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Directing the growth and alignment of biliary epithelium within extracellular matrix hydrogels

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Abstract

Three-dimensional (3D) printing of decellularized extracellular matrix (dECM) hydrogels is a promising technique for regenerative engineering. 3D-printing enables the reproducible and precise patterning of multiple cells and biomaterials in 3D, while dECM has high organ-specific bioactivity. However, dECM hydrogels often display poor printability on their own and necessitate additives or support materials to enable true 3D structures. In this study, we used a sacrificial material, 3D-printed Pluronic F-127, to serve as a platform into which dECM hydrogel can be incorporated to create specifically designed structures made entirely up of dECM. The effects of 3D dECM are studied in the context of engineering the intrahepatic biliary tree, an often-understudied topic in liver tissue engineering. Encapsulating biliary epithelial cells (cholangiocytes) within liver dECM has been shown to lead to the formation of complex biliary trees *in vitro*. By varying several aspects of the dECM structures' geometry, such as width and angle, we show that we can guide the directional formation of biliary trees. This is confirmed by computational 3D image analysis of duct alignment. This system also enables fabrication of a true multi-layer dECM structure and the formation of 3D biliary trees into which other cell types can be seeded. For example, we show that hepatocyte spheroids can be easily incorporated within this system, and that the seeding sequence influences the resulting structures after seven days in culture.

Statement of Significance—The field of liver tissue engineering has progressed significantly within the past several years, however engineering the intrahepatic biliary tree has remained a

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Author contributions

P.L.L.: Designed experiments, optimized 3D printing methods, performed all imaging work, analyzed image quantification data, wrote the manuscript, and designed figures. M. Y.: Optimized and performed image quantifications and imaged dECM gels under SEM. J. S.: Performed rheological tests and analyses. R. N. S.: Principle investigator, assisted in experimental design, methods optimization, interpretation of data, manuscript writing and editing.

Disclosure statement

R.N.S. has financial interests in Dimension Inx, LLC which could potentially benefit from the outcomes of this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.actbio.2018.12.039>.

significant challenge. In this study, we utilize the inherent bioactivity of decellularized extracellular matrix (dECM) hydrogels and 3D-printing of a sacrificial biomaterial to create spatially defined, 3D biliary trees. The creation of patterned, 3D dECM hydrogels in the past has only been possible with additives to the gel that may stifle its bioactivity, or with rigid and permanent support structures that may present issues upon implantation. Additionally, the biological effect of 3D spatially patterned liver dECM has not been demonstrated independent of the effects of dECM bioactivity alone. This study demonstrates that sacrificial materials can be used to create pure, multi-layer dECM structures, and that strut width and angle can be changed to influence the formation and alignment of biliary trees encapsulated within. Furthermore, this strategy allows co-culture of other cells such as hepatocytes. We demonstrate that not only does this system show promise for tissue engineering the intrahepatic biliary tree, but it also aids in the study of duct formation and cell-cell interactions.

Keywords

Liver; Tissue engineering; 3D printing; Decellularized; Extracellular matrix; Hydrogel; Bile duct

1. Introduction

The field of liver tissue engineering has seen numerous advances in recent years [1,2]. Any number of these approaches have succeeded in restoring hepatic function in animal models, however many of these approaches focus on manipulating hepatocytes, often in ectopic sites. Restoring hepatocyte function has the potential to treat metabolic or synthetic disorders or acute liver failure, however they rely on an intact and functional biliary tree to ultimately remove the bile secreted by hepatocytes. Cholestatic liver disease, however, is the leading cause of liver failure with over 15% of liver transplants due to cholangiopathies, diseases of the biliary tree which cannot be alleviated by restoring hepatocyte function [3]. The biliary tree itself functions to store and modify bile in addition to transporting it to the duodenum. While there have been recent successes in creating portions of the extrahepatic biliary tree [4], strategies for engineering the intrahepatic biliary tree has not progressed significantly [5].

The intrahepatic biliary tree is a complex network permeating every level of the liver anatomy, from the hilum to the lobule. Development of the biliary tree is intimately tied to the extracellular matrix (ECM) [6,7]. The presence of ECM, specifically basement membrane proteins, allow biliary epithelial cells (cholangiocytes) to polarize and eventually form a lumen into which bile will be transported. Researchers have attempted to harness the innate structure and bioactivity of liver ECM by perfusing whole decellularized organs with cholangiocytes [8], however these approaches have not led to the same level of success as endothelial cells or hepatocytes [9–12]. Our lab has previously demonstrated the potential of liver decellularized ECM (dECM) hydrogels to induce the spontaneous formation of complex biliary trees *in vitro* on a scale not demonstrated in Matrigel or type 1 collagen gels [13–18]. However, these structures are disorganized and completely encased within hydrogels, precluding addition of other cell types. Strategies need to be developed that can harness the bioactivity of dECM in an organized manner that facilitates co-culture along with biologically relevant designs.

The utility of 3D printing in tissue engineering applications has greatly expanded in recent years [19,20]. The ability to precisely place both cells and biomaterials in three dimensions has revolutionized the complexity with which tissue engineers can reproducibly fabricate tissues that approach what is found in nature. 3D printing holds promise for the intrahepatic biliary tree, as its feature sizes range from millimeters in diameter in the hepatic duct, to tens of microns in diameter in the Canals of Hering [21]. However, the 3D printability of dECM hydrogels is less than ideal, prompting many researchers to design systems that allow dECM to be incorporated into the bioink as an additive [22], or synthetically recreating aspects of its functionality [23]. Multiple laboratories have also devised methods that allow for printing of weak hydrogels into a supportive hydrogel bath [24–26]. In the present study, we employ a sacrificial poloxamer Pluronic F-127 as a support structure to control the directionality and geometry of *in vitro*-formed biliary trees. Pluronic F-127 has often been used as a sacrificial or ‘fugitive’ ink which can serve as a mold for tubular structures such as microvasculature or epithelial tissue [27–29]. We take advantage of the ease with which F-127 can be printed, creating sacrificial structures that allow for the simultaneous incorporation of a cholangiocyte-laden dECM hydrogel. This work builds upon our previous work investigating the phenotype and function of these biliary trees by studying how the 3D structure influences the dECM hydrogel properties and resulting duct alignment in a manner that enables co-culture. This work also defines a system that contributes to the growing body of knowledge emphasizing the importance of the biliary tree in liver tissue engineering.

2. Materials and methods

2.1. Liver decellularization and digestion

Livers were harvested from 3 to 4 month old female Yorkshire pigs, and were decellularized and digested as previously described [13,30,31] in a method initially developed by Freytes et al [32]. Briefly, tissues were minced into ~3 mm cubes and subjected to alternating washes of 1 mg/mL sodium dodecyl sulfate and ultrapure water. Decellularized extracellular matrix (dECM) pieces were then lyophilized and milled into a fine powder and digested at 10 mg/mL dECM in 0.01 M HCl and 1 mg/mL pepsin from porcine mucosa (Sigma) for 48 h. Pepsin digests were stored at –80 °C until further use.

2.2. Cell culture, encapsulation, and spheroid formation

Immortalized mouse small cholangiocytes [33] expressing eGFP or mCherry were expanded as previously described [13]. Cells were trypsinized and encapsulated within cold dECM pregel. Pre-gels were formulated as described [13] by neutralizing with 1 M NaOH, buffering with 10x PBS, and kept on ice until 3D printing. HUH7 human hepatocellular carcinoma cell line was expanded as previously described [34]. HUH7 cells were formed into approximately 200 µm diameter spheroids using agarose microwell molds (Microtissues, catalog number 12–256). After incubation overnight within the agarose microwells, spheroids were gently washed 3X with warm PBS, followed by incubation in serum-free medium containing 5 µM CellTracker Red CMTPX (Thermo Fisher) for 30 min. Spheroids were then agitated to dislodge from microwells and transferred to microtubes and allowed to settle before resuspension in culture medium for seeding onto 3D printed

cholangiocyte-laden dECM scaffolds. HUH7 spheroids were seeded either immediately after cholangiocyte/dECM printing or after 7 days of cholangiocyte/dECM culture.

2.3. 3D-printing cholangiocyte-laden dECM and sacrificial Pluronic F-127

A schematic outlining the dual printing process is outlined in Fig. 1. A solution of 400 mg/mL Pluronic F-127 (Sigma) was aseptically prepared, stored at 4 °C in the liquid state, and loaded into a sterile 3D printing cartridge. The cartridge was then allowed to gel and warm to room temperature prior to printing. A cholangiocyte-laden dECM pre-gel was prepared as described above and loaded into a separate refrigerated sterile 3D printing cartridge, which was maintained at 4 °C before and during printing. All 3D modeling and design of scaffolds was performed in AutoCAD (Autodesk). All 3D printing was performed on a 3D Bioplotter (EnvisionTEC). The F-127 was 3D printed using 200 µm or 150 µm diameter nozzles at pressures of either 0.9 bar or 5 bar, respectively. Cell/dECM pre-gel was then either printed into the F-127 structures, or F-127 structures were stored at room temperature in a humidified and sterile environment until cell/dECM pre-gel would be infused by hand. In the case of co-printing, cell/dECM pre-gel was immediately co-printed into the F-127 structure. Composite F-127/dECM structures were then incubated for 1 hr at 37 °C to allow dECM to gel. Structures were then washed 5 times with 4 °C PBS to liquify and remove sacrificial F-127. Printed dECM structures were incubated in growth medium overnight, with medium switched to William's Medium E supplemented with 0.5% FBS, penicillin/streptomycin, 100 nM insulin (Sigma), and 5. µM hydrocortisone (Sigma) [13].

2.4. Confocal and multiphoton imaging

Live samples were imaged using a Nikon A1 Confocal Laser Scanning Microscope with an incubator attachment. Live samples were also imaged using a liquid immersion Nikon A1+ Multiphoton microscope. To confirm second harmonic generation (SHG) of collagen fibrils present within dECM, samples were subjected to 800, 920, and 950 nm wavelength laser emission. Structures visible under 800 and 950 nm emission but not visible under 920 nm were deemed to be collagen fibril SHG, while structures visible under all wavelengths are autofluorescence [35]. Samples of varying strut width were imaged under 800 nm emission to allow for visualization of SHG and eGFP cholangiocytes simultaneously. Only back-ward SHG is reported, as forward SHG yielded no new information.

2.5. Image quantification and statistical analysis

Initial image acquisition, stitching, and processing was performed in NIS Elements software (Nikon). Subsequent analysis of duct structures was performed in Imaris (Bitplane). Imaris automatic Filament Tracer function was employed to create a computational model of duct-like structures. 3D reconstructions of structures such as cells in 2D on the culture dish or on the exterior surface of the dECM hydrogel strut were manually deleted to obtain an accurate representation of ducts within a scan area. Statistics on angle of orientation, duct tortuosity, and duct branching angle were gathered for struts of 1, 2, 4, and 8 mm in diameter, as well as angled struts of 30°, 90°, and 150°, and for multi-layered structures. Angle statistics were grouped into 30° bins and normalized to the total number of ducts within a scan area.

2.6. Rheotogicat analysis

Rheological characterization of hydrogels was performed following a recommended protocol for hydrogels for tissue engineering applications [36]. Testing was performed on an Anton-Paar MCR 302 rheometer with a 25-mm parallel-plate fixture under strain-controlled conditions. Before sample loading, the lower Peltier cell was set to 4 °C. After sample loading and lowering of the measuring system, mineral oil was applied to the edges of the fixture and the system was enclosed within a solvent trap to prevent sample dehydration. Once the rheological measurement was started, the Peltier cell was rapidly ramped up to 37 °C, kept at 22 °C for 2 h and then ramped to 37 °C, or kept at 15 °C and then ramped to 37 °C. All samples were prepared fresh. Time sweeps were performed for 120 min. at 0.1% strain and 10 rad/s.

2.7. Scanning electron microscope (SEM) imaging

Struts of thicknesses 1 mm, 2 mm, 4 mm, and 8 mm were prepared and imaged under SEM as previously described [13]. Briefly, samples were fixed with 2% glutaraldehyde with 3% sucrose and dehydrated using an ethanol series starting with 30% ethanol and ending with 100%. Samples were critically point dried, osmium coated, and imaged using a LEO Gemini 1525.

2.8. Statistical analysis

Computational image analysis as well as rheological measurements were performed in triplicate. Statistical significance was determined using paired, 2-tailed Student's T-Tests.

3. Results

3.1. Duct alignment is controlled by strut width

Strut widths of 1 mm, 2 mm, 4 mm, and 8 mm and their degree of control of duct alignment in the direction of extrusion were first tested (Fig. 2A). Qualitatively, there was no reduction in amount of ducts formed per gel volume in 3D constructs compared to bulk cast gels. Nearly all encapsulated cells either proliferated, formed duct-like structures, or both. Quantification of duct orientation angle relative to the direction of extrusion (0°) indicated widths of 1 mm or less led to the most alignment, indicated by duct angles near 0° or 180° (Fig. 2C). Strut widths of 8 mm begin to show duct orientation perpendicular (near 90°) to the direction of extrusion. Duct alignment was hypothesized to be a result of collagen fibril alignment. When visualizing with multiphoton microscopy, the number of assembled collagen fibrils evident by second harmonic generation (SHG) decreases as strut width decreases (Fig. 2B). This was possibly due to a slower rate of heating imparted by a larger volume of 4 °C dECM pre-gel. However, rheological analysis of different heating rates of dECM indicates that the final storage and loss moduli are identical regardless of temperature ramping (Supplemental Fig. 1). However, SEM imaging of struts of varying thickness (Supplemental Fig. 2) indicate a reduced porosity in 1 and 2 mm thick struts and thicker fibers and larger pores in 4 mm and 8 mm thicknesses. Additionally, cracks and folds in the bulk gel structure are visible in certain samples.

3.2. Geometric control over 3D duct formation

F-127 struts at 1 mm widths oriented at acute (30°), obtuse (150°), or right (90°) angles were printed to determine the limits of the possible geometries aligned ducts will follow (Fig. 3A, B). Assigning a base strut of 0° initially leads to ~50% of ducts aligned near 0° or 180° (Fig. 3B, C). The orientation of the ducts within the second strut leads to peaks at 30°, 90°, or 150° respectively, while a higher proportion of orientation angles are present at angles greater than the printed structure as ducts round corners (Fig. 3C).

Large two-dimensional (2D) grid structures were printed to test if duct geometry could be repetitively controlled on a larger scale (Fig. 4A). Culture after 7 days indicates that the shape fidelity of the 2D dECM grid is reduced, as pore corners become rounded (Fig. 4B). Furthermore, while ducts may be aligned in narrow struts between pores, ducts within strut intersections appeared randomly oriented (Fig. 4C).

As an alternative, a sacrificial structure that would lead to a 3D grid of dECM was designed (Fig. 5A). After 7 days of culture, shape fidelity was maintained, as evident by sharp pore corners (Fig. 5B), while struts in the same plane showed rounded corners. To further illustrate that the two overlapping struts are in separate planes, cholangiocytes expressing two different fluorescent proteins (eGFP - green, or mCherry - red) were co-printed in perpendicular orientations (Fig. 5D). Fig. 5D shows a volume view of this image, indicating struts have not sagged into one another after 7 days in culture. Image quantification of separate channels indicates near perpendicular orientation of the two struts (Fig. 5E).

3.3. Hepatocyte-cholangiocyte co-culture

HUH7 human hepatocyte spheroids were seeded 1 day after initial dECM printing. After 7 days in culture, cholangiocytes near dense hepatocyte areas remained viable but did not proliferate and branch (Fig. 6A). This contrasts with areas of sparse HUH7 concentration, where ducts grew and branched as normal. HUH7 spheroids seeded after 7 days of cholangiocyte monoculture indicated that hepatocytes can be seeded without obvious adverse effects to existing more mature duct structures (Fig. 6B).

4. Discussion

Decellularized extracellular matrix (dECM) hydrogels have become powerful tools for tissue engineers to recapitulate organ-specific extracellular milieu in a reproducible and potentially translatable manner [37]. Several different decellularization and post-processing strategies have arisen to further tune bioactivity and mechanical properties to specific applications. However, limitations common to bulk gel culture such as nutrient diffusion and scale up have prompted researchers to devise new methods to manipulate dECM for complex organ engineering. 3D printing hydrogels is a central challenge in extending 3D printing technology to traditional tissue engineering [38,39]. Hydrogel bioactivity and printability (extrudability, self-supporting, etc) are often at odds with one another [40]. While 2D grids can be created by extruding dECM gels from a nozzle [41,42], creating multi-layer, dECM-only constructs where subsequent layers do not sag into the first is challenging. Thus, this has led several laboratories to develop support structures into which weak hydrogels can be

extruded. One method is to hot-melt print a poly(capro-lactone) framework wherein dECM hydrogels can be infused in a layer-by-layer fashion [41,42]. This approach has advantages in creating very large size scales, in addition to demonstrating the combinatorial biological effects of both dECM and 3D printing in the context of aligned myotubes for skeletal muscle tissue engineering [43,44]. However, issues may arise when implanting a mechanically stiff thermoplastic into a mechanically sensitive organ such as the liver, which may fibrose if exposed to excessive ECM stiffness [45,46].

In this study, we have taken a similar approach to 3D printing dECM hydrogels by employing a co-printed sacrificial support material, under the initial goal of controlling the formation of duct-like structures. We employ Pluronic F-127, a routinely-used sacrificial 3D-printable material which displays reversible gelation and liquification properties at room temperature (23 °C) and refrigerated (4 °C) temperatures, respectively. While there have been indications that Pluronic F-127 will negatively affect cell viability and proliferation if left in culture [47,48], several successive washes of cold buffer are sufficient to remove nearly all Pluronic from the construct such that there is no observable effect on *in vitro* tissue formation [28,29,44]. With no adverse effects on cell function, liquid dECM pre-gel can then be co-printed or infused by hand into the F-127 structure. The irreversible gelation of dECM hydrogels allow for the removal of F-127, leaving behind a pure 5 mg/mL dECM structure submerged in culture media.

To inform the design of future structures, certain parameters of scaffold design needed to be determined initially. We therefore first investigated the width at which ducts will most likely align in the direction of extrusion. Qualitatively, no reduction in the amount of ducts formed within 3D patterned gels was observed compared to previous experiments [13] using cast gels. This indicates that the confinement within a 3D dECM strut does not influence viability. We subsequently determined smaller strut widths led to increased alignment. However, larger widths led to alignment perpendicular to the direction of extrusion, possibly indicating width contraction of the dECM gel. We initially hypothesized that duct alignment resulted from collagen fibril alignment within the dECM gel. Multiphoton microscopy was chosen to visualize both collagen fibrils via second harmonic generation (SHG) and eGFP-expressing encapsulated cholangiocytes. While we observed enhanced alignment of ducts in short width (1 mm) struts, we observed only autofluorescence and no SHG. SHG was observed to increase with increasing strut width, leading us to hypothesize that, because of the larger dECM volume in wider struts, the slower the construct's temperature will increase to 37 °C once placed inside an incubator. Such a change in fibril assembly would lead to a change in hydrogel mechanics, which would be observable via rheological measurements. After testing three different temperature ramping profiles, we observed the same storage and loss moduli once 37 °C was reached, leading us to conclude the reduction in SHG is not a result of the rate of temperature change. We instead speculate that the reduction in SHG may be a manifestation of a similar phenomenon observed in solvent-based 3D printing [49]. In the article by Guo et al, larger diameter struts displayed an increase in polymer crystallinity due to slower solvent evaporation and increased mobility of polymer chains in a larger strut diameter. The reduced SHG in our structures may indicate that, within short strut widths, insufficient collagen molecules are available to diffuse and assemble into fibrils detectable with SHG. Regardless, ducts do show alignment within these struts, which is possibly due to

some other aspect such as tension or nutrient availability within dECM. Under SEM, however, we observe increased fiber thickness and pore size as strut width increases. Occasionally, folds in the bulk gel were observed under SEM, leading us to hypothesize that some aspect of the overall gel structure itself, such as tension or confinement, is leading to the alignment. However, the dense packing in smaller widths and the folds themselves could also be artifacts of the SEM preparation process.

The logical progression from alignment experiments is to test the limits of what geometry ducts will follow. Changing the z height of struts is likely to simply lead to taller gel with duct alignment still dependent on strut width. We instead sought to explore design parameters, such as different angles and multi-layered structures, more applicable to the complex architecture of the biliary tree. The biliary tree itself is highly tortuous [50] and formation of duct-like structures along a variety of geometries must be demonstrated if we are to engineer one. To that end, we designed simple 1 mm wide angled struts and quantified the resulting duct structures. As expected, most ducts were aligned in the directions of the two primary struts, with a small number of ducts measured at larger angles as they rounded the corners. One caveat to representing computational image analysis of duct orientation angle as a histogram is that statistical significance can be artificially inflated simply by changing bin size. In addition to the significance between individual bins, we would like to also draw attention to the overall trend in duct alignment. When exploring more complicated architectures such as two-dimensional (2D) grids, however, we note several shortcomings. Primary among them is reduced shape fidelity of the dECM structure, which led to random duct orientation at strut intersections. While this strategy enables coculture and seeding of a second cell type in the pores, 2D structures are ultimately limited in the available designs for coculture. Furthermore, such 2D arrays can easily be fabricated using other methods such as PDMS stamps, and have been shown to enhance cell viability and function when hepatocyte co-cultures are patterned into cords [51,52]. However, there are limitations in size scale and the engineered complexity available for 2D strategies.

Sacrificial materials are more powerfully leveraged when creating 3D structures. A number of researchers have developed methods to 3D print weak hydrogels into baths that allow for gel curing and tissue culture [24–26]. This strategy enables true layer-by-layer fabrication of multiple weak hydrogels at higher resolutions within an embedding medium which can be eventually removed. The chief difference from the present study is that sacrificial F-127 is immediately removed before extending culture time, leaving a multi-layer pure dECM structure surrounded by culture medium. To the best of our knowledge, this is the first time a self-supporting multi-layer structure composed of such a low dECM weight fraction (5 mg/mL) has been reported. The primary advantage to this approach is that a suspension of cells, or spheroids, can be seeded into 3D dECM at any point during culture, allowing for interpenetration of cells within the structure. This would not be possible if the entire structure were embedded within a gel or were supported by permanent thermoplastics. The multi-layer capability of our approach also leads to better shape fidelity than simple 2D grid structures. While low weight fraction dECM gel struts sag after removal of culture medium, addition of replacement media allows the structure to restore its original shape, enabling seeded cell interpenetration. Sufficiently mature tissue with addition of several cell types would eventually be robust enough for manipulation and surgical implantation.

In our previous studies, we have shown lumen formation and transport of fluorescent bile salts into the lumen interior of these duct-like structures [13]. However, the lumen are discrete and discontinuous, leading us to believe that the duct structures themselves are not quite mature. Co-culture with hepatocytes has been shown by others to enhance *in vitro* duct formation possibly due to the hepatocytes' bile secretion and paracrine signaling [15]. Furthermore, in the context of liver tissue engineering, explorations of cholangiocyte-hepatocyte co-culture have been limited [15,16,53]. Anatomically, bile canaliculi between hepatocytes lead to the Canals of Hering and then to bile ductules lined by small (immature) cholangiocytes which lead to intrahepatic ducts lined by large (mature, functional) cholangiocytes, ultimately terminating in the common hepatic duct [54]. The goal of these co-culture studies was to investigate the effects that hepatocytes have on the *in vitro* development of bile duct structures within dECM and to determine if it is possible to recreate a scenario that can recapitulate the hepatocyte-cholangiocyte interaction naturally seen in liver tissue (i.e. formation of the Canals of Hering). Our initial experiments indicate that seeding of HUH7 cells immediately after cholangiocyte/dECM printing may lead to localized nutrient competition. This is evident by reduced duct formation in areas of high HUH7 density, and normal duct formation in other areas. If the HUH7 cells were secreting soluble factors that stifled duct formation, the entire structure would have shown this effect instead of just in localized regions. We subsequently showed that seeding HUH7 spheroids after first culturing the cholangiocyte/ dECM structures for 7 days of culture to allow for cholangiocyte branching is an alternative option. One day after seeding the hepatocytes within the duct-containing structures, the ducts showed no signs of deterioration. This approach may be more mimetic of what occurs in the developing liver, as ductal plate cholangiocytes will begin to mature and polarize prior to adjacent hepatoblast to hepatocyte differentiation [6,7]. We hypothesize that prolonged (several weeks) culture would lead to hepatocyte proliferation, however proliferation of *encapsulated* cholangiocytes appears to halt after several weeks in culture (not shown).

5. Conclusion

We have shown that the formation of complex biliary trees can be controlled *in vitro* using 3D printed dECM hydrogels and sacrificial support structures. Image quantification of the resulting ducts indicate a strong degree of control in several 2D geometries. We show that a 3D structure composed of only dECM hydrogel can be achieved using sacrificial materials, and this serves as a proof of concept that can lead to the creation of organic 3D structures based on the biliary tree. Furthermore, we demonstrate this system facilitates 3D patterned co-culture of hepatocytes and cholangiocytes. While human/mouse hybrid tissues obviously do not occur in nature, the cholangiocyte and hepatocyte cell lines in this study demonstrate the feasibility of this approach to control duct formation while enabling patterned co-culture of hepatocytes. These cell lines serve as easily expandable models for primary isolated cells or for proliferative stem cell-derived sources, as both arise from bipotent hepatoblasts during development. Both hepatocytes and cholangiocytes line the Canals of Hering (CoH), a unique feature of the liver lobule, and CoH recreation *in vitro* is something tissue engineers have yet to achieve. Co-culture of hepatocytes and cholangiocytes can therefore allow us to answer several questions about liver biology, such as if a phenotypic intermediate

(hepato-blast, hepatic stem cell) is necessary for CoH formation [55]. Future studies may incorporate hepatoblasts that develop into ducts inside scaffold struts and into hepatocytes within scaffold pores, enabling culture of other cell types within the liver such as vascular or sinusoidal endothelial cells as well as stellate cells and Kupffer cells. Successfully recreating all levels of the liver microstructure will require sophisticated designs and precise placement of the correct cell types and biomaterials in three dimensions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematic of the dual printing process. A. A 3D structure of Pluronic F-127 is first 3D printed. B. Cholangiocyte-laden dECM hydrogel is co-printed into the interior of an F-127 structure either by hand or using a 3D printer. C. The dual material structure is first incubated at 37 °C for 1 h to gel the dECM, followed by several washes of 4 °C to liquefy and remove the F-127. D. After 7 days in culture, a biliary tree in the shape of the dECM structure will arise, which can then theoretically be seeded with other cell types such as hepatocytes.

Fig. 2.

Strut width influences duct alignment. A. Printed Pluronic F-127 structures in increasing widths. B. Cholangiocyte duct structures (green) and collagen fibrils visualized with second harmonic generation (blue). C. Quantified duct orientation angle relative to the direction of extrusion. Error bars $\pm$ SD, * = $p < 0.05$, $n = 3$. Scale bars = 25 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Controlling duct formation along different angles. A. Printed Pluronic F-127 structures into acute, right, and obtuse angles. B. Maximum intensity Z-projections of resulting duct structures after 7 days in culture. Dotted lines indicate 0° references for orientation angle alignment quantification. C. Quantified duct orientation angle relative to 0° reference. Error bars $\pm$ SD, * = $p < 0.05$, $n = 3$. Scale bars = 500 μ m.

Fig. 4. Controlling duct formation in a 2D grid. A. Schematic of the 2D grid printing process. B. Maximum intensity Z-projection and bright field image of strut intersection at 1 day and at 7 days (C) in culture. Scale bars = 250 μm .

Fig. 5. Controlling duct formation in a 3D grid. A. Schematic of the 3D grid printing process. Layers 1–3 of F-127 control the first layer of dECM, layer 4 isolates what will become second dECM layer printed with layers 5–7 of F-127. B. Maximum intensity Z-projection of resulting duct-structures after 7 days in culture. Curved corners (→) indicate struts are in the same plane, sharp corners (*) indicate struts are in separate planes. C. Maximum intensity Z-projection of the intersection of 2 struts printed with either eGFP or mCherry cells, and its

rotated volume view (D) indicating struts have not sagged. E. Quantified duct orientation angle of separate channels.. Error bars $\pm$ SD, * = $p < 0.05$, $n = 3$. Scale bars = 250 μm .

Fig. 6.

Enabling hepatocyte/cholangiocyte co-culture. A. Maximum intensity Z-projection of hepatocyte (red) and cholangiocyte (green) co-culture after 7 days in co-culture. * indicate areas of high hepatocyte seeding density flanked by low duct formation, # indicate areas of low hepatocyte seeding density flanked by normal duct formation. Dotted lines outline cholangiocyte-laden dECM struts. B. Maximum intensity Z-projection of hepatocytes and cholangiocyte co-culture, where hepatocytes are seeded after 7 days of cholangiocyte only culture. Scale bars = 250 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)