

# HEPATIC TISSUE ENGINEERING

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## **INTRODUCTION**

Liver tissue engineering aims to provide novel therapies for liver diseases and to create effective tools for understanding fundamental aspects of liver biology and pathologic processes. Approaches range from bio-mimetic *in vitro* model systems of the liver to three-dimensional implantable constructs. Collectively, these cell-based approaches endeavor to replace or enhance organ transplantation, which is the current standard treatment for liver diseases in most clinical settings. However, the complexity of liver structure and function as well as the limited supply of human hepatocytes pose unique challenges for the field. This chapter reviews advances in the field of liver tissue engineering within the context of current therapies for liver diseases and clinical alternatives such as cell transplantation strategies and extracorporeal bioartificial liver devices.

## **CURRENT TREATMENTS FOR LIVER FAILURE**

Liver failure, representing the cause of death for over 40,000 individuals in the United States annually, [1] can result from acute or chronic end-stage liver diseases. Current treatments for liver failure include administration of fluids and serum proteins, but these continue to be largely palliative. Liver transplantation is the only therapy proven to directly alter mortality, and therefore, remains the standard of care for liver disease patients. In order to maximize the therapeutic benefits of the limited supply of transplantable livers, a number of surgical techniques have been investigated, including the use of non-heart-beating donors or split liver transplants from cadaveric or living donors [2]. Partial liver transplants take advantage of the body's ability to regulate liver mass and the innate capability of mammalian livers to undergo significant regeneration [3]. However, although partial liver transplants have demonstrated some effectiveness, liver regeneration is difficult to regulate in clinical settings, and biliary and vascular complications are major concerns in these procedures [2]. Despite these surgical advances in expanding single donor livers into multiple grafts, the ballooning discrepancy between the number of livers available and the number of patients requiring liver transplants [4] indicates that organ transplantation alone is unlikely to fulfill the increasing demand for transplant-grade organs. Furthermore, patients who do receive transplants are subjected to the costs and complications associated with major surgery as well as a life-time of immunosuppressive regimens. Consequently, alternative approaches are actively being pursued. These include non-biological extracorporeal systems, such as hemoperfusion, hemodialysis, plasma exchange, and plasmapheresis over charcoal or resins [5-7]. They have shown only limited success, presumably due to the narrow range of functions supported by these acellular devices. Recapitulation of a more substantial number of the liver's purported 500+ functions will likely be needed to offer effective liver support.

### **Cell-based therapies**

To provide the large array of known and currently unidentified liver functions, cell-based therapies have been proposed as an alternative to both liver transplantation and strictly non-biological systems. [8]. These cell-based therapies range from approaches that provide temporary support, such as bioartificial liver (BAL) devices, to more permanent interventions, such as cell transplantation and implantable tissue engineered liver constructs (Figure 1).

Extracorporeal devices primarily aim to offer transient support during liver regeneration or to serve as a bridge to transplantation. These devices process the blood of patients in a manner analogous to kidney dialysis systems. Substantial efforts have been invested in developing

extracorporeal BAL devices containing hepatic cells to supply the multitude of essential liver functions. There are four main categories of BAL devices [9-10]: 1) hollow-fiber devices, 2) flat plate and monolayer systems, 3) perfusion bed or porous matrix devices, and 4) suspension reactors; each of these general designs exhibit innate advantages and disadvantages. Overall, a clinically useful BAL device must be scalable to therapeutic levels and exhibit key properties such as efficient bidirectional mass transfer and maintenance of cell viability and liver functions. Several BAL devices have been tested in clinical settings and researchers continue to improve device and trial designs. Ultimately, even if current BAL systems do not yet represent effective therapeutic options, information gained from these studies, along with advancements in cell sourcing and functional maintenance of hepatocytes *ex vivo*, promises to empower the next generation of devices.

In addition to temporary support, more permanent cell-based therapies are being actively developed to replace damaged or diseased liver tissue. One such approach is the transplantation of isolated hepatocytes, which has been demonstrated to be safe and in some cases, effective, in both animal models and human trials [11-13]. Hepatocyte transplantation therapy is less invasive than organ transplantation [14] and could circumvent immunosuppressive regimens through the use of autologous cells. In rodent models, transplanted hepatocytes were further demonstrated to exhibit substantial proliferative capacity [12, 15-17]. This *in vivo* proliferation of transplanted cells is highly dependent on the presence of a “regenerative” environment, which can be provided by transgenic injury, partial hepatectomy, or the introduction of hepatotoxic agents prior to cell transplantation. The feasibility of hepatocyte transplantation is limited by the availability of appropriate cell populations as only mature hepatocytes have been repeatedly and consistently shown to provide sufficient rescue of liver functions [18] and only organs deemed inappropriate for transplantation can be perfused to yield scarce supplies of these cells. This constraint of limited availability of highly functional hepatocytes is unfortunately universal to all cell-based approaches for liver disease treatment.

Another emerging therapeutic approach for liver failure is based on the development of implantable tissue engineered hepatocellular constructs. Similar to cell transplantation, this strategy relies on transplanted hepatocytes to perform liver functions. Tissue engineering approaches further consider that hepatocytes are known to be anchorage-dependent; thus to maximize cell viability and functionality, hepatocytes are cultured *ex vivo* to form “organoids”, immobilized on scaffolds, or encapsulated in aggregates prior to surgical implantation in a number of anatomical sites, including the spleen, liver, pancreas, peritoneal cavity and mesentery, and subcutaneous tissues [19-20]. Proposed constructs have utilized scaffolds of various composition and architecture, both of which clearly influence hepatocyte survival and function. Despite advances in key aspects of hepatocyte maintenance *in vitro*, implantable systems remain largely experimental due to a number of obstacles that must be overcome before qualifying as a viable clinical modality. Specifically, hepatic tissue engineering shares many of the limitations of BAL devices and cell transplantation, but additionally faces challenges in establishing transplant vasculature and promoting transplant integration and remodeling. Details of these features will be discussed in later sections.

## **CELL SOURCING**

Studies into cell-based therapies suggest great promise but progress has been hindered by the propensity of hepatocytes to lose both phenotypic functions and the ability to proliferate *in vitro* [21-22]. Thus, the continued elucidation of molecular mediators that regulate hepatocyte

function and proliferation will be critical for the advancement of cell-based therapies and their routine use in clinics to treat compromised liver functions. In addition, the potential of alternative cell sourcing approaches, based on stem cell differentiation and reprogramming, are active areas of investigation.

### **Mature Hepatocytes**

Primary human hepatocytes are functionally the most robust cell type for cell-based therapies for liver diseases [8, 23]. Within their native microenvironments *in vivo*, human hepatocytes have phenomenal proliferative capability. Following resection of two-thirds of the liver through a surgical procedure known as partial hepatectomy (PHx), the residual mature cell populations, comprised mainly of hepatocytes, are able to proliferate and restore lost liver mass [24]. This full regenerative response can be seen after each of at least 12 sequential PHx's [25]. To demonstrate the clonogenic potential of the hepatocyte itself, mouse models were generated in which livers were rendered incapable of supporting animal life through experimentally induced defects. Healthy hepatocytes injected into these compromised livers can proliferate, generate nodules of normal hepatocytes, and rescue the animals [26]. As low as 1000 normal hepatocytes were found to be sufficiently therapeutic. Furthermore, cells from newly formed nodules of normal hepatocytes can be isolated and serially transplanted, through as many as four generations, to rescue other animals. Mathematical calculations based on this model predict that a single hepatocyte can undergo at least 34 cell divisions to give rise to  $1.7 \times 10^{10}$  cells, suggesting that a single rat hepatocyte can generate 50 rat livers of 300 million hepatocytes each [27].

Various attempts have been made in the last several decades to harness *ex vivo* this tremendous replication potential of mature human hepatocytes (Figure 2). It is recognized that proliferating hepatocytes *in vivo* are presented a complex and dynamic mixture of soluble factors via the blood while maintained within an interactive support system of extracellular matrix (ECM) and non-parenchymal cells. Thus, early studies focused on providing select key components to *in vitro* culture systems, including humoral and nutritional supplements as well as ECM and supportive cell types [28]. To specifically promote hepatocyte expansion *in vitro*, primary cultures have been treated with serum and cytosol collected from livers that underwent PHx [29], and with more defined soluble factors including various growth factors [30-31], sugars [30], amino acids [30], hormones [31-32], vitamins [30, 33], serum proteins [30, 34], and trace metals [30, 34]. The effect of any individual supplement on hepatocyte proliferation can be difficult to directly determine, as the effect depends on the state of the hepatocyte, which is synergistically determined by the combination of all culture components [28]. Nevertheless, investigations have yielded a multi-factor media formulation, which can be used for moderate expansion of rat hepatocytes through a dedifferentiated bi-potential intermediate [30]. Non-soluble culture components such as different ECM [30, 35] and supportive cell types [35-38] have also been examined for mitogenic effects on hepatocytes. These include physiologic liver ECM proteins, and non-physiologic tumor-secreted protein mixtures in different configurations, in addition to co-cultures of hepatocytes with various intrahepatic and extrahepatic cell types, both live and dead. Many different combinations of culture components have been shown to support moderate expansion of rat hepatocytes although translation of these findings to human cultures has not been reported.

Human cells are critical for cell-based therapies due to substantial species-specific differences between animal and human hepatocellular functions including apolipoprotein

expression, metabolic regulation of cholesterol, and phase I detoxification enzymes [39-41]. To overcome the growth limitations of primary human cells, investigations are underway to develop highly functional human hepatocyte cell lines. A common approach is to introduce oncogenes through retroviral transduction. The simian virus 40 tumor antigen gene (SV40 Tag) is a common immortalization agent, whose product binds to cell cycle regulator proteins Rb and p53 [42-43]. Cell lines have also resulted from spontaneous immortalization of hepatocytes in co-cultures or collagen gel sandwich cultures [44], and additionally can be derived from liver tumors, as in the case of the HepG2 hepatoma cell line [45]. Although these cell lines are growth-competent, they introduce new safety concerns and typically underperform primary cells in terms of liver functions [46-47]. The principal safety concern is the transmission of oncogenic agents to the host, especially in the case of implanted cells. To address this, researchers have developed mechanisms to inactivate transduced oncogenes through temperature-sensitive SV40 Tag [48], Cre-*loxP*-mediated oncogene excision [49], and suicide genes such as herpes simplex virus thymidine kinase (HSV-tk) [50].

Another intriguing approach for human hepatocyte expansion, particularly as a model system, is the transplantation of human hepatocytes into genetically-altered mouse strains [17, 51-53]. This strategy takes advantage of the *in vivo* mitogenic environment, known to orchestrate many rounds of hepatocyte replication and can be generated through experimentally induced defects to host livers. Such defects can be produced by large amounts of urokinase, which can be abnormally over-expressed under the influence of the albumin promoter in hepatocytes [3, 12, 51, 54]. While effective as a hepatic xeno-repopulation system, these mice are fragile and present only a limited time window for transplantation. Alternatively, Grompe and colleagues have produced regeneration-inducing liver defects through an experimentally introduced deficiency in the catabolic enzyme fumarylacetoactate hydrolase (Fah). After pretreatment with a urokinase-expressing adenovirus, Fah-deficient mice can be very receptive hosts to human hepatocytes [17]. Findings from these animal studies suggest that human hepatocytes do retain their considerable proliferation potential upon isolation and can expand given the appropriate stimuli. However, similar to the use of hepatocyte cell lines, the therapeutic utility of hepatocytes expanded in animal models is limited by safety concerns such as the transmission of pathogenic agents and the incorporation followed by expression of animal glycoproteins on human hepatocyte cell surfaces.

Ultimately, sustainable proliferation of highly functional human hepatocytes could generate patient-specific cell populations. These cells can be used to provide sufficient autologous materials for cell-based treatments, thus circumventing post-surgical immunosuppressive regimens. *In vitro*, the ability to expand human hepatocytes can enable drug therapies to be selected according to the characteristics of individual patients, thus minimizing adverse drug reactions.

### **Stem Cells and Progenitor Populations**

Due to limitations in mature hepatocyte expansion *in vitro*, alternative cell sources are being pursued. These include various stem cell populations, which can self-renew *in vitro* and exhibit pluripotency or multipotency and thereby serve as a possible source of hepatocytes, as well as other non-parenchymal liver cells.

Studies have shown that embryonic stem cells can be induced to differentiate down the hepatic lineage in culture through the carefully orchestrated addition of various growth factors, and when supported by the appropriate ECM [55-57]. More recently, studies are also exploring

in more scope and detail the functional capacity of these differentiated populations, both *in vitro* and *in vivo* [58-60]. Such endeavors are being guided by improved insight into how different cell types are specified in embryonic development. This insight is typically gained through observations of cellular responses to individual inductive signals. Zaret and colleagues have further investigated how different inductive signals interrelate and have reported complex, dynamic signaling networks that could help explain incomplete cell programming in stem cell differentiation protocols [61].

In addition to embryonic stem cells, a wide range of fetal and adult progenitor cell types have been explored. Continuing investigations are focused on determining the differentiation potential and lineage relationships of these populations. Fetal hepatoblasts are liver precursor cells present during development that exhibit a bipotential differentiation capacity, defined by the capability to generate both hepatocytes and bile duct epithelial cells [62]. Furthermore, within the adult liver, a rare percentage of resident cells have been demonstrated to exhibit properties consistent with their designation as adult hepatic stem cells [63-64]. It has been suggested that these cells represent precursors to adult progenitor cells, termed oval cells, which share phenotypic markers and functional properties with fetal hepatoblasts. In adult livers suffering certain types of severe and chronic injury, oval cells can mediate liver repair through a program similar to hepatic development [65-66]. Various cell lines exhibiting characteristics comparable to fetal hepatoblasts and oval cells have been developed, for example, lines derived from mouse E14 embryos by Weiss and colleagues. These bipotential mouse embryonic liver (BMEL) cells are proliferative, can be induced to be hepatocyte-like or bile duct epithelial-like *in vitro* [67], and can home to the liver to undergo bipotential differentiation *in vivo* within a regenerative environment [68].

Outside the liver, there may also exist multipotent stem/progenitor-like cells that are of therapeutic and biomedical interest [69]. For example, multipotent adult progenitor cells (MAPCs) derived from the bone marrow have been shown to generate hepatocyte-like cells *in vitro* [70]. Similarly, various mesenchymal stem cell preparations have been reported to give rise to cells exhibiting many characteristics of mature liver cells [71-74], including the ability to engraft *in vivo*; however, the extent of functional liver repopulation has been modest [69]. Other sources of extrahepatic liver cell progenitors include human amniotic fluid and membranes, which may contain cells capable of hepatic differentiation [75-79].

### **Reprogrammed Adult Cells**

Fully differentiated adult cells, such as skin cells, were recently demonstrated to be reprogrammable to a undifferentiated, pluripotent state through forced expression of reprogramming factors Oct3/4 and Sox2 along with either Klf4 [80-83] or Nanog and Lin28 [84]. These reprogrammed cells are termed induced pluripotent stem (iPS) cells and highly resemble embryonic stem (ES) cells, sharing many characteristics such as significant self-renewal capabilities *in vitro* and pluripotent differentiation potential. However, iPS cells offer an additional advantage of sourcing from adult somatic cells for the generation of patient-specific cell populations, potentially enabling therapies to be developed according to the characteristics of an individual patient. Work done by Duncan and colleagues, as well as other researchers, demonstrated that through iPS reprogramming and a subsequent multistep differentiation protocol, skin cells can give rise to hepatocyte-like cells, which not only exhibit a variety of hepatocyte-specific functions *in vitro*, but can also be induced to generate intact fetal livers in mice *in vivo* [85-87].

As a parallel strategy, work done by Melton and colleagues has demonstrated that it is also possible to directly reprogram one adult cell type into another, without an undifferentiated pluripotent intermediate. Similar to the use of master transcriptional regulators in the reprogramming to iPS cells, the expression of a key set of transcription factors in pancreatic exocrine cells *in vivo* induced conversion into cells that highly resemble  $\beta$ -cells [88]. These findings raise future possibilities for deriving hepatocytes directly from another adult cell type.

Ultimately, understanding the mechanisms governing the fates of stem and progenitor cell populations can empower the development of cell-based therapies. However, many challenges remain, including the ability to program differentiation completely. Furthermore, regardless of the cell source, phenotypic stabilization of hepatocytes *ex vivo* remains a primary issue. Accordingly, the development of robust *in vitro* liver models is an essential stepping-stone towards a thorough understanding of hepatocyte biology and improved effectiveness of cell-based therapies for liver disease and failure.

## **IN VITRO PLATFORMS AND APPLICATIONS**

An important component of liver tissue engineering is the development of *in vitro* hepatocyte culture platforms. Such cultures can be used for applications aimed at studying fundamental hepatocyte biology, understanding and developing remedies for liver pathophysiology, and evaluating the liver metabolism and toxicity of pharmaceutical drug candidates. A summary of previously developed liver platforms is provided in Table 1. When selecting a platform for a particular application, it is crucial to consider the necessary model criteria given the specific strengths and weaknesses of each approach.

Within these model systems, isolated primary hepatocytes are generally considered the most appropriate cell source; however, primary hepatocytes are notoriously difficult to maintain in culture due to a rapid decline in viability and liver-specific functions post-isolation [89-91]. Research has thus focused on providing the stimuli necessary to maintain the hepatocyte phenotype, and this research is gradually giving way to a systems-level picture of the molecular signals that furnish phenotypic stability of hepatocytes. In this section, we focus on methods that have been developed to stabilize the hepatocyte phenotype. We discuss in parallel how such models have been used in tissue engineering applications including drug development and disease modeling.

### **2D Culture Platforms**

Several parameters of two-dimensional culture can be modulated to enhance primary hepatocyte morphology, survival, and liver-specific functions. Three such parameters are culture medium, extracellular matrix, and heterotypic interactions with non-parenchymal cells.

Culture media supplemented with serum and physiological factors such as hormones, corticosteroids, growth factors, vitamins, amino acids, or trace elements [90, 92], as well as non-physiological factors such as phenobarbital and dimethylsulfoxide [93-94], have been shown to modulate the hepatocyte phenotype. Hepatocytes have also been maintained in media without serum [95]. Investigators utilizing a co-culture system (co-culture configurations discussed in detail below), with endothelial cells in serum-free medium under high (95%) oxygen, recently demonstrated support of hepatocyte gene expression and drug metabolism functions better than co-culture in serum medium at 21% oxygen [96]; furthermore, the oxygenated model s stabilized more quickly. Jindal et al. have additionally identified the amino acid proline as the key factor

secreted by endothelial cells in co-culture responsible for mediating the acceleration of hepatocyte recovery [97].

Extracellular matrix (ECM) plays an important role in hepatocyte culture; ECM preparations of different composition and topology have different effects on hepatocyte morphology and function. For instance, the presence of collagen I on a substrate enhances hepatocyte attachment, although hepatocyte spreading on adhesive substrates is often associated with loss of liver-specific functions [98]. Culture of hepatocytes on a monolayer of “biomatrix”, a complex ECM mixture extracted from the liver, has been shown to improve hepatocyte function over culture on a monolayer of pure collagen [98-99]. To screen in greater throughput the effects of various ECM proteins on hepatocyte physiology, a microarray platform was developed by Flaim et al. [100]. This system enabled investigation of the synergistic impact of ECM combinations on hepatocyte function with potential implications for the crosstalk between integrin signaling pathways initiated by various ECM molecules. Monolayers of ECM are, however, not the only means of presenting ECM molecules to hepatocytes. In the standard “double gel” configuration, hepatocytes are sandwiched between two layers of collagen gel. In this format, hepatocytes demonstrate desirable morphology and liver functions for approximately 1 week [90]; rat hepatocytes in particular show P450 induction and a contiguous, anastomosing network of bile canaliculi indicative of polarized structures [22, 101]. Limitations of this format include the fact that phase I/II detoxification processes typically become imbalanced over time [102], and that an ECM gel above the hepatocytes may inhibit the diffusion of paracrine signals or other molecular stimuli in the culture medium. Additionally, surface modifications such as polyelectrolyte chemistries have been tested for effects on hepatocyte function *in vitro* [103-104]. Specifically, Chen et al. developed a two-dimensional model consisting of polyelectrolyte multilayers that enables independent variation of both substrate mechanical compliance and ligand presentation. By enabling optimization of chemical and mechanical cues, such culture techniques could prove useful in rational design of culture platforms for applications such as tissue engineering.

Heterotypic interactions with non-parenchymal cells have been used successfully to preserve the viability, morphology, and function of hepatocytes from a range species for several weeks. Through extensive studies beginning with initial work by Guguen-Guillouzo and colleagues [105], the “rescue” of hepatocytes within co-culture settings has been demonstrated utilizing a wide variety of non-parenchymal cells from both within and outside the liver, and across species barriers, suggesting that the mechanisms responsible for stabilization are conserved [106]. Overall, substantial experimental efforts continue to explore various co-culture systems as potential models of physiologic and pathophysiologic processes in the liver. Furthermore, the identification of the important mechanisms underlying stabilization within non-parenchymal co-cultures could provide the basis for the addition of key factors and increased functionality within hepatocyte-only culture platforms.

### **3D Spheroid Culture**

Certain substrates promote the aggregation of cultured hepatocytes into three-dimensional spheroids and can affect functionality [107-111]; this is potentially due to the retention of a 3D cytoarchitecture, the presence of ECM surrounding the spheroids, and the formation of homotypic cell-cell contacts between neighboring hepatocytes [112]. On non-adhesive surfaces, for example, hepatocytes aggregate over 1-2 days first into smaller spheroids of ~50  $\mu\text{m}$  which over weeks gradually fuse into larger 150-175  $\mu\text{m}$  spheroids [98]. These

spheroids have functions superior to standard collagen monolayer culture [113-114]. On Matrigel (a laminin-rich basement membrane extract), hepatocytes also form spheroids that retain hepatic functions [115-116], though it is difficult to pinpoint the cause of these effects due to the contamination of Matrigel with proteins, hormones, and growth factors [90, 117]; further, Matrigel-based platforms suffer from the gradual imbalance of phase I/II detoxification processes (CYP450 decline) over a few days in culture [102]. Upon transfer to static collagen surfaces, the spheroids disassemble and the hepatocytes spread and dedifferentiate [118-119]. Spheroid cultures have also been produced with support cells. A recent study produced an array of spheroids made from fetal mouse liver cells containing fetal hepatocytes and other liver cell types; hepatospecific function and differentiation induction were enhanced by co-culturing these spheroids with non-parenchymal feeder cells [120]. Other methods such as rotation have been used to make hepatocyte spheroids. Recently, a rocking method was employed to produce spheroids [121], generating spheroids faster and with fewer non-adherent hepatocytes than rotational methods, exhibiting preserved stable expression for many typical liver-specific genes.

Spheroids can in turn be encapsulated to control cell-cell interactions. In one method, spheroids suspended in methylated collagen are syringe-extruded into terpolymer solution to form microcapsules [122], but other methods have made use of various synthetic and natural scaffolds. Spheroid cultures have been utilized for both small-scale [91, 123-124] and large-scale bioreactor systems [125]. While spheroid cultures can demonstrate desirable liver functions, there are several limitations including the fusion of small spheroids into larger aggregates and death in the center of such aggregates due to limiting influx of nutrients and efflux of waste products. Thus, platforms for optimizing spheroid size and handling are under ongoing development.

### **Bioreactor Cultures**

Though useful for many applications, the types of *in vitro* models described above provide a relatively homogeneous view of liver function. *In vivo*, there is a significant distribution of hepatic functions along the length of the sinusoid associated with translobular gradients in nutrients, oxygen, hormones, and ECM. Some bioreactor cultures attempt to capture these differences. In order to study the effects of oxygen variation across the liver lobule, a small-scale, parallel-plate bioreactor was developed that exposes hepatocyte/non-parenchymal co-cultures to a steady-state oxygen gradient [126]. These cultures were able to replicate the heterogeneous expression distribution of the drug metabolism enzymes CYP2B and CYP3A observed *in vivo*, and expression could be controlled with chemical inducers and growth factors. Furthermore, exposure of the culture to acetaminophen caused greatest cell death in areas of low oxygen, replicating the centrilobular death pattern observed *in vivo*.

Bioreactors have also been produced to culture hepatic aggregates. One device consisted of a 1 cm<sup>2</sup> planar polymer scaffold with 900 micro-containers that could each culture a uniformly-sized 3D hepatic aggregate [123-124]. The aggregates were perfused via a pore laser-drilled within each micro-container and retained desirable morphology and liver functions for 2 weeks. Another device consists of hepatic spheroids cultured in an array of micro-channels etched into silicon wafers using deep reactive ion etching [91]. In this system, culture medium was passed across the top of the array, enabling spheroids to retain liver-specific characteristics for 2-3 weeks in culture, as assessed by gene expression profiling, protein expression, and activity of drug metabolism enzymes [91]. Recently, Domansky et al. have integrated multiple perfused bioreactors into a multiwell plate format in which each bioreactor houses hundreds of

microscale hepatic monolayers; the format of this device could be applied towards studying perfused tissue units in high-throughput [127].

Bioreactors have been developed in several other studies as well. A flat plate bioreactor was designed to study the effects of oxygenation and shear stress on hepatocyte function [128]. Additionally, alternative bioreactor configurations have been developed to minimize shear stress effects, and have included, for example, grooved substrates to protect hepatocytes from shear, or adjacent channels separated by a gas-permeable membrane to decouple oxygen exchange and volumetric flow rate [129-130]. Such bioreactors can also be scaled for clinical applications; in one particular demonstration, grooved culture substrates were stacked in a radial flow bioreactor [131]. To improve oxygen delivery, collagen sandwich co-cultures of hepatocytes and non-parenchymal cells from the liver were cultured in a 96-well perfused micro-bioreactor with a biocompatible, gas-permeable membrane [89]; hepatocytes in this system maintain liver functions such as albumin and urea expression, expression of phase I/II detoxification enzymes, and inducible expression of CYP1A1. Finally, bioreactors may be used to study more dynamic physiological processes than is possible in conventional culture platforms; for example, a recent bioreactor device describes the ability to monitor invasion of metastatic cells into hepatic parenchyma by recreating relevant features of the liver tissue such as fluid flow and length scales [132]. Collectively, bioreactors enable complex control over hepatocyte culture parameters [133-135] and in turn hepatocyte function; as such, they will continue to be useful in studying liver biology and in applications such as drug development.

### **Microtechnology Tools**

Microtechnology tools afford micron-scale control of tissue architecture as well as cell-cell and cell-matrix interactions, facilitating investigations of the mechanisms underlying tissue development and function [136]. Based on methods used in the semiconductor industry, microtechnology approaches allow fine control over cell adhesion, shape, and multi-cellular interactions [137]. Consequently, they are enabling studies of biological phenomena at cellular length scales [138-139], as well as techniques for miniaturizing and parallelizing biomedical assays (e.g. DNA microarrays, microfluidics) [140-141].

In order to study the effects of homotypic and heterotypic cell-cell interactions between hepatocytes and non-parenchymal cells, a photolithographic cell patterning technique was employed to make micropatterned co-cultures in which hepatocyte islands of controlled diameters were surrounded by non-parenchymal cell [106]. Initially employed for rat hepatocyte culture, the highest levels of liver-specific functions occurred at an intermediate island diameter, implying that optimal function results from an optimal balance of homotypic/heterotypic interactions. Using soft lithography, this co-culture pattern has been recently miniaturized and adapted into a multi-well format to serve as a microscale human liver tissue model for drug development [140]. The utility of this platform for drug development has been shown through gene expression profiles, phase I/II metabolism, canalicular transport, secretion of liver-specific products, and susceptibility to hepatotoxins. Fine control over the spatial distribution of cells is also demonstrated in a recent platform developed by King et al. which uses a microfabricated device with quantitative live cell imaging to measure gene expression in real-time of individual living cells [142]; the tool is used to investigate gene expression changes in the course of hepatic inflammation.

Another application of microtechnology is the use of microfabrication and microcontact printing techniques to develop a microarray containing hepatocyte spheroids of a uniform size

[143]; this method reduces aggregate heterogeneity and minimizes cell necrosis resulting from oxygen/nutrient depletion or waste accumulation. These spheroids retain liver functions including expression of liver-enriched transcription factors, albumin secretion, and expression of urea cycle enzymes. Another application of microtechnology tools in building multicellular hepatic structures is the formation of hepatic tissue sheets by release of confluent hepatocytes from surfaces coated with the temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm) [144].

Microtechnology tools can also be used to create culture platforms in which stimuli are dynamically modified, in contrast to typical culture platforms in which stimuli are static; such tools should enable investigation and optimization of cellular responses that exhibit spatiotemporal components and thus contribute to tissue engineering applications. Microfluidic devices are typical examples of systems that permit spatiotemporal control over delivery of nutrients and other soluble mediators to cultured cells. Recently, Chao et al. describe a microfluidic platform that simulates flow through the liver to predict the *in vivo* hepatic clearance of pharmaceutical compounds [145]. Dynamic cellular responses can also be interrogated using other nascent microfabrication approaches. In one example, a mechanically actuated “comb” device was fabricated that enabled investigation of cell-cell interactions by permitting micron-scale temporal control of cell-cell interactions [146]. When used to study hepatocyte-stromal cell interactions, this platform revealed that phenotypic stabilization of phenotypes by the non-parenchymal cells required direct contact for hours followed by a sustained short-range paracrine signal.

The fine spatial and temporal control afforded by microtechnology tools has already accelerated studies of basic liver biology and applications that were impossible without such methods.

### **Application of *In Vitro* Liver Models: Studying Liver Pathophysiology**

*In vitro* hepatocyte cultures and co-cultures have been utilized to investigate various physiological and pathophysiological processes, including host response to sepsis, mutagenesis, xenobiotic toxicity, response to oxidative stress, lipid metabolism, and induction of the acute phase response [106]. Among the many applications of *in vitro* liver tissue models is the study of the behavior of pathogens that target hepatocytes and screening for therapeutics of the associated diseases. Hepatitis C virus (HCV) and malaria are two such pathogens.

Hepatitis C virus is an enveloped RNA virus whose genome consists of a single positive-stranded RNA that replicates in the cytoplasm of infected hepatocytes without integrating into the host genome. The first *in vitro* model enabling studies of replication and screens for small molecule inhibitors of the replicative enzymes of HCV consisted of a subgenomic replicon stably-transfected into carcinoma cells [147]; with this system, however, it was not possible to study the complete viral life cycle as the structural proteins were omitted from the replicon. Collectively, at this time, researchers were unable to find a viral genotype capable of executing the full viral life cycle *in vitro*. In 2001, Kato et al. [148] found and sequenced a genotype-2a strain of HCV that caused fulminant hepatitis in a Japanese patient. This genotype was named JFH-1, and in 2005 it was shown that JFH-1 and a chimeric variant were able to complete the entire viral life cycle in the Huh7 carcinoma cell line and do so more robustly in certain Huh7 sublines [149-151]. More recently it has become possible to study HCV infection in primary human hepatocytes [152-154]. Ploss et al. [154] demonstrate that a microscale human liver tissue model [140] is capable of recapitulating the entire viral life cycle and can act as drug screening platform for compounds that suppress HCV replication.

*Plasmodium* infection is responsible for malaria disease, and distinct species, such as *P. falciparum* and *P. vivax*, are associated with different degrees of severity and patterns of pathogenesis. After transmission of the *Plasmodium* sporozoites to the human blood circulation from mosquito saliva, the sporozoites infect hepatocytes in the liver. There, they eventually proliferate and differentiate into merozoites which then go on to infect red blood cells, where they rapidly amplify in number. *In vitro* liver models have the potential to enable studies of the hepatocyte stage of the malarial life cycle and presumably vaccine development and screening of small molecules that inhibit viability or proliferation of the parasite in its liver stages. Several lines of research have explored recently this possibility, for example, in a two-dimensional collagen monolayer culture model of primary human hepatocytes, investigators were able to recapitulate the complete liver development stage of *P. falciparum* [155] and *P. vivax* [156]. A similar culture model has been used to characterize the mechanistic basis of CD81-dependent invasion of hepatocytes by *Plasmodium*, with the conclusion that SR-BI enhances permissiveness to infection by increasing plasma membrane cholesterol and organizing CD81 into an entry-favorable configuration [157]. van Schaijk et al. use this model to show that disruption of the p52 gene in *P. falciparum* leads to arrest in the liver stages of development, potentially providing a source of genetically attenuated sporozoites for vaccination purposes [158]. By reproducing key physiologic properties of liver tissue, *in vitro* liver models enable applications such as drug development and the study of liver pathophysiology. As discussed in this section, numerous culture configurations have been attempted which each serve different functions. In pursuing a particular application, investigators must decide what critical aspects of the *in vivo* liver tissue must be replicated in their systems, informing the selection of an appropriate model.

## **IMPLANTABLE ENGINEERED TISSUE CONSTRUCTS**

Transplantation of hepatocytes to perform liver functions shows great potential for the treatment of liver disease and in the development of humanized liver mouse models, but direct injection of cells is associated with variable seeding efficiency and poor long-term survival and engraftment. Hepatocyte delivery in a tissue-like structure that preserves cell attachments could increase engraftment efficiency, reduce the need for a repopulation advantage in donor cells and reduce the overall lag phase before clinical benefit is attained [159-161]. Thus, hepatic tissue engineering technologies, which seek to generate liver-like tissue *in vitro* prior to *in vivo* implantation, may provide an alternative delivery method to transplantation of suspension cells, as well as a means to implant cells and/or additional biological cues that interact with the host and ultimately serve to improve liver function.

Implantable engineered hepatic tissues have typically been created by immobilizing or encapsulating hepatocytes using biomaterial scaffolds. As such, scaffold properties and cell sourcing are both critical in the development of engineered tissue. Though great strides have been made in this field, many issues must be addressed before implantable hepatic tissue becomes clinical reality. As work in this field advances, careful attention needs to be paid to issues that dictate the ultimate clinical translation of these therapies.

### **Scaffold Properties**

Highly functional 3-D implantable liver tissue will likely require dense population with functional and stable hepatocytes, while also facilitating the transport of nutrients and large

macromolecules. Cell seeding and nutrient transport are ultimately dictated by scaffold properties, which include material and chemical modifications, porosity, and 3-D architecture.

### **Material and Chemical Modifications**

The choice of material determines the physicochemical and biological functions of the scaffold. For example, natural, biologically-derived materials containing binding sites for cell attachment can enhance function since hepatocytes are anchorage-dependent cells. Hepatocytes have been attached to collagen-coated dextran microcarriers and transplanted intraperitoneally into two different Gunn rat genetic models for replacement of liver-specific functions [162]. Microcarriers provide a platform for cell attachment and enhanced the survival and function of the transplanted hepatocytes. Cellulose [163-165], gelatin [166], and gelatin–chitosan composite [167] microcarrier chemistries have also been explored for hepatocyte attachment. Additionally, hepatocytes have been encapsulated within natural, extracellular matrix-derived scaffolds, including the collagen gels [168-169], hyaluronic acid [170], peptides [171], or alginate and alginate-based composites [172-175]. Microencapsulation of hepatocytes in these types of systems can facilitate hepatocyte aggregation and improve function. For instance, alginate-based encapsulation platforms have been shown to support hepatocyte spheroid culture [172-173, 175] and thus have been proposed for use in implantable constructs.

Synthetic polymers have afforded hepatic tissue engineers improved control over scaffold physicochemical and biological properties. The most common synthetic polymers utilized in the generation of porous tissue engineering constructs are polyesters such as poly(L-lactic acid) (PLLA) and poly(D,L-lactide-co-glycolide) (PLGA). These materials are biocompatible and biodegradable, support hepatocyte culture, and have been widely used as scaffolds for hepatocyte transplantation [176-186]. A key advantage of these polyesters is the ability to finely tune its degradation time, based on the relatively contribution of the PLLA versus PLGA components, which each exhibit distinct hydrolysis kinetics. Material modifications of PLGA scaffolds have also been shown to improve hepatocyte functionality. Specifically, addition of hydrophilic poly(vinyl alcohol) (PVA) into PLGA scaffolds enhanced hepatocyte seeding [180]. Alkali hydrolysis and extracellular matrix coating of PLGA constructs can similarly enhance hepatocyte attachment [184-185, 187]. Importantly, a composite PLLA–PLGA scaffold coated with PVA supported long-term engraftment of hepatocytes after transplantation in the mesentery in a rodent injury model [179]. Despite these advances, the accumulation of hydrolytic degradation products upon PLLA and PLGA degradation has been shown to produce an acidic environment within the scaffold and initiate peptide degradation, stimulate inflammation, or result in poor tissue engraftment [188-189]. As such, groups have explored methods to control peptide degradation in PLGA as well as explore alternative synthetic polymer-based systems for use in tissue engineering.

The synthetic hydrogel system based on poly(ethylene glycol) (PEG), has also been widely utilized for various tissue engineering applications [190-208] including, recently, for hepatocellular platforms [209]. PEG-based systems are particularly useful in tissue engineering due to their high water content (which give them similar mechanical properties to tissues), hydrophilicity and resistance to protein adsorption, biocompatibility, and capacity for customization through the modification of chain length and the addition of bioactive elements [208, 210]. An additional advantage of PEG is that it can be polymerized through photocrosslinkable diacrylate (DA) endgroups in the presence of cells, which provides for the generation of 3-D constructs with uniform cellular distribution. Studies examining PEG-DA

hydrogel encapsulation of hepatic cells have utilized immortalized hepatocytes, hepatoblastoma cell lines and primary hepatocytes, and together have shown that tailoring the design of the hydrogel network dictates hepatocyte survival and function [211-213]. Taken together, these results demonstrate that synthetic hydrogel systems represent promising platforms for implantable constructs as well as investigating *in vitro* hepatocellular responses in a model 3-D environment.

Although synthetic polymer scaffolds offer numerous advantages, the absence of natural cell-binding sequences in these systems often limit their capacity to promote cell adhesion. The inert nature of synthetic systems can also be used as a tool to study the basic biology of hepatocellular adhesion, by facilitating the controlled incorporation of biologically active elements aimed at regulating different aspects of cell function. Multiple approaches have been explored for modulating hepatocyte interactions with synthetic platforms by non-specific adsorption or chemical conjugation of biological molecules, including the incorporation of 1) extracellular matrix molecule coatings [184-185], 2) various sugar residues such as lactose and heparin [214] and galactose [215-216], 3) poly(*N-p*-vinylbenzyl- 4-*O*  $\beta$ -d-galactopyranosyl-d-glucosamine) (PVLA) in PLLA scaffolds [181, 217-218], and 4) epidermal growth factor (EGF) in poly(ethylene terephthalate) (PET) fabric scaffolds [218]. Each of these modifications have been implicated in improving hepatocyte adhesion within polymer scaffolds and, therefore, highlight modifications of the polymer scaffold backbone that influence hepatocyte processes. As an alternative to the incorporation of entire biomolecules, polymer scaffolds can also be conjugated with bioactive adhesive peptide sequences. Adhesive peptides, which interact with integrin receptors in the cell membrane, have been extensively utilized to promote cell attachment within polymer networks. For example, inclusion of the RGD peptide sequence within biomaterial scaffolds dramatically influences the adhesion and function of a diverse assortment of cell types [219]. In experiments using hepatocytes, grafting RGD peptides to PLLA scaffolds similarly enhanced hepatocyte attachment [186]. Notably, RGD conjugation also significantly improved the long-term stability of primary hepatocyte function in PEG hydrogels [211]. Incorporation of additional adhesive peptides that bind other integrins might further enhance hepatocyte function within synthetic polymer substrates. Additionally, the incorporation of hydrolytic or protease-sensitive peptide sequences (such as matrix metalloproteinase-sensitive peptide sequences) into hydrogel networks as degradable linkages has been shown to permit cell-mediated degradation and remodeling of the gel [199, 201, 220-223]. Although these systems have not yet been applied to hepatocellular technologies, it is interesting to note that liver regeneration proceeds in conjunction with a distinctive array of remodeling processes such as protease expression and extracellular matrix deposition [224-226]. Therefore cell-mediated material degradation could provide a mechanism for the efficient integration of implantable constructs. The ability to modify biomaterial scaffold chemistry by introducing biologically active factors will likely allow for fine-tuned regulation of cell function and graft-host interactions.

### **Porosity**

Many natural and synthetic implantable tissue engineering approaches utilize porous scaffolds, which provide mechanical support and often biological cues for growth and morphogenesis. The material's pore size can be controlled over several orders of magnitude, thus allowing materials to be tailored for purposes such as the optimization of protein exchange [211], cell-cell interaction and survival [211, 227], or the promotion of wound repair and tissue

ingrowth [228]. Collagen or various alginate and chitosan composites are the most frequently used biologically-derived scaffold materials for hepatocyte tissue engineering [168, 172-175, 227, 229-242]. Collagen sponges have been utilized as scaffolds in a diverse array of cell systems and also as substrates for promoting wound repair [243-249]. In a study in which hepatocytes were seeded onto collagen scaffolds exhibiting a variety of pore sizes (10–82  $\mu\text{m}$ ), pore size was found to be an important factor regulating cell spreading and cell–cell interactions [227]. Additionally, alginate scaffolds with pore sizes of approximately 100  $\mu\text{m}$  have been shown to encourage spheroidal aggregation of hepatocytes owing to the weakly adhesive properties of the material, and spheroid formation in turn promoted hepatocyte stabilization [230]. Variations in alginate formulation, such as an alginate–galactosylated chitosan–heparin composite system [241], can further enhance cell aggregation and viability [172, 174, 229, 240-241]. A variety of porous synthetic materials such as PLLA and PLGA have also been used in hepatic tissue engineering, as detailed above. Porous, acellular scaffolds are normally seeded using gravity or centrifugal forces, convective flow, or through cellular recruitment with chemokines [250-252]. However, incorporation of hepatocytes into scaffolds is hindered by insufficient or non-homogeneous cellular distribution and by the relatively immotile nature and limited proliferation of hepatocytes *ex vivo*. Additionally, despite the fact that porous scaffold systems continue to be explored for use in engineered liver tissue, many of these scaffold architectures contain pores that are many times larger than individual hepatocytes, essentially making them 2-D surfaces from the hepatocyte perspective. This may limit the ability of porous scaffolds to fully recapitulate 3-D cues.

### **3-D Architecture**

An alternative approach for tissue-engineered scaffolds strives to more closely mimic *in vivo* microarchitecture. The 3-D architecture of native tissues influences cellular function, mechanical properties of the tissue, and the integration of grafted engineered tissue with the host. The ability to fabricate cellular scaffolds with highly defined structure could facilitate the recapitulation of the appropriate microscale environment for cell viability, cell function, and cell–cell interactions, as well as desired macroscale properties that determine mechanical properties and nutrient delivery.

Porous scaffolds were initially produced by solvent casting or particulate leaching methods. These techniques did not permit for pre-designation of the internal scaffold structure or pore connectivity. More recently, CAD-based rapid prototyping strategies have been developed that allow for defined control by utilizing multiple assembly modes, including fabrication using heat, light, adhesives, or molding, as reviewed elsewhere [253]. Briefly, 3-D printing with adhesives combined with particulate leaching has generated porous PLGA scaffolds that were used for hepatocyte attachment [254]. Microstructured ceramic [255] and silicon scaffolds [256-257] have additionally been proposed as systems for the culture of hepatocytes. Furthermore, molding and microsyringe deposition have been utilized to fabricate specified 3-D PLGA structures [258].

Microfabrication techniques have also been employed to pattern cells within natural and synthetic hydrogels. For example, microfluidic molding has been used to create biological gels containing patterned cells in multi-layer structures [259-260]. In addition, syringe deposition and micropositioning were recently used to generate patterned gelatin hydrogels containing hepatocytes [261]. The ability to polymerize synthetic PEG hydrogels using UV-initiation allows for the use of photolithography to generate hydrogel networks with defined microscale patterns.

In this process, patterned masks printed on transparencies localize UV exposure to selected regions of the prepolymer solution and therefore dictate the structural features of the resultant hydrogel. Photolithography-based techniques have been employed to pattern biological factors [262], produce hydrogel structures with a variety of shapes and sizes [263-264], and build multilayer cell networks [213]. Hydrogel photopatterning is thus ideal for the regulation of scaffold architecture at the multiple size scales required for engineered hepatocellular constructs. As one example, perfusion of hepatocellular constructs is known to improve hepatocyte functions [215, 254]; therefore, photopatterning of PEG hydrogels containing hepatocyte–fibroblast co-cultures was used to create a branched 3-D network that was cultured under flow conditions for improved oxygen and nutrient transport and encapsulated hepatocyte functions [211]. At the microscale, dielectrophoretic gradient field gradients have been employed to pattern hepatocytes and fibroblasts within a pre-polymer solution of PEG prior to photoencapsulation [265]. The combined utility of photopatterning and dielectrophoresis-mediated cell patterning thus allows the construction of hepatocellular hydrogel structures with an organization defined at both the macroscale and cellular scale. Finally, the recent introduction of a new family of PEG-based photodegradable hydrogels, which allow selective degradation of hydrogels using light, will allow real-time manipulation of spatial features and mechanical properties at the microscale. Such materials create new opportunities to study the effects of changes in scaffold microarchitecture, chemistry, and mechanics over the course of culture time [266]. In summary, the ability to dictate scaffold architecture coupled with advances in scaffold material properties, chemistries, and the incorporation of bioactive elements will serve as the foundation for the future development of improved tissue-engineered liver constructs.

### **Cell-sourcing for implantable liver tissues**

Cell sourcing for implantable engineered liver tissue faces challenges similar to cell transplantation. Specifically, hepatocytes cultured in three-dimensions within biomaterial scaffolds lose phenotypic functions and the ability to proliferate *in vitro* in the absence of the appropriate biological cues [212]. In the effort to optimize both phenotypic function and cell proliferation, various groups have explored the use of mature primary hepatocytes, hepatic progenitor cells, and non-parenchymal supporting cells in tissue engineered constructs.

### **Mature hepatocytes *versus* hepatic progenitor cells**

Most tissue engineering strategies outlined in the scaffolds section above have utilized immortalized hepatocytes, hepatoblastoma cell lines, or primary mature hepatocytes as the cell source [190, 211-212, 261, 267-270]. Of these, primary hepatocytes are the preferred cell type for clinical therapies due to safety concerns associated with cell immortalization and potential tumor formation [271]. Similar to whole organs, however, the supply of primary hepatocytes is limited [212]. Additionally, the limited proliferative capacity of mature hepatocytes *in vitro* often results in low cell density in engineered tissues and ultimately limits the success of hepatic tissue engineering. In the attempt to identify more proliferative hepatocyte cell sources, recent work has turned to testing the efficacy of the wide variety of hepatic progenitor cell populations (as described in detail above) in engineered tissues. As one example, bipotential mouse embryonic liver (BMEL) cells have been shown to survive and differentiate towards the hepatic lineage in PEG hydrogels [211]. Additionally, mouse embryonic stem cell-derived hepatocytes have been implanted in a tissue engineered assist device and shown to improve mouse hepatic failure [272].

While hepatic progenitor populations are an exciting alternative to primary hepatocyte cell sources, work to further refine the unknown advantages and disadvantages of proliferative hepatic progenitors *versus* fully mature hepatocytes in tissue engineering is necessary. For example, it is currently unknown whether the proliferative capacity of hepatocyte progenitors in tissue engineered constructs will result in superior engraftment, tissue density, and tissue function following implantation compared to constructs containing fully functional mature hepatocytes. In fact, a major concern in the use of progenitor cell populations for implantation therapy is that these cells may exhibit uncontrolled cell proliferation and/or differentiation towards undesired cell fates [273]. Implantation of proliferative hepatic progenitors could indeed result in “over-population” of tissue engineered constructs. In the worst situation, implantation of undifferentiated pluripotent cell types could result in teratoma formation [273]. Current work therefore speaks to address issues such as whether progenitor cell populations need to be pre-differentiated to cells dedicated to the hepatic lineage prior to culture in three-dimensional scaffold materials and implantation [272], whether hepatic progenitors need to be fully mature and exhibit no expression of fetal programs prior to implantation, and whether signals can be engineered strategically into the scaffold to guide appropriate differentiation within the scaffold itself [274-276]. In summary, a wealth of exciting new hepatocyte progenitor cell sources has recently become available for use in implantable liver devices, and future studies will be needed to evaluate their therapeutic potential.

### **Cell-cell interactions**

Similar to two-dimensional culture, homotypic [211, 277] and heterotypic [211, 213, 278] interactions have been found to be essential in the maintenance of hepatocyte function and survival in culture in biomaterial scaffolds. For example, pre-aggregation of primary hepatocytes or BMEL progenitor cells in PEG gels enhanced both cell survival upon encapsulation and also hepatocyte function [211], suggesting that homotypic cell interactions are important in this system. Numerous groups have also co-encapsulated non-parenchymal cell populations such as fibroblasts, endothelial cells, mesenchymal stem cells, and stellate cells with hepatocytes in biomaterial scaffolds, and some studies have shown improved hepatocyte function in the presence of non-parenchymal cell populations [211, 279-286]. For example, co-encapsulation of primary hepatocytes and 3T3 fibroblasts in PEG hydrogels has been shown to improve hepatocyte survival and function [211]. Hepatocytes have also been shown to affect the morphogenesis and phenotype of non-parenchymal cells in biomaterial scaffolds. For example, when hepatocytes and microvascular endothelial cells are cultured on a collagen gel scaffold in a microfluidic device, endothelial morphogenesis is dependent on diffusion from one cell compartment to the other [287]. Additionally, 3-D culture of hepatocytes with liver sinusoidal endothelial cells allows for maintenance of the SE-1 marker in the endothelial cells, an indication of persistence of liver-specific endothelial cell phenotype [278]. Taken together, these results show that non-parenchymal cell populations have played a significant role in the development of robust engineered liver tissue to date.

Future iterations of engineered liver tissue need to further refine the non-parenchymal cell types that are necessary in implantable constructs. For example, the non-parenchymal cell types utilized in liver tissue engineering to date have typically been non-human and non-native to the liver (e.g., J2-3T3 mouse fibroblasts [211, 278]). Clinical impact of engineered liver devices will be accelerated through the use and study of human cell sources. Additionally, ease of large-scale manufacturing will likely be enhanced by minimizing the number of cell types required in

any given device. In order to reduce the number of cell types in a given device, a detailed mechanistic understanding of the cues involved in cell-cell interactions as well as liver development and regeneration is necessary. Techniques to micropattern the spatial configuration of multiple cell types with respect to one another [265], precisely regulate the spatial and temporal release of growth factors and morphogens [275], and model cell signaling networks [288-290] should aid in determining the mechanistic roles of different cell types and biological cues. Such knowledge could be used to design engineered therapies that incorporate key signaling molecules but limit the number of exogenous cell types needed to improve hepatocyte survival and function in engineered tissues [291]. Thus, cell-cell interactions have been shown to be important in the development of engineered liver tissue and the exact molecular mechanisms responsible for the functional benefits derived from cell-cell interactions is an area of investigation that will be critical to the design of advanced liver tissue engineered devices.

### **Clinical Translation for Human Therapy**

Prior to the translation of implantable device therapies to the clinic, animal models must be developed that adequately assess the safety and efficacy of these therapies. These tissues will also need to integrate with the patient's vascular and biliary systems. Finally, it will be essential that implantable engineered therapies are composed of immune-compatible cell and material components that are fit for use in humans.

### **Assessment in animal models**

Prior to the translation of any cell-based liver to the clinic, the safety and therapeutic efficacy of these therapies must be demonstrated in animal models. Animal models for testing these therapies include genetic, toxic, ischemic, partial hepatectomy, and total hepatectomy models. Several extensive reviews outline important criteria used in the development of animal models of fulminant hepatic failure [292-294]. These criteria include reproducibility, reversibility, liver failure-induced cell death, and presence of sufficient time interval for diagnosis and therapeutic intervention [295]. Genetic models of liver injury include the urokinase plasminogen activator overexpression (uPA<sup>++</sup>/SCID) and FAH knockout mouse models [296-297]. Chemical induced injury models include exposure to toxic doses of carbon tetrachloride, D-galactosamine, or acetaminophen, both of which induce localized centrilobular necrosis [298-299]. Chemical induced injury models are especially useful for testing the efficacy of cell-based liver therapies because these models most closely mimic acute liver injuries commonly found in humans (e.g. drug toxicity). Surgically induced injury models include partial hepatectomy and have been widely employed because the injury stimulus is well-defined [300]. Despite the fact that the hepatectomy model is less clinically relevant (with the exception of liver resection patients), it serves as a well-controlled system to examine the importance of regenerative cues in the engraftment of hepatic constructs implanted in extrahepatic sites [300]. To this end, the optimal implantation site of implantable engineered tissue will also need to be determined. Tissue engineered constructs are frequently evaluated after implantation into subcutaneous or mesenteric spaces due to the ease of surgical access and improved imaging options. Early work in injury damage models suggested that orthotopic transplantation was necessary for hepatocyte survival due to interaction with 'hepatotropic factors' available in the portal vein. However, the effectiveness of implanted extrahepatic scaffold based systems in supporting hepatic function following hepatectomy has been demonstrated in some studies with rodent models [272, 300-301] in which constructs were implanted subcutaneously, peritoneally,

or in the fat pad, suggesting that injury cues originating in the host liver can reach extrahepatic sites. The utilization of numerous surgical and chemical animal models will be instrumental in testing the efficacy of cell-based liver therapies. Concurrently, knowledge of the mechanisms of liver injury and regeneration obtained from these experiments will influence the future design of next-generation engineered liver tissue.

### **Integration with host tissue vasculature and biliary system**

To derive maximal therapeutic benefit from implantable engineered liver tissue, grafted tissue must integrate with the host tissue, and in particular, with the host vasculature and biliary system. Within the normal liver environment, hepatocytes are supplied by an extensive sinusoidal vasculature [302]. This vasculature allows for the efficient transport of nutrients to the highly metabolic hepatocytes. A significant challenge in the design of implantable liver constructs is the need to sustain thick implanted tissue in the face of transport limitations prior to the establishment of functional vasculature. One strategy is to incorporate “pre-formed” vasculature into engineered constructs prior to implantation. For example, microfabricated vascular units could be created and followed by surgical anastomosis during implantation [257, 302]. Polymer molding using microetched silicon has produced channel networks with capillary dimensions [257]. Additionally, recent muscle engineering studies have demonstrated that prevascularization of engineered tissue using endothelial and mesenchymal cells (in addition to muscle cells) *in vitro* improves survival and vascular integration of engineered tissue with host tissue after implantation [303-305]. A second strategy is to incorporate angiogenic factors within the implanted scaffolds so that these factors can recruit the ingrowth of host vasculature immediately upon implantation. Specifically, integration of cytokines that play critical roles in angiogenesis, such as VEGF [306-307], bFGF [308], and VEGF in combination with PDGF [309], promotes the recruitment of host vasculature to implanted constructs. A final strategy is to “prime” the implantation site through pre-vascularization. For example, pre-implantation of VEGF releasing alginate scaffolds prior to hepatocyte seeding enhances capillary density and improves engraftment [310].

Finally, inclusion of excretory capabilities associated with the biliary system may be necessary in future engineered liver tissue. To date, studies have focused on the developing *in vitro* models that exhibit biliary morphogenesis and recapitulation of the appropriate polarization and bile canaliculi organization [311-313], as well as platforms for engineering artificial bile duct structures [314]. Future studies will determine whether the inclusion of biliary elements is necessary in implantable liver tissue.

### **Immune Response**

Similar to whole liver or cell transplantation, an understanding of the host immune system responses following transplant of tissue-engineered constructs will be paramount to the success in translating these therapies to the clinic. To minimize the host immune response to implantable constructs, all parenchymal and non-parenchymal cells populating engineered tissue should be entirely human. Similarly, implantable tissue should be free of xenogenic materials. Towards this end, engineered tissues must be cultured under serum-free conditions and should not contain naturally-derived xenogenic biomaterials [315-316].

The road to the ultimate immuno-compatible implantable liver therapy may be multi-tiered and will likely parallel that of cell transplantation therapies. For first-generation therapies, immunosuppressive treatments could be combined with the establishment of allogenic human

primary hepatocyte or ES-derived hepatocyte cell banks that contain immunologically diverse phenotypes [317]. Next-generation therapies may be populated by autologous cell sources such as iPS-derived hepatocytes and therefore reduce the need for rigorous immunosuppressive treatment. Furthermore, harnessing the liver's ability to induce antigen-specific tolerance [318-319] could improve the immune acceptance of engineered grafts. Overall, immune biology challenges will be critical in the successful translation of cell-based therapies. Multiple options exist for building immune-compatible cell therapies, and careful attention to these issues during the design and development of implantable engineered liver tissue will facilitate the ease and efficiency of clinical translation of these therapies.

## CONCLUSION

Substantial advances have been achieved in the field of liver tissue-engineering, as shown by the concurrent improvements in bio-mimetic *in vitro* liver systems and implantable hepatocellular constructs. This progress is enabled by integrating knowledge bases from various disciplines, including fundamental liver biology, medicine and biomedical engineering. Although many challenges remain, our evolving understanding of key regulators of liver function and regeneration promises to lay solid ground work for the next generation of clinically effective tissue engineered liver systems.

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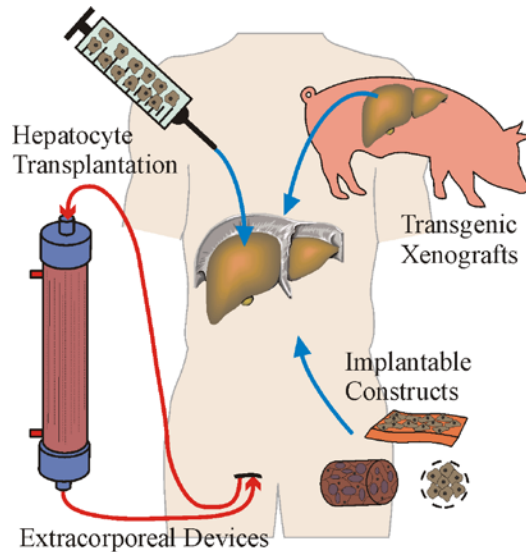
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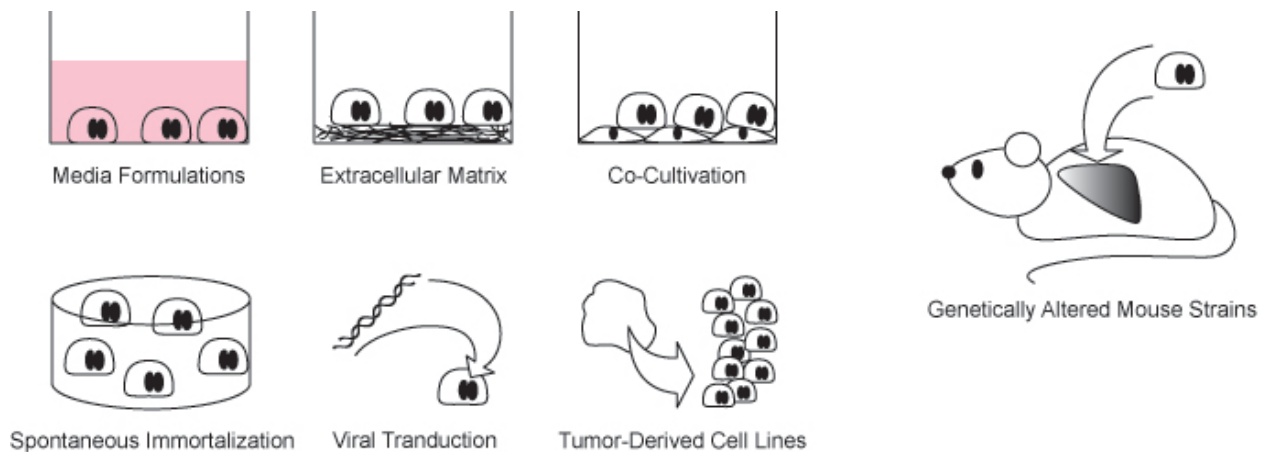
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**Figure 1.** Cell-based therapies for liver disease and failure. Extracorporeal BAL devices provide temporary support by processing patient blood and plasma. More permanent interventions include transplantation of isolated mature hepatocytes and hepatocellular constructs. Transgenic animal tissue have also been pursued. Reproduced from Allen *et al.* (2001) with permission.



**Figure 2.** *Ex vivo* expansion of mature human hepatocytes. Approaches have focused on providing essential culture components, generating hepatocyte cell lines and leveraging *in vivo* regenerative environments.

**Table 1. Summary of liver platforms**

<b>Platform</b>	<b>Pros</b>	<b>Cons</b>
<b>Perfused whole organs, wedge biopsies, and precision-cut liver slices</b>	More normal <i>in vivo</i> microenvironment and architecture. Can use human liver samples [89, 320-321]	Short-term viability (<24 hours), limited nutrient/oxygen diffusion to inner cell layers, Low availability, Inter-donor variability [90]
<b>Purified liver fractions (organelles, membranes) and single enzyme systems</b>	Used in high-throughput systems to identify enzymes involved in the metabolism of new pharmaceutical compounds [322-323]	Lack gene expression and full cellular machinery to execute liver functions
<b>Cell lines from hepatoblastomas or immortalization of primary hepatocytes</b>	Reproducible, inexpensive models of hepatic tissue [324-328]	Abnormal levels and repertoire of hepatic functions [91]
<b>Isolated primary hepatocytes in suspension or cultured upon extracellular matrix</b>	Functionally more representative of normal liver than cell lines. Can cryopreserve to overcome inter-donor variability	Difficult to maintain in culture due to dedifferentiation, Low availability [89-91]
<b>Engineered Tissue Models</b>	Improved stabilization of primary hepatocytes and better reproduction of normal tissue architecture [140, 211, 329]. Can be translated to <i>in vivo</i> through implantation [179]	Models can be challenging and expensive to fabricate