9

LIVER TISSUE ENGINEERING

Learning Objectives

After completing this chapter, students should be able to:

1. Describe the structure and function of the liver.
2. Describe symptoms associated with acute liver failure, along with potential treatment options.
3. Discuss orthotopic and partial liver transplantation.
4. Briefly describe the molecular and cellular events that take place during liver regeneration after partial hepatectomy.
5. Briefly discuss the process of liver development during human development.
6. Discuss design considerations for liver tissue engineering.
7. Describe the process scheme to bioengineer artificial liver tissue.
8. Describe the differentiation of hES cells, iPS cells, bone marrow MSCs, and hepatic stem cells to form hepatocytes.
9. Discuss robotic protein printing, photo-responsive culture surfaces, and PDMS stencils for spatial control of hepatocytes.
10. Describe different biomaterial platforms that have been used for the fabrication of artificial liver tissue.
11. Describe strategies for the fabrication of artificial liver tissue.

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12. Describe strategies to support vascularization of artificial liver tissue.
13. Describe perfusion systems that have been developed to support the culture of artificial liver tissue.
14. Discuss the role of spheroid culture in liver tissue engineering.

CHAPTER OVERVIEW

We begin this chapter with a discussion of the structure and function of the mammalian liver. We then look at some liver disorders, with particular attention to acute liver failure. This is followed by a discussion on liver transplantation, and we proceed to describe the chronic shortage of donor livers. We next provide a brief description of liver regeneration and development. This is followed by a list of design criteria for the fabrication of artificial liver tissue. We next provide a general process flow sheet for liver tissue engineering. We provide a discussion of the use of stem cells for liver tissue engineering, including strategies that have been used to drive the differentiation fate of stem cells to form hepatocytes. We next look at surface patterning technologies and biomaterial platforms as applied to the field of liver tissue engineering. This is followed by a discussion on strategies that have been used to support the fabrication of 3D artificial liver tissue. We follow this up with a discussion of vascularization of artificial liver tissue and bioreactors to support the culture of artificial liver tissue. We end this chapter with a discussion of spheroid culture of hepatocytes and a comparison of this technology with tissue engineering.

9.1 STRUCTURE AND FUNCTION OF THE LIVER

Introduction—The liver is a component of the digestive system and performs many functions necessary for digestion. The liver is the largest internal organ in humans, weighing an average of three pounds. In the previous two chapters, we studied the trachea and the bladder that are hollow organs; in comparison, the liver is a solid organ. Hepatocytes are the primary functional cell type found in the liver; they are responsible for most of the functional properties of the liver. Under normal physiological conditions, hepatocytes have a slow rate of turnaround and are not very proliferative. However, in response to injury, the liver has a remarkable regenerative capacity, and in rats, the loss of liver tissue by partial hepatectomy to remove two-thirds of the organ is compensated for by an increase in the rate of hepatocyte proliferation within two weeks.

Liver Function—The liver has several functions related to digestion. Hepatocytes in the liver make a fluid known as bile, which contains cholesterol, bile acids, and bilirubin and aides in the digestion process by breaking down fats to fatty acids (1–5). Bile is made in the liver by hepatocytes and is transported to the gallbladder for storage via the bile canaliculi and the hepatic ducts. The liver is also the primary site for the storage of glycogen, vitamins, and minerals; and is responsible for

metabolism of fats, proteins, and carbohydrates, and excretion of metabolic waste products. The liver also plays an important role in the synthesis of components of the blood, including plasma proteins and clotting agents.

Liver Structure—Anatomically, the liver consists of four lobes that are known as the left, right, caudate, and quadrate lobes (6). The functional unit of the liver is known as the lobule and consists of hepatocytes, blood vessels, nerves, and bile ducts—all of which are uniformly arranged. A central vein is located at the center of the each lobule; the central veins come together to form the hepatic veins, which in turn feed into the inferior vena cava. The blood that exits the liver follows the aforementioned path. The portal triad is located at the ends of lobules and consists of three primary structures: the hepatic bile duct, hepatic portal vein, and hepatic artery. In addition to these three primary structures, the portal triad also contains nerve tissue and lymphatic vessels.

Liver Blood Supply—The liver has a dual blood supply from the hepatic portal vein and the hepatic artery (7–13). The hepatic artery delivers oxygenated blood from the circulatory system, while the hepatic portal vein delivers blood from the small intestines containing nutrients. About three-quarters of the blood entering the liver is from the hepatic portal vein, while the remaining amount enters from the hepatic artery. Blood exits the liver via the central vein that is located in the middle of the liver lobule and feeds into the hepatic veins.

Liver Innervation—Sympathetic nervous stimulation to the liver is from the thoracic nerves T7-T12 of the spinal cord, while parasympathetic nervous stimulation is from the dorsal motor nucleus of the vagus nerve, which is located in the dorsal brainstem (14,15).

Hepatocytes—Hepatocytes are the primary functional cells of the liver and perform many of the functions that have been described (16,17). As expected, hepatocytes have been the focus of much research in tissue engineering, as we will study in subsequent sections of this chapter. The structure of hepatocytes is consistent with their role in energy metabolism; they contain a single round nucleus and numerous mitochondria. Consistent with the role of hepatocytes in protein synthesis, hepatocytes contain large amounts of rough endoplasmic reticulum, which in turn has large amounts of ribosomes that are necessary for protein synthesis. Hepatocytes are also functionally coupled to other hepatocytes via an extensive network of gap junctions that occupy as much as three percent of the cell membrane surface. The gap junctions allow intracellular cellular communication between neighboring cells and support the growth, proliferation, and function of hepatocytes and hence, liver function. The predominant gap junction proteins are connexin32 and connexin26.

9.2 ACUTE LIVER FAILURE

Definition of Acute Liver Failure—Acute liver failure (ALF) is defined as the onset of hepatocellular dysfunction in the absence of pre-existing liver disease characterized by coagulopathy and encephalopathy within 8 weeks of the hepatic insult (18–24). The onset of ALF is sudden without any pre-existing liver conditions,

and progression of the disease over time can lead to multiorgan failure, which can result in patient mortality. Acute liver failure (ALF) is a rare disease, as classified by the NIH Office of Rare Disease Research, with an incidence rate of one to six cases per year for every one million people in the developed world. The incidence for ALF in developing countries is more difficult to estimate, though it is expected to be significantly higher. Although the incidence of ALF is low, the consequences are significant and the mortality rate is very high, often exceeding 50%.

ALF Symptoms—Jaundice is one of the earlier symptoms of ALF, and can be easily recognized by the skin becoming yellowish. This change in color is due to an accumulation of bilirubin, which is a byproduct of red blood cells' deterioration. Other symptoms of ALF are linked to a loss of liver function and include a loss of metabolic function, decreased gluconeogenesis (formation of glucose from molecules other than carbohydrates) leading to hypoglycemia (reduction in blood glucose concentration), and decreased ammonia clearance leading to hyperammonemia (increase in blood ammonia concentration). While ALF primarily affects the liver, the manifestation and progression of ALF can lead to dysfunction in many organs, including the heart, lungs, kidneys, and brain. ALF can also lead to coagulopathy, which impairs the mechanism responsible for blood clotting, and encephalopathy, which refers to damage of brain function.

Classification of ALF—There are three classification schemes that are used to describe ALF (25–27): hyperacute liver failure, acute liver failure, and subacute liver failure. In each of these three cases, there are differences in the time from jaundice to encephalopathy, the severity of jaundice and coagulopathy, and the survival rate without emergency liver transplantation. As we progress from hyperacute liver failure to subacute, there is an increase in the time from jaundice encephalopathy, an increase in the severity of the jaundice, and a decrease in the severity of coagulopathy. There is also a decrease in the survival rate of the patients without emergency liver transplantation as the disease progresses from hyperacute to subacute liver failure.

Causes of ALF—ALF is caused by a significant loss of liver function, brought about by apoptosis of hepatocytes, the primary functional cells of the liver. The liver is responsible for numerous critical functions, most of which are carried out by the hepatocytes. Therefore, any loss in the number of hepatocytes will directly correlate with a decrease in liver function. Apoptosis of hepatocytes during ALF can be brought about by many different agents, some of which include viral infection, primarily by the hepatitis virus, and drug induced injury, particularly by nonprescription acetaminophen. ALF by viral infection is more predominant in developing nations, while ALF by drug-induced injury is more prevalent in developed countries.

Treatment Strategies for Patients with ALF—The primary treatment options for patients with ALF include pharmacological intervention, organ transplantation, and the use of mechanical support devices. High dose N-acetylcysteine (NAC) is used for the treatment of ALF that has been induced by an overdose of acetaminophen, and has shown to be effective when used during the early stages of ALF. Later stage ALF may require liver transplantation, and since the progression of ALF is

rapid, emergency transplantation is usually required. While effective, emergency liver transplantation has a lower rate of success compared to elective liver transplantation. Mechanical liver support devices are also used for the treatment and management of ALF, although such devices are primarily used as a bridge to transplantation.

9.3 LIVER TRANSPLANTATION

Introduction—Liver transplantation is the standard of care for adult patients with end stage liver failure (28–35). Due to the regenerative capacity of the liver, many different transplantation strategies have been developed. The most common method has been orthotopic liver transplantation (OLT), in which case donor livers harvested from cadavers are transplanted to patients after complete removal of the damaged or diseased liver. In addition to OLT, other methods have been developed for liver transplantation. Some of these methods include the use of partial liver grafts obtained from living donors (living donor liver transplantation, LDLT) or the use of a single liver divided into two allografts that are transplanted into two patients (split-liver transplantation, SLT).

Liver Transplantation Statistics for Adult Patients—The most recent year for which transplantation data was available from the US Department of Health and Human Services Scientific Registry of Transplant Patients was 2011 (36–38). This data shows that close to 12,000 adult patients were on the waiting list for a liver transplant (Figure 9.1a), with around 6000 patients receiving a transplant (Figure 9.1b). The data further shows a mortality rate of just over 10% for patients on the waiting list for a liver transplant (Figure 9.1c). However, in patients who are able to receive a liver transplant, the survival rate has been high, with a one-year survival rate of greater than 80%, a three-year survival rate of greater than 75%, and a five-year survival rate just under 70% (Figure 9.1d). Therefore, while liver transplantation has saved numerous lives, there remains a chronic shortage of donor livers with a high mortality rate of patients on the waiting list.

Liver Transplantation using Partial Liver Segments—Livers for use in adult liver transplantations primarily originate from deceased donors, while a very small fraction are from living donors, as can been seen in Figure 9.1b. Living donor liver transplantation (LDLT) is performed in pediatric patients or in adults with a small size. The regenerative capacity of the liver allows transplantation from living donors; a portion of the liver is removed from the donor and transplanted into the recipient, from whom the diseased and/or damaged liver has been completely removed. In both cases, the liver is able to regenerate and provide functional support for both the donor and recipient of the liver. Within a time period of 8–12 weeks, normal liver volume is restored in both the donor and the recipient, as a result of the regenerative capacity of the liver. This is particularly beneficial for pediatric patients, for whom size-matched livers are often difficult to source; the use of partial liver grafts provides an option for these patients. In addition to LDLT, another strategy that has been used for partial liver transplantation is

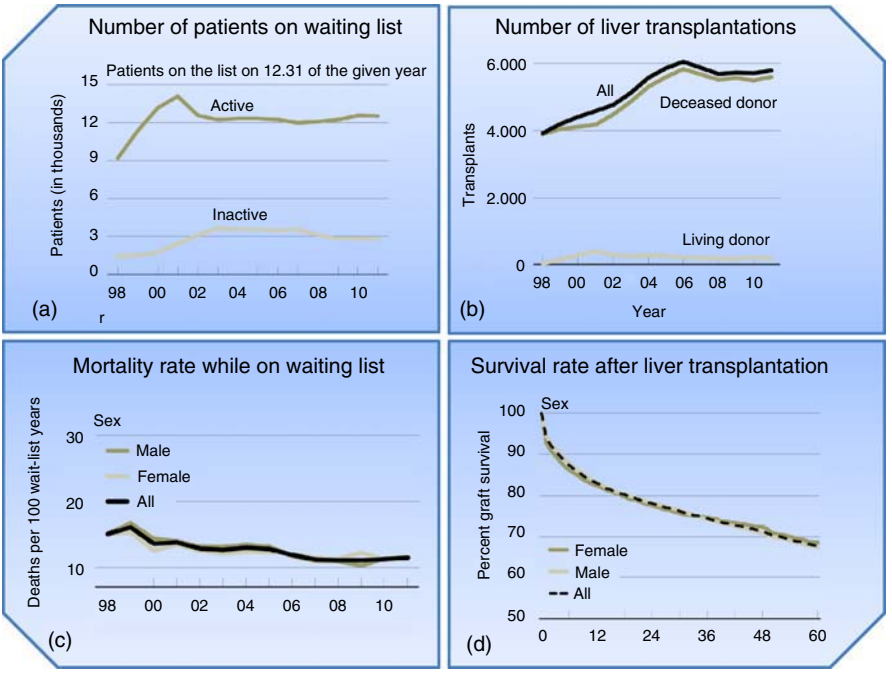

Figure 9.1 Statistics for Liver Transplantation—(a) Number of Patients on Waiting List—There are about 12,000 patients currently on the waiting list for a liver transplant. **(b) Number of Liver Transplantations**—Just under 6000 patients are able to receive a liver transplant. **(c) Mortality Rate While on Waiting List**—The mortality rate of patients while waiting for a liver transplantation is in excess of 10%. **(d) Survival Rate after Liver Transplantation**—For patients who do receive a liver transplantation, the survival rate is high: reported to be about 75% at the three-year time point. *Note*–The data presented here has been obtained from the Organ Procurement and Transplantation Network (OPTN) and Scientific Registry of Transplant Recipients (SRTR). The data and analyses reported in the 2011 Annual Data Report of the Organ Procurement and Transplantation Network and the US Scientific Registry of Transplant Recipients have been supplied by the Minneapolis Medical Research Foundation and UNOS under contract with HHS/HRSA. The authors alone are responsible for reporting and interpreting these data; the views expressed herein are those of the authors and not necessarily those of the US Government.

split-liver transplantation (SLT), in which a single adult cadaveric liver is divided or split into two, and the two pieces serve as transplantation grafts, one for an adult patient and one for a pediatric patient. As in the case for LDLT, regeneration of the transplanted grafts results in restoration of normal liver function for both recipients.

Indications for Adult Liver Transplantation—In the previous section, we looked at ALF, which can lead to end stage liver failure and require liver transplantation. ALF is the primary cause of 5%–6% of all liver transplants in the US. Other diseases which can require liver transplantation include chronic

liver failure due to cirrhosis caused by hepatitis C virus (HCV), hepatocellular carcinoma (HCC), and liver diseases related to alcohol consumption, which accounted for one in six transplants in 2011.

Quality of Life after Liver Transplantation—After undergoing liver transplantation, the patient undoubtedly extends his/her life. Cases of patients who have lived for more than 30 years post-liver transplantation have been reported. However, there is a decrease in the quality of life for patients after liver transplantation. There is a tendency for liver transplant patients to have a reduced social life and a decrease in physical activity due to excessive fatigue and poor sleep quality. An increase in the rate of depression for patients after liver transplantation has also been observed. Complications resulting from immunosuppression therapy are also present, some of which include hypertension, new-onset diabetes mellitus, and dyslipidemia. Obesity, renal disease, and an increase in the risk of cancer are additional factors that may affect the quality of life of liver transplant patients.

9.4 LIVER REGENERATION

The mammalian liver has a remarkable regenerative capacity, and after partial hepatectomy to remove 70% of the liver tissue, it is able to completely recover lost functionality (39–48). The increase in tissue mass is primarily due to an increase in the number of mature hepatocytes. Recruitment of stem cells does not appear to play a significant role in the regenerative capacity of the liver. This unique characteristic of the mammalian liver has allowed the development of liver transplantation techniques using liver segments, as we have seen in the previous section. In addition, understanding the molecular mechanisms leading to the regenerative capacity of the liver has far-reaching implications in tissue engineering and can be exploited to support the tissue fabrication process.

The regenerative capacity of the mammalian liver is due to the proliferative capacity of hepatocytes. After partial hepatectomy to remove 70% of the liver tissue, cells in the remaining 30% of the tissue undergo one round of cell division, doubling the number of cells and resulting in an increase of tissue mass. This process increases the tissue mass of the liver to about 60% of the original mass; a subset of the cells undergo a second round of cell division, which allows recovery of the entire tissue mass lost during partial hepatectomy. In addition to supporting functional recovery after partial hepatectomy, the hepatocytes are able to maintain normal function of the liver during the recovery phase. This is indeed a remarkable characteristic of the liver.

There are changes in liver hemodynamics after partial hepatectomy due to changes in blood flow regimes, and these hemodynamic changes are important in initiating liver regeneration. This is followed by a complex cascade of molecular and cellular changes along with activation of several intracellular signaling pathways, which orchestrate the functional recovery of the liver. These changes affect both the cellular and extracellular components of the liver tissue. One of the earlier events of liver regeneration is the breakdown and remodeling of the extracellular

matrix, which is important to release the cells to support cell proliferation and increase in cell number. The breakdown of the liver extracellular matrix is brought about by several matrix metalloproteinases, which are upregulated during the regeneration process. The breakdown of extracellular matrix components result in release of several growth factors, which are stored locally, with hepatocyte growth factor (HGF) being one such example. HGF has proliferative effects and acts by binding to the cell surface receptor *cMet*.

In addition to changes in the extracellular matrix, there are significant intracellular molecular events that take place during the liver regeneration process. Partial hepatectomy leads to an increase in the expression of more than 100 genes; this increase orchestrates the proliferative response of hepatocytes. Shortly after partial hepatectomy, there is an increase in the expression of Stat3 and NFkB, which are important signaling molecules that trigger the proliferative response of the hepatocytes. The purpose of the regenerative response of the liver is to restore normal functionality after partial hepatectomy, and this is achieved in part by an increase in the rate of proliferation of hepatocytes. However, as this process continues, there is also a need to stop the proliferation of hepatocytes once functional recovery has been accomplished. Termination of the regenerative response makes use of a complex feedback system between growth factors, extracellular matrix components, and the cells. There are several compounds that participate in the process, with transforming growth factor-β1 (TGF-β1) being one such example. TGF-β1 production is increased by stellate cells in response to HGF. However, proliferating hepatocytes become resistant to TGF-β1, which plays a part in the termination of the regenerative process.

9.5 LIVER DEVELOPMENT

The liver develops from cells in the endoderm, and one of the early events of liver development is the expression of albumin, transthyretin, and α-fetoprotein; the expression of these proteins is also used as a marker for liver function during tissue engineering studies (49–51). During the early stages of liver development, hepatoblasts, which are early stem cells giving rise to hepatocytes, undergo a complex series of steps which include proliferation, cell migration, and loss of adhesion. Several transcription factors regulate this process, including Hex, Prox-1, Tbx3, HNF-6, and OC-2. In addition, BMP and FGF signaling is important for cell proliferation. Once the endoderm cells have been specified, a liver diverticulum forms at day 22 in humans; the endoderm cells are known as hepatoblasts at this stage. The hepatoblasts give rise to a pseudostratified epithelium and proliferate to form a tissue bud, which is delineated by a basement membrane which contains laminin, collagen IV, nidogen, fibronectin, and heparan sulfate proteoglycan. The hepatocytes then migrate away from the epithelial lining of the endoderm, travel through the basement membrane, and invade the septum transversum. During later stages of liver development, the hepatoblasts give rise to mature hepatocytes under a careful gene expression pattern.

9.6 DESIGN CONSIDERATIONS FOR LIVER TISSUE ENGINEERING

In the previous two chapters, we looked at the design considerations for tracheal and bladder tissue engineering. Many of the design considerations that were presented for tracheal and bladder tissue engineering also apply for the fabrication of artificial liver tissue. In the previous two chapters, we also presented an overarching design statement for tracheal and tissue engineering, which also applies for liver tissue engineering: "*bioengineered liver tissue should be similar in form and functional to a mammalian liver*."

The specific design considerations for liver tissue engineering are: 1) functionality: albumin synthesis is used as an early indicator of artificial liver function, 2) biocompatibility, 3) nonimmunogenic and minimal inflammatory response, 4) nontoxic and noncarcinogenic, 5) avoidance of collapse by reasonable strength, 6) support cell engraftment, 7) support neovascularization, 8) possibility of growth, 9) resistance to fibroblastic and bacterial invasion, 10) standardized easy and short fabrication, 11) customizable and low cost, 12) easy surgical handling, 13) provide physiological environment such as ECM, 14) minimal necessity of donors and accessibility, 15) the results of engraftment are predictably successful, 16) provide or support epithelial resurfacing, 17) must not dislocate or erode over time, and 18) permanent constructions.

9.7 PROCESS OF BIOENGINEERING ARTIFICIAL LIVER TISSUE

Introduction—In Chapter 1, we presented a general scheme to bioengineer artificial tissue, and in the previous two chapters we applied this scheme for tracheal and bladder tissue engineering. In this section, we will adopt the general scheme for tissue engineering toward the fabrication of artificial liver tissue (Figure 9.2). Our discussion will focus on general points based on what we have learned about liver structure and function in the previous few sections. We will structure our discussion to answer one question: *based on what we know about liver structure and function, what strategies can be implemented to fabricate artificial liver tissue?* In subsequent sections, we will look at specific examples from the recent literature of different methods that have been adopted to bioengineer artificial tissue.

Cell Sourcing for Liver Tissue Engineering—The liver has a remarkable regenerative capacity, which is due to hepatocyte proliferation in response to partial hepatectomy. This provides a unique option for cell sourcing that may not be available for other tissue systems. A tissue biopsy from the patient can be used to isolate and expand primary hepatocytes during culture, and these cells then can be used to bioengineer artificial tissue; this strategy will provide an autologous cell source for the fabrication of artificial liver tissue. Other sources of cells for liver tissue engineering are from the differentiation of stem cells, including hES and iPS cells. Stem cells have not been used extensively in tracheal and bladder tissue engineering, but have been used for the fabrication of artificial liver tissue; we look at some specific examples in a subsequent section.

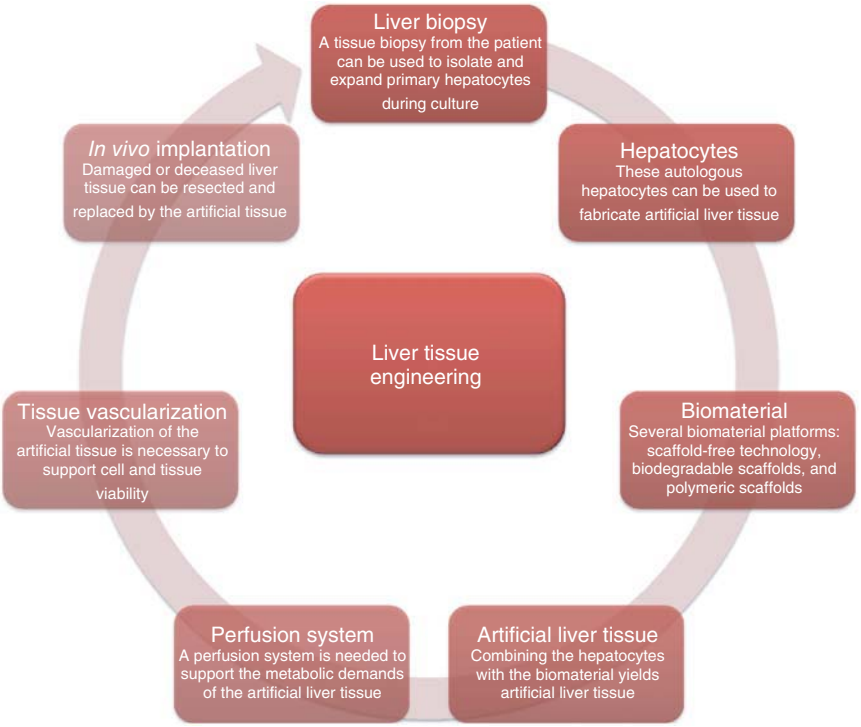

Figure 9.2 Overview of Liver Tissue Engineering—Primary hepatocytes are isolated from a liver biopsy and cultured and expanded using controlled *in vitro* conditions. These cells are then cultured within a 3D scaffold to support the formation of artificial liver tissue. Vascularization of artificial tissue is required to support metabolic activity, and the newly formed blood vessels are perfused using custom bioreactors. Artificial liver tissue fabricated using this process can be implanted *in vivo* to support the functional activity of damaged or diseased livers.

Biomaterials for Liver Tissue Engineering—In the previous two chapters, we looked at biomaterials that have been used for tracheal and bladder tissue engineering; as we have seen, acellular scaffolds have been a preferred biomaterial to support artificial tissue development. The trachea and the bladder are hollow structures and therefore require materials that have a high mechanical strength. However, for liver tissue engineering, the requirements for mechanical strength are not as high. Therefore, many other biomaterials platforms have been tested: scaffold-free technologies, biodegradable scaffolds, and polymeric scaffolds have been used to support the fabrication of 3D artificial liver tissue.

Bioreactors for Liver Tissue Engineering—In the previous two chapters, there was an evident lack of interest in the development of bioreactors for tracheal and bladder tissue engineering. However, this is not the case for liver tissue engineering, in which bioreactor technology has been an integral part of the development process. The liver has a very high metabolic activity to support synthesis of thousands

of proteins. Therefore, it is self-evident that perfusion systems are necessary to support the metabolic demands of artificial liver tissue. Electrical stimulation and mechanical stretch are not essential for the development of liver tissue. Liver tissue is not excitable and therefore does require electrical stimulation to maintain function. Similarly, the liver is not exposed to large hemodynamic loads during normal tissue function, hence, mechanical stretch is not required during the tissue fabrication process.

Vascularization of Artificial Liver Tissue—Since liver tissue is known to be highly metabolic, vascularization of the artificial tissue will be necessary to support cell and tissue viability. During our discussion of vascularization strategies in an earlier chapter, we looked at several *in vitro* and *in vivo* strategies being developed for tissue engineering. Many of these strategies, including additional novel methods, have been developed to support the neovascularization of liver tissue, and we will study these in a subsequent section.

In Vivo Implantation—Development of patches of liver tissue can be used directly as a graft for implantation in the host. Damaged or diseased liver tissue can be resected from the patient and replaced by tissue segments that have been fabricated in the laboratory. Coupling between the implanted and the host liver tissue can lead to recovery of lost tissue functionality and restore normal liver function.

The discussion in this section has been designed to provide a general scheme to bioengineer artificial liver tissue. As we study specific examples, we will see how these elements come together, along with many other novel methods, to bioengineer artificial tissue.

9.8 STEM CELLS FOR LIVER TISSUE ENGINEERING

In Chapter 2, we studied stem cell engineering and looked at several sources for stem cells and strategies to regulate the differentiation fate of these cells toward specific cell lineages. In this section, we will study specific examples that have been implemented to drive the differentiation fate of stem cells toward a hepatic lineage. Specially, we will study human embryonic stem cells, induced pluripotent stem cells, hepatic stem cells and mesenchymal stem cells. There have been several strategies published in the recent literature describing different strategies to drive the differentiation fate of these stem cells toward a hepatic lineage. For illustrative purposes, we will describe one strategy for each of the four stem cell types.

Embryonic Stem Cells for Liver Tissue Engineering—The strategy to drive the differentiation of ES cells toward a hepatic lineage has been to mimic the process as it happens during embryogenesis (52). It may be recalled that during embryogenesis, early stem cells are first differentiated toward the three germ layers—the ectoderm, the mesoderm, and the endoderm—which then give rise to different organ and tissue systems. It may be further recalled that the liver is derived from endodermal cells. Therefore, the strategy for liver tissue engineering has been to first drive the differentiation ES cells toward endodermal cells, followed by differentiation of endodermal cells toward a hepatic lineage. In one specific example, a three-step

process was used to drive the differentiation of mouse ES cells to form hepatocytes. The first two steps were designed to differentiate mouse ES cells to endodermal cells and then to form hepatocytes; the third step was designed to support functional maturation of hepatocytes. ES cells were cultured in the presence of Activin A for three days, followed by culture in the presence of aFGF and sodium butyrate for an additional five days; these steps were designed to drive the differentiation fate of ES cells to endodermal cells and then to hepatocytes, respectively. Hepatocyte maturation was accomplished by culturing the differentiated cells in culture media containing HGF for five days, followed by OSM and Dex for an additional five days.

Induced Pluripotent Stem Cells for Liver Tissue Engineering—The concept of iPS cells may be recalled from our earlier discussion of stem cell engineering in Chapter 2. Mature somatic cells like fibroblasts are transformed to an embryonic lineage, which can be differentiated to multiple cell types. As iPS cells resemble ES cells, the differentiation strategy to form hepatocytes from iPS cells has been similar to that for ES cells. In a recent study, a series of chemical conditioning steps were used to drive the differentiation of iPS cells to form hepatocytes (53). iPS cells were first differentiated to form definitive endodermal cells and then progressively differentiated to form more specialized cells. Definitive endodermal cells refer to early endodermal cells that differentiate to form all tissue/organs derived from the endoderm, while anterior definite endodermal (ADE) cells are more specialized and only give rise to the liver, pancreas, lungs, and thyroid. Using this strategy, various chemical compounds were used to differentiate the iPS cells to form early endodermal cells, ADE cells, and then hepatic progenitor stem cells. This was followed by differentiation of the hepatic progenitor stem cells to form immature hepatocytes and then mature hepatocytes.

Hepatic Stem Cells for Liver Tissue Engineering—Hepatic stem cells are present in the human liver and have the potential to differentiate into hepatocytes to support liver function in cases of injury and disease. Hepatic stem cells can be isolated and maintained in culture, and several strategies have been developed to support the differentiation of these cells to form hepatocytes. Hepatic stem cells are isolated from liver tissue specimens using an enzymatic digestion process and are cultured on the surface of a feeder layer of fibroblast cells. Chemical conditioning was used to drive the differentiation of hepatic stem cells to form hepatocytes using a two-step process (54). In the first step, the stem cells were conditioned with epidermal growth factor to form immature hepatocytes. In the second step, the immature hepatocytes were conditioned with HGF to support the formation of mature hepatocytes.

There are significant differences in the strategies that have been used to drive the differentiation of ES cells and iPS cells when compared with differentiation strategies for hepatic stem cells. In the case of ES and iPS cells, conditions were optimized to drive the differentiation of the stem cells to form endodermal cells; this was followed by differentiation of the endodermal stem cells to form hepatocytes. The differentiation strategy was designed to mimic embryogenesis. However, when compared with ES and iPS cells, hepatic stem cells have limited differentiation

potential and are committed to forming either hepatocytes or bile duct epithelium. Therefore, chemical conditioning can be used to drive the differentiation of these cells toward a hepatic lineage without the intermediate step to generate endodermal stem cells.

Bone Marrow-Derived Mesenchymal Stem Cells for Liver Tissue Engineering—We have studied bone marrow MSCs for tracheal and bladder tissue engineering in the previous two chapters. Due to the many advantages these cells offer, they have also been used for applications in liver tissue engineering. In one specific example, bone marrow MSCs were isolated from human donors and maintained and expanded under controlled *in vitro* conditions (55). The MSCs were cultured in a 3D nanofiber scaffold that was fabricated using PCL as the polymer; electrospinning was used as the biomaterial fabrication technology. As controls in the study, MSCs were also cultured on the surface of monolayer 2D tissue culture plates. Differentiation of MSCs to form hepatocytes was accomplished using a two-step chemical conditioning process, as in the previous examples. In the first step, HGF and DEX were used to drive the differentiation of MSCs to form immature hepatocytes; this was followed by chemical conditioning using OSM to support the formation mature hepatocytes. The hepatocytes formed during 3D culture of MSCs were shown to have higher functional performance when compared with hepatocytes formed during 2D culture of the MSCs. This study showcased a novel application of tissue engineering technology to regulate the differentiation of stem cells to a specific cell lineage, and serves to highlights the advantages of 3D culture when compared to monolayer 2D culture.

9.9 SURFACE PATTERNING TECHNOLOGY FOR LIVER TISSUE ENGINEERING

Introduction—During our discussion of tissue fabrication technology in Chapter 4, we looked at strategies to regulate the spatial distribution of cells. Cell and organ printing, soft lithography, and surface patterning were some examples of techniques that have been used to control the spatial distribution of cells. The primary advantage of these technologies is the ability to regulate the placement of different cell types and extracellular matrix components during the tissue fabrication process; this in turn results in artificial tissue that is closer in form to mammalian tissue. In this section, we will study three examples that have been used to regulate the spatial distribution of hepatocytes relative to fibroblasts.

Robotic Protein Printing—A new method was developed to control the placement of extracellular matrix components on the culture surface (56). This method, known as robotic protein printing, allows 2D spatial control of the placement of proteins; cells then bind to the protein using cell-surface integrins (56). This process not only promotes the spatial alignment of cells but also elicits very specific cell-matrix interactions.

In one study, robotic protein printing was used to develop a novel co-culture system using hepatocytes and fibroblasts (56). Collagen was first printed in a specific

configuration on the surface of glass slides that had been modified and prepared to support robotic protein printing. A cell suspension of hepatocytes was placed on the surface of the printed culture surface; the hepatocytes attach to regions at which collagen was printed. A cell suspension containing fibroblasts was then added to the glass slide with the hepatocytes attached. The fibroblasts attached to regions of the culture surface where hepatocytes did not attach. Using this method, the 2D spatial distribution of the hepatocytes and fibroblasts was controlled by placement of collagen. In addition to regulating spatial placement of multiple cell types, this process offers many other advantages, including the formation of a multi-cellular culture system and promoting cell-cell and cell-matrix interactions.

Photoresponsive Culture Surface—Another very interesting way to regulate spatial distribution of cells is via photoresponsive culture surfaces. Using this technology, the culture surface is coated with a photoresponsive polymer, which under normal conditions does not support cell attachment (57). At the start of the process, the entire culture surface is coated with photoresponsive polymer; this process results in a culture surface that does not support cell adhesion. Specific regions of the culture surface are then exposed to UV radiation, which causes a change in the configuration of the photoresponsive polymer; this in turn results in the culture surface switching to "cell friendly."

In one study, hepatocytes were added to the culture surface and preferentially attached to the regions that were coated with the photoresponsive polymer PEG and treated with UV light (57). Selective areas of the culture surface that did not have hepatocytes attached were exposed to a second round of UV treatment. A second cell type, which in this case was fibroblasts, was added to the treated regions of the tissue culture plate. Using this method, the placement of different cell types was regulated on a 2D culture surface. As in the previous example, this process offers advantages of promoting cell-cell and cell-matrix interactions.

PDMS Stencils for Spatial Positioning of Hepatocytes—Another method to spatially regulate hepatocytes and fibroblasts has been via the use of PDMS stencils (58). Using this technology, a stencil is fabricated on PDMS using soft lithography; the stencil can be generated in any pattern or configuration. The PDMS stencil is then placed on the culture surface, and cells can be added to the culture surface; the cells attach to regions of the culture surface not protected by the pattern on the stencil; the PDMS stencil serves to guide the placement of cells on the culture surface.

In one example, the PDMS stencil was used to generate a specific pattern, and hepatocytes were added to the culture surface (58). The hepatocytes attached to regions of the culture surface not protected by the stencil. The second cell type, which in this case was fibroblasts, was added to the culture surface. As before, the fibroblasts attached to regions of the tissue culture surface not protected by the PDMS stencil. In order words, the fibroblasts attach directly on top of the hepatocytes, thereby creating a bilayer structure. The PDMS stencil was removed, leaving a controlled pattern of hepatocytes and fibroblasts. The spatial positioning of the cells is regulated by the pattern of the stencil, and as can be envisioned, many different configurations can be created.

9.10 BIOMATERIAL PLATFORMS FOR LIVER TISSUE ENGINEERING

During our discussion of biomaterial platforms in Chapter 3, we looked at four strategies: scaffold-free methods, polymeric scaffolds, biodegradable hydrogels, and acellular tissue grafts. We also looked at the relative advantages and disadvantages of each strategy. During our discussion of tracheal and bladder tissue engineering in the previous two chapters, we saw that a subset of these biomaterial platforms have been tested to support the fabrication of artificial tracheas and/or bladders. The rigid design constraints placed by the hollow structures of the mammalian trachea and the mammalian urinary bladder, has limited the application of some of these technologies. For example, scaffold-free methods have not matured to the point at which they can meet the design requirements of mechanical strength and stability to support artificial bladders. The case of liver tissue is different, as the requirements for mechanical strength are not as high. However, mechanical stability is still a critical design requirement, as the biomaterial needs to support hepatocyte culture and remodeling. Therefore, it comes as no surprise that there has been a very high degree of interest in testing many different biomaterial platforms to support the fabrication of artificial liver tissue. All four biomaterial platforms that we have studied earlier—scaffold-free technology, polymeric scaffolds, biodegradable hydrogels, and acellular tissue grafts—have been used to support liver tissue fabrication. In the next section, we will look at specific examples of these four biomaterial platforms to support the fabrication of 3D artificial liver tissue.

9.11 FABRICATION OF 3D ARTIFICIAL LIVER TISSUE

Introduction—There have been numerous strategies implemented for the fabrication of artificial liver tissue, and during the course of this discussion, we will have an opportunity to apply many of the principles we learned in earlier chapters. Many of the technologies we have discussed have been tested in liver tissue engineering, including scaffold-free technologies, polymeric scaffolds, biodegradable hydrogels, and acellular matrix. In this section, we will look at four examples of methods that have been used to bioengineer 3D artificial liver tissue: cell sheet engineering, alginate scaffolds, poly(l-lactic acid) (PLLA) scaffolds, and acellular matrices.

Cell Sheet Engineering to Bioengineer Artificial Liver Tissue—As we have seen before, the primary advantage of cell sheet engineering is that external scaffolding is not required; rather, the cells make their own extracellular matrix, which is used to support 3D tissue formation and remodeling. We have discussed the method of tissue fabrication before, and the same method has been used to support the fabrication of artificial liver tissue (59). Primary hepatocytes were plated on a temperature-sensitive culture surface. When the cells are maintained at 37°C, the properties of the culture surface support cell adhesion; at this stage, the culture surface is "cell-friendly." This promotes the formation of a cohesive cell monolayer, which

is referred to as a cell sheet. A decrease in culture temperature changes the properties of the culture surface, making it "cell-unfriendly." The cohesive cell monolayer detaches from the culture surface and remains intact, resulting in the formation of artificial liver tissue.

Porous Alginate Scaffolds for Liver Tissue Engineering—During our discussion of biomaterials for tissue engineering in Chapter 3, we studied biodegradable hydrogels and their use to support tissue fabrication. Collagen, fibrin, matrigel, and alginate are examples of biodegradable hydrogels that have been used extensively in tissue engineering. These are all naturally occurring compounds and have extracellular matrix components similar to that of mammalian tissue; this similarity supports cell-matrix interaction. In this particular study, matrigel was used as the biomaterial; alginate is the monovalent salt of alginic acid and is a block polymer of β-D-mannuronic acid and α-L-guluronic acid (60). The properties of alginate can be carefully controlled based on processing conditions; resulting in control of the porosity, 3D scaffold architecture, degradation kinetics, and material properties. In this example, porous sponges with an average pore size of 100–150 μm were fabricated using alginate, and primary hepatocytes were seeded into the scaffold using direct injection technology. The cellularized scaffolds were maintained in culture for two weeks and shown to support the formation of artificial liver tissue.

Porous PLLA Scaffolds for Liver Tissue Engineering—During our discussion of biomaterials for tissue engineering, we compared porous polymeric scaffolds to biodegradable hydrogels. As we discussed earlier, the primary advantage of polymer scaffolds was the ability to control the properties of the scaffold by changing processing conditions, polymer composition, and/or addition of cross-linking agents, to name a few. The primary drawback of polymeric scaffolds was the lack of specific binding sites for integrin-mediated cell binding. In this study, PLLA scaffolds were used to support the fabrication of artificial liver tissue (61). The strategy used in this study was similar to the one described for alginate scaffolds. Porous scaffolds were fabricated using PLLA and cellularized with primary hepatocytes. Direct cell injection was used as the cellularization technology; over time in culture, this strategy resulted in the formation of artificial liver tissue.

Acellular Scaffolds for Liver Tissue Engineering—In the previous two chapters, we have looked at several examples using acellular scaffolds to support the fabrication of artificial tracheal and bladder tissue. Acellular scaffolds are fabricated by the removal of cells from naturally occurring tissue; after removal of all cellular components, an intact extracellular matrix is left behind. This ECM has the right composition and distribution of proteins to support artificial tissue fabrication. Due to these advantages, acellular scaffolds have found extensive applications in the tissue engineering literature. In this particular study, acellular scaffolds were fabricated by decellularization of porcine liver specimens (62). Cellularization of the acellular scaffolds was performed via direct injection of primary hepatocytes. As in the previous cases, over time in culture, this method resulted in the fabrication of artificial liver tissue.

9.12 VASCULARIZATION FOR LIVER TISSUE ENGINEERING

Introduction—Vascularization is important to support cell viability in any given tissue system, and in Chapter 5, we studied several strategies to induce vascularization in bioengineered artificial tissue. In this section, we will look at the applications of these principles to support the fabrication of vascularized artificial liver tissue. We will look at two specific examples from the literature, both using *in vivo* vascularization methods, coupled with controlled release of angiogenic growth factors.

Prevascularized Scaffolds for Liver Tissue Engineering—In one study, several elements of neovascularization were incorporated into fabricating vascularized liver tissue. A novel scaffold was fabricated using alginate and was embedded with microspheres designed for the controlled release of vascular endothelial growth factor (VEGF) (63). The alginate scaffold was designed to support neovascularization and the growth and functionality of hepatocytes. The objective of this study was to use the alginate scaffold as the biomaterial during the tissue fabrication process. The purpose of the microspheres was to provide a mechanism for the controlled release of VEGF into the local culture environment; VEGF is known to increase the rate of neovascularization in mammalian tissue. VEGF was encapsulated in custom microspheres with known degradation kinetics; as the microspheres degrade, VEGF was gradually released into the local culture environment. The rate of release of VEGF was reported to be 8–10 ng/day over a two-week period. The purpose of the VEGF was to promote neovascularization of the alginate scaffold. Therefore, the novelty in scaffold design should be appreciated, as it serves two critical functions—supporting hepatocyte culture and activity (a function performed by the alginate) and promoting neovascularization (a function performed by the controlled release of VEGF).

The next step in the process was prevascularization of the scaffold. This was achieved by implantation of the scaffold, without seeding any hepatocytes, onto the liver lobes of recipient rats. In this particular study, *in vivo* vascularization strategies were implemented, as discussed in Chapter 5. This means that neovascularization of the implanted tissue was a result of the host response and not due to any external user intervention. The presence of VEGF enhanced neovascularization of the implanted tissue, as measured by the capillary density. It should be noted that the prevascularization of the scaffold was undertaken prior to implantation of the hepatocytes. Through this method, when the hepatocytes were implanted, a vascular bed was in place to support the metabolic activity of the cells.

After a seven-day implantation period, primary hepatocytes were injected into the prevascularized scaffold while the scaffold remained implanted at the original site. The scaffold was not explanted before hepatocytes were injected; instead, the hepatocytes were injected into the scaffold while it was still implanted onto the liver lobe of the recipient rats. At various intervals after scaffold cellularization, the scaffolds were recovered and processed for histological assessment. As controls, hepatocytes were also injected into scaffolds that were prevascularized in the absence of the controlled release of VEGF. As expected, the functional performance

of the prevascularized scaffold in the presence of VEGF was superior to the controls' performance.

In Vivo Vascularization of Scaffolds for Liver Tissue Engineering—In this study, polylactic acid (PLA) was used as the biomaterial and was engineered to form porous discs designed to support the culture and viability of hepatocytes (64). The polymer was first coated with bFGF, an angiogenic agent known to support neovascularization in mammalian tissue. The PLA scaffold was designed to support controlled release of bFGF into the local environment, as was the case in the previous example; however, the release kinetics were different, and most of the bFGF was released within the first 72 hours. Hepatocytes were seeded onto the scaffold and then implanted *in vivo*; as in the previous example, controlled release of the bFGF was designed to support neovascularization of the liver patch during development and maturation. This was indeed the case, as two weeks after implantation, vascularized liver tissue was formed. During the course of this study, neovascularization progressed in parallel with liver tissue formation and maturation, meaning that both happened at the same time. In the previous example, a prevascularized scaffold was used for liver tissue formation. These studies were not conducted side-by- side, and therefore, a direct comparison cannot be made between the two. However, the differences between the two methods should be appreciated, along with the novelty associated with each one.

9.13 BIOREACTORS FOR LIVER TISSUE ENGINEERING

Bioreactors are critical to support the tissue fabrication process. We studied bioreactor design in great detail in Chapter 6. During our discussion of tracheal and bladder tissue engineering, we noted a significant absence of bioreactor technology during the tissue fabrication process. While bioreactors have not been extensively used in the development of mammalian tracheal and bladder tissue, they have been used during the fabrication of artificial livers. This provides our first opportunity to study the application of bioreactor technology during the tissue fabrication process. Liver tissue is highly metabolic due to the number of functions performed; as such, perfusion is important to support the high metabolic activity of hepatocytes. Electrical stimulation is not important and, in fact, not required for the fabrication and/or culture of artificial liver tissue due to the non-excitable nature of the tissue. Similarly, mechanical stretch is not important, as liver tissue is not constantly exposed to changes in the hemodynamic environment (as is the case for the cardiovascular system). In this section, we will look at two specific bioreactor systems that have been developed to support the culture of artificial liver tissue.

Perfusion Culture of Artificial Liver Tissue—As one example, a perfusion system was developed to support the culture of artificial liver tissue. The liver tissue was fabricated by direct injection of primary hepatocytes into a porous PLGA scaffold (65). The perfusion system consisted of a reservoir to hold the artificial tissue constructs, a reservoir to hold the cell culture media, and a peristaltic pump. Cell culture media was perfused to the artificial liver tissue, and spent media was

recirculated through the system. The perfusion system was housed in a cell culture incubator to regulate temperature and pH. As expected, culture of artificial liver tissue in the presence of continuous media flow significantly enhanced functional performance when compared with controls that were maintained under static culture conditions.

Dual-Compartment Perfusion System for Liver Tissue Engineering—A second perfusion system was developed to support the culture of artificial liver tissue. In this system, artificial liver tissue was fabricated by direct injection of primary hepatocytes within a porous hybrid scaffold fabricated using collagen and PLGA (66). As in the previous example, the system consisted of culture vessels to accommodate artificial liver tissue and cell culture media. A peristaltic pump was used for perfusion of cell culture media to the tissue specimens, and spent media was recirculated through the system. The tissue specimens were maintained in a cell culture environment for temperature and pH regulation. The novelty of this perfusion system was the development of a dual-chamber compartment to accommodate two different samples. A porous membrane separated the two compartments, which allowed the flow of soluble factors between the two compartments. Artificial liver tissue and stellate cells, also cultured on the 3D scaffolds, were maintained in each of the two compartments; it was hypothesized that the release of soluble factors from stellate cells would enhance the functional performance of the artificial liver tissue. It was demonstrated that the presence of the stellate cells did, in fact, have a significant impact on the functional performance of the artificial liver tissue.

9.14 SPHEROID CULTURE FOR LIVER TISSUE ENGINEERING

During the course of our discussion of tissue engineering for the development of artificial liver tissue, we have looked at the isolation and culture of primary hepatocytes using monolayer 2D culture. Using monolayer cell culture techniques described in Chapter 2, primary cells are isolated after enzymatic digestion of tissue specimens, and these cells are maintained on the surface of tissue culture plates. The culture conditions are optimized to support the proliferation and expansion of primary cells and are subpassaged to increase cell yield. This strategy for monolayer 2D cell culture has been used extensively for tissue engineering studies, as we have seen earlier in this chapter and during our discussions for the fabrication of artificial trachea and bladder tissue. In addition to the examples presented in this book, monolayer cell culture has been used extensively for almost all tissue fabrication efforts, and is a core technology required to bioengineer 3D artificial tissue.

During our discussion of 2D monolayer cell culture, we looked at some limitations of this technique. Under normal physiological conditions, cells are maintained in 3D, and the 3D culture environment is important to maintain cell/tissue function and support 3D tissue architecture. During 2D monolayer culture, cells maintain partial functionality due to lack of complete cell-cell and cell-matrix interactions seen during normal mammalian tissue function. This functionality is one major

advantage of developing 3D tissue engineering models that mimic many of the physiological cues seen during normal mammalian tissue function.

In order to address this limitation, culture techniques have been developed to support the culture of primary cells in 3D aggregates known as spheroids; this technique has been referred to as spheroid culture. In this technique, the culture surface is modified with agents that change the properties of the surface, making them "cell-unfriendly." When the cells are cultured on these "unfriendly" surfaces, they do not attach to the culture surface; rather, the cells remain in suspension and form aggregates or spheroids. As the cells are maintained in a 3D environment during spheroid culture, they exhibit a higher degree of functionality than cells that are maintained during monolayer 2D culture.

Spheroid culture is a technique used to culture cells in spheroids or aggregates to support cell functionality. There are similarities between spheroid culture and tissue engineering, as both require 3D culture of cells. However, spheroid culture techniques are primarily used to maintain cell phenotype and function during culture and are not often used to design therapeutic strategies or to support artificial tissue or organ fabrication. Therefore, spheroid culture should be considered a specialized method of cell culture designed to increase cell phenotype and functionality.

Spheroid culture has been used extensively to support the culture of hepatocytes. Several methods have been described in the literature to support the formation of spheroids using hepatocytes. In one example, the culture surface was coated with poly (2-hydroxyethyl methacrylate), which prevents cell adhesion and promotes the formation of spheroids of hepatocytes (67). In another study, the culture surface was also coated with poly (2-hydroxyethyl methacrylate) to prevent cell adhesion; however, this strategy was coupled with gentle mixing to further prevent the hepatocytes from attaching to the culture surface (68). In another example, spheroid culture of hepatocytes was promoted by maintaining cells in a custom bioreactor that was designed to provide continuous rotation of cells, thereby preventing attachment to a culture surface. In this example, primary hepatocytes were maintained in a bioreactor designed to culture the cells in the presence of an oscillatory frequency, which was shown to support spheroid formation. It was also demonstrated that spheroid culture of hepatocytes was associated with a decrease in cell death and increase in cell function when compared to hepatocytes that are maintained under standard monolayer 2D conditions (69). In another example of spheroid culture of hepatocytes, the primary cells were cultured by entrapment within a synthetic thermoreversible extracellular matrix. The functional performance of primary hepatocytes was enhanced when maintained in spheroid culture, as compared to monolayer 2D culture of the cells (70).

SUMMARY

Current State of the Art—The field of liver tissue engineering is considerably mature; it is more mature than the fields of tracheal and bladder tissue engineering. Many resources have been invested in developing strategies to fabricate artificial

liver tissue, as we have seen throughout the course of this chapter. There has been a large amount of research invested in driving the differentiation fate of stem cells to form hepatocytes. Induced pluripotent stem cells, embryonic stem cells, adipose-derived mesenchymal stem cells, and hepatic stem cells have all been evaluated, with chemical conditioning being the preferred differentiation strategy. Similarly, considerable resources have been invested in evaluating different biomaterial platforms to test suitability for applications in liver tissue engineering. Some of the biomaterials platforms used to fabricate artificial liver tissue include scaffold-free methods, polymeric scaffolds, biodegradable hydrogels, and acellular grafts. There have been several strategies for vascularization of artificial tissue that involve implantation of artificial liver tissue coupled with angiogenic growth factors to enhance blood vessel formation. Bioreactor technology has also been developed to support the culture of artificial liver tissue and has primarily been focused on fabrication of perfusion systems to support the metabolic activity of hepatocytes.

Thoughts for Future Research — While there has been considerable progress in the field of liver tissue engineering, there are several areas that could benefit from additional research. A large amount of research has been published in the recent literature describing the differentiation of stem cells to form hepatocytes, particularly using chemical compounds. Due to this extensive knowledge base, we now have an understanding of the drivers of stem cell differentiation to form hepatocytes; this knowledge base can be expanded, and chemical factors can be coupled to bioreactors to deliver controlled perfusion protocols. The combined use of chemical compounds and fluid stresses resulting from media perfusion can increase differentiation efficiency and/or support the formation of mature hepatocytes. Another area of research that requires attention is the development of perfusion systems to support the metabolic activity of artificial liver tissue. Most of the systems developed thus far require the use of a cell culture incubator for regulation of processing variables. In addition, the current generation of perfusion systems does not have the capacity to monitor the functional performance of the artificial liver tissue in real- time or make use of noninvasive monitoring technology. The development of perfusion systems that operate independently of a cell culture incubator, with inline monitoring of processing variables, provide greater control over the system. In addition, the incorporation of real-time, noninvasive monitoring of the functional performance of artificial liver tissue will provide significant information about 3D tissue remodeling and functional reorganization in response to media perfusion. This information can be used to regulate perfusion variables. For example, as the metabolic activity of the artificial liver tissue increases or decreases, this information can be monitored in real- time and can be used to increase or decrease the flow rate of the culture media.

PRACTICE QUESTIONS

1. Describe the structure and function of the mammalian liver.
2. Describe the causes, symptoms, and treatment options for acute liver failure.

3. Discuss various strategies that have been used for liver transplantation. What are the relative advantages and disadvantages of each of these strategies?

4. Describe the molecular mechanism of liver regeneration.

5. Discuss development of the liver during embryogenesis.

6. The liver has a remarkable regenerative capacity. However, this capability has not been widely used to support the fabrication of artificial liver tissue. Based on your understanding of liver regeneration and liver tissue engineering, develop a strategy that makes use of liver regeneration to bioengineer artificial liver tissue.

7. In this chapter, we described a general process scheme to bioengineer artificial liver tissue. Develop a process scheme to bioengineer artificial livers, starting with a tissue biopsy and working up to the fabrication of artificial tissue. Provide a description for cell sourcing, material selection, and scaffold cellularization, and justify and explain your choice for each of the three.

8. During our discussion of stem cells for liver tissue engineering, we looked at several strategies to drive the differentiation fate of stem cells to form hepatocytes. However, all strategies were based on the use of growth factors or other chemical compounds. Develop a strategy that does not rely solely on the use of growth factors or other chemical compounds to drive the differentiation fate of induced pluripotent stem cells to form hepatocytes.

9. During our discussion of biomaterial platforms for liver tissue engineering, we looked at scaffold-free methods, polymeric scaffolds, and biodegradable hydrogels to support the fabrication of artificial liver tissue. Which one of these biomaterial platforms is more suited to support the fabrication of artificial liver tissue and why?

10. We looked at several models of artificial liver tissue, all of which used direct cell injection to populate the 3D scaffold with cells. Discuss the relative advantages and disadvantages of direct cell injection as a strategy to support scaffold cellularization. Develop an alternative strategy for scaffold cellularization to support the fabrication of artificial liver tissue. Describe the strategy and explain the relative advantages and disadvantages of the selected scaffold cellularization strategy when compared with direct cell injection.

11. In Chapter 4, we looked at cell and organ printing as strategies that have been used to support the fabrication of artificial tissue. Discuss the feasibility of using cell and organ printing to support the fabrication of artificial liver tissue. Describe the advantages and disadvantages of using this technology in liver tissue engineering. Develop a strategy to implement cell and organ printing to support artificial liver tissue fabrication.

12. During our discussion of bioreactors for liver tissue engineering, we looked at two examples of perfusion systems that were developed to support the metabolic activity of artificial liver tissue. Both of these perfusion systems were designed to function inside of a cell culture incubator, which provided temperature and pH regulation. Another strategy in the development of perfusion systems is to engineer sensors and feedback loops for regulation of processing variables within the perfusion system. Discuss the relative advantages and disadvantages of each of these two strategies. Design a perfusion system with embedded sensors and feedback loops.

13. During our discussion of vascularization strategies for liver tissue engineering, we looked at two examples that utilized *in vivo* methods to support liver vascularization. It may be recalled from our discussion in Chapter 5, that *in vivo* and *in vitro* strategies can be used to support the neovascularization of artificial tissue. Discuss the relative advantages and disadvantages of *in vivo* and *in vitro* strategies for the vascularization of liver tissue. Develop an *in vitro* strategy to support the vascularization of artificial liver tissue.

14. During our discussion of tissue fabrication strategies for liver tissue engineering, we looked at one example of scaffold-free technology. In this example, cell sheet engineering was used to support the fabrication of artificial liver tissue. It may be recalled from our discussion in Chapter 4 that self-organization strategies have also been used to support the fabrication of artificial tissue. Compare cell sheet engineering and self-organization strategies as technologies to support the fabrication of artificial liver tissue. Develop a method to bioengineer artificial liver tissue using self-organization technology.

15. We discussed spheroid culture of primary hepatocytes. This method involves the culture of primary hepatocytes in aggregates, thereby supporting 3D culture of the cells. There are similarities and differences between spheroid culture and 2D monolayer culture. There are also similarities and differences between spheroid culture and tissue engineering. Discuss the similarities and differences between spheroid culture and monolayer cell culture. Also discuss the similarities and differences between spheroid culture and tissue engineering.

REFERENCES

1. Oppe TE. Liver Function in the Newborn Infant. Biochem. Clin. 1964;3:5–11.
2. Hoe CM, Wilkinson JS. Liver function: a review. Aust. Vet. J. 1973 Mar;49(3):163–9.
3. Lathe GH. Liver function in the newborn. Med. Chir Dig. 1974;3(3):215–20.
4. Reichen J, Paumgartner G. Excretory function of the liver. Int. Rev. Physiol. 1980;21:103–50.
5. Corless JK, Middleton HM, III. Normal liver function. A basis for understanding hepatic disease. Arch. Intern. Med. 1983 Dec;143(12):2291–4.

6. Israel J, London WT. Liver structure, function, and anatomy: effects of hepatitis B virus. Curr. Top. Microbiol. Immunol. 1991;168:1–20.
7. Michels NA. Variations in blood supply of liver, gallbladder, stomach, duodenum and pancreas; summary based on one hundred Dissections. J. Int. Coll. Surg. 1945 Nov;8:502–4.
8. Michels NA. Variations in blood supply of liver, gallbladder, stomach, duodenum and pancreas. Anat. Rec. 1946 Mar;94:481.
9. Wakim KG. The blood supply of the normal liver. Proc. Staff. Meet. Mayo Clin. 1953 Apr 22;28(8):218–27.
10. Popper HL, Jefferson NC, Wulkan E, Necheles H. Bile secretion and blood supply of the liver. Am. J. Physiol. 1955 May;181(2):435–8.
11. Michels NA. The ever varied blood supply of the liver and its collateral circulation. J. Int. Coll. Surg. 1957 Jan;27(1):1–17.
12. Woodburne RT. Segmental anatomy of the liver: blood supply and collateral circulation. Med. Bull. (Ann. Arbor) 1962 Jul;28:189–99.
13. Sleight DR, Thomford NR. Gross anatomy of the blood supply and biliary drainage of the canine liver. Anat. Rec. 1970 Feb;166(2):153–60.
14. McCuskey RS. Anatomy of efferent hepatic nerves. Anat. Rec. A Discov. Mol. Cell Evol. Biol. 2004 Sep;280(1):821–6.
15. Berthoud HR. Anatomy and function of sensory hepatic nerves. Anat. Rec. A Discov. Mol. Cell Evol. Biol. 2004 Sep;280(1):827–35.
16. Klover PJ, Mooney RA. Hepatocytes: critical for glucose homeostasis. Int. J. Biochem. Cell Biol. 2004 May;36(5):753–8.
17. Sell S. The hepatocyte: heterogeneity and plasticity of liver cells. Int. J. Biochem. Cell Biol. 2003 Mar;35(3):267–71.
18. Herrera JL. Management of acute liver failure. Dig. Dis. 1998 Sep;16(5):274–83.
19. Berry P, Wendon J. Recognition and early management of acute liver failure. Acute. Med. 2005;4(3):92–8.
20. O'Grady J. Modern management of acute liver failure. Clin. Liver Dis. 2007 May;11(2):291–303.
21. Williams R. Acute liver failure–practical management. Acta Gastroenterol. Belg. 2007 Apr;70(2):210–3.
22. Stravitz RT, Kramer DJ. Management of acute liver failure. Nat. Rev. Gastroenterol. Hepatol. 2009 Sep;6(9):542–53.
23. Craig DG, Lee A, Hayes PC, Simpson KJ. Review article: the current management of acute liver failure. Aliment. Pharmacol. Ther. 2010 Feb 1;31(3):345–58.
24. D'Agostino D, Diaz S, Sanchez MC, Boldrini G. Management and prognosis of acute liver failure in children. Curr. Gastroenterol. Rep. 2012 Jun;14(3):262–9.
25. O'Grady JG, Williams R. Classification of acute liver failure. Lancet 1993 Sep 18;342(8873):743.
26. Williams R. Classification, etiology, and considerations of outcome in acute liver failure. Semin. Liver Dis. 1996 Nov;16(4):343–8.
27. Rosmawati M. Aetiology and classification of acute liver failure. Med. J. Malaysia 2005 Jul;60 Suppl B:125–6.

28. Saidi RF. Current status of liver transplantation. Arch. Iran Med. 2012 Dec;15(12):772–6.
29. Carlisle EM, Testa G. Adult to adult living related liver transplantation: where do we currently stand? World J. Gastroenterol. 2012 Dec 14;18(46):6729–36. PMCID:PMC3520161.
30. Yang X, Gong J, Gong J. The value of living donor liver transplantation. Ann. Transplant. 2012 Dec 31;17(4):120–4.
31. Bodenheimer HC, Jr., Okun JM, Tajik W, Obadia J, Icitovic N, Friedmann P, Marquez E, Goldstein MJ. The impact of race on organ donation authorization discussed in the context of liver transplantation. Trans. Am. Clin. Climatol. Assoc. 2012;123:64–77. PMCID:PMC3540608.
32. O'Mahony CA, Goss JA. The future of liver transplantation. Tex. Heart Inst. J. 2012;39(6):874–5. PMCID:PMC3528242.
33. Zazula CP. Liver transplantation: the illusion of choice. Virtual. Mentor. 2012 Mar;14(3):269–71.
34. Wakade VA, Mathur SK. Donor safety in live-related liver transplantation. Indian J. Surg. 2012 Feb;74(1):118–26. PMCID:PMC3259172.
35. Lobritto S, Kato T, Emond J. Living-donor liver transplantation: current perspective. Semin. Liver Dis. 2012 Nov;32(4):333–40.
36. Organ Procurement and Transplantation Network and Scientific Registry of Transplant Recipients 2010 Data Report. American Journal of Transplantation 2012 Jan;12:1–154.
37. Issue S. Organ Procurement and Transplantation Network and Scientific Registry of Transplant Recipients 2011 Data Report introduction. American Journal of Transplantation 2013 Jan;13:8–10.
38. Issue S. Organ Procurement and Transplantation Network and Scientific Registry of Transplant Recipients 2011 Data Report preface. American Journal of Transplantation 2013 Jan;13:5–7.
39. Tarla MR, Ramalho FS, Ramalho LN, Silva TC, Brandao DF, Ferreira J, Silva OC, Zucoloto S. A molecular view of liver regeneration. Acta Cir. Bras. 2006;21 Suppl 1:58–62.
40. Tarla MR, Ramalho F, Ramalho LN, Silva TC, Brandao DF, Ferreira J, Silva OC, Zucoloto S. Cellular aspects of liver regeneration. Acta Cir. Bras. 2006;21 Suppl 1:63–6.
41. Tanimizu N, Miyajima A. Molecular mechanism of liver development and regeneration. Int. Rev. Cytol. 2007;259:1–48.
42. Michalopoulos GK. Liver regeneration. J. Cell Physiol. 2007 Nov;213(2):286–300. PMCID:PMC2701258.
43. Michalopoulos GK. Liver regeneration: alternative epithelial pathways. Int. J. Biochem. Cell Biol. 2011 Feb;43(2):173–9. PMCID:PMC2888836.
44. Jia C. Advances in the regulation of liver regeneration. Expert. Rev. Gastroenterol. Hepatol. 2011 Feb;5(1):105–21.
45. Gilgenkrantz H, Collin de lA. New insights into liver regeneration. Clin. Res. Hepatol. Gastroenterol. 2011 Oct;35(10):623–9.
46. Papadimas GK, Tzirogiannis KN, Mykoniatis MG, Grypioti AD, Manta GA, Panoutsopoulos GI. The emerging role of serotonin in liver regeneration. Swiss. Med. Wkly. 2012;142:w13548.

47. Karkampouna S, Ten DP, Dooley S, Julio MK. TGFbeta signaling in liver regeneration. Curr. Pharm. Des. 2012;18(27):4103–13.
48. Carnovale CE, Ronco MT. Role of nitric oxide in liver regeneration. Ann. Hepatol. 2012 Sep;11(5):636–47.
49. Shin D, Monga SP. Cellular and molecular basis of liver development. Compr. Physiol. 2013 Apr;3(2):799–815.
50. Zong Y, Stanger BZ. Molecular mechanisms of liver and bile duct development. Wiley. Interdiscip. Rev. Dev. Biol. 2012 Sep;1(5):643–55.
51. Wilkins BJ, Pack M. Zebrafish models of human liver development and disease. Compr. Physiol. 2013 Jul 1;3(3):1213–30.
52. Zhou M, Li P, Tan L, Qu S, Ying QL, Song H. Differentiation of mouse embryonic stem cells into hepatocytes induced by a combination of cytokines and sodium butyrate. J. Cell Biochem. 2010 Feb 15;109(3):606–14.
53. Hannan NR, Segeritz CP, Touboul T, Vallier L. Production of hepatocyte-like cells from human pluripotent stem cells. Nat. Protoc. 2013 Feb;8(2):430–7. PMCID:PMC3673228.
54. He ZP, Tan WQ, Tang YF, Feng MF. Differentiation of putative hepatic stem cells derived from adult rats into mature hepatocytes in the presence of epidermal growth factor and hepatocyte growth factor. Differentiation 2003 Jun;71(4–5):281–90.
55. Kazemnejad S, Allameh A, Soleimani M, Gharehbaghian A, Mohammadi Y, Amirizadeh N, Jazayery M. Biochemical and molecular characterization of hepatocyte-like cells derived from human bone marrow mesenchymal stem cells on a novel three-dimensional biocompatible nanofibrous scaffold. J. Gastroenterol. Hepatol. 2009 Feb;24(2):278–87.
56. Revzin A, Rajagopalan P, Tilles AW, Berthiaume F, Yarmush ML, Toner M. Designing a hepatocellular microenvironment with protein microarraying and poly(ethylene glycol) photolithography. Langmuir 2004 Apr 13;20(8):2999–3005.
57. Kikuchi K, Sumaru K, Edahiro J, Ooshima Y, Sugiura S, Takagi T, Kanamori T. Stepwise assembly of micropatterned co-cultures using photoresponsive culture surfaces and its application to hepatic tissue arrays. Biotechnol. Bioeng. 2009 Jun 15;103(3):552–61.
58. Cho CH, Park J, Tilles AW, Berthiaume F, Toner M, Yarmush ML. Layered patterning of hepatocytes in co-culture systems using microfabricated stencils. Biotechniques 2010 Jan;48(1):47–52. PMCID:PMC3147300.
59. Ohashi K, Yokoyama T, Yamato M, Kuge H, Kanehiro H, Tsutsumi M, Amanuma T, Iwata H, Yang J, Okano T, et al. Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. Nat. Med. 2007 Jul;13(7):880–5.
60. Glicklis R, Shapiro L, Agbaria R, Merchuk JC, Cohen S. Hepatocyte behavior within three-dimensional porous alginate scaffolds. Biotechnol. Bioeng. 2000 Feb 5;67(3):344–53.
61. Torok E, Lutgehetmann M, Bierwolf J, Melbeck S, Dullmann J, Nashan B, Ma PX, Pollok JM. Primary human hepatocytes on biodegradable poly(l-lactic acid) matrices: a promising model for improving transplantation efficiency with tissue engineering. Liver Transpl. 2011 Feb;17(2):104–14.
62. Lang R, Stern MM, Smith L, Liu Y, Bharadwaj S, Liu G, Baptista PM, Bergman CR, Soker S, Yoo JJ, et al. Three-dimensional culture of hepatocytes on porcine liver tissue-derived extracellular matrix. Biomaterials 2011 Oct;32(29):7042–52.

63. Kedem A, Perets A, Gamlieli-Bonshtein I, Dvir-Ginzberg M, Mizrahi S, Cohen S. Vascular endothelial growth factor-releasing scaffolds enhance vascularization and engraftment of hepatocytes transplanted on liver lobes. Tissue Eng. 2005 May; 11(5–6):715–22.
64. Lee H, Cusick RA, Browne F, Ho KT, Ma PX, Utsunomiya H, Langer R, Vacanti JP. Local delivery of basic fibroblast growth factor increases both angiogenesis and engraftment of hepatocytes in tissue-engineered polymer devices. Transplantation 2002 May 27;73(10):1589–93.
65. Fiegel HC, Havers J, Kneser U, Smith MK, Moeller T, Kluth D, Mooney DJ, Rogiers X, Kaufmann PM. Influence of flow conditions and matrix coatings on growth and differentiation of three-dimensionally cultured rat hepatocytes. Tissue Eng. 2004 Jan;10(1–2):165–74.
66. Wen F, Chang S, Toh YC, Arooz T, Zhuo L, Teoh SH, Yu H. Development of dual-compartment perfusion bioreactor for serial coculture of hepatocytes and stellate cells in poly(lactic-co-glycolic acid)-collagen scaffolds. J. Biomed. Mater. Res. B Appl. Biomater. 2008 Oct;87(1):154–62.
67. Landry J, Bernier D, Ouellet C, Goyette R, Marceau N. Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. J. Cell Biol. 1985 Sep;101(3):914–23. PMCID:PMC2113699.
68. Wang YJ, Li MD, Wang YM, Ding J, Nie QH. Simplified isolation and spheroidal aggregate culture of rat hepatocytes. World J.Gastroenterol. 1998 Feb;4(1):74–6.
69. Nyberg SL, Hardin J, Amiot B, Argikar UA, Remmel RP, Rinaldo P. Rapid, large-scale formation of porcine hepatocyte spheroids in a novel spheroid reservoir bioartificial liver. Liver Transpl. 2005 Aug;11(8):901–10.
70. Park KH, Song SC. Morphology of spheroidal hepatocytes within injectable, biodegradable, and thermosensitive poly(organophosphazene) hydrogel as cell delivery vehicle. J. Biosci. Bioeng. 2006 Mar;101(3):238–42.