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Concise review: Cell therapies for hereditary metabolic liver diseases - Concepts, clinical results, and future developments
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ABSTRACT

The concept of cell-based therapies for inherited metabolic liver diseases has been introduced for now more than 40 years in animal experiments, but controlled clinical data in humans are still not available. In the era of dynamic developments in stem cell science the “right” cell for transplantation is considered as an important key for successful treatment. Do we aim to transplant mature hepatocytes or do we consider the liver as a stem/progenitor driven organ and replenish the diseased liver with genetically normal stem/progenitor cells? Although conflicting results from cell tracing and transplantation experiments have recently emerged about the existence and role of stem/progenitor cells in the liver, their overall contribution to parenchymal cell homeostasis and tissue repair is limited. Accordingly, engraftment and repopulation efficacies of extrahepatic and liver derived stem/progenitor cell types are considered to be lower compared to mature hepatocytes. Based on these results we will discuss the current clinical cell transplantation programs for inherited metabolic liver diseases and future developments in liver cell therapy.

Hepatocytes or stem cells: which cell drives parenchymal liver maintenance and regeneration of the liver?

The the long prevailing view in liver physiology and pathophysiology has localized a hepatic stem/progenitor cell compartment at or near the Canals of Hering, in periductular glands and in extrahepatic bile duct structures [1-3]. Some researchers found proof that the stem/progenitor cells countinuously generate hepatocytes and bile duct cells and maintain the normal turnover of parenchymal liver cells [4]. According to the streaming concept stem/progenitor cell derived hepatocytes continously migrate from the periportal areas towards central vein structures, mature and express differential metabolic activities. Cell fate tracing experiments have attributed Sox9 expressing cells with stem/progenitor cell properties in the liver as well as in several other epithelial organs [5-7]. More recent data challenge this view and provide evidence for a more complex situation in the liver. Following acute injury of the liver a $CK19^+$, $Sox9^+$ and $LGR5^+$ cells emerge in the liver and generate hepatocytes [8]. These cells were identified randomly throughout the liver and could not be traced in the unperturbed liver. Although $CK19^+$,$Fogl1^+$,$LGR5^+$ cells could still be derived from a (not identified) stem cell in the liver another study indicates that mature hepatocytes can dedifferentiate and aquire properties of a liver stem/progenitor cell including the expression of progenitor cell and bilary lineage associated cell surface markers. “Knock down” of the Hippo/YAP signalling pathway in hepatocytes resulted in emergence of liver stem/progenitor cells. Upon restauration of YAP expression at least some progenitor cells turned back to the hepatocyte phenotype indicating a bidirectional role of the Hippo/YAP pathway. Isolated mature hepatocyte derived stem/progenitor cells in this study demonstrated self-renewal and engraftment capacity at a single cell level [9].

Regardless of the nature and phenotype of the stem/progenitor cells the overall contribution of these cells in various injury models has been questenioned. After partial hepatectomy and acute injuries the liver regenerates by replication of mature hepatocytes [10-12]. Chronic injuries, in which replication capacity of mature hepatocytes are exhausted or chemically blocked, small oval shaped cells expressing stem cell and biliary lineage markers appear in periportal areas [13-15]. It was hypothesized that these cells form hepatocytes and regenerate the liver. This view was challenged in murine cell fate tracing models [16-18]. They show that non-hepatocytes in the liver do not significantly contribute to hepatocyte regeneration in

acute as well as various chronic injury liver models, which have previously been found to induce stem/progenitor cells. The results have recently been confirmed in a chimeric liver transplantation model [19]. Although cells with characteristic stem/progenitor markers were induced in chronic liver injury the contribution to hepatocyte regeneration was neglectable. Additionally, all of these studies did not find evidence for stem/progenitor contribution to hepatocyte maintenance in normal livers.

Engraftment and repopulation in chimeric liver transplantation models

After intraportal or intrasplenic injection engraftment and integration into the hepatic cords of primary mature hepatocytes has been demonstrated in many animal species and in humans. Hepatocytes are entrapped into liver sinusoids and cross the endothelial barrier most likely by mechanical forces. The transplanted cells connect to neighboring hepatocytes by re-expression of gap junction proteins, survive long term and respond to growth stimuli [20]. Engraftment in the adult liver similar to mature hepatocytes has also been demonstrated for fetal hepatoblasts, embryonic and induced pluripotent stem derived hepatocyte-like cells as well as hepatic cells derived from direct reprogramming protocols [21-23]. In a competitive liver repopulation mouse model (heterozygous alb-uPA mice) engraftment and repopulation capacities of mature hepatocytes were superior to early and late hepatoblasts as well as ES- and iPS- derived hepatocyte-like cells [24]. Any of the tested postnatal non-hepatocytes such as hematopoietic stem cells or mesenchymal stromal cells did not engraft long term and did not repopulate the liver. The data indicate that stem/progenitor derived cells should be forced to mimic primary hepatocytes as close as possible for proper engraftment and repopulation (**Figure 1**). Rat hepatoblasts and human/rat liver derived stromal cells were shown to engraft and gradually replace resident hepatocytes in the normal liver [21,25]. In our own experiments this phenomenon has not been observed for mouse and human hepatoblasts or stromal cells after transplantation into unperturbed livers of mice so far. Signals in the liver, which maintain continuous growth and termination of proliferation, should be identified to support this concept.

Clinical experience with hepatocyte transplantation in hereditary metabolic liver disease

From the clinical point of view, transplantation of hepatocytes or hepatocyte-like cells may represent an alternative to orthotopic liver transplants for the correction of genetic disorders resulting in metabolically deficient states. The aim of hepatocyte transplantation in metabolic disease is to partially replace the missing function without the need to replace the whole organ. Almost 30 children and adults who received liver cell therapy for metabolic liver disease are reported in literature [26-28]. Clinical therapies up to now have been performed by infusing fresh or cryopreserved primary hepatocyte suspensions, which were isolated from donated organs. The availability of high quality liver tissue for cell isolation, however, has slowed the widespread application of this therapy. Furthermore, the clinical situation of target patients is rarely immediately life threatening and often acceptable conventional therapies are available. Therefore, the potential benefit must be carefully weighed against any possible complications, such as side effects from immunosuppression, hepatocyte embolisation of the pulmonary vascular system, sepsis or hemodynamic instability.

The results of hepatocyte transplantation for several metabolic liver diseases have been encouraging with demonstrable therapeutic effects, although long-term correction of metabolic deficiencies in the majority of cases has not been reported. Therapeutic benefit has been reported in a girl with Crigler–Najjar Syndrome Type I, which is a recessively inherited metabolic disorder characterized by severe unconjugated hyperbilirubinaemia [29]. Isolated hepatocytes were infused through the portal vein and partially corrected plasma bilirubin levels for more than 11 months. Similarly, a 9-year-old boy received 7.5×10^9 hepatocytes, infused via the portal vein, which resulted in a decrease in of bilirubin levels from 530 ± 38 µmol/L (mean ± SD) before to 359 ± 46 µmol/L [30] after transplantation. Hughes et al. also reported a 40% reduction in bilirubin levels in a Crigler–Najjar Syndrome Type I patient following transplantation of hepatocytes [31]. Although these data demonstrate efficacy and safety, however, a single course of cell application seems not sufficient to correct Crigler–Najjar Syndrome Type I completely.

Sustained response was reported in a patient with argininosuccinate lyase deficiency after repeated hepatocyte transplantation. Engraftment of the transplanted cells was analyzed in repeated liver biopsies for more than 12 month by fluorescence in situ hybridization for the Y-chromosome and by measurement of tissue enzyme activity [32]. Promising results have also been obtained in a 47-year-old woman suffering

from glycogen storage disease type 1a, an inherited disorder of glucose metabolism resulting from mutations in the gene encoding the hepatic enzyme glucose-6-phosphatase [33]. 2×10^9 ABO-compatible hepatocytes were infused into the portal vein. Nine months after cell transplantation, her metabolic situation had clearly improved. Successful hepatocyte transplantation has also been achieved in a 4-year-old girl with infantile Refsum disease, an inborn error of peroxysome metabolism, leading to increased levels of serum bile acids and the formation of abnormal bile acids [34]. A total of 2×10^9 hepatocytes from a male donor were given during eight separate intraportal infusions. Abnormal bile acid production (for instance pipecholic acid) had decreased by 40% after 18 months. Hepatocyte transplantation has been used successfully to treat inherited factor VII deficiency [35]. Two brothers (aged 3 months and 3 years) received infusions of 1.1 and 2.2×10^9 ABO-matched hepatocytes into the inferior mesenteric vein. Transplantation clearly improved the coagulation defect and decreased the necessity for exogenous factor VII to approximately 20% of that prior to cell therapy. As with the other metabolic liver diseases, hepatocyte transplantation has been shown to provide a partial correction of urea cycle defects. Patients showed clinical improvement, reduced ammonia levels and increased production of urea [28, 36-39].

Based on these initial results two major programs, which are sponsored by biotech companies, currently evaluate safety and efficacy of liver cell therapy for inherited metabolic liver disease in controlled clinical trials. The Cytonet program utilizes cryopreserved isolated primary human hepatocytes for intraportal application in patients with urea cycle disorders. The Promethera program isolates and propagates a liver stromal cell for transplantation in various hereditary metabolic diseases.

The Cytonet program

The Cytonet investigational medicinal product “Human Heterologous Liver Cells” (HHLivC) is being developed as an advanced therapy medicinal product for the treatment of urea cycle disorders, a rare disorder, which is characterized by inherited deficiencies of enzymes involved in the urea cycle. HHLivC consists of a cryopreserved dispersion of liver cells prepared for intraportal administration. HHLivCs are isolated from non-transplantable donor organs and refined in a manufacturing process under Good Manufacturing Practices (GMP) conditions [40]. For long-term storage in the vapor phase over liquid nitrogen the final study

medication is prepared with a cryopreservation solution and subsequently filled in single final medication bags. The study medication is infused via the portal vein through branches of the inferior or superior mesenteric vein. $3x10^8$ viable liver cells/kg body weight are infused in equal fractions over period of six days. After initial applications of HHLivCs in four patients with urea cycle disorders with promising results a total of 21 patients with Ornithine Transcarbamylase (OTC), Carbamoylphosphat-Synthetase I (CPS1) and Argininosuccinate Synthase Deficiencies (ASSD) at the age of 0-5 years have subsequently been recruited for two pivotal studies (CCD02 and CCD05) in Germany, USA and Canada [39]. Primary efficacy endpoints as defined by the incidence of severe (>500µM) and moderate (>250µM) hyperammonemic events are compared with matched historical controls (n = 63 patients). In addition, diagnostic studies were conducted to investigate and establish an *in vivo* ^{13}C-ureagenesis assay. Performance of the urea cycle cannot be easily assessed *in vivo*, since *in vitro* enzyme activity does not always reflect *in vivo* metabolic flux in UCD patients [41]. Furthermore, established biochemical markers such as plasma urea, ammonia or glutamine concentrations do not reliably indicate the functioning of the urea cycle because the plasma concentrations of these metabolites also depend on other factors, mainly the diet, conservative therapy or trigger factors like e.g. infections. Therefore, direct outcome parameters, which are unaffected by concomitant treatments and diet, are desirable to determine the effectiveness of HHLivC therapy *in vivo*. The ^{13}C-ureagenesis assay is able to directly measure the patient's metabolic capability to produce urea from oral sodium acetate. This ureagenesis assay was adopted for the studies based on published protocols [42, 43] and is used as an additional efficacy endpoint in studies CCD02 and CCD05. To allow patients access to the new treatment option that enables prevention or delay of hyperammonemic crises in this pediatric population with a high medical need, the Market Approval Application was submitted to the EMA in December 2013 based on currently available interim data.

The Promethera program

HepaStem® from Promethera Biosciences, a Belgian biotech company, consists of a Heterologous Human Adult Liver Progenitor Cells (HHALPC) suspension, which is generated from normal adult human liver tissue. The cells are described as fibroblastic in morphology expressing mesenchymal as well as hepatocytic markers

and can be expanded from cultured primary hepatocytes [25, 44]. Preclinical studies in animals have shown safety and engraftment in recipient livers. The first transplantation of HepaStem® in a 3-year-old girl suffering from ornithine carbamoyltransferase (OTC) deficiency showed 3% engraftment after 100 days as determined by Y chromosome FISH immunostaining. The clinical outcome of this patient was not reported [45] in the publication. At the annual meeting of the Society for the Study of Inborn Errors of Metabolism (SSIEM) in October, 2014, safety of the treatment was demonstrated in 14 patients with urea cycle defects and in 6 patients with Crigler-Najjar Syndrome. Analysis of clinical efficacy in the patients suffering from urea cycle defects showed variable results [46].

Future developments to close the bottle necks of liver cell therapies

Clearly, one of the most obvious bottle necks for wide spread distribution of liver cell therapies for metabolic liver diseases is the availability of high quality cells for transplantation. Applying more advanced differentiation protocols supported by fine-tuned cytokine and small molecule supplementation or microRNA modulation, the derivation of hepatocyte-like cell from ES and iPS cells has made considerable progress [47-50], although maturity of the cells in various differentiation protocols is still lacking and engraftment after transplantation is low. With iPS technology the production of unlimited numbers of patient-derived or immunologically compatible hepatocyte-like cells can be envisioned and genetic enginering technology will allow the correction of disease-causing mutation in patient-derived iPS cells as recently demonstrated for alpha-antitrypsin deficiency-specific iPS cells [51-53]. As proof of concept for safety and applicability of iPS cell based therapies, iPS cells from a mouse model of hereditary tyrosinemia (fumarylacetoactetehydrolase deficiency, $FAH^{-/-}$–mice) have been be genetically corrected by a lentivirally delivered construct and such engineered iPS cells have been subjected to tetraploid embryo aggregation experiments [54]. The resulting newborn mice did neither show signs of FAH-deficiency nor growth of iPS cell related tumors and exhibited a normal live span.

Recently, major advances in the generation of human iPS cells have been achieved and the elucidation of preferential stoichiometries of the four reprogramming factors [55, 56] inspired the design of suitable polycistronic vectors for the delivery of the reprogramming factors [57]. Importantly, human iPS cells can now be generated by vectors, which do not integrate the transcription factors into the genome of the target cells, or can even be induced by small molecule compounds [58-61]. Although insertional mutagenesis by integrating vectors can be avoided, the cells still carry the risk of “a mutational history”, when they are derived from postnatal tissues.

Isolation protocols for ES and iPS derived hepatocytes have been developed, but the risk of teratoma formation after transplantation due to remaining stem cells remains and provides a hurdle for clinical grade manufacturing. To avoid the state of pluripotency a partial reprogramming approach has been developed. Those protocols result in the generation of hepatic multipotent progenitor-like cells, which can still be expanded and utilized for differentiation toward hepatocytes or cholangiocytes [62]. This direct reprograming approach attempts to generate cells exhibiting a hepatic phenotype by over-expression of combinations of liver-enriched transcription factors.

Several groups have now demonstrated the induction of a mature hepatic phenotype in mouse and human fibroblasts and shown transplantability of the cells in liver repopulation animal models [63-66]. Although many functional features of primary hepatocytes could be demonstrated, fidelity of global gene expression of the so-called iHeps has been questioned [67, 68]. Furthermore, no data are available on genetic stability and tumorigenicity of the cells in transplanted animals. However, the concept of transprogramming somatic cells across the respective germ-layer lineages is rather young and similar obstacles need to be overcome for the direct induction of neural cels, cardiac cells, and blood cells [69].

For stable and long term phenotypic correction in a variety of hereditary metabolic liver diseases engraftment of the transplanted cells must be improved. A repeated application of cells has been shown to increase the engraftment in animals, a procedure, which has already been incorporated in the Cytonet® protocol. Further increase of engraftment can be achieved by pharmacological interventions with vasodilatotors, which increase the capacity of liver sinusoids for infused hepatocytes or the application of anti-inflammatory drugs such as indomethacin [70-72]

Various methods and genetic animal models, which harness a selective advantage of transplanted cells over resident cells, are widely available and demonstrate a high degree of stable cellular repopulation after hepatocyte transplantation. Most of these priniciples, however, are not applicable in humans. A combination of liver irradiation/portal ischemia and portal venous embolization has been successfully tested in rats and non-human primates and shown to increase engraftment compared to conventional transplantation [73, 74]. Concerns about long-term consequences in neonates and small children, however, have slowed the translation of these techniques into clinical practice.

Summary

Approved protocols for the isolation, cryopreservation and storage of primary human hepatocytes from donated liver organs have now been developed. Controlled trials with those cells in standardised clinical protocols will soon show, whether hepatocyte infusions can achieve clinical benefits in defined patient populations with hereditary metabolic liver diseases. The engraftment efficacy of primary hepatocytes may already be good enough to show significant clinical benfits in some indications such as “early onset” urea cycle disorders. For other disorders such as LDL deficiency,

clinically applicable liver repopulation strategies are needed to stably correct the clinical phenotype. Alternative cell sources, which are most likely based on ES, iPS or direct programming technologies will likely replace the isolated primary cells in the future, if safety and engraftment efficacy can be further increased.

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Legends to Figures:

Fig. 1: Competetive repopulation fitness of various human cell sources in heterozygous immunodeficient uPA – mice. (A) alb-uPAtg mice express the urokinase-type plasminogen activator (uPA) in heptaocytes, resulting in activation of metalloproteinases, breakdown of extracellular matrix, and fibrin dissolution. In heterozygous alb-uPAtg mice a few endogenous hepatocytes (light brown) could silence the transgene and compete with transplanted EGFP-transgenic cells (green) to repopulate the liver. **(B)** Extent of liver tissue repopulation subject to various cell transplant sources. aHSC, adult murine hematopoietic stem cells ($1x10^6$ cells, n=21 animals); MSC, murine mesenchymal stromal cells derived from bone marrow ($1x10^6$ cells, n=23 animals); ES/iPS-HLC, murine embryonic or induced pluripotent stem cell derived hepatocyte-like cells ($1x10^6$, n=9 animals, fHBC (ED 11.5d), murine fetal hepatoblasts at day 11.5 post conception ($1x10^6$ cells, n= 7 animals); murine fetal hepatoblasts at day 13.5 post conception ($5x10^5$ cells, n= 4 animals, $1x10^6$ cells, n=4 animals); adult HC: adult murine hepatocytes($5x10^5$ cells, n= 5 animals, $1x10^6$ cells, n=5 animals). Results were partially adapted from Haridass et al. [24].

Fig 2. Generation of hepatocyte-like cells from various cellular sources. Embryonic stem cells (ES) can be directly differentiated into hepatocyte-like cells (HLC). Somatic cells (SC) such as fibroblasts are induced by transcription factors (TFs) to become induced pluripotent stem cells (iPS). Similar to ES cells HLC can be generated by cytokines and supportive media compositions. Somatic cells can be partially programmed by transduction of TFs into endoderm progenitor cells, which have bipotential (hepatocyte-like cells, cholangiocyte-like cells) differentiation capacity. Somatic cells can be directly converted by TFs into hepatocyte-like cells and further differentiated by cytokines and media.