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Organoid Transplant Approaches for the Liver

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Abstract

Organoid technology is a state-of-the-art cell culture tool that has revolutionized study of development, regeneration, and diseases. Human Liver Organoids (HLOs) are now derived from either adult stem/progenitors or pluripotent stem cells (PSCs), emulating cellular diversity and structural symphony akin to the human liver. With the rapid rise in decompensated liver disease conditions only treated by liver transplant therapy, HLOs represent an alternate source for transplantation to address the ongoing shortage of grafts. Although ongoing advancements in bioengineering technology have moved the organoid transplant approach to the next level, sustained survival of the transplanted tissue still eludes us towards functional organ replacement. Herein, we review the development of HLOs, and discuss promises and challenges on organoid transplant approaches.

Keywords

liver organoid; transplantation; transplantation sites; applications; safety; efficacy

Introduction

The incidence of diseases of civilization has accelerated astronomically over the past few decades [1]. Chronic liver disease is one such disease that accounts for approximately

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Conflict of interest

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2 million deaths annually due to factors such as imbalanced diet, excessive alcohol consumption, drug induced injury, viral infection and genetic mutations [2–4]. The rapid rise in these diseases has led to a shortage of donors for organ transplantations. Since 1983, orthotopic liver transplantation has been considered as the only treatment for patients who have lost their liver functions. As of 2020, there were still 107,603 patients on the waiting list, with 39,035 transplant recipients, and only 18,316 donors available at the time [5, 6]. Unfortunately, there was a 25% reduction in liver donors due to various reasons, leading to a crunch in overall liver transplantation [7]. Moreover, a majority of these chronic liver disease patients fail to meet the criteria in place for transplantation. Thus, there is a critical need to come up with alternative solutions to the shortage crisis and failure of acceptance affecting cadaveric transplants.

With a goal to supplement organ transplant needs, various cell culture methods have been formulated for developing renewable and transplantable hepatocytes [2]. For example, the discovery of epigenetic reprogramming has sparked significant advancements into understanding cell fate acquisition, thereby, turning the focus towards directed differentiation or direct reprogramming of adult stem/progenitors or PSCs into hepatocyte-like cells [8]. Another area of interest includes the development of three-dimensional miniature liver models, called human liver organoids, grown from adult or pluripotent stem cells, which have both structural and functional resemblance to *in vivo* organs (Fig. 1). Thus, hepatocytes generated *in vitro*, and organoids have attracted a great deal of attention, as reports have indicated their efficacious potential when transplanted. Organoid based approach is of particular interest here as the patient's own cells can be used to generate unlimited amount of polarized hepatocytes existing in organized hepatic tissues accompanied by the emergence of secretory, metabolic and excretory functions. Additionally, recent advances of organoid technology can develop vasculature allowing them to engraft quickly and survive longer periods, thus supporting multi-faceted challenges informed by cell transplantation approaches [9, 10].

Organoids contain a collection of specific cell types that develop from stem cells or organ progenitors through self-organization by cell sorting and spatially restricted lineage commitment, in a manner similar to *in vivo* [11]. In 1907, Wilson first reported the phenomenon of isolating sponge cells self-assembled to form entire organisms [12]. In the 2000s, there were many reports of 3D generation of the intestine and cerebral cortex, which led to their dissemination in various clinical applications [13, 14]. Following these advancements the organoid field has never looked back, spreading to multiple organs such as the retina, kidney, lung, and liver [15]. Excitingly, in 2017 induced pluripotent stem cells (iPSC) derived retinal pigment epithelial (RPE) cells were transplanted in human patients with age-related macular degeneration (AMD). However, no organoids have been transplanted clinically with a goal to restore liver functions.

The liver is a complex organ with a variety of functions. Hepatocytes are the main epithelial component, accounting for 80% of the adult liver volume and over 60% of total cells in the liver; they are responsible for metabolism, protein secretion, detoxification, and bile production [16]. It has been known for some time that mature liver cells are extremely difficult to grow *in vitro*. Chick embryonic livers were reconstituted in 1960, yet hepatocytes

were made from mouse and human embryonic stem cells only recently in 2004 [17, 18]. Reports of liver organoid generation quickly followed in the 2010s [19, 20]. Although the goal of organoid research is the elucidation of developmental processes and construction of pathological organs *in vitro* with hopes of developing and testing therapeutic options, there is also an increasing enthusiasm to utilize organoids in future clinical applications. The success of transplantation depends on many factors, including the location, the preconditioning method, and the use of immunosuppressive agents in the recipient liver. For example, administration through the portal vein, splenic arteries, and intraperitoneal sites have each shown therapeutic benefits, but the risk of increased portal pressure, gastric or splenic infarction, and microemboli in the lungs remain high [21]. Therefore, the next step is to resolve these shortcomings to turn organoid transplantation into reality.

Past and present efforts to complement liver transplantation

Past and present cell therapies for liver diseases can be divided into four main categories: (i) hepatocyte transplantation (ii) non-parenchymal cell transplantation (iii) hepatic progenitor transplantation and (iv) bioartificial liver trials (Fig. 2 A). These techniques are extensively studied in animals and translated into clinics, providing a foundational lesson for shaping future organoid therapy.

Hepatocyte transplantation

Hepatocyte transplantation (HTx) is a well-studied technique that has been applied both preclinically and clinically to treat diseases such as Crigler–Najjar syndrome (CNS), Urea cycle disorders (UCD), and Factor VII deficiency [22]. In this procedure hepatocytes are usually obtained from human deceased donors or partially resected liver samples using enzymatic perfusion techniques. The cells are transplanted immediately or cryopreserved for future use [23]. Mito *et al.* were the first to transplant hepatocytes in humans through the splenic pulp, however no clinical improvements were observed [24]. Approximately 10^9 to 10^{10} hepatocytes are usually transplanted into the liver through the portal vein; it is accessed either transhepatically, by an umbilical vein or by a peripheral mesenteric vein using a catheter or implantable device [25, 26]. HTx has been reported to ameliorate CNS by decreasing total bilirubin from 26.6 to 14 mg/dL, wherein transplanted hepatocytes were still viable after 11 months in the patient liver [27]. Numerous patients have undergone HTx for UCDs, and in some cases, reinstituted enzymatic activity led to a decrease in ammonia and an increase in urea levels [28]. Factor VII deficiency was also mitigated in two brothers using HTx, decreasing the need for Factor VII by 20% in these patients [29]. Following these advances, several clinical trials with HTx to treat liver-based metabolic deficiencies have been approved and are awaiting results (ClinicalTrials.gov, Identifier: NCT01345578 and NCT01465100). Overall, HTx trials have shown that hepatocyte based therapies has the potential to treat at least some hereditary liver diseases. However, the main drawback of HTx is the lack of engraftment and host immune responses that preclude its use in the clinics [23].

Non-parenchymal cell transplantation

Mesenchymal stem cell transplantations (MSC Tx) were initiated in the 1990s to modulate immune reactions. MSCs maintain specific ECM components and secrete soluble factors to control immune cells [30]. The MSCs are obtained from a variety of sources including the bone marrow and umbilical cord blood, and usually cultured for 4 generations before being transplanted or cryopreserved [31]. Shortly after that, this technique was suggested for the treatment of liver cirrhosis and fibrosis. MSCs in the range of 10^5 to 10^9 are infused by intravenous, intrahepatic, or intrasplenic injection, however it is unclear whether this therapy contributes to liver regeneration in the long period [32, 33]. In 2017, MSC Tx was reported to treat liver graft rejection; it was found that liver functions and allograft histology were improved in addition to Treg/ Th17 ratio, $CD4^+$ T-cell activation [34]. Lin *et al.* also found improvements in survival rate, liver function, and reduction in infection when bone marrow derived MSCs were transplanted in acute-on-chronic liver disease patients [35]. More recently in 2018, liver cirrhosis patients exhibited enhancement of Child-Pugh scores, MELD (Model for End-Stage Liver Disease) scores and liver functions after intravenous injection of umbilical cord MSCs [36]. There are multiple clinical trials utilizing MSCs for liver disease treatment in different phases now showing promises [37]. Moroni *et al.* have shown the feasibility and efficacy of autologous macrophage therapy in liver cirrhosis patients. The clinicians injected 10^9 cells in 9 patients and reported a reduction in MELD scores over 90 days [38]. Several clinical trials are also underway to test the reversal of liver cirrhosis using autologous endothelial progenitor cells. The endothelial progenitor cell delivery studies utilize the hepatic artery and portal vein route for administration of $CD133^+$ cells (ClinicalTrials.gov, Identifier: NCT01333228 and NCT03109236). Thus, non-parenchymal cell transplantation has established itself as a supporting technique to modulate immune function and hepatocyte survival in transplantations. That said, many clinical trials are yet to bear any substantially positive results due to the poorly defined nature of the MSCs [32].

Hepatic progenitor transplantation

Hepatic progenitor cells (HPCs), while their physiological nature is widely debated, can regenerate to produce hepatic parenchymal cells, hepatocytes, and/or bile ductular epithelial cells following injury. The progenitor cells are mainly obtained from resected liver samples by means of enzymatic digestion and cell separation techniques. The cells are then grown for a couple generations before being transplanted [39]. HPCs have advantages compared to adult hepatocyte because they can differentiate into hepatocytes and cholangiocytes [40, 41]. Several studies have also suggested that HPCs have a minimum risk of alloreactivity and can be directed towards the required lineage based on clinical needs [42, 43]. Khan *et al.* have reported transplanting HPCs through the hepatic artery to manage hyperbilirubinemia in biliary atresia. The authors observed a threefold drop in total bilirubin after just one month [44]. In 2019, human $CD34^+$ progenitor cells were transplanted in five patients through the portal vein or hepatic artery, and these patients exhibited marked improvements in serum bilirubin and albumin levels [45]. Khan et al. have also supplemented their past efforts by transplanting human fetal liver-derived stem cells in 25 patients, reporting a significant decrease in bilirubin, creatinine, and MELD scores after 6 months [46]. Very few clinical trials have been approved for cirrhotic patient with human fetal liver cell

transplantation. One trial used splenic artery injection under radiological guidance with up to 10^9 cells, which resulted in slightly improved Child-Pugh score and no serious adverse events (ClinicalTrials.gov, Identifier: NCT01013194). Promethera Bioscience has developed Hepastem, human adult liver progenitor cells, for phase II transplantation in CNS, UCD, and other chronic liver failure patients. The company has reported transplantation in over 40 patients with up to 73% survival rate who had minor complications [47, 48]. Hepatic progenitor cells can be effectively transplanted into the liver as was shown in hepatocyte therapy approach. Nonetheless, safety and efficacy remains the prime factor preventing HPCs from being used in the clinic as they play a major role in development of primary liver cancers [49].

Bioartificial liver (BAL) trials

A BAL device is an extracorporeal liver support system that performs regular functions and detoxification. Most BAL systems in clinical trials I to III have been reported to encapsulate clonal derivatives of hepatocellular carcinoma cells known as C3A cell line, or freshly isolated porcine hepatocytes in a hollow fiber and extracellular matrix or a bioreactor [50]. In 2002, molecular adsorbent re-circulating system (MARS) and plasma exchange was used to treat liver failure in 60 patients, showing only a 10% mortality rate in the patients [51]. Prometheus standard medical therapy (SMT) was used to treat 68 chronic liver patients, however no improvements in survival were observed [52]. Lee *et al.* treated 6 acute liver failure patients with the LifeLiver BAL system, and reported decrease in serum ammonia and MELD scores [53]. Several improvements were reported in 2020; Wu *et al.* reported the fabrication of a whole liver by integrating gelatin and hepatocytes in a decellularized matrix, while Hou *et al.* described a chitosan/gelatin scaffold that had higher hepatocyte viability (50 % increase) and mechanical strength [54, 55]. In 2018, scientists reported that Extracorporeal cellular therapy (ELAD), a hollow fiber based immortalized C3A human cell line system, was used on 96 alcoholic hepatitis patients with promising results. The results indicated that patients below 46.9 years and MELD < 28 had higher survivability with ELAD, however the study failed to show better results for the elderly [56]. Subsequently, ELAD was used in 78 patients, but the study failed after 600 days as survival dropped below 70% [57]. Additionally, human ESC (Embryonic Stem Cell) and iPSC derived cells have been considered for BAL devices for a long time [58]. Most recently, Takeishi *et al.* used hiPSC derived hepatocytes, endothelial cells, and biliary epithelial cells to repopulate a liver scaffold and transplanted it into immunocompromised rats [59]. There are several clinical trials underway now with ELAD, potentially advanced by organoid based system with more metabolic functionality. Yet, the myriad of adverse effects and the inability of BAL to improve survival over longer periods overshadow its potential [60].

Liver organoids

There has been remarkable progress in cell based liver disease treatments over the last decade, although no clinical trial has progressed to mainstream medical therapies. Most clinical trials have yet to increase survivability in elderly patients. This uncertainty has fueled the rise of human liver organoids (HLOs), 3D self-organizing assemblies derived from adult stem/ progenitor cells or PSCs that differentiate into single or multi-tissue

structures. These organoids have shown encouraging developments over the years. The different types of these HLOs are discussed below (Fig. 1).

Epithelial organoids

Epithelial organoids are derived from a single germ layer, and small fragments of these organoids are capable of regenerating into entire organoids (Fig. 1) [61]. The first reported generation of a primitive hepatic organoid was using highly proliferative rat hepatocytes in 1999 by Mitaka et al. These pseudo-organoids did not proliferate or have complex functional capacities [62, 63]. Following this advance, Huch *et al.* in 2013 developed a culture system of leucine-rich-repeat-containing G-protein-coupled receptor 5+ (Lgr5+) murine bile duct stem cell derived organoids that grew indefinitely *in vitro* (Table 1) [19]. The investigators also reported that clonal hepatocytes were generated from human epithelial cellular adhesion molecule + (EpCAM+) cells in the periphery of the adult bile ducts [41]. The production methods of organoids continue to advance further. Due to the improved oxygenation in the spinner flasks, organoids derived from Lgr5+ adult human stem cells rapidly proliferated to reach an average 40-fold cell expansion after 2 weeks, compared with a 6-fold expansion in static cultures [64]. Hu *et al.* have successfully synthesized hepatocytes derived from fetal and adult cells with 3D culture by collagenase perfusion [65]. Yamamoto *et al.* reported on the development of induced hepatocytes from murine dermal fibroblasts that formed 3D aggregates, wherein re-aggregation was important for final maturation and was activated by Hippo signaling [66]. Further development in technique and culture media led Prior *et al.* to successfully isolate bipotent Lgr5+ human embryonic hepatoblasts which retain their ability to form hepatocytes or cholangiocyte organoids [67].

Multi-tissue and multi-organ organoids

Multi-tissue organoids are derived from co-culture of at least two germ layers or co-differentiation of PSCs, whereas multi-organ organoids are more complex, and incorporate multiple organs with interconnected structures (Fig. 1) [61]. In 2013, 3D transplantable and vascularized HLOs were first created from human iPSCs [20]. After inducing hepatoblasts from human iPSCs and mixing them with stromal cells such as mesenchymal cells and vascular endothelial cells, the cells self-aggregated and self-organized into a spherical liver bud [20]. Thus, the generated HLOs lacked long-term self-renewal, but the organoids efficiently vascularized within 48 hours and engrafted to produce albumin and were involved in the metabolism of drugs, bile, and cholesterol, resembling the fetal liver at around 2^{nd}-3^{rd} trimester stage. A single-cell RNA sequencing analysis of the iPSC derived HLOs revealed that each cell type had a distinct sign of maturation that depended on the endothelial-mesenchymal-endodermal communications [68]. In another method, Stevens *et al.* bio-fabricated seeds of human liver tissue, which are essentially multi-tissue organoids, by engineering human primary hepatocytes, endothelial cells, and fibroblasts based on cell signaling networks [69]. More recently, iPSC generated endoderm derived HLOs were shown to acquire more functional characteristics of mature hepatocytes *in vitro* [70]. Koike *et al.* have succeeded in constructing hepato-biliary-pancreatic (HBP) connections in hiPSC derived organoid culture by emulating the foregut-midgut boundary, a prospective region where HBP precursors segregate out to form individual organs [71]. Even with these advances, one limitation of using iPSCs is that they can become genetically unstable due

to extensive proliferation and exposure to reprogramming factors [72]. However, proper monitoring and current Good Manufacturing Practice (cGMP) can be used to overcome this problem. Thus, organoids are the potential tools to address the current transplantation needs.

Transplantation of Liver organoids at different sites

Preclinically, liver organoids have been transplanted into various sites *in vivo* (Fig. 2B) (Table 1 and 2). There are various sites of engraftment: liver, mesentery, kidney capsule, and omentum, but a complete replacement of liver function is yet to be reported. Numerous surgical techniques have been developed that could be applied to transplant organoids with varying regimes of drugs and immune-deficient organoids to prevent graft rejection. Therefore, various reports of successful transplantation have been recorded, and some of the transplantations are described below.

Orthotopic (Liver)

The methods for delivery into the liver are divided into three main routes: a splenic, or portal vein injection, or liver implantation. Huch et al reported that transplantation of Lgr5+ stem cell-derived murine liver organoid by splenic injection into FRG mice increased survival (Table 1) [19]. Researchers have also reported that human adult liver EpCAM+ cell derived HLOs showed albumin production in mice with liver injury (Table 2) [41]. Stevens *et al.* also transplanted human artificial liver “seeds” into mice experiencing liver failures and succeeded in expanding these multi-tissue organoids compared to randomly organized cells [69, 73]. HiPSC generated endoderm derived HLOs have also been efficiently engrafted in mice livers, and they expressed human albumin at even day 32 following a single intrasplenic injection [70]. Zhou *et al.* attempted to inject ROSA26 C57BL/6 murine single cells or organoids by splenic injection to implant them into the liver directly. The result showed that an organoid injected by direct orthotopic implantation showed limited signs of engraftment, and turned necrotic over time due to graft rejection [74]. Hu *et al.* injected HLOs derived from fetal liver as single cells into immunodeficient mice with a damaged liver, in the form of a splenic injection. Post-injection human albumin in the serum had risen 200-fold to more than 200 μg/ml on average, leading us to believe this therapeutical method can successfully repopulate damaged livers [65]. Other reports have shown that orthotopic administration of liver organoids from hESC, hiPSC, and canine hepatic progenitors to mice, rats, and dogs with liver damage respectively, reduced AST and ALT levels and improved liver function [75–77]. Our group has transplanted iPSC derived HLOs into the liver, kidney, and brain of immunodeficient mice, and extensive vascularization was observed leading to blood perfusion and eventual hepatocyte maturation. The transplantation of this liver bud into immunodeficient liver failure mice significantly improved their survival rate [20, 78, 79]. More recently, Tsuchida *et al.* posited that portal vein injection of fetal liver and hiPSC derived HLOs was safe and effective in treating rat chronic liver damage. They also reported about 70% replacement of the damaged liver after 120 days with an almost 100% survival rate [80]. The intravenous transplantation techniques are limited in their capacity for the treatment of intrahepatic injury, but the limitations can be overcome by techniques involving biliary reconstruction. Recently, it has been reported that primary cholangiocyte derived organoid replacement in the liver and bile ducts of mice with extrahepatic biliary injury has

improved the prognosis [81, 82]. All in all, orthotopic liver transplantation has shed much needed light on the cellular and functional requirements needed for the treatment of liver diseases.

Ectopic

Mesentery—At the time of this publication, we are the only group to transplant hiPSC derived liver buds (LBs) into the mesentery. The human serum albumin levels in the blood 30 days after transplantation are higher when transplanted into the proximal mesentery (>1000 ng/ml) compared to the distal mesentery (<200 ng/ml) [78]. The mice exhibited human-specific drug metabolism after about 30 days. Additionally, the survival rate of ganciclovir-induced liver failure TK-NOG mice 30 days after the transplantation of LBs was >60% when compared to sham [20]. Thus, transplantation of liver organoids in the mesentery not only induces maturation but also adds therapeutic benefits.

Cranial window—Cranial window is a well-studied technique in brain physiology that has been repurposed to study vascular biology and engineering due to high vascularity and ease of optical access. Takebe *et al.* reported transplantation of hiPSC derived liver organoids into NOD/SCID mice to intravitally assess their vascularization potential. It was observed that the endothelial cells in transplanted organoids evolved functional anastomosis to recipient blood vessels at 48 hrs, increasing the chances of engraftment and maturation [83].

Kidney capsule—The transplantation of HLOs into the subrenal capsule is one of the most studied sites and technically the easiest for investigating vascularization, growth and maturation [84]. Furthermore, human albumin in the blood 30 days after transplantation was similar to those transplanted into the proximal mesentery (>800 ng/ml) [78, 83]. There have been reports claiming that both the survival rate and liver functions were improved after functional iPSC derived HLOs were transplanted into the renal capsule of mice with acute liver failure [85]. Almost 70% of the mice showed recovery from acute liver failure upon the HLO transplantation and human albumin was detected at the concentration of 1128±338.1 ng/ml and 988.2±660.3 ng/ml at 2 and 7 days after transplantation. Most recently, Harrison *et al.* showed a scalable method for PSC derived HLO production that was transplanted in the subrenal capsule with Matrigel and FGF2 [86].

Omentum—Saito *et al.* described transplantation of murine liver organoids into a pocket under the kidney capsule and the omentum of BALB/cAJcl mice. These organoids were generated by mouse-immortalized hepatocytes and nonparenchymal cells in a radial flow bioreactor. The expression of albumin mRNA increased at 8 weeks after the transplantation of the murine organoids in the omentum and kidney [84]. Therefore, the omentum serves as a site for maturation of liver organoids.

Ectopic transplantation of HLOs has taught us a lot regarding the niche, vascularization, and cellular support required for proper engraftment. However, from a biological perspective, a perfect site for transplantation of HLOs to complement the liver is yet to be discovered. For clinical application, we must also consider less invasive routes for administration of the organoids and safety of patients with poor systemic conditions. However, it is

noteworthy that intra-portal administration is common in some hepatocyte transplantation studies [87]. The omentum is another alternative candidate for transplantation site since it has ample blood vessels for vascularization, and its proximity and unique microenvironment is amenable to liver development.

Applications of transplanted HLOs

Organoid transplantation has opened up new avenues of research in hopes of supporting disease treatment and liver transplantation (Fig. 3). Extensive experimentations in the field have borne many facets of utility:

Modeling development

Most HLOs are fetal in nature and express immature liver markers present in fetal development, therefore organoids are the perfect tool to understand the developmental process by tracking their maturation. For example, we recently applied single cell RNA sequencing technology to analyze human iPSC derived multi-cellular organoids over time, highlighting a unique role of inter-cellular communication during liver development, otherwise inaccessible in human. Increasing evidence indicate that the inclusion of stromal population is essential for post-transplant development of hepatic vascularization and function [20, 78, 83, 88]. Nie *et al.* reported that endothelial and mesenchymal cells played a major role in liver development and regeneration. The authors' data suggests that endothelial and mesenchymal cells provide a unique microenvironment to promote hepatic gene expression, and thereby regeneration in acute liver failure mice [85]. Transplanted iPSC derived HLOs indicated that VEGF crosstalk potentiates the endothelial network thereby promoting hepatoblast differentiation [68]. Recent developments in organoid technology and transplantation techniques have led to a better understanding of the multilineage communication inherent to liver development.

Disease modeling

Kras mutant murine liver organoids have been used to model Cholangiocarcinoma (CCA). The p3 gene was deleted in these organoids and transplanted subcutaneously in mice. The organoids formed tumors that bore a striking resemblance to CCAs [89]. Mori *et al.* transplanted HLA-A2 human hematopoietic stem cells and HLA mismatched human liver bud organoids into humanized mice to model alloimmune response. The researchers found that human T cells invaded the HLOs to induce graft rejection [90]. Finally, genetically modified tumorigenic murine liver organoids were transplanted in NOD-SCID mice to study tumor development. The resulting neoplastic tissue spread to form cholangiocarcinoma, however dual blockade of FF-Erk signaling significantly reduced tumor volume [91]. Additionally, our group has found human specific metabolism of ketoprofen or debrisoquine in HLOs transplanted in TK-NOG mice [20]. Even though HLOs are being used in drug screening, these transplantation procedures present an opportunity to study the systemic effects of various drugs in trial. Combined, these studies demonstrate the utility of liver organoids in the study of human diseases and investigation of therapeutic targets.

Auxiliary therapy

Organoids are already being used in different organs for multiple clinical trials as an auxiliary therapy to treat metabolic diseases by complementing as little as 1% of functional tissue [73]. Additionally, Cao *et al.* devised a method to transplant tumorigenic murine liver organoids into NOG mice by subcutaneous implantation. The scientists tested anti-cancer drugs on the organoids and showed that they reduce proliferation [92]. Another group generated hepatocyte-like organoids from hiPSCs and transplanted them by intrasplenic route in athymic nude rats. The liver-failure rats had higher survival rates due to human albumin secretion and improvements in ALT and AST levels [93]. In 2020, COMMD1-Deficient canine liver organoids were genetically restored using lentiviral transduction. The canine organoids were transplanted in an intrahepatic injury model using a portal catheter, which resulted in long-term survival of the organoids and led to improved excretion of copper in bile [94].

Organ replacement

Organ replacement is being attempted in multiple studies to replace whole parts of organs as a functional unit and to treat diseases. Zhang *et al.* reported hiPSC derived PFG that formed HLOs. The authors transplanted the HLOs in Alb-TRECK/SCID mice and showed that this recued the mice from liver failure [95]. In 2010, another group showcased the regenerative effect of fetal liver and iPSC HLOs by portal vein injections in a rat Retrorsine/Partial Hepatectomy model. The transplantation results exhibited an incredible 70% replacement of the damaged liver. The authors were also able to detect albumin 14 days post-transplantation verifying the method as a potential treatment for chronic liver disease [80]. More recently, it was shown that human cholangiocyte derived organoids could reconstruct the extrahepatic biliary tree. When these organoids were transplanted in extrahepatic biliary injury mouse model, the cells from the organoid replaced the native bile duct and resulted in increased bile excretion [81]. The same group also succeeded in transplanting the organoids in an *ex vivo* human liver from deceased donors undergoing normothermic perfusion. The organoid grafted livers had repaired intrahepatic ducts and were maintained for up to 100 hours without any major complications [82].

Challenges ahead

As with many new technologies, organoids have many limitations and challenges that need to be overcome before moving to a clinical setting. Clinical safety, efficacy and ethical guidelines have yet to be established for liver organoid transplantation, but effective interdisciplinary collaboration between clinicians, researchers, and pharmaceutical industries will facilitate the proper protocol development. There are also fears of HLOs undergoing genetic instability leading to the formation of teratomas, however that has largely been assuaged even with long term cultures [41]. The results of hepatocytes transplantation for many metabolic liver diseases such as CNS Type 1, glycogen storage disease type 1a have been encouraging, but persistent recovery of at least ~1 to 5% of total liver function remains a major challenge, which is thought to be sufficient for clinical improvement. The transplanted hepatocytes engrafted into the liver and continued to confer functional augmentation for a few months at best [87]. Another key limitation of organoids is the lack

of innate cellular diversity, and their global maturation over time. Proper environment and support which include differential and incremental exposure to hormones, oxygen, nutrients, and growth for differentiation and proliferation is an equally important issue, but this too can be addressed by mixing in specific cells that provide a unique niche to confer precise trophic effects [96]. Stromal cells aid in this context by forming connective tissue [97]. Furthermore, there is a crucial need to develop more robust and well-defined biomaterials for proper maturation of organoids [98]. Ye *et al.* have made great strides in this regard by developing a chemically define hydrogel that is capable of promoting proliferation and differentiation to induce specific liver functions in HLOs [99].

One of the most important aspects is mitigating hypoxia that becomes more and more difficult with the size and complexity of organoids. Recent data suggest that liver sinusoidal cells can promote vascularization and thus support survival over long periods of time [100]. Yanagi *et al.* attempted transplantation of 3D printed scaffold free LBs in ligated and transected liver parenchyma. The authors reported that the LBs did not survive long due to non-viable parenchyma and lack of vasculature [101]. However, there have been great strides in addressing the deficiency of angiogenetic features and implantation over the years [102]. In 2018, a microsurgical method enabled bleeding free engraftment of 3D printed LBs in rat liver [103]. Another major challenge is translating the therapeutic experiments to the clinic since results do not always conform between species. Nevertheless, Sampaziotis *et al.* have shown much promise by transplanting cholangiocyte organoids to repair bile ducts in deceased human transplant donor livers, similar to mice, ex vivo using fluoroscopic guidance [82]. Although there is a dire need for transplants, the cost, safety and ethical concerns of HLOs have not been addressed in the long run. Even though safety is a prime issue, recent developments in several models have shown time and again that when controlled properly liver organoids can be safely transplanted in a clinical setting without any risk of integration at extrahepatic sites [104].

Conclusion

HLOs have become a versatile tool for understanding the development and pathology of the liver with a wide range of potential clinical applications including personalized medicine and cell therapy. For this purpose, the cooperation of physicians, biologists, bioengineers, and specialists, has become necessary [105]. Clinical applications and basic research with HLOs have the potential to grow tremendously over the next decade, especially in liver transplantation. Organoid therapy may be a powerful adjunctive or alternative to deceased donor or living donor liver transplantation in the future because of its ability to suppress liver damage. This therapy may be effective for the recipient of liver transplantation, or patients who are ineligible for liver transplantation, or even as organ preservation in donor livers to inhibit damage or to promote regeneration.

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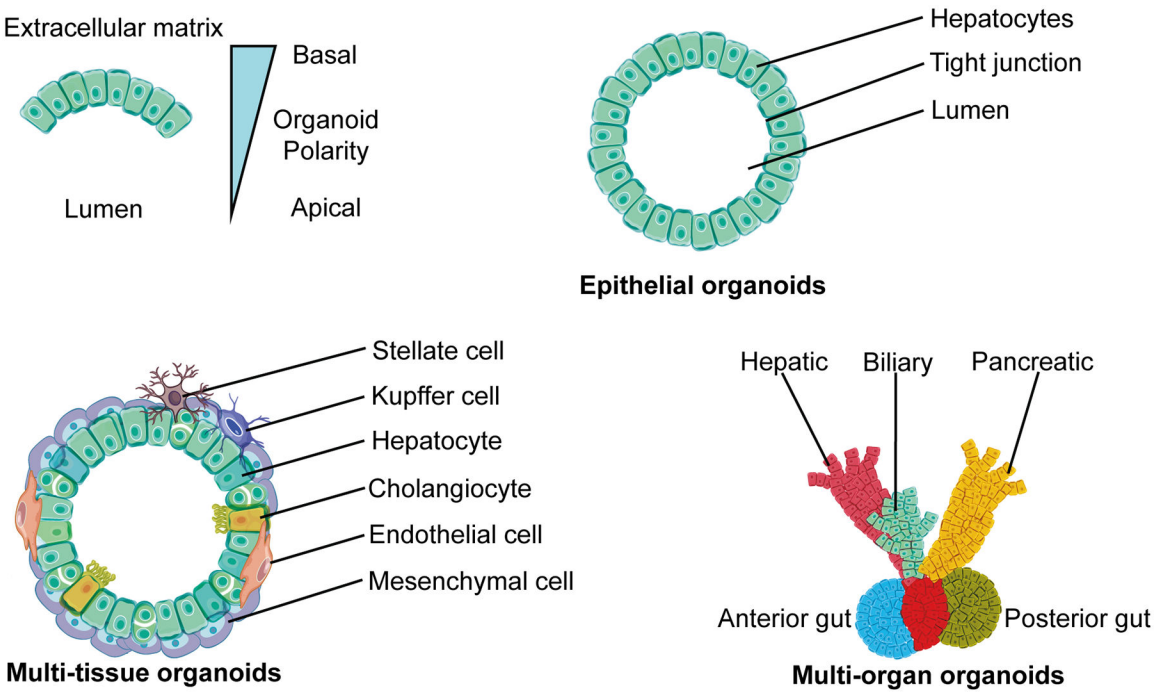

Figure 1. Types of human liver organoids.

Epithelial organoids are derived from either adult stem/ progenitor cells or PSCs and differentiate into a single type of cell; these organoids only have basic functions of hepatocytes. Multi-tissue organoids are derived from either PSCs or combined with stromal cells to generate at least two germ layers; these organoids are capable of complicated functions of the liver and can vascularize to survive longer. Multi-organ organoids are the most complex and encompass multiple interconnected organ like structures that function separately; these organoids have been derived from PSCs only.

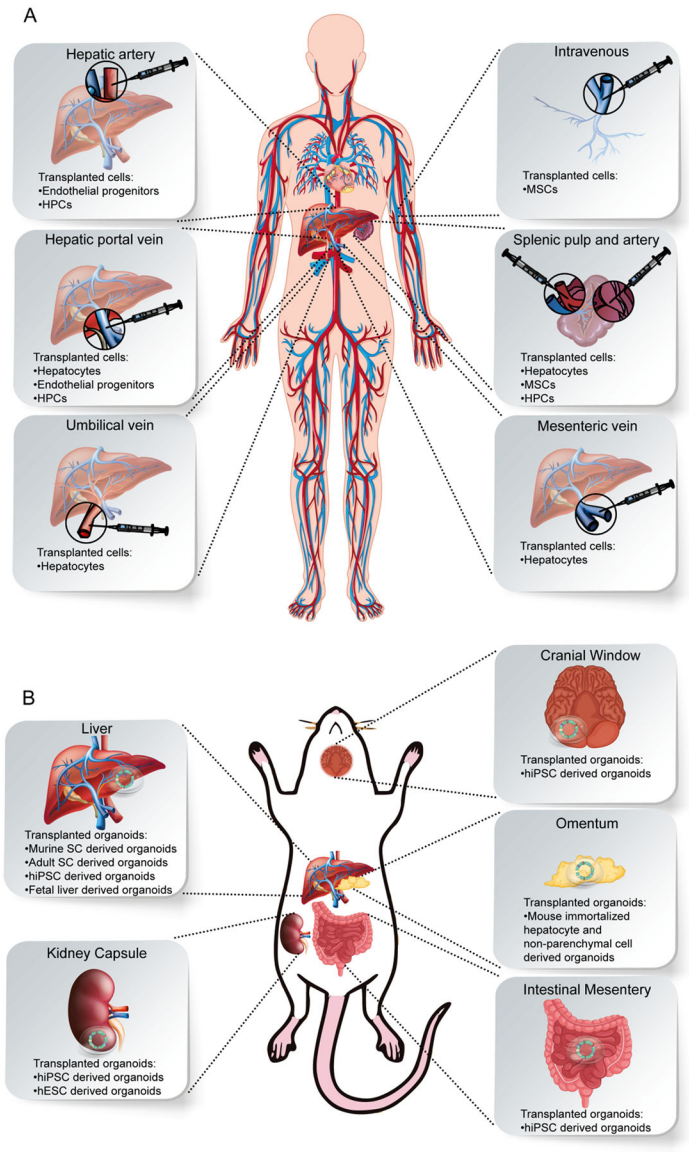

Figure 2.

Hepatocyte and organoid based transplant approaches. A) Transplantation of hepatocytes in clinics. B) Transplantation of liver organoids in animals.

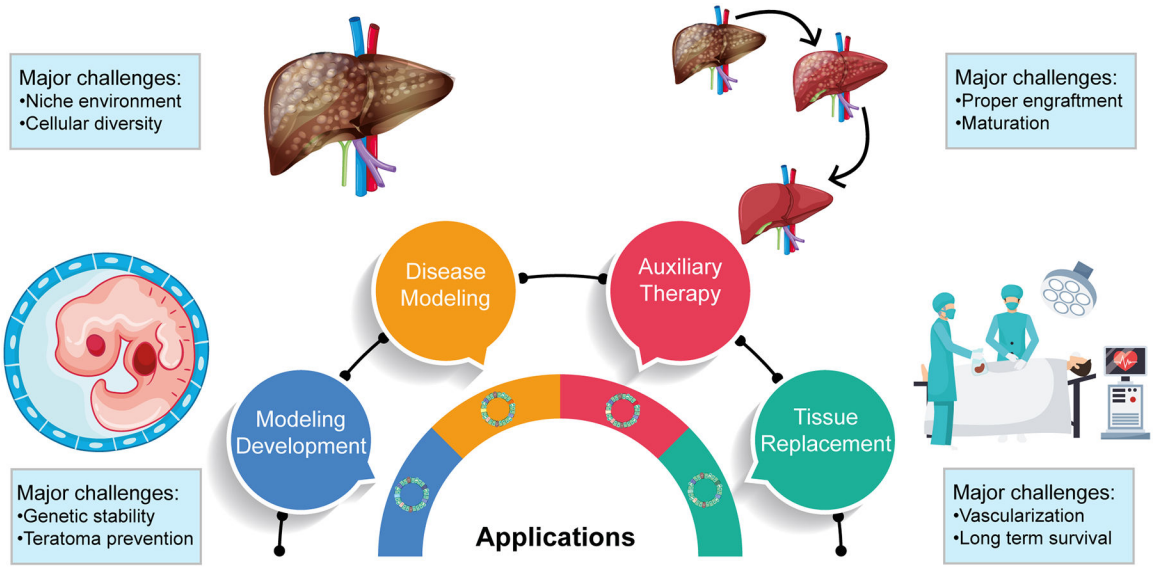

Figure 3.

Applications of transplanted human liver organoids derived from hPSCs, and their major challenges.

Table 1.

Post-transplant features of animal derived liver organoids *in vivo*.

Database			**Search term used**			**Hits**
PubMed			**((liver organoid) OR (liver bud)) AND (organoid transplant)**			**72**
Organoids	**Recipient**	**Transplantatio n site**	**Purpose**	**Survival**	**Liver function**	**References**
Mouse immortalized organoids	BALB/c nude mice	Subrenal capsule, omentum	Maturation and Engraftment	—	Albumin production+	[84]
Mouse Lgr5+ stem cell derived organoids	FRG mice	Intrasplenic injection	Therapeutic	Improved	—	[19]
ROSA26 C57BL/6 mouse liver organoids	Wild-type C57BL/6 mice	Liver	Maturation and Engraftment	Limited	—	[74]
Mutant murine liver organoids	C57BL/6 and NSG mice	Subcutaneous injection	Disease Modeling	Worsened	—	[89]
Murine liver tumor organoids	NOG mice anti-cancer treated	Subcutaneous	Therapeutic	Improved	—	[92]
Murine hepatobiliary organoids	FRG mice	Vascularized chambers	Therapeutic	Improved	Albumin production+	[100]
Canine liver organoid	COMMDI-deficient Beagle–Bedlington	Intrahepatic injection	Therapeutic	—	Cu excretion+	[94]
Tumorigenic mouse liver organoids	NOD-SCID mice	Liver, subcutaneous	Disease Modeling	Worsened	—	[91]

Table 2.

Post-transplant features of human liver organoids *in vivo*.

Database	Search term used					Hits
PubMed	((human liver organoid) OR (human liver bud)) AND (organoid transplant)					43
Organoids	**Recipient**	**Transplantatio n site**	**Purpose**	**Survival**	**Liver function**	**References**
Human iPSC (hiPSC) derived liver bud organoids	Ganciclovir treated TK-NOG mice	Mesentery, cranium, subrenal capsule	Maturation, Engraftment, and Therapeutic	Improved	Albumin production+	[20, 78, 79, 83, 88]
Human EpCAM+ duct cell derived organoids	Retrorsine/CCl4-treated Balbc/nude mice	Intrasplenic injection	Maturation and Engraftment	—	Albumin production+	[41]
3D printed human LBs	Wild type Lewis rats	Liver	Maturation and Engraftment	—	Albumin production+	[101]
hiPSC derived liver bud organoids	NOD/SCID mice	Cranium	Vascularization	—	Albumin production+	[68]
hiPSC derived liver organoids	Diphtheria toxin-induced Alb-TRECK/SCID mice	Subrenal capsule	Therapeutic	Improved	Serum ALT and AST levels decreased	[85]
hiPSC derived liver organoids	Alb-TRECK/SCID mice	Subrenal capsule	Therapeutic	Improved	Albumin production+	[95]
hiPSC derived liver organoids	Retrorsine treated FNRG mice	Intrasplenic injection	Therapeutic	—	Albumin production+	[65]
hiPSC derived HBPOs	NSG mice	Subrenal capsule	Maturation and Engraftment	—	—	[71]
hiPSC derived liver organoids	NSG mice	Intrsplenic injection	Therapeutic	—	Albumin production+	[70]
hiPSC derived liver organoids	Athymice nude rats	Intrsplenic injection	Therapeutic	Improved	Serum ALT and AST levels decreased	[93]
hiPSC derived liver organoids	Immunocompromised rats	Liver, intraportal	Therapeutic	Improved	Albumin production+	[80]
hiPSC derived liver organoids	Piglets	Portal vein injection	Safety and Efficacy	—	Albumin production+	[104]
Human amniotic mesenchymal stem cells	CCl4-treated CD1 mice	Intrsplenic injection	Therapeutic	Improved	Albumin production+	[106]
hPSC derived liver organoids	NSG mice	Subrenal capsule	Maturation and Engraftment	—	Albumin production+	[86]
hiPSC-derived cholangiocyte organoids	NSG mice, ex vivo human liver	Intrahepatic ducts	Therapeutic	Improved	Bile production+	[81, 82]
HLA mismatched hiPSC liver bud organoids	NOG-HLA-A2Tg mice	Liver	Disease Modeling	—	Albumin production+	[90]
Human CCA organoids	NSG mice	Subcutaneous	Disease Modeling	—	—	[97]