BIOENGINEERED LIVER ASSEMBLOIDS WITH ZONATION

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Abstract

There are a number of pressing issues that the creation of a biomimetic liver culture may be able to solve including catching hepatic mal interactions that are currently missed in preclinical drug screening and offering an alternative solution in the shortage of organs for liver transplant.[1-4] There are multiple hepatic models that have been created to overcome these issues. However, of the many considerations that need to be taken in the creation of a biomimetic liver model, many fail to capture the functional zone-patterning that is found *in vivo*. Here is detailed the creation of a hepatic assembloid model that incorporates zone-specific human pluripotent stem cell derived hepatocytes for the recapitulation of zone-patterning in the liver tissues. Use of our lab's z-wire plate and PGS z-wire scaffold allows for the formation of elongated 3D tissues that resembles the overall morphology of the liver acinus and facilitate the spontaneous development of an aligned vascular-like network. Sustained hepatocyte-specific function in these tissues are promising indicators for application of the hepatic models in drug screening, disease modelling, and regenerative medicine.

Preface

The manuscript, "*Bioengineered Liver Assembloids with Zonation"* has been included in this thesis as it has been prepared for publication. This work has been conducted from May 2019 through July 2021. My contributions to this manuscript include performing the experiments, analyzing the results, and preparation of the manuscript. The work that is contained in this writing constitutes the work that has been conducted towards fulfillment of my Master of Applied Sciences degree.

Contributions to this manuscript: T.L.J.S. performed the experiments, analyzed the results, and prepared the manuscript, L.E.P.E contributed to image collection, C.C. helped with tissue culture maintenance, S.H. envisioned the concept and provided hPSCs, B.Z. envisioned the concept, supervised the work and prepared the manuscript.

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I would like to thank my supervisor Dr. Boyang Zhang for taking a chance on me as an undergraduate student, and for his continued guidance through a year that was anything but normal.

I would like to thank Dr. Wenzi Ckurshumova for believing in my abilities, and whose relentless encouragement convinced me to pursue graduate studies.

I would like to thank my grade 12 biology teacher Mr. Stephen Merner for his mentorship. Without it I would have never pursued a university education or a path in biology.

I would like to take this small space to recognize Henrietta Lacks. The size of her unknowing contribution to the health and medical fields cannot begin to be put into words. If not for her relentless advocation for her own health, medical research would not be nearly as advanced as it is today. While her story has been a driving force behind increasing autonomy in health care, 70 years later women continue to have to fight to have their health concerns heard. May her name continue to live on in our memories and her story be present in our minds with each moment spent in the lab.

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List of Abbreviations and Symbols

2D	Two-dimensional
3D	Three-dimensional
ALF	Acute Liver Failure
BC	Bile Canaliculi
DILI	Drug Induced Liver Injury
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extra Cellular Matrix
ECs	Endothelial Cells
ESCs	Embryonic Stem Cells
EMEM	Minimum Essential Medium
FBs	Fibroblasts
FBS	Fetal Bovine Serum
FDA	Federal Drug Association
HCs	Hepatocytes
HSCs	Hepatic Stellate Cells
hPSCs	human Pluripotent Stem Cells
hiPSCs	human induced Pluripotent Stem Cells
hPSC-ECs	human Pluripotent Stem Cell derived Endothelial Cells
hPSC-HCs	human Pluripotent Stem Cell derived Hepatocytes
HUVEC	Human Umbilical Vein Endothelial Cells
KCs	Kupffer Cells
LR	Liver Resection
LSECs	Liver Sinusoid Endothelial Cells
NPCs	Non-Parenchymal Cells
PDMS	Polydimethylsiloxane
PGS	Poly(glycerol sebacate)
PHHs	Primary Human Hepatocytes
PS	Polystyrene
ROI	Reactive Oxygen Intermediates

Chapter 1. Introduction

There has been a major push in research towards the development of *in vitro* tissue cultures that emulate innate human systems. Of particular interest in this conquest of biological design has been in the development of biomimetic liver tissue cultures. The reason for this is due to the significant value that such a culture may present in the fields of regenerative medicine, drug discovery and disease modeling. World-wide there is an overwhelming demand for liver transplants, many of which go unmet.[3] In early stages of drug discovery there is major oversight of hepatic mal interactions due to the currently employed methods used in hepatic drug screening.[5] The oversight of hepatotoxicity in pre-clinical trials accounts for 22% of safety related clinical trial failures and 32% of post-market attrition.[6] In disease modelling, hepatic cultures either do not capture the patient-specific characteristics of a disease or can not be cultured for long enough to assess the therapeutic effect of repeat dose administration.[5, 7, 8]

A biomimetic liver culture that employs human pluripotent stem cell derived hepatocytes (hPSC-HCs) may present a solution to each of these pressing issues. Such a culture could be utilized at an early stage in pharmaceutical testing to reveal mal interactions that the drug may have *in vivo* later during human introduction.[9, 10] It may also be used for *in vivo* hepatic repair, significantly reducing, or even potentially replacing the need to rely on human organ donors.[1, 11] Finally, hPSCs patient specificity and potential for long-term culture means that these cultures may be used to effectively study and assess individual disease treatment plans.[8, 11]

1.1. Thesis Objective

In this thesis, the development of a hPSC-derived assembloid three-dimensional (**3D**) biomimetic liver model that incorporates zone-specification is discussed. The focus of the work that has been undertaken investigates the feasibility of the creation of such a tissue culture with anticipated end use in drug discovery, disease modelling, and regenerative medicine. It is reasoned that the publication of the results discussed within will add to the lacking collective knowledge of hPSC-HCs in culture, and readily increase accessibility of the creation of zone-patterned hepatic tissues following the novel culturing technique of assembloid biofabrication. Immunohistochemical staining, histological sectioning and staining, and ELISA albumin assay were used to assess structure and function of the tissues.

1.2. Thesis Outline

The work that has been undertaken is covered in four chapters. Chapter 1 provides an introduction into the thesis topic as well as an outline for the thesis. Chapter 2 is a literature review which provides the reader with context for the research work. The review touches on liver anatomy and physiology, diseases of the liver, hepatic models used in pre-clinical drug discovery, considerations to be taken in hepatic model development, and a few notable recent advancements in hepatic tissue culture development. Chapter 3 constitutes the body of the research. This chapter incorporates a replica of the manuscript: "*Bioengineered Liver Assembloids with Zonation*", as it has been prepared for publication, and is preceded by a short introduction to the work. This text contains the results and discussion of the work as well as the methods and materials used. Chapter 4 concludes the thesis with a look at the significance of the work, and future work to be conducted with the hepatic model.

Chapter 2. Literature Review

2.1. Liver Anatomy and Physiology

The basic unit of the liver is the hepatic lobule; a hexagonal building-block whose repeated structure forms the four lobes that compose the liver. At each corner junction of an individual lobule is a hepatic triad made up of a portal venule, bile ductule, and hepatic arteriole. The portal venule and hepatic arteriole of each of the six hepatic triads drain through individual sinusoids to meet at the central vein, at the middle of a lobule (**Figure 1**).[12]

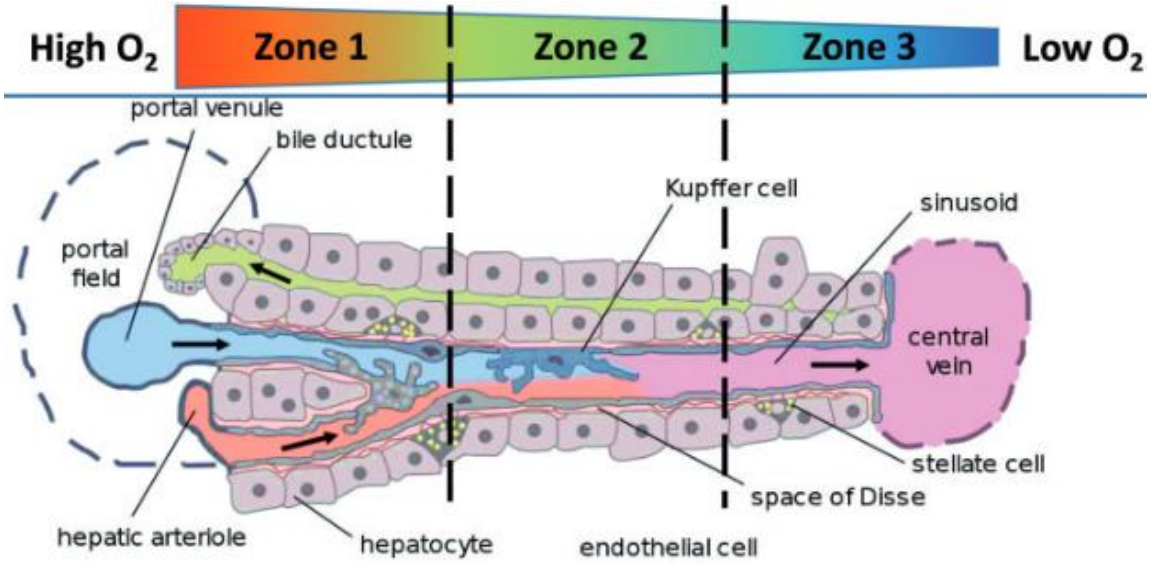

Figure 1. Schematic of the liver acinus. Modified from [13]

2.1.1. Liver Acinus Cellular Composition

The liver sinusoids are lined by fenestrated liver sinusoidal endothelial cells (**LSECs**) which are further surrounded by a layer of hepatocytes (**HCs**).[14] HCs, the liver's parenchymal cells, make up the majority of the cells within the liver, accounting for 60-

70% of cell number and up to 80% of the cellular volume.[15] Separating the LSEC and HC layers is a substantial extracellular matrix (**ECM**) where the Hepatic Stellate Cells (**HSCs**), responsible for ECM remodeling and vitamin A storage, can be found.[12] Kupffer Cells (**KCs**), the liver's resident macrophages, can be found in the sinusoidal space.[14] Together, LSECs, HSCs, and KCs make up the liver's non-parenchymal cells (**NPCs**) and the three cell types account for the remaining percentage of the liver's cells by number and volume.[15] Together, these structures constitute the liver acinus (**Figure 1**).

2.1.2. Liver Zonation

The architecture of the liver sinusoid creates a natural oxygen and metabolite gradient that progresses from the hepatic triad to the central vein. Those cells closest to the hepatic triad in the periportal region have access to the most oxygen-rich and nutrient-rich bloods that blend from the hepatic artery and the portal vein. This region is called zone 1. The cells towards the central vein receive the oxygen and nutrient depleted blood. This region is called zone 3. Differences in blood oxygen, hormone, and substrate levels along the liver sinusoid as well as differing cell-cell and cell-ECM interactions results in morphological and histochemical differences in all cell types that give rise to the acinus's zone designations (**Figure 1**).[16] Some notable functional differences in HCs include glutathione peroxidase production, albumin synthesis, and bile acid export carrier formation, largely attributed to zone 1 HCs, and xenobiotic metabolism largely attributed to zone 3 HCs (**Table 1**).[13, 16, 17] Zone 2, the transitional region between zones 1 and 3, serves interdisciplinary roles as the functional shift occurs.[13]

Table 1. Zone 1 and zone 3 hepatocyte functional roles. Adapted from [16]

	Parenchymal cells				
	Periportal zone		Perivenous zone		
Physiologic function	Metabolic function	Enzyme or protein involved	Physiologic function	Metabolic function	Enzyme or protein involved
Oxidative energy metabolism		Succinate dehydrogenase			
Glucose output	1. Glucosefrom pyruvate (gluconeogenesis)		Glucose uptake	1. Glucose to pyruvate (glycolysis)	
	2. Glucose from glycogen	Glucose-6-phosphatase		2. Glycogen from glucose	Glucokinase
	3. Glycogen from pyruvate	Phosphoenolpyruvate carboxykinase		3. Glycogen to pyruvate	Pyruvate kinase type L
Urea formation	Urea from amino acid nitrogen and from NH_3	Carbamoyl phosphate synthetase	Glutamine formation	Glutamine from NH_3	Glutamine synthetase[a]
Protective metabolism	1. Glutathione peroxidation	Glutathione peroxidase	Xenobiotic metabolism	1. Monooxy-genation	Cytochrome P450
	2. Glutathione conjugation	(Glutathione level)		2. Glucuroni-dation	UDP-glucuronosyl transferase
Plasma protein synthesis		Albumin α2-Macroglobulin Fibrinogen	Plasma protein synthesis		a-Fetoprotein Angiotensinogen α1-Antitrypsin
Cholesterol synthesis	Hydroxymethyl glutaryl-CoA reductase[b]				
Bile formation	Taurocholate uptake carrier				
	Bile acid export carrier				

2.1.3. Biliary Network

Bile canaliculi (**BC**) are the smallest branches of the liver's excretion system which ultimately diverts into the small intestine. Through here blood constituents that are too large to be filtered through the kidneys, as well as xenobiotics metabolites are expelled.[18, 19] The BC flow counter-current to the sinusoid, draining into the bile ductule of the hepatic triad.[20] The biliary network which spans the entirety of the liver acinus is naturally formed by HCs with HCs constituting the lining of the BC (**Figure 1, 2**).[21] Connecting the canaliculi to the bile ductule is the Canal of Herring which is lined by liver progenitor cells. The remainder of the biliary network including the bile ductule is lined by cholangiocytes. As the branches are traced back to the common hepatic duct which is the biliary tree's connecting component to the duodenum, the size of the cholangiocytes increase.[22]

The formation of BC is made possible by the polarization of HCs and the formation of tight junctions between HCs.[18, 23, 24] The basolateral membranes of HCs are exposed to the ECM that connects to the sinusoidal spaces and the apical membranes are found between adjacent HCs.[18] While canaliculi have an average diameter of 1 – 2 μm, diameter of the canaliculi in the periportal region are greater than those found perivenous. This is to accommodate a greater volume of transport that occurs in this region as well as the greater production of bile in zone 1 since it is in this region that bile acid export carriers are primarily produced.[16, 20]

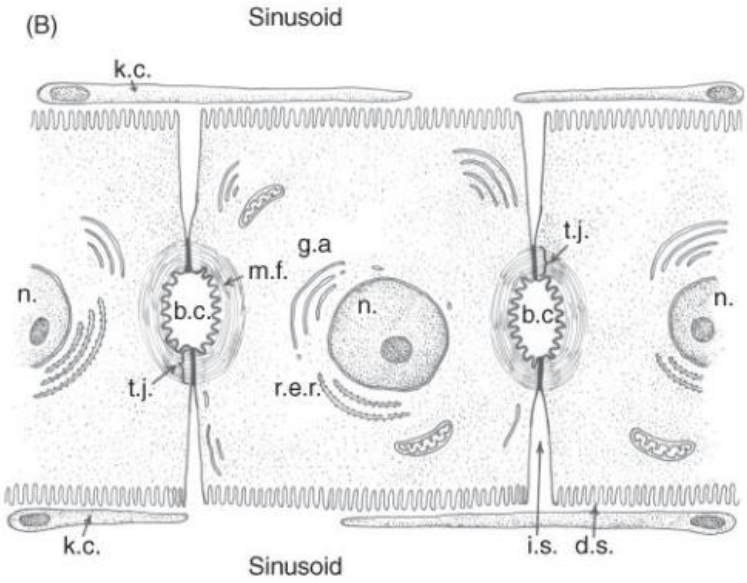

Figure 2. Schematic of bile canaliculi location. Bile canaliculi (b.c.) form between adjacent hepatocytes and are sealed by tight junctions (t.j.). Adapted from [22]

2.2. Diseases of the Liver

The liver is generally regarded as a self-healing organ, and for the most part this is true. Studies that have analyzed the regenerative capabilities of the liver typically follow patients that have undergone liver resection (**LR**) surgery.[3, 25, 26] A standard LR removes approximately 70% of the liver mass.[25] Through these studies the high functional reserve of the liver has been elucidated. HCs are typically in a quiescent state but during liver damage such as that seen in LR, the HCs in the remaining healthy liver mass function to maintain homeostasis while simultaneously devoting resources to regenerate the lost mass.[25, 26] Studies also suggest that regeneration can be attributed to all mature cell populations of the liver including all NPCs and cholangiocytes.[26]

Typically, damaged liver is restored to 100% of its initial volume.[25, 26] In rodents this restoration occurs in a matter of weeks, whereas for humans this process can take up to three months with an initial burst of proliferation seen in the first seven days following damage.[25] Some factors such as age can limit the liver's restorative abilities, impacting the percentage of cells involved in proliferation and therefore the speed of recovery.[26, 27] There are also afflictions that impede the liver's restorative abilities by causing extensive or irrevocable damage to the liver tissue in which the damage is too great for the remaining cells to recover. These afflictions are detailed in this section.

2.2.1. Chronic Liver Disease

Chronic liver disease, or liver cirrhosis, is damage to the liver tissue that occurs due to the sustained activation of the wound healing response. This process results in the uncontrolled replacement of the healthy ECM constituents with high levels of collagens I and III, also known as the scarring of the liver. HSCs are heavily implicated in this disease as they are responsible for the remodelling of the liver's ECM. Liver scarring leads to the capillarization of the LSECs, blockage of the vital endothelial fenestrations. With capillarization HCs are unable to obtain oxygen and nutrients and as a result they die.[28]

The disease, with multiple inciting factors such as hepatitis B, hepatitis C, excessive alcohol consumption, and non-alcoholic fatty liver disease, can be attenuated at an early stage by the removal or control of the inciting factor, allowing the remaining healthy cells to proliferate and restore the damaged tissue.[12] However, due to the liver's high functional reserve individuals will often remain unaware of their affliction until an advanced stage of liver scarring, liver cirrhosis, has been reached. When a state of liver cirrhosis has been reached the damage is too extensive for the liver to recover.[12, 29] With no other treatment available liver cirrhosis remains the leading reason for liver transplantation.[12] However, donor demand outweighs availability and therefore there is a great need for an alternative course of remediation for these individuals.[1-3, 30]

2.2.2. Acute Liver Failure

Acute liver failure (**ALF**) is the rapid decline of liver functionality. 50% of ALF cases can be attributed to drug hepatotoxicity, drug induced liver injury (**DILI**).[30] DILI is among the leading reasons for safety related drug attrition during clinical testing and post-market introduction.[31, 32] With the cost of bringing a single drug to market reaching over $1 billion USD the withdrawal of a drug candidate in late stages is highly unfavourable.[33, 34] There are two types of DILI: intrinsic and idiosyncratic. Intrinsic DILI is dose dependent and often predictable. Idiosyncratic DILI on the other hand is hard to predict, and the mode of liver injury is not well-understood.[35]

Intrinsic DILI is the type of liver injury that is most seen in acetaminophen overdose.[31, 35] In the case of many xenobiotic interactions, hepatocellular damage first presents in the HCs of zone 3. This is due to zone 3 HCs being responsible for the metabolism of drugs which can result in the production of reactive oxygen intermediates (**ROI**), while zone 1 HCs are primarily responsible for the production of glutathione peroxidase which is attributed to the detoxification and reduction of ROI. The greater concentration of ROI in zone 3 and ROI protective agent in zone 1 leaves zone 3 HCs susceptible to damage by the ROI.[16]

Idiosyncratic injury is the type of DILI that is commonly attributed to hepatotoxicity oversight in pre-clinical culture screening.[35] It is not as well understood as intrinsic DILI as it does not appear to be dose dependent, and often does not present directly following drug administration but rather weeks or months later.[31, 35] Its unpredictable nature makes it

difficult to screen for with strategies currently used in pre-clinical drug trials, and therefore typically is not noticed until end stage clinical trials or post-market introduction.[34, 36] One line of thought suggests that idiosyncratic injury may be due to an autoimmune-like response induced by drug presence, which aligns with the fact that idiosyncratic injury is individual specific.[31]

Cholestatic injury, the impairment of bile flow out of the liver, can be caused by either intrinsic or idiosyncratic reaction.[37] In intrinsic injury, drugs cause damage to the HC bile salt export protein causing intracellular accumulation of bile acids. This build-up can eventually lead to hepatocyte death.[34] The less frequent but none-the-less severe case of vanishing bile duct syndrome is an idiosyncratic form of cholestatic injury that presents as the name suggests.[38] Cholestatic injury is one of the leading reasons for drug-induced hepatotoxicity, and a leading reason for safety related late-stage drug attrition in the drug discovery process.[36]

2.3. Current Hepatic Models used in Pre-Clinical Drug Testing

Current techniques used to culture hepatic cells for the cell testing stages of drug discovery are markedly simplistic when compared to the innate human liver anatomy. The methods of hepatic culture do not recapitulate well the *in vivo* microenvironment, therefore they do not entirely capture the functionality of the liver.[4] The high level of dissimilarity between human liver structure and function compared to those of animals that are used in pre-clinical trials can account for the poor predictability of these trials.[39] It is for these

reasons that there is such a high level of oversight in hepatic mal interactions with potential pharmaceutic compounds during the early stages of drug screening.[4, 5]

Current cell culturing techniques used in drug screening include monolayers, sandwich cultures, and hepatic spheroids (**Figure 3**).[15] Primary cells are currently considered to be the gold standard for use with these cultures.[7, 18, 40] The reason for the use of these culturing methods is their relative ease of development, low cost, and high throughput capabilities which is important for early-stage drug screening.[8, 40, 41] In the order listed above, the techniques grow increasingly in cost as well as in the complexity of culturing. However, they also progress in their similarity to the innate liver environment.[15] Evidently there is a trade-off between culturing cost, and the relative effigy of culture to the organ it is developed to represent. Although a more complex culture may call for greater upfront capital, investing in more complex culturing techniques has the potential to save both time and money from early detection of what could otherwise be overlooked hepatotoxic interactions. More complex cell cultures have the potential to expedite the process of bringing a product to market by catching required reformulation significantly sooner than would otherwise be discovered.[9, 42]

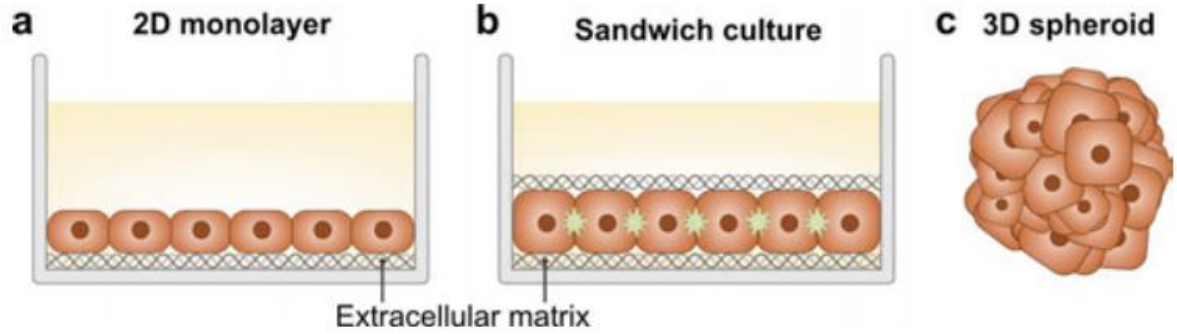

Figure 3. Common hepatic models used in pre-clinical drug-screening. Adapted from [15]

2.3.1. Monolayer Culture

Monolayers are the simplest form of the three cultures discussed here. They are two-dimensional (**2D**) cultures, a single layer of cells grown on a base layer of ECM (**Figure 3**).[15] They are easy to produce and operate and can be developed at a relatively low cost. For these reasons, cultures can be used for high throughput drug screening.[5] However, their utility is limited to screening drugs for a couple days at most.[8, 15] When the gold standard of hepatic cells, primary human hepatocytes (**PHHs**), are cultured in this format, they undergo rapid dedifferentiation and functional decline, losing their ability to metabolize and transport drugs as well as their ability to produce albumin.[5, 7, 8, 15, 40, 41] As a result, cultures are typically used for 4–72-hour drug incubation tests at most.[8, 15]

2.3.2. Sandwich Culture

Sandwich cultures are slightly more complex than monolayer cultures. Here, a second layer of ECM is added on top of the 2D cell layer (**Figure 3**).[15] This added layer of ECM creates a more *in vivo* like culture environment, allowing the cells to form either cell-cell or cell-ECM connections on all sides.[18] These interactions result in a decrease in metabolism downregulation and cell dedifferentiation compared to a monolayer format.[8, 15, 40] PHHs and hiPSC-HCs have been shown to polarize in sandwich cultures, meaning that they are capable of forming BC.[18, 21, 23, 40] However, one study that assessed BC formation in PHH sandwich cultures found there to be BC leakage regardless of the type of ECM used, which presents an issue if attempting to use the cultures for long-term excretion studies.[18] PHHs in sandwich culture can typically be maintained for upwards of two weeks

before substantial functional downregulation and cell dedifferentiation is seen, therefore cultures are typically used for short-term studies of transport and uptake.[40]

2.3.3. Spheroid Culture

Spheroid cultures are formed by the self-aggregation of cells in culture.[15] Rather than growing in a 2D layer of cells, these cultures form into 3D spheres.[15] This culturing method encourages the production of ECM by the incorporated cells.[15] The high level of cell-cell contact and the naturally formed ECM allows for the preservation of mature hepatic phenotype and function in PHHs for much longer than is seen in the other two culture methods.[11, 15, 36, 40, 43] 3D spheroids are capable of sustaining PHH albumin secretion, metabolizing enzyme production and function, and cell polarization for up to five weeks.[11, 15, 36, 40, 43] Due to the benefits that they offer over monolayer and sandwich cultures, they have largely been used for longer-term toxicity screening.[40, 43] However, spheroid cultures are prone to the development of a necrotic core due to poor oxygen and nutrient transport into the inner cell mass, therefore spheroid size must be regulated carefully.[8]

2.3.4. Animal Testing

The use of animal models in pre-clinical drug screening has been mandated by the Federal Drug Association (**FDA**) since 1938.[39] Lately, the utility of animal models has come into question. With a high degree of dissimilarity between the liver structure and metabolic activity of humans and the animals used in testing, the prediction accuracy that animal models have in detecting hepatotoxic events has been compared to tossing a coin, or random chance.[39, 44] Concerns then arise for participants involved in clinical trials being at risk of experiencing hepatotoxic events.[5, 39] The reverse case of formulations with potential

therapeutic effects in humans being discontinued due to hepatic mal interactions seen in animal models is also a concern.[9, 39]

Animals have been used as test cases for centuries, largely due to the ethical concerns that surround subjecting humans to the same experimental trials.[9] However, as time goes on, humans have progressively questioned the ethics of the use of animal models for these studies, creating great controversy on the subject.[4, 5] Ethics aside, the cost of animal studies is high both in terms of money and the time that pre-clinical animal trials add to the drug development process.[39] Between the poor predictability, rising ethical concerns, and high cost of animal studies it may be time for this practice to be supplemented with an alternative, or discontinued altogether.

2.4. Considerations of Hepatic Culture Development

2.4.1. Cells and Cell Sources

A major design component to be considered when creating a tissue culture is the type of cells to be used. There are three cell sources that may be considered for HCs: primary human cells, carcinoma or immortalized cell lines, and stem cells.[5, 45] The choice of cell type depends on availability, accessibility, and the specific end-goal of the culture with consideration for the question that one wishes to answer.

PHHs are considered to be the gold standard of cell choice in drug studies due to the near innate level of functionality that they are capable of providing.[5, 7, 18, 40, 45] However, the utility of these cells is highly dependent on the method under which they are cultured, with

their survival and retained functional levels ranging from a couple days to several weeks, culture method dependent.[11, 36, 40, 43]

PHHs use is limited by their availability and the complications surrounding long-term storage of the cells.[5, 7, 8, 11, 36] These cells must be harvested directly from the liver, limiting the accessibility of the cell source.[5, 7, 11] Advancements in cryopreservation of PHHs have increased viable yield to greater than 90% following one year in liquid nitrogen, however issues remain with the thawed cell's drug clearance performance.[5] PHHs are also patient specific, retaining the properties of the individual from which they were harvested.[7, 8, 36] Therefore, in pre-clinical drug screening which requires testing against phenotypes representative of the broader population, the use of PHHs from different sources is required.[34] Together, these issues attribute to a final problem with PHHs use; the high cost of obtaining and using this cell type, which presents another accessibility barrier to many labs.[36]

Hepatic carcinoma or immortalized cell lines overcome many of the limitations of PHHs.[36] These cells are a nearly limitless source that can be cryopreserved for long periods of time with little impact on function.[5, 8] The main drawbacks of this cell source are their abnormal functional levels, and their limited genetic variability as each line is derived from a single source.[5, 7, 8] Some more popular and well-established cell lines include HepG2, Huh7, and HepaRG.[5] Most have little to no CYP450 expression, with the one exception of HepaRG which has CYP3A4 production levels comparable to PHHs.[5, 7] The cell lines listed are capable of BC formation, but this is not the case for all available hepatic cell lines.[24, 36] Overall, this cell source is not ideal for use as a drug screening tool as they tend to have an

unnatural response to drug insult that can be attributed to their hepatoma origins.[5, 7] These cells often show levels of resistance that are much greater than that seen in PHHs.[5] These cells are best reserved for proof-of-concept designs and experimentation.[7]

The final source option, stem cells, include embryonic, adult, and induced pluripotent stem cells. Embryonic stem cells (**ESCs**) were first discovered in 1981.[2] These cells are collected from the inner mass of blastocysts that are grown *in vitro*, can be differentiated into most cell types, and replicated indefinitely.[46] In theory, ESCs are an invaluable resource for regenerative medicine, however, much like with routine organ transplant, there is the fear of allogenic rejection following implantation.[46, 47] ESCs are also shrouded with a high level of controversy due to the cell's origins.[48] Adult stem cells are collected from developed structures such as bone marrow, liver, or skin, and are specific to the tissue from which they were collected.[47] These cells also have great theoretic potential as they can be collected from an individual for differentiation for personal medicine purposes, however these cells are limited by their accessibility and poor growth *in vitro*.[47]

Human induced pluripotent stem cells (**hiPSCs**) are collected from an individual from easily accessible sources such as urine, blood, or skin, and are reverted to a state that mimics ESCs.[23, 46, 49] From here, hiPSCs too can be differentiated into most cell types, and replicated indefinitely.[46] With hiPSCs there is a need to ensure complete differentiation of these cells prior to implantation due to the risk of teratoma formation by non-differentiated cells.[47] Methods of differentiation need to be refined and there has been great investment into the development of methods for establishing mature hiPSC-HCs.[1, 10, 49] These cells are gaining in their functional capabilities and are being touted for their potential in drug

discovery and regenerative medicine.[4, 10, 46] With their ease of collection and patient-specificity, these cells present as a viable option for the study of idiosyncratic DILI.[4] Differentiated hiPSCs currently lack availability due to the novelty of the cell source and the rigorous processes that can be involved in the differentiation of the cells, however as their popularity grows so does the research into making the differentiation process more robust and accessible.[5, 45]

Studies that have looked at the incorporation of NPCs in hepatic cultures have found a number of benefits to their inclusion. In general, NPC incorporation has been found to prevent HCs dedifferentiation and promote hepatocyte-specific function in 3D cultures. Like HCs, the gold standard of NPCs are primary cells which also come at a high price. PHHs and primary NPCs can either be harvested together or separately, however NPC specific extraction typically results in a low cell yield.[5]

Primary LSECs are not stable when grown *in vitro* and they are difficult to cryopreserve. Typically, primary cells Human Umbilical Vein Endothelial Cells (**HUVEC**) are used in their place. HUVEC inclusion has been demonstrated to improve HC-specific function in PHHs including CYP450 activity and albumin synthesis. An immortalized LSEC cell line, TMNK-1, exists and co-culture of these cells with ESC-derived HCs have demonstrated to improve HC-specific function, however when cultured these cells reflect the pathogenesis of liver fibrosis as they lack fenestrations.[5]

KCs may be beneficial to include in liver disease models as these cells play an important role in inflammatory response and cytokine production. An immortalized cell line of KCs exists, however *in vitro* cultures lack the components that function to keep KCs quiescent *in vivo*, therefore these cells exhibit a greater level of inflammatory response than would be seen *in vivo*. HSCs are implicated for their role in liver fibrosis progression and in HC proliferation following liver damage. There are a number of HSC cell lines, but like the immortalized KCs their activation differs from the patterns seen *in vivo*.[5]

Both LSECs and HSCs have established methods for derivation from hiPSCs which offer a promising alternative that overcomes the barriers associated with alternate sources for these cell types. One study that looked at the co-cultivation of these hiPSC-derived NPCs with hiPSC-derived liver progenitor cells found the presence of the NPCs to increase the maturation of the HCs.[50] While the incorporation of these cell types is ideal for full recapitulation of *in vivo* conditions, the inclusion of these cells in culture is not always necessary and may act only to increase culture complexity. The decision for inclusion or exclusion should reflect the end goal for the culture model.

2.4.2. Platform Considerations

Platform considerations in regard to design and materials should also be made while bearing in mind the specific biological question that one wishes to answer. A model should only be as simple or as complex as necessary to answer the specific biological question in mind.[5] Monolayer, sandwich, and spheroid culture designs all have utility in the process of drug screening as discussed earlier. However, there are still ways in which culture designs

can be improved to better represent the *in vivo* environment. Some notable recent advancements in culture design are discussed in the next section.

There are considerations to be made surrounding the materials used in building a cell culture. Glass, polystyrene (**PS),** and polydimethylsiloxane (**PDMS**) are all common materials used for tissue culture plates or bases due to the high level of biocompatibility that they offer.[51] However, the type of material used may depend on the cells in culture. For example, primary HSCs have been found to activate when they are cultured on plastic so these materials should be avoided for use with these cells.[5] The reason for cell culture is also a determinant in the material choice. Materials such as glass or PS are not good options for applications that require reconfigurations, manipulability, or frequent reworking due to the rigidness of the materials. PDMS however is a great choice for preliminary design of a culturing model or method due to the ease of manipulation of the material and it's relatively low cost.[52] The use of PDMS should be avoided in drug screening due to the absorbent properties of the material, whereas glass and PS are both good choices for this application.[51]

Another common place that materials are incorporated in cell cultures is as scaffolds. Here, the considerations are even more important since scaffolds are frequently implanted with tissue cultures used for regenerative purposes, and therefore need to be compatible with the *in vivo* environment.[53] Common materials used as scaffolds include hydrogels such as collagen,[53] and synthetic polymers such as poly(glycerol sebacate) (**PGS**).[54] The available scaffold materials are highly variable, and selection should be made depending on the end goal of the culture. Hydrogels for example offer a growth environment that mimics the structure of ECM, but they are prone to batch-to-batch variability.[53, 55] PGS is

an attractive scaffold material due to its relatively low cost and its ease of control of tailoring it's mechanical properties.[54]

2.5. Recent Advances in Cell Culturing Techniques

Recognition of the unsustainability of current pre-clinical drug trials, and the increasing imbalance of organ donors to recipients has spurred advancements of a number of hepatic cell culture designs and techniques. These cultures vary greatly in their methods of creation, and their intended end applications. Some notable hepatic cell culture designs and techniques are discussed in this section.

2.5.1. Microfluidic Organ-on-a-Chip Devices

Liver-on-a-Chip culture models are microfluidic platforms that can consist of one or multiple chambers with cells seeded in layers on the chamber interior. In the multi-chamber design, there is typically an interface that connects the chambers by a semi-permeable membrane. These devices are perfusable, allowing for directional fluid flow, and diffusion across the membrane of a multi-chamber device.[56] One such Liver-Chip designed by Jang et al. as a drug screening tool that was seeded with PHHs in one chamber, and LSECs, KCs, and HSCs in the other was found to remain functionally viable for up to 14 days.[57] One beneficial aspect of these models is the ability to link multiple organ chips together to form Human-body-on-Chip systems.[58] In these designs multiple organ chips are connected so that models of the differing systems can function together to process xenobiotics, as would be seen in the human body.[58] Problems with these chip designs include their low throughput capabilities, and the requirement of external equipment such as pump and tubing which can require active maintenance to ensure continual flow through the chip devices.[8, 58] The main

benefit that Liver-on-a-Chip designs offer over other culturing techniques is the ability to study the effects of perfusion through the cell culture.[13, 57]

2.5.2. Bio-printing

Bio-printing is the highly controlled placement of cell-containing bioink in culture, or the controlled construction of scaffolds that are later to be seeded with cells. Both of these methods are used to recreate the architecture of a given organ.[59] Goulart et al. used bioprinting techniques to create hepatic constructs using hiPSCs. They found that by using bio-printing for the controlled placement of hiPSC spheroid-containing bioink in the formation of donut shaped cultures the cells were able to retain their functionality for longer than cultures created with bioink containing dissociated hiPSCs.[60] Different techniques of bio-printing exist, and all feature their own positive and negative aspects. Inkjet bioprinting is lower cost, offers relatively high resolution, and quick printing times, but is prone to nozzle clogging and prints at low cell densities. Extrusion based bioprinting is capable of printing at greater cell densities, however is more costly than Inkjet, has lower resolution, and is slower to print.[61] The main benefit that bio-printing offers over other liver culturing methods is the fine control over structure construct, such as the ability to create aligned vasculature that is indicative of the liver sinusoid architecture.[62]

2.5.3. Assembloids

Assembloids compared to chip devices and bio-printing are a novel culturing concept. Simply, assembloids are multiple 3D spheroids or organoids that have been assembled together in a controlled manner. Therefore, they are subject to the same problems as seen

in 3D spheroid cultures. The end goal of these cultures is to achieve a system that incorporates multiple cell types in a controlled manner. The coculture of cell types in assembloids has been shown to increase cell functional lifespan as compared to individual spheroid or organoid cultures.[63] One notable assembloid system developed by Kim et al. is a human bladder assembloid. This multi-layer 3D organoid consists of an inner layer of epithelium, a middle layer of stromal cells, and an outer muscle layer. These assembloids were developed with the end goal of studying disease and regeneration *in vitro*.[64] Another notable assembloid developed by Anderson et al. is a cortico-motor complex that is the integration of three hiPSC-derived spheroid cultures. Here, a human cortical spheroid is connected to a human spinal spheroid, which is further connected to a human skeletal muscle spheroid. These cultures were created to study disease and human development and were found to remain functional for up to 10 weeks.[65]

2.6. Summary

The current issues surrounding hepatic drug screening and regenerative medicine exemplify the need for an alternative solution to these problems. These issues are complex and the considerations that are needed in developing a solution to begin to solve and overcome them are extensive. It is seemingly the case that with such complex and diverse problems there is no one solution for all. It is much more likely that multiple hepatic culturing methods and designs will be used collectively as solutions in these multiple applications. Therefore, each new design, advancement, or finding should be taken with as much consideration as the last.

Chapter 3. Research

3.1. Manuscript Introduction

In the following manuscript is described the development of a biomimetic liver acinus tissue culture using zone-specific hPSC-HCs and PGS scaffolds. This cell culture incorporates zone-patterning through the use of zone 1 and zone 3 specific hPSCs following the recently developed culturing techniques of assembloid biofabrication. Using this culture design, we demonstrated the formation of an aligned vascular-like network, and successfully incorporated bile ductule representative hPSC-derived cholangiocyte cysts into the hepatic tissues. These zone-patterned hPSC-derived tissue cultures show promise for use as a telling screening tool for DILI in pre-clinical drug testing, as a model for personalized disease modelling and remedy screening, and the use of a PGS scaffold creates the possibility of application to personalized tissue repair.

3.2. Manuscript

The following manuscript appears as it has been prepared with the intention of publication in the scientific journal, Biofabrication or Advanced Healthcare Materials. It has not yet been submitted for review.

Bioengineered Liver Assembloids with Zonation

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Abstract

Biomimetic liver cultures have great potential in the fields of drug discovery and regenerative medicine. Here, zone-patterned human pluripotent stem cell derived hepatic assembloids are fabricated using PGS z-wire scaffolds and a novel plate design. The z-wire scaffold and plate well basins support the production of elongated 3D tissue cultures and make possible the spontaneous formation of an aligned vascular-like network. Coculture of patterned zone-specific human pluripotent stem cell derived hepatocytes show retention of zone-specific CYP3A4 expression, and continual increasing levels of albumin synthesis. Theses factor are promising indicators of long-term culture maintenance and cell maturity. When bile ductule representative human pluripotent stem cell derived cholangiocyte cysts are added to the cultures they are partially engulfed into the tissue. This novel hepatic tissue design has potential in application of drug discovery, disease modelling, and regenerative medicine.

Introduction

Research into the development of *in vitro* three-dimensional (3D) tissue cultures that aim to emulate human tissues has flourished in recent years. These engineered cultures have gained popularity due to their significant potential in regenerative medicine and drug discovery [1, 2]. Of particular interest has been in the development of engineered liver tissues. For drug development, liver toxicity is one of the leading causes of clinical failure and drug withdrawal from the market [3]. For regenerative medicine, there is need for both an *in vitro* platform for studying liver afflicting disease, and an alternative treatment of

advanced liver disease as the leading remedy of transplantation has a necessity that outweighs availability [4-7]. Access to stem cell derived biomimetic liver tissues could provide a solution to these pressing issues [8].

Accurately recreating the microarchitecture of any organ is essential to capture the cell-cell and cell-extracellular matrix (ECM) interactions that play an important role in cellular function, survival, and metabolic activity [2]. However, the ability to replicate the microarchitectures of liver has remained elusive due to the complex nature of the structure. Recently, attention has turned towards recreating the liver acinus as these structures are not only responsible for the basic functional processes within the liver, but through the approach of modular tissue engineering they may be used as building blocks for constructing larger liver tissues for regenerative medicine (**Figure 1a**) [9].

There have been great strides made towards the advancement of hepatic cultures for intended end use in drug development, implantation, and disease modelling. Advancements in culturing techniques and stem cell technologies have allowed for the recapitulation of liver microarchitecture that previously was not possible [10]. Notable effort has been put into the incorporation of vasculature in culture. Some make use of the controlled patterning of endothelial cells for the development of aligned endothelial cell cords, while others rely on the spontaneous assembly of endothelial cells in culture into structures resembling vascular networks [2, 11, 12]. Greater understanding on the importance of bile canaliculi presence in hepatic tissue cultures has shifted sights towards development of cultures and culture platforms where this characteristic feature is able to form [4, 11, 13, 14]. Developments in micropatterning has paved way for the fine control over cell placement

and tissue construction [5, 13]. Liver-on-a-Chip devices have the impressive ability to allow for the modelling of flow through the liver sinusoid in a highly controlled manner [15, 16].

With these excellent advancements in hepatic culture design there is one component that many of these cultures fail to consider, and that is the zonation of the liver acinus. The innate architecture of the liver sinusoid creates a natural oxygen and metabolite gradient that progresses from the hepatic triad to the central vein. Differences in blood oxygen, hormone, and substrate levels along the liver sinusoid as well as differing cell-cell and cell-ECM interactions results in morphological and histochemical differences that give rise to zone designations (**Figure 1a**) [17].

The recent conception of spatially controlled organization of multiple cell types known as assembloid tissue cultures has opened the possibility for development of a 3D liver microtissue with zone-patterning. Notable assembloids created to date include a functional human cortico-motor complex [18], and human bladder [19]. Herein, we describe the formation of the first hepatic assembloid developed in the image of the liver acinus. These cultures are created by the assembly of human pluripotent stem cell (hPSCs) derived zone 1 and zone 3 specific cells. To produce the elongated shape of the liver sinusoid, we used our lab's multi-well z-wire plate and z-wire scaffolds previously shown to allow for the formation of elongated 3D tissue cultures (**Figure 1b, c)** [20].

Results & Discussion

To produce elongated hepatic tissues in the z-wire plates, we first optimized the cell assembly process in the z-wire wells. Here, HepG2, GFP-HUVEC, and primary human fibroblasts were cocultured at a density of 0.45 million cells per well to consistently and reliably compact to form a singular tissue in the long and narrow z-wire well basins. *In vivo*, hepatocytes are found to make up approximately 60-70% of the liver by cellular content [21]. In our HepG2 tissue cocultures, we opted for a much lower seeding percentage of 45%. The decision for this discrepancy was made due to the continued expansive growth seen with the HepG2 in the tissue cultures beginning at day 4 (**Figure 2a**).

Importantly, our tissue cultures do not require the use of hydrogel which are usually derived from animal sources and are prone to batch-to-batch variation [22]. Rather the cells are capable of self-assembly with the support of the poly(glycerol sebacate) (PGS) z-wire scaffold alone. PGS is an FDA approved, cost effective, biocompatible and biodegradable polymer [23, 24]. Due to it's tunable mechanical properties PGS has been used for various purposes including hard and soft tissue generation, and drug delivery [23]. Our z-wire scaffolds are designed to fit into the z-wire plate well basins where they provide a slightly malleable support structure for cells to compact around, and ultimately form elongated tissue cultures (**Figure 1d-f**). In testing the degradability of our PGS, we found a 2.7 - 3.4 mg block of PGS to completely degrade in six month's time when kept in PBS at 37ºC. This rate of degradation makes the polymer an ideal candidate for both short- and long-term drug testing and long-term implantation for regenerative medicine.

Cell compaction begins within the first 24-hours following seeding and is completed by day 3 (**Figure 2a**). The result of the compaction of the cells around the z-wire scaffolds is an elongated tissue that resembles the overall morphology of the liver acinus (**Figure 1a, 2a**). The PGS z-wires are semi-rigid structures which work to resist the contractive forces exerted by the cells and allows for the cultures to remain elongated in the long and narrow z-wire well basins (**Figure 2b**).

Levels of tissue albumin production was assessed using ELISA assay to test for any toxic effect in our hepatic tissues induced by the incorporation of the PGS scaffolds. Media was collected at day 9 in HepG2 cocultures that both did and did not contain z-wire scaffolds. Albumin production results show there to be no significant difference between the two conditions, indicating that the incorporation of the z-wire scaffold does not have a significant toxic effect on the hepatic tissue cultures (**Figure 2b**).

The inclusion of endothelial cells (ECs) in hepatic culture has been shown to enhanced hepatocyte specific function [25]. Studies that have looked at the importance of EC arrangement have shown that while clustered groupings of ECs quickly become apoptotic, the tubular arrangement of ECs improves lifespan by at least 8 days [26]. Additionally, the presence of endothelial networks in tissues have shown to enhance anastomosis in tissue implants [2]. We assessed the roles of both the z-wire-induced tensile force and the contractive forces induced by the incorporation of fibroblasts in the formation of vascular structures in our tissues. The GFP-HUVEC were visualized using a Cytation 5 Cell Imaging Multimode reader. By day 3 the vasculature structures had stabilized (**Figure 2c**). We found the presence of both the scaffold and fibroblasts to be necessary for the elongation and

alignment of a vasculature-like network along the length of the z-wire scaffold (**Figure 2b-d**). Cultures lacking a scaffold developed elongated vascular networks that lacked orientation while cultures without fibroblasts lacked the necessary tension for vascular elongation. In the latter condition, ECs arranged into multiple clustered spheres throughout the tissue (**Figure 2b, c**). Various publications have reported the development of hepatic cultures that incorporate the spontaneous creation of EC networks or the patterning of aligned endothelial cords; however, we believe our tissue cultures to be the first instance of the spontaneous formation of an aligned vascular-like network in a 3D hepatic tissue culture [2, 11, 12, 27, 28].

The functional differences that are found between zones in the liver are an important component to model in a hepatic tissue. The actions of each zone work together to accomplish the liver's crucial tasks. For example, in the process of toxin breakdown and removal, it is primarily zone 3 that is responsible for cytochrome p450 production and therefore toxin processing, and zone 1 that is primarily responsible for the production of bile transporters for the removal of waste and by-products. Therefore, the ability to replicate these zone-specific functions *in vitro* is necessary in a biomimetic culture that is used in screening for drug effects and drug toxicity. The incorporation of zone-specification in hepatic cultures is an aspect that has largely been overlooked until recently. Liver-on-a-Chip models have been created to incorporate liver zonation; however, current models use mostly cell lines or primary cells [16, 29]. Primary human hepatocytes fall short in their potential for long term use as they have a tendency to dedifferentiate *in vitro* and survive for only a short period in culture, and cell lines lack in their genetic variability and their

abilities to properly emulate the functions of primary hepatocytes [25, 30-32]. HPSC-derived hepatocytes (hPSC-HCs) show promise in replacing cell lines and primary cells, especially for use in long-term functionality applications, including drug discovery [32]. HPSC-HCs have been demonstrated to retain their levels of functionality for longer periods of time, which is essential for repeat dose experimentation that looks at the long-term effects of drug and toxin exposure [33]. Therefore, we developed an hPSC-derived assembloid tissue culture that incorporates the specificities of both zone 1 and zone 3 into a singular tissue.

To incorporate zone-specific patterning, it was necessary to develop a stepwise seeding method to work with our z-wire plates (**Figure 3d**). Briefly, with the plate tilted left on a 30^{o} angle in the horizontal axis, the first cell mixture is directed into the bottom corner of the z-wire containing well basins, followed by centrifugation on this angle. The plate is then tilted 30^{o} in the opposite direction for the directed seeding of the second cell mixture into the right corner of the well basins. Here, the plate is allowed to rest for the passive aggregation of the second cell mixture. This method of cell patterning was first tested with the previously established HepG2 coculture. Two seeding mixtures were prepared where one set of HepG2 had been fluorescently tagged using CellTracker Red dye. The two mixtures were seeded onto z-wire scaffolds following the stepwise seeding protocol. Fluorescent imaging in the days following seeding revealed a maintained gradient across the tissue culture from one cell seeding mixture to the other (**Figure S1a**).

The hPSC-HCs were differentiated to be specific for either zone 1 or zone 3. These cells show zone specific indicators as would be seen *in vivo*. Zone 1 specific cells for example express greater levels of G6P, whereas zone 3 specific cells express greater levels of CYP3A4. The ability to capture these functional variations between zone 1 and zone 3 hepatocytes is essential in developing a biomimetic hepatic tissue culture since these regional functional nuances and their interplay are essential to how the liver performs it's activities [17]. Transitioning to the use of zone-specific hPSC-HCs first required the re-optimization of seeding conditions in our customized well plates. HPSC-HCs seeded at 0.7 million cells per well were found to compact well to encapsulate the z-wire scaffolds in the z-wire well basins (**Figure 3a, b**). To visualize sustained patterning in the hPSC-HC cultures, zone 3 specific hPSC-HCs were fluorescently tagged with GFP. Imaging of these tissue cultures in the days following seeding revealed that patterning of the cells can be visualized for at least 9 days (**Figure 3c, e**).

It is well known that the coculture of endothelial cells with primary and immortalized hepatocytes improves hepatic function, however the impact of endothelial inclusion with hPSC-HCs is inconclusive as research into the subject is still minimal [25]. Takebe et al. successfully incorporated HUVEC into their spontaneously forming hiPSC liver bud 3D aggregates for the development of rudimentary vessel networks. Presence of HUVEC in culture advanced levels of hiPSC maturity in comparison to HUVEC-lacking models [12]. Jin et al. cocultured HUVEC and iPSC-HCs in a 3D decellularized liver ECM. They too were successful in forming a vessel-like network in their organoids which they found improved hepatic functionality compared to HUVEC-lacking conditions [27]. However,

attempts to incorporate HUVEC into our hPSC-HC tissues resulted in the dissociation of the tissues and the HUVEC were not able to align as was found in the HepG2 cocultures (**Figure S1b**). It is hypothesized that these findings were due to the inclusion of ECGM2 in the media mixture used with these cultures. While the ECGM2 was included for the sustainment of the HUVEC, it is likely that the highly sensitive hPSC-HCs reacted poorly to growth factors in the media. More appropriate ECs for the inclusion in hPSC-HCs cultures may be hPSC-derived endothelial cells (hPSC-ECs) which can be derived from the same source as hPSC-HCs [34].

As a marker of functionality in adult hepatocytes, albumin production is commonly used as an indicator of maturity in the differentiation of hPSCs into hepatocyte-like cells [34, 35]. Levels of albumin production were assessed in our hPSC-HCs tissue cultures over 9 days. Results in albumin production over these days show a pattern of increasing albumin secretion levels. This trend is promising for the continued health and functionality of the hPSC-HCs in culture (**Figure 3f**). Assessment of longer-term albumin production is necessary to compare the utility of our culture method to PHHs for the purpose of long-term drug assays. In the short-term, these results show a similar level of production to PHH albumin secretion by multiple studies that have looked at PHHs in the same time frame [13, 36].

Non-fluorescently tagged hPSC-HCs tissues that were fixed after 9 days of culture were used in the immunostaining of DAPI with CYP3A4, albumin, or e-cadherin (**Figure 3g**). Immunostaining for CYP3A4 revealed sustained patterning of cell types in the tissues. In these tissues, CYP3A4 level of fluorescence can be seen at a greater intensity in the zone 3

cells than in zone 1 cells. This pattern agrees with the innate liver physiology where CYP3A4 is primarily produced in zone 3 [17]. Immunostaining for both albumin and e-cadherin showed similar, high levels of expression across the z-wire tissues. As indicators of maturity, visualization of albumin and e-cadherin are promising indicators for the preserved differentiation of the hPSC-HCs, and greater CYP3A4 expression in zone 3 is promising for the retention of the cell's zone-specific properties.

In vivo, there is the natural formation of the biliary network's smallest leg, the bile canaliculi. Bile canaliculi are formed by and between healthy hepatocytes and are used as a means of excretion (**Figure 4a**) [37-39]. In certain *in vitro* hepatic cultures, bile canaliculi are also found to naturally form by and between hepatic cells where tight junctions are able to form [14, 40, 41]. Bile flow through bile canaliculi is counter-current to the liver sinusoid with constituents exiting the liver acinus periportal through the bile ductule, which branches to the remainder of the biliary tree (**Figure 4a**) [38]. The common hepatic duct is the 'trunk' of the tree that connects the network to the duodenum for excretion. From the bile ductule to the common hepatic duct, the biliary tree is lined by cholangiocyte epithelial cells [42]. To complete the picture of the acinus's contiguous biliary network, we incorporated into the tissue cultures hPSC-derived cholangiocyte cysts, representing the bile ductule of the hepatic triad (**Figure 4b, d, e**)

On day 3, after the tissues had the opportunity to compact around the scaffold, hPSC-derived cholangiocyte cysts were placed into the z-wire wells on the left end of the tissues, closest to zone 1 cells (**Figure 4c**). However, this method does not allow precise control of the placement of the cyst and more optimization is needed to improve accuracy. When

cholangiocyte cysts were placed touching the hepatic tissue, the cysts were partially enveloped by the hPSC-HCs in the days following seeding (**Figure 4d, e**). Day 9 cholangiocyte cyst containing tissues were preserved and prepared for histology assays. Staining for H&E, e-cadherin, and Masson's Trichrome was conducted. In one sample a cholangiocyte cyst can be seen located on the zone 3 end of the tissue (**Figure 4f**). The tight junctions that are visualized with e-cadherin stain is suggestive of bile canaliculi formation between adjacent hPSC-HCs. The more prominent e-cadherin staining around the edges of the tissue is due to the greater tension that is naturally exerted on the cells closest to the surface. It is also possible that this staining pattern is due to lower levels of oxygen and nutrients permeating deep into the inner tissue mass.

The results that have been seen with our zone-specific hPSC-HC tissues are promising. The patterning of the zone-specific cell types and the sustained functional levels that have been seen are suggestive of the high applicability of these tissue cultures to drug testing applications. While hPSCs are more easily sourced than PHHs, longer-term functional tests are required to determine the functional utility of the cultures compared to PHH cultures for long-term drug-screening purposes. The patient-specificity that these cultures offer promises an accurate and telling disease model for personalized remedy screening. Finally, used singularly or as a modular building block, the hPSC and PGS containing tissue cultures present as a practicable option for implantation for hepatic regenerative purposes (**Figure S1c**).

Limitations

The tissue cultures in their current state are not without their limitations. It is still necessary to incorporate vasculature into the hPSC-HCs tissues to assess the functional impact that the incorporation of endothelial cells has on the hPSC-HCs. The inclusion of vasculature has been demonstrated to be beneficial in the process of angiogenesis and perfusion of tissues upon implantation [2, 12]. Therefore, it is ideal for our tissues to incorporate this feature for intended future applications. It is also necessary to visualize the formation of the bile canaliculi in our tissue cultures to ensure that there is the proper development of an excretory system in the tissues. This feature is important for a tissue culture to have if it is to be used for long-term drug assays. One way this can be accomplished is staining for CD26 which highlights the structures [37]. Finally, there needs to be a functional drug analysis of the tissue cultures to assess their predictability and therefore their utility in drug assays.

Conclusion

We have used the emerging tissue culturing technique of assembloid bio fabrication to create what is the first 3D hepatic culture to incorporate hepatic zone-specification. We have demonstrated the ability for aligned vascular-like network formation in our hepatic design and have shown the potential for the integration of cholangiocyte cysts into the tissues. CYP3A4 expression and albumin secretion are promising for sustained zone-specificity, and long-term hepatocyte-specific functional preservation. We anticipate a broad scope of applications for our tissue system design including utilization in pre-clinical drug trials, disease modelling, and use in regenerative medicine.

Methods

Polymer Synthesis

Poly(glycerol sebacate) (PGS) was produced using a previously established protocol [43]. Briefly, the polymer was created from a polycondensation reaction with equimolar glycerol (sigma, G5516-1L) and sebacic acid (Sigma, 283258-250G). The two reagents were reacted under Nitrogen purge at 120oC for 1 hour, followed by vacuum for 24 hours.

z-Wire Scaffold Fabrication

An array of 56 embossed z-wire structures was designed in AutoCAD and an SU-8 master mould of the design was formed using standard photolithography techniques. Polydimethylsiloxane (PDMS, Sylgard 186, 217054, The Dow Chemical Company) was then used to create a z-wire scaffold mould from the SU-8 master mould. The PDMS z-wire features were coated with evaporated trichloroperfluoroocytl silane (Sigma, 448931-10 g). The PGS polymer was spread onto the PDMS mould, filling the z-wire structure indents. Excess polymer was removed and the PGS containing PDMS mould was baked at 145oC for 16 hours. The crosslinked PGS z-wires were manually removed from the PDMS mould and stored in PBS at 4oC. Characterization of the z-wire scaffold mechanical properties were conducted as presented previously [20].

Plate fabrication

A customized 384-well plate design featuring funnel-shaped wells was rendered using AutoCAD software. The negative of the construct was taken to create a mould for the plate design. The mould was 3D printed using a Stratasys 3D printer with Tangoblack plus ink. Following printing the mould was submerged in a NaOH solution for 2 days, dried, baked

for 1 day at 80oC, then finally coated with trichloroperfluoroocytl silane (Sigma, 448931-10 g). Polydimethylsiloxane (PDMS, Sylgard 186, 217054, The Dow Chemical Company) plates were then formed using the 3D printed mould, and hard-baked at 300oC for 20 minutes before being cut with PDMS punch into circles containing 5 of the funnel-shaped wells. These cut-outs were then fixed to the wells of a 24 well polystyrene cell culture plate (VWR, 734-2325) using additional PDMS glue in a base-to-catalyst ratio of 5:1. These wells were first filled with 70% ethanol, then a z-wire was placed in the center well of each 5-well cut out. The well plates were then centrifuged at 1000 RPM for 30s, forcing the scaffolds to the bottom of each well. Well plates were left to sterilize in 70% ethanol for 2 hours. 70% ethanol was removed, and plates washed 2-3 times with PBS. Wells designated for cell seeding were treated with an anti-adherence rinsing solution (07010, STEMCELL technologies) for 30 minutes to prevent cell attachment. The anti-adherence solution was removed, and plates were washed 2-3 times with PBS, which was slowly switched to culture media over 2-3 media changes.

Cell culture

Green fluorescent protein human umbilical vein endothelial cells (GFP-HUVECs) were purchased from Angio-Proteomie (CAP-0001GFP). The GFP-HUVECs were cultured in endothelial cell growth medium 2 (ECGM2, C-22011, Promo Cell). Human primary cardiac fibroblasts were purchased from Cedarlane labs (C-12375). The fibroblasts were cultured in a Dulbecco's modified Eagle's medium (DMEM, 319-005-CL, Wisent Bioproducts) containing 10% (v/v) fetal bovine serum (FBS, Gibco), 1% (v/v) Hepes (100 U mL-1, Gibco), and 1% (v/v) penicillin-streptomycin (100 mg mL-1, Gibco).

Hepatocellular carcinoma cells (HepG2s) were purchased from the American Type Culture Collection (ATCC, CRL-10741). The HepG2s were cultured in an Eagle's minimum essential medium (EMEM, 30-2003, ATCC) containing 10% (v/v) fetal bovine serum (FBS, Gibco), 1% (v/v) Hepes (100 U mL-1, Gibco), and 1% (v/v) penicillin-streptomycin (100 mg mL-1, Gibco). Hepatocyte tracking was accomplished by staining cells with CellTracker Red CMTPX (C34552, Thermo Fisher Scientific) following the supplier's protocol. Human pluripotent stem cell (hPSC) derived zone-specific hepatocytes (hPSC-HCs) and hPSC derived cholangiocyte cysts were provided by Dr. Ogawa's group at the McEwen Stem Cell Institute, University Health Network. HPSCs were cultured in hepatic maturation medium containing 0.1% Y-27632 (STEMCELL Technologies, 72304).

Cell seeding and device operation

A mixture of 45% HepG2, 45% GFP-HUVEC, and 10% primary human cardiac fibroblasts were used in the formation of the immortalized tissue cultures. Cells were cultured in a ratio of 1:1 (v/v) EMEM and ECGM2 media. Cells were suspended in culture media at 40 million cells mL-1 and pipetted into wells at the equivalent of 0.45 million cells per well. The plate was then centrifuged for 1 minute at 150 G to aggregate the cells to the bottom of the wells, embedding the z-wire scaffolds where present. For zone-specific iPSC-derived hepatocyte cultures, hPSC-HCs were suspended in culture media at 40 million cells mL^{-1}. The plate was tilted at a 30^{o} angle and zone 1 specific hPSC-HCs were pipetted into wells at the equivalent of 0.35 million cells per well. While remaining tilted at 30^{o} the plate was then centrifuged for 1 minute at 150 G to aggregate the cells to one side of the bottom of the well. The plate was then place on a 30^{o} angle tilt in the opposite direction and zone 3

specific hPSC-HCs were pipetted into wells at the equivalent of 0.35 million cells per well, for a cumulative 0.7 million cells per well. The plates were placed in an incubator. Immortalized tissue cultures were placed on a rocker with a stage tilt of 30^{o} programmed to change direction every 15 minutes. Media was changed every other day. The cells self-aggregated and compacted around the z-wire scaffolds over 3 days. FIJI was used to measure tissue length from brightfield images. The GFP-HUVEC were imaged with confocal microscope (Nikon A1 confocal with ECLIPSE Ti microscope), and a Cytation5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Inc., Montreal, Canada).

Quantification of Vessel Alignment

At day 3, immortalized tissue cultures were imaged with a Cytation 5 Cell Imaging Multi-Mode reader (BioTek Instruments, Inc., Montreal, Canada) as shown in figure 4a. Images were processed in Adobe photoshop to highlight culture vasculature as shown in supplementary figure S1d. Images were analyzed in FIJI to assess vascular circularity and orientation. Low circularity and a tendency to orient in the horizontal plane indicate alignment of vasculature in the tissue cultures.

Immunofluorescent staining

To prepare tissues for immunofluorescent staining, tissues were first fixed in 4% (w/v) paraformaldehyde (158127-100G, Sigma-Aldrich) in PBS overnight at 4^{o}C. Tissues were washed and left in PBS overnight at 4oC. They were then permeated and blocked with 10% FBS and 0.1% Triton X-100 in PBS for 1 hour at room temperature. The tissues were then stained for Cytochrome P450 3A4 (ab3572, Abcam), E-Cadherin (ab1416, Abcam), Albumin (A80-129A, Bethyl Laboratories), CK7 (ab68459, Abcam), or DAPI (D9542-

5MG, Sigma-Aldrich) following standard procedures. The tissues were then washed and imaged using a confocal microscope (Nikon A1 confocal with ECLIPSE Ti microscope).

Histology Sectioning

To prepare tissues for histology sectioning and staining, tissues were fixed in 10% (w/v) Formalin for two days at 4^{o}C. Tissues were then placed in histology cassettes and immersed in 70% ethanol until embedding in paraffin wax. The tissues were then sectioned and stained with hematoxylin and eosin, E-cadherin, Albumin, and CYP3A4.

Albumin assay

In the immortalized tissues, culture media was collected and replaced with fresh 1:1 (v/v) EMEM and ECGM2 media every other day at set volumes. For the hPSC-HCs tissues, culture media was collected and replaced with fresh hepatic maturation medium every other day at set volumes. Quantification of secreted albumin was conducted using the Bethyl's human albumin ELISA quantification Set (Bethyl Laboratories, Inc., Cat# E80-129) according to the manufacturer's protocol.

Statistical Analysis

For all experiments normality and equality of variance was tested using SigmaPlot. One-way ANOVA test with Holm-Sidak correction at $p < 0.05$ and a power greater than 0.90, or two-tailed unpaired t-test with 95% confidence interval was used to determine statistical significance. Means and standard deviations are plotted in each graph. '*' indicates significant differences with $p < 0.05$. '**' indicates significant differences with $p < 0.01$. sample size (n) for each experiment is described in figure captions. A minimum of $n = 3$ independent samples were used in each experiment.

Acknowledgements

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Author contribution

T.L.J.S. performed the experiments, analyzed the results, and prepared the manuscript, L.E.P.E contributed to image collection, C.C. helped with tissue culture maintenance, S.H. envisioned the concept and provided hPSCs, B.Z. envisioned the concept, supervised the work and prepared the manuscript.

Competing financial interests

None.

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Figures

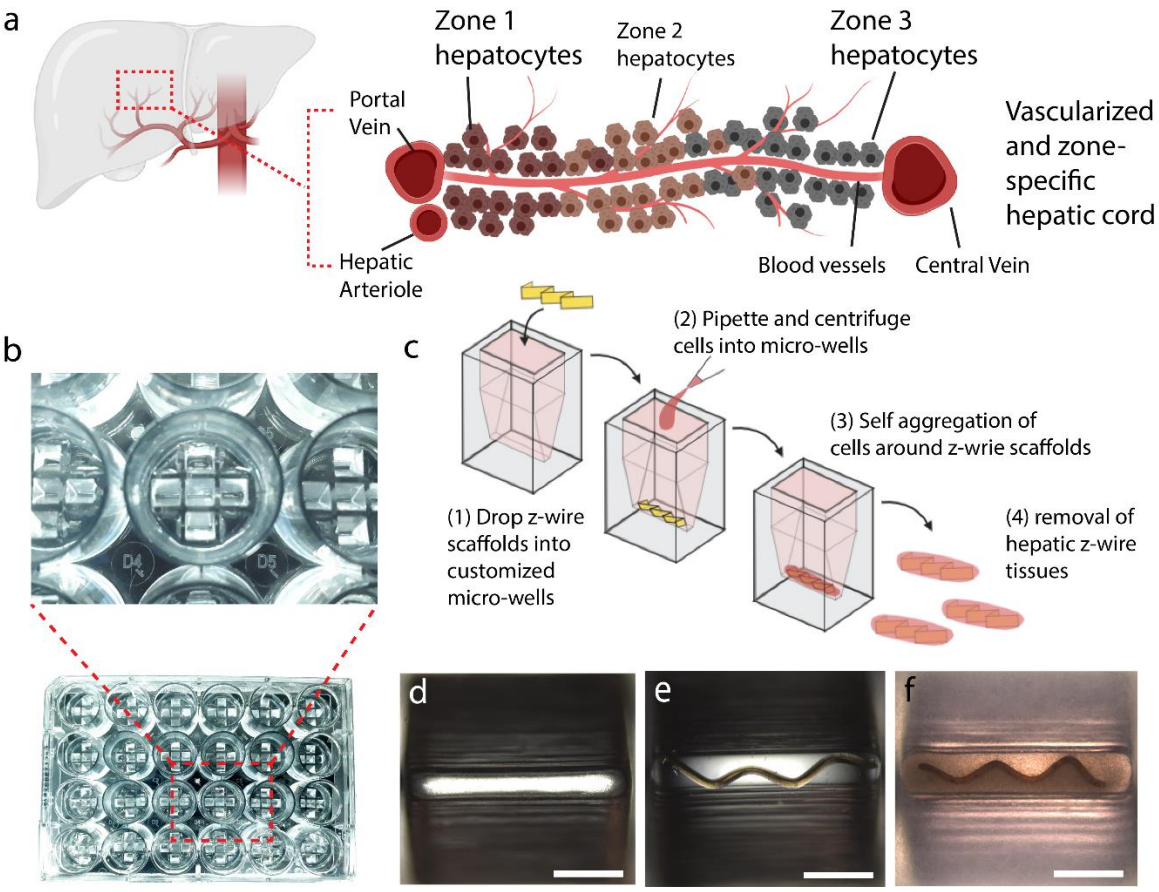


Figure 1. Overview design and assembly of hepatic cord z-wire tissues. a, Illustration of human liver. Enhanced section shows vascularized liver cord with indication of zone-specific regions. **b**, PDMS cut-outs from customized 3D-printed 384-well plate with funnel shaped wells for the facilitation of tissue assembly, fit and fixed into a standard 24-well polystyrene plate. **c**, Schematic of hepatic tissue production process 1) placing of PGS z-wire scaffold, 2) cell seeding, 3) tissue aggregation, 4) removal of assembled tissues from wells. **d-f**, Image of funnel-shaped 3D printed customized well (d) without z-wire scaffold, (e) with z-wire scaffold, and (f) with z-wire scaffold and seeded cells. Scale bar, 1mm.

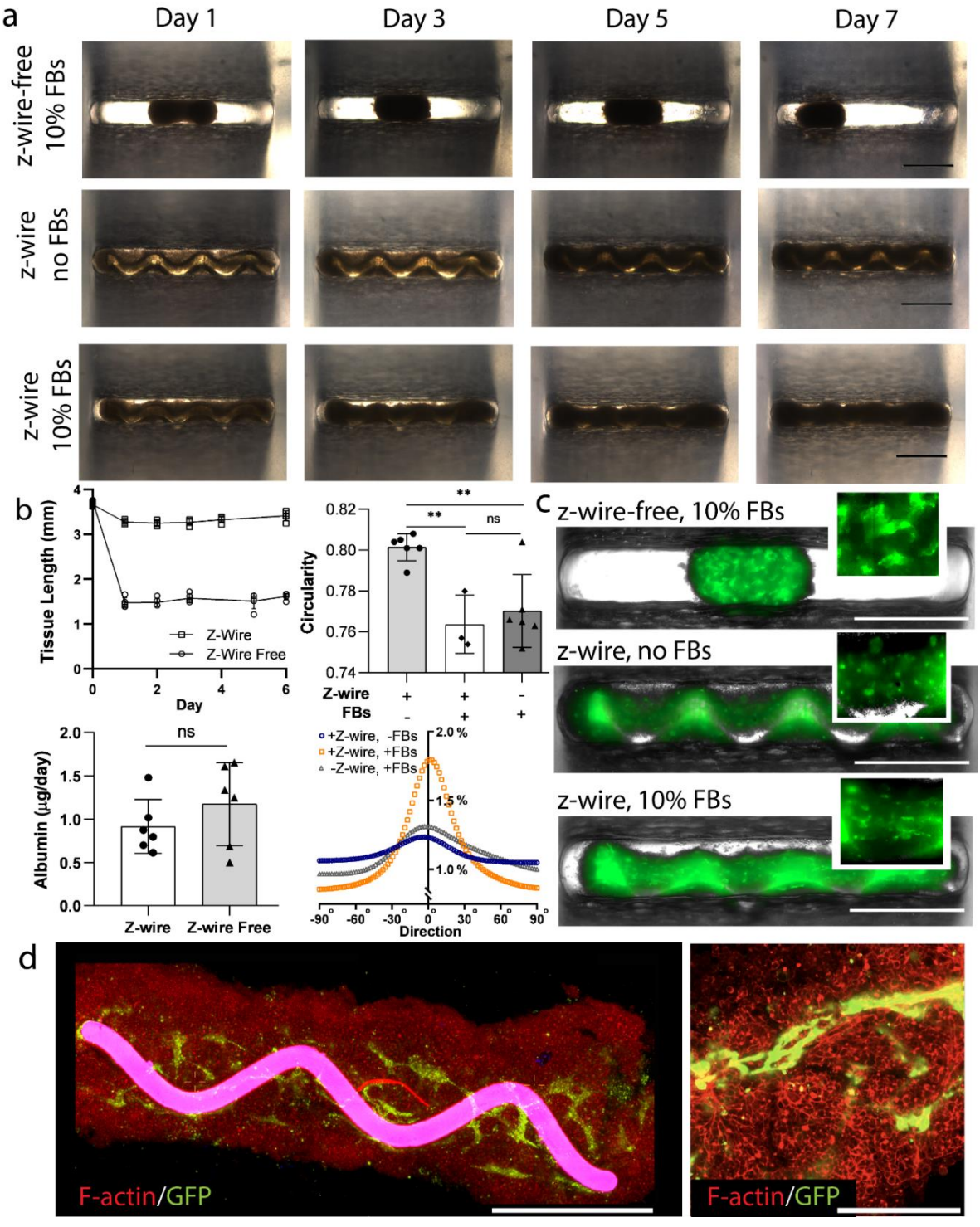

a
Day 1
Day 3
Day 5
Day 7
z-wire-free 10% FBs
z-wire no FBs
z-wire 10% FBs
b
Tissue Length (mm)
Day
Z-Wire
Z-Wire Free
Circularity
Z-wire
FBs
ns
**
Albumin (µg/day)
Z-wire
Z-wire Free
+Z-wire, -FBs
+Z-wire, +FBs
-Z-wire, +FBs
Direction
c
z-wire-free, 10% FBs
z-wire, no FBs
z-wire, 10% FBs
d
F-actin/GFP
F-actin/GFP

Figure 2. Formation of aligned vasculature in immortalized cell culture hepatic tissues. a, Brightfield time series images of assembly of vascularized liver tissues under varying seeding conditions. Scale bar, 1mm. **b**, Quantification of tissue structure and functionality, (1) Quantification of hepatic tissue length (mm) with (n = 4) and without (n = 5) z-wire scaffold, (2) Circularity of tissue vasculature on a scale of 0 to 1, with 1 indicating perfect circularity, without scaffold (n = 6), with scaffold and without FBs (n = 6), and containing both scaffold and FBs (n =3), (3) Production of albumin (μg/day) at day 9 of culture in HepG2 cocultures with (n = 6) and without (n = 6) z-wire scaffold, (4) Vascular structure orientation, where 0^o indicates alignment in the horizontal axis, without scaffold (gray, n = 6), with scaffold and without FBs (blue, n = 6), and containing both scaffold and FBs (orange, n = 3). ** denotes statistical difference between groups with $p < 0.01$. **c**, GFP (green) fluorescent images of HUVEC vasculature development under varying cell seeding conditions, with enhanced view of vasculature morphology, (1) without z-wire scaffold, (2) with z-wire scaffold, without FBs, (3) containing z-wire scaffold and FBs. Final images are stitched from multiple images. Scale bars, 1 mm. **d**, Fluorescent images of liver tissue with vasculature-like structures, containing z-wire scaffold and FBs. GFP-endothelial cells (green) and stained for F-actin (red). Final images are stitched from multiple images. Scale bars, 1 mm and 200 μm.

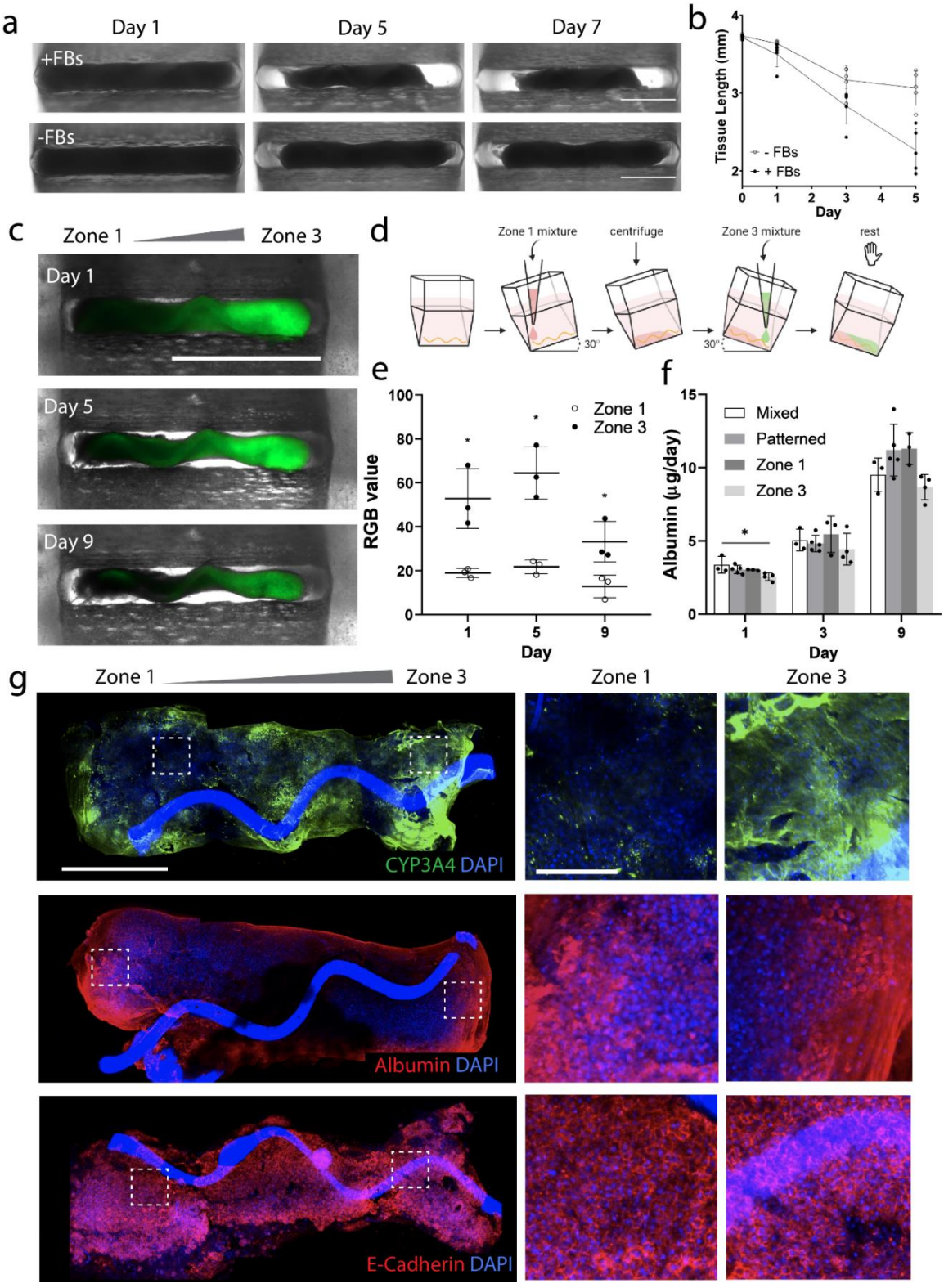
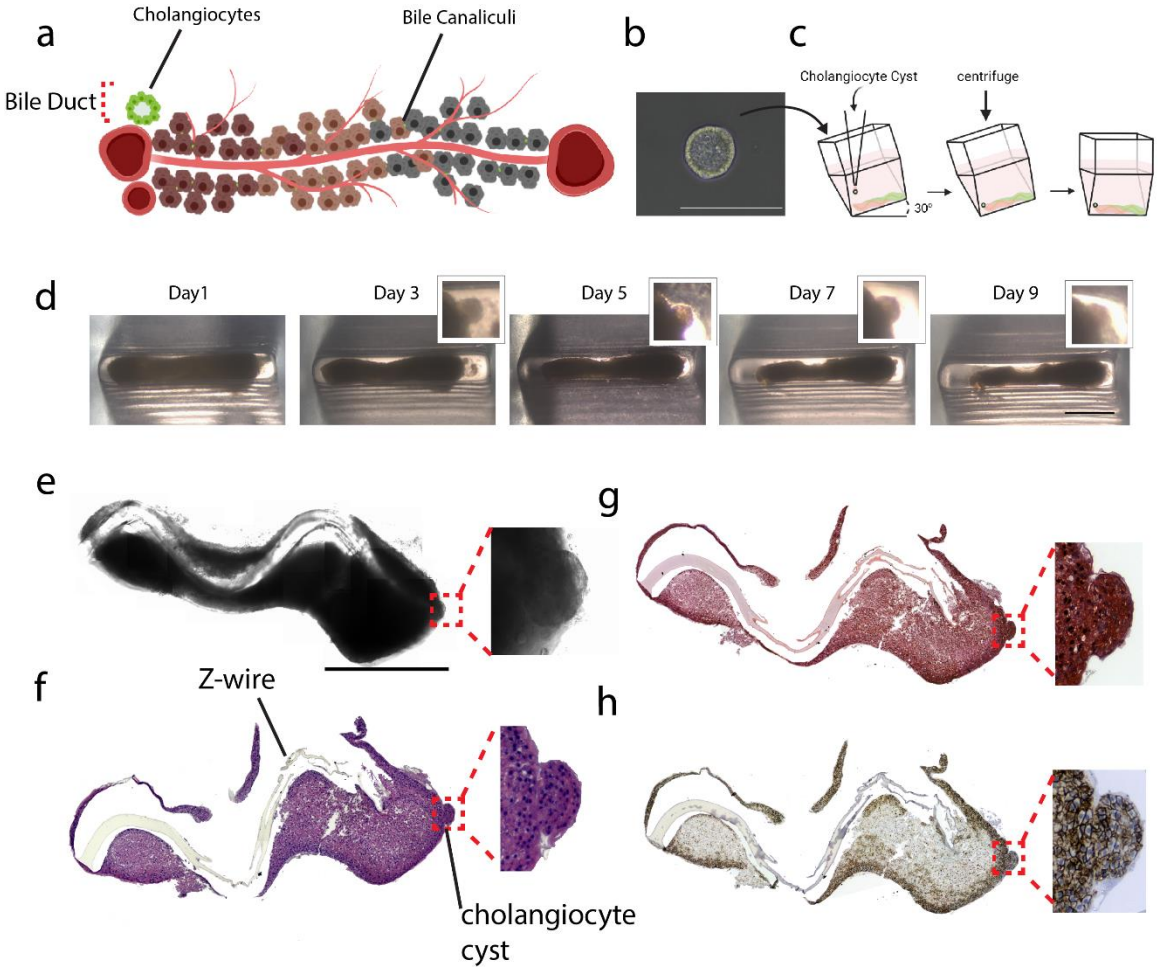

a
Day 1
Day 5
Day 7
+FBs
-FBs
b
Tissue Length (mm)
- FBs
+ FBs
Day
c
Zone 1
Zone 3
Day 1
Day 5
Day 9
d
Zone 1 mixture
centrifuge
Zone 3 mixture
rest
30°
30°
e
RGB value
Zone 1
Zone 3
Day
f
Albumin (µg/day)
Mixed
Patterned
Zone 1
Zone 3
Day
g
Zone 1
Zone 3
Zone 1
Zone 3
CYP3A4 DAPI
Albumin DAPI
E-Cadherin DAPI

Figure 3. Formation of human pluripotent stem cell derived zone-specific hepatocyte patterned liver tissue. a, Brightfield time series images of hPSC-HC tissue formation with and without FBs. Scale bar, 1 mm. **b**, Quantification of liver tissue length (mm) with and without FBs over time (n = 5). **c**, Florescent time series images of pattern seeded zone-specific hPSC-HCs where zone 3 cells have been tagged with GFP (green). Scale bar, 2 mm. **d**, Schematic of stepwise seeding procedure for patterned tissues. **e**, Median RGB values and standard deviation from intensity profiles of zone 1 and zone 3 of pattern seeded zone-specific hPSC-HCs, where zone 3 cells have been fluorescently tagged with GFP (green), n=3. * indicates statistical difference between zone 1 and zone 3 groups with $p < 0.05$. **f**, Albumin production (μg/day) in varying hPSC-HC seeding conditions. Seeding conditions: equally mixed zone 1 and zone 3 specific hPSC-HCs (n = 3), patterned zone 1 and zone 3 specific hPSC-HCs created following stepwise seeding method (n = 5), zone 1 specific hPSC-HCs alone (n = 3), and zone 3 specific hPSC-HCs alone (n = 4). * indicates statistical difference between groups with $p < 0.05$. **g**, Fluorescent images of patterned zone- specific hPSC-HCs following 9 days of culture. Tissues stained for DAPI (blue) and CYP3A4 (green), or Albumin (red), or E-cadherin (red). Images taken with confocal microscope. Final images are stitched from multiple images. Enhanced figures display intensity in zone 1 and zone 3 specific cell regions. Scale bars, 1mm and 200 μm.


a
Cholangiocytes
Bile Canaliculi
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b
c
Cholangiocyte Cyst
centrifuge
30°
d
Day1
Day 3
Day 5
Day 7
Day 9
e
g
f
Z-wire
cholangiocyte
cyst
h

Figure 4. Incorporation of human pluripotent stem cell derived cholangiocyte cyst into hPSC-HCs patterned liver cord. a, Depiction of the liver's bile ductule and bile canaliculi structural organization. **b**, Brightfield image of hPSC-derived cholangiocyte cyst, scale bar = 250 μm. **c**, Schematic of seeding procedure for incorporation of cholangiocyte cyst into zone-specific hPSC-HCs patterned z-wire tissues. **d**, Time series brightfield images of development of zone-specific hPSC-HCs patterned tissue with hPSC-derived cholangiocyte cyst incorporation at day 3 of culture. Enhanced view of cholangiocyte cyst. Scale bar, 1 mm. **e**, Brightfield image of fixed zone-specific hPSC-HCs patterned tissue with hPSC-derived cholangiocyte cyst. Scale bar, 1 mm. Enhanced view shows location of cholangiocyte cyst. Image stitched from multiple images. **f-g**, Histology sectioning of fixed zone-specific hPSC-HCs patterned tissue with hPSC-derived cholangiocyte cyst. Stained for (f) H&E, (g) Masson's Trichrome, (h) E-cadherin.

Supplementary Materials

Bioengineered Liver Assembloids with Zonation

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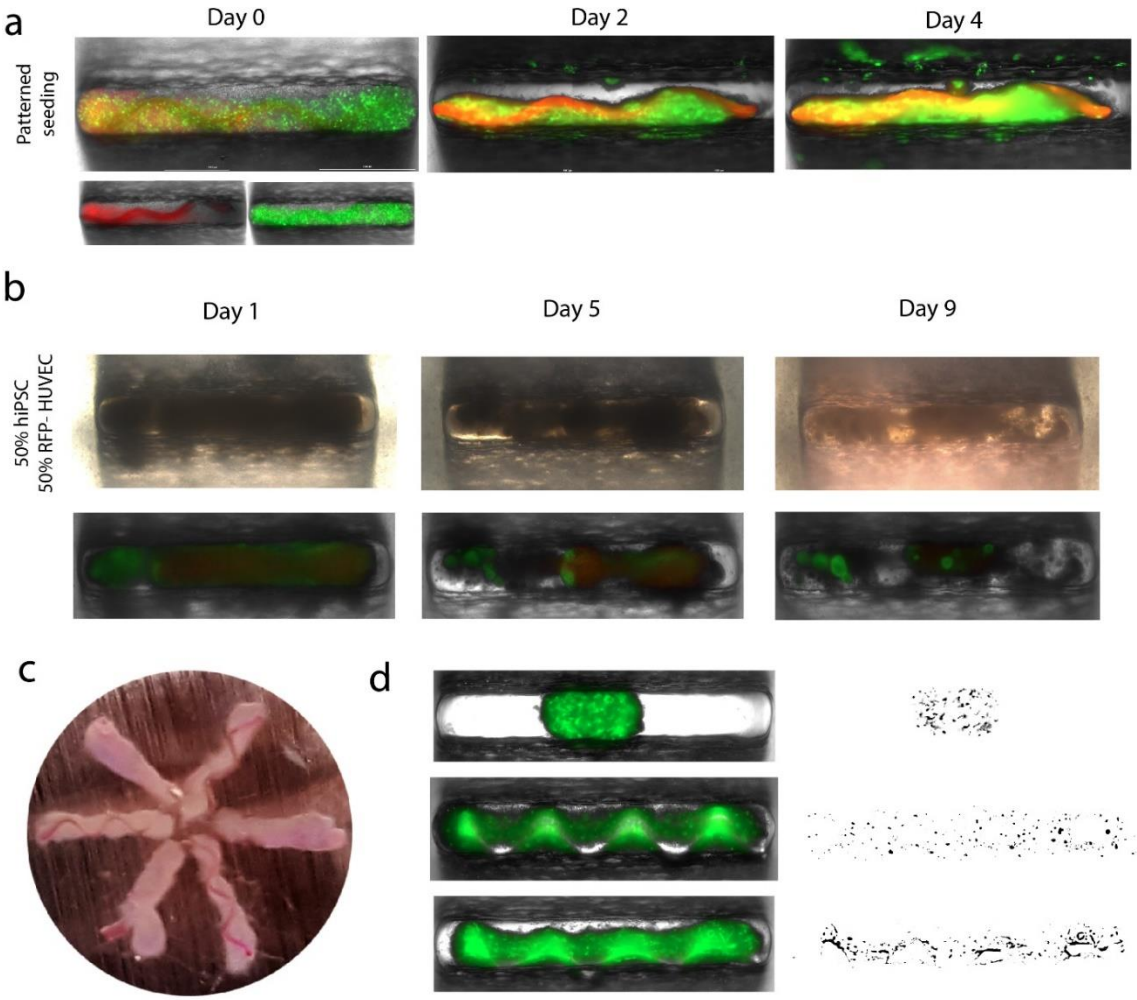

Supplementary Figure 1

a, Time series of brightfield images of patterned seeding of immortalized tissue culture with GFP-HUVEC (green) and half of HepG2 tagged with Red Celltracker Dye (red). **b**, Time series of brightfield images of coculture of hPSC-HCs and RFP-HUVEC (red). **c**, controlled patterned organization of HepG2 coculture z-wire tissues removed from z-wire well plate at day 9, embedded in collagen gel. **d**, Processing of figure 2c florescent plate reader images of HepG2 cocultures containing GFP-HUVEC (green) for the quantification of vasculature formation.

Chapter 4. Conclusion

4.1. Significance

This work describes what is the first zone-patterned hPSC-derived hepatic tissue cultures to be created using the novel tissue culturing technique of assembloid biofabrication. The research herein has value in adding to the collective knowledge of hPSC-HC cultures, a topic on which there is limited published work. The detailed method of patterned cell seeding is a novel technique that may increase accessibility to the creation of zone-patterned hepatic tissues. Intended applications of this work is discussed in depth in the manuscript. Briefly, the novel zone-specific patterning of zone 1 and zone 3 hPSC-HCs is highly applicable to pre-clinical drug screening, the patient-specificity offered by the hPSCs conceivably makes these cultures of use in telling disease modelling and remedy screening, and the use of PGS scaffolds opens the possibility of culture use as an implant for regenerative medicine. The full realization of what this novel tissue design has to offer could lead the way for future hepatic culture model designs.

4.2. Future Work

The tissue cultures require further structural and functional analysis to fully realize their potential for the intended applications. First, while results are suggestive of bile canaliculi formation, visualization of the structures is required for proof of their development in the hepatic cultures. We plan on further studying tissue function via gene expression analysis. Here, cultured tissues will be sectioned in half so that each zone-specific region may be analyzed separately. Gene analysis would look at markers of cell maturation and functional preservation of the zone-specific cell types following coculture. The tissues also need to be

assessed for their utility in drug screening. The hepatic models will be subject to known hepatotoxins and monitored for functional changes. Further future work includes assessing the tissue's potential as a modular building block for larger form hepatic tissue implantation. Here, individual tissues will be patterned in a hepatic lobule formation. Further growth of these cultures arranged in this orientation could provide a means of developing larger hepatic structures for personalized hepatic repair. Finally, there are plans for further recapitulation of the *in vivo* liver acinus via the introduction of hPSC-ECs for the development of aligned vasculature in the hPSC hepatic models. Studies following the incorporation of this cell type will need to be conducted to assess structural arrangement of these cells, and the functional impact that coculture has on the hPSC-HCs.

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