Liver Tissue Engineering

Iris Pla-Palacín, Natalia Sánchez-Romero, Sara Morini, Daniela Rubio-Soto, and Pedro M. Baptista

Contents

1 Introduction 144
1.1 Brief Description of Acute, Inborn Errors of Metabolism and End-Stage Liver Disease Worldwide 145
1.2 Liver Transplant Worldwide 147
1.3 Bottlenecks and Limitations 147
1.4 Solutions Developed Throughout the Years to Increase Organ Availability 148

Iris Pla-Palacín and Natalia Sánchez-Romero contributed equally with all other contributors.

I. Pla-Palacín · D. Rubio-Soto
Instituto de Investigación Sanitaria Aragón (IIS Aragón), Zaragoza, Spain

N. Sánchez-Romero
Instituto de Investigación Sanitaria Aragón (IIS Aragón), Zaragoza, Spain

Cytes Biotechnologies, Barcelona, Spain

S. Morini
Instituto de Investigación Sanitaria Aragón (IIS Aragón), Zaragoza, Spain

Department of Bioengineering and iBB-Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal

P. M. Baptista (✉)
Instituto de Investigación Sanitaria Aragón (IIS Aragón), Zaragoza, Spain

Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Zaragoza, Spain

Instituto de Investigación Sanitaria de la Fundación Jiménez Díaz, Madrid, Spain

Departamento de Ingeniería Biomédica y Aeroespacial, Universidad Carlos III de Madrid, Madrid, Spain

Fundación ARAID, Zaragoza, Spain
e-mail: pmbaptista@iisaragon.es

D. Eberli et al. (eds.), *Organ Tissue Engineering*, Reference Series in Biomedical Engineering, https://doi.org/10.1007/978-3-030-44211-8_2

2 Tissue Engineering 150
2.1 First Approaches Used for Liver Tissue Engineering 150
2.2 Whole-Liver Scaffolds (Decellularization) 151
2.3 Cellular Components, Their Source, and Role in Generating Hepatic Tissue 153
2.4 Bioreactors for Liver Tissue Engineering 155
2.5 In Vivo Results of Liver Tissue-Engineered Constructs 159
3 Future Perspectives 159
3.1 Liver Organoids 159
3.2 Liver Buds 161
3.3 3D Bioprinting 162
4 Regulatory Landscape for Tissue-Engineered Livers 163
4.1 Food and Drug Administration (FDA) 163
4.2 European Medicines Agency (EMA) 164
4.3 Ministry of Health, Labour, and Welfare (MHLW) 164
5 Conclusions 165
References 166

Abstract

Up to date, liver transplantation is the only definitive cure for patients with end-stage liver disease. However, donor organs are limited, and several patients succumb to liver failure before a suitable donor is found. Liver tissue engineering and regenerative medicine are promising new technologies that can help reduce the burden of liver shortage by increasing the number of organs available for transplantation. In this chapter, we focus on various aspects of liver bioengineering, describing current liver diseases and the most relevant liver models used in liver tissue engineering, with particular attention to liver decellularization and recellularization processes. Also, we highlight the importance of bioreactor systems to allow a dynamic growth of all the cells seeded in liver scaffolds. Furthermore, we describe new cell culture systems that help us to pave the way from the bench to the bedside, as well as the regulatory and technological challenges related to liver bioengineering.

1 Introduction

The liver is the second largest organ in the human body. With an approximate weight of 1.2–1.5 kg in an adult human, the liver constitutes the most significant gland of the organism.

It is involved in many vital functions like the regulation of the energetic metabolism, processing all the nutrients; in the synthesis of essential proteins and enzymes needed during the digestion and normal organism behavior; in the maintenance of hormone balance; and in the detoxification and elimination of compounds for a correct incorporation of amino acids, carbohydrates, lipids and vitamins, and their storage.

The liver is considered a metabolic organ and an exocrine gland, too, because of its role in metabolic and bloodstream secretion functions. Furthermore, because of its capacity for bile production and secretion, it is also considered as an exocrine gland.

This organ has the particularity of receiving a double blood contribution: 80% enters through the portal vein, with low oxygenation and providing the liver with all the substances drained from the abdominal organs. The remaining 20% enters through the hepatic artery, carrying well-oxygenated blood. Arterial and venous blood converge both at the hepatic sinusoid, which allows the transvascular interchange between the blood and parenchymal cells.

It is anatomically divided into eight independent segments, and its functional morphologic unit is the hepatic lobule, organized surrounding the central vein (CV). Hepatic cords and hepatic sinusoids are surrounding this CV. In the apex of the hepatic lobule is located the portal trial, formed by a hepatic artery, a portal vein, and a bile duct. Repetitions of this functional unit form the hepatic tissue.

Furthermore, many types of cells coexist in the liver tissue: the parenchymal cells, which are the hepatocytes, constitute 80% of the tissue. Non-parenchymal cells constitute the remaining 20% and include liver sinusoidal endothelial cells (LSEC), ductular cells (DC), Kupffer cells (KC), stellate cells (HSC), and Pit cells.

1.1 Brief Description of Acute, Inborn Errors of Metabolism and End-Stage Liver Disease Worldwide

Liver diseases are any conditions that lead to liver inflammation or damage, affecting thus its normal function. Many factors like infections, exposure to drugs or toxic compounds, autoimmune processes, or genetic malignancies can cause these. The effects include inflammation, scarring, blood clotting, bile duct obstructions, and liver failure. The following table (Table 1) summarizes most liver diseases:

Liver disease causes approximately two million deaths per year worldwide: one million originated by cirrhosis complications and the remaining million because of viral hepatitis or hepatocellular carcinoma (Mokdad et al. 2014). Cirrhosis is the 11th most common cause of death worldwide, with 1.16 million global deaths per year. Liver cancer represents the 16th most common cause of death, with 788,000 global deaths (Asrani et al. 2019).

Some global data analyzed from 1990 to 2010 suggest that Latin America, the Caribbean, Middle East, and North Africa have the highest percentage of deaths because of liver disease. Egypt, Moldova, and Mongolia have the highest cirrhosis mortality rate in the world. India represents an 18,3% of cirrhosis death in the world, followed by China with 11%. Central Asia, the Russian Federation, and Europe's mortality are increasing.

Almost any chronic liver disease leads to cirrhosis. Globally, the leading causes are hepatitis B and C virus and alcohol. Other causes can be immune liver diseases, drugs, cholestatic disease, among others. The causes vary between different countries: in Western and industrialized countries, alcohol and nonalcoholic fatty liver disease are the leading causes, whereas hepatitis B is the main cause in developing countries (Asrani et al. 2019; Lim and Kim 2008; Lozano et al. 2012).

Acute liver failure (ALF) is a rare disorder with a high mortality rate (Bernal et al. 2010). It can be produced by viral causes, predominantly in developed countries; in

Table 1 Characteristics and causing agents of different hepatic diseases. (Source: Adapted from (AACC 2019))

Liver disease	Characteristics	Causing agents
Acute liver failure	A rapid decrease in liver function	Drugs, toxins, liver diseases
Alcoholic liver disease	Liver damage which can lead to fatty liver, alcoholic hepatitis, or cirrhosis	Abusive alcohol intakes
Autoimmune liver disease	Body's immune system attacks liver cells	Primary biliary cirrhosis, autoimmune hepatitis
Biliary obstruction	Bile duct blocking	Trauma, tumors, inflammation
Cirrhosis	Liver scarring	Chronic hepatitis, alcoholism, chronic bile duct obstruction
Genetic diseases	Gene mutation	Hemochromatosis, Wilson disease
Hepatitis	Acute or chronic liver inflammation	Virus, alcohol, drugs, toxins
Infections	Can originate liver damage and/or bile duct blockage	Virus, parasites
Liver cancer	Abnormal growth of liver cells	Cirrhosis, chronic hepatitis, virus

the USA and Western Europe, drug-induced injury is the leading cause, and there are many other cases with no clear origin. ALF can be associated with multiorgan failure.

Nonalcoholic fatty liver disease (NAFLD) is an increasing health problem associated with diabetes and obesity that affects one-third of adults in developed countries (Cohen et al. 2011).

This clinicopathological condition comprises a broad spectrum of liver damage, ranging from simple steatosis to steatohepatitis, fibrosis, and cirrhosis in patients who do not abuse alcohol. This illness starts with an abnormal accumulation of triglyceride droplets within the hepatocytes, leading to hepatic steatosis. This stage is usually self-limited, but it can progress to nonalcoholic steatohepatitis (NASH) due to hepatic injury because of hepatocyte death due to ballooning, inflammatory infiltrate, or collagen deposition (fibrosis). NASH can then develop a cirrhotic stage, where hepatocytes are replaced by scar tissue, decreasing hepatic function and altering blood flow. There are several degrees of fibrosis, being the last step the progression of the cirrhotic stage to hepatocellular carcinoma (HCC).

Hepatocellular carcinoma is usually the result of a complication of liver diseases, commonly liver cirrhosis (Fattovich et al. 2004). The WHO estimates that in the 2000, primary liver cancer is supposed the fifth most prevalent illness in men and the ninth in women, representing about the 5,6% of all human cancers (Parkin et al. 2001), with 564,000 new cases per year and expected to increase by 2020 (Bosch et al. 2004).

The Barcelona-Clínic Liver Cancer (BCLC) has classified HCC in liver cirrhotic patients into five different stages: stage 0 (very early stage), stage A (early stage), stage B (intermediate stage), stage C (advanced stage), and stage D (terminal stage), and nowadays it is utilized by many centers.

1.2 Liver Transplant Worldwide

According to the data published in the *Newsletter Transplant*, handled by the NTO (National Transplant Organization) in collaboration with the World Health Organization, Spain revalidates its world leadership for the 26 consecutive years, with a rate of 47 donors per million population (p.m.p). This country provides 19,2% of organ donations in the EU and 6,4% of the registrations worldwide (34,096). Spain also maintains the leadership in transplants, with 113,4 p.m.p, above the USA, which has 109,7 p.m.p. In 2018, there were 135,860 organs transplanted worldwide. This number means an increase of 7,2% to the previous year (which was 126,670). From these, 89,823 were kidney transplants, 30,352 liver transplants, 7,626 heart transplants, 5,497 lung transplants, 2,342 where pancreas transplants, and 220 intestine transplants. By the end of 2018, there were 56,399 European people on the waiting list. According to these data, ten patients died per day waiting for an available organ in the EU.

The donation rate has increased in the last years worldwide: there are 31,7 donors p.m.p in the USA; Australia reached 20,8 p.m.p and Canada 21,9 p.m.p. Russia figures its rate in only 4 donors p.m.p, and Latin America reached 9,5 donors p.m.p.

The following table (Table 2) recapitulates data from a liver transplant in 2017 (data obtained from the Newsletter Transplant):

Table 3 summarizes the number of patients in the waiting list (WL) for a liver transplant and the number of patients who died in 2017 (data obtained from the Newsletter Transplant):

1.3 Bottlenecks and Limitations

As mentioned above, liver transplantation means the only effective treatment option for patients suffering from end-stage liver disease, acute hepatic failure, and hepatocellular carcinoma. Although short-term graft and recipient survival outcomes have improved, thanks to advancements in the surgical technique, perioperative management, and immunosuppressive therapy, there are still two main limitations in liver transplantation: the first one and the most important is organ shortage. That is

Table 2 Number of liver transplant worldwide

Country	Number of transplanted livers
Australia	282
Canada	585
European Union	7.984
Latin America	3.288
New Zealand	55
Russian Federation	438
Saudi Arabia	226
USA	8.082

Table 3 The number of patients waiting for a liver transplant and *exitus* number of total patients in 2017 across the world

Country	Number of patients in the WL	*Exitus* number
Australia	482	11
Canada	–	74
European Union	16.064	990
Latin America	8.112	1.087
New Zealand	–	–
Russian Federation	1.666	141
Saudi Arabia	658	–
USA	24.178	1306

why significant efforts are being developed to increase the existing scarce donor pool. This effort has derived in the use of liver allografts from donors after cardiac death in combination with extended criteria donors. The goal is to get a better selection of donors, selecting not only the adequate ones but has also helped in the development of mechanical perfusion strategies (Jadlowiec and Taner 2016). The second limitation is the long-term recipient's complications after liver transplantation. Infections, allograft failure, cardiovascular events, or renal failure are the most common causes of later mortality after liver transplantation, which derive from long-term immunosuppression (Watt et al. 2010). Substantial efforts are being developed to improve the recipient's long-term outcomes.

1.4 Solutions Developed Throughout the Years to Increase Organ Availability

Due to organ shortage, it became necessary to find alternative therapies to treat liver failure. Extracorporeal liver support systems for patients suffering from liver failure consist of temporary relief developed to speed up liver recovery from injury or as a bridge to transplantation. There are two types of devices for temporal support: artificial livers (AL), which use nonliving components to remove toxins accumulated in the blood or plasma due to liver failure, using membrane separation associated with columns or sorbents. On the other hand, bioartificial liver (BAL) provides not only liver detoxification but also synthetic functions by combining chemical procedures and bioreactors containing cells to maintain the liver function (Carpentier et al. 2009). Appropriate cell choice for BAL devices is still under investigation.

Primary human hepatocytes represent the ideal source for replacing liver function, but they still are difficult to maintain in vitro. Primary porcine hepatocytes, on the contrary, enable the availability of large quantities of cells but with the immunologic and infectious problems that they carry. This is the reason why human or human-derived cells are more desirable than animal cells. Other cell types investigated are immortalized cells [like C8-B (Cai et al. 2000), HepZ (Werner et al. 2000), HH25 (Kono et al. 1995). . .], which are easily cultivated in vitro and maintain liver-

specific functions. Their problem still resides in their potential transmission of oncogenic substances to the patient (Allen et al. 2001; Carpentier et al. 2009). Up to date, BAL is still under investigation. Although some of them have FDA authorization for clinical development (like HepaMate, ELAD, or Excorp Medical), there is still a lot to do regarding their capacity to provide and replace liver functions. Significant issues have to be overcome, like cost, cell availability, or maintenance of cell viability that have delayed their appearance on the clinic.

Split livers represent another choice. This technique means a way to increase the number of cadaveric donor organs for children and adults: it provides a left lateral (to be transplanted into a child) and a right-extended liver graft (to be transplanted into one adult). Furthermore, the outcomes showed comparable results as those in whole organ liver transplantation (Broering et al. 2004). Nevertheless, the main problem with split livers resides in that it is a sophisticated variant of liver transplantation that requires a very high level of technical and logistical skills and an extensive knowledge of possible anatomic variations. It also needs a reliable judgment on graft quality and also an optimal graft-recipient size match (in order to avoid the small-size liver syndrome). However, in the latest years, new technologies like organ and tissue engineering have emerged not in order to maintain liver function while injury but for the creation of new organs in vitro able to be transplanted.

Another potential alternative to liver transplantation is allogeneic hepatocyte transplantation (Iansante et al. 2018; Laconi et al. 1998) to restore hepatic function once engrafted in the recipient's liver. Hepatocytes can be isolated from different sources: whole donor livers unsuitable for transplant, from liver segments after split liver transplantation, or from neonatal and fetal livers (which could provide very high hepatocyte quality). The advantages of these techniques include the fact that it is less invasive and less expensive than surgery, and it can be performed repeatedly if necessary. Cryopreserved cells are also available when necessary, and because this procedure requires few cell quantities, different recipients could beneficiate from the same donor organ. On the contrary, the limitations reside in the difficulty of isolating and maintaining high-quality hepatocytes (Ibars et al. 2016; Stephenne et al. 2007). Liver cell engraftment is usually poor (approximately 0,1–0,3%) (Wang et al. 2002), and allogeneic rejection may be the leading cause of failure in cell graft function.

Mesenchymal stem cells (MSC) are another important source to consider. They are an attractive source due to their capacity to proliferate and differentiate in vitro and their anti-inflammatory and immune status (Iansante et al. 2018). Furthermore, there are several tissues containing hMSC: bone marrow, adipose tissue, and umbilical cord. Some authors have recently isolated MSCs from the liver, called liver-derived human MSCs, which can be transdifferentiated toward hepatocyte-like cells (Najimi et al. 2007). However, their role is still under investigation in liver-recipient repopulation and providing satisfactory hepatic function.

Tissue-engineered whole livers are becoming very promising too. By combining tissue engineering techniques and biology, autologous livers might be able to be created in vitro and transplanted, eliminating thus the problem of an organ donor, waiting list, and rejection.

2 Tissue Engineering

2.1 First Approaches Used for Liver Tissue Engineering

Tissue engineering is one of the most promising fields in regenerative medicine. As described in 1993 by Robert Langer and Joseph Vacanti, it is the conjugation of biomaterials (synthetic or naturally derived) with cells, in order to generate tissue constructs that can be implanted into patients to substitute a lost function and maintain or gain new functions (Langer and Vacanti 1993). The current paradigm is suitable for the engineering of thin constructs like the bladder, skin, or blood vessels. In the specific case of the liver, the 3D architecture and dense cellular mass require novel tissue engineering approaches and the development of vascularized biomaterials, in order to support thick tissue masses and be readily transplantable. Additionally, to the vascular support for large tissue masses, hepatocyte function maintenance represents the ultimate aim in any organ engineering or regenerative medicine strategy for liver disease. Hepatocytes are known to be attachment-dependent cells and lose rather quickly their specific functions without optimal media and ECM (extracellular matrix) composition and cell-cell contacts. Also, the function and differentiation of liver cells are influenced by the 3D organ architecture (Mooney et al. 1992).

In the last two decades, multiple strategies for the culture of adult hepatocytes in combination with several types of 3D, highly porous polymeric matrices, have been attempted (Fiegel et al. 2008; Kim et al. 2000b; Lin et al. 2004; Linke et al. 2007; Tong et al. 1990). However, in the absence of vasculature, restriction in cell growth and function is joint due to the limitations in nutrient and oxygen diffusion. Some of these problems are being now partially overcome with the development of bioreactors that provide continuous perfusion of culture media and gases, allowing a 3D culture configuration and hepatocyte function maintenance (Gerlach et al. 1994; Torok et al. 2001, 2006).

The tissue engineering concept has several advantages over the injection of cell suspensions into solid organs. The matrices provide sufficient volume for the transplantation of an adequate cell mass up to whole-organ equivalents. Transplantation efficiency could readily be improved by optimizing the microarchitecture and composition of the matrices, as well as by attaching growth factors and extracellular matrix molecules to the polymeric scaffold, helping to recreate the hepatic microenvironment (Mooney et al. 1992). The use of naturally derived matrices has also proved to be very helpful in hepatocyte culture (Lin et al. 2004). These matrices, besides preserving some of the microarchitecture features of the tissues that they are derived from, also retain bioactive signals (e.g., cell-adhesion peptides and growth factors) required for the retention of tissue-specific gene expression (Kim et al. 2000a; Voytik-Harbin et al. 1997). Additionally, cell transplantation into polymeric matrices is, in contrast to cell injection into tissues and organs, a reversible procedure since the cell-matrix constructs may be removed if necessary.

Finally, heterotopic hepatocyte transplantation in matrices has already been demonstrated in long-term studies (Johnson et al. 1994; Kaufmann et al. 1999).

Nonetheless, initial engraftment rates are suboptimal. One of the reasons for this is the absolute requirement of the transplanted hepatocytes for hepatotrophic factors that the liver regularly receives through its portal circulation (Starzl et al. 1973). Thus, the development of a tissue-engineered liver construct capable of being orthotopically transplanted is essential.

2.2 Whole-Liver Scaffolds (Decellularization)

One other alternative is based on the decellularization process to generate a whole-organ scaffold. These have to be later recellularized using different cell types to get a functional bioengineered organ to be transplanted into recipient animals (Fig. 1). The strategy of decellularization of extracellular matrices has induced the development of other disciplines such as cell biology, tissue engineering, and regenerative medicine further than the implementation of more simple naturally derived biomaterials. The resultant scaffolds can be considered to be cell-derived matrices, which are a compound of natural proteins, macromolecules, and other components such as associated growth factors. They can recapitulate the native ECM, thanks to the conservation of its organization and composition. As these scaffolds are derived from the ECM, they provide biological and mechanical support, and above all, a template of the organ, so that cells can migrate, attach, proliferate, organize, and

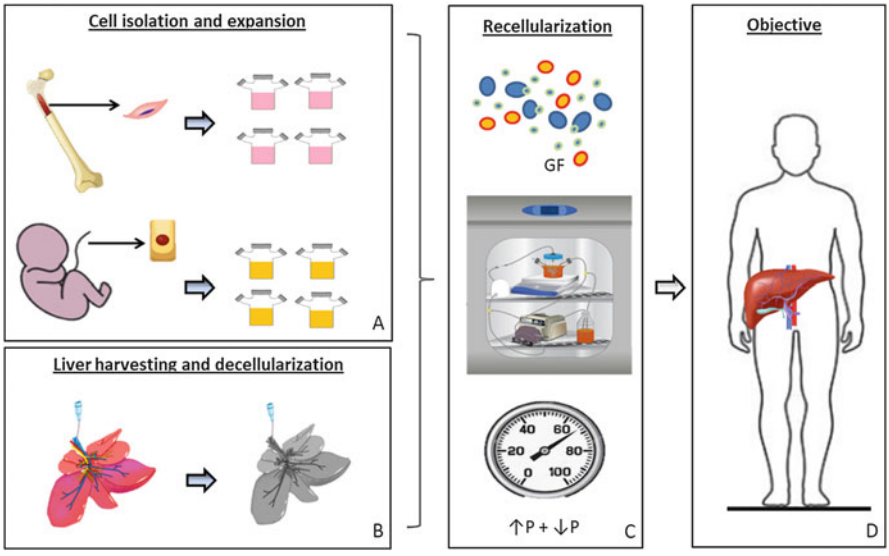

Fig. 1 Components of liver bioengineering. The process needs two components: cells, which are isolated and long-scale in vitro expanded (**a**), and a scaffold, which is decellularized (**b**) and recellularized with the isolated cells assembled in a bioreactor system, in combination with growth factors and different pressure conditions (**c**). The goal is the creation of a whole human bioengineered liver able to be transplanted into patients who require a new organ (**d**)

differentiate at the same time that it provides paracrine factor production (Robb et al. 2018).

Currently, decellularized tissues have some applications in the clinic in different areas. It is the case of orthopedics, dentistry, surgery, and even in the cardiovascular field (Parmaksiz et al. 2016). Nonetheless, the replacement of solid organs as the liver is not a clinical reality, yet as they are considerably larger than thinner tissues, their three-dimensional structure is much more complex, and they need a robust vascular network so that it can stay functional. In the field of whole-liver engineering, human, rat, and pig livers have been used as the primary sources to obtain scaffolds. However, some studies show that the spleen can also be used (Xiang et al. 2015). The generation of bioengineered livers able to replicate its native function has some limitations, including the formation of a patent vascular network and the vast expansion of cells to get the required number so that a minimal degree of function can be achieved. The first one is needed so that the blood flow becomes normal because the ECM without cellular components stimulates the formation of blood clots since it is considerably thrombogenic. However, the advances that have been made in the recellularization process give rise to an increase in vascular patency. Although this technology is expected to be used in the long term to treat patients suffering from end-stage liver failure, in recent years, some advances have been made in the field of whole-liver engineering. Researches have described different protocols that can be used to decellularize an organ in order to obtain a specific tissue-derived matrix. The strategies used are based on physical, chemical, mechanical, or enzymatic methods and even on a combination of them to maximize the efficacy of the process. The most common approximation is based on the use of detergent solutions to be perfused through the portal vein using peristaltic pumps so that the cellular content is removed.

At the same time, the extracellular matrix and the vascular tree are preserved, becoming one of the main advantages of this technology. It has been reported that the ultrastructure and composition are conserved, as well as the bile duct system (Baptista et al. 2011; Fukumitsu et al. 2011). At the same time, the microarchitecture and essential bioactive signals are maintained, which are considered complex to be replicated in vitro. This fact is quite relevant, taking into account that they are essential for cells to be viable, functional, and to differentiate. Two detergent solutions have been widely used: sodium dodecyl sulfate (SDS) (Buhler et al. 2015) and Triton X-100 (Baptista et al. 2011; Barakat et al. 2012; Crapo et al. 2011). However, there are also other agents such as enzymes, proteases, or acids that can be used. For the latter, the most widely used ones are peracetic acid, ethylenediaminetetraacetic acid, or deoxycholic acid, but alkaline solutions can also be perfused, such as sodium hydroxide. They may be used alongside with enzymes as DNase I or trypsin and physical agents as pressure and temperature.

On the one hand, for the first one, high hydrostatic pressure can be used or even supercritical CO_2. On the other hand, when the temperature is used, freeze-thaw cycles may be implemented. In the protocols published so far, the reagents mentioned above are used in multiple concentrations and even in different combinations (Faulk et al. 2015; Wang et al. 2017). Researchers have also reported the existence of

diverse strategies to perfuse the detergent solutions including the use of the portal vein (Buhler et al. 2015), the portal vein together with the hepatic artery (Baptista et al. 2011; Barakat et al. 2012; Ko et al. 2015), and even the inferior *venae cavae* (Shupe et al. 2010). At the same time, both the hepatic artery and the portal vein are used in the recellularization procedure (Faulk et al. 2015; Wang et al. 2017).

Uygun and his co-workers have been able to decellularize ischemic rat livers implementing the perfusion of SDS through the portal vein using different concentrations (0.01%, 0.1%, and 1%) for 24 h each of them (Uygun et al. 2010). As a result, they obtained a scaffold without cells characterized by being translucent and by maintaining the main physical structure of the organ. On the other hand, Baptista and colleagues have reported the use of a protocol that depends on the size of the liver and its structure (Baptista et al. 2011). In this case, different animal models have been studied, including mice, rabbits, and pigs. In this case, SDS is not used, but it is based on the perfusion of a detergent solution together with other agents. Briefly, the authors identified high efficacy when 1% Triton X-100 with 0.1% ammonium hydroxide was perfused through the portal vein after perfusing a volume of deionized water equal to 40 times the liver. The authors reported that the matrices obtained were transparent, with the vascular tree visible. Finally, other authors, as De Kock et al., have described protocols in which both detergent solutions are used. They were able to decellularize a rat liver in just an hour using first 1% Triton-X 100 half of the time and 1% SDS during the second half an hour, with perfusion through the portal vein. This strategy gave rise to translucent liver scaffolds with no cellular component and able to endure fluid flows, thanks to the preservation of its architecture (De Kock et al. 2011).

2.3 Cellular Components, Their Source, and Role in Generating Hepatic Tissue

Once the decellularization procedure has been performed, to obtain a functional organ, it is necessary to seed different types of cells in the generated scaffold. To do so, they have to be sterilized and incorporated into a designed bioreactor system to be recellularized. As it was mentioned before, obtaining the required number of cells from each required type is one of the main limitations of this strategy.

As in the case of decellularization, there are different protocols reported and already available for the recellularization. Soto-Gutiérrez and collaborators compared three of them by studying direct parenchymal injection, multistep infusion, and continuous perfusion (Soto-Gutierrez et al. 2011). The first one, as its name indicates, is based on injecting the cells directly into the hepatic lobes of the scaffold, while the continuous perfusion strategy requires perfusing the obtained cells in suspension together with the culture medium using a bioreactor system to control its flow rate across the liver scaffold. Both the multistep infusion and the continuous perfusion involve delivering cells in small but multiple batches separated by intervals of time equally separated. The conclusion of this study indicated that the best

results were achieved when the multistep infusion strategy was used as seeding conditions.

On the other hand, cell number is another critical parameter to be taken into account (Fukumitsu et al. 2011). In order to mimic liver functionality and morphology, its spatial distribution has to be recreated with the appropriate concentration and cellular type. Different cells can be used in the field of tissue engineering and regenerative medicine, including primary hepatocytes, which can be human or porcine, human hepatic cell lines, and immortalized hepatocytes, as well as pluripotent stem cells or fetal progenitors.

Regarding the cell types that have to be used to generate relevant hepatic tissue in vitro, the gold standard is primary human liver cells, isolated from liver tissue or whole livers that did not fulfill the conditions to be used in transplantation. Their relevance comes from their source, the human liver, and because consequently, they give rise to the functionality of this organ in vivo. Thus, in the toxicological and pharmacological research, their use can provide predictive results. However, multiple donors are required what causes differences in the characteristics of the cells used, such as the age, sex, or liver damage of each one of them. These variations give rise to some deviations in the experimental results that make it difficult to standardize the generated models. Indeed, the mentioned differences determine the success of the isolation process, as well as other factors as cell isolation conditions or intraoperative factors. During the mentioned procedure, enzymatic digestion may be performed, requiring the use of collagenase and other enzymes, and plastic culture dishes are often used. However, these conditions change some characteristics of these cells, like morphology or gene expression, due to a process known as dedifferentiation that represents a practical limitation. Some studies have been carried out to identify the best strategy to overcome it improving the survival of these cells and their functionality. The alternatives evaluated include the modification of the signals from the microenvironment by including soluble factors in the culture medium. The cell media can be modified with hormones, growth factors, or vitamins, in order to modulate the functionality of hepatocytes (Guillouzo 1998; Kidambi et al. 2009; Miyazaki et al. 1985).

The similarities between pigs and humans make it possible to use primary porcine hepatocytes as the availability of human sources is limited. Conversely, some disadvantages need to be considered. The xenogeneic character of these cells makes it possible to transfer zoonotic diseases and to identify an incompatibility between proteins. Subsequently, more appropriate cell sources have to be identified. To overcome the limitations mentioned above, human hepatocyte cell lines have been considered as an alternative. They have been immortalized using different approximations as transfection using simian virus 40 T antigen (Li et al. 2005). However, the potential to give rise to tumorigenic effects has not been assessed sufficiently in these studies. The immortalized cell lines derived from the liver that are more widely used include the HepaRG, HepG2, and Hep3B, among others (Guguen-Guillouzo et al. 2010). The first one, derived from a human hepatoma, conserves the expression of liver-specific functions, membrane transporters, and nuclear receptors (Aninat et al. 2006), presenting a stable karyotype and high

proliferative capacity. Even more critical, this cell line has revealed that it can provide consistent and reproducible data as a result of different experiments. Still, its expression of liver-specific functions is lower than the one that can be identified for primary hepatocytes (Marion et al. 2010).

Another approximation that has been evaluated is the co-culture of primary human hepatocytes with non-parenchymal cells, such as Kupffer cells, liver endothelial cells, and hepatic stellate cells. The reason to do so is that researchers have identified that these cells have a critical role for liver function and in processes like acute inflammation or chronic liver diseases. As an example, some investigations have tried to obtain co-cultures with Kupffer cells to evaluate the reaction of hepatocytes in a pro-inflammatory environment (Nguyen et al. 2015).

Stem cells have also been considered to generate hepatic tissue as, in theory, they represent an unlimited cell source. Autologous liver stem cells are the leading type of cells used, but others are also being studied, such as induced pluripotent stem cells (iPSCs), mesenchymal stem cells, and humanized hepatocytes (Agmon and Christman 2016). The main advantage of iPSCs is their autologous character and that they are considered to be limitless being able to repopulate a scaffold with the appropriate size. Furthermore, these cells reduce the limitation that can be identified when others are used, such as ethical considerations when embryonic stem cells are used. Hepatocyte-like cells can also be obtained over-expressing transcription factors (Huang et al. 2011) specific from the hepatocyte lineage, starting with adult cells giving rise to induced hepatocyte-like cells or iHep. Further research is required to improve this technology, but the results obtained so far are promising.

Other cell sources have also been used, such as umbilical vein endothelial cells, both from a human and porcine source. In 2017, Mao and his co-workers were able to achieve complete coverage of the vascular network with appropriate patency in a porcine model using these cells (Mao 2017).

The number of cells that need to be used to generate functional hepatic tissue is not the only important factor. The cellular type is also a crucial parameter as it determines the flow-seeding conditions that have to be used. Then, the most appropriate speed has to be identified to optimize cellular engraftment. Hence, there are still relevant issues to be addressed to obtain functional organs before it becomes a clinical reality.

2.4 Bioreactors for Liver Tissue Engineering

Bioreactors are extensively used in tissue engineering for the development of tissue-engineered constructs. A bioreactor represents the central essence of any biochemical process in which plant, microbial, enzymes, or mammalian cell systems are used for the manufacture of a broad range of biological products. The primary function of a bioreactor is to provide a controlled in vitro environment for tissue generation and growth while mimicking the mechanochemical regulation that cells and tissues experience in vivo in their native environment. Ideally, and in the context of tissue engineering, bioreactors serve as a system able to allow the uniform seeding of

cells to a scaffold, control the physiological conditions in the cell culture medium (i.e., nutrients, oxygen levels, temperature, pH), supply sufficient metabolites, and provide physiologically relevant signals in the form of mechanical loads (Altman et al. 2002; Freed and Vunjak-Novakovic 2000). Bioreactors are broadly used almost at any step of the generation of a tissue-engineered liver construct. Before their large-scale expansion or seeding in a scaffold, primary cells are isolated from autologous or allogeneic liver tissue or whole organ and cultured under static conditions in T-flasks or Petri dishes to let them recover from the isolation and purification stress and to obtain the adequate large mass of cells required for the creation of the final desired bioproduct.

An appropriate design of the bioreactor system is crucial to create better tissue-engineered products, such as artificial organs for transplantation. Each decision made in the design of the bioreactor may strongly impact the overall process performance. Theoretically, any bioreactor useful for tissue engineering purposes has to be robust enough to maintain the cultured cells or tissues for long periods, structured to deliver nutrients, growth factors, peptides, and gases into the culture system in order to encounter and sustain the needs of the cultured cells or tissues, easily cleanable, and operator friendly. From the performance point of view, the most inescapable hindrance regarding the biological design of a liver bioreactor is meeting the basic metabolic requirements of the cells or tissue.

Oxygen tension is finely controlled in the liver, varying along the sinusoid from 85 μM periportal to approximately 45 μM pericentral. Hepatocytes consume oxygen at 10- to 100-fold the rates of most cells, so there is a desperate need to balance oxygen consumption with oxygen delivery to guarantee an optimal hepatocellular function (Ebrahimkhani et al. 2014). Each hepatocyte contains over 1500 mitochondria, which consume oxygen at a rate of 0.3–0.9 nmol/sec/million cells (Nahmias et al. 2007), while an average rate of oxygen utilization by many other cells is about 2–40 picomol/sec/million cells (Wagner et al. 2011). This issue represents a considerable challenge in 3D cultures, as the oxygen gradient across a layer of five cell diameters, which represents a distance of approximately 120 μm, ranges in the liver from normoxic to hypoxic (Ebrahimkhani et al. 2014).

Another crucial step in the development of liver constructs for liver replacement is represented by the seeding of a vast amount of liver cells uniformly on or throughout a scaffold. Furthermore, the adherent cells require appropriate amounts of nutrients, oxygen, peptides, and growth factors to survive and proliferate and adequate chemo/physical signals to organize and differentiate and to generate cellular structures and metabolic zonation resembling that of the liver. On the one hand, a high initial cell seeding might favor tissue formation (Dvir-Ginzberg et al. 2003), but on the other hand, it would require a high amount of organ tissue for the isolation of the cells and more time to obtain an adequate number of cells to seed. Moreover, a uniform initial cell distribution on the scaffold is needed to avoid spatial variation in nutrients, oxygen, and metabolite concentrations that would condition the survival and metabolism of cells at different positions in the scaffold (Catapano and Gerlach 2007).

When designing a bioreactor for liver engineering, it is imperative to consider the diffusion distance existing between cells and the blood. Indeed, liver cells are sensitive to waste metabolites and have essential nutrient requirements that need a small diffusion distance (i.e., within a few hundred microns in the sinusoids) (Catapano and Gerlach 2007). It is an enormous challenge to provide a system to supply essential substrates (e.g., oxygen, glucose, and amino acids) or clear waste metabolites (e.g., CO_2, ammonia, urea, lactate) from liver cells in large 3D constructs and a central prerequisite to promote cell growth, differentiation, and long-term survival (Catapano 1996; Martin et al. 2004; Martin and Vermette 2005).

Plenty of efforts have been made to develop bioreactor designs adapted for liver cell culture. Hollow fiber cell culture bioreactors have been developed in the 1970s and used until today for the generation of high concentration of cell-derived products, such as monoclonal antibodies, recombinant proteins, growth factors, viruses, and virus-like particles. Nowadays, these reactors include control of pH, oxygen, fluid dynamics, and medium exchange, allowing the production of high numbers of bioproducts. The traditional hollow fiber bioreactor is a three-dimensional cell-culturing system based on small, semipermeable capillary membranes arranged in a parallel array. These membranes are bundled and housed within tubular polycarbonate shells to create the bioreactor cartridges containing two compartments: the intracapillary space (IC) within the hollow fibers and the extracapillary space (EC) surrounding the IC. The culture medium is pumped through the IC space outside the fibers while delivers molecular components with the cells cultured and expanded into the EC space. Here, the exchanges occur in a manner resembling some features of capillary blood-tissue exchanges in vivo, being the membranes the core of the bioreactor itself. As these membranes are permeable, it is possible to control the molecular exchange properties between the nutrient fed and tissue compartment through the selection of membrane permeability properties. These bioreactors have been applied to liver cell culture, practically from the beginning. Wolf C. et al. firstly describe that a rat hepatoma cell line could carry out bilirubin conjugation in hollow fiber culture with either 10 kDa or 50 kDa cutoff membranes (Wolf and Munkelt 1975), with speculation that the rates of conjugation may be limited by the transport of the bilirubin carrier protein albumin across the membrane. Next, Jauregui H. et al. found that primary rat cells cultured up to 45 days in a 7-cm-long hollow fiber reactor with 520–0.3 mm diameter fibers maintained almost half the diazepam-metabolizing capacity after 10 days provided the perfusion medium was equilibrated with 30% oxygen atmosphere (Jauregui et al. 1994). Due to the similarity between hollow fiber cell culture bioreactors and blood dialysis units, liver culture in hollow fiber bioreactors appeared as a potentially promising approach for extracorporeal liver support to provide metabolic capacity in synthesis of urea and metabolism of toxic metabolites like bilirubin (Demetriou et al. 2004; Jauregui et al. 1994; Rozga et al. 1993, 1994; Struecker et al. 2014).

Stirred-tank bioreactors have mainly been used in the large-scale culture of mammalian cells to create a suitable microenvironment, where variables are tightly controlled, such as pH and oxygen levels. With the appropriate design of impellers, the fluid mechanic microenvironment in these systems can provide relatively uniform

low-shear mixing, and the commercial availability of reactor systems makes them accessible for general use (Morini et al. 2018). When a stirred-tank bioreactor is used for growing adherent cells, specific microcarriers are used as a supporting surface for cell attachment. Alternatively, adherent cells are grown as aggregates in suspension.

The ability of hepatocytes to form aggregates when seeded on low-adhesive surfaces, and for aggregates to maintain liver-specific function better compared to cells in monolayer cultures, has been described and demonstrated (Powers 1997). The utility of stirred-tank bioreactors for efficient formation of relatively uniform, functional spheroidal aggregates of primary rat hepatocytes that exhibit polarization of bile canaliculi was already illustrated over 20 years ago (Wu et al. 1996). Recently, Alves and colleagues established and tested a perfused bioreactor system for the long-term maintenance of primary cultures of human hepatocyte spheroids from three different donors. They found that using this method, the generated hepatocyte spheroids reproducibly recapitulated in vivo hepatic functions and structure, despite inter-donor variability. They hypothesized that these reproducible time-course profiles were made possible because of the tight control of critical environmental variables at physiological values. Moreover, they observed that the spheroid's inner structure resembled the liver architecture, with functional bile canaliculi-like structures and liver-specific markers (such as CYP450 expression and phase II and III drug-metabolizing enzyme gene expression and transport activity). This system constitutes an ideal long-term culture platform for analyzing hepatic function for drug development tests (Tostoes et al. 2012).

Commercially available microcarriers with different sizes and composition can also be used for large-scale cell culture for many different anchorage-dependent cell types. Microcarriers differ in their porosity, specific gravity, optical properties, presence of animal components, and surface chemistry and can be made of different materials (i.e., glass, polystyrene plastic, collagen, alginate, dextran) which can influence cellular behavior, phenotype, morphology, and proliferation. The main advantages of microcarrier technology reside in providing a larger surface-area-to-volume ratio for the growth of anchorage-dependent cells in a suspension culture system, ease of scale-up, and the ability to monitor and control various physiological conditions when used in a bioreactor. Several attempts have been made to take advantage of microcarrier technology for liver tissue engineering purposes. Gao Y. et al. used Cytodex-3 microcarriers to cultivate high-density human liver cell line Cl-1 to improve the cultivation efficiency and yield and evaluated specific functions of liver cells periodically. They observed that the human liver cell line Cl-1 can be cultivated to a high density on these microcarriers and has better biological functions, such as albumin and urea synthesis and diazepam transformation, along with the prolonging of the cultivation (Gao et al. 1999). Zhang L. and colleagues reported that rat hepatocytes cultivated on chitosan microcarriers cross-linked by oxidized lactose retained the spherical shape as they have in vivo. Moreover, liver-specific functions such as albumin secretion and glucose metabolism were stably maintained for 7 days in culture, and the metabolic activity of hepatocytes cultured on these specific microcarriers was higher than those of hepatocytes cultured on chitosan microcarriers cross-linked by glutaraldehyde and on Cytodex-3 (Zhang et al. 2003).

2.5 In Vivo Results of Liver Tissue-Engineered Constructs

Hepatic tissue engineering using primary hepatocytes has been considered a new exciting approach for the treatment of different classes of liver diseases. Hepatocyte-based therapies have been experimentally and clinically explored and generate new interest in the bioengineering of alternative liver systems in vivo based on the manipulation of in vitro-modified hepatocyte cultures (Griffith and Naughton 2002; Ohashi et al. 2001; Strom and Fisher 2003). Ohashi and co-workers developed a method to engineer a uniformly continuous sheet of hepatic tissue using isolated primary hepatocytes cultured on temperature-responsive surfaces. Sheets of hepatic tissue transplanted into the subcutaneous space resulted in efficient engraftment to the surrounding cells, with the formation of two-dimensional hepatic tissues that stably persisted for longer than 200 days. The engineered tissues also showed several characteristics of liver-specific functionality. They described this technology as simple, minimally invasive, and free of potentially immunogenic biodegradable scaffolds (Ohashi et al. 2007).

Clinical trials on hepatocyte transplantation have demonstrated long-term safety, but donor hepatocyte engraftment and restoration of failing host livers have not been adequate to reduce the need for organ transplantation (Dhawan et al. 2010). One alternative could be represented by the use of hydrogels reproducing the biochemistry of tissue-specific ECM proteins. ECM hydrogels were derived from decellularized rat livers and employed for both 2D-plate coatings and in vivo hepatocyte transplantation. Primary rat hepatocytes cultured on a liver ECM hydrogel-coated substrate exhibited higher viability and improved hepatic functions compared to cells cultured on a non-coated or collagen type I-coated substrate. Besides, liver ECM hydrogels engineered with rat hepatocytes maintained the hepatic phenotype and function after in vivo transplantation (Lee et al. 2014).

3 Future Perspectives

In the last years, the advances and improvements regarding the technology of liver bioengineering have been used to understand the hepatic regeneration mechanism better, and it has facilitated the development of different in vitro models near-physiological ex vivo culture system. In this section, different in vitro models showing a more precise control over the liver cell microenvironment will be discussed.

3.1 Liver Organoids

In the middle of the last century, different authors began to use the term “organoids” (Vendrely 1950), and this nomenclature started to appear in various publications in the 1960s. The advances in the organoid field over the last decade are the consequence of years of work to understand more profoundly the role of progenitor cells, self-organization of dissociated tissues, and ECM biology.

Usually, the term organoids are used to refer to a range of 3D culture systems that resemble the modeled organ to varying extents (Prior et al. 2019). Specifically, an organoid is an in vitro 3D cellular cluster derived from tissue-resident stem/progenitor cells, embryonic stem cells (ESCs), or induced pluripotent stem cells (iPSCs) capable of self-renewal and self-organization that recapitulate the functionality of the tissue of origin (Huch and Koo 2015; Lancaster and Knoblich 2014; Prior et al. 2019). Considering this definition, the organoid culture requires the isolation of stem/progenitor cells, either pluripotent stem cells or tissue-resident cells from embryonic stages of adult tissues, which are cultured by using specific medium supplemented with growth factors that recreate the signals to give rise to the specific tissue. Also, the organoid cultures require a specialized physical environment, and it usually involves culturing cells in suspension, on an air-liquid interface, or embedded in a suitable ECM such as Matrigel. Thus, when the isolated cells are cultured with the correct growth medium and a specific physical environment, they can follow intrinsic developmental or homeostatic/repair programs to proliferate and self-organize into 3D organoid structures, allowing the use of these models in different areas of knowledge. For example, the use of organoids has high relevance in disease modeling, as alpha-1 antitrypsin (A1AT) deficiency, an example of a monogenic liver disease that affects the liver parenchyma. Recently, the group led by Hans Clever showed that organoids derived from adult liver tissue from patients with A1AT deficiency had been successfully developed and recapitulated critical aspects of this disease: accumulation of protein aggregates and reduced ability to block elastase activity. They also showed signs of ER stress, such as phosphorylation of eIF2α, among other aspects, showing similar characteristics to what had been observed in the original biopsy from patients. Thus, organoids from A1AT-deficiency patients can be expanded in vitro and mimic the in vivo pathology recapitulating critical features of the disease in vitro and enabling further understanding of the disease processes (Huch et al. 2015). Another area of interest where liver organoids have been exploited is for personalized medicine use, as HCC.

Nuciforo et al. reported the generation of long-term organoid cultures from tumor needle biopsies of HCC patients with various etiologies and tumor stages. These HCC organoids showed a preserved morphology, as well as the expression pattern of HCC tumor markers, and preserved the genetic heterogeneity of the originating tumors. This study showed that liver cancer organoids could be used to test sensitivity to sorafenib, providing a tool for developing tailored therapies (Nuciforo et al. 2018).

After the examples described above, it is easy to understand the relevance of liver organoids. It is focused on its applications since this type of culture shows an improved understanding of hepatic regenerative pathways and the development of in vitro systems to mimic both the expansion and differentiation of hepatocytes. As a consequence of the relevance in its applications, the organoids were named "Method of the year 2017" by *Nature Methods* showing the interest and promise of this rapidly expanding field to provide new experimentally tractable and physiologically relevant models of organ development, human pathologies, and paving the way for therapeutic applications (Methods 2018; Prior et al. 2019).

3.2 Liver Buds

Cell-based therapy has been proposed as a useful alternative to orthotopic liver transplantation. Organ bud progenitor cells play essential roles in organ development. These cells have a remarkable capacity for rapid cell growth and differentiation into multi-lineage cells.

A liver bud is defined as the primordial cellular diverticulum of the embryonic foregut endoderm that gives rise to the parenchyma of the liver. Hepatic progenitor cells (HPCs), or liver bud progenitors, are specified from foregut endoderm at embryonic day (E)9.5 in mice. These is followed by a massive HPC expansion with a 10,000-fold increase in population, doubling from E9.5 to E13.5 in mice (Koike et al. 2014; Takebe et al. 2013). This tremendous growth is regulated by signals secreted from neighboring mesenchyme such as hepatocyte growth factors (Matsumoto et al. 2001), bone morphogenetic proteins (Rossi et al. 2001), and fibroblast growth factors (Serls et al. 2005) and by transcriptional networks that act intrinsically in the HPCs, such as Tbx3 (Suzuki et al. 2008), Smad2/3 (Weinstein et al. 2001), and beta-catenin (Micsenyi et al. 2004). However, the mechanism regulating this intensive and transient amplification in developing liver bud is mainly unknown.

Organoid technology represents a revolutionary paradigm toward therapy but is not yet applied in humans, due to lack of reproducibility and scalability. Several attempts have been made to overcome these limitations. Takebe and colleagues created a scalable organ bud production platform from human-induced pluripotent stem cells (iPSC). First of all, they identified three progenitor populations that can effectively generate liver buds in a highly reproducible manner: hepatic endoderm, endothelium, and septum mesenchyme. Furthermore, they achieved human scalability by developing an Omni-well-array culture platform for mass-producing homogeneous and miniaturized liver buds on a clinically relevant large scale. Vascularized and functional liver tissues generated entirely from iPSCs significantly improved subsequent hepatic functionalization potentiated by stage-matched developmental progenitor interactions, enabling functional rescue against acute liver failure via transplantation (Takebe et al. 2017).

Yanagi and co-workers have made another attempt. They reported a novel transplantation method for liver buds that were grown in vivo involving orthotopic transplantation on the transected parenchyma of the liver, which showed prolonged engraftment and marked growth in comparison with heterotopic transplantation. Furthermore, they demonstrated a method for rapidly fabricating scalable liver-like tissue fusing hundreds of liver bud-like spheroids using a 3D bioprinter. The ex vivo-fabricated human liver-like tissue exhibited self-tissue organization and engraftment on the liver of nude rats (Yanagi et al. 2017).

At the moment, no one succeeded in assembling human liver buds containing HSCs and LSECs. Recently, Li J. et al. described a reproducible, easy-to-follow, and comprehensive self-assembly protocol to generate 3D human liver buds from naïve MSCs, MSC-derived hepatocytes, and HSC- and LSEC-like cells. By optimizing the ratio between these different cell lineages, the cell mixture self-assembled into 3D

human liver buds within 72 h in vitro and exhibited similar characteristics with early stage murine liver buds. In a murine model of acute liver failure, the mesenteric transplantation of self-assembled human liver buds effectively rescued animal death and triggered hepatic amelioration effects that were better than the ones observed after splenic transplantation of human hepatocytes or naïve MSCs. Besides, transplanted human liver buds underwent maturation during injury alleviation, after which they exhibited a gene expression profile signature similar to one of adult human livers (Li et al. 2018).

3.3 3D Bioprinting

Three-dimensional bioprinting is an innovative technology based on the successive addition of small quantities of biomaterials and cells which are deposited layer by layer. It comprises different technologies to obtain artificial constructs with a high precision level (Murphy and Atala 2014).

The biomaterials used can be natural polymers such as collagen and fibrin, synthetic polymers, and even decellularized matrices. Indeed, two different approaches can be followed when 3D bioprinting technology is used (Tiruvannamalai-Annamalai et al. 2014). On the one hand, the traditional approach of top down, which is based on seeding the cells into a porous scaffold to promote cell proliferation and scaffold degradation, gives rise to the engineered tissue. The other modular approach, or bottom-up, cells, together with biomaterials, are printed using the tridimensional technology to obtain the engineered tissue. The first one has different limitations that are solved in the second one, such as diffusion limitations and slow vascularization. However, most purists consider that just the modular approach represents three-dimensional bioprinting. The number of applications of 3D printing in medicine has increased over the past years. It can be used today to obtain customized implants, pre-surgery models, prostheses, exoskeletons, and even in drug discovery, delivery, and dosage forms (Klein et al. 2013). Hence, the uses of this technology belong to two different categories: the production of tissues and organs and research in the pharmaceutical area. For the latter, 3D bioprinting has been used to determine if a particular drug could work as a treatment for a specific patient as a screening technology (Banks 2013). Some strategies can be used to overcome some of the limitations of the process itself. For example, cells can also be printed using liver spheroids to protect them from the effect of shear stress as it can be generated along with the procedure (Bhise et al. 2016).

Currently, 3D bioprinting has the potential to produce complex organs, such as the liver, with a high density of cells (Zhang et al. 2017). Nevertheless, there are some limitations still to be solved. It is the case of achieving the appropriate vascular network. However, it can considerably simplify the process compared with traditional approaches. Due to the described characteristics of this technology, some companies have decided to invest in it, like Organovo™.

They have been able to generate 3D vascularized liver constructs using different types of cells, including HSC, LSEC, and hepatocytes. They have identified high

cellular viability, as well as robustness for drug metabolism, resembling native hepatic lobules. At the end of 2016, this company announced that it would investigate the use of 3D bioprinting technology to obtain human liver tissue that could be transplanted directly to human patients (Delivery 2016). Their plan was focused on obtaining a clinical solution for acute-on-chronic liver failure and pediatric errors in metabolism. The preclinical studies that have been carried out demonstrated that after 60 days of implantation, there was sufficient vascularization, as well as engraftment and functionality. The next step was to test this approach to humans. However, due to the difficulty of a long-term function of non-vascularized bioprinted tissues after transplantation in larger animal models, the company investors declared that they would not proceed further, and the company stopped its research activities in May 2019.

4 Regulatory Landscape for Tissue-Engineered Livers

In legal terms, the regulatory landscape of liver tissue engineering and regenerative medicine, in general, is uninterruptedly moving toward a more favorable situation, allowing the creation and commercialization of new innovative products that can improve the quality of life of patients in need. These types of products are challenging due to the novelty, complexity, and technical specificity, so it is essential to understand the regulations that guarantee the quality and safety of these novel products. Next, three different regulatory agencies will be described: the Food and Drug Administration (FDA) in the USA, the European Medicines Agency (EMA) in Europe, and the Ministry of Health, Labour, and Welfare (MHLW) in Japan. All these organizations have similar objectives, but their systems of operation are different, and the approval of one of them does not imply the endorsement by the other (Bertram et al. 2013).

4.1 Food and Drug Administration (FDA)

In the USA, the FDA's Center for Biologics Evaluation and Research is the regulatory agency responsible for guaranteeing the safety, purity, potency, and effectiveness of many biologically derived products. All these functions are performed by the six centers and the several offices in which the FDA is divided.

The field of tissue engineering is englobed under the term "tissue-engineered medical products" (TEMP). This new terminology was defined in a standard document of the American Society for Testing and Materials, and it was included in the FDA-recognized consensus standards database (ASTM F2312-11). The term TEMP can consist of a variety of different constituents as cells, scaffolds, device, or any combination of these, and the FDA classifies these products as combination products. The US Congress recognized the existence of combination products when it enacted the Safe Medical Device Act of 1990, and it was defined in the 21 Code of Federal Regulation 1271 Part C 210/211/820 (CFR; FDA). The offices involved in

the evaluation of TEMPs include the Office of Combination Products (OCP), the Office of Regulatory Affairs (ORA), and the Office of Orphan Products (OOP). Notably, the Office of Combination Products (OCP) determines the primary mode of action (PMOA). PMOA establishes its regulatory and product development framework and assigns it to the proper center to lead the review of that product, with the other two centers providing input (Montagne et al. 2011). The PMOA is such an essential concept that the FDA published a docket in August 2005 entitled *Definition of Primary Mode of Action of a Combination Product*. The PMOA is defined as "the single model of action of a combination product that provides the most important therapeutic effect of the combination product" (Food and Drug Administration 2005).

4.2 European Medicines Agency (EMA)

The EMA is the agency of the European Union in charge of the evaluation and supervision of medicinal products.

Under the term, advanced therapy medicinal products (ATMP) are englobed in the field of liver tissue engineering. ATMP is defined as being a somatic cell therapy medicinal product (SCTMP), a tissue-engineered product (TEP), a gene therapy medicinal product (GTMP), or a combined ATPM (EMA 2018).

Considering the progress of liver tissue engineering in particular and regenerative medicine in general, in 2007, the European Parliament and Council of the European Union (EU) issued an amendment to Directive 2001/83/EC and Regulation No. 776/2004 to include regulatory provisions for ATMPs defined in Regulation EC No 1394/2007. This regulation established that when a product contained viable cells or tissues, the pharmacological, immunological, or metabolic action of those cells or tissues has to be considered as the principal mode of action of the product (EPC 2007). To offer high-level expertise to assess the quality, safety, and efficacy of ATMPs, the EMA established a multidisciplinary committee called the committee for advanced therapies (CAT). This committee is responsible for reviewing applications for marketing authorization for advanced therapy medicinal products (EMA 2017). The application for the authorization of an ATMP is supervised down in Article 6 of Regulation No 726/2004 (EMA 2004), and among other requirement, the application should also include the description of the product design method by the Annex 1 to Directive 2001/83/EC (EPC 2001).

4.3 Ministry of Health, Labour, and Welfare (MHLW)

The Ministry of Health, Labour, and Welfare (MHLW) is the regulatory body that oversees food and drugs in Japan, which includes creating and implementing safety standards for medical devices and drugs (JPMA 2015).

Japan established that liver tissue engineering under a regulatory landscape is considered as cellular and tissue-based product. In Japan, the approved

cell/tissue-engineered products were regulated as medical devices by adapting existing legislation under clause 2 of the Pharmaceutical Affairs Law (PAL) (Yano et al. 2015). These types of products are intended to be used for reconstruction, repair, or formulation of a structure or function of the body and treatment or prevention of disease or to be inducted into human cells for gene therapy. The underlying technical requirements to assure the quality and safety of these cellular and tissue-based products are specified in Notification No. 0912006 of the PFSB dated September 12, 2008.

In conjunction with the MHLW, the Pharmaceuticals and Medical Device Agency (PMDA) is an independent agency that is responsible for the in-country representation, certification processes, licensing, and quality assurance systems (Jokura et al. 2018). The PMDA consist of 25 offices, and the office of cellular and tissue-based products is the one in charge to confirm clinical trial notifications and adverse drug reactions, and it also conducts reviews required for approval, reexaminations, and reevaluations of regenerative medical products (cellular and tissue-based products and gene therapy products), preliminary reviews for approval or verification based on the Cartagena Protocol, and quality review of antibody preparations (JPMA 2017).

5 Conclusions

This chapter is focused on state-of-the-art strategies for liver tissue engineering. Through the different sections, it has been explained the current need of available organs for human liver transplantation, emphasizing the importance of achieving an optimal method of liver decellularization and recellularization to create new organs, as well as the development of innovative and efficient models for the study of the liver trying to recreate the physiological aspects found in vivo. The regulatory landscape is also discussed to have a global idea concerning the creation and commercialization of tissue-engineered products that can improve the quality of life of patients in need.

Even though regenerative medicine has exponentially increased in the last years, giving new hopes to the development of effective treatments for liver diseases, further work has to be performed in order to obtain functional therapies and models that represent the liver to its fullest. Critical components to consider in the liver tissue engineering field include the proper technology to obtain a high-quality scaffold after decellularization, as well as the identification, selection, and large-scale production of the most suitable cell sources for the most effective scaffold seeding.

Optimal recellularization is also a crucial step to generate a clinically functional organ. To accomplish this goal, bioreactors are an excellent tool to control all the process parameters. Nevertheless, their configuration is critical for the control of vital parameters such as oxygen, nutrient supply to cells, the control of biochemical concentrations, and gradients. However, it is essential to highlight that large scaffolds are complex and highly depend on cell metabolism and bioreactor

configurations. Up to date, a complete recellularization using all the necessary liver cell types and the generation of a fully functional liver has not been accomplished.

In the last years, the technological advances in liver bioengineering have been used to understand the hepatic regeneration mechanism better, and it has facilitated the development of different in vitro models and ex vivo culture systems. Through this chapter, we have described relevant sophisticated engineering tools, as liver organoids, liver buds, and 3D bioprinting technology. All these in vitro models show a more precise control over the liver cell microenvironment, and they allow us to enhance our knowledge of disease development and progression, demonstrating faithful recapitulation of disease pathways in vitro.

The rapid advances and improvements achieved in tissue engineering require rigorous control over the laws and regulations of each country. For these reasons, the regulatory landscape of some regulatory agencies as the FDA, EMA, and MHLW has been intensely discussed.

In conclusion, liver tissue bioengineering requires a multidisciplinary approach, with biologists, clinicians, and bioengineers working closely to understand further how liver cells can self-organize to build the liver, one of the most complex organs in our body, in order to get a transplantable liver for those in need.

Acknowledgments This work was supported by Instituto de Salud Carlos III, through a predoctoral fellowship i-PFIS IFI15/00158 (I.P-P), by MINECO through a Torres Quevedo Grant fellowship PTQ-17-09267 (N.S-R), and by a predoctoral fellowship from Fundação para a Ciência e a Tecnologia Portugal through PD/BD/144057/2015 (S.M) and PMB and was supported with the project PI15/00563 and PI18/00529 from Instituto de Salud Carlos III, Spain, with project CIBEREHD EHD16PI02 from CIBERehd, Spain, and with the project LMP226_18 from the Government of Aragon, Spain.

References

AACC (2019). https://labtestsonline.org/conditions/liver-disease

Agmon G, Christman KL (2016) Controlling stem cell behavior with decellularized extracellular matrix scaffolds. Curr Opin Solid State Mater Sci 20:193–201

Allen JW, Hassanein T, Bhatia SN (2001) Advances in bioartificial liver devices. Hepatology 34:447–455

Altman GH, Lu HH, Horan RL, Calabro T, Ryder D, Kaplan DL, Stark P, Martin I, Richmond JC, Vunjak-Novakovic G (2002) Advanced bioreactor with controlled application of multi-dimensional strain for tissue engineering. J Biomech Eng 124:742–749

Aninat C, Piton A, Glaise D, Le Charpentier T, Langouet S, Morel F, Guguen-Guillouzo C, Guillouzo A (2006) Expression of cytochromes P450, conjugating enzymes, and nuclear receptors in human hepatoma HepaRG cells. Drug Metab Dispos 34:75–83

Asrani SK, Devarbhavi H, Eaton J, Kamath PS (2019) Burden of liver diseases in the world. J Hepatol 70:151–171

ASTM F2312-11 (2020) Standard Terminology Relating to Tissue Engineered Medical Products. https://www.astm.org/Standards/F2312.html.

Banks J (2013) Adding value in additive manufacturing: researchers in the United Kingdom and Europe look to 3D printing for customization. IEEE Pulse 4:22–26

Baptista PM, Siddiqui MM, Lozier G, Rodriguez SR, Atala A, Soker S (2011) The use of whole organ decellularization for the generation of a vascularized liver organoid. Hepatology 53:604–617
Barakat O, Abbasi S, Rodriguez G, Rios J, Wood RP, Ozaki C, Holley LS, Gauthier PK (2012) Use of decellularized porcine liver for engineering humanized liver organ. J Surg Res 173:e11–e25
Bernal W, Auzinger G, Dhawan A, Wendon J (2010) Acute liver failure. Lancet 376:190–201
Bertram T, Hellman KB, Bayon Y, Ellison S, Wilburn S (2013) The regulatory imperative: international perspective. Tissue Eng Part B Rev 19:191–193
Bhise NS, Manoharan V, Massa S, Tamayol A, Ghaderi M, Miscuglio M, Lang Q, Shrike Zhang Y, Shin SR, Calzone G et al (2016) A liver-on-a-chip platform with bioprinted hepatic spheroids. Biofabrication 8:014101
Bosch FX, Ribes J, Diaz M, Cleries R (2004) Primary liver cancer: worldwide incidence and trends. Gastroenterology 127:S5–S16
Broering DC, Schulte am Esch J, Fischer L, Rogiers X (2004) Split liver transplantation. HPB (Oxford) 6:76–82
Buhler NE, Schulze-Osthoff K, Konigsrainer A, Schenk M (2015) Controlled processing of a full-sized porcine liver to a decellularized matrix in 24 h. J Biosci Bioeng 119:609–613
Cai J, Ito M, Westerman KA, Kobayashi N, Leboulch P, Fox IJ (2000) Construction of a non-tumorigenic rat hepatocyte cell line for transplantation: reversal of hepatocyte immortalization by site-specific excision of the SV40 T antigen. J Hepatol 33:701–708
Carpentier B, Gautier A, Legallais C (2009) Artificial and bioartificial liver devices: present and future. Gut 58:1690–1702
Catapano G (1996) Mass transfer limitations to the performance of membrane bioartificial liver support devices. Int J Artif Organs 19:18–35
Catapano G, Gerlach JC (2007) Bioreactors for liver tissue engineering. In: Ashammakhi N, Reis R, Chiellini E (eds) Topics in tissue engineering. Vol 3. pp 1–41
CFR F. Code of Federal Regulations Title 21 Parts 210 and 211. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm
Cohen JC, Horton JD, Hobbs HH (2011) Human fatty liver disease: old questions and new insights. Science 332:1519–1523
Crapo PM, Gilbert TW, Badylak SF (2011) An overview of tissue and whole organ decellularization processes. Biomaterials 32:3233–3243
De Kock J, Ceelen L, De Spiegelaere W, Casteleyn C, Claes P, Vanhaecke T, Rogiers V (2011) Simple and quick method for whole-liver decellularization: a novel in vitro three-dimensional bioengineering tool? Arch Toxicol 85:607–612
Delivery DDa (2016) Organovo introduces 3D bioprinted human liver as leading therapeutic tissue in preclinical development. https://ir.organovo.com/news-releases/news-release-details/organovo-introduces-3dbioprinted-human-liver-leading/
Demetriou AA, Brown RS Jr, Busuttil RW, Fair J, McGuire BM, Rosenthal P, Am Esch JS 2nd, Lerut J, Nyberg SL, Salizzoni M et al (2004) Prospective, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. Ann Surg 239:660–667. discussion 667–670
Dhawan A, Puppi J, Hughes RD, Mitry RR (2010) Human hepatocyte transplantation: current experience and future challenges. Nat Rev Gastroenterol Hepatol 7:288–298
Dvir-Ginzberg M, Gamlieli-Bonshtein I, Agbaria R, Cohen S (2003) Liver tissue engineering within alginate scaffolds: effects of cell-seeding density on hepatocyte viability, morphology, and function. Tissue Eng 9:757–766
Ebrahimkhani MR, Neiman JA, Raredon MS, Hughes DJ, Griffith LG (2014) Bioreactor technologies to support liver function in vitro. Adv Drug Deliv Rev 69–70:132–157
EMA (2004) Regulation No 726/2004 of the European Parliament and of the Council of 31 March 2004 laying down community procedures for the authorization and supervision of medicinal products for human and veterinary use establishing a European Medicines Agency
EMA (2017) EMA reflection paper on classification of advanced therapy medicinal products, EMA/CAT 600280/2010 rev.1

EMA (2018) Advanced therapy medicinal products (ATMPs) and ATMP Regulation. https://www.ema.europa.eu/en/documents/presentation/presentation-advanced-therapy-medicinal-products-atmps-atmp-regulation-patrick-celis_en.pdf
EPC (2001) Directive 2001/83/EC of the European Parliament and of the council of 6 November 2001 on the Community code relating to medicinal products for human use
EPC (2007) Regulation (EC) No 1394/2007 of the European Parliament and of the council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004
Fattovich G, Stroffolini T, Zagni I, Donato F (2004) Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 127:S35–S50
Faulk DM, Wildemann JD, Badylak SF (2015) Decellularization and cell seeding of whole liver biologic scaffolds composed of extracellular matrix. J Clin Exp Hepatol 5:69–80
FDA, H.P. FDA, USA. Regulation of human cells, tissues, and cellular and tissue-based products (HCT/Ps). 21 CFR, Parts 1270 and 1271
Fiegel HC, Kaufmann PM, Bruns H, Kluth D, Horch RE, Vacanti JP, Kneser U (2008) Hepatic tissue engineering: from transplantation to customized cell-based liver directed therapies from the laboratory. J Cell Mol Med 12:56–66
Food, and Drug Administration, H.H.S (2005) Definition of primary mode of action of a combination product. Final rule. Fed Regist 70:49848–49862
Freed LE, Vunjak-Novakovic G (2000) Tissue engineering bioreactors, 2nd edn. Academic, London
Fukumitsu K, Yagi H, Soto-Gutierrez A (2011) Bioengineering in organ transplantation: targeting the liver. Transplant Proc 43:2137–2138
Gao Y, Xu XP, Hu HZ, Yang JZ (1999) Cultivation of human liver cell lines with microcarriers acting as biological materials of bioartificial liver. World J Gastroenterol 5:221–224
Gerlach J, Unger J, Hole O, Encke J, Muller C, Neuhaus P (1994) Bioreactor for long-term maintenance of differentiated hepatic cell functions. ALTEX 11:207–215
Griffith LG, Naughton G (2002) Tissue engineering – current challenges and expanding opportunities. Science 295:1009–1014
Guguen-Guillouzo C, Corlu A, Guillouzo A (2010) Stem cell-derived hepatocytes and their use in toxicology. Toxicology 270:3–9
Guillouzo A (1998) Liver cell models in in vitro toxicology. Environ Health Perspect 106(Suppl 2):511–532
Huang P, He Z, Ji S, Sun H, Xiang D, Liu C, Hu Y, Wang X, Hui L (2011) Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature 475:386–389
Huch M, Koo BK (2015) Modeling mouse and human development using organoid cultures. Development 142:3113–3125
Huch M, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MM, Ellis E, van Wenum M, Fuchs SA, de Ligt J et al (2015) Long-term culture of genome-stable bipotent stem cells from adult human liver. Cell 160:299–312
Iansante V, Mitry RR, Filippi C, Fitzpatrick E, Dhawan A (2018) Human hepatocyte transplantation for liver disease: current status and future perspectives. Pediatr Res 83:232–240
Ibars EP, Cortes M, Tolosa L, Gomez-Lechon MJ, Lopez S, Castell JV, Mir J (2016) Hepatocyte transplantation program: lessons learned and future strategies. World J Gastroenterol 22:874–886
Jadlowiec CC, Taner T (2016) Liver transplantation: current status and challenges. World J Gastroenterol 22:4438–4445
Jauregui HO, Naik S, Santangini H, Pan J, Trenkler D, Mullon C (1994) Primary cultures of rat hepatocytes in hollow fiber chambers. In Vitro Cell Dev Biol Anim 30A:23–29
Johnson LB, Aiken J, Mooney D, Schloo BL, Griffith-Cima L, Langer R, Vacanti JP (1994) The mesentery as a laminated vascular bed for hepatocyte transplantation. Cell Transplant 3:273–281

Jokura Y, Yano K, Yamato M (2018) Comparison of the new Japanese legislation for expedited approval of regenerative medicine products with the existing systems in the USA and European Union. J Tissue Eng Regen Med 12:e1056–e1062

JPMA (2015) Quality and safety assurance for drug products or medicinal devices with processed (homogeneous) human-derived cells or tissue. http://www.jpma.or.jp/english/parj/pdf/2015.pdf

JPMA (2017) Pharmaceutical administration and regulations in Japan. http://www.jpma.or.jp/english/parj/pdf/2017.pdf

Kaufmann PM, Kneser U, Fiegel HC, Kluth D, Herbst H, Rogiers X (1999) Long-term hepatocyte transplantation using three-dimensional matrices. Transplant Proc 31:1928–1929

Kidambi S, Yarmush RS, Novik E, Chao P, Yarmush ML, Nahmias Y (2009) Oxygen-mediated enhancement of primary hepatocyte metabolism, functional polarization, gene expression, and drug clearance. Proc Natl Acad Sci U S A 106:15714–15719

Kim BS, Baez CE, Atala A (2000a) Biomaterials for tissue engineering. World J Urol 18:2–9

Kim SS, Sundback CA, Kaihara S, Benvenuto MS, Kim BS, Mooney DJ, Vacanti JP (2000b) Dynamic seeding and in vitro culture of hepatocytes in a flow perfusion system. Tissue Eng 6:39–44

Klein GT, Lu Y, Wang MY (2013) 3D printing and neurosurgery – ready for prime time? World Neurosurg 80:233–235

Ko IK, Peng L, Peloso A, Smith CJ, Dhal A, Deegan DB, Zimmerman C, Clouse C, Zhao W, Shupe TD et al (2015) Bioengineered transplantable porcine livers with re-endothelialized vasculature. Biomaterials 40:72–79

Koike H, Ueno Y, Naito T, Shiina T, Nakata S, Ouchi R, Obana Y, Sekine K, Zheng YW, Takebe T et al (2014) Ring1B promotes hepatic stem/progenitor cell expansion through simultaneous suppression of Cdkn1a and Cdkn2a in mice. Hepatology 60:323–333

Kono Y, Yang S, Letarte M, Roberts EA (1995) Establishment of a human hepatocyte line derived from primary culture in a collagen gel sandwich culture system. Exp Cell Res 221:478–485

Laconi E, Oren R, Mukhopadhyay DK, Hurston E, Laconi S, Pani P, Dabeva MD, Shafritz DA (1998) Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. Am J Pathol 153:319–329

Lancaster MA, Knoblich JA (2014) Organogenesis in a dish: modeling development and disease using organoid technologies. Science 345:1247125

Langer R, Vacanti J (1993) Tissue engineering. Science 260:920–926

Lee JS, Shin J, Park HM, Kim YG, Kim BG, Oh JW, Cho SW (2014) Liver extracellular matrix providing dual functions of two-dimensional substrate coating and three-dimensional injectable hydrogel platform for liver tissue engineering. Biomacromolecules 15:206–218

Li J, Li LJ, Cao HC, Sheng GP, Yu HY, Xu W, Sheng JF (2005) Establishment of highly differentiated immortalized human hepatocyte line with simian virus 40 large tumor antigen for liver based cell therapy. ASAIO J 51:262–268

Li J, Xing F, Chen F, He L, So KF, Liu Y, Xiao J (2018) Functional 3D human liver bud assembled from MSC-derived multiple liver cell lineages. Cell Transplant:963689718780332

Lim YS, Kim WR (2008) The global impact of hepatic fibrosis and end-stage liver disease. Clin Liver Dis 12:733–746. vii

Lin P, Chan WC, Badylak SF, Bhatia SN (2004) Assessing porcine liver-derived biomatrix for hepatic tissue engineering. Tissue Eng 10:1046–1053

Linke K, Schanz J, Hansmann J, Walles T, Brunner H, Mertsching H (2007) Engineered liver-like tissue on a capillarized matrix for applied research. Tissue Eng 13:2699–2707

Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY et al (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380:2095–2128

Mao SA (2017) Sustained in vivo perfusion of a re-endothelialized tissue engineered porcine liver. Int J Transplant Res Med 3:2572–4045

Marion MJ, Hantz O, Durantel D (2010) The HepaRG cell line: biological properties and relevance as a tool for cell biology, drug metabolism, and virology studies. Methods Mol Biol 640:261–272
Martin Y, Vermette P (2005) Bioreactors for tissue mass culture: design, characterization, and recent advances. Biomaterials 26:7481–7503
Martin I, Wendt D, Heberer M (2004) The role of bioreactors in tissue engineering. Trends Biotechnol 22:80–86
Matsumoto K, Yoshitomi H, Rossant J, Zaret KS (2001) Liver organogenesis promoted by endothelial cells prior to vascular function. Science 294:559–563
Methods N (2018) Method of the Year 2017: organoids. Nat Methods 15:1
Micsenyi A, Tan X, Sneddon T, Luo JH, Michalopoulos GK, Monga SP (2004) Beta-catenin is temporally regulated during normal liver development. Gastroenterology 126:1134–1146
Miyazaki M, Handa Y, Oda M, Yabe T, Miyano K, Sato J (1985) Long-term survival of functional hepatocytes from adult rat in the presence of phenobarbital in primary culture. Exp Cell Res 159:176–190
Mokdad AA, Lopez AD, Shahraz S, Lozano R, Mokdad AH, Stanaway J, Murray CJ, Naghavi M (2014) Liver cirrhosis mortality in 187 countries between 1980 and 2010: a systematic analysis. BMC Med 12:145
Montagne K, Huang HY, Ohara K, Matsumoto K, Mizuno A, Ohta K, Sakai Y (2011) Use of liposome encapsulated hemoglobin as an oxygen carrier for fetal and adult rat liver cell culture. J Biosci Bioeng 112:485–490
Mooney D, Hansen L, Vacanti J, Langer R, Farmer S, Ingber D (1992) Switching from differentiation to growth in hepatocytes: control by extracellular matrix. J Cell Physiol 151:497–505
Morini S, Sánchez-Romero N, Plá IP, de la Maza Arnal PS, Almeida M, Verscheijden L, Almeida JI, Luè A, Llorente S, Almeida H, Royo Dachary P, García Gil A, Serrano-Aulló T, Baptista PM (2018) Liver tissue engineering. In: Cabral J, da Silva CL (eds) Bioreactors for stem cell expansion and differentiation. Taylor & Francis, Boca Raton
Murphy SV, Atala A (2014) 3D bioprinting of tissues and organs. Nat Biotechnol 32:773–785
Nahmias Y, Berthiaume F, Yarmush ML (2007) Integration of technologies for hepatic tissue engineering. Adv Biochem Eng Biotechnol 103:309–329
Najimi M, Khuu DN, Lysy PA, Jazouli N, Abarca J, Sempoux C, Sokal EM (2007) Adult-derived human liver mesenchymal-like cells as a potential progenitor reservoir of hepatocytes? Cell Transplant 16:717–728
Nguyen TV, Ukairo O, Khetani SR, McVay M, Kanchagar C, Seghezzi W, Ayanoglu G, Irrechukwu O, Evers R (2015) Establishment of a hepatocyte-kupffer cell coculture model for assessment of proinflammatory cytokine effects on metabolizing enzymes and drug transporters. Drug Metab Dispos 43:774–785
Nuciforo S, Fofana I, Matter MS, Blumer T, Calabrese D, Boldanova T, Piscuoglio S, Wieland S, Ringnalda F, Schwank G et al (2018) Organoid models of human liver cancers derived from tumor needle biopsies. Cell Rep 24:1363–1376
Ohashi K, Park F, Kay MA (2001) Hepatocyte transplantation: clinical and experimental application. J Mol Med (Berl) 79:617–630
Ohashi K, Yokoyama T, Yamato M, Kuge H, Kanehiro H, Tsutsumi M, Amanuma T, Iwata H, Yang J, Okano T et al (2007) Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. Nat Med 13:880–885
Parkin DM, Bray F, Ferlay J, Pisani P (2001) Estimating the world cancer burden: Globocan 2000. Int J Cancer 94:153–156
Parmaksiz M, Dogan A, Odabas S, Elcin AE, Elcin YM (2016) Clinical applications of decellularized extracellular matrices for tissue engineering and regenerative medicine. Biomed Mater 11:022003
Powers MJ (1997) Cell-substratum adhesion strength as a determinant of hepatocyte aggregate morphology. Biotechnol Bioeng 53(4):415–26. https://doi.org/10.1002/(SICI)1097-0290(19970220)53:4<415::AIDBIT10>3.0.CO;2-F.

Prior N, Inacio P, Huch M (2019) Liver organoids: from basic research to therapeutic applications. Gut 68(12):2228–2237 https://doi.org/10.1136/gutjnl-2019-319256
Robb KP, Shridhar A, Flynn LE (2018) Decellularized matrices as cell-instructive scaffolds to guide tissue-specific regeneration. ACS Biomater Sci Eng 4:3627–3643
Rossi JM, Dunn NR, Hogan BL, Zaret KS (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev 15:1998–2009
Rozga J, Williams F, Ro MS, Neuzil DF, Giorgio TD, Backfisch G, Moscioni AD, Hakim R, Demetriou AA (1993) Development of a bioartificial liver: properties and function of a hollow-fiber module inoculated with liver cells. Hepatology 17:258–265
Rozga J, Morsiani E, Lepage E, Moscioni AD, Demetriou AA, Giorgio T (1994) Isolated hepatocytes in a bioartificial liver: a single group view and experience. Biotechnol Bioeng 43:645–653
Serls AE, Doherty S, Parvatiyar P, Wells JM, Deutsch GH (2005) Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. Development 132:35–47
Shupe T, Williams M, Brown A, Willenberg B, Petersen BE (2010) Method for the decellularization of intact rat liver. Organogenesis 6:134–136
Soto-Gutierrez A, Zhang L, Medberry C, Fukumitsu K, Faulk D, Jiang H, Reing J, Gramignoli R, Komori J, Ross M et al (2011) A whole-organ regenerative medicine approach for liver replacement. Tissue Eng Part C Methods 17:677–686
Starzl TE, Francavilla A, Halgrimson CG, Francavilla FR, Porter KA, Brown TH, Putnam CW (1973) The origin, hormonal nature, and action of hepatotrophic substances in portal venous blood. Surg Gynecol Obstet 137:179–199
Stephenne X, Najimi M, Ngoc DK, Smets F, Hue L, Guigas B, Sokal EM (2007) Cryopreservation of human hepatocytes alters the mitochondrial respiratory chain complex 1. Cell Transplant 16:409–419
Strom S, Fisher R (2003) Hepatocyte transplantation: new possibilities for therapy. Gastroenterology 124:568–571
Struecker B, Raschzok N, Sauer IM (2014) Liver support strategies: cutting-edge technologies. Nat Rev Gastroenterol Hepatol 11:166–176
Suzuki A, Sekiya S, Buscher D, Izpisua Belmonte JC, Taniguchi H (2008) Tbx3 controls the fate of hepatic progenitor cells in liver development by suppressing p19ARF expression. Development 135:1589–1595
Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, Zhang RR, Ueno Y, Zheng YW, Koike N et al (2013) Vascularized and functional human liver from an iPSC-derived organ bud transplant. Nature 499:481–484
Takebe T, Sekine K, Kimura M, Yoshizawa E, Ayano S, Koido M, Funayama S, Nakanishi N, Hisai T, Kobayashi T et al (2017) Massive and reproducible production of liver buds entirely from human pluripotent stem cells. Cell Rep 21:2661–2670
Tiruvannamalai-Annamalai R, Armant DR, Matthew HW (2014) A glycosaminoglycan based, modular tissue scaffold system for rapid assembly of perfusable, high cell density, engineered tissues. PLoS One 9:e84287
Tong JZ, Bernard O, Alvarez F (1990) Long-term culture of rat liver cell spheroids in hormonally defined media. Exp Cell Res 189:87–92
Torok E, Pollok JM, Ma PX, Kaufmann PM, Dandri M, Petersen J, Burda MR, Kluth D, Perner F, Rogiers X (2001) Optimization of hepatocyte spheroid formation for hepatic tissue engineering on three-dimensional biodegradable polymer within a flow bioreactor prior to implantation. Cells Tissues Organs 169:34–41
Torok E, Vogel C, Lutgehetmann M, Ma PX, Dandri M, Petersen J, Burda MR, Siebert K, Dullmann J, Rogiers X et al (2006) Morphological and functional analysis of rat hepatocyte spheroids generated on poly(L-lactic acid) polymer in a pulsatile flow bioreactor. Tissue Eng 12:1881–1890
Tostoes RM, Leite SB, Serra M, Jensen J, Bjorquist P, Carrondo MJ, Brito C, Alves PM (2012) Human liver cell spheroids in extended perfusion bioreactor culture for repeated-dose drug testing. Hepatology 55:1227–1236

Uygun BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F et al (2010) Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat Med 16:814–820

Vendrely C (1950) Contribution to the cytochemical study of nucleic acids of some cell organoids. Arch Anat Histol Embryol 33:113–155

Voytik-Harbin SL, Brightman AO, Kraine MR, Waisner B, Badylak SF (1997) Identification of extractable growth factors from small intestinal submucosa. J Cell Biochem 67:478–491

Wagner BA, Venkataraman S, Buettner GR (2011) The rate of oxygen utilization by cells. Free Radic Biol Med 51:700–712

Wang LJ, Chen YM, George D, Smets F, Sokal EM, Bremer EG, Soriano HE (2002) Engraftment assessment in human and mouse liver tissue after sex-mismatched liver cell transplantation by real-time quantitative PCR for Y chromosome sequences. Liver Transpl 8:822–828

Wang Y, Nicolas CT, Chen HS, Ross JJ, De Lorenzo SB, Nyberg SL (2017) Recent advances in decellularization and recellularization for tissue-engineered liver grafts. Cells Tissues Organs 203:203–214

Watt KD, Pedersen RA, Kremers WK, Heimbach JK, Charlton MR (2010) Evolution of causes and risk factors for mortality post-liver transplant: results of the NIDDK long-term follow-up study. Am J Transplant 10:1420–1427

Weinstein M, Monga SP, Liu Y, Brodie SG, Tang Y, Li C, Mishra L, Deng CX (2001) Smad proteins and hepatocyte growth factor control parallel regulatory pathways that converge on beta1-integrin to promote normal liver development. Mol Cell Biol 21:5122–5131

Werner A, Duvar S, Muthing J, Buntemeyer H, Lunsdorf H, Strauss M, Lehmann J (2000) Cultivation of immortalized human hepatocytes HepZ on macroporous CultiSpher G microcarriers. Biotechnol Bioeng 68:59–70

Wolf CF, Munkelt BE (1975) Bilirubin conjugation by an artificial liver composed of cultured cells and synthetic capillaries. Trans Am Soc Artif Inter Organs 21:16–27

Wu FJ, Friend JR, Hsiao CC, Zilliox MJ, Ko WJ, Cerra FB, Hu WS (1996) Efficient assembly of rat hepatocyte spheroids for tissue engineering applications. Biotechnol Bioeng 50:404–415

Xiang JX, Zheng XL, Gao R, Wu WQ, Zhu XL, Li JH, Lv Y (2015) Liver regeneration using decellularized splenic scaffold: a novel approach in tissue engineering. Hepatobiliary Pancreat Dis Int 14:502–508

Yanagi Y, Nakayama K, Taguchi T, Enosawa S, Tamura T, Yoshimaru K, Matsuura T, Hayashida M, Kohashi K, Oda Y et al (2017) In vivo and ex vivo methods of growing a liver bud through tissue connection. Sci Rep 7:14085

Yano K, Watanab N, Tsuyuki K, Ikawa T, Kasanuki H, Yamato M (2015) Regulatory approval for autologous human cells and tissue products in the United States, the European Union, and Japan. JSRM 1:45–56

Zhang L, Pan J, Li J, Wu W, Yu Y (2003) Studies on the preparation of chitosan microcarriers cross-linked by oxidized lactose and culture of primary hepatocytes. Artif Cells Blood Substit Immobil Biotechnol 31:293–301

Zhang YS, Yue K, Aleman J, Moghaddam KM, Bakht SM, Yang J, Jia W, Dell'Erba V, Assawes P, Shin SR et al (2017) 3D bioprinting for tissue and organ fabrication. Ann Biomed Eng 45:148–163