Concise Review: Updated Advances and Current Challenges in Cell Therapy for Inborn Liver Metabolic Defects

**Mustapha Najimi, Florence Defresne, Etienne M. Sokal**

**Key Words.** Liver • Liver regeneration • Transplantation • Hepatocyte differentiation • Engraftment • Mesenchymal stem cells • Cell transplantation

**ABSTRACT**

The development of liver cell transplantation (LCT), considered a major biotechnological breakthrough, was intended to provide more accessible treatments for liver disease patients. By preserving the native recipient liver and decreasing hospitalization time, this innovative approach has progressively gained interest among clinicians. LCT initially targets inborn errors of liver metabolism, enabling the compensation of deficient metabolic functions for up to 18 months post-transplantation, supporting its use at least as a bridge to transplantation. The rigorous clinical development and widespread use of LCT depends strongly on controlled and consistent clinical trial data, which may help improve several critical factors, including the standardization of raw biological material and immunosuppression regimens. Substantial effort has also been made in defining and optimizing the most efficient cell population to be transplanted in the liver setting. Although isolated hepatocytes remain the best cell type, showing positive clinical results, their widespread use is hampered by their poor resistance to both cryopreservation and in vitro culture, as well as ever-more-significant donor shortages. Hence, there is considerable interest in developing more standardized and widely accessible cell medicinal products to improve engraftment permanency and post-cell transplantation metabolic effects.

**SIGNIFICANCE**

In this therapeutic approach to liver disease, new solutions are being designed and evaluated to bypass the documented limitations and move forward toward wide clinical use. Future developments also require a deep knowledge of regulatory framework to launch specific clinical trials that will allow clear assessment of cell therapy and help patients with significant unmet medical needs.

**INBORN ERRORS OF LIVER METABOLISM**

Inborn errors of metabolism comprise various diseases that can cause symptoms related to metabolic defects as early as the neonatal stage. Each metabolic disease displays specific characteristics, but signs generally include lethargy, decreased feeding, vomiting, acidosis, and seizures [1]. Early diagnosis and appropriate treatment, including dietary prescriptions and pharmacologic treatments enabling the excretion of toxic metabolites, are crucial to limit neurologic impairment. Nevertheless, orthotopic liver transplantation (OLT) remains the gold standard for a variety of life-threatening diseases [2]. In this review, we have focused our attention on two inborn errors of metabolism that are the primary targets of liver cell therapy: Crigler-Najjar (CN) syndrome and urea cycle defects (UCDs).

CN syndrome is a rare inborn error of liver metabolism with an estimated incidence of 0.6–1.0 cases per million live births. In healthy individuals, unconjugated bilirubin is conjugated with glucuronic acid in hepatocytes to form water-soluble bilirubin glucuronides that are subsequently excreted via the bile. This process is catalyzed by a specific hepatic enzyme isoform (1A1) belonging to the uridinediphosphoglucuronate glucuronyltransferase (UGT) family. This enzyme’s activity is specifically impaired in CN syndrome, leading to abnormal bilirubin conjugation [3–5]. Two forms of the disease have been documented: type 1 (CN1), associated with severe jaundice and significant risk of neurologic impairment, and type 2 (CN2), associated with decreased serum bilirubin concentrations and less risk of neurologic dysfunction. CN1 patients rapidly develop jaundice, and unconjugated serum bilirubin levels promptly increase after birth, reaching 20–45 mg/dl [6, 7]. This results in kernicterus and neurologic sequelae, which can ultimately cause severe disability or death. CN2 patients exhibit no signs of liver disease at birth. Their UGT activity is only deficient...
(rather than absent), leading to a milder form of the syndrome, with total serum bilirubin levels of 6–20 mg/dL. Although jaundice can develop later on, it is usually mild, and patients generally survive into adulthood without neurologic or intellectual impairment. CN patients are primarily treated with phototherapy, which converts unconjugated bilirubin into water-soluble bilirubin isomers that are subsequently excreted via the bile and has been successfully used for years in controlling bilirubin levels.

UCD patients exhibit impaired nitrogen detoxification/arginine synthesis caused by defects in urea cycle enzymes [8]. UCDs are recognized as orphan diseases, with a prevalence of 1 in 7,400–13,200 people in Europe. All forms exhibit an autosomal recessive inheritance pattern, with the exception of ornithine transcarbamylase deficiency (OTCD), which is X-linked. Because of severe deficiency or total absence of any of the enzyme activities (carbamoyl phosphate synthetase I, OTC, argininosuccinate synthase, argininosuccinate lyase [ASL], and arginase 1), in the urea cycle or cofactor producer (N-acetylglutamate synthase), ionized ammonia (NH₄⁺) and other precursor metabolites accumulate during the first days of life. Given the high susceptibility of the central nervous system to NH₄⁺ toxicity, UCD patients remain at high risk of metabolic decompensation and irreversible neurologic damage, with long-term impairment and disabilities [9]. Infants with severe UCD rapidly develop cerebral edema at birth, with related signs of lethargy, anorexia, hyper- or hypoventilation, hypothermia, seizures, abnormal posturing, and coma.

**Hepatocytes: Fully Functional Metabolic Units With Potential Stemness Features**

Liver is one of the human body’s key organs, involved in several crucial functions, including the storage and biosynthesis of coenzymes and cofactors, metabolism, excretion, and uptake of nutrients, xenobiotics, and waste products. Hepatocytes represent the liver’s predominant cell population by both number and mass, occupying more than 80% of the parenchymal area [10]. These cells also have the unique capacity of displaying a particular polarity phenotype different from other epithelial cells [10]. With their polygonal shape, they communicate with blood system via the sinusoidal domain, with neighboring cells via the lateral pole, and with the biliary system via the canalicular domain. As a result, hepatocytes are pivotal functional entities, each cell representing its own unique and fully active metabolic unit. Depending on their position in the plate and acinus, hepatocytes exhibit heterogeneous structure, function, and shape. Highly developed intracytoplasmic structures and organelles enable hepatocytes to simultaneously coordinate several complex and crucial functions. They are able, for example, to monitor urea cycle metabolic activities and modulate the levels of ammonia and amino acids derived from the intestine, as well as bilirubin conjugation [11]. Mature hepatocytes have been clearly shown to primarily contribute to liver regeneration via both proliferation and hypertrophy [12]. Furthermore, in response to injuries, hepatocytes have been found to exhibit high plasticity and lose mature cell features (dedifferentiation) while acquiring stem/progenitor cell phenotypes [13].

**Rationale Behind Liver Cell Transplantation**

The complexity of tasks performed by the liver to ensure both its own function and that of other organs has instigated numerous therapeutic strategies designed to counteract its defects. These include chemical drugs, specific diets, and surgical interventions. OLT remains by far the most widely accepted, clinically validated therapeutic approach for liver diseases, especially when conservative treatments have failed. The rarity of donor organs, however, limits the availability of OLT to only a small fraction of patients. Increasing waiting times for grafts contribute to mortality, currently estimated at 15%-20% in children, and others have no access at all to this approach for numerous reasons [14]. Furthermore, OLT has its own limitations, including the immediate inherent risk of the procedure for both recipients and living donors and progressive fibrosis of the graft as reported by long-term follow-up data [15]. These limitations prompted the development of innovative strategies able to temporarily or definitively support liver function without heavy and invasive interventions.

LCT has been proposed as an alternative therapy that offers an interesting concept: liver cells, as carriers of the deficient metabolic functions, can be infused easily because of the liver’s unique blood supply and architecture [16]. LCT has been validated in several animal models, demonstrating the ability of transplanted syngeneic hepatocytes to correct enzymatic defects and thereby enhance recipient survival. In different animal models, transplanted hepatocytes have been convincingly shown to integrate into the recipient livers and support hepatic function. Liver structural or functional defects were mimicked by means of surgical models, such as after partial or total hepatectomy, with administration of pharmacologic treatments such as CCl₄, or the use of genetically modified animals such as the FAH⁺/− mouse, a model of type 1 tyrosinemia, or the Gunn rat, a model of CN syndrome [17]. In the aftermath of these experimental successes, LCT has generated increasing interest for its application in human liver diseases, especially those for which OLT is not the ultimate treatment [18].

**How Does Liver Cell Transplantation Work?**

LCT is a technique aimed to restore liver function deficiencies, be they congenital or acquired, and consists in transplanting a liver cell suspension via the portal vein system into the patient. When intact, the vascular access allows transplanted cells to reach liver sinusoids via the bloodstream. Entrapped infused cells then integrate into the recipient parenchyma after crossing the sinusoidal endothelium and start restoring the deficient liver function. If conditions are favorable, the engrafted cells will proliferate, spread, and restore parenchymal structure and function over the long term. By preserving the native liver, LCT is by far less radical and invasive than OLT, and cell infusions can be repeated with no harm caused to the patient. Furthermore, the cell suspension infusion procedure is simple, thus significantly decreasing hospitalization time while avoiding potential mortality/morbidity risks [16, 19].

**Raw Tissue Material**

The liver cells used are isolated from cadaveric donors primarily in the form of resected segments recovered after split or reduction hepatectomy. Several parameters are critical in this process, potentially significantly influencing both the quality and yield of recovered liver cell suspensions, including the donor’s medical history, preservation conditions, and the isolation procedure.
Donor liver availability remains a major obstacle for the clinical development of LCT programs, as it does for OLT. Trauma-affected livers that are unsuitable for surgical procedures may be considered usable solely if their processing has been optimized for the isolation of high-quality cell suspensions. Living donor liver transplantation can be ethically considered once the technique has been clinically validated.

Isolation Procedure

The hepatocyte suspensions used in LCT are obtained after clinical-grade processing of the liver. This is performed by experienced practitioners following good manufacturing practices (GMP) (Fig. 1). Livers/liver segments are dissociated using collagenase, according to the well-documented and understood two-step perfusion technique [20, 21]. Hepatocytes are isolated from the recovered crude cell suspension after filtration and low-speed centrifugation (Fig. 1). The quality of the obtained hepatocyte population is evaluated in terms of viability, metabolic activity, and sterility levels. Isolated hepatocytes are directly transplanted, preserved in University of Wisconsin cold solution for subsequent use, or cryostored for later infusions. To date, our center in Brussels has processed 118 livers or liver segments and has been able to isolate a mean of 17.4 ± 5 million cells per gram of tissue. The quality of supplied cell suspension is a critical factor in LCT, and efforts are currently being made to efficiently preserve highly viable and metabolically functional hepatocytes until a recipient candidate is identified.

Based on the literature, the lack of a standardized definition of the human hepatocyte and its characterization for LCT, as well as a detailed description of the purity of the recovered cell suspension, is significantly impeding efforts to standardize and optimize the isolation protocol. It is also crucial to define assays that quickly enable efficient investigation of infectious pathogens in the recovered cell suspensions, before their use in the clinical setting. In 2011, a consensus paper was published by several experts in the field [22]. The consortium addressed the strategies that would need to be pursued for improving LCT efficacy. To standardize liver cell products, the implementation of donor selection criteria, along with quality control of the evaluation assays used in the different centers specializing in liver cell transplantation, appears to be a first step to obtain a correlation between liver cell quality and post-transplantation long-term efficiency at both metabolic and engraftment levels [22]. To achieve this aim, a retrospective study investigating these parameters is required.

Preservation of the Cell Suspension

Long-term preservation is a critical issue for the clinical development of LCT, because access to cell suspensions and complete quality control monitoring can be easily scheduled. Cryopreservation is considered the best option for long-term hepatocyte storage [23]. Preclinical investigations on cryopreserved/thawed hepatocytes showed them to exhibit behavior similar to freshly isolated cells after in vivo transplantation, as well as externally supporting liver functions in bio- artificial liver devices [23]. However, investigations are still ongoing to improve cell-viability parameters, which are always significantly altered after thawing. Current strategies focus on improving cryopreservation media and protocols. Furthermore, any improvement demonstrated in vitro should prompt further investigation into its impact on cell engraftment and survival in vivo.

Cell Infusion

In preparation for transplantation, freshly isolated and cryopreserved/thawed hepatocytes are suspended in a formulation medium at a specific concentration after quality control. For liver metabolic defects, 200–400 million hepatocytes per kilogram of body weight are injected, theoretically to achieve 5%–10% of the recipient hepatic mass. The infusion can be scheduled over one or several sessions. A defined range of 30–100 million cells per kilogram and an infusion rate of ≤ 8 ml/kg/hour should be respected per infusion session [24]. The recipient’s liver is accessed via catheter placement in the portal vein or one of its branches (surgically or after intervention radiology). The catheter can be external or internal, depending on the cell infusion schedule. In newborn infants, the catheter can be directly inserted into the umbilical vein. The patency of the catheter is regularly monitored using Doppler ultrasound, with its position checked using contrast Doppler after injection of microbubbles, monitoring their progression toward the portal vein and liver.

During cell infusion, several parameters should be examined, including blood flow, portal pressure, and microbiological quality. Coagulation parameters have been recently added to those that should be monitored, because hepatocytes have been shown to display a tissue factor-dependent procoagulant effect in vitro [25]. Specific transient D-dimer level increases were also detected in one CN patient receiving LCT, although no change was observed in other coagulation parameters. In view of these findings, N-acetylcysteine supplementation is now added to the formulation medium. All hepatocyte transplantations performed in our center were conducted with no safety issues. The major complications that might have occurred were portal vein thrombosis, bleeding, or fatal infection [24]. These complications can be avoided by careful selection of candidates and monitoring of portal pressure and blood flow during infusions. When freshly isolated hepatocytes are used, prophylactic antibioticotherapy may be administered if the bacterial culture results from the donor suspension are still pending.

Follow-Up of Cell-Transplantation Efficiency

Cell-transplantation efficiency correlates with safety features, and transplanted cells are thus tracked early after infusion. By labeling a small portion of the transplanted cells, it is possible to track their early distribution in ectopic tissues, primarily the lung [26]. Long-term tissue distribution follow-up is still lacking at the clinical level. Regarding engraftment follow-up, a vast amount of data from preclinical studies indicates that transplanted cells are first entrapped in the liver sinusoids [27]. After passing the endothelial barrier, surviving cells integrate into the recipient liver parenchyma, a useful microenvironment for the transplanted cells to maintain or upregulate hepatic gene expression.

Cell engraftment rate analysis can be assessed by means of recipient tissue and serum analyses. In experimental models, engrafted cells can be tracked easily using species-specific immunologic staining of the recipient liver tissue or by labeling the donor cells using genetic markers or other approaches.

To assess the engraftment and repopulation potential of human liver cells, animal models have been extensively used and continuously developed, such as immunodeficient mice either crossed or not with animals displaying functional or structural liver diseases [28]. Because immunosuppressant agents do alter hepatocyte engraftment, and because most clinical transplantations do concern allogeneic cells, immunocompromised models
appropriately allow for significant xenogeneic cell engraftment [24]. Lessons learned from in vivo studies have highlighted the need to use small animals to efficiently investigate the mechanisms governing cell engraftment, in addition to evaluating positive and negative modulators by assessing long-term functionality of transplanted cells. In addition, large animals are needed because they better mimic clinical conditions, and because several critical issues such as safety, repeatability, and infusion device optimization can be more easily addressed on larger animals. For the manipulated cells, all data should be carefully analyzed, specifically if phagocytosis and cell fusion occur after transplantation. Needless to say, any cell manipulation should be accompanied by rigorous checks of the impact on cell identity and function before transplantation.

In the clinical setting, transplanted cell engraftment in patients with inborn errors of liver metabolism is indirectly verified by measuring the donor-derived functional protein or its activity’s product in the liver or serum. This can be monitored in the hospital using blood or urine samples recovered at different times postinfusion. By monitoring recipient human leukocyte antigen class I antigen levels with mismatched donor human leukocyte antigen hepatocyte transplantation, it may be possible to assess the extent of liver chimerism [29]. For sex-mismatched transplantation, engrafted cells may be detectable using DNA techniques such as fluorescence in situ hybridization [30]. Metabolic efficiency is typically evaluated using surrogate markers, such as NH4 and glutamine for UCD [1] or bilirubin for CN disease [31]. A more convincing demonstration of cell engraftment is evidence of de novo metabolic activity. This can be revealed by evaluating carbon-13 incorporation into the urea for UCD patients [32] or bilirubin mono- and diconjugate levels in CN patients [33].

Most patients infused by means of isolated hepatocytes were listed for OLT. The LCT procedure is still in its exploratory phase, without being routinely used at the clinical level. All patients at the Brussels center have benefited from OLT, with no sensitization documented so far. Neither rejection episodes nor specific complications were reported, even for those who were transplanted using hepatocytes isolated from more than two different donors (i.e., OTC-deficient patient transplanted with hepatocytes from two donors [34], ASL-deficient patient transplanted with hepatocytes from three donors [35], CN patient transplanted with hepatocytes from two donors) [36]. Screening of the literature did not reveal straightforward information relating to such issues by other centers in the field.

**Clinical Experience of Hepatocyte Transplantation**

**Brussels Center Experience**

Over the last decade, we performed hepatocyte transplantation on 11 pediatric patients presenting with various inborn errors of liver metabolism (Table 1). We performed the first European
The two other children with UCD (both with OTCD) received only cryopreserved hepatocytes and showed no improvement, thus calling into question the quality of the cryopreserved/thawed hepatocytes used. As a matter of fact, a small and transient effect on ammonia levels, with no subsequent impact on urea production, was observed shortly after infusion, compared with what has been reported in other patients transplanted in our own center and others. Given that both patients were female heterozygotes with low residual OTC expression and activity, the quantity of infused cells was probably too small to result in significant improvements in OTC enzymatic activity and urea production. In addition, one of the patients showed perilobular microvacuolar steatosis, which could have hampered cell engraftment. This observation correlated with the absence of detecting Y-positive donor cells as demonstrated by fluorescence in situ hybridization. Also, the transplanted cells could have been rejected owing to an inefficient immunosuppression regimen.

In the CN cases, two children received freshly isolated and cryopreserved hepatocytes [36]. The first was a 9-year-old girl who exhibited a reduction in serum bilirubin values from 17.5 to 13.6 mg/dl postinfusion (lowest value 11.4 mg/dl). The second was a 1-year-old girl who also displayed significantly reduced serum bilirubin values from 17.6 to 13.3 mg/dl (lowest value 6 mg/dl). This patient’s phototherapy schedule was also shortened from 10 to 8 h. Unfortunately, the effects did not remain in either child for more than 6 months, and both underwent OLT. Other metabolic disease patients have undergone liver cell transplant with variable results [24].

THE FUTURE OF LIVER CELL-BASED THERAPIES

It is now well accepted that LCT has shown moderate and transient efficiency in both animal model studies and clinical case reports. The reduced invasiveness of the technique compared with OLT, its schedulability thanks to the potential use of cryopreserved cell suspensions, and its safety together justify its use, at least as a bridge to transplantation. Presently, it is still difficult to draw strong conclusions regarding the efficacy of hepatocyte transplantation because no clinical trial of sufficient size has thus far been performed. Cytonet (Weinheim, Germany) is currently conducting phase II clinical trials in Europe and the United States using cryopreserved hepatocytes for children with OTC and carboxymethyl phosphate synthetase I deficiencies [39]. Preliminary data are expected to be available in the second quarter of 2016.

To extend this promising technique to routine clinical use, it is crucial that all developments or improvements made to this approach take into account its primary advantage as a noninvasive method. To improve LCT efficacy, several strategies have been appraised, including improving raw material quality, hepatocyte cryopreservation protocols, and novel cell sources that could substitute isolated hepatocytes and optimize both the quality and quantity of cell suspensions for transplant. The domino concept involved in transplanting hepatocytes isolated from one metabolically diseased liver to a patient exhibiting a different metabolic defect was demonstrated to be feasible and efficient [40], and should be considered for increasing the pool of raw material. Other strategies are focusing more on the recipient liver and ways to ensure durable acceptance of transplanted donor cells.

---

Table 1. Crigler-Najjar and urea cycle defect patients who benefited from liver cell transplantation in the Brussels center

| Pathology                  | Age (yr) | Cells used                                      | Post-transplantation effects                                                                 | References        |
|----------------------------|----------|------------------------------------------------|---------------------------------------------------------------------------------------------|-------------------|
| OTC deficiency             | 14 mo    | Cryopreserved hepatocytes                       | Decrease of ammonia, urea increase, psychomotor improvement                                | Stéphenne et al., 2005 [38] |
| ASL deficiency             | 3.5 yr   | Freshly isolated and cryopreserved hepatocytes  | Decrease of ammonia, detection of donor cells in liver, enzymatic activity, psychomotor improvement | Stéphenne et al., 2006 [35] |
| OTC deficiency             | 5 yr     | Cryopreserved hepatocytes                        | No benefit                                                                                 | Not published     |
| OTC deficiency             | 2 yr     | Cryopreserved hepatocytes                        | No benefit                                                                                 | Not published     |
| OTC deficiency             | 3 yr     | Cryopreserved stem/progenitor cells              | Decrease of ammonia and orotic acid levels, detection of male donor cells in two different biopsies | Sokal et al., 2014 [34] |
| CN syndrome                | 9 yr     | Freshly isolated and cryopreserved hepatocytes   | Decrease of serum bilirubin levels                                                        | Lysy et al., 2008 [36] |
| CN syndrome                | 1 yr     | Freshly isolated and cryopreserved hepatocytes   | Decrease of serum bilirubin levels                                                        | Lysy et al., 2008 [36] |
| CN syndrome                | 23 yr    | Cryopreserved stem/progenitor cells              | Slight decrease in serum bilirubin levels after 3 months                                   | Not published     |

Abbreviations: ASL, argininosuccinate lyase; CN, Crigler-Najjar; OTC, ornithine transcarbamylase.
The transient LCT efficacy observed at the clinical level may be indicative of either a delayed extinct function or an elimination of transplanted cells, or both. Although very few details are available regarding cell-induced immune responses, rejection after hepatocyte transplantation seems to differ from that documented post-OLT [41]. Receptor activation by Fas and tumor necrosis factor ligands has been shown to be involved in inducing both hepatocyte apoptosis and rejection [42]. Although cell labeling and noninvasive quantified tracking have been used to evaluate cell homing and detection a few days after transplantation, long-term detection for estimating late rejection still remains a major challenge [26].

Further investigations on the immune responses of transplanted cells and a deeper knowledge regarding the pathways involved in the rejection/acceptance processes may help us better understand long-term engraftment mechanisms and result in fine-tuned prevention approaches by optimizing prophylaxis and immunosuppression regimens. Preconditioning of the recipient liver before cell transplantation has been proposed as a potential strategy to improve engraftment efficiency and durable survival of transplanted cells [22]. Strategies that have been used to prime the host liver targeted both recipient cell death and blockade of cell proliferation, combined with subsequent strong mitogenic stimuli. However, most protocols could not be extrapolated to clinical settings. Combining regional transient portal ischemia and irradiation has been shown to be more suitable for the clinical setting, although standardized implementation of such procedures still needs further optimization [22].

Freshly isolated primary hepatocytes remain the optimal cell candidates. Although they are significantly dependent on donor availability, reconstituting their niche for better quality in vitro could help improve their survival and functional quality while avoiding cryopreservation and the consequent alterations [43].

Most clinical trials conducted so far have used isolated hepatocytes, with limited functional post-transplantation effects. Immediately after isolation, viability (because of anoikis) and several mature differentiated liver cell functions were shown to be significantly decreased, which may lower the transplanted hepatocytes' engraftment level. Strategies have endeavored to bioengineer liver cells for clinical purposes by generating an extracellular microenvironment for hepatocytes. To this end, both synthetic compounds and extracellular matrices from decellularized livers have been used. Reconstruction of these 3D organoids clearly revealed their potential to significantly prolong both hepatocytes' survival and metabolic functions [44]. This observation highlights the usefulness of such liver bioengineered models for transplantation and pharmaco-toxicological screening studies [45]. Recently, organoids using induced pluripotent stem cells (iPSCs) have been transplanted into ectopic sites, thereby revealing their ability to form three-dimensional vascularized and functional liver structures [46]. Large-scale culture of these organoids in bioreactors may certainly help improve cell transplantation schedulability, as well as the quality of cells to be delivered. By maintaining cell-cell interactions, organoid structures may also be more protected against cryopreservation [47]. Standardizing the requirements for the generated organoids (such as nutrients, oxygen delivery, and cell-biomaterial combinations) is mandatory for efficient and durable post-transplantation functionality [48]. Ongoing studies will likely pave the way to help us understand how mature hepatocytes maintain their initial features depending on the extracellular matrix (ECM) and nature of the critical bioactive molecules.

The post-thawing quality of cryopreserved hepatocytes should also be well characterized, including determination of survival features, shelf life, purity, and immunogenicity characteristics. Advances must also be made in cryopreservation protocols for in vitro nonproliferating cells, as well as in pretransplantation formulation media, using, for example, biocompatible and biodegradable scaffolds that could improve survival immediately after infusion. Given that medicine and regulatory agencies have approved the use of cryopreserved hepatocytes as validated models for drug-metabolizing enzyme studies, improvement of cryopreservation protocols has been subject to extensive investigations. Initially, prior cryopreservation protocols focused on slow freezing strategies. New innovative approaches including vitrification to avoid the crystalline state, with or without encapsulation to confer mechanical protection, are currently under investigation using mainly nonhuman isolated hepatocytes. Vitrification, for instance, has been shown to maintain nonhuman hepatocyte attachment efficiency, in addition to hepatocytes' metabolic function. To date, vitrification of rat hepatocytes that were previously attached has been shown to be more effective compared with hepatocytes in suspension [49]. Recently, native antifreeze glycoproteins have been shown to significantly increase the viability of human embryonic liver cells after thawing, owing to their ability to inhibit ice recrystallization [50]. For future validation of this procedure on human hepatocytes, rigorous consideration of standardized accessibility and possible clinical translation appears mandatory. The cotransplantation of supportive nonparenchymal cells with hepatocytes may be an option, as demonstrated by several studies reporting improved homing and engraftment when using hepatic stellate cells or liver sinusoidal endothelial cells [51, 52].

Establishing appropriate inclusion/exclusion criteria for both available and discarded livers could counteract the increasing donor shortage and competition with OLT. We need to clearly define and standardize optimal handling and care of the LCT selected grafts, cell injection route, and immunosuppression regimen across all centers developing this field. Currently, tacrolimus is administered in first line, offering the benefit of being well tolerated, with very few side effects.

Over the last few years, extensive research has been conducted into the development of alternative cell sources to replace mature hepatocytes. Stem/progenitor cells are strong contenders, extensively investigated because of their well-documented proliferation and plasticity characteristics. Given that hepatocyte-like cells can be efficiently generated from pluripotent stem cells, embryonic stem cells and iPSCs are currently being evaluated to decipher both liver development and deficient underlying mechanisms, in addition to being alternative cell populations in liver cell-based therapy [53]. Efforts are being made to develop stringent differentiation models strongly associated with stable genetic signature and expression profiles [54]. Accordingly, novel hepatocytic markers involved in ECM modulation, as well as cell-cell and ECM-cell interactions, have been proposed as standard markers for predicting in vitro performance and long shelf-life of GMP-compliant cell suspensions. Other strategies have been aimed at isolating hepatic progenitor cells that are activated after mature hepatocytes have lost their regenerative potential. Under such liver injury settings, the number of these progenitor cells was shown to significantly increase...
in response to hepatocyte apoptosis and senescence, which may facilitate their isolation and further analyses in the dish. This is the case for the E3 ubiquitin ligase Mdm2 mouse model (AhCreCmMdm2flox/flox), in which hepatic progenitor cells were shown to display bilineage differentiation potential, along with significant repopulation of the damaged hepatic parenchyma [55]. A deep understanding of hepatocyte subpopulations may also help to evaluate their proliferation, differentiation, and repopulation potential. Very recently, a subpopulation of pericentral and diploid hepatocytes has been documented to participate in hepatocyte renewal in normal liver homeostasis [56].

Naive and genetically manipulated cells have been studied in vitro and in different animal models in vivo. Recent improvements in molecular biology tools enable the introduction of targeted modifications within any genomic sequence into living cells and organisms. Such highly targeted genome editing (including clustered regularly interspaced short palindromic repeat [CRISPR] technology) may efficiently delete or insert specific sequences, thereby allowing for significantly modifying endogenous genes, even in cells and organisms known to be hard to manipulate [57, 58]. Using a CRISPR-Cas9 system, human pluripotent stem cells have been efficiently manipulated in vitro [59], whereas the FAH mutation was corrected in vivo in hepatocytes of a mouse model pertaining to human hereditary tyrosinemia [60]. Ongoing studies primarily aim to improve delivery techniques and repair efficiency. This will likely foster the clinical development of techniques used to treat human monogenic liver defects.

Figure 2. Potential key advantages of mesenchymal stem cell use for liver disease treatment. Abbreviation: OLT, orthotopic liver transplantation.
functional or structural, as well as its severity and chronicity. The complexity of the liver regeneration process and involved cell types may therefore suggest the recruitment of different stem cell compartments. For liver metabolic diseases, and particularly those displaying intransigent alterations, tissue replacement is crucial. For correcting the disease, cell transplantation is dependent on hepatocytic functional differentiation of the stem cells used and their ability to sufficiently provide the deficient enzyme [65]. Finally, the optimal number of stem cells believed to enable 1%-2% of the recipient liver mass to be attained should be better outlined in studies on both metabolic benefit and durable survival.

When conducting a search using the key words mesenchymal stem cells and liver diseases, 48 studies were found on the clinicaltrials.gov Web site, primarily concerning acquired liver diseases. Mesenchymal stem cells from bone marrow, menstrual blood, umbilical cord, adipose tissue, and liver have been used. With respect to liver metabolic diseases, only Wilson’s disease, homozygous familial hypercholesterolemia (bone marrow MSCs), UCD, and CN syndrome (liver MSCs) were documented as being targeted by MSCs. Promethera Biosciences, a spinoff of the Université Catholique de Louvain, is currently investigating liver mesenchymal cells in both UCD (n = 14) and CN syndrome (n = 6). Safety has been proven using different Hepastem doses, whereas cell product potency is to be demonstrated in the approved phase llb/III trial that started in late 2015.

Most of the MSCs translated to clinical use did not display any safety complications (Fig. 2). Their potency was clearly shown at the preclinical level, although it is still under investigation in several clinical trials. The development and wide clinical use of liver stem cell therapy may certainly benefit from our deeper understanding of the expansion and storage of MSCs from other tissues, including umbilical cord and bone marrow. This will enable us to overcome the scarcity of donor material, yet supportive data are required regarding the stability of the stemness quality of the MSCs used after large-scale culture, if higher doses are needed. The development and fine-tuning of stem cell therapy programs is a work in progress, regularly contributed to by data gathered in studies investigating stem cell biology, liver regeneration processes, and tissue reconstitution mechanisms.

**CONCLUSION**

LCT is a very promising approach for treating inborn errors of liver metabolism, offering several advantages for the patient, including its noninvasive and safe aspects. After demonstrating interesting results at the clinical level, new solutions for this therapeutic approach are currently being designed and evaluated to bypass the documented limitations and extend liver cell use to wider clinical applications. These future developments also require a deep knowledge of regulatory frameworks to launch specific clinical trials that will allow us to assess the beneficial role of cell therapy in patients with unmet medical needs.

**AUTHOR CONTRIBUTIONS**

M.N., F.D., and E.M.S.: manuscript writing, final approval of the manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

E.M.S. has uncompensated intellectual property rights, is a compensated consultant, has compensated research funding, and has uncompensated stock options. The other authors indicated no potential conflicts of interest.

**REFERENCES**

1. Raghueer TS, Garg U, Graf WD. Inborn errors of metabolism in infancy and early childhood: An update. Am Fam Physician 2006;73:1981–1990.
2. Fagiuoli S, Daina E, D’Antiga L et al. Monogenic diseases that can be cured by liver transplantation. J Hepatol 2013;59:595–612.
3. Servedio V, d’Apolito M, Maiorano N et al. Spectrum of UGT1A1 mutations in Crigler-Najjar (CN) syndrome patients: Identification of twelve novel alleles and genotype-phenotype correlation. Hum Mutat 2005;25:325.
4. Maruo Y, Verma IC, Matsui K et al. Conformational change of UGT1A1 by a novel missense mutation (p.L131P) causing, Crigler-Najjar syndrome type I. J Pediatr Gastroenterol Nutr 2008;46:308–311.
5. Sneath N, Bakker CT, de Knecht RJ et al. Crigler-Najjar syndrome in the Netherlands: Identification of four novel UGT1A1 alleles, genotype-phenotype correlation, and functional analysis of 10 missense mutants. Hum Mutat 2010;31:52–59.
6. Lee WS, McKiernan PJ, Beath SV et al. Bile bilirubin pigment analysis in disorders of bilirubin metabolism in early infancy. Arch Dis Child 2001;85:38–42.
7. Bosma PJ. Inherited disorders of bilirubin metabolism. J Hepatol 2003;38:107–117.
8. Scaglia F, Brunetti-Pierri N, Kleepo S et al. Clinical consequences of urea cycle enzyme deficiencies and potential links to arginine and nitric oxide metabolism. J Nutr 2004;134(suppl):2775S–2782S; discussion 2796S–2797S.
9. Nassogne MC, Héron B, Touati G et al. Urea cycle defects: Management and outcome. J Inherit Metab Dis 2005;28:407–414.
10. Mutsch A. The unique polarity phenotype of hepatocytes. Exp Cell Res 2014;328:276–283.
11. O’Brien PJ, Chan K, Silber PM. Human and animal hepatocytes in vitro with extrapolation in vivo. Chem Biol Interact 2004;150:97–114.
12. Miyaoa Y, Ebato K, Katoh H et al. Hyper- trophy and unconventional cell division of hepatocytes underlie liver regeneration. Curr Biol 2012;22:1166–1175.
13. Chen Y, Wong PF, Sjeklocha L et al. Mature hepatocytes exhibit unexpected plasticity by direct dedifferentiation into liver progenitor cells in culture. Hepatology 2012;55:563–574.
14. Kamath PS, Wiesner RH, Malinchoc M et al. A model to predict survival in patients with end-stage liver disease. Hepatology 2001;33:464–470.
15. Struecker B, Raschzok N, Sauer IM. Liver support strategies: Cutting-edge technologies. Nat Rev Gastroenterol Hepatol 2014;11:166–176.
16. Jorns C, Ellis EC, Nowak G et al. Hepatocyte transplantation for inherited metabolic diseases of the liver. J Intern Med 2012;272:201–223.
17. Gilgenkrantz H. Rodent models of liver repopulation. Methods Mol Biol 2010;640:475–490.
18. Bonavita AG, Quaresma K, Cotta-de-Almeida V et al. Hepatocyte xenotransplantation for treating liver disease. Xenotransplantation 2010;17:181–187.
19. Najimi M, Smets F, Sokal EM. Hepatocyte transplantation: Current and future developments. Curr Opin Organ Transplant 2008;12:503–508.
20. Strom SC, Jirtle RL, Jones RS et al. Isolation, culture, and transplantation of human hepatocytes. J Natl Cancer Inst 1982;68:771–778.
21. Berardinis S, Lombard C, Evraerts J et al. Gene expression profiling and secretome analysis differentiate adult-derived human liver stem/progenitor cells and human hepatic stellate cells. PLoS One 2014;9:e86137.
22. Puppi J, Strom SC, Hughes RD et al. Improving the techniques for human hepatocyte transplantation: Report from a consensus meeting in London. Cell Transplant 2012;21:1–10.
23. Stéphenne X, Najimi M, Sokal EM. Hepatocyte cryopreservation: Is it time to change the strategy? World J Gastroenterol 2010;16:1–14.
24. Smets F, Najimi M, Sokal EM. Cell transplantation in the treatment of liver diseases. Pediatr Transplant 2008;12:6–13.
25. Stéphenne X, Nicastro E, Eckhoudt S et al. Bivalirudin in combination with heparin to control mesenchymal cell procoagulant activity. PLoS One 2012;7:e42819.
26. Defresne F, Tondreau T, Stéphenne X et al. Biodistribution of adult derived human liver stem cells following intraportal infusion.
in a 17-year-old patient with glycogenosis type 1A. Nucl Med Biol 2014;41:371–375.

27 Gupta S, Rajavashri P, Sokhi R et al. Entry and integration of transplanted hepatocytes in rat liver plates occur by disruption of hepatic sinusoidal endothelium. Hepatology 1999;29:509–519.

28 Weber A, Groyer-Picard MT, Franco D et al. Hepatocyte transplantation in animal models. Liver Transpl 2009;15:7–14.

29 Ng IO, Chan KI, Shek WH et al. High frequency of chimerism in transplanted livers. Hepatology 2003;38:989–998.

30 Wang L, Chen YM, George D et al. Engraftment assessment in human and mouse liver tissue after sex-mismatched liver cell transplantation by real-time quantitative PCR for Y chromosome sequences. Liver Transpl 2002;8:822–828.

31 Jansen PL. Diagnosis and management of Crigler-Najjar syndrome. Eur J Pediatr 1999;158 (suppl 2):S89–S94.

32 Mew NA, Yudkoff M, Tuchman M. Stable isolates in the diagnosis and treatment of inherited hyperammonemia. J Pediatr Biochem 2014;4:57–63.

33 Scharschmidt BF, Blanckaert N, Farina FA et al. Measurement of serum bilirubin and its mono- and diconjugates: Application to patients with hepatobiliary disease. Gut 1982;23:643–649.

34 Sokal EM, Stéphane X, Ottolenghi C et al. Liver engraftment and repopulation by in vitro expanded adult derived human liver stem cells in a child with ornithine carbamoyltransferase deficiency. JIMD Rep 2014;13:65–72.

35 Stéphane X, Najimi M, Sibille C et al. Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency. Gastroenterology 2006;130:1317–1323.

36 Lysy PA, Najimi M, Stéphane X et al. Liver cell transplantation for Crigler-Najjar syndrome type I: Update and perspectives. World J Gastroenterol 2008;14:3464–3470.

37 Sokal EM, Smets F, Bourgeois A et al. Hepatocyte transplantation in a 4-year-old girl with peroxisomal biogenesis disease: Technique, safety, and metabolic follow-up. Transplantation 2003;76:735–738.

38 Stéphane X, Najimi M, Smets F et al. Cryopreserved liver cell transplantation controls ornithine transcarbamylase deficient patient while awaiting liver transplantation. Am J Transplant 2005;5:2058–2061.

39 Alexandrova K, Griesel C, Barthold M et al. Large-scale isolation of human hepatocytes for therapeutic application. Cell Transplant 2005;14:845–853.

40 Stéphane X, Debray FG, Smets F et al. Hepatocyte transplantation using the domino concept in a child with tetrabioterin non-responsive phenylketonuria. Cell Transplant 2012;21:2765–2770.

41 Bumgardner GL, Gao D, Li J et al. Rejection responses to allogeneic hepatocytes by reconstituted SCID mice, CD4, KO, and CD8 KO mice. Transplantation 2000;70:1771–1780.

42 Mignon A, Guidotti J.E, Mitchell C et al. Selective repopulation of normal mouse liver by Fas/CD95-resistant hepatocytes. Nat Med 1998;4:1185–1188.

43 Stéphane X, Najimi M, Ngoc DK et al. Cryopreservation of human hepatocytes alters the mitochondrial respiratory chain complex 1. Cell Transplant 2007;16:409–419.

44 Mitaka T, Ooe H. Characterization of hepatic-organoid cultures. Drug Metab Rev 2010;42:472–481.

45 Leite SB, Roosens T, El Taghdouini A et al. Novel human hepatic organoid model enables testing of drug-induced liver fibrosis in vitro. Biomaterials 2016;78:1–10.

46 Takebe T, Sekine K, Enomura M et al. Vascularized and functional human liver from an iPSc-derived organ bud transplant. Nature 2013;499:481–484.

47 Magalhães R, Nagraha B, Pervaiz S et al. Influence of cell culture configuration on the post-cryopreservation viability of primary rat hepatocytes. Biomaterials 2012;33:829–836.

48 Booth C, Soker T, Baptista P et al. Liver bioengineering: Current status and future perspectives. World J Gastroenterol 2012;18:6926–6934.

49 Wang X, Magalhães R, Wu Y et al. Development of a modified vitrification strategy suitable for subsequent scale-up for hepatocyte preservation. Cryobiology 2012;65:289–300.

50 Leclère M, Kwok BK, Wu LK et al. C-linked antifreeze glycoprotein (C-FAGBP) analogues as novel cryoprotectants. Bioconjug Chem 2011;22:1804–1810.

51 Ding BS, Nolan DJ, Butler JM et al. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. Nature 2010;468:310–315.

52 Dusabineza AC, Najimi M, van Hul N et al. Hepatic stellate cells improve engraftment of human primary hepatocytes: A preclinical transplantation study in an animal model. Cell Transplant 2015;24:2557–2571.

53 Szolcndiack D, Farnworth SL, Lucendo-Villarín B et al. Accurate prediction of drug-induced liver injury using stem cell-derived populations. Stem Cells Translational Medicine 2014;3:141–148.

54 Villarín BL, Cameron K, Szolcndiack D et al. Polymer supported directed differentiation reveals a unique gene signature predicting stable hepatocyte performance. Adv Healthc Mater 2015;4:1820–1825.

55 Lu WY, Bird TG, Boulter L et al. Hepatic progenitor cells of biliary origin with liver regeneration capacity. Nat Cell Biol 2015;17:971–983.

56 Wang B, Zhao L, Fish M et al. Self-renewing diploid Asn2(+) cells fuel homeostatic renewal of the liver. Nature 2015;524:180–185.

57 Joung JH, Sander JD. TALENs: A widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol 2013;14:49–55.

58 Sander JD, Joung JH. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 2014;32:347–355.

59 Mali P, Yang L, Esvelt KM et al. RNA-guided human genome engineering via Cas9. Science 2013;339:823–826.

60 Yin H, Xue W, Chen S et al. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. Nat Biotechnol 2014;32:551–555.

61 Najimi M, Khoo DN, Lysy PA et al. Adult-derived human liver mesenchymal-like cells as a potential progenitor reservoir of hepatocytes? Cell Transplant 2007;16:717–728.

62 Nombela-Arrieta C, Ritz J, Silberstein LE. The elusive nature and function of mesenchymal stem cells. Nat Rev Mol Cell Biol 2011;12:126–131.

63 Petersen BE, Bowen WC, Patrene KD et al. Bone marrow as a potential source of hepatic oval cells. Science 1999;284:1168–1170.

64 Banas A, Yamamoto Y, Teratani T et al. Stem cell plasticity: Learning from hepatogenic differentiation strategies. Dev Dyn 2007;236:3228–3241.

65 Hengstler JG, Brulport M, Schommann W et al. Generation of human hepatocytes by stem cell technology: Definition of the hepatocyte. Expert Opin Drug Metab Toxicol 2005;1:61–74.