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Thinking Out Loud

# Biofabrication of Autologous Human Hepatocytes for Transplantation: How Do We Get There?

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Directed differentiation of hepatocytes from induced pluripotent stem cells (iPSCs) holds promise as source material for treating some liver disorders. The unlimited availability of perfectly differentiated iPSC-derived hepatocytes will dramatically facilitate cell therapies. While systems to manufacture large quantities of iPSC-derived cells have been developed, we have been unable to generate and maintain stable and mature adult liver cells ex vivo. This short review highlights important challenges and possible solutions to the current state of hepatocyte biofabrication for cellular therapies to treat liver diseases. Successful cell transplantation will require optimizing the best cell function, overcoming limitations to cell numbers and safety, as well as a number of other challenges. Collaboration among scientists, clinicians, and industry is critical for generating new autologous stem cell-based therapies to treat liver diseases.

**Key words: Hepatocyte proliferation; Liver regeneration**

## INTRODUCTION

The demand for transplantable livers has increased progressively, outpacing the supply of live and cadaveric organs; this results in longer waiting time for the patients, reducing their prospective survival rate[1]. Additionally, it is projected that the demand for livers will increase by 10% in 10 years and 23% in 20 years[1]. Several strategies have been explored to increase the number of livers available for transplantation: i) The use of marginal donors (e.g., donors over the age of 60; donors with greater than 30% hypernatremia or macrosteatosis, donors with positive serologies for the hepatitis C or hepatitis B virus, donors with a cold ischemia time of greater than 12 h, non-heart-beating donors, and grafts from split livers or living related donors)[2]; ii) machine perfusion approaches to resuscitate marginal donor livers[3]; and iii) novel cell therapies to induce tolerance[4]. However, if successful, these approaches are estimated to have limited impact on the organ donor pool[1]. New regenerative approaches to investigating the liver organogenesis seek to provide novel insights into liver repopulation. Liver cell therapy has been under intensive investigation for decades[5] and seen as a promising potential alternative to orthotopic liver transplantation. We have learned that the ideal candidates for this kind of therapy are individuals with acute liver failure and liver-based inborn errors of metabolism[6,7]. Indeed, published experiences of liver cell therapy in the treatment of these conditions have shown promising results[5]. However, only partial correction of metabolic disorders has been achieved, and liver cell therapy has not shown to reliably circumvent the need for traditional organ transplant. While a detailed clinical report is beyond the scope of this article, it is noted that significant barriers persist limiting broader implementation of liver cell therapy that has been highlighted in a recent report that included a preclinical and clinical

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approach[8]. The most important hurdles identified, besides the well recognized lack of hepatocyte source for transplantation, were i) the ability to monitor the function and rejection of transplanted cells and ii) the ability to control rejection episodes in vivo. Thus, given the short-term graft survival and immunological hurdles that have been identified in the latest hepatocyte allotransplantation trials, autologous gene-corrected and fully functional hepatocytes would be a desirable solution.

The aim of this minireview is to critically evaluate the current state of biofabricating autologous human hepatocytes, underscoring important advancements that have occurred in the expanding field of cell engineering and discuss new exciting technologies that have the potential to change the landscape of liver replacement once and for all.

## CHALLENGES IN GENERATING AUTOLOGOUS HUMAN HEPATOCYTES FOR TRANSPLANTATION

### *Induced Pluripotent Stem Cell-Derived Hepatocytes*

Induced pluripotent stem cells (iPSCs) can be expanded indefinitely and differentiate into all cell types of the body, while maintaining genetic stability and are therefore a promising cell source of functional autologous hepatocytes[9]. Human iPSCs are generated by forced expression of specific pluripotency transcription factors[10]. Great progress has been made in exploring the differentiation capacity of iPSCs toward the hepatocyte lineage. Generating hepatocyte-like cells (HLCs) from iPSC is necessary taking into account multiple factors related to the differentiation process: extracellular matrix, media, and small molecules and supplements to provide adequate and in a timely manner cell signaling to guide pluripotent stem cells toward a definitive endoderm and finally hepatocyte-like cells. Great progress has been made in exploring the differentiation capacity of PSCs toward the hepatocyte lineage. Although specific culture conditions can differ, most protocols share a general three-step strategy based on liver development studies. The three basic steps are endoderm induction, generation of hepatic progenitors or hepatoblasts, and hepatocyte maturation. The endoderm induction is characterized by the use of activin A, FGF, and BMP4, which, together with the activation of WNT3, signal trigger the generation of endoderm cells[11–15]. The WNT pathway activation promotes FOXA2 activation via SOX17[16]. The differentiation to hepatic progenitor or hepatoblasts is generated by the addition of HGF that induce the expression of HNF4 [17–19] and fetoprotein[20]. Other hepatic inducers include FGF2, FGF4, and BMP4. Finally, hepatocyte maturation is the most critical step during generation of HLCs. Commonly oncostatin M (an interleukin-6-related protein) and dexamethasone (a glucocorticoid) are used to induce the expression of hepatocyte markers, such as albumin, CK18, some cytochrome p450, SERPINA1, ASGPR1, C/EBP , and UGT1A1[21]. Additionally, some functional assays, such as urea production, LDL uptake, and albumin production are used to evaluate the HLCs to primary hepatocytes[22]. Unfortunately, stem cell-derived HLCs do not possess all functions of mature hepatocytes. Liver maturation is complex and dynamic; full maturation in humans can take as long as 2 years from birth and involves expression of several signaling pathways, such as those responsible for bile acid synthesis, drug metabolism, and amino acid transport. Current efforts in many laboratories are focused on the study of components that promote hepatocyte differentiation and maturation including growth factors, transcription factors, microRNAs, small molecules, and the microenvironment. Despite progress in advancing the differentiation of human stem cells into hepatocytes in vitro, cells that replicate the ability of human primary adult hepatocytes to proliferate and completely replace livers for clinical applications have not been achieved[9,23]. Moreover, HLCs usually show a fetal phenotype and function[24,25].

To generate a functional hepatocyte, it is necessary to take into consideration the developmental pathways and the developmental microenvironment where interactions with other cells and their extracellular matrix may provide specific maturation cues[25–27]. However, despite the successful use of growth factors and cytokines to design protocols for definitive endoderm and approaches to promote hepatocyte specification, the implementation of approaches for final hepatocyte maturation remains elusive[25–27]. Components that promote hepatocyte maturation including the dynamic changes that occurred after birth in the human liver (microbiota, circulation, nutrition) that could be potentially used for in vitro approaches have been discussed elsewhere[22].

### *Gene-Edited Hepatocytes: Essential for Autologous Transplantation*

CRISPR/Cas9 system was reported for the first time in 1987[28], but it was not until 2005 that it was postulated as an immune adaptive system to multiple species of bacteria[29]. The natural function of this system is recognizing foreign DNA sequences in the bacterial genome and promoting host protection through the destruction of these sequences. However, the major milestone in the development of CRISPR/Cas9 was its application in mammalian cells to potentially treat multiple genetic diseases.

One could imagine two future scenarios. The first and more logical scenario is to genetically correct the defect directly in the patient via viral delivery systems [e.g., adeno-associated virus (AAV)]; however, this approach has been more challenging[30]. The targeting efficiency in somatic cells has been relatively modest (<1%) and failed to yield a distinct phenotype[31]. Alternatively, in

vivo selection was required to enrich the gene-edited cells[32], limiting its application to only those genes that conferred a selective growth and survival advantage in vivo [e.g., fumarylacetoacetate hydrolase (Fah)]. Other disadvantages that have been detected using AAV are related to its known inflammatory properties following systemic administration that often provoke liver toxicity and rapid immune clearance of gene-modified cells[33] and the likelihood of undesired genome editing in nontarget tissues or nonspecific off-target editing[34,35]. These limitations justify the development and validation of robust tissue-specific in vivo gene-editing platforms (e.g., lipid nanoparticles[36,37], mRNA[38]) that further minimize the risk of off-target gene editing in nontarget genes and/or nontarget tissues in the near future. The second scenario that one could imagine is where liver tissue could be collected from patients with monogenic diseases, and gene correction could be achieved in vitro using the CRISPR/CAS9 system. Those cells, which successfully underwent functional gene correction, could then be selected and amplified in vivo using xenograft animal models to grow hepatocytes. A critical mass of modified, mature hepatocytes would be produced, which would enable the original patient/donor to receive an autologous hepatocyte transplantation of their own genetically corrected cells. Hurdles to this approach would need to be overcome, including exposing patients to risky surgical resections and quickening the process of gene correction and selection, as isolated human hepatocytes are known to undergo rapid dedifferentiation in vitro. However, other cell sources could be used (e.g., human iPSC-derived hepatocytes).

The number of studies that use human primary hepatocytes for gene editing is limited. This is related to the lack of efficient repopulation of animal models to evaluate these cells in vivo. Wang et al. reported for the first time the knockdown of the *PCSK9* gene in human hepatocytes in vivo using the AVV- CRISPR/Cas9 system in FRG mouse model with a 47% efficiency[39]. Despite these promising results, the use of AVV has been related to hepatic injury due to AVV-induced immunogenicity[40]. The implementation of an alternative less immunogenic delivery system for CRISPR/Cas9, such as lipid nanoparticles, might resolve the immunological damage.

CRISPR/Cas9 gene editing can be used for the treatment of monogenic liver diseases, caused by loss-of-function mutations or deficient gene expression. The most recent approaches in hepatocyte gene editing have been used for treatment of metabolic diseases in in vivo systems. Yin et al. demonstrated the application in vivo of CRISPR/Cas9 system to correct the *Fah* mutation in a model of hereditary tyrosinemia type 1 (HT1), using hydrodynamic injection of Cas9-encoded plasmid and ssDNA donor template. This approach resulted in 33% corrected hepatocytes[32]. In a similar study, Yang et al. corrected ornithine transcarbamylase (OTC) deficiency, through the administration of AAV containing a CRISPR/Cas9 system in neonatal OTC mice; they showed a reversion of the mutation in 10% of hepatocytes[41]. Interestingly, the same protocol applied to adult OTC mice resulted in 1.7% corrected hepatocytes, which was insufficient to rescue the OTC phenotype[20]. These results suggest a different pattern of response to gene editing in relation to hepatocyte maturation state.

If human iPSC-derived hepatocytes were to be used, then we envision the requirement of collection of any cells (e.g., blood, skin fibroblast, epithelial cells, etc.) from patients with monogenic diseases, as there are well-establish protocols for in vitro culture and expansion of these types of cells. After that, these cells could be reprogrammed to pluripotency to generate iPSCs, and gene correction could be achieved in vitro using the CRISPR/Cas9 system. Moreover, screening and selection for the best clones after gene editing would not be a burden. Resulting iPSCs can be screened and then undergo hepatocyte-directed differentiation. These HLCs could be amplified in vivo using xenograft animal models to grow hepatocytes. A critical mass of modified, mature hepatocytes would be produced, which would enable autologous hepatocyte transplantation (Fig. 1).

### *In Vivo Bioreactors to Expand Autologous Hepatocytes*

To improve the amount and the maturation stage of the HLCs generated from iPSCs, animals could be used as in vivo bioreactors to mature and biofabricate large amounts of functional human hepatocytes for transplantation. As discussed below, liver tissue could be collected from patients with monogenic diseases, hepatocytes could be isolated, and gene correction could be achieved in vitro using the CRISPR/CAS9 system. Those cells that successfully underwent functional gene correction could then be selected and amplified in vivo using xenograft animal models to grow hepatocytes. A sufficient cellular mass of modified, mature hepatocytes would be produced, which would enable the original patient/donor to receive an autologous hepatocyte transplantation of their own genetically corrected cells. In fact, human hepatocyte gene correction has been reported[39,42] (Fig. 1).

Genetically modified animals are the most suitable models to be used as a bioreactor. These models provide transplanted hepatocytes a strong advantage related to growth and repopulation of the host liver. Overexpression of an albumin–urokinase type plasminogen activator (uPA) fusion construct, which increases uPA concentration promoting liver damage, enables the transplanted cells to reconstitute over 90% of the liver mass[43]. In recent years, the most used animal model to evaluate the liver repopulation is the FRG; this mouse carries three knockout

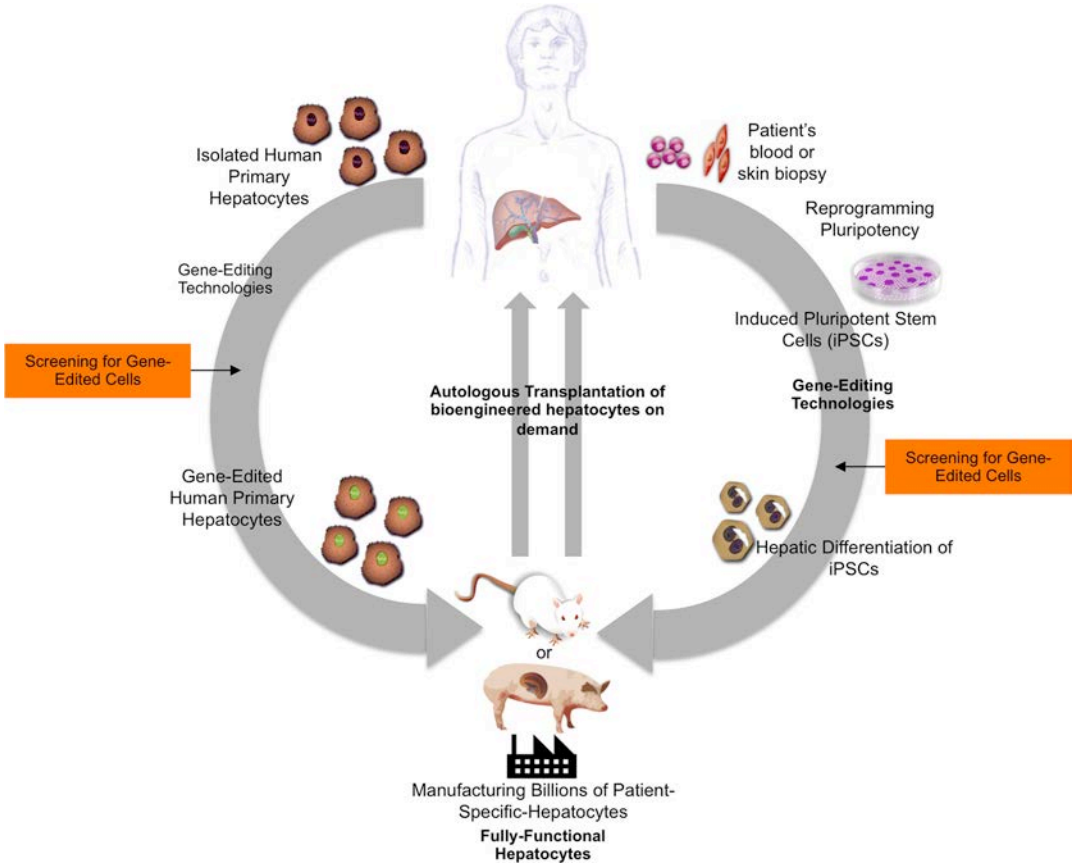


**Figure 1.** Schematic representation of manufacturing approach of autologous hepatocytes for transplantation. Primary hepatocytes are isolated from the patient and edited with CRISPR/Cas9 to correct the pathogenic genomic alteration ex vivo. Then the edited primary hepatocytes are select and multiplied in a "bioreactor" to be transplanted in patients. Alternatively, fibroblasts/blood cells are isolated from the patient and edited for pathogenic mutations using CRISPR/Cas9 ex vivo. The cells are then screened and reprogrammed to produce induced pluripotent stem cells (iPSCs) in vitro. These corrected iPSCs are differentiated to generate hepatocytes, which are transplanted into a "bioreactor" to produce functional hepatocytes that can be used for transplantation in patients.

genes: fumarylacetoacetate hydrolase knockout (*Fah*−/−), recombinase 2 gene (*Rag2*) knockout, and IL2 receptor chain gene (Il2rg) knockout. *Fah*−/− modification offers the possibility to control liver injury using (2-nitro-4-trifluoro-methyl-benzoyl)-1, 3 cyclohexanedione (NTBC), which avoids the accumulation of degradation products of phenylanine and tyrosine, whereas Rag2 and Il2rg knockout reduce the action of the immunological system on the transplanted hepatocytes[44].

In relation to the previous mentioned approaches, Zhu et al. reported for the first time a method to overcome the pluripotent state to generate HLCs[45]. The process involved reprogramming the fibroblasts with OCT4, SOX2, and KLF4 to induce a plastic state, and then after 3 days the hepatocyte differentiation protocol was continued. The cells showed the common HLC features such as albumin production, HNF4 expression, and cytochrome activity. The authors argued that this approach reduced the tumor risk related to the expression of the pluripotency-specific genes. However, after transplantation (9 months) into FRG mice, the cells only achieved a 2% liver repopulation. Importantly, this study showed that the HLCs generated in vitro could mature after transplantation, showing a gene expression profile similar to adult hepatocytes after laser capturing of liver repopulated nodules.

In order to generate large amounts of human hepatocytes in live bioreactors, mice naturally would not be suitable for this purpose due to the size restrictions. The pig is a more appropriate model because of its similarity in size, anatomy, and biology to humans[46]. It has been reported that generated and characterized FAH−/− pigs[47] could be used to generate human hepatocytes. Moreover, severe combined immunodeficiency (SCID) pigs have been generated (naturally tolerant of xenotransplantation)[48] that could potentially be used to produce human hepatocytes in combination with systems to exacerbate their liver repopulation abilities. Hurdles to this approach would need to be overcome, including establishment of

facilities and protocols necessary to obtain clean piglets and the high cost associated to it[49]. One more potential less costly and more practical option is the use of rats as bioreactors. Assuming that SCID rats preconditioned to repopulate its liver with human hepatocytes can be created, one can easily obtain $5–6 \times 10^8$ viable cells per rat liver. A total of $2.0 \times 10^8$ viable cells/kg is a hepatocyte dose that is proven to be safe and, to a certain degree, effective in humans[8]. Most candidates for cell therapy are small children with genetic liver disorders[8]; thus, only two to four rat livers would be necessary to treat a pediatric patient (weighting up to 12 kg).

Culturing and gene correction of human iPSCs is an unexplored path with little research performed. Some evidence of the application of human iPSCs to correct metabolic and genetic disorders has been seen in in vitro conditions[50–52]. Zhang et al. have described a differentiation protocol to produce gene-corrected human iPSCs to treat Wilson's disease, which is caused by a mutation in the ATP7B gene resulting in a defective protein transporter in the liver[51]. Gene correction reversed the effects of this genetic disease in vitro. In a similar fashion, Yusa et al. used a combination of zinc finger nucleases (ZNFs) and *piggyBac* technology in human iPSCs to achieve biallelic correction of a point mutation (Glu342Lys) to correct $_1$-antitrypsin deficiency. They reported this method as being more efficient than CRISPR-mediated systems by preventing contamination of the host genome with residual nonhuman sequences[50].

The major challenge in using gene editing is off-target point mutations where the base pairing between the target site and the sgRNA of Cas9 may not be perfect, leading to cleavage at sites with multiple mismatches[53]. A major concern of this mosaicism is the potential of tumor development in vivo. Point mutations can also occur while culturing human iPSCs in vitro. Yoshihara et al. have reported the presence of de novo mutations that can occur due to genetic reprogramming of somatic cells to produce iPSCs[54]. These mutations are underrepresented but can cause severe DNA damage and have tumorigenic potential. The type of somatic cells and the method used to make iPSCs can also affect the occurrence of point mutations[8]. Araki et al. generated nuclear transfer embryonic stem cells (ntESCs) from mouse embryonic fibroblasts (MEFs) and tail-tip fibroblasts (TTFs) and found fewer point mutations in TTF-derived iPSCs[55]. It continues to be a major hurdle that needs to be overcome to make autologous transplantation with gene-corrected cells a reality.

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