RICE UNIVERSITY

Engineering Vascularized Hepatic Tissue in Bioactive Poly(ethylene glycol)-based Hydrogels

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
Doctor of Philosophy

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Houston, TX

December 2013
ABSTRACT

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Transport of oxygen and nutrients to cells within engineered tissues remains one of the most significant challenges in tissue engineering. This challenge has led researchers to seek new strategies to engineer vascularized tissues. Co-cultures of endothelial cells and pericytes can be used to form microvascular networks in bioactive scaffolds, and these networks have been shown to be perfusable and capable of anastomosis with host vasculature. These co-cultures are prevalent in the literature; however, little investigation has been done into the combination of cell-formed microvasculature with parenchymal cells. In our work, we used a co-culture approach to grow microvascular networks in a biomimetic poly(ethylene glycol) (PEG) hydrogel, in the presence of functional hepatocytes. Through the simultaneous encapsulation of three cell types – endothelial cells, pericyte precursors, and hepatocytes – in our biomimetic PEG system, we successfully engineered vascularized hepatic tissue. These vascularized tissues exhibited two distinct benefits when compared to non-vascularized controls. First, incorporation of the vasculogenic cells led to significant improvements in hallmark hepatocyte functions. Hepatocytes encapsulated alongside the vasculogenic cells demonstrated improved albumin synthesis and cytochrome P450 enzyme activity. These improvements result from physical and chemical cues from non-parenchymal cells, which regulate hepatocyte function in vivo and in vitro. Second, the cell-formed microvasculature led to improved mass transport within the hydrogel. In a microfluidic culture system designed to investigate the functionality of the cell-formed microvasculature, we demonstrated that the cell-formed networks are capable of
anastomosis with prefabricated channels within the device. Further, transport through these networks significantly increased the distance from a media channel over which hepatocyte viability was supported. Our results suggest that a combination of prefabricated conduits and cell-formed microvasculature may be influential in the scaling up of engineered tissues.
ACKNOWLEDGMENTS

Thank you to Dr. Jennifer West for her guidance and mentorship. I truly appreciate her effort and consideration in allowing myself and several colleagues to remain at Rice to complete our studies when the lab moved last year. Thank you to my committee members, Dr. Jane Grande-Allen and Dr. Daniel Harrington, for their support and advice throughout this process. And thank you to all of the West Lab members that I’ve worked alongside, especially Michael Cuchiara, who mentored me in the lab.

Thank you to Mykel Green, an excellent undergraduate researcher that I had the pleasure to mentor for two summers. Thank you to Xin Qu, a visiting post-doc who briefly assisted with some of the work presented in this thesis. And thank you to Maureen Aliru, Mallory Pierpoint, and Kylie Witherel. The opportunity to mentor these undergraduate students in senior design both led to a chapter of my thesis and sparked my interest in a career in teaching. My career path would be different if not for my experiences with undergraduates at Rice.

Thank you to all of the friends I’ve met in Houston. You all made my time here more enjoyable and memorable than I could have imagined. Thank you to Rice for providing such a wonderful community to work in. Thank you to my family for supporting me as I moved across the country. And finally, thank you to my wife, Halle, who has supported me throughout my tenure at Rice and in everything that I do.
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CHAPTER 1 – BACKGROUND AND PROJECT INTRODUCTION

1.1 Vascularization of Engineered Tissues

1.1.1 Motivation

One of the biggest challenges facing tissue engineers is the vascularization of engineered tissues and organs. Diffusion of oxygen and other essential nutrients can support cellular survival and function only over short distances, on the order of 150 μm\(^1,2\). Consequently, tissue engineering successes to date have been limited to thin or avascular tissues, such as skin, bladder\(^3\), trachea\(^4\), and cartilage\(^5\). In order to successfully engineer larger tissues that can survive after implantation, a vascular network or similar feature must be created in order to deliver oxygen and nutrients to functional cells throughout an entire construct. This is particularly important for the engineering of complex, highly vascularized organs, such as liver and lung.

Attempts to achieve vascularization of tissue engineered constructs have traditionally fallen into two major categories: \textit{in vivo} vascularization, where the materials are designed to induce ingrowth of host vasculature upon implantation, and prevascularization, where vessels or channels are created in the implant prior to implantation. \textit{In vivo} vascularization is typically not an option for engineered replacements for larger tissues (e.g. liver), as the sprouting and spreading of new vessels (angiogenesis) occurs at a rate of less than 1 mm/day\(^6\). Prevascularization techniques are of greater interest here, as the work presented herein involved the \textit{in vitro} engineering of tissues with cell-formed microvascular networks. The tissue engineering of the microvascular network, like most other tissues, follows the traditional paradigm consisting of cells, a biomaterial scaffold, and bioactive factors. Through the use of this paradigm, researchers have made great progress in the engineering of tubule networks that
resemble native capillary beds. Figure 1-1 summarizes the different approaches that have been taken to achieve vascularization of tissue engineered constructs.

Figure 1-1: Vascularization approaches in tissue engineering. Approaches for vascularizing engineered tissues include: (a) growth factor delivery, (b) vascular progenitor cell implantation, (c) implantation of degradable biomaterials, such as bioactive PEG hydrogels, (d) implantation of biomaterials with prefabricated channels, and (e) combination approaches, such as bioactive scaffolds incorporating growth factors and cellular elements. Reproduced from Phelps and Garcia.7

1.1.2 Microvascular Biology and Development

Capillaries are the smallest blood vessels found in the body, measuring 5-10 μm in diameter, and consist of three main components: endothelial cells, pericytes, and basement membrane. These microvessels form beds that connect arterioles to venules, bridging the gap between these larger vessels and facilitating gas, nutrient, and waste exchange between blood and cells. Capillary lumens are formed by monolayers of endothelial cells, with diameters generally consisting of 2-3 cells. Pericytes are analogous to the smooth muscle cells (SMCs) found in larger vessels, as they are responsible for mural cell functions. Pericytes line capillaries
and control vessel diameter, help control blood flow, and regulate permeability. The basement membrane of capillaries is rich in laminin and collagen and is generally found between the endothelium and a single layer of supportive pericytes.

The formation of new capillaries occurs through two major processes: vasculogenesis and angiogenesis. Vasculogenesis refers to the de novo formation of capillaries, generally beginning with endothelial progenitor cells (EPCs). The vasculogenesis process is principally responsible for capillary formation during development. Angiogenesis refers to the sprouting of new vessels from existing capillaries. Angiogenesis is particularly important to the branching of new capillary networks during healing processes. Pericytes play a major role in both vasculogenesis and angiogenesis, as they are recruited by endothelial cells to support newly formed microvessels. Figure 1-2 summarizes the vasculogenesis and angiogenesis processes and clearly depicts endothelial cell and pericyte organization in capillaries.

Figure 1-2: Formation of new capillaries in vivo. New capillaries can be formed (A) by endothelial progenitor cells through vasculogenesis, or (B) by endothelial cells forming new vessel sprouts from an existing capillary, as in angiogenesis. (C) In both cases, pericytes are recruited to stabilize the newly formed capillaries. Reproduced from Moon et al.
1.1.3 Growth Factors Involved in Vasculogenesis and Angiogenesis

The phenomena involved in vasculogenesis and angiogenesis, including progenitor cell recruitment, vascular sprouting, and pericyte recruitment are driven by various environmental cues. Most notably, several important growth factors have been implicated in the vasculogenesis and angiogenesis processes. In vasculogenesis, basic fibroblast growth factor (bFGF)\textsuperscript{11} and vascular endothelial growth factor A (VEGF-A)\textsuperscript{12} have been implicated in the recruitment and differentiation of endothelial progenitor cells, leading to the formation of \textit{de novo} tube-like networks known as capillary plexuses. In addition to promoting progenitor recruitment and differentiation, these growth factor gradients also play a major role in establishing capillary network characteristics, including density, branching, and vessel diameter.

In angiogenesis, VEGF is widely considered to be the most important growth factor, as gradients in VEGF concentration drive endothelial cell migration, proliferation, and differentiation\textsuperscript{13}. VEGF expression is heavily mediated by hypoxia, through hypoxia-inducible factors (HIF-1 and HIF-2)\textsuperscript{14}. VEGF levels are typically elevated in tumors, leading to rapid formation of new vessels; however, these rapidly formed vessels are generally leaky in nature\textsuperscript{15}. As in vasculogenesis, bFGF also plays a major role in angiogenesis. Specifically, bFGF gradients drive endothelial cell proliferation, migration, and expression of important angiogenic enzymes (matrix metalloproteinases) and integrins\textsuperscript{16}. Like VEGF, bFGF levels are mediated by hypoxia, as macrophages release bFGF in response to hypoxia\textsuperscript{17}.

While VEGF and bFGF signaling drive the early stages of vasculogenesis and angiogenesis, including primitive capillary plexus formation and branch sprouting, platelet-derived growth factor (PDGF) signaling drives the later stages of these processes. Specifically, PDGF-BB has been implicated in the recruitment of pericytes\textsuperscript{18}, the formation of anastomoses
between new and existing vessels\textsuperscript{19}, and increased production of ECM and basement membrane by pericytes\textsuperscript{20}. Together, these phenomena serve to stabilize newly formed vessels, demonstrating the importance of PDGF in the later stages of vasculogenesis and angiogenesis. The well-defined roles of these various growth factors in capillary formation enable engineers to use combinations of these factors to drive the formation of microvascular networks in engineered tissue.

1.1.4 Biomaterial Design for \textit{in Vivo} Vascularization

The most common technique used to vascularize tissue engineered constructs is \textit{in vivo} vascularization. When a cellular scaffold is implanted, hypoxia induced by the implanted cells typically induces spontaneous vascularization; however, this process is usually too slow, and most cells die before new vessels reach interior parts of the scaffold\textsuperscript{7}. Tissue engineering researchers have developed countless techniques that speed up the neovascularization process, with varying levels of success. These techniques can be broken down into two categories: scaffold design and growth factor delivery.

Scaffold architecture significantly affects the rate of vascularization after implantation. Most notably, vessel ingrowth into scaffolds with pore sizes greater than 250 $\mu$m occurs faster than in scaffolds with smaller pore sizes\textsuperscript{21}. In addition to pore size, the interconnectivity of pores is extremely important to vessel ingrowth, as new vessel formation requires abundant cell migration through scaffold pore structures\textsuperscript{22}. Consequently, fabrication techniques that allow for precise control of the porous nature of scaffolds have become popular in tissue engineering\textsuperscript{23}. Finally, the use of degradable biomaterials enhances vessel ingrowth, as cells can more easily migrate through a degrading scaffold\textsuperscript{24}.
In addition to scaffold design, pro-angiogenic growth factor delivery is often used by tissue engineers to increase scaffold vascularization rate after implantation. The formation of new blood vessels can be accelerated through scaffold-mediated delivery of growth factors, such as VEGF and bFGF. Stabilization of newly formed vessels can be induced through delivery of other growth factors, including PDGF and transforming growth factor β (TGF-β). Combined delivery of VEGF and PDGF has demonstrated positive effects on the rate of scaffold neovascularization and on the stability and maturity of the newly formed vessels. The delivery of angiogenic growth factors can be achieved in many different ways, including addition of recombinant growth factors to implantable scaffolds, growth factor gene delivery, and implantation of cells genetically modified to overproduce specific growth factors. Finally, some of the best neovascularization results were obtained when growth factor release from a scaffold was coupled to cell migration. In one such case, improved endothelial cell proliferation and progenitor cell maturation were observed when the release of VEGF from a fibrin matrix was coordinated with cellular enzyme activity.

1.1.5 Prevascularization – Microfabricated Channels

Due to the shortcomings of in vivo vascularization, there has been extensive research into potential techniques for vascularizing engineered tissues prior to implantation. The native microvasculature bridges arterial and venous blood flow, enabling transport of gases, nutrients, and waste between blood and tissue. In the human body, the average functional cell lies no farther than 30 μm from a capillary. The thin walls and high surface area of capillaries facilitate diffusive transport between blood and functional cells. These parameters are used by engineers in order to design techniques that aim to adequately vascularize engineered tissue.
One approach to prevascularization forgoes the creation of actual blood vessels. Instead, microfabrication-based techniques are used to create networks of vessel-like channels throughout a material. Techniques such as replica molding and soft lithography have been used to fabricate networks of channels that mimic native vasculature and microvasculature. Typically, a negative master is created in the shape of the desired channel network, and then a biomaterial is molded to the negative master to produce the channels in relief. Poly(dimethylsiloxane) (PDMS) is the most commonly used biomaterial in these molding-based techniques. This molded material can then be sealed to glass, an unpatterned slab of the same biomaterial, or additional patterned biomaterial layers to form patent vessel networks\textsuperscript{32}. Recently, more complex channel networks have been fabricated in PDMS using direct-write laser technology\textsuperscript{33,34}. In one such case, channel networks were fabricated at multiple depths and with changing diameters, in order to best mimic native capillary networks\textsuperscript{34}.

Due to the highly organized and repetitive nature of the vasculature that supports hepatocyte function in the liver, the use of microfabrication-based techniques is appealing for hepatic tissue engineering. The Vacanti group leads this charge, as they have engineered multiple hepatic constructs with microfabricated vessels that mimic the native microvasculature. In one such construct, standard soft lithography was used to pattern PDMS into branching vessel structures. The vessel structures were designed to mimic the physical flow characteristics that are observed in native hepatic tissue\textsuperscript{35}. Another hepatic construct fabricated by this group involved more complex fabrication techniques, as separate PDMS channels and culture chambers were divided with a nanoporous membrane that mimics the fenestrated endothelium of liver sinusoids\textsuperscript{36}. This device was connected to live rat circulation and demonstrated capacity as a liver-assist device\textsuperscript{37}. Although the use of PDMS enabled precise patterning of the hepatic
microdevices, PDMS does not support long-term implantation. Figure 1-3 summarizes the Vacanti group’s attempts to fabricate biomimetic microvasculature into hepatic tissue.

**Figure 1-3: Engineering of biomimetic vasculature in hepatic microdevices.** (A) Branching channels were fabricated for a hepatic microdevice, with 6 outer inlets draining into one central outlet. (B) Pressure and shear stress levels in the fabricated channels mimic native vasculature. (C) A hepatic microdevice was designed that mimics the separation of hepatocyte cords by highly fenestrated sinusoids. (D) This microdevice was connected to rat circulation, demonstrating hepatocyte function and viability over 1 week. (E) A cross-section of the hepatic microdevice depicts the separation of the hepatocyte culture chamber (H) from the fabricated vascular network (V) by a nanoporous membrane (M) that functionally mimics fenestrated liver sinusoids. All devices were fabricated using PDMS and soft lithographic techniques. Recreated from Hogansan et al.\textsuperscript{35}, Carraro et al.\textsuperscript{36}, and Hsu et al.\textsuperscript{37}.

The possibilities associated with microfabricated vascular networks are tremendous; however, for a number of reasons, most current efforts in this field are limited to *in vitro* culture studies, *ex vivo* organ assist devices, and other benchtop type applications. The formation of
complex networks that mimic native microvasculature generally requires complex fabrication techniques, which often cannot be applied to the most biocompatible scaffolds. Materials that are easily microfabricated and pattern often lack the mass transport properties to support diffusion in and out of microfabricated channels. For example, it is relatively easy to pattern PDMS scaffolds, in order to incorporate vessel networks; however, PDMS is not suitable for implantation or supporting long term cell survival, due to insufficient mass transport, nondegradability, and some cytotoxicity. Recent work has begun to address these issues, as the molding of degradable, more biocompatible materials has been explored.

Poly(glycerol sebacate) (PGS), a biodegradable elastomer, has been successfully molded to form microvascular-like channels, using a similar process as described for PDMS. These channel networks have been endothelialized using human umbilical vein endothelial cells (HUVECs) and stacked to form 3D channel networks that might support prolonged cell survival throughout a slab material. These studies using PGS demonstrated microvascular-like network formation in biodegradable matrices; however, the bulk of the scaffolds were not seeded with cells. The fabrication of vascular networks in cell-seeded materials, including collagen, fibrin, and agarose, has also been demonstrated. In one such instance, gelatin was micromolded in the shape of a desired vascular network, then a cellular hydrogel (collagen or fibrin) was formed around the gelatin network, and finally the gelatin was melted and flushed away, leaving the vessel structures in relief within the cellular hydrogel. The authors of this work demonstrated the formation of hydrogels with encapsulated fibroblasts and endothelial cell-lined channel networks. In another instance of forming channel networks in a cell compatible material, a cellular agarose hydrogel was molded to a negative master and sealed to an agarose hydrogel slab, in order to form 2D channel networks in a cellular material. In both of these
studies involving cellular materials, flow parameters and support of cell survival were evaluated. Although the results of these studies are promising, little has been done in terms of implanting these cellular, vascularized constructs. Figure 1-4 summarizes these studies, in which microvascular-like channels were formed in cell compatible materials.

**Figure 1-4: Microfabricated channels in cell compatible materials.** (A) A channel network that mimics a native capillary bed was fabricated in PGS. (B) The PGS capillary network was successfully endothelialized with HUVECs. (C) Channel networks were fabricated in cellular collagen hydrogels, using micromolded gelatin as a sacrificial element. (D) These collagen hydrogels were stacked to form tissues with channel networks designed to support a 3D tissue construct. (E) Live (green) and dead (red) cells, as a function of distance from a perfused microfabricated channel in an agarose hydrogel, at 0 and 72 hours. Medium flow supports cell survival within approximately 150 μm of the fabricated channel. Recreated from Fidkowski et al.\textsuperscript{39}, Golden and Tien\textsuperscript{42}, and Ling et al.\textsuperscript{43}.

A recent study from Miller et al. has introduced a new method for forming channels within scaffolds to serve as functional vasculature. A 3D printing apparatus was used to fabricate
filament networks that consisted of a carbohydrate glass. Cells were then encapsulated around these filament networks in various polymer systems (PEG, fibrin, Matrigel, alginate, and agarose). Upon submersion in culture medium, the carbohydrate glass networks dissolved leaving hollow tubes. The authors demonstrated successful perfusion and endothelialization of these tubes, and they were able to perfuse constructs containing primary hepatocytes and maintain hepatocyte viability near the perfused tubes. Figure 1-5 summarizes the major findings of this study. In a subsequent study by Baranski et al. by many of the same authors, endothelial cell cords were formed in molds and were then encapsulated in larger hydrogels containing hepatocytes. These constructs with aligned endothelial cords were implanted into mice and improved hepatocyte function and viability were observed in comparison to constructs with random endothelial networks.44

1.1.6 Prevascularization – Cell-formed Microvascular Networks

Recently, the use of cells as tools to prevascularize tissue engineered constructs has been explored. Endothelial cells, often in combination with pericytes or other vascular support cells, have been shown to generate microvascular networks in both 2D and 3D culture. Many cellular co-cultures have demonstrated the ability to form microvascular networks when seeded on or encapsulated in bioactive scaffolds. Accordingly, tissue engineers have attempted to use encapsulated cells and an in vitro culturing period as a prevascularization strategy for biomaterial scaffolds. Many of these studies have generated promising results, as robust microvascular networks grown in vitro have demonstrated the capacity to improve in vivo outcomes of implanted biomaterials.
Figure 1-5: Perfusion and endothelialization of fabricated channels. (A) A 3D printer was used to create a carbohydrate lattice. Cells were encapsulated around the lattice in various polymers and the lattice was dissolved to create a perfusable vascular architecture. (B) Inlet ports were printed to allow the printed vascular networks to be perfused with blood. (C) Endothelialization was achieved by injecting HUVECs into the vascular architecture. (D) Primary hepatocyte survival was supported near the perfused, endothelialized tubes. Recreated from Miller et al.\textsuperscript{45}.

Tubulogenic cultures have been used in attempts to prevascularize biomaterials for many tissue replacement applications, including bone, skin, and cardiac muscle\textsuperscript{46}. In a study by Rouwkema et al., co-cultures of human mesenchymal stem cells (hMSCs) and HUVECs were grown using a scaffoldless spheroid approach for 10 days \textit{in vitro}, then implanted subcutaneously in mice, in an attempt to produce vascularized bone. HUVEC inclusion in the hMSC spheroids led to formation of 3D microvascular networks and a 4-fold increase in alkaline phosphatase (an osteogenic marker) expression by hMSCs. Unfortunately, significant anastomosis was not observed between host and implanted vasculature\textsuperscript{47}. A study by Tremblay et
*al.* involved skin tissue engineering through the encapsulation of human keratinocytes, human dermal fibroblasts, and HUVECs in a collagen sponge. After 7 days in culture, the skin substitutes were implanted as skin grafts on mice. Graft vascularization was observed 4 days after implantation, and perfusion of implant-derived vessels was also evident\(^{48}\). Finally, a study by Kelm *et al.* involved the gravity-enforced self-assembly of micro-tissues with encapsulated cardiomyocytes. These micro-tissues were then coated in HUVECs, cultured for 7-14 days, assembled into an agarose scaffold, and implanted into rat pericardia. Perfused vessels were observed in the implant 60 hours after implantation; however, the vessels could not be identified as host- or implant-derived\(^{49}\).

Figure 1-6 depicts some of the promising results observed in these cellular prevascularization studies. Although these studies all used HUVECs as the endothelial source for prevascularization, similar studies have used other cell types, including endothelial progenitor cells (EPCs)\(^{50}\), human dermal microvascular endothelial cells (HDMECs)\(^{51}\), and human embryonic stem cells (HESCs)\(^{52}\).

**Figure 1-6: Prevascularized scaffolds for implantation.** (A) HUVECs formed 3D microvascular networks in hMSC micro-tissues\(^{47}\). (B) Capillary like structures formed 60 hours after implantation of skin substitutes prevascularized using HUVECs\(^{48}\). (C) Micro-tissues (green) encapsulating cardiomyocytes and prevascularized with HUVECs seamlessly integrated into the rat myocardium (red)\(^{49}\). Recreated from Rouwkema *et al.*\(^{47}\), Tremblay *et al.*\(^{48}\), and Kelm *et al.*\(^{49}\).
The use of a co-culture consisting of HUVECs and 10T½ cells (murine pericyte precursors) has been studied extensively. The tubulogenic nature of this co-culture was first described by Koike et al., who noted that the HUVEC-10T½ co-culture forms more robust and longer lasting microvessels than most other tubulogenic cultures. This group also demonstrated that while HUVECs alone form ephemeral tubule networks, the inclusion of 10½ cells stabilizes the vessel structures and tremendously prolongs regression. Figure 1-7 depicts the major findings of this initial study of the HUVEC-10T½ co-culture.

**Figure 1-7: Tubulogenic nature of the HUVEC-10T½ co-culture.** Fluorescent micrographs of (a) tubule networks formed by E-GFP expressing HUVECs (green) and (b) tubule networks formed by E-GFP expressing HUVECs (green) perfused *in vivo* with rhodamine dextran (red). (c) Vessel density is significantly greater over time for co-culture (blue line) than for HUVECs alone (fuchsia line). (d) Fluorescent micrograph of tubule networks formed by HUVECs (unlabeled) and E-GFP expressing 10T½ cells (green) perfused *in vivo* with rhodamine dextran (red) after 4 weeks in culture. Reproduced from Koike et al.

The C3H 10T½ cell line has been used frequently in cellular research, particularly in the investigation of various differentiation processes. First derived from murine embryonic tissue in
1972, 10T½ cells are multipotent mesenchymal cells that resemble fibroblasts. These progenitor cells demonstrate resistance to spontaneous differentiation and high susceptibility to chemically induced differentiation. In response to treatment with 5′-azacytidine, 10T½ cells have undergone both myogenic and adipogenic differentiation in otherwise standard culture medium. Under more specialized culture medium conditions, these cells have differentiated into osteogenic, chondrogenic, and myofibroblast cell types. For the work summarized herein, differentiation of 10T½ cells into mural cells (i.e. smooth muscle cells or pericytes) is of the greatest interest.

The co-culture of HUVECs and 10T½ cells has been shown to produce stable vessel-like structures, both in vivo and in vitro, with differentiated 10T½ cells providing pericyte functions. The transformation of 10T½ cells into pericytes and recruitment of the cells to vessel structures are driven by this co-culture scenario, in a process that is mediated by PDGF, transforming growth factor β (TGF-β), and the heterotypic cell-cell interactions with HUVECs. Development of smooth muscle cell morphology and expression of pericyte markers (e.g. α-smooth muscle actin, calponin, etc.) by 10T½ cells demonstrate this mural differentiation. Other growth factors, most notably bFGF, have been shown to inhibit the TGF-β mediated mural differentiation of 10T½ cells. Ultimately, through the use of appropriate culture medium and co-culture with endothelial cells, 10T½ cells provide an excellent source of pericytes for in vitro vascularization.

This early work was important in demonstrating the vasculogenic nature of the HUVEC-10T½ co-culture; however, the tubulogenesis in these studies was observed in collagen hydrogels implanted in vivo. Consequently, the cues responsible for driving HUVEC migration and organization, 10T½ cell differentiation into pericytes, and other phenomena involved in
vasculogenesis were unclear. A bioactive PEG hydrogel system has been developed that supports HUVEC-10T½ co-culture vasculogenesis\textsuperscript{60}. Since PEG itself resists protein adsorption and does not directly interact with cells, only the biological modifications engineered into a PEG scaffold are capable of driving cellular behaviors. Accordingly, the hydrogel developed to support co-culture vasculogenesis was proteolytically degradable, due to insertion of an MMP-sensitive peptide into the PEG backbone, and functionalized with PEG-tethered adhesive peptides (PEG-RGDS). When encapsulated in this bioactive hydrogel and cultured in medium supplemented with vasculogenic growth factors, the HUVEC-10T½ co-culture readily formed pre-vascular networks, degrading the PEG matrix and depositing laminin and type IV collagen in the process. These cell-formed tubule networks remained stable in culture for up to 30 days\textsuperscript{60}. Figure 1-8 depicts the process used to generate microvascular networks in biomimetic PEG hydrogels.

![Figure 1-8: Tubulogenic co-culture in a cell adhesive and proteolytically degradable PEG hydrogel.](image)

Since the development of the bioactive PEG hydrogel that supports HUVEC-10T½ co-culture vasculogenesis, significantly more work has been done involving the encapsulation of this co-culture in bioactive PEG hydrogels. Much of this work has focused on the effects of
various pro-angiogenic and pro-vasculogenic growth factors on the in vitro and in vivo behavior of the co-culture, when implanted in proteolytically degradable PEG hydrogels. First, the effects of PDGF-BB on tubule formation were evaluated. It was determined that covalently immobilizing PDGF-BB in the previously described bioactive PEG hydrogels led to increased tubule formation on hydrogel surfaces, but no increase in tubule formation was observed when the co-culture was encapsulated in these hydrogels. Next, the pro-angiogenic nature of these PDGF-BB modified hydrogels was determined using a modified corneal micropocket assay for angiogenesis. In this assay, proteolytically degradable hydrogels containing soluble and/or covalently immobilized growth factors are implanted into a micropocket created within the cornea of Flk1-myrc::mCherry transgenic mice, which express a red fluorescent protein that is specific to endothelial cells. Since the cornea is otherwise avascular, vessel ingrowth into the cornea and implanted hydrogel occur only due to the angiogenic nature of the implanted gel. Covalently immobilizing PDGF-BB in the bioactive hydrogels proved to induce a greater angiogenic response when paired with soluble PDGF-BB delivery, as compared to soluble PDGF-BB alone. In later studies, EphrinA1, another pro-angiogenic growth factor, was covalently immobilized in bioactive PEG hydrogels and also proved to induce a greater vessel growth into the cornea when paired with soluble PDGF-BB delivery, as compared to soluble PDGF-BB alone. Finally, anastomosis between cell-formed tubules and native mouse vessels was observed in a process that involved the implantation of a pro-angiogenic acellular hydrogel to initiate vessel formation, excising that hydrogel after 2 weeks in the cornea, and then implanting a pro-angiogenic hydrogel with encapsulated bEnd.3 endothelial cells and 10T½ cells. This study proved that the microvascular networks formed by vasculogenic co-cultures are capable of anastomosis with host vasculature. Figure 1-9 depicts a summary of the results of
these studies, which involved covalently modifying proteolytically degradable PEG hydrogels with pro-angiogenic and pro-vasculogenic growth factors.

**Figure 1-9: Growth factor immobilization in proteolytically degradable PEG hydrogels.** PDGF-BB was conjugated to a monoacrylated PEG spacer (A) and covalently immobilized in proteolytically degradable PEG hydrogels. Fluorescent micrographs of bioactive PEG hydrogels with encapsulated HUVECs and 10T½ cells, immunostained for PECAM (green) and smooth muscle α-actin (red), demonstrate no significant difference in tubule formation between gels without (B) and with (C) immobilized PDGF-BB. Pro-angiogenic hydrogels were implanted in the corneal micropockets (D), and significantly less vessel ingrowth was observed in hydrogels without immobilized PDGF-BB (E), as compared to hydrogels with immobilized PDGF-BB (F). A 3D rendering depicts anastomosis (red arrow) between a cell-formed tubule (green) and a native mouse vessel (red) (G), with red blood cells present in the anastomosed network (yellow arrow). Recreated from Saik et al.\(^\text{64}\), Poché et al.\(^\text{65}\), and Saik.\(^\text{67}\).

The HUVEC-10T½ co-culture has also been encapsulated in proteolytically degradable PEG hydrogels, in a dual-channel microfluidic culture device. The major goals of this work revolved around improved mass transport and vascularization in engineered tissue and the combination of microfabricated channels with cell-formed microvascular networks. Early work in a single-channel flow device confirmed that vessels must be spaced every 100-200 μm in an
engineered tissue in order to maintain cell survival and function.\textsuperscript{68} This was later confirmed in a dual-channel PDMS flow device, where medium and buffer were flowed in a countercurrent fashion along opposing sides of a bioactive PEG hydrogel slab, with encapsulated HUVEC-10T½ co-culture. Robust tubule networks were observed near the flow of medium; however, cell survival and organization were low farther from the nutrient source. Further, improved mass transport properties were observed in hydrogels with cell-formed tubule structures, as compared to acellular hydrogels. Finally, perfusion of gels with fluorescent dextran demonstrated mass transport through cell-formed vessels, suggesting that connections were established between microfabricated and cell-formed channels.\textsuperscript{63} Figure 1-10 summarizes the major findings of these studies, in which cell-formed tubule networks were combined with microfabricated channels.

1.1.7 Shortcomings of Previous Cellular Attempts at Prevascularization

Although the formation of robust microvascular networks has been demonstrated repeatedly, by different groups and using different combinations of cells, there exists a major gap in the literature concerning the use of these networks to support parenchymal cells. The work combining tubulogenic cultures with parenchymal cells has been mostly limited to skin\textsuperscript{69} and bone\textsuperscript{47} tissue engineering, where the importance of vascularization is less than clear. In studies that have combined tubulogenic cells with parenchymal cells that require significant interaction with vasculature (e.g. hepatocytes), vasculogenesis has not been observed in the presence of parenchymal tissue. These studies have generally involved the coating of parenchymal micro-tissues with endothelial cells, then combining micro-tissues into a larger tissue.\textsuperscript{70} Due to these shortcomings, our work has focused on growing vascular networks through parenchymal tissue, thereby demonstrating the potential of cell-formed microvasculature to improve the function of
engineered tissues. The ability to grow these cell-formed networks through parenchymal tissue demonstrates the capability of these vascularization techniques to support cell survival in tissue engineered implants, on biologically relevant size scales. The work presented involves the engineering of vascularized hepatic tissue and examining the relationship between hepatocytes and vascular cells. An overview of the field of hepatic tissue engineering is presented in the following section.

Figure 1-10: Combining cell-formed and microfabricated channels to improve mass transport in engineered tissues. (A) Soft lithography was used to create a PDMS culture chamber. A bioactive PEG hydrogel with encapsulated HUVEC-10T½ co-culture was photopolymerized in the chamber, creating two channels on opposing sides of the hydrogel slab. Medium and buffer were flowed in a countercurrent fashion through these channels. After 4 days of culture, the hydrogel was immunostained for PECAM (green), smooth muscle α-actin (red), and DAPI (blue). Near the medium channel, the cells formed robust tubule networks (B), while in the middle of the gel, cell migration and organization was nonexistent (C). In other studies, high molecular weight fluorescent dextran was used to perfuse hydrogels after 4 days in culture. Mass transport profiles of acellular hydrogels (D) and gels that had been remodeled by cells for 4 days (E) demonstrate the improved mass transport in gels with cell-formed tubule networks. Co-localization of fluorescent dextran (red) with tubule structures (green) (F) indicates that mass transport is occurring through tubule structures in remodeled gels. Recreated from Cuchiara et al. 33,71.
1.2 Hepatic Tissue Engineering

1.2.1 Motivation

The liver is the largest internal organ in the human body and is responsible for a vast number of important functions, including blood glucose maintenance, regulation of proteins, fats, and vitamins, digestive functions, and biotransformation of toxic substances. Because the liver plays an important role in so many biological functions, the consequences of liver disease are often widespread and very serious. Liver transplantation has proven to be an effective option for many suffering with liver disease; however, issues such as availability and immunogenicity often preclude liver transplantation. A liver transplantation involves the complete replacement of a patient’s liver with part of a liver from a living donor or all or part of a liver from a recently deceased donor. These procedures are performed due to a number of diseases, including hepatitis B and C, cancer, cirrhosis, and various genetic and autoimmune diseases. About 75% of patients live more than five years after liver transplantation; however, rejection and infection are major concerns for these procedures. Approximately 6,000 liver transplants are performed each year in the United States, and there are currently around 16,000 Americans on the waiting list for a liver transplant. Consequently, the need for tissue engineered liver is tremendous, as engineered hepatic tissue may be an excellent option when liver transplantation has failed or is not an option.

1.2.2 Liver Biology

Despite the tremendous size of the liver, it is made up of around one million smaller functional units called lobules. These structures are repeated throughout the liver and are responsible for the organ’s many functions. Blood supply to the lobules is achieved through
elaborate branching of the two major vessels that provide the liver with oxygenated blood, the hepatic portal vein and the hepatic (portal) artery. The liver also contains a ductal system for the transport of bile to and from the gall bladder and intestines. A schematic of the liver is depicted in Figure 1-11. By combining functional liver cells with other cells found in the liver, and recapitulating the lobular organization seen in vivo, researchers aim to engineer functional liver tissue that is suitable for human implantation. The following sections outline the physiology and tissue architecture of native liver, which guide efforts by tissue engineers to create functional liver tissues in the lab.

**Figure 1-11: Structure of the liver.** Blood is delivered to the liver through branching of the hepatic artery and hepatic portal vein. Blood returns to the heart from the liver via the hepatic vein. The liver also contains a duct system that transports bile to and from the gall bladder and intestines.

### 1.2.2.1 Cell Types

The predominant cell type that makes up liver tissue is the hepatocyte. Hepatocytes are the chief functional cell of the liver, with millions of hepatocytes making up each lobule and accounting for more than 70% of the cytoplasmic mass of the liver. Hepatocytes play major roles in protein synthesis and storage, detoxification, lipid metabolism and synthesis, and
carbohydrate metabolism. Other cell types found in the liver predominantly make up the non-parenchymal structures, including blood vessels, lymphatic vessels, and bile ducts and canaliculi. Like other blood vessels in the body, the arterial, venous, and sinusoidal structures in the liver consist of endothelial and mural cells (i.e. vascular smooth muscle cells and pericytes). Ito cells (hepatic stellate cells) are specialized pericytes that are found in the space of Disse, between sinusoids and hepatocytes. Ito cells play a major role in the fibrotic events associated with many liver diseases, such as cirrhosis\textsuperscript{77}. Finally, Kupffer cells are stellate macrophages that line sinusoids, destroying ingested bacteria that have entered the blood\textsuperscript{78}. Due to the scope of the research presented in this document, particular interest is placed on the relationship between hepatocytes and vascular cells.

1.2.2.2 Tissue Organization and Hepatic Microenvironment

The liver predominantly consists of repeating structural units called lobules, which are separated from each other by thin layers of connective tissue. Lobules are often roughly hexagonal in shape, with vascular supply and bile ducts located at the vertices and a central vein at the center. The vessels found at the vertices of lobules include the portal triad: a branch of the hepatic portal vein, a branch of the hepatic artery, and a bile duct, in addition to lymphatic vessels. The lobule consists mostly of hepatocytes, which are arranged into cords and separated by heavily fenestrated sinusoids. These sinusoids carry blood from the periphery of the lobules to the central vein and contain little or no basement membrane or other connective tissue, allowing for extensive interaction between the blood and surrounding hepatocytes\textsuperscript{79}. Figure 1-12 depicts the structure of the hepatic functional unit, focusing on the organization of hepatocytes and the
vasculature. The intricate and repetitive organization of hepatic tissue indicates that the liver has evolved in order to best carry out its diverse biological functions.

Figure 1-12: Schematic of liver functional unit. Part of a hepatic lobule is pictured, demonstrating the flow of blood in the hepatic functional unit. Blood enters from branches of the portal vein and hepatic artery and then flows through sinusoids (highly fenestrated capillary-like structures) that separate cords of hepatocytes. Hepatocytes are able to detoxify substances from the blood and secrete substances back into the blood. Sinusoids drain into the central vein, at the center of the lobule. Reproduced from Lee et al.80.

The lobule was the first structure to be identified as the functional unit of the liver; however, more recent definitions have emerged that redraw the boundaries of the hepatic functional unit. Criticisms of the traditional lobule definition claim that there is no basis for using the central vein, the drainage vessel, as the center of the functional unit. Consequently, two other definitions of the liver functional unit exist: the portal lobule and the acinus. The older of these two definitions, the portal lobule, defines the functional unit as a triangle, with vertices at three central vein branches, centered at a single portal area. The portal lobule definition places importance on afferent blood supply, via branches of the hepatic portal vein and hepatic artery, and bile drainage, via bile ducts, by placing the relevant structures at the center of the functional
The most recent definition of the hepatic functional unit, the acinus, relates structural units to the terminal branches of hepatic triad vessels, rather than to the triad vessels themselves. The acinus is generally defined as a diamond, with one pair of vertices at two central veins and the other pair of vertices near two portal areas. The acinus definition places importance on the metabolic and secretory functions of liver tissue. The lobular structure of hepatic tissue and the different definitions of the functional unit are depicted in Figure 1-13. Histological images of human liver are displayed in Figures 2-14 and 2-15. Figure 1-14 includes histological images of the different functional unit definitions, while Figure 1-15 includes histological images of important vascular features of the hepatic microenvironment.

Figure 1-13: Lobule structure and definitions. Hepatic tissue is divided into roughly hexagonal slabs of tissue called lobules. The “classical” lobule definition (bold outline) defines the functional unit of the liver as one of these hexagonal tissue slabs, the tissue drained by a single central vein. The portal lobule definition (dotted triangle) defines the functional unit of the liver as the tissue supplied with blood and drained of bile via a single periportal space (PS), which contains branches of the hepatic portal vein and hepatic artery, in addition to a bile duct. The hepatic acinus definition (solid diamond) defines the functional unit of the liver as tissue supplied with blood and drained of bile via the smallest branches of the hepatic triad vessels. The acinus is often separated into three zones (I, II, and III) based on the order in which hepatocytes receive afferent blood. Hepatocytes in zone I are close to arterioles, thus they contact highly oxygenated and unfiltered blood. Hepatocytes in zone III receive much less oxygen and are responsible for functions that require little to no oxygen, including drug detoxification via cytochrome P450 enzymes. Reproduced from the University of Virginia School of Medicine.
**Figure 1-14: Lobule histology.** Hematoxylin and eosin (H&E) staining of a human liver section demonstrates the structure of the three lobule classifications: (A) the classic lobule, (B) the portal lobule, and (C) the acinus. Recreated from The University of Oklahoma Health Sciences Center. 

**Figure 1-15: Histology of hepatic vasculature.** H&E staining of a human liver section demonstrates the structure of important vascular components found in the hepatic functional unit, including (A) the portal triad (branch of hepatic portal vein, branch of hepatic artery, bile duct), (B) the sinusoids, and (C) the central vein. Recreated from The University of Oklahoma Health Sciences Center.

Hepatocyte function is modulated by the various cues present in the cellular microenvironment. The hepatic microenvironment consists of various cell types and matrix components, which are arranged in a way that is repeated throughout the liver. In general, hepatic tissue is highly cellular and contains less ECM than many other parenchymal tissues. The ECM present in hepatic tissue, which is important in bridging the space between the highly fenestrated sinusoids and cords of hepatocytes, is similar to basement membrane in structure and composition. Consequently, type IV collagen, laminin, and heparan sulfate proteoglycans (*e.g.* perlecan) are prevalent in the hepatic ECM. Type I collagen, type III collagen, and fibronectin are also present in the matrix surrounding hepatocytes, but in lower quantities. Hepatocytes are responsible for some heparan sulfate proteoglycan synthesis; however, Ito cells are responsible
for the majority of collagen and laminin deposition. In the hepatic microenvironment, a variety of cell-matrix and cell-cell interactions influence growth and behavior of hepatocytes.

Hepatocyte interactions with the ECM primarily take place through integrin interactions with fibronectin, laminin, and collagen. Several in vitro studies have demonstrated that hepatocyte-matrix interactions modulate cellular growth, morphology, and gene expression. In one such study, integrin binding events were shown to activate the G0 to G1 transition of the cell cycle in hepatocytes, and cell spreading was implicated in the G1 to S phase transition. Consequently, hepatocyte growth in vivo is highly dependent on cell-matrix interactions. Other studies have demonstrated that hepatocytes non-preferentially adhere and spread on various ECM components (fibronectin, laminin, type I collagen, type IV collagen) in vitro, but that basement membrane components (e.g. laminin and type IV collagen) promote specific hepatocyte functions (e.g. enzyme activity) while diminishing DNA synthesis and other growth processes. The basement membrane-like hepatic ECM also plays a major role in the presentation of various growth factors to hepatocytes. Type IV collagen has been shown to bind TGF-β1, enhancing and prolonging its activity. Heparan sulfate proteoglycans bind bFGF, masking its activity and ultimately releasing the active growth factor upon matrix remodeling. And finally, laminin contains 25 epidermal growth factor (EGF)-like repeats, which demonstrate tremendous mitogenic properties upon cleavage from laminin. To summarize, the complex interactions between hepatocytes and their basement membrane-like ECM are intimately involved in the cellular growth, differentiation, and overall function.

Cell-cell interactions also play a major role in governing cellular growth, migration, and other behaviors in the hepatic microenvironment. Two types of cell-cell interactions are particularly important in hepatic tissue: (1) homotypic cell-cell interactions amongst hepatocytes
and (2) heterotypic cell-cell interactions between hepatocytes and various non-parenchymal cells. Due to the tight packing of hepatocytes in the liver, homotypic cell-cell interactions are prevalent and facilitate robust signaling between hepatocytes. In one study, decreasing hepatocyte plating density diminished cytochrome P450 activity in primary human hepatocytes, which demonstrates the importance of robust homotypic cell-cell interactions in hepatocyte function. More interesting, however, are the interactions between hepatocytes and other cell types in the liver. Hepatocytes form cell-cell interactions with various non-parenchymal cells in the liver, most commonly Ito cells, Kupffer cells, and biliary epithelial cells. These heterotypic cell-cell interactions have been implicated in enzyme localization, hepatocyte differentiation, liver disease, and fibrotic response to injury and play a major role in the coordination of total organ function. While the precise mechanisms by which these heterotypic cell-cell interactions affect hepatocyte behavior mostly remain unclear, the high dependence of hepatocytes on these interactions has spurred the use of various co-culture systems for tissue engineering, drug toxicity screening, and other research applications.

1.2.2.3 Biological Functions

More than 500 different liver functions have been identified, and the most important biological functions can be broken down into four categories: 1) blood glucose maintenance, 2) regulation of proteins, fats, and vitamins, 3) digestive functions, and 4) biotransformation. The liver’s role in blood glucose homeostasis involves (a) the storage of excess glucose as glycogen and (b) synthesizing glucose, through glycogenolysis and gluconeogenesis, in response to low glucose levels. Ammonia is produced as a toxic byproduct of gluconeogenesis, but the liver readily converts ammonia into urea, which is easily secreted in the urine. The liver’s role in
regulating proteins, fats, and vitamins involves synthesis, storage, metabolism, and other processes. Excess carbohydrates and proteins are converted into fat in the liver, which can be stored or packaged for use in other parts of the body. In addition, many important proteins are synthesized in the liver, including albumin. Packaging of nutrients, vitamins, cofactors, and enzymes also takes place primarily in the liver. The digestive functions of the liver are based on the secretion of bile, which is produced by hepatocytes and collected in biliary networks. Bile is stored in the gall bladder and secreted as a digestive enzyme into the small intestine, where its major roles are to help digest fats and to neutralize stomach acid\textsuperscript{04}.

Biotransformation is perhaps the function most commonly associated with the liver. Biotransformation refers to changes made in the chemical structure of compounds by living organisms. In general, the liver functions to transform endogenous and xenobiotic (exogenous) compounds, rendering these compounds less toxic, more water soluble, and easier to excrete. Most hepatic transformation is performed by two phases of enzymes, which have broad substrate specificity. Phase I enzymes perform preliminary modifications to chemical compounds, generally adding simple functional groups (\textit{e.g.} amine, carboxyl, hydroxyl, thiol, \textit{etc.}). Cytochrome P450 enzymes, the most notable enzymes in drug detoxification, are phase I enzymes involved in hepatic biotransformation. Phase II enzymes are not required for detoxification of all compounds; however, they add an amino acid or other moiety to phase I metabolites that lack sufficient solubility in water. Upon detoxification by these two phases of enzymes, chemical compounds are easily secreted in the bile or urine\textsuperscript{95}.
1.2.3 Hepatic Tissue Engineering Overview

Hepatic tissue engineering involves the fabrication of tissues that closely mimic the *in vivo* behavior of liver. The ultimate goals of liver tissue engineering can be separated into two categories. First, engineered tissue that closely resembles native hepatic tissue can be used to improve drug toxicity studies for pre-clinical trials. When hepatocytes are cultured in conditions that more closely resemble the *in vivo* microenvironment, the cellular response to drugs is a better indicator of actual hepatotoxicity\(^96,97\). Second, engineered hepatic tissues may serve as treatments to a number of diseases that affect the liver, including cirrhosis, cancer, hepatitis, and several genetic disorders that affect children. Success for this second goal is still likely decades away; however, current research in hepatic tissue engineering begins to lay the groundwork for future full-organ engineering. Current research in the field focuses on a number of key concepts, including cell source, biomaterial selection, and complex techniques to better mimic native tissue (*e.g.* patterning, cocultivation, *etc.*).

1.2.3.1 Cell Sources for Hepatic Tissue Engineering

Researchers have considered many different cell types for use in engineered hepatic tissue. Options include human hepatocytes and hepatocyte progenitors, various stem cells, hepatic cell lines, and animal cells. In any case, the number of cells required to fabricate a liver that would sufficiently replace an adult human liver is likely between \(10^{10}\) and \(10^{11}\) cells\(^94\). Mature human hepatocytes do not readily proliferate in *ex vivo* culture\(^98\), making it extremely difficult to obtain adequate cell numbers for implantation. Hepatocyte progenitors and stem cells can be difficult to obtain and may require extremely long culture periods to expand sufficient cell populations\(^99\). Significant progress has been made in differentiating human embryonic stem cells.
into hepatic cells\textsuperscript{100–102}; however, the use of embryonic stem cells is associated with ethical concerns and risks of tumorigenicity. Cell lines established from human hepatocellular cancer and immortalized hepatocyte cell lines have been used for engineered hepatic tissue\textsuperscript{70,103}; however, the use of cell lines is associated with significant tumorigenic concerns. Finally, animal cells such as porcine hepatocytes have been considered for hepatic tissue engineering, but the risk of disease transfer is likely too great and complete reproduction of human cell function is uncertain\textsuperscript{104}. Table 1-1 summarizes the different cell types that have been or may be used in hepatic tissue engineering, focusing on the availability and risks associated with each cell type.

Table 1-1: Summary of cell sources for human hepatic tissue engineering

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Source</th>
<th>Availability</th>
<th>Concerns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human hepatocytes</td>
<td>Rejected livers from organ donors</td>
<td>Scarce</td>
<td>Availability; non-proliferative \textit{ex vivo}</td>
</tr>
<tr>
<td>Human hepatoblasts</td>
<td>Fetal or neonatal livers</td>
<td>Scarce</td>
<td>Availability; maturation takes time</td>
</tr>
<tr>
<td>Human hepatic stem cells</td>
<td>Cadaveric livers</td>
<td>Potentially sufficient</td>
<td>Maturation takes time</td>
</tr>
<tr>
<td>Human embryonic stem cells</td>
<td>Various sources</td>
<td>Unlimited</td>
<td>Ethical concerns; tumorigenic</td>
</tr>
<tr>
<td>Human induced pluripotent stem (iPS) cells\textsuperscript{105}</td>
<td>Generated from various cell types</td>
<td>Unlimited</td>
<td>Tumorigenic</td>
</tr>
<tr>
<td>Human Wharton’s jelly cells\textsuperscript{106}</td>
<td>Umbilical cord Wharton’s jelly</td>
<td>Unlimited</td>
<td>Tumorigenic; ethical concerns</td>
</tr>
<tr>
<td>Human hepatoma cell line (e.g. HepG2)</td>
<td>Culture collection (e.g. ATCC)</td>
<td>Unlimited</td>
<td>Reduced hepatic functions; tumorigenic</td>
</tr>
<tr>
<td>Human immortalized hepatocytes</td>
<td>Culture collection (e.g. ATCC)</td>
<td>Unlimited</td>
<td>Reduced hepatic functions; tumorigenic</td>
</tr>
<tr>
<td>Porcine hepatocytes</td>
<td>Porcine livers</td>
<td>Unlimited</td>
<td>Transmission of zoonoses\textsuperscript{94}</td>
</tr>
</tbody>
</table>

1.2.3.2 Biomaterials in Hepatic Tissue Engineering

Tissue engineers choose scaffold materials based on the desired properties of their construct. These properties include degradability, biological interaction with cells, and
mechanical properties. Scaffolds fall into two major categories, natural and synthetic, based on their origin. The following paragraphs summarize the use of natural and synthetic scaffolds in tissue engineering, discuss a special class of scaffolds known as hydrogels, and summarize traditional scaffold selection for hepatic tissue engineering.

Natural scaffolds, which generally consist of ECM proteins found naturally in the body, have been widely used in tissue engineering. These scaffolds often most closely mimic the native chemical and physical environment of cells; however, they can be very difficult to characterize, as they generally consist of large and varied proteins. Natural materials that have been used in tissue engineering include collagen\textsuperscript{107}, fibrin\textsuperscript{108}, Matrigel\textsuperscript{109}, agarose\textsuperscript{110}, and alginate\textsuperscript{111}. Natural scaffolds are inherently cell adhesive, cell degradable, and bioactive in nature; therefore, cells encapsulated in these scaffolds can readily migrate, degrade matrix, and synthesize new ECM, leading to overall tissue remodeling. Although these properties make natural scaffolds ideal for tissue engineering, there are also significant drawbacks to using these materials. First, it is generally difficult to control the mechanical properties of these materials. Natural scaffolds are generally much softer than their synthetic counterparts, which makes handling, culture, and implantation more difficult. These scaffolds, although derived from native proteins, are also generally much softer than native tissue. Next, it is difficult or impossible to control ligand presentation to cells. Natural scaffolds generally consist of large and varied proteins, thus cell-matrix interactions vary significantly, making it more difficult to determine the roles of different signals in controlling cell behavior. Finally, natural scaffolds generally consist of proteins retrieved from cadavers, animals, or other tissue sources. Consequently, implanted natural scaffolds present greater risks of disease transmission and unfavorable immunogenic reaction.
Synthetic scaffolds, which generally consist of branching synthetic polymers, have also been widely used in tissue engineering. In comparison to natural materials, synthetic scaffolds present significantly less risk of adverse immune reaction upon implantation, and disease transmission is generally not an issue. Although it is difficult to incorporate all of the chemical intricacies of the native ECM into synthetic scaffolds, these materials offer the ability to precisely control the chemical and mechanical properties of a scaffold for tissue engineering. The polymers that form the basis of synthetic scaffolds are typically bioinert in nature; therefore, these polymers must be modified in order to achieve bioactivity. Most importantly, in order to use synthetic scaffolds for tissue engineering or remodeling, these scaffolds must be engineered to be cell adhesive and biodegradable. The most common approach used to render synthetic scaffolds cell adhesive is to modify these synthetic polymers with ECM proteins or peptide sequences from these proteins that play major roles in cell adhesion. Fibronectin, collagen, and other proteins have been incorporated into various synthetic scaffolds in order to enable cell adhesion. Adhesive peptides derived from ECM proteins, including RGD, YIGSR, IKVAV, and REDV, have also been incorporated into various synthetic scaffolds to enable cell adhesion. These peptides lack the complex functions associated with entire ECM proteins, are easy to synthesize, are easily tethered to polymer chains, and limit non-specific interactions, making them excellent alternatives to using entire ECM proteins to modify synthetic scaffolds. Some synthetic scaffolds are inherently biodegradable, as they naturally degrade over time after implantation, via hydrolysis. In other cases, biodegradability is engineered into synthetic scaffolds by engineering block copolymers, with two polymer chains connected by a small degradable moiety. This moiety may be engineered to be susceptible to hydrolytic or enzymatic degradations.
Hydrogels, which may be natural or synthetic in nature, have demonstrated tremendous applicability in tissue engineering. Due to their water retaining properties and tissue-like mechanical properties, hydrogels may better mimic native tissue than all other types of scaffolds. A number of synthetic polymers have been used to fabricate scaffolds for engineered tissues, including PEG\textsuperscript{122}, poly(vinyl alcohol) (PVA)\textsuperscript{123}, and poly[N-(2-hydroxypropyl) methacrylamide] (PHPMA)\textsuperscript{124}. PEG is likely the most commonly used hydrogel material for tissue engineering applications. PEG hydrogels offer a number of advantages that make them ideal for tissue engineering applications. First, PEG has been approved by the U.S. Food and Drug Administration (FDA) due to its non-toxic and non-immunogenic nature\textsuperscript{125}. Next, PEG hydrogels are extremely hydrophilic, which renders these hydrogels resistant to protein adsorption. This resistance to protein adsorption allows PEG to serve as a blank slate, such that all desired bioactivity must be engineered into a PEG hydrogel. This property allows for precise control over the chemical ligands that are displayed to encapsulated cells. Finally, altering PEG concentration or molecular weight allows these hydrogels to be tuned to meet desired mechanical properties.

Hepatic tissue engineering is one of the most established branches of the tissue engineering field. Consequently, dozens of different material scaffolds have been used in hepatic tissue engineering research. Natural materials including type I collagen\textsuperscript{126}, Matrigel\textsuperscript{127}, alginate\textsuperscript{128}, and many others have been used for engineered hepatic tissue. Alternatively, synthetic materials used for hepatic tissue engineering include poly(L-lactic acid) (PLLA)\textsuperscript{129}, poly(lactic-co-glycolic acid) (PLGA)\textsuperscript{127}, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)\textsuperscript{130}, PEG\textsuperscript{131}, and many others. For most tissue engineering applications, a degradable or highly porous matrix is desired, as cells must migrate and remodel tissue in order for the therapy
to be successful. Hepatocytes, on the other hand, are not highly migratory and can function in non-degradable matrices. More recent work has begun to combine scaffolding techniques with spatial patterning techniques, such as microcontact printing and photopatterning\textsuperscript{131}. These techniques can be used to control tissue organization or to control adhesion areas for hepatocytes, in attempts to recreate aspects of the native hepatocyte environment. Figure 1-16 depicts one such instance, in which photopatterning is used to control the spatial organization of a bioactive scaffold.

**Figure 1-16:** Multilayer photolithographic fabrication of hepatocyte-laden hydrogels. (A) An additive, photolithographic process was used to fabricate multilayer, biomimetic liver constructs. The photopatterning process encapsulated hepatocytes into PEG diacrylate (PEGDA) hydrogels doped with cell-adhesive ligands. (B) Three photopatterns were designed and used to fabricate each of the three repeating layers (red, green, and blue). (C) A phase contrast image of the 3D hepatic tissue mimic demonstrates the intricately patterned and layered microstructure. Reproduced from Tsang et al.\textsuperscript{131}.
1.2.3.3 Cocultivation of Hepatocytes with Other Cell Types

In the body, hepatocytes exist in a well-defined niche that includes several different cell types and structures. *In vivo*, hepatocytes exist in close proximity to sinusoidal vessels, which consist of endothelial cells and are lined by pericytes (Ito cells) and stellate macrophages (Kupffer cells). Accordingly, hepatocytes rely heavily on heterotypic cellular interactions which regulate most important hepatocyte functions. The need for these heterotypic interactions is preserved in *in vitro* culture. Previous work in static cell culture has demonstrated positive functional and morphological effects of co-culturing hepatocytes with epithelial cells\textsuperscript{132}, fibroblasts\textsuperscript{133,134}, and endothelial cells\textsuperscript{135}. These studies have involved plating hepatocytes either concurrently with other cell types or plating hepatocytes on over-confluent layers of other cell types. Hepatocytes in these 2D co-culture systems have demonstrated improved and prolonged enzyme activity, protein synthesis, and other hepatocyte functions. More recent work has begun to combine co-culturing techniques with spatial patterning techniques, such as microcontact printing\textsuperscript{136,137} and photopatterning. Figure 1-17 depicts one such instance, in which co-culture and microfabrication-based techniques are combined.

Due to the role of the HUVEC-10T½ co-culture in this thesis, interactions between 10T½ cells and hepatocytes are also of interest. There are no studies that have characterized the effects of hepatocytes on 10T½ cell differentiation; however, these two cell types have been combined to promote *in vitro* culture of hepatocytes. Co-culture with fibroblasts and other non-parenchymal cells have demonstrated improved function, viability, and stability of primary hepatocytes cultured *in vitro*. Consequently, primary hepatocytes have been cultured on monolayers of 10T½ cells to enhance hepatocyte function and prolong culture\textsuperscript{138}. Although the effects of hepatocytes on 10T½ cells have not been characterized, differentiation of other
mesenchymal stem cells into hepatocyte-like cells has been demonstrated, despite the endoderm origin of hepatocytes\textsuperscript{139,140}. Consequently, interactions with hepatocytes may have significant effects on 10T½ cell differentiation, but these effects are difficult to predict.

**Figure 1-17: Microfabricated co-cultures of hepatocytes and fibroblasts.** (A) Patterned hepatocyte islands without fibroblasts, (B) hepatocyte-fibroblast co-culture 24 hours after fibroblast seeding, and (C) hepatocyte-fibroblast co-culture 5 days after fibroblast seeding. Fibroblasts enhance survival and proliferation of hepatocytes. Recreated from Revzin et al.\textsuperscript{137}.

### 1.2.3.4 “Bottom-up” Tissue Engineering

“Bottom-up” tissue engineering refers to the combination of many small engineered tissues to form one larger, functional tissue. Micro-tissue engineering allows for the control of the cellular microenvironment, and many micro-tissues can be combined (often after a culture period) to form an organ-like structure that still maintains the micron and millimeter-scale features that are essential to proper cellular function. “Bottom-up” approaches often lead to engineered tissues that closely mimic the structure of native tissue, as highly repetitive functional units are common within organs, *in vivo*. McGuigan and Sefton presented an interesting example of “bottom-up” tissue engineering, in which a basic vasculature was used to separate hepatic tissue modules. HepG2 cells were cultured in collagen hydrogel pillars, which were then coated in HUVECs. After the HUVECs formed confluent layers on the collagen pillars, these modules were packed together within a microfluidic culture chamber and perfused with culture medium or blood. The spaces between the hepatic modules formed interconnected vessel-like networks.
that delayed clotting times in these engineered hepatic tissues. Figure 1-18 summarizes this “bottom-up” approach to hepatic tissue engineering.

**Figure 1-18:** A bottom-up approach to engineering vascularized hepatic tissue. (A) Collagen hydrogel pillars seeded with HepG2 cells were coated with HUVECs and packed together to form a perfusable hepatic tissue. (B) A light micrograph depicts a collagen module seeded with HepG2 cells, prior to HUVEC coating. (C) A fluorescent micrograph of vascular endothelial (VE)-cadherin (green) within a collagen module, 7 days after seeding with HUVECs, demonstrates confluence of HUVECs on the module surface. Recreated from McGuigan and Sefton.

### 1.2.3.5 Quantitative Metrics for Analyzing Engineered Hepatic Tissue

Due to the well-defined metabolic and secretory functions of hepatocytes, the function of engineered hepatic tissue can be evaluated using several simple assays. Although there are dozens of assays that could be used, several tests have become standard measures of hepatocyte function. First, in order to evaluate the metabolic activity and biotransformation capacity of hepatocytes, simple assays are performed to measure activity of cytochrome P450 enzymes. There are simple molecules, such as 7-ethoxy-4-trifluoromethylcoumarin (EFC), which form
fluorescent byproducts after degradation by cytochrome P450 enzymes; therefore, enzymatic activity can be assayed by taking simple fluorescent measurements of culture medium. Another commonly used test used to evaluate hepatocyte function is a urea secretion assay. Like cytochrome P450 activity, urea secretion is a hepatocyte-specific behavior. The urea secretion test evaluates the capacity of hepatocytes to convert toxic compounds (e.g., ammonia) into urea, which is easily secreted. Finally, an assay for albumin synthesis is commonly used to evaluate hepatocyte function. Again, albumin synthesis is a hepatocyte-specific behavior, and it relates to the biosynthetic capacity of the cells. These assays are very useful tools for hepatic tissue engineers, as they allow for quick and simple evaluation of engineered hepatic constructs.

1.2.4 Liver Biochips

In addition to the future potential for tissue engineered hepatic tissue that can be safely implanted in humans, a major impetus for hepatic tissue engineering is the improvement of drug toxicity assays. Historically, the cytotoxicity of drug candidates has been measured by exposing monolayers of hepatocytes in culture to a drug and evaluating the survival and metabolic function of the cells. Later, sandwich cultures, where hepatocytes are cultured between porous scaffolds, were developed to better mimic in vivo exposure of cells to drugs. Improved mass transport in hepatic tissue models has been achieved through the use of microfluidic devices with intricate hepatocyte perfusion mechanisms. This represents a major step in improving these toxicity studies, as these microfluidic devices are able to closely mimic the manner in which hepatocytes are exposed to potentially toxic compounds in vivo. Recently, these microfluidic culture devices have been shown to provide the best alternative to human and
animal testing\textsuperscript{147–151}. These microfluidic devices range from simple perfusion systems to complex biomimetic approaches.

Hepatic biochips have also been designed to facilitate multiplexing, meaning that dozens of potential drug candidates can be screened simultaneously. One such device, which housed hepatocyte co-cultures in an intricately designed PDMS chamber, was successfully used to screen 27 drug compounds simultaneously\textsuperscript{149}. These assays, which expose hepatocytes to drug candidates, generally monitor the activity of cytochrome P450 enzymes in hepatocytes, which are particularly important to drug detoxification. Hepatocyte viability and proliferation are also generally monitored. Ultimately, as culture models for drug toxicity become more similar to native tissue, they become better predictors of \textit{in vivo} toxicity, thereby diminishing the need for animal testing, which is far more complex and costly\textsuperscript{152}. Figure 1-19 depicts some of the microfluidic hepatocyte culture devices that have been developed for drug screening applications.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure19.png}
\caption{Microfluidic hepatocyte culture chips for drug toxicity screening. (A) Fluorescent micrograph of hepatocytes cultured in a microfluidic device demonstrated formations of bile canalicular structures\textsuperscript{147}. (B, C) Closely spaced PDMS structures have been used to mimic fenestrations in liver sinusoids, separating medium/blood flow channels from hepatocyte culture chambers. The microfluidic sinusoid was designed with a nutrient transport channel surrounding a cell culture area. These two features were separated by microfabricated PDMS structures, which are spaced 1-2 μm from each other. These small spaces allowed for diffusive transport between cells and the flow channel, while limiting convective transport. Reproduced from Goral \textit{et al.}\textsuperscript{147}, Lee \textit{et al.}\textsuperscript{70}, and Toh \textit{et al.}\textsuperscript{151}.}
\end{figure}
1.2.5 Conclusions

To date, successes in hepatic tissue engineering have been limited due to the inability to successfully engineer vascular networks throughout tissue implants. Due to the tremendous number of hepatocytes that must be implanted in order to provide adequate liver function, a tissue engineered liver must be quite large; therefore, a robust vascular network is necessary for such an implant, and this network must quickly anastomose with native vasculature. Additionally, great strides have been made in improving hepatocyte-based culture studies for studying drug toxicity. The use of microfluidic devices has been a major factor in these improvements, as have other methods that allow these assays to better mimic \textit{in vivo} hepatocyte exposure to drug molecules. There is room, however, for continued improvement in mimicking the hepatic microenvironment during the engineering of hepatic tissue for drug toxicity studies. The work presented addresses several of the major shortcomings of hepatic tissue engineering, as the use of cell-formed vascular networks improved mass transport in engineered hepatic tissue and provided essential heterotypic cellular interactions that mimic the native hepatocyte microenvironment. Bioactive PEG hydrogels were used as scaffolds for our hepatic tissue engineering efforts. These hydrogel technologies are reviewed in the following section.

1.3 Project Introduction

The research project presented herein represents our attempts to engineer vascularized hepatic tissue using a bioactive PEG hydrogel system. Our work addresses the shortcomings of current research in the vascularization of engineered tissues, as we investigated the ability of cell-formed microvascular networks to support parenchymal cell viability within a scaffold. In addition, our work addresses a need in the field of hepatic tissue engineering, as we begin to look at the potential for perfusion of larger tissues through cell-formed microvasculature in
conjunction with prefabricated conduits. Finally, we continued our study of cell formed microvasculature by developing a platform for temporal control over growth factor presentation within our bioactive PEG hydrogels. The project aims are briefly summarized below.

**Aim 1: To characterize a vasculogenic and hepatic tri-culture under static conditions**

A novel tri-culture was developed that combined a vasculogenic co-culture with hepatic cells. After appropriate culture conditions were determined, our work focused on understanding the interactions between vascular and hepatic cells. A combination of 2D culture studies and encapsulations within a bioactive PEG scaffold were performed to observe the behaviors of the cells in the tri-culture. Our results demonstrated the positive effects of vascular cells on hepatocytes and the ability of the tri-culture to spontaneously form vascularized hepatic tissue in a bioactive PEG hydrogel.

**Aim 2: To analyze a vasculogenic and hepatic tri-culture under dynamic conditions**

After characterizing the vasculogenic and hepatic tri-culture in bioactive PEG hydrogels under static culture, we analyzed these engineered tissues within a dynamic culture system. Medium flow was delivered in a controlled fashion to slab hydrogels containing hepatic cells. Hepatic viability was compared between vascularized and non-vascularized hydrogels in order to demonstrate the ability of cell-formed microvasculature to improve mass transport within engineered tissues.

**Aim 3: To develop a platform that allows for the temporal control of tethered ligand presentation within PEG hydrogels**

The affinity between streptavidin and biotin was used to develop a platform that allowed growth factors to be tethered into PEG hydrogels after the initial polymerization event. Briefly,
PEG chains were attached to streptavidin, allowing streptavidin to be tethered into the PEG matrix upon the photoencapsulation of cells. Biotinylated growth factors were then added at later time points and became tethered into the gels through the streptavidin-biotin interaction. Our studies demonstrated the ability to control the onset of tubule formation by endothelial cells in response to VEGF delivery.
CHAPTER 2 – SYNTHESIS OF HYDROGEL COMPONENTS

2.1 PEG Hydrogel Overview

Poly(ethylene glycol) (PEG) hydrogel-based scaffolds are widely used in tissue engineering for a number of reasons. The hydrophilic nature of PEG makes it resistant to protein adsorption, which allows the material to be bioinert upon implantation. PEG has been approved by the United States Food and Drug Administration for dozens of applications (e.g. contact lenses, surface coatings, drug delivery devices, etc.) due to the lack of biological response to the implanted polymer. Its resistance to protein adsorption also allows PEG to serve as a blank slate, onto which bioactivity may be specifically engineered. Finally, PEG hydrogels can be tuned to match the mechanical properties of native soft tissues.

Due to the inherent bioinert nature of PEG, chemical modifications are often made to the material to give the polymer biological properties. Biological molecules can be incorporated non-covalently into PEG hydrogels by combining them with the hydrogel precursor prior to polymerization. This technique is useful for drug delivery or the controlled release of bioactive factors. For the purpose of forming a bioactive scaffold, creating PEG hydrogels that maintain controlled levels of bioactivity is essential. This is best achieved by incorporating biological molecules into the PEG hydrogel through covalent modifications to the PEG chains. Covalent modification of PEG chains with bioactive molecules is prevalent throughout the literature and has been achieved using various chemistries. Heterobifunctional PEG molecules can be used that are first conjugated to biomolecules and then can be crosslinked into a hydrogel network via the functionality at the other end of the PEG chain. Conjugation to amine groups using n-hydroxysuccinimide (NHS) or similar chemistry and conjugation to free thiols represent the most frequently employed methods for PEGylation of biomolecules.
For the purpose of tissue engineering, creating PEG scaffolds that are cell adhesive and degradable by cells is necessary. Incorporation of PEG chains modified with cell adhesive peptides, such as RGDS, has been used to render PEG hydrogels adhesive to cells\textsuperscript{159,164}. Furthermore, peptide sequences that are degradable by cellular enzymes have been incorporated into the PEG backbone of hydrogels. These hydrogels allow cells to degrade and migrate through the material\textsuperscript{154,159}. The development of PEG hydrogels that allow for both adhesion and degradation by cells has made PEG a widely used and versatile base material for tissue engineers.

The work outlined in this chapter involves the synthesis of different PEG hydrogel components that were used in the studies outlined in this thesis. A few preliminary studies were performed using PEG that had not been modified with any biological molecules; however, the vast majority of our work involved hydrogels consisting of PEG chains that were tethered to peptides or proteins. This characterizes one of the greatest advantages of working with PEG: that the biological properties of the scaffold can be tuned by simply varying the components of the precursor solution. By first mixing PEG chains with different biological functionalities in a precursor solution, we are then able to polymerize a hydrogel with distinct biological properties.

2.2 Overview and Synthesis of Hydrogel Components

2.2.1 PEGDA

Poly (ethylene glycol) diacrylate (PEGDA) is often used as the basis for hydrogel materials, as reactions between terminal acrylate groups can be used to link polymer chains. PEGDA is synthesized by adding acrylate groups to the ends of PEG chains. These acrylate groups are reactive, such that in the presence of an activated free radical photoinitiator PEGDA
chains can be crosslinked into an insoluble hydrogel matrix. PEGDA was used briefly in some preliminary studies presented in this thesis; however, the majority of our work dealt with PEG chains modified with biological molecules which are described later in this chapter.

PEGDA with molecular weight of 6kDa was synthesized as previously described\textsuperscript{165}. Briefly, dry 6kDa PEG (Sigma-Aldrich, St. Louis, MO) was dissolved at a concentration of 0.1M in anhydrous dichloromethane (DCM) with 0.4M acryloyl chloride and 0.2M triethylamine (TEA). The reaction was performed overnight, under argon and with constant stirring. The reaction mixture was then washed with 1.5M K$_2$CO$_3$ and allowed to separate into aqueous and organic phases, in order to remove HCl. Next, the organic phase was dried using anhydrous MgSO$_4$ and PEGDA was precipitated out of solution in cold diethyl ether. The resulting PEGDA was filtered, dried under vacuum, and crushed into a powder. Efficient acrylation of PEG was confirmed using proton nuclear magnetic resonance spectroscopy (\textsuperscript{1}H-NMR, Bruker, Billerica, MA). Figure 2-1 depicts the reaction by which PEG chains are terminally acrylated to form PEGDA.

\begin{center}
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\includegraphics[width=\textwidth]{PEGDA_synthesis.png}
\caption{PEGDA synthesis. Terminal hydroxyl groups on PEG chains react with carbonyl groups on acryloyl chloride to form PEGDA. Reaction takes place in DCM with TEA.}
\end{figure}
\end{center}

\textbf{2.2.2 Cell-adhesive PEG-RGDS}

Cell-adhesive moieties and other bioactive factors must be incorporated into PEG hydrogels in order for these scaffolds to support tissue formation and remodeling. In order to
incorporate biomolecules into the PEG matrix, these molecules can first be conjugated to monoacrylated-modified PEG chains. Acrylate-PEG-succinimidy l carboxymethylene (PEG-SCM) is one of the polymers used for biomolecule conjugation, as its amine-reactive SCM group can react with amine groups in bioactive peptides, while its acrylated terminus enables photo-crosslinking into PEGDA hydrogels. N-hydroxysuccinimide (NHS) and succinimidyl valerate (SVA) are other amine-reactive groups that similarly may be coupled to monoacrylated PEG and used to conjugate bioactive proteins or peptides to PEG chains. The PEG chain provides distance between the crosslinking point and the attached bioactive factor, ensuring that steric hindrance does not negatively impact bioactivity\textsuperscript{166}.

The cell adhesive peptide RGDS was conjugated to PEG-SCM as previously described\textsuperscript{159}. Briefly, the RGDS peptide (American Peptide, Sunnyvale, CA) was reacted with PEG-SCM (MW 3.4 kDa, Laysan Bio, Arab, AL) in anhydrous dimethyl sulfoxide (DMSO) with diisopropylamine (DIPEA). The reaction used a 1.1:1 molar ratio of RGDS:PEG and a 2:1 ratio of DIPEA:PEG. The reaction was carried out for 24 h under argon and constant mixing. The resulting PEG-RGDS was purified via dialysis, frozen, and lyophilized. Conjugation efficiency and purity were confirmed using a gel permeation chromatography (GPC) system with a 5\textmu m PLgel column (Polymer Laboratories, Amherst, MA) and evaporative light scattering (ELS) and ultraviolet (UV) detectors. Figure 2-2 depicts the reaction by which the cell adhesive peptide RGDS is conjugated to PEG-NHS.
Figure 2-2: PEG-RGDS conjugation. Acryloyl-PEG-NHS is reacted with the RGDS peptide in dimethyl sulfoxide (DMSO) and N,N-diisopropylethylamine (DIPEA) to form monoacrylated PEG-RGDS. The NHS group reacts with the amine terminus of the RGDS peptide. A similar reaction is used for conjugating bioactive proteins to PEG; however, different reaction conditions must be used to ensure that proteins do not denature or unfold.

2.2.3 Proteolytically Degradable PEG-PQ-PEG

In order to for cells to migrate through PEG hydrogels, thus remodeling and forming tissue, the hydrogels must be degradable. The two ester linkages in PEGDA are minimally susceptible to hydrolysis at physiologic pH, thus cell migration in PEGDA hydrogels is generally not possible\textsuperscript{167}. Rather than fabricating gels that degrade via hydrolysis, proteolytically degradable PEG derivatives can be synthesized that are degraded by cellular proteolytic enzymes that are naturally involved in cell migration and tissue synthesis processes\textsuperscript{154,159}. Gels consisting of these degradable PEG derivatives, therefore, have degradation characteristics paired with cellular migration, creating the ideal environment for tissue formation. Similar to the conjugation of RGDS to PEG described above, synthesis of degradable PEG derivatives involves PEG-SCM and a bioactive molecule. In this case, two PEG-SCM chains are conjugated to a peptide (GGGPQGIWQGK, abbreviated PQ), which is susceptible to degradation by matrix metalloproteinases 2 and 9 (MMP-2 and -9)\textsuperscript{159}, in order to form PEG-PQ-PEG.

PEG-PQ-PEG was synthesized as previously described\textsuperscript{66}. Briefly, solid phase peptide synthesis was performed to produce the GGGPQGIWQGK peptide. An Apex396 peptide synthesizer (Aapptec, Louisville, KY) and fomc-protected amino acids were used for the
synthesis, and the resulting peptide was purified via dialysis. After purification, matrix-assisted laser desorption ionization time of flight mass spectroscopy (MALDI-ToF, Bruker, Billerica, MA) was performed to confirm the molecular weight of the purified peptide. The PQ peptide was reacted with PEG-SCM (MW 3.4 kDa, Laysan Bio) in anhydrous DMSO with DIPEA. The reaction used a 1:2.2 molar ratio of PQ:PEG and a 2:1 ratio of DIPEA:PEG. The ratio of PQ peptide to PEG was chosen in order to form block co-polymers consisting of the peptide flanked by monoacrylated PEG chains. The monoacrylated PEG-SCM molecules were able to react with both the amine terminus of the peptide and the amine in the lysine side group (near the carboxyl terminus), thus creating these block co-polymers. The reaction was carried out for 24 h under argon and constant mixing. The resulting PEG-PQ-PEG was purified via dialysis, frozen, and lyophilized. Conjugation efficiency and purity were confirmed using GPC as described above. Figure 2-3 depicts the reaction by which the MMP-degradable PQ peptide is conjugated between monoacrylated PEG chains.

**Figure 2-3: Synthesis of enzymatically degradable PEG-PQ-PEG.** PEG-SCM is reacted with the MMP-sensitive peptide (GGGPQGIWGQGK) in DMSO and DIPEA, in a 2:1 ratio, to form acrylate-PEG-GGGPQGIWGQGK-PEG-acrylate (PEG-PQ-PEG). The SCM groups react with the amine terminus of the PQ peptide and the primary amine in the side chain of the terminal lysine residue of the PQ peptide. Since the resulting PEG-PQ-PEG molecules are terminally acrylated, PEG-PQ-PEG is photopolymerizable under similar conditions as PEGDA.

### 2.3 Crosslinking Conditions and Cell Encapsulation

Although there are other methods for crosslinking PEG scaffolds, such as Michael-type addition\textsuperscript{168}, our work has focused on free-radical based photopolymerization of terminally
acrylated PEG molecules. This process is very rapid and is driven by the formation of micelle-like structures by terminal acrylate groups, due to hydrophobic interactions. Photoinitiator molecules included in polymer precursor solutions generate free radicals when exposed to a corresponding wavelength of light, and this free radical initiates polymerization and chain elongation events. Polymerization nodes form, where multiple PEG chains are crosslinked at a single point. As crosslinks form, an insoluble PEG hydrogel matrix is generated, as depicted in Figure 2-4.

**Figure 2-4: PEGDA network formation schematic.** The following structures occur spontaneously during PEGDA photo-crosslinking: (a) crosslinked PEG chain, (b) unreacted acrylate groups, (c) cyclic PEG crosslinks, (d) PEG chain entanglement, and (e) PEG tethered bioactive molecules (if included in precursor solution). Crosslinking of enzymatically degradable PEG occurs in the same fashion (e.g. pictured PEG chains could contain degradable peptides). Reproduced from Beamish *et al.*

The crosslinking conditions used for PEG hydrogels enable cell encapsulation with low cytotoxicity. Cells are suspended in the aqueous polymer precursor solution and are physically encapsulated in the PEG matrix upon photo-crosslinking. A number of different photoinitiators, which are sensitive to different wavelengths of light, have been used to encapsulate cells in PEG hydrogels. For the work presented in this document, Irgacure 2959, eosin Y, and 2,2-dimethoxy-2-phenylacetophenone (DMAP) photoinitiator systems are of particular interest. In a
study examining the cytotoxicity of various photoinitiator systems, 3T3 fibroblasts exposed to Irgacure 2959, a photoinitiator sensitive to ultraviolet light, at concentrations up to 0.1\% (w/w), demonstrated high viability (greater than 80\%)\textsuperscript{170}. In another study, porcine pancreatic islets were encapsulated in PEG hydrogels using a number of different photoinitiator systems. Islet viability of greater than 90\% was observed when eosin Y, a photoinitiator sensitive to green light, was used at concentrations of 5-20 mM\textsuperscript{171}.

Finally, a major advantage of the photopolymerization and encapsulation process is the ability to photopattern PEG hydrogels. Cellular hydrogel structures or PEG tethered biomolecules can be photopatterned in two\textsuperscript{131,165,172} or three dimensions\textsuperscript{173}, using mask- or scanning-based processes. These patterning techniques allow for precise control of the physical and chemical surroundings of cells encapsulated in bioactive PEG hydrogels, allowing for the fabrication of engineered tissues that more closely resemble native tissue on macro- and microscopic levels. In work presented later in the thesis, we use photopatterning to create hydrogel tissue beds between microfluidic channels.
CHAPTER 3 - HEPATIC TISSUE ENGINEERING IN BULK

POLY(ETHYLENE GLYCOL) HYDROGELS

3.1 Introduction

Tissues in the body typically consist of multiple cell types organized in such a way as to produce desired functions. The precise organization of cells within tissues establishes heterotypic cell-cell interactions, the physical and chemical signals shared between cells of different types, which are often essential to tissue or organ function. A key class of heterotypic cell-cell interactions, those between parenchymal and non-parenchymal cells, is observed in tissues throughout the body. These interactions are particularly important to overall tissue function, as non-parenchymal cells can modulate many activities of the functional parenchymal cells, including proliferation, differentiation, and protein expression. Study of the interactions between parenchymal and non-parenchymal cells allows for the effective use of co-culture in tissue engineering, in order to engineer tissues that more closely mimic the behavior of their \textit{in vivo} counterparts.

Hepatic tissue is an excellent example of a tissue that depends greatly on heterotypic cell-cell interactions, as hepatocyte function is highly regulated by interactions with numerous other cell types. \textit{In vivo}, hepatocytes exist in a well-defined microenvironment and interact with several different cell types, including those that constitute capillaries and larger blood vessels. These cellular interactions can be physical (\textit{e.g.} cell-cell and cell-secreted matrix interactions) and chemical (\textit{e.g.} secreted factors from non-parenchymal cells) in nature, and have been shown to affect several hepatocyte functions, including biotransformative enzyme activity and protein synthesis\textsuperscript{134}. The effects that vascular cells have on hepatocytes are of particular interest because the metabolic and protein synthesis functions of hepatocytes are so closely tied to their direct
access to the blood supply. Various non-parenchymal cell types including fibroblasts\textsuperscript{133,136}, endothelial cells\textsuperscript{135}, and other liver-specific cell types\textsuperscript{132} have demonstrated beneficial effects on hepatocytes in culture; however, the vast majority of these studies have been performed in two-dimensional culture. In order to successfully engineer liver tissue, however, it is important to understand the role of these cellular interactions in a three-dimensional tissue replacement. For instance, hepatocytes may be affected differently by endothelial cells forming three-dimensional vascular structures than they are by endothelial cells in two-dimensional cell culture. In this work, we combine hepatocytes with a vasculogenic co-culture in a biomimetic hydrogel to begin to investigate the interactions between hepatic and vascular structures within tissue engineered therapeutics.

One common vascularization technique in tissue engineering research involves the use of endothelial cells to form microvasculature-like networks in biomaterial scaffolds. Myriad sources of endothelial cells have been used to form these networks, and co-cultures of endothelial cells with pericytes or other mesenchymal cells are also common\textsuperscript{38}. Human umbilical vein endothelial cells (HUVECs) and 10T½ cells, mesenchymal pericyte precursor cells of murine origin, were co-cultured in collagen hydrogels in one such study that demonstrated the importance of vascular support cells in endothelial cell tubulogenesis. In this study, 10T½ cells were shown to behave as pericytes and were necessary for the long-term stability of endothelial tubule structures\textsuperscript{53}. Despite continued work with this co-culture and other cellular methods for vascularization, there remains a void in the literature. There is a striking lack of research that has studied the role of cell-formed microvasculature in supporting functional cells in a tissue engineered implant. By combining hepatocytes and a vasculogenic co-
culture, we begin to investigate the potential for cellular prevascularization in bulky, metabolically active tissues.

This study builds upon previous work from our research group that has demonstrated the ability to engineer functional microvascular networks in bioactive poly(ethylene glycol) (PEG) hydrogels using a co-culture of HUVECs and 10T½ cells. Select studies from our group have demonstrated the long-term stability (at least 30 days) of these tubule networks in a PEG hydrogel system and the improved mass transport properties of PEG scaffolds after prevascularization with the HUVEC-10T½ co-culture. Other groups have used PEG-based hydrogels as scaffolds for engineered hepatic tissue; however, these efforts have lacked the vascular network or other transport system necessary for implantation of hepatic tissue. The work presented in this chapter begins the investigation into the use of these cell-formed microvascular networks to support functional, parenchymal cells in tissues engineered in PEG hydrogel scaffolds.

We have thoroughly studied the combination of hepatocytes with the vasculogenic HUVEC-10T½ co-culture, both in 2D and 3D cultures. First, cells from a hepatocyte-like cell line (HepG2 cells) were combined with the HUVEC-10T½ co-culture. We used this novel tri-culture to gain an understanding of the role of different culture media on 2D and 3D culture systems with both vascular and hepatic elements. In a bioactive PEG hydrogel, this tri-culture spontaneously formed both vascular networks and functional hepatic structures; however, few improvements were observed in HepG2 functions. Additional studies replaced the HepG2 cells with primary rat hepatocytes. Functional benefits of the tri-culture were more apparent with the primary cells, as both protein synthesis and cytochrome P450 enzyme (CYP) activity were enhanced due to interactions with the vasculogenic cells. Ultimately, hepatocytes were combined
with the vasculogenic co-culture while maintaining vascular network formation and improving hepatocyte function, which represents an essential first step towards the engineering of hepatic tissue that is capable of anastomosis with host vasculature upon implantation.

3.2 METHODS

3.2.1 Cell Maintenance

HUVECs (Lonza, Walkersville, MD) were cultured in endothelial growth medium 2 (EGM-2, Lonza) supplemented with 2 mM L-glutamine, 1 MU L⁻¹ penicillin, and 100 mg L⁻¹ streptomycin. 10T½ cells (American Type Culture Collection [ATCC], Rockville, MD) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 1 MU L⁻¹ penicillin, and 100 mg L⁻¹ streptomycin. Both cell types were incubated at 37°C and 5% CO₂, with culture medium replacement and subculturing performed as necessary. HUVECs were used for experiments at passages 3-5, and 10T½ cells were used at passages 16-18.

HepG2 cells (ATCC, Rockville, MD) were cultured in Minimum Essential Medium (MEM, Invitrogen, Carlsbad, CA) with 2mMGlutaMAX™ (L-glutamine dipeptide) supplemented with 10% FBS, 1 MU L⁻¹ penicillin, and 100 mg L⁻¹ streptomycin. HepG2 cells were incubated at 37°C and 5% CO₂, with culture medium replacement and subculturing performed as necessary. HepG2 cells were used for experiments at passages 3-10.

Primary rat hepatocytes (Invitrogen, Carlsbad, CA) were obtained in frozen stocks and were used immediately after thawing. Hepatocyte thawing and plating medium (Invitrogen), which was supplemented with 5% FBS, was used for the first six hours of hepatocyte culture,
both in two-dimensional culture and in hydrogel encapsulation. Hepatocyte growth medium (Invitrogen), which was serum-free, was used for the remainder of hepatocyte culture periods.

3.2.2 Two-dimensional Culture Studies with HepG2 Cells

Although the tri-culture system was ultimately encapsulated in bioactive PEG hydrogels, some of the preliminary work was first completed in standard 2D culture on tissue culture polystyrene (TCPS). Most significantly, 2D culture studies were used to help determine the medium formulation that is best suited to support the survival and function of the different cell types. In previous work, endothelial growth medium (EGM) was used for the HUVEC-10T½ co-culture\textsuperscript{71}. Although this medium is intended for use with endothelial cells, no changes were made due to the 10T½ cells, as the soluble signals (\textit{i.e.} pro-angiogenic growth factors) in EGM may help drive 10T½ cells towards the desired pericyte lineage. The effect of the growth factors present in EGM on HepG2 cells, however, was much less clear. Consequently, a series of studies were conducted to determine optimal culture conditions for the tri-culture. HepG2 cells are typically cultured in minimum essential medium (MEM), which is quite different from EGM as it contains no soluble growth factors. Fortunately, both the HUVEC-10T½ co-culture and HepG2 cells are typically cultured with serum and antibiotics present.

First, the basic effects that other cell types have on the HepG2 cells were evaluated. A review of the literature regarding hepatocytes reveals a number of different co-culture approaches that have been employed to enhance hepatocyte function. Most commonly, hepatocytes are co-cultured with fibroblasts\textsuperscript{133,136}, but there has also been some work with endothelial cells and other cells found in the liver\textsuperscript{134}. For this study, cells were seeded in a 48-well plate in the following groups: HepG2 cells alone, HepG2 cells co-cultured with 3T3
fibroblasts, 3T3 fibroblasts alone, HepG2 cells tri-cultured with HUVECs and 10T½ cells, and the HUVEC-10T½ co-culture alone. A 50/50 blend of EGM and MEM was supplemented with 100 μM7-ethoxy-4-trifluoromethylcoumarin (EFC, Figure 3-1), a molecule that yields a fluorescent byproduct when degraded by cytochrome P450 enzymes in hepatocytes, and added to all wells. Consequently, the relative metabolic activity of the HepG2 cells was assayed by collecting culture media over time and measuring fluorescence using a fluorimeter (excitation at 385 nm, emission at 502 nm). Media samples were collected and analyzed every 24 hours for four days. The results of this study were used to demonstrate the effects that the HUVEC-10T½ co-culture has on the metabolic activity of HepG2 cells. Statistical comparisons between groups were made using an unpaired student’s t-test, accounting for the Bonferroni correction.

![Figure 3-1: EFC structure](image)

Second, the effects of different media formulations on the cells in the tri-culture were determined. HUVECs, 10T½ cells, and HepG2 cells were seeded in a 48-well plate. HUVECs were seeded at a density four times greater than 10T½ cells. HepG2 cells were seeded at various densities, with ratios of co-culture:HepG2 cells ranging from 1:1 to 30:1. The final number of HepG2 cells in each well was 1,000, with the number of HUVECs and 10T½ cells varying from well to well. Control wells were seeded with 1,000 HepG2 cells, with no vascular cells. Twelve wells were seeded for each individual ratio of co-culture:HepG2 cells, which allowed for four medium conditions to be tested on three wells. After allowing all of the cells to attach to the
TCPS, the various medium conditions were applied to the wells. The media that were tested were combinations of EGM and MEM, and each was supplemented with 100 μM EFC. EGM was combined with MEM in ratios of 1:0, 2:1, 1:2, and 0:1. Culture media samples were collected and analyzed using a fluorimeter (ex. 385, em. 502) every 24 hours for four days. The results of these fluorescent readings were used to demonstrate the effects of the different media formulations on the metabolic activity of HepG2 cells (both in mono- and tri-culture). Statistical comparisons between groups were made using an unpaired student’s t-test, Bonferroni correction. In addition to collecting culture media daily, phase images were taken of the cells using a standard inverted microscope. These images were used to qualitatively analyze the behavior of the HUVEC-10T½ co-culture. As the concentration of EGM is decreased in the culture medium, it is likely that the formation of tubules by the co-culture also decreases. Accordingly, this study was used to find an ideal culture medium formulation, which limited negative impact on both hepatocyte function and tubule formation. Further studies were needed to devise more complex media schemes (i.e. changing media types mid-study), but these studies are not outlined here and followed similar protocols as those described above.

3.2.3 Three-dimensional Culture Studies with HepG2 Cells

The behavior of cells in 3D culture can be completely different than in 2D culture. Consequently, after learning a great deal about the HUVEC-10T½-HepG2 tri-culture in the 2D culture studies, some of that work was repeated in 3D culture. For these studies, a cell-adhesive PEG hydrogel system was used. PEG diacrylate (PEGDA) and PEG-RGDS were synthesized as summarized earlier (Chapter 2) and as previously described\(^{159,165}\). Cells were suspended in hydrogel precursor solutions consisting of 6 kDa PEGDA (10% w/v) supplemented with PEG-
RGDS (3.5 mM) and photoinitiator (Irgacure 2959). Droplets (5 μL each) of the precursor solutions were pipetted into glass-bottom well plates, and the hydrogels were photopolymerized using UV light, thus encapsulating the cells. Cell densities were based on the results of the 2D culture studies, with the number of HepG2 cells held constant from gel-to-gel. Hydrogel droplets were subjected to culture medium conditions as described above for 2D culture. Both 2D studies outlined, one to determine the effects of other cell types on HepG2 cell function and one to determine the effects of culture medium formulation on HepG2 function, were performed on cell-encapsulated hydrogels as described above for 2D culture. Statistical comparisons between groups were made using an unpaired student’s t-test, accounting for the Bonferroni correction.

3.2.4 Engineering Vascularized Hepatic Micro-tissues with HepG2 Cells

After completing the aforementioned 2D and 3D assays to determine optimal culture conditions, these conditions were used in initial attempts to engineer vascularized hepatic micro-tissues. Cell-adhesive and cell-degradable PEG hydrogels were used as the scaffold for the tri-culture. PEG-RGDS and enzymatically degradable PEG-PQ-PEG were synthesized as summarized earlier (Chapter 2) and as previously described. HepG2 cells, HUVECs, and 10T½ cells were maintained in culture until encapsulation. Hydrogel precursor solutions were prepared by dissolving PEG-PQ-PEG at 50 mg/mL (5% w/v) and PEG-RGDS at 14 mg/mL (3.5 mM) in HEPES buffered saline (HBS) with triethanolamine (TEOA, 15 μL/mL), eosin Y photoinitiator (10 μM), and N-vinyl-pyrrolidone (NVP, 3.5 μL/mL). HUVECs were added to the hydrogel precursor solution at a concentration of 2.4x10^7 cells/mL, 10T½ cells at 6x10^6 cells/mL, and HepG2 cells at 1x10^6 cells/mL. HepG2 mono-culture and HUVEC-10T½ co-culture precursors were prepared as controls.
Methacrylated coverslips were prepared by thoroughly cleaning coverslips in piranha solution (3:1 solution of \( \text{H}_2\text{SO}_4:\text{H}_2\text{O}_2 \)), incubating coverslips in 3-(Trimethoxysilyl)propyl methacrylate (85 mM, Sigma-Aldrich, St. Louis, MO) in ethanol (pH adjusted to 4.5 with acetic acid) for 72 h, and baking coverslips for 1 h at 90°C. Hydrogels were formed by pipetting 5 μL of precursor-cell suspension between a methacrylated coverslip and a glass slide and then using white light to photopolymerize the solutions (20 second exposure). Hydrogels were transferred to a 24-well plate and cultured using the conditions determined in the 2D and 3D culture studies. This method is depicted in Figure 3-2, and the overall approach is summarized in Figure 3-3.

After the cellular hydrogels were transferred to a well-plate, culture media samples were collected daily to monitor the function of the HepG2 cells. In addition to the previously outlined assay, which uses EFC degradation to monitor CYP activity, an additional assay was performed to monitor albumin synthesis. The secretion of albumin by HepG2 cells was measured over time using a sandwich enzyme-linked immunosorbent assay (ELISA). This assay was used monitor the biosynthetic activity of HepG2 cells, which represents another essential function of engineered hepatic tissue. These assays (CYP activity and albumin biosynthesis) were used to demonstrate the function of HepG2 cells in engineered hepatic tissues, both with and without the HUVEC-10T½ co-culture. Statistical comparisons between groups were made using an unpaired student’s \( t \)-test, accounting for the Bonferroni correction. Consequently, these assays began to demonstrate effects of the HUVEC-10T½ co-culture, and the tubules formed by these cells, on the function of parenchymal cells in an engineered tissue.
Figure 3-2: Fabrication of individual 5 μL micro-tissues. The process depicted was used to fabricate each individual micro-tissue. Attachment of these tissues to methacrylated cover glasses facilitates tissue handling, culture, immunostaining, and imaging.

Figure 3-3: Tri-culture encapsulation in proteolytically degradable PEG hydrogels. HUVECs, 10T½ cells, and HepG2 cells will be encapsulated in cell adhesive, proteolytically degradable PEG hydrogels. Recreated from Cuchiara.⁶³
In addition to functional assays, cellular morphology and the overall structure of the engineered hepatic micro-tissues were of great interest. The architecture of the engineered hepatic tissues was completely controlled by the self-organization of the three cell types. Cellular morphology and overall tissue architecture were observed in 3D through the use of a confocal microscope (Carl Zeiss Inc., LSM 5 Live, 20X objective, 405, 488 and 532 nm excitations) after tissue staining. After a given amount of time in culture (~ 1 week), the hepatic micro-tissues were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. After three 10 min rinses in PBS (pH 7.4), gels were incubated in a Triton X 100 solution (0.5% in PBS) for 15 min at room temperature, in order to permeabilized the encapsulated cells. After three 10 minute rinses in PBS, the hydrogels were blocked in a donkey serum solution (5% in PBS) overnight at 4°C. Primary antibodies were selected to bind protein markers in the three cell types. HUVECs express platelet endothelial cell adhesion molecule (PECAM, also known as CD-31) on the cell surface, differentiated 10T½ cells express α-smooth muscle actin (α-SMA) intracellularly, and HepG2 cells express FoxA2/HNF3β in the nucleus. The PECAM antibody (goat anti-human, Santa Cruz Biotechnology, Dallas, TX) was diluted at 1:200, the α-SMA antibody (rat anti-mouse, Sigma-Aldrich, St. Louis, MO) at 1:300, and the FoxA2/HNF3β antibody (rabbit anti-human, Cell Signaling Technology, Danvers, MA) at 1:500 in a donkey serum solution (1% in PBS) and added to the gels. Gels were incubated in the primary antibody solution for 24 hr at 4°C. Gels were then rinsed in PBS 4 times, at 1 hr per rinse. Secondary antibodies (Life Technologies, Grand Island, NY) coupled to Alexa Fluor 488 (donkey anti-rabbit), 555 (donkey anti-rat), and 647 (donkey anti-goat) dyes were used to stain FoxA2/HNF3β, α-SMA, and PECAM, respectively. The attached fluorophores were chosen to match the excitation peaks of the lasers on the confocal microscope (488, 532, and 635 nm). After 3 final rinses in PBS, the
micro-tissues were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 2µM) to label nuclei and imaged on the confocal microscope. At least three representative stacks of images were taken of each micro-tissue, at randomly selected locations. Both fluorescent (immunostaining and DAPI) and differential interference contrast (DIC) images were acquired at each location. Image stacks were acquired in the z-dimension (along axis of illumination) with 1-µm spacing between each image.

The images acquired with the confocal microscope were analyzed both qualitatively and quantitatively. First, qualitative analysis of the immunostained micro-tissues enabled the characterization of the tissue architecture that occurs spontaneously when the HUVEC-10T½-HepG2 tri-culture is encapsulated in an enzymatically degradable PEG hydrogel. Images were evaluated for examples of interesting cell morphologies (e.g. relative spreading of HepG2 cells as compared to vascular cells), heterotypic cell-cell interactions (e.g. endothelial cell-pericyte relationship), homotypic cell-cell interactions (e.g. aggregation of hepatocytes), and overall architecture of the micro-tissues. Next, quantitative analysis of the PECAM staining in the co-culture and tri-culture hydrogels was performed in order to determine the effects of HepG2 inclusion on tubule formation. Vessel density was quantified by observing the fraction of pixels that stained positive for PECAM. Vessel densities were compared between co-culture and tri-culture gels using an unpaired student’s t-test.

3.2.5 Two-dimensional Culture Studies with Primary Hepatocytes

HUVECs, 10T½ cells, and a 4:1 ratio of HUVECs to 10T½ cells were plated in individual wells of a 24-well cell culture-treated plate (Invitrogen) at total cell concentrations of 2x10^5 cells/well. These cells were cultured in endothelial growth medium and allowed to grow
until sufficient matrix had been deposited to allow for primary hepatocyte culture in these wells (roughly 3.5 weeks). At this point, primary rat hepatocytes (Invitrogen) were seeded in these wells at a concentration of $4 \times 10^5$ cells/well, per the vendor’s protocol. Hepatocytes were also plated in wells coated with type I collagen (BD Biosciences) as a control, as these surfaces were recommended by the cell vendor for primary hepatocyte culture. After six hours in culture, hepatocyte plating medium was aspirated and replaced with serum-free growth medium.

After hepatocytes were seeded on the cell-secreted matrix and collagen surfaces, they were maintained in culture for approximately three weeks in hepatocyte growth medium (Invitrogen). During this culture period, the CYP activity and albumin synthesis of hepatocytes were monitored in order to evaluate hepatocyte bioactivity. A CYP substrate, EFC, was used to evaluate the enzymatic activity of the hepatocytes, as deethyla
tion of EFC by CYP enzymes results in a fluorescent byproduct, 7-hydroxy-4-trifluoromethylcoumarin (HFC), that can be measured using a fluorimeter (ex. 385 nm, em. 502 nm). Every 3-4 days EFC was added to the culture medium at a concentration of 100 μM, and, after three hours of cell exposure to EFC, the culture media samples were collected and evaluated using a fluorometric plate reader (Molecular Devices, Sunnyvale, CA). Absorbance values were compared to a standard curve of known HFC concentrations, in order to determine the concentration of the HFC metabolite formed by the hepatocytes in culture. Separate culture medium aliquots were taken every 4-5 days, in the absence of EFC, and were assayed for albumin content using an ELISA kit (Innovative Research, Novi, MI). CYP enzyme and albumin synthesis data were collected over the course of approximately three weeks for each of the cellular substrate groups and the collagen control.
3.2.6 Three-dimensional Culture Studies with Primary Hepatocytes

Micro-tissues were prepared in a similar fashion to those prepared in the HepG2 studies. HUVECs and 10T½ cells were maintained in culture and trypsinized just prior to use. Hydrogel precursor solutions were prepared by dissolving PEG-PQ-PEG at 50 mg/mL and PEG-RGDS at 14 mg/mL in HEPES-buffered saline with TEOA (15 μL/mL), eosin Y photoinitiator (10 μM), and NVP (3.5 μL/mL). HUVECs were added to the hydrogel precursor solution at a concentration of 2.4x10⁷ cells/mL, 10T½ cells at 6x10⁶ cells/mL, and primary hepatocytes at 3x10⁶ cells/mL. Hepatocyte mono-culture and HUVEC-10T½ co-culture precursors were also prepared using the same concentrations of each cell type. Hydrogels were formed by pipetting 10μL of precursor-cell suspension between a methacrylated coverslip and a glass slide and then using white light to photopolymerize the solution. Hydrogels (n = 4 per cell group) were immediately transferred to a 24-well plate, immersed in culture medium, and maintained at 37°C and 5% CO₂.

After cells were encapsulated in the bioactive hydrogels, they were maintained in culture for approximately three weeks. During this culture period, the CYP activity and albumin synthesis of encapsulated hepatocytes were monitored in order to evaluate hepatocyte bioactivity. These assays were performed using the same protocols described for the two-dimensional assays, with media samples collected and analyzed every 3-5 days.

In addition to functional assays, cellular morphology and the overall structure of the engineered hepatic micro-tissues were of great interest. The architecture of the engineered hepatic tissues was completely controlled by the self-organization of the three cell types. After 7 days in culture, cellular hydrogels were fixed in 4% PFA, permeabilized in 0.5% Triton X 100, and blocked in 5% donkey serum. Incubations with primary and secondary antibodies were
performed subsequently in 1% donkey serum. The primary antibodies used were goat anti-PECAM (Santa Cruz Biotechnology, Santa Cruz, CA), used to label the cell membranes of HUVECs, and rabbit anti-FoxA2/HNF3β (Cell Signaling Technology, Danvers, MA), used to label the nuclei of hepatocytes. The secondary antibodies used were donkey anti-goat coupled to Alexafluor 647 and donkey anti-rabbit coupled to Alexafluor 488. Images were obtained using a confocal microscope (Zeiss LSM 5-LIVE) equipped with a 20X objective (NA 0.8). Fields of view measuring approximately 0.1 mm² in area were obtained in vertical z-stacks, with approximately 75 slices obtained with 1-μm spacing between slices. Final images were obtained by generating maximum intensity projections of these z-stacks using ImageJ software.

3.2.7 Statistical Methods

Statistical comparisons between control and experimental groups in two-dimensional and three-dimensional CYP activity and albumin synthesis assays were made using unpaired t-tests, accounting for multiple comparisons using the Bonferroni correction. In order to compare all control and experimental groups simultaneously for the two-dimensional assays involving primary hepatocytes, one-way AVOVA tests were performed with Tukey’s post hoc analysis.

3.3 Results and Discussion

3.3.1 2D and 3D Cell Culture Studies with HepG2 Cells

The HUVEC-10T½-HepG2 tri-culture was established and culture conditions were determined that best suit this tri-culture. In this section, preliminary data involving this novel tri-culture are presented, demonstrating the potential for using cell-formed microvascular networks to promote transport in engineered tissues containing parenchymal cells. These preliminary
studies set out to establish the plausibility of the simultaneous culture of these three cell types, while maintaining their individual functions.

As a first step, a multitude of *in vitro* tests were performed in order to begin to understand the effects of the different cell types on each other and to establish culture conditions that best suit the HUVEC-10T½-HepG2 tri-culture. Figure 3-4 depicts the relative CYP activity of HepG2 cells, both in 2D culture on TCPS and in 3D culture in cell-adhesive PEG hydrogels, in the presence of non-parenchymal cell types. Inclusion of HUVECs and 10T½ cells led to increased CYP activity of HepG2 cells cultured on TCPS. Inclusion of 3T3 fibroblasts had no significant effects on CYP activity of HepG2 cells. Statistical comparisons between groups were made using unpaired student’s *t*-tests, accounting for the Bonferroni correction. These results demonstrate potential functional benefits of culturing hepatocytes with non-parenchymal cells (HUVECs and 10T½ cells). Hepatocytes are known to depend heavily on interactions with other cell types found in hepatic tissue^{91,95,132,134,136,149}; therefore, these results are not surprising.

![Graph showing EFC metabolism by HepG2 cells](image)

**Figure 3-4: EFC metabolism by HepG2 cells.** Relative CYP enzyme activity of HepG2 cells was measured after 24 hours in 2D cell culture on TCPS and after 24 hours in 3D culture in cell-adhesive PEG hydrogels. Tri-culturing HepG2 cells with HUVECs and 10T½ cells led to increased CYP activity on TCPS (*p < 0.05*). Co-culturing HepG2 cells with 3T3 fibroblasts had no significant effect.
Continuing with *in vitro* evaluation of the HUVEC-10T½-HepG2 tri-culture, different media conditions were tested on these cells cultured in 2D on TCPS. Various blends of MEM and EGM were used for the HUVEC-10T½-HepG2 tri-culture, and media samples were collected after 24 hours and assayed to determine CYP activity of HepG2 cells (Figure 3-5). The HUVEC-10T½ co-culture was used as a negative control. The results demonstrate that increasing the concentration of MEM in the culture medium significantly increases the CYP activity of HepG2 cells, in a dose dependent fashion. These results agree with the literature, as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), which are both components of EGM, have been shown to decrease CYP gene expression in hepatocytes\textsuperscript{175,176}. The results of this study demonstrate that some of the components of EGM may be detrimental to HepG2 function, which presents difficulty for the HUVEC-10T½-HepG2 tri-culture, as these medium components are needed to drive tubule formation by the HUVECs and 10T½ cells.

![Figure 3-5: Effect of EGM on HepG2 bioactivity.](image)

Relative EFC metabolism of the HepG2-HUVEC-10T½ tri-culture was measured after 24 hours in 2D cell culture on TCPS, using different blends of HepG2 culture medium (MEM) and HUVEC culture medium (EGM). Increasing the amount of EGM present in culture medium diminished the cytochrome P450 activity of HepG2 cells (p < 0.01). HUVEC-10T½ co-culture served as a negative control and demonstrated negligible EFC metabolism.
In order to overcome the difficulty presented by the differential effects of EGM observed for HepG2 cells and the tubulogenic co-culture, a study was performed using transient media conditions to demonstrate the recovery of HepG2 function after initial culture in EGM. Once again, CYP activity of HepG2 cells was used as a benchmark for cellular function. In this study, the HUVEC-10T½-HepG2 tri-culture was seeded and cultured for 72 hours in MEM, EGM, or a 50/50 blend of the two media types. Culture in EGM led to a 90% decrease in HepG2 CYP activity after 72 hours in culture, as compared to culture in MEM. At this point media was changed for all cultures, with half of the cultures that had previously been cultured in EGM remaining in EGM and all other cultures remaining in or switching to MEM. Within the next 72 hours, cultures with previously diminished CYP activity fully recovered when cultured in MEM. In fact, after these 72 hours, cultures that had begun under tubulogenic media conditions demonstrated improved CYP activity, as compared to cultures that had remained in MEM for the entire 6 days of the study. This suggests that the formation of tubule-like structures by the HUVECs and 10T½ cells may enhance the bioactivity of HepG2 cells. Figure 3-6 depicts the results of this study.

### 3.3.2 Vascularized Hepatic Micro-tissues with HepG2 Cells

Having gained a preliminary understanding of the HepG2-HUVEC-10T½ tri-culture and appropriate culture conditions for this cellular combination, we set out to engineer vascularized hepatic tissue using these cell types. Micro-tissues (5 μL) were fabricated as described above. Briefly, cells were encapsulated in cell-adhesive, enzymatically degradable PEG hydrogels. HepG2 cell mono-culture, HUVEC-10T½ co-culture, and HepG2-HUVEC-10T½ tri-culture micro-tissues were fabricated and cultured under conditions established in previous in vitro
studies. The media was collected after 1, 2, and 5 days in culture and was analyzed for HepG2 cell albumin synthesis using an ELISA assay. As shown in Figure 3-7, albumin synthesis was not significantly affected by the inclusion of HUVECs and 10T½ cells.

**Figure 3-6: Culture media schemes for hepatic tri-culture.** Relative EFC metabolism of the HepG2-HUVEC-10T½ tri-culture was measured over time in 2D cell culture on TCPS, using different culture media schemes. In some cases, culture media type was changed after 72 hours in culture. At each time point (after initial 72 hours in culture, then at 24, 48, and 72 hours beyond those initial 72 hours), EFC metabolism is normalized to the tri-culture cultured in MEM for the duration of the study. The bars farthest to the left represent the tri-culture cultured in MEM for the entire 6 days of the study. The next group of bars represents cells cultured in EGM for the entire 6 days of the study. The third group of bars represents cells cultured in EGM for the first 72 hours of the study, then switched to MEM for the remaining 3 days. Finally, the last group of bars represents cells cultured in a 50/50 blend of EGM and MEM for the first 72 hours of the study, then switched to MEM for the remaining 3 days. The results demonstrate that EGM or a MEM/EGM blend can be used at the beginning of tri-culture to induce tubule formation, and that MEM can be used later in culture to allow recovery of HepG2 metabolic function.

In addition to functional outputs, the architecture of the engineered tissues was of great interest. In these engineered micro-tissues, the tissue architecture was essentially governed by the behavior of the different cells within the bioactive hydrogel. After one week in culture, the micro-tissues were fixed and immunostained with antibodies to PECAM, α-SMA, and
FoxA2/HNF3β for HUVECs, 10T½ cells, and HepG2 cells, respectively. After immunostaining, the micro-tissues were counterstained with DAPI to label nuclei and imaged on a confocal microscope.

**Figure 3-7: Albumin synthesis by encapsulated HepG2 cells.** Albumin synthesis by HepG2 cells was unaffected by the inclusion of the HUVEC-10T½ co-culture. In both mono- and tri-culture, HepG2 cells synthesized albumin at an approximate rate of 15 ng/day.

Figure 3-8 depicts the spontaneous organization of the HepG2-HUVEC-10T½ tri-culture in an enzymatically degradable PEG hydrogel. The tissue architecture observed in tri-culture gels demonstrates that HepG2 cells form cell clusters, while HUVECs form tubule networks that intertwine these clusters. The results suggest that these cell-formed tubule networks may present a possible mass transport mechanism for delivery of nutrients and waste to and from hepatocytes in our engineered tissues. These results are also quite significant when compared to the literature, as in spite of the tremendous amount of work that has been done involving cell-formed microvascular networks, there has very little work in which these networks have been grown through a parenchymal tissue\(^{46}\). Additionally, the many previous attempts at engineering
vasculature for hepatic tissue engineering have rarely demonstrated such intimate connection and proximity between vasculature and hepatocytes.\textsuperscript{35,37,80,177}

Figure 3-8: Spontaneous organization of HepG2 tri-culture. The HepG2-HUVEC-10T½ tri-culture was encapsulated in an enzymatically degradable PEG hydrogel and cultured for 1 week. The engineered tissue was immunostained and imaged using a confocal microscope. The tissue architecture observed in tri-culture gels demonstrates that HepG2 cells form cell clusters, while HUVECs form tubule networks that intertwine these clusters. HepG2 cells (violet) were identified using an antibody against nuclear marker FoxA2, while HUVECs (green) were identified using a membrane marker, PECAM. Image is a projection of a 60-μm z-stack; SMA and DAPI staining are not pictured.

Figure 3-9 compares the spontaneous organization of the three different types of cellular micro-tissues that were fabricated. HepG2 cells in mono-culture formed relatively small cell clusters (~50 μm diameter), while the clusters formed in tri-culture were much larger (100-200μm diameter). This difference may be due to the enhanced degradation of the hydrogel by the additional cells, or it may be due to effects of these tubulogenic cells on the HepG2 cells. In both co-culture and tri-culture gels, the HUVECs and 10T½ cells formed tubule networks.
Comparisons between these tubule networks were made by quantitatively measuring the vessel densities in both co-culture and tri-culture micro-tissues. The results of this analysis, which are displayed in Figure 3-10, demonstrate essentially identical vessel density (~37%) in co-culture and tri-culture micro-tissues. This demonstrates that the inclusion of HepG2 cells does not significantly affect the tubulogenic nature of the HUVEC-10T½ co-culture. As was observed qualitatively, the quantitative results demonstrate the ability to effectively grow cell-formed tubule networks in an engineered tissue containing functional parenchymal cells. Studies presented later in the thesis (Chapter 5) begin to look at the role of these cell-formed microvascular networks in improving nutrient delivery to functional HepG2 cells.

**Figure 3-9: Spontaneous organization of mono-, co-, and tri-culture micro-tissues (HepG2).** Fluorescent images of immunostained (A) HepG2 cells, (B) HUVEC-10T½ co-culture, and (C) HepG2-HUVEC-10T½ tri-culture were acquired after 1 week encapsulated in enzymatically degradable PEG hydrogels. HepG2 cells formed cell clusters, both in mono-and tri-culture, while the HUVEC-10T½ co-culture formed tubule networks. HepG2 cells (violet) were identified using an antibody against nuclear marker FoxA2, while HUVECs (green) were identified using a membrane marker, PECAM. Images are projections of ~60-μm z-stacks taken with a confocal microscope.
3.3.3 2D and 3D Cell Culture Studies with Primary Hepatocytes

After seeing interesting tissue morphologies but limited functional benefits when tri-culturing HepG2 cells with HUVECs and 10T½ cells, we opted to investigate the combination of the vasculogenic cells with primary hepatocytes. HepG2 cells are typically good functional models of hepatocytes, as they have retained key functions such as CYP enzyme activity and protein synthesis; however, due to their cancerous origin and immortalization, they fail to recapitulate a great deal of primary hepatocyte behavior. Consequently, by working with primary hepatocytes instead of HepG2 cells, we hoped to observe a greater functional dependence on interactions with non-parenchymal cells. As was done for HepG2 cells, primary hepatocytes were combined with the vasculogenic co-culture in both 2D and 3D culture systems in order to probe for the effects of these cellular interactions.

The 2D studies were modeled after previous studies that investigated the role of co-culture in primary hepatocyte function\textsuperscript{133,138}. Rather than seeding primary hepatocytes
simultaneously with non-parenchymal cells, hepatocytes were seeded afterwards. Two-dimensional culture substrates were prepared by seeding non-parenchymal cells (HUVECs, 10T½ cells, and a 4:1 co-culture of these two cell types) in 24-well plates. These cells were grown for approximately 3.5 weeks, allowing them to secrete ample ECM that could serve as a substrate for primary hepatocyte attachment. When primary hepatocytes were seeded on these substrates, they generally exhibited enhanced CYP activity and albumin synthesis, as compared to hepatocytes seeded on type I collagen. This phenomenon was amplified as time in culture increased, suggesting that HUVECs and 10T½ cells may play a role in prolonging the functionality of primary hepatocytes.

Figures 3-11, 3-12, and 3-13 depict the CYP activity of primary hepatocytes seeded on substrates secreted by HUVECs, 10T½ cells, and a 4:1 ratio of HUVECs to 10T½ cells, respectively. In each of these figures, the CYP activity of primary hepatocytes seeded on type I collagen was used as a control and baseline. The CYP activity levels were normalized to the number of cells seeded in each case.
Figure 3-11: Hepatocyte CYP activity on HUVEC-secreted substrates. CYP activity was mostly unchanged in hepatocytes seeded on substrates secreted by HUVECs, as compared to hepatocytes seed on type I collagen substrates. Statistically significant differences, as determined using t-tests and accounting for the Bonferroni correction, are denoted by (*).

Figure 3-12: Hepatocyte CYP activity on 10T½ cell-secreted substrates. At and beyond 10 days in culture, CYP activity was significantly greater in hepatocytes seeded on substrates secreted by 10T½ cells, as compared to hepatocytes seed on type I collagen substrates. Statistically significant differences, as determined using t-tests and accounting for the Bonferroni correction, are denoted by (*).
Figure 3-13: Hepatocyte CYP activity on vasculogenic co-culture-secreted substrates. At and beyond 10 days in culture, CYP activity was significantly greater in hepatocytes seeded on substrates secreted by a 4:1 co-culture of HUVECs to 10T½ cells, as compared to hepatocytes seed on type I collagen substrates. Statistically significant differences, as determined using t-tests and accounting for the Bonferroni correction, are denoted by (*).

For each time point of the 2D CYP activity assay, a one-way ANOVA was performed to compare all of the control and experimental groups. In each case, Tukey’s post hoc test was used to identify significant differences between the groups. Table 3-1 summarizes the results of these statistical tests. A single plus sign (+) is used to indicate significantly greater CYP activity than the collagen control. A double plus sign (++) is used to indicate significantly greater CYP activity than the collagen control and the HUVEC experimental group. A zero (0) indicates no significant difference from the collagen control. The 10T½ and co-culture experimental groups were never significantly different from each other.
Figures 3-14, 3-15, and 3-16 depict the total albumin secretion of primary hepatocytes seeded on substrates secreted by HUVECs, 10T½ cells, and a 4:1 ratio of HUVECs to 10T½ cells, respectively. In each of these figures, the total albumin secretion of primary hepatocytes seeded on type I collagen was used as a control and baseline. The albumin secretion levels were normalized to the number of cells seeded in each case.

![Graph](image)

**Figure 3-14: Hepatocyte albumin secretion on HUVEC-secreted substrates.** At and beyond 13 days in culture, albumin secretion was significantly greater in hepatocytes seeded on substrates secreted by HUVECs, as compared to hepatocytes seeded on type I collagen substrates. Statistically significant differences, as determined using t-tests and accounting for the Bonferroni correction, are denoted by (*).
Figure 3-15: Hepatocyte albumin secretion on 10T½ cell-secreted substrates. At and beyond 13 days in culture, albumin secretion was significantly greater in hepatocytes seeded on substrates secreted by 10T½ cells, as compared to hepatocytes seeded on type I collagen substrates. Statistically significant differences, as determined using *t*-tests and accounting for the Bonferroni correction, are denoted by (*).

Figure 3-16: Hepatocyte albumin secretion on vasculogenic co-culture-secreted substrates. At all observed time points, albumin secretion was significantly greater in hepatocytes seeded on substrates secreted by a 4:1 co-culture of HUVECs to 10T½ cells, as compared to hepatocytes seeded on type I collagen substrates. Statistically significant differences, as determined using *t*-tests and accounting for the Bonferroni correction, are denoted by (*).
For each time point of the 2D albumin secretion assay, a one-way ANOVA with Tukey’s post hoc test was performed to compare all of the control and experimental groups. Table 3-2 summarizes the results of these statistical tests. A single plus sign (+) is used to indicate significantly greater albumin secretion than the collagen control. A double plus sign (++) is used to indicate significantly greater albumin secretion than the collagen control and the HUVEC experimental group. A zero (0) indicates no significant difference from the collagen control. The 10T½ and co-culture experimental groups were never significantly different from each other.

### Table 3-2: Hepatocyte albumin secretion comparisons on 2D substrates

<table>
<thead>
<tr>
<th></th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 13</th>
<th>Day 17</th>
<th>Day 19</th>
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<td>HUVEC</td>
<td>0</td>
<td>0</td>
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<td>Co-culture</td>
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+ Significantly greater than collagen control
++ Significantly greater than collagen control & HUVEC group

The 2D investigation into the effects of the HUVEC-10T½ co-culture on primary hepatocyte function demonstrated that these non-parenchymal cells are capable of improving and prolonging the hallmark functions of primary hepatocytes. When primary hepatocytes were seeded on substrates secreted by 10T½ cells or the HUVEC-10T½ co-culture, CYP activity was maintained at fairly consistent levels over approximately 3 weeks. In comparison, hepatocytes seeded on type I collagen, which is the most commonly used substrate for primary hepatocyte culture, demonstrated decreasing CYP activity, particularly after 1 week in culture. At the final time point (19 days), hepatocytes on the cell-secreted substrates exhibited CYP activity levels more than double the levels of hepatocytes on type I collagen.

The albumin secretion data from the 2D investigation are similarly promising. Primary hepatocytes seeded on cellular substrates produced albumin at more consistent levels over
approximately 3 weeks than hepatocytes seed on type I collagen. Primary hepatocytes seeded on substrates secreted by HUVECs or 10T½ cells exhibited significantly improved albumin secretion over the final 4 time points of the study. Primary hepatocytes seeded on substrates secreted by the HUVEC-10T½ co-culture exhibited significantly improved albumin secretion at all 6 time points. At the final time point (22 days), primary hepatocytes on the cellular substrates had secreted between 2.5 and 4 fold the amount of albumin as hepatocytes on type I collagen. The results of the 2D studies are in agreement with the literature, as the ability of various non-parenchymal cells to both enhance and prolong hepatocyte function in 2D culture is well-established\textsuperscript{91,132,134,176}. Further, albumin secretion in these studies was in agreement with \textit{in vitro} albumin secretion as reported in the literature, which ranges from 1-20 pg/cell/day\textsuperscript{91,132,178}.

After observing significant improvements in primary hepatocytes in response to HUVECs and 10T½ cells in 2D culture, we wanted to investigate if these trends would persist in our bioactive PEG hydrogel system. Much less work has been done regarding the functional benefits of hepatocyte co-culture with non-parenchymal cells in 3D culture, so the expectations were not as clear as in the 2D work. Also, functional benefits were not observed in our 3D studies involving HepG2 cells, but our 2D work demonstrated that primary hepatocyte functions are affected more by the vasculogenic cells than HepG2 cell functions.

The positive effects of the HUVEC-10T½ co-culture on the CYP enzyme activity and albumin synthesis of hepatocytes that were observed in two-dimensions were conserved after transition to a three-dimensional hydrogel platform. Co-encapsulation of hepatocytes with the HUVEC-10T½ co-culture in our cell-adhesive and enzymatically-degradable PEG hydrogel led to enhanced CYP activity and albumin secretion. Figure 3-17 and 3-18 depict the CYP activity and total albumin secretion of primary hepatocytes encapsulated with HUVECs and 10T½ cells.
(tri-culture). Primary hepatocytes encapsulated alone (mono-culture) served as the control. Both CYP activity and total albumin secretion were normalized to the number of hepatocytes encapsulated. HUVECs and 10T½ cells, when encapsulated without hepatocytes, did not register any CYP activity or albumin secretion.

As in the two-dimensional culture study, the enhancement in hepatocyte function was more prevalent as time in culture increased. CYP activity diminished drastically over 3 weeks when hepatocytes were encapsulated alone; however, inclusion of the HUVEC-10T½ co-culture significantly slowed this decline. At the final time point (22 days), CYP activity levels were more than double in the tri-culture gels than in the mono-culture controls. Albumin secretion levels also decelerated over the 3-week study in primary hepatocytes encapsulated alone. Primary hepatocytes encapsulated with the HUVEC-10T½ co-culture secreted albumin at more consistent levels over 3 weeks, producing more than double the total amount of albumin over the course of the study. These results suggest that the inclusion of HUVECs and 10T½ cells in a PEG hydrogel-based approach to hepatic tissue engineering may lead to improved and prolonged functionality of encapsulated hepatocytes.

3.3.4 Vascularized Hepatic Micro-tissues with Primary Hepatocytes

In addition to functional outputs, the architecture of the engineered tissues was of great interest. Hepatic micro-tissues (5 μL) were fabricated in the same manner as in the HepG2 studies and the 3D functional assays for primary hepatocytes. In these engineered micro-tissues, the tissue architecture was essentially governed by the behavior of the different cells within the bioactive hydrogel. After one week in culture, the micro-tissues were fixed and immunostained with antibodies to PECAM, α-SMA, and FoxA2/HNF3β for HUVECs, 10T½ cells, and primary
hepatocytes, respectively. After immunostaining, the micro-tissues were counterstained with DAPI to label nuclei and imaged on a confocal microscope.

Figure 3-17: Hepatocyte CYP activity in mono- and tri-culture hydrogels. At and beyond 16 days in culture, CYP activity was significantly greater in hepatocytes co-encapsulated with a 4:1 co-culture of HUVECs to 10T½ cells in bioactive PEG hydrogels, as compared to hepatocytes encapsulated alone. Statistically significant differences, as determined using t-tests and accounting for the Bonferroni correction, are denoted by (*).

Figure 3-18: Hepatocyte albumin secretion in mono- and tri-culture hydrogels. At and beyond 10 days in culture, albumin secretion was significantly greater in hepatocytes co-encapsulated with a 4:1 co-culture of HUVECs to 10T½ cells in bioactive PEG hydrogels, as compared to hepatocytes encapsulated alone. Statistically significant differences, as determined using t-tests and accounting for the Bonferroni correction, are denoted by (*).
Figure 3-19 depicts the spontaneous organization of the three different types of cellular micro-tissues that were fabricated. Primary hepatocyte cells in mono- and tri-culture appeared healthy but not proliferative, as they did not form clusters as HepG2 cells did. In both co-culture and tri-culture gels, the HUVECs and 10T½ cells formed tubule networks. The microvasculature-like structures that were formed when HUVECs and 10T½ cells were co-encapsulated in cell-degradable and cell-adhesive PEG hydrogels demonstrate great structural similarity to structures that were formed when primary hepatocytes were encapsulated in conjunction with these two cell types. This demonstrates that the inclusion of primary hepatocytes did not significantly impede tubule formation by the HUVEC-10T½ co-culture. This adds to our growing evidence that combining this vasculogenic co-culture with a functional parenchymal cell neither prevents the formation of microvasculature-like networks nor does it prevent proper parenchymal cell function.

Figure 3-19: Spontaneous organization of mono-, co-, and tri-culture micro-tissues (primary hepatocytes). Fluorescent images of immunostained(A) primary hepatocytes, (B) HUVEC-10T½ co-culture, and (C) hepatocyte-HUVEC-10T½ tri-culture were acquired after 1 week encapsulation in enzymatically degradable PEG hydrogels. Primary hepatocytes were markedly less proliferative than HepG2 cells, both in mono-and tri-culture, while the HUVEC-10T½ co-culture formed tubule networks. Primary hepatocytes (violet) were identified using an antibody against nuclear marker FoxA2, and HUVECs (green) were identified using a membrane marker, PECAM. Images are projections of ~60-μm z-stacks taken with a confocal microscope.
3.4 Conclusions

In summary, we have established two novel tri-cultures that combine vasculogenic cells with hepatic cells. These tri-cultures are promising for hepatic tissue engineering applications, as they aim to provide hepatocytes with the capillary access and interactions with non-parenchymal cells that direct hepatocyte behaviors in vivo. Cells from the HepG2 cell line showed little functional dependence on the vasculogenic cells; however, the vasculogenic HUVEC-10T½ co-culture had a positive impact on the bioactivity of primary hepatocytes, both in 2D culture and in a 3D hydrogel construct. Most notably, the presence of these non-parenchymal cells seemed to prevent the deterioration of basic hepatocyte functions over time. In our bioactive PEG hydrogel system, the vasculogenic co-culture remained capable of forming microvasculature-like tubule structures in the presence of hepatocytes, which introduces the possibility of growing spontaneously vascularized hepatic tissue, in vitro.

The incorporation of a functional microvasculature into engineered hepatic tissue has significant implications in the integration of hepatic tissue both into living hosts and into microfluidic “liver-on-a-chip” devices. Previous work has demonstrated that microvascular networks grown in a scaffold, in vitro, are capable of anastomosis with host vasculature upon implantation. In addition, it has been shown that microvascular structures formed by a co-culture of HUVECs and 10T½ cells in a bioactive PEG scaffold are capable of anastomosis with microfabricated channels within an adjacent poly(dimethylsiloxane) (PDMS) construct. By using cells to form microvascular networks in immediate proximity to encapsulated hepatocytes, one establishes a de facto transport system that could support hepatocyte function and viability, both in vitro and in vivo. The results of these studies demonstrate a functional benefit of
including vascular cells in engineered hepatic tissue; however, further studies (Chapter 4) begin to address the benefit added by the microvasculature-like structures formed by these cells.
CHAPTER 4 – Dynamic Culture of Engineered Hepatic Tissues

4.1 Introduction

Despite recent improvements in the clinical application of tissue engineered therapeutics, the need for a perfusable vasculature continues to prevent the use of engineered tissues for the replacement of tissues other than thin or sparsely vascularized tissues\(^3,^{10,46}\). Implantation of bulky engineered tissues that lack the mass transport properties to carry nutrients from surrounding vasculature to the construct interior typically develop necrotic cores due to cell death within the construct\(^{179}\). The pre-vascularization of engineered tissues – the \textit{in vitro} formation of networks capable of anastomosis with host vasculature – has shown promise in improving the penetration of nutrients into larger constructs, thus decreasing necrotic core size\(^{46}\). Recent work has demonstrated the integration of cell-formed microvascular networks with prefabricated conduits within a polymer, which exhibits the role that combinatorial pre-vascularization techniques may play in improving mass transport in engineered tissues\(^{45,71}\).

Although culturing cells in a bioactive scaffold in a static 3D environment begins to model native cell behavior, dynamic culture involving controlled nutrient delivery and waste removal from cells provides a more accurate model of \textit{in vivo} phenomena. In the static studies presented earlier (Chapter 3), hepatic micro-tissues were merely submerged in culture medium in well plates. This may significantly diminish the positive effects that cell-formed tubule networks have on hepatocyte function, as the role of tubule networks in distributing nutrients throughout a tissue is not needed in a submerged system. A system in which culture medium is delivered only to the surfaces of engineered tissues will better demonstrate the role that the tubule networks must play: to improve the mass transport throughout the construct such that hepatocytes farther from a nutrient supply may continue to survive and function. Figure 4-1 depicts the differences
between static and dynamic culture, especially the disparate access to culture medium by encapsulated cells. In the studies presented in this chapter, an enzymatically degradable PEG hydrogel, in which the HUVEC-10T½-HepG2 tri-culture was encapsulated, was cultured within a microfluidic device.

**Figure 4-1: Hydrogel culture – static vs. dynamic.** In *vitro* culture of hydrogel micro-tissues is very different in a microfluidic culture system (dynamic) than in a well plate (static). In static culture, the hydrogel (light blue) is completely submerged in culture medium (red); consequently, nutrients freely diffuse throughout the entire scaffold. In our dynamic culture system, the hydrogel (light blue) is exposed to culture medium only at its two interfaces with flow channels (red and dark blue). Nutrients must penetrate the hydrogel from these interfaces in order to reach interior cells. This occurs to a small extent through simple diffusion, and our aim to vascularize engineered tissues ought to improve nutrient perfusion into the hydrogel tissues.

In this chapter, we present data related to the dynamic culture of hepatic micro-tissues formed in cell adhesive and degradable PEG hydrogels. We demonstrate that the tubule networks formed by HUVECs and 10T½ cells are capable of improving nutrient perfusion into the hydrogel tissues and thus improve the viability of encapsulated HepG2 cells at significant distances from the culture medium channels. In addition, we observe the tissue architecture spontaneously formed under dynamic culture of the HUVEC-10T½-HepG2 tri-culture hydrogel.
Finally, our promising results exhibit that an approach to tissue prevascularization that combines cell-formed microvasculature with prefabricated channels within a polymer may play a role in the scaling up of engineered tissues.

4.2 Methods

4.2.1 Cell Maintenance

HUVECs (Lonza, Walkersville, MD) were cultured in endothelial growth medium 2 (EGM-2, Lonza) supplemented with 2 mM L-glutamine, 1 MU L\(^{-1}\) penicillin, and 100 mg L\(^{-1}\) streptomycin. 10T½ cells (American Type Culture Collection [ATCC], Rockville, MD) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 1 MU L\(^{-1}\) penicillin, and 100 mg L\(^{-1}\) streptomycin. Both cell types were incubated at 37°C and 5% CO\(_2\), with media replacement and subculturing performed as necessary. HUVECs were used for experiments at passages 3-5 and 10T½ cells were used at passages 16-18.

HepG2 cells (ATCC, Rockville, MD) were cultured in Minimum Essential Medium (MEM, Invitrogen, Carlsbad, CA) with 2mM GlutaMAX\(^{TM}\) (L-glutamine dipeptide) supplemented with 10% FBS, 1 MU L\(^{-1}\) penicillin, and 100 mg L\(^{-1}\) streptomycin. HepG2 cells were incubated at 37°C and 5% CO\(_2\), with media replacement and subculturing performed as necessary. HepG2 cells were used for experiments at passages 3-10.

4.2.2 Synthesis of PEG Derivatives

The synthesis of PEG derivatives was described in Chapter 2. Briefly, acrylate-PEG-succinimidyl valerate (acrylate-PEG-SVA) was reacted with several bioactive molecules, which
yielded proteins and peptides attached to monoacrylated PEG chains. PEG-RGDS and enzymatically degradable PEG-PQ-PEG were synthesized as summarized earlier (Chapter 2) and as previously described\textsuperscript{64,159}. PEG-RGDS and PEG-PQ-PEG were purified via dialysis and lyophilized prior to use.

4.2.3 Microfluidic Device Fabrication

A poly(dimethylsiloxane) (PDMS) microfluidic culture device was prepared using standard photolithography and soft lithography. First, photoresist masters were prepared as masters, against which PDMS would be molded to form the dynamic culture chambers. Glass slides were thoroughly cleaned using piranha solution (3:1 solution of H\textsubscript{2}SO\textsubscript{4}:H\textsubscript{2}O\textsubscript{2}) and dehydrated in preparation for spin coating. SU-8 2100 (Microchem, Newton, MA) was applied to the glass slides and spun coat at 1,000 rpm for 40 seconds, which leveled the photoresist at a thickness of approximately 350 μm. A mask aligner (SUSS MicroTec, Garching, Germany) was used to align the photomask depicted in Figure 4-2 and to cure the photoresist in the regions defined by the photomask. Uncured photoresist was thoroughly rinsed away and the final photoresist masters were cleaned with ethanol and dried. Individual photoresist masters were used to prepare up to ten PDMS microfluidic culture chambers.

Figure 4-2: Photomask for PDMS flow device fabrication. This transparency mask was used to generate the silicon master, to which PDMS was molded and cured. Consequently, the structures shown in white above make up the channels, injection ports, and culture chamber present in the final PDMS devices. The channels are 1 mm in diameter, and the culture chamber measures 10 mm x 3 mm.
After creating the photoresist master, PDMS was molded against the master to create a dynamic culture device. First, a PDMS (Sylgard 184) prepolymer was prepared by mixing a silicon elastomer and curing agent in a 15:1 w/w ratio. This prepolymer was then degassed in a vacuum oven, poured over the silicon master, and cured for 3 hours at 60°C. The cured PDMS was then peeled away from the silicon master and trimmed, and access ports were created using a 1 mm biopsy punch. The PDMS culture chamber was then washed thoroughly in ethanol and sealed to a glass slide for storage.

4.2.4 Dynamic Culture of Engineered Hepatic Tissues

The PDMS culture chamber was first treated with photoinitiator (2,2-dimethoxy-2-phenylacetophenone, 300 mg/mL in NVP) for 5 min in order to render the PDMS surfaces conducive to interfacial polymerization of the bioactive PEG hydrogels. This was done in order to allow the fabricated hydrogel tissues to completely span the culture chamber of the PDMS device (i.e. no space left between the hydrogel and the walls of the PDMS culture chamber). The PDMS device was then rinsed in 100% ethanol, rinsed in ultrapure H₂O, and sealed to glass slides that had been previously treated with SigmaCote (Sigma-Aldrich, St. Louis, MO).

A hydrogel precursor solution was be prepared by dissolving PEG-PQ-PEG at 50 mg/mL (5% w/v) and PEG-RGDS at 14 mg/mL (3.5 mM) in PBS with Irgacure 2959 photoinitiator (3 mg/mL). Both HepG2 mono-culture and tri-culture flow studies were performed. In the case of the tri-culture, HUVECs were added to the hydrogel precursor solution at a concentration of 2.4x10⁷ cells/mL, 10T½ cells at 6x10⁶ cells/mL, and HepG2 cells at 1x10⁶ cells/mL. In monoculture flow studies, only HepG2 cells were added to the hydrogel precursor solution. Next, 200 μL of precursor-cell suspension was injected into the PDMS housing, and a simple 1 mm
photomask was aligned with the culture chamber. The precursor solution was then exposed to UV light for 5 minutes, forming a hydrogel slab that spanned the culture chamber. This polymerization event created two channels on opposite sides of the hydrogels that are in series with the microfabricated channels. These channels were each connected to a syringe pump and media reservoir from which flow was initiated at a rate of 10 μL/min. The hydrogel was then cultured for 96 hours under these dynamic conditions. Figure 4-3 depicts a cross-section of the culture device after the flow of medium begins. Figure 4-4 summarizes the fabrication of this dynamic culture system, beginning with the microfabrication process and ending with dynamic culture of an engineered micro-tissue hydrogel. Several flow studies were performed, both for HepG2 mono- and tri-culture, both to confirm that results were consistent and to allow for multiple types of staining and analysis. Additionally, a culture study was performed using the HepG2 mono-culture without flow, by simply filling the channels with culture medium. This study was used as a control for the flow studies.

**Figure 4-3: Schematic of dynamic culture system.** A hydrogel slab was polymerized within a PDMS microfluidic device, forming two channels through which culture media flowed in a countercurrent fashion. On the left, a cross-section of the flow device is depicted (flow directions were in and out of the page). On the right, a bottom view of the flow device is depicted (flow directions were left and right), showing the face of the device that is sealed to glass. In both images, grey sections denote PDMS, red sections are media flow channels, and blue sections are the hepatic micro-tissue hydrogels polymerized between the channels.
A PDMS culture chamber was fabricated using soft lithography. Next, a precursor solution containing proteolytically degradable PEG, PEG tethered RGDS, photoinitiator, and the hepatic tri-culture was injected into the PDMS chamber. A photomask was then aligned and used to crosslink a hydrogel slab, establishing two flow channels between the hydrogel and PDMS. Finally, the gel was rinsed, the device was resealed to glass, and media flow was initiated in the channels.

4.2.5 Viability Staining and Quantification of Dynamically Cultured Engineered Tissues

After 96 hr of dynamic culture, the HepG2 cells encapsulated in the bioactive PEG hydrogels were stained using viability and nuclear markers. First, the PDMS culture chamber was unsealed from the cover glass and inverted so that the hydrogel faced upwards. Next, a
A live/dead staining solution was prepared by combining a live stain (calcein AM, 2 μM) and dead stain (ethidium homodimer-1, 4 μM) in PBS containing DAPI (2 μM). This solution was used to simultaneously label all cell nuclei (via DAPI) and differentiate between live and dead cells. Approximately 200 μL of the live/dead/DAPI solution was pipetted onto the hydrogel, ensuring that the entire gel was submerged. After one hour incubation, the gel was thoroughly rinsed 4-5 times with PBS to remove excess stain. This assay was performed for HepG2 mono-culture flow studies (flow and no flow) as well as the HepG2 tri-culture flow study.

After staining, the gels were imaged using a confocal fluorescence microscope (Carl Zeiss Inc., LSM 5 Live, 20X objective, 405, 488 and 532 nm excitations). For each hydrogel slab, images were acquired and tiled to generate complete cross-sections of the gels, in the direction perpendicular to which flow occurred. Because the gels spanned the 3 mm distance between the flow channels, 10 square images of approximately 300 μm in length/width were captured for each cross-section. Three cross-sections were acquired for each gel, and each was acquired from near the center of the flow device (i.e. far from inlets and outlets of the culture chamber). Figure 4-5 depicts the orientation and location of the images captured of the hydrogel within the microfluidic culture device. All images captured were vertical stacks of approximately 150 μm thickness, with 1 μm spacing between image slices. Each image contained three distinct channels: DAPI, live stain, and dead stain. Projections of z-stack images were prepared using ImageJ (NIH) for image presentation but were not used during the counting of live and dead cells.
For each flow study, viability images were captured along three cross-sections of the cultured hepatic micro-tissue. Ten image stacks, with images roughly 300 μm square, were captured with n = 3 for each flow study. These images were used to determine HepG2 viability as a function of distance from the media channels. Dashed areas alongside the HepG2 micro-tissue depict the surfaces of the hydrogel that were in contact with media flow during the culture period.

Viability levels were calculated by dividing the total number of live HepG2 cells by the total number of HepG2 cells in each image captured. In the HUVEC-10T½-HepG2 micro-tissue, the viability levels of only HepG2 cells were calculated. Due to the high level of apoptosis associated with vasculogenesis during lumen clearing, which overlaps with the time period of culture, viability of the microvasculature-forming cells was not of interest. DAPI staining and DIC images were used in coordination to morphologically identify and count HepG2 cells, and live/dead staining was used to identify each HepG2 cell as either alive or dead. Figure 4-6 depicts the manner in which HepG2 cells were differentiated from tubule-forming cells during the live/dead quantification. These HepG2 viability levels were then compared to the corresponding viability levels from the mono-culture micro-tissue. Statistical comparisons between all viability studies were performed using an unpaired student’s t-test, with significance level of 0.05.
Figure 4-6: Identification of HepG2 cells within the tri-culture. In order to identify HepG2 cells within the tri-culture for viability quantification, several imaging channels were used in coordination. The live stain (left, green) and DIC images (not depicted) were used to morphologically identify tubule structures (examples in violet ovals) and HepG2 clusters (yellow ovals). Dead stain (center, red) was notably high along tubule structures due to the role of apoptosis in tubule formation. DAPI (right, blue) was used to count the total number of HepG2 cells. Note: not all tubule structures and HepG2 clusters are identified above.

4.2.6 Immunohistochemical Staining of Dynamically Cultured Engineered Tissues

Imaging of the immunohistochemical staining was performed in a similar fashion as the live/dead staining, as cross-sections of the cultured hydrogels perpendicular to the direction of flow were compiled (as previously shown in Figure 4-4). The methods and antibodies used for the immunohistochemical staining were identical to those presented in section 3.2.4. Briefly, after 96 hr of dynamic culture, the HepG2 tri-culture hydrogel slab was fixed in 4% paraformaldehyde (PFA) and immunostained. HUVECs were stained using an antibody against PECAM and HepG2 cells using an antibody against FoxA2/HNF3β. Secondary antibodies coupled to Alexa Fluor 488 and 647 dyes were used to best match the excitation peaks of the lasers on the confocal microscope. After immunostaining, the tri-culture hydrogel was imaged on a confocal fluorescence microscope (Carl Zeiss Inc., LSM 5 Live, 20X objective, 488 and 635 nm excitations). Three cross-sections were acquired for each gel, and each was acquired from
near the center of the flow device. All images captured were vertical stacks of approximately 100 μm thickness, with 1 μm spacing between image slices. Projections (maximum pixel value method) of z-stacks were prepared using ImageJ (NIH).

4.3 Results and Discussion

In these studies, HepG2 micro-tissues were polymerized as slabs of 3 mm width, 10 mm length, and approximately 350 μm height within a PDMS microfluidic device. Culture medium was flowed, in a countercurrent manner, along the two long faces of the hydrogel slab, between the hydrogel and the PDMS culture chamber. The other four faces of the hydrogel slab were directly in contact with the PDMS chamber. Consequently, the only sources of nutrients available to the cells in the hydrogel slab were the two streams of medium. Cells at the interior of the hydrogel were as far as 1.5 mm from a nutrient source, which is much too far for the simple diffusion of nutrients to support cell survival and function. Accordingly, convective mass transport must occur through cell-formed tubule networks in order for these interior cells to survive. HepG2 mono- and tri-culture flow studies were performed, in addition to a study in the absence of flow.

4.3.1 Viability of HepG2 Cells in a Bioactive PEG Hydrogel After Static Mono-culture

As a control, HepG2 cells were encapsulated in a degradable and cell-adhesive PEG hydrogel within the PDMS microfluidic device and cultured for 96 hours in the absence of flow. Flow channels were filled with culture medium but no flow was initiated. The lack of flow prevented the replacement of nutrient-depleted culture media with fresh media, and diffusion of nutrients into the gel occurs more slowly in the absence of perpendicular flow. After culture, the
gel was stained using the live/dead/DAPI solution as described above in section 4.2.5. Figure 4-7 depicts the quantification of the live/dead staining for the static HepG2 micro-tissue, as a function of the distance from one of the flow channels.

Figure 4-7: HepG2 viability within a hydrogel cultured in a PDMS culture chamber without flow. The average viability levels of HepG2 cells in a degradable and cell-adhesive PEG hydrogel were calculated for each 300 μm section of the micro-tissue, beginning at one media channel and progressing towards the next. After 96 hours of culture without flow, HepG2 viability was high (over 80%) in the 300 μm sections of hydrogel adjacent to the media channels, less than 60% in the next 300 μm, and essentially zero at all other locations. Average viability levels are plotted against the midpoints of the imaging sections.

As expected, viability of HepG2 cells within the hydrogel micro-tissues was very low away from the media sources. Viability was high (greater than 80% on average) in the images captured immediately adjacent to each source of media, which corresponds to the first 300 μm of
the hepatic micro-tissue. At distances between 300 and 600 μm from the media sources, average HepG2 viability was just over 50%. At all distances greater than 600 μm from the media sources, HepG2 cells were almost exclusively dead (viability less than 10%). Morphological differences were also apparent as the distance from the media channel was increased. The HepG2 cells nearest the media channels were more likely to proliferate and form clusters; however, this behavior diminished significantly away from the media channels and was completely absent at distances greater than 600 μm from the media sources. In the next study performed, flow was initiated in the media channels and carried out throughout the 96 hours of culture. The flow served to both replenish the nutrients in the culture medium and drive diffusion of these nutrients into the HepG2 micro-tissue.

4.3.2 Viability of HepG2 Cells in a Bioactive PEG Hydrogel After Dynamic Mono-culture

The first experimental flow study performed involved the encapsulation of HepG2 cells alone in a degradable and cell-adhesive PEG hydrogel between two counter-current media channels. Dynamic culture was carried out for 96 hours at media flow rates of 10 μL/min. After culture, the gel was stained using the live/dead/DAPI solution as described above in section 4.2.5. Figure 4-8 depicts the live/dead staining in one cross-section of the dynamic HepG2 micro-tissue, spanning from one flow channel to the other. Figure 4-9 displays the live/dead quantification for the dynamic HepG2 micro-tissue, as a function of the distance from one of the flow channels. The no-flow control viability is included on this figure as a control.
Figure 4-8: Live/dead cross-section of HepG2 cells within a dynamically cultured hydrogel. Ten projections of viability image stacks were combined to depict the live/dead staining of one cross-section of the HepG2 micro-tissue hydrogel after 96 hours of dynamic culture. The media channels ran along the left and right sides of the panel, and the scale bar denotes 150 μm.

Figure 4-9: HepG2 viability within a dynamically cultured hydrogel. The average viability levels of HepG2 cells in a degradable and cell-adhesive PEG hydrogel were calculated for each 300 μm section of the micro-tissue, beginning at one media channel and progressing towards the next. After 96 hours of dynamic culture, HepG2 viability was high (over 70%) in the 600 μm adjacent to the media channels, less than 30% in the next 300 μm, and essentially zero at all other locations. In both the 300-600 μm and 600-900 μm sections, viability was significantly greater than HepG2 cells in corresponding sections of the no-flow control (p < 0.05, t-test). Average viability levels are plotted against the midpoints of the imaging sections.
As in the no-flow control, viability of HepG2 cells within the hydrogel micro-tissues was very low far away from the media sources; however, the system with flow supported HepG2 viability over significantly greater distances than the no-flow control. Viability was high (greater than 80% on average) in the images captured immediately adjacent to each source of media, which corresponds to the first 300 μm of the hepatic micro-tissue. At distances between 300 and 600 μm from the media sources, average HepG2 viability remained greater than 70% and was significantly greater than the corresponding cells in the no-flow control. Some HepG2 cells remained viable at distances between 600 and 900 μm from the media sources (roughly 25% viability), which again was significantly greater than the corresponding cells in the no-flow control. At distances greater than 900 μm from the media sources, HepG2 cells were almost exclusively dead (viability less than 10%). Morphological differences were also apparent as the distance from the media channel was increased. The HepG2 cells nearest the media channels were more likely to proliferate and form clusters, as this behavior was most prevalent within 600 μm of the media channels. This clustering diminished significantly away from the media channels and was completely absent at distances greater than 750 μm from the media sources.

The results of the dynamic culture study involving a HepG2 mono-culture micro-tissue demonstrate the role of flow in the delivery of nutrients to tissue beds. However, in the absence of a functional microvasculature, delivery of these nutrients is wholly dependent on diffusion away from the fabricated channels and into the hydrogels. In the next study performed, a HUVEC-10T½-HepG2 tri-culture micro-tissue was cultured under these dynamic conditions in order to demonstrate the role of functional microvascular structures in improving mass transport within an engineered tissue.
4.3.3 Viability of HepG2 Cells in Bioactive PEG Hydrogel After Dynamic Tri-culture

The final experimental flow study performed involved the encapsulation of the HUVEC-10T½-HepG2 tri-culture in a degradable and cell-adhesive PEG hydrogel between two counter-current media channels. Dynamic culture was carried out for 96 hours at media flow rates of 10 μL/min. After culture, the gel was stained using the live/dead/DAPI solution as described above in section 4.2.5. Figure 4-10 depicts the live/dead staining in one cross-section of the dynamic HepG2 micro-tissue, spanning from one flow channel to the other. Figure 4-11 displays the quantification of the live/dead staining for the dynamic HepG2 micro-tissue as a function of the distance from one of the flow channels. The HepG2 mono-culture viability data is included in this figure as a control.

Figure 4-10: Live/dead cross-section of the tri-culture within a dynamically cultured hydrogel. Ten projections of viability confocal image stacks were combined to depict the live/dead staining of one transverse cross-section of the HUVEC-10T½-HepG2 micro-tissue hydrogel after 96 hours of dynamic culture. The media channels ran along the left and right sides of the panel, and the scale bar denotes 150 μm.
The average viability levels of HepG2 cells co-encapsulated with the HUVEC-10T½ co-culture in a degradable and cell-adhesive PEG hydrogel were calculated for each 300 μm section of the micro-tissue, beginning at one media channel and progressing towards the next. After 96 hours of dynamic culture, HepG2 viability was high (over 75%) in the 900 μm adjacent to the media channels, less than 30% in the next 300 μm, and essentially zero in the middle 600 μm. In the 300-600 μm, 600-900 μm, and 900-1200 μm sections, viability was significantly greater than HepG2 cells in corresponding sections of the mono-culture control (p < 0.05, t-test). Average viability levels are plotted against the midpoints of the imaging sections.

The results of the dynamic culture study involving the HUVEC-10T½-HepG2 tri-culture micro-tissue demonstrate the role of functional microvascular structures in the delivery of nutrients to tissue beds. As in the HepG2 mono-culture flow study, viability of HepG2 cells within the hydrogel micro-tissues was high (greater than 80% on average) in the images captured immediately adjacent to each source of media, which corresponds to the first 300 μm of the hepatic micro-tissue. At distances between 300 and 600 μm from the media channels, average
HepG2 viability remained greater than 80% and was significantly greater than the corresponding cells in the HepG2 mono-culture micro-tissue. Unlike the HepG2 mono-culture study, HepG2 cells in the tri-culture hydrogel remained largely viable (roughly 80%) at distances between 600 and 900 μm from the media channels, which again was significantly greater than the corresponding cells in the mono-culture micro-tissue. At distances between 900 and 1200 μm from the media channels, some HepG2 cells in the tri-culture micro-tissue remained viable (roughly 29%). This again was significantly greater than the corresponding cells in the mono-culture micro-tissue, which were essentially all dead at these distances from the media channels. At distances greater than 1200 μm from the media sources, HepG2 cells in the tri-culture micro-tissue were almost exclusively dead (viability less than 10%).

The results of the three viability studies within the PDMS culture device demonstrate the role of fluid flow in the delivery of nutrients to an engineered tissue. HepG2 cells in a degradable and cell-adhesive PEG hydrogel remained viable over greater distances from media sources when flow was present in the device. Viability gains were more pronounced when the HUVEC-10T½ co-culture was co-encapsulated with HepG2 cells, which allowed microvascular structures to form that facilitated nutrient flow into the engineered tissue. In fact, the distance over which HepG2 viability was supported in the hydrogel micro-tissues that included the HUVEC-10T½ co-culture (roughly 1 mm) was essentially double the distance over which HepG2 viability was supported in the no-flow control. This result exhibits that improving the mobility of culture medium (or blood in the case of implantation) significantly increases the sizes of tissue engineered constructs that can remain viable and provides important design criteria for construction of future engineered hepatic tissues.
4.3.4 Organization of Dynamic Tri-culture Tissues

After completing the viability analysis of the HepG2 micro-tissue dynamic culture studies, an additional tri-culture flow study was performed in order to observe the organization of the tissue formed in the hydrogel. While tubule and hepatic structures were clearly visible in the viability staining, it was difficult to observe the tissue structure using the live/dead stain alone. Consequently, after 96 hours of dynamic culture, a HUVEC-10T½-HepG2 tri-culture hydrogel was fixed and immunostained as described in the methods. A confocal fluorescence microscope was used to capture image stacks across an entire cross-section of the hydrogel, similar to the fashion in which viability images were captured. Figure 4-12 depicts the fluorescent images that were captured of the tri-culture micro-tissue after dynamic culture. An image captured of an area of the hydrogel ranging from 600 to 900 μm from a media channel is shown in greater detail, as it demonstrates that tubule formation and HepG2 clustering are still supported at these greater distances from the nutrient source.

4.4 Conclusions

The studies presented in this chapter add to the growing evidence that a combination of prefabricated and cell-grown networks may be capable of supporting larger tissue engineered constructs upon implantation. Flow through prefabricated channels (those molded in PDMS in these studies) was shown to drive diffusion into adjacent tissue beds (HepG2 micro-tissues in these studies). Furthermore, the inclusion of the HUVEC-10T½ co-culture in the HepG2 micro-tissues supported HepG2 viability out to approximately 1 mm from the media channels. This result suggests that the tubule structures formed by this co-culture are capable of connecting to
prefabricated conduits and transporting oxygen and nutrients into the tissue bed as previous studies have suggested\textsuperscript{45,71}.

![Figure 4-12: Immunostained cross-section of the tri-culture within a dynamically cultured hydrogel. Ten projections of image stacks were combined to depict the immunostaining of one cross-section of the HUVEC-10T½-HepG2 micro-tissue hydrogel after 96 hours of dynamic culture (bottom). The violet stain depicts HepG2 cells (FoxA2) and the green stain marked HUVECs (PECAM). A single image is shown in greater detail below, demonstrating the formation of tubule structures and HepG2 clusters at distances between 600 and 900 µm from the media channel. The media channels ran along the left and right sides of the panel, and the bottom scale bar denotes 150 µm.](image)

Studies presented earlier in the thesis demonstrated the functional benefits of co-encapsulating hepatocytes with the HUVEC-10T½ co-culture in bulk hydrogel studies. These studies demonstrated that the heterotypic cell-cell interactions were in themselves beneficial. The dynamic studies performed in this chapter exhibit the other major benefit of the novel tri-culture system: the HUVEC-10T½ co-culture generated microvascular structures that allowed HepG2 viability to persist at greater distances away from a nutrient source. Future studies should begin
to look at implanting combinations of fabricated channels and cell-grown microvascular networks \textit{in vivo} in order to study the size scales over which tissue engineered constructs can remain viable and functional.
CHAPTER 5 - Development of a Dynamically Tunable Hydrogel for Post-encapsulation Ligand Presentation

5.1 Introduction

Tissue engineering efforts that aim to combine multiple cell types in order to form complex tissues, such as our efforts to create vascularized hepatic tissue, are constrained by need for separate cues for different cell types and desired behaviors. For instance, developing vasculature and parenchyma requires different sets of cues, which differ both in identity and the time frame in which they ought to be delivered. These constraints create the need for biomaterials that are more active and controllable. For instance, biomaterials that can be used to alter the type, onset, and duration of biological ligand presentation would be useful for tissue engineers.

Poly (ethylene glycol) (PEG) hydrogels offer several advantages for tissue engineering research in comparison to scaffolds consisting of natural proteins such as collagen. Perhaps most notably, PEG hydrogels allow researchers to precisely control the presentation of biological molecules to encapsulated cells. By coupling biological molecules to PEG chains and crosslinking these modified PEG chains into bulk hydrogels, cells can be encapsulated in PEG hydrogels that are modified with covalently tethered bioactive molecules.

Cells encapsulated in PEG hydrogels can be exposed to biologically active molecules in two distinct fashions. First, soluble proteins or peptides can be added to culture medium and allowed to diffuse into the hydrogel. Second, proteins or peptides can be covalently tethered into the hydrogel network. Previous studies have shown that cells react differently to soluble and tethered proteins. Proteins and peptides tethered to hydrogels are better analogs of biological
cues present in the extracellular matrix. Tethered molecules have also been shown to have prolonged bioactivity, as compared to soluble factors dissolved in culture medium\textsuperscript{160,180}.

The incorporation of tethered proteins into PEG hydrogels is typically performed concurrently with the initial hydrogel crosslinking. Proteins are PEGylated and then mixed in with the polymer precursor solution, such that they are crosslinked into the matrix upon polymerization. Tethering of different growth factors in PEG matrices has been shown to have significant effects on encapsulated cells, such as the stimulation of endothelial cell tubulogenesis by VEGF\textsuperscript{64,181}. After the initial polymerization event, however, tethering of proteins becomes more complicated. Fewer free acrylate groups are available for subsequent crosslinking reactions and exposing encapsulated cells to additional photoinitiator is not ideal. We set out to develop a platform that would facilitate tethering proteins into PEG hydrogels after the original polymerization event in an efficient and cell compatible manner.

Streptavidin and biotin are used often in biology and engineering due to their high binding affinity to one another\textsuperscript{182,183,184}. Streptavidin is a tetrameric protein extracted from bacteria (\textit{Streptomyces avidinii}) with a molecular weight of 52.8 kDa\textsuperscript{185}. Biotin, sometimes referred to as vitamin B7 or vitamin H, is a small molecule (244.31 Da) that serves as a coenzyme for carboxylase enzymes involved in the synthesis of fatty acids, amino acids, and sugars\textsuperscript{182}. Streptavidin molecules contain four distinct biotin binding sites (one on each of the four identical monomers that make up the protein), and the dissociation constant for these binding events is roughly $4 \times 10^{-14}$ M. The affinity between streptavidin and biotin is one of the strongest non-covalent interactions found in nature, and this affinity is based on hydrogen bonding, physical structure, and van der Waals interactions\textsuperscript{186}.
In this work, we have developed a PEG hydrogel system that can be modified with bioactive peptides or proteins after the initial polymerization event. This was accomplished by tethering streptavidin within the PEG matrix upon encapsulation. At a later time, biotinylated peptides or proteins were added to buffer or culture medium and allowed to diffuse into the hydrogel network and bind to the streptavidin molecules. Unbound biotinylated molecules were then rinsed away, leaving a hydrogel with tethered bioactive molecules. Figure 5-1 displays a schematic of the dynamically tunable hydrogel network developed and characterized in these studies. Given the scope of this thesis, we were particularly interested in the use of the biotin/streptavidin platform to delay the delivery of VEGF in order to further control endothelial cell tubulogenesis. Herein, we investigate using temporal VEGF delivery as a cue to initiate the vascularization of PEG hydrogels and engineered hepatic tissue.

![Schematic of dynamically tunable PEG hydrogel](image)

**Figure 5-1:** Schematic of dynamically tunable PEG hydrogel. Tethering PEG-Streptavidin into the degradable (PEG-PQ-PEG) and cell-adhesive (PEG-RGDS) hydrogel system enables biotinylated biomolecules (*e.g.* growth factors) to be incorporated into the matrix after the polymerization and cell encapsulation event. Schematic is not to scale.

## 5.2 Methods

### 5.2.1 Cell Maintenance

HUVECs (Lonza, Walkersville, MD) were cultured in endothelial growth medium 2 (EGM-2, Lonza) supplemented with 2 mM L-glutamine, 1 MU L⁻¹ penicillin, and 100 mg L⁻¹
streptomycin. 10T½ cells (American Type Culture Collection [ATCC], Rockville, MD) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 1 MU L⁻¹ penicillin, and 100 mg L⁻¹ streptomycin. Both cell types were incubated at 37°C and 5% CO₂, with media replacement and subculturing performed as necessary. HUVECs were used for experiments at passages 3-5 and 10T½ cells were used at passages 16-18.

HepG2 cells (ATCC, Rockville, MD) were cultured in Minimum Essential Medium (MEM, Invitrogen, Carlsbad, CA) with 2mM GlutaMAX™ (L-glutamine dipeptide) supplemented with 10% FBS, 1 MU L⁻¹ penicillin, and 100 mg L⁻¹ streptomycin. HepG2 cells were incubated at 37°C and 5% CO₂, with media replacement and subculturing performed as necessary. HepG2 cells were used for experiments at passages 3-10.

5.2.2 Synthesis of PEG Derivatives

The synthesis of PEG derivatives was described in Chapter 2. Briefly, acrylate-PEG-succinimidyl valerate (acrylate-PEG-SVA) was reacted with several bioactive molecules, which yielded proteins and peptides attached to monoacrylated PEG chains. PEG-RGDS and enzymatically degradable PEG-PQ-PEG were synthesized and characterized as summarized earlier (Chapter 2) and as previously described. Streptavidin (Sigma-Aldrich, St. Louis, MO) was also PEGylated via reaction with acrylate-PEG-SVA to form acrylate-PEG-streptavidin (PEG-Strep). This reaction was completed using a 1:3 molar ratio of streptavidin to acrylate-PEG-SVA in order to maximize the number of protein molecules PEGylated without tethering too many PEG chains to a single streptavidin molecule (which could result in decreased biotin affinity). PEGylation of streptavidin was confirmed and characterized via gel
electrophoresis by running the PEG-Strep on a Tris-HCl polyacrylamide gel (4-15%, Biorad, Hercules, CA). PEG-RGDS, PEG-PQ-PEG, and PEG-Strep were purified via dialysis and lyophilized prior to use.

5.2.3 Biotinylation of RGDS and VEGF

The adhesive peptide RGDS and the bioactive protein VEGF were used as model molecules for these studies. In order to tether these bioactive molecules into PEG hydrogels containing streptavidin, they first had to be biotinylated. This was accomplished using a commercially available reagent, EZ-Link NHS-PEG12-Biotin (Thermo Scientific, Rockford, IL), which is depicted in Figure 5-2. Biotinylation was achieved by simply combining this reagent with a protein or peptide in PBS, as the NHS (N-hydroxysuccinimide) ester reacted with amines within the protein or peptide. The reagent provides a 12-unit PEG spacer arm that physically separates the biotin from the protein or peptide target, which helps prevent steric hindrance for the biotin-streptavidin interaction. Biotinylation of RGDS and purity of the dialyzed product were confirmed using GPC, using the apparatus described in Chapter 2 (section 2.3.2). Biotin-RGDS and biotin-VEGF were purified via dialysis and lyophilized prior to use.

![Image of biotinylation reagent](http://www.piercenet.com/browse.cfm?fIdID=01030914)

**Figure 5-2: Biotinylation reagent.** The biotinylation reagent utilizes an NHS ester to couple to amines within bioactive proteins or peptides. A 12-unit PEG spacer physically separates the biotin molecule from its target to prevent steric hindrance upon binding to streptavidin. Image courtesy of Thermo Scientific: (http://www.piercenet.com/browse.cfm?fIdID=01030914).
5.2.4 Fluorescent Tagging of Biotin-RGDS

For proof of concept studies, fluorescent imaging was used to confirm the tethering of biotinylated peptides to PEG matrices containing PEG-Strep. In order to accomplish this, the biotinylated RGDS peptide was fluorescently tagged to allow for visualization within a hydrogel. First, the RGDS peptide was biotinylated as described in the previous section. Next, fluorescent tagging was accomplished using a commercially available reagent, Alexa Fluor 488 carboxylic acid 2,3,5,6-tetrafluorophenyl ester (AF488-TFP, Invitrogen, Carlsbad, CA). AF488-TFP was reacted with biotin-RGDS in dimethylformamide at a 10:1 molar ratio for 2 hr under constant mixing, as previously described, resulting in a fluorescently tagged and biotinylated RGDS peptide (biotin-RGDS-AF488). Biotin-RGDS-AF488 was purified via dialysis and lyophilized prior to use.

5.2.5 Tethering Fluorescent Biotin-RGDS to PEG Hydrogels with PEG-Streptavidin

After biotinylating and fluorescently tagging the RGDS peptide, a proof of concept study was completed to confirm that RGDS could be tethered into a prefabricated PEG hydrogel. Hydrogel precursor solutions were prepared by dissolving PEGDA at 50 mg/mL (5% w/v) and PEG-Strep at 1.27 mg/mL (20 μM) in HEPES buffered saline (HBS, pH 7.4) with triethanolamine (TEOA, 15 μL/mL), eosin Y photoinitiator (10 μM), and N-vinyl-pyrrolidone (NVP, 3.5 μL/mL). Methacrylated coverslips were prepared as previously described (section 3.2.4). Hydrogels were formed by pipetting 5 μL of precursor solution between a methacrylated coverslip and a glass slide and then using white light to photopolymerize the solutions into hydrogels (20 second exposure). Hydrogels were transferred to a 24-well plate and incubated in PBS for 24 hours.
Biotin-RGDS-AF488 solutions were prepared at concentrations of 5, 10, and 20 μM in PBS (pH 7.4). These solutions, along with a PBS negative control, were added to wells within the 24-well plate containing individual PEGDA hydrogels with PEG-Strep (20 μM). The labeled peptides were allowed to diffuse into the hydrogels for one hr under gentle agitation. The hydrogels were then washed four times in PBS to remove unbound peptide. After washing, the gels were imaged using a confocal fluorescence microscope (Carl Zeiss Inc., LSM 5 Live, 10X objective, 488 nm excitation). The average fluorescence intensity of the gels was computed using ImageJ (NIH). Student’s t-tests, accounting for the Bonferroni correction, were used to compare the average fluorescent intensities of the groups.

5.2.6 Testing the Bioactivity of Biotin-VEGF

VEGF was biotinylated as described in section 5.2.3. Next, HUVECs were seeded in standard 24-well plates at a concentration of 10,000 cells/well and allowed 6 hours to adhere. EGM-2 culture medium prepared without VEGF supplementation was used for cell seeding. After 6 hr, biotin-VEGF was added to the culture medium of the experimental group (n=6). Unmodified VEGF was added to culture medium in the positive control wells (n=6), and no VEGF was delivered in the negative controls (n=6). Both biotinylated and unmodified VEGF were delivered at two different concentrations: 10 and 100 ng VEGF / mL. The cells were allowed to proliferate for 24 hr, at which point the nuclei were labeled with DAPI (2 μM). An inverted fluorescence microscope (Zeiss Axiovert 135) was used to acquire several images of the HUVEC nuclei in each well under 10X magnification. These images were used to count the number of cells in experimental and control wells. Finally, the levels of proliferation in the biotin-VEGF and unmodified VEGF groups were normalized to the VEGF-free control to give a
relative measure of the bioactivity of the VEGF treatments. This was accomplished by counting cells to determine the increase in cell number, then normalizing the increase in cell number to the increase that occurred without VEGF (i.e. a value of 1 for the experimental groups would correspond to no change in proliferation in comparison to VEGF free control).

5.2.7 Cell Encapsulation in Degradable PEG Hydrogels with PEG-Streptavidin

After confirming both the ability to tether biotinylated VEGF into the hydrogels and the bioactivity of the biotinylated VEGF, I set out to determine the effects of tethering VEGF into a PEG hydrogel after initial encapsulation of endothelial cells. HUVECs were maintained in culture until encapsulation as described in section 5.2.1. Hydrogel precursor solutions were prepared by dissolving PEG-PQ-PEG at 50 mg/mL (5% w/v), PEG-RGDS at 14 mg/mL (3.5 mM), and PEG-Strep at 1.27 mg/mL (20 μM) in HBS (pH 7.4) with TEOA (15 μL/mL), eosin Y (10 μM), and NVP (3.5 μL/mL). Control precursor solutions were also prepared with all components except PEG-Strep. HUVECs were added to the hydrogel precursor solutions at a concentration of 2.4x10^7 cells/mL. Methacrylated coverslips were prepared as previously described (section 3.2.4). Hydrogels were formed by pipetting 5 μL of precursor-cell suspension between a methacrylated coverslip and a glass slide and then using white light to photopolymerize the solutions into hydrogels (20 second exposure). Hydrogels were transferred to a 24-well plate and cultured in EGM-2 prepared without VEGF supplementation.

Six hr after encapsulation, the culture media on HUVEC-laden hydrogels were supplemented with biotin-VEGF, unmodified VEGF, or a PBS control. Both VEGF groups (biotinylated and unmodified) received a dose of 100 ng/mL. These experimental groups and controls were applied to both hydrogels with and without PEG-Strep in order to investigate the
effects of VEGF that might become physically entrapped within the hydrogel versus that which actually tethers to the hydrogel through the biotin-streptavidin interaction. After 3 hr incubation to allow the VEGF to penetrate the gels, the culture media were replaced with VEGF-free EGM-2. Four washes with this VEGF-free medium were conducted over several hours to ensure unattached VEGF did not remain in the culture media.

After 72 total hr in culture, gels were fixed and immunostained in order to observe tubule formation by the HUVECs. A primary antibody against platelet endothelial cell adhesion molecule (PECAM, also known as CD-31) and a secondary antibody coupled to Alexa Fluor 488 were used to label HUVECs within the gels. After immunostaining, the gels were imaged on a confocal fluorescence microscope (Carl Zeiss Inc., LSM 5 Live, 20X objective, 488 nm excitation). At least three representative stacks of images were taken of each gel, at randomly selected locations. Image stacks were acquired in the z-dimension (along axis of illumination) with 1-μm spacing between each image. Projections of the image stacks were prepared by taking the maximum value of each pixel using ImageJ (NIH).

5.2.8 Delayed VEGF Presentation through Biotin-VEGF Tethering to PEG Hydrogels

After confirming that biotin-VEGF tethered into a degradable and cell-adhesive PEG hydrogel shortly after encapsulation of HUVECs was capable of stimulating HUVECs to form tubule networks, we set out to discover if tubule formation could be stimulated several days after HUVEC encapsulation. The methods described in the previous section were repeated exactly, with one difference. Rather than adding biotin-VEGF to the hydrogels shortly after encapsulation, biotin-VEGF was added on day 4 (unbound growth factor was rinsed away as described in the previous section). Gels were fixed, immunostained, and imaged 72 hours after
delivery of biotin-VEGF, or a total of 7 days after encapsulation. Some gels were fixed and immunostained at day 4 (without biotin-VEGF treatment), to demonstrate the extent of tubule formation and HUVEC spreading present prior to VEGF delivery.

5.2.9 Delayed Tubule Formation Around Hepatic Structures

After demonstrating that tubule formation could be delayed by several days by tethering biotin-VEGF into HUVEC-laden gels 4 days after encapsulation, we hypothesized that these methods may be applied to the hepatic and vasculogenic tri-culture that is central to this thesis. The methods described in the previous section were essentially repeated; however, the HUVEC-10T½-HepG2 tri-culture was encapsulated rather than HUVECs alone. Hydrogel precursor solutions were prepared by dissolving PEG-PQ-PEG, PEG-RGDS, and PEG-Strep in HBS with TEOA, eosin Y, and NVP. HUVECs were added to the hydrogel precursor solutions at a concentration of 2.4×10⁷ cells/mL, 10T½ cells at 6×10⁶ cells/mL, and HepG2 cells at 1×10⁶ cells/mL. Methacrylated coverslips were prepared as previously described (section 3.2.4). Hydrogels were formed by pipetting 5 μL of precursor-cell suspension between a methacrylated coverslip and a glass slide and then using white light to photopolymerize the solutions (20 second exposure). Hydrogels were transferred to a 24-well plate and cultured in a 1:1 ratio of MEM and EGM-2 (without VEGF).

On day 4, the culture media were supplemented with biotin-VEGF (100 ng/mL). After 3 hr incubation to allow the VEGF to penetrate the gels, the culture media were replaced with VEGF-free EGM-2. Four washes with this VEGF-free medium were conducted over several hours to ensure unattached VEGF did not remain in the culture media. After 72 additional hr in culture (day 7 overall), gels were fixed and immunostained in order to observe tubule formation.
and hepatic structures. HUVECs were stained using an antibody against PECAM and HepG2 cells were stained using an antibody against FoxA2/HNF3β, as described in section 3.2.4. Secondary antibodies coupled to Alexa Fluor 488 and 647 dyes were used to best match the excitation peaks of the lasers on the confocal microscope (Carl Zeiss Inc., LSM 5 Live, 20X objective, 488 and 635 nm excitations). After immunostaining, the gels were imaged on a confocal fluorescence microscope. At least three representative stacks of images were taken of each gel, at randomly selected locations. Image stacks were acquired in the z-dimension (along axis of illumination) with 1 μm spacing between each image.

5.3 Results and Discussion

5.3.1 Incorporation of Biotin-RGDS into PEG Hydrogels in a Dose-dependent Fashion

As a proof of concept of the newly developed platform for incorporating biomolecules into PEG hydrogels, a fluorescently tagged RGDS peptide was biotinylated and added to PEGDA hydrogels containing PEG-Strep. After sufficient rinsing, the gels were imaged using a confocal microscope and relative fluorescent measurements were recorded. The results demonstrate that the fluorescent RGDS peptide was tethered into the hydrogel in a dose-dependent fashion via the biotin-streptavidin platform. Figure 5-3 displays the fluorescent images of three hydrogels that were doped with differing concentrations of biotinylated fluorescent RGDS peptide, and Figure 5-4 depicts the fluorescent quantification of these images.
Figure 5.3. Tethering of fluorescent RGDS into PEGDA hydrogels. Biotinylated and fluorescently tagged RGDS was incorporated into PEGDA hydrogels containing PEG-Strep (20 μM) at three different concentrations (5, 10, and 20 μM). Edge artifacts (most apparent in center image) were neglected for quantification.

Figure 5.4. Fluorescent quantification of RGDS tethered into PEGDA hydrogels. Biotinylated and fluorescently tagged RGDS peptide was incorporated into PEGDA hydrogels containing 20 μM PEG-Strep in a dose dependent fashion. All experimental groups are significantly different from all other groups (p < 0.05, t-test). The PEGDA control (red bar) demonstrates that biotinylated RGDS was not significantly retained in hydrogels that did not contain PEG-Strep.

5.3.2 Bioactivity of Biotin-VEGF

Before encapsulating cells in hydrogels modified with PEG-Strep and a tethered growth factor, we set out to confirm growth factor bioactivity using standard 2D culture. After treating
HUVECs with low (10 ng/mL) and high (100 ng VEGF/mL) concentrations of both biotinylated and unmodified VEGF, cells were counted to observe changes in proliferation. The higher concentration of both biotinylated and unmodified VEGF led to significant increases in HUVEC proliferation relative to the VEGF-free control ($p < 0.05$, unpaired $t$-test). Biotinylated and unmodified VEGF were not statistically different from each other at either concentration. The lower VEGF concentrations had no significant effect on HUVEC proliferation. Consequently, the higher biotin-VEGF concentration was used for subsequent studies that investigated the effects of tethered biotin-VEGF on HUVECs encapsulated in PEG hydrogels. Figure 5-5 depicts the factors by which HUVEC proliferation changed in response to both biotinylated and non-biotinylated VEGF, in comparison to controls not treated with VEGF.

**Figure 5-5: Biotin-VEGF enhances HUVEC proliferation.** Both biotinylated and unmodified VEGF at the higher concentration (100 ng/mL) significantly increased HUVEC proliferation over 24 hours ($p < 0.05$, $t$-test), as compared to HUVECs in VEGF-free EGM-2.
5.3.3 Tubule Formation in PEG Hydrogels Modified with Biotin-VEGF

Having demonstrated that the synthesized biotin-VEGF was bioactive (at approximately the same level as unmodified VEGF), we set out to test the effects of tethering this growth factor into PEG hydrogels after the initial photopolymerization event. HUVECs were encapsulated in degradable and cell-adhesive PEG hydrogels, some of which included covalently immobilized PEG-Strep. Shortly thereafter, each gel was subjected to one of three treatments: biotin-VEGF, unmodified VEGF, or PBS. The treatments took place for 3 hr, in order to allow the proteins time to diffuse into the hydrogel, prior to thorough rinsing and returning gels to VEGF-free culture medium. Gels were immunostained and imaged after 3 days in culture.

HUVECs in gels treated with PBS did not demonstrate robust tubule formation, as VEGF is required for the tubulogenesis process. Similarly, HUVECs in gels without PEG-Strep did not demonstrate robust tubule formation, as the biotinylated growth factors were not well retained in the hydrogel after rinsing. HUVECs in gels that had PEG-Strep but received unmodified VEGF treatments also did not demonstrate robust tubule formation, as the VEGF was unable to tether to the hydrogel and was rinsed away. Only HUVECs in gels with PEG-Strep and treated with biotin-VEGF demonstrated robust tubule formation, as the biotin-VEGF was able to remain in the hydrogel after the treatment and rinsing periods due to its strong interaction with the covalently immobilized PEG-Strep. Figure 5-6 demonstrates the proper functioning of the PEG-Strep and biotin-VEGF system, as robust tubule formation only occurred when both molecules were present, allowing the VEGF to become physically tethered within the hydrogel.
HUVECs were encapsulated in cell-degradable and cell-adhesive PEG hydrogels (some also containing PEG-Strep). Robust tubule formation was present after 3 days in culture in gels with PEG-Strep that were treated with biotin-VEGF shortly after encapsulation (left panel). Robust tubule formation was not observed in gels with PEG-Strep that were treated with unmodified VEGF (center) nor in gels without PEG-Strep (right).

5.3.4 Delaying Tubule Formation in PEG Hydrogels via Delayed Biotin-VEGF Tethering

After demonstrating that the addition of biotin-VEGF to bioactive PEG hydrogels containing PEG-Strep led to tubule formation by the HUVECs immediately following encapsulation, we set out to investigate if this tubule formation could be delayed. Given the aim of these studies – to develop a dynamically tunable PEG hydrogel to allow for temporal control over ligand presentation – we were interested in investigating the effect of the vasculogenic growth factor at a later time point. This was accomplished by essentially repeating the methods from the previous study; however, the delivery of biotin-VEGF was delayed until the fourth day of the study. Some gels were fixed, immunostained, and imaged after the delay, to observe the HUVECs prior to biotin-VEGF attachment. The remaining gels were fixed, immunostained, and imaged 72 hours after biotin-VEGF attachment to the PEG-Strep molecules within the gels.
The confocal images captured demonstrate the ability to delay the onset of tubule formation by delaying the tethering of VEGF into the hydrogel. Gels fixed and imaged at day 4, prior to treatment with biotin-VEGF, demonstrated minimal HUVEC spreading and little to no tubule formation. Gels fixed and imaged at day 7, 72 hours after biotin-VEGF was tethered into the gels, however, demonstrated robust tubule network formation by the encapsulated HUVECs. These results demonstrate the success of the developed platform for dynamic addition of bioactive molecules to PEG hydrogels. Figure 5-7 depicts the HUVECs prior to (left) and three days after (right) the tethering of biotin-VEGF into the hydrogels.

![Day 4](image1.png) ![Day 7](image2.png)

**Figure 5-7: Delaying tubulogenesis via delayed tethering of VEGF into hydrogels.** HUVECs (PECAM, green) were encapsulated in cell-degradable and cell-adhesive PEG hydrogels that also contained PEG-Strep. Tubule formation was not present after 4 days in culture in gels cultured in VEGF-free EGM-2 (left panel). Three days after modifying these gels with biotin-VEGF, robust tubule formation was observed in the HUVEC-laden hydrogels (right).

### 5.3.5 Delayed VEGF Tethering to Initiate Tubulogenesis in Hydrogels with Hepatic Cells

After demonstrating that tubule formation by HUVECs could be delayed using the biotin/streptavidin platform for tethering bioactive molecules, we were interested in applying this platform to our efforts in hepatic tissue engineering. Previous studies demonstrated that the
HUVEC-10T½-HepG2 tri-culture spontaneously formed both hepatic and microvascular structures when cultured in degradable and cell-adhesive PEG hydrogels. For some applications, it may prove to be beneficial to grow these structures at different time points. Consequently, the use of the newly developed biotin/streptavidin platform for delayed growth factor delivery was employed to delay tubulogenesis in gels containing the tri-culture.

The previous study, which used only HUVECs, was essentially repeated with all three cell types encapsulated simultaneously. Hydrogels that were fixed, immunostained, and imaged after 4 days of culture in VEGF-free medium exhibited HepG2 clusters but little HUVEC spreading and no tubulogenesis. Gels that were fixed, immunostained, and imaged after 7 days (72 hours after tethering of biotin-VEGF into the hydrogels) of culture demonstrated robust tubule formation around hepatic clusters. The cellular organization in these gels was similar to what was seen when hepatic and microvascular structures were grown simultaneously. These results exhibit the potential to grow microvasculature at later time points in the process of engineering vascularized hepatic tissue. Figure 5-8 depicts the tri-culture hydrogels prior to (left) and three days after (right) the tethering of biotin-VEGF into the gels.

5.4 Conclusions

The methods and results in this chapter have established a platform for tethering biomolecules into PEG hydrogels after the original polymerization event. The combination of PEGylated streptavidin and biotinylated biomolecules allows us to delay the presentation of physically tethered factors to encapsulated cells. Previous studies have shown the benefits of physically tethered biomolecules, as compared to soluble factors; therefore, this platform should retain many of the same advantages in comparison to simply adding biomolecules to culture
media at later time points. There are a number of applications in tissue engineering in which this platform for delayed ligand presentation could prove to be useful. Biological proteins and peptides are used in tissue engineering to elicit desired behaviors from cells; however, stimulating these behaviors at the onset of the *in vitro* culture period (at the point of encapsulation) is not always ideal. For instance, growth factors including VEGF, platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) have been used by tissue engineers to stimulate microvascular network formation by endothelial cells; however, these growth factors may have undesired effects on parenchymal cells such as hepatocytes. FGF has been shown to accelerate hepatocyte proliferation while inhibiting hallmark hepatocyte functions such as cytochrome P450 enzyme activity. This example demonstrates that as tissue engineers increase the complexity of implantable constructs (*e.g.* increase the number of cell types or desired behaviors), precise control over bioactive factor presentation becomes increasingly important.

Specifically, our work has focused on using the developed platform to gain additional control over tubulogenesis by HUVECs and the HUVEC-10T½ co-culture. Our results demonstrate that these cells are responsive to biotinylated growth factors, both in 2D culture and when these biotinylated factors are tethered into a hydrogel network. Tubule formation by HUVECs was stimulated by tethering biotin-VEGF into PEG hydrogels after the encapsulation event, and our results demonstrate that this stimulation could occur immediately after encapsulation or several days later.
Figure 5-8: Delaying tubulogenesis in hydrogels with hepatic cells. HUVECs (PECAM, green), 10T½ cells, and HepG2 cells (FoxA2, violet) were encapsulated in cell-degradable and cell-adhesive PEG hydrogels that also contained PEG-Strep. Tubule formation was not present after 4 days in culture in gels cultured in VEGF-free medium; however, HepG2 clusters were observed (left panel). Three days after modifying these gels with biotin-VEGF, robust tubule formation was observed in the tri-culture hydrogels (right).

The final study of this chapter further demonstrates the flexibility of the hepatic and vasculogenic tri-culture that is central to this thesis. We previously demonstrated that this tri-culture spontaneously forms microvascular structures that surround hepatic clusters when encapsulated in degradable and cell-adhesive PEG hydrogels. The studies from this chapter allowed us to demonstrate that the formation of microvascular structures could be delayed until after hepatic clusters have formed, through the use of PEG-Strep and delayed biotin-VEGF delivery. There may be applications in vascularized tissue engineering in which this could be useful. For instance, when thinking about potentially implanting these hepatic tissues into a host animal, it may be desirable to grow hepatocytes for extended periods prior to implantation. These long culture periods would often come with the risk of tubule network regression; however, using delayed delivery of VEGF, tubule formation could be initiated closer to implantation. To
conclude, the developed platform establishes an additional level of control over the vasculogenic process within PEG hydrogels that we hope to use in future tissue engineered therapeutics. Furthermore, the platform more generally provides tissue engineers with a means to controlling the temporal expression of tethered peptides and growth factors within a construct, which should provide for more nuanced control over cell behavior.
CHAPTER 6 – CONCLUSIONS AND FUTURE DIRECTIONS

The results of the studies presented in this thesis add to the evidence that cell-grown microvascular networks present an opportunity for the scaling up of tissue engineered constructs. Using the liver as a model tissue, we have demonstrated that microvasculature-like networks formed by a vasculogenic co-culture are capable of improving mass transport within a biomimetic PEG hydrogel, such that hepatocyte viability and function can be maintained over greater distances. In addition, we’ve shown that the HUVEC-10T½ co-culture provides beneficial cues that enhance and prolong the function of primary hepatocytes seeded in a PEG hydrogel-based engineered tissue. The results of the studies performed add to the growing evidence that a combination of prefabricated conduits and cell-formed microvasculature will lead to significant increases in the size of engineered tissues that can be implanted and remain wholly viable and functional. Our results establish several future directions in which this work could be taken, including further in vitro scaling up of the engineered hepatic tissues, implantation of the vascularized hepatic hydrogels, and the use of the vasculogenic co-culture to support additional parenchymal cell types.

The dynamic culture studies performed demonstrate that cell-formed tubule networks are capable of significantly improving the distances over which cells can remain viable within a tissue engineered construct. In the presence of the HUVEC-10T½ co-culture, HepG2 cells remained highly viable for several hundred additional microns from the culture media channels, in comparison to non-vascularized controls. Although this seems like a small step forward, these results combined with the efforts of earlier work continue to establish important constraints for engineers attempting to create large-scale perfused tissues. Our results demonstrate that tubules formed by the HUVEC-10T½ co-culture are capable of supporting high parenchymal cell
viability over distances of approximately 1 mm from a media source. This establishes a basis for the spacing of prefabricated channels when combining prefabricated and cell-formed conduits for the purpose of engineered tissue perfusion. New efforts should be made to fabricate scaffolds with conduits spaced such that all encapsulated cells are within 1 mm of a prefabricated conduit. New techniques have recently been developed to accomplish this and have shown promising results. In order to continue the work presented herein, new microfluidic culture systems should be developed that incorporate more channels, possibly in branching patterns to recapitulate vascular trees in the body. In vitro tools should continue to be useful in assaying the extent to which engineered tissues can be scaled up using combinations of prefabricated channels and cell-formed microvessels; however, the ultimate goal remains to implant these larger, vascularized scaffolds to truly analyze the success of these engineered tissues.

The PDMS culture chamber used for the dynamic culture studies presented in this thesis served as an analog for the delivery of blood to tissue beds that occurs in the body. However, in order to truly examine the use of cell-formed microvasculature and prefabricated conduits as a means for perfusing engineered tissues, these constructs must be implanted into hosts. Previous work indicates that the microvascular networks formed by the HUVEC-10T½ co-culture should be capable of anastomosis with host vasculature upon implantation. Future studies should focus on implanting tissues engineered within our bioactive hydrogel system, which incorporate physically fabricated conduits, microvasculature formed in vitro by the HUVEC-10T½ co-culture, and functional hepatocytes. In vivo studies should be designed to assess the success with which these engineered tissues are incorporated into the host. After the implantations, engineered tissues should be explanted at various time points for analysis. This should include sectioning and imaging to determine the extent to which the implanted microvasculature and conduits have
connected to host vasculature. In addition, viability and functional staining should be performed to determine if implanted tissues remain viable throughout the tissue bulk. It’s clear that in vivo studies will be essential for determining the size scales and time frames over which these prevascularized engineered tissues can remain viable and functional after implantation.

The static and dynamic culture studies presented in this thesis exhibit the benefits of combining hepatocytes with a vasculogenic co-culture within a bioactive PEG hydrogel. Through both beneficial heterotypic cell-cell interactions and improved mass transport to hepatocytes throughout a hydrogel scaffold, the vasculogenic HUVEC-10T½ co-culture enhanced our hepatic tissue engineering efforts. Future studies should be performed to determine if these benefits carry over to parenchymal cell types other than hepatocytes. Microvasculature plays an essential role throughout the body and supports bulky parenchyma in the pancreas, lung, and other bulky organs. When investigating the benefits of a vasculogenic co-culture on a new parenchymal tissue, the studies presented in this thesis could rapidly be repeated. First, two-dimensional culture studies should be performed to begin to understand the compatibilities between different cell types, the media conditions needed for multiple cell types, and any functional benefits that may arise due to heterotypic cellular interactions. Next, a new tri-culture (e.g. HUVECs, 10T½ cells, and pancreatic beta cells) should be encapsulated in the bioactive PEG hydrogel system. Ideally, after optimizing the number of cells of each type to encapsulate, the cells will spontaneously form a vascularized and functional tissue, as was the case in our studies involving hepatocytes. Finally, dynamic culture should be used to assess the role of cell-formed microvasculature in changing mass transport in the engineered tissue. In the case of engineered pancreatic tissue, these assays could be very interesting as tools to study how effectively the engineered tissue senses glucose levels and produces insulin. Due to the role of
microvasculature in essentially all functional tissues in the body, the hepatic tri-culture system presented in this thesis could ideally be adapted to fit many diverse tissue engineering efforts.

Future successes in tissue engineering depend on strategies that will allow cells throughout tissue- and organ-sized constructs to remain viable and functional after implantation. Our results and the work of many others have begun to suggest that combinations of polymer fabrication techniques and cellular solutions may provide an answer to this challenge. A combination of larger prefabricated conduits and cell-formed microvasculature has begun to show promise in tissue engineering research\textsuperscript{44,45,71}, as it smartly recapitulates the body’s own vascular structure. Larger vessels are difficult to grow \textit{in vitro} using cellular techniques, so they are replaced with physically fabricated conduits. Microvasculature, on the other hand, is difficult to recreate through physically fabricated polymer channels, thus cellular techniques are used to fill in the spaces between larger conduits. Consequently, these combinatorial or hybrid approaches to vascularization and tissue perfusion represent some of the most promising approaches to successfully engineering large tissue replacements.
CHAPTER 7 – BIBLIOGRAPHY


