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A decade of progress in liver regenerative medicine

Jingwei Zhang; Xin Zhao; Liguo Liang' Jun Li*; Utkan Demirci**; ShuQi Wang***

Abstract

Liver diseases can be caused by viral infection, metabolic disorder, alcohol consumption, carcinoma or injury, chronically progressing to end-stage liver disease or rapidly resulting in acute liver failure. In either situation, liver transplantation is most often sought for life saving, which is, however, significantly limited by severe shortage of organ donors. Until now, tremendous multi-disciplinary efforts have been dedicated to liver regenerative medicine, aiming at providing transplantable cells, microtissues, or bioengineered whole liver via tissue engineering, or maintaining partial liver functions via extracorporeal support. In both directions, new compatible biomaterials, stem cell sources, and bioengineering approaches have fast-forwarded liver regenerative medicine towards potential clinical applications. Another important progress in this field is the development of liver-on-a-chip technologies, which enable tissue engineering, disease modeling, and drug testing under biomimetic extracellular conditions. In this review, we aim to highlight the last decade's progress in liver regenerative medicine from liver tissue engineering, bioartificial liver devices (BAL), to liver-on-a-chip platforms, and then to present challenges ahead for further advancement.

Keywords

LiverTissue engineeringBioartificial liver devices (BAL)Liver-on-a-chip

1. Introduction

The liver, as a vital organ, plays an essential role in protein synthesis and xenobiotic metabolism. Although the liver has a high degree of regenerative capacity, drugs, toxins, viral infections, cancer, or injury can still result in permanent tissue damage and liver function impairment, which will eventually lead to end-stage liver disease or rapidly cause acute liver failure [1]. To save patients' lives, liver transplantation is often conducted for long-term therapeutic efficacy [2], which is, however, considerably limited by the lack of immunologically compatible donor organs [3]. In addition, short-term and long-term immunosuppression needs to be administrated and maintained in patients, which inevitably deteriorates patient's health conditions and increases healthcare cost [4]. Compared to liver transplantation, hepatocyte transplantation, which is less invasive and can be performed repeatedly, has been used to treat acute liver failure [5]. However, hepatocyte transplantation suffers from a low degree of engraftment (less than 30%), and this method cannot restore long-term liver functions [5]. Thus, liver tissue engineering approaches have been extensively explored to provide transplantable microtissues or whole bioengineered liver for long-term hepatic functional restoration.

Until now, liver tissue engineering has significantly progressed in engineering cells, biomaterials and tissue architectures to fabricate transplantable liver microtissues [6], [7], [8], [9] or bioengineered whole liver [10], [11] over the last decade. Cell sources range from primary hepatocytes to induced pluripotent stem cells (iPSCs) derived hepatocyte-like cells, which are amenable to cell cultivation at a large scale and offer immunological compatibility for allogeneic transplantation. Biomaterial wise, hydrogel, gelatin methacryloyl (GelMA), and polyethylene

glycol (PEG) have been evaluated in construction of 3D extracellular matrix (ECM), which is essential to provide spatial architectures and mechanical cues for stem cell differentiation and hepatocyte maturation. More importantly, perfusion-based culture systems have been introduced into 3D tissue culture, as opposed to traditional static 2D cell culture, to mimic extracellular environment in vivo for efficient mass exchange and cellular communication, especially in long-term culture [12]. It has been demonstrated that liver microtissues generated from stem cells with fine adjustment of biophysical and soluble factors exhibit enhanced liver functions in pigs [3] and mice [13], [14]. Besides liver microtissues, bioengineered whole liver has also been clinically attractive, and pioneered research in this field has shown that decellularized whole liver can be reseeded with functional hepatocytes to rescue animals with induced liver failure [10], [11].

Apart from liver tissue engineering, other liver regenerative medicine-based strategies such as bioartifical liver (BAL) devices [15] and liver-on-a-chip platforms [16], [17] have been developed to mainly provide extracorporeal support [18] and pharmacological testing [19], [20], respectively. Based on blood dialysis to remove metabolic wastes, BAL is incorporated with functional hepatocytes to partially restore a patient's liver functions while waiting for liver transplantation [18]. It has been clinically proven that temporal extracorporeal support offered by BAL for patients can provide beneficial effects on treating patients with liver diseases in clinical trials as previously reviewed [18]. In contrast to bulky BAL devices, the liver-on-a-chip platform is a miniaturized microscale device integrated with the capacity to precisely control temporal and spatial distribution of nutrients and growth factors, as well as delivery of physiological stimuli to cells under perfusion-based culture conditions [19], [20]. Depending on the type of cells, scaffolds, growth factors, and biomechanical stimuli, the liver-on-a-chip platform can be adapted for versatile applications such as disease modeling, stem cell differentiation, drug screening, toxicity testing, and so on. Clearly, these two types of devices have significantly impacted the clinical and basic research related to liver regenerative medicine.

In this review, we therefore aim to summarize the progress of liver regenerative medicine including both regenerating transplantable liver microtissues and creating in vitro liver models for regeneration purposes from the last decade. In detail, we will focus on liver regenerative medicine including basic and translational research in liver tissue engineering, bioartificial liver devices (BAL) for extracorporeal support in clinical practice, and liver-on-a-chip platforms for drug testing (Fig. 1). We will first present the advances in four key components, namely cells, scaffolds, soluble factors and biophysical cues for liver tissue engineering [8], [21], [22], and their applications in generating transplantable liver microtissues or bioengineered whole liver. We will then further discuss the advances and applications of BAL and liver-on-a-chip technologies in the domain of liver regenerative medicine, benefiting from latest development of dynamic 3D cell culture systems. Finally, we envision the challenges ahead and research endeavor needed for further advancing liver regenerative medicine.

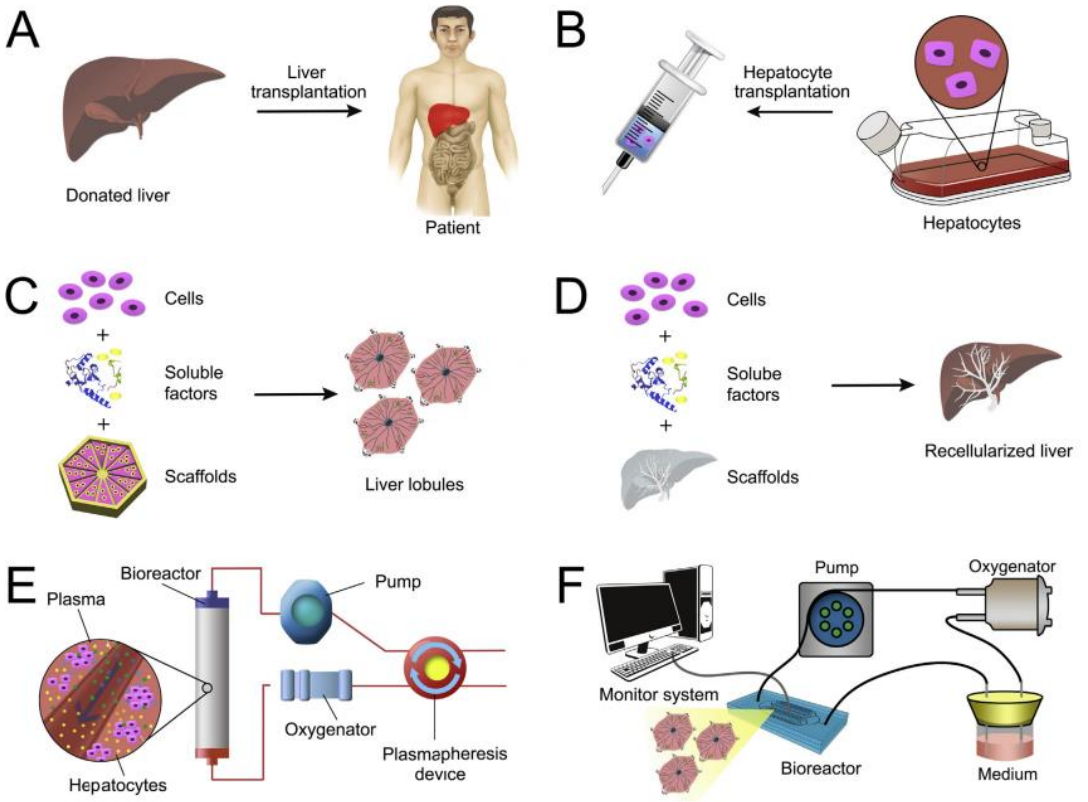

Fig. 1. Liver regenerative medicine-based approaches. (A) Liver transplantation. (B) Cell transplantation. (C) Regeneration of liver microtissues/organoids. (D) Bioengineered whole liver. (E) Bioartificial liver system (BAL). (F) Liver-on-a-chip.

2. Liver tissue engineering

In general, two highly interacting components, namely cells and extracellular environment [23], [24], [25], [26], [27], are considerably investigated in tissue engineering. Cells including mature cells, progenitor cells or stem cells can be modulated to provide physiological functions as their counterparts in vivo. On the other hand, extracellular environment can be designed to recapitulate the hierarchy milieu of native tissues with consideration of biomechanical, biochemical and biophysical cues [28], [29], [30], [31], [32]. We, hereby, present the latest advances in liver tissue engineering with respect to cells, scaffolds, soluble factors, and biophysical factors.

2.1. Essential components for liver tissue engineering

2.1.1. Cells

Nowadays, primary hepatocytes remain the first choice for liver tissue engineering, because freshly isolated hepatocytes still maintain drug-metabolizing capacity in vivo. However, primary hepatocytes, when cultured in vitro, gradually lose their morphology and liver-specific functions such as carbohydrate metabolism, protein synthesis and cytochrome P450 activity. To overcome these drawbacks, a great deal of research effort has been focused on enhancing hepatocyte functions via developing 2D matrix [33], 3D scaffolds [34], [35] and perfusion-based microfluidic systems [36]. For instance, to maintain key specific functions of primary hepatocytes, a monolayer coating of collagen was applied in a 2D culture system [33]. The hepatocytes, cultured on 2D collagen coating, maintained a typical honeycomb morphology and showed augmented gene expression (e.g., vimentin, Zinc finger E-box-binding homeobox 1 (ZEB1) and snail-1). Further, primary hepatocytes, seeded on 3D scaffolds such as hydrogels [34] and polymer scaffolds [36] showed enhanced hepatocyte aggregation, spreading, and metabolic functions [34]. In a 3D perfusion-based

microfluidic system, which constantly removes metabolic wastes and replenishes nutrients, cell morphology, cell viability and cell-cell fusion were significantly enhanced even when primary human hepatocytes were cultured in the absence of biochemical matrices for two weeks [37]. In another study, it was found that the dynamic flow maintained and stabilized the hepatocyte function through collagen secretion [38]. Despite the advances in culturing conditions, the use of primary hepatocytes still faces considerable technical obstacles in liver tissue engineering, since they need to be freshly isolated from patients for immune compatibility, and they tend to lose replication capacity over time in vitro.

To generate an unlimited supply of functional hepatocytes, a variety of stem cells, including embryonic stem cells (ESCs) [39], mesenchymal stem cells (MSCs) [40] and iPSCs [7], have been investigated. ESCs are derived from the inner cell mass of blastocysts, and they have self-renewal capability and pluripotency to differentiate into almost all cell types. A number of studies have shown that ESCs can be induced to differentiate into hepatocytes by chemical stimulation [41], [42]. For example, ESCs differentiated into hepatoblasts when cultured in basal media supplemented with biochemical factors such as insulin and sodium selenite in a stepwise manner at sequential differentiation stages [41]. The derived hepatoblasts expressed its characteristic markers and liver-specific transcriptional factors such as AFP/hepatocyte nuclear factor 4α (HNF4α), EpCAM/HNF4α, and pan-cytokeratin/forkhead box protein A2 (FoxA2) during prolonged culture, indicative of the establishment of partial liver functions. In another study, cells differentiated from human ESCs, owing to enhanced interactions among cells in a 3D culture system, exhibited morphological and ultrastructural characteristics of primary hepatocytes under SEM and TEM [42]. In addition, enhanced and prolonged expression of liver-specific proteins such as albumin (ALB), phosphoenolpyruvate carboxykinase, and asialoglycoprotein receptor 1 was observed in the 3D aggregates [42]. These studies indicate the feasibility to differentiate hepatocytes from ESCs.

MSCs isolated from bone marrow [3], [43], [44], adipose tissue [29], and umbilical cord [4] have also been successfully differentiated into hepatocytes in vitro[40] and in vivo [3]. For example, MSCs, derived from mouse bone marrow, exhibited enhanced hepatocyte-specific expressions of AFP, aspartate transaminase (AST) and urea when they were cultured on decellularized scaffolds with addition of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) in vitro [40]. In a recent study, human bone marrow MSCs (hBMSCs), which showed typical phenotypes and the capacity to differentiate into multiple cell lineages, were successfully implanted into pigs for treating fulminant hepatic failure [3]. Both undifferentiated hBMSCs and human-derived hepatocytes were observed in pigs' liver tissues after 3 days of implantation, whereas only human-derived hepatocytes were observed after 7 days of implantation. Further, lipoproteins and total cholesterol rebounded to the baseline after 5–7 days of implantation. The results indicated that the implanted hBMSCs differentiated into human-derived hepatocytes and exhibited synthetic functions after 7-day implantation in pigs. In addition, approximately 4.5% and 4.7% of total hepatocytes were derived from the implanted hBMSCs after 7 and 14 days of implantation, respectively.

iPSCs derived from a patient's somatic cells hold great promise in liver tissue engineering. A key study by Takebe et al. showed that iPSCs can be self-organized into 3D iPSC liver buds (iPSC-LBs) by applying co-culture of hepatic endoderm cells derived from human iPSCs, human MSCs and

human umbilical vein endothelial cells (HUVECs) [7]. As demonstrated, the results of immunostaining and gene-expression revealed a correlation between in vitro grown iPSC-LBs and in vivo liver buds. In another study, iPSCs were differentiated into mature hepatocyte-like cells using microRNA122 (miR122) integrated with polyurethane-graft-short-branch polyethylenimine copolymers, which were embedded in amphiphatic carboxymethyl-hexanoyl chitosan [45]. The results showed that miR122 effectively accelerated the hepatic differentiation of iPSCs resulting in hepatocyte-like cells with mature functions. Moreover, human iPSC-derived hepatic progenitor cells (hiPSC-HPCs) with HUVECs and MSCs were embedded in a hydrogel-based 3D culture system, which was designed to have microscale hexagonal architectures [8]. The co-cultured hiPSC-HPCs in this 3D culture system showed enhanced liver-specific gene expression of HNF4α, mouse anti-human Transthyretin, and ALB, as well as hepatocyte functions after 10 days. Alternatively, murine or human fibroblasts can be transformed into hepatocytes by direct programming [46], [47]. For example, human induced hepatocytes (hiHeps) were obtained by transduction via lentiviral vectors that expressed forkhead box protein A3 (FoxA3), hepatocyte nuclear factor 1-alpha (HNF1α), and HNF4α [46]. The obtained hiHeps were expandable in vitro and displayed functional characteristics of mature hepatocytes including CYP450 activity.

Clearly in liver tissue engineering, there is a shift in cell sources from primary hepatocytes to stem cells due to the latter's remarkable attributes such as renewability, pluripotency, and immune compatibility. Furthermore, a number of studies have been conducted to explore the feasibility of stem cell transplantation for potential clinical treatment of liver failure. For example, hepatocyte-like cells induced from fibroblasts were transplanted in mice via intrasplenic injection [47]. In this study, fibroblasts obtained from the tail-tip of a mouse underwent hepatic conversion by transducing the gene of transcription factors of GATA binding protein 4 (Gata4), HNF1α, and FoxA3 via lentiviral vectors. Hepatocyte-like cells not only demonstrated hepatocyte morphology after transduction, but also restored liver functions in approximately 50% of mice receiving cell transplantation. In another study, intravenously injected hBMSCs were able to rescue pigs with induced fulminant hepatic failure (FHF) [3]. This study revealed that transplantation of hBMSCs via intrahepatic portal vein suppressed fatal cytokine storms in pigs with induced FHF, and stabilized 6 typical liver function biomarkers (i.e., indirect bilirubin, direct bilirubin, total bilirubin, total bile acid, glycylproline dipeptidyl aminopeptidase and aspartate transaminase) within 7 days. All pigs died in the non-treated control group within 3.22 days, whereas 13 out of 15 FHF pigs receiving stem cell transplantation survived up to 6 months.

2.1.2. Scaffolds

Biomaterial scaffolds are often used as substrates for cell culture and as carriers to deliver soluble factors for in vitro studies and in vivo implantation [41], [48], [49], [50], [51]. For liver tissue engineering, hydrogel-based scaffolds [52], electrospun nanofiber scaffolds [53], [54], and decellularized liver scaffolds [40], [55], [56] have been employed to provide an appropriate ECM for improving hepatic differentiation of stem cells as well as for maintaining hepatocyte functions (Fig. 2).

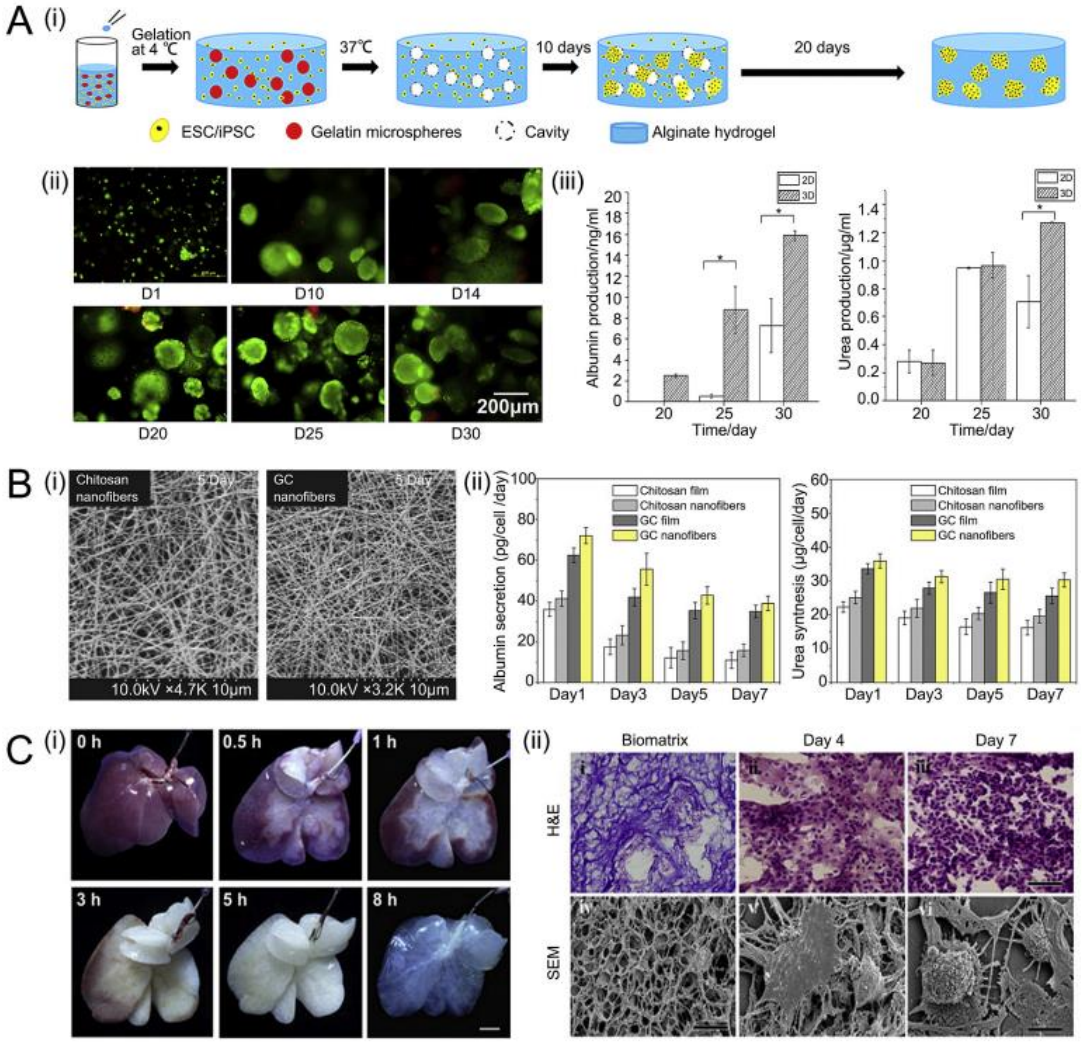

Fig. 2. Scaffolds for liver tissue engineering. (A) Hydrogel scaffolds Adapted with permission from Ref. [52]. (i) Schematic of ESCs and iPSCs encapsulated in a 3D alginate hydrogel scaffolds, and (ii) live/dead staining of iPSCs during a long-term culture period (30 days), and (iii) production of albumin and urea from iPSCs. (B) Electrospun nanofiber scaffolds. Adapted with permission from Ref. [53]. (i) SEM images of electrospun nanofiber scaffolds made of chitosan and galactosylated chitosan (GC), and (ii) production of albumin and urea from hepatocytes seeded on chitosan and GC-based nanofiber scaffolds for day 1, 3, 5 and 7. (C) Decellularized liver scaffolds. Adapted with permission from Ref. [40]. (i) Preparation of 3D decellularized liver scaffolds from a whole rat liver, and (ii) H&E staining and SEM images of decellularized liver scaffolds before and after recellularization with hepatocytes for days 4 and 7.

Hydrogels are the most commonly used scaffolds for 3D tissue engineering, owing to their particular space-filling porosity, scaffold topography, and biocompatibility [52]. To provide porous 3D scaffolds for liver regeneration, a 3D micro-cavitary hydrogel system was developed to encapsulate murine iPSCs that were subsequently induced to endodermal lineage and hepatic lineage (Fig. 2A) [52]. Gelatin microspheres were mixed with an alginate solution containing iPSCs at 4 °C, and gelation was achieved with the introduction of calcium chloride. Upon increasing the temperature to 37 °C, gelatin microspheres melted and formed microcavities, which allowed efficient nutrient exchange and rapid cell growth in a larger living space compared to non-cavity alginate hydrogels. Over a 30-day 3D culture period, iPSCs underwent endodermal induction, hepatic differentiation and hepatoblast maturation with specific growth factors. The results showed that iPSCs were viable over the course of 3D culture and produced significantly higher levels of urea and ALB on Day 20, compared to 2D monolayer culture [52]. In another study, iPSCs-derived endothelial cells and hepatocytes were assembled in fibers via multi-interfacial polyelectrolyte complexation to mimic their spatial configuration in ECM [13]. The results showed that endothelial cells in the same hydrogel scaffolds improved hepatocyte function as evidenced by ALB production and facilitated

vascularization after transplantation in a mouse model. These two studies demonstrated the utility of hydrogel scaffolds for liver tissue engineering.

Electrospun nanofibers can be used as 3D scaffolds to maintain stable liver-specific gene expression and metabolic functions of primary hepatocytes [53], [54]. For instance, galactosylated chitosan (GC) electrospun nanofibrous scaffolds were successfully fabricated to provide suitable mechanical properties for culturing primary hepatocytes (Fig. 2B) [53]. Mouse hepatocytes cultured on the GC nanofiber scaffolds showed higher levels of ALB secretion, urea synthesis, and cytochrome P450 enzyme activity, compared to cells cultured on GC film. In another study, electrospun nanofiber scaffolds of poly-L-lactic acid (PLLA) and gelatin were used to induce hepatic differentiation of MSCs [54]. The immunofluorescence staining results showed that the MSCs cultured on the electrospinning nanofiber scaffolds exhibited liver-specific markers such as alpha-fetoprotein (AFP), ALB, and cytochrome-18. Additionally, MSCs cultured on PLLA/gelatin electrospun nanofiber scaffolds showed enhanced liver specific gene expression of AFP, ALB and CY-18, compared to cells cultured on PLLA electrospun nanofiber scaffolds.

Decellularized liver scaffolds, which can be obtained by removing hepatocytes and nonparenchymal cells, also play an important role in liver regenerative medicine [40], [55], [56]. The 3D decellularized liver scaffolds provide biophysical and biochemical factors, which can facilitate hepatic lineage differentiation of stem cells, cell attachment, and tissue formation in the presence of grow factors [40], [57]. For example, decellularized 3D liver scaffolds (DCS) derived from mice have been used to investigate the hepatic differentiation of MSCs in vitro and liver functions in vivo (Fig. 2C) [40]. In this study, MSCs cultured on DCS with addition of biochemical factors exhibited enhanced liver-specific gene expressions of AFP and ALB. In addition, the differentiated MSCs showed polygonal morphology with larger microvilli, a significantly reduced ratio of nucleus to cytoplasm, and a great amount of cytoplasmic structures. When MSCs pretreated with DCS and biochemical factors were transplanted into carbon tetrachloride (CCl4) - injured mice, a significantly enhanced survival rate and hepatocyte functions were observed, compared to the non-transplantation group. Recently, mouse decellularized liver scaffolds have also been applied to culture iPSC-derived hepatocytes (iPSC-Heps) [55]. As reported, decellularized mouse liver scaffolds recellularized with porcine iPSC-Heps showed enhanced hepatic differentiation following stem cell transplantation, suggesting the feasibility of using iPSCs for liver regeneration in vivo. However, the use of xenogeneic-decellularized scaffolds may induce immunological rejection in patients receiving transplantation of xenografts, and their use is also loomed out in ethical debate.

2.1.3. Soluble factors

As discussed above, 3D liver tissue engineering requires spatiotemporal distribution of soluble factors for hepatic differentiation of stem cells and long-term culture of hepatocytes. The effect of soluble factors on hepatocytes has been previously reviewed [58]. Here, we focus on soluble factors that impact proliferation of stem cells (i.e., ESCs, iPSCs and MSCs), hepatic differentiation, and hepatocyte maturation in a time-dependent manner (Table 1).

Table 1
Soluble factors for hepatic differentiation of stem cells. ActA: activin A, BMP4: bone morphogenetic protein-4, FGF: fibroblast growth factor, OCM: oncostatin M, HGF: hepatocyte growth factor, DEX: dexamethasone, ITSP: insulin-transferrin-selenium.

Cell type	Soluble factors									References
	Definitive Endoderm		Hepatic Endoderm		Hepatic Progenitor		Further Maturation			
mESCs/ miPSCs	ActA (100ng/mL) + BMP-4 (10ng/mL) + FGF2 (20ng/mL) + B27 (2%)	D5	BMP4 (20ng/mL) + FGF2 (10ng/mL) + B27 (2%)	D10	HGF (20ng/mL) + B27 (2%)	D15	OCM (20ng/mL)	D20		Ref 52
	Mesendoderm		Hepatic Lineage		Hepatic Cells					
mMSCs	ActA (100ng/mL)	D3	HGF (20ng/mL)	D8	OCM (10ng/mL)	D13				Ref40
	Definitive Endoderm		Hepatic Endoderm		Hepatic Progenitor		Further Maturation			
hiPSCs	ActA (100ng/mL) + BMP-4 (10ng/mL) + FGF2 (20ng/mL) + B27 (2%)	D5	BMP4 (20ng/mL) + FGF2 (10ng/mL) + B27 (2%)	D10	HGF (20ng/mL) + B27 (2%)	D15	OCM (20ng/mL)	D20		Ref8
	Mesendoderm		Hepatic Lineage		Hepatic Cells					
	ActA (100ng/mL)	D3	HGF (20ng/mL)	D8	OCM (10ng/mL)	D13				Ref13/Ref77
	Endodermal Induction		Hepatic Induction		Maturation Steps					
	ActA (100ng/mL) + Wnt 3a (50ng/mL) + HGF (10ng/mL	D3	L-glutamine (1mM) + 2-mercaptoethanol (0.1mM) + Nonessential amino acids (1%) + Dimethyl sulfoxide (1%)	D8	OCM (20ng/mL) + DEX (0.5nM/mL) + ITSP (50mg/mL)	D20				Ref59
	Endodermal Induction		Hepatic Specfication		Hepatoblast Expansion		Hepatic Maturation			
	Albumin fraction V (0.5mg/ml) + ActA (100ng/mL)	D2	BMP2 (20ng/mL) + FGF4 (30ng/mL)	D6	HGF (20ng/mL) + KGF (20ng/mL) OCM (10ng/mL) + DEX (0.1nm/mL)	D11	Glutamx (1mM/L) + Nonessential amino acids (1%) + beta-mercaptoethanol (0.1mM)	D14		Ref45

In order to induce hepatic differentiation of ESCs, a series of soluble factors can be applied at various stages [7], [52], [59]. For instance, a three-stage induction method was successfully developed to differentiate ESCs into functional hepatocytes using FGF4 plus bone morphogenetic protein-2 [41]. In this study, the differentiated ESCs expressed enhanced specific liver markers and functions such as production of ALB and inducible cytochrome P450 activity. It was also reported that soluble factors of activin A (actA), acid fibroblast growth factor, and hepatocyte growth factor (HGF) were absorbed on the surface of polyethyleneimine (PEI) nanoparticles for directing the hepatic differentiation of ESCs [41]. The PEI-delivered soluble factors enabled ESCs to exhibit significantly enhanced hepatocyte function as indicated by elevated gene expression of AFP and ALB.

In addition, soluble factors are involved in the regulation of proliferation and differentiation of MSCs [58]. It is known that EGF and FGF enhanced the proliferation rate of MSCs [60], whereas nicotinamide, insulin-transferrin-selenium, recombinant Human Oncostatin-M (OSM) and dexamethasone induced hepatic differentiation of MSCs [61]. For instance, collagen I spots immobilized with HGF induced 10–20 fold higher expression of hepatocytes-specific genes in MSCs including albumin, alpha-fetoprotein and alpha 1 antitripsin, compared to the control group (collagen I only) [62]. In another study, EGF and bFGF enhanced hepatocyte-specific expressions of AFP, ALB, and urea in MSCs cultured on decellularized scaffolds [40].

Mounting evidence has shown that soluble factors play a central role in the hepatic differentiation of iPSCs [7], [52], [59]. For instance, soluble factors such as actA, HGF, OSM and EGF were applied at four major stages to induce differentiation of iPSCs into hepatocytes [52]. Immunofluorescence staining and qPCR analysis showed that the iPSCs expressed stage-specific markers when these soluble factors were applied in culture [8], [52]. In another study, B27, activin A, HGF, and oncostatin M (OCM) were applied to induce hepatic differentiation of iPSCs in a hydrogel 3D culture system. Hepatic maturation was observed at day 20, and the hepatic lineage characteristics were maintained till day 30 [52]. It has also been reported that approximately 80% iPSCs differentiated into hepatic endoderm cells when stimulated by biochemical cues, i.e.,

dexamethasone, HGF and OSM, as indicated by a typical hepatic marker of HNF4α [7]. Therefore, these studies underline the importance of soluble factors to stem cells especially on hepatic differentiation.

2.1.4. Biophysical factors

Biophysical factors are critical for providing 3D culture conditions for cell proliferation and differentiation of stem cells. Therefore, understanding of cell responses to biophysical factors is integral to the design of biomaterial scaffolds for liver tissue engineering. Here, we focus on the effect of biophysical factors from scaffolds (e.g., pore structure and stiffness) [63], [64] and shear stress imposed by fluid flow [63], [64] on cell migration, proliferation, hepatic differentiation, and tissue formation.

It has been reported that the pore size and porosity of scaffolds can be controlled by tuning the moisture content in biodegradable poly (D, L-lactide-co-glycolide) (PLGA) during fabrication [64]. The pore size of microporous scaffolds, such as 0.4, 1.0, 3.0 and 8.0 μm, showed varying capacity to control the migration of hepatic stellate cells (HSCs) on the PLGA scaffolds [63]. After 14 days of culture, the coverage of HSCs on the surface of microporous membranes with varying pore sizes (i.e., 0.4, 1.0, 3.0 and 8.0 μm), was 0% ± 0%, 63.6% ± 11.6%, 75.9% ± 10.8%, and 81.4% ± 4.3%, respectively. The membrane with a pore size of 1.0 mm was critical to construct a hepatocyte-HSC-endothelial cell (EC) layer that mimics the liver histological structure in vivo [63]. It was observed that hepatic HSCs, ECs, and hepatocytes formed layered tissues only when EC-HSC interactions were controlled by a micro-porous membrane having a pore size of 1.0 mm. It was further demonstrated that hepatocytes exhibited improved hepatocyte functions when they were cultured on membranes with defined topography, as compared to hepatocytes cultured on a single layer of membrane [64]. Such a culture model using porous membranes may provide a new approach to reconstructing multilayered hepatic tissues with highly specific liver functions in vitro.

Apart from pore size and porosity, stiffness is another important physical character that affects cell physiology and mechanics. Cellular responses to the stiffness of substrates were investigated from the aspects of cell spreading, proliferation, and dedifferentiation. In a recent study, a hyaluronic acid scaffold was successfully fabricated to mimic the stiffness of cellular microenvironment in the liver [65]. The scaffolds having stiffness from 0.6 to 4.6 kPa were fabricated by adjusting the extent of crosslinking of hyaluronic acid hydrogel. Cell attachment, viability, and structure of actin cytoskeleton were improved with enhanced stiffness up to 4.6 kPa, whereas a greater ability of cell detachment and movement occurred on softer substrates such as 0.6 and 1.2 kPa. In another study, cells in alginate gel (ALG) beads with a stiffness of 21 kPa proliferated faster than those in ALG beads with a stiffness of 70 or 105 kPa. Following 16 days of culture, under a stiffness of 21, 70, and 105 kPa, the cell count increased by 4.45, 3.29, and 3.09 folds, respectively [66]. Other factors such as substrate topography at a micro/nano scale and roughness may also play an important role in modulating cell phenotypes [44], [67]. Further understanding of how the architecture of scaffolds affects cellular behavior would maximize the efficacy of cell-based treatment and liver tissue engineering strategies.

Shear stress is another critical factor affecting hepatocyte-specific functions such as ALB production

and urea secretion in flow-based culture systems [17], [68]. For instance, a magnetically controlled microfluidics culture system developed using a microfabrication technology provides controllable shear stress as experienced in vivo [69]. In this study, the shear stress in the microfluidics culture system was at 8 gynes cm−2, which is within the magnitude of shear stress of physiological blood flow. Under this shear stress, hepatocytes expressed an enhanced level of urea and ALB production. In a recent study, a microfluidics device with a proper shear stress, which was controlled by the concentration of PEG, was fabricated to investigate the cellular behaviors of hepatocytes in both monolayer culture and 3D spheroid culture systems [68]. The shear stress generated by PEG at 0.1 M and 0.25 M did not show any effects on cellular morphology or adhesion of hepatocytes in the monolayer culture system. However, a decreased shear stress obtained by PEG at 0.05 M induced enhanced liver-specific gene expression (e.g., CYP1A2, multidrug resistance-associated protein 1 (Mrp1) and glucuronosyltransferase1A5) of hepatocytes in the 3D spheroid culture system.

These studies clearly demonstrated that the biophysical factors such as pore size, stiffness, and shear stress can be used to modulate cellular behaviors in 3D liver tissue engineering. Thus, precise control of these parameters, in conjunction with soluble factors, would be of great value in differentiating hepatic lineages from stem cells and in guiding hepatocyte proliferation and maturation.

2.2. Liver microtissues/organoids

Microtissues and organoids share the multicellular characteristics of cellular aggregates in a confined 3D structure; organoids particularly contain multiple types of cells derived from stem cells recapitulating native organ structure and function to a higher degree [7]. Both have shown various applications such as disease modeling, drug screening and potentially clinical implantation. Here, we highlight recent studies on fabrication of liver microtissues and organoids for liver regenerative medicine.

So far, liver microtissues have been commonly fabricated via microscale patterning [22], manipulative assembly [7] and 3D printing [8]. For example, hepatocytes encapsulated in poly(ethylene glycol) (PEG) hydrogels were microscale-patterned in islands, which were surrounded by fibroblasts embedded in PEG hydrogels [22]. Further cellular organization was patterned using a dielectrophoretic technique, forming a hexagonal liver lobule-like structure. Through enhanced cell-to-cell and cell-to-matrix interaction, the fabricated microtissues exhibited enhanced hepatocyte functions such as albumin secretion, urea synthesis and glycogen storage [22]. Moreover, human hepatocytes and mouse fibroblasts encapsulated in PEG diacrylate showed sustained hepatocyte function when they were implanted in immune-competent humanized mice ectopically. The results also revealed the drug metabolic profile and drug-drug interactions of human liver in a humanized mouse model [70]. Another strategy to fabricate liver microtissues is based on assembly of cell-laden hydrogels by magnetic or acoustic manipulation [69]. For example, magnetic microcryogels containing HepRG and NIH 3T3 fibroblasts were assembled to form 3D hepatic microtissues. The design of co-culture of hepatic microtissues via magnetic assembly not only enhanced the hepatocyte function, but also allowed separation of hepatocytes for downstream hepatotoxicity testing. Recently, acoustics-based cell-laden hydrogel assembly has enabled the production of liver organoids [71]. By altering the acoustic wave frequency, cell spheroids were

assembled into a variety of structural patterns at acoustic nodes (Fig. 3A) [72]. In this study, heterogeneous cell types including primary hepatocytes, HUVECs and fibroblasts embedded in fibrin hydrogel were assembled via acoustic wave in a few seconds, and formation of bile canaliculi and hepatocyte cell junctions was observed in the following co-culture. As demonstrated, micro-patterning and cell manipulation can serve as a versatile toolbox for constructing liver microtissues and organoids.

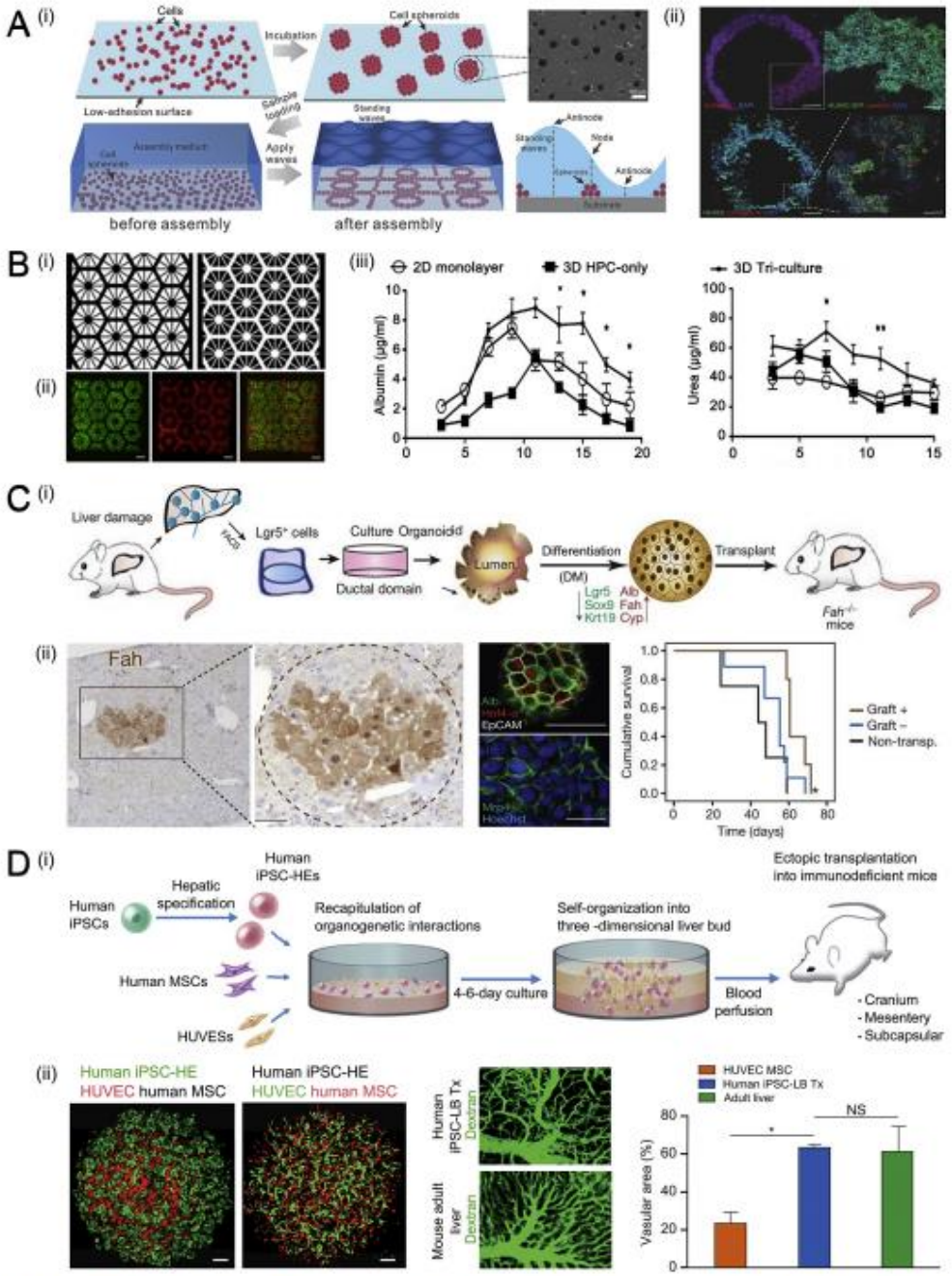

Fig. 3. Liver microtissues/organoids. (A) Liver organoids were assembled using acoustics-based cell-laden hydrogel. Adapted with permission from Ref. [71]. (i) Schematic of formation of liver organoids on a low-adhesion surface, and (ii) Positive staining of collagen type I, IV and vascular basal lamina in the assembled liver organoids. (B) Liver microtissues were fabricated using 3D bioprinting. Adapted with permission from Ref. [8]. (i) 3D bioprinted microtissue scaffolds containing lobule structures (top left) and vascular structures (top right), (ii) fluorescence staining of hiPSC-HPCs (green) and supporting cells (red) in patterned liver microtissues, and (iii) production of albumin and urea from hiPSC-HPCs and supporting cells. (C) Liver organoids for transplantation into Fah$^{-/-}$ mutant mice. Adapted with permission from Ref. [9]. (i) Schematic of regenerated liver organoids for

Apart from microscale patterning and manipulative assembly, 3D printing can be used to engineer liver microtissues. 3D bioprinting allows for even higher freedom in constructing 3D hepatic microtissues with defined morphology and precise cellular organization [67], [73], [74]. This

technology enables direct deposition of cells and biochemical factors in biomaterials (e.g. collagen, matrigel, or alginate), thus allowing the study of cell-scaffold interactions in a 3D microenvironment [75]. For example, hydrogel-based 3D printing scaffolds embedded with iPSC-derived hepatic progenitor cells (iPSC-HPCs), HUVEC and adipose-derived stem cells were fabricated in a microscale hexagonal architecture (Fig. 3B) [8]. The 3D bioprinted scaffolds showed significantly higher levels of ALB secretion and urea production from day 5 to day 15 compared to 2D monolayer culture or 3D culture of HPC only. In another study, hydrogel (e.g., gelatin) concentration, printing temperature, pressure and speed, and cell density were optimized to prepare porous scaffolds that allowed encapsulation of HepG2 cells with more than 97% of cell viability after 3D bioprinting [67]. Furthermore, hepatocytes, HUVECs, and fibroblasts were bioprinted in polycaprolactone that enhanced their mechanical properties compared to hydrogels. The co-culture of these types of cells not only maintained hepatocyte functions (e.g., production of albumin and urea), but also facilitated the formation of vascular networks [76]. Therefore, 3D cell-laden bioprinting is promising for regenerating biomimetic microtissues or even whole liver for regenerative applications.

Hepatic organoids also hold great potential to regenerate functional and transplantable liver grafts from stem cells using organoid technologies. In 2013, Huch and colleagues demonstrated that Lgr5+ liver stem cells isolated from damaged liver tissue were able to differentiate and expand in vitro, forming liver organoids and further differentiating into hepatocytes and biliary duct cells (Fig. 3C) [77]. Clonal expansion of Lgr5+ liver stem cells was achieved in culture media supplemented with an agonist, Rspo1 R-spondin 1 (RSPO1), coincided with marked up-regulation of Wnt signaling. Differentiation of Lgr5+ liver stem cells into hepatocyte and biliary duct cells resulted in hepatic morphological structure, but also exhibited hepatocyte functions such as albumin secretion and glycogen storage. Intrasplenic implantation of liver organoids in Fah−/- mutant mice led to engraftment of Lgr5+ liver stem cell-derived hepatocytes, and more importantly increased the survival rate. In another study, co-culture of human iPSCs, HUVECs, and MSCs resulted in vascularized and functional liver buds, which shows great promise of generating hepatic organoids for liver transplantation (Fig. 3D) [7]. The co-culture of these three types of cells facilitated organogenetic interactions and vascularization, and more importantly led to self-organization into 3D liver buds. Following transplantation in mice, the vascular networks in human liver buds connected to host vessels and restored liver functions, enabling ALB secretion and human-specific metabolites to drug exposure. Additionally, enhanced survival rates were observed in liver failure mouse models with ectopic transplantation of functional human liver buds. In another recent study, human fibroblast/hepatocyte cellular sheets obtained in vitro were transplanted under mouse skin for subcutaneous liver tissue engineering [14]. The close interaction between fibroblasts and primary hepatocytes helped maintain the phenotype and function of primary hepatocytes (e.g., ALB and alpha 1-anti-trypsin), but also significantly enhanced formation of vascular networks. These studies clearly demonstrated the great potential of leveraging hepatic organoids, which resemble with native tissues to a greater degree, for liver regeneration.

2.3. Bioengineered whole liver

Bioengineered whole liver has always been clinically attractive because of its therapeutic potential for liver transplantation, which is a long-term treatment option. As to this goal, tremendous efforts have been made to decellularization and recellularization of the whole liver, hoping that

parenchymal cells can reside in their native morphological structure, and thus can revive the liver-specific functionalities and proliferative capacity. In 2010, Uygun and colleagues showed that perfusion of sodium dodecyl sulfate and then Triton-x 100 through the portal vein effectively removed cellular components of rat livers and retained the 3D native matrix with preserved vascular networks (Fig. 4A) [10]. Recellularization of adult rat hepatocytes in the acellular architecture using a 4-step seeding protocol maintained high cell viability and metabolic function for 5 days. The following auxiliary transplantation of the bioengineered whole liver graft into a rat showed that engrafted hepatocytes maintained their seeding positions and cellular morphology under the sheer stress of blood flow. More importantly, liver-specific functions such as secretion of albumin and urea, and the CP450 activity exhibited levels comparable to in vitro, demonstrating the feasibility of implanting a whole liver graft in rats.

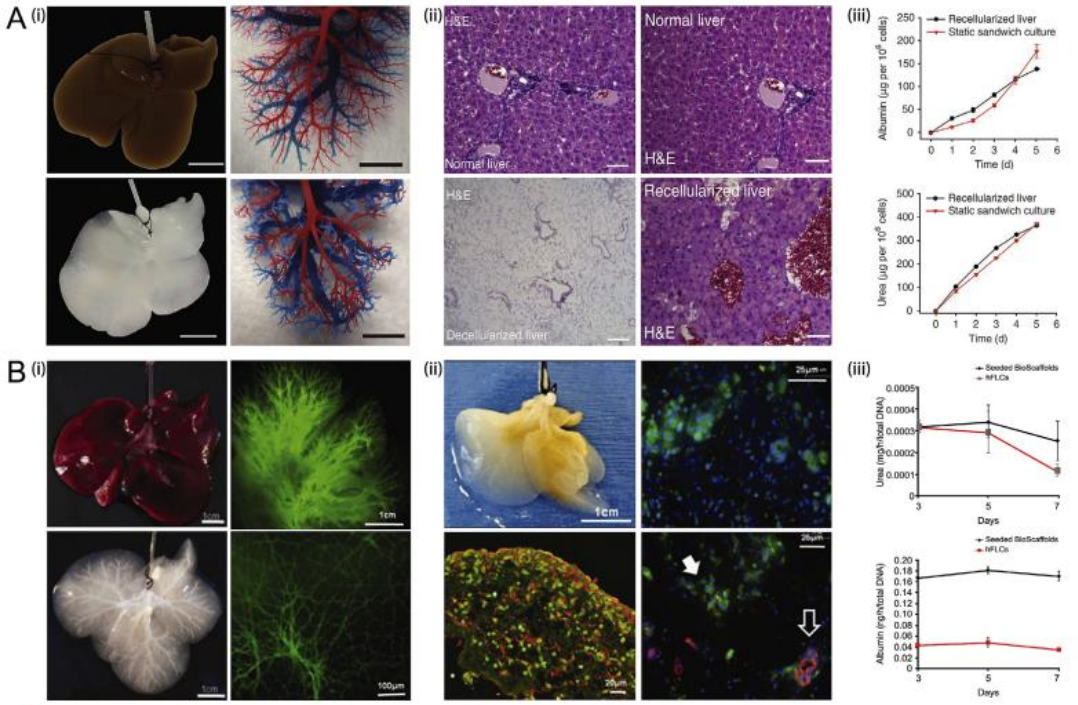

Fig. 4. Bioengineered whole liver. (A) Rat recellularized liver graft. Adapted with permission from Ref. [10]. (i) Representative images of rat liver before and after decellularization, and corrosion cast model of normal liver and decellularized liver, (ii) immunohistochemical staining of the normal liver, decellularized liver and recellularized liver, and (iii) production of albumin and urea of recellularized liver with human fetal liver cells under static sandwich culture. (B) Recellularized ferret liver graft. Adapted with permission from Ref. [11]. (i) Representative images and vascular tree of normal liver and decellularized liver of ferret, (ii) representative images and immunohistochemical staining of recellularized liver, and (iii) production of albumin and urea of recellularized liver and hepatocytes cultured in petri dishes.

In 2011, Baptista and colleagues also reported a bioengineered whole liver through decellularization using Triton X-100 and ammonium hydroxide, followed by recellularization of the bioscaffolds with HUVECs and human fetal liver cells through well-preserved vascular networks (Fig. 4B) [11]. This humanized rat liver was cultured in a bioreactor system with continuous flow of growth factors and gas exchange, showing hepatic morphological and functional characteristics. Of note, the use of fetal liver cells containing progenitor cells allowed for the differentiation of mature hepatocytes, biliary epithelial cells and endothelial cells. To establish a functional vascular network within a decellularized porcine liver, anti-endothelial cell antibody was conjugated to the inner wall of blood vessels prior to recellularization of endothelial cells [78]. As a consequence, uniform attachment of endothelial cells to blood vessels and even the capillary bed was achieved, which significantly reduced the degree of platelet adhesion during the in vitro blood perfusion. Upon transplantation into pigs, the vascularized porcine liver was able to endure the shear stress caused by blood flow and to prevent thrombosis. In another study, instead of the use of anti-endothelial cell antibody, a

heparin-gelatin mixture was utilized to promote the efficiency of endothelial cell attachment and to prevent thrombosis [57]. The approach also successfully resulted in transplantation of bioengineered livers in pigs with reconstructed vascular networks. Clearly, these studies represent ongoing efforts to reestablish a functional vasculature of decellularized whole liver and subsequently to recellularize functional hepatocytes for potential clinical applications.

3. BAL

BAL is mainly designed to provide extracorporeal support to patients who suffer from liver failure and wait for liver transplantation. In addition to plasma exchange, BAL is generally incorporated with a bioreactor containing a large amount of hepatocytes that synergistically offset a patient's damaged liver functions. Here, we mainly discuss the recent advancement of BAL devices with respect to biological component, bioreactor design, and perfusion mechanism, as well as their application in preclinical studies [79], [80].

For example, hepatocytes are incorporated into a bioreactor in combination with an extracorporeal BAL system capable of providing important liver functions such as oxidative detoxification, biotransformation and protein synthesis [81], [82]. Recently, a choanoid fluidized bed bioreactor was developed with primary hepatocytes cultured on alginate-chitosan scaffolds (Fig. 5A) [83]. The primary hepatocytes were grown in 3D alginate gel-based microcapsules (with diameter ranging between 700 and 1000 μm), which maintained hepatocyte functions and protected hepatocytes from being injured by host immune cells. In a porcine model of FHF induced by D-galactosamine, the fluidized bed bioreactor significantly increased the survival time (72.9 ± 4.72 h) compared to the untreated FHF group (54.8 ± 3.98 h) as a control. Furthermore, a fluidized bioreactor with diversion of microcapsule suspension was developed with increased cell viability, CYP1A2 and CYP3A4 activity, and gene expression of CYP450 and ALB [16]. In this bioreactor, turbine guide vanes were applied to reduce the damage of hepatocyte-containing microcapsules and to decrease void volume, yielding enhanced performance at varying flow rates. In another study, a bioreactor with stacked sandwich culture plates was fabricated for hepatocyte culture in a BAL system (Fig. 5B) [84]. The sandwich-based bioreactor allowed serial perfusion through each cell plate to increase the cell-fluid contact area for effective oxygen exchange and nutrition delivery. Hepatocytes cultured in this bioreactor maintained prolonged viability and liver specific functions (e.g., production of ALB and urea) for 7 days.

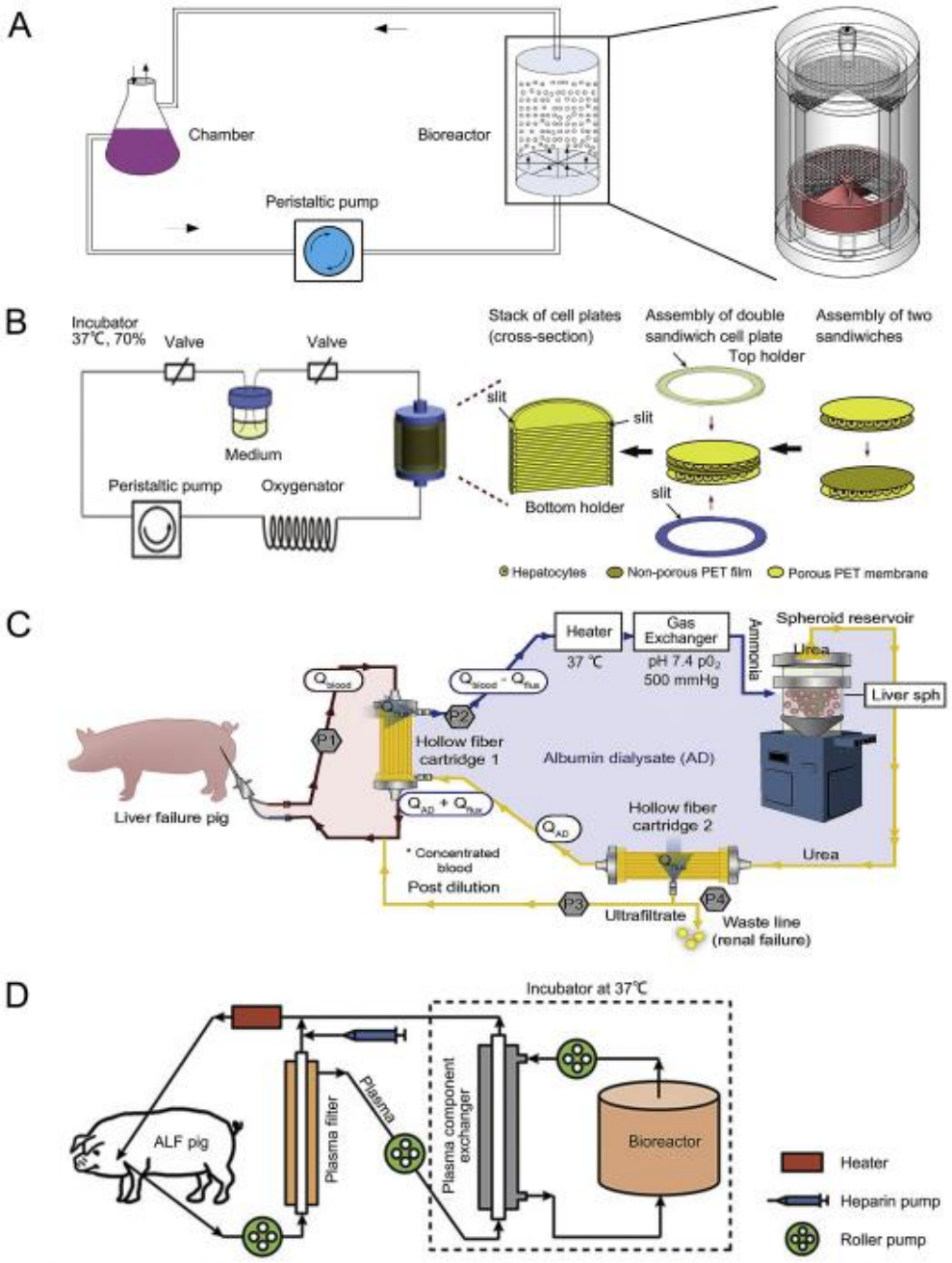

Fig. 5. Bioartificial liver (BAL) devices. (A) A diversion-type microcapsule suspension fluidized bed bioreactor with cultured C3A hepatocytes. Adapted with permission from Ref. [15]. (B) A perfusion-based macro-scale bioreactor with stacked sandwich hepatocyte cell plates. Adapted with permission from Ref. [84]. (C) A spheroid reservoir bioartificial liver with hepatocytes cultured in hollow fiber cartridges. Adapted with permission from Ref. [85]. (D) A hiHep-BAL support system with functional hepatocytes cultured in a macro-scale bioreactor. Adapted with permission from Ref. [86].

Recently, a spheroid reservoir BAL was evaluated in a preclinical trial using a porcine model with D-galactosamine induced acute liver failure (ALF) (Fig. 5C) [85]. In this BAL, hepatocyte spheroids were retained in a bioreactor by a nanopore-sized membrane, which was tethered with two hollow fiber cartridges to remove detoxification products. During extracorporeal therapy, hepatocyte spheroids maintained functionality for at least 24 h. ALF pigs treated with the spheroid-based BAL had a survival rate of 83% (n = 6), whereas standard treatment or no-cell device resulted in a survival rate of 0% (n = 6) and 17% (n = 6), respectively. Although the efficacy was affected by hepatocyte dosage, membrane pore size and duration of therapy, this study clearly demonstrated the feasibility and efficacy of spheroid-based BAL for treating ALF. In another extracorporeal BAL, up to 3 billion

human functional hepatocytes (hiHeps) obtained from lineage conversion were used to treat ALF in pigs (Fig. 5D) [86]. The large amount of hiHeps were expanded in Hyperflasks and then perfused into a multi-layer, radial-flow bioreactor. A plasma filter and a plasma exchanger were used to remove metabolic wastes. In the preclinical trial, hiHep-BAL successfully maintained low levels of AST, ALT, ammonia and bilirubin, compared to No-BAL or Empty-BAL in pigs with induced ALF. Furthermore, human ALB and alpha-1-antitrypsin were detected in pig sera, showing that they were synthesized by hiHeps and then secreted into ALF pigs. As documented, 7 out of 8 ALF pigs were rescued by providing metabolic detoxification and synthetic functions from the hiHep-BAL. Taken together, BAL systems have shown significant efficacy in treating ALF in porcine models, holding promise for clinical translation.

4. Liver-on-a-chip

Long-term culture is essential to maintain the hepatocyte phenotype and spheroids, and to support associated multiple applications. To sustain the phenotype of hepatocytes and liver-specific functions in long-term culture, perfusion-based cell culture techniques have been introduced in microfluidics devices with fine-tuning of physical, chemical and biological cues, leading to the development of organ-on-a-chip technologies [19], [20]. Here, we summarize the application of liver-on-a-chip technologies for investigating cell-cell interaction and drug screening, as well as integration of sensors for real-time monitoring of cells responses under physiological or pharmaceutical testing conditions.

The key to liver-on-a-chip technologies is to offer dynamic fluidic flow in a 3D culture environment and to deliver soluble factors in a controlled manner. For instance, a low-cost liver cell culture microfluidics device was utilized to create microfluidic flow over 3D multi-cell culture, and to continuously evaluate the cellular behavior of primary hepatocytes for 14 days [16]. The microfluidic device offered a bidirectional fluid flow via gravity by placing on a rocking platform. The result showed that primary hepatocytes co-cultured with fibroblasts, stellate cells and Kupffer cells under the bidirectional flow for 14 days had increased metabolic activity, as well as production of ALB and urea compared to static culture. In another study, a vertical-flow compaction microfluidic device was fabricated to mimic intra-abdominal pressure and portal pressure as hepatocytes experience in vivo (Fig. 6A) [17]. Under this vertical perfusion-based condition, hepatocytes were compacted and resembled in vivo-like cuboidal morphology, exhibiting hepatocyte polarity and enhanced production of ALB and urea, compared to static culture conditions [17]. These results demonstrate that fluidic flow plays a pivotal role in increasing the viability of hepatocytes and in enhancing hepatocyte functions in long-term 3D culture by mimicking physiological conditions.

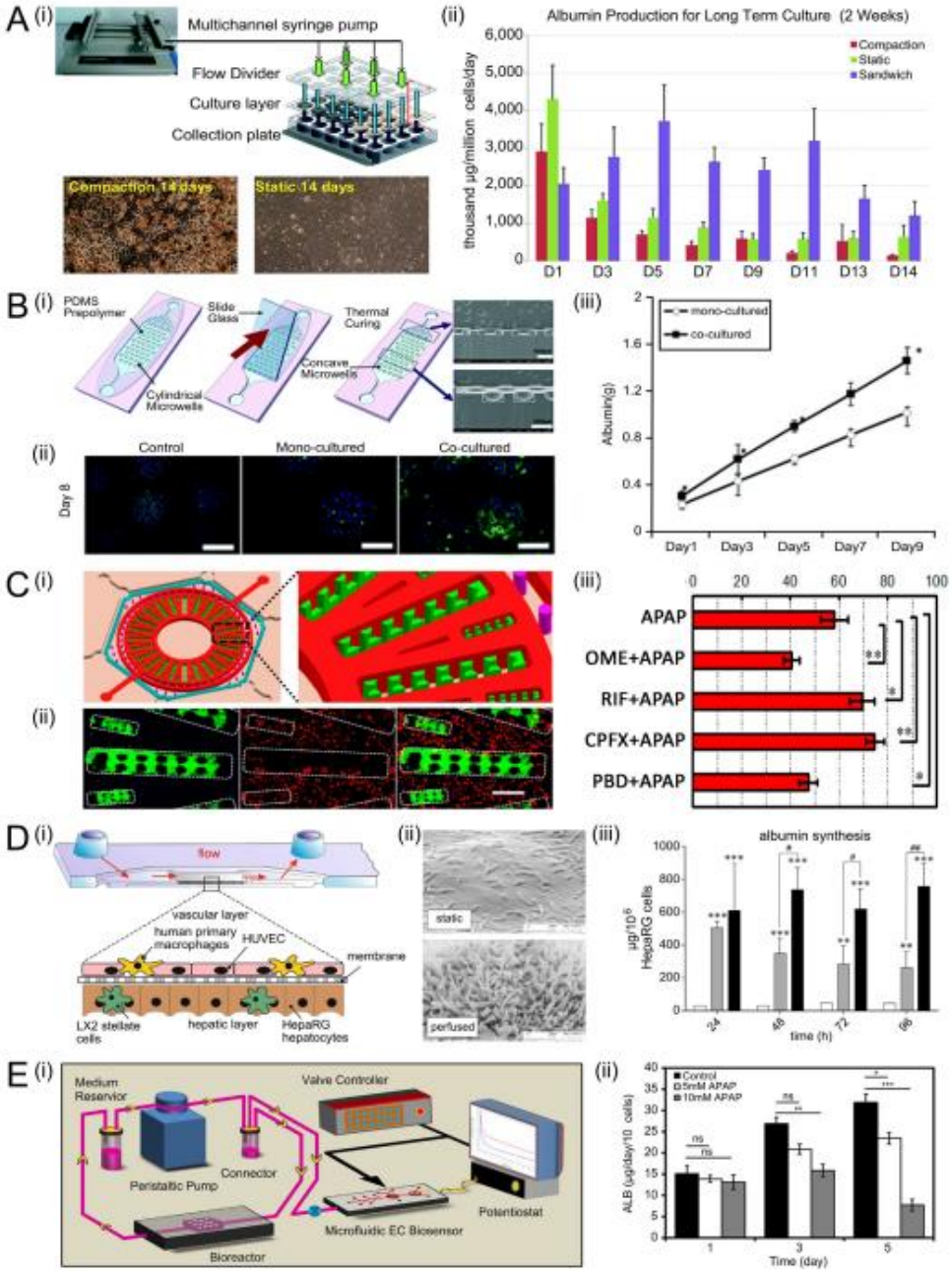

Fig. 6. Liver-on-a-chip devices. (A) A vertical flow compaction microfluidic device. Adapted with permission from Ref. [17] (i) Schematic of the microfluidic device connected with a multichannel syringe pump, (ii) phase contrast (10x) images of hepatocytes under compaction and static culture conditions, and (iii) production of albumin and urea from hepatocytes under compaction and static culture conditions. (B) Concave microwell array-based microfluidic device. Adapted with permission from Ref. [89]. (i) Schematic of the PDMS-based concave microwell arrays, (ii) immunostaining of albumin, and (iii) production of albumin and urea in both mono-cultured (hepatocytes) and co-cultured spheroids (hepatocytes and hepatic stellate cells). (C) Biomimetic microfluidic device with liver microarchitecture. Adapted with permission from Ref. [94]. (i) Schematic of the biomimetic

Liver-on-a-chip technologies have proven to be practical and useful in controlling the delivery of soluble factors to primary hepatocytes and thus in enhancing hepatocyte phenotype and functions. For example, a microfluidic perfusion device was modeled and experimentally established to deliver transforming growth factor-beta1 (TGF-beta1) from gelatin microspheres in a controlled manner to support the function of primary hepatocytes in vitro [87]. The controlled perfusion of TGF-beta1 using a microfluidics device enhanced liver-specific functions of primary hepatocytes. After 7 days of cell culture upon the stimulation of TGF-beta1, primary hepatocytes exhibited significantly enhanced ALB secretion and phase I/II enzymatic activities compared to co-culture of hepatocytes and fibroblasts. In another study, a reconfigurable microfluidic device was designed to deliver HGF

from collagen spots aiming at characterizing the signal communication among hepatocytes [88]. The results showed that HGF released from collagen spots increased autocrine production of HGF encapsulated in these collagen spots and then further enhanced hepatocyte phenotype from neighboring hepatocytes in a paracrine manner. Clearly, liver-on-a-chip technologies offer a versatile toolbox to precisely deliver soluble factors to hepatocytes for multiple tissue engineering applications.

Liver-on-a-chip technologies have also been increasingly used to culture 3D liver spheroids and to maintain prolonged hepatocyte functions in a continuously perfused manner. For example, a liver-on-a-chip device was designed to study the interaction between hepatocytes and hepatic stellate cells (HSCs) (Fig. 6B) [89]. In the device configuration, hepatocytes and HSCs were seeded in a flat chamber and a concave chamber, respectively. Both chambers were then connected, and the cells were cultured under continuous flow. This liver-on-a-chip device enabled formation of hepatocytes-HSCs spheroids and maintained long-term culture for 13 days. The results showed that co-cultured hepatocyte-HSC spheroids in this device were able to enhance hepatocyte functions (e.g. production of ALB and urea as well as cytochrome P450 activity) compared to mono-culture conditions. To minimize cell loss or detachment from liver spheroids, a constrained spheroids culture device has been developed by immobilizing liver spheroids between a thin membrane and a cover slip [90]. The surface of both sides was modified with PEG and galactose to better form and maintain liver spheroids. In this sandwich configuration, perfusion had little effect on cell loss, and significantly elevated hepatocyte functions were observed. Recently, iPSC-derived hepatocytes alone or in co-culture with supporting cells have been cultured in a C-trap chip allowing for evaluation of hepatocyte functions under different flow rates [91]. The results showed that flow rates varying from 24 to 540 μL/h did not significantly affect hepatocyte functions in this C-trap device. As demonstrated, the versatile design of microfluidic devices can be therefore leveraged to engineer conditions for generating and culturing liver spheroids.

So far, liver-on-a-chip technologies have been utilized for drug testing, since perfusion-based 3D culture can maximally mimic in vivo conditions than conventional 2D cell culture. For example, a digital platform based on microfluidic organoids for drug screening has been developed for testing the dosage effect of acetaminophen on apoptosis of liver organoids [92]. In this microfluidic device, HepG2 and NIH-3T3 fibroblast cells were encapsulated in hydrogels, forming liver organoids and showing enhanced hepatocyte functions than 2D culture systems. As documented in this system, 5 mM of acetaminophen did not cause significant toxic effects, whereas 10 and 20 mM of acetaminophen did, compared to the non-treatment controls, clearly showing a dose-dependent effect of acetaminophen on liver organoids. In another study, bioprinted hepatic spheroids in GelMA were also tested with acetaminophen in a liver-on-a-chip platform [93]. Under prolonged cultured conditions with continuous fluidic flow for up to 30 days, bioprinted 3D hepatic spheroids maintained secretion of ALB, alpha-1-antitrypsin, and other hepatocyte markers. Treatment of 15 mM of acetaminophen resulted in significantly decreased metabolic activity and cell death. In a recent study, drug-drug interactions were tested in a liver-on-a-chip platform with liver lobule-like microtissues (Fig. 6C) [94]. Pretreatment with omeprazole or probenecid for 48 h prior to treatment with acetaminophen for 24 h exacerbated the toxicity of acetaminophen, whereas pretreatment with rifampicin or ciprofloxacin alleviated the toxicity of acetaminophen. These studies have shown the

proof-of-concept of leveraging liver-on-a-chip technologies for hepatotoxicity testing and potential new drug development.

Recently, liver-on-a-chip technologies have merged with biosensors to real-time monitor the morphology and functions of hepatocyte or hepatocyte spheroids while in culture [36], [95]. On one hand, the liver-on-a-chip module can provide sufficient nutrition, 3D microenvironment, and cell-cell interfaces, as well as yield functional liver organoids comprising all main liver cell types (Fig. 6D) [36]. On the other hand, a real-time luminescence-based microfluidic biosensor enabled the measurement of oxygen consumption of hepatocytes, which is a key parameter of the metabolic activity, during cell culture. The real-time biosensor showed that the oxygen consumption of hepatocytes increased when the perfusion rate was elevated, indicating that the metabolic activity of hepatocytes was affected by the perfusion rate. For continuous monitoring of cellular functions, a liver-on-a-chip platform integrated with magnetic microbead-based (MB-based) biosensors was developed with the capacity to automatically measure hepatocyte-secreted biomarkers (Fig. 6E) [95]. In this study, the MBs-based microfluidic biosensor showed continual monitoring of ALB production of human primary hepatocyte spheroids, which were cultured in liver-on-a-chip microfluidics devices. Multiple physical, biochemical and optical sensors have also been integrated into an organ-on-chips platform for automated and continuous monitoring of cellular behaviors [96]. Thus, the liver-on-a-chip technologies integrated with biosensors provide an invaluable toolbox to investigate human hepatocellular physiology under conditions close to the in vivo physiological environments.

5. Conclusion and perspectives

Over the last decade, liver disease remains one of the most important human health issues, which has driven liver regenerative medicine to strive forward prominently. As summarized in this review, liver regenerative medicine-based approaches such as cell transplantation, tissue engineering, BAL and liver-on-a-chip have made significant progress due to the capability to engineer cells and scaffolds, emerging biofabrication technologies, biosensing technologies, etc. Particularly for liver tissue engineering, renewable cell sources (e.g., ESCs, MSCs, and iPSCs), biomimetic engineering of native and synthetic scaffolds, and spatiotemporal control of biochemical and biomechanical cues have synergistically made it possible to regenerate functional hepatic microtissues/organoids. In addition, co-culture of human iPSCs (iPSC-HEs), HUVECs, and MSCs facilitates organogenetic interactions and vascularization, leading to self-formation of 3D liver buds, which have successfully increased the survival rate of diseased mouse models. As such, striking progresses have been made by tissue engineering approaches towards fabricating transplantable tissues for liver restoration in vivo. Meanwhile, BAL and liver-on-a-chip technologies have also benefited from these progresses to provide extracorporeal support, disease modeling and drug testing, and so on.

Despite fast advances in liver regenerative medicine, several key challenges, in our opinion, need to be fully addressed prior to clinical translation. First, it remains challenging to fully recapitulate native liver tissue microstructures through microfabrication techniques or self-organization of hepatic organoids. Although 3D bioprinting and decellularized liver scaffolds allow for reconstruction of ECM at a higher degree, physiologically relevant cell density and organization can hardly be achieved in vitro, given the complexities of cell varieties (fibroblasts, stellate cells

and Kupffer cells) and cell-cell interface for maintenance of hepatocyte phenotype and function. Second, it is challenging to reestablish liver tissue extracellular microenvironment in vitro where hepatocytes and nonparenchymal cells reside, since liver physiology at a cellular level remains elusive. It may rely on sophisticated 3D modeling to reveal in vivo extracellular microenvironment with consideration of biophysical factors such as porosity, stiffness, shear stress, etc. as well as dynamic spatiotemporal distribution of soluble factors under liver physiological conditions. Third, it is of challenge to engraft liver microtissues/organoids or whole bioengineered liver and maintain functionality post transplantation. To be functional, the implanted graft, as an integral component, should be anastomosed with the host vascular networks, be compatible with the host immune system, and regulated through nervous and endocrine systems, which, however, has not been systemically investigated. Despite these major challenges and other technical barriers, we envision that full understanding and close biomimicry of the liver biology, in the context of communication with human immune system, endocrine system, nervous system and other organs in a dynamic and highly interacted manner, would provide invaluable insights into liver regenerative medicine.

Conflicts of interest

U. D. is a founder of, and has an equity interest in: i) DxNow Inc., a company that is developing microfluidic and imaging technologies for point-of-care diagnostic solutions, and ii) Koek Biotech, a company that is developing microfluidic IVF technologies for clinical solutions. U.D.'s interests were viewed and managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict-of-interest policies. Correspondence and requests for materials should be addressed to U.D.

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