Development of an Endothelial Cell Niche in Three-Dimensional Hydrogels

by

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A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy
Department of Chemical Engineering and Applied Chemistry
University of Toronto

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Abstract

Three-dimensional (3D) tissue models have significantly improved our understanding of structure/function relationships and promise to lead to new advances in regenerative medicine. However, despite the expanding diversity of 3D tissue fabrication methods, in vitro approaches for functional assessments have been relatively limited. Herein, we describe the guidance of primary endothelial cells (ECs) in an agarose hydrogel scaffold that is chemically patterned with an immobilized concentration gradient of vascular endothelial growth factor 165 (VEGF165) using multiphoton laser patterning of VEGF165. This is the first demonstration of this patterning technology to immobilize proteins; and the first demonstration of immobilized VEGF165 to guide endothelial cell growth and differentiation in 3D environments. It is particularly compelling that this 3D hydrogels provide an excellent biomimetic environment for stem cell niche, thereby offering a new approach to study stem cell biology. In this thesis, we focused on the retinal stem cell niche, investigating cellular interactions between retinal stem and progenitor cells (RSPCs) and endothelial cells (ECs). By using this 3D in vitro model, we demonstrated the synergistic interactions between RSPCs and ECs wherein RSPCs migrated into 3D gels only in the presence of ECs and RSPCs stabilized EC tubular-like formations. Moreover, we
characterized the contact-mediated effects of ECs on RSPC fate in terms of proliferation and differentiation.
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1 Background and Introduction

(Section 1.6 Aizawa, Y., Owen, S., Shoichet, M.S., Polymer Used to Influence Cell Fate In 3-D Geometry: New Trends, Progress in Polymer Science (Invited review), submitted)

1.1 Overview

Biomaterials provide a technological platform for launching biomedical applications in regenerative medicine. Particularly, the stem cell-based therapy using engineered biomaterials has the potential to significantly improve cellular therapies in tissue engineering. Tissues that can now be engineered using stem cells comprise a diverse range from epithelial surfaces (skin, cornea and mucosal membranes) to skeletal tissues. Despite significant contributions, most applications for complex tissues such as central nervous system have yet to achieve clinical success. A main reason for the lack of successful stem cell therapies is the difficulty in fully recreating the maintenance and control of the native stem cell niche. Therefore, understanding of the cellular mechanisms guiding stem cell behavior in vivo is required to achieve consistent and effective stem cell treatments. Most techniques, however, for uncovering mechanisms controlling cell behavior have been developed using two-dimensional (2D) cell cultures. Considering the cellular functional and morphological difference between 2D and three-dimensional (3D) cultures, it is essential to develop 3D bioactive materials with spatially and temporally controlled patterning of biomolecules like that seen in vivo.

An ongoing research effort in the laboratory of Dr Molly Shoichet focuses on the use of photolabile agarose hydrogels to study cellular behavior of stem cells including retinal stem and progenitor cells (RSPCs) through biomimetic strategies of the stem cell niche in 3D. Recent discovery has demonstrated that endothelial cells (ECs), a key cellular component of blood vessels, play an important role within the stem cell niche, such as that of: neural stem cells
(NSCs), hematopoietic stem cells (HSCs) and adipose stem cells (ASCs). Despite similarities in the structures of capillary vessels within both the retinal stem cell niche to neural stem cell niche, studies of functional interactions between ECs and RSPCs have neither been investigated in vitro nor in vivo. Furthermore, the lack of RSPC-specific markers in vivo hampers functional studies on the retinal vascular niche, that is, it is difficult to detect and/or distinguish RSPCs from other cell types in the ciliary margin. Here, the overarching goal of this project is to develop a 3D co-culture in vitro model to ultimately elucidate the effects of cellular interactions between RSPCs and ECs. In Chapter 2, we developed a 3D culture system using photolabile agarose hydrogels in which a VEGF165 concentration gradient was formed to guide the growth of primary endothelial cells. In Chapter 3, we advanced this system to include the co-culture of endothelial cells with retinal stem/progenitor cells, thereby providing insight into the interactions between ECs and RSPCs, which may be relevant to the stem cell niche.

1.2 Hypothesis and Objectives
The stated hypothesis governing this body of work is:

*Adult mouse brain-derived endothelial cells cultured with adult retinal stem and progenitor cells guide their proliferation and differentiation when co-cultured together in three-dimensional hydrogels.*

To test this hypothesis, the following objectives were set:

1. Develop a three-dimensional in vitro endothelial cell model using photolabile agarose hydrogels.

   - Immobilize GRGDS cell adhesive peptides and VEGF165 gradients within defined volumes of agarose hydrogels using a multiphoton patterning technique.
   - Guide ECs in the patterned agarose hydrogels and evaluate EC growth depending on
concentration gradients of immobilized VEGF165.

2. Develop a 3D co-culture model in order to understand the effects of endothelial cells (ECs) on adult retinal stem and progenitor cells (RSPCs) in 3D environments.

- Evaluate the 3D co-culture model by culturing both RSPCs and ECs on the cell-adhesive, VEGF165 gradient agarose hydrogels.
- Investigate the effects of ECs on RSPC proliferation and differentiation in 3D and 2D.

1.3 Adult Stem Cells in Tissue Engineering

1.3.1 Overview

The term ‘tissue engineering’ represents a concept that focuses on the regeneration of neotissues from cells with the support of biomaterials and growth factors [1]. This interdisciplinary field has attracted much attention as a new therapeutic approach. Although organ transplantation such as heart, liver and kidney is a highly successful therapy for many devastating diseases aimed at replacing lost or severely damaged tissues or organs, problems in current organ transplantation remain because of a shortage of donated organs and immune rejection [2]. In addition, there are many incurable tissue disorders caused by disease or injury, for example, retinal degeneration and spinal cord injury [1, 3]. Therefore, the potential to treat these diseases and physical defects with tissue engineering therapies holds great promise for tissue or organ repair with the ultimate goal to regenerate and restore normal function. Current research in tissue engineering involves a combination approach which integrates cells, scaffolds, cytokines and growth factors. Particularly, stem cell based therapies including embryonic, tissue specific adult, and induced pluripotent stem cells, in tissue engineering hold high therapeutic promise based on the possibility of stimulating their expansion and differentiation into functional progeny that could
regenerate injured tissues and organs in humans. Manipulating stem cells both in vitro and in vivo for tissue or organ repair holds great promise for overcoming supply problems of traditional organ transplantation.

1.3.2 Tissue Specific Adult Stem Cells

Adult stem and progenitor cells in most tissues/organs of mammalian organisms reside in a “niche” and provide critical functions in homeostatic maintenance by replenishing the mature cell types within the tissues in which they reside [4, 5]. Their discovery has sparked great interest and enthusiasm for their use in cellular and tissue engineering therapies [6, 7]. The stringent regulation of the balance between a quiescent and dividing state of adult stem cells is mediated via activation of a complex network of diverse signaling mechanisms[5, 8]. Some of these signaling factors include hormones and a variety of growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), sonic hedgehog, Wnt/β-catenin, Notch, and bone morphogenic proteins (BMPs)[8-10]. These growth factors up-regulate the self-renewal and/or differentiation of adult stem cells under specific physiological and pathological conditions [5, 8]. According to a hierarchical model, the symmetric division of adult stem cells may lead to their expansion within the niches. In contrast, their asymmetric division may generate one stem cell and one more committed proliferative daughter progenitor cells. The transit-amplifying/intermediate cells in turn can give rise to further differentiated cells and terminally differentiated mature cells with specialized functions within the tissue/organ from which they originate [5, 8]. Therefore, understanding of the components of stem cell niches and how the niche translates tissue state into stem cell behavior will provide a rational basis for developing cell-based therapies.
1.3.3 Current Applications and Future Perspectives

Stem cells can be used to restore tissue function either as integrated participants in the target tissue or as vehicles that deliver complex signals to a target tissue without actually integrating into the tissue itself. Transplantation of hematopoietic stem cells (HSCs) is the gold standard for restoring tissue function by engraftment of the stem cells directly into target tissue [4]. Clearly, there are very different requirements for the replacement of cardiac or neural tissue. Replaying morphogenic programs to drive complex tissue relationship is a daunting prospect, casting doubt on these efforts. Recent evidence, however, suggests that engineered scaffolds can assist cell replacement therapy to create tissue constructs [11]. For example, cardiomyocytes assemble into functional units that coordinate synchronous impulse propagation on biocompatible thin films in vitro and can be shaped into 3D structures [12]. Also, matrices can be generated with graded concentration of signaling and tethering molecules that enable heterologous cells to assemble in an organized manner [13]. Thus, cell-intrinsic morphogenic properties and engineered scaffolds to assist them offer the potential for more complex tissue complex tissue reconstruction in the future [14]. However, we need to better understand organ morphogenesis to fully exploit this possibility. The interface of development and stem cell biology with tissue engineering ultimately promises to transform regenerative medicine.

1.4 Cellular Functions of Vasculature

1.4.1 Vascular Development and Angiogenesis

The circulatory system is the first embryonic system to develop, and its correct development and early function is absolutely critical for survival of the embryo. The earliest ECs arise in the yolk sac from a group of cells called angiogenic clusters [15]. These clusters consist of hemangioblasts, which differentiate into hematopoietic cells at the interior and ECs lining the
cluster periphery. Subsequent vessels develop by the distinct processes of vasculogenesis and angiogenesis. Vasculogenesis denotes the *de novo* formation of blood vessels during embryogenesis with progenitor cells migrating to sites of vascularization, differentiating into endothelial cells and organizing the primary capillary plexus. The budding of new capillary branches from pre-existing blood vessels is referred to as angiogenesis, and it involves complex interactions between vascular cells and the corresponding extracellular environment [16]. Angiogenesis mediates vascular development during subsequent stages of embryogenesis, continuing throughout development until the final adult vasculature has formed [17]. As a result of these vascular developmental processes, a network of vessels capable of supporting growth and survival of other newly differentiating tissues is formed. As the embryonic blood vessels become established and various tissues differentiate, ECs continue to undergo numerous changes, generating functionally distinct, tissue-specific vascular beds. ECs of the brain vasculature [18], and to a certain extent, the retinal vasculature [19], form tight junctions that result in the formation of the blood-brain and blood-retinal barriers. These barriers help maintain homeostasis in the neural microenvironment by limiting fluctuations in plasma composition, and by preventing circulating agents such as neurotransmitters and other small molecules from entering the brain or retina and disrupting neural function [18, 19]. The appropriate guidance of vessels and development of these tissue-specific differences is critical for proper tissue function.

The process in vasculogenesis and angiogenesis involves ECs undergoing a series of steps, including degradation of the basement membrane by secreted proteases, migration (sprouting) of the ECs, alignment, proliferation, lumen formation, branching, and anastomosis [20]. Angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are two potent cytokines that are involved during several stages of angiogenesis [21, 22]. One of the most studied factors that controls blood vessel formation and function is vascular
endothelial growth factor A (VEGF-A) [23, 24]. VEGF-A exists in several isoforms which bind with different affinities to VEGF receptors (VEGFRs). VEGF165, in particular, is a critical angiogenic factor [25]. Developing sprouts are composed of ECs with unique phenotypes and respond to VEGF165 quite differently depending on the surrounding microenvironment. The binding of VEGF165 to its receptor, VEGFR2, causes EC proliferation and migration. Tip cells are highly migratory, display numerous filopodia, and have been shown to migrate up a concentration gradient of VEGF165 [25, 26]. Moreover, tip cells are trailed by stalk cells, which proliferate and self-organize to form lumens, when cultured in 3D, in response to VEGF-A [27]. In addition to secreted factors, extracellular matrix (ECM) proteins are also key contributors to vessel growth. ECM ligands bind to specific integrin receptors and promote EC adhesion and migration [28, 29].

1.4.2 Vascular Contributions to Stem Cell Niches

Blood vessels are generally required to provide cells and tissues with oxygen and nutrients. However, the finding that endothelial cells (ECs), a key cellular component of blood vessels, are likely to have fundamental roles in neurogenic niches suggests a special role of vascular cells beyond their role as suppliers of oxygen and nutrients [30]. Due to the significance of endothelial cells in the stem cell niche, a new term “vascular niche” has been created recently and it has been defined as “a microenvironment where endothelial cells affect the behavior of adjacent cells” [30].

Adult neurogenesis is assumed along the lifespan of the organism by multipotent neural stem and progenitor cells (NSPCs) with an astroglia-like cell phenotype found within two specific brain regions: the subventricular zone (SVZ) of lateral ventricle in the forebrain and dentate gyrus of the hippocampus [31]. Interestingly, NSPCs in the hippocampus are found in close proximity to
the tips of capillaries [32], and NSPCs in the SVZ lie adjacent to the ependymal layer that lines the lateral ventricles and blood vessels [33]. These ECs have been demonstrated to express laminin to hold NSPCs and secrete factors such as brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) that promote neuroblast proliferation and/or survival. Furthermore, NSPCs appear to regulate ECs as well. It has been suggested that NSPCs harvested from the ischemic SVZ promote angiogenesis in vitro [34].

Recent studies have shown bi-directional interactions between stem cells and the vascular niche does not only occur in the nervous system. For example, in the HSC niche, ECs influence HSC maintenance by expressing adhesion molecules such as E-selectin, P-selectin and VCAM-1 as well as by secreting chemokines such as stem cell factor (SCF), transforming growth factor-β (TGF-β), interleukin 8 (IL-8) and IL-11 [35-37]. Moreover, the stromal vascular fraction (SVF) of adipose tissue is known to include progenitor cells with extensive plasticity and a capacity to differentiate into various musculoskeletal lineages as well as into neuronal and endothelial cells [38]. In particular, the endothelial component in SVZ was reported to produce neovascularization in an in vivo ischemic model [39], and the human adipose tissue development is associated with extension of the capillary network as well as adipocyte hypertrophy and hyperplasia [40].

1.5 Retinal Stem and Progenitor Cells in Regenerative Medicine

1.5.1 Overview

Diseases of the retina and retinal function can lead to permanent loss of visual function for which there is no definitive treatment. The detrimental impact of vision loss on quality of life and activities of daily living has been well documented and affects the entire age spectrum. Retinitis pigmentosa (RP) affects the pediatric and young adult population, and is the leading cause of inherited retinal degeneration-associated blindness [41]. Irreversible photoreceptor death or loss
of function is common to all of the pathologies including age-related macular degeneration [42] and diabetic retinopathy [43]. Current therapies for vision loss have focused predominantly on pharmacological treatments. While these therapies show promise in limiting the pathophysiologic advancement of the disease, they do not represent a restorative approach. Recent studies of stem cell transplantation shows promise for reconstituting the damaged cellular populations of the retina as a cellular transplantation therapy [44, 45]. One of the key advantages of using stem cells is their potential to differentiate into any type of cell, including retinal neurons and RPE. However, there have been limitations in adult sub-retinal transplantations, including cellular survival and host tissue integration.

1.5.2 Adult Retinal Stem and Progenitor Cells

During embryonic development of the eye, a group of founder cells in the optic vesicle gives rise to multipotent progenitor cells that generate all the neurons and the Muller glia of the mature retina. In most vertebrates, a small group of retinal stem and progenitor cells (RSPCs) persists at the margin of the retina, near the junction with the ciliary epithelium [46-48]. These cells from the ciliary body have been isolated and grown in vitro for extended periods and the cultured cells express many of the markers of retinal progenitors and their progenies express proteins normally present in subtypes of retinal neurons [46-48]. However, it is not clear how these cell types relate to the “founder” cells of the optic vesicle that produce all the progenitors of the retina [3, 46, 47].

When the ciliary body from adult mice is dissociated into single cells by enzymatic treatment and cultured in serum-free medium, cells proliferate and form pigmented sphere colonies regardless of growth factor conditions. These pigmented sphere colonies (PSCs) can be subcloned for at least six generations, which may depend on endogenous FGF2 [46]. When the PSCs are cultured under conditions known to promote retinal cell differentiation, some cells migrate away from PSCs and begin expressing the pan-neuronal marker microtubule-associated
protein 2 (MAP2) and neurofilament, while other cells express the astrocytic maker glial fibrillary acidic protein (GFAP). Later, differentiated cells contain a small number of nestin-positive cells that remain confined to the centres of the colonies and express several markers observed in different differentiated retinal cells: rod photoreceptors (RetP1, ROM-1, 309L, Rho1D4,D2P4) bipolar cells (Protein kinase C, Chx10), retinal ganglion cells (β tubulin) and Muller glia (10E4) [46]. Although these retinal stem cells could provide a potential cell therapy for retinal degeneration, it is known that they have no regenerative capacity in vivo although the retina in cold-blooded vertebrates continues to grow throughout adult life in response to injury by the proliferative addition of new neurons from the germinal zone [49]. The strategies of stimulating endogenous RSPCs in mammalian cells remain to be investigated for the treatments of retinal degenerative disease.

1.5.3 Retinal Stem and Progenitor Cell Niche

The ciliary body, where adult RSPCs are localized is composed of two distinct epitheliums: inner layers and outer layers. The inner layer of the ciliary body epithelium consists of non-pigmented cells, whereas the outer layer contains pigmented cells, which include RSPCs [46]. According to anatomical studies of the ciliary body, the outer layers include a highly dense three-dimensional network of capillaries lined by endothelial cells that are fenestrated and lack continuous tight junctions [50, 51]. These capillaries in the ciliary body are, therefore, very permeable to macromolecules as well as to ions and fluids [52]. Interestingly, it has been demonstrated that proliferative NSPCs in the SVZ of the brain tightly make contact with the vasculature at sites that lack astrocyte endfeet and pericyte coverage. In other words, these blood vessels are also fenestrated capillaries similar to ciliary body blood vessels. There has been no report of cellular interactions between RSPCs and ECs in the ciliary body, and studies of interactions between RSPCs and ECs have been limited due to lack of specific RSPC markers in vivo.
1.5.4 Retinal Vascular Development

Mouse retinal developmental vascularization occurs post-natal when well-defined vascular plexuses form in a highly reproducible temporal and spatial pattern [53]. In mice, retinal vessels emerge from the optic nerve head immediately after birth and, during the first postnatal week, grow radially toward the retinal periphery forming the laminar superficial vascular plexus [54]. Around postnatal day 8, branches from the superficial vessels sprout and begin formation of the deep vascular plexus. These branches migrate toward the posterior retina until reaching the outer edge of the inner nuclear layer where they anastamose laterally and form the secondary, deep vascular plexuses [54]. Finally, during the third week after birth, an intermediate vascular plexus is formed at the inner edge of the inner nuclear layer. Importantly, growth factors play a prominent role during this retinal vascular development. A simplified model of endothelial cell guidance mechanisms has been proposed and are described as follows [55]: “1. Retinal vascular development is initiated when astrocytes emerge from the optic nerve head. 2. VEGF expression by the astrocytes, along with the coordinated expression of other growth factors including IGF-1, GlIGH, PDGF, and bFGF initiates endothelial cell proliferation and formation of the superficial vascular plexus. Specifically, tip cell filopodia are stabilized by the interactions of VEGF receptor and cadherin molecules with VEGF and R-cadherin adhesion molecules localized near or on the surface of the underlying astrocytes. 3. Directed endothelial cell migration is followed by recruitment of pericytes and other mural cells, mediated by the specific expression of PDGF-B localized to the endothelial cells. 4. Subsequent adherence and activation of leukocytes then initiates selective endothelial cell apoptosis and vascular pruning, resulting in the final characteristic superficial retinal vascular patterns.” With regard to formation of the deep retinal vascular plexuses, although little is known about the mechanisms that guide ECs, hypoxia in the
deeper retina is likely to be the main driving force for formation of the deep retinal vascular plexuses. As a result, the production of growth factors within the neural retina is increased, subsequently initiating the formation of the deep vascular plexuses. Interestingly, it has been reported that vascular targeting bone-marrow-derived cells also migrate and localize to regions of deep vascular plexus formation, even prior to vascularization of those regions.

1.5.5 Selection of Endothelial Cells

In order to establish an endothelial cell niche in 3D hydrogels, primary brain-derived endothelial cells are utilized in this thesis. It has not been determined whether brain derived endothelial cells can substitute for retina derived endothelial cells to investigate molecular interactions between RSPCs and ECs in co-culture system. However, it has been demonstrated that both the endothelial blood-retina and the blood brain barrier share common features such as low fluid-phase endocytosis and tight junctions with high electrical resistance, limiting transcellular and paracellular flux [56]. Moreover, the capillary vessels in the SVZ of the brain and in the ciliary margin of the retina are fenestrated. Notwithstanding similarities in function and structure, we acknowledge that there may be differences between the endothelial cells in the brain and retina. While technical limitations prohibited the use of retina-derived endothelial cells in these studies, we are confident that the similarities in these two cell types have allowed us to probe the interactions between ECs and RSPCs and that these interactions are meaningful. An alternate source to primary brain-derived endothelial cells is immortalized human retina-derived endothelial cells or mouse and human embryonic derived endothelial cells. Primary mouse brain-derived endothelial cells were utilized as proof of concept studies to develop a patterned endothelial cell hydrogel since these cells predominantly express VEGFR-2 and VEGF165 is an active growth factor to stimulate survival, proliferation, and migration.
1.6 Design of 3D in vitro scaffolds

1.6.1 Overview

Tissue engineering is the process of creating functional three-dimensional (3D) tissues using scaffolds or devices that facilitate cell growth, organization, and differentiation [57]. In particular, scaffolds made from natural and synthetic polymers have been used to drive the formation and maintenance of 3D tissue structures that can be tailored to specific applications. The interactions of cells and biomaterials comprise a dynamic regulatory system responsible for tissue regeneration, thus 3D biomaterial scaffolds with spatially controlled features are essential in tissue development for ultimate application in regenerative medicine [58].

The in vivo extracellular matrix (ECM) is comprised of several different biopolymers, encompassing a wide range of biological, chemical, and mechanical properties. The composition, organization, and assembly of these components at the molecular level gives the ECM its properties, which are unique for each tissue type [59]. Cellular behavior is guided by interactions that occur between cells and their local microenvironment, and this interrelationship plays a significant role in determining physiological functions. Thus, the natural ECM is an attractive model for design and fabrication of bioactive scaffolds for tissue engineering.

There is a growing, insightful body of literature demonstrating the significant differences in cell phenotype that arise when cells are cultured on traditional two-dimensional (2D) surfaces as compared to their native 3D microenvironment [58]. 2D substrates are, by definition, flat static materials that lack the heterogeneity that is found in vivo, and therefore do not adequately recapitulate the 3D nature of native tissue microenvironments.

Bioengineering approaches have been developed to fabricate novel bioactive scaffolds for tissue engineering [13, 14]. Particularly, hydrogels have been studied intensively and used as tissue engineering scaffolds because they can provide a hydrated, three-dimensional environment
similar to soft tissues that allow the diffusion of nutrients and cellular waste through elastic networks [60, 61].

This review explores material designs and fabrication approaches that are leading the development of 3D bioactive hydrogels as tissue engineering scaffolds. As the fundamental biology of the cellular microenvironment is often the inspiration for material design, the review focuses on modifications to control bioactive cues such as adhesion molecules and growth factors, and summarizes the current applications of biomimetic scaffolds that have been used in vitro as well as in vivo.

1.6.2 Mimicking the Extracellular Matrix

Over the last decade, bioengineering approaches have been developed to mimic the cellular microenvironment, thereby providing insight into the natural interactions between cells and the ECM. This section addresses the recent progress in material design and fabrication approaches that are leading to the development of bioactive three-dimensional hydrogels as tissue engineering scaffolds.

The in vivo extracellular matrix (ECM) is a hydrogel-like structure comprised of biopolymers with a wide range of biological, chemical and mechanical properties. In addition, the ECM is a reservoir of matricellular proteins (e.g. laminin, fibronectin); soluble growth factors (e.g. neurotrophin-3); and matrix-bound factors (e.g. basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF)). Together, these ECM components play a critical instructive role mediating key cell functions such as: cell adhesion, growth factor binding, proteolytic degradation, and mechanical support. Inclusion of these factors within natural, synthetic or semi-synthetic materials allows for recapitulation of the functions of the natural ECM in orchestrating cell proliferation, migration, and invasion. Sulfated glycosaminoglycans (GAGs) are among the main biopolymers found in the ECM [62]. Hyaluronan (HA) is a ubiquitous non-sulfated GAG,
and present in all connective tissue as a major constituent of the ECM. HA has a key role in morphogenesis and is therefore an important factor in tissue engineering. Recently, Shoichet et al designed covalently-crosslinked HA gels by taking advantage of Diels-Alder click cycloaddition chemistry [63]. HA was modified with maleimide functional groups and reacted with difunctional PEG furan to yield HA crosslinked gels where the mechanical and swelling properties were tuned by the amount of crosslinker as depicted in **Figure 1-1**.

![Figure 1-1](image-url)

**Figure 1-1** Error! Reference source not found.: Schematic Representation of the Formation of the Diels-Alder HA-PEG Hydrogels by Crosslinking HA-Furan with (Maleimide)$_2$-PEG. (Reproduced with permission from Biomacromolecules 12, 2011, 824-830)

To enhance cell interaction, the ubiquitous RGD cell adhesive peptide was covalently bound by similar coupling chemistry. Previously, Prestwich *et al.* developed a covalently-crosslinked,
synthetic ECM using hyaluronan (HA) [64]. In this approach, the disulfide hydrazide 3,3’-di(thiopropionyl) bishydrazide (DTPH) is first used to modify the carboxyl groups of HA, chondroitin, or heparin. Second, the disulfide bonds are reduced with dithiothreitol (DTT) to give the thiol-modified macromonomers such as HA-DTPH, chondroitin-DTPH, and heparin-DTPH. Third, the monomers are cross-linked by the electrophilic addition of thiol-ene to form hydrogels [62]. **Figure 1-2** illustrates the basic chemistry of the three-step process.
Alternatively, crosslinking with difunctional electrophiles can be accomplished in the presence or absence of cells, to give biocompatible hydrogels. Using this strategy, Prestwich et al. further demonstrated the synthesis of cell-adhesive hydrogels by cross-linking thiol-modified gelatin (gelatin-DTPH) with HA-DTPH to support cell attachment, growth and proliferation in 3D culture [64]. Their work has expanded to include the development of matrices composed of co-crosslinked HA-DTPH, chondroitin-DTPH and heparin-DTPH, and more recently formulations with thiol-modified HA, gelatin, and heparin in order to control the release rate of growth factors [65]. As heparin sulfate proteoglycans are major bFGF binding molecules, these heparin-modified hydrogels have been employed for the controlled release of bFGF. Results demonstrate that chemical immobilization of heparin and chondroitin to HA give a half-life for bFGF release of over one month in vitro.

In a separate approach, Heilshorn et al. reported a genetic strategy to prepare polypeptide-based ECM hydrogels [66]. In this study, multiple repeats of tryptophan-rich residues and proline-rich peptide domains were encoded in a modular genetic construct using recombinant protein technology. The two domains associate into anti-parallel β-sheet structures to form a physically crosslinked hydrogel. This strategy allows simple and gentle cell encapsulation without compromising cell viability and without the use of any crosslinking agents or environmental triggers. Similar results have been shown in other studies in the development of well-defined, three-dimensional structures based on molecular interactions (hydrogen bonds, disulfide bonds, electrostatic and ionic interactions, etc.) [67].
1.6.2.1 Incorporating Biochemical Signals

In the ECM, some growth factors are active in the bound state, while others are active only after being released by enzymatic cleavage of the matrix. In either case, growth factors may be synthesized and sequestered in the extracellular matrix for immediate activity, or for liberation and activity at a much later time as regulated by the enzymatic demand of cells in the environment. Hubbell et al. attempted to exploit and mimic growth factor-matrix interactions using fibrin hydrogels and peptides derived from transglutaminase enzyme factor XIIIa [68].

Fibrin is a biopolymeric gel that plays an important role in natural wound healing and medicine. It is formed by the enzymatic polymerization of fibrinogen at most sites of injury, and fibrin can be formed for therapeutic purposes either from purified fibrinogen or from processed blood plasma. Fibrinogen monomers are generated from the dimeric form by proteolytic activity of thrombin. The transglutaminase enzyme factor XIIIa then covalently crosslinks the fibrinogen monomers into polymer networks of fibrin. Once a crosslinked fibrin network is formed, the subsequent degradation of the fibrin is tightly controlled. One of the key molecules in controlling the degradation of fibrin is α2-plasmin inhibitor. By using these enzyme properties, Hubbell et al. demonstrated the increased extension of neurites within fibrin gels modified with exogenous peptides derived from ECM (e.g. laminin and collagen) or proteins (e.g. nerve growth factor) by either synthesizing them or expressing them as bipartite fusions, one part being a domain that serves as a substrate for factor XIIIa and the other being the bioactive domain of interest.

Hubbell’s recent work has explored the use of a fibrin-binding variant of VEGF, with an intervening plasmin-sensitive linker [69]. Whereas VEGF$_{121}$ simply mixed within fibrin and diffused out within a few hours, the engineered variant form of VEGF$_{121}$ was quantitatively bound within fibrin and remained there until liberated by active plasmin as depicted in Figure 1-3. This result also demonstrated that bound VEGF$_{121}$ induces endothelial cell (EC)
proliferation, as well as endothelial progenitor cell maturation into endothelial cells. Indeed, the matrix-bound forms are seen to be more effective than native VEGF\textsubscript{121} at promoting maturation.

Figure 1-3: Native VEGF\textsubscript{121} is freely diffusible in the aqueous milieu of the fibrin matrix and is released by passive, diffusive burst. (Reproduced with permission from Journal of Controlled Release 101, 2005, 93-109)

Cellular migration and architectural assembly are driven by extracellular spatial and temporal biomolecular cues. In biological tissues, two important classes of spatial molecular mechanisms are responsible for guiding cell motility and organization: chemotaxis and haptotaxis. By mimicking these microenvironments in ECM, the Shoichet group has advanced cell guidance
strategies using growth factor bound photoactive hydrogels formed from agarose - a naturally derived polysaccharide extracted from agarophyte seaweeds [70]. Using nitrobenzyl-protected thiols, patterns are produced within agarose hydrogels by uncaging sulfhydryl groups upon exposure to conventional He/Ne 325 nm laser source. These exposed thiols react readily within maleimide-terminated peptides and proteins, yielding peptide-/protein-modified agarose gels localized throughout specific volumes for the study of neurite outgrowth. Shoichet et al later expanded this approach to three-dimensional patterns using two-photon patterning techniques by chemically modifying agarose with thiol-protected 6-bromo-7-hydroxycoumarin Figure 1-4.

![Figure 1-4: Conjugation of photochemically masked thiol to polysaccharide backbones.](figure)

(Reproduced with permission from Chemistry of Materials 20, 2008, 55-60)

This approach allows the creation of more complex patterned gels, including the production of islands (<20 µm³) at defined depths that can be linked to create a variety of geometries (Figure 1-5) [71].
Recent studies by the Shoichet lab demonstrated that a concentration gradient of VEGF$_{165}$ was immobilized within defined volumes of the agarose hydrogel, by increased exposure to multiphoton light, thereby creating a concentration gradient of coumarin-deprotected agarose-thiol groups which were available to react with maleimide-modified VEGF$_{165}$ [27]. By mimicking the cues that guide ECs during vascular development, ECs were shown to follow an immobilized VEGF$_{165}$ concentration gradient in a 3D hydrogel, with tip and stalk cells identified and tubular-like structures formed.
One important aspect of current research is consideration of cell-cell interactions. In addition to chemical and physical properties of the surrounding ECM as described above, cellular functions in vivo are influenced by interactions with nearby cells. Previous studies by Bhatia et al. reported that hepatocytes, found in liver tissue, were stabilized by co-culturing with fibroblasts, inducing the liver-specific functions and preserving the maximal levels of functional integrin expression [72]. These co-cultured hepatocytes preferentially adhered to poly(ethylene glycol) (PEG)-based hydrogels photo-patterned with cell adhesive peptides, resulting in higher levels of albumin and urea, indicative of hepatocyte functionality.

Interestingly, recent work has implicated a strong functional interaction between neural progenitor cells (NPCs) and endothelial cells within the stem cell niche and has shown spatial proximity between established neural and vascular networks [73]. Lavik et al. demonstrated that hydrogels formed by crosslinking PEG with polylysine around salt-leached polylactic-co-glycolic acid (PLGA) supports the co-culture of ECs and NPCs in vivo by promoting the stabilization of microvascular networks [74]. The authors suggested that the mechanism involved in these interactions can be investigated further using 3D hydrogel scaffolds; therefore, synthetic scaffolds are promising platforms for stem cell culture in vitro, providing the ability to elucidate complex biological mechanisms of the stem cell niche.

### 1.6.2.2 Regulating Matrix Degradation

In addition to hormonal cues and biochemical signaling, the proteolytic degradation of the natural ECM is an essential feature of a variety of biological processes, such as cell migration, tissue repair and remodeling. Most ECM proteins, including collagen, fibrin, fibronectin, and laminin have specific cleavage sites for degradation by enzymes, such as matrix metalloproteinases (MMPs), plasmin, and elastase. In a specific example, proteolytic degradation
of the ECM is one of the key processes in angiogenesis where extensive endothelial cell proliferation requires degradation of the extracellular matrix to permit migration and tube formation [75]. Several enzymes, including MMPs, degrade the proteins that keep the vessel walls solid, which allows the endothelial cells to escape into the interstitial matrix. This course of ECM degradation is highly controlled and coordinated because unguarded dissolution of ECM would result in a loss of the integrity, and thereby function, of the microvasculature [75].

In recreating suitable matrices to recapitulate these processes, it is critical to include components that allow for the natural remodeling of the ECM as seen in the cells’ native environment. Recent work by Burdick et al. demonstrated three-dimensional, spatially controlled remodeling in patterned hydrogels using hyaluronic acid (HA) and enzyme-sensitive peptides [76]. Unlike the entire protein structure, which is subject to denaturation and degradation, short peptide sequences have the advantage of being relatively stable for modification, tunable for cell binding, and easy to be synthesized in large scale. In Burdick’s study, a two-step protocol was used to develop crosslinked hydrogels. In a first step, crosslinked hydrogels were synthesized via Michael-type addition reactions between multi-acrylate HA macromers and bifunctional MMP-degradable peptides and cell-adhesive peptides [76]. A photo-initiator was mixed together in this first step. The mixtures were then exposed to UV light to initiate free radical photopolymerization of the remaining acrylate groups. The resulting hydrogels were expected to prevent remodeling if non-degradable covalent crosslinks were incorporated; however, remodeling would be encouraged if degradable crosslinks were incorporated. As the secondary crosslinking is initiated by light, spatially distinct zones of remodeling were created as seen in Figure 1-6.
Figure 1-6: Photopatterning of AHA hydrogels. a) Sequentially crosslinked hydrogel photopatterned using a high resolution photomask. Inset images show the top and bottom surfaces of hydrogels patterned with 250 µm stripes. b) Quantification of photopattern fidelity at the top and bottom gel surfaces. (Reproduced with permission from Biomaterials 31, 2010, 8228-8234)

When human mesenchymal stem cells (hMSCs) or chick aortic arches were encapsulated in patterned hydrogels, outgrowth of cells, as a result of gel remodeling were observed in biodegradable versus restrictive regions of the gels. The results suggest that proteolytic degradation is necessary to support cellular spreading and differentiation. Similar studies have been investigated into the development of bioactive hydrogels incorporating proteolytic peptide sequences using poly(ethylene glycol) (PEG) as a backbone. Although PEG is neither cell adhesive nor biodegradable, PEG hydrogels have been modified with bioactive molecules, such as cell adhesive and enzyme-sensitive peptides. Anseth et al. reported incorporation of a cysteine-containing, bifunctional peptide, CPENFFRGD into PEG hydrogels by thiol-acrylate photopolymerization [77]. This peptide has the RGD motif for cell adhesion and the sequence of PENFF for MMP-13-sensitive cleavage, both of which are important for the differentiation of human mesenchymal stem cells (hMSCs). Peptides like collagen-derived GPQGIAGQ [78] and
peptide library-derived GPQGIWGQ [79] have also been used to make MMP-sensitive PEG hydrogels, while fibrin-derived YKNRD and VRN have been used to make plasmin-sensitive PEG hydrogels.

For cell transplantation studies in tissue engineering, scaffolds are often designed to degrade over time to provide space for new tissue formation. In each case, the degradation rate of scaffolds should match with new tissue regeneration at the defect site. If the degradation is more rapid than the tissue regeneration, the scaffolds will lose their carrier function for cell growth. Conversely, if the degradation is too slow, the scaffolds will impede tissue regeneration. Efforts by Mooney et al. have been made to achieve this balance by synthesizing cell-adhesive alginate hydrogels with tunable degradation rates as artificial constructs for the replacement of muscle [80].

Alginate is a naturally derived polysaccharide composed of linearly assembled (1-4) linked β-mannuronic acid (M) and α-L-guluronic acid (G) monomers. Alginate gels are formed when blocks of G-monomers and divalent cation (e.g., Ca\(^{2+}\)) interact to form ionic bridges between different polymer chains. Alginate gels are considered biocompatible and have been used to transplant cells in a variety of applications, yet the degradation of typical alginate hydrogels is very slow and poorly controlled. In order to overcome this problem, Mooney et al. utilized alginates with bimodal molecular weight distributions, one of which has undergone partial oxidation of the polymer to facilitate subsequent hydrolytic breakdown. When alginate is oxidized by reacting with sodium periodate, the carbon-carbon bond of the cis-diol group in the uronate residue is cleaved, which creates hydrolytically labile bonds in the polysaccharide (Figure 1-7).
Figure 1-7: Periodate oxidation of alginate creates open chain adducts that are susceptible to hydrolytic scission. (Reproduced with permission from Biomaterials 26, 2005, 2455-2465)

This approach provides control over the degradation rate by varying the degree of oxidation where increased degrees of oxidation result in accelerated rates of degradation [80]. Primary skeletal muscle cells encapsulated in this gel demonstrated higher proliferation in degradable than in non-degradable gels, indicating that the degradability of the gels influences cell fate in 3D culture [81].

1.6.2.3 Controlling Mechanical Properties

The ECM acts not only as a bioactive environment as described above, but also as a mechanical support to organize cells into specific tissues and control cell behavior. Tissues are exquisitely sensitive to mechanical forces (e.g. hemodynamic forces in blood vessels, and tension in skin and muscle), which are transmitted through ECM to individual cells [59]. Particularly, many studies have investigated the influence of mechanical stimuli on cell shape over the past years, demonstrating that cell shape is intimately related to gene expression. Although the molecular mechanisms by which cell shape change is translated into biochemical signals is currently not known, several studies suggest that cells can be switched between entirely different gene programs through alterations of ECM structure or mechanics, independent of growth factor or integrin binding [82]. In order to mimic the mechanical aspects of natural tissue, collagen, the most abundant protein in mammals, has been used to enhance the functionality of engineered tissues. There are 29 different forms of collagen in the body, the most ubiquitous of which is type I collagen. Comprised of triple α-helices, collagen self-assembles to form a fibrillar
structure [83]. The self-assembling tendencies of type I collagen have led to it being used in tissue engineering. The two major limitations of collagen-based scaffolds are their weak nature and their extensive contraction by encapsulated cells. One group, led by Khademhosseini, has sought to address both of these drawbacks by mixing collagen with photo-crosslinkable methacrylated hyaluronic acid (MeHA) [84]. By changing the methacrylation and concentration of the HA, the moduli of the hydrogels can be varied, and the authors demonstrated that these composite hydrogels achieve an increase in strength, failure stress, stiffness and thus, provide better mechanical control than collagen. Enhanced mechanical properties also resulted in higher levels of NIH-3T3 cell viability upon cell encapsulation.

Important work by Discher et al. demonstrated that the mechanical properties of the matrix impact the differentiation profile of mesenchymal stem cells (MSCs) in two-dimensional culture [85]. By preparing collagen-coated polyacrylamide gels that mimicked the elasticity of various tissues, the MSCs differentiated into lineages that corresponded to the stiffness of the native environment. Recent studies by Schaffer and Healy et al. also demonstrate the dependence on matrix rigidity for adult neural progenitor cells derived from the hippocampus [86]. The authors synthesized interpenetrating polymer networks (IPNs) through the sequential, free-radical polymerization of poly(acrylamide) using TEMED and ammonium persulfate as catalysts. From this approach, the authors were able to vary the modulus within a range of 10-10,000 Pa, while allowing for surface modification of the gel networks to modulate cell adhesion (Figure 1-8).
These results suggest that neural progenitors are directed to neurons on softer gels (100-500 Pa) and glial cells on harder gels (1,000-10,000 Pa).

Interestingly, using neural stem/progenitor cells derived from the subventricular zone of adult rats, Shoichet’s lab found similar results using methacrylamide-modified chitosan where rigidity was controlled using a photolabile crosslinker [87]. In order to gain insight into the mechanism of response to cells in 3D culture, the Shoichet lab modified methacrylamide chitosan scaffolds with cell adhesive RGD peptides and interferon-gamma [88], which had been shown to promote differentiation of neural stem/progenitor cells to neurons [89]. Scaffolds with encapsulated cells were photocrosslinked using the cytocompatible photo-initiator 2,2-dimethoxy-2-phenylacetophenone. The NSPCs within modified gels differentiated preferentially to neurons.

To gain further perspective on the role of mechanical properties in 3D, the Anseth group has been exploring chemical modifications of poly(ethylene glycol) (PEG)-based hydrogel materials [90-92]. PEG is an important polymer for biomedical applications, such as drug delivery and
tissue engineering, because it is biocompatible, has low immunogenicity, and reduces protein adsorption. PEG can be synthesized with both linear and branched (multi-arm or star) structures. PEG-diol can be modified to introduce different functional groups, such as: carboxyl, amine, thiol, azide, vinyl sulfone, and acrylate. Several crosslinking methods have been used to synthesize PEG hydrogels, including Michael-type addition and click chemistry. In the work by Anseth et al., a photocleavable cross-linking diacrylate macromer was firstly synthesized by attaching a photodegradable, nitrobenzyl ether-derived moiety, to PEG-\textit{bis}-amine [91]. Photodegradable PEG hydrogels were then synthesized by redox-initiated, free radical polymerization with PEG monoacrylate in phosphate-buffered saline (PBS) (Figure 1-9).

Figure 1-9: Synthesis of photodegradable hydrogel for tuning gel mechanical properties. A) The base photodegradable acrylic monomer. B) The photodegradable cross-linking macromer composed of PEG (black), photolabile moieties (blue), and acrylic end groups (red). C) Macromer was polymerized with PEGA creating gels connected by PEG with photolabile groups (blue boxes). (Reproduced with permission from Science 324, 2009, 59-63)
Upon UV irradiation, the PEG is released. As the storage modulus is proportional to the hydrogel crosslinking density, the degradation rate and resulting material properties, such as stiffness, were predictably manipulated with light intensity and wavelength. The authors demonstrated that the morphology of encapsulated human mesenchymal stem cells (hMSCs) was regulated in real-time using these photodegradable PEG hydrogels. Anseth et al. recently suggested a sophisticated alternative strategy to synthesize PEG hydrogels with tunable moduli using copper-free, azide-alkyne click chemistry. In this approach, a four-arm PEG tetraazide is reacted with a bis(cyclooctyne)-peptide to form multifunctional hydrogels [92]. By using the biocompatible cyclooctyne molecule, the cycloaddition of alkyne-azide occurs in the absence of a catalyst. Furthermore, crosslinking can be controlled spatially and temporally with exposure to light. The mechanical properties of the gel can be tuned by altering the length of the PEG arms as well as the stoichiometric ratio of azide:alkyne on the PEG and peptide cross-linker, respectively. For example, the gels containing the lowest molecular weight PEG showed the highest moduli and lowest swelling whereas those with the highest molecular weight PEG provided the lowest moduli and highest swelling. Through the bioorthogonal thiol-ene click reaction, biomolecules were also conjugated to the hydrogel backbone without changing the network structure. While this may be useful to probe 3D cell behavior, no cell data has been presented to date.

1.6.3 Current Applications

1.6.3.1 In Vitro
Studies using natural or synthetic materials have begun to elucidate the role of tissue structure and 3D organization of niche cues on differentiation, proliferation, migration, matrix deposition, development and pathogenesis [13]. By designing polymeric biomaterials with the appropriate properties and providing a controlled microenvironment for cells, 3D scaffolds hold great promise for decoupling comprehensive microenvironment variables and effectively
understanding the physiological systems *in vivo*. Moreover, these 3D engineered scaffolds may be used for *in vitro* screening applications, and some have already been shown to provide an excellent model where pharmaceuticals can be tested prior to animal studies [58].

The Bhatia group has created a 3-D photo-crosslinked PEG hydrogel platform as a high-throughput assay using dielectrophoretic forces (DEP) as shown in Figure 1-10 [93].

Figure 1-10: Fabrication method and examples of DCP hydrogels. a) Cells in prepolymer solution are introduced into transparent chambers and localized to micropatterned gaps. UV light is used to polymerize the hydrogel. b) Electrical field strength model. c-f) Embedded fibroblast clusters shown in hydrogels. g) A bilayered hydrogel containing distinct layers of fibroblasts (rings and clusters). (Reproduced with permission from Nature Methods 2006, 3, 369-375)

The DEP system allows precise control over single cells to investigate cell shape, organization and interactions at micro-scale resolution. In this system, living cells are arrayed under DEP within the uncross-linked PEG solution. Single cells were encapsulated by photo-crosslinking PEG, forming clusters with precise size and shape within the hydrogels, and maintaining 3D microstructures with high cell viability during the cell culture period. This 3D cellular
microarray system allows for the investigation of cell-cell interactions that resemble *in vivo* behavior. Bhatia *et al.* later demonstrated that 3D structures of bovine articular chondrocytes exhibit altered matrix biosynthesis compared with the ones in 2D culture; cumulative matrix proteins synthesized by chondrocytes were decreased in a dose-dependent manner with increasing cluster size [94].

In the past few years, 3D scaffolds have been designed for applications in cancer where the goal is to better understand tumor progression, metastasis and provide a better tool for screening therapeutics *in vitro*. Despite significant contributions, conventional 2-D cell culture systems poorly reflect tumor niches. 3-D culture systems are designed to bridge the gap between *in vitro* and *in vivo* cancer models by retaining the *in vivo* phenotype through mimicking the structure of the tumor microenvironment. Although commercially available Matrigel® matrix has been used in 3D tumor studies, Matrigel® is ill-defined and inconsistent in composition, making results difficult to interpret.

The Mooney group has created a simple 3D human tumor model using poly(lactide-co-glycolide) (PLG) [95]. The PLG scaffolds provide a biocompatible porous culture system which the authors used to investigate the micro-environmental conditions representative of tumors *in vivo*. Using this 3D model, they showed aspects of cancer progression, demonstrating the relevance of this culture system to *in vivo* tumor characteristics [95].

1.6.3.2 *In Vivo*

*In vivo*, the ultimate function of the scaffold is to facilitate wound-healing and regeneration. Therefore, the tissue engineered construct must be designed to foster local tissue growth as well as to promote integration with the host tissue. Integration with the host tissue requires the use of biocompatible material and connectivity between implanted constructs/cells and host tissue. One of the limitations of cell-scaffolds is that the cells that are more than 100-200 µm away from the
vascular network die due to lack of oxygen and nutrients. Recent studies by the Langer group demonstrated that pre-vascularized scaffolds supported survival of transplanted cells [96]. In this study, endothelial cells were seeded together with fibroblasts and myoblasts (muscle cells) into a scaffold comprised of 50% poly(L-lactic acid) (PLLA) and 50% poly(lactic-co-glycolic acid) (PLGA). The PLGA was selected for its rapid degradation profile, allowing for cellular ingrowth, whereas the PLLA was selected to provide mechanical support to 3D structures [97]. In addition, the blended polymers were fabricated using a salt-leaching process, exhibiting highly porous network that allowed endothelial cells to form vascular networks within the scaffolds. Results show that after implantation into nude mice, the pre-vascularized scaffold successfully integrated with host microvessels and, importantly, promoted viability of the implanted muscle cells [96].

Recent studies by the Song group have investigated injectable self cross-linkable polyphosphazene hydrogels [98]. In this study, thiol- and acrylate-based polyphosphazenes were prepared as shown in Figure 1-11.
These blended polymers exhibited a solution state at low temperature and a transparent gel state at physiological temperature due to physical entanglements and chemical crosslinking via Michael addition of thiols across acrylate double bonds, providing control over mechanical properties. Physical crosslinking brought the reactive double bond of acrylate together with thiol groups in the polymer network, and facilitated the fast gelling transition through chemical crosslinking. As expected, the rate of gel degradation depended on the degree of crosslinking.

In separate studies in the Shoichet lab, an injectable hydrogel of hyaluronan and methylcellulose...
(HAMC) has been used for *in vivo* applications [99, 100]. HAMC has been shown to be biocompatible, biodegradable, inverse thermal gelling and easily injectable through fine 30-34 gauge needles, resulting in minimally-invasive surgery. Studies have shown that HAMC is a promising gel for localized delivery of therapeutic agents to the spinal cord and brain, and stem cells to the retina [101-103]. Importantly, HAMC has demonstrated some therapeutic benefit on its own where it has promoted healing and attenuated the inflammatory response in the CNS [100].

1.6.4 Future Perspectives

Tissue engineering presents the possibility of creating or regenerating various organs or organ-like structures for potential therapeutic intervention. Since the concept was first proposed by Langer and Vacanti [1], the use of living cells in combination with biodegradable scaffold materials has yielded several clinical successes in the recreation of a wide variety of tissues, such as cartilage, bone and blood vessels. In addition, engineered tissues have been created as *in vitro* 3D physiological models, providing more biologically relevant complexity than traditional 2D cultures. In these strategies, scaffolds have been designed to incorporate both biochemical and mechanical cues in an attempt to reconstruct tissues that resemble the native structures, whether the application is *in vitro* or *in vivo*.

Despite recent advances in the development of bioactive hydrogels, several challenges still remain including recapitulating the dynamic cellular microenvironment in the design of scaffolds. Particularly with the advent of stem cells, their inherent capacities to self-renew and differentiate into multiple cell types may provide novel therapeutic strategies.

The Shoichet and Zandstra groups have collaborated to demonstrate that encapsulating embryonic stem cells in chemically modified agarose hydrogels including immobilized VEGF can drive their hematopoietic differentiation under defined conditions in bioreactors [104]. This
*in vitro* model has been shown to be predictive of the temporal and microenvironmental events that occur *in vivo* during embryonic development.

Extending *in vitro* stem cell-niche engineering to *in vivo* cellular strategies poses additional challenges. Stem-cell transplantation requires a scaffold that regulates the presentation of ligands, is sensitive to stimuli, provides structural support, and has the ability to induce cell migration or invasion into the scaffold. Stupp and co-workers recently developed peptide amphiphilic assemblies, which formed long cylindrical nanofibers that crosslinked in the presence of salts [105]. Importantly, the nanofibres formed highly oriented hydrogels via a liquid-crystalline phase, resulting in guided cell growth *in vivo*. Due to strong interfibril interactions, the hydrogels also exhibited high stability. This injectable material supported the growth of neural stem and progenitor cells as well as human mesenchymal stem cells along the fiber axis. The promise of such scaffolds *in vivo* is just beginning to be explored as a means to deliver stem cells.

Innovative biomaterials strategies will continue to drive the field to engineer better tissue scaffolds. Engineered polymers can be used as a platform to better mimic the stem cell niche, allowing for multiple stimuli and many cell types to be explored individually or in combination. Control over the ECM will facilitate investigation of cell biology, and allow the complexity of the system to be evaluated in terms of individual components, including soluble signals, cell-substrate interactions, and cell-cell contacts. The 3D bioengineered matrices will be advanced for continued use both *in vitro* as a model of disease progression and *in vivo* for tissue regeneration.
2  Endothelial cell guidance in 3-D patterned scaffolds


2.1  Abstract

We describe the guidance of primary endothelial cells in an agarose hydrogel scaffold that is chemically patterned with an immobilized concentration gradient of VEGF165 using multiphoton laser patterning of VEGF165. This is the first demonstration of this patterning technology to immobilize proteins; and the first demonstration of immobilized VEGF165 to guide endothelial cell growth and differentiation. It is particularly compelling that, in this 3D patterned hydrogel, endothelial cells differentiate to tip-like and stalk-like cells, having the morphology that is observed in vivo. Thus this 3D patterning methodology provides an excellent biomimetic environment for endothelial cell growth, thereby offering a new approach to build engineered tissues and to study endothelial cell biology.

2.2  Introduction

A vascular network is required to provide engineered tissue constructs with the oxygen and nutrients needed for survival.[106, 107] Endothelial cells (ECs), key cellular components of blood vessels, play important roles in the stem cell niche and thus their function in regenerative strategies extends beyond the vascular system.[73, 108, 109] To better understand the role of ECs in the stem cell niche, representative in vitro models of EC growth are required. Most studies of ECs have been performed in two-dimensional (2D) tissue culture;[110, 111] however, given the morphological and functional differences exhibited by these cells in 2D and three-dimensional (3D) cultures[112], the development of well-defined 3D culture models is necessary to better investigate vascular development.
During vascular development, the leading front of the sprout exposes a tip cell with numerous filopodia that express receptors to sense secreted and cell-bound guidance cues provided by surrounding cells.[23, 25] One of the most studied factors that controls blood vessel formation and function is vascular endothelial growth factor A (VEGF-A).[17, 23, 25, 113] VEGF-A exists in several isoforms which bind with different affinities to VEGF receptors (VEGFRs).[24, 114] VEGF165, in particular, is a critical angiogenic factor. The binding of VEGF165 to its receptor, VEGFR2, causes EC proliferation and migration.[23, 25, 114, 115] Tip cells have been shown to migrate up a concentration gradient of VEGF165.[23, 25, 116, 117] Moreover, tip cells are trailed by stalk cells, which proliferate and self-organize to form lumens, when cultured in 3D, in response to VEGF-A.[23, 25, 116, 117]

In this study, we designed a 3D culture system, comprised of concentration gradients of human VEGF165 in hydrogels, to guide endothelial cell growth with the ultimate goal of enhancing regenerative strategies and laying the foundation to investigate their role in the stem cell niche. By mimicking the cues that guide endothelial cells in vivo, we show, for the first time, that ECs follow an immobilized VEGF165 concentration gradient in a 3D hydrogel, with tip-like and stalk-like cells identified and tubular-like structures formed. Previously, only cell-adhesive cues have been studied for EC growth in 3D and this has limited the morphology of tip and stalk cells formed.[118]

### 2.3 Materials and Methods

#### 2.3.1 Immobilization of VEGF165 and GRGDS Peptides on Photolabile Agarose

Agarose was modified with a photolabile thiol protected- 6-bromo-7-hydroxycoumarin as described previously [71]. Modification and immobilization of VEGF165 was performed based
on the previously described method [119]. Briefly, VEGF165 (100 μL, 500 μg mL⁻¹) was dissolved in 2-(N-morpholino) ethanesulfonic acid (MES) buffer (50 mM, pH 6; Sigma-Aldrich, Oakville, ON, Canada) including NaCl (400 mM), 1,2-propanediol (10 wt%). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 50 μL, 44 mM; Sigma-Aldrich) and N-hydroxysulfosuccinimide (sulfo-NHS, 50 μL, 48 mM; Pierce Biotechnology, Rockford, IL, USA). After mixing and reacting the above reagents at room temperature for 15 min, Alexa Fluor 594 hydrazide sodium salt (100 μg) dissolved in DMSO (10 μL, Invitrogen, Carlsbad, CA) and 4-(4-N-maleimidophenyl) butyric acid hydrazide (MPBH, 7 mM, Pierce Biotechnology, Rockford, IL) in sodium acetate buffer (200 μL, 100mM, pH 5.5) were added to the VEGF165 for 75 min at room temperature. The maleimide- and Alexa Fluor-modified VEGF165 (MI-VEGF165-f) was purified with AKTA-FPLC (Amersham Pharmacia, Piscataway, NJ) using Sephadex G-25 (Sigma-Aldrich) column (10 X 200 mm, Amersham Pharmacia) equilibrated in sodium phosphate buffer (100 mM, pH 7.0) including NaCl (400 mM), propanediol (5 wt%).

Conjugation of MI-VEGF165-f to photolabile agarose hydrogel was investigated using coumarin-agarose (100 μL, 0.75 wt%) gelled in a 96-well plate. For covalent immobilization of the MI-VEGF165-f, photolabile agarose solution and MI-VEGF165-f solution in PBS were mixed and irradiated for 2 min with a UV lamp (EFO X-Cite XC60000) after forming the hydrogel. Controls for MI-VEGF165-f adsorption were treated identically without irradiation of photolabile agarose. Non-covalently bound MI-VEGF165-f was removed by washing the hydrogels in PBS for 2 days. Cell-adhesive peptides, maleimide-glycine-arginine-glycine-aspartic acid-serine dissolved in PBS (mi-GRGDS, 100 μL, 0.5 mg mL⁻¹, Ana Spec, San Jose, CA) were added to each well and the entire plate was irradiated for 3 min with a UV lamp to form GRGDS-agarose hydrogels. Unreacted mi-GRGDS was rinsed by successive washing in PBS for 12 h.
2.3.2 Creation of VEGF165 immobilized gradient within the hydrogels

VEGF165 was modified with MPBH and Alexa Fluor 488 hydrazide sodium salt (Invitrogen) as described above. A solution of photolabile agarose (0.3 wt%) containing MI-VEGF165-f solution (8 µg mL\(^{-1}\)) was pipetted into a 1 mm glass cuvette (Starna Cells, Inc, Atascadero, CA). After cooling at 4 °C for 40 min to ensure complete gelation, the cuvette was mounted on the stage of a Leica TPS SP2 confocal microscope equipped with a Spectra-Physics Mai Tai broadband Ti-Sapphire laser, tuned to 740 nm [71]. After focusing the laser to a plane in the interior of a gel at low power, the Leica software was used to define a region of interest (300 µm x 300 µm) and the laser power was increased through software controls to the maximum available. A macro program was previously written using Leica software to create concentration gradient patterns of the desired shape and dimension [71]. After patterning, gel samples were immersed in PBS buffer to remove unreacted MI-VEGF165-f for 2 days. mi-GRGDS including tris(2-carboxyethyl) phosphine hydrochloride (Sigma-Aldrich) in PBS (100 µL, 0.5 mg mL\(^{-1}\)) was then added to each cuvette and immobilized as described above using confocal laser patterning. Unreacted mi-GRGDS was rinsed by successive washing in PBS for 12 h.

2.3.3 Cell culture

Brain derived endothelial cell lines (bEnd3, ATCC, Manassas, VA, USA) were routinely grown in DMEM supplemented with fetal bovine serum (FBS, 10% Gibco-Invitrogen, Carlsbad, CA, USA) and penicillin-streptomycin (P/S, 100 µg mL\(^{-1}\), Sigma-Aldrich) in a 25 cm\(^2\) tissue culture flask (VWR, Mississauga, ON) at 37 °C and 5% CO\(_2\). Cell numbers and viability were determined with a haemocytometer and the trypan blue exclusion assay (Sigma-Aldrich) before seeding. Primary mouse brain derived endothelial cells were isolated (see Appendix B for isolation protocol) and cultured in DMEM/F12 medium (Gibco-Invitrogen) containing FBS
(10%), horse serum (10%, Gibco-Invitrogen), heparin (100 μg mL⁻¹; Sigma-Aldrich), endothelial cell growth supplement (100 μg mL⁻¹; Sigma-Aldrich) and P/S (100 μg mL⁻¹) in a 24-well plate coated with murine collagen. Experiments were performed on cells at passage 1 and 2.

2.3.4 MTT assay

The MTT Proliferation Assay (Promega, Madison, WI, USA) was performed following the manufacturer’s protocol on bEnd3 cells cultured on the hydrogels containing either immobilized VEGF165, physically adsorbed VEGF165, or soluble VEGF165 (n=3). After 3 days of culture, the labeling mixture (40 μL) was added to each well containing the hydrogel immersed in the media (200 μL). The plate was incubated at 37 °C and 5% CO₂ for 1 h. Then supernatant (100 μL) was added by pipette from each well and put into a new plate. The absorbance was measured at 490 nm using a UV plate reader (Molecular Device, Sunnyvale, CA).

2.3.5 Immunocytochemistry and imaging

The following primary antibodies were purchased from Abcam (Cambridge, MA) for the immunocytochemical studies: monoclonal rat anti-CD31 (1:10); polyclonal chicken anti-laminin (1:500); and monoclonal rabbit anti-beta catenin (1:250). For all immunocytochemical procedures, appropriate controls were obtained by omission of the relevant primary antibody. Cells on the hydrogel were fixed with PBS solution containing PFA (4%) for 1 h and then washed with PBS. After cell membrane permeation and blocking by treating with BSA (1.5 wt%) and Triton X-100 solution (0.2%) at room temperature for 1 h, each specific primary antibody solution described above, was added for 5 h at room temperature. After washing with PBS, samples were exposed to goat Alexa 633 anti-chicken, goat Alexa 488 anti-rabbit IgG (1:200, Invitrogen) and goat Cy3 anti-rat IgG (1:100 Jackson Immunoresearch, West Grove, PA) for 5 h at room temperature and then washed with PBS. For imaging, fluorescent Z-series image
stacks were captured on Zeiss MicroImaging LSM510 microscope using a water-immersion 40x numerical aperture plan Apochromat objective and compiled into three-dimensional rendering with LSM software (University of Toronto, Faculty of Medicine, ON, Canada). Excitation wavelengths were 488 nm (Argon laser), 543 nm and 633 nm (He-Ne laser) for Alexa 488, Cy3, and Alexa 633 respectively. For all EC guidance studies, images of tubule-like structures were photographed using a BX 61-microscope (Olympus) using a 20 x objective.

2.3.6 Statistical Analysis
All statistical analyses were performed using JMP IN 5.1 (SAS Institution Inc., Cary, NC). Differences among groups were assessed by one-way ANOVA with the Tukey’s post hoc analysis to identify statistical differences among three or more treatments. All data are presented as mean ± SD.

2.4 Results and Discussion
In order to study the guidance of endothelial cells in 3D, agarose hydrogels were photochemically patterned with multi-photon lasers [71] to immobilize a series of concentration gradients of VEGF165. To promote cell adhesion, agarose was also modified with the ubiquitous cell adhesion peptide, glycine-arginine-glycine-aspartic acid-serine (GRGDS). Based on previous studies with neural stem cells, we hypothesized that the peptides and growth factors would be stably immobilized and remain bioactive after immobilization.[70, 119] These comprised our first studies.

VEGF165 was covalently bound to agarose and compared to soluble VEGF165 and adsorbed VEGF165 in terms of endothelial cell proliferation. Agarose was chemically modified with cysteine-protected 6-bromo-7-hydroxycoumarin,[71] which, upon excitation with either UV light or a pulsed infrared laser, yielded agarose-sulphide. VEGF was modified with 4-(4-N-
maleimidophenyl) butyric acid hydrazide (MI) [119] and Alexa Fluor 594 hydrazide (f), yielding MI-VEGF165-f which allowed Michael-type addition to agarose-thiol and facilitated quantification by fluorescence of immobilized VEGF165. By comparison to a standard curve of fluorescence intensity, 924 ± 25 ng mL⁻¹ of MI-VEGF165-f was immobilized to agarose-sulphide (100 μL) whereas only 79 ± 15 ng mL⁻¹ of MI-VEGF165-f was physically adsorbed. The bioactivity of the covalently-bound agarose-VEGF165 was characterized relative to adsorbed and soluble VEGF controls by the MTT cell proliferation assay using VEGFR2+ brain-derived endothelial (bEnd3) cells.[120] Soluble VEGF165 controls were prepared by mixing VEGF165 solution including 50 ng or 100 ng with 100 μL agarose. After 3 days of culture, ECs cultured on agarose-VEGF165 (924 ng mL⁻¹) showed a similar proliferation profile to those cultured in soluble 1000 ng mL⁻¹ VEGF165 treatment (n=3, Figure 2-1) yet a significantly different proliferation profile from those cultured in soluble 500 ng mL⁻¹ VEGF165 (p<0.05, n=3) or in the absence of VEGF165 (p<0.05, n=3).
Figure 2-1: Analysis of bioactivity of immobilized VEGF165 relative to no VEGF165, or soluble VEGF165 in agarose hydrogels by MTT cell proliferation assay. Data are expressed as the fold changed in UV absorbance relative to the control (no VEGF165 treatment). ECs cultured on agarose-VEGF165 (924 ng mL\(^{-1}\)) showed a similar proliferation profile to those cultured in soluble 1000 ng mL\(^{-1}\) VEGF165 controls, yet significantly different profile from cultures with no VEGF165, or 500 ng mL\(^{-1}\) soluble VEGF165. (The different letters represent significant differences as measured by ANOVA, p<0.05)

In addition, physically adsorbed MI-VEGF-f did not stimulate bEnd3 cells and was not statistically different from the control treatment without VEGF165 (n=3, Figure 2-2). Thus, covalently-immobilized VEGF165 remained bioactive.
Figure 2-2: Analysis of bioactivity of physically adsorbed VEGF165 relative to no VEGF165 in agarose hydrogels by the MTT cell proliferation assay. The UV absorbance data are expressed as fold change relative to the control (no VEGF165 treatment). The treatment of cellular response to physically adsorbed MI-VEGF165 was not statistically different from the control treatment with No VEGF165.

Since aggregates of ECs promote tubular formation due to cell-cell and cell-extracellular matrix interactions, [121, 122] we tested the suitability of GRGDS-agarose for cell penetration and tubule-like structure formation. Primary endothelial cells that were isolated from the microvessels in the brains of adult mice [123] and cultured as EC aggregates,[124] were seeded on the GRGDS-agarose hydrogels. In the first 12 h of culture, sprouts were observed protruding from the aggregates into the hydrogels. After 24 h, ECs started forming tubule-like structures within the gel. Stalk-like and tip-like cells were first identified based on their morphology (Figure 2-3a) and then were further characterized by staining for junctional proteins with β-catenin, and for vessel wall extracellular matrix with laminin. We captured fluorescent confocal image stacks along the z-axis of the hydrogels to create 3D representations of the vessels (Figure 2-3b,c and d). We observed lumen-like structures in the organized EC cultures, suggesting that these 3-D cultured ECs in agarose were in the process of tubulogenesis (Figure 2-4).
Figure 2-3: Representative images of primary endothelial cells from the brain microvascularature extend tip and stalk-like cells into GRGDS agarose hydrogels and form tubule-like structures: a) Phase-contrast microscopy image of an EC aggregate cultured on top of the GRGDS hydrogel after one day has stalk-like cells (arrow at top) and tip-like cells (arrow at bottom) growing into the agarose gel (arrows point the surface of the hydrogels Scale bar = 100 μm). Confocal projected images of these primary ECs immunostained with primary antibodies: b) β-catenin for junctional proteins, c) laminin for extracellular matrix, d) combined staining of β-catenin and laminin. (Scale bar = 20 μm) (A series of individual images are shown in Appendix C)
Figure 2-4: Tubule-like formations of ECs in 3D agarose hydrogels: a) Lumen formation was observed in EC tubule-like structures (+ points a cavity in EC Scale bar = 100 μm). The region of lumen formation in image a) was magnified in b) where the beginning of lumen formation is more easily observed by confocal image of ECs immunostained by laminin. (Scale bar = 20 μm).

Having established the suitability of GRGDS-agarose hydrogels for EC migration and bioactive immobilization of VEGF165, we then created and tested the guidance capacity of a series of immobilized VEGF165 concentration gradients in agarose. Using a multiphoton Ti/sapphire confocal laser, a gradient of VEGF165 was immobilized within a defined volume in the agarose hydrogel, taking advantage of photolabile coumarin-protected agarose-sulphide groups and maleimide-modified VEGF165. We designed a series of vertical, linear concentration gradient volumes of fluorescently-tagged VEGF165 having dimensions of 300 μm x 300 μm x 600 μm (L x W x D, Figure 2-5a). The gradients were created by controlling the scanning number and scanning regions and quantified based on the fluorescence intensity: 2.48, 1.65 and 0.99 ng mL⁻¹ μm⁻¹ (Figure 2-5b).

To study endothelial cell guidance in the immobilized VEGF165 concentration gradient hydrogels, GRGDS peptides were immobilized at a uniform concentration of 2.08 μM in the same volume using the confocal patterning technique (Error! Reference source not found.).
Dissociated primary ECs were seeded on top of the cell-adhesive, gradient agarose hydrogels, and formed small aggregates on top of the patterned volumes within the first 12-24 h of culture prior to penetrating the gels. Interestingly, after 3 days of culture, primary ECs had penetrated the agarose hydrogels to a depth of more than 200 μm and formed tubule-like structures in agarose hydrogels having VEGF165 gradients of both 1.65 and 0.99 ng mL⁻¹ μm⁻¹ (Figure 2-5c and d). In contrast, ECs cultured on the 2.48 ng mL⁻¹ μm⁻¹ VEGF gradient hydrogel developed a few sprouts from the EC aggregates, but showed no evidence of tubular formation in the gel (data not shown). Considering that the matrix-bound VEGF gradient in vivo is regulated by interstitial flow [26], there is a physiological gradient range to which tip endothelial cells can respond. Our results suggest that the steeper 2.48 ng mL⁻¹ μm⁻¹ VEGF gradient may have saturated the VEGFR2 receptors on endothelial tip cells, thereby limiting their guidance response to this gradient.

To test whether the EC guidance observed resulted from the immobilized VEGF165 gradients, primary ECs were cultured on patterned GRGDS peptide hydrogels either in the absence of VEGF165 (Figure 2-5e) or in the presence of homogeneously immobilized VEGF165 (ie., no gradient): 195 ± 98 ng mL⁻¹, 521 ± 106 ng mL⁻¹, or 722 ± 172 ng mL⁻¹ (Error! Reference source not found.). In all cases, tubular-like sprouts grew from EC aggregates; however, these penetrated the agarose to only a limited depth (<30-50 μm), just below the surface. In contrast, the mean ± standard deviation tubule extension was 195±21 μm (n=6) for an immobilized VEGF165 concentration gradient of 1.5-1.8 ng mL⁻¹ μm⁻¹ and a starting concentration of 250-300 ng mL⁻¹ (Fig 3c). The mean ± standard deviation tubule extension was 210±41 μm (n=5) for an immobilized VEGF165 concentration gradient of 0.8-1.0 mL⁻¹ μm⁻¹ and a starting concentration of 230-300 ng mL⁻¹ (Figure 2-5d).
b) 

(1) \( y = 2.4835x + 299.62 \) 
\( R^2 = 0.9557 \)

(2) \( y = 1.6506x + 316.86 \) 
\( R^2 = 0.9067 \)

(3) \( y = 0.9907x + 308.00 \) 
\( R^2 = 0.9756 \)
Figure 2-5: Creation of an immobilized gradient of VEGF165 in the agarose hydrogel to guide primary EC growth. a) Confocal image of the gradient of fluorescently tagged immobilized VEGF165. As the number of scans by the confocal laser increases from top to bottom, the fluorescent intensity increases correspondingly, reflecting the greater number of deprotected thiol groups that react with maleimide modified VEGF165 (Scale = 600 μm). b) Quantification of a gradient of immobilized VEGF165 in the agarose hydrogel based on the standard curve of fluorescently tagged VEGF. Each gradient was calculated at (I) 2.48, (II) 1.65 and (III) 1.00 ng mL$^{-1}$ μm$^{-1}$. c-e) Phase-contrast microscopy of cross-sectional images of primary ECs growing into the hydrogels after culturing for 3 d. ECs formed aggregates on top of the patterning areas (arrows point the surface of the hydrogels) and then were guided into the agarose hydrogels, in tubular-like formations, following the VEGF165 gradients: c) 1.65 ng mL$^{-1}$ μm$^{-1}$, d) 1.00 ng mL$^{-1}$ μm$^{-1}$; EC growth was minimal into the agarose hydrogel control with no VEGF165 immobilized. Scale bars (=100 μm) are parallel to the Z-direction in the gels.
Figure 2-6: ECs exhibited tubular-like sprouts from aggregates, yet grew into agarose hydrogels only to a depth of 30-50μm (arrows point to the surface of the hydrogels) when VEGF was immobilized at uniform concentrations (not as a gradient): a) 195 ± 98 ng mL\(^{-1}\); b) 521 ± 106 ng mL\(^{-1}\); and c) 722 ± 172 ng mL\(^{-1}\). (Scale bar = 100 μm).

Figure 2-7: Quantification of fluorescent labeled GRGDS peptides using a two photon laser. GRGDS was immobilized at a uniform concentration in the same volume where VEGF165 was immobilized.

gradient manner. To gain greater insight into the 3D tubule-like structures, the 1.65 ng mL\(^{-1}\) μm\(^{-1}\) VEGF165 concentration gradient agarose hydrogels were stained for: ECs with CD31, junctional
proteins with β-catenin and vessel wall extracellular matrix with laminin. Fluorescent confocal image stacks along the z-axis of the hydrogels show 3D representations of the vessels (Error! Reference source not found.).

Figure 2-8: Tubular formations of ECs guided into the 3D GRGDS-agarose hydrogels following a VEGF165 gradient of 1.65 ng mL⁻¹ μm⁻¹, having a starting concentration of 316 ng mL⁻¹ (as shown in Figure 3c). Confocal images were taken at cross-sectional views of ECs immunostained with primary antibodies: a) CD31 for ECs; b) β–catenin for junctional proteins; c) laminin for extracellular matrix proteins; and d) combined image of a, b, c. Scale bars (= 20 μm) are parallel to the Z-direction in the gels.
To explore the impact of the VEGF165 concentration on the elongation of the tubular-like structures, a series of agarose hydrogels were synthesized with the same immobilized VEGF165 gradient (of 1.8 ng mL$^{-1}$ μm$^{-1}$) and different initial VEGF165 concentrations: 566 ng mL$^{-1}$, and 256 ng mL$^{-1}$ (Error! Reference source not found.). We observed that ECs did not migrate beyond depths where the VEGF165 concentration was 600-800 ng mL$^{-1}$, suggesting that the VEGFR2 receptors on endothelial stalk cells may have been saturated and thus they were neither proliferating and nor promoting EC migration. While the Kd of soluble VEGF165 for VEGFR2 is 75-125 pM, our data suggest a significantly higher saturation of 15.7-20.9 μM, indicating that VEGF165 immobilization may affect its binding to VEGFR2.
Figure 2-9: ECs were guided into tubule-like structures in the hydrogels and the migration distance was regulated by the concentration of immobilized VEGF165. a) Quantification of the gradient of immobilized VEGF165 in the agarose hydrogels was based on a standard curve of fluorescently tagged VEGF165. A similar gradient of immobilized VEGF165 (1.8 ng mL\(^{-1}\) μm\(^{-1}\)) was designed in the agarose hydrogels, with different initial concentrations of immobilized hVEGF165: (I) 566 ng mL\(^{-1}\) or (II) 256 ng mL\(^{-1}\). b-c) Phase-contrast microscopy images of primary ECs growing into the hydrogels after 3 d of culture (arrows point the surface of the hydrogels). ECs migration seemed to be limited by the concentration of VEGF165 present at a given depth. EC migration was limited to b) 200-250 μm and c) 50-100 μm, which correspond to approximately 600-800 ng mL\(^{-1}\) VEGF165 according to a) (Scale bar = 100 μm).

2.5 Conclusions

In summary, we demonstrated that ECs can be guided to form tubule-like structures by a gradient of immobilized VEGF165 in 3D hydrogels. By using coumarin chemistry and a multiphoton patterning technique, maleimide-modified VEGF165 and GRGDS were immobilized within agarose-sulphide hydrogels. The ECs showed stereotypical tip and stalk-like cell morphology in the agarose gels and exhibited tubule-like formation as they migrated in response to the VEGF165 gradient. The effect of VEGF165 concentration and concentration gradients on tubule-like structure in 3D environments has not been previously reported. This 3D model of EC growth may be useful for engineered tissues and to study the stem cell niche. Ultimately, the ability to fabricate 3D-patterned scaffolds opens up a broad array of applications in tissue engineering.
3 Development of a Endothelial Cell Niche Using 3D Hydrogels

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3.1 Abstract

The role of endothelial cells (ECs) in the adult retinal stem and progenitor cell (RSPC) niche has been largely unexplored; yet, ECs have a significant role in the adult neural stem cell niche. Furthermore, most studies have been performed in 2D cell cultures, lacking the 3D microenvironment found in vivo. To better mimic the stem cell microenvironment in vivo, engineered polymeric scaffolds were synthesized to examine the role of ECs on RSPC fate in 3D. Our results demonstrate that ECs influence migration, proliferation and differentiation of RSPCs. ECs promote RSPC migration, yet, inhibit proliferation and differentiation of RSPCs when they are co-cultured in 3D environments. To our knowledge, this is the first time that the role of ECs on RSPC fate has been demonstrated in a biomimetic 3D model where ECs adopt morphologies similar to those observed in vivo. This novel biomimetic vascular niche model should provide significant insight into the RSPC in vivo niche.

3.2 Introduction

Adult stem cells are regulated by signals from both adjacent differentiated cell types and extracellular matrix molecules, which collectively define the stem cell “niche”[11, 125, 126]. In order to elucidate the complex biological mechanisms of the stem cell niche, biomimetic strategies must evolve to include some of this complexity including 3D presentation of the extracellular matrix (ECM) and culture of multiple cell types.

Adult mammalian retinal stem and progenitor cells (RSPCs), isolated from the pigmented ciliary epithelium of the eye [46-48], show the two cardinal properties of self-renewal and
multipotentiality, having the capacity to differentiate into rod photoreceptors, bipolar cells, retinal ganglion cells and Müller glia [46, 48]. Interestingly, the retina in cold-blooded vertebrates continues to grow throughout adult life by the proliferative addition of new neurons at the rim of the retina from a germinal zone at the ciliary margin [49]. However, endogenous stimulation of adult mammalian RSPCs has not yet been shown.

Endothelial cells (ECs) have been shown to have fundamental roles in neurogenic niches beyond their vascular roles as suppliers of oxygen and nutrients [30]. For example, it has been shown that neural stem and progenitor cells (NSPCs) in the subventricular zone (SVZ) of the brain are found in close proximity to capillary tips [33]. ECs have been shown to secrete factors, supporting neurogenesis of NSPCs [32, 73]. The co-localization of ECs and stem and progenitor cells is by no means unique to the brain [127], and regulatory signals generated by ECs appear to influence stem cell fate in various tissues, including the hematopoietic system [4, 10] and adipose tissue [6, 40].

In the RSPC niche, the ciliary body is composed of two distinct epithelia: inner layers and outer layers [46]. The inner layer of the ciliary body epithelium consists of non-pigmented cells whereas the outer layer contains pigmented cells, which include RSPCs. Importantly, the outer layer includes a highly dense 3D network of capillaries lined by ECs [50, 51], suggesting a role of ECs in the RSPC niche; however, understanding the in vivo interactions between ECs and RSPCs has been limited by the lack of RSPC-specific markers.

Bioengineering approaches have been developed to fabricate novel bioactive scaffolds for tissue engineering [13, 14]. Hydrogels provide a hydrated, 3D environment similar to soft tissues that allow the diffusion of nutrients and cellular waste through elastic networks [60, 61]. Although commercially available Matrigel® matrix has been used in 3D studies [128], Matrigel® is ill-
defined and inconsistent in composition, making results difficult to interpret. ECs have been previously shown to grow into GRGDS-modified agarose hydrogels having a linear concentration gradient of immobilized VEGF165 [129]. In this 3D hydrogel environment, ECs exhibit morphological and functional differences (relative to 2D cultures), forming tubular-like structures with tip-like and stalk-like cells identified. Here, using this well-defined spatially-controlled platform, we investigate the interactions between ECs and RSPCs in a defined co-culture system. This bioengineered scaffold allows the investigation of cell-cell interactions in 3D, thereby providing important insights to the in vivo niche.

### 3.3 Materials and Methods

#### 3.3.1 Scaffold fabrication and immobilization of cell adhesive peptides and VEGF165

Agarose was modified with a photolabile thiol protected-6-bromo-7-hydroxycoumarin as described previously [71]. Modification and immobilization of VEGF165 was performed as previously described with minor modifications [119, 129]. A solution of photolabile agarose (0.3 wt%) containing maleimide- and Alexa Fluor-modified VEGF165 (MI-VEGF165-f) solution (8 μg/mL) was pipetted into a 1 mm glass cuvette (Starna Cells, Inc, Atascadero, CA). After cooling at 4 °C for 40 min to ensure complete gelation, the cuvette was mounted on the stage of a Leica TPS SP2 confocal microscope equipped with a Spectra-Physics Mai Tai broadband Ti-Sapphire laser, tuned to 740 nm [71]. After focusing the laser to a plane in the interior of a gel at low power, the Leica software was used to define a region of interest (300 μm × 300 μm) and the laser power was increased through software controls to the maximum available. A macro program was previously written using Leica software to create concentration gradient patterns of the desired shape and dimension [71]. After patterning, gel samples were immersed in PBS buffer to remove unreacted MI-VEGF165-f for 2 days. Mi-GRGDS including tris(2-
carboxyethyl) phosphine hydrochloride (Sigma-Aldrich) in PBS (100 μL, 0.5 mg/mL) was then added to each cuvette and immobilized as described above using confocal laser patterning. Unreacted mi-GRGDS was rinsed by successive washing in PBS for 12 h.

3.3.2 EC and RSPC Migration

We examined the migratory effect of ECs on RSPCs by confocal imaging using our engineered matrix in which the 3D relationship of cells is preserved. Descriptions of the isolation and culture of primary endothelial cells (ECs) from the brain of adult mice and primary retinal stem and progenitor cells (RSPCs) from the ciliary epithelium of adult mice are available in the later section. 3D co-culture was performed in the co-culture medium comprised of retinal serum free media (SFM) [46, 48] containing 2% fetal bovine serum (FBS), 2% horse serum (Gibco-Invitrogen), 20 μg/mL heparin (Sigma-Aldrich), 20 μg/mL endothelial cell growth supplement (ECGS) (Sigma-Aldrich) and 100 μg/mL P/S. Dissociated primary ECs (10,000 cells per cuvette) and RSPCs (10,000 cells per cuvette) were seeded on top of the cell-adhesive agarose hydrogels including VEGF165 gradient or no VEGF165 gradient. In order to distinguish RSPCs from ECs, RSPCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using CellTrace™ CFSE Cell Proliferation Kit (Invitrogen). We maintained the hydrogels for 14 d in 0.5 mL medium per cuvette, changed every 4 d, in a humidified 5% CO₂ incubator at 37°C. As a control, RSPCs were cultured alone separately for the same period in the identical cell culture medium.

Based on the observation that laminin expression was highly abundant and localized where RSPCs and ECs were in contact, we examined whether adult RSPCs express the laminin receptor α6β1 integrin by immunofluorescence staining, described in the later section. In addition, in order to test whether α6β1 integrin was important for RSPCs to bind to ECs, ECs (Passage 2-4)
were grown in 48-well plates in DMEM/F12 plus 10% FBS, 10% horse serum, 100 μg/mL heparin, 100 μg/mL ECGS to 80% confluence. Medium was changed to serum-free medium 2 d before co-culture. RSPCs labeled with CFSE were pre-incubated with GoH3-blocking antibody 1:50 (Beckman Coulter), β1 integrin-blocking antibody (BD), or IgG control antibody (BD). To assess adhesion, the medium was changed 1 h after plating, washing away non-adhered cells; the remaining attached CFSE positive cells were counted.

For all immunocytochemical procedures, appropriate controls were obtained by omission of the relevant primary antibody.

### 3.3.3 Effects of ECs on RSPC Proliferation and Differentiation

Dissociated primary ECs (10,000 cells per cuvette) and RSPCs (10,000 cells per cuvette) were seeded on top of the cell-adhesive, VEGF165 gradient agarose hydrogels under the co-culture medium. In order to distinguish RSPCs from ECs, RSPCs were labeled with CFSE, and ECs were labeled using the CellTrace™ Far Red DDAO-SE Kit (Invitrogen). We maintained the hydrogels for 14 d in 0.5 mL medium per cuvette, changed every 4 d, in a humidified 5% CO₂ incubator at 37°C. We investigated RSPC proliferation and differentiation with immunofluorescence staining, described in detail in the SI text. Next, we investigated the effects of ECs on RSPC fate in 2D culture by co-culturing ECs and RSPCs either directly or indirectly. Descriptions of direct and indirect RSPC and EC co-culture systems are available in the later section. We investigated RSPC proliferation as well as differentiation with immunofluorescence staining and reverse transcription polymerase chain reaction (RT-PCR), described in detail in the SI text. For all immunocytochemical procedures, appropriate controls were obtained by omission of the relevant primary antibody.
3.3.4 Statistical Analysis

Statistical significance of 3D and 2D culture samples were tested with n = 3 samples. Differences among groups were assessed by one-way ANOVA with the Tukey’s post hoc analysis to identify statistical differences among three or more treatments at p < 0.05. A student’s t-test was used to statistically compare less than three treatments. All data are presented as mean ± SD.

3.3.5 Isolation of Endothelial Cells (ECs) and Culture System

Primary brain-derived ECs were isolated by following a protocol (see Appendix B) [27]. These are cultured in DMEM and F12 (Gibco-Invitrogen) medium containing 10% FBS, 10% horse serum (Gibco-Invitrogen), 100 μg/mL heparin (Sigma-Aldrich), 100 μg/mL endothelial cell growth supplement (Sigma-Aldrich) and 100 μg/mL P/S in a 24-well plate coated with murine collagen (BD Biosciences, San Jose, CA, USA). Experiments were performed on cells at passage 2 - 4.

3.3.6 Isolation of Retinal Stem/Progenitor Cells (RSPCs) and Culture System

RSPCs were derived from the ciliary epithelium of adult mice (minimum 6 weeks old) as described previously with minor modifications [46, 48]. Cells were plated in serum free media (SFM) on non-adherent 6-well tissue culture plates (Nunc; Thermo Fisher Scientific, Rochester, NY) at a density of 10-20 cells/μL. Floating spheres of cells were selected after 7 days of primary culture and plated on laminin (50 ng/mL) coated 24-well plates (Nunc) in SFM plus 1% FBS, FGF2 (10 ng/mL, human recombinant; Sigma, Burlington, Ontario) and heparin (2 ng/mL; Sigma) before co-culture studies to expand RSPC populations.
3.3.7 Direct RSPCs and ECs Co-culture

Confluent brain ECs were trypsinized and plated at a density of 50,000 cells per well using 8 Well Lab-Tek Chamber Slide (Nunc) pre-coated with murine collagen (BD Biosciences). The ECs were cultured for 1 d in the EC culture medium. The medium was then removed, and ECs were rinsed before co-cultured with RSPCs. A total of 20,000 RSPCs were plated directly onto the EC cultures. RSPC and EC co-cultures were grown in the co-culture media (SFM including 2% FBS, 2% horse serum, 20 μg/mL heparin, 20 μg/mL ECGS). As a control, RSPCs were cultured alone separately for the same period and in the same medium. RSPC proliferation and differentiation were analyzed after 5 weeks of culture by immunofluorescence staining, and reverse transcription polymerase chain reaction (RT-PCR).

3.3.8 Indirect RSPC and EC Co-culture Using Transwells

RSPCs were harvested by trypsinization, and plated on laminin-coated 24 well plates at a density of 20,000 cells per well. ECs were trypsinized and plated into the upper compartment of transwell inserts (0.4 mm pore size, BD Falcon, Franklin Lakes, NJ, USA), at a density of 50,000 cells per 0.3cm² area and expanded in a serum containing EC medium for 1 d before co-culture study. RSPCs and ECs were both rinsed with SFM, and the upper transwell compartments containing ECs were placed into the RSPC culture well. Indirect RSPC and EC co-cultures were maintained in the co-culture media and harvested, and RSPCs were processed separately for immunofluorescence staining and RT PCR. As a control, RSPCs were cultured alone. For conditioned medium experiments, approximately 50,000 EC cells per well of a 24-well plate were incubated with the co-culture medium, and the supernatant was collected every 4 days and then stored frozen at -80 °C until further use.
3.3.9 Immunofluorescence Analysis

For all immunocytochemical procedures, appropriate controls were obtained by omission of the relevant primary antibody.

The following primary antibodies were used for the immunocytochemical studies: rat anti-CD31 (1:10; Abcam); chicken anti-laminin (1:500; Abcam); and mouse anti-VE Cadherin (1:25; Abcam), mouse anti-rhodopsin (Rho1D4, 1:00; Millipore), mouse anti-RPE65 (1:250; Millipore), mouse anti-Ki-67 (1:10; BD Pharmigen), mouse anti-Pax6 (1:400; Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-Cralbp (1:500; Abcam, Cambridge, MA), rabbit anti-calbindin (1:1000; Millipore), rabbit anti-Otx2 (1:500; Millipore), goat anti-Brn-3b (1:50; Santa Cruz Biotechnology, INC), rat anti-integrin α6 (1:50; Millipore) and rat anti-integrin β1 (1:25; Millipore). The following Alexa-tagged secondary antibodies (Molecular Probes, Invitrogen) were used (1:400): goat anti-chicken 568, goat anti-rabbit 568, anti-mouse 568, and anti-mouse 633. For all immunofluorescence procedures, appropriate controls were obtained by omission of the relevant primary antibody. Cells on the substrates were fixed with PBS solution containing 4% PFA for 30 min in 3D culture and 20 min in 2D culture, and then washed with PBS including 0.2 wt% BSA solution. After cell membrane permeation and blocking by treating with BSA (1.5 wt%) and Triton X-100 solution (0.3%) at room temperature for 1 h, each specific primary antibody solution, as described above, was added overnight at 4 °C. After washing with PBS, samples were exposed to each secondary antibody overnight at 4 °C in 3D culture or for 2 h in 2D culture, and then washed with PBS buffer including 0.2 wt% BSA solution. For 2D coculture studies, chamber slides or 24 well plates were counterstained with DAPI mounting media (Fluoro-gel; Electron Microscopy Sciences, Hatfield, PA). For 3D imaging, fluorescent Z-series image stacks were captured on Zeiss MicroImaging LSM510 microscope using a water-immersion 40X numerical aperture plan Apochromat objective and compiled into three-
dimensional rendering with LSM software (University of Toronto, Faculty of Medicine, ON, Canada). Excitation wavelengths were 488 nm (argon laser), 543 nm and 633 nm (He-Ne laser) for Alexa 488, 568, and 633 respectively. For 2D imaging, chamber slides were imaged using a 20 X objective on the upright microscope (Olympus, BX61) equipped with a monochrome camera (Photometrics Cool Snap HQ, Roper Scientific, Tucson, AZ) as well as spinning disc confocal imaging system (Zeiss Axio Observer Z1). 24 well plates were imaged using a 20X objective on the inverted microscope (Zeiss Axio Observer Z1). Quantitative analysis was performed by counting a total cell number of at least 200 cells per well in randomly selected fields using DAPI labeling. Each determination was performed in triplicate, and each experiment was repeated at least three times.

3.3.10 Reverse Transcription Polymerase Chain Reaction (RT PCR) Analysis

Total RNA was extracted with using the RNaseaueous micro kit (Ambion, Austin, TX), treated with RNase-free DNase I, and reverse transcribed with ThermoScript™ RT-PCR System (Invitrogen), according to the instructions of the manufacturer. PCR amplifications were performed as follows: 2 min at 94 °C followed by 35 cycles consisting of 30 s at 94 °C, 30 s at specific temperature as listed below, 40 s at 72 °C and a final 5 min extension at 72 °C. The PCR products were separated by electrophoresis on a 2.0% agarose gel and detected under UV illumination. For each set of primers, control amplification was performed without addition of reverse transcriptase to exclude the possibility of genomic DNA contamination. Primers used for PCR are as follows: Pax6-forward 5’-CCATCTTTGCTTGGGAAATCC-3’ and reverse 5’-TCATCCGAGTCTTCTCCATTGG-3’ (56°C, 310bp), Otx2-forward 5’-GAGAGGAGGTGGCACTGAAAATC-3’ and reverse 5’-CCCCTAGGATGGAGGAAGTTGGAGC-3’ (56°C, 391bp), rhodopsin-forward 5’-
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TCACCACCACCCTCTACACA-3' and reverse 5'-TGATCCAGGTGAAGACCACA-3' (60°C, 216bp), calbindin1-forward 5'-GCAACCATAGGACTTCACCC-3' and reverse 5'-GCTACCTCCCTACCAAGC-3' (58°C, 215bp), and S15-forward 5' - TTCCGCAAGTTTCCTACC-3' and reverse 5'-CGGCCGGCCATGCTTTACG-3' (60 °C, 361 bp).

3.4 Results

Vertical, linear concentration gradient volumes of fluoresceinly-tagged VEGF165, having dimensions of 300 μm × 300 μm × 600 μm (L × W × D), were synthesized in GRGDS-functionalized agarose, as previously described [129]. A schematic of the experimental setup is shown in Figure 3-1. The gradients were created by controlling the number and volume of two photon-scanned regions and quantified based on the fluorescence intensity. A VEGF165 concentration gradient of 1.5–1.8 ng/mL/μm with an initial concentration of 250–300 ng/mL was synthesized.

Figure 3-1: Schematic of the co-culture experimental setup of RSPCs and ECs. (A) Using a multiphoton laser patterning technique, maleimide-modified VEGF165 gradient and maleimide-modified GRGDS were immobilized within agarose-sulphide hydrogels. (B) RSPCs and ECs were plated on the cell-adhesive, VEGF165 gradient agarose in the co-culture medium. (C) Both
RSPCs and ECs formed aggregates on top of the patterned volumes and started penetrating agarose hydrogels by following the VEGF165 concentration gradient.

In order to examine the cellular behavior of RSPCs and ECs when they are co-cultured, dissociated adult mouse primary brain microvascular ECs and adult mouse RSPCs, dissected from the ciliary epithelium, were seeded on top of the GRGDS cell-adhesive, VEGF165 gradient agarose hydrogels using the co-culture medium of: 2% fetal bovine serum (FBS), 2% horse serum, 20 μg/mL heparin, 20 μg/mL endothelial cell growth supplement (ECGS) and 100 μg/ml penicillin/ streptomycin (P/S). In order to distinguish RSPCs from ECs, RSPCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using the CellTrace™ CFSE Cell Proliferation Kit. After seeding ECs and RSPCs on top of agarose hydrogels, they formed small aggregates on top of the patterned volumes, within the first 24 h of culture, prior to penetrating the gels. Controls had RSPCs alone and ECs alone cultured separately on identical VEGF-gradient, GRGDS-agarose hydrogels. After 1 d, RSPCs cultured alone formed aggregates on top of the patterned volumes, yet did not migrate into the hydrogels. As previously observed, ECs cultured alone penetrated the agarose hydrogels and formed tubule-like structures in the agarose hydrogels after 3 d of culture; however, ECs retracted their leading process and reversed direction toward the EC aggregates localized on the surface after 5 d of culture (in 11 out of 12 samples). In the co-culture of ECs and RSPCs, ECs migrated up the VEGF165 concentration gradient after 3 d of culture and, surprisingly, RSPCs migrated along the EC tubular-like formations as shown in Figure 3-2A. Moreover, in contrast to ECs cultured alone, ECs retained their tubular-like structures for 14 d, as shown in Figure 3-2B, and with which RSPCs were closely associated. To gain greater insight into the 3D spatial relationship between ECs and RSPCs co-cultured in the VEGF165 concentration gradient GRGDS-agarose hydrogels, EC specific junctional proteins were immunostained with VE cadherin and vessel wall extracellular
matrix was stained with laminin. Fluorescent confocal image stacks along the z-axis of the hydrogels (Figure 3-2B) show 3D representations of cellular interactions: ECs are in process of forming tubular-like structures and RSPCs are migrating along the ECs. Although we frequently detected the filopodia of endothelial tip-like cells in the EC-RSPC co-culture system, a hallmark of angiogenesis during the first 3 d of culture, we did not detect them after 5 d. These results suggest that ECs provide directional guidance to RSPCs, and that the association of RSPCs with ECs stabilizes EC tubular-like structures within the hydrogels for a longer time period than ECs cultured alone. When ECs and RSPCs were cultured on patterned GRGDS peptide hydrogels in the absence of VEGF165, ECs and RSPCs migrated only a short distance (< 30-50 μm) into the hydrogels, just below the surface as shown in Figure 3-2C. Interestingly, in the absence of the VEGF165 gradient, both ECs and RSPCs made a U-turn toward the aggregates on top of the hydrogels after 3 d of culture, suggesting that RSPC migration is regulated by that of ECs which is in turn dependent on a VEGF165 concentration gradient.
Figure 3-2: Representative images of RSPCs migrating along ECs in 3D agarose hydrogels. (A) Confocal 3D images show both ECs migrating into the GRGDS-, VEGF165 gradient- agarose hydrogels and RSPCs closely associated with the ECs after 3 days of co-culture. Cells were immunostained with primary antibodies: a) VE cadherin (blue) for junctional proteins expressed by ECs; b) laminin (red) for the extracellular matrix of ECs; c) CFSE (green) to label RSPCs prior to co-culture with ECs; and d) a merged image showing the combined staining of VE cadherin, laminin and CFSE labeled RSPCs shows the close association of ECs and RSPCs (scale bar = 20 µm). (B) Confocal 3D images of ECs that had migrated and formed tubular-like structures in the GRGDS-, VEGF165 gradient-agarose hydrogels after 14 days of culture. RSPCs were detected where laminin expression was highly abundant. RSPCs appeared to stabilize EC tubular structures. Cells were immunostained with primary antibodies as in (A): a) VE cadherin (blue) for junctional proteins; b) laminin (red) for EC ECM; c) CFSE (green) for RSPCs; and d) merged staining of VE cadherin, laminin and CFSE-labeled RSPCs (scale bar = 50 µm). (C) Confocal 3D images of ECs and RSPCs growing into GRGDS-agarose hydrogels in the absence of VEGF165 gradients. ECs and RSPCs retracted toward the aggregates on top of the hydrogels after 3 days of co-culture (white arrow points to the top of the hydrogels): a) ECs immunostained with CD31 (blue); b) RSPCs labeled with CFSE (green); c) merged images of CD31-labeled ECs and CFSE-labeled RSPCs (scale bar = 50 µm).

The strong cellular interactions between ECs and RSPCs observed in 3D may be explained by a chemical and/or physical hypothesis. For example, trophic factors produced by ECs may promote the proliferation and/or differentiation of the RSPCs, lending credence to a chemical hypothesis. Alternatively, the tubular-like EC structures may act as physical scaffolds that guide the migrating RPSCs. It is well known that the ECM plays a critical role in regulating stem cell differentiation, migration and proliferation. The ECM interacts with cells via cell-surface receptors such as integrins and provides a site for growth factor binding, a substrate for cell attachment and spreading, contact guidance for cell migration, and a scaffold that serves as the building blocks of tissues.
In terms of the physical guidance hypothesis, it is important to note that laminin is expressed largely by ECs and that laminin expression was highly abundant and localized at the EC-RSPC interface (Figure 3-2B). Moreover, the α6β1 integrin, one of the laminin receptors, was widely expressed by cultured RSPC neurospheres (see Figure 3-3 and Figure 3-4).

Figure 3-3: Representative confocal images of α6 integrin immunostaining of RSPC spheres cultured on laminin-coated plates: a) α6 integrin (green), b) DAPI (blue), c) merged staining of α6 integrin and DAPI.

Figure 3-4: Representative confocal images of β1 integrin immunostaining of RSPC spheres cultured on laminin-coated plates: a) β1 integrin (green), b) DAPI (blue), c) merged staining of β1 integrin and DAPI.
To determine whether RSPCs express the laminin α6β1 integrin receptor when they migrated along ECs, the GoH3 antibody, which recognizes the α6 integrin, was found to co-express with the most laminin-positive elements and also labeled RSPCs lying on ECs (see Figure 3-5).

Figure 3-5: Representative 3D confocal images of α6 integrin immunostaining of RSPCs migrating along tubular-forming ECs in GRGDS- VEGF165 gradient-agarose hydrogels. The α6 integrin detected most laminin-positive elements and also labeled RSPCs closely associated with ECs: a) laminin (green) for extracellular matrix; b) α6 integrin (blue); c) RSPCs labeled with CFSE (red); and d) merged staining of laminin, α6 integrin and CFSE labeled RSPCs (scale bar = 20 µm).
Similarly, we observed the β1 integrin antibody at the laminin-positive elements where RSPCs bound to ECs (Figure 3-6A). To test whether α6β1 integrin is important for RSPCs to bind to ECs, CFSE labeled RSPCs were either pre-incubated in an anti-α6 or anti-β1 or simply plated on top of 80% confluent EC monolayers. Most of the dissociated CFSE-labeled RSPCs did not bind to the EC monolayer when pre-incubated in either GoH3 (anti-α6) or anti-β1 prior to plating whereas most bound to the EC monolayer when these integrin receptors were available (controls, Figure 3-6B). Thus, RSPCs express a key receptor that enables them to bind to the laminin-rich EC surface and the attractive molecules derived from ECs promote RSPC-EC interactions. These results provide new insights into the potential mechanisms that regulate RSPC migration along ECs: the laminin ECM produced by the endothelial cells allows α6β1-integrin expressing RSPCs to bind and migrate along the ECs.
Figure 3-6: α6β1 integrin is expressed in RSPCs and required for RSPCs to adhere to laminin expressed by ECs. (A) Representative 3D confocal images of β1 integrin immunostained RSPCs migrating along with tubular-forming ECs in the GRGDS-, VEGF165 gradient- agarose hydrogels. β1 integrin detected most laminin-positive elements and also labeled RSPCs in close
association with the ECs: a) laminin (blue) for EC extracellular matrix; b) β1 integrin (red) expressed by RSPCs; c) CFSE (green) labeled RSPCs; and d) merged staining of laminin, β1 integrin and CFSE labeled RSPCs (scale bar = 20 µm). (B) Blocking with anti-α6 integrin (GoH3) or anti-β1 integrin inhibited the adhesion of RSPCs (CFSE+ cells) to EC monolayers demonstrating the importance of EC-laminin and RSPC-laminin receptors, α6β1, for EC-RSPC interactions. Data are mean ± SD (**p<0.001, n=3 separate experiments, with each experiment done in triplicate and a minimum of 200 cells counted).

In terms of the chemical cue hypothesis where ECs guide RSPC proliferation and differentiation through contact-mediated signaling, the autocrine and/or paracrine factors secreted by ECs were examined in terms of RSPC proliferation and differentiation. Previous studies using PCR analysis demonstrated that RSPCs cultured in 3D GRGDS-agarose hydrogels exhibited mRNA expression specific to differentiated retinal cells including: Brn3b+ ganglion cells, rhodopsin+ photoreceptors, GFAP+ Müller glia cells and PKCα+ bipolar cells in addition to undifferentiated nestin+ retinal progenitor cells after 14 days of culture (see Appendix D). In order to distinguish RSPCs from ECs in our co-culture experiments, RSPCs were labeled with CFSE, and ECs were labeled using the CellTrace™ Far Red DDAO-SE Kit. After 14 days of co-culture in VEGF165-gradient GRGDS-agarose hydrogels, ECs formed tubular-like structures that were apparently stabilized by the close association of RSPCs. The co-cultured cells were fixed and immunostained with Ki67 for proliferation and with a series of markers to determine the differentiated retinal progeny. Interestingly, 84.5 ± 6.7% of RPSCs were Pax6+ progenitor cells (Error! Not a valid bookmark self-reference.A) with only a low percentage of Otx2+ mature bipolar cells or immature rod/bipolar progenitors (1.2 ± 0.5 %). There were neither markers for proliferative RSPCs nor for mature differentiated retinal cell phenotypes, suggesting that ECs may, in addition to affecting the migration, regulate the differentiation and proliferation of RSPCs.
To better define the signaling between ECs and RSPCs, 2D co-culture experiments were performed. RSPCs were cultured with ECs in either direct contact or indirect contact. In the direct co-culture study, RSPCs were plated directly on EC cultures whereas in the indirect co-culture study, RSPCs were plated on laminin-coated 24-well plates and ECs were plated on collagen-coated transwell inserts in the same well. In this way, RSPCs and ECs shared the same growth medium with facile diffusion of molecules throughout the culture yet no physical contact between the two cell types. As a control, RSPCs were cultured alone but still in the same co-culture medium. After 5 weeks of culture, the RSPCs were analyzed using immunofluorescence staining and RT-PCR.

As shown in Table 3.1, RSPCs in control monocultures differentiated to Otx2+Rho1D4-mature bipolar cells or immature rod/bipolar progenitors, Calbindin+ horizontal cells, and Rho1D4+ photoreceptors. Interestingly, the expression of Pax6 was significantly reduced to 33.6±3.5% in control cultures at 5 weeks compared to that of 78.6 ± 6.3% at day 0 (Error! Not a valid bookmark self-reference.B). The expression of these retinal lineage markers was similar to previous reports of the differentiation potential of the RSPCs [46, 48] with the exception of no Müller glia detected in our assay. This may be due to the addition of horse serum and/or endothelial cell growth supplements to the medium used in these studies.

In contrast to the differentiation profile observed for RSPC monocultures, most of the RSPCs growing in the direct EC contact and indirect EC co-cultures remained undifferentiated, exhibiting 78.4±4.1% of Pax6+ cells in direct and 76.9±1.0% in indirect co-culture systems. In these co-culture systems, there was no significant difference in the Pax6+ progenitor cell population compared to day 0 (Error! Not a valid bookmark self-reference.B; n=3, p<0.001).

Confocal images in Error! Not a valid bookmark self-reference.C demonstrate that RSPCs
maintain an undifferentiated state, with CFSE labeled RSPCs expressing Pax6 when cultured in direct contact with CD31-stained ECs. Low levels of Otx2 expressing cells were observed after 5 weeks of co-culture; however, 10.8±3.1% of Otx2+ cells were observed prior to co-culture studies. This immunofluorescence analysis was consistent with mRNA expression for all markers except cells showed very low levels of Rho1D4+ immunostaining yet no rhodopsin mRNA expression in the indirect co-culture system. This may be due to the difference in sensitivity of analysis. Consistent with the 3D co-culture results, Ki67+ proliferative RSPCs were not detected in direct contact co-culture systems after 1, 2 and 5 weeks of culture. In contrast, a low percentage of proliferating cells was observed in both indirect co-culture systems and control RSPC monoculture systems after 5 weeks of culture, although the percentage of proliferative RSPC populations decreased from time 0: 34.1±4.0% at day 0 decreased to 5.6±1.7% in indirect co-culture and to 2.5±0.3% in RSPC monocultures. In a separate experiment, to gain further insight into EC-derived soluble factors, when RSPCs were cultured in EC-conditioned medium, RSPCs did not seem to differentiate, keeping a high percentage of Pax6+ cells, yet a low percentage of proliferating RSPCs, which is similar to the indirect co-culture results described in Table 3.1. This result confirmed that soluble factors derived from ECs play a critical role in regulating RSPC differentiation. Taken together, we demonstrate that ECs influence the RSPC differentiation prolife: ECs maintain RSPCs in an undifferentiated state and physical direct contact with ECs inhibits RSPC proliferation.
Table 3.1: Quantitative evaluation of RSPC differentiation profiles at 5 weeks. RSPC differentiation profiles cultured: alone, indirectly with ECs, directly with ECs, and in the conditioned media from ECs. The number of cells is expressed as a percentage of total DAPI-positive nuclei in cultures (n=3 separate experiments, with each experiment done in triplicate and a minimum of 200 cells counted, mean ± standard deviation reported).

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Control</th>
<th>Indirect co-culture with ECs</th>
<th>Direct co-culture with ECs</th>
<th>RPCs in conditioned media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progenitor cells (Pax6)</td>
<td>33.6±3.5%</td>
<td>76.9±1.0%</td>
<td>78.4±4.1%</td>
<td>70.2±2.3%</td>
</tr>
<tr>
<td>Bipolar cells (Ob2+/Rho-)</td>
<td>27.5±0.2%</td>
<td>8.8±2.1%</td>
<td>9.9±2.1%</td>
<td>8.9±2.1%</td>
</tr>
<tr>
<td>Horizontal cells (Calbindin)</td>
<td>11.3±3.6%</td>
<td>&lt; 1.0%</td>
<td>Not detected</td>
<td>&lt; 1.0%</td>
</tr>
<tr>
<td>Photoreceptors (Rho1D4)</td>
<td>14.9±3.3%</td>
<td>2.0±1.3%</td>
<td>Not detected</td>
<td>7.1±2.6%</td>
</tr>
<tr>
<td>Ki67 (Proliferation)</td>
<td>2.5±0.3%</td>
<td>5.6±1.7%</td>
<td>Not detected</td>
<td>3.0±0.1%</td>
</tr>
</tbody>
</table>
Figure 3-7: RSPCs remain undifferentiated when they are co-cultured with ECs. (A) Representative 3D confocal images of Pax6 immunostaining of RSPCs migrating along tubular-forming ECs in GRGDS-, VEGF165 gradient-agarose hydrogels at day 14: a) RSPCs express the retinal progenitor marker Pax6 (red); b) RSPCs labeled with CFSE (green); c) ECs labeled with Far Red DDAO-SE (blue); and d) merged staining of Pax6 and CFSE labeled RSPCs with DDAO-SE labeled ECs (scale bar = 20 µm). (B) Comparison of the percentage of Pax6+ cells at day 0 vs. RSPCs cultured alone at 5 weeks vs. RSPCs co-cultured indirectly (i.e. without contact) with ECs at 5 weeks vs. RSPCs co-cultured in direct contact with ECs at 5 weeks. The percentage of Pax6+ cells was significantly reduced in control cultures at 5 weeks compared to day 0. In contrast, RSPCs co-cultured with ECs (either through direct or indirect contact) maintained their undifferentiated state at 5 weeks of co-culture. Data are mean ± SD (**p<0.001, n=3 separate experiments, with each experiment done in triplicate and a minimum of 200 cells counted). (C) Representative confocal images of Pax6 immunostaining of RSPCs co-cultured with 2D EC monolayers at 5 weeks: a) RSPCs expressing the retinal progenitor marker, Pax6 (orange); (b) RSPCs labeled with CFSE (green) are co-localized with Pax6+ cells; and (c) merged staining of Pax6, CFSE labeled RSPCs, ECs stained with CD31 (red) and DAPI (blue). (scale bar = 49 µm). (D) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of RSPCs: lane 1) RSPCs cultured alone at day 0; lane 2) RSPCs cultured alone for 5 weeks; lane 3) RSPCs co-cultured indirectly (i.e. without contact) with ECs for 5 weeks; and lane 4) RSPCs co-cultured in direct contact with ECs for 5 weeks. RSPCs grown alone expressed a range of differentiated retinal cell markers, including Otx2 (mature bipolar cells or immature rod/bipolar progenitors), Rhodopsion (photoreceptor), Calbindin1 (horizontal cells); although, in a separate experiment, Calbindin1 was also detected on ECs cultured alone. In contrast, RSPCs
co-cultured with ECs remained undifferentiated, expressing the retinal progenitor marker, Pax6, as well as Otx2, similar to RSPC expression at day 0.

### 3.5 Discussion

Our understanding of the importance of endothelial cells and the vascular system within the stem cell niche is emerging. Although the spatial and functional relationships between RSPCs and ECs within the ciliary margin of the retina are unknown, cell-cell interactions through ECM and integrins are critical in the neural stem cell niche of the adult forebrain SVZ [30, 33]. In the SVZ, laminin is a major component of the ECM around blood vessels, and NSPCs express the laminin receptor, α6β1 integrin [33, 130]. Previous studies by Tanentzapf et al. demonstrated that integrin-laminin interactions keep NSPCs in the vascular niche, and that this interaction impacts lineage regulation [131].

There have been several studies that elucidate the interactions between NSPCs and ECs. For example, under physiological conditions, NSPCs migrate along the rostral migratory stream (RMS) toward the olfactory bulbs in close association with blood vessels, supporting proliferation and neurogenesis of NSPCs [132]. Recent studies showed that brain-derived neurotrophic factor (BDNF), derived from blood vessels, promote NSPC migration in the RMS under physiological conditions [33]. In addition, it has been reported that NSPCs promote EC differentiation and vessel formation by diffusible VEGF and hypoxia inducible factor-1 α (HIF-1α) [133]. Furthermore, Lavik et al. demonstrated that transplanting both ECs and NSPCs promotes the stabilization of microvascular networks in vivo [74]. Using VEGF165 gradient-tethered cell-adhesive hydrogels, we showed that RSPCs migrate along ECs and stabilize EC assembly into tubule formations. Importantly, most of the migrating RSPCs were associated with strong laminin-containing ECM derived from ECs. RSPCs express laminin receptor, α6β1
integrin, and this receptor plays a critical role in RSPC adhesion to ECs. In contrast, RSPCs cultured alone did not migrate, suggesting that the ECM derived from ECs plays a pivotal role in regulating RSPC behavior, which in turn impacts EC behavior. Thus it is highly plausible that the functional reciprocal signaling between RSPCs and ECs, governed by common growth factors, stabilizes EC tubular formations. While the lack of specific RSPC markers in vivo hampers functional studies on the retinal vascular niche, these results suggest that our 3D in vitro model is a promising platform to study the detailed molecular mechanisms underlying the guidance of RSPCs and stabilization of ECs.

Our initial observation of RSPC migration with ECs, which is similar to that between NSPCs and ECs, led us to investigate whether ECs regulate RSPC differentiation as well as proliferation. Surprisingly, our studies showed that RSPCs remain undifferentiated in both direct and indirect co-culture systems. These results suggest that paracrine interactions regulate RSPC differentiation. This is the first demonstration to show that interactions between ECs and RSPCs regulate the differentiation potential of RSPCs. Although the molecular signals mediating interactions between RSPCs and ECs remain to be fully elucidated, a number of signaling pathways that are involved in retinal development and vascular stabilization have been reported [3, 134, 135]. We discuss several signaling mechanisms which may be involved in the regulation of RSPCs and ECs in Chapter 4.

Our results demonstrate that direct cellular contact with ECs inhibits proliferation of RSPCs when co-cultured in 3D as well as 2D. Interestingly, we did not observe this same result with indirect co-cultures, indicating that EC-derived soluble factors did not inhibit proliferation. This indicates that the interactions between ECM and integrin receptors play a key role in RSPC proliferation. A number of studies have reported that integrin binding regulates cell fate via
modulation of intracellular signaling pathways which in turn regulate transcriptional activity [136, 137]. For example, during early embryogenesis, ECM is considered to be important in self-renewal and subsequent differentiation of embryonic stem (ES) cells [95, 137]. Hayashi et al. demonstrated that ECM-integrin signaling inhibited self-renewal of ES cells cultured on fibronectin and laminin via the extracellular signal-regulated kinase (ERK)1/2 activity [137]. The integrin subunits expressed by proliferating RSPCs may provide further insight. Our results suggest that ECs regulate RSPC differentiation through soluble factors; however, it is possible that cell-contact may also play a role yet is masked by the absence of proliferation in the EC-RSPC direct co-cultures.

Engineered polymers can be used as a platform to better mimic the stem cell niche, allowing for multiple stimuli and many cell types to be explored individually or in combination. We demonstrate here that our 3D in vitro scaffold provides more biologically relevant complexity than typical 2D culture systems, resembling the cellular microenvironment in terms of cellular assembly and migration, and allowing soluble signals, cell-substrate interactions, and cell-cell contacts to be probed. These results provide a foundation for future studies on the stem cell niche and may find ultimate therapeutic use as well.
3.6 Conclusions

In summary, we demonstrated that ECs can be guided to form tubule-like structures by a gradient of immobilized VEGF165 in 3D hydrogels. By using coumarin chemistry and a multiphoton patterning technique, maleimide-modified VEGF165 and GRGDS were immobilized within agarose-sulphide hydrogels. The ECs showed stereotypical tip and stalk-like cell morphology in the agarose gels and exhibited tubule-like formation as they migrated in response to the VEGF165 gradient. The effect of VEGF165 concentration and concentration gradients on tubule-like structure in 3D environments has not been previously reported. This 3D model of EC growth may be useful for engineered tissues and to study the stem cell niche. Ultimately, the ability to fabricate 3D-patterned scaffolds opens up a broad array of applications in tissue engineering.

4 Conclusions and Future Recommendations

4.1 Overall Discussion

The principal goal in regenerative medicine is to promote tissue regeneration and healing after injury or disease. Tissue engineering presents the possibility of creating or regenerating various organs or organ-like structures for potential therapeutic intervention by using living cells in combination with biodegradable scaffold materials. Sources of cells for implantation include autologous cells from the patient, allogeneic cells from a human donor who is not
immunologically identical to the patient, and xenogeneic cells from a different species [138]. Each category may be further delineated in terms of whether the cells are adult or embryonic stem cells or a mixture of differentiated cells at different stages of maturation (including rare stem and progenitor cells). Some approaches use cell mixtures, whereas others rely on separation or enrichment of stem cells [139]. Particularly, stem cells hold great promise for treating damaged tissue where the source of cells for repair is extremely limited or not readily accessible. However, we still need better understanding to identify stem cells and their progenies, better ways to expand them in culture, and more research to see whether there is a way to direct stem cell differentiation along specific lineages.

Many biomaterials have been designed as 3D scaffolds in order to elucidate stem cell biology using a wide variety of materials, synthetic or natural in the past. The strategy of the development of bioactive materials is mimicking the microenvironments in stem cell niches, providing the spacial and temporal controlled cues that specifically govern stem cell fate determination [106]. While a variety of tissue engineering strategies to synthesize biomimetic scaffolds developed are discussed in Section 1, it is likely that a combination of these strategies is necessary to create the engineered extracellular environments to control stem cell functions.

Juxtacrine cell-cell communications, propagated by the physical association of two cells, provide a persistent morphogenic cue, while the diffusion of soluble signals from neighboring cells, paracrine signaling, transiently affects proximal populations of cells. The method of association between neighboring cell populations represents a significant difference in commitment to cooperate. These interactions between cell populations influence a range of stem cell behavior, including the induction of programs of differentiation and promotion of expansion and self-renewal properties. Mature cell populations physically associate, through adherent junctions,
with stem cells in native niche. For example, endothelium regulates neural stem cell differentiation in the brain as well as hematopoietic stem cells’ self-renewal in the bone marrow. It has been demonstrated that a prevascular network in engineered biomaterial constructs accelerate blood supply of hypoxic zones within the graft, resulting in increased survival of transplanted stem cells [140].

In order to elucidate stem cell biology, engineered polymers can be also used as a coculture platform to better mimic the stem cell niche, allowing for multiple stimuli and many cell types to be explored individually or in combination. Therefore, considering the importance of ECs, it is essential to develop an innovative 3D endothelial cell niche *in vitro* to investigate the role of ECs within stem cell niches. Our bioengineered scaffold allows the investigation of cell-cell interactions in 3D, thereby providing important insights to the *in vivo* niche.

Future challenges in tissue engineering and biomaterial strategies are likely to include extending *in vitro* stem cell-niche engineering to *in vivo* cellular strategies to further enhance regeneration after injury or disease. With regard to retinal degenerative diseases, biomaterials are likely to be incorporated into a retinal stem cell strategy to promote regeneration. Either endogenous retinal stem and progenitor cells could be stimulated through the delivery of the appropriate factors and stimuli, or exogenous retinal stem cells could be transplanted within biomaterial-based scaffolds [102]. Therefore, understanding the retinal stem niche using a 3D *in vitro* model will facilitate regeneration of retina after injury or disease.

In Chapter 2, we describe the synthesis of a novel 3D endothelialized hydrogel using photolabile agarose hydrogels. In terms of the range of immobilized VEGF165, these studies are performed with high VEGF concentration range - 300 to 1000 ng/ml. However, free VEGF typically is used at ~10-100 ng/ml. In this study, ECs cultured on RGD agarose hydrogels did not respond to
lower concentration such as 10-100 ng/ml. It has been demonstrated that interplay between integrin and VEGF regulate EC proliferation [141]. Considering that the ECs are usually cultured on the extracellular matrix, such as 5-10 µg/cm² collagen, it might be possible that the sensitivity and/or activity of VEGF165 receptors on ECs are impaired by relatively smaller immobilized concentration of GRGDS, leading to higher concentration range of VGEF165.

The mechanical microenvironment presents another dimension that influences cell behavior, especially for anchorage-dependent cell lineages. However, how substrate stiffness influence cell behavior on a molecular-level remains largely unknown. EC adhesion, migration, and differentiation are also known to be influenced by a mechanical component [142, 143]. We found in this study that ECs migrated into the gels when agarose hydrogel concentration was 0.3 wt%. On 0.5 wt% or 0.4 wt% agarose hydrogel substrates, ECs did not respond to the immobilized VEGF165 in the same fashion as on the 0.3 wt%. EC growth and migration is affected by the hydrogel mechanical property, which is directly related to the hydrogel concentration. Higher concentration gives a stronger matrix, but makes the gel harder for ECs to infiltrate. Thus, substrate stiffness is an important criterion in biomaterial modification, in addition to functionalization with proteins and/or peptides, in order to mimic the in vivo environment. In order to investigate mechanical effects on EC migration, rheological strength should be measured.

With regard to stabilization of EC tubular formation within the gels, we observed that ECs retained tubular-like structures for 3-4 d at the longest time. Afterwards, ECs retracted their leading process and reversed direction toward the surface. We assume that this might be due to the absence of supporting cells, such as pericytes, smooth muscle cells and fibroblasts. These supporting cells are known to secrete ECM and soluble factors important to maintain vessel
formations by regulating EC proliferation, differentiation and migration [144, 145]. For example, a number of studies demonstrated that ECs promote extensive outgrowth of tubular sprouts for a longer period when co-cultured with fibroblasts within collagen hydrogels [145, 146]. Furthermore, when we seeded brain-derived endothelial (bEnd3) cells (100% EC population) on top of the patterned hydrogels, they did migrate into the gels, yet they did not assemble tubular-like formations. In contrast, primary endothelial cells derived from microvessels in the brain exhibited tubular-like formations. We calculated the percentage of purity in primary ECs after dissections. As we passaged primary ECs, the purity of EC populations was increased. Although it exhibited high purity (~80%) around passage 2 and 3, we still detected other supporting cell types including astrocytes, smooth muscle cells, and fibroblasts. These results indicate that the communications between ECs and other supporting cells contribute to EC outgrowth and cellular tubular assembly inside the hydrogels. Further studies of co-culture ECs with supporting cells may enhance our understanding of the cell-cell interactions and molecular mechanisms involved in injury or healing as well as promote developing more effective strategies to enhance or inhibit angiogenesis.

The co-culture system developed in Chapter 3 provides cellular interactions between RSPCs and ECs where ECs adopt morphology similar to that observed in vivo. We have shown that RSPCs migrate along ECs and stabilize EC assemblies into tubule formations. Importantly, we found that these cellular interactions are mediated via ECM and integrin α6β1, and this receptor plays a critical role in RSPC adhesion to ECs. Prior studies of the ciliary body anatomy showed the presence of a dense network of blood vessels and endothelial basal lamina wherein retinal stem cells are localized [51]. Wang et al. have demonstrated that laminin is expressed largely by vascular cells in the ciliary body of human eyes and laminin chains of α1 and β2 are highly abundant and localized around blood vessels [51]. Although this study demonstrated the
interactions mediated through integrin α6β1 and ECM, it is highly possible that other laminin receptors including α1, α2, α3, α7 and β4 might be also involved in these cellular interactions between RSPCs and ECs.

Furthermore, surprisingly, contact mediated interactions with ECs inhibit RSPC proliferation and soluble factors derived from ECs inhibit RSPC differentiation. Recent evidence demonstrated that under physiological conditions, NSPCs migrate along the rostral migratory stream (RMS) toward the olfactory bulbs in close association with blood vessels, supporting proliferation and neurogenesis of NSPCs. Although we observed similar RSPC migration along ECs to NSPCs migrating with ECs, we did not observe any proliferative effects. One possible explanation for this is an ECM-integrin mediated effect as we discussed in Chapter 3. In addition, it has been demonstrated by Shen et al. that proliferative NSPCs in the SVZ of the brain make tight contacts with the vasculature at sites that lack astrocyte end feet and pericyte coverage [33]. Following this observations, Tavazoie et al. have also shown that regeneration of NSPCs occurs at these sites where circulating small molecules in the blood can enter due to fenestrated properties of capillaries in SVZ of the brain [147]. Thus, the fenestrated vasculature might be a key component of the adult SVZ neural stem cell niche; however, it is unclear which circulating molecules improve neurogeneration. Therefore, the lack of proliferation may be observed due to the absence of blood flow in our 3D co-culture system.

While we demonstrated the contact mediated interactions between RSPCs and ECs via laminin and integrins, the biological mechanism of RSPC migration along ECs remains to be investigated. For example, our studies did not demonstrate whether individual RSPC migrates together with individual EC (co-migration) or whether RSPCs follow along the EC tubular-like formations (post-migration). In order to investigate this, real-time live cell imaging could be used.
to elucidate these interactions. In the co-migration, RSPCs should migrate simultaneously with ECs. In the post-migration, we should observe RSPCs migrate along ECs after (or while) the ECs form tubular-like structures. Additionally, in the co-migration, cell-adhesive molecules such as cadherins should be expressed between RSPCs and ECs, and immunofluorescence analysis might be able to detect these biological molecules. In the post-migration, we can investigate whether extracellular matrix and/or growth factors secreted by ECs stimulate RSPC migration along the EC tube-like structures.

Molecules originally identified during neuronal development are continuously being identified as players during vascular development as well. For example, in the developing central nervous system (CNS), ventricular zone cells produce vascular endothelial growth factor, which attracts vessel growth toward them. Thus, vascular cells are close to CNS germinal zones throughout life, and it has been suggested that they form a niche for neural stem cells. Although it is becoming clear that the retinal vasculature has evolved to use some of the same guidance cues used by neurons during earlier stages of retinal development as described in Chapter 1, it remains to be investigated whether retinal vasculature development contributes to a niche formation for retinal stem cells in the ciliary margin. In addition, our results indicate that ECs contribute to maintaining RSPCs in the quiescent state, inhibiting differentiation and proliferation of RSPCs. This might suggest that endothelial cells localized in the ciliary margin may have a functional role as inhibitors for RSPC proliferation and differentiation, and in vivo studies should be performed in order to evaluate our findings and elucidate these biological mechanisms in the future. Some of these recommendations are discussed in Chapter 4.

A major limitation in 3D in vitro co-culture systems is the difficulty of performing quantitative analysis. Our model is no exception, but we were able perform quantitative immunostaining analysis of RSPC differentiation profiles. In order to overcome this problem, Bhatia et al. have
been developing an automated 3D co-culture system [148]. This system allows the creation of 3D µ-tissues by encapsulating multiple cell types within PEG suspensions, which allow large sample numbers. Multiparametric µ-tissue cytometry enables quantification of fluorescent reporter expression within populations of intact µ-tissues and sorting-based enrichment of subsets for subsequent studies. Although this system does not include biological components within the hydrogels (which can allow spatial or temporal control of cell behaviour), it might be necessary to combine our technologies with their high-throughput micro-tissue techniques to assess cellular fate changes for high sample size and populational analysis of 3D biology in vitro as well as in vivo.

The research tool described in Chapter 3 can also find applications in examining the cellular interactions between the other cell types. For example, it can be developed into an in vitro model to investigate the cell-cell interactions between Schwann cells and neurons following injury or during regeneration by patterning growth promoting factors. There studies can be further extended to examine co-cultures of neurons from the spinal cord and oligodendrocytes. They may help to understand the mechanisms leading to degeneration following injury to the nervous system or in neurodegenerative diseases, as well as developmental disorders.

## 4.2 Conclusions

The original hypothesis for this work was:

*Adult mouse adult brain-derived endothelial cells cultured with adult retinal stem and progenitor cells guide their proliferation and differentiation when co-cultured together in three-dimensional hydrogels.*

Several objectives were set in order to test this hypothesis and are revisited with the summary of the work presented to meet these objectives.
1. Develop a three-dimensional *in vitro* endothelial cell model using photolabile agarose hydrogels.

In the context of this study, a novel 3D endothelial cell niche was developed using photolabile agarose hydrogels and characterized. This 3D *in vitro* model allows ECs to assemble into tubule formations following a concentration gradient of immobilized VEGF165, resembling the vascular microenvironment *in vivo*.

Specifically:

1. A concentration gradient of VEGF165 was covalently immobilized within defined volumes of agarose hydrogels by using laser confocal patterning of coumarin-protected thiols, which, once deprotected, reacted with maleimide-modified VEGF165 via Michael-type addition.

2. ECs were guided into the GRGDS-agarose hydrogels, forming tubular-like structures following a concentration gradient of immobilized VEGF165.

3. Concentration gradients (steepness ranging from 0.99 to 1.65 ng mL\(^{-1}\) μm\(^{-1}\)) were optimized to guide ECs into the gels. ECs did not migrate beyond depths that had VEGF165 concentrations greater than or equal to 600-800 ng mL\(^{-1}\).

4. ECs migrated only short distances into the gels when VEGF165 was immobilized at a constant concentration (and without a gradient).

2. Develop a 3D co-culture model in order to understand the effects of endothelial cells (ECs) on adult retinal stem and progenitor cells (RSPCs) in 3D environments.

A co-cultured model for investigating interactions between RSPCs and ECs was developed whereby they are embedded within 3D patterned hydrogels including a concentration gradient of
immobilized VEGF165. By using this 3D co-culture model, we demonstrated that soluble factors derived from ECs inhibit differentiation of RSPCs, and contact mediated interactions with ECs inhibited RSPC proliferation.

Specifically:

5. In the co-culture of ECs and RSPCs, ECs migrated up the VEGF165 concentration gradient, and RSPCs migrated along the EC tubular-like formations.

a. Laminin was expressed largely by ECs, and laminin expression was highly abundant and localized at the EC-RSPC interface.

b. α6β1 integrin, one of the laminin receptors, was widely expressed on RSPCs when they migrated along ECs.

6. Soluble factors, derived from ECs, inhibited differentiation of RSPCs. Contact mediated interactions with ECs inhibited RSPC proliferation.

4.3 Major contributions

In this thesis, we demonstrated that the guidance of primary endothelial cells (ECs) in an agarose hydrogel scaffold that is chemically patterned with an immobilized concentration gradient of VEGF165 using multiphoton laser patterning of VEGF165. This is the first demonstration of this patterning technology to immobilize proteins; and the first demonstration of immobilized VEGF165 to guide endothelial cell growth and differentiation in a 3D environment.

Furthermore, by using this 3D endothelial cell niche model, co-culturing ECs and RSPCs, we were able to examine the role of ECs on RSPC fate in a 3D environment. Our results demonstrate that ECs influence proliferation and differentiation of RSPCs. ECs inhibit
proliferation and differentiation of RSPCs when they are co-cultured in a 3D environment. To our knowledge, this is the first time that the role of ECs on RSPC fate has been demonstrated. Moreover, this is the first time that interaction of ECs and RSPCs has been explored in a 3D model where ECs adopt morphologies similar to those observed in vivo. This novel biomimetic vascular niche model should provide significant insight into the RSPC in vivo niche.

4.4 Recommendations for future work

4.4.1 Further investigating of EC guidance in 3D environments

1. As described in Chapter 2, we demonstrated that ECs followed an immobilized VEGF165 concentration gradient in a 3D hydrogel, with endothelial tip-like and stalk-like cells indentified. In this study, we demonstrated the morphological differences of tip and stalk cells forming tubular-like formations (Figure 2.3). Additionally, immunocytochemistry could be used to distinguish between stalk and tip cells by using endothelial tip cell markers such as platelet derived growth factor-B (PDGFB). Since it is known that several gene expressions in tip cells are higher including VEGFR2, Dll3, VEGFR3 in addition to PDGFB compared with adjacent stalk cells [149], it would be interesting to investigate the difference of these gene expression levels and also between 2D and 3D cultures.

2. As described in Chapter 2, in order to optimize the concentration of immobilized VEGF165, we tested different concentrations of soluble VEGF165 on EC proliferation. We observed EC proliferation in the range of 500-1000ng/ml when ECs were cultured on GRGDS agarose hydrogels (Figure 2-1). Since these experiments were performed without serum and all 3D experiments were carried out using EC media including serum (2%FBS, 2% horse serum, 20ug/ml ECGS and heparin), it might be interesting to
investigate the range of VEGF165 concentrations on EC proliferation under EC media to see whether the presence of serum change the range of concentration of VEGF165 on EC proliferation.

3. We observed that EC penetrations into the agarose hydrogels were limited to less than 200 μm. In order to create a microenvironment for ECs to remodel the hydrogel matrix and deposit appropriate extracellular matrix, it would be interesting to base the adhesion biochemical channels in a proteolytically degradable hydrogel material such as hyaluronan. Presumably cells would be able to invade deeper into such hydrogel matrices and follow the biochemical pathways.

4. We described in Chapter 2 that ECs did not migrate beyond depths where the VEGF165 concentration was 600-800 ng mL⁻¹. We suggested that this might be caused by saturation of VEGFR2 receptors on endothelial stalk cells, preventing EC proliferation and migration. To further confirm this, primary anti-VEGFR2 can be added to block the receptors on ECs before seeding cells on the hydrogels in order to investigate whether this blocking specifically inhibits EC proliferation and migration following VEGF165 gradients.

5. We found that ECs cultured alone did not stabilize their tubular structures after 3-4 d. This might be due to the saturation of VEGFR2 and lack of further biochemical cues to guide ECs. Considering that addition of RSPCs to ECs helped stabilized ECs, it may be interesting to investigate whether addition of supporting cells such as fibroblasts or smooth muscle cells can stabilize ECs for a longer period.
4.4.2 Further investigation of interactions between RSPCs and ECs

1. Although it has not reported that RSPCs and ECs labeled using fluorescent dyes alter the migration ability of the cells, it should be confirmed by using yellow or green fluorescent muted mice.

2. In order to prove whether our findings are endothelial cell specific effects or tissue-(or species) specific endothelial cell effects, other feeder cells, such as primary human umbilical vein endothelial cells, primary bovine pulmonary artery endothelial cells, a mouse brain endothelial cell line, vascular smooth muscle cells, NIH3T3 fibroblasts should be co-cultured with RSPCs to investigate whether these cells influence RSPC proliferation and/or differentiation.

3. We still need to refine specific cellular interactive biomolecules derived from ECs that influence RSPC fate change. Recent studies demonstrated that SVZ ependymal and vascular cells produce and secrete active PEDF which stimulates self-renewal of NSCs and induces molecular state of undifferentiation, with increased expression of the Notch effectors Hes1 and Hes 5, and of the HMG-box transcription factor Sox2 [150]. Considering that ciliary margin where RSPC are localized is filled with capillary vessels similar to that of SVZ, it might be interesting to investigate whether soluble factors such as pigmented epithelial derived factor (PEDF) influence RSPC proliferation and differentiation.

4. Although we focused on the characterization of RSPC differentiation and proliferation when they are in contact with ECs, it remains to be investigated how the presence of RPSCs influences EC fate. Interestingly, we observed that the presence of
RSPCs stabilized EC tubular-like formations. Our results have led to the notion that angiogenesis might stimulate the migration of RSPCs, but the converse may also be true, that is, that the neurogenic response is vasculotrophic, and thereby critical for the stabilization of new vasculature. It might be interesting to investigate whether RSPCs could provide trophic support for EC in culture, leading to EC differentiation and vessel formation.

5. Although we demonstrated in Chapter 3 that mRNA expressions were consistent with immunofluorescence results, mRNA expressions of ECs, when directly co-cultured with RSPCs, should be investigated by culturing ECs with RSPC-conditioned media in order to confirm that ECs do not change and/or express any of RSPC markers, such as Pax6, Otx2, and Rhodopsion when co-cultured with RSPCs.

6. In Chapter 3, we demonstrated that EC might regulate RSPC differentiation. It has been similarly reported that cell-cell contacts and paracrine factors from ECs inhibit the differentiation of stromal cells (e.g. multipotent mesenchymal stem and progenitor cells) via Wnt signaling, most prominently Wnt1, Wnt 4, and Wnt 10a [151]. Furthermore, addition of recombinant Wnt ligand or the Wnt signaling agonist inhibited adipogenic differentiation of adipose stromal cells in the absence of ECs. Retinal histogenesis occurs from the center to the periphery, such that peripheral retina contains the least committed retinoblasts, while the central retina contains the mature retinoblasts, the postmitotic, differentiated retinal precursors and the earliest differentiated retinal neurons. In terms of the retinal developmental process, it has been reported that the differential expression of Wnt molecules correlates with a potential role for retinoblast differentiation. For example, the presence of Wnt-5a and
Fz-4 in peripheral retina suggests a role for these molecules in maintenance of multipotent progenitors and not in specifying cell function. Wnt/beta-catenin reporter is also expressed in a peripheral-to-central gradient, being present at robust levels in the retinoblasts in the peripheral margin. Furthermore, in terms of retinal stem and progenitor cell maintenance, Wnt signaling has been also shown to play a pivotal role in regulating RSPC differentiation[135]. Particularly, it has been suggested that Wnt2b signaling is involved in maintaining retinal stem and/or progenitor cells in an undifferentiated state by preventing their differentiation mediated through Notch[135, 152]. Therefore, it might be interesting to investigate whether endothelial cell effect would be accompanied by increased expression of factors involved in Wnt 2b signaling by analyzing mRNA expression.

7. With respect to mechanisms that might play a role in RSPC-mediated vascular stabilization, it is known that Notch signaling is involved in retinal development. Notch signaling suppresses the proliferation of differentiated ECs, maintaining them in a quiescent state of differentiation-associated cell-cycle arrest, and contributes to adult vascular homeostasis [153]. Furthermore, Notch seems to limit the branching process of angiogenesis by inhibiting endothelial tip cell formation and maintaining the adjacent stalk cell phenotype [154]. Interestingly, ECs co-cultured with RSPCs in our 3D model did not exhibit the filopodia of endothelial tip-like cells after 3 d of culture, suggesting a role for Notch signaling in these cultures. Given these observations, it might be interesting to investigate whether the RSPC-specific effect would be accompanied by increased expression of factors involved in Notch signaling by analyzing mRNA expression.
8. In order to prove the biological relevancy of our *in vitro* findings to physiological conditions *in vivo* (ECs regulate RSPC proliferation and differentiation, as well as RSPCs stabilize EC tubular-like formations), we need to assess our findings with *in vivo* experiments. For example, finding biomolecules secreted from ECs, which cause the inhibition of RSPC proliferation and differentiation, may enhance stimulation of RSPCs, promoting proliferation and/or differentiation, by injecting their antagonists in the ciliary margin *in vivo*.

9. As described in Chapter 3, RSPCs and ECs were cultured 1:1 ratio. We briefly observed that RSPC did not migrate out when cell number ratio, RSPCs/ECs was below 0.5. In contrast, when RSPCs/ECs was above 0.5, RSPCs tended to migrate out with ECs, yet it was harder to observe the interactions between ECs and RSPCs. Therefore, it will be interesting to investigate whether different cell ratio of RSPCs/ECs change RSPC and/or EC fates in terms of proliferation and differentiation and migration.

10. As described in Appendix D, it has been reported that RSPCs differentiated into mature retina phenotype including photoreceptors, bipolar cells, and Muller glia cells when they are cultured in 3D agarose hydrogels under 1%FBS. In addition to this report, it would be beneficial to obtain RSPC differentiation profile when RSPCs are cultured under EC media (not 1%FBS) in 3D agarose hydrogels as a future work to confirm our 3D cultured observations (ECs inhibit RSPC differentiation).
References


## Appendix A: Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bEnd3:</td>
<td>Brain Endothelial Cell Line 3</td>
</tr>
<tr>
<td>EC:</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECGS:</td>
<td>Endothelial Cell Growth Supplement</td>
</tr>
<tr>
<td>ECM:</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGF:</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>FBS:</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FGF:</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>GRGDS:</td>
<td>Glycine-Arginine-Glycine-Aspartic acid-Serine</td>
</tr>
<tr>
<td>HSC:</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>NSPC:</td>
<td>Neural Stem and Progenitor Cell</td>
</tr>
<tr>
<td>RSPC:</td>
<td>Retinal Stem and Progenitor Cell</td>
</tr>
<tr>
<td>SVZ:</td>
<td>Subventricular Zone</td>
</tr>
<tr>
<td>VEGF:</td>
<td>Vascular Endothelial Growth Factor</td>
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Appendix B: Dissection protocol of mouse brain derived endothelial cells

This protocol of endothelial cell isolation has been generously provided by Dr. Moreno (University of Ottawa).

B.1 Mouse brain endothelial cell dissection protocol

Materials

6-10 CD1 mice at 6 weeks of age

50 mL DMEM-F12 media and 50 mL HANKS

40 µm nylon mesh filter

Dounce tissue grinder

30% Dextran solution

0.1% collagenase-dispase including 20 U/ml Dnase

murine collagen IV

Procedure

1. Collect and rinse 6-10 mouse brains using ice-cold DMEM-F12.

Cerebrum (Cerebral cortices devoid of cerebella and white matter) is dissected under sterile conditions.

2. The cortices are cut into small pieces. Only solid tissue should be homogenized by Dounce tissue grinder until it becomes mixture becomes pink, and no large brain chunks are left.

3. Centrifuge the homogenized 200 x g for 5 min.

4. After removal of the supernatant, resuspend 15ml of 18% dextran solution and centrifuge it at 10000 x g for 10 min.
5. Discard the foamy myelin layer and dextran supernatant and resuspend the pellet with HANKS.

6. Pass through 40 µm nylon mesh filter and wash the filter with HANKS. (Microvessels retained on the filter would be dislodged by inverting the filter and rinsing with HANKS)

7. Immediately after the microvessels are obtained, place them in 60 mm Petri dish in 5 ml of 0.1% collagenase-dispase and 20 U/ml DNAse at 37°C for 90 min with occasional agitation.

8. Pass through a 40 µm cell strainer to remove the longer vessels fragments (which tend to contain more contaminating pericytes).

9. Resuspend them in complete culture medium (DMEM-F-12 containing 10% plasma-derived horse serum, 10% fetal bovine serum, 1% antibiotic-antimycotic, 100 ug/ml heparin, and 100 ug/ml endothelial cell growth supplement).

10. Plate at density of 8x10^3 vascular fragments/cm^2 onto tissue culture dishes coated with murine collagen IV.

5. Change the media after 24h and every 2-3 days afterwards.

*Endothelial cell purification protocol*

In order to increase the percentage of endothelial cell population, the following protocol can be added before the procedure # 9 described above.

*Materials*

Dynal Manetic particle Concentrator

anti-mouse PECAM-1-precoated Dynabeads

*Procedure*

1. Incubate vessels fragments with anti-mouse PECAM-1-precoated Dynabeads at 4 °C from 30 min with constant rotation. Then separate bead-bound vessel fragments from residual-free perivascular cells in a Dynal Manetic particle Concentrator.
2. Wash dynabeads using EC medium and collect by magnet and plate them onto collagen I-coated tissue culture plate.
Appendix C: Endothelial cell tubular-like formation

Figure C1. A series of confocal images captured along the z-axis of the hydrogels (shown in Figure 2-3) Primary endothelial cells from the brain microvascularature extend tip and stalk-like
cells into GRGDS agarose hydrogels and form tubule-like structures. ECs were immunostained with primary antibodies: β-catenin for junctional proteins (green), laminin for extracellular matrix (red). (Scale bar = 20 µm)
Appendix D: Retinal stem and progenitor cell differentiation profile in 3D RGD-agarose hydrogels

Figure D1. RSPC differentiation profile in 3D and 2D with varying substrate stiffness (0.75, 1.25 and 1.75%) in GRGDS-agarose hydrogels. RSPCs were cultured for 14 days in SFM containing 1% FBS. On day 14, cells were lysed and assayed for mRNA of specific retinal markers using qPCR (Y-axis is mRNA expression relative to expression of 18S rRNA). (A) the photoreceptor marker, Rhodopsion (B) the immature ganglion marker, beta-III tubulin (C) the bipolar cell marker, PKCα (D) the glial cell (Muller glia) marker, GFAP (E) the mature ganglion marker, Brn3b (F) retinal stem and progenitor marker, Nestin. Rhodopsion was significantly enriched on softer substrates in both 3D and 2D cultures. Conversely, stiffer substrates (1.75%) generated...
higher expression of beta-III tubulin and Brn3b mRNA and GFAP in both 3D and 2D cultures. Nestin and PKCα expression remained consistent irrespective of the substrate stiffness.

(Reproduced with permission from the Master’s thesis of Shoeb Ahsan, “Investigating of adult retinal precursor cell behavior in response to soluble factors and varying substrate stiffness in two and three dimensional scaffold”, 2010, p48)