REGENERATIVE HEPATOLOGY:

IN THE QUEST FOR A MODERN PROMETHEUS?

Authors: Margarita Papatheodoridi[1], Giuseppe Mazza[1] and Massimo Pinzani[1]

Affiliations: University College London, Institute for Liver and Digestive Health

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Corresponding author:

Massimo Pinzani,

Sheila Sherlock Liver Unit and UCL Institute for Liver and Digestive Health,

Royal Free Hospital and UCL, London, UK.

Email: m.pinzani@ucl.ac.uk

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Abbreviations: acute liver failure (ALF), bioartificial liver (BAL), end-stage liver disease (ESLD), hepatocyte-like cells (HLC), hepatocyte transplantation (HT), induced pluripotent stem cells (iPSCs), orthotopic liver transplantation (OLT), three-dimensional (3D)

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ABSTRACT (max 200words)

As liver-related morbidity and mortality is rising worldwide and orthotopic liver transplantation (OLT) remains the only standard-of-care for end-stage liver disease or acute liver failure, shortage of donor organs is becoming more prominent. Importantly, advances in regenerative Hepatology and liver bioengineering are bringing new hope to the possibility of restoring impaired hepatic functionality in the presence of acute or chronic liver failure.

Hepatocyte transplantation and artificial liver-support systems were the first strategies used in regenerative hepatology but have presented various types of efficiency limitations restricting their widespread use.

In parallel, liver bioengineering has been a rapidly developing field bringing continuously novel advancements in biomaterials, three dimensional (3D) scaffolds, cell sources and relative methodologies for creating bioengineered liver tissue. The current major task in liver bioengineering is to build small implantable liver mass for treating inherited metabolic disorders, bioengineered bile ducts for congenital biliary defects and large bioengineered liver organs for transplantation, as substitutes to donor-organs, in cases of acute or acute-on-chronic liver failure.

This review aims to summarize the state-of-the-art and upcoming technologies of regenerative Hepatology that are emerging as promising alternatives to the current standard-of care in liver disease.

INTRODUCTION

The last two decades have witnessed great progress in the research field named “Regenerative Hepatology”, in which scientists search alternative approaches to fight liver disease and restore impaired liver function. In general, regenerative medicine constitutes an interdisciplinary branch of biomedical research, which focuses on developing science and tools to repair or replace damaged tissues or organs. Major contributions in this effort derive from tissue engineering, stem cell biology and additive manufacturing.

Liver disease occurs for various causes ranging from viral hepatitis and drug toxicity to fat accumulation and autoimmune and/or cholestatic inflammation. Besides the very recent introduction of effective antiviral treatments for hepatitis C virus (HCV) infection, there is no medical treatment able to eliminate the cause of liver damage and halt disease progression to cirrhosis, end-stage liver disease (ESLD) and possibly liver cancer[1], despite the well-known regenerative ability of liver tissue. Consequently, liver disease burden is rising and, to date, the relative annual mortality reaches approximately 2 million deaths worldwide[2]. In clinical practice, orthotopic liver transplantation (OLT) remains the sole treatment option for ESLD or acute liver failure (ALF) and represents the second most common solid organ transplantation. However, only 10% of the global needs for transplantation are currently met, mainly due to scarcity of donor organs[2,3], while post-operative complications and necessary long-term immunosuppression treatments carry additional risks and suboptimal quality of life for the patient[4,5]. To this end, applications of regenerative medicine and tissue bioengineering are emerging as possible alternative to OLT.

The first “regenerative” strategy, introduced more than twenty years ago, is hepatocyte transplantation (HT) aiming to serve as a bridge to OLT or to prolong survival in

ESLD[6,7]. Nonetheless, the clinical applicability of HT has been restricted mainly due to limited efficacy and several limitations regarding cell sources, engraftment and repopulation efficiency[8,9]. Moreover, in the same years, several devices, designed mainly to replace the detoxifying function of the liver (a sort of liver dialysis) have been tested in clinical practice with controversial efficiency and applicability. More recently, extracorporeal bio-artificial liver devices aiming to provide liver detoxification together with key synthetic hepatic functions have been increasingly proposed. Although most of the proposed systems could be applicable in clinical practice, this development has been so far extremely slow with very high production costs and unclear efficacy in clinical trials, which have been, on the other hand, characterised by limited and often not homogeneous patient cohorts[10–12].

In the last decade and in parallel with the slow development and establishment of HT and bio-artificial liver devices, the field of liver bioengineering has been rapidly advancing towards the development of biomaterials, expandable cell sources and relative methodologies for developing functional liver tissues ex vivo or in vitro. These involve improvements in the biochemical and biomechanical features of three dimensional (3D) scaffolds to be employed as an essential bioactive environment for seeding cells, novel cell sources, challenging techniques to enhance cell growth and culture methods in a 3D liver bioengineered mass. The current major tasks in liver bioengineering are to build a small implantable liver mass for treating inherited metabolic disorders, bioengineered bile ducts for congenital biliary defects and large bioengineered liver organs that could be transplanted and substitute donor-organs in cases of acute or acute-on-chronic liver failure. Needless to say, tissue bioengineering projects in Hepatology seem quite ambitious and radical, but the reported success in

developing solid organs such as trachea, skin and bladder certainly contribute to maintain hopes and expectations at the highest level.

This review is aimed at summarizing the most recent advancements of regenerative Hepatology (**Figure 1**) from clinical to translational and basic research level and, in particular, focusing on cell transplantation and therapies, BAL devices and liver bioengineering for 3D-microtissues and whole liver regeneration.

CURRENT ALTERNATIVES TO LIVER TRANSPLANTATION

Hepatocyte transplantation (HT) and cell therapies

HT was originally introduced as a potential alternative to OLT and it has been proven beneficial in selected patients with inborn metabolic errors or ALF [8,13,14]. Donor livers unsuitable for transplantation, surgically resected hepatic tissue or even deceased donor organs with prolonged warm ischemia time, have so far represented common sources of primary hepatocytes. Cell isolation is performed according to a standardised collagenase three-step perfusion protocol[15,16] by cannulating the major hepatic vessels with an average yield of 3-20 x 10^6 hepatocytes per gram of liver tissue. Subsequently, viability and functionality of the hepatocytes are assessed. In practice, hepatocytes should be ABO compatible, show viability rates >50-60% and the presence of basic metabolic functions before transplantation. Hepatocytes can be sequentially infused in the liver (preferably via the portal vein), spleen or peritoneal cavity. Several infusion attempts may be needed and therefore the use of long-term intravascular catheter, i.e. intraportal port-a-cath, is usually considered. Cryopreservation is a crucial step to allow scalability of the procedure and it is usually achieved by storing the cells in liquid nitrogen after the addition of a permeable cytoprotectant (dimethyl sulfoxide 10-12%), followed by gradual temperature decrease in a controlled rate freezer-box that contains the cell vials. This method allows to safely store isolated hepatocytes until their clinical use. The process of thawing should be performed rapidly at 37^o C to avoid crystal formation, however cell viability and functionality is usually affected, except for the cases when foetal and neonatal hepatocytes are used[17].

When compared with OLT, HT can be provided to multiple recipients using a single donor source matched for blood type. In addition, the microvasculature and the biliary

network of the host organ is preserved thus potentially favouring organ recovery even in cases of extensive liver damage as in most cases of ALF[18]. However, low cell viability and proliferation rates following cryopreservation, instant blood-mediated inflammatory reaction (IBMIR) and limited cell engraftment [13,19] are the main reasons preventing a more widespread use of HT. Therefore, although there have been a significant number of studies presenting HT in hepatic inborn errors of metabolism [20–34] and ALF[35–39], the applicability and effectiveness of this approach are still debated. This is also supported by the fact that, in inborn metabolic diseases, the effective correction of metabolic errors has been always limited and/or short-lasting due to lack of cell engraftment in the recipient therefore not excluding the urgent need of OLT after the HT procedure[18,40].

Considering these limitations and technical obstacles, the most recent developments in HT have been characterized by the introduction of technical solutions aimed at improving hepatocyte sourcing including stem cells differentiated towards hepatic lineage, their proliferative capacity and engraftment rate associated with strategies to monitor hepatocyte survival and function after HT[18]. Along these lines, several research groups have proposed to substitute allogenic primary hepatocytes with embryonic, mesenchymal or autologous induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells (HLCs), while others insist on the value of using primary hepatocytes for direct transplantation. Therefore, efforts have been made to enhance hepatocyte viability[41], to improve cell yield and survival[42,43] and to more precisely assess hepatocyte functionality before and after HT[44]. In addition, other technical approaches have been proposed to improve engraftment in the host liver, which is considered the main challenge of the whole approach. Accordingly, irradiation of the host liver has been studied in both rats and humans showing some benefit regarding

cell proliferation[45]. Even better results have been obtained by employing the combination of focal radiation with hepatic cell growth stimuli (hepatic mitogen GC-1) in mice[46]. Other approaches include gene therapy to provide a selected repopulating advantage to hepatocytes[47] or strategies to prevent immunological activation that would hamper engraftment[48]. Finally, monitoring hepatocyte fate after infusion and understanding if alterations of liver enzyme/function tests following HT are caused by the transplanted cells or by the background liver disease represents an additional challenge of HT. Recent findings on the donor-specific antibody's predictive ability on graft loss in solid organ transplantation could be applicable also to HT[49], while non-invasive imaging techniques using various cell-labelling particles can allow cell localization and post-HT monitoring[50,51].

Artificial liver support and bioartificial liver (BAL) devices

A different approach to support liver disease patients in need of transplantation is provided by artificial or bio-artificial liver (BAL) systems. The use of artificial liver devices attempting to replace the hepatic detoxification function through a system of filters has been proposed for several years. Despite some limited clinical utility, the main drawback of these systems is the lack of necessary support to the metabolic and synthetic functions of the liver[10]. A very recent meta-analysis[52] assessed 25 randomised controlled trials evaluating the effectiveness of extracorporeal artificial liver support systems in 1796 patients. The results showed reduced overall mortality and severity of hepatic encephalopathy in patients with ALF or acute-on-chronic liver failure who were on artificial liver support compared to those who were not. However, it was uncertain if the risk of complications, such as hypotension, bleeding,

thrombocytopenia and line infection was affected and it was concluded that larger clinical trials are warranted[52].

Later, cell-based BAL systems were also introduced[53]. Essential functions of a BAL should include ammonia detoxification, elimination of bile components, xenobiotic metabolism and synthesis of albumin and coagulation factors. In addition to these basic requirements and from a more technical standpoint, multicellularity, microarchitecture and zonation, vasculature, sufficient oxygen supply and bile removal should be provided by state-of-the-art BAL systems[10,54]. Since these systems require large amounts of primary hepatocytes, which, as previously mentioned, are scarce, cell lines or xenobiotic cells, mainly from pigs, are most commonly used to provide a sufficient hepatocyte-like cellular mass for these devices. Two BAL systems have been more extensively used in clinical practice and have undergone clinical trials in the last decade: the ELAD[55] and the HepatAssist[56], which use human hepatoblastoma cell line (HepG2)/C3A and healthy porcine hepatocytes in hollow-fiber membrane bioreactors, respectively. However, none of them has shown to significantly improve overall survival and none has obtained Food and Drug Administration (FDA) approval to date.

More recently, alternative cell sources for BAL devices have been evaluated in preclinical studies. These include stem cells[57] and hiPSCs[58], although these models have not been tested in humans yet. On the other hand, other BAL models have been designed to incorporate the cells in different types of synthetic matrix, such as nonwoven polyester[59] or alginate beads[60], in order to provide a 3-dimensional (3D) support and enhance their synthetic efficiency. Indeed, preclinical data from a GMP-designed clinical-scale BAL machine including HepG2 cells cultured in alginate beads as 3D-organoids showed promising results. In particular, this BAL developed at

University College London (UCLBAL) successfully improved 3-day survival, as well as coagulation and brain oxygenation parameters, reduced vasopressor requirements and lowered metabolic acidosis levels in a porcine model of irreversible liver failure[60].

NEW PERSPECTIVES FOR ALTERNATIVES TO LIVER TRANSPLANTATION

Liver bioengineering

While the research on artificial and bio-artificial liver support continues and could lead to more sophisticated and effective systems, it is implicit that this type of approach will be always limited to temporal bridging to OLT and, in the most fortunate cases, spontaneous recovery of a sufficient hepatic function. On the other hand, the possibility of engineering transplantable organs or at least implantable tissues opens up a new era in regenerative Hepatology.

The key paradigm of liver bioengineering is to combine various cell types within suitable biomaterials to recreate a complex 3D-environment that could resemble human liver tissue in terms of organization and functionality. Below we will discuss the various platforms and technologies used in bioengineering applications, as well as the necessary cell sources and culture methods. Finally, we will define the main objectives of this field in terms of possible clinical translations.

a. **Bioengineering platforms: From hydrogels and scaffolds to decellularized whole livers**

The addition of a third dimension to cell cultures, compared to conventional two-dimensional cultures on plastic surfaces, significantly improves cell functionality by providing a more appropriate microenvironment for the expression of the cell physiological phenotype [61]. In this context, tissue engineering has explored numerous

artificial or natural materials to create hydrogels or scaffolds that could serve as the suitable 3D background-niche for bioengineered liver constructs. Regardless of the method (hydrogel or scaffold) and type of biomaterial (natural or artificial), it should be noted that the creation of a 3D structure incorporating liver cells does not guarantee for a successful application of liver bioengineering without the presence of a fourth dimension provided by the native liver extracellular matrix (ECM)[62]. The ECM characterises less than 3% of the normal liver tissue but has a fundamental role in providing cohesiveness, leveraging cell polarization, gene expression and differentiation[63]. Liver ECM mainly consists of collagen type I and III (large fibrils), IV (net structure), V and VI (small fibrils), glycoproteins such as laminin and fibronectin, elastins, glycosaminoglycans and proteoglycans[64]. In the pathological process known as hepatic fibrogenesis, the relative proportion of the different ECM components is progressively altered with an excess of what is defined “fibrillary ECM”, i.e. denoting an overabundance of fibrillary collagens produced in excess by hepatic myofibroblasts[65]. Therefore, it is quite clear that every modification in the ECM composition alters liver structure and functionality and it is of paramount importance to preserve native ECM properties in materials used in liver bioengineering[66]. Furthermore, another characteristic which can affect biological behaviour of bioengineered liver tissues is the stiffness of the biomaterial, since it is known to affect cell growth and differentiation[67].

Development of novel biomaterials that can be used as hydrogels has brought up new opportunities to advance cell culture and tissue engineering techniques, by mimicking ECM properties and enhancing cell adhesion and growth when compared to conventional 2D culture systems[68,69]. A variety of well-characterized hydrogels based on natural or synthetic materials is currently available. Artificial polymers such as

polyethylene glycol (PEG), poly-l-lactic acid (PLLA), polycaprolactone (PCL), and polyacrylamide have been used to produce hydrogels for liver bioengineering[70–72]. Being artificial, these materials are easy to produce on large scale. They are also economically and commercially convenient, in addition of getting a relatively easier FDA approval[62]. However, they might lead to significant reduction of cell survival and growth[68,73] due to the lack of cell-instructive signals. On the other hand, polysaccharides-based (agarose, alginate, cellulose etc) or ECM-inspired biomaterials such as fibrin or collagen-based materials (i.e. Matrigel), are characterized by better biomimetic properties than the synthetic materials (PEG, PLLA, PCL etc)[62]. However, beyond the selection of materials based on their biophysical and biochemical properties, the optimal choice needs to consider the context and finalities of the final application[69]. Nonetheless, none of these biomaterials can generate the biochemical and architectural complexity of a fully assembled human liver ECM microenvironment and, indeed, synthetic scaffolds and hydrogels are characterized by limited hepatocyte viability and function[73].

An attractive “natural” solution to this problem is offered through the decellularization of liver tissues and organs. This method consists of the complete removal of the cellular component of the tissue while preserving the properties of the native ECM[74]. Acellular liver tissue can serve as the ideal scaffold maintaining intact tissue architecture, and micro- and macro-molecular ECM composition. In addition, decellularized tissue can be dried, lyophilized and then reconstituted to create a liver ECM specific-hydrogel [68,75].

The methodology for the decellularization for whole organs was pioneered by Ott et al.[76] in 2008 with the decellularization of a mouse heart, while preserving the vascular network, ECM composition and 3D architecture of the native tissue. Since 2008,

several protocols for decellularization have been proposed with the use of physical, chemical and biological agents and according to the distinct features of different organs. Regardless of the method applied, it is crucial to maintain balance between cellular removal and preservation of ECM composition and structure, as excessive exposure to decellularization reagents could damage the ECM matrix, thus causing biomolecule denaturation and/or the micro-architectural degradation[77].

Currently, perfusion decellularization represents the state-of-the-art approach to obtain the decellularization of a whole liver. According to this technique, the native complex vascular tree provides the best thoroughfare to homogenously diffuse reagents inside the tissue[68]. By applying the perfusion decellularization protocol, it has been shown that whole liver scaffolds can be obtained from the livers of small and large animals[78–84]. In 2015, the first successful attempt of decellularizing a human liver (left lobe and whole organ) was achieved by Mazza et al.[85] by using a retrograde, two-step, perfusion flow-rate methodology, cannulating the organ via the inferior vena cava. This strategy proved to be effective in preserving the fine organ architecture and the liver ECM composition as shown in scanning electron microscopy and proteomic analysis, respectively[85,86].

Decellularizing a whole human liver has represented a key step forward towards obtaining ideal natural scaffolds and has opened new perspectives in whole human liver engineering. The whole human liver acellular scaffold can sufficiently provide not only a 3D-background with fine vasculature for nutrient delivery but also maintain the micro-environmental features that allow parenchymal and non-parenchymal cells to grow, proliferate, differentiate and exert their function[68,86,87].

Finally, although xenogeneic livers have also been widely used in hepatic bioengineering, there are rational reservations regarding the interspecies differences

in the 3D structure, ECM composition and stiffness. Moreover, biocompatibility and immunogenicity issues should be considered, while differences in the vascular structure between human liver and animal livers might have detrimental hemodynamic consequences that would render a transplanted engineered liver incompatible. In view of all this, it is relevant to further stress that the ideal source of biomaterials for liver tissue engineering is clearly represented by healthy human liver.

b. Bioengineering technologies for creating liver tissue

Tissue engineering involves a variety of techniques to produce bioengineered 3D constructs combining different cell types with the appropriate 3D biomaterials. Cell microencapsulation technology, one of the first methods introduced in this field, practically consists in cells immobilization within a polymeric semi-permeable membrane that allows the bidirectional diffusion of molecules such as oxygen, nutrients and growth factors as well as the outflow of essential hepatic products (e.g. albumin, coagulation factors etc.) and waste product [88,89]. Although there are encouraging results employing primary hepatocytes encapsulated in alginate beads, unanswered questions still remain regarding the long-term viability of the encapsulated cells both in *in vitro* cultures and *in vivo* after implantation[90,91].

A technique with increased popularity in the field of tissue engineering is 3D bioprinting, which relies on 3D printers able to adequately mix cells within a biocompatible material (generally defined bio-ink) for the *in vitro* manufacturing of high precision complex bio-structures. Multiple techniques including laser, inkjet or extrusion-based bioprinting have been employed in recent studies in which hepatic cell lines, such as HepG2, HUVEC or HepRG cells, or primary human or murine hepatocytes were bio-printed with synthetic and natural materials. However, cell

survival in the bio-printed constructs was rather inhomogeneous ranging from 2 to 60 days[92–95]. A liver specific bio-ink, recently developed employing human liver ECM, has provided improved cell viability and albumin secretion in bio-printed constructs of hepatic cell lines or primary hepatocytes when compared with constructs of the same cells in nanocellulose[96]. In addition, recent data from bioprinting organoids derived from human liver iPSCs on alginate/pluronic hydrogel blends demonstrated improved hepatic functionality and prolonged survival in vitro, compared to single cell dispersion[97].

Once the methodology of tissue decellularization is established, the next key technical development in tissue bioengineering is represented by the repopulation of the 3D scaffold. This process, called recellularization, appears particularly challenging especially when considering the recellularization of whole organs with the variety of parenchymal and non-parenchymal cell types typical of the hepatic tissue.

Various methods of recellularization have been proposed. These include direct parenchymal injection, continuous perfusion, and multistep infusion. Based on the accumulated experience, it is accepted that the multistep infusion technique leads to increased cell engraftment and the achievement of a satisfactory level of hepatic function, including albumin production, urea metabolism and cytochrome P450 induction[98]. While it has been shown that recellularization can be achieved in small cubes of decellularized liver tissue, evidence relative to the recellularization of whole human livers derives from studies on decellularized xenograft organs [75]. However, as of today, there are no reports on the recellularization of a whole human liver scaffold. Undoubtedly, such development poses difficulties mainly due to the large number of cells needed, the limitations in the re-endothelization of the scaffold vascular network and the lack of fully automated bioreactors. Notably, whole organ re-endothelization

prior to hepatocytes reseeding represents a crucial point, since insufficient endothelial lining leads to intravascular thrombosis induced by the activation of platelet and the whole coagulation cascade[75]. Along these lines, Baptista et al. reported that both the direction of the perfusion flow from the portal vein and a high flow rate (12ml/min) led to the best cellular distribution through the parenchyma and re-endothelization after 7 days of culture[84] .

In addition to this, the presence of a balanced proportion of parenchymal and non-parenchymal cellular within the recellularization procedure plays a key role promoting the correct engraftment and functionality of parenchymal cells[83]. Finally, a fundamental issue that needs to be addressed when designing an engineered whole liver graft is the integrity of the biliary tree. It is estimated that the daily bile production is approximately 750mL and the majority is secreted by hepatocytes[99]. However, differentiation and maturation of hepatocytes at the point that they are able to secrete bile still represents a major challenge for liver bioengineering. Lately, positive results have been reported by Baptista et al., who showed that foetal hepatoblasts can differentiate into biliary and hepatic lineages when seeded in decellularized livers[84]. It has also been suggested that employing 3D scaffold of native ECM will favour organ-specific cell-ECM communication positively affecting the maturation of foetal hepatocytes into cholangiocytes and hepatocytes[100].

c. Cell sources for liver bioengineering: stem cells and organoid technologies

As mentioned previously, primary hepatocytes do not represent the ideal option either for cell transplantation or for liver bioengineering applications. On the other hand, cell lines are initially useful to test the feasibility of novel bioengineering applications, since

they can generate a standardised and inexpensive cell population that can easily proliferate and retain some basic hepatocellular functions[7]. However, with the perspective of creating bioengineered liver constructs for implantation or transplantation, cell lines are clearly unsuitable because of their immortalized/tumorigenic nature. Therefore, different types of stem cells have been extensively explored in liver engineering studies as potential HLCs sources. Several studies have reported the use of adult, mesenchymal or even embryonic stem cells as the seeding population of hydrogels, scaffolds or xenogenic whole livers[7,75]. Unfortunately, up to now, none of these attempts have resulted in satisfactory outcomes (**Table 1**).

More recently, the possibility of obtaining HLCs from iPSCs has emerged as a promising and almost inexhaustible cell source that could generate both parenchymal and non-parenchymal cells to be employed in liver bioengineering[101]. IPSCs are developed from human somatic cells (e.g obtained from skin cells or PBMCs) that are reprogrammed to the pluripotent state and characterized by an *in vitro* differentiation capacity to HLCs under specific stimuli[102]. To date, there are various protocols for reprogramming and generating iPSCs[103,104] and for differentiating them to hepatic progenitor cells[105,106]. However, there is no conclusive evidence that they can be fully differentiated and functional *in vitro*.

On the other hand, one of the big benefits of iPSCs is that they enable autologous cell transplant, thus avoiding the possibility of immune rejection and eliminating the need for autologous hepatocyte transplantation or immunosuppression. However, the use of autologous iPSCs still remains complicated in terms of manufacturing, upscaling and quality control compared to the use of well validated hIPSCs lines from a cell bank.

Recent advances in genetically engineered hIPSCs lines not expressing HLA class I and overexpressing HLA E could overcome this issue. Of note, personalised approaches such as matching the donor with the recipient or even manipulate human leukocyte antigen (HLA) expression of the cells used are currently proposed to avoid possible immune reactions after iPSC transplantation[107].

The extremely large number of cells needed to recreate the complexity of human livers definitely requires some important considerations. Approximately, 300 billion cells are present in the adult human liver[108,109], with the vast majority (70-80%) consisting of hepatocytes[110,111] and the rest represented by cholangiocytes and various non-parenchymal cell types[111,112]. In order to create a bioengineered organ with sufficient functional capability, all cell types are necessary to be allocated in the appropriate proportions[112,113]. Since it is considered that 30% is the critical mass necessary for ensuring liver function, the estimated minimum number of hepatocytes or HLCs to be engrafted would be 80 billion cells[109]. In order to obtain these large numbers of cells, various culture platforms may be applied that could enable this scale of *in vitro* cell expansion[114,115], although they may be time- and resource-consuming. Although stem cells or iPSCs derived HLCs might offer a better comprehensive solution, the process still requires extensive expansion of the cell population with still not completely defined effects on cell differentiation and maturation.

Liver organoids represent a novel 3D-culture approach that allows expansion and differentiation of stem cell-derived HLCs. The term “organoids” has been introduced almost a decade ago to describe a self-organizational level of 3D-culture development[116,117]. Based on their proliferative capacity, organoids can yield approximately 1 million cells from one single stem cell in two month time[116]. Another

advantage of organoids is their genetic stability, as the karyotype of the cells seems normal after several months in culture. Moreover, a whole genome-sequencing analysis showed almost no mutations in 3-month culture, in contrast to other HLC culture systems such as iPSCs which are prone to acquire mutations and may be therefore at increased risk of carcinogenesis[116,118]. Finally, it has been shown that organoids are bipotent with regards to *in vitro* differentiation towards mature hepatocytes and cholangiocytes, depending on the culture medium employed[116]. The first proof-of-concept studies by Huch et al. suggested the usefulness of liver organoids for direct transplantation in a mouse model of Tyrosinemia type I, after ex-vivo gene therapy of the mouse cells grown into organoids. Subsequently murine and human foetal or paediatric primary hepatocytes were used for liver organoids (called hep-orgs), which showed growth for multiple months and reserved key morphological, genetic and functional hepatocyte properties. Along the same lines, Hu et al.[119] showed the feasibility of growing human hepatocyte organoids as well, while Ouchi et al. presented an approach to develop multi-cellular human liver organoids from iPSCs and ESCs, that can simulate the progression of liver statosis to inflammation and fibrosis[120]. Moreover, Levy et al. demonstrated the long-term culture and expansion of human hepatocytes up to 10^{16} cells from a single human hepatocyte isolate[121]. ~~Of note~~ In another study, human liver organoids highly repopulated damaged mouse livers and showed high levels of albumin production after 90 days of transplantation, recapitulating successfully the hepatocyte response after partial hepatectomy[119]. Finally, Sampaziotis and colleagues created human extrahepatic cholangiocyte organoids expressing key biliary markers and preserved significant cholangiocyte functionality. These human cholangiocyte organoids were then used to seed biodegradable scaffolds and showed similar organization to the human biliary

epithelium, while they also succeeded to repair extrahepatic biliary damage when implanted in mice[122]. Almost a year later, the same group seeded bioengineered scaffolds with cholangiocyte organoids and created bioengineered bile ducts *in vitro*[123]. This pioneering work of combining organoid and tissue engineering technology definitely offers new perspectives in the regenerative Hepatology agenda. Future studies are needed to show if organoids can constitute an inexhaustible cell source that could supply liver bioengineering applications and be used for reseeding liver tissue and organ scaffolds with mature hepatocytes and cholangiocytes[124].

Objectives of liver tissue engineering towards clinical translation

The objectives of liver bioengineering fundamentally reflect the current aims of regenerative Hepatology (**Figure 1**), and particularly the creation of feasible alternatives to liver transplantation. In particular, the developments in regenerative Hepatology and liver bioengineering may allow to cure paediatric patients with genetic errors that cause inherited metabolic liver disease or those with congenital defects such as biliary atresia in addition to adult patients with ALF or ESLD of any aetiology.

i. Inherited metabolic liver disease

Considering the limitations of HT and the need of further assessing the efficiency and safety of gene-modified autologous or HLC-hIPSCs cell therapy, engineered implantable liver tissues might represent a safer, more effective and durable alternative treatment approach for these patients[75]. The cornerstone of developing liver micro-tissues that could be implanted and overcome the innate metabolic error is to provide functional hepatocytes or HLCs that can supply the essential missing protein/factor with consequent long-term survival.

ii. Congenital biliary defects

Congenital biliary defects, and particularly biliary atresia, have not been yet addressed by regenerative Hepatology. However, following the latest breakthroughs in organoid technology and bioengineering[122,123], there might be possibilities to use constructed biliary ducts to improve and even resolve the main anatomical abnormalities. This would definitely provide a long-term viable solution for a substantial proportion of liver paediatric patients that would otherwise be directed to transplantation at a very early age.

iii. Acute liver failure

Acute liver failure (ALF) is the past and present key target clinical condition for regenerative Hepatology. Indeed, all the tools and methodologies so far established have been considered for the treatment of ALF. The significant limitations of HT or BAL technologies have led to the experimenting liver bioengineering applications in patients with ALF. Specifically, small scale bioengineered liver constructs or even better a whole bioengineered liver could serve as a bridge-to-transplantation or a bridge-to-self-recovery and potentially offer a better solution in terms of functionality, non-immunogenicity and long-term engraftment.

iv. End-stage chronic liver disease

Finally, a major ambition of regenerative Hepatology and liver bioengineering is to find a solution for end-stage liver disease, which is the clinical condition of the vast majority of patients in need of liver transplantation. Therefore, a key objective of liver bioengineering is to develop a 30% bioengineered liver mass by employing human scaffolds reseeded with human cells (“all human engineered liver construct”)[75]. If this is successful, human liver tissue engineering will make a consistent step forward towards meeting the demand of organs needed for liver transplant.

CONCLUSIONS

Regenerative medicine is a multidisciplinary field in rapid development and with major aspirations of success, especially in the field of Hepatology where the scarcity of donor organs for OLT necessitates the need to search viable alternatives. Ongoing research in the field of bioengineering is exponentially increasing with the hope of successful results in the near future, allowing the translation of basic research achievements to clinical practice. The anticipated rapid clinical translation requires the development of a new class of hepatologists with strong translational skills and scientific competence from basic to clinical. Therefore, there is a great opportunity to learn new skills in order to be ready to follow up the forthcoming advancements[125]. Pursuing a career in regenerative medicine requires various kinds of expertise from bioengineering to stem cell biology and from bioinformatics to clinical medicine. It appears that the next-generation of hepatologists will be more trained in novel biotechnologies and fully dedicated to personalised-medicine.

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Figure legends

Figure 1. Status and recent advancements of regenerative Hepatology from clinical to translational and basic research level. Regenerative Hepatology can offer alternatives to liver disease patients, for whom the current standard-of-care would only be liver transplantation. Primary hepatocyte transplantation and artificial liver support systems have been already introduced in clinical practice, while new perspectives of liver bioengineering applications are expected to be ready for clinical translation in the near future.