Liver disease burden

Fatal liver disease accounts for \( \sim 2 \) million deaths annually worldwide and has steadily increasing rates over the years [1]. Liver failure can be divided into three major categories: (1) acute liver failure (ALF) that presents as a rapid loss of liver function in patients without preexisting liver disease, (2) chronic liver disease due to metabolic dysfunction, and (3) chronic liver failure accompanied by tissue remodeling and scarring.

ALF is a rare syndrome with an annual incidence of less than 10 cases per million people in the developed world. In the United States, \( \sim 2000 \) cases of ALF are diagnosed each year [2]. It commonly develops in healthy adults in their 30s. Patients with ALF usually present with abnormal liver biochemistry, coagulopathy, and encephalopathy. The causes vary geographically. Damage due to drug exposure (e.g., acetaminophen) is the most common cause in the West, while in large parts of the East, viruses (e.g., Hepatitis A and E) are the most prominent cause of ALF [3]. Clinically, ALF can be subdivided based on the period of time between the appearance of jaundice and onset of hepatic encephalopathy. Data from O’Grady et al. proposed the following classification: hyperacute for periods between 0 and 7 days, acute for periods between 7 and 28 days, and subacute for periods between 4 and 12 weeks [4]. In hyperacute cases the cause is usually acetaminophen toxicity or viral infection. Subacute cases that evolve slowly often result from idiosyncratic drug-induced liver injury (DILI). Even though patients with a subacute presentation have less coagulopathy and encephalopathy, paradoxically they have a consistently worse medical outcome than those with a more rapid onset of the disease [5].

Chronic liver disease develops on the background of a constant injurious insult, either resulting from a metabolic disorder or a number of etiologies that lead to widespread tissue remodeling and pathologic deposition of extracellular matrix (ECM). Inborn liver-based errors of metabolism are life-threatening conditions caused by genetic defects in single enzymes or transporters and lead to blockade of a specific metabolic pathway. While they can be accompanied by progressive fibrosis and cirrhosis, such as in the case of \( \alpha_1 \)-antitrypsin ZZ deficiency, hemochromatosis, Wilson’s disease, and hereditary tyrosinemia [6], the liver parenchyma often remains intact. Some examples of metabolic disorders with an intact parenchyma include hypercholesterolemia, Crigler–Najjar syndrome, ornithine transcarbamylase deficiency, organic acidurias, and hyperoxaluria [7]. In a biopsy the lack of parenchymal destruction often leads to a delayed diagnosis, exposing the patient to sequelae. In all cases of liver disease the lack of FDA-approved noninvasive biomarkers makes it challenging to diagnose and treat liver diseases.

Chronic liver disease occurs in the setting of nonalcoholic fatty liver disease (NAFLD). NAFLD is marked by hepatic steatosis and is related to the presence of metabolic syndrome in association with obesity, diabetes, and/or arterial hypertension [8]. A subset of NAFLD patients
will develop signs of nonalcoholic steatohepatitis (NASH), a more severe condition associated with lobular inflammation and hepatocellular ballooning and can lead to fibrosis and cirrhosis [9]. In NAFLD the liver is unable to utilize carbohydrates and fatty acids properly, leading to toxic overaccumulation of lipid species. These metabolites induce cellular stress, injury, and death, which predispose the liver to sequelae such as cirrhosis and hepatocellular carcinoma [10].

In the United States the number of NAFLD cases is projected to expand from 83.1 million in 2015 (~26% of the population) to 100.9 million by 2030 (~28% of the population) [11]. An increasing percentage of these cases are projected to be classified as NASH, rising from 20% to 27% of adults with NAFLD during this interval [12]. While diagnosing NASH at an early stage remains a challenge, multiplexed protease-activated nanosensors have demonstrated utility in monitoring NASH progression and treatment response in a 3,5-diethylcarbonyl-1,4-dihydrocollidine model of fibrosis in mice. With further development, these noninvasive readouts can be used to diagnose disease.

### Current state of liver therapies

In order to mitigate the clinical burden of liver disease, several therapeutic strategies have been undertaken (Fig. 40.1).

#### Extracorporeal liver support devices

Liver failure is associated with abnormal accumulation of numerous endogenous substances such as bilirubin, ammonia, free fatty acids, and proinflammatory cytokines [13]. Extracorporeal liver support devices have been developed to detoxify the blood and plasma in order to bridge patients to liver transplantation (LT) or allow the native liver to recover from injury. Artificial liver (AL) devices use nonliving components for detoxification, such as membrane separation or sorbents, to selectively remove toxins but have limited clinical use because they do not replace the synthetic and metabolic roles of the liver [13]. Bioartificial liver (BAL) devices, on the other hand, contain a cell-housing bioreactor that aims to provide the detoxification and synthetic functions of the liver and are an ongoing topic of clinical investigation. Current versions are either based on hollow fiber cartridges [14–16] or on perfused three-dimensional (3D) matrices [17]. BALs, just like other hepatocyte-based therapies, face many challenges, such as the lack of readily available functional cell sources and the loss of cell viability and phenotype during the treatment process.

#### Biopharmaceuticals

In the setting of ALF, N-acetyl cysteine (NAC) is FDA-approved to reduce the extent of liver injury after acetaminophen overdose [18]. In the setting of chronic liver disease, however, most of the FDA-approved therapies are for hepatitis A, B, and C. A detailed listing can be found in Table 40.1. While a few treatments have shown moderate efficacy, there are currently no biopharmaceuticals that are approved for NAFLD, NASH, or cirrhosis. Glitazones, for example, upregulate adiponectin, an adipokine with antisteatogenic and insulin-sensitizing properties [19]. Vitamin E, an antioxidant, can prevent liver injury by blocking apoptotic pathways and protecting against oxidative stress [19]. Despite clinical studies of a large number of therapeutic candidates, no single agent or combination has shown improvement to liver-related morbidity and mortality in patients with NASH. Until a drug is FDA-approved for NASH indications, lifestyle modifications and optimizing metabolic risk factors are the best medical-treatment options for these patients.

#### Liver transplantation

The first attempt at human LT took place at the University of Colorado on March 1, 1963 but turned out to be unsuccessful [20]. Based on the pioneering work of Thomas Starzl, the first extended survival of a human recipient after LT was achieved on July 23, 1967 with a 19-month-old female patient with hepatocellular carcinoma who survived.
13 months before succumbing to metastatic disease [21]. After the initial success of the surgery, advancements were made to improve donor organ quality, recipient selection, operative and perioperative management, immunosuppression and infectious complications. These advancements have made orthotopic LT the primary treatment for end-stage liver disease and certain cancers. These transplants have 1-year patient survival rates over 80% [22]. However, many challenges remain, including donor organ shortages, recipients with more advanced disease at transplant, a growing need for retransplantation, and adverse effects associated with long-term immunosuppression. To overcome a growing imbalance between the supply and demand of donor livers, transplant centers have developed strategies to expand the donor pool. These strategies include live donor LT [23], split-LT [24], and extended criteria for donor livers [25]. Despite all these efforts, the number of liver transplants has not increased in the last decade.

### TABLE 40.1 List of FDA-approved therapies for chronic liver diseases.

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Year approved</th>
<th>Indication(s)</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heplisav-B</td>
<td>2017</td>
<td>Hepatitis B</td>
<td>Combines hepatitis B surface antigen with a proprietary Toll-like receptor 9 agonist to enhance the immune response</td>
</tr>
<tr>
<td>Mavyret</td>
<td>2017</td>
<td>HCV genotype 1–6</td>
<td>Fixed-dose combination of glecaprevir, an HCV NS3/4A protease inhibitor, and pibrentasvir, an HCV NS5A inhibitor</td>
</tr>
<tr>
<td>Vosevi</td>
<td>2017</td>
<td>Hepatitis C</td>
<td>Fixed-dose combination of sofosbuvir, an HCV nucleotide analog NS5B polymerase inhibitor, velpatasvir, an HCV NS5A inhibitor, and voxilaprevir, an HCV NS3/4A protease inhibitor</td>
</tr>
<tr>
<td>Ocaliva</td>
<td>2016</td>
<td>Primary biliary cholangitis</td>
<td>FXR agonist</td>
</tr>
<tr>
<td>Zepatier</td>
<td>2016</td>
<td>HCV genotype 1 or 4</td>
<td>Fixed-dose combination product containing elbasvir, an HCV NS5A inhibitor, and grazoprevir, an HCV NS3/4A protease inhibitor</td>
</tr>
<tr>
<td>Cholbam</td>
<td>2015</td>
<td>Bile acid synthesis and peroxisomal disorders</td>
<td>Primary bile acid synthesized from cholesterol in the liver</td>
</tr>
<tr>
<td>Daklinza</td>
<td>2015</td>
<td>HCV genotype 3</td>
<td>Inhibitor of NS5A, a nonstructural protein encoded by HCV</td>
</tr>
<tr>
<td>Technivie</td>
<td>2015</td>
<td>HCV genotype 4</td>
<td>Fixed-dose combination of ombitasvir, an HCV NS5A inhibitor, paritaprevir, an HCV NS3/4A protease inhibitor, and ritonavir, a CYP3A inhibitor</td>
</tr>
<tr>
<td>Olysio</td>
<td>2013</td>
<td>Hepatitis C</td>
<td>Small molecule orally active inhibitor of the NS3/4A protease of HCV</td>
</tr>
<tr>
<td>Sovaldi</td>
<td>2013</td>
<td>Hepatitis C</td>
<td>Inhibitor of the HCV NS5B RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>Incivek</td>
<td>2011</td>
<td>HCV genotype 1</td>
<td>Inhibitor of the HCV NS3/4A serine protease</td>
</tr>
<tr>
<td>Victrelis</td>
<td>2011</td>
<td>HCV genotype 1</td>
<td>Inhibitor of the HCV NS3 serine protease</td>
</tr>
<tr>
<td>Viread</td>
<td>2008</td>
<td>Hepatitis B</td>
<td>Oral nucleotide analogue DNA polymerase inhibitor</td>
</tr>
<tr>
<td>Tyzeka</td>
<td>2006</td>
<td>Hepatitis B</td>
<td>Inhibitor of HBV DNA polymerase</td>
</tr>
<tr>
<td>Baraclude</td>
<td>2005</td>
<td>Chronic hepatitis B with evidence of active viral replication</td>
<td>Small-molecule guanosine nucleoside analog with selective activity against HBV polymerase</td>
</tr>
<tr>
<td>Hepsera</td>
<td>2002</td>
<td>Chronic hepatitis B with evidence of active viral replication</td>
<td>Inhibitor of HBV DNA polymerase</td>
</tr>
<tr>
<td>Pegasys</td>
<td>2002</td>
<td>Chronic hepatitis C with compensated liver disease</td>
<td>Binds to and activates human type 1 interferon receptors</td>
</tr>
<tr>
<td>Peg-intron</td>
<td>2001</td>
<td>Chronic hepatitis C</td>
<td>Binds to and activates human type 1 interferon receptors</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>2001</td>
<td>Chronic hepatitis C</td>
<td>Synthetic nucleoside analog with antiviral activity</td>
</tr>
<tr>
<td>Twinrix</td>
<td>2001</td>
<td>Hepatitis A and B</td>
<td>Recombinant vaccine</td>
</tr>
</tbody>
</table>

FXR, Farnesoid X receptor; HBV, hepatitis B virus; HCV, hepatitis C virus; NS3, nonstructural protein 3.
An alternative to human LT is xenotransplantation, though it has been clinically intractable due to concerns about immunological rejection and zoonotic pathogen transfer. With the advent of accessible genetic engineering technologies to circumvent the aforementioned challenges, the breeding efficiency of animals can be leveraged to mass-produce tissue for human organ transplants. Niu et al. applied CRISPR-Cas9 to inactivate all 62 copies of porcine endogenous retroviruses, thus paving the way for pig-to-human transplants [26]. Relatedly, Längin et al. genetically engineered porcine heart xenografts and demonstrated long-term pig-to-baboon orthotopic transplantation [27].

**Hepatocyte transplantation**

Given the several drawbacks of LTs, alternative strategies have been pursued. A potential alternative to LT is allogeneic hepatocyte transplantation (HT). Transplanted cells can provide the missing or impaired hepatic function once engrafted. Given their synthetic and metabolic capabilities, mature hepatocytes are the primary candidates for liver cell transplantsations. HT offers several advantages over LT. It is less invasive and can be performed repeatedly to meet metabolic requirements. Furthermore, multiple patients can be treated with a single dissociated donor tissue, and harvested cells can be cryopreserved for later use on an as-needed basis.

The first experimental attempt of HT was done in 1976 to treat an animal model for Crigler–Najjar syndrome type I [28]. Along with other observations, it led to the first transplant of autologous hepatocytes in 10 patients with liver cirrhosis in 1992 in Japan [29]. Since then, reports have been published on more than 100 patients with liver disease treated by HT worldwide [30]. Human HT has resulted in partial correction of a number of liver diseases, including urea cycle disorders [31], factor VII deficiency [32], glycogen storage disease type 1 [33], infantile Refsum disease [34], phenylketonuria [35], severe infantile oxalosis [36], and ALF [37]. HT faces several limitations: limited supply of high-quality mature hepatocytes, freeze–thaw damage due to cryopreservation, poor cellular engraftment (estimated to be from 0.1% to 0.3% of host liver mass in mice after infusion of 3%–5% of the total recipient liver cells) [30], and allogeneic rejection.

Clinically, the most widely used administration route for HT is through the portal vein or one of its branches. Hepatocytes traverse the sinusoidal vasculature and create transient occlusions. The occlusions lead to vascular permeabilization which allows transplanted cells to reach the liver parenchyma [35]. The number of cells that are injected intraportally and subsequently engraft is a function of portal pressure and liver architecture. Thus other administration routes have been explored for patients with cirrhosis who have high portal pressures due to fibrosis.

In animal studies, hepatocytes transplanted into the spleen proliferate for extended periods of time and display normal hepatic function. The spleen has been shown to be well-suited for hepatocyte engraftment because it functions as a vascular filter and provides an immediate blood supply [30]. The peritoneal cavity represents an attractive administration route as it is easily accessible and can house a large number of cells. Due to cell number requirements associated with metabolic compensation, it has been used in patients with ALF [38]. As an alternative to the portal vein, spleen and peritoneum, the lymph node (LN) has also been shown to demonstrate engraftment of donor hepatocytes [39,40]. While this strategy has not been utilized in the clinic yet, the preclinical data is promising.

**Current clinical trials**

Several pathways have been implicated in the biology and pathogenesis of NAFLD development: insulin resistance, lipotoxicity, oxidative stress, altered immune/cytokine/mitochondrial functioning, and apoptosis. New therapeutic modalities are being developed to target many of these pathways. For a detailed overview of NAFLD-targeted drugs that are currently in the clinical trial pipeline, please refer to Younossi et al. [41].

**In vitro models**

To build high-fidelity cellular models and therapies, components of the native liver microenvironment must be incorporated (Fig. 40.2). The liver’s highly organized structure is key to its role as a complex tissue supporting myriad synthetic and metabolic functions. In addition to hepatocytes the main parenchyma of the liver, there are several nonparenchymal cell types such as liver sinusoidal endothelial cells, Kupffer cells, cholangiocytes, and stellate cells. In each lobule of the liver an array of parallel hepatocyte cords are sandwiched between the sinusoid, carrying circulating blood, and the bile duct, carrying hepatocyte-secreted bile acids. Notably, this arrangement dictates a unique set of architecturally driven cell–cell and cell–matrix cues, which gives rise to liver-specific phenotypes. Gradients of physicochemical stimuli along the sinusoid drive zonal phenotypes with disparate metabolic and synthetic functional profiles [42]. Interrupting the natural order of cell arrangement in the liver is directly connected with diseases discussed in the “Liver disease burden” section. In this chapter, we will primarily focus on human platforms, which are biologically distinct from animal-derived cell models of the liver that are reviewed more in depth elsewhere [43].
Two-dimensional liver culture

Hepatocytes are responsible for more than 500 metabolic and synthetic functions of the human body, often categorized broadly as protein synthesis and secretion, detoxification, bile synthesis, and nitrogen metabolism. Primary hepatocytes quickly lose their phenotype and function after a few days in traditional monolayer culture and require a collagen-coated surface for adherence and survival [6]. In contrast, when primary human hepatocytes (PHHs) are cultured between two layers of collagen gel (i.e., sandwich culture configuration), they retain viability, polarity and many axes of relevant metabolic and synthetic function [44,45]. Guguen-Guillouzo et al. found that a random coculture with a liver epithelial cell line was sufficient to support hepatic albumin secretion, suggesting the importance of heterotypic cell interactions for long-term ex vivo culture [46]. Bale et al. demonstrated that other nonparenchymal liver cells can better recapitulate hepatic response to inflammatory stimuli, through higher order intercellular interactions captured in a multicellular platform [47]. It was later discovered that a micropatterned architecture consisting of hepatocyte-filled islands surrounded by a nonbiomimetic cell type and mouse fibroblasts can also stabilize hepatocytes, suggesting the existence of conserved coculture signals across species. These micropatterned cocultures (MPCCs) enable the study of DILI and hepatotropic pathogen infection for several weeks in vitro [48–50]. Furthermore, Davidson et al. added hepatic stellate cells to the traditional MPCC to create an in vitro model of NASH [51].

Three-dimensional liver constructs

Commonly, 3D hepatic cultures consist of primary cell or induced pluripotent stem cell (iPSC)-derived spheroid and organoid cultures, which are typically embedded in ECM-based hydrogels [55]. Spheroids and organoids can be manufactured using a variety of techniques, such as microwell mold-based technologies, which offer a high degree of composition and size control but are difficult to scale. Spinning flasks and bioreactors can produce large populations of spheroids, though they are typically non-uniform in size and function. Bell et al. showed that primary human hepatic spheroids fabricated and cultured in microwell plates serve as useful models of hepatotoxicity and multiple liver pathologies [56].

Despite the utility of two-dimensional (2D) hepatic cultures in screening assays, a wealth of literature suggests that they are dissimilar to hepatocytes in vivo. Specifically, 2D formats, even with overlaid collagen matrix, are more flattened than their native cuboidal architecture. On a subcellular level, this translates to major differences in cytoarchitecture, which is linked to aberrant polarization and nonphysiological behavior [52,53]. Griffith and Swartz have described the improved presentation of relevant biochemical and mechanical cues in 3D cultures, typically cell-laden hydrogels, compared to traditional 2D cultures [54].

FIGURE 40.2 Advances in hepatic tissue engineering. Traditional tissue culture approaches such as the addition of extracellular matrix, soluble factors, cocultivation with supporting cell types, hanging drop, microwell molding, and nonadhesive surfaces have enabled the early study of hepatocyte phenotype in vitro in both 2D and 3D cultures. The advent of technologies from disciplines such as chemical engineering and electrical engineering has led to a new level of control for hepatic tissue cultures, such as micropatterning to template cell interactions, microphysiological systems to study the impact of bioactive perfusate, polymeric biomaterials for constructing 3D cell-laden grafts, perfusion technologies for decellularization/recellularization strategies, and 3D printing for scalable engineering of cellular grafts. 2D, Two-dimensional; 3D, three-dimensional.
cocultures of PHHs and fibroblasts, which can be embedded in agarose, fibrin, or polyethylene glycol hydrogel scaffolds. The resulting tissue constructs support hepatic function in vitro and in vivo after ectopic transplantation into the peritoneal cavity [57]. Furthermore, Stevens et al. demonstrated that implanting a tissue seed, consisting of hepatic aggregates and vascular cords, into an FRNG [fumarylacetoacetate hydrolase-deficient (Fah−/−), recombinase activating gene-deficient (Rag1−/−), nonobese diabetes (NOD), and interleukin-2 receptor γ chain-deficient (II2γ-null)] mouse model of hereditary tyrosinemia leads to a 50-fold expansion in serum human albumin and formation of perfusable vessels after 80 days of cycled exposure to regenerative stimuli [58]. Relatively, Takebe et al. constructed liver buds consisting of iPSC-derived hepatocyte-like cells (HLCs), mesenchymal stem cells, and human umbilical vein endothelial cells; mesenteric transplantation of these human liver buds resulted in vascularization and rescued a lethal TK-NOG mouse model of liver injury [59].

To create clinically viable engineered liver constructs, cell sourcing (discussed further in the “Cell sourcing” section), and clinical-scale manufacturing of organoids and spheroids must be addressed. Toward this end, Takebe et al. constructed a large-scale liver bud microwell culture platform, enabling the formation of 10⁸ liver buds [60].

**Physiological microfluidic models of liver**

Despite improvements to in vitro liver platforms as model systems and implants, static cultures lack physiologically relevant dynamic components. The native liver’s dynamic physiology arises from blood circulation and multiorgan crosstalk. Thus to improve biological fidelity, many have attempted to create microfluidic models of the liver [61]. By leveraging techniques from the semiconductor manufacturing industry such as soft lithography, groups have fabricated microphysiological systems with preformed channels to allow for perfusion of nutrients and to aid in waste removal [62–67]. These so-called liver-on-chip platforms allow for the study of biochemical and mechanical cues such as growth factor gradients and shear stress. Lee et al. demonstrated that the presence of human stellate cells and application of shear via flow enabled the formation of stable hepatic spheroids on chip [68]. Furthermore, by linking microfluidic channels between multiple tissue models, multiorgan phenomena such as drug metabolite toxicity and disease progression can be captured in vitro, which is not possible in traditional cultures [69–71].

**Controlling three-dimensional architecture and cellular organization**

Another approach to improving the functionality of tissue-engineered constructs is to more closely mimic in vivo microarchitecture by generating scaffolds with a highly defined material and cellular architecture, which would provide better control over the 3D environment at the microscale.

A range of rapid prototyping and patterning strategies have been developed for polymers using multiple modes of assembly, including fabrication using heat, light, adhesives, or molding, and these techniques have been extensively reviewed elsewhere [72]. For example, 3D printing with adhesives combined with particulate leaching has been utilized to generate porous poly(lactic-co-glycolic acid (PLGA) scaffolds for hepatocyte attachment [73], and microstructured ceramic [74] and silicon scaffolds [75,76] have been proposed as platforms for hepatocyte culture. Furthermore, molding and microsyringe deposition have been demonstrated to be robust methods for fabricating specified 3D PLGA structures toward the integration into implantable systems [77].

Microfabrication techniques have similarly been employed for the generation of patterned cellular hydrogel constructs. For instance, microfluidic molding has been used to form biological gels containing cells into various patterns [78]. In addition, syringe deposition in conjunction with micropositioning was recently illustrated as a means to generate patterned gelatin hydrogels containing hepatocytes [79]. Patterning of synthetic hydrogel systems has also recently been explored. Specifically, the photopolymerization property of poly(ethylene glycol) (PEG) hydrogels enables the adaptation of photolithographic techniques to generate patterned hydrogel networks. In this process, patterned masks printed on transparencies act to localize the ultraviolet exposure of the prepolymer solution and, thus, dictate the structure of the resultant hydrogel. The major advantages of photolithography-based techniques for patterning of hydrogel structures are its simplicity and flexibility. Photopatterning has been employed to surface pattern biological factors [80], produce hydrogel structures with a range of sizes and shapes [81,82], as well as build multilayer cellular networks [83,84]. Consequently, hydrogel photopatterning technology is ideally suited for the regulation of scaffold architecture at the multiple length scales required for implantable hepatocellular constructs. As a demonstration of these capabilities, photopatterning of PEG hydrogels was utilized to generate hepatocyte and fibroblast coculture hydrogels with a defined 3D branched network, resulting in improved hepatocyte viability and functions under perfusion [85]. More recently, a “bottom-up” approach for fabricating multicellular tissue constructs utilizing DNA-templated assembly of 3D cell-laden hydrogel microtissues demonstrates robust patterning of cellular hydrogel constructs containing numerous cell types [86]. Also, the additional combination of photopatterning with dielectrophoresis-mediated cell patterning enabled the construction of hepatocellular hydrogel
structures organized at the cellular scale. Overall, the ability to dictate scaffold architecture coupled with other advances in scaffold material properties, chemistries, and the incorporation of bioactive elements (discussed further in the “Extracellular matrix for cell therapies” section) will serve as the foundation for the future development of improved tissue-engineered liver constructs that can be customized spatially, physically, and chemically.

**In vivo models**

While there have been impressive advances in cell culture models of the human liver, experimental animal models still play an important role in the effort to engineer liver therapies. Commonly performed surgeries such as bile duct ligation and partial hepatectomy are experimentally tractable models of acute liver injury, yet they are of little clinical relevance. Drug-induced (e.g., carbon tetrachloride, acetaminophen, or thioacetamide) hepatotoxicity to induce necrotic lesions is more recapitulative of human pathophysiology, but the phenotype is difficult to reproduce [87]. In addition, modeling chronic liver injury in animal models is problematic because they tend to rapidly correct severe hepatic damage after a few days, which is not representative of human disease progression and resolution [88].

In order to model a human-like context for liver injuries, it is necessary to develop improved, controlled models of human liver injury. Such animal models can be useful for the evaluation of human liver biology and the preclinical performance of candidate therapies and drugs [89,90]. To accomplish this, human hepatocytes can be transplanted orthotopically in immunocompromised mice with no liver injury via the injection of a cell solution and are useful for modeling human-specific drug metabolism, liver injury, and hepatotropic infections. However, on average, HT exhibits poor levels of engraftment (~10%–30%) [91].

Transplanted hepatocytes have the ability to expand preferentially if the host is compromised by injury or genetic modification. The first genetically engineered mouse model to demonstrate this was the Alb-uPA mouse that carries a uroplasminogen activator under an albumin promoter, causing liver injury and failure [92]. Aiming to improve on the Alb-uPA system, a transgenic model of hereditary tyrosinemia I was developed, in which a genetic knockout of FAH leads to the hepatotoxic accumulation of fumarylacetoacetate [93]. FAH knockout mouse injury initiation and duration can be controlled through the administration of a small molecule drug [2-(2-nitro-3-trifluoro-methylbenzoyl)-1,3-cyclohexane-dione] in the drinking water. Another inducible model called TK-NOG, which expresses thymidine kinase under an albumin promoter, causes hepatocyte ablation following activation by ganciclovir treatment [94]. In the AFC8 injury model, induction by the small molecule AP20187 drives caspase-8-initiated apoptosis of hepatocytes modified to express FK506 under an albumin promoter [95]. For all of the above models, engraftment rates surpassing 70% have been observed.

A classical study in parabiotic rats in the 1960s revealed that hepatic injury results in the expression of systemic, soluble signals that have the potential to drive liver regeneration [96]. Despite decades of research, the complex signaling cascade driving liver regeneration is still not well understood but has found utility in ectopic humanized mouse models. Chronic liver injury often presents with high portal pressures, which can reduce engraftment levels during an orthotopic transplantation. Thus transplantation to ectopic sites is clinically attractive. Ectopic grafts that anastomose to the host vasculature can interact with regenerative stimuli from the host liver, causing expansion and proliferation of the transplanted human hepatocytes. Initially, ectopic transplantation was demonstrated in the LN [40] and spleen [97], and later in the subcutaneous space and mesenteric fat pad, both of which offer ease of accessibility for manipulation and noninvasive imaging [58,59,98].

Taken together, the range of liver injury animal models are an essential tool for studying various perturbations to normal liver biology and building implantable tissue constructs to address acute and chronic liver failure. The field is just beginning to uncover mechanisms that control liver regeneration in various disease and injury contexts. The discovery of new soluble regenerative signals will be central to advancing therapies that have the potential to improve the supply of donor tissue.

**Cell sourcing**

**Cell number requirements**

The development of cell-based therapies poses myriad challenges, partially stemming from the scale of the liver. An adult human liver is estimated to possess 241 billion hepatocytes, 24 billion stellate cells, and 96 billion Kupffer cells [99]. Sourcing such enormous cell numbers using current technologies is not feasible.

However, many human HT studies suggest that clinical intervention is possible with a fewer number of cells and offer critical insights to help us determine minimum cell numbers. In a review, Fisher and Strom cataloged 78 different human HT studies, detailing both the input cell number and a qualitative description of functionality [30]. Correlating these, we can broadly surmise that to correct inborn errors of metabolism, at least 1–10 billion hepatocytes are needed. However, for ALF, that number grows to 5–20 billion cells. For liver cirrhosis, HTs have largely been unsuccessful (discussed further in the “Hepatocyte transplantation” section); therefore it is unclear what the
cellular requirements for cirrhosis are. While injection of hepatocytes is not the same as implantation within a scaffold, these studies serve as useful inputs into more complex physiological models.

In order to get us closer to these numbers, many different cell sources have been explored.

**Immortalized cell lines**

Immortalized hepatocyte cell lines can be derived from liver tumor tissue or directly from primary hepatocytes in vitro. The prominent lines utilized today are HepG2, derived from hepatocellular carcinoma; HepaRG [100], a human bipotential progenitor cell line; C3A, derived from HepG2s; and Huh7, derived from liver tumor [101]. Several other fetal and adult hepatic cell lines have also been established, typically using a combination of viral oncogenes and the human telomerase reverse transcriptase protein [102]. However, these cell lines lack the full functional capacity of primary adult hepatocytes and there is a risk that oncogenic factors could be transmitted to the patient, limiting their use as a cell source for transplantation therapies.

**Primary cells**

Unlike immortalized lines, PHHs can provide a whole host of human liver-specific function. PHHs, however, are limited in supply, and their phenotype is difficult to maintain in vitro. Many methods have been developed for maintaining long-term functionality of hepatocytes through the use of a variety of configurations and biomaterial constructs, which are further discussed in the “In vitro models” section. Due to limitations in the supply of mature hepatocytes, many groups have attempted to promote the expansion and proliferation of PHHs in vitro. Peng et al. have shown that TNFα promotes the expansion of hepatocytes in 3D cultures and enables serial passaging and long-term culture for more than 6 months [103]. In a similarly notable study, Hu et al. identified an optimal cell culture cocktail consisting of B27 supplement (without vitamin A), R-spondin, CHIR99021 (a Wnt agonist), NAC, nicotinamide, gastrin, epidermal growth factor (EGF), TGFα, fibroblast growth factor (FGF)7, FGF10, HGF, a TGFβ inhibitor (A83-01), and ROCK inhibitor that led to long-term 3D organoid culture of PHHs [104].

**Fetal and adult progenitors**

Given their ability to differentiate into diverse lineages both in vitro and in vivo, iPSC and human embryonic stem cell cultures can also be utilized to generate HLCs. Various differentiation protocols have been applied to these cultures to yield cell populations that exhibit some phenotypic and functional characteristics of hepatocytes [62,105—108]. These populations are termed HLCs because of their expression of fetal proteins and fetal-like cytochrome P450 profiles [109]. While they are distinct from mature adult hepatocytes, HLCs can still serve as a potential cell source in very specific contexts.

In addition to pluripotent cells, bipotential progenitor cells can also serve as a source for hepatocytes. Huch et al. delineated conditions that allow for long-term expansion of adult bile duct-derived EpCAM + bipotential progenitor cells from the human liver [110]. The expanded cell population attained using their protocol is stable at the chromosomal level and can be converted into functional HLCs in vitro and in vivo [110].

**Reprogrammed hepatocytes**

HLCs can also be generated using direct reprogramming of mature cell types. For example, several groups have demonstrated the feasibility of reprogramming fibroblasts into HLCs without a pluripotent intermediate [111–113]. Cheng et al. demonstrated that a combination of nuclear factors can stimulate the conversion of hepatoma cells to HLCs [101]. These findings raise the future possibility of deriving human HLCs directly from another adult cell type.

**Extracellular matrix for cell therapies**

The ECM of the liver provides a structural scaffold with bioactive cues that modulate hepatic function and promote vascularization. Collagen and fibronectin are the major structural components of the liver ECM. Along with other nonstructural proteins, these components participate in integrin-mediated signaling between cells and their surrounding matrix. Hepatocytes are sensitive to their ECM, and it has been demonstrated that the presence of abnormal amounts and/or types of ECM components correlates with the onset and progression of liver fibrosis [114].

ECM scaffolds for hepatic tissue engineering are useful for constructing 3D tissue models and as a delivery vehicle for implants. Polymeric biomaterial hydrogels gained popularity as an engineering tool for recapitulating a physiologically relevant 3D tissue niche. Aside from creating a permissive environment for hepatocyte survival and growth, ECM scaffolds for hepatic tissue engineering also enable the formation of biliary and vascular networks that will be further discussed in the “Vascular and biliary tissue engineering” section. Broadly speaking, ECM scaffolds can be constructed using synthetic and/or naturally derived polymers.
Natural scaffold chemistry and modifications

A wide range of natural biomaterial polymers spanning polysaccharides (e.g., dextran and chitosan), peptides (e.g., collagen and fibrin), decellularized ECM (dECM), and composites of these have been employed as hepatic tissue—engineering scaffolds [55,57,58,115—121]. The advantages of biologically derived materials include their biocompatibility; naturally occurring cell adhesive moieties; and, in the case of decellularization, native architectural presentation of ECM molecules. However, naturally derived biomaterials have several barriers to use in the clinic, primarily due to lot-to-lot variability and xenogeneic origin.

The choice of material determines the physicochemical and biological properties of the scaffold. For example, early efforts in developing implantable hepatic constructs utilized collagen-coated dextran microcarriers that enabled hepatocyte attachment since hepatocytes are known to be anchorage-dependent cells. The intraperitoneal transplantation of these hepatocyte-attached microcarriers resulted in successful replacement of liver functions in two different rodent models of genetic liver disorders [122]. Subsequently, collagen-coated or peptide-modified cellulose [120,123], gelatin [124], and gelatin—chitosan composite [125] microcarrier chemistries have also been explored for their capacity to promote hepatocyte attachment. On the other hand, materials that are poorly cell adhesive such as alginate [115] have been exploited for their utility in promoting hepatocyte—hepatocyte aggregation (i.e., spheroid formation) and phenotypic stabilization within these scaffolds. Collectively, the size of engineered tissues created by these approaches is limited by oxygen and nutrient diffusion to only a few hundred microns in thickness.

To address this constraint, recent work has sought to use decellularized whole organ tissue as a matrix for liver tissue engineering. The decellularization process utilizes perfusion-based technologies to remove cells from donor tissues but preserve the structural and functional characteristics of the native underlying tissue. Recent advances in decellularization protocols have yielded scaffolds with native liver ECM composition, growth factor presentation, vascular structure, and biliary network architecture [126—128]. To date, seeding protocols have achieved up to 95% efficiency of recellularization with relevant cell populations (e.g., hepatocytes, vascular cells and bipotent hepatic progenitors); resulting recellularized grafts exhibited liver-specific function, and survival after transplantation in rodents [121,126,129]. Furthermore, cell-laden, xeno-derived dECM scaffolds are compatible with immunocompetent animal models [128]. However, given the shortage of donor tissue, the wide use of dECM scaffolds is unlikely.

Synthetic scaffold chemistry

In contrast to biologically derived material systems, synthetic materials enable precisely customized architecture (porosity and topography), mechanical and chemical properties, and degradation modality and kinetics, which are known to drive cell behavior. Synthetic materials that have been explored for liver tissue engineering include poly(l-lactic acid) (PLLA), PLGA, poly(ε-caprolactone), and PEG [85,98,130—135]. Polymers such as PLLA and PLGA are the most common synthetic polymers utilized in the generation of porous tissue-engineering constructs. These materials are biocompatible, biodegradable, and have been used as scaffolds for HT [132,136]. A key advantage of PLGA is the potential to finely tune its degradation time due to differences in susceptibility to hydrolysis of the ester groups of its monomeric components (lactic acid and glycolic acid). However, the accumulation of hydrolytic degradation products has been shown to produce an acidic environment within the scaffold which initiates peptide degradation and stimulates inflammation, which may affect hepatocyte function [137]. Consequently, as alternatives to macroporous scaffold systems, approaches aimed at the efficient and homogeneous encapsulation of hepatocytes within a fully 3D structure have been explored. In particular, hydrogels that exhibit high water content and thus similar mechanical properties to tissues are widely utilized for various tissue-engineering applications, including hepatocellular platforms. Synthetic, PEG-based hydrogels have been increasingly utilized in liver tissue-engineering applications due to their high water content, hydrophilicity, resistance to protein adsorption, biocompatibility, ease of chemical modification, and the ability to be polymerized in the presence of cells, thereby enabling the fabrication of 3D networks with uniform cellular distribution [138]. PEG-based hydrogels have been used for the encapsulation of diverse cell types, including immortalized and primary hepatocytes and hepatoblastoma cell lines [85,98,135]. The encapsulation of primary hepatocytes requires distinct material modifications [e.g., 10% w/v PEG hydrogel, inclusion of RGD adhesive motifs] as detailed below, as well as, analogous to 2D coculture systems, the inclusion of nonparenchymal supporting cell types such as fibroblasts and endothelial cells [135].

Modifications in scaffold chemistry

The relatively inert nature of synthetic scaffolds allows for the controlled incorporation of chemical/polymer moieties or biologically active factors to regulate different aspects of cellular function. Chemical modifications such as oxygen plasma treatment or alkali hydrolysis of PLGA [139,140], or the incorporation of polymers such as
poly(vinyl alcohol) or poly(\(N\)-\(p\)-vinylbenzyl-4-\(O\)-\(β\)-D-galactopyranosyl-\(O\)-glucosamide) (PVLA) into PLGA or PLLA scaffolds [132,141,142] have improved hepatocyte adhesion by modulating the hydrophilicity of the scaffold surface [143]. Biological factors may include whole biomolecules or short bioactive peptides. Whole biomolecules are typically incorporated by nonspecific adsorption of ECM molecules such as collagen, laminin, or fibronectin [139,144]; covalent conjugation of sugar molecules such as heparin [145,146], galactose [131,147], lactose [145] or fructose [148]; or growth factors such as EGF [149]. Alternatively, short bioadhesive peptides that interact with cell surface integrin receptors have been extensively utilized to promote hepatocyte attachment in synthetic scaffolds. For example, conjugation of the RGD peptide to PLLA has been shown to enhance hepatocyte attachment [150], whereas RGD modification significantly improved the stability of long-term hepatocyte function in PEG hydrogels [98,135]. The additional incorporation of adhesive peptides that bind other integrins may serve as a way to further modulate and enhance hepatocyte function within synthetic polymer substrates. Moreover, Stevens et al. demonstrated that integration of matrix metalloproteinase-sensitive peptide sequences into hydrogel networks as degradable linkages has been shown to enable cell-mediated remodeling of the hydrogel [151].

The capacity to modify biomaterial scaffold chemistry through the introduction of biologically active factors will likely enable the finely tuned regulation of cell function and interactions with host tissues important for implantable systems.

Porosity

A common feature of many implantable tissue-engineering approaches is the use of porous scaffolds that provide mechanical support, often in conjunction with cues for growth and morphogenesis. Collagen sponges, various alginate and chitosan composites, and PLGA are the most commonly used porous scaffolds for hepatocyte culture and are generally synthesized using freeze-dry or gas-foaming techniques. Pore size has been found to regulate cell spreading and cell–cell interactions, both of which can influence hepatocyte functions [116], and may also influence angiogenesis and tissue ingrowth [152]. Porous, acellular scaffolds are normally seeded using gravity or centrifugal forces, capillary action, convective flow, or through cellular recruitment with chemokines, but hepatocyte seeding is generally heterogeneous in these scaffolds [153,154].

Vascular and biliary tissue engineering

Beyond compatibility with hepatic cell types, scaffolds should also be conducive to vasculature formation. Relying on vascularization by the host is not sufficient for large tissue constructs required for the clinic, because cells that are not near capillary structures (>150–200 \(\mu\)m) are at a risk for necrosis after a matter of hours due to a lack of oxygen, nutrient availability, and waste transport. In this section, we discuss composite approaches toward building scalable, vascularized constructs.

Vascular engineering

Approaches to engineering vessels can generally be categorized as bottom-up induction of vascular assembly and top-down fabrication of vascular conduits [155]. Bottom-up vascular engineering approaches are built upon the idea of neovascularization, or new vessel formation. Vessel formation can occur by angiogenic sprouting, the formation of vessels branching off of an existing blood vessel, or vasculogenesis, the self-assembly of single endothelial cells or progenitor cells into lumened vessels. Despite the ability of single vascular cells to coalesce to enable self-assembly, vasculogenesis is accelerated by coculture with supporting stromal cells, such as fibroblasts, mesenchymal stem cells, and pericytes [155–157]. Studies exploring angiogenesis and sprouting events suggest that chemical gradients, fluid-driven shear stress, and hypoxia are key players in vessel formation [158–161].

Top-down fabrication approaches dictate geometry and architecture, rather than driving self-assembly. Polymer molding using microetched silicon has been shown to generate extensive channel networks with capillary dimensions, though it is not amenable to high-throughput manufacturing [162]. While direct printing of cells can be cytotoxic, 3D printing has become a popular approach for fabricating hollow channels that enable vascular cell seeding. The challenge lies in using this approach to build patent capillary beds, which are 5–10 \(\mu\)m in diameter. While two-photon polymerization has an impressively high feature size resolution at 100 nm, the trade-off between build volume (i.e., building constructs large enough for clinical impact), build speed (i.e., impacting manufacturability), and printer resolution renders it inappropriate for most applications in tissue engineering. Alternate printers using direct-ink writing (DIW) of viscoelastic materials have emerged as a powerful tool for the fabrication of patterned hydrogel constructs. DIW can achieve minimum feature size resolutions from 1 to 250 \(\mu\)m, depending on the ink “building block” size [163]. However, DIW printing requires yield-stress fluid inks with restrictive viscosities (10^2–10^6 mPa s), such that they fluidize under stress but regain the original shear elastic modulus after printing [163]. Kolesky et al. demonstrated that DIW and fugitive inks were useful for multimaterial printing as well as...
construction of thick (>1 cm), vascularized tissues [164,165]. Miller et al. demonstrate the use of thermal microexrusion approach to create sacrificial sugar glass lattices that can be embedded in various cell-laden biomaterials, evacuated and subsequently lined with vascular cells [166].

Taken together, self-assembly driven formation of a capillary bed can be combined with printing of larger vessels to enable the fabrication of a fully vascularized tissue construct. Song et al. used 3D printing to create curved vascular channels using a fugitive ink [159]. The vascular cells lining the channel were able to degrade the surrounding hydrogel matrix and undergo sprouting when exposed to angiogenic factors. They further demonstrated that the curvature and complexity of printed vasculature impacted the extent of sprouting, which will be an important consideration for vessel patterning in future clinical applications [159].

**Host integration**

For vascularized constructs to survive after implantation the engineered vessels must anastomose with the host vasculature. To date, the exact mechanism that drives integration with the host is not well understood [155]. A number of studies have elucidated the contribution of cytokines important in angiogenesis and recruitment of host vasculature in implant constructs, such as vascular EGF (VEGF) [136], basic FGF [167], and VEGF in combination with platelet-derived growth factor [168]. Furthermore, preimplantation of VEGF releasing alginate scaffolds prior to hepatocyte seeding was demonstrated to enhance capillary density and improve engraftment [169]. In addition, while building an AL tissue construct, Stevens et al. demonstrated that the coimplantation of parallel endothelial cell cords with PHH and fibroblast spheroids led to better hepatic performance and survival when compared to an implant with nonpatterned endothelial cells, suggesting that there may be an optimal templated endothelial geometry that enables vascularization and host integration in vivo [58]. Surgical anastomosis poses an alternative to biologically driven anastomosis, though this approach requires invasive surgery and access to suture-able vessels both in the graft and in the host. Strategies to incorporate vasculature into engineered constructs include the microfabrication of vascular units with accompanying surgical anastomosis during implantation [75,170].

In addition to interactions with the vasculature, integration with other aspects of host tissue will constitute important future design parameters. For instance, incorporation of hydrolytic or protease-sensitive domains into hepatocellular hydrogel constructs could enable the degradation of these systems following implantation [151]. Of note, liver regeneration proceeds in conjunction with a distinctive array of remodeling processes such as protease expression and ECM deposition. Interfacing with these features could provide a mechanism for the efficient integration of implantable constructs. Similarly to whole liver or cell transplantation, the host immune response following the transplant of tissue-engineered constructs is also a major consideration. Immunosuppressive treatments will likely play an important role in initial therapies, although stem cell–based approaches hold the promise of implantable systems with autologous cells. Furthermore, harnessing the liver’s unique ability to induce antigen-specific tolerance [171] could potentially represent another means for improving the acceptance of engineered grafts.

**Biliary network engineering**

Importantly, future iterations of hepatic grafts should include a biliary system, which is responsible for excretory functions. In a similar vein, we envision that a combination of “top-down” manufacturing, such as the aforementioned technologies for generating patent vascular conduits, and “bottom-up” approaches, which could involve leveraging biological phenomena that drive biliary morphogenesis, will be useful for building a biliary network.

The biliary tree is a complex, 3D network of tubular conduits of various sizes and properties. The liver contains an intrahepatic compartment that is lined by biliary epithelial cells, termed cholangiocytes, that aid in the modification and removal of hepatocyte-secreted bile. Even though the blood vessel—fabrication approaches delineated above have not yet been applied to engineering bile networks for implantable liver constructs, advances in cholangiocyte sourcing methods have made it feasible. Sampaziotis et al. identified a protocol for directed differentiation of human iPSCs into cholangiocyte-like cells [172]. In 2017 the same group provided the first proof-of-concept study to reconstruct the gallbladder wall and repair the biliary epithelium using human primary cholangiocytes expanded in vitro [173]. Furthermore, current studies are focused on the development of in vitro models that exhibits biliary morphogenesis and recapitulates appropriate polarization and bile canaliculi organization [174–176], as well as platforms for the engineering of artificial bile duct structures [177,178].

**Conclusion and outlook**

Traditionally, hepatic tissue—engineering research has focused on designing the microenvironment to support a stable hepatic phenotype. As concomitant advances in pluripotent cell research and polymer chemistry have been actualized, new cell sources and extracellular
matrices have been added to the pipeline. This interdiscipli-

nary synergy has been the driving force behind the de-

velopment of tissue-engineered grafts with long-term sur-

vival and growth. In order to inch closer to the regen-

erative medicine north star of an ex vivo engineered graft that can serve as a replacement for the native organ, there are several areas to consider for improvement: (1) vascular-

ization of thick, dense grafts through a combination of self-assembly and bioprinting; (2) engineering of the hepatic graft to prevent immune rejection in allogeneic and xenogeneic settings; (3) improved understanding of metabolic and cellular requirements of various liver dis-

eases; (4) development of scalable, renewable cell sources that do not compromise the functional capabilities of cells; (5) leveraging of animal injury models as bioreac-

tors for cell sourcing; and (6) upscaling of grafts in a manner that is compatible with FDA’s Good Manufacturing Practice standards.

References


