



Three-dimensional cell-based strategies for liver regeneration

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Abstract: Liver regeneration and the development of effective therapies for liver failure remain formidable challenges in modern medicine. In recent years, the utilization of 3D cell-based strategies has emerged as a promising approach for addressing these urgent clinical requirements. This review provides a thorough analysis of the application of 3D cell-based approaches to liver regeneration and their potential impact on patients with end-stage liver failure. Here, we discuss various 3D culture models that incorporate hepatocytes and stem cells to restore liver function and ameliorate the consequences of liver failure. Furthermore, we explored the challenges in transitioning these innovative strategies from preclinical studies to clinical applications. The collective insights presented herein highlight the significance of 3D cell-based strategies as a transformative paradigm for liver regeneration and improved patient care.

Introduction

The liver is a crucial organ in the human body that performs various essential functions for maintaining overall health and well-being. However, despite its importance, the liver is highly susceptible to damage and disease [1]. Factors such as drugs, aging, inflammation, fibrosis, and cirrhosis can impair liver regeneration and cause irreversible liver failure [2,3]. For patients with end-stage liver failure, the only definitive treatment is orthotopic liver transplantation (OLT), which has been the only therapeutic option since 1983. Nevertheless, the demand for OLT consistently outpaces the available supply of donor organs, leading to post-operative complications such as thrombosis, biliary disorders, graft rejection, and elevated morbidity and mortality rates among patients [4,5]. Therefore, much hope has shifted to alternative therapies that can prevent or reverse liver failure.

Over the years, researchers and medical professionals have explored various strategies to address this condition. These approaches have been extensively examined in previous reviews [6–8]. In Fig. 1, we provide a concise overview of the advantages and drawbacks of these strategies leading up to the era of 3D cell technologies. Subsequently, we present current 3D cell-based technologies

for liver regeneration, as well as the challenges and future perspectives of using 3D cell-based strategies for liver therapy.

Liver Disease Requiring Regenerative Therapy

The liver possesses an extraordinary capacity to regenerate after injury, however, this capacity can be overwhelmed by a wide range of liver diseases, typically categorized as chronic liver diseases and acute liver failure. Chronic liver diseases such as non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, viral hepatitis, and genetic disorders may induce persistent hepatic injury, leading to liver fibrosis. Liver-related mortality escalates significantly with advancing fibrosis [9], paving the way for the development of severe liver cirrhosis or hepatocellular carcinoma, ultimately culminating in end-stage liver failure [10]. In recent years, acute-on-chronic liver failure (ACLF) in hospitalized patients with chronic liver disease has been increasing globally, leading to a significant rise in mortality rates [11]. Furthermore, acute liver failure induced by drugs, toxins, and other factors may progress to end-stage liver failure. In such cases, regenerative therapy holds the potential to facilitate the restoration of liver function.

Cell Sources for 3D Cell Cultures

Primary hepatocytes

Hepatocytes, as the principal functional entities of the liver, comprising a substantial proportion of the liver mass,

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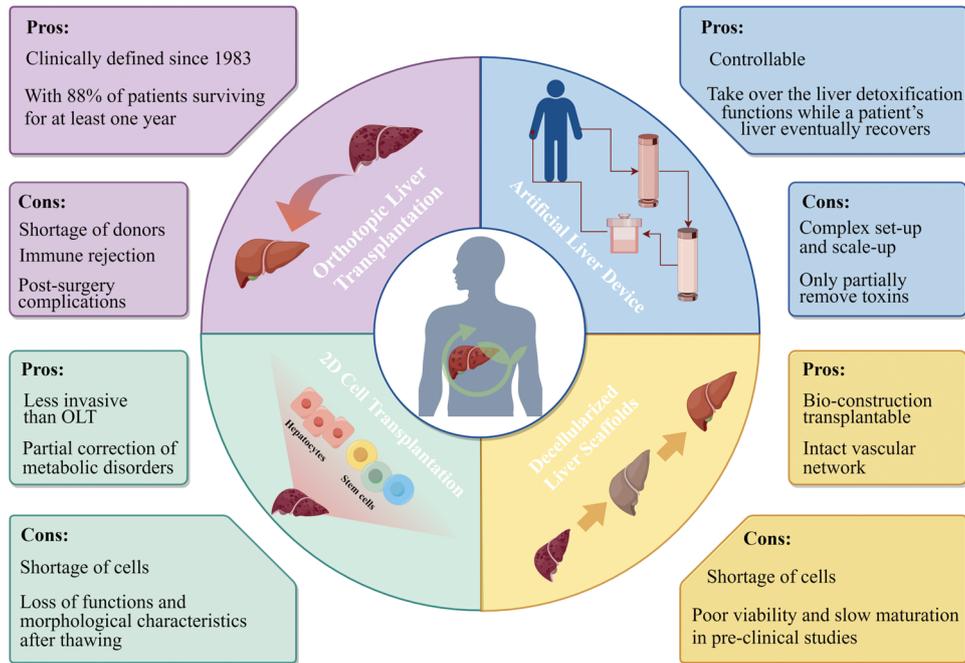


FIGURE 1. Advantages and drawbacks of liver therapy and regeneration approaches prior to the emergence of 3D cell technologies. The strategies include orthotopic liver transplantation, artificial and bio-artificial liver devices, cell transplantation, decellularized liver scaffolds.

representing up to 80%. They possess a repertoire of functional characteristics reflective of the liver and can be transplanted into recipients for the restoration of hepatic function, obviating the need for intricate surgical procedures [12,13]. Despite being widely regarded as the gold standard in research, primary hepatocytes in 2D cultures possess drawbacks that significantly restrict their utilization in clinical applications, as their susceptibility to loss of viability, and function during *in vitro* cultivation or subsequent cryopreservation. Although some scientists have made successful attempts in long-term cultivation of hepatocytes in 3D culture environments [14,15], a consistent cell source remains elusive primarily due to the scarcity of available donor livers. Therefore, it is essential to define and validate novel sources of functional hepatic cells (Fig. 2).

Stem cells

To address the scarcity of hepatocytes, researchers are actively engaged in the pursuit of generating functional hepatocytes from diverse stem cell populations, including mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) [16].

MSCs represent a multipotent type of stem cells that can be obtained from a variety of tissues. They express major histocompatibility complex class I (MHC-I) and class II (MHC-II) molecules, making them well-suited for allogeneic transplantation without causing rejection [17,18], in addition to their immunomodulatory properties [19–21], making them highly suitable for transplantation purposes. The mechanisms underlying the therapeutic effects mediated by MSCs involves their differentiation into multiple cell lineages, migration towards damaged tissues, immunomodulatory actions, and a wide array of biologically active factors, including cytokines, chemokines, hormones,

growth factors, and miRNAs that promote cell survival and proliferation [22,23]. However, the constrained migration and suboptimal viability of transplanted MSCs [24,25] have prompted investigators to delve into alternative stem cell sources for the purpose of liver regeneration.

ESCs originate from the inner cell mass of a blastocyst that possess unique pluripotency and self-renewal capabilities [26]. These cells possess the exceptional capability of undergoing indefinite division *in vitro* while retaining their potential to differentiate into all types of cells present in an adult body [27,28]. Hence, ESCs hold the potential to offer an inexhaustible supply of transplantable cells to replace or regenerate damaged tissue. Although the clinical application of ESCs for extensive expansion and differentiation into hepatocytes remains a significant challenge owing to lingering uncertainties concerning their acquisition and potential tumorigenic risks, scientists have developed several effective strategies, such as growing ESCs in 3D fibrous matrices or organoids system [29,30]. Another concern is that ESCs are unfortunately mired by ethical controversies.

iPSCs have been demonstrated to exhibit remarkable similarity to ESCs in regard to their transcriptional programs, chromatin modification profiles and overall chromatin configurations [31–33]. As iPSCs can be derived directly from adult tissues, they present the advantage of bypassing the need for embryo destruction and can be personalized for each patient. The advent of iPSCs has thus emerged as a focal point in the realm of stem cell research [34]. Numerous studies have documented the successful differentiation of iPSCs into diverse array of liver cell types, encompassing hepatocytes, endothelial cells, cholangiocytes, and stellate cells [35–37]. Concurrently, the growing fascination with self-assembling or matrix-guided 3D organoids has opened new avenues towards the

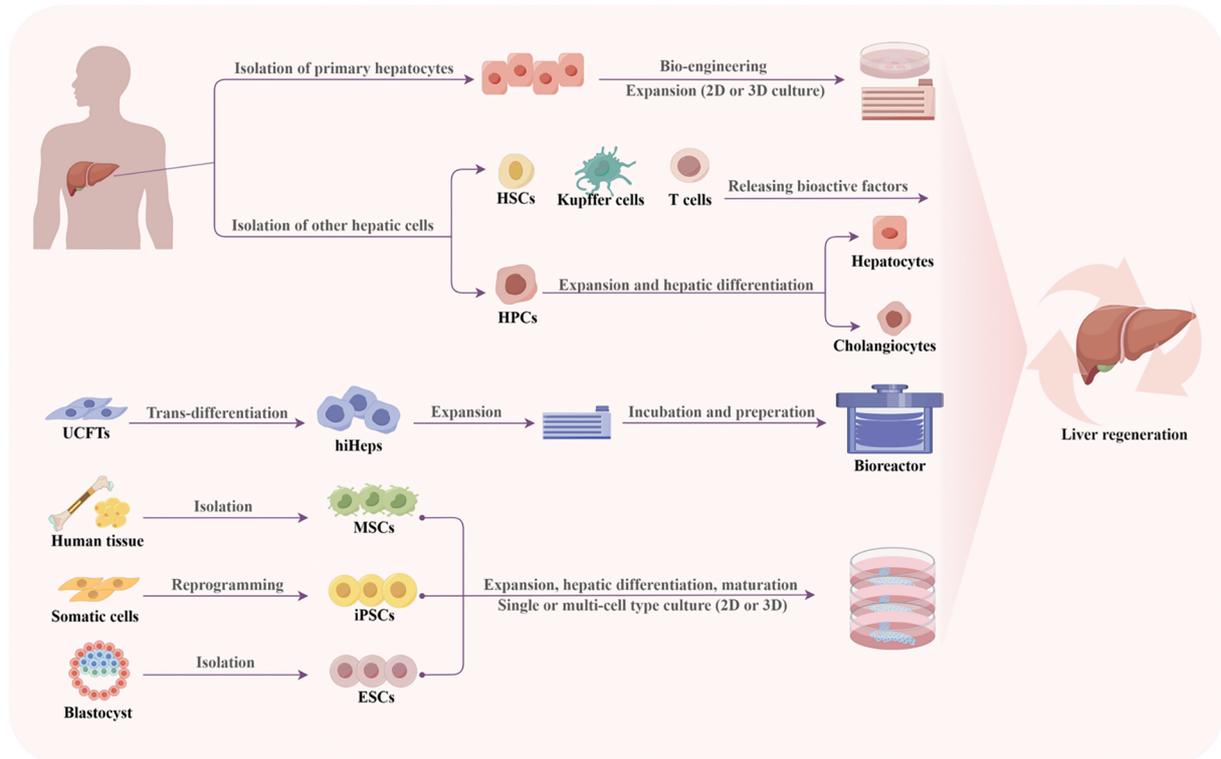


FIGURE 2. Schematic representation of various cell sources in 3D cell cultures for liver therapy and regeneration. Primary hepatocytes can be converted to liver progenitor-like cells to enable expansion, while stem cells can be isolated, reprogrammed, and differentiated into hepatic cells. HSCs: hepatic stellate cells; HPCs: hepatic progenitor cells; UCFTs: human umbilical cord fibroblasts; hiHeps: Human-induced hepatocytes; MSCs: mesenchymal stem cells; iPSCs: induced pluripotent stem cells; ESCs: embryonic stem cells.

development of functional bioartificial livers [38]. However, it is crucial to note that while initially believed to eliminate the risk of immune rejection in transplants, accumulating evidence suggests that certain iPSC-derived cells may still provoke immune responses [39,40].

Other hepatic cells

Hepatic progenitor cells (HPCs) represent a class of bipotential cells that are renowned for their exceptional capacity to simultaneously proliferate and differentiate into both hepatocytes and cholangiocytes [41]. HPCs are regarded as a promising cellular reservoir for liver regeneration. Nevertheless, the clinical application of HPCs is impeded by the potential risk of hepatocellular carcinoma development [42,43]. Human-induced hepatocytes (hiHeps) are derived through the direct reprogramming of human fibroblasts. These hiHeps could be easily expanded and maintained hepatocyte-specific functions, including the secretion of plasma proteins and activity of P450 enzymes [44]. The hiHep-based bioartificial liver system demonstrated promising outcomes in both a porcine liver failure model and patients undergoing extended liver resection [45]. Hepatic stellate cells (HSCs) comprise 15% of all hepatocytes and play a pivotal role in the synthesis and secretion of collagen and other extracellular matrix components. HSCs orchestrate liver regeneration by producing a diverse array of growth factors, cytokines, and glycoproteins that are activated by proinflammatory cytokines and transformed into myofibroblasts [46]. Kupffer cells, referred to as hepatic macrophages, play a pivotal role

as the liver's primary immune defense against injurious particles and chemical agents that infiltrate the portal vein. Kupffer cells are responsible for releasing crucial bioactive factors for regulating hepatocytes [47,48].

Development and Application of 3D Cell-Based Strategies for Liver Regeneration

Over the past decades, the aforementioned stem or progenitor cells have exhibited the potential to undergo differentiation into hepatocyte progenitor-like cells or hepatocyte-like cells, thus presenting a potential avenue for treating liver diseases through both preclinical and clinical investigations [49–52]. Traditional two-dimensional (2D) cell cultures have provided crucial insights into liver biology, yet they often fail to accurately replicate the intricate spatial and biochemical cues present in the native liver microenvironment [53]. In contrast, 3D cell-based strategies have the ability to mimic crucial interactions between cell-cell and cell-matrix, leading to a transformative paradigm shift as they successfully replicate the dynamic complexity of liver tissue. The integration of primary hepatocytes, stem cells, and innovative culture techniques provides novel opportunities to restore liver function and improve patient outcomes.

Spheroids

Spheroids, which represent dense 3D cell aggregates, serve as optimal building blocks for regenerative medicine. Given their capacity to facilitate tissue formation, spheroids hold potential

applications in areas such as wound healing or correcting congenital defects [54]. Approaches to guide spheroid behavior can be categorized as *ex vivo* or *in situ* [55]. *Ex vivo* manipulation of spheroids typically begins during the monolayer culture phase prior to spheroid formation. During this stage, cells undergo priming through exposure to pharmacological agents or other soluble bioactive factors, while the modulation of microenvironmental factors, like cell confluency and localized oxygen levels, can prompt differentiation and enhance cell survival. *In situ* instruction of spheroids often experience limited viability, swift dedifferentiation, and propensity for migration from the injection site [56]. Biomaterials play a pivotal role in facilitating the localization of cells at the targeted delivery site, while also providing instructive cues to regulate and guide cellular behavior. These biomaterials can be customized to emulate the characteristics of the extracellular matrix (ECM), thereby eliciting stimulation of cell proliferation and enhancement of cell survival. This allows for quicker delivery of therapies and conservation of valuable resources [57].

In preclinical studies using animal models, researchers have explored the prospective capacity of spheroids to amplify and potentiate regenerative processes within the liver. Sun et al. demonstrated that spheroids of human umbilical cord-derived MSCs (hUC-MSCs) exhibited enhanced migratory capabilities towards the injured liver when contrasted with hUC-MSCs cultured in a two-dimensional (2D) manner. Notably, these hUC-MSCs were found to effectively stimulate liver regeneration and facilitate repair in mice afflicted by liver injury [58]. Hepatocyte spheroids of human were effectively engrafted into the livers of mice with viral hepatitis following intrasplenic injection [59]. Collagen fiber-based 3D spheroids comprising adipose-derived stem cells effectively maintained cellular functionality and the capacity for paracrine secretion. Furthermore, transplantation of these spheroids mitigated thioacetamide (TAA)-induced liver cirrhosis in murine models [60]. Spheroids derived from stem cells of human exfoliated deciduous teeth were successfully grafted into the livers of mice afflicted with carbon tetrachloride (CCl₄)-induced liver fibrosis. In this transplantation, the spheroids engrafted well, leading to enhanced liver function, and demonstrating antifibrotic efficacy in mice [61]. Innovatively, some researchers have utilized 3D printing to construct hepatocyte spheroids, thereby fabricating functional liver tissue that replicates intricate duct and sinusoid microanatomy [62,63]. Zhang et al. devised a method for fabricating spheroids with precise quantities of distinct cell types using microfluidic flow cytometric printing. These precision spheroids exhibit enhanced variability and functional consistency in comparison to randomly formed spheroids [64].

Organoids

Organoids represent a condensed and simplified rendition of an organ, often originating from stem cells. They may encompass diverse cell types that spontaneously self-organize *in vitro*, recapitulating the morphology and intricate biological interplays within a living organism [65].

Here, we elucidate the recent endeavors directed toward constructing next-generation organoids and outline the current potential of utilizing mature organoids in liver regeneration.

Generation of liver organoids

ESCs and iPSCs are preferred candidates for liver organoid generation due to their proliferation and differentiation characteristics. For instance, Wang et al. presented a study detailing the generation of hepatic organoids derived from ESCs with expandable attributes, utilizing entirely defined medium devoid of serum and feeder cells. Remarkably, these organoids were capable of expanding for up to 20 passages, facilitating large-scale growth. Furthermore, upon transplantation, they exhibit a notable capacity for repopulating injured livers within *Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-} (FRG) mice, subsequently differentiating into mature hepatocytes *in vivo* [66]. In 2022, Messina and colleagues successfully developed human iPSC-derived hepatocytes organoids. Specifically, the process involved the initial differentiation of human iPSCs into hepatoblasts through the application of growth factors and cytokines. Subsequently, hepatoblasts were developed into iPSC-derived hepatocytes organoids using hepatic growth factor (HGF), oncostatin M, vitamin K and dexamethasone [67].

There are some parameters that are crucial to control during the construction of 3D organoids. Firstly, liver organoids require exposure to specific growth factors, including Wnt, fibroblast growth factor, HGF, and bone morphogenetic protein, at precise timings to effectively promote hepatic progenitor survival and their differentiation [68]. The ECM also plays a critical role in the formation of organoids. While Matrigel has been widely employed for organoid culture, its application is limited by factors like complexity, batch-to-batch variability, and its origin from mouse tumor cells. Consequently, various alternatives are under exploration, such as decellularized liver ECM, diverse synthetic hydrogels, as well as peptide and recombinant protein matrices [69]. Lastly, designed geometries like micropatterning or microfluid dynamics represent strategies that can be harnessed to facilitate the growth of cells and organoids [70].

Co-culture of the liver organoids

Employing a single cell type in organoids culture ensures the proliferation and self-organization of a homogenous cellular population, thereby simplifying the formation process. However, co-culturing multiple cell types, including endothelial cells, HSCs and MSCs can significantly enhance the functional maturity and complexity of the organoids, leading to a better mimicry of the liver organ structure [71,72]. In 2013, Takebe et al. made the pioneering report of generating a three-dimensional vascularized and functionally active human liver using human iPSCs. Specifically, they demonstrated that iPSCs, co-cultured alongside MSCs and human umbilical vein endothelial cells (HUVECs), could spontaneously self-organize into three-dimensional iPSC-liver buds (organoids) which exhibited an endothelial network and expressed hepatic-specific marker genes [71]. In 2019, Pettinato et al. interlaced iPSCs with human

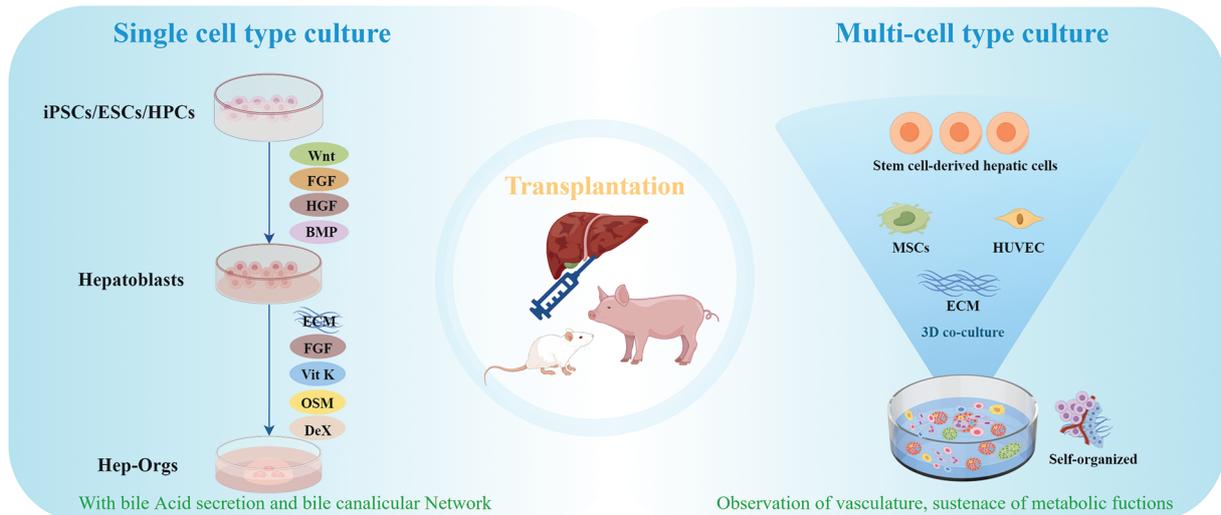


FIGURE 3. Generation of hepatic organoids for transplantation. In single cell type culture, stem cells were differentiated in the presence of several factors like wnt, FGF, HGF and BMP, and developed into hepatic organoids using HGF, OSM, vitamin K and Dex. In multi-cell type culture, co-culturing multiple cell types, including stem cell-derived hepatic cells, and endothelial cells, can significantly enhance the functional maturity and complexity of the organoids. FGF: fibroblast growth factor; HGF: hepatic growth factor; BMP: bone morphogenetic protein; ECM: extracellular matrix; Vit K: vitamin K; OSM: oncostatin M; DeX: dexamethasone; HUVEC: human umbilical vein endothelial cells.

adipose microvascular endothelial cells, resulting in an increased differentiation yield and significant enhancements across a diverse spectrum of hepatic functions [73]. In 2021, Tanimizu et al. generated a functional hepatobiliary tubular organoid through the co-cultivation of mouse hepatocyte progenitors and cholangiocytes. Remarkably, this organoid exhibited the acquisition and sustenance of metabolic functions, including the secretion of albumin and activities of cytochrome P450, persisting over an extended duration [74] (Fig. 3).

Applications of organoids in regenerative medicine

Principally, organoids serve as valuable exploration tools, acting as platforms for drug screening and providing insights into the intricate processes underlying human development and diseases [75,76]. For regenerative medicine, the ultimate objective is to achieve the transplantation of tissue-specific organoids, which will facilitate the recovery or enhancement of tissue function. Despite the extensive exploration of regenerative medicine for liver diseases over numerous decades, several challenges persist. Transplanted organoids must exhibit long-term survival, compatibility with extensive expansion, and genetic stability [77].

Several studies have demonstrated that organoids transplantation is a promising strategy for cell-based therapy

for liver regeneration (Table 1). Huch et al. established a durable human liver organoid culture, and the cells exhibit the capability to be readily transformed into functional hepatocytes both *in vitro* and upon transplantation *in vivo* [78]. Tsuchida et al. verified the safe transplantation of human iPSC-derived liver organoids via the portal vein, with concurrent ligation of the ductus venosus [52]. Sampaziotis et al. delivered remarkable evidence, showcasing the potential of cholangiocyte organoids to repair human biliary epithelium. This groundbreaking work introduces a therapeutic 3D stem cell-based approach for repairing ischemic bile ducts, potentially providing the potential for organoid-based therapies targeting various cholangiopathies [79]. Furthermore, organoids can be derived from patients with genetic diseases, and subsequently, genetically modified organoids hold the potential for transplantation into patients to alleviate disease phenotypes [80]. An illustrative instance of this strategy involves the application of Retinitis Pigmentosa GTPase Regulator (RPGR) gene-edited organoids in tackling retinitis pigmentosa. This strategy has demonstrated the restoration of defects characteristic of the disease, such as photoreceptor reduction [81]. Here, we summarized the typical studies that performing human liver organoid transplantation in the past decade (Table 1).

TABLE 1

Studies performing human liver organoid transplantation

Cell type	Organism	Outcome	Reference
Human pluripotent stem cells (hPSC) derived cholangiocytes	NOD-SCID-IL2ry ^{-/-} ; NOD Scid Gamma (NSG) mice	Duct-like structures displaying cholangiocyte characteristics were formed within 6 to 8 weeks	[82]
Lgr5+ liver stem cells	Mice with acute liver failure	Limited engraftment and albumin secretion up to 40 days	[78]

(Continued)

Table 1 (continued)

Cell type	Organism	Outcome	Reference
Induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells and stromal cells	Immunocompetent mice	Successful engraftment and robust function, but induced fibrosis	[83]
iPSC derived cells	Alb-tk-nog mice	Significantly improving survival, increasing albumin and decreasing alpha fetoprotein (AFP). Effective detoxification up to 60 days after transplantation	[84]
Primary hepatocytes, endothelial cells, and stromal cells	FAH ^{-/-} NOD mice with liver injury	Expansion of more than 50-fold was witnessed in mice with injured livers, observation of a perfused vasculature	[85]
iPSCs derived endoderm, endothelial cells, and mesenchymal cells	Mice with acute liver failure	20-day mark post-transplant, in contrast to a mere 30% for the control group	[86]
Hepatocytes	Fah ^{-/-} NOD Rag1 ^{-/-} Il2rgnull mice	Proliferate extensively after engraftment	[87]
iPSCs-derived EpCAM ⁺ endodermal progenitors	Immunodeficient NSG mice with acute liver damage	Passaged for ≥6 months in vitro, engraft and express albumin in the mouse liver for ≥32 days	[88]
iPSC-derived hepatobiliary cells	NOD-SCID mice	Survived for more than 8 weeks	[89]
Fetal liver cells	Rats performed with a two-thirds partial hepatectomy	The repopulation rate was up to 70% after 120 days	[90]
iPSC-derived hepatic endodermal cells, endothelial cells, and mesenchymal cells	10-day and 28-day old piglets	Patent ductus venosus (PDV) ligation before transplantation of organoids can inhibiting the extrahepatic translocation	[52]
Primary cholangiocytes	NSG mice with biliary injury; <i>Ex vivo</i> perfusion human liver grafts	Mice survived for 104 days after engraftment, repair human intrahepatic ducts after transplantation	[79,91]
hPSC-derived cholangiocytes	TK-NOG mice	Generated ductal structures in the liver and expressed biliary markers for at least 6 weeks	[92]
Co-hepato/pancreatic stem/progenitors	NRG/FAH-KO mice Piglets (10 kg)	Rescue mice with tyrosinemia, successful engraftment and Matrix metalloproteinases (MMPs) expression in the liver of piglets for 2 weeks	[93,94]
Induced hepatic stem cells and endothelial cells	Mice with cholestatic liver fibrosis	Transplanted in the kidney, ameliorate cholestatic liver fibrosis with no tumor formation	[95]
Engineered iPSC-derived cells	FVIII-deficient HA mice	Survived in mice for at least 2 weeks, reduced bleeding events and time	[96]
Primary human hepatocytes dedifferentiated progenitor-like cells	Fah ^{-/-} /Rag2 ^{-/-} /Il2rg ^{-/-} (FRG) mice	Enhanced engraftment, repopulated 49.2% of the liver parenchyma at 5 months after transplantation	[97]
hPSC line AG27-derived hepatocytes, Hepatic stellate cells (HSCs), Kupffer cells and endothelial cells	Immunodeficient NSG mice	Human albumin was consistently detected over a 5-week period in mouse blood samples	[98]

Bioscaffolds

Bioscaffolds can mimic the ECM environment of the organ and can effectively induce cell growth and differentiation. As a result, these bioscaffolds possess the capacity to adequately support the colonization of host cells, meeting up the requirements of regeneration and repair. In general, bioscaffolds can be classified into two main groups: natural and synthetic. Natural bioscaffolds, such as collagen, chitosan, silk fibroin, or Polyhydroxyalkanoates (PHAs) are derived from biological sources and demonstrate exceptional biocompatibility and bioactivity. On the other hand, synthetic

bioscaffolds, which include polymers like polylactic acid (PLA), polyglycolic acid (PGA), or polycaprolactone (PCL), are artificially produced, allowing for precise manipulation of their physical and chemical characteristics [99–101]. Here, we delve into a discussion of two representative bioscaffolds used in the field of liver regeneration.

3D encapsulation of cells in a collagen hydrogel has shown potential for enhancing maturation compared to 2D culture. Hussein et al. demonstrated that the combining of 3D techniques, namely guided aggregation and microencapsulation, with liver differentiation protocols,

constitutes a robust strategy for producing fully mature and functional hepatocytes. This innovative approach presents a sustainable and limitless source of hepatocytes [102]. Song et al. successfully engrafted iPSC-derived hepatocyte-like cells (iPS-H) with stromal cells into immunocompetent mice. This accomplishment was realized through the encapsulation of the cells within biocompatible hydrogel capsules. The secretion of human albumin and α 1-antitrypsin by encapsulated iPS-H/stromal cell aggregates attained a level comparable to the primary human hepatocyte/stromal cell control [83].

However, achieving a complete recapitulation of the entire biochemical and architectural intricacies of the natural microenvironment remains a challenge. Decellularized scaffolds from xenogeneic animals and discarded human livers offers a promising approach to tackle this issue. Decellularized liver scaffolds can retain the functional attributes of the native microvascular and bile drainage networks within the liver, along with the essential growth factors required for both angiogenesis and liver regeneration [103]. The techniques for producing decellularized liver scaffolds encompass physical, chemical, and enzymatic treatments, often employed in combination. Currently, substantial strides have been taken towards

ensuring safety and clinical applicability. Detailed reviews are available elsewhere [104,105]. The next critical steps are the recellularization of the parenchyma and reendothelialization of the vascular lumen to generate transplantable and functional liver grafts. In the past decade, various proof-of-concept studies have been conducted to assess the viability of repopulation of liver grafts in both small and large animal models (Table 2).

Bioprinting

3D bioprinting, a technique that employ computer-aided processes to precisely assemble cells and biomaterials into precise 3D structures, has emerged as a promising avenue for replicating hepatic tissue and facilitating translational endeavors. Bioprinting can be broadly classified as extrusion-based, inkjet-based, or light-assisted bioprinting. Notably, 3D-bioprinted liver models are commonly classified as either static scaffold-based models or dynamic liver-on-chip models [116]. Diverse forms of scaffolds featuring intricate structures have been designed for the exploration of liver regeneration. Kang et al. utilized a preset extrusion bioprinting technique to generate multicellular and multi-material structures comprising hepatic cells, endothelial cells, along with a

TABLE 2

Summary of decellularized liver grafts in transplantation studies

Recipient animal	Infused cells	Outcome	Reference
Mice (lethal dose of carbon tetrachloride (CCl ₄) induced liver damage)	Mice bone marrow-derived mesenchymal stem cells (MSCs)	5 out of 8 mice survived, exhibiting glycogen storage and albumin production	[106]
Mice (CCl ₄ induced liver damage)	Huh7, Human umbilical venous endothelial cells (HUVECs), Human bone marrow mesenchymal stem cells (hBMSCs)	The patch could be incorporated into the recipient mouse liver, lower the levels of injury markers	[107]
Syngeneic rats	Primary rat hepatocytes	Flow through the graft was limited, thrombus formation was reduced	[108]
Rats (immune compromised)	Human induced pluripotent stem cells (iPSC)-derived hepatocytes, endothelial cells, biliary epithelial cells	Remained functional for 4 days after auxiliary liver transplantation	[109]
Rats (CCl ₄ induced liver damage)	Rat hepatocyte spheroids	Viable for at least 10 weeks <i>in vivo</i> , albumin synthesis, glycogen storage and cytochrome P 3A4 activity were highly expressed, new blood vessels formed	[110]
Rats	HUVECs, hBMSCs and mouse hepatocyte cell line	Achieved a survival time of 81.38 ± 4.263 min in rats (n = 8) subjected to complete hepatectomy	[111]
Yorkshire pigs	Re-endothelialized by conjugating anti-endothelial cell antibodies	Withstand physiological blood flow and maintained for up to 24 h	[112]
Pigs (30–40 kg)	HUVEC or PUVEC cells	Patent vascular inflow, absence of gross thrombus, and patent post-explant portal venogram after 72 h of <i>in vivo</i> hemoperfusion	[113]
Immunosuppressed pigs	HUVECs	Continuous perfusion of the revascularized bioengineered livers for over two weeks	[114]
Pigs (28–36 kg, surgically induced acute liver failure)	HUVECs, primary porcine hepatocytes	Slowed ammonia accumulation during <i>in vivo</i> perfusion, maintenance of canonical endothelial and hepatocyte markers	[115]

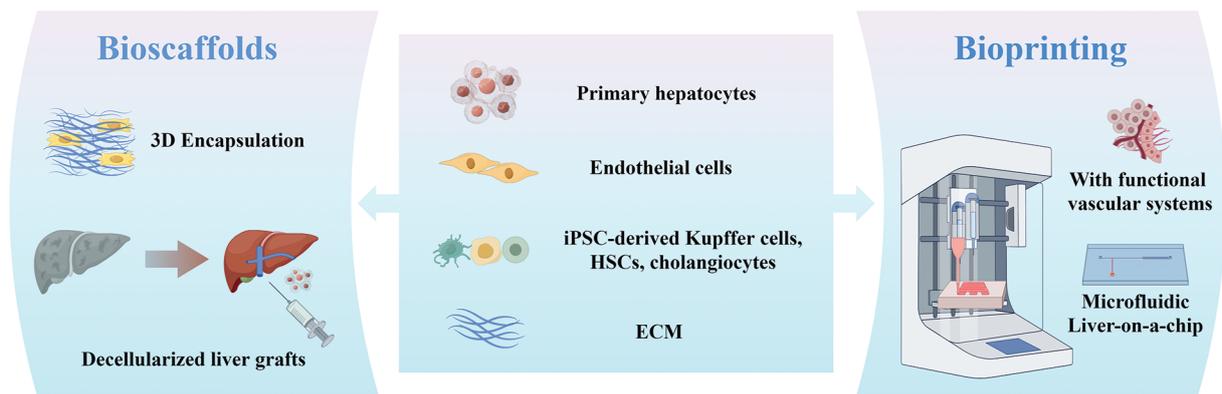


FIGURE 4. Schematic summarizing bioscaffolds and bioprinting strategies for liver therapy and regeneration. 3D encapsulation of cells within biocompatible hydrogel capsules has shown potential for enhancing maturation compared to 2D culture. Decellularized scaffolds from xenogeneic animals and discarded human livers offers a promising approach to recapitulate the microenvironment. 3D-bioprinted liver models, especially liver-on-chip, offers the capacity to recreate more intricate microenvironments like temperature, pH, cell shear stress and waste removal.

lumen. This construct, comprising hepatic lobules within a densely vascularized framework, facilitated the fabrication of tissue at both micro- and macro-scales (Fig. 4). Such an approach can be pivotal in developing expansive 3D tissue constructs for multiscale tissue engineering purposes [117]. In a recent study, 3D-bioprinting was employed to construct hepatorganoids using HepaRG cells and bioink. Following transplantation into Fah-deficient mice, these hepatic organoids exhibited the formation of functional vascular systems, which consequently enhanced material transport and liver function. Consequently, the survival rate of the mouse model afflicted with liver injury exhibited significant improvement [118].

Unlike conventional static platforms, the integration of bioprinting with dynamic microfluidic liver chips offers the capacity to recreate intricate microenvironments encompassing factors like temperature, pH, cell shear stress, nutrient supply, oxygen gradient, and waste removal. Although the primary focus of research in the field of liver-on-chip technology is directed towards the application of these platforms for drug screening and disease modeling, a recent study introduced a novel microfluidic liver system that integrates hiPSCs-derived hepatocytes-laden microparticles and semipermeable microtubes. This system exhibited high cell viability, functional regeneration, and effective circulation system. Building upon these merits, a liver chip integrated with multiple microfluidic liver systems was employed in a rabbit model of acute liver failure. This intervention not only mitigated inflammation, but also facilitated the production of serum proteins, ultimately leading to improved survival rates [119].

Challenges towards Clinics

In recent years, numerous clinical trials focusing on liver transplantation using 2D stem cells have emerged. Concurrently, there are also an increasing number of clinical studies on the application of organoids or liver-on-chips for predicting treatment response. However, to our knowledge, neither organoids nor 3D bioprinted liver constructs have been implemented in clinical trials for liver transplantation.

Recent years have witnessed great advances in the application of 3D cell-based strategies for liver regeneration in animal models. A plethora of studies have affirmed the superiority of 3D cell culture over the traditional 2D approach [120]. Consequently, there exists promising potential in the utilization of spheroids, organoids, and 3D liver constructs for advancing the treatment of liver failure. The critical obstacles impeding translation from laboratory innovations to clinical reality are discussed below (Fig. 5).

Scale-up and manufacturing challenges

Transitioning from small-scale laboratory experiments to large-scale production of functional liver tissue constructs presents a substantial manufacturing challenge. Consistency in quality, reproducibility, and scalability is essential for guaranteeing the safety and effectiveness of liver constructs on a human scale. For spheroids or organoids, culture conditions that seamlessly integrate with the large-scale manufacturing platforms need to be developed. Indeed, the commonly used Matrigel carries potential risks for tumorigenesis and immune reactions due to its mouse origin, and batch-to-batch variability further complicates its reliable usage. Synthetic hydrogels, like polyethylene glycol (PEG), offer enhanced reliability and safety. However, cells within these hydrogels often exhibit restricted liver functionality [121]. Thus, it is necessary to develop reliable and safety ECM for generating reproducible and functional organoids. Furthermore, ensuring adequate oxygenation and nutrient exchange throughout the entire tissue construct, as well as the complex microenvironment and metabolic demands, are crucial for the viability and functionality of liver constructs.

Cell sources and immunological challenges

The choice between allogeneic and autologous cell sources in clinical applications involves careful consideration of factors, such as immune compatibility, treatment timeline, availability, and ethical considerations. In cases of patients with acute liver failure, the feasibility of utilizing autologous cells for readily available regenerative medicine solutions could be limited. Meanwhile, the use of allogeneic cells carries the inherent risk of inciting immune reactions within

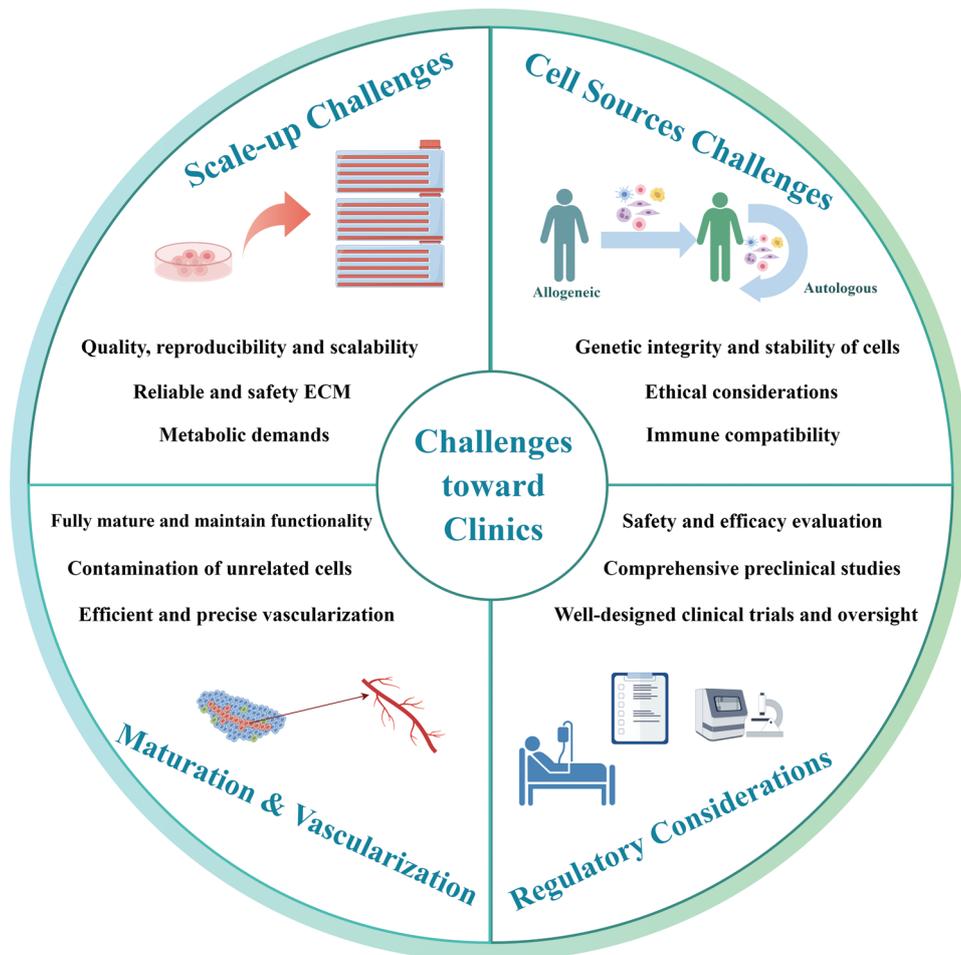


FIGURE 5. Challenges of 3D cell-based strategies for liver therapy and regeneration, from laboratory innovations to clinical reality. It is imperative for researchers to substantiate the safety and efficacy of these therapies through comprehensive preclinical studies, rigorous safety evaluation and well-designed clinical trials.

the host's physiology, possibly culminating in graft rejection or analogous immune-mediated intricacies [122]. Genetic engineering techniques can be employed to manipulate allogeneic donor cells to diminish immunogenicity, presenting a promising avenue, albeit accompanied by a spectrum of inherent challenges [123].

Functional maturation and vascularization

Hepatocytes or stem cell-derived cells in 3D cultures often struggle to fully mature and maintain their functionality over time. For instance, the presence of unrelated cells or undifferentiated stem cells or within organoids can give rise to the subsequent development of undesirable and potentially hazardous tissue formations characterized by genetic abnormalities [124]. This issue highlights concerns regarding the safety and potential tumorigenicity associated with organoids. To address this challenge, the genetic stability of organoid cells should undergo regular check. The cells utilized for generating organoids should undergo purification by microdissection methods to ensure separation from their surrounding stromal components. Modulating the microenvironment composition, particularly by manipulating the presence of growth factors, can effectively guide the differentiation of organoids towards the desired pathways [125].

Vascularization is critical for the growth and efficient exchange of factors within organoids and liver constructs. Ensuring efficient vascularization within 3D liver tissue constructs remains a significant challenge. Although the vascularization of bioprinted liver scaffolds has been generated successfully in a few models, there remains a need for the development of advanced technologies that enable more precise bioprinting of small-diameter blood vessels. Currently, spheroids and organoids still encounter limitations in establishing proper vascularization upon engraftment. To address this, incorporating endothelial cells or HUVECs as organoid components may presents a promising strategy [71]. Exploring transplantation and cytokine induction methods for *in vivo* vascularization should receive more attention.

Ethical and regulatory considerations

Ethical concerns pertaining to the origin, potential hazards, and long-term consequences of transplanted liver grafts introduce intricate questions. Participants may face risks including tumorigenesis, adverse immunogenic responses, and infections [126]. Therefore, the progression of 3D cell-based liver regeneration therapies from laboratory breakthroughs to clinical implementation demands rigorous safety evaluations and regulatory approval. To attain

regulatory approval and ensure patient welfare, it is imperative to substantiate the safety and efficacy of these therapies through well-designed clinical trials and comprehensive preclinical studies. At present, no established clinical or ethical guidelines are in place to evaluate the safety of 3D cell-based liver grafts for clinical trials. Researchers could gain insights from analogous fields like cell therapy, for addressing matters such as cell sourcing, risk evaluation, patient selection criteria, trial design and oversight.

Conclusions

Over the last decade, the evolution of 3D cell-based strategies for liver regeneration has been swift and substantial, bearing tremendous potential and breakthroughs in the realms of hepatology and regenerative medicine. As technology continues to evolve and our understanding of cellular interactions deepens, overcoming the aforementioned challenges is only a matter of time. The integration of multiple cell types within 3D constructs will mirror the intricate liver microenvironment, enhancing our understanding of liver diseases and tissue functionality. Biomimetic environments within these cultures will foster cell maturation and function, while breakthroughs in vascularization will ensure an efficient nutrient and oxygen supply. Advanced imaging and gene editing techniques will provide unprecedented insights and control, accelerating the development of safer and more effective treatments. With these advancements, the translation of 3D cell-based strategies into clinical trials will inch closer, potentially reshaping the landscape of liver disease treatment and regeneration.

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