	induced multipotent progenitor cells
iMPC-Heps	iMPC-derived hepatocytes
iPSCs	induced pluripotent stem cells
iPSC-Heps	iPSC-derived hepatocytes
MSCs	mesenchymal stem cells
MUP	major urinary protein
NK	natural killer
NOD	non-obese diabetic
NOG	NOD/SCID/IL2RG
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione
FAH	fumarylacetoacetate hydrolase
pHeps	primary human hepatocytes
RAG2	recombination-activating gene 2
SCID	severe combined immune deficiency
SCi-Heps	spherical culture iHeps
SIRPα	signal-regulatory protein α
tet	tetracycline
TK	thymidine kinase
uPA	urokinase type plasminogen activator

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Fig. 1.

Assays used to assess the therapeutic efficacy of lab-made hepatocytes.

Fig. 2.

Comparison of HSA and liver repopulation levels between recent studies using lab-made hepatocytes and studies using pHeps that reported near-complete liver repopulation. For each study ALB levels were correlated with % liver repopulation. Studies are labeled with the number of transplanted cells (M = million), cell type, animal model, first author and year of publication. The left panel shows results from all studies. The right panel is a double-log plot showing only results from studies using lab-made hepatocytes (highlighted area in left panel). [^]Anti-asialo-GM1 antibody. [°]Tacrolimus. *Equivalent % liver repopulation of liver buds transplanted under the kidney capsule was calculated as the ratio of 2.9 million iPSC-Heps to an estimated 50 million hepatocytes in the mouse liver. **Transplantation by intrahepatic injection. ***% liver repopulation was normalized by estimating that the engrafted median lobe contains 30% of the total liver mass.