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3D Printing for Liver Tissue Engineering: Current Approaches and Future Challenges

Phillip L. Lewis[1,2] · **Ramille N. Shah**[1,2,3,4,5]

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Abstract Recent developments in 3D printing have greatly accelerated progress in the field of liver tissue engineering by enabling the fabrication of more tissue-mimetic structures capable of restoring function. A variety of 3D printing and additive manufacturing techniques ranging from stereolithography to direct ink writing have shown great promise in liver tissue engineering and the study of cellular interactions. Despite these advances, however, there is significant room for improvement. Furthermore, because of the enormous capabilities of 3D printing, methods to analyze complex heterogeneous tissues in vitro have yet to be perfected. Investigations into the ability of 3D printing to recreate the macro- and microstructural components of the liver are still in their infancy. These specific issues need to be addressed in combination with massive scale up if 3D-printed tissue-engineered livers are to reach clinical relevance.

Keywords Bioprinting · 3D printing · Additive manufacturing · Liver tissue engineering

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✉ Ramille N. Shah
ramille-shah@northwestern.edu

Phillip L. Lewis
philliplewis2015@u.northwestern.edu

[1] Department of Biomedical Engineering, Northwestern University, 2145 Sheridan Rd, Evanston, IL 60208, USA

[2] Simpson Querrey Institute, Northwestern University, 303 E Superior St, Chicago, IL 60611, USA

[3] Department of Materials Science and Engineering, Northwestern University, 2145 Sheridan Rd, Evanston, IL 60611, USA

[4] Department of Surgery (Transplant Division), Feinberg School of Medicine, Northwestern University, 676 N. Saint Clair St, Chicago, IL 60611, USA

[5] Comprehensive Transplant Center, Feinberg School of Medicine, Northwestern University, 676 N Saint Clair St, Chicago 60611, IL, USA

Introduction

Liver disease affects 25 million patients with over 25,000 deaths per year in the USA [1]. Currently, the only treatment for end-stage liver disease is transplantation, of which there is a critical shortage of available donor tissue. Tissue engineering (TE) aims to produce viable tissues or whole organs to supplement the transplant deficit [2]. Advances in tissue engineering over the past several decades have produced a myriad of creative approaches that range from ex vivo liver dialysis to whole organ engineering that utilize a number of new and developing technologies [3]. One such technology that has expanded into the field of tissue engineering is three-dimensional (3D) printing [4]. 3D printing enables the fabrication of more complex scaffolds with better control over uniformity, architecture, shape, porosity, and pore connectivity. Organs of a repetitive microstructure such as the liver are particularly amenable to 3D printing technologies. Due to the relative novelty of 3D printing technology within tissue engineering, primary research has been focused on biomaterial and methods development and optimization [5••]. Advanced additive manufacturing (AM) techniques have also led to the development of complex microfluidic systems for modeling normal and pathologic systems, in addition to extracorporeal bioartificial livers for prolonging patient survivability until a transplant is available [6]. The focus of this review is to address AM technologies that are currently being used, issues with characterizing engineered constructs, attempts at

recreating liver architecture, and future directions in the field of 3D printing liver tissue engineering.

Liver Structural and Functional Complexity

The liver is responsible for an enormous array of complex functions, each of which will need to be addressed before tissue-engineered livers are ever brought to the clinic. Advantages of 3D printing technology in liver tissue engineering are leveraged more effectively when considering the lobule architecture (Fig. 1). The lobule is a roughly hexagonal feature flanked at each vertex by the portal triad consisting of branches from the portal vein, hepatic artery, and bile duct. Inscribed within each lobule is the central vein, which anastomose to hepatic veins that ultimately empty into the vena cava. The circulatory and ductular architecture of the liver divides it into eight segments relevant in segmentectomy for cancer resection or living donor liver transplantation [7]. Because of its size and complexity, an entire engineered liver will require billions of cells and an enormous amount of time to acquire the necessary knowledge to engineer relevant-sized functional tissue and is at minimum several decades away. More near-term approaches would target candidates for living donor liver transplantation and thus tailor organ-level and vessel-level structure to specific liver segments. However, even this is a massive scale-up from current achievements. A possibly more feasible engineering approach may be to fabricate individual units that can be manufactured in parallel and subsequently assembled for on-demand transplantation, with only moderate sacrifices to vessel and duct architecture that still facilitate surgical anastomosis.

Liver Tissue Engineering and Additive Manufacturing

A variety of AM techniques, each having their own advantages and disadvantages, have found their way into liver tissue engineering [5••, 8]. Those methods most often employed by tissue engineers fall into broad categories that are either focused energy-based or deposition-based. They may be used alone, in conjunction with another AM technique, or with established TE scaffold fabrication methods. Each has numerous nuances and requires optimization in their use for different tissue engineering applications.

Selective Laser Sintering (SLS)

Energy-based methods are able to produce highly detailed architectures but are limited in overall material choice. Selective laser sintering (SLS) is a powder bed-based energy AM technique that fuses individual polymer particles via melting. Poly (caprolactone) powder mixed with sodium chloride as a porogen can be processed using SLS to produce a precise interconnected pore network and subsequently avidin-modified to enhance cell attachment [9]. Feature size using this technique was only able to reach 4 mm, greater than that

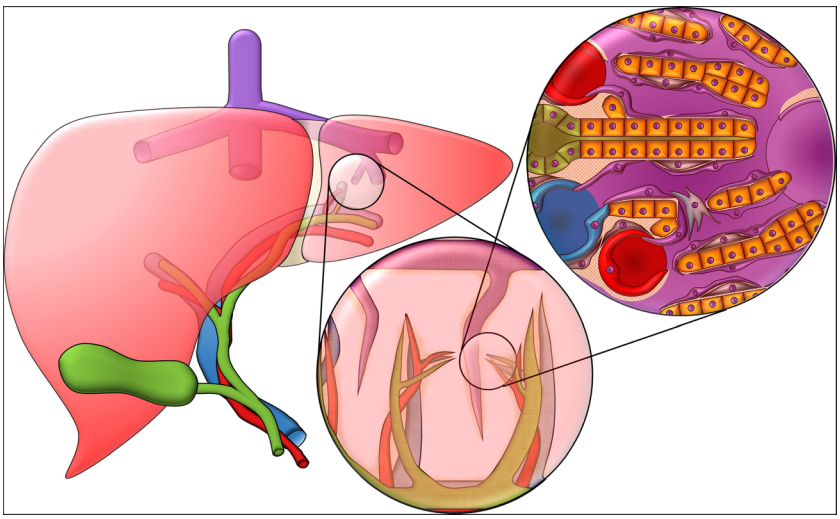

Fig. 1 Liver structural hierarchy and concerns with 3D printing and tissue engineering. Organ level: The transplanted organ needs to be of the correct size to sufficiently restore or supplement liver function in a transplant recipient. Placement of duct and vasculature needs to be in anatomically correct organizations to facilitate surgery. This requires massive scale-up of current approaches, along with digitizing and printing of patient scans. Vasculature level: Large diameter vessels and ducts must be robust enough for surgical anastomosis, necessitating strong printed structural materials. Smaller-diameter vessels need to oriented in order to be conducive to lobule formation, corresponding on an interpenetrating network of portal triads and central veins. Directional flow of bile and blood is a major engineering concern. All intervening space must be filled with parenchyma (lobules). Lobule level: Patterning lobule structures requires spectacular spatial resolution (<50 μm). All other approaches must incorporate bioactive matrices to encourage lobule morphogenesis. These approaches must incorporate multiple cell types and biomaterials that will encourage zonal specificity. Scaling from lobule to vasculature levels is the quintessential challenge in liver tissue engineering

of the liver lobule, which is a maximum of 3 mm in diameter in humans. Furthermore, melting and solidification of thermoplastic polymers via SLS (or other methods) yield stiff, brittle structures as a result of induced polymer crystallinity [5••]. Such properties are not particularly amenable to liver tissue engineering due to mechanical property mismatch.

Stereo Lithography (SLA)

Lithographic techniques are often employed to precisely pattern the location of cells. Individual hepatocytes and endothelial cells have been patterned into a lobule-like structure using SLA and dielectrophoretic patterning [10, 11]. Maskless SLA has been used to photopolymerize polyethylene glycol diacrylate (PEG-DA) and HepG2 cells into 3D shapes [12]. SLA has also been used to produce poly (dimethyl siloxane) (PDMS) molds to enable mass-production of architecturally defined structures, as demonstrated with hybrid chitosan-gelatin scaffolds [13]. While there is a cost to scaffold resolution and structural complexity, scaffold material choice is greatly expanded as a variety of scaffold materials can be cast into a PDMS mold. PDMS itself can be used to form detachable sheets using SLA [14]. SLA can also be used to specifically polymerize or immobilize certain moieties on, or within, a bulk substrate. For example, the ubiquitous tripeptide adhesion molecule arginine-glycine-aspartate (RGD) was immobilized on PEG-DA and acryloyl-PEG-RGDS in the presence of hepatocytes and a photoinitiator [15]. Subsequent bioreactor perfusion culture resulted in increased hepatocyte function compared with unpatterned controls, but albumin and urea syntheses were on par with 2D culture. SLA was also used as a pre-processing technique to produce HepG2 spheroids which were subsequently encapsulated within fibroblast-laden RGD-modified gels as a method to study cell-cell interactions [16]. Limitations of SLA are reached ultimately with difficulties in 3D scalability and biocompatibility of residual photoinitiators. The high temperatures of energy-based approaches for scaffold fabrication also lead to enormous limitations in biomaterial choice and often the preclusion of cell encapsulation.

Inkjet Printing

By far, the most versatile and common AM techniques that have become most synonymous with 3D printing are deposition-based. The first uses of 3D printing for tissue engineering applications were born out of inkjet printing, a method more familiar with 2D printing [17]. Inkjet printing consists of microscopic droplets of fluid being ejected through an opening onto a substrate. This can be scaled in three dimensions by simply moving the substrate. Traditional inkjet methods utilized the vaporization of a bubble of ink to eject a droplet. More cell and biomaterial-friendly approaches have since been developed. HepG2 cells were successfully printed using piezoelectric inkjet printing onto 2D collagen gels utilizing a Pluronic surfactant [18]. Inkjet printing is a popular choice for in vitro modeling and screening, as highly precise deposition of multiple cell, protein, and biomaterial solutions is possible [19, 20]. However, limitations with inkjet printing arise again with biomaterial choice, as the process requires low viscosity materials. Furthermore, maintaining a dispersion of high concentrations of cells or additives such as fibrous proteins or micro- or nanoparticles is difficult in low-viscosity solutions and requires consistent stirring for resuspension [4].

Fused Deposition Modeling (FDM)

Most consumer 3D printing platforms are based upon fused deposition modeling (FDM), wherein a material is extruded through a robotically controlled nozzle as subsequent layers and adjacent strands are fused with one another via temperature or solvent melting. High temperatures or the presence of solvents prevents cell encapsulation, but produces scaffolds that can be populated with cells by traditional top seeding. Scaffolds with uniform, sub-millimeter pore geometries can be fabricated using traditional tissue engineering materials such as poly (caprolactone) or poly (L-lactic acid) [21, 22]. Solvent droplets can also be deposited into powder beds, in a manner similar to SLS, to fuse individual polymer particles. One of the first approaches using FDM for liver tissue engineering utilized poly (lactic co-glycolic acid), along with a sodium chloride porogen to produce macro and microporous structures [23]. FDM printed structures are often composed of thermoplastics, which prevent cell encapsulation and rarely match the mechanical properties of soft tissue. FDM approaches also ultimately require top seeding of cells, which may present issues of inhomogenous cell distribution, especially when producing thick, cell-dense organs such as the liver.

Direct Ink Writing

Direct ink writing (DIW) is a similar but distinct technique from FDM in that solvent drying or cooling is not a requirement post extrusion. This principle of DIW is therefore employed in the development of a wide variety of inks that allow for cell encapsulation, the vast majority of which are hydrogels. Hydrogel inks that are self-supporting and retain user-defined features, however, are difficult to develop primarily due to the competing requirements that the material be extrudable and at the same time self-supporting. Methods around this limitation have incorporated support structures. For example, FDM printed PCL scaffolds were fabricated, throughout which fibroblast-laden atelocollagen gels were patterned, followed by primary hepatocyte seeding to create

a patterned 3D co-culture [24]. In such a system, a rigid support scaffold is necessary due to the slow gelation kinetics of collagen solutions. Support scaffolds typically vary drastically in mechanical properties from liver tissue, which highlights the need to develop post-processing stabilization treatments for 3D-printed hydrogels to enhance the mechanical properties of the hydrogels themselves. Cell-friendly post-printing treatments are a popular way to stabilize hydrogel structures to be self-supporting or for long-term in vitro culture. The cross-linking of alginate when in the presence of calcium ions is an attractive base material for cell-encapsulated DIW. Alginate can be used as a binder to print a number of cell-friendly pre-polymers within a calcium ion bath which can be subsequently stabilized in a manner dependent on the encapsulated pre-polymer [25]. Residual alginate can be dissolved via treatment with an aqueous calcium chelator yielding a structure composed of only cells (ex. fibroblasts, endothelial cells, and hepatocytes) and the pre-polymer. To explore the effects of printing parameters on cell viability, Chang et al. extruded HepG2-laden alginate into calcium chloride baths [26]. Cell viability was, unsurprisingly, inversely related to extrusion pressure and directly related to nozzle diameter, although recovery after certain nozzle/pressure conditions was noted. Future experiments into the effects on cell viability and function will necessitate evaluating other biomaterials, as alginate is characteristically bioinert to mammalian cells. Addition of gelatin to alginate at a 2:3 ratio allowed a primary hepatocyte-laden ink to be extruded at 10 °C and subsequently stabilized with calcium chloride [27]. Utilization of gelatin as a base material allows for control of ink thickness, and therefore printability, as a function of decreasing temperature. Pure hepatocyte-gelatin solutions have been printed into large (>2 mm in height) structures, but required post-printing stabilization with a harsh glutaraldehyde wash [28]. A gelatin-chitosan mixture has been processed in a similar method by following sodium tripolyphosphate cross-linking with glutaraldehyde [29]. Mixing of gelatin and fibrinogen takes advantage of gelatin's printability as well as the cell-friendly thrombin-induced cross-linking of fibrinogen [30]. Of note, however, is that the above gelatin-based approaches employ polymer weight percentages above 10 %, which often present nutrient diffusion issues with encapsulated cells. A lower, diffusion-friendly gelatin concentration (less than or equal to 5 %) was successfully printed in the presence of a PEG-based cross-linker yielding self-supporting, well-defined, and multi-layered constructs [31]. This method allows for post-printing UV cross-linking of methacrylated gelatin (GelMA) to create more robust structures with prolonged degradation rates. Furthermore, fibrinogen was also able to be printed using this method and secondarily crosslinked using thrombin to tailor the mechanical and degradation properties. In a similar method, 100-μm diameter aggregates of primary hepatocytes, Kupffer cells, and stellate cells were printed within thiol-modified gelatin, hyaluronic acid, or decellularized ECM and spontaneously cross-linked PEG-DA [32]. UV polymerization of PEG-alkyne and thiols acted as a secondary stabilization method, although feature sizes were relatively large (>1 mm), and multi-layered structures were not demonstrated in this approach. Functionalization of gelatin by addition of methacrylate to its amine groups is a common method to yield photopolymerizable gelatin in the presence of a photoinitiator. Photoinitiators, however, are almost exclusively toxic and are a significant drawback to GelMA or PEG-DA-based printing. Billiet et al. managed to demonstrate higher viability of printed HepG2-laden GelMA by substituting the standard photoinitiator Irgacure 2959 with VA-086 [33]. Effects on HepG2 viability of UV exposure time, pressure, and nozzle diameter and type were also examined here, in addition to rheological properties. Cell concentration was found to have a surprising influence on ink mechanical properties. This is significant because ink rheology significantly affects its ability to extrude from a nozzle. In a different extrusion approach, which is less dependent on gel rheology, HepG2 co-cultured with NIH3T3 mouse fibroblasts were encapsulated within GelMA and photopolymerized within a glass capillary tube prior to extrusion [34]. This method, however, has limitations in the length of the tube able to be formed and its inherent labor intensive nature to build thick 3D constructs.

Cell Aggregate Printing

Another approach to create printed tissue structures was developed by Forgacs et al. wherein spherical aggregates of cells are printed onto removable support structures that serve to mechanically brace the structure during printed tissue maturation [35]. Aggregates are then allowed to fuse after printing to create a material-free scaffold. This technology has since been licensed to Organovo in the form of the Novogen MMXbioprinter™[36, 37]. Utilizing the cell-aggregate method of printing, Organovo has managed to create stable (>40 days in vitro) liver tissue constructs geared towards acute and/or chronic toxicity screening. The aggregates printed are composed of multiple cell types including commercially available human endothelial cells, stellate cells, and either primary isolated or induced pluripotent stem cell (iPSC)-derived hepatocytes. Endothelial cells and hepatocytes are demonstrated to form sinusoid-like microvascular structures upon histological analysis. Hepatocytes demonstrate phenotypic stability and increased viability when cultured in much smaller spheroids [38]. A number of liver tissue engineering approaches utilize smaller hepatocyte spheroids, including some hydrogel-based 3D printing systems [32]. Cell aggregate printing is an attractive prospect for an organ with the cell density and parenchymal homogeneity as the liver; however, limitations are ultimately reached when scaling such a method to produce relevant-sized transplantable organs. Furthermore, recreating the hierarchical structure of the liver's multiple vascular and ductular systems presents a major challenge to tissue

engineers and materials scientists and warrants the development of new biomaterials, which can be designed with the ideal architecture, mechanical properties, and biological signaling that can lead to a truly functioning engineered liver transplant.

Challenges in Characterization

While some approaches to 3D printing for liver tissue engineering may hold more promise than others, the feasibility of clinical implementation ultimately depends on assessments of function. Depending upon the novelty of the methodology, different approaches may employ varying levels of analysis. Because the liver is responsible for such a wide array of functions, simplifications in functional assessment are necessary. Additionally, due to the relative novelty of the field of liver tissue engineering and 3D printing in general, biomaterial characterization and methods optimization have thus far received more attention than tests of liver functionality. For example, in more method-based and biomaterial-focused investigations, hepatocyte viability is commonly the only measured quantity. There are challenges in experimental design when it comes to performing more thorough assessments of functionality. For example, simply stating that secretion (of albumin, urea, etc.) is taking place indicates that the hepatocyte phenotype is present; however, it does not give insight into the homogeneity or heterogeneity of the resulting 3D-engineered tissue. A number of methods need to be specifically tailored in order to more fully analyze the structure and function of complex tissues generated in vitro.

3D Imaging

Three-dimensional printing has the potential to create large, uniform tissue constructs to a scale not demonstrated before. Assessment of entire constructs to evaluate cellular organization and tissue morphogenesis will therefore require development of refined and specialized 3D imaging systems. Even highly sophisticated tissue imaging techniques, such as multiphoton microscopy, can only image to a maximum tissue depth of approximately 500 μm within the native liver [39]. Confocal laser scanning microscopy is commonly employed to image 3D tissue-engineered constructs. The aspect of the construct being imaged, however, is often simply viability stains, cell tracker dyes, or fluorescent proteins. The advantages of these markers is that they are stable long-term (i.e., resist photobleaching) and are particularly bright. This allows their imaging through relatively thick or mildly translucent constructs; however, z-corrected laser intensity modification is necessary for confocal imaging deeper within samples [40••]. Immunofluorescent (IF) imaging has the potential to better spatially characterize tissue morphogenesis and function within a tissue construct, especially one whose microstructure is as complex as the liver lobule. Imaging of large 3D constructs is therefore an ongoing field of development. Fluorescently labeled secondary antibodies are often not nearly as bright as viability stains, cell tracker dyes, or fluorescent proteins due to their low concentrations within stained samples. Issues with IF imaging of large 3D constructs include the limitation of working focal depth at higher magnification and the rapid bleaching of fluorescent antibodies with increased laser intensities. Laser scanning confocal and multiphoton microscopy both have more potential than simple viability or live cell tracking, but will require significant optimization utilizing new sophisticated methods. Immunostaining of 3D-printed tissue-engineered constructs for the time being is largely reserved to histological processing.

Histological Analysis

Scaffold fixation and processing methods can be highly variable and dependent on scaffold biomaterial composition. Of paramount concern is the preservation of the 3D-printed biomaterial structure, otherwise any inferences about the influence it has on cellular behavior are lost. Thus, there are numerous histological processing concerns. 3D-printed constructs composed of certain polymers (ex. thermoplastics) may be sensitive to chemicals commonly used in histology, such as xylene used to dissolve paraffin in formalin-fixed paraffin-embedded (FFPE) samples [41]. Dehydration of 3D-printed high water content (>95 %) hydrogel scaffolds can also result in warping and distortion of printed and/or cellular structures. Methods developed for biomaterials common in tissue engineering, but not necessarily 3D printed, are often employed [42]. Structure can be better preserved by embedding in plastics such as JB-4 or within OCT compound for cryosectioning [23]. Implantation of the construct within an animal model can also lead to the infiltration of cells and matrix, making the scaffold more amenable to traditional histological processing [43].

In - Vivo Performance

Biocompatibility of a material is often confirmed with surgically simple subcutaneous implantation experiments. Implantation of tissue-engineered liver constructs at ectopic sites is an attractive treatment for synthetic diseases, such as hemophelia [44]. However, functional assessment of 3D-printed liver tissue-engineered constructs in vivo has remained particularly limited. Because of the inherent novelty of the field, the majority of experimental rigor is focused on biomaterial development, printing methods, and in vitro performance, and not on the potential of the construct as a therapeutic. Nevertheless, several studies using some variant of 3D printing have evaluated engineered constructs in vivo [43, 45]. Maintenance of fragile scaffold structure fidelity upon

implantation into the mesenteric parametrial fat pad was achieved by embedding the construct in a polypropylene surgical mesh. Upon implantation, anastomosis of host and graft vasculature was confirmed with species-specific immunohistochemical staining for CD31. Intravital imaging of hepatocytes and anastomosed vasculature was performed with luciferase transfection and FITC-dextran perfusion, respectively. The goal of these studies was to achieve effective vascularization, however, and did not assess restoration, modulation, or increase in hepatic function.

Challenges in Assessing Co-Cultures

The cellular heterogeneity of any organ is a strong impetus for using co-culture as the basis for organ engineering. Hepatocytes show significant improvements in function and viability when cultured with liver non-parenchymal cells or other cell types including fibroblasts, stellate cells, mesenchymal stem cells, macrophages (Kupffer cells), biliary epithelial cells (cholangiocytes), endothelial cells, and liver sinusoidal endothelial cells (LSECs). Cells within co-cultures are typically imaged using cell tracker dyes for short-term analysis [25, 31] and fluorescent proteins for long term [43, 45, 46]. Flow cytometric analysis is a powerful technique often employed to analyze the effects of co-cultures, as different cell populations can easily be differentiated by certain unique surface markers [15, 47]. Practical difficulties arise when attempting to isolate cells suspended within a hydrogel network. Methods to digest or dissociate the biomaterial while leaving the cells unaffected need to be tailored to each unique biomaterial system. This requirement is at odds with the necessity of scaffolds to be rigid and self-supporting enough to allow for multiple printed layers.

Engineering the Complex Macro- and Microstructural Complexity of the Liver

Each organ, like the human body, has a genetically predetermined architectural blueprint. 3D printing larger structures of an organ such as its large diameter arterial, venous, or ductular vasculature can be achieved with 3D printing. Indeed patient scans are routinely printed for surgical practice, although large diameter vasculatures vary little from patient to patient [48]. The majority of the challenges associated with solid organ tissue engineering stem from the need to engineer the organ's functional unit. In the context of the liver, this is the lobule. The lobule contains all cell types found in the liver: hepatocytes, LSECs, vascular endothelial cells, cholangiocytes, stellate cells, Kuppfer cells, and natural killer cells [49]. While technologies do exist that can spatially pattern individual cells and small biomaterial volumes in three dimensions, the process would take an enormous amount of time to print an entire liver. Furthermore, there are limited studies that have incorporated multiple cell types within one construct and therefore limited knowledge on what spatial configuration of cells will result in a fully functioning unit. A harmony between printed structure and tissue morphogenesis, mediated by scaffold architecture and bioactivity, therefore, needs to be developed.

Vasculature

By far, the paramount challenge in solid organ tissue engineering is recreating vasculature [50]. Hepatocytes of the liver are particularly sensitive to hypoxia and nutrient depletion. The vessels of the liver lobule, excluding the portal vein, hepatic artery, and central vein, are classified as sinusoids [51, 52]. Sinusoids are lined with undiaphragmed fenestrated endothelial cells and are discontinuous with one another, exposing the blood directly to hepatocyte surfaces. Sinusoids retain a thin matrix layer between hepatocytes and LSECs, but lack a basement membrane. This is in contrast to the branches of the hepatic vein, hepatic artery, portal vein, and bile ductules whose basement membranes are intact. Recreation of capillary networks in vitro is routine and is now used to verify a pure endothelial cell population [53]; however, sinusoids are phenotypically distinct from typical capillaries seen in other tissues. Large-diameter blood vessel tissue engineering is also reaching maturity and does not necessitate a sophisticated technology such as 3D printing [54]. The chief challenges lie in engineering the intermediary between large-diameter blood vessels and capillary-sized vessels [50]. Several researchers have found promise in utilizing 3D printing of sacrificial, or "fugitive", materials around which a bulk cell-laden material can be cast [40••, 46]. Removing printed sacrificial structures results in a hollow network, which can then be perfusion-seeded with endothelial cells. Kolesky et al. demonstrated this approach, along with multi-material and cell printing, using a Pluronic F127 ink which liquefies at reduced temperatures, leaving open, designed, channel networks [46]. Another approach used carbohydrate glass printed into a number of defined structures, around which agarose gels containing primary hepatocytes and fibroblasts were cast [40••]. Carbohydrate glass sacrificial inks can then simply be dissolved with water and perfused with HUVEC-laden media. Viability of hepatocytes was demonstrated nearer to vessel walls, in addition to capillary branching from fabricated vessels. A similar method was used to connect two channels via induction of angiogenic sprouting [55] or to demonstrate barrier formation [56]. Molds of SLA-fabricated PDMS have been used to form EC cords around which hepatocyte spheroid-laden gels can be cast and intraperitoneally implanted [43, 45]. The use of primary isolated LSECs has thus far been very limited in tissue engineering [57–59]. Within the context of liver 3D-printed tissue engineering, LSECs have

remained elusive in lieu of more readily available (and in some cases more translatable) sources such as HUVECs and circulating endothelial progenitor cells (EPCs). The capacity of HUVECs or EPCs to differentiate into LSECs is unknown.

Lobule Zonal Specificity

While all hepatocytes are capable of performing all functions of the liver, hepatocytes in different areas of the liver lobule are more specified for certain functions [60••]. The lobule itself is arbitrarily divided into three zones. Hepatocytes in Zone 1, or periportal hepatocytes, are geared toward β-oxidation, cholesterol, and urea biosynthesis. Hepatocytes in Zone 3, or pericentral hepatocytes, are geared toward lipogenesis, ketogenesis, xenobiotic metabolism, and synthesis of bile acids, heme, and glutamine. Hepatocytes in Zone 2 display a gradient of function between both Zone 1 and 3. The primary modulator of lobule zonation is assumed to be based on differential concentration signaling factors, nutrients, and oxygen partial pressure within sinusoids, as well as wnt/β-catenin signaling, along the portal-central axis [61, 62]. Induction of differential zonal functions in vitro has been demonstrated with different ECM culture substrates [63, 64] or in complex bioreactor systems [65]; however, this knowledge has yet to be applied to 3D printing. Indeed lobular zonal specificity is hardly addressed in the liver tissue engineering community partially due to the difficulty of isolating zonal specific hepatocytes [66] and due to the presumption that hepatocytes will autonomously zonate themselves once functional tissue is formed.

Biliary Tree

The majority of liver tissue engineering applications have focused on manipulating hepatocytes and other non-parenchymal cells in extrahepatic sites [44, 67]. While these approaches have potential to treat liver synthetic and metabolic disorders, they ultimately rely on a physiologically intact biliary epithelium to transport bile out of the blood stream and into the small intestine. To date, there have been a very limited number of tissue engineering approaches to regenerating the biliary epithelium [68–71]. No attempts have been made at regenerating the intrahepatic biliary epithelium, despite the in vitro morphogenic quality of isolated cholangiocytes [72]. 3D printing of bioactive matrices has the capacity to induce the formation of the finer branches (i.e., Canals of Hering) of the biliary tree, while printing the larger diameter ducts. Barriers to widespread use of cholangiocytes in tissue engineering stem from their prohibitively difficult isolation procedures [73, 74]. Once handling of these cells is more widespread, attempts at recreating the biliary tree are likely to be more common. Until then, most liver tissue-engineering experiments will fall slightly short of producing transplantable tissue, opting instead to produce what are characterized as bioartificial liver assist devices. Furthermore, if an engineered organ is to be transplantable, it needs to recreate all aspects of the anatomy in a way that also facilitates routine surgical handling and host integration.

Conclusions

The clinical success of solid organ tissue engineering will require optimization of a number of factors specific to the organ in question. Creation of transplantable liver tissues will obviously require recapitulation of traditionally assessed liver functions such as xenobiotic metabolism, protein synthesis and secretion, bile secretion, as well as fatty acid and carbohydrate metabolism. 3D printing is a unique technology that can allow for the creation of macroscopic vascular and ductular structures while guiding the morphogenesis of their microscopic counterparts. The capacity to create uniform solid tissue constructs may be feasible for the first time in the history of tissue engineering due to 3D printing. The field thus far has geared itself towards the development of novel biomaterials and patterning strategies. However, sufficient methods need to be developed that assess the in vitro function of these constructs and their subsequent in vivo capacity to restore function. As the field matures and the accessible palette of printable bioinks expands, so too will the level of sophistication and success of 3D-printed functional liver constructs.

Compliance with Ethical Standards

Conflict of Interest Phillip L. Lewis and Ramille N. Shah declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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