Chapter

Optimization of a Decellularization/ Recellularization Strategy for Transplantable Bioengineered Liver

Quanyu Chen, Xiaolin You, Jiejuan Lai, Shifang Jiang, Hongyu Zhang and Lianhua Bai

Abstract

The liver is a complex organ that requires constant perfusion for the delivery of nutrients and oxygen and the removal of waste in order to survive. Efforts to recreate or mimic the liver microstructure via a ground-up approach are essential for liver tissue engineering. A decellularization/recellularization strategy is one of the approaches aiming at the possibility of producing a fully functional organ with in vitro-developed construction for clinical applications to replace failed livers, such as end-stage liver disease (ESLD). However, the complexity of the liver microarchitecture along with the limited suitable hepatic component, such as the optimization of the extracellular matrix (ECM) of the biomaterials, the selection of the seed cells, and development of the liver-specific three-dimensional (3D) niche settings, pose numerous challenges. In this chapter, we have provided a comprehensive outlook on how the physiological, pathological, and spatiotemporal aspects of these drawbacks can be turned into the current challenges in the field, and put forward a few techniques with the potential to address these challenges, mainly focusing on a decellularization-based liver regeneration strategy. We hypothesize the primary concepts necessary for constructing tissue-engineered liver organs based on either an intact (from a naïve liver) or a partial (from a pretreated liver) structure via simulating the natural development and regenerative processes.

Keywords: tissue engineering, decellularization, recellularization, thrombogenicity, hemocompatibility, partial hepatectomy transplantation

1. Introduction

The liver is the largest internal organ in the human body, accounting for approximately 2–5% of the total body volume [1, 2]. Physiologically, the liver possesses over 500 different functions [3] and any severe damage could be life-threatening, such as that caused by ESLD, including acute liver failure and chronic liver disease.

In modern times, the failure of solid organs, such as ESLD caused by injury or disease, has become a major challenge in clinics [4]. According to the U.S. Centers for Disease Control and Prevention (https://www.cdc.gov/), in 2014, 38,170 people died of ESLD. Currently, orthotopic liver transplant (OLT) is an ideal therapy for ESLD. However, a shortage of liver organ donors severely limits OLT usage. The Department of Health and Human Services in the United States has estimated that (https://optn.transplant.hrsa.gov) 22 people on the National Transplant Waiting List die each day, while one person is added to the waiting list every 10 min. Additionally, people fortunate enough to receive an organ transplantation have to suffer from the lifelong use of immunosuppressants against chronic rejection. Therefore, new technologies are eagerly needed to create a transplantable liver [5]. Tissue engineering is a mixed field that aims to fabricate functional organs in vitro [6]. Over the decades, great progress has been achieved in the laboratory, and even some livers have been used in clinics [7]. Tissue engineering by using a decellularization/recellularization strategy, which maintains the architecture, vascular system, and ECM components, has been shown to be a promising tool for solid organs, such as liver.

Liver tissue engineering by using decellularization/recellularization strategy (**Figure 1**) involves biomimicking the architecture and physiological features of the native liver. The procedure generally needs three major components: a scaffolding platform, seed cells, and a 3D microenvironment. Despite the numerous advances over the years, it is still an enormous challenge to fabricate a liver organ [8]. Generating liver organ-specific 3D structure scaffold to keep as much as original biochemical, physiochemical, and biomechanical ECM microenvironment is the one of the main hurdles in liver engineering field [9]. Such physiological 3D structure also plays a remarkable role in influencing seeded cell long-term survival and complex liver tissue mass formation [10]. To achieve this, scientists have been working with different scaffolding systems for liver tissue engineering. Studies have shown that a construction strategy based on a combination of a decellularized naïve liver matrix and recellularization with seed cells has led to constructs that match human organs in size and structure. However, the present constructs still only fulfill

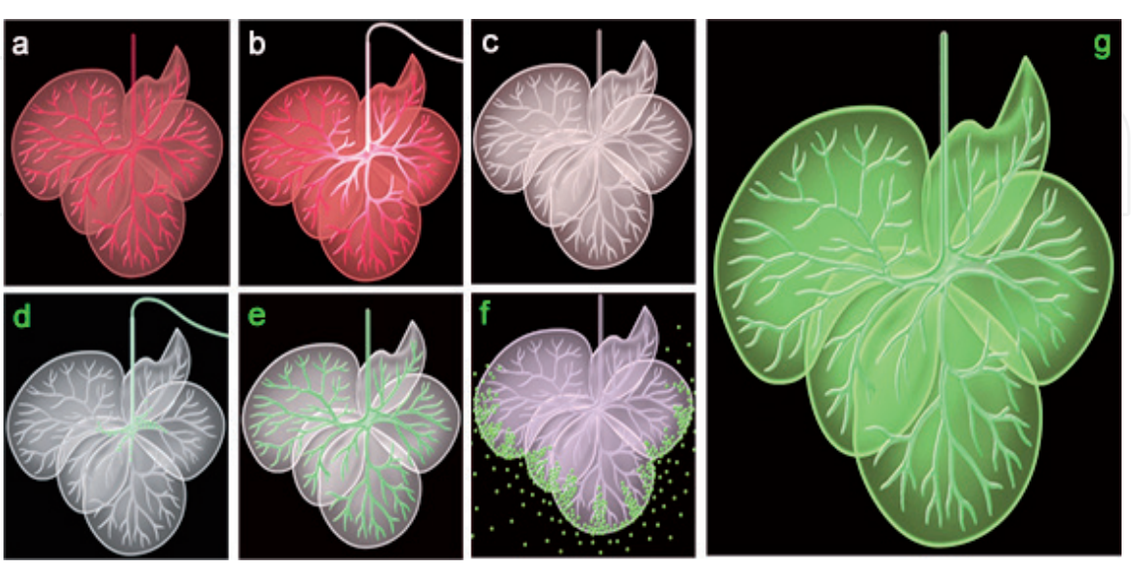

Figure 1.
The decellularization/recellularization strategy in liver tissue engineering. Mammals donor-derived livers undergo a process of decellularization to obtain decellularized liver scaffolds (DLS) (step a–c), and then recellularized seed cells are placed onto the scaffolds (step d–f). Finally, the recellularized scaffolds are placed into 3D culture conditions in a bioreactor to construct liver-like tissues or organs with an overall structure and vasculature (step g).

partial functions of the liver. The preservation of a functional ECM during decellularization, cellular differentiation [11], and a lack of endothelial-lying vascular networks limits the long-term functional integration of constructs after in vivo transplantation. As techniques continue develop, some methods with the potential to overcome these challenges should be explored in the near future, which will further boost the development of a tissue-engineered liver with improved functions. In this chapter, we have tried to focus on the possibility of liver tissue engineering by using a decellularization/recellularization strategy and to describe the current advancements made in the field to address a possible clinical transplantation.

2. Decellularization-based scaffold biomaterials

The term "biomaterials" traditionally means a nonliving substance used for a medical purpose. As the technology of biomaterials developed, the definition expanded to include substances to control the biological environment of cells and tissues for increased compatibility with a host to allow for colonization, proliferation, and differentiation of cells while maintaining their specific morphologies, configurations and avoiding immunological rejection. Based on the increasing knowledge of ECM biology, scaffold biomaterials can be grouped as synthetic materials, natural materials, or a decellularized matrix [12]. Moreover, modifications have been made to enhance the biologically active signals of scaffolds, leading to improved cell attachment, survival, and tissue formation [13, 14].

Biomaterials with required properties have been well studied from synthetic materials. For instance, a nanofibrous matrix made of poly and poly-embedded growth factors was transplanted into animals and restored cardiac regeneration by promoting vascularization [15]. Zawaneh et al. have reported the design of an injectable synthetic and biodegradable polymeric biomaterial consisting of polyethylene glycol and a polycarbonate of dihydroxyacetone that is easily extruded through narrow-gage needles, biodegrades into inert products, and is well tolerated by soft tissues [16]. Those chemically and biologically modified synthetic materials could result in a better way to mimic and control seed cell responses [17, 18]. Another advantage of synthetic materials is their easier to predict and control the degradation of synthetic scaffolds. However, despite this wealth of knowledge, the ability of synthetic biomaterials to support cell attachment, or induce phenotypic expression is much lower than that of natural biomaterials [19–21]; thus, natural biomaterials have been extensively studied [22].

Natural biomaterials include collagen, alginate, and chitosan. These types of biomaterials are inherently able to facilitate for seed cell attachment, proliferation, and functional differentiation, thus they hold significant promise for liver tissue engineering [23, 24]. However, traditional natural materials have poor inherent bioactivity, acidic byproducts, etc., and alone cannot rebuild the complex architecture of solid organs like liver. Other limitations include their unpredictable degradation kinetics; generally, weak mechanical strength, and risk of evoking an immune response [25], etc. also need to be considered.

Decellularized scaffolds (matrices) being natural biomaterials, which are deprived of cellular components while maintaining their original architecture and vascular system, have been widely studied and used in more complex tissue engineering [26]. In the case of liver tissue engineering, the use of decellularization/recellularization strategy was inspired by a pioneer study of heart tissue engineering from the Ott group in 2008 [27]. After that, liver tissue engineered by using this approach has been fabricated [28–30]. Compared to those derived from other synthetic or natural biomaterial scaffolds, the decellularized liver scaffold (DLS)

mostly preserves the native complex liver ECM components, spatial microstructure, and perusable vascular architecture [31, 32] as more "biocompatible ways" for seed cells attaching and reorganizing on a complex 3D level [33]. Therefore, the DLS might have more favorable advantages than other scaffolds for clinical application although the biocompatibility signal between ECM of the DLS and seed cells is still unclear. Scientists have recellularized stem cells onto the natural 3D DLS and have found that these culturing cells not only survive better in the scaffold structure than their culturing in 2D environment but also differentiate into functional cells as well [34]. Zhang et al. seeded adult mouse liver hepatic stem/progenitor cells onto the DLS that generated from naïve liver (nDLS) and cultured the complex in bioreactor, which formed a liver-like construction. Importantly, the nDLS/cell construction was able to repair a cirrhotic liver and even replace the failure liver [35].

Although many studies have been performed in the DLS field for liver tissue engineering [36–40], unfortunately, because of the nDLS being a lack of "active microenvironmental" support in existing ECM components, the optimization of the nDLS biomaterials become an important procedure for improving the skill of liver tissue engineering. Many protocols have been applied to modify the non-bioactive decellularized scaffolds. The application of a variety of growth factors [41] to promote the survival, proliferation, and differentiation of cells, like insulin-like growth factor 1 thought to promote hepatic cell differentiation from bone marrow-derived mesenchymal stem cells, vascular endothelial growth factor applied to enhance the vascularization of tissue-engineered tissues or organs. Additionally, the complex synergistic and antagonistic actions between different kinds of growth factors in vivo, more attention should be paid to the combined and sequential application of different growth factors. Consideration of optimizing the ECM of nDLS foir its behave like "naïve liver regenerative niche" might be a nice way to induce liver-like tissue formation spontaneously both in vitro and in vivo. Based on this, recently, Yang et al. has presented a very interesting experiment: the authors generated an acellular liver scaffold from pretreated naïve liver. They pretreated a naïve liver by performing a 30–55% partial hepatectomy, and the liver was maintained in vivo for 3–5 days until acute liver regeneration occurred, which allowed for the generation of the scaffold from the regenerative liver (rDLS) (**Figure 2**). These rDLS retain a variety of higher level of supporting growth factors for liver spontaneous regeneration as compared to that of nDLS, including their collagens, growth factors (HGF, TGF-α, IL-6, b-FGF, VEGF), glycosaminoglycans, antithrombotic proteins, and other matrix proteins [42]. Since the novel rDLS possesses a natural liver regenerative microenvironment, so-called "bioactive" ECM, it has shown more efficiency than nDLS in promoting primary hepatocyte survival and antithrombotic activity. Notably, when recellularized the rDLS with intrahepatic stem/progenitor cells and cultured them in 3D environment, a more likely liver organ was formatted as compared to the nDLS recellularized with the same stem/progenitor cells, after transplanted into recipients [42]. This pioneer study demonstrated that "bioactive" scaffolds of the rDLS obtained from a regenerative liver possess an advanced natural "active state niche" as compared to nDLS ("still state niche") for promoting primary hepatocyte survival, resistance to thrombosis, and liver-like organ construction. Other forms of bioactive factors are also involved in liver tissue engineering, like microRNAs, etc. [43, 44]. Furthermore, it needs to be mentioned that the advantage of highly conserved each specific ECM protein of decellularized scaffold among species of which the ECM are recognizable within and between species largely without immune rejection [45, 46] when properly processed to remove cellular antigens that would induce an immune rejection without damaging the ECM.

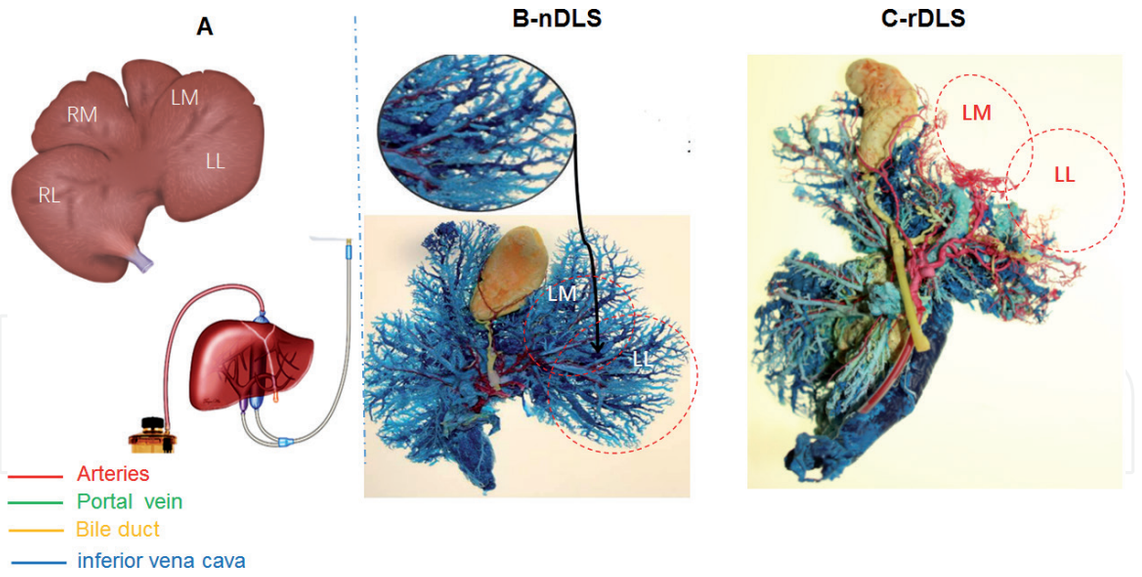

Figure 2.
Generation of a porcine decellularized liver scaffold from naïve livers and livers that had undergone partial hepatectomy (PHx). (A) Perfusion procedure for liver organ decellularization. (B) Blood-vessel tree of a decellularized scaffold from a naïve liver (nDLS). (C) Blood vessel tree of a decellularized scaffold from a partial hepatectomy (PHx) liver (rDLS).

3. Seed cells response to the natural three-dimensional-decellularized liver biomaterial scaffold

Cellular components are an integral part of any tissue engineering. In the case of the liver, it is important to find appropriate cells, such as hepatocytes or stem cells and to seed them into biomaterial scaffolds to regenerate liver tissues or organs [47]. Appropriate seed cells contain parenchymal such as hepatocytes, cholangiocytes, and supportive cells like liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, and pit cells [48]. Hepatocytes account for 60–65% of a liver's cell population [1, 2, 49], which is important for liver tissue engineering. If it is difficult to obtain patient-derived hepatocytes, along with challenging isolation, culture, and the low yields of these cells in vitro [50], stem cells are required for liver tissue engineering [51, 52].

Stem cells are generally grouped as embryonic stem cells (ESCs), somatic stem cells (SSCs), and inducible pluripotent stem cells (iPS) [53]. ESCs have a higher regenerative capacity and can be manipulated to differentiate into other cell types [54, 55]. For liver tissue engineering, ESCs are considered beneficial for the purpose of cell differentiation. For instance, epithelial cells differentiate from ESCs, which could cover the interior of vessels (arteries, veins, and capillaries) of DLS, and the interior of vessels is one of the major players of the angiogenesis process in physiological and pathological conditions involved in thrombus resistant effects. Due to the ethical problems with ESCs, tetratomics and expanded adult human hepatocytes [56], iPS are described as an alternative for adult human hepatocyte differentiation [57–61]. More studies about iPS are under active investigation at present [62], but dozens of publications regarding iPS-derived hepatic lineages have varied from report to report, making it difficult to compare the relative successes of the various modified protocols in enhancing hepatocyte differentiation [63, 64]. Moreover, cultured human hepatocytes often upregulate inappropriate immature markers, such as alpha-fetal protein (AFP). Consequently, any comparisons made to these altered adult hepatocytes may make the candidate immaturely appear more strongly functional than they truly are. Indeed, an examination of published accounts reveals that many protocols lead to fetal hepatocyte-like cells, but in some

cases, the characterization reported is not sufficient to determine the fetal versus mature nature of the resulting differentiated hepatic cells. Given the seemingly fetal nature of iPS-derived hepatic cells produced to date, it is apparent that additional, careful modification of differentiation protocols is still required for further investigation before clinical implementation. Somatic stem cells could overcome the obstacles caused by ECSs, thereby making them more appropriate for liver tissue engineering [65, 66].

SSCs are composed of intrahepatic SSCs and extrahepatic SSCs. Bone marrow-, umbilical-, and fat tissue-derived mesenchymal stem cells are well accepted extrahepatic SSCs [67–69], while oval cells, especially neuro-glial antigen 2 (Ng2)-expressing cells ($Ng2^+$HSP), are currently identified as intrahepatic stem/progenitor cells. Isolation of the $Ng2^+$HSP should be completed by using a specific protocol [70]. Other sources of SSC behaviors seeded in the DLS have also influenced liver tissue engineering. Several studies have demonstrated that liver-derived mesenchymal stem cell (MSC)-like cells can differentiate into hepatocytes and cholangiocytes in nDLS and that the functional differentiation of MSCs in certain situations could be an alternative approach for an engineered liver organ transplantation in the treatment or replacement of ESLD [35, 71]. Our recently studied animal models have revealed that intrahepatic MSC-like SSCs repaired injured livers better than extrahepatic MSCs [unpublished]. Contrary to past hypotheses, extrahepatic bone marrow-derived MSCs do not seem to directly differentiate themselves into hepatocytes, in particularly in vivo, compared to local (liver) MSC-like cells, such as above mentioned the $Ng2^+$HSP. As the $Ng2^+$HSP has been demonstrated to have a role in tissue repair [70] and failed liver replacement [35] in liver cirrhosis murine model, we recently further demonstrated that the intrahepatic $Ng2^+$HSP cells are capable of more efficiency than extrahepatic BM-MSCs in self-renewal and hepatocyte and cholangiocyte differentiations (unpublished) (**Figure 3**). Interestingly, by using the $Ng2^+$HSP, Zhang et al. have successfully reconstituted a liver construct in vitro

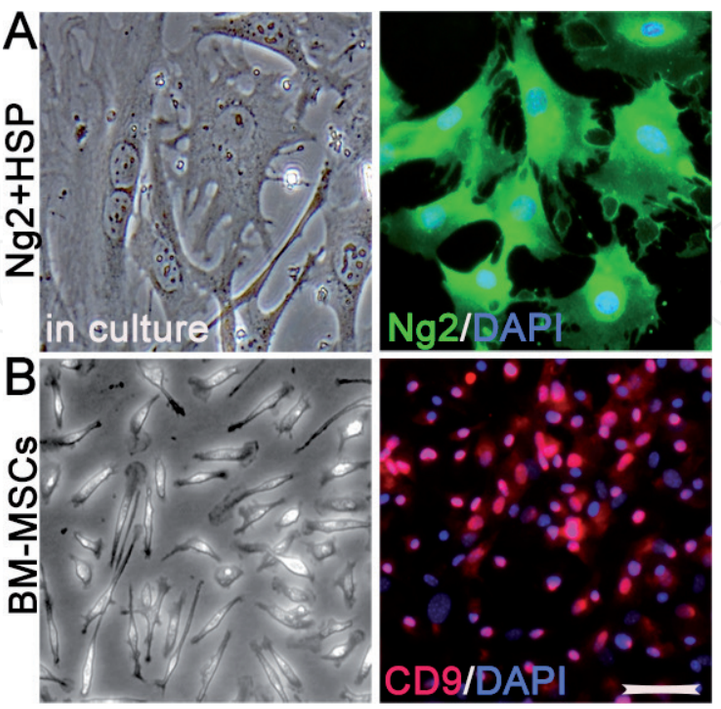

Figure 3.
Murine intrahepatic and extrahepatic mesenchymal stem cells (MSCs). (A) Cultured and immunofluorescently stained of intrahepatic neuro-glial antigen 2 (Ng2)-expressing mesenchymal stem cell (MSC)-like stem/progenitor cells ($Ng2^+$HSP). (B) Cultured and immunofluorescently stained identical bone marrow (BM)-derived-MSCs (BM-MSCs), as visualized by optical microscopy, scale bar = 100 μM.

that is very similar to a naïve liver organ [35]. In addition, the immuno-modulatory, anti-inflammatory, antiapoptotic, and angiogenic properties of the intrahepatic MSC-like $Ng2^{+}HSP$ in the liver still need to be further investigated for liver tissue engineering.

4. Decellularization/recellularization strategy-based liver construction

With the development of decellularization approaches, such as the detergent perfusion technique, whole decellularized scaffolds from liver organs have been produced DLS with an ECM structure and bioactive components being used fabricate bioengineered liver tissues, thus serving as a platform for liver organ bioengineering. Within the past several decades, numerous accomplishments have been driven by the development of these construction strategies. To date, decellularization-based liver construction strategies are constantly advancing such as maintaining complete hepatic vessel networks [72].

Despite the well-conserved macroscopic structure of a liver organ obtained by using decellularization, it is still difficult to avoid some disruption to the ECM composition and ultrastructure through decellularization, which leads to impairment of the natural 3D microenvironment, for example, an impairment of glycosaminoglycans within the ECM by enzymes [73] can cause altered stiffness. Therefore, improved measures for preserving the integrity of the ECM during the decellularization process are required [74, 75]. An functional engineered liver tissue usually uses stem cells or progenitor cells that need to differentiate into multiple kinds of repair cells, which is a challenge to directly seed cells to colonize in relevant sites of DLS to induce their differentiation into specific cell types. Whether an engineering formed liver organ can successfully fulfill its functions depends not only on its physically decellularized scaffold structure but also on an effective recellularization. Therefore, how to populate seed cells like differentiated hepatocytes from different kinds of seed cells or stem cells themselves onto the DLS needs to be carefully considered. In particular, how to manipulate the DLS to enhance the targeted specific colonization of cells to specific areas of DLS such as perfused endothelial cells [76–78], has drawn much attention. To ensure the long-term survival of an engineered liver by allowing exchanges of oxygen, nutrients, and disposal of metabolic waste [79], a functional vascular network and thrombosis after transplantation also needs to be considered. Despite the conservation of the general vascular structure by DLS, the formation of a functional vascular network remains a challenge for liver organ construction. The mainstream strategy to fabricate an engineered liver organ with a functional vascular network includes also the procedure of prevascularization. The initial approaches have been successfully used in spontaneous lineage of endothelial cells in DLS vascular networks after recellularization with stem cells [80–82] to challenge thrombosis after transplantation when exposed to blood, thus leading to localized organ failures [83]. There are two nice approaches showed that endothelialization of vasculature and immobilization of heparin on nDLS could reduce its incidence of thrombosis [84]. More recently, from a pretreated naïve liver obtained rDLS, exhibited except for strong promoting primary hepatocyte survival but also antithrombosis more effect [biomaterials 2018]. Notably, after transplantation guiding the rDLS/cells complex forms complex liver-like tissues (geometries) more effective on rDLS than on nDLS (**Figure 4**), meanwhile combined with better organization of endothelial lineage in rDLS than in nDLS [42]. This suggests that rDLS possesses an advanced "bioactive natural regeneration state niche" relative to the nDLS, which preserves a "still state niche." Therefore, the spontaneous manipulation of the ECM on DLS is a more promising strategy for decellularization-based

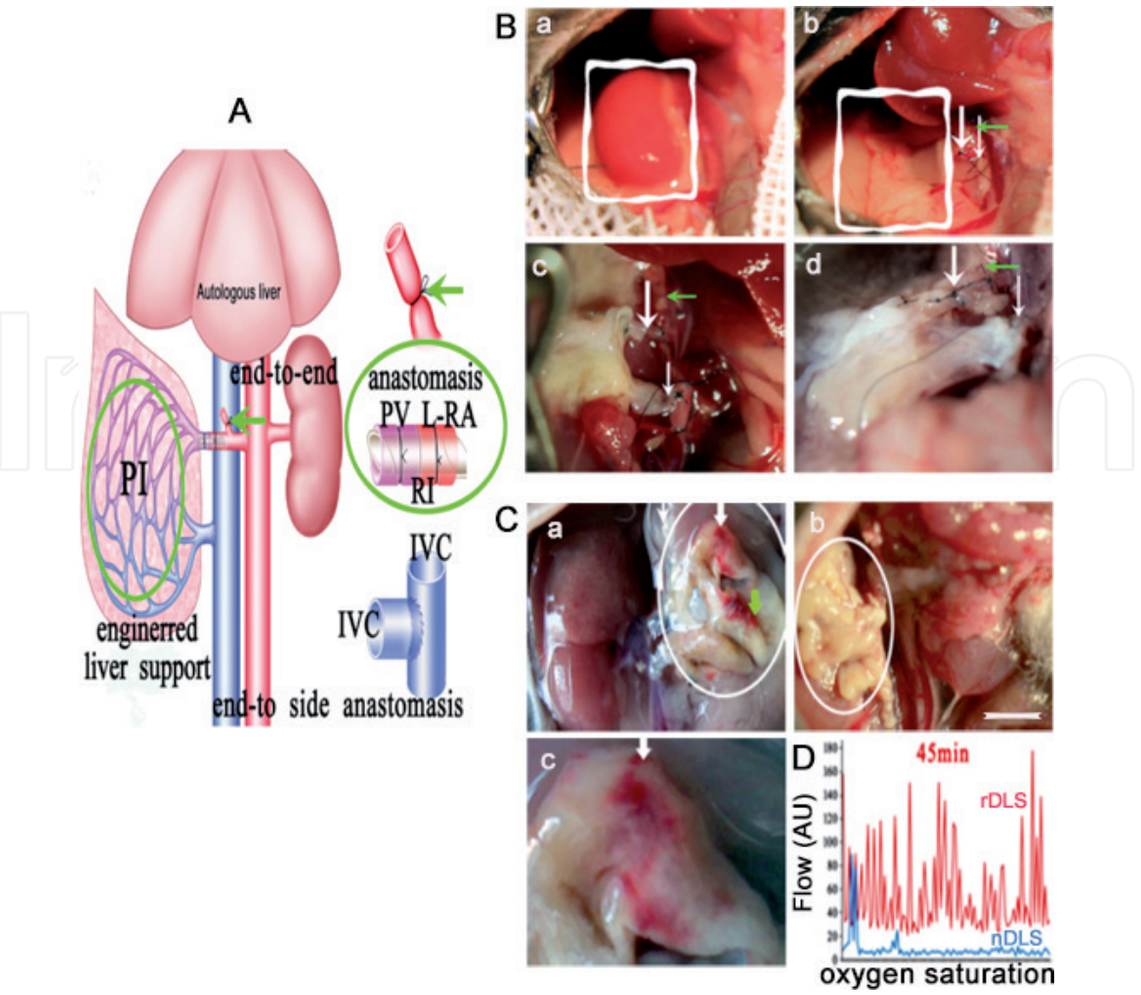

Figure 4.
Comparison of the murine liver-lobule-like tissue construction formation between rDLS and nDLS after portal-renal arterialized auxiliary heterotopic liver transplantation. (A) Schematic of the procedure. The left green cycle indicates the DLS, and the right green cycle indicates the end-to-end anastomosis of the PV (scaffold)-L-RA (recipient). The green arrows in the panels indicate the right-RA. The right bottom cartoon shows the end-to-side anastomosis of the IVC (scaffold)-IVC (recipient). (Ba–d) Exposure of the right-side kidney (the square indicates the kidney) (a). Nephrectomy of the right-side kidney (the square indicates the lack of kidney) (b). The cell-loaded DLS where the kidney was removed (the bold arrow indicates the PV, thin arrow indicates the IVC, and the green arrow indicates a right renal artery (right-RA)). The left-side renal artery (L-RA) was connected to the PV with cross-clamping of the PV and the IVC of the recellularized scaffold (c). The noncell loaded DLS was connected to the recipient by the same procedure as the cell-loaded DLS where the kidney was removed (d). (Ca–c) DLS seeded with $Ng2^{+}$HSP cells formed a liver-lobule-like construct in rDLS (a and b) after approximately 20–40 days (a, indicated as a cycle), for two lobes with better blood patency (b), represented with a white arrow; there was no visible blood flow in the nDLS loaded with $Ng2^{+}$HSP cells for the same time (c). (D) Blood flow velocity (flow, arbitrary unit, AU) was measured in rDLS and nDLS at 45 min within 100 s after the operation by a near-infrared-LDF system, scale bar = 50 μM.

liver tissue. In the future, the objective of a decellularization-based liver construction strategy could be based on generating a 3D decellularized biomaterial scaffold with natural "regenerative bioactive niche" for the seed cell attachment, proliferation and differentiation of cells, and developing a transplantable "new" liver in vitro that maintains the structures and functions of a naïve liver.

In summary, compared with other strategies that can only fabricate partial structures, a decellularization/recellularization-based liver tissue engineering strategy enables the construction of the liver structures with complete blood vessel network at a clinically relevant scale, thus becoming a more promising approach for liver tissue engineering. However, in order to provide a promising route for developing a functional bioartificial liver with potential applications for humans by

such strategy, several questions must be answered: (1) Is the use of a decellularized liver matrix the only possible solution? (2) What kinds of cells need to be chosen for recellularization? Extrahepatic cells? or possibly resident stem/progenitors cells? (3) What is the optimal decellularized liver scaffold (DLS)? (4) What is the length of time for incubation in a bioreactor? (5) Would the technique be applicable to a human liver with its extensively sinusoidal surface?

5. Conclusion and challenges

Clearly, decellularization/recellularization through the development of in vitro and in vivo tissue and organ models for liver bioengineering are advancing strategies. This, combined with multidisciplinary team-workers performing focused, systematic studies to address critical questions, is essential for the success of this strategy. The following critical issues might need to be addressed before clinical applications: (1) preservation and modification of a functional ECM structure to better mimic the regenerative niche; (2) selection of effective seed cell sources for recellularization; (3) modification of blood-vessel networks for "endothelialized DLS"; (4) long-term survival by preventing from thrombosis and functions after transplantation; and (5) immune rejection. In the coming years, many new techniques will be explored, which are expected to have the potential to address these challenges.

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Abbreviations

ESLD	end-stage liver disease
ECM	extracellular matrix
3D	three-dimensional
OLT	orthotopic liver transplant
DLS	decellularized liver scaffold
nDLS	the decellularized scaffold that generated from naïve liver
rDLS	the decellularized scaffold that generated from pretreated liver
HGF	hepatic growth factor
TGF-α	transforming growth factor-alpha
IL-6	interleukin 6
b-FGF	fibroblast *growth* factor-beta
VEGF	vascular endothelial growth factor
ESCs	embryonic stem cells
SSCs	somatic stem cells
iPS	inducible pluripotent stem cells
$Ng2^{+}HSP$	neuro-glial antigen 2 (Ng2)-expressing cells
MSC	mesenchymal stem cell

Author details

Quanyu Chen[1,2], Xiaolin You[2], Jiejuan Lai[2], Shifang Jiang[2], Hongyu Zhang[2] and Lianhua Bai[1,2]*

1 Key Laboratory of Freshwater Fish Reproduction and Development, Ministry of Education, Laboratory of Molecular Developmental Biology, School of Life Sciences, Southwest University, Chongqing, China

2 Hepatobiliary Institute, Southwest Hospital, The Army Medical University, Chongqing, China

*Address all correspondence to: qqg63@outlook.com

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