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Repopulation of intrahepatic bile ducts in engineered rat liver grafts

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Abstract

Engineered liver grafts for transplantation with sufficient hepatic function have been developed both in small and large animal models using the whole liver engineering approach. However, repopulation of the bile ducts in the whole liver scaffolds has not been addressed yet. In this study, we show the feasibility of repopulating the bile ducts in decellularized rat livers. Biliary epithelial cells were introduced into the bile ducts of the decellularized liver scaffolds with or without hepatocytes in the parenchymal space. The recellularized grafts were cultured under perfusion for up to 2 days and histological analysis revealed that the biliary epithelial cells formed duct-like structures, with the viable hepatocyte mass residing in the parenchymal space, in an arrangement highly comparable to the native tissue. The grafts were viable and functional as confirmed by both albumin and urea assay results and the gene expression analysis of biliary epithelial cells in recellularized liver grafts. This study provides the proof-of-concept results for rat liver grafts co-populated with parenchymal and biliary epithelial cells.

Keywords

Biliary Epithelial Cells; Hepatocytes; Whole-Liver Engineering; Decellularization; Recellularization

INNOVATION

Engineering of transplantable liver grafts, through in-vitro decellularization and recellularization, has emerged as a feasible strategy to provide alternatives for donor livers and address the donor organ shortage in transplantation. While much attention has been given to recreating the parenchyma in engineered liver grafts, repopulation of the biliary tree has been neglected so far. The presented work, for the first time, demonstrates an innovative

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AUTHOR CONTRIBUTIONS

Y.C., M.L.Y. and B.E.U. developed the concept and designed the experiments. Y.C., J.D. performed the experiments. Y.C. and B.B. analyzed the data. Y.C., B.B. and B.E.U. wrote the manuscript. All authors commented on the manuscript.

DISCLOSURES

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way to engineer rat liver grafts through heterogeneous recellularization of decellularized rat liver scaffolds with hepatocytes and biliary epithelial cells. We employed two separate routes to introduce biliary epithelial cells and hepatocytes into the scaffolds. Biliary epithelial cells were injected into the biliary tree through the extrahepatic bile duct and hepatocytes were distributed into parenchyma through injection into the portal vein. This innovative cell seeding strategy resulted in a cellular arrangement similar to native tissue architecture and the co-populated grafts were viable and functional. These results provide proof-of-concept evidence for engineering liver grafts that have the biliary component along with the parenchymal cells leading the path for developing fully functional engineered liver grafts for transplantation.

INTRODUCTION

The concept of whole liver engineering has drawn attention in recent years offering a solution to the severe shortage of donor organs for transplantation, which is a growing health concern. Orthotopic transplantation is the definitive treatment for end-stage liver failure; however, the gap between the transplantation demand and the organ availability has grown extremely over the past two decades. According to the Organ Procurement and Transplantation Network (OPTN), over 114,000 patients are waiting for transplant in the United States as of September 2018, while only 34,770 transplants were performed in 2017¹. The short supply of donor livers leads to increased mortality among patients waiting for a transplant^{2,3}. Several social and technical strategies have been suggested to solve this problem, including paired organ donation acceptance, “reward” system and proper utilization of marginal organs⁴. Among possible alternatives, whole organ engineering stands out as an innovative solution as it allows for the potential redemption of donor organs found unsuitable for transplantation for the generation of patient specific organs⁵.

Despite efforts of over more than 20 years in tissue engineering, the difficulty of reproducing the native architecture and function of a complex organ such as liver has been an obstacle for clinical application of engineered tissues⁶. Whole liver engineering, however, takes advantage of the natural tissue matrix composition and architecture in scaffold preparation. Whole liver scaffold is prepared through a process called decellularization where the discarded liver is perfused with detergents to remove the cellular components leaving behind an acellular structure composed of extracellular matrix (ECM) components, such as type I/IV collagen, glycosaminoglycans (GAGs), fibronectin, laminin, and growth factors; and the natural microvascular structure is well preserved^{7,8}. Therefore, unlike traditionally engineered tissues, scaffolds obtained via whole organ engineering allow for connecting the tissue directly with the blood circulation upon transplantation, allowing for immediate circulation, thus instant nutrient and oxygen delivery to the cells seeded inside. The acellular whole liver scaffold is repopulated with healthy cells to create a functional tissue that can be transplanted. One advantage of whole liver engineering approach is that the immune rejection issue can be potentially solved by engaging patient-specific liver cells such as those derived from induced pluripotent stem cells (iPSCs) in the process of recellularization^{9,10}. Differentiation of iPSCs has been established for hepatocytes^{11–13} and more recently for cholangiocytes^{14–16}. We have shown that human iPSCs induced to differentiate on decellularized liver matrix differentiate into hepatocytes more efficiently than those on

Matrigel indicating feasibility of this strategy¹⁰. Together with complete removal of host DNA, the decellularized ECM is less likely to result in an immune response, unlike the scaffolds prepared using synthetic biomaterials, as most of the ECM proteins are highly conserved among species¹⁷.

Whole organ engineering approach has been applied to a number of solid organs including the liver, in small animals and on the large clinical scale^{18–20}. While there have been a few successful and promising studies showing successful recellularization with hepatocytes and endothelial cells, and short-term transplantation, some aspects of whole liver engineering need further investigation for clinical application and translation. One of the challenges to be resolved is to rebuild a functional biliary tree within the engineered liver which has not been addressed yet. Bile, secreted by hepatocytes, is a yellow-green, basic solution containing bile salts, bile pigments, cholesterol, neutral fats, phospholipids, and various electrolytes. It emulsifies lipid during the digestion process²¹. However, some components of bile may be toxic if it is not directed into bile ducts for removal²². One example is bilirubin, which is the product of red blood cell breakdown and is taken up by hepatocytes, conjugated to either glucuronic acid or sulfate and is subsequently secreted into the bile canaliculus as part of the bile and is excreted from the body. If cells are not present to line the intrahepatic bile ducts to enable the flow of bile out of the liver, bilirubin would accumulate in the blood leading to hyperbilirubinaemia²³. While some degree of hyperbilirubinaemia might be tolerated without inducing major harm on the patient or the graft, bilirubin toxicity may become a chronic problem²⁴. Biliary epithelial cells, also known as cholangiocytes, line the intrahepatic bile ducts and transport bile to the common bile duct, thus removing it from liver cells²⁵. Cholangiocytes also participate in bile modification such as alkalization through ion exchange, fluid and electrolytes secretion and reabsorption²⁶. Therefore, the incorporation of a bile-modification and draining system is necessary in order to build a lasting artificial liver that is capable of maintaining the diverse metabolic activities. This reveals the necessity of the reconstitution of the intrahepatic as well as extrahepatic biliary ducts during the process of whole liver engineering. There have been a few studies on the reconstruction and replacement of diseased bile ducts, which mostly focused on the engineering of the extrahepatic bile ducts. Some of the recent studies utilized artificial scaffolds composed of bioabsorbable polymers, biocompatible polymer mixtures (polycaprolactone (PCL) and poly(lactide-co-glycolic acid) (PLGA)) and other synthetic polymers such as polyglycolic acid (PGA)^{27–29}, whereas others used tissue-engineered vessels to reconstruct the extrahepatic biliary tree³⁰. In this study, we performed heterogeneous recellularization by repopulating the decellularized rat liver scaffolds with cholangiocytes and primary hepatocytes. The whole biliary structure, including intrahepatic biliary tree, was engineered by retrograde infusion of cholangiocytes through the common bile duct. The recellularized liver tissue was evaluated morphologically (histology), and via gene expression analysis for cholangiocyte functional markers. In grafts co-populated with hepatocytes and cholangiocytes, hepatocyte functions were assessed by measurement of albumin and urea secretion.

MATERIALS AND METHODS

Animals and liver harvest

All animal care, handling, and surgical procedures were in accordance with the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital. Livers were harvested from female Lewis rats (Charles River Laboratories, Wilmington, MA) weighing 150–200 g. The portal vein and the common bile duct were cannulated with an 18-gauge catheter and a 28-gauge catheter, respectively. The livers were perfused with 20 mL of PBS through the portal vein, after cutting the inferior vena cava and before excising from the body cavity, in order to clear the blood. Harvested livers were stored at -80°C until further use.

Decellularization

Liver decellularization and sterilization were performed according to our previously established protocol^{20,31}. Harvested livers were perfused through the portal vein with a series of sodium dodecyl sulfate (SDS) (Sigma-Aldrich, St. Louis, MO) solutions at an ascending concentration gradient. The decellularized grafts were stored in PBS containing 2% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO), 10 $\mu\text{g/mL}$ gentamicin (Sigma-Aldrich, St. Louis, MO), and 2.5 $\mu\text{g/mL}$ amphotericin B (Sigma-Aldrich, St. Louis, MO) at 4°C for up to 2 weeks until further use.

Corrosion casting

The anatomical view of the vascular structure as well as the biliary structure of the decellularized liver matrix (DLM) scaffold was demonstrated by performing a simple corrosion casting after the decellularization by using a corrosion kit (Polysciences, Inc., Warrington, PA). The base solution was mixed with the catalyst and the curing agent according to manufacturer's instructions. The mixtures with a color of red, and green were injected into the scaffold through the portal vein, and the bile duct, respectively. The DLM was immersed in PBS during the whole process and pictures were taken after overnight incubation in ice bath.

Cholangiocyte culture

A master cell bank and a working cell bank were established with normal rat cholangiocytes (NRCs) (courtesy of the Cholangiopathies Laboratory of Mayo Clinic). NRCs were resuscitated from cryopreservation at passage 19 and cultured in T25 collagen coated flasks (Corning, Corning, NY) at 37°C and 5% CO_2 . The culture medium (Supplementary Table 1) was changed daily.

NRC cultures were harvested when the confluency reached 80–100%. The cells were detached from the culture flask using 0.05% trypsin (Invitrogen, Carlsbad, CA) and subsequently used in recellularization or passaged to a new T25 flask.

Cholangiocyte characterization

Cytokeratin-7 (CK7), a cholangiocyte marker^{32,33}, was used for the characterization of cholangiocytes via immunocytochemistry (ICC). As the primary and secondary antibodies,

200 µg/mL mouse monoclonal IgG anti-CK7 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX) and 2 mg/mL Alexa Fluor 568 goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA) were used, respectively. The cholangiocyte culture was fixed via formalin. The primary antibody and secondary antibody were diluted 1:200 and 1:500 respectively following the manufacturer's recommendations. Cells were counterstained with DAPI (Life Technologies, Carlsbad, CA) for 10 minutes. The immunofluorescent images were acquired using Zeiss 200M microscope.

Recellularization with cholangiocytes only

The scaffold was flushed with PBS before cell seeding. In recellularizations with cholangiocytes only, no cells were introduced into parenchymal space, therefore, the liver scaffold was only perfused with PBS instead of culture media prior to the seeding. A total number of 1 or 1.8 million NRCs at passage 20 were injected into the common bile duct cannula in the DLM scaffold at a density of 4 million cells per mL using an insulin syringe. During the injection, it could be observed that the pink-colored cell suspension propagated to the intrahepatic bile ducts, indicating an effective seeding in the liver graft. The DLM scaffold seeded with NRCs was incubated statically at 37°C and 5% CO₂ for 1 hour and subsequently transferred to a closed sterile bioreactor chamber (Supplementary Fig. 1). The recellularized livers were perfused through the portal vein cannulation with 60–80 mL perfusion media (Supplementary Table 2) at a flow rate of 2 mL/min, to maintain a low shear stress. The culture was kept for one day at 37°C and 5% CO₂.

Recellularization with hepatocytes and cholangiocytes

NRCs and freshly isolated rat hepatocytes (Cell Resource Core at Massachusetts General Hospital) were used in this part of the study. For co-populated grafts, the DLM scaffold was flushed with PBS and NRC culture media before cell seeding. The NRCs were seeded in the DLM scaffold in the same manner as described above. The NRCs were cultured in the DLM statically submerged in culture medium in a petri-dish at 37°C and 5% CO₂ overnight. Adult primary rat hepatocytes were seeded in the NRC repopulated and blank DLM scaffolds (hepatocyte only controls) through the portal vein the next day¹⁸. Briefly, cholangiocyte populated and blank scaffolds were placed into two parallel perfusion setups and perfused for 30 minutes prior to the hepatocyte seeding with high glucose DMEM supplemented with 10% FBS and 2% penicillin-streptomycin. A total of 71 ± 11 freshly isolated rat hepatocytes, at a density of 10 million cells per mL with a viability of 90–95%, were infused into the decellularized liver in four subsequent injections with 10-minutes intervals as previously described¹⁹. In total, 6 co-populated grafts were used for morphological assessment, 5 co-populated grafts were used for albumin and urea analyses, and 5 hepatocyte only grafts served as controls.

The perfusate was collected at the end of the seeding and the number of hepatocytes that were retained in the DLM scaffold was determined by subtracting the cells in the perfusate from the total seeding number; 74–97% of hepatocytes were retained in the scaffolds. The recellularized liver grafts were transferred and kept immersed in culture medium and cultured in a sterile closed bioreactor for 2 days while perfused through the portal vein cannulation. The perfusion flow rate was kept at 8 mL/min and the culture system was

incubated at 37°C and 5% CO₂. Same culture medium as cholangiocytes recellularization was used and changed daily.

Histology and immunohistochemistry

The native liver tissue and recellularized liver grafts were fixed with 10% formalin for 24 hours, embedded in paraffin, and processed at the Specialized Histopathology Services Core at MGH for staining with hematoxylin and eosin (H&E), 1:100 diluted cytokeratin-7 (CK7) (Abcam, Cambridge, UK) with 1:250 diluted biotinylated goat secondary anti-rabbit (Vector Laboratories, Inc., Burlingame, CA) and 1:25 diluted Ki-67 (Leica Biosystems, Wetzlar, Germany) with mouse HRP (DAKO Agilent, Santa Clara, CA). The dilutions of antibodies were selected following manufacturer's recommendations for application in immunohistochemistry. The slides were imaged using Nikon Eclipse E800 and Hamamatsu NanoZoomer-XR, and subsequently processed with NDP.view 2 from Hamamatsu Photonics.

RNA isolation and analysis of gene expression

Cholangiocyte functional markers were tested via gene expression analysis of recellularized grafts using quantitative reverse transcription polymerase chain reaction (qRT-PCR). DLM scaffold recellularized with cholangiocytes was flushed with PBS in order to remove culture medium in the graft and then was snap-frozen in liquid nitrogen, grinded using a mortar and pestle set (Fisher Scientific, Waltham, MA). Total RNA was isolated from the ground tissue and from the plate-cultured cholangiocytes using an RNA isolation kit (Macherey-Nagel, Germany). The quantity of total RNA was measured by a spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Every 100 ng RNA was reverse transcribed to cDNAs with reverse transcription kit (Promega, Madison, WI). The synthesized cDNA was used for quantitative RT-PCR with 6 primers to assess the gene expression of sodium/hydrogen exchanger isoform 1 (NHE-1) and isoform 2 (NHE-2), cystic fibrosis transmembrane conductance regulator (CFTR), Cl⁻/HCO₃⁻ anion exchange protein isoform 2 (AE-2), hepatocyte nuclear factor 6 (HNF-6), gamma-glutamyl transferase (GGT). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. A quantitative PCR system (ViiA 7, Life Technologies, Carlsbad, CA) was used to amplify the synthesized cDNAs. The initial denaturation was done at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 20 seconds, primer annealing at 55°C for 30 seconds, and primer extension at 68°C for 1 minutes. A final melt cycle consisting of 15 seconds at 95°C, 1 minute at 55°C, and 15 seconds at 95°C was included at the end. The relative mRNA expression was quantified in triplicate using comparative Ct (Ct) method. Custom primers were synthesized by the DNA Core at the Massachusetts General Hospital. The sequences of the primers are listed in Supplementary Table 3.

Albumin and urea assays

Albumin and urea assays were conducted to determine the levels of albumin production and urea synthesis in the recellularization culture media as indicators of hepatocyte metabolism. Samples were collected daily. The albumin level was determined by a direct, competitive enzyme-linked immunosorbent assay (ELISA) method, and the urea concentration was determined using a commercially available kit (Stanbio Laboratory, Boerne, TX).

Statistical analysis

The quantitative data are presented as the mean $\pm$ standard error of the mean (s.e.m.) from at least three repeats per group. The qRT-PCR data were assessed using unpaired t-test. The urea and albumin production were analyzed via paired t-test. All statistical analyses were performed via Graphpad Prism software and p-values less than 0.05 were considered to be statistically significant.

RESULTS

Liver decellularization

The rat livers gradually turned translucent during the decellularization process and transparent liver scaffolds were obtained at the end of 72 hours of perfusion (Fig. 1a). As the cells were washed off from the liver, the gross morphology of the liver was retained. The decellularized liver matrix (DLM) scaffolds were characterized by H&E staining. As shown in Fig. 1b, compared to the native rat liver where hepatocytes were lined along the sinusoids, the DLM scaffold had an acellular matrix with empty honeycomb-like structures, which are the niche for liver cells, confirming the removal of the cells. Following the same decellularization protocol, we have shown previously that the DLM scaffold contained DNA that is less than 5% of the native liver (0.44 ± 0.07 vs. 0.02 ± 0.01 $\mu\text{g}/\text{mg}$ tissue) and the most abundant cellular protein, b-actin, was undetectable by Western blot analysis confirming efficient decellularization³¹. The presence of ECM components such as collagen, glycosaminoglycans and basement membrane proteins (e.g., laminin, fibronectin and collagen type IV) and matrix bound growth factors in the DLM was also reported^{18,19,31}. In this report, the DLM scaffold was further characterized via corrosion casting, which captured the vascular and biliary architecture of the scaffold (Fig. 1c). Portal (red), and biliary (green) circulatory systems were preserved with visible microcirculatory branches. The complete biliary structure, including the common bile duct and the intrahepatic biliary branches embedded in four lobes in close proximity to portal vasculature was well preserved. We employed the common bile duct (black arrowhead, Fig. 1c) to inject the rat cholangiocytes into the DLM scaffold and repopulated only the intrahepatic biliary ducts and used portal vein to introduce hepatocytes into the parenchymal space.

Recellularization of decellularized liver scaffolds

Prior to recellularization, rat cholangiocytes, demonstrating an epithelial morphology (Supplementary Fig. 2), were stained for CK7, which is a cytokeratin specifically expressed by cholangiocytes. Immunofluorescence staining confirmed the biliary epithelial phenotype through a distinct positive signal for the cholangiocyte marker CK7 (Supplementary Fig. 2). These cells were grown to confluency and used in recellularization experiments. In order to determine the number of cholangiocytes needed to fully line the biliary tree in the DLM scaffold in approximately 3 days, we considered the average surface area of bile ducts in a normal rat liver and the doubling time of normal rat cholangiocytes^{32,33} and estimated that an initial seeding of 1.8 million cells in 300 μL was required. However, this seeding density led to occlusion of the bile ducts as seen in histology analysis. We reduced the initial seeding to 1 million cells which yielded biliary spaces fully lined with cells, without any occlusion

of the ducts (Supplementary Fig. 3). Therefore, in subsequent recellularization experiments, we used 1 million NRCs.

The recellularized grafts were assessed via H&E staining with respect to morphological analysis. Samples of the heterogeneous recellularization (scaffolds recellularized with both cholangiocytes and hepatocytes, $n = 6$) were collected from different anatomical locations of the liver, including left lateral lobe, medial lobe, right lateral lobe, superior right lateral lobe, inferior right lateral lobe, caudate lobe, and central area two days after hepatocyte seeding. Histology analysis revealed that both NRCs and hepatocytes were sporadically distributed in patches throughout the recellularized grafts as shown in Fig. 2a. All the regions mentioned above had hepatocytes populated in, and there was no distinct difference of cell distribution among lobes.

Hepatocytes were found in parenchymal space, forming dense colonies (Fig. 2b). Cholangiocytes were found within the ductules, exhibiting distinctive higher nucleus to cytoplasm ratio when compared to hepatocytes³⁴. IHC-CK7 analysis confirmed that the cells within the ductules were indeed NRCs which stained positive for CK7, while the hepatocytes remained negative (Fig. 3). The CK7 positive cells, cholangiocytes, were restricted within the biliary structure, while the parenchyma remained CK7 negative, indicating the parenchymal spaces were populated with hepatocyte as shown in Fig. 3a–c. It was also observed that hepatocytes and cholangiocytes resided rather close, separated by a thin layer of ECM. The comparison of the histology results of native (Fig. 3d) and recellularized (Fig. 3a,b) livers show that NRCs and hepatocytes seeded in the scaffolds reconstituted the cellular architecture highly comparable to the native tissue. Magnified images showed that the CK7 positive cells were orderly aligned, lining up the ductules (Fig. 3e). Surrounded by the CK7 positive cells, empty lumen could be easily spotted. The CK7 positive cells remained viable and proliferative as confirmed by positive Ki-67 staining. Altogether, the observation of the duct-like structures with CK7 positive cells propitiously validated a successful biliary tree reconstruction and the co-existence of hepatocytes and NRCs in the recellularized graft demonstrated success of the heterogeneous recellularization approach to achieve physiologically relevant cellular architecture.

Characterization of recellularized grafts

In order to characterize the viability of the recellularized grafts, we first investigated the expression of genes indicative of biliary function in grafts recellularized with cholangiocytes only. We have selected gamma-glutamyl transferase (GGT), cystic fibrosis transmembrane conductance regulator (CFTR), hepatocyte nuclear factor 6 (HNF-6), anion exchange protein 2 (AE-2), Na^+/H^+ exchanger isoforms 1 and 2 (NHE-1, NHE-2) as marker proteins and quantified the expression of these genes in recellularized grafts 24 hours post seeding and compared the results to those obtained from plate culture controls. As shown in Fig. 4, the cholangiocytes seeded inside the DLM scaffolds expressed the biliary markers at levels comparable to or higher than plate culture controls. Specifically, the expression levels of GGT, HNF-6, CFTR and NHE-1 did not demonstrate a statistically significant change among the control and recellularization groups. On the contrary, there was a significant

increase in the expression levels of NHE-2 and AE-2 in the recellularized liver scaffolds with a fold increase of 11.2 ± 1.9 and 2.1 ± 0.3 , respectively.

Hepatic functionality of the recellularized grafts was assessed through measurement of albumin and urea production during perfusion culture of grafts recellularized with hepatocytes and cholangiocytes, which was compared to grafts repopulated with hepatocytes only. The co-populated grafts had higher production of albumin and urea than grafts with hepatocytes only, but it was not found to be statistically significant (Fig. 5). Both grafts continued to produce albumin and urea for two days as the cumulative levels of both metabolites increased during the culture. We also performed plate cultures to assess the quality of hepatocytes from that particular isolation. The results of these plate cultures are reported in Supplementary Fig. 4.

DISCUSSION

In the past decade, a new approach has evolved to potentially solve the donor organ shortage problem: whole organ engineering through decellularization of a discarded organ and subsequent recellularization of the resulting scaffold with healthy cells. This fairly new approach has been applied to several organs, including heart and liver²⁴. Different cell types are present in the liver, and together, they maintain various metabolic functions of the liver. Cholangiocytes, the epithelial cells of the bile duct, are responsible for removal of the bile from the liver, which, for the most part, have been neglected in the liver tissue engineering studies. In addition, most of the bile duct engineering studies focus on the reconstruction of extrahepatic bile duct only. In this study, native rat livers were harvested and decellularized through perfusion with detergent solutions. Decellularized liver grafts were recellularized with hepatocytes as well as cholangiocytes. The intrahepatic biliary tree were repopulated with rat cholangiocytes through injection into the bile duct and the liver parenchyma was repopulated via perfusion of primary rat hepatocytes into the portal vein. The recellularized grafts were cultured under perfusion for up to 2 days. The grafts were analyzed via histology, immunohistochemistry (IHC), gene expression and urea and albumin production.

In the recellularization study, in five out of six grafts, cholangiocytes were found to be evenly distributed in both intrahepatic (peripheral) and central regions and only in one case, they were only found in central area which may be due to an anatomical difference in that particular liver. In addition, cholangiocytes specifically lined the bile duct without going into the parenchymal space. It is possible that the ductal retention of the cells is a combined result of the seeding strategy and the microarchitecture of the biliary network in the scaffolds. After decellularization, the biliary tree network in the DLM scaffolds remains almost impermeable and closed at ends of the smallest ductules such that any liquid injected into the extrahepatic bile duct remains within the biliary tree and does not diffuse into the parenchyma when kept under constant pressure. Therefore, when cholangiocyte suspension was injected in a volume sufficient to only fill the bile ducts (~250 μ L), and were allowed to attach statically for 1 hour without removing the syringe to maintain the pressure of the injection, the cells remained within the ductules and did not diffuse into the parenchymal space. As a result, it was possible to achieve bile duct restricted seeding of cells.

The hepatocyte colonies were found in an adequate distance to the NRC colonies separated by a thin layer of ECM structure. Hence, the cellular architecture was anatomically comparable and physiologically plausible. However, the integration of bile canaliculi with the bile ducts remains to be confirmed for successful transport of bile from hepatocytes through the cholangiocytes and into the bile ducts. In addition, we have found that the hepatocytes located near the vasculature had larger nuclei than those found relatively distant to the vessels. Cells away from the vessels had smaller or shrunk nuclei, which is an indication that the cells might be pyknotic, i.e., they might be displaying chromatin condensation as a result of necrosis or apoptosis. This may be a result of insufficient distribution of oxygen and other nutrients throughout the graft and will need to be resolved by adjusting the perfusion pressure and oxygen delivery. One final concern is the possibility of the proliferative cholangiocytes (Ki67 positive) to occlude the bile duct post seeding needs to be addressed for the long-term functional success of the engineered liver grafts.

The gene expression analysis revealed that cholangiocytes expressed genes of functional proteins in recellularized grafts, which may indicate active biliary metabolism (Fig. 4). Cholangiocytes in the recellularized grafts demonstrated GGT expression, which indicated potential cholangiocyte functionality as GGT activity is one of the key enzymatic functions of cholangiocytes and has been shown to increase in response to ductal structure development^{35,36}. The expression of CFTR was also preserved in recellularized grafts, which plays a role in the bile flow and secretion and is a commonly used marker for bile functionality^{15,37}. Among the hepatocyte nuclear factor (HNF) family members, HNF-6 has a critical role in the regulation of metabolic functions of biliary epithelial cells and is especially important for proper development of intra- and extrabiliary tree³⁸. The expression level of this transcription factor was conserved in the recellularized grafts. NHE-1 expression was higher in recellularized grafts than in 2D plate culture, NHE-1 mediates acid extrusion and prevents intracellular H⁺ accumulation³⁹. NHE-2 plays a role in fluid and sodium chloride absorption from the bile duct lumen, which could potentially explain the increase in the expression level of this transporter in the recellularized graft as the cholangiocytes in this group were in a three-dimensional, physiologically relevant bile duct environment. Interestingly, AE-2 expression increased as well. AE-2 is a pH regulatory protein involved in epithelial acid-base mediation through secretion of HCO₃⁻^{39,40}. Diminished AE-2 activity was correlated with cell injury and biliary tree damage while increased intracellular pH levels lead to increased AE-2 activity. It is possible that the difference between the pH levels among the groups led to the slight increase in the AE-2 expression in recellularized grafts. Overall, these results suggested that cholangiocytes remained viable and continued to express genes of functional proteins in the recellularized liver matrices. In addition, hepatocytes seeded with cholangiocytes inside the DLM scaffolds secreted albumin and urea during perfusion culture indicating their viability and function. Compared to hepatocyte only group, scaffolds recellularized with cholangiocytes and hepatocytes demonstrated slightly higher levels of cumulative albumin production and urea secretion as shown in Fig. 5.

In this study, the functional assessment of cholangiocytes was done indirectly through the measurement of gene expression levels for functional biliary proteins. While gene

expression does not equate to functional metabolism, it still demonstrates that the cells are viable and expressing functional proteins which may translate into active metabolism. The ultimate functional assessment of a liver graft with biliary component would be to demonstrate the biliary flow in the liver using an imaging technique such as cholangiography. In this proof-of-concept study, we were not able to show the functional integration of biliary system with the parenchyma. It should be noted that the recellularized grafts were only kept under perfusion for 2 days as a preliminary assessment of the feasibility of the approach. It is critical that the co-populated grafts be evaluated in the long-term cultures, which would enable demonstration of viability and function of grafts including bile secretion. Once the long-term functionality is achieved, addition of endothelial cells and testing the recellularized grafts in vivo for heterotopic transplantation are the next steps toward creating a fully recellularized transplantable liver graft.

CONCLUSION

In this work, we have demonstrated for the first time, whole liver grafts co-populated with hepatocytes and cholangiocytes. Successful recellularization of the graft was achieved by introducing cholangiocytes through the biliary entry in a retrograde manner, as well as perfusing hepatocytes through the portal vein. Cholangiocytes formed duct-like structures, with the viable hepatocyte mass residing in the parenchymal space, in an arrangement highly comparable to the native tissue. Both albumin and urea assay results confirmed hepatocyte functionality and the gene expression analysis of cholangiocytes in recellularized liver grafts indicated viability and sustained gene expression of functional proteins. In summary, we presented the proof-of-concept study for engineered liver grafts harboring the biliary component. Future studies will include assessment of biliary functions such as secretion of bile in long term cultures and in vivo transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Decellularization of rat livers.

(a) Livers were harvested with the portal vein and the bile duct cannulated. After one freeze/thaw cycle, the liver was gently perfused with a series of detergent solutions through the portal vein for 3 days. The color of the liver changed from reddish brown to white resulting in a translucent scaffold at the end of Day 3. Pictures were taken at 0, 24, 48, and 72 hours.

(b) Histology (H&E staining) of the decellularized liver matrix scaffold showed complete removal of cells compared to native livers while the vascular structure was preserved. Scale bars 100 μm .

(c) Corrosion cast model of the decellularized scaffold showing that biliary (green) and portal (red) circulatory trees were preserved. Black arrows indicate the smaller bile ductules preserved near the periphery following the portal vein. For recellularization, NRCs were injected into the cannulated bile duct (arrowhead) and hepatocytes were injected through the portal vein. Media was perfused through the graft via portal vein.

Figure 2. Histology of recellularized liver grafts.

H&E staining of recellularized liver grafts co-copulated with hepatocytes and cholangiocytes showed the following. **(a)** Cholangiocytes are located within the ductules (black arrows) and hepatocytes were distributed throughout the parenchyma (red arrowheads) 2 days post hepatocyte seeding. **(b)** Some parts of the grafts were densely populated with hepatocytes. Scale bars 100 μm .

Figure 3. Repopulation of biliary tree in recellularized liver grafts.

(a–c) Representative images of liver sections in recellularized liver grafts stained for the cholangiocyte marker CK7 (brown), displaying that the ductal structures were repopulated by cholangiocytes adjacent to the parenchymal space, where the hepatocytes reside. (d) Native liver section stained for CK7 reveals comparable extent of cholangiocyte presence among the parenchymal cells. (e) A close-up view of a biliary ductule lined with CK7 positive cholangiocytes. Cholangiocytes within the ductule stained positive for Ki-67 (brown), indicating that the cells remained proliferative within the recellularized grafts. Scale bars: (a,b,d) 250 μm , (c) 100 μm , (e) 50 μm .

Figure 4. Assessment of biliary function of recellularized liver grafts via qRT-PCR. The expression of biliary functional markers GGT, CFTR, HNF-6 and NHE-1 in recellularized liver grafts were statistically not different from those measured in 2D controls. Fold expression levels of AE-2 and NHE-2 were significantly higher in the recellularized liver than in plate cultures (* $p < 0.05$ by unpaired t-test, $n = 3$).

Figure 5. Hepatic metabolism via albumin and urea production.

Albumin and urea production in recellularized liver grafts co-populated with cholangiocytes (1 million); 45 ± 5 million hepatocytes were seeded with 50 mL perfusion media. The engineered grafts co-populated with hepatocytes and cholangiocytes produced albumin and urea in perfusion culture at levels higher than grafts populated with hepatocytes only. [N.S. (non-significant) by paired t-test, $n = 5$.]