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In situ expansion of engineered human liver tissue in a mouse model of chronic liver disease

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Abstract

In spite of the vast collective experience in tissue engineering, control of both tissue architecture and scale are fundamental translational roadblocks. An experimental framework that enables

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investigation into how architecture and scaling may be coupled is needed. Here, we introduce an approach called ‘SEEDs’ (‘in Situ Expansion of Engineered Devices’), in which we fabricate a structurally organized engineered tissue unit that expands in response to regenerative cues after implantation. We find that tissues containing pre-patterned human primary hepatocytes, endothelial cells, and stromal cells in degradable hydrogel expand over 50-fold over the course of 11 weeks in animals with liver injury, with concomitant increased function as characterized by the production of multiple human liver proteins. Histologically, we observe the emergence of stereotypical microstructure via coordinated growth of hepatocytes in close juxtaposition with a perfused, chimeric vasculature. Importantly, we demonstrate the utility of this platform for probing the impact of multicellular geometric architecture on tissue expansion in response to regenerative cues. This approach represents a hybrid strategy that harnesses both biology and engineering to deploy a limited cell mass more efficiently than either approach could do in isolation, and thus offers a new convergent paradigm for tissue engineering.

Introduction

Advances in tissue engineering have enabled the generation of numerous tissue types that can recapitulate many aspects of native organs, bringing closer the promise that engineered tissues may ultimately replace whole organ transplantation^{1,2}. However, constructing complex solid organs remains a major physical and biological challenge. For engineered tissues that can be fed by diffusion of nutrients from the environment, such as the cornea and skin, thick tissues with low metabolic requirements, such as cartilage, or small-scale endocrine tissues such as beta cells of the pancreas, the recapitulation of complex tissue architecture has not been a limiting factor for clinical translation^{1,2}. However, providing structural organization is likely important for large, metabolically active solid organs such as the heart, kidney, and liver. For example, the liver contains over 100 billion hepatocytes, all positioned within 50 microns of the circulation³. The organization of the circulation and its lining endothelium are integral aspects of the functional organ, and are critical to the delivery of vital nutrients to the entire parenchyma of tissue, as well as to the cell-cell interactions that define juxtacrine and paracrine signals that drive diverse processes such as embryological development, organ function, and regeneration^{4,5}.

A variety of approaches have been examined to promote vascularization and architectural structural control of solid engineered tissues. For example, inclusion of randomly-organized or patterned endothelial cells has improved both the engraftment rate and persistence of metabolic tissues^{6–8}. Decellularization of native structures^{9,10} and biofabrication techniques such as microtissue molding^{8,11} and bioprinting^{12–14} have provided further means to organize cells into scalable vascularized architectures, but all of these approaches offer limited utility for large solid organs because tissues must be perfused rapidly (within ~1 hr of tissue assembly) to prevent ischemic injury.

A different approach may be to nucleate a ‘seed’ of an organ derived from mature cell populations that can grow by *in situ* expansion, as seen both in hepatic embryogenesis wherein primordial tissue buds grow into vascularized organs, and also in regenerative adult responses wherein hepatocytes and their vasculature undergo coordinated expansion^{5,15–17}.

As a step towards this goal, cells have been expanded after engraftment in solid organs through manipulation of cellular signaling pathways^{18–20} or by creating a repopulation advantage for graft cells using injury models, such as in models of chronic liver injury^{21–25}, and in some cases in coordination with self-organizing vascular cells²⁶. However, each of these methods depend upon the ability of grafted cells self-organize to form larger, organized tissue structures, and leave open the question as to the role of controlled multicellular architectural interactions during the expansion and ultimate function of grafted tissues.

In this study, we focus on the fabrication of a tissue seed of controlled initial multicellular architecture, with the goal of specifying a nominal engineered microenvironment that can support the *in situ* expansion of its cellular components in response to systemic regenerative cues after implantation. Our efforts were informed by biological reports of the importance of paracrine signaling between hepatocytes, endothelial cells, and stromal cells in both liver development and regeneration processes^{5,15–17}, as well as by our prior engineering efforts that demonstrated context-dependent cell signaling in microfabricated tissue microenvironments using a range of biomaterials^{7,11,27–30}. Our combined approach yielded an engineered, fully human tissue seed composed of human endothelial cells, hepatocytes, and fibroblasts in a degradable hydrogel that engrafts ectopically in mice and expands over 50-fold *in situ* upon initiation of regenerative stimuli. The resultant human organoid phenocopies several aspects of native liver structure and function including perfused vascular networks, self-assembled structures resembling bile ducts, and a repertoire of circulating human hepatocyte blood products. Importantly, we demonstrate that this platform can be used to dissect the role of multicellular tissue architecture on the ultimate function of expanded seeds in response to regenerative cues.

Results

Construction of human tissue seeds

We first sought to create an engineered ‘tissue seed’ candidate, by pre-arranging a combination of human cell types in a format that would allow for its expansion *in situ*, in response to regenerative cues. Previous work had demonstrated the importance of paracrine signals between hepatocytes, endothelial cells, and stromal cells^{5,15–17} in regeneration, as well as the requirement for defined spatial organization of engineered tissues for optimizing cellular function^{7,8,11}. We therefore opted to incorporate human hepatocytes, endothelial cells, and stromal cells in structurally organized tissue seeds. To do this, we first used microwell technology to create aggregates of a defined size, composed of combinations of human hepatocytes and normal human dermal fibroblasts (NHDFs; Fig. 1A)¹¹. Similarly, microtissue molding was used to create patterned endothelial cord structures⁸ from human umbilical vein endothelial cells (Fig. 1B). This fabrication process was adaptable for scaled construction of larger tissues using a bioprinting process that we developed previously (Suppl Fig. 1)¹³. Finally, we co-encapsulated hepatic cellular aggregates with endothelial cords in a fibrin hydrogel to create tissue seeds which were suitable for ectopic implantation in the intraperitoneal mesenteric fat of mice (Fig. 1B).

As an initial characterization step, short-term *in vitro* tests showed that the addition of NHDFs to hepatocyte aggregates enhanced albumin production, a measure of hepatic function, in a dose-dependent manner (Suppl Fig. 2) and up to approximately 6-fold prior to encapsulation with endothelial cords (Fig. 1C). Similarly, upon implantation in uninjured nude mice, the albumin promoter activity⁷ of tissue seeds bearing aggregates of both hepatocytes and NHDFs was enhanced over 8-fold compared to those with aggregates without NHDFs (Fig. 1D). Thus, the geometrically patterned combination of human hepatocyte, endothelial, and stromal cells yielded candidate human liver tissue seeds that could survive engraftment *in vivo*.

Human hepatocytes in ectopic tissues expand after injury to the host liver

We hypothesized that leveraging host-derived, soluble regenerative stimuli^{17,31} in recipient animals would trigger hepatic tissue seeds to grow ectopically. Accordingly, we implanted fully humanized tissue seeds onto the mesenteric fat of fumarylacetoacetate hydrolase-deficient (Fah^{-/-}) mice that had been backcrossed to nonobese diabetic (NOD), recombinase activating gene-deficient (Rag1^{-/-}), interleukin-2 receptor gamma chain-deficient (Il2r γ null) strains, known as FNRG mice, an immune-deficient mouse model of hereditary tyrosinemia type I^{21,32,33}. This mouse strain experiences progressive liver failure unless treated with the small molecule NTBC. NTBC was administered continuously to control animals ('- Regenerative Stimulus' animals; Fig. 2A) or cycled on/off to induce liver damage ('+ Regenerative Stimulus' animals). Animals were sacrificed and grafts were retrieved at 80 days following tissue seed implantation. Grafts were readily located in the mesenteric fat pad using the suture as a landmark.

To determine whether ectopic tissue seeds had expanded in animals with regenerative signals compared to controls, we identified human hepatocytes by immunostaining against human cytokeratin-18 (Ck-18), an intermediate filament expressed by hepatocytes, and arginase-1 (Arg-1), an enzyme that catalyzes the hydrolysis of arginine to ornithine and urea^{34,35}. Grafts from control animals contained small cellular aggregates containing Ck-18 and Arg-1-positive cells dispersed within the hydrogel (Fig. 2B, **left**). In animals that underwent cycles of injury, visibly larger hepatic grafts were composed of densely packed Ck-18 and Arg-1-positive cells (Fig. 2B, **right**). Fibrin hydrogel remnants were identified at the periphery of grafts. The Ck-18-positive surface area in tissue seed grafts was quantified by a blinded observer using morphometric analysis in histologic sections. Hepatic tissue seed grafts covered significantly more surface area in animals with regenerative stimuli compared to control animals (Fig. 2C, **top**, **p < 0.01**). By assuming the grafts to be spherical, we extrapolated graft volume based on surface area measurements and calculated an average 11-fold graft expansion in animals with regenerative stimuli compared to control animals (Fig. 2C, **bottom**, **p < 0.05**).

To assay for active proliferation in tissue seeds 80 days following implantation, sections were double stained using antibodies against both Ck-18 and Ki67, a nuclear protein associated with cellular proliferation. We identified numerous Ck-18 and Ki67 double-positive cells with round nuclei characteristic of hepatocytes (Fig. 2D, **left**), as well as rare double-positive cells actively undergoing mitosis (Fig. 2D, **center**). When compared with

control animals, 4-fold more Ck-18 and Ki67 double-positive cells were observed in grafts from animals with regenerative stimuli (Fig. 2D, **right**, $p < 0.01$).

To test whether our candidate tissue seeds also respond to regenerative cues after acute liver injury, we implanted seed grafts in the mesenteric fat of athymic mice. After a one week engraftment period, mice were subjected to two-thirds partial hepatectomy of the host liver and pulsed every 12 hours with EdU to label cells in the S phase of the cell cycle. One week after liver injury, animals were sacrificed and engrafted tissues were excised, sectioned and double-immunostained using antibodies that recognize EdU and Ck-18+ to identify hepatocytes in the S-phase of the cell cycle. Grafts subjected to regenerative signals induced by hepatectomy injury contained significantly more EdU and Ck-18 double-positive cells compared to controls (Suppl Fig. 3). Taken together, our results demonstrate that in the context of endogenous liver injury, ectopic liver seeds containing primary hepatocytes proliferate to enlarge the graft. On the basis of these findings, we propose to define the product of our current approach as hepatic SEEDs, for ‘in Situ Expansion of Engineered Devices’.

Characterization of human hepatic function of ectopic grafts

To evaluate the functional characteristics of the expanded hepatic SEEDs, we focused our studies on two major axes of liver function that are vital for life, synthesis and drug metabolism, and also performed a more global analysis of SEED phenotypes. We first assayed for the synthesis axis by testing for the presence of human proteins in mouse serum. In our system, human albumin was detectable in mouse serum as early as the first time point (day 3) and rose over 50-fold from day 3 to the endpoint of the experiment in animals with regenerative stimuli (Fig. 3A–B). The maximum human albumin level detected in a single animal with regenerative stimuli was 105 µg/ml. Human serum albumin levels began to diverge between the treatment groups at approximately day 20, and SEEDs in animals exposed to regenerative stimuli produced significantly greater albumin than those in control animals (Fig. 3A–B; 10-fold difference at the endpoint of the experiment). In addition to human albumin, blood drawn from animals subjected to regenerative stimuli contained significantly elevated levels of human transferrin, alpha-1-antitrypsin, and fibronectin relative to controls (Fig. 3C, $p < 0.05$). These results suggest that human hepatocytes in ectopic tissue seed grafts are functional and synthesize more human proteins when in the presence of regenerative signals, compared to their counterparts in uninjured animals.

In order to assess an additional functional axis, as well as gauge the potential utility of SEEDs for studies of drug metabolism, we characterized the expression and induction of human drug-metabolizing enzymes and other key liver-specific genes (i.e., transcription factors, albumin) in expanded grafts. We first collected RNA from SEEDs explanted from injured host mice, as well as from samples of human liver, and human primary hepatocyte, HUVEC, and NHDF controls for RNA-Seq analysis. We then assessed the expression levels of 50 genes that represent varying hepatic gene classes (e.g. CYP3A4 and 2B6 for cytochrome P450 activity, SULT1A1/2A1 for sulfotransferase/Phase II activity, SLCO1A2/1B1 for anion transporter activity, ABCB/ABCG for ATP-binding transporters, APOB/APOE for lipoprotein biosynthesis, ALB for biosynthesis, HNF4A/G as key

transcription factors) that were expressed in both human liver and primary human hepatocyte control RNA samples (transcript count ratio > 1e-6). Read counts across groups were normalized to human primary hepatocyte controls to create an expression heat map (Fig. 3D). 47/50 of these liver-specific genes were expressed in explanted SEED grafts, compared to 18/50 genes expressed in unexpanded HUVEC and NHDF RNA samples. Genes from each of the major hepatic drug metabolism pathways were generally expressed in SEEDs at similar levels to human liver, such as Phase I cytochrome P450 enzymes (Fig. 3E), Phase II enzymes such as sulfotransferases (Fig. 3F), and Phase III anion and ATP-binding transporters (Fig. 3G,H). In addition to hepatic gene expression studies, we tested the ability of SEEDs to upregulate key drug metabolism enzymes in response to a known human CYP450 inducer⁷. To this end, we administered Rifampin or vehicle control to animals bearing both expanded SEED grafts and injured livers, euthanized the animals, and collected RNA from explanted SEEDs. We found that Rifampin induced CYP3A4 expression in SEEDs, a highly liver-specific phenomenon indicative of mature hepatocyte function. Together these studies demonstrate that SEEDs express drug-metabolizing enzymes, and the expression of these enzymes can be enhanced after administration of the known inducer, Rifampin.

Finally, we sought to interrogate the transcriptional profile of SEEDs more globally. We first used Ingenuity Pathway Analysis to assess the fraction of genes known to be downstream of given transcription factors that were differentially regulated between expanded SEEDs and HUVEC/NHDF control cells. This analysis identified distinct transcriptional regulation in SEEDs by hepatocyte transcription factors in the HNF1, 3, and 4 families, as well as C/EBP, compared to HUVEC/NHDF controls (Suppl Fig. 4A), which supports the interpretation that the hepatocytes present in expanded SEEDs display a lineage-appropriate phenotype. Furthermore, since SEEDs are composed of primary hepatocytes, HUVECs, and NHDFs, we then sought to test whether expression profiles from each of these three cell types were detectable in SEEDs after expansion. Hierarchical clustering of expression RNA-seq profiles obtained from samples of expanded SEEDs, pure human primary hepatocytes, human liver, and pure populations of cultured NHDFs and HUVECs demonstrated that SEEDs cluster between the primary hepatocyte/human liver samples and non-parenchymal HUVEC/NHDF cell lines, consistent with an intermediate phenotype driven by the presence of each of these three cell types within the expanded graft (Suppl Fig. 4B).

Taken together, our results demonstrate that human hepatocytes in ectopic tissue SEED grafts retain a hepatic phenotype and are functional, as characterized by synthesis and drug metabolism, two major axes of hepatic function that are necessary for life.

Characterization of hepatic graft morphology

Our earlier-generation synthetic tissues, when implanted into uninjured hosts, were characterized histologically by the presence of disperse hepatic aggregates within fibrin hydrogels upon explant^{7,8,11}. In our current studies, we hypothesized that cells would self-organize in response to the presence of regenerative stimuli as the tissue seeds expanded. Immunohistological characterization revealed that the expanded SEEDs in animals with regenerative stimuli contained densely packed polyhedral cells resembling hepatocytes,

many of which were binucleated (Fig. 4A; Hematoxylin & Eosin). These cells stained positively for Ck-18 and Arg-1, both of which are normally expressed in human hepatocytes (Fig. 4B,C). Graft hepatocytes in expanded SEEDs were organized into dense aggregate-like units that in some cases exhibited structure reminiscent of hepatic cords in the normal human liver (Fig. 4B, white star). Furthermore, hepatic units in expanded SEEDs were arranged within a syncytium of interconnected lacunae containing endovascular stroma and lined with collagen III, which lines hepatic cords in the space of Disse in the human liver (Fig. 4D, Reticulin stain). In addition, SEEDs grafts were examined for the expression of multidrug resistance-associated protein 2 (MRP2, also known as ABCC2), which is selectively transported to the apical (i.e., canalicular) domain of hepatocytes in human liver. We observed that hepatocytes in expanded liver SEED grafts exhibit polarized expression of MRP2 (Fig. 4C, red). Tissue seed grafts also contain bile canalicular-like structures between adjacent hepatocytes characteristic of normal liver structure (Fig. 4C, white arrows), as well as larger vacuolar structures lined with MRP2 (Fig. 4C, white stars).

Further characterization with hematoxylin and eosin staining revealed that expanded tissue seed grafts also contained duct-like structures resembling bile ducts (Fig. 4E, arrows). To further examine whether biliary epithelial-like cells were present in ectopic grafted tissues, tissue sections were immunohistochemically stained for expression of both Ck-18 (a cytokeratin expressed by hepatocytes and biliary epithelial cells) and Ck-19 (a cytokeratin expressed by biliary epithelial cells but not hepatocytes). Cells organized in ductal structures stained positively for both Ck-18 and Ck-19, suggesting that these cells exhibit biliary epithelial-like characteristics (Fig. 4F, **left and center**). Notably, Ck-18 and Ck-19 double-positive ductal structures were typically located within connective tissue and adjacent to huCD31-positive blood vessels, many of which contained Ter-119 positive erythroid cells (Fig. 4F, **right**). To further confirm both the biliary epithelial cell-like phenotype and whether these cells were of human origin, tissue sections were stained for human Ck-18 and a second cytokeratin expressed on biliary epithelial cells but not hepatocytes, human Ck-7. Ungrafted mouse control livers did not stain with either human marker, whereas positive control human liver tissue contained Ck-18 and Ck-7 double-positive cells in ductal structures (Suppl Fig. 5). Ductal structures in ectopic grafts stained positive for both Ck-18 and Ck-7, further confirming that they were comprised of human cells with an epithelial cell phenotype (Suppl Fig. 5). We hypothesized that biliary-like epithelial cells identified in SEEDs grafts may have arisen at least partially from contaminating biliary epithelial cells present in cryopreserved human hepatocyte lots. To test this hypothesis, we immunostained for CK-19 expression using cells from primary hepatocyte lots immediately upon thawing. We identified Ck-19-positive cells in both of the human hepatocyte primary cell lots used in this study (0.16% and 0.13% of total cells in hu8085 and NON lots, respectively, Suppl Fig. 6), suggesting that self-assembling biliary-like structures may have been at least partially derived from these cells. These results demonstrate the presence of human biliary epithelial-like cells that have self-assembled to form ductal-like structures at an ectopic location within human tissue seeds, and these ductal structures are commonly associated with other classic features of portal triads, such as vasculature and connective tissue. Taken together, pre-patterned hepatocytes in SEEDs that were implanted in animals exposed to regenerative

stimuli self-assembled upon expansion to create densely packed, ectopic hepatic tissue that exhibit several microstructural hallmarks typically associated with human liver.

Concomitant expansion of vessels containing human endothelial cells

Since lacunae in the human liver form the vascular sinusoidal network that feeds hepatocytes with blood, we wondered whether interconnected lacunae observed in hepatic SEEDs contained red blood cells, and whether regenerative cues would promote expansion of the blood pool. We noted numerous cells resembling red blood cells in lacunae of expanded seed grafts by H&E staining (Fig. 4A), and we further confirmed the identify of such cells by staining for Ter-119, an erythrocyte marker (Fig. 5A). We also quantified total blood area and observed that grafts from animals subjected to liver injury contained significantly more blood compared to those from control animals (Fig. 5A, $p < 0.05$), suggesting that the blood pool coordinately expanded with the expansion of hepatic SEEDs in animals with regenerative stimuli.

The presence of organized red blood cells in expanded tissue SEEDs suggested that vascular networks might be present in these grafts, a hypothesis that is consistent with our observation of human albumin and other synthetic products in the host circulation. While numerous existing ectopic engraftment models have been shown to recruit host-derived vascularization, it is also known that human hepatocytes and vascular cells expand in concert during liver regeneration^{4,5,36}. These observations led us to hypothesize that the pre-patterned, human endothelial cells within hepatic SEEDs may also expand in response to regenerative cues. To test these hypotheses, we immunostained graft sections with antibodies recognizing human CD31 (endothelial cells), Ter-119, and Arg-1. Numerous vessels lined in part by human endothelial cells were identified throughout the grafts in injured animals (Fig. 5B). Incubation of explanted graft sections in a solution containing lectins that bind specifically to human or mouse endothelium demonstrated that vessels were lined with both human and mouse endothelial cells (Suppl Fig. 7). In many cases, these vessels contained Ter-119-positive erythroid cells (Fig. 5B). Blood vessels lined with human CD31-positive endothelial cells were located in the lacunae between and within hepatic units (Fig. 5B, **center**). Grafts in hosts with regenerative stimuli included more vessels containing human endothelial cells compared to grafts in control animals (Fig. 5B, $p < 0.01$). Ki67-positive human endothelial cells were present, but rare (generally less than one Ki67 and huCD31 double-positive cell at the graft faces of all 1 mm sections), suggesting most endothelial cells were not undergoing active cell cycle progression at the time of tissue explant (Suppl Fig. 8). Taken together, these results demonstrate that human vascular networks carrying blood coordinately expanded with the SEED's hepatic mass in animals with regenerative stimuli.

SEED architecture impacts expansion in response to regenerative stimuli

Since tissue architecture ultimately defines the cell-cell contacts and paracrine signaling gradients that drive cellular phenotype and function, we hypothesized that relative cellular positioning in SEEDs would impact expansion in response to regenerative stimuli. We thus sought to determine whether our platform could be used to dissect of the role of architectural cellular patterning in mediating implant expansion in response to regenerative stimuli. We created three constructs using varying arrangements of human hepatocytes, endothelial cells,

and stromal cells. Three configurations were tested, including 1) all three cell types randomly organized as single cells within fibrin hydrogels, 2) all three cell types aggregated to create tri-cell aggregates, and suspended randomly within fibrin hydrogels, and 3) the original SEED architecture in which hepatocytes and stromal cells were patterned in aggregates and embedded in fibrin alongside organized endothelial cords (Fig. 6A). Constructs were implanted in the mesenteric fat of FNRG mice, and NTBC was cycled on/off to induce liver damage in all groups. Ck-18+ hepatic graft size of explanted grafts appeared to increase in animals with SEEDs, but this difference did not reach significance (Fig. 6B–C). Nonetheless, human tissue graft function, as measured by the levels of human albumin and transferrin in mouse serum, was significantly enhanced in SEED constructs (Fig. 6C, middle and right). To further assess the relative importance of the inclusion of different cell types in mediating SEED expansion, we next removed NHDFs and/or endothelial cords from SEED constructs, respectively, prior to implantation in FNRG mice and initiating liver injury. We found that each cellular element in SEEDs had a positive impact on human albumin produced in the mouse serum, and this effect reached significance in the presence of all three cell types (Fig. 6D).

Finally, we sought to explore the mechanism by which cell patterning might play a role in tissue expansion. We created tissues containing either randomly organized endothelial cells or endothelial cells patterned in endothelial cords and analyzed the expression of key ‘angiocrine’ genes, which have been shown previously to be upregulated in endothelial cells and enhance hepatocyte proliferation during liver regeneration^{4,5}, immediately after cord formation *in vitro*. We found that mRNA expression of multiple angiocrine factors were upregulated in tissues containing patterned endothelial cords compared to those with randomly organized endothelial cells (Fig. 6E). These data suggest that expansion of SEEDs may be driven in part by enhanced expression of angiocrine signals. Together, these analyses demonstrate that tissue architecture influences implant expansion, and that the observed SEED expansion may be partially mediated by enhanced expression of angiocrine cues.

Discussion

In this study, we explore the concept of constructing the seed of an organ from mature cell populations that coordinately grow following implantation. These SEEDs (in Situ Expansion of Engineered Devices) are composed of human parenchymal cells, vascular cells, and stromal cells in a specified architecture in a degradable biomaterial that collectively favor self-organized expansion in response to regenerative stimuli. We show that hepatic SEEDs can engraft and expand ectopically by 50-fold over time, and even demonstrate emergence of self-organized biliary epithelial structures within the newly-formed, vascularized hepatic parenchyma.

The composition of our tissue seeds was influenced by previous work in both biology and engineering that probed the multicellular paracrine signaling loops that exist between hepatocytes, endothelial cells, and stromal cells. For example, HGF, TGF α , Wnt2, HGF, and angiopoietin-2 communicate signals from stellate cells or endothelial cells to neighboring hepatocytes that are known to be critical for normal organogenesis and regeneration^{4,5,36,37}. Since paracrine signals are influenced by the spatial proximity between different cell types,

engineers have further developed microfabrication technologies to better understand how microstructural arrangement of cells within the tissue impacts tissue signaling and function. In the case of the liver, such studies have shown that culturing hepatocytes in spheroids or aggregates with other hepatocytes^{7,11}, stromal cells in direct contact with hepatocytes^{7,11,30}, and endothelial cells near to but not in contact with hepatocytes^{7,11} leads to optimal hepatic function in engineered platforms. In our current focus on the fabrication of a tissue seed, the goal was not to mimic the structure of the tissue, but rather to specify an engineered environment that could stabilize parenchymal function and direct *in situ* cellular expansion in response to systemic regenerative cues after implantation. We used our architectural patterning capabilities to create human hepatic tissues with varying cellular patterning confirmations and then probed whether structural architecture impacted the ultimate function of expanded tissues. Our results demonstrated that patterning stromal cells in immediate proximity to hepatocytes within cellular aggregates, and the inclusion of distinct endothelial cords near to but not touching hepatic aggregates in the hydrogels resulted in SEEDs with elevated function following expansion, relative to alternate initial arrangements of the same cell types.

In addition to local signals from neighboring cell types, liver SEEDs in our studies responded to systemic regenerative signals after injury to the mouse liver. A systemic effect of liver injury on a distant uninjured organ was first described by seminal parabiosis studies linking the circulation of two animals³⁸ and more recently in studies grafting hepatocytes ectopically into the lymph nodes and other locations^{26,39–41}. Expansion of primary adult human hepatocytes in our studies demonstrate that signals generated in the mouse in response to injury can communicate with human tissue seeds, suggesting that at least some relevant mouse growth factors can cross-react with cognate receptors on human cells. The signals mediating this interaction likely include growth factors known to control hepatocyte proliferation in regeneration and development, such as hepatocyte growth factor (HGF)^{17,42,43}. Next generation biomarkers⁴⁴ might further enable elucidation of regenerative cues present in human disease, such as liver cirrhosis. If regenerative cues in human disease are insufficient to induce robust expansion of the hepatic SEEDs developed here, additional microenvironmental cues such as microbeads releasing small molecules or growth factors might be incorporated to trigger hepatocyte proliferation and expansion^{45,46}.

Notably, ectopic synthetic tissue grafts in our studies also contained biliary epithelial-like cells that frequently self-organized into structures resembling bile ducts. The origin of these structures remains an open question. While rare biliary-lineage cells were present in the human primary hepatocyte isolates, there have also been recent documentations of adult human hepatocytes giving rise to this lineage during liver injury in other systems^{47,48}. Our observation of biliary duct-like structures in ectopic liver tissue grafts thus requires additional characterization, but nonetheless represents an important first step towards creating ectopic human livers with hallmark anatomic features of the native liver.

To date, most cell-based strategies targeted at treating liver diseases have involved direct engraftment of hepatocytes into the host liver⁴⁹. The efficacy of this approach is likely to be limited in the majority of patients with end stage liver disease because cirrhosis and fibrosis limit cellular engraftment. We and others are pursuing the development of a therapeutic

‘satellite liver’ that could be implanted in an ectopic location in a patient and provide functional support to the failing liver^{26,39–41,50,51}. Although human induced pluripotent stem (iPS) cell-derived hepatocyte-like cells have been engrafted ectopically in a prior study, iPS-derived cell therapies must still address a variety of concerns, including challenges in creating mature cell populations that exhibit robust adult functions, the potential for teratoma, and the time needed for cellular expansion and differentiation. The present study represents a complementary approach, in which we hijack ‘regenerative cues’ from adult liver injury to stimulate the expansion of an ectopic adult human tissue. Our studies here shed light on the importance of refined construct engineering as a factor that was previously overlooked in enabling expansion of adult human hepatocytes.

We believe this work sets the stage for an alternate paradigm for scale-up of engineered organs – one that hijacks native developmental, injury, or regenerative circuitry to expand pre-fabricated constructs *in situ*. However, more work must be done before SEEDs are ready for the clinic. First, while our studies demonstrated that expanded SEEDs exhibit a plethora of adult hepatic functions, additional studies must be performed to further analyze the capability of SEEDs to recapitulate the over 500 adult liver functions (for example, production of human specific drug metabolites and further analysis of biliary function). Second, while SEED expansion is augmented using engineering tools such as geometric patterning and material cues in the study here, expansion may be additionally supported by inclusion of additional microenvironmental factors, such controlled delivery of mitogens^{20,52}. Third, to attain the ~10 billion hepatocytes necessary to treat several forms of liver disease, SEEDs will need to be further scaled in size, likely in combination with other technologies – one can envision bioprinting of tissue seeds followed by post-implantation expansion as an alternative to printing a whole human organ *in vitro*. Finally, future studies should be undertaken to determine the extensibility of the SEEDs concept to other forms of liver injury, such as cirrhosis. Taken together, we believe that this work enables fundamental studies on regenerative signaling between endothelial and parenchymal cells and represents a new *in situ* expansion approach to create scaled tissues for regenerative medicine.

Materials and Methods

Study Design

Sample size justification for all studies was performed using power analysis. N = 3 – 4 was used for each group for *in vitro* experiments, and N = 6 – 9 animals per group was used for *in vivo* experiments. The overall objective of this study was to test whether human engineering liver tissue ‘SEEDs’ engrafted ectopically expand over and remain functional in mice with liver injury. Hence, treatments for *in vivo* studies generally included two groups of mice, both of which were implanted with SEEDs constructs and then subsequently randomly divided into one of two groups – one receiving ongoing liver injury and an uninjured control group. Measurement techniques (endpoints) were designed to assess hepatocyte phenotype and function based on a variety of established endpoints. All histomorphometric analyses were performed by a blinded observer. Studies were repeated at least three times.

Cell culture

Primary cryopreserved human hepatocytes (Lot NON, 35 year old, Caucasian, Female, Celsis or Lot Hu8085; 1 year old, female, Caucasian, Invitrogen) were maintained in high-glucose DMEM (Cellgro) containing 10% (vol/vol) FBS (Gibco), 1% (vol/vol) ITS supplement (insulin, transferrin, sodium selenite; BD Biosciences), 0.49 pg/mL glucagon, 0.08 ng/mL dexamethasone, 0.018 M Hepes, and 1% (vol/vol) penicillin–streptomycin (pen-strep; Invitrogen). Primary human umbilical endothelial cells (HUVECs; Lonza; passages 4–7) were maintained in dishes in EGM-2 media (Lonza). Normal human dermal fibroblasts (NHDFs; Lonza; passages 4–8) were cultured in DMEM with 10% (vol/vol) FBS and 1% (vol/vol) pen-strep.

Fabrication of micropatterned ‘tissue seeds’

Engineered liver tissue seeds were fabricated using previously described methods⁸. To create hepatic aggregates, human primary hepatocytes were thawed and immediately plated into AggreWell micromolds along with NHDFs and incubated overnight. To create endothelial cords, HUVECs were suspended at a density of 3 million HUVECs per ml of 2.5 mg/ml liquid collagen (BD Biosciences) and centrifuged into polydimethylsiloxane (PDMS) channels. Collagen was polymerized, and constructs were incubated in EGM-2 media for 4 hours to allow for cord formation. Endothelial cord arrays were then embedded in 10 mg/ml fibrin (human thrombin, Sigma; bovine fibrinogen, Sigma). Hepatic aggregates (approximately 100 hepatocytes and 200 dermal fibroblasts per aggregate) were suspended in 10 mg/ml fibrin at a concentration of 90,000 aggregates/ml fibrin and added in a second layer over endothelial cords in order to fully encase the cords in hepatic aggregates and fibrin gel. Synthetic tissues were cut with a 6 mm biopsy punch immediately prior to implantation. Each seed tissue contained approximately 150,000 human hepatocytes, 300,000 dermal fibroblasts, and 150,000 HUVECs upon implantation.

For bioprinted constructs (Supp Fig. 1), sacrificial lattices of carbohydrate glass were constructed using a custom built 3D printer containing a heated nozzle as described previously¹³, (left), embedded within a fibrin hydrogel, and dissolved using PBS to leave open channels (center). Channels were filled by pipetting a slurry containing HUVECs and neutralized collagen using methods and cell densities described above.

Implantation and induction of liver injury

All surgical procedures were conducted according to protocols approved by The Rockefeller University and Massachusetts Institute of Technology Institutional Animal Care and Use Committees. Eight- to twelve-week-old female NCR nude (Taconic; for uninjured mouse studies in Figure 1) or FAH(–/–) backcrossed to NOD, Rag1(–/–), and Il2r γ (null) (FNRG) mice^{21,32,33} were anesthetized using isoflurane, and the synthetic tissue constructs were sutured to the mesenteric parametrial fat pad (1 tissue per animal for nude mouse studies; 4 tissues per animal for FNRG studies). The incisions were closed aseptically, and the animals were administered 0.1 mg/mL buprenorphine every 12 h for 3 d following surgery. NTBC was withdrawn from animals’ drinking water immediately following synthetic tissue implantation and for 14 days following implantation. NTBC was then administered for four

days and then cycled off/on in 14 days-off and 3–4 days-on increments for the remainder of the experiment.

For hepatectomy studies, partial hepatectomy liver injury was performed one week following implantation of SEEDs. Prior to hepatectomy surgery, mice were administered 5 mg/kg carprofen subcutaneously. Partial hepatectomy was then performed as described previously⁵³ with slight modifications. Specifically, the left lateral and left median lobes were excised, sparing the gall bladder. The abdomen was washed with saline prior to closing the peritoneum with vicryl sutures. Following hepatectomy, animals were injected with 50 mg/kg EdU (5-ethynyl-2'-deoxyuridine; ThermoFisher Scientific) daily until sacrifice at seven days following hepatectomy.

Bioluminescence imaging

To enable noninvasive imaging of the survival of functional hepatocytes, primary human hepatocytes were transduced in suspension culture immediately upon thawing with a lentiviral vector expressing firefly luciferase under the human albumin promoter (pTRIP.Alb.IVsb.IRES.tagRFP-DEST, gift of Charles Rice, The Rockefeller University) before SEEDs fabrication. For viral transduction, concentrated virus was diluted 1:5 into hepatocyte media containing HEPES buffer (20 mM; Invitrogen) and polybrene (4 $\mu\text{g ml}^{-1}$, Invitrogen) in six-well ultra-low-attachment plates (Corning). Immediately before bioluminescence imaging, mice were injected intraperitoneally with 250 μl of 15 mg ml^{-1} D-Luciferin (Caliper Life Sciences) and imaged using the IVIS Spectrum (Xenogen) system and Living Image software (Caliper Life Sciences).

Biochemical assays

Throughout the experiment, mice were bled retro-orbitally, blood was collected, and serum was separated by centrifugation. Serum levels of human albumin were determined by an enzyme-linked immunosorbent assay (ELISA) using goat polyclonal capture and HRP-conjugated goat anti-human albumin detection antibodies (Bethyl laboratories). At the time of sacrifice for some animals, blood was retrieved via cardiac puncture and collected in clot-activating tubes. Serum levels of human albumin (Bethyl), human alpha-1-antitrypsin (Bethyl), and human fibronectin (Boster) were determined by ELISA.

Tissue harvesting and immunohistochemistry

Animals were sacrificed at the termination of the experiment (80–84 days). Tissue was harvested from the intraperitoneal space, and explants were fixed in 4% (vol/vol) paraformaldehyde (PFA) for 48 h at 4 °C. Explants were sectioned into approximately 1 mm sections by hand and then dehydrated in graded ethanol (50–100%), embedded in paraffin, and sectioned using a microtome (6 μm) for immunohistochemical staining. All morphometric analyses (e.g., graft size) were performed on stained 6 μm sections from the surface of all 1 mm sections of each graft.

For gross visualization of tissue, sections were stained with hematoxylin and eosin (H&E). For visualization of type III collagen, sections were stained with reticulin/nuclear fast red stain (Dako). For identification of primary human hepatocytes, sections were incubated with

primary antibodies against human Ck-18 (mouse, 1:25; Dako) or arginase 1 (rabbit, Arg-1, 1:400; Sigma) and followed with species-appropriate secondary antibodies conjugated to Alexa 647. To determine graft size, Adobe Photoshop was used to quantify the number of Ck-18 positive pixels in each graft. For identification of primary human hepatocytes in active cell cycle phases, sections were blocked using M.O.M. Blocking Reagent and normal donkey serum, then incubated with primary antibodies against human Ck-18 (mouse, 1:25; Dako) and Ki67 (rabbit, 1:500; Abcam) and followed with species-appropriate secondary antibodies conjugated to Alexa 555 and 647.

For identification of primary human hepatocytes and human bile canaliculi, sections were blocked using M.O.M. Blocking Reagent (Vector Laboratories) and normal donkey serum, then incubated with primary antibodies against human Ck-18 and human MRP2 (rabbit, 1:100; Abcam) and followed with species-appropriate secondary antibodies conjugated to Alexa 555 and 647. For identification of primary hepatocytes, human endothelial cells and mouse red blood cells, sections first were blocked using M.O.M. Blocking Reagent and normal donkey serum and then immunostained using primary antibodies against human arginase 1 (rabbit, Arg-1, 1:400; Sigma), human CD31 (mouse, 1:20; Dako), and Ter-119 (rat, 1:100; BD Pharmingen), respectively. Signal was visualized after incubation with secondary goat anti-IgG1–Alexa 555, donkey anti-rat–Alexa 488, and donkey anti-rabbit–Alexa 647 antibodies (Jackson ImmunoResearch). For identification of primary human hepatocytes and biliary cells, sections were blocked using M.O.M. Block Reagent and normal donkey serum, then immunostained with primary antibodies against human Ck-18 and Ck-19 (rabbit, 1:250; Abcam) or human CK7 (rabbit, 1:300; Novus) and followed with species-appropriate secondary antibodies conjugated to Alexa 555 and 647. Positive control human adult liver sections were purchased from Abcam. For identification of biliary cells in human hepatocyte cell lots, 50,000 freshly thawed cells from each human hepatocyte lot were deposited on glass slides using a Cytospin Cytocentrifuge and then immunostained for Ck-19, as described above. Images were obtained using a Nikon Eclipse Ti microscope, Nikon 1AR Ultra-Fast Spectral Scanning confocal microscope, or Zeiss AxioCam HRm Stereoscope.

For SWITCH tissue clarification, 1 mm thick sections of explanted expanded SEED grafts were treated with glutaraldehyde and then cleared with 200 mM SDS using methods we developed previously⁵⁴. To visualize mouse versus human vessels, cleared SEED graft sections were incubated in a solution of 500 µg/ml lectin from *Helix pomatia* agglutinin (PHA) conjugated to Alexa 488 (Sigma-Aldrich) and 100 µg/ml lectin from *Ulex europaeus* agglutinin (UEA-1) conjugated to TRITC (Vector laboratories) in PBS, which have been shown previously to bind to mouse or human endothelial cells, respectively⁸. Images were obtained using an Olympus 10× CLARITY immersion medium 0.6 NA objective.

RNA-Seq and bioinformatics

Total RNA was extracted from explanted SEEDs grafts (n=3), primary cryopreserved human hepatocytes (immediately after thawing, n = 2), human liver (n = 1), HUVECs (n = 1), and NHDFs (n = 1) using Qiagen RNeasy Mini kits. RNA was passed through initial quality control using an Agilent BioAnalyzer, poly-A purified and converted to cDNA using the

Illumina Tru-Seq protocol, run on SPRI-works system (Beckman Coulter) using custom barcodes for library preparation, enriched by PCR, and submitted for Illumina sequencing. 40nt paired-end sequencing was then performed on an Illumina HiSeq 2000. A standard pipeline was used for quality control of HiSeq outputs, consisting of gathering fastqc and tag count statistics at the flowcell level, as well as individual fastqc on each sample.

The fastq files for each sample were aligned to both human reference genome hg19 using STAR⁵⁵. HTSeq⁵⁶ was used to obtain transcript counts from the SAM outputs of the STAR alignment, using Ensembl gene annotations. The counts data was normalized for read depth and analyzed using the DESeq2 package in R, clustering was performed using the pheatmap package, and plots were produced in R and GraphPad Prism. Healthy adult human liver control RNA was obtained commercially (LifeTech and CloneTech).

Statistical analysis

All data are expressed as the mean $\pm$ SEM. Statistical significance ($p < 0.05$) was determined using a Student's T-test (two-sided) or One-way ANOVA followed by Tukey's post hoc test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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promoter activity was enhanced in implanted tissue seeds containing hepatic aggregates composed of hepatocytes and NHDFs compared to tissues containing aggregates with only hepatocytes, six days following implantation (D; $p < 0.01$, p/s = photons/second; ROI = Region of Interest). All scale bars are 400 μm .

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Figure 2. Human liver tissue seed grafts expand after host liver injury

Human liver tissue seeds were implanted onto the mesenteric fat of FAH(-/-) on NOD, Rag1(-/-), Il2rγ(-/-) (FNRG) mice, which experience liver injury unless treated with NTBC. NTBC was provided continuously ('- Regenerative Stimulus'; control) or cycled (14 days off / 3–4 days on pattern) to induce host liver injury ('+ Regenerative Stimulus') after engraftment of ectopic synthetic tissues (A). Immunostaining of tissue seed grafts retrieved at 80 days post-implantation revealed Ck-18 and Arg-1 positive grafts in animals with and without regenerative stimulus (B, scale bars 100 μm). Histomorphometry revealed significantly greater Ck-18-positive graft area and volume in animals with regenerative

stimuli compared to controls (C). Numerous Ki67 and Ck-18 double-positive graft cells (D, left, arrows) and rare Ki67 and Ck-18 double-positive cells undergoing mitosis (D, right, arrows) were identified in grafts. Grafts in animals with regenerative stimuli exhibited a significantly greater number of Ki67 and Ck-18 double-positive cells compared to uninjured animals (D, right, scale bars 10 μ m). * $p < 0.05$, ** $p < 0.01$.

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Figure 3. Function of human liver tissue seed grafts

Mice were implanted with SEEDs, and host liver injury was induced by cycling the removal of NTBC (14 days off, followed by 3–4 days on – grey bars). Blood samples were collected weekly via retro-orbital bleeding. Human albumin in mouse serum was significantly greater in animals with regenerative stimuli compared to controls (A, one-way ANOVA; B, average albumin represents albumin levels for each animal averaged across all timepoints). Human transferrin, alpha 1 antitrypsin, and fibronectin levels in mouse serum were greater in animals with regenerative stimuli compared to controls at 80 days following implantation (C). RNA analysis of explanted SEEDs grafts demonstrated that 47/50 liver-specific genes

were expressed in explanted SEED grafts compared to 18/50 genes expressed in HUVEC and NHDF cellular RNA (D). Genes from each of the major hepatic drug metabolism pathways were expressed in SEEDs at levels similar to human liver, such as Phase I enzymes (E), Phase II enzymes (F) and Phase III anion and ATP-binding transporters (G,H). Mice with SEEDs were injected intraperitoneally with 25 mg/kg Rifampin solution or vehicle control daily for 3 days and again 1 hour prior to sacrifice. Rifampin induced CYP3A4 expression in expanded SEEDs compared to mice injected with vehicle control (I). * $p < 0.05$, *** $p < 0.001$.

Figure 4. Characterization of human tissue seed graft morphology

Immunohistochemical staining of tissue seed grafts from animals with regenerative stimuli sacrificed at day 80 revealed densely packed polyhedral cells resembling hepatocytes (A, H&E) that stained positive for both ARG-1 and Ck-18 (B, C). These hepatocytes were organized into units that sometimes self-organized to exhibit cord-like structures (B, star). Hepatocytes were polarized, forming MRP2-positive bile canaliculi-like structures between hepatocytes (arrows, inset) as well as larger vacuoles lined with MRP2 (C, stars). Hepatic units were surrounded by a syncytium of interconnected lacunae lined with collagen III (D, reticulin). Tissue seed graft sections stained with hematoxylin and eosin contained duct-like structures that resembled bile ducts (E, arrows), and cells in these ductal-structures stained positive for Ck-19, which marks biliary epithelial cells (F, left). Ductal structures were typically located adjacent to blood vessels lined with human CD31-positive endothelium and containing Ter-119-positive blood (F, right). All scale bars 25 μm.

Figure 5. Characterization of hepatic tissue seed graft vascularization

Lacunae between hepatocytes contained Ter-119-positive red blood cells (A, left, green), and tissue seed grafts in animals with regenerative stimuli contained significantly more blood compared to control animals (A, right). Blood vessels containing Ter-119 positive red blood cells (white) and lined in part by human endothelial cells (red) were identified in tissue seed grafts (B, left), and grafts from animals with regenerative stimuli contained significantly more human CD31-positive blood vessels compared to controls (B, right). All scale bars 25 μ m, * $p < 0.05$, *** $p < 0.001$.

Figure 6. Tissue architecture impacts function of SEEDs after expansion

Human liver tissues were created in which hepatocytes, HUVECs, and NHDFs were randomly organized as single cells within fibrin hydrogels (A, left), hepatocytes, HUVECs, and NHDFs were aggregated to create tri-cell aggregates, which were then randomly seeded within fibrin hydrogels (A, center), or 3 hepatocytes and NHDFs were patterned together in aggregates, and HUVECs in endothelial cords followed by molding together to form SEEDs (A, right). All three architectural conformations produced Ck-18+ hepatic grafts after expansion (B). Graft sizes measured by histomorphometry appeared to be enhanced in SEEDs, but this difference was not significant (C). Hepatic function as measured by human

albumin and transferrin production was significantly enhanced in SEEDs (C, center, right). Inclusion of both NHDFs and HUVECs in SEEDs was necessary for maximal hepatic function after expansion (D). A comparison of the gene expression patterns present in engineered constructs with either random HUVECs or endothelial cords one day after formation of the tissues *in vitro*, revealed that pre-organization of HUVECs into endothelial cords resulted in increased expression of several key angiocrine genes (E). * $p < 0.05$, Scale bars 100 μm).

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