Cardiac Fibroblasts as a Support for Endothelial Cell Sprout Formation in Engineered Cardiac Tissue

A thesis

submitted by

Rachel L Twardowski

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Abstract

Cardiac tissue engineering offers a promising approach to regenerating post-MI myocardial tissue; however, a primary impediment in tissue engineering lies in the inability to adequately vascularize a tissue scaffold. Cardiac tissue constructs require a closer proximity to a nutrient and blood supply than many other tissues due to the higher metabolic demands of myocardium, further decreasing the required distance to a capillary bed. It is known that endothelial cells (ECs) require a support cell to form mature patent lumens both in vivo and in vitro, and it has been demonstrated that pericytes, vascular smooth muscle cells and mesenchymal stem cells (MSCs) are able to support the formation of mature tubules/vessels. Cardiac fibroblasts (CFs) provide important electrical and mechanical components to the myocardial tissue and are present in the native cardiac cell population, but to date have not been sufficiently studied for their role in angiogenesis in heart. Three different approaches were used to study the ability of CFs to aid in the formation of EC sprouts: 1) varying the co-culture ratio of neonatal CFs to ECs to best match the *in vivo* conditions, while comparing the results to EC sprout formation in MSC co-cultures, 2) studying the differences of EC proliferation and sprout formation in co-culture with CFs at different developmental life points: fetal, neonatal and adult and 3) the use of a decellularized vessel as a central conduit for angiogenesis in a fibrin construct. As a model of myocardial tissue, we co-cultured different concentrations of various cell types in fibrin hemispheres in appropriate combinations of their specific media to determine the optimal sprout formation through DNA analysis, flow

cytometry and immunohistology (IH). Our hypothesis was that cardiac fibroblasts can support endothelial cell sprout formation as well or better than mesenchymal stem cells, which are considered to be a "gold standard" for the stabilization of EC sprouts in engineered tissues. Our results indicated that ECs tended to form longer and more numerous sprouts in culture with neonatal CFs, but formed more multi-cellular sprouts in culture with MSCs, which is more indicative of the *in vivo* process. Additionally, we found that EC proliferation was best when cultured with neonatal and adult CFs over the proliferation rate with fetal CFs. In summary, cardiac fibroblasts are able to provide a support for the formation of endothelial cell sprouts and could aid in the eventual vascularization of a cardiac tissue scaffold.

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Chapter 1: Introduction

The work in this thesis is divided into three separate, but complementary projects. The first project includes the study of endothelial cell cultured with two different support cell types: mesenchymal stem cells and neonatal cardiac fibroblasts. The first part of this study involves the selection of an optimal culture medium through the analysis of total cell growth and endothelial cell sprout formation. The second part involves culturing endothelial cells with three different ratios of support cells to endothelial cells in order to find the optimal concentration of support cells that leads to the best mimic of *in vivo* endothelial cell sprout formation. The second project involves the study of cardiac fibroblasts at three different developmental life points: fetal, neonatal and adult and their ability to support endothelial cell proliferation and sprout formation. The third project demonstrates initial work to recellularize a previously decellularized vessel and the use of this vessel as a central conduit for angiogenesis in a three dimensional fibrin construct. These three projects will fully connect in the future as an *in vivo* implantable pre-vascularized cardiac tissue constructs for patients of different ages. A brief description of each of the chapters is below:

Chapter 2 introduces the necessary background material for each of these projects and explains with is already understood about the topics, while provoking further investigation into other areas.

Chapter 3 describes the development and design of a fibrin construct to support the culture of endothelial cells with the two different support cell types: MSCs

and cardiac fibroblasts. Moreover, we measure total cell proliferation, EC proliferation and EC sprout formation in these two support cell types in order to determine whether CFs can serve as an adequate support cells for in vitro pre - vascularization of an engineered myocardial construct.

Chapter 4 describes the isolation and study of cardiac fibroblasts at three different developmental life points (fetal, neonatal and adult), in addition to their interaction with endothelial cells in co-culture. Since CF phenotype and protein expression changes with developmental age, we sought to investigate whether the age of the CF population would influence EC proliferation and sprout formation.

Chapter 5 describes the initial set-up and experiments for the recellularization of a decellularized vessel and the fabrication of a fibrin construct with the vessel as a central conduit.

Chapter 6 summarizes conclusions drawn from this work, in addition to the future directions this work might take.

Chapter 2: Background

2.1 Clinical Relevance: The Need For A Vascularized Tissue Engineered Scaffold

Advances in medicine and tissue engineering have led to a considerable aging population; therefore, a substantial clinical need for tissue-engineered organs exists, based on the dramatic shortage of organs for transplant. According to the United Network for Organ Sharing (UNOS), the waiting list of organ transplant candidates is over 110,000 [1]. The number of patients requiring an organ transplant far outweighs the number of allogeneic organ transplants available. Although numerous academic and industry biotechnology laboratories around the world are designing experiments based on the optimal combination of cells, scaffold biomaterials and biochemical factors to create different types of engineered organs, there are significant shortcomings. The primary impediment in the clinical translation of many tissue engineered constructs lies in the inability to adequately vascularize the tissue scaffold [5]. In order to maximize cell survival and optimize effective nutrient and gas diffusion, cells and tissues *in vivo* must be located within 100-200μm of a capillary bed [2]. This diffusion distance is influenced by many factors, including the metabolic rate of the tissue.

In addition to the need for vascularized tissue scaffolds for organ transplants, there is a considerable need for vascularized cardiac tissue scaffolds. Cardiovascular disease is one of the leading causes of death in the United States, accounting for over 616,000 deaths, or 25.4% of deaths in the US in 2007 [3]. After a myocardial infarction (MI) a portion of the left ventricle becomes necrotic

resulting in a negative remodeling process that leaves the tissue scarred. Current pharmacological and surgical treatments aimed at treating the heart post-MI are limited in the ability to remodel the necrotic left ventricle and often seek to reduce the workload of the injured heart to help maintain its function [4]. Tissue engineering offers a novel approach to potentially regenerate the necrotic myocardial tissue; there have been numerous studies aimed at utilizing cellular, mechanical and biocompatible components to do this [6-7].

Cardiac tissue constructs require a closer proximity to a nutrient and blood supply due to the high metabolic demands of constant muscular contraction. This increased metabolic demand further decreases the necessary diffusion distance between the tissue and blood supply. Although the maximum diffusion limit in the human heart is about 100μm, in the adult mammalian heart cardiomyocytes are located no more than 2-3μm from myocardial capillary endothelial cells (MyoCapE) to maintain paracrine cross-talk with cardiomyocytes and facilitate nutrient and oxygen transfer through the blood [51]. Cardiac endothelial cells outnumber cardiomyocytes 3:1 in the human heart, although the volume of cardiomyocytes is much greater than ECs [79]. In the left ventricular wall and papillary muscle of the adult rat heart the capillary-to-cardiomyocyte ratio varies from .9-1.1 and the intercapillary distance in the ventricular wall was reported to be 20.2μm [77]. The distance of ~1μm between capillary endothelial cells and cardiomyocytes allows for a diffusion radius of 3-12μm for each capillary endothelial cell to neighboring cardiomyocytes [78]. Based on this information it

is clear that pre-vascularization of cardiac tissue constructs is critical to their survival and function upon implantation *in vivo.*

Furthermore, highly vascularized tissues, such as the myocardium, liver and skeletal muscle requires a readily available blood supply, further reducing the effective thickness of these engineered tissues [8-9]. The immediate vascularization of an implantable tissue scaffold is crucial for its survival and function, as the addition of vasculature and a capillary network will help to maintain cell viability in the tissue by optimizing oxygen and nutrient delivery to the cells beyond the diffusion limit [10]. Nonetheless, the process of ingrowth of the host vasculature into an implanted tissue is often too slow—on the order of days. Therefore, creating a prevascularized scaffold provides a greater prospect for success, allowing for rapid (<1 day) anastomosis of the host vasculature [8]. The *in vitro* prevascularized scaffold can anastomose to the host, allowing for improved delivery of oxygen and nutrients, thus enabling the scaffold to better retain its function [11]. Following the native process of angiogenesis within the tissue, the prevascularized tissue scaffold will begin to anastomose with vascular networks from the host blood supply.

2.2 The Process of Angiogenesis

During early embryonic development, as the vascular network begins to form, endothelial cells (ECs) begin to assemble into tree-like tubular structures, creating blood vessels. These vessels eventually permit the transport of fluids,

oxygen, and nutrients and allow for the circulation of cells, hormones and gases [12-13]. The creation of these tubular networks is via sprouting angiogenesis [14]. The work pioneered by Judah Folkmann suggested that the inhibition of angiogenesis would be an effective strategy for the treatment of human cancer, which led to a significant search for the factors that induce angiogenesis.

The endothelial vasculature of the fetus forms during embryonic development by both angiogenesis and vasculogenesis. The process of angiogenesis is defined as the sprouting of new capillaries or vessels from pre-existing vessels, and is a process used by nearly all vessels within the developed vascular system of vertebrates. Vasculogenesis or the *de novo* formation of embryonic blood vessels involves the differentiation, migration and coalescence of endoderm-derived endothelial progenitors—or angioblasts—to create a primordial vascular network. This development involves five main stages: formation, stabilization, branching, pruning and specialization [68]. A mature vasculature consists of an elaborate hierarchy of arteries, arterioles, capillaries, venuoles and veins, which promote the circulation of oxygenated blood between the heart, lungs and tissues of the body (Fig. 1) [15]. As the blood vessels begin to assemble, endothelial cells become specified to either an arterial or venous fate and unite to generate the first embryonic blood vessels, the dorsal aorta and cardinal vein, forming the cardiovascular system—the first organ system developed during vertebrate embryogenesis [16]. The angioblasts then segregate to form blood islands, generating a primitive, interlaced network of arterial and venous plexi. After vasculogenic assembly, the angiogenic remodeling of the

dorsal aorta, cardinal vein and venous plexi occurs, forming the complex hierarchy of arteries, arterioles, capillary beds, venuoles and veins. In order to stabilize this network, mural cells (pericytes, vascular smooth muscle cells and mesenchymal stem cells) are recruited [16]. The mural cells also aid in the further maturation of the vascular network and lead to the sprouting of lymphatic endothelial cells to form the lymphatic system. The diversification of the vessels occurs as signals from local tissues influence the endothelial cells to take on tissue-specific characteristics. For example, endothelial cells destined for the brain will create the microvasculature of the blood-brain barrier, while pancreatic endothelial cells will form the endocrine vasculature and myocardial vascular endothelial cells will form the coronary vasculature (Fig. 1B).

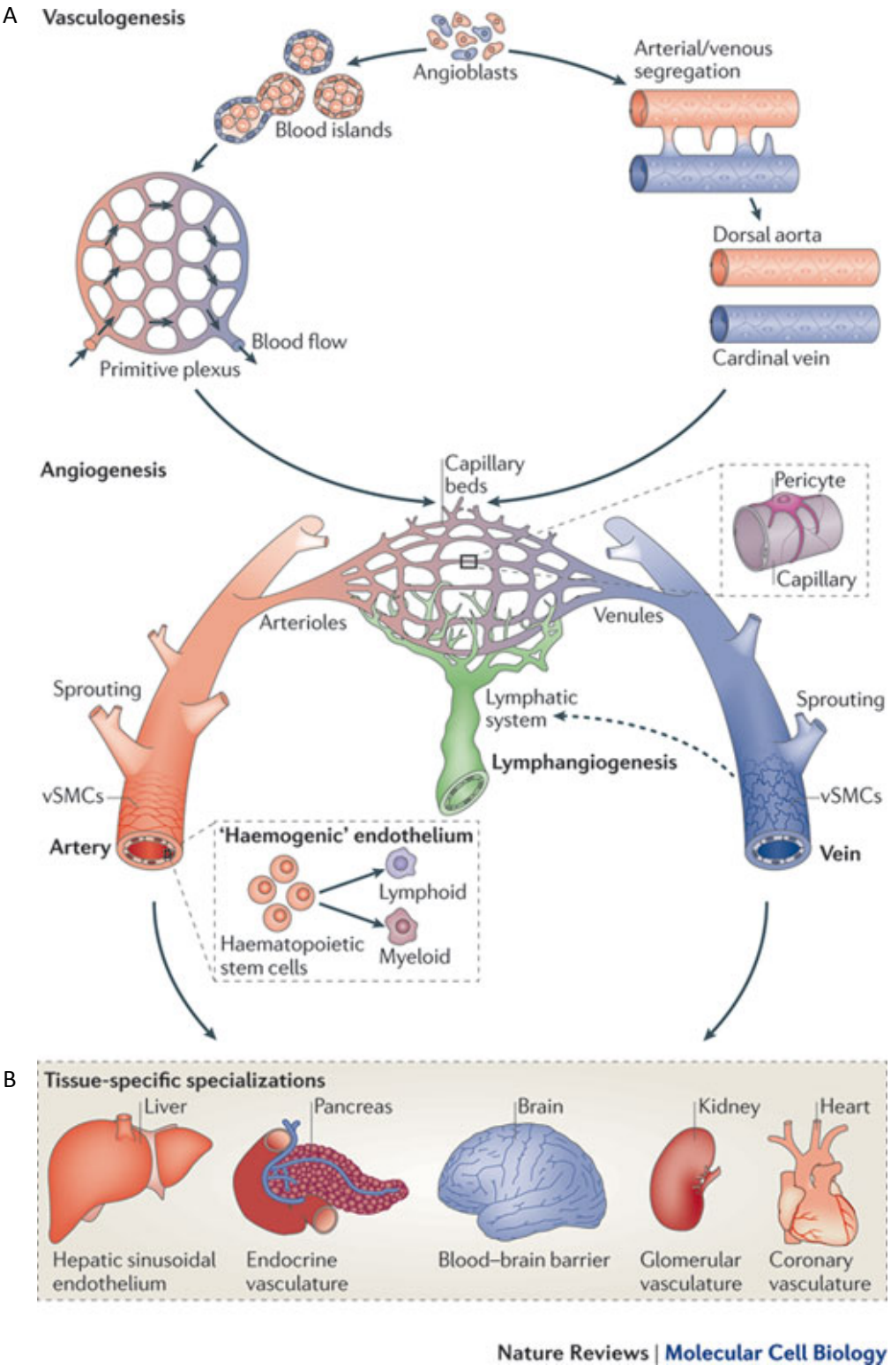

Figure 1: Process of Vasculogenesis and Angiogenesis- the development of a functional vascular network [15]

In quiescent vessels, endothelial cells maintain a cobblestone-like monolayer of inactive cells that line the lumen of blood vessels. The ECs support this phenotype until angiogenic signals are detected which induce fundamental changes in the behavior of the ECs (Fig 2), causing them to loosen their cell-cell contacts and activate proteases to degrade the surrounding basement membrane (Fig. 2b) [14]. Vessel destabilization and vascular remodeling is thought to occur as a result of the disruption of the angiopoietin-1 (ANG1) signaling via the TIE2 (or TEK) receptor Tyrosine kinase by the antagonist angiopoietin-2 (ANG2) [18]. The Ang-Tie signaling axis controls vessel quiescence in adults and regulates later vessel maturation steps of the angiogenic cascade.

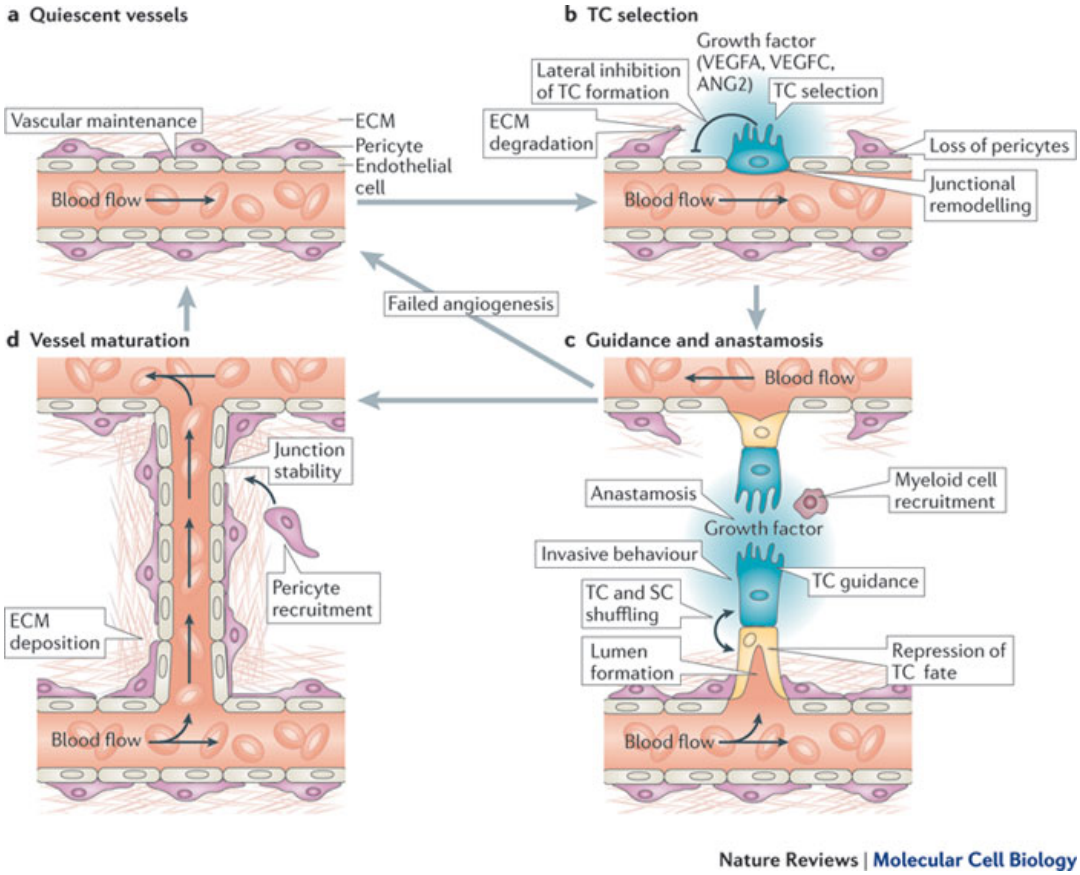

Figure 2: Mechanism of EC sprouting *in vivo* [15]

The initiation of endothelial cell sprouting involves one endothelial cell responding to the angiogenic stimulus by extending a filopodia, then migrating outward from the parent vessel, while still remaining connected to its neighbors [17]. This process involves different phenotypes of endothelial cells, known as "Tip" and "Stalk" cells and will be described in greater detail in the next section.

2.3 The Formation of Tip and Stalk Cells

Only a small portion of the endothelial cells that "sense" the angiogenic ANG-TIE stimuli will lead the sprouting of new vessels. These cells are known as "tip cells" and extend numerous filopodia extensions into the surrounding extracellular matrix. The filopodia will be guided through the microenvironment by angiogenic stimuli [15]. As the tip cell moves further from the parent vessel, neighboring cells remain attached to the tip cell and migrate behind it. Figure 3 illustrates the formation of tip cells and EC filopodia with trailing stalk cells behind it. The process of angiogenesis and vascular development is initially dependent on endothelial tip cell invasion, followed by cell maturation and lumen formation.

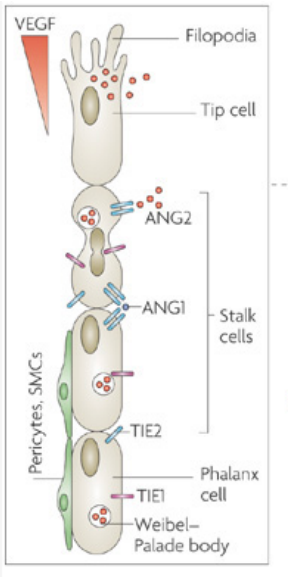

Figure 3: Formation of Tip and Stalk Cells [18]

The endothelial cells that trail the tip cells are known as "stalk cells". Stalk cells are less motile, but more proliferative than tip cells. The stalk cells are critical in the support of the extending sprouting vessels, generating the trunk of

the new vessels and establishing the vascular lumen of growing vessels [17]. Once the tip cells contact other ECs, tight EC-EC junctions are generated, fusing with adjacent vessels to form a continuous (patent) lumen, allowing blood flow to occur [19]. Table 1 below demonstrates important characteristics of tip and stalk cells.

Characteristic	Tip Cell	Stalk Cell
Response to VEGF-A	Induced by VEGF-A; rarely proliferates	Proliferate when stimulated with VEGF-A
Basement Membrane	Limited BM deposition	Deposits a BM
Vascular Lumens	Lacks a vascular lumen; creates flilopodia extensions	Forms vascular lumens
mRNA Expression	↑ PDGFR, Dll4, Flt4	↑ Jag1, Flt1, Robo4, PAR3
Migration	Specialized for guided migration	Limited migration capacities, but can be induced to become new tip cells
Cell Junctions	Lack tight cell junctions	Form tight EC-EC junctions to facilitate vascular lumen formation

Table 1: Molecular and phenotypical differences of tip and stalk cells [15].

During angiogenesis, there is an upregulation of vascular endothelial growth factor-A (VEGFA) and VEGFC with increased signaling activity of the VEGF receptor, VEGFR2, which activates the formation of tip cells [15]. Delta-like ligand 4 (Dll4) is a Notch ligand critical to the development of normal vasculature. Mammals lacking Dll4 expression demonstrate severe endothelial remodeling and die early in gestation. Activation of Notch upon ligand binding to Dll4 laterally inhibits tip cell formation in the adjacent ECs (Fig. 2b). Tip cell sprouting is further facilitated by VE-Cadherin mediated loosening of the EC-EC junctions. The initiation of partitioning-defective 3 (PAR3 or PARD3) signaling mediates vascular lumen morphogenesis in the stalk cells and allows for the maintenance of connectivity in the developing lumen (Fig. 2c) [15].

2.4 The Role of Growth Factors and Signaling in Angiogenesis and Vessel Formation

The Notch signaling pathway plays numerous roles in angiogenesis from the initial vascular plexus formation to the formation of early arteries and veins, as well as eventual support cell recruitment and downstream vascular remodeling [42]. Mammals express four Notch receptors: Notch 1-4 and five Notch ligands: Delta-like ligand (Dll) 1, Dll3, Dll4, Jagged1 (Jag1) and Jagged2 (Jag2). For vascular development to occur Notch1, Dll4 and Jg1 must be present. The suppression of the aforementioned receptor and ligands results in the death of the embryo. The suppression or genetic deletion of the other receptors and ligands are not lethal, however result in decreased or no arterial differentiation, lack of vascular smooth muscle cell (vSMC) maturation (loss of Notch3) and a decrease in mural cell differentiation (loss of Jag1) [43-45]. During angiogenesis Notch signaling regulates the tip and stalk cell dynamics by affecting VEGF-A. VEGF-A is induced by hypoxic conditions and expressed by many tissues [17]. The EC that produces more Dll4 than its neighbor will remain the tip because it can suppress the same response in competing neighbors via Notch signaling. Notch signaling also regulates vSMC and support cell recruitment to vessels through the upregulation of platelet derived growth factor receptor-β (PDGFR-β) [46].

Endothelial cells deposit their own basement membrane (BM) in order to regulate vessel maturation. The basement membrane is a thin layer of specialized extracellular matrix (ECM), about 50-100nm thick. This ECM provides support for endothelial and epithelial cells and allows for the accumulation of von

Willebrand's Factor, which is a secretion from the endothelial Weibel-Palade bodies [47]. vWF aids in EC hemostasis and the adhesion, migration and recruitment of leukocytes to the endothelium. The BM is composed of type IV collagen, fibronectin and laminin, which are also components of myocardial ECM, in addition to collagen I, III and V [87].

Growth factors also play an important role in angiogenesis and tissue vascularization. The first pro-angiogenic related growth factor, isolated by Gospodarowicz in 1974, was basic Fibroblast Growth Factor (bFGF), referred to as an endothelial mitogen. A mitogen, typically in the form of a protein, is a chemical substance that promotes cell division and triggers mitosis. bFGF promotes proliferation and differentiation of endothelial, smooth muscle (SMCs) and fibroblasts cells; all common cells types located in blood vessels [22]. Additionally, bFGF serves as a heparin-binding protein, and helps initiate endothelial capillary formation. The VEGF family is known for aiding in endothelial cell proliferation, decreasing EC apoptosis, increasing permeability and aiding in the migration of ECs *in vivo*. Secreted from platelets, Platelet-Derived Growth Factor (PDGF) helps promote vessel formation by recruiting SMCs to the endothelial linings of vessels [20]. Table 2 below illustrates the function of many key growth and signaling factors related to angiogenesis, along with the diffusion and absorption time course of the factor and other issues or effects of the factor.

Growth or Signaling Factor	Function	Time Course Issues and Other Notes
VEGF	Initiator of endothelial capillary formation; ↑ permeability, ↑ Plasminogen activators, EC proliferation, EC apoptosis ↓, EC migration and *in vivo* angiogenesis	Short half-life--degrades rapidly. Abundant amount can cause vascular leakage
bFGF	Induces proliferation of ECs and SMCs; initiates formation of endothelial capillaries; aids in binding of heparin	Diffuses quickly and must be observed for controlled release
PDGF	Released from platelets; aids in vessel maturation and recruits SMCs to endothelial linings; stimulates *in vitro* chord formation	Excessive amounts lead to vessel destabilization
ANG1	Signaling factor, aids in regulating vessel homeostasis; stabilizes newly formed capillaries and promotes EC migration and sprouting	Endothelial hyperplasia induced by over-expression of signaling factor; leads to reduced vessel leakage
FGF- 1	EC proliferation, Plasminogen activators ↑, promotes adhesion molecules and EC migration	Diffuses quickly
MMP	ECM degradation, formation of microvessels during angiogenesis	Too many MMPs can generate angiostatin, blocking the growth of new vessels
TGF-β	Promotes vessel stabilization, *in vitro* tube formation, *in vivo* angiogenesis in inflammatory environment	Low doses required for tube formation; too much can result in ↓EC proliferation, Plasminogen activators ↓, increased EC apoptosis
NO	↑ Permeability, promotes release of FGF, induces EC proliferation	Too much nitric oxide results in tissue toxicity. Typically paired with VEGF.
DSL	Delta, Serrate, LAG2: Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged 1 (Jag1) and Jagged 2 (Jag2) are type 1 cell-surface proteins with multiple tandem epidermal growth factor repeats.	Dll4 is typically expressed in prenatal vascular endothelium and is normally only expressed postnatally in tumor vessels.
Notch Signaling	There are four Notch receptors in mammals (Notch1-Notch4) that regulate the formation of Tip and Stalk cells and endothelial cell specification.	Dll4/Notch signaling pathway negatively regulates VEGF-induced EC proliferation, migration and sprouting

Table 2: Growth Factors that Stimulate Angiogenesis [20-21]

2.5 Endothelial And Support Cell Source

In order to complete the process of new blood vessel formation, ECs must be supported by another cell source [23]. The recruitment of support cells is important for the maturation and stabilization of endothelial cell vasculature *in vivo*. Support cells can vary in cell lineage based on the tissue-specific

vasculature. For example, in the brain, pericytes in addition to astrocytes are recruited to wrap around the endothelium and stabilize the microvasculature. The endothelial cells form the inner lining of the blood vessel wall and the support cells envelope the surface of the vascular lumen (Fig. 4).

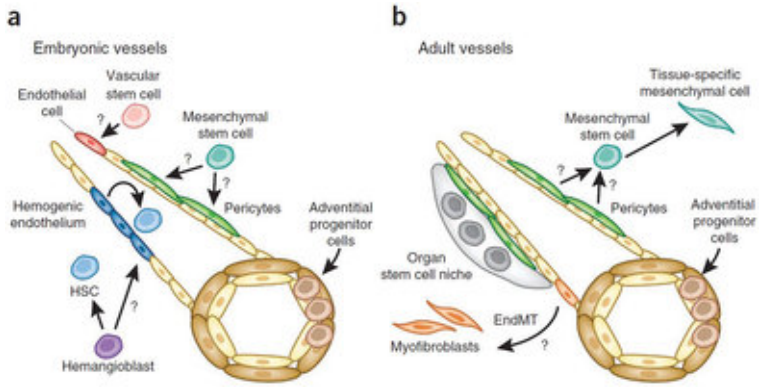

Figure 4: Pericyte origin and stabilization of ECs [66]

Based on the above literature, the two principal components to fabricating a practical engineered scaffold are the vascularization architecture and the cells to be placed within the scaffold. A key limitation in creating a vascularized tissue scaffold is the ability to support stable endothelial cell (EC) sprout formation and subsequent formation of perfusable lumens. Pericytes or vascular smooth muscle cells (vSMCs) provide this support *in vivo* [24-25] however, mesenchymal stem cells (MSCs) [26], adipose derived stem cells (ASCs) [27] and fibroblasts (FCs) [11] have been studied for their ability to support EC vessel formation, stabilization and maturation over time.

For the past four decades, the majority of research in angiogenesis has focused on the EC component and the interaction of pericytes, with minimal research on other support cell populations. However, without the presence of

pericyte or support cells, EC-only vessels become hemorrhagic and begin to recede.

Pericytes, first discovered in 1873, are also known as mural cells or Rouget cells after Charles Rouget, who discovered them [41]. These cells possess a cell body with a prominent nucleus and low cytoplasm content, in addition to several long processes that embrace the abluminal wall of the endothelium (Fig. 5). The pericyte processes embed themselves within the BM of microvessels [48]. These processes typically span several ECs and bridge connections with neighboring capillary branches.

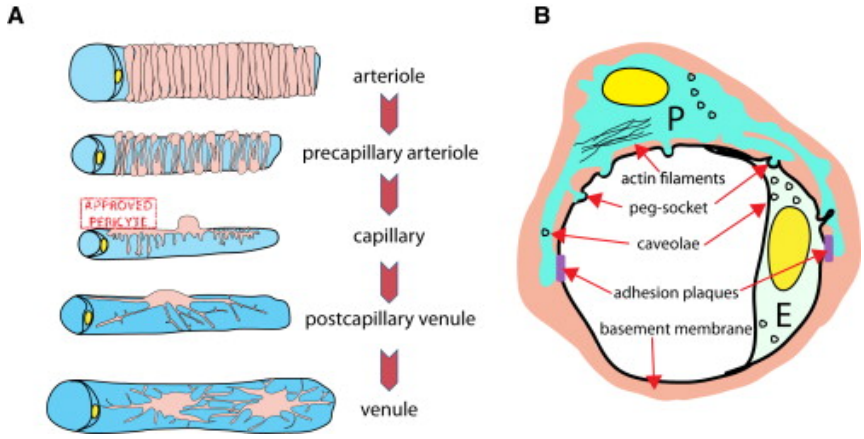

Figure 5: The Pericyte Cell. A) Pericytes supporting larger and smaller vessels B) The interaction between a pericyte (P) and endothelial cell (EC) [49]

It was initially thought that the only function pericytes provided was that of support, however, it is now understood that pericytes also communicate with ECs through paracrine signaling pathways and direct physical contact [49]. This contact, aided by the direct connections of gap junctions between ECs and

pericytes enable the exchange of ions and small molecules [24]. The connections between ECs and pericytes are usually referred to as peg-socket contacts in which the pericyte cytoplasmic processes (pegs) are inserted into the endothelial invaginations (sockets) [49]. It is widely accepted that pericytes belong to the same lineage of cells as vSMCs, however, there is no single molecular marker to identify pericytes and distinguish them from vSMCs or other mesenchymal cells.

The density and number of pericyte cells differs with respect to the function of the organ in which the vasculature is located. For example, pericytes are abundant on larger vessels like arteries, arterioles and veins, but sparse on capillaries and venuoles (Fig 5A) [49]. Vasculature in the central nervous system is covered for the most part by pericyte support cells at a 3:1 ratio between pericyte and endothelial cells [46]. Conversely, lower pericyte to endothelial cell ratios have been reported for other tissues like human skeletal muscle, which has about a 100:1 endothelial to pericyte ratio [24, 50]. Like the highly vascularized central nervous system, the ratio of pericytes to endothelial cells in the myocardium ranges from 2:1 to 3:1 [81]. Although cardiomyocytes comprise the largest cell volume of the heart, pericytes account for ~$3.6x10^7$ cells/cm^3 [81].

The recruitment of pericytes to stabilize developing vasculature occurs through the interaction between at least four known pathways: platelet-derived growth factor-β (PDGF-β)—PDGF receptor-β (PDGFR-β), sphingosine-1-phosphate-1 (S1P1)—endothelial differentiation sphingolipid G-protein-coupled receptor-1 (EDG1), Ang1-Tie2 and transforming growth factor-β (TGF- β) [67]. The endothelial cells release PDGFβ in response to the upregulation of VEGF,

thus facilitating the recruitment of pericytes and support cells. The pericyte or support cells must have a PDGFR-β receptor in order to fulfill the interaction with the ECs and allow for support cell proliferation and migration to the site the immature vasculature [68]. Sphingosine-1-phosophate receptor (S1PR) controls the EC-pericyte interactions by communicating with S1P to trigger cytoskeletal, adhesive and functional changes between the ECs and pericytes, as well as promoting pericyte adhesion to the EC junctions. In ECs, S1P1 controls the trafficking of N-cadherin to the abluminal side of the EC. S1P1 deficiency leads to weakened or no EC-pericyte contacts on the ECs [68]. Aside from the aforementioned Ang1-Tie2 and PDGF-β—PDGFR-β interactions, TGF-β signaling plays an important role in the stimulating pericytes induction, differentiation, proliferation and migration, in addition to promoting the production of extracellular matrix [68].

Although the majority of support cell research covers pericytes, it has been elucidated that other cell types can provide the same support. Other support cells used in co-culture with endothelial cells include stem cells—embryonic progenitor cells, mesenchymal stem cells and haematopoitic stem cells, among others—lung and dermal fibroblasts and smooth muscle cells. The use of mesenchymal stem cells and cardiac fibroblasts as a support cell is discussed in more detail below.

2.6 Strategies for Vascularizing Tissues

As previously mentioned, there is a significant need for vascularized tissue scaffolds, when creating thick tissues. Thick tissues beyond the dimensions of the diffusion limit (>100-200μm) require oxygen and nutrients to be delivered via convection or perfusion, rather than diffusion alone [2]. A prevascularized tissue has the potential to reduce the time of functional vascular perfusion to the host. Different strategies have been used for creating *in vitro* and *in vivo* vasculature, including prevascularizing *in vitro* tissue scaffolds [11], the formation of *de novo* vasculogenesis *in vivo* [56], the creation of organized endothelial cell layers or sheets [80] and the entrapment of cell aggregates/spheroids [82]. The *in vitro* prevascularization of tissue scaffolds involves the creation of a three-dimensional vascularized network that can then be implanted onto a tissue, *in vivo* [11]. In the *in vivo* approach, a non-vascularized tissue engineered scaffold is implanted onto the host and the *de novo* vascularization of the scaffold occurs [56]. It has been demonstrated in many studies that host cells are able to build a perfusable vascular network into suitable biomaterial scaffolds. The polysurgery technique of fabricating tissue engineered cell sheets combines biomaterials with either endothelial cells on their own or in co-culture [82]. Cell sheets are confluent monolayers of cells that are removed from tissue culture plates and layered onto the host tissue [83]. Endothelial cell aggregates or spheroids can be cultured as clusters in a biomaterial scaffold to produce capillary-like sprouts. EC spheroids have been cultured with growth factors and other support cells including

osteoblasts and fibroblasts, demonstrating the development of lumen-like sprouts, but lacking anastomosis with the host vasculature upon implantation *in vivo* [82].

The success in creating a functional vasculature *in vitro* lies in the ability to select the proper cell source, biomaterial and bioreactor to create a tissue engineered organ. The specific selection of these components depends on the type of tissue being engineered. The bioreactor system must provide physical stimulation to mimic the tissue environment and provide perfusion to improve cell viability, while the cells must be expandable. Additionally, it is preferable that they are harvested directly from the patient [20].

Cellular based tissue engineered constructs focus on the co-culture of multiple progenitor cell types along with native cell types in the tissue construct. Stem cells hold great potential as a component to tissue regeneration, however, they must be co-cultured with another cell type due to their insufficient expression of angiogenic factors [53]. Levenberg, *et al.* constructed three-dimensional vascularized skeletal muscle from fibroblasts, myoblasts and endothelial cells to demonstrate improved cell survival *in vivo* [52]. Furthermore, Arnold Caplan, *et al*. pioneered the idea that MSCs can act as pericytes in the perivascular system—although not all pericytes are MSCs—supporting wound healing and vascularization processes [88-89].

Tissue engineering typically requires and artificial extracellular matrix to act as a scaffold for cells to proliferate and differentiate on, while a new tissue is formed. The biomaterial selected for the scaffold is important as it forms the

backbone of the scaffold. The degradation rate of the material is one of the most significant characteristics to consider because a degradation rate that is too rapid or too slow can jeopardize the mechanical integrity of the scaffold [54]. Commonly used biomaterials include collagen, hyaluronic acid (HA), Matrigel™, polyethylene glycol (PEG), fibrin and several others. Matrigel™ is a decellularized matrix derived from mouse sarcoma cells. It is commonly used to develop *in vitro* vascularized tube networks [58] and is part of a common assay for endothelial cells ability to generate tubules [57]. Nevertheless, Matrigel is developed from a cancerous cell source containing a composite of growth factors and other matrix bound bioactive signals, leaving the material largely uncharacterized [58] and likely unusable from a regulatory standpoint.

PEG, PEG-vinyl sulfone (PEG-VS) and PEG-diacrylate (PEGDA) hydrogels have been developed with different crosslinking reactions to aid in better vascularization [59]. These hydrogels are functionalized with matrix metalloproteinase cleavable peptide sequences, growth factors and cell adhesion domains like RGD and they have been studied *in vitro* and *in vivo*. The additional binding of growth factors, like VEGF and RGD allows for faster anastomosis of the scaffold to the host and more rapid vascularization post-implantation [60].

Porous biodegradable polymeric scaffolds like poly (D, L)-lactic-co-glycolic acid (PLGA) and hyaluronic acid (HA) have been widely used for regenerating tissues and organs [90]. The open porous structure of PLGA allows cells to be seeded in the scaffold and cultured while the scaffold slowly degrades. While the HA, which is a naturally occurring polysaccharide is a major

component of the ECM in connective tissues and present in synovial fluids, will interact with cells through various surface receptors [59].

Hydrogels, which contain water-soluble based components, can act as tissue engineered scaffolds by either chemical or physical cross-linking. One hydrogel of note is collagen, which is the most widely distributed class of proteins in humans and mammals [91]. As a naturally occurring biodegradable biomaterial, selected for its ability to cross-link with other proteins and polymers, collagen is used as a scaffold for my tissue engineered constructs. Since collagen is one of the most abundant proteins in nature, it can be extracted from almost every living animal; however, the most common forms of collagen used in tissue engineered constructs come from bovine skin and tendons, rat tail and porcine skin.

Another hydrogel that is central in tissue engineering is fibrin, a biopolymer of the monomer fibrinogen [92]. Fibrin can be produced from the patient's own blood and used as an autologous scaffold, dramatically decreasing the patient's risk of foreign body reaction or infection. Fibrin has many favorable features as a biological scaffold, including its ability to bind to tissues, the fact that it is biodegradable and it has biocompatible properties. Its involvement in the wound healing process, the fact that it can stimulate the release of angiogenic growth factors such as VEGF and bFGF and the fact that fibrin can be degraded and remodeled, makes it a good scaffold for engineered tissue constructs (107). Furthermore, fibrin has many adhesive capabilities and allows for efficient and high cell seeding, in addition to uniform cell distribution within the gel. Fibrin is

made up of two sets of three polypeptide chains Aα, Bβ, and γ. Fibrin is formed after thrombin cleaves the fibrinopeptide A from the Aα and fibrinopeptide B from the Bβ chains, which allows for a conformational change and the exposure of a polymerization site [93]. Fibrin can serve as a temporary matric to facilitate the entry of fibroblasts and macrophages, which then degrade the fibrin and deposit their own ECM, remodeling the tissue scaffold (107). Since fibrin is easily degraded by cells, the cells are able to degrade the fibrin and are stimulated to deposit their own ECM in place of the fibrin, ultimately allowing for the tissue constructs to contain the cells from the patient and the ECM that these cells have created.

2.7 Fabricating a Vascularized Cardiac Tissue Scaffold

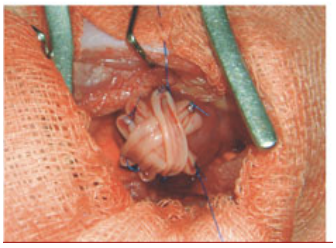

Figure 6: *In vivo* implantation of a construct from the Zimmermann group [70]

Myocardial tissue is highly vascularized with a dense capillary network. In the rat there are $9x10^7$ EC/cm^3, while there are only $2.7x10^7$ cardiomyocytes/cm^3 in the left ventricle of the myocardium [79]. The generation of three-dimensional engineered myocardial tissue has the potential to improve the function of the damaged myocardium and lead to better cell retention as compared to cell injection. Myocardial tissue constructs have the ability to improve myocardial function post-MI through the injection of biomaterials [69] or the creation of a cardiac tissue scaffold to place over the infracted area [70]. Figure 6 is an

example of an engineered myocardial tissue created by Zimmermann, et al. The construct was developed to generate contractile forces consistent with those in the myocardium [6]. Other labs have worked to vary the biophysical cell signals to match the multiple signals present in the native heart [7]. Furthermore, proangiogenic factors, such as VEGF and insulin growth factor-1 (IGF-1) can be added to the constructs to aid in prevascularization [71].

Cells respond to their environment, making it necessary for the tissue engineered construct to mimic the native environment of the host tissue. In order for the myocardial cells to function normally—as they would in a non-infarcted region of the myocardium—the surrounding environment must be made of a matrix that matches the components of the healthy tissue. One way to mimic this environment is by starting with the decellularized matrix of the heart and populating it with cardiogenic cells—work pioneered by Ott, et al—which will induce the functional assembly of cell *in vitro* [94]. Another approach by Radisic et al, is to mimic the mechanical properties of the heart through the use of a perfusion bioreactor system in which the scaffold will form a contractile cardiac patch [95].

Another study of interest was completed by Morritt, et al, in which a cardiac tissue construct consisting of rat cardiomyocytes in Matrigel was implanted in a chamber with an arteriovenous blood vessel loop, subcutaneously into the groin [96]. The chamber was harvested at 1, 4 and 10 week time points. This study demonstrated that the constructs that the latter two time points contracted spontaneously as a result of the blood pumping through the chamber.

Furthermore, the constructs exhibited cardiomyocytes that had differentiated and formed networks interconnected cells within the ECM of the construct.

2.8 Cardiac Fibroblasts Structure and Function

Cardiac fibroblasts play many roles in the heart, one of the most critical being the maintenance of normal cardiac function via ECM synthesis and maintenance. In addition, cardiac fibroblasts promote better contraction of the cardiomyocytes [31] and compaction and alignment of stem cell derived engineered cardiac tissue [85]. In terms of their clinical relevance for use in cardiac repair, CFs could be isolated from a biopsy of the right atrial appendage [32] along with cardiac progenitor cells used to create a functional muscle construct. Although studies have been done using a co-culture or tri-culture of endothelial cells with cardiomyocytes [35], stem cell derived cardiomyocytes [34], embryonic [34] dermal or lung fibroblasts [11] and other pericyte cells, limited studies have been completed to investigate the regeneration of vasculature in myocardial tissue using cardiac fibroblasts. In addition, cardiac fibroblasts, like other fibroblast cells have been shown to secrete angiogenic factors, furthering the benefit to co-culturing FCs with endothelial cells [37, 40].

When developing a tissue engineered construct for the heart, the cell source must be considered. The myocardium consists of cardiomyocytes, endothelial cells, cardiac fibroblasts and smooth muscle cells. Although cardiomyocytes make up the largest volume of cells, cardiac fibroblasts account for the largest cell population and are the source of matrix metalloproteases,

extracellular matrix production, growth factors and paracrine signaling with cardiomyocytes and other cells [28]. Furthermore, CFs play a key role in the structure and biochemical function of the myocardium, including myocardial remodeling after a cardiac event (ie. myocardial infarction or plaque formation). CFs are also integral in the process of hypertrophy of cardiomyocytes; and respond to changes in the extent and composition of cardiac ECM [38]. Studies have shown that CFs secrete angiogenic factors and are able to regulate the sprouting of vascular endothelial cells *in vivo*. Among the angiogenic factors that CFs secrete are VEGF, FGF, TGF-β_1, thrombospondin-1 and PDGF [84]. These angiogenic factors play a role in the stimulation, proliferation and maturation of ECs in the myocardium. For these reasons, CFs serve as an interesting cell type to explore for the creation of vascularized engineered myocardial tissue.

2.9 Mesenchymal Stem Cells as a Vascular Support Cell in the Myocardium

Mesenchymal stem cells (MSCs) are, at times, considered to be similar to pericyte cells, or precursors to pericytes [65]. MSCs are multipotent stem cells that are versatile in that they can differentiate into many different cell types: osteoblasts, chondrocytes and adipocytes; plus they play an important role in the wound healing and bone repair process [65]. MSCs will migrate to the site of damage and colonize around the wound. As the MSCs repair the wound, they differentiate and proliferate in response to different cytokines and growth factors released from neighboring cells [61-62]. Furthermore, MSCs support

neovascularization through the release of proangiogenic factors such as angiopoetin-1, bFGF and VEGF [63]. MSCs express many smooth muscle cell markers *in vivo*, including myocardin, a cardiac and smooth muscle-specific transcription coactivator, which is upregulated through the cell-cell contact with endothelial cells [64].

As previously mentioned, many studies have been done which illustrate that role MSCs play as a support for EC vascularization and sprout formation [88, 89]. Moreover, much of the work pioneered by Caplan, et al revealed that MSCs share many of the same markers and characteristics of pericytes, among which include their role in wound healing and focal adhesion.

2.10 Summary

Cardiac tissue engineering offers a promising approach to regenerating post-MI myocardial tissue; however, a primary impediment in tissue engineering cardiac tissue lies in the inability to adequately vascularize the tissue scaffold. The purpose of this thesis is to investigate the use of Cardiac Fibroblasts as a support for Endothelial Cell sprout formation in engineered myocardial tissue. As a model of myocardial tissue, different concentrations of CFs and MSCs were co-cultured with ECs in fibrin hemispheres in appropriate combinations of their specific media to determine the optimal sprout formation through DNA analysis, flow cytometry and immunohistological image analysis. Later three different developmental life points—fetal, neonatal and adult—of CFs were studied to

assess their ability to support EC sprout formation as CFs are a dynamic population of cells that change throughout normal development. Finally, the process of angiogenesis in *in vitro* culture was studied by decellularizing a vessel, recellularizing it with membrane-labeled endothelial cells and observing the outgrowth of cells into a fibrin hydrogel.

Chapter 3: Cardiac Fibroblasts as a Support for EC Sprout Formation

3.1 Introduction

Cardiovascular disease is one of the leading causes of death in the United States, accounting for over 600,000 deaths in the US in 2007 [3]. After a myocardial infarction (MI) a portion of the left ventricle becomes necrotic, resulting in a negative remodeling process that leaves the tissue scarred. This scar tissue in turn leads to reduced contractile properties of the heart due to muscle loss from the ventricular wall. Current pharmacological and surgical treatments aimed at treating the heart post-MI lack the ability to regenerate the heart tissue and more often seek to reduce the workload of the injured heart to help maintain its function. Tissue engineering offers a novel approach to potentially regenerate myocardial tissue, and there have been a significant number of studies aimed at utilizing cellular, biophysical [7] and mechanical components [6]. However, many of these approaches are limited in size to thicknesses on the order of 200 microns, as they rely on simple diffusion to deliver oxygen and nutrients to the cells.

The inability to form functional vasculature in tissue scaffolds is a primary impediment in tissue engineering [5, 9]. The immediate vascularization of an implantable tissue scaffold is crucial for its survival and function, as the addition of a capillary network helps to maintain cell viability in the tissue by optimizing oxygen and nutrient delivery to the cells beyond the diffusion limit [10]. The *in vitro* prevascularized scaffold can anastomose to the host, allowing for better delivery of oxygen and nutrients, thus enabling the scaffold to better retain its function [11]. Furthermore, it is known that support cells are important for the

formation of stable endothelial cell (EC) sprouts and subsequently vessels. In order to complete the process of new blood vessel formation, ECs must be supported by another cell source [23]. Pericytes or vascular smooth muscle cells (vSMCs) provide this support *in vivo* however [24, 18], mesenchymal stem cells (MSCs) [19], adipose derived stem cells (ASCs) [20] and fibroblast cells (FCs) [11] have also been studied for their ability to support EC vessel formation, stabilization and maturation over time.

When creating a cardiac tissue scaffold, progenitor cells are typically the most clinically relevant source for the formation of functional myocardial tissue. Cardiac progenitor cells can be isolated from a biopsy of the patient's myocardium (e.g., the right atrial appendage (RAA) [32]); while other induced pluripotent stem cells can be derived from the skin fibroblasts of a patient [86]. Studies have demonstrated that cardiac fibroblasts improve contractile force in engineered cardiac constructs [31]. Moreover, when engineered cardiac tissues are created from pluripotent stem cell derived cardiomyocytes, it has been demonstrated that they require the presence of CF like cells in order to have appropriate tissue formation and function [87]. In both of the options for cell source described above, CFs can be either isolated from the heart biopsy along with the progenitors or derived via differentiation from the iPS cells. Although studies have been done using a co-culture or tri-culture of endothelial cells with cardiomyocytes [35], stem cell derived cardiomyocytes [34], embryonic fibroblasts [34], dermal fibroblasts, lung fibroblasts [11] and other pericyte cells, limited studies have been completed to investigate the regeneration of vasculature

in engineered myocardial tissue using cardiac fibroblasts. This is especially critical given that studies have confirmed that CFs secrete angiogenic factors necessary for the maturation and stabilization of endothelial cell sprouts and ultimate lumen formation [84].

Our hypothesis was that cardiac fibroblasts can support endothelial cell sprout formation as well or better than mesenchymal stem cells, which are considered to be a "gold standard" for the stabilization of EC sprouts in engineered tissues [27]. To test this hypothesis, endothelial cells were co-cultured with cardiac fibroblasts and mesenchymal stem cells in order to determine whether cardiac fibroblasts can provide as good of support for endothelial cell sprout formation as MSCs. Sprout formation was measured by converting the fluorescent images of the stained fibrin constructs into binary images and measuring the lengths of each sprout in ImageJ. Our results indicate that ECs form more numerous sprouts when cultured with CFs, but have more multi-cellular sprouts when cultured with MSCs, indicating that mesenchymal stem cells are better able to support the adhesion and protrusion activities of endothelial cells typical demonstrated during angiogenesis. Flow cytometry was performed on the cells after 10 days of culture in the fibrin constructs, which illustrated that endothelial cells in co-culture with cardiac fibroblasts were able to proliferate better than when in culture with the mesenchymal stem cells. The fact that ECs form more numerous sprouts in the CF culture is likely directly related to the fact that CFs were better able to support EC proliferation compared to MSCs. In summary, CFs lead to increased EC proliferation and longer, more numerous

sprouts than MSCs, while MSCs allow for more multicellular sprouts, which is more indicative of the *in vivo* process.

3.2 Materials and Methods

3.2.1 Cell Culture

Rat Aortic Endothelial Cells (rAoECs) were purchased from Cell Applications (San Diego, CA) at passage 3 and cultured for use in experiments from P5-P7. The rAoECs were cultured in complete rAoEC medium (EBM-2; Lonza Walkersville, Walkersville, MD) with the addition of EGM-2 SingleQuots at 37^{o}C, 5% CO_2. Cardiac cells were isolated from neonatal (postnatal day 2 to 3) Sprague Dawley rats using a type II collagenase solution as previously described [72]. Neonatal Cardiac fibroblasts (nCFs) were isolated from this mixed cardiac cell population using the pre-plate method [73] and cultured in nCF media (DMEM, 15% Fetal Bovine Serum, 1% penicillin/streptomycin; Invitrogen, Grand Island, NY). When cultured in the fibrin hydrogels, the nCF media was supplemented with 1% ε-aminocaproic acid (ACA)—to control fibrinolysis, 0.1% insulin (Invitrogen, Grand Island, NY) and 50μg/ml ascorbic acid (Sigma Aldrich, St. Louis, MO)—both to promote ECM production. When entrapped in fibrin constructs the CFs were used from passage P5-P10. Rat Mesenchymal Stem Cells (rMSCs) were purchased from Texas A&M University at passage 6 and cultured in MSC media (alpha-MEM, 20% fetal bovine serum, 1% penicillin/streptomycin and supplemented daily with 2% L-Glutamine; all from

Invitrogen) and used in experiments from passage 8-12. For all three cell types in this study, medium was changed three times per week and cells were harvested at 80% confluence via 0.25% trypsin-EDTA (1x) treatment (Invitrogen) for use in experiments.

3.2.2 Characterization of Cell Lines

In order to characterize the cell lines prior to implantation in the constructs, flow cytometric analysis was conducted on the cells in two-dimensional culture. The cells were labeled with FITC-conjugated mouse monoclonal anti-rat CD31 (ab33858; Abcam, Cambridge, MA) diluted to 1:10 (Endothelial Cells), PerCP-conjugated anti-rat vimentin (vimentin (V9), Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:50 (Cardiac Fibroblasts) and APC-conjugated CD90 anti-rat (BioLegend) diluted at 0.06µg in 100µL (MSCs). Antibody labeling was carried out for 30 minutes, incubated at 4°C, followed by two washes in autoMacs® Running Buffer (Miltenyi Biotech, Auburn, CA). The cells were characterized using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) at the Tufts Medical Center Flow Core facility using CellQuest Software (Becton Dickinson, Franklin, NJ). All FACS data was analyzed using the FlowJo software (FlowJo, Ashland, OR).

3.2.3 Creation of Fibrin Co-Cultured Hydrogels

Fibrin gel hemispheres (550µl) were fabricated in 12-well tissue culture plates, in order to study the interactions between the endothelial cells and the support cells in 3-D. The fibrin hydrogels were created by combining the cell solutions with bovine fibrinogen in 20mM HEPES-buffered saline, bovine thrombin with 2N Ca^{2+} and DMEM. The fibrin solution contains a 4:1:1 ratio of fibrinogen to cells to thrombin (Sigma Aldrich, St Louis, MO). For the medium studies, hemispheres were created in a final gel concentration of 3.3mg/ml with 2.5 x 10^5 cells/mL initial concentrations. Prior to the addition of the fibrin hydrogel, the plates were scored with a #13 metal biopsy punch (Fisher Scientific, Waltham, MA) in order to aid in the creation of the fibrin hemisphere shape [74]. The fibrin solution was allowed to polymerize for 20 minutes before culture medium was added. The hemispheres were cultured in 12-well plates with 2 mL of medium (replaced three times per week) in a cell culture incubator. Experiments were run in quadruplicates ($n = 4$).

3.2.4 Fibrin Hydrogels for Co-Culture Media Studies

In order to determine the best media combination for optimal endothelial cell and support cell formation, ECs were cultured at a 1:1 ratio with either Cardiac Fibroblasts or Mesenchymal Stem Cells as support cells in the following media conditions (listed in Table 3 below): 100% Endothelial Cell Media, 1:1 Endothelial Cell Media: Support Cell media and 100% Support Cell media.

Endothelial cells were seeded in fibrin hydrogels with all five media ratios, as controls. To determine the optimal media content, DNA Assays were run and fluorescent images for sprout formation were analyzed.

Cell Type	100% EC Media	100% CF media	100% MSC Media	EC:CF media	EC:MSC media
100% EC	X	X	X	X	X
EC:CF	X	X		X	
EC:MSC	X		X		X

Table 3: Experimental design of media studies

3.2.5 Fibrin Hydrogels for Varied Co-Culture Studies

Once the optimal media ratio was determined, the concentration of endothelial cells to support cells was varied in order to determine the best ratio of support cells to endothelial cells for optimal endothelial cell sprout formation. Three ratios of endothelial cells to support cells were considered for both cardiac fibroblasts and mesenchymal stem cells as support cells (demonstrated in Table 4 below): 3:1 Support Cells: Endothelial Cells, 1:1 Support Cells: Endothelial Cells and 1:3 Support Cells: Endothelial Cells in 1:1 endothelial cell: support cell media. Endothelial cells were seeded in fibrin hydrogels as a control with 100% endothelial cell media and 1:1 Endothelial Cells: Support Cell media. The number of endothelial cells was held constant at $2x10^5$ cells/mL fibrin solution for all of the conditions.

EC-CF varied co-culture (EC:CF media)	EC-MSC varied co-culture (EC:MSC media)	EC (control)
75% CF: 25% EC	75% MSC: 25% EC	100% EC media
50% CF: 50% EC	50% MSC: 50% EC	EC:CF media
25% CF: 75% EC	25% MSC: 75% EC	EC:MSC Media

Table 4: Cell Ratios in varied co-culture study

3.2.6 Two Dimensional Fluorescent Image Acquisition and Analysis

Prior to encapsulation in the fibrin hydrogels, endothelial cells were dyed with PKH 26 (Sigma Aldrich), a red fluorescent cell membrane stain or CellTracker Green (Invitrogen), used as a membrane label to track endothelial cell sprout formation and growth. The fibrin hydrogel constructs were cultured for 7-10 days, after which high-resolution images of the hemispheres from each condition were captured on an Olympus IX81 microscope at 10x and 20x magnifications. The endothelial cells were also stained with Von Wilibrand's Factor (vWF, Abcam. Cambridge, MA) prior to fluorescent imaging to verify co-localization of vWF and PKH26. Multiple images (n=3-5) were acquired from random locations for each condition. The length of each sprout was measured in the XY projections from segments of each endothelial cell sprout (defined as branch point to branch point) using ImageJ (NIH, Bethesda, MD). The majority of the sprouts lacked a significant curvature, thus a straight line was drawn from one branch point to another branch point. The average sprout length for each construct condition was determined and plotted against the ratio of support cells to endothelial cells for each support cell condition. In order to determine whether the endothelial cells sprouts were multicellular, the number of endothelial cell nuclei in each sprout was also counted.

3.2.7 Immunohistochemical and immunofluorescent Staining

In order to look at endothelial sprout formation and the role of the support cells in this process, constructs underwent immunohistological analysis. For

fluorescent imaging, constructs were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) at 4^{o}C for 3 h, rinsed with PBS three times for 5 min each and then permeabilized with 0.1% Triton-X solution for 10 minutes. After permeablization, constructs were washed with 1x PBS, blocked with 5% Donkey Serum (Sigma Aldrich) for one hour and stained with α-smooth muscle actin (1:500 α-SMA; ab18147 Abcam, Cambridge, MA), for nCFs and MSCs or von Wilibrand's Factor (1:200 vWF; ab6994 Abcam, Cambridge, MA) as an EC marker. Alexa Fluor 555 donkey anti-rabbit and Dylight 488 donkey anti-mouse were used as secondary antibodies for vWF and α–SMA, respectively (1:500, Jackson ImmunoResearch, West Grove, PA). When entrapped in the fibrin hydrogel the rCFs take on a myofibroblast morphology, verified by the presence of α–SMA [75]. One to two constructs per condition and experiment were processed for histological analysis. The samples were analyzed with an Olympus IX81 inverted fluorescent microscope or by confocal microscopy on a Leica SP2 confocal microscope equipped with an Argon laser (Wetzlar, Germany). The resulting images (n=2-4 per sample) were analyzed for endothelial cell sprout formation.

3.2.8 Characterization of Cell Number

The final cell count of all constructs was quantified using a DNA assay as previously described [83]. After 7-10 days in culture, two to three constructs per condition for each experiment were sterilely set aside. The numbers of cells per

construct were quantified using a modified Hoechst assay [36]. Samples were digested in 0.5mg/mL proteinase K in digestion buffer (100mM Tris, 50mM EDTA, pH 7.4) overnight at 56^{o}C. The assay was performed using a 1:4 ratio or 1:10 of digested sample to TNE buffer (Tris-NaCL-EDTA buffer) depending on the concentration of cells in each construct and 100uL of Hoechst dye solution was added to each well in a 96-well clear bottom, black microplate. The 96-well plate was then analyzed on a microplate reader (Bio-Tek Instruments, Inc, Winooski, VT) at 360nm excitation/460nm emission. Cell numbers were determined from DNA content assuming 7.6pg of DNA per cell, as previously described [76].

To analyze the proliferation of endothelial cells in the fibrin constructs after the 7-10 day culture period, the constructs underwent a modified type-II collagenase digestion. The collagenase was prepared at 50mg/mL in 1xPBS-glucose, following the same procedure as the neonatal cardiomyocytes isolation. The collagenase was warmed and then added at 1mL solution per construct and incubated at 37^{o}C for 7 minutes. The solution was pipetted multiple times against the fibrin construct to break it up more and then added to a 50mL conical. Another 1mL of collagenase solution was added to each construct and incubated for another 7 minutes. This process was repeated 5-6 times until the entire fibrin construct was digested. Stop media which contains 5% FBS and 1% penicillin/streptomycin in DMEM (Invitrogen) was added to the conical with the collagenase + cell solution between each step to stop the collagenase reaction. Once the fibrin construct was completely broken down, the collagenase + cell

solution was filtered through a 70μm cell strainers (Fisher Scientific) and spun down at 200g for 5 minutes at 4°C. The solution was aspirated and the cells were re-suspended in autoMACS® Buffer and the flow cytometry procedure was carried out as stated above.

3.2.9 Statistical Analysis

All results are expressed as the mean ± the standard deviation. Statistical analysis of all data was conducted in SigmaPlot (Systat Software, Inc, San Jose, CA). When comparing more than two groups, a one-way or two-way analysis of variance (ANOVA) was used, followed by a post hoc Tukeys test. The Tukeys t-test was used to compare the means between two groups. Significant levels were set at $p < 0.05$. At least three images of each condition were analyzed and multiple images were acquired from each construct per condition. At least two samples per group for cell count from the DNA assay were tested.

3.3 Results

3.3.1 Endothelial Cell Co-Culture Media Experiments

3.3.1.1 Cell Count in Mixed Media Studies

The effect of the growth medium on the proliferation of the endothelial cells and the support cells was examined, as indicated in Table 5 below. In order to find the optimal medium for the culture of both the endothelial cells and the

support cells, we evaluated the cell growth rate of ECs, MSCs and nCFs in various ratios of medium. The ECs were cultured on their own, in the five different types of medium as a control. After 7-10 days in co-culture, the total co-culture population appeared to proliferate the best when cultured in a 1:1 ratio of EC media: Support Cell media (Fig 7), although the highest total endothelial cell count per day was found in the 100% MSC medium culture ($p<0.003$ for cell number in 100% MSC media compared to 100% nCF medium). The constructs in both the co-cultured and EC-only conditions were viewed under bright-field and fluorescent microscopy and it was determined that the 1:1 ratio of EC medium to Support Cell medium was the optimal mixture to support both cell types.

Cell Type	100% EC Media	100% CF media	100% MSC Media	EC:CF media	EC:MSC media
100% EC	X	X	X	X	X
EC:CF	X	X		X	
EC:MSC	X		X		X

Table 5: Support Cell Media Study

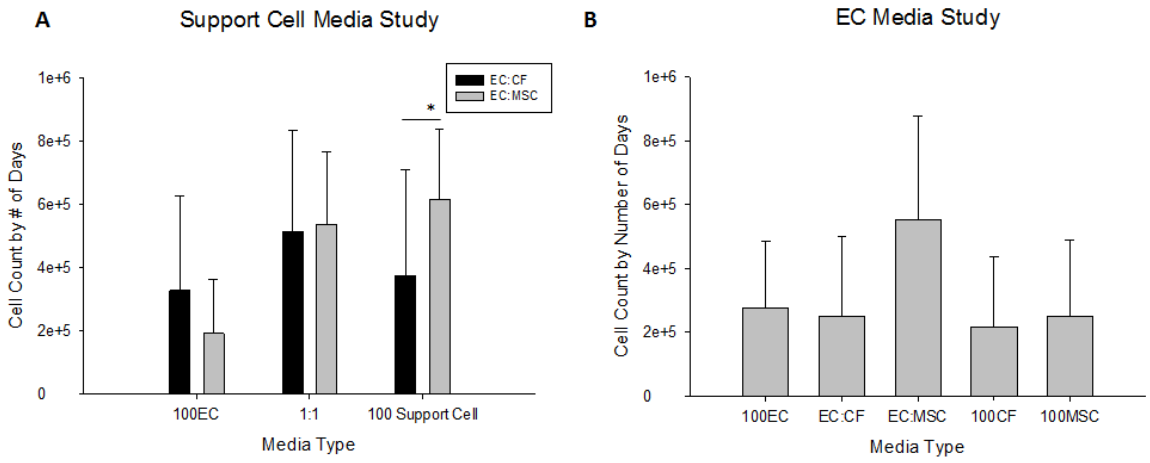

Figure 7: Support Cell Media Study. A) Endothelial Cells cultured with MSCs and nCFs in 100% EC media, 100% Support Cell media and a 1:1 ratio of EC:Support Cell media. In 100% Support Cell media, cell number is significantly greater in 100% MSC media vs 100%CF media ($p<0.003$) B) ECs cultured alone as a control for cell growth in various media solutions.

When the ECs were cultured alone, the highest cell number (standardized by the number of days in culture) was found in the EC:MSC group, although there was no statistically significant difference between any of the groups.

3.3.1.2 Sprout Formation in Mixed Media Study

Even though the ability for both cell populations to proliferate in co-culture is important, the purpose of this study was to assess which population is best for rAoEC sprout formation. Through the fluorescent imaging of rAoECs pre-membrane labeled with PKH26 and stained with vWF and the support cells stained with alpha-Smooth Muscle Actin (α-SMA), the average number of sprouts, average sprout length and number of nuclei was found.

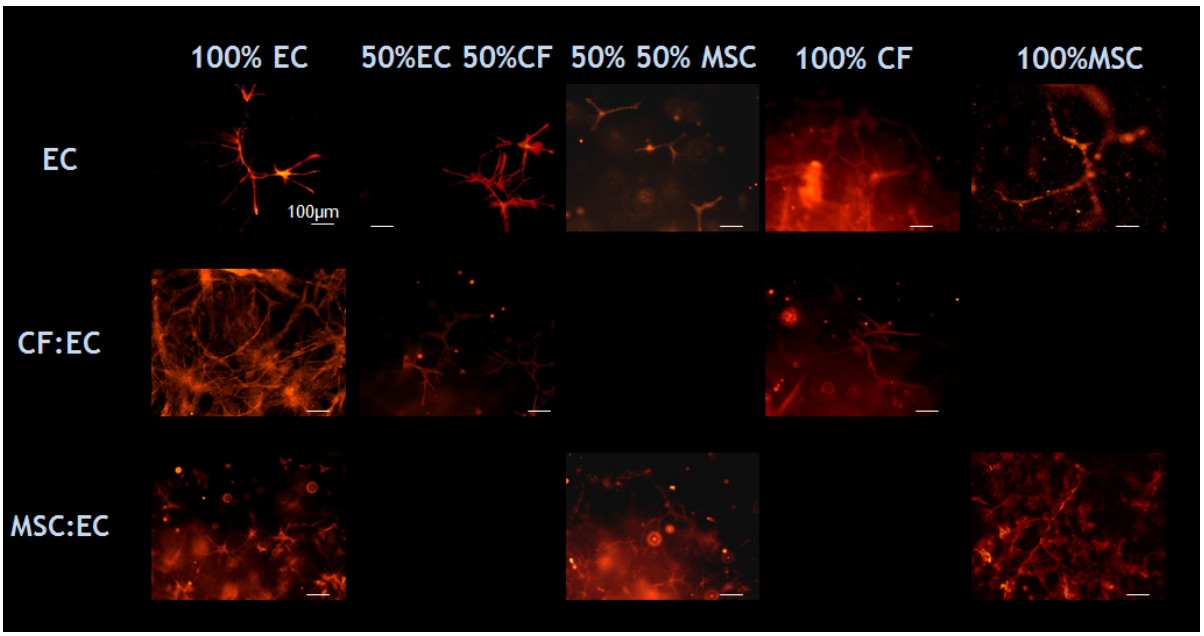

Figure 8: Representative images of fluorescently stained constructs for each media and cell type condition. ECs are stained with von Willibrand's Factor

The number of endothelial sprouts formed in each media condition was quantified using ImageJ (Fig. 9). After analyzing the fluorescent images (Figure 8), it was clear that endothelial cells were able to form sprouts in all conditions (Fig. 10B). However, the ECs formed the longest sprouts when co-cultured with the cardiac fibroblasts (Fig. 10A). Conversely, when cultured with the MSCs, endothelial cells formed more abundant, multi-cellular sprouts (Figures 11 and 12).

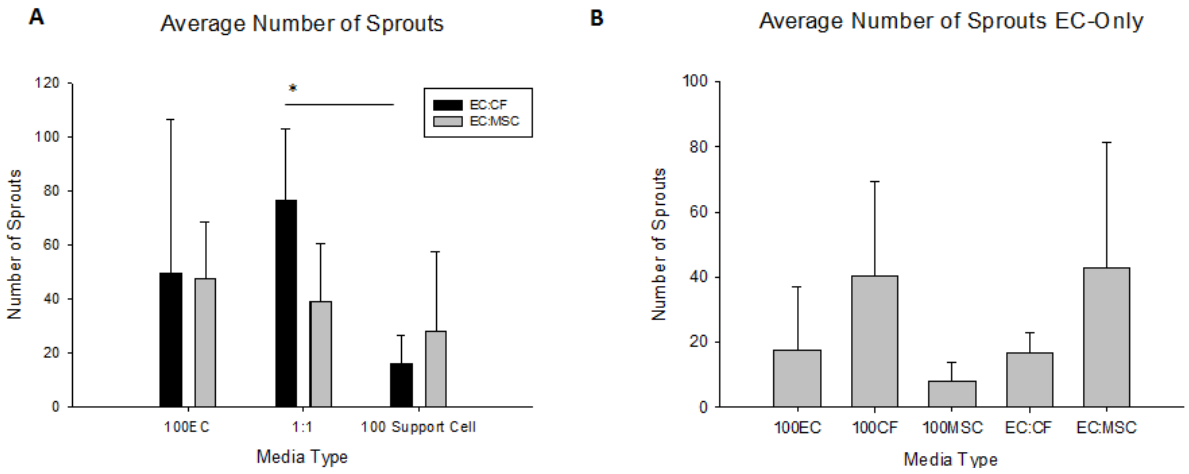

Figure 9: Average number of sprouts in 3-D culture. A) ECs co-culture with support cells in mixed media study (P<0.025). B) Average sprout number in EC-only culture

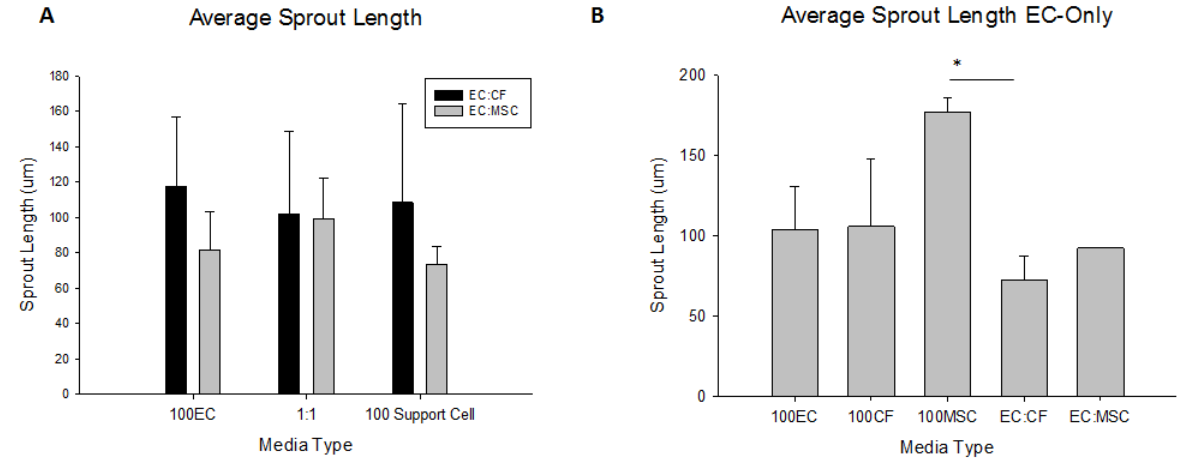

Figure 10: Average sprouts length in 3-D culture. A) ECs co-culture with support cells in mixed media study. B) Average sprout length in EC-only culture (P = 0.027).

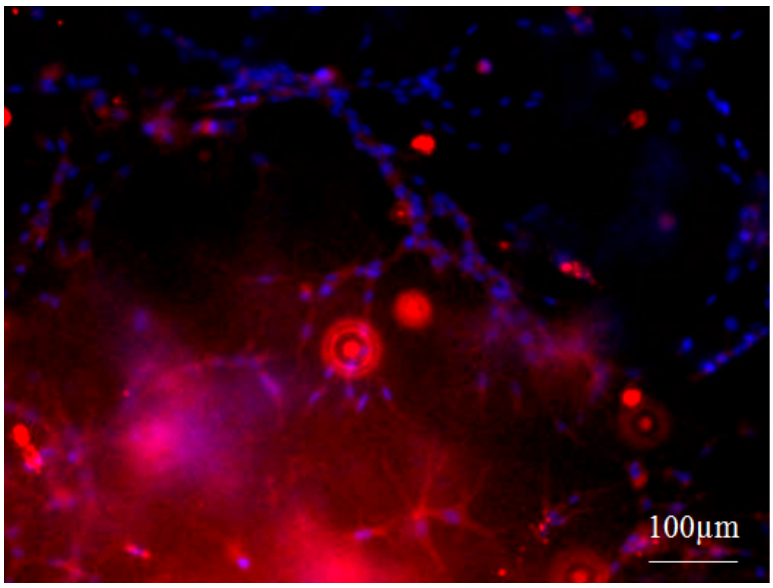

Figure 11: Representative images of fluorescently stained of MSC-EC 50%MSC-50%ECmedia illustrating the multi-cellularity of the EC sprouts in culture with MSCs. Red= von Willibrand's Factor; Blue = Hoechst nuclei stain

After completing the Analysis of Variance (ANOVA) on the mean values, it became apparent that the differences in the average number of branches in each support cell condition was not great enough to exclude the possibility that these differences occurred due to random chance. Furthermore, the affect of the different cell type in the construct does not depend on what type of media is present with respect to the average number of sprouts. In addition, the post-hoc Tukey's test revealed that within the EC:nCF condition, the average number of sprouts is greatest in the 50%nCF-50%EC media condition as compared to the 100% nCF media condition (P=0.025).

When considering the average lengths of each sprout there is no statistically significant difference between the different support cell types after allowing for the effects of different types of media (P=0.125), nor is there a

significant difference in the different type of media used when considering the differences in support cell type (P=0.883). Nevertheless, in all media conditions, the endothelial cells form the longest sprouts when co-cultured with the nCFs. When ECs were cultured alone, they formed the longest sprouts in the 100% MSC media condition. The 100% MSC media condition allowed for a significantly greater sprout length than the 50%nCF-50%EC media condition (P=0.027). When considering the mean values for all media conditions for the EC-only culture, the differences in the mean sprout lengths is greater than would be expected by chance (P=0.035).

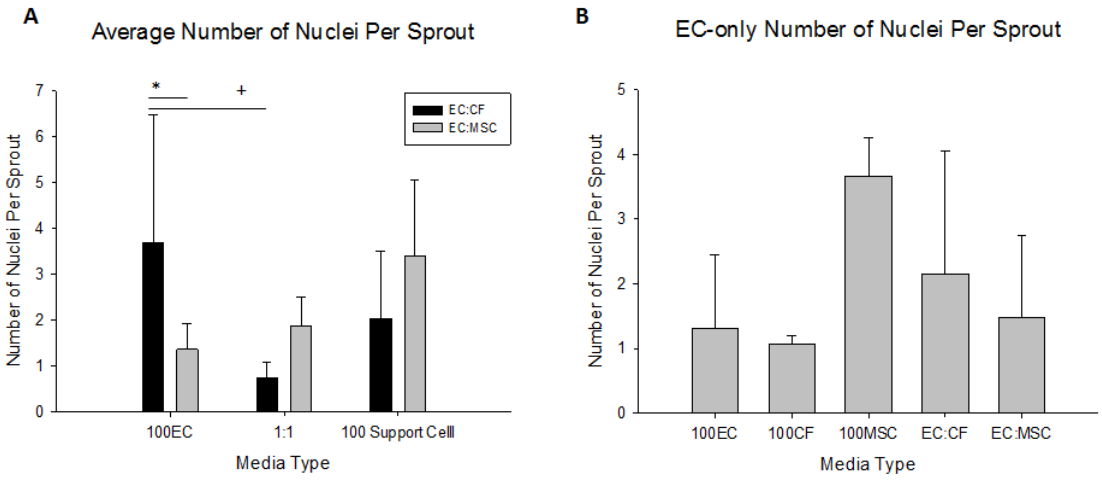

Figure 12: Average number of sprouts. A) EC co-culture with support cells in mixed media study (* P=0.007, + P=0.002). B) EC-only culture

The average number of nuclei per sprout is dependant on the support cell type present within a specific media type. This can be seen in Figure 12A for ECs in co-culture with support cells and in Figure 12B for EC-only culture. There is a statistically significant interaction between the support cell type and the media type (P=0.009). For the support cell co-culture conditions, the most nuclei per sprout was demonstrated in the EC:nCF condition when the constructs were

cultured with 100% EC media. This condition illustrated a significantly greater number of nuclei per sprout compared to the EC:MSC (P=0.007) and the EC:nCF condition in the 50%nCF-50%EC media condition (P=0.002). The EC-only culture demonstrated the most abundant number of nuclei per sprout when cultured with 100%MSC media and in the 50%nCF-50%EC media condition, although there was not a statistically significant difference between the groups.

3.3.2 Endothelial Cell Varied Co-Culture Concentration Experiments

3.3.2.1 Total Cell Count in Varied Concentration Studies

After the optimal media concentration was selected, a subsequent set of experiments was carried out with the total number of cells in co-culture being varied. The three different conditions of varied co-culture, along with the initial number of cells seeded in the constructs are listed in Table 4 below. Similar to the mixed media studies discussed previously, the total cell number after 10 days in culture was calculated after running a DNA assay on the constructs (Figure 13A). Because the initial number of cells seeded in the constructs varied by condition, the total number of doublings for each condition was considered (Figure 13B).

Ratio of Support Cell: Endothelial Cell	Initial Support Cell Number	Initial Endothelial Cell Number	Total Cell Number
3:1	330,000	110,000	440,000
1:1	110,000	110,000	220,000
1:3	36,630	110,000	146,630

Table 6: Ratio of Support Cells to Endothelial Cells with the total initial cell number and the number of each cell type seeded in the constructs.

As illustrated in Figure 13 below, the total cell number after ten days in culture does not depend on the cell type, or the concentration of support cells to endothelial cells. The difference in the mean values of the doublings for each condition among the different levels of Support Cell to EC ratio is significantly greater (P = <0.001). The conditions with less support cells (1:3 and 1:1 conditions) result in more doublings. When compared to the 3:1 condition for both support cell types, the 1:1 and 1:3 ratio of support cells to ECs results in a statistically greater number of doublings (P<0.001). Within the MSC support cell condition, the 1:3 ratio of Support Cell to EC resulted in a significantly greater number of doublings than the 3:1 and 1:1 conditions (P<0.001 and P=0.027, respectively), and the 1:1 condition resulted in a significantly greater number of doublings when compared to the 3:1 condition. While within the nCF support cell type, the 1:3 and 1:1 condition leads to a significantly greater number of doublings compared to the 3:1 condition (P=0.003 and P=0.035, respectively).

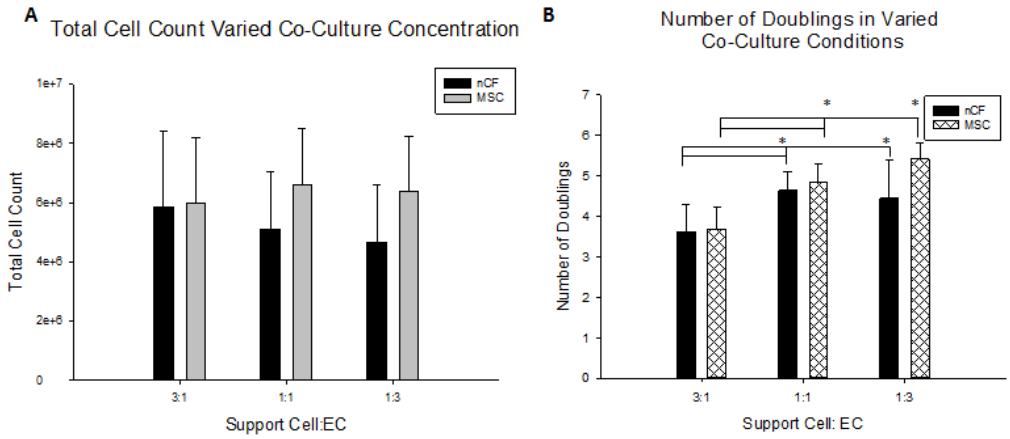

Figure 13: Varied Co-Culture Concentration total cell count. A) Total cell count of varied co-culture studies. B) The number of doublings in varied co-culture conditions. In the 1:3 condition, the MSCs have a greater number of doublings than the nCF condition (P= 0.065).

3.3.2.2 Flow Cytometry of Constructs in Varied Co-Culture

Prior to encapsulating the ECs and support cells in constructs, flow cytometry was conducted. Figure 14 below demonstrates the percent of endothelial cells in 2D culture stained with CD31 (Fig 14A), CD90 (Fig 14B), Vimentin (Fig 14C), DDR2 (Fig 14D) and CD31 + CD90 (Fig 14E). The ECs demonstrated a strong affinity for CD31 with 99.4% of the events marking for CD31 in 2D culture. When the ECs were stained with CD31 and CD90 at the same time, the ECs still demonstrated a strong stain for CD31 at 96.7%. In constructs, the ECs still had a strong affinity for CD31, with 96.7% of the events marking $CD31^{+}$. Vimentin, which is an intermediate filament protein expressed in mesenchymal cells marked 43% of the ECs, while only 1.4% of the ECs demonstrated a CD90 binding.

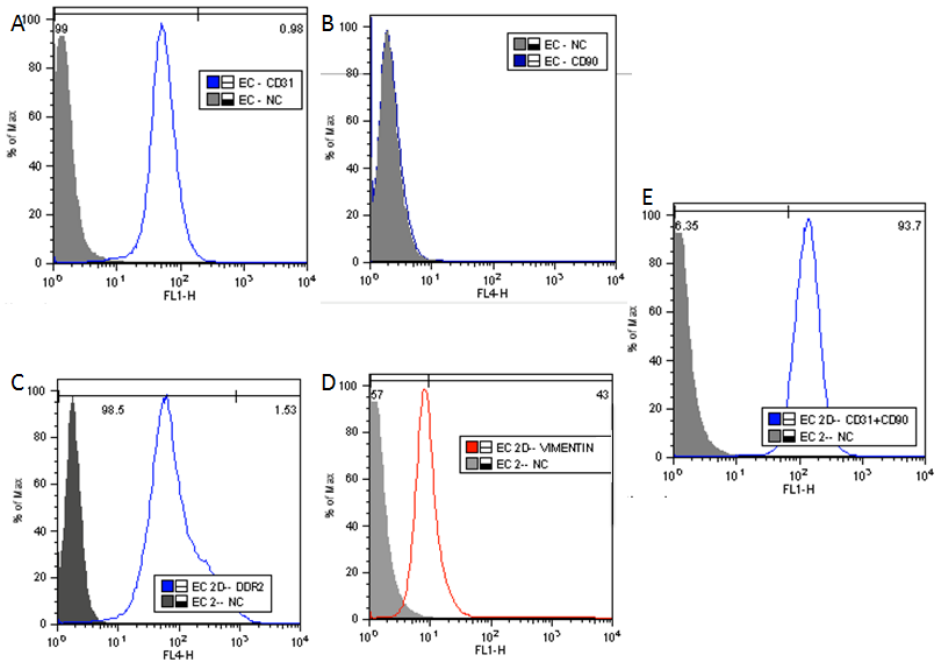

Figure 14: Flow cytometry histograms of Endothelial Cells in 2D culture stained with A) CD31 B) CD90 C) Vimentin D) DDR2 and E) CD90+ CD31. The gray shadow indicates the negative control and the blue or red lines indicates the antibody stain

Another goal of the flow cytometry study was to attempt to quantify the percent of ECs in the constructs after 10 days. In order to determine if the ECs changed morphology in the constructs, and if the cells could be removed from the fibrin hydrogel without significant cell loss, ECs were cultured on their own in the fibrin constructs for ten days. After digesting the constructs, the results from flow cytometry demonstrated that the ECs could be adequately removed from the constructs and stained with the same antibodies used on the ECs in 2D culture. In 3D culture, 96.7% of ECs stained for CD31, while less than 1% of ECs stained for CD90. When the ECs were cultured with 50%EC-50%CF media and 50%EC-50%MSC media they still had a strong affinity for CD31 (98.9% and 96.8%), which is shown in Table 7 below. There was relatively no difference between the forward and side scatter of the EC constructs cultured in the mixed media as compared to the 100% EC media. Additionally, the fluorescent shift of the $CD31^+$ cells also looks the same (visible in the dot plots of Figure 15), meaning there is no change in EC morphology in culture with the mixed mediums. The two markers that were considered for Cardiac Fibroblasts were Vimentin and DDR2, however, both marked the ECs in the constructs at 43% and 96.3% respectively (Figure 16).

Media Type	% $CD31^+$
EC 100% EC m	96.7
EC 50/50CF m	98.9
EC 50/50MSC m	96.8

Table 7: Percent of ECs that were $CD31^+$ after analysis by flow cytometry

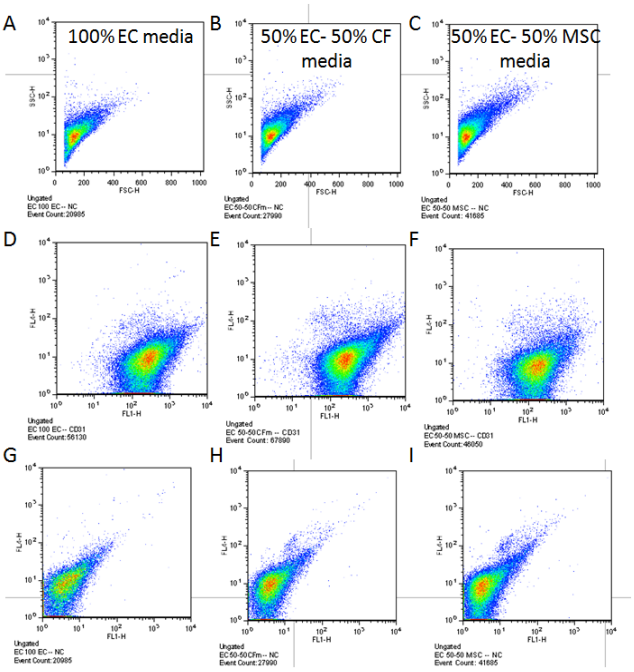

Figure 15: Flow cytometry dot plots of Endothelial Cells in 3D constructs A-C) Forward vs Side Scatter D-F) CD31^{+} cells (Fl1 vs Fl4) G-I) Negative control of construct (Fl1 vs Fl4)

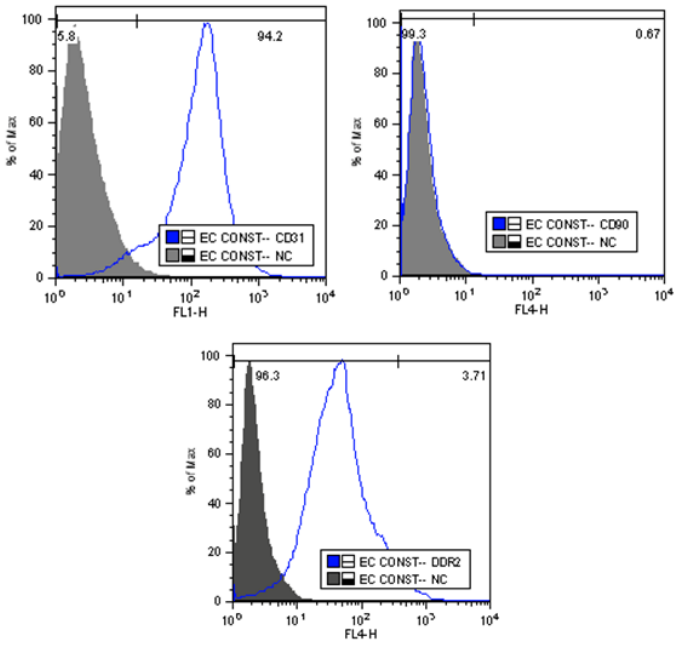

Figure 16: Flow cytometry histograms of ECs in fibrin constructs stained with A) CD31 B) CD90 and C) DDR2. The gray shadow indicates the negative control and the blue lines indicate the antibody stain

To test antibodies for their ability to bind to Cardiac Fibroblasts, nCFs were stained with CD31 (Fig. 17A), DDR2 (Fig 17B) and Vimentin (Fig 17C). The nCFs did not stain for CD31 (~1.3% binding to CD31), but did mark for DDR2 (93.5% binding) and Vimentin (70%). Likewise, the MSCs were also stained with CD90 and CD31 in 2D culture (Figures 18A and 18B, respectively). Because MSCs are not a completely homogenous cell population, a slightly lower percent of the MSCs stained for CD90 in 2D culture (91.1%), compared to the percent of ECs that stained for CD31, while only 1.3% of MSCs stained for CD31 in 2D culture.

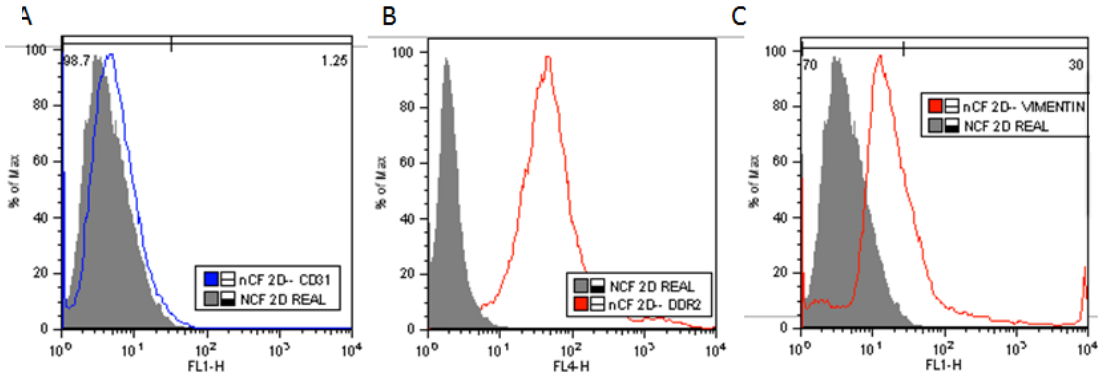

Figure 17: Flow cytometry histograms of nCFs in 2D culture stained with A) CD31 B) DDR2 and C) Vimentin

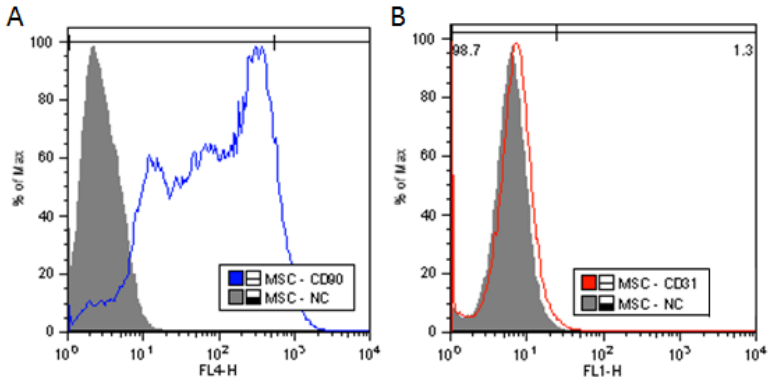

Figure 18: Flow cytometry histograms of MSCs in 2D culture stained with A) CD90 B) CD31

As a result of the lack of a strong marker for CFs, the nCF-EC constructs were stained with CD31 only. Moreover, the co-culture of ECs and MSCs were stained with both CD31 and CD90. Figure 19 below are representative images of the dot plots and histograms obtained from the 50% Support Cell- 50%EC conditions stained with CD31, during flow cytometry. For the example, in the nCF:EC constructs only 19.4% of the cells stained for CD31, while 80% did not stain for anything. Furthermore, in the MSC:EC constructs only 10.2% of the cells stained for CD31. Multiple experiments were run and the average percent of ECs in constructs was obtained (illustrated in Table 8), as previously mentioned.

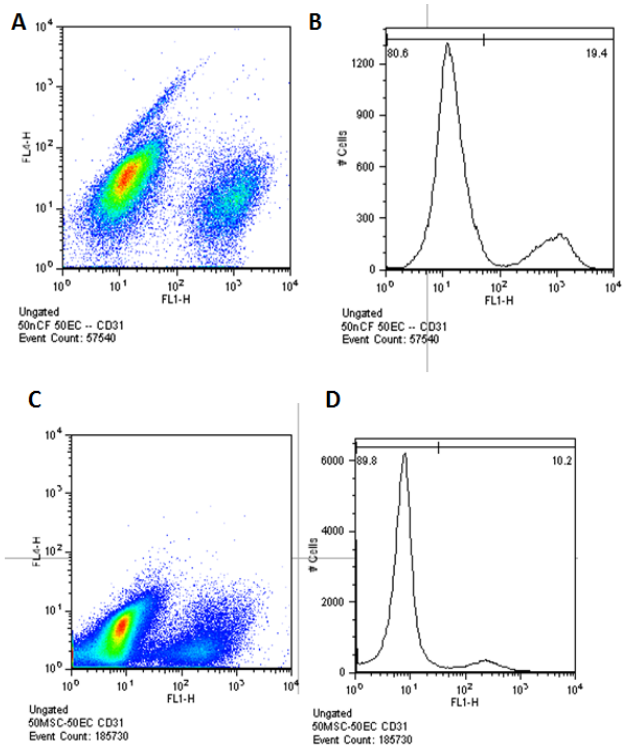

Figure 19: Flow cytometry dot plots and histograms of ECs in fibrin constructs with nCFs and MSCs stained with CD31 A) dot plot of 50nCF 50EC B) histogram of 50nCF 50EC C) dot plot of 50MSC 50EC and D) histogram of 50MSC 50EC

EC Average	Average	StDev
100 EC 100EC m	96.70%	3.68
75nCF 25EC	18.97%	2.66
50nCF 50EC	41.97%	25.73
25nCF 75EC	52.92%	18.58
75aCF 25EC	15.33%	1.67
50aCF 50Ec	32.13%	24.67
25aCF 75Ec	57.18%	22.34
75fCF 25EC	5.12%	0.79
50fCF 50EC	6.19%	1.27
25fCF 75EC	13.25%	4.71
75MSC 25EC	13.17%	8.43
50MSC: 50EC	20.61%	16.33
25MSC 75EC	59.53%	7.93

Table 8: Average percent of EC in each culture condition including standard deviation for each condition (n=2-6).

3.3.2.3 EC Proliferation in Varied Concentration Studies

Although the total cell growth in co-culture is important, the purpose of these experiments is to ascertain which support cell type is optimal for EC growth and sprout formation. To do this, the proliferation of ECs in co-culture must be considered. The proliferation of ECs was calculated by combining flow cytometry measurements on the digested constructs (Figure 20) with the DNA data.

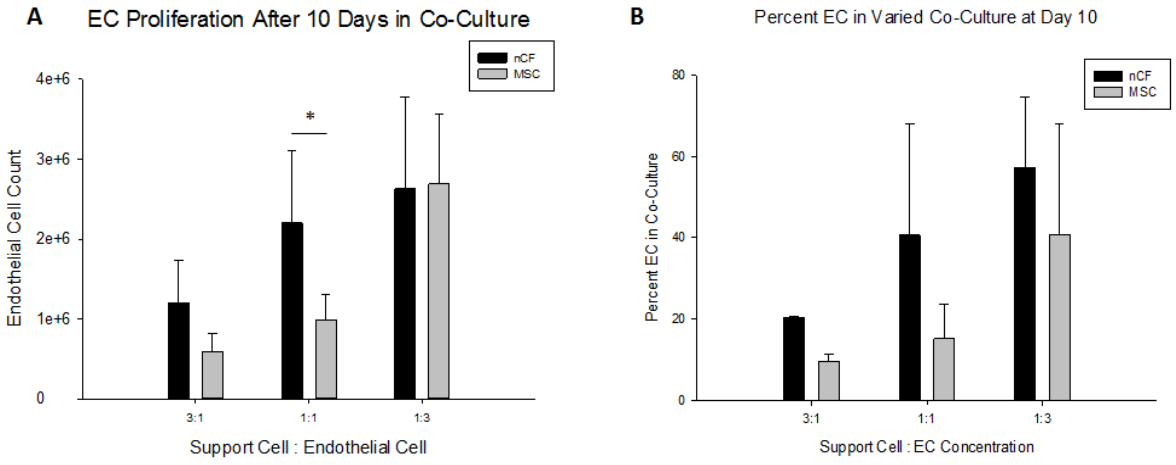

Figure 20: EC growth after 10 days in co-culture. A) EC proliferation in co-culture (P=0.001). B) Percent of ECs present in co-culture after 10 days.

At Day 10 there was a significantly greater number of ECs in the nCF:EC condition than the MSC:EC condition (P=0.001). This is especially significant since the conditions were initially run with an equal number of ECs to start. As a support cell, nCFs allow for a significantly greater proliferation of ECs by Day 10 than MSCs do (P=0.014). Table 9 below illustrates the initial percent of ECs in each co-culture condition, the final percent of ECs in the nCF and MSC conditions and the percent change of ECs in co-culture.

Concentration	3:1	1:1	1:3
Initial Percent	25%	50%	75%
nCF Final Percent	18.97%	41.97%	52.92%
MSC Final Percent	13.17%	20.61%	59.53%
nCF Percent Change	24%	20.80%	29.44%
MSC Percent Change	52.12%	60.66%	20.62%

Table 9: Initial and final percent of EC in Varied Concentration Co-Culture, including percent change of ECs

3.3.2.4 Sprout Formation in Varied Co-Culture Concentration Study

The total number of sprouts, average number of nuclei per sprout and average sprout length were calculated in the varied co-culture concentration study in the same manner as for the mixed media study. Images were analyzed in ImageJ. Figure 21 demonstrates representative stained fluorescent images of ECs in co-culture with both nCFs and MSCs.

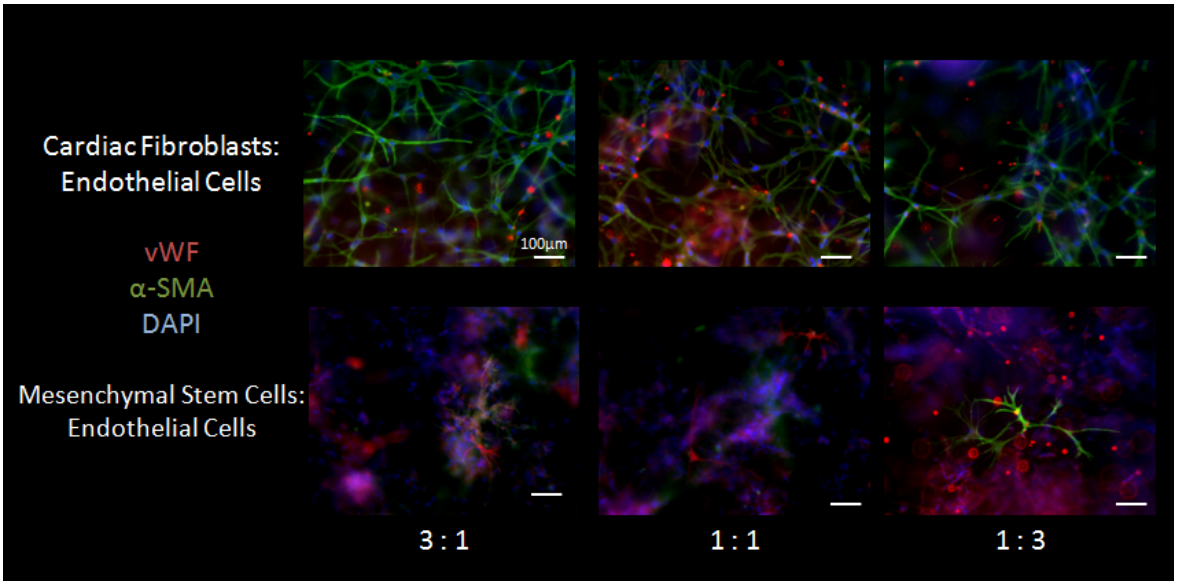

Figure 21: Representative fluorescent images of ECs in varied concentration co-culture with nCFs and MSCs

The total number of sprouts was greatest for all concentrations of nCFs to ECs, as compared to the conditions where the MSCs served as support cells (Figure 22). The results from the different support cell types indicate that there is a significant difference between the support cell type and the ratio of support cells to endothelial cells ($P<0.001$). In the 1:1 condition of Support Cells: ECs, the nCFs allowed for a significantly greater number of sprouts to grow versus the MSCs ($P=0.007$), while in the 3:1 condition the significance of the number of sprouts was even greater between the nCFs and the MSCs ($P=0.005$). There was no significant difference at the 1:3 ratio between cell types.

Although none of the support cell conditions demonstrated a significant difference in length, the nCF support cell conditions illustrated a trend of longer sprouts (Figure 23). In the EC-only culture, the ECs demonstrated the longest sprouts when cultured in 50%MSC 50%EC medium. When the effect of ratio is not considered via ANOVA there is a significant difference in sprout length depending on the Support cell type (P=0.023).

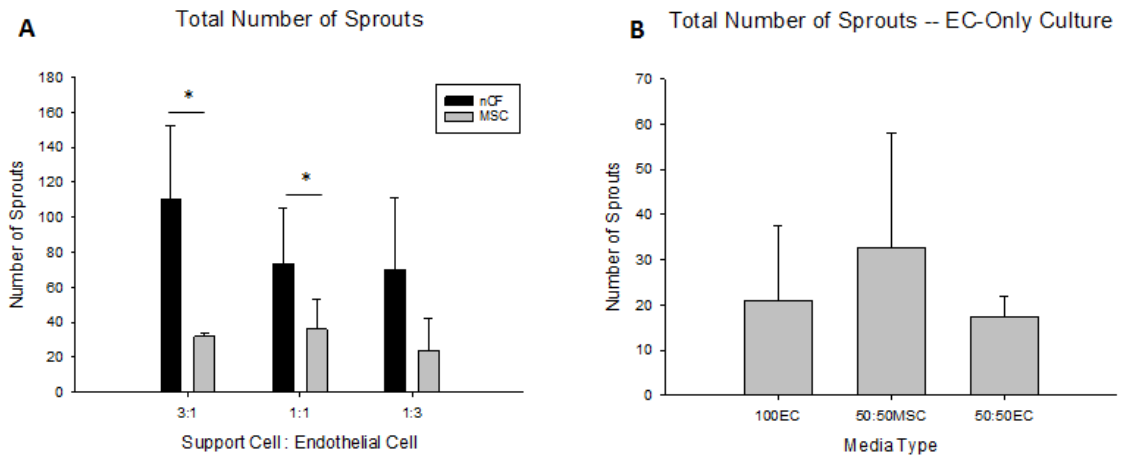

Figure 22: Average number of sprouts per construct. A) Total number of sprouts in the varied support cell co-culture conditions. In the 1:1 Support Cell: EC condition, nCFs as a support cell allow for a significantly greater number of sprouts compared to MSCs (P <0.007). In the 3:1 condition, nCFs allow for a significantly greater number of sprouts compared to MSCs (P<0.005). B) Total number of sprouts in EC only culture.

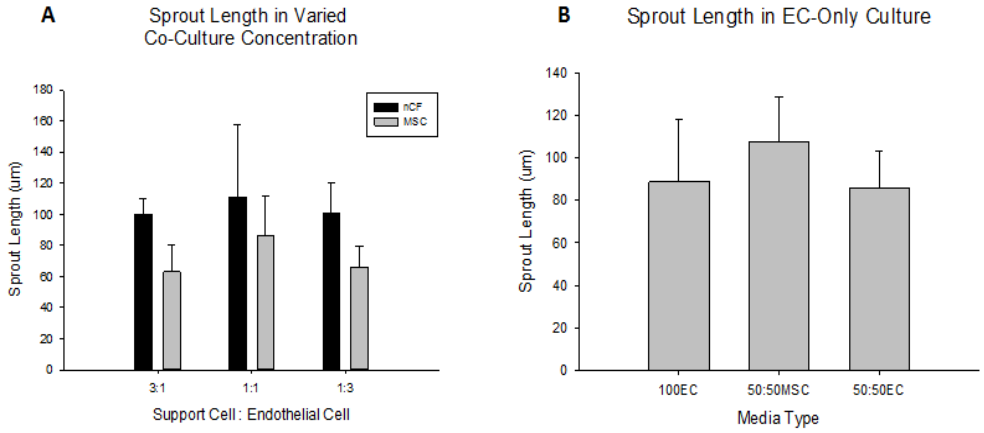

Figure 23: Average Sprout Length in Varied Concentration Co-Culture. A) ECs in co-culture with a varied concentration of support cells B) Average sprout length in EC-only culture

Aside from considering the total sprout number and average length of each sprout, we also recorded the average number of nuclei per sprout. In Figure 24, the average number of nuclei per sprout is displayed for both support cell co-culture (Fig 24A) and EC-only culture (Fig 24B). The ECs are able to form the most multi-cellular sprouts when cultured with MSCs. The number of multi-cellular sprouts is significantly greater in the 1:1 MSC:EC and 1:3 MSC:EC conditions as compared to the nCF-EC co-culture of those ratios ($P<0.001$ and $P=0.019$, respectively). There is no difference in the EC only condition.

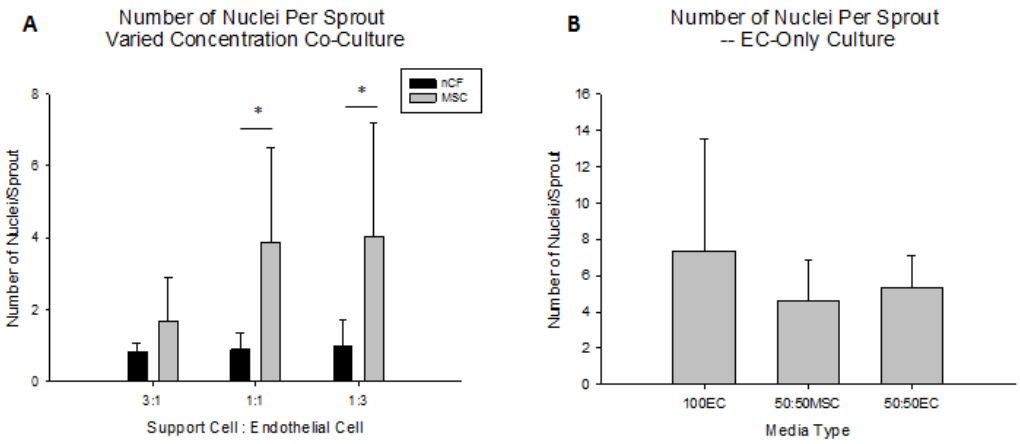

Figure 24: The average number of nuclei per sprout when the ratio of support cells to endothelial cells is varied. A) The number of nuclei per sprout in varied support cell ratio co-culture studies. B) The number of nuclei per sprout in endothelial cell-only control studies.

3.4 Discussion

In order to develop a thick three-dimensional engineered tissue construct, a vascularization system must be fabricated. This system will aid in the transport of oxygen and nutrient delivery within the tissues, quickly after implantation. Several groups have demonstrated that the pre-vascularization of tissue constructs by EC vessel network formation prior to implantation, aids in more rapid anastomosis of the construct to the host [56, 59].

Although many studies have established the necessity of support cells in the formation of EC sprouts, they have failed to utilize a cell source native to the myocardium [11, 56, 10]. In this study we compared the *in vitro* culture of EC-only, and ECs cultured with nCFs and MSCs as support cells to study the potential for nCFs to serve as a support for EC sprout formation in 3D tissue constructs. The primary hypothesis of these studies was the neonatal CFs provide an as good support for EC sprout formation as MSCs, which are the "gold standard" of support cells for EC maturation *in vitro* [27].

3.4.1 Total Cell Count and EC Proliferation

The first set of experiments conducted in this chapter focused on determining the optimal medium concentration to support the growth of both the endothelial cells and support cells. The results of these experiments (Figure 7) indicated that endothelial cells were able to grow in all cell conditions. However, the highest number of endothelial cells was demonstrated in the 50%EC-

50%MSC media condition. A reason for this could be that EC media contains 2% fetal bovine serum, while MSC medium contains 20% FBS. When the mediums are mixed, the ECs are receiving 11% serum, a much greater amount than in their normal growth medium. Other studies have been done to show that when ECs are cultured in higher serum media, they proliferate at a more rapid rate [97], likely explaining our results.

3.4.2 Flow Cytometry

After conducting flow cytometry studies of the ECs in the varied support cell ratio experiments, it became evident that ECs proliferated best when in culture with the neonatal CFs and maintained a higher percentage of ECs in culture. When cultured with the MSCs as a support cell there was a significant decrease in the percent of ECs remaining in the constructs after ten days. A reason for this could be that the MSCs are inhibiting EC proliferation, while the nCFs foster an environment for better EC proliferation.

The lack of a specific marker for fibroblasts (and CFs in particular) has long been a limitation in studying CFs *in vivo*. A common label for CFs is an anti-vimentin antibody, which reacts with the intermediate filaments of the fibroblasts [100]. Initially Vimentin was selected as markers for CFs during flow cytometric analysis; however, Vimentin can also mark vascular endothelial cells [99]. Vimentin can still be used as an immunofluorescent stain for CFs *in vitro* due to the differences in morphology of CFs and ECs *in vitro*. A more specific marker

for CFs is the collagen receptor, Discoidin Domain Receptor 2 (DDR2), which we also studied as a CF marker; however, it also bound to the endothelial cells, and did not completely mark all of the Cardiac Fibroblasts. For this reason, we were not able to select a good marker for the CF population. Because the CFs did not stain for CD31, we carried out the rest of the flow cytometry on the constructs with CFs using only CD31 staining.

3.4.3 EC Proliferation

In the first set of media experiments, the proliferation and growth of endothelial cells was studied through bright field images of the cells in EC-only constructs and flow cytometry analysis on co-culture constructs. The effects of the media on the cells growth was tested and it was determined that both cell types grew best in the 50%EC-50%Support Cell condition. Flow cytometry of the constructs was not conducted until after the 50%EC-50%MSC and the 50%EC-50%Cf media was selected, however later studies indicated that the EC-only constructs still maintained a strong stain for CD31 in both 50%EC-50%Support cell medias compared to the 100%EC media, indicating the cells were not changing morphology in the other mediums.

The presence of VEGF is important for the formation of new vessels during embryogenesis and vasculogenesis. VEGF helps mediate the induction of EC proliferation and differentiation in vascular structures. Increased levels of VEGF also aid in the formation and elongation of tip cells, which is important in

the angiogenic process [101]. When endothelial cells are confluent, or cultured in a more confluent setting, there is an increase in the cell-cell contact of the ECs to other ECs or other cells. This increased contact creates a barrier between the VEGF receptors on the ECs and the VEGF [101]. These confluent cell layers then respond poorly to the proliferative signals of VEGF. In order to create stable vessels, Dll4-Notch signaling represses the VEGFR signaling and the stalk cells no longer form tip cells [15]. The suppression of VEGFR decreases proliferation of ECs, but allows the ECs to form more stalk cells, which in turn leads to the creation of a more mature vessel. Although the total cell count was higher when ECs were cultured with MSCs as compared to the nCF conditions, the EC proliferation was much lower in the 3:1 and 1:1 support cell ratios. A reason for this could be that the ECs have a decreased response to VEGF as a result of a suppression of the VEGFR, thus causing a decrease in EC proliferation. Another possible reason that the ECs proliferated less in culture with the MSCs could be that the MSCs inhibit contact EC-EC contact because the MSC population is greater in those cultures (there are more MSC cells than EC cells) or that the ECs cannot form strong cellular connections with the MSCs due to the strong MSC-MSC contact. Furthermore, there could be other paracrine signaling effects that cause a decrease in the proliferation of the ECs.

3.4.4 Sprout Analysis

Although there is a trend that the nCFs aid in longer EC sprout formation, the MSCs allow for more multicellular sprouts, which is more indicative of the *in vivo* process (102). A reason for this could be the cell-cell contacts that MSCs form with ECs that might be lacking between the EC-nCFs or other paracrine signaling effects between the ECs and the MSCs. Another factor could be the higher serum concentration of MSC media (11% for EC-MSC media vs 8.5% FBS in EC-CF media), which would lead to increased proliferation of ECs when cultured alone and thus lead to more multicellular sprouts. However, one would expect that if the ECs were forming more sprouts and the sprouts were longer with the nCFs, then these sprouts would have more cells and thus be more multi-cellular. Moreover, the EC proliferation was greater in the 3:1 and 1:1 nCF condition than in the MSC condition for the varied co-culture experiments. An important question to investigate further is: why are there more endothelial cells in the 3:1 and 1:1 nCF co-cultured conditions and the sprouts are more abundant, but there are less multi-cellular sprouts?

The use of fibrin as a scaffold should be taken into consideration when discussing EC sprout formation. Fibrin, which can be harvested from the plasma of a patient and used as an autologous scaffold source has many intrinsic angiogenic properties (107). Although the myocardial wall is not made up entirely of fibrin, the fibrin scaffold can be used as a temporary scaffold. The CFs will degrade the fibrin over time, as they produce ECM. A fibrinolytic inhibitor ε-aminocaproic acid (ACA) was added to the culture medium to control the rate of

degradation of the fibrin. The intent was not to have the ACA completely stop degradation but to time it so that the CFs would break down the fibrin at an appropriate rate to match the ECM production, which would then form a large portion of the scaffold for the myocardial constructs. Furthermore, it is possible that the CFs degrade the fibrin at a faster rate than the MSCs, which could be releasing more angiogenic factors through the constructs, thus increasing EC sprout formation.

Chapter 4: Cardiac Fibroblast Life Point Study

4.1 Introduction

The cells of the developing embryo proliferate and differentiate more rapidly than postnatal cells. Studies have been done comparing embryonic cardiac fibroblasts to adult CFs to explain that fetal CFs support cardiomyocyte proliferation better than adult CFs (103). Fetal cardiac fibroblasts secrete more growth factors than adult CFs, including herparin-binding epidermal growth factor (HB-EGF) which has been shown to induce proliferation of cardiomyocytes (103). Conversely, adult CFs induced more hypertrophy and less proliferation of the cardiomyocytes, which is more consistent with the postnatal shift from rapid proliferation to sustained cell growth (104). While these studies point to important differences in CF interactions with cardiomyocytes, to date no one has studied the effects of developmental age on CF interaction with ECs.

Our hypothesis was that cardiac fibroblasts from different life stages will have varying effects on endothelial cell sprout formation. During early embryonic development, the body is undergoing more rapid vascularization through the process of vasculogenesis and angiogenesis. We hypothesize that fetal CFs secrete more growth factors that lead to more rapid proliferation of endothelial cells and could thus result in more abundant EC sprouts. Conversely, we hypothesize that adult CFs, which represents an older developmental life point, will lead to less EC proliferation and thus result in less abundant EC sprouts (but may be more supportive of multicellular sprout formation because of the quiescent nature of the vasculature in older age). To test this hypothesis,

endothelial cells were co-cultured with fetal, neonatal and adult cardiac fibroblasts and mesenchymal stem cells in order to determine whether different developmental life points of cardiac fibroblasts lead to differences in the proliferation of endothelial cells.

Our results indicate that ECs are able to proliferate best when cultured with neonatal cardiac fibroblasts as compared with the proliferation of ECs in culture with fCFs and aCFs. Furthermore, after completing conditioned media studies, it is apparent that ECs proliferate more when cultured in with 50%EC-50%MSC media and in the nCF conditioned media group, indicating that paracrine signaling is a likely cause for the observed alterations in EC proliferation. Flow cytometry illustrated that endothelial cells in co-culture with either an equal ratio of neonatal or adult cardiac fibroblasts to endothelial cells, or a greater ratio (75% EC-25% nCF or aCF) were able to proliferate better than when in culture with the mesenchymal stem cells and fetal CFs. In summary, there appears to be a difference in the angiogenic factors that cardiac fibroblasts secrete at different developmental life points.

4.2. Materials and Methods

4.2.1 Cell Culture

As described in Chapter 3 (3.2.1), ECs were purchased from Cell Applications and used in these studies from Passage 4-7. Rat Mesenchymal Stem Cells were purchased from Texas A&M University. Neonatal Cardiac Fibroblasts were isolated from the ventricles of neonatal day 2-3 Sprague Dawley rats following the same pre-plate method as previously described. Fetal cardiac fibroblasts were isolated from embryonic day 17 Sprague Dawley fetal rats using the same procedure and were used in experiments from P4-P10. Adult cardiac fibroblasts (aCF) were isolated by removing the heart from an adult female Sprague Dawley rat, removing the atrium and mincing the ventricles into 1-2mm pieces. The pieces of myocardium were plated in a petri-dish with 10mL of media for 3 days to allow the CFs to migrate out of the tissue explants. After 3 days, the media was carefully aspirated off the petri-dish and replaced with fresh fibroblast media. The tissue pieces were left in the petri dish for one week, until the adult CFs were confluent. At which point, the tissue pieces were discarded and the cells were passaged via 0.25% trypsin-EDTA (1x) treatment for use in experiments. Adult cardiac fibroblasts were used in experiments from Passage 3-6.

4.2.2 Characterization of Cell Lines

As with the cells in Chapter 3, all cell lines in this study were characterized through flow cytometry, in 2-D culture prior to implantation in the

constructs. The cells were labeled with FITC-conjugated mouse monoclonal anti-rat CD31 (ab33858; Abcam, Cambridge, MA) diluted to 1:10 (Endothelial Cells), PerCP-conjugated anti-rat vimentin (Vimentin (V9), Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:50 (Cardiac Fibroblasts) and APC-conjugated CD90 anti-rat (BioLegends) at 0.06ug/100uL (MSCs). Antibody labeling was carried out for 30 minutes, incubated at 4° C, followed by two washes in autoMacs® Running Buffer (Miltenyi Biotech, Auburn, CA). The cells were characterized using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) at the Tufts Medical Center Flow Core facility using CellQuest Software (Becton Dickinson, Franklin, NJ). All FACS data was analyzed using the FlowJo software (FlowJo, Ashland, OR).

4.2.3 Creation of Fibrin Co-Cultured Hydrogels

Fibrin gel hemispheres (550µl) were fabricated in 12-well tissue culture plates, in order to study the interactions between the endothelial cells and the support cells in 3-D. For the purpose of this study, the fibrin hydrogels were fabricated in the same way as in Chapter 3. The hemispheres were cultured in 12-well plates with 2 mL of medium (replaced three times per week) in a cell culture incubator. Experiments were run in quadruplicates (n = 4). Three ratios of endothelial cells to support cells were considered for the cardiac fibroblasts (fCF, nCF and aCF) and mesenchymal stem cells as support cells: 3:1 Support Cells: Endothelial Cells, 1:1 Support Cells: Endothelial Cells and 1:3 Support Cells:

Endothelial Cells in 1:1 endothelial cell: support cell media. Endothelial cells were seeded in fibrin hydrogels as a control with 100% endothelial cell media and 1:1 Endothelial Cells: Support Cell media. The number of endothelial cells was held constant at $2x10^5$ cells/mL fibrin solution for all of the conditions.

4.2.4 Two Dimensional Fluorescent Image Acquisition and Analysis

Prior to encapsulation in the fibrin hydrogels, endothelial cells were membrane-labeled with PKH 26 (Sigma Aldrich), a red fluorescent cell membrane stain or CellTracker Green (Invitrogen), used as a membrane label to track endothelial cell sprout formation and growth. The fibrin hydrogel constructs were cultured for 10 days, after which high-resolution images of the hemispheres from each condition were captured on an Olympus IX81 microscope at 10x and 20x magnifications. The endothelial cells were also stained with Von Wilibrand's Factor (vWF) or PECAM-1 (CD31) prior to fluorescent imaging to verify co-localization of vWF and PKH26/CellTracker Green. Multiple images (n=3-5) were acquired from random locations for each condition. The length of each sprout was measured in the XY projections from segments of each endothelial cell sprout (defined as branch point to branch point) using ImageJ (NIH, Bethesda, MD). The majority of the sprouts lacked a significant curvature, thus a straight line was drawn from one branch point to another branch point. The average sprout length for each construct condition was determined and plotted against the total number of sprouts for each condition type. In order to determine whether the

endothelial cells sprouts were multicellular, the number of endothelial cell nuclei in each sprout was also counted.

4.2.5 Immunohistochemical and Immunofluorescent Staining

In order to look at endothelial sprout formation and the role of the support cells in this process, constructs underwent immunohistological analysis. For fluorescent imaging, constructs were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) at 4^{o}C for 3 h, rinsed with PBS three times for 5 min each and then permeabilized with 0.1% Triton-X solution for 10 minutes. After permeablization, constructs were washed with 1x PBS, blocked with 5% Donkey Serum (Sigma Aldrich) for one hour and stained with α-smooth muscle actin (1:500 α-SMA; ab18147 Abcam, Cambridge, MA), for nCFs and MSCs or von Wilibrand's Factor (1:200 vWF; ab6994 Abcam, Cambridge, MA) as an EC marker. Alexa Fluor 555 donkey anti-rabbit and Dylight 488 donkey anti-mouse were used as secondary antibodies for vWF and α–SMA, respectively (1:500, Jackson ImmunoResearch, West Grove, PA). When entrapped in the fibrin hydrogel the rCFs take on a myofibroblast morphology, verified by the presence of α–SMA [75]. One to two constructs per condition and experiment were processed for histological analysis. The samples were analyzed with an Olympus IX81 inverted fluorescent microscope or by confocal microscopy on a Leica SP2 confocal microscope equipped with an Argon laser (Wetzlar, Germany). The resulting images (n=2-4 per sample) were analyzed for endothelial cell sprout formation.

4.2.6 Characterization of Cell Number

Final cell count of all constructs was quantified using a DNA assay as previously described [83]. After 10 days in culture, two constructs per condition for each experiment were sterilely set aside. The numbers of cells per construct were quantified using a modified Hoechst assay [36]. Samples were digested and analyzed as explained above, in Chapter 3.

To analyze the proliferation of endothelial cells in the fibrin constructs after the 10 day culture period, the constructs underwent the same modified type-II collagenase digestion as explained above. Subsequently, the flow cytometry procedure was carried out as stated above in section 3.2.2 and 3.2.7.

4.2.7 Conditioned Media Study

To assess growth factor release and paracrine signaling from the support cells, a conditioned media study was carried out. Rat cardiac fibroblasts were cultured in fibroblast media (DMEM, 15% FBS and 1% penicillin-streptomyocin) and MSCs were cultured in MSC media (α-MEM, 20%FBS, 1% penicillin-streptomyocin) for 24-48 hours. Five milliliters of conditioned media was collected from a T75 flask, into a 15mL conical and allowed to settle for 5 minutes at room temperature. The purpose of allowing the media to settle is that any dead cells mixed with the media could potentially be removed. The conditioned media contained 30% Support Cell conditioned media, 20% fresh support cell medium and 50% Endothelial Cell media (EBM-2 + BulletPack), as

illustrated in Table 10 below. The experiment was conducted in a 24 well plate with 2.5 x 10^3 ECs per well. EC media was used as a control for n=6 wells. As a control for each support cell media type, a 1:1 ratio of Support Cell media: EC media was placed in wells for each support cell type (n=3) while conditioned media for each support cell type was added to 3 wells, each. Each well of the 24 well plate received 1mL of media.

Media Type	EC : Support Cell Media	EC:CF Conditioned Media	EC:CF Conditioned Media
EC Media	2.5 mL EC media	2.5 mL EC media	2.5 mL EC media
Support Cell Media	2.5 mL either CF or MSC media	1 ml CF media	1 ml MSC media
Conditioned Media		1.5 mL aCF, nCF or fCF conditioned media	1.5 mL MSC conditioned media

Table 10: Conditioned media experimental set-up

4.2.8 Statistical Analysis

All results are expressed as the mean ± the standard deviation. Statistical analysis of all data was conducted in SigmaPlot (Systat Software, Inc, San Jose, CA). When comparing more than two groups, a one-way or two-way analysis of variance (ANOVA) was used, followed by a post hoc Tukeys test. The Tukeys t-test was used to compare the means between two groups. Significant levels were set at $p < 0.05$. At least three images of each condition were analyzed and multiple images were acquired from each construct per condition. At least two samples per group for cell count from the DNA assay were tested.

4.3 Results

4.3.1 Total Cell Count and Number of Doublings in Culture

The effect of the different concentrations of support cells to endothelial cells, as well as the different cell types on the proliferation of the endothelial cells was examined, as indicated in Table 11 below. In order to examine the growth and proliferation of both the support cells and endothelial cells in co-culture we evaluated the cell growth rate of ECs, nCFs, fCFs, aCFs and MSCs in 3 different ratios, in co-culture for ten days. The initial cell count for each condition is listed in Table12 below. The ECs were also cultured on their own in three different types of media: 100% EC media, 50% CF-50% EC media and 50% MSC-50%EC medium.

Support Cell	75% Support Cell 25% EC	50% Support Cell 50% EC	25% Support Cell 75% EC
Fetal CF	X	X	X
Neonatal CF	X	X	X
Adult CF	X	X	X
MSC	X	X	X

Table 11: Experimental design for developmental life point study

Ratio of Support Cell: Endothelial Cell	Initial Support Cell Number	Initial Endothelial Cell Number	Total Cell Number
3:1	330,000	110,000	440,000
1:1	110,000	110,000	220,000
1:3	36,630	110,000	146,630

Table 12: Initial number of cells seeded in constructs for each condition at Day 0.

After 10 days in co-culture the total co-culture population was the greatest when the ECs were cultured with the fetal CFs (Figure 25). When analyzing the total cell number in co-culture by ANOVA, it is apparent that the mean values for all ratios in the fCF condition are significantly greater than those of the nCF and aCF conditions (P=0.021 and P=0.043, respectively). Because the initial cell number in each condition is different, these results are not all that surprising. However, when considering the interactions between the support cell types within each group, it was confirmed that the total cell number was significantly greater in the 3:1 Support Cell: EC condition when the ECs were cultured with fCFs versus the nCFs and MSCs (P=0.044, P=0.027).

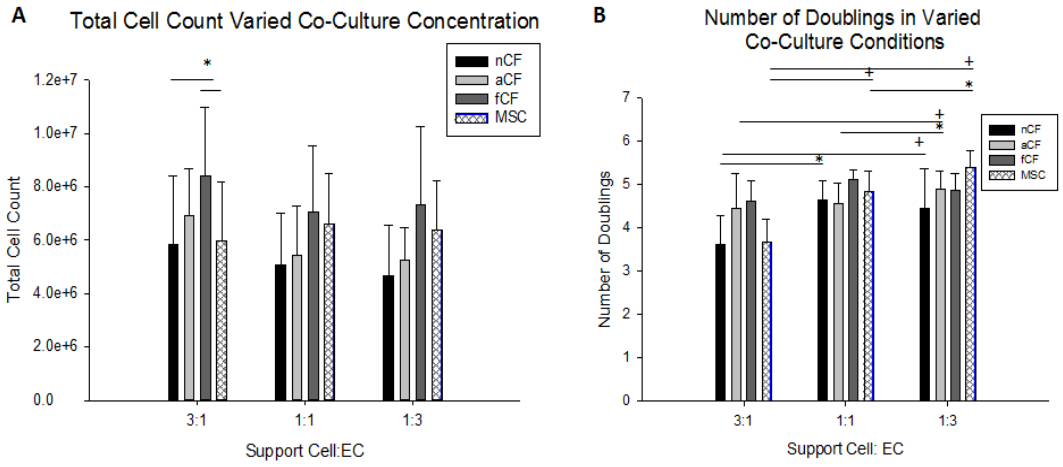

Figure 25: DNA Analysis of all cells in culture at Day 10. A) Total cell count in varied co-culture at Day 10 (P<0.05) B) Number of doublings in varied co-culture conditions (*P<0.05, $^{+}$P<0.001).

While these results are interesting, because of the differences in initial cell number between the ratio conditions, we also considered the number of doublings for each condition. The total number of doublings of each condition was then

directly compared within the different ratios of support cells to ECs (Figure 25B). The difference in the mean values of the number of doublings among the different levels of support cell concentration is greater than would be expected by chance after allowing for effects of differences in cell type. There is a statistically significant difference ($P = <0.001$) between the three different support cell concentrations. Furthermore, the difference in the mean values of the number of doublings among the different support cell conditions is greater than would be expected by chance after allowing for effects of differences in support cell concentration in co-culture. There is a statistically significant difference ($P = 0.020$) between the different support cell types. The number of doublings in the nCF support cell condition is greatest in the 1:1 condition, with a statistically significant difference between the 1:1 and 3:1 group ($P=0.014$) and the 1:3 and 3:1 group ($P<0.001$). The MSC condition also demonstrated a significantly greater number of doublings in the 1:3 condition as well as the 1:1 versus 3:1 condition. In the aCF culture, both the 1:3 and 1:1 condition had a significantly larger number of doublings as compared to the 3:1 ratio ($P<0.001$ and $P=0.045$). The fCF conditions did not have any significant difference between the number of doublings within or between each group.

4.3.2 Flow Cytometry of Varied Co-Culture Constructs

In order to find the final percent of endothelial cells and support cells in each construct, a few constructs from each condition were digested for flow

cytometry. Figures 26, 27 and 28 below illustrate the percent of ECs in each construct when co-cultured with CFs at different developmental life points and MSCs as a support cell control for the three different co-culture ratios: 3:1, 1:1 and 1:3. Each set of images illustrates the dot plots and histograms for the constructs staining for CD31. The red in the dot plot demonstrates a higher cell population in that area.

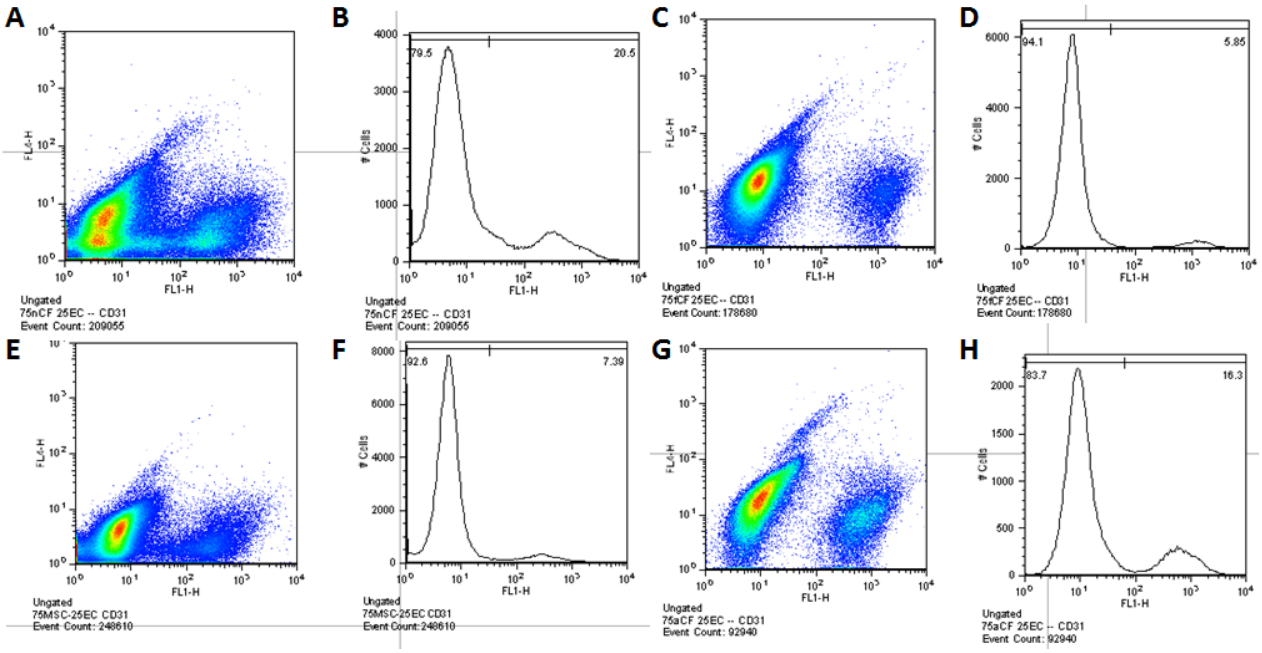

Figure 26: Representative images of the 3:1 ratio of Support Cells to Endothelial Cells. A-B) nCF: EC, C-D) fCF: EC, E-F) MSC: EC, G-H) aCF: EC

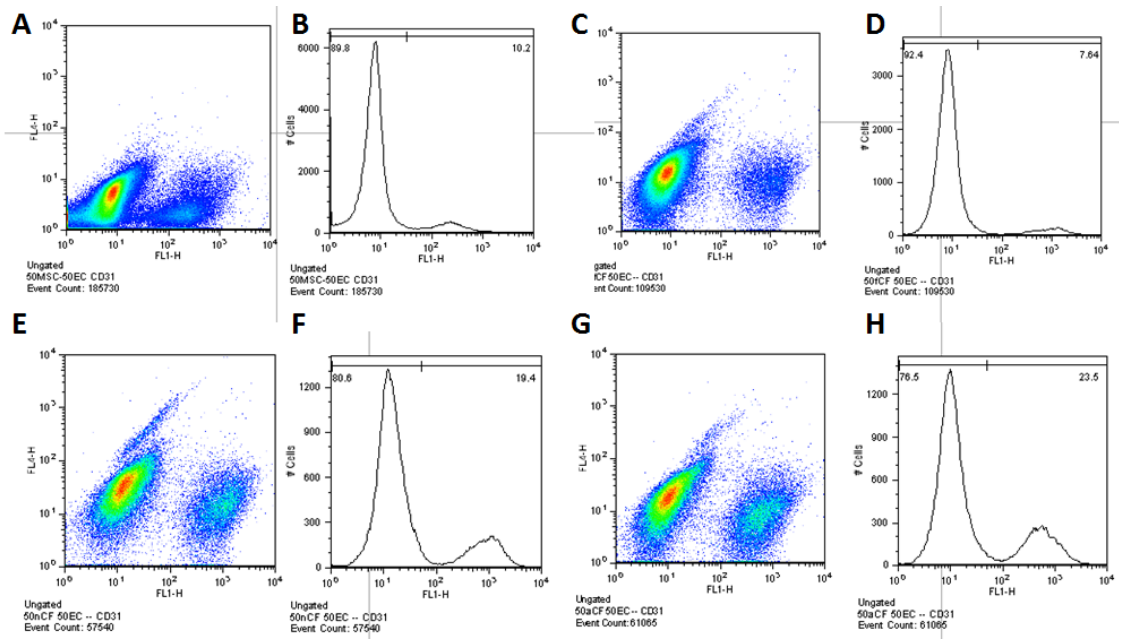

Figure 27: Representative images of the 1:1 ratio of Support Cells to Endothelial Cells. A-B) nCF: EC, C-D) fCF: EC, E-F) MSC: EC, G-H) aCF: EC

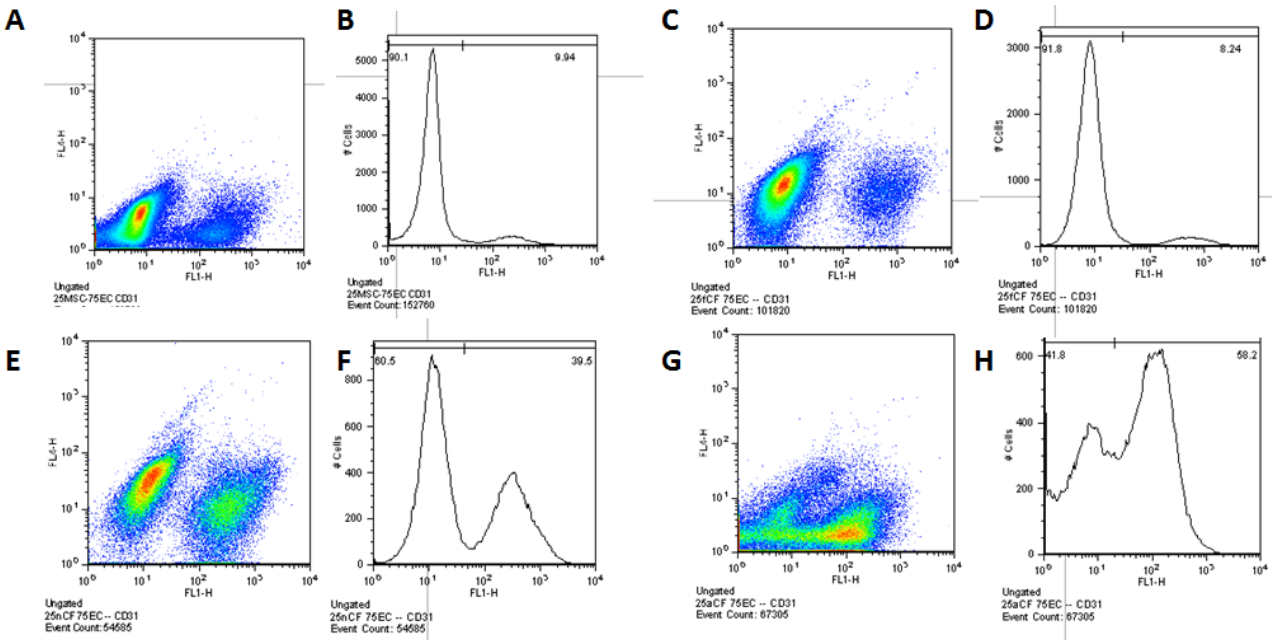

Figure 28: Representative images of the 1:3 ratio of Support Cells to Endothelial Cells. A-B) nCF: EC, C-D) fCF: EC, E-F) MSC: EC, G-H) aCF: EC

4.3.3 EC Specific Proliferation in Culture

While the total cell growth in co-culture is important, the purpose of these experiments is to ascertain which support cell type is optimal for EC growth and proliferation, which will eventually lead to EC sprout formation. The proliferation of ECs was calculated after running flow cytometry on the digested constructs (Figure 29). The results indicate that ECs proliferate best when cultured with neonatal CFs in the 3:1 and 1:1 conditions and have the lowest proliferation when co-cultured with the fetal CFs. When comparing the average EC proliferation values for all cell types, ECs proliferate best in the nCF conditioned when compared to the fCF ($P<0.001$) and MSC ($P=0.014$) conditions. Moreover, ECs experienced a significantly higher proliferation in the adult CF and MSC culture when compared to the fetal CF culture ($P<0.001$ and $P=0.002$ respectively). Within the 1:1 co-culture condition, neonatal CFs allowed for better EC proliferation compared to fCFs ($P=0.008$) and MSCs ($P=0.001$). In the 1:3 condition, MSCs, aCFs and nCFs all allowed for better EC proliferation compared to fCFs ($P<0.001$ for all support cell conditions). Within each support cell type, other than the fCF condition, there was a significant difference between the concentration of support cells to ECs and the support cell type. Within the aCF condition, there was significantly greater EC proliferation in the 1:3 condition versus the 3:1 condition ($P=0.002$). The MSC support cell condition allowed for greater EC number in the 1:3 versus 3:1 and 1:1 conditions ($P<0.001$). For the nCF condition there was a significantly greater difference between EC

proliferation in the 1:3 and 1:1 conditions when compared to the 3:1 condition (P=0.002 and p=0.034).

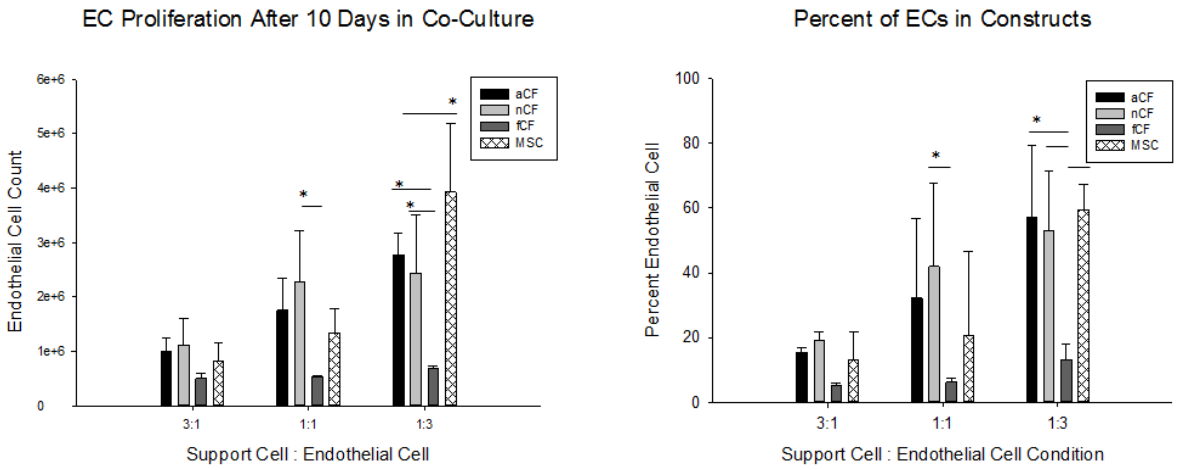

Figure 29: EC proliferation in co-culture with aCF, nCF, fCF and MSCs as support cells. A) EC proliferation after 10 days in culture (*P<0.02), B) Percent of ECs in constructs after 10 days

We also looked at the ECs in each condition in terms of the percentage of the total cell number that were ECs after the 10days in culture. Note that for 1:3 the number of ECs in culture start at 25%, for 1:1 EC initial concentration starts at 50% and 3:1 start at 75%. When comparing the proliferation of ECs in co-culture, it is also interesting to compare the initial percent of ECs in culture, with the final percent of ECs in culture, compared to total cell number. Figure 29B illustrates the percent of ECs present in each condition at Day 10 after flow cytometry was conducted on the constructs. Table 13 below illustrates the final percent and percent change of ECs in each condition in the constructs after 10 days.

Support Cell Type	3:1		1:1		1:3	
	Final Percent	Percent Change	Final Percent	Percent Change	Final Percent	Percent Change
fCF	5.12%	79.50%	6.19%	87.60%	13%	82.33%
nCF	18.97%	24%	41.97%	20.80%	52.92%	29.44%
aCF	15.33%	38.67	32.13%	53.85%	57.18%	29.97%
MSC	13.17%	52.12%	20.61%	60.66%	59.53%	20.62%

Table 13: Final Percent and Percent Change of EC in Varied Concentration Co-Culture

4.3.4 Endothelial Cell Conditioned Media Study

To study whether the support cells released angiogenic paracrine signaling factors that influenced EC proliferation and growth, a 2D conditioned media study was carried out (Figure 30). DNA analysis of the cells after 7 days demonstrated that the ECs had the highest cell count when cultured with an equal ratio of EC to MSC medium. For the conditioned medium conditions, there was a trend that ECs proliferated more when in culture with neonatal fibroblast conditioned media and MSC conditioned media.

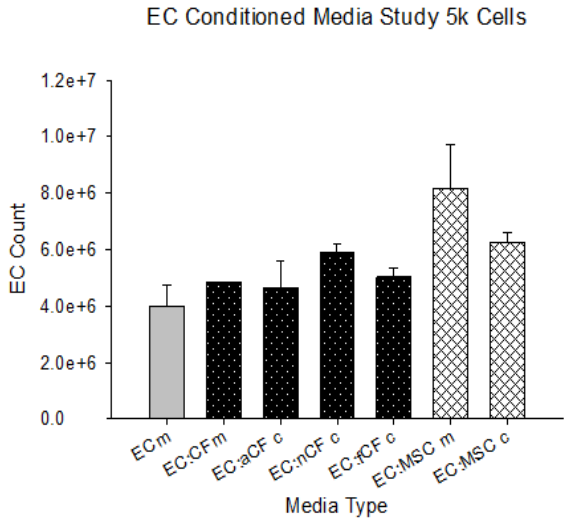

Figure 30: Endothelial cells grown in 2D culture in the presence of unconditioned and conditioned media

4.3.5 Endothelial Cell Sprout Formation

Fluorescent imaging of endothelial cells cultured with both adult and fetal CFs indicate that endothelial cells do form sprouts in both conditions. Figure 31 below shows endothelial cells stained with CD31. Although complete analysis of these images has not been done, initial studies indicate that endothelial cells form sprouts better in the adult CF condition than the fetal CF condition.

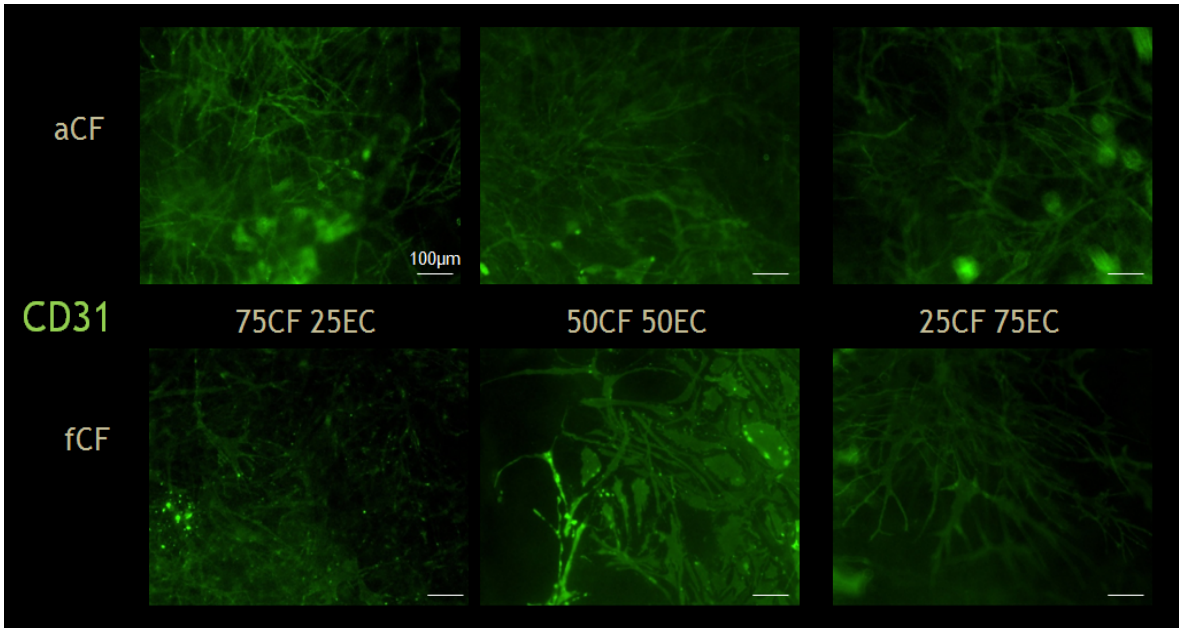

Figure 31: Representative images of ECs cultured with aCFs and fCFs in fibrin constructs. ECs are stained with CD31. All images are at 10X.

4.3.6 Antibody Staining for Cardiac Fibroblasts

In order to visualize possible differences between adult and neonatal cardiac fibroblasts, both cell types were cultured on glass cover slips, fixed and then stained for common cardiac fibroblast and myofibroblast markers: Vimentin, DDR2 and α-SMA. Two different passages (P3 and P8) of adult CFs were stained to compare the difference in antibody expression at different passages of adult CFs. The results are illustrated by the representative images in Figure 32 below.

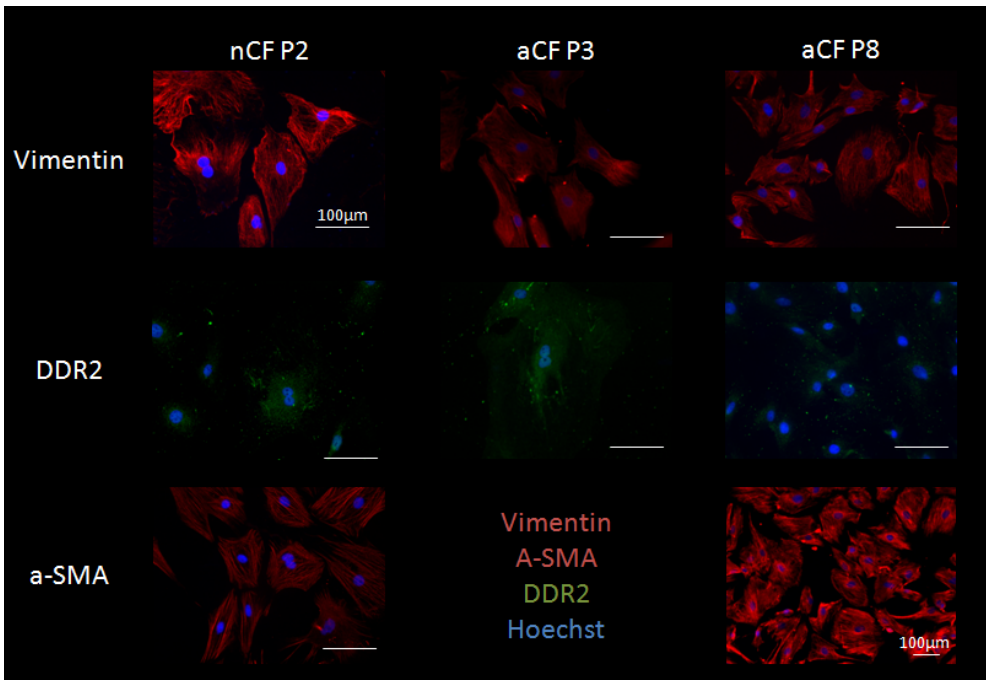

Figure 32: Neonatal and adult CFs stained with common fibroblast and myofibroblast markers, Vimentin, DDR2 and α-SMA. All images are at 20X, except aCF P8 α-SMA is at 10X. Scale bar = 100µm for all images.

4.3.7 Confocal Imaging of Endothelial Cell-Support Cell Construct

Initial confocal imaging demonstrates potential lumen formation in fibrin constructs. Figure 33 at right is a representative confocal image of a 50aCF-50EC construct stained with vWF and Alexa Fluor 488 secondary antibody. The white arrows point to the potential formation of a lumen within the construct.

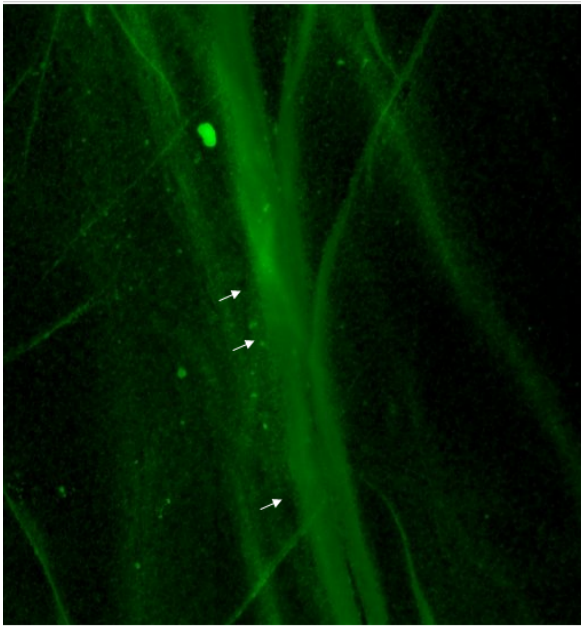

Figure 33: Fibrin construct with a 1:1 ratio co-culture of aCF:EC. ECs are stained with vWF. White arrows indicate potential lumen formation in the construct

4.4 Discussion

During embryonic development, the fetus is undergoing a rapid process of vasculogenesis and angiogenesis, while this process slows down in later life (105). The purpose of this study was to ascertain whether cardiac fibroblasts at different developmental life points were better able to support endothelial cell proliferation. A strong motivation for this study is the fact that cells can be harvested from the biopsies of neonates and adults, allowing for the formation of vascularized scaffolds for patients at different developmental stages. Pediatric cells behave much differently than adult cells do, therefore understanding the ability of cells from different developmental points to support vascularization, is important.

From the results, we found that the total cell population in the constructs was highest in the fetal CF support cell condition. However, when considering the EC proliferation, the fCF condition allowed for the lowest proliferation of ECs. This implies that fCFs were proliferating rapidly in the fibrin constructs. From 2D culture experiments, it was observed that aCFs proliferate at a slower rate than the other cell types, which is indicative of cell growth *in vivo*. Qualitative analysis of the fluorescent images indicates that ECs are able to form the longest and most abundant sprouts when in culture with adult CFs as compared to fetal CFs.

4.4.1 Total Cell Proliferation

As an individual ages, the growth of cells slows down (105). With this in mind, it would be expected that the fetal CFs proliferate at a greater rate than the neonatal and adult CFs. The rate of proliferation of the different CF populations explains one reason why the fCF population has the greatest total cell number at day 10 in culture. The developmental age of the rats that the MSCs were isolated from were adult, which could affect the paracrine signaling factors released from the MSCs (as compared to MSCs isolated at an earlier developmental stage).

4.4.2 Flow Cytometry and EC Specific Proliferation

Flow cytometry data indicates that like the nCFs, the adult and fetal CFs do not mark for CD31 and demonstrate a greater forward and side scatter than the ECs, indicative of their larger and more complex cell size and shape. From this data it has been demonstrated that the ECs proliferate best when in culture with the nCFs and MSCs. Moreover, because the fCFs proliferate at such a rapid rate, they may be taking nutrients away from the ECs and taking up the space in the construct that the ECs would need to spread out and sprout, thus reducing their ability to proliferate.

Furthermore, during early embryonic development, the cells are forming organs and vasculature at a more rapid rate, which would explain why the fCF condition has the greatest total cell proliferation–but since the embryo is also forming more vasculature at this time it would be expected that there would also

be more EC proliferation. Our data does not support this hypothesis. As previously mentioned, the number of support cells or pericytes found on cardiac vasculature is typically 1 support cell to 2-3 ECs [81]. This would explain why the ECs proliferate best in the 1:3 condition as compared to the 3:1 condition. Furthermore, the ECs are able to form more cell-to-cell contacts between each other and with the support cells in the 1:3 culture because less cells are seeded in this culture and there are a higher ratio of endothelial cells to total cells in the culture. The higher cell-cell contact between the ECs would likely allow the ECs to better sense the presence of VEGF and other angiogenic growth factors, which will lead to increased proliferation.

4.4.3 Conditioned Medium

To ascertain whether CFs and MSCs secrete growth factors that induce or inhibit endothelial cell proliferation, ECs were cultured in 2D culture with fresh EC: Support Cell media and the conditioned media of neonatal, fetal and adult CFs and MSCs. Initial results of the EC conditioned media study indicated that EC proliferation is greatest in the unconditioned EC:MSC media, and is also slightly greater in the MSC and nCF condition media. The increase in proliferation in the conditioned media groups could be a result of paracrine signaling between growth factors in the conditioned media and the endothelial cells. Growth factors that can influence EC proliferation include VEGF and an upregulation of the Notch signaling pathway [15, 24].

4.4.4 Sprout Formation Analysis

Early embryonic development is characterized by rapid vascularization and cell growth, beginning with the *de novo* formation of the dorsal aorta, blood islands and endocardium of the heart, accomplished through vasculogenesis (106). Embryonic cells cause the early endothelial cells to aggregate in order to give rise to the endothelium, leading to the direct migration of ECs through the embryo, to segregate into vascular cords. Because fetal and embryonic cells aid in this initial vascularization, it would be expected that the sprout formation in the fCF conditions would be rapid. Although EC sprouts were visible during initial qualitative analysis of the fluorescent images, the sprouts did not appear to be as abundant or as thick and mature as those in the aCF culture. A possible reason for this could be that fetal CFs induce EC proliferation and initial formation of tip cells through the release of greater amounts of VEGF, but the adult cells could signal better EC stalk formation. This could explain why thicker sprouts were visible in the aCF conditions. Further studies that probe for paracrine signaling factors could explain whether this is the case.

Chapter 5: Recellularization of Decellularized Vessels As A Starter Conduit For Angiogenesis

5.1 Introduction

The process of angiogenesis is the formation of new blood vessels from pre-existing vessels. Under this same principle, a vessel can be excised from a patient, decellularized and recellularized with healthy endothelial cells. The aorta and femoral arteries have many branch points, which extend off of it. When the vessel is excised, cleaned and decellularized, these branch points can serve as a starting point for EC sprout formation within a tissue construct. This vessel can then be grown in culture, and once vascularized, it can be re-implanted into the patient. Because the cells, vessel and biomaterial can all be excised from the patient, there is a decreased chance of the patient rejecting the tissue engineered scaffold.

The goal of this study was to use a decellularized vessel as a starter conduit for vascularization of a tissue scaffold. The principal idea of this study was to recellularize the decellularized vessel with membrane ECs and observe the endothelial cells forming vasculature from the branch points of the vessel. While not accomplished in this thesis the data laid out below demonstrate the feasibility of this approach and detail the methods for future work in implementing it in engineered cardiac constructs.

5.2 Materials and Methods

5.2.1 Excision of a Vessel

The aorta, carotid and femoral arteries were harvested from adult female and male Sprague Dawley rats, during the removal of the heart. The animals used for this purpose had previously been euthanized using CO_2, followed by a bilateral thoracotomy. All Institutional Animal Protocols were followed for the euthanization of the animals and the harvest of the heart.

5.2.2 Decellularization Process

After the aorta, carotid and femoral arteries were excised from the animal, any remaining adipose tissue and pericardium was dissected from the aorta. The branching vessels of the aorta were trimmed to tiny micrometer stubs. The vessels were then decellularized using a 1% sodium dodecyl sulfate (SDS) solution, which is an ionic detergent previously shown to decellularized ECM components of mammals [97]. The rat aorta was placed in a 50mL container full of 45 mL 1% SDS in 1X PBS and stirred continuously overnight. The vessels were subsequently rinsed with 1X PBS for 24 hours. The vessels were then stored in diH_2O at 4^oC.

5.2.3 Re-cellularization Process

After decellularization of the vessel was complete, the vessel was sterilized by injecting 70% isopropanol through a syringe into the lumen of the vessel in a bioculture hood. The isopropanol was allowed to dry. Then the vessel was rehydrated with sterile 1X PBS for 30 minutes. After this process was complete, endothelial cells between Passage 4-7 were trypsinized, spun down and suspended in 250uL of endothelial cell media for the recellularization of an aorta or 100uL of media for the recellularization of a carotid artery. Figure 34 below illustrates the set-up of the recellularization system. One needle was closed off with a leur-lok plug and the entire set-up was submerged in a 50mL conical full of 30mL of media. Media was added to the conical to create positive pressure against the walls of the aorta. The cell-media solution was injected into the other end of the needle and into the vessel. The conical with the vessel set-up was loaded into a rotisserie and placed in the incubator at 37^{o}C for 16-24 hours. The following day the vessel was removed from the system, with care taken not to jostle the cells off the vessel walls. A small piece was removed to be imaged to ensure

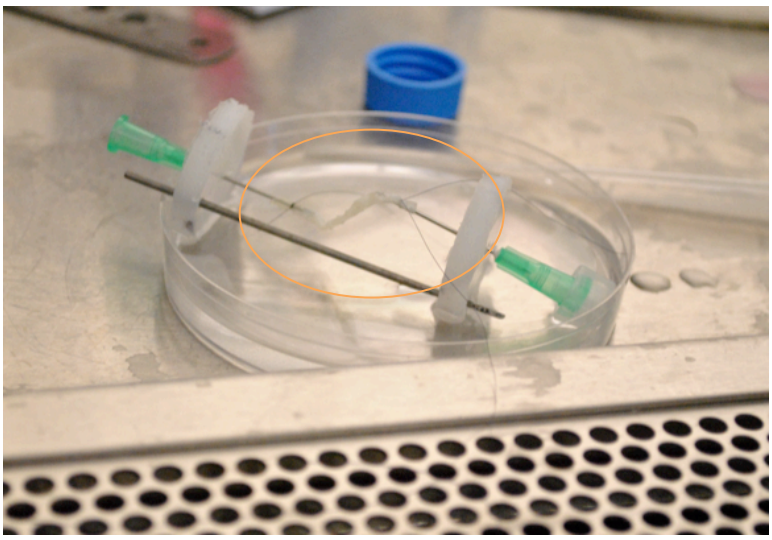

Figure 34: Bioreactor set-up to recellularize central vessel

that endothelial cells stuck to the vessel. The vessel was then cut into 1mm long sections and placed in the fibrin hydrogels.

5.2.4 Creation of a Fibrin Construct with a Central Vessel

Prior to placing the vessel in the fibrin construct, the fibrin solution was made in a volume to match the space the construct would be filling. Different variations of the central vessel study were generated: fibrin hemispheres on a 33mm glass bottom petri-dish with a central vessel suspended inside, 3D polyethylene boxes with a central vessel connected to a 25g needle and fibrin constructs in a 6 well plate with a vessel attached to two 25g needles. Depending on the size of the space being filled with fibrin, the amount of fibrin varied. For studies in which endothelial cells were seeded outside the vessel in the fibrin constructs, ECs were seeded with a support cell at a 1:1 ratio with $2.5x10^6$ total cells/mL fibrin solution. The three different vessel types used for this study—aorta, carotid and femoral arteries—received the same amount of cells by volume, but less cell solution was injected into the smaller vessels.

5.2.5 Cell Culture of Fibrin Constructs

Careful attention was paid while changing the media for the constructs to ensure that the fibrin construct was left intact. The media used for the experiments matched the cell type in culture. For example, the EC-only fibrin constructs were fed with 100%EC media, while the constructs with both EC-nCF or EC-MSC were given 50%EC-50%nCF and 50%EC-50%MSC media, respectively.

5.3 Results

5.3.1 Fluorescent Imaging of the Decellularized Vessel

To demonstrate the inherent auto-fluorescence of the decellularized vessel, an aorta was sliced open and imaged on cover glass. Figure 35 below shows an image of an unstained aorta at 180ms of fluorescence with the GFP and TRITC filter and 40ms for the DAPI filter. Figure 35B is an image of a decellularized heart post-paraffin slicing.

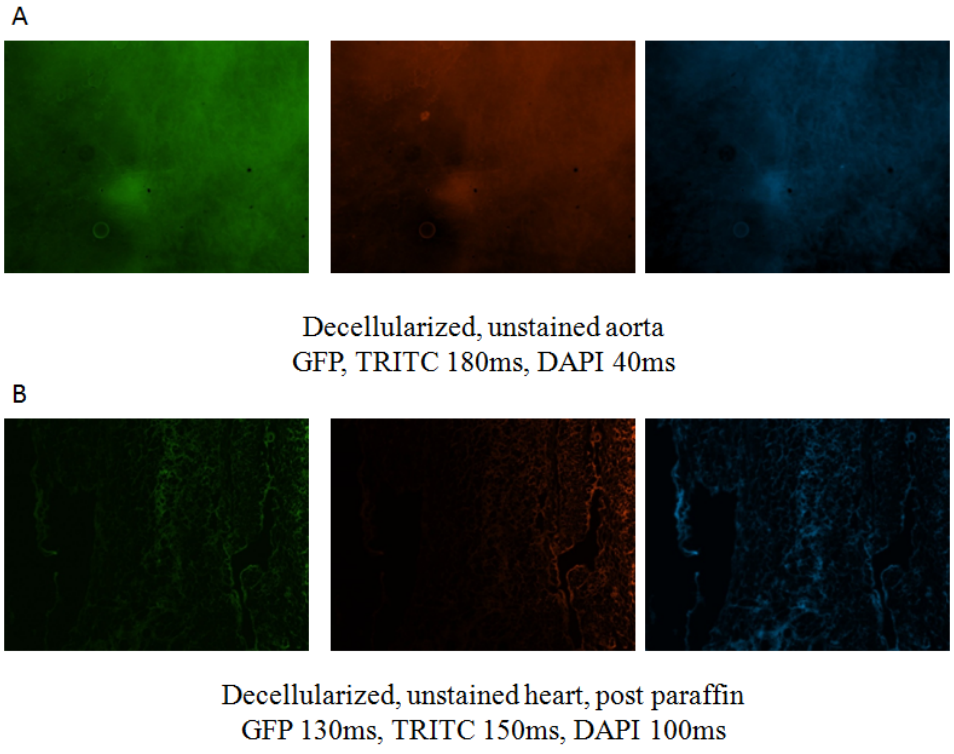

Figure 35: Autofluoresence of decellularized vessel and adult rat heart A) autofluoresence of unstained, decellularized aorta at 4X.

5.3.2 Creation of a Re-cellularization System

The creation of a system to recellularize the vessels took several iterations. The final version is illustrated below in Figure 36. Subsequent to the sterilization

of the decellularized vessel, it is attached to two 25 gauge needles, with sutures, so it does not fall off during rotation, on the recellularization system (Fig 36A). The system is then loaded into a 50mL conical and (Fig. 36B) medium is added to the conical to add positive pressure against the vessel prior to the injection of the cell-media solution into the conical (Fig. 36C). Once the cell-media solution was loaded into the conical it was rotated sideways on a rotisserie over night.

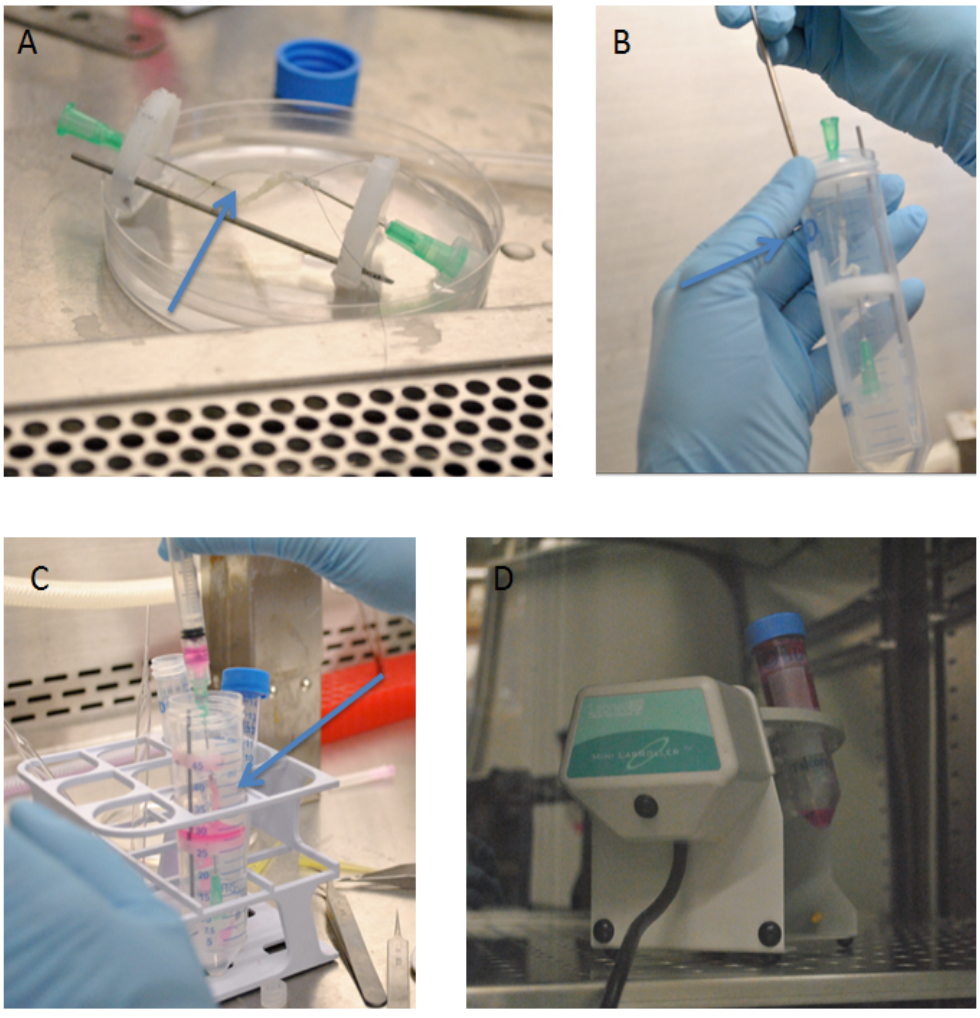

Figure 36: Recellularization system set-up. A) Vessel is attached to two 25g needles with sutures. B) Recellularization bioreactor was loaded into a 50mL conical and C) media was added to the conical prior to the addition of the cell-media solution. D) The bioreactor was then loaded onto the rotisserie so it could be rotated in the incubator overnight.

After the vessel was rotated in the bioreactor overnight, the bioreactor was removed from the incubator and the vessel was extracted from the needles. A small portion of the vessel was cut at the end to be imaged to ensure that the endothelial cells stuck to the vessel. Figure 37 below demonstrates the ability of the recellularization process to seed endothelial cells on the inside surface of the vessel. In Figure 37A it is apparent that the PKH26 labeled ECs, visible as circular cells are adherent to the inner surface of the vessel, while there are no cells present on the outer surface of the vessel. The red fluorescence in Figure 37B is the autofluoresence of the vessel.

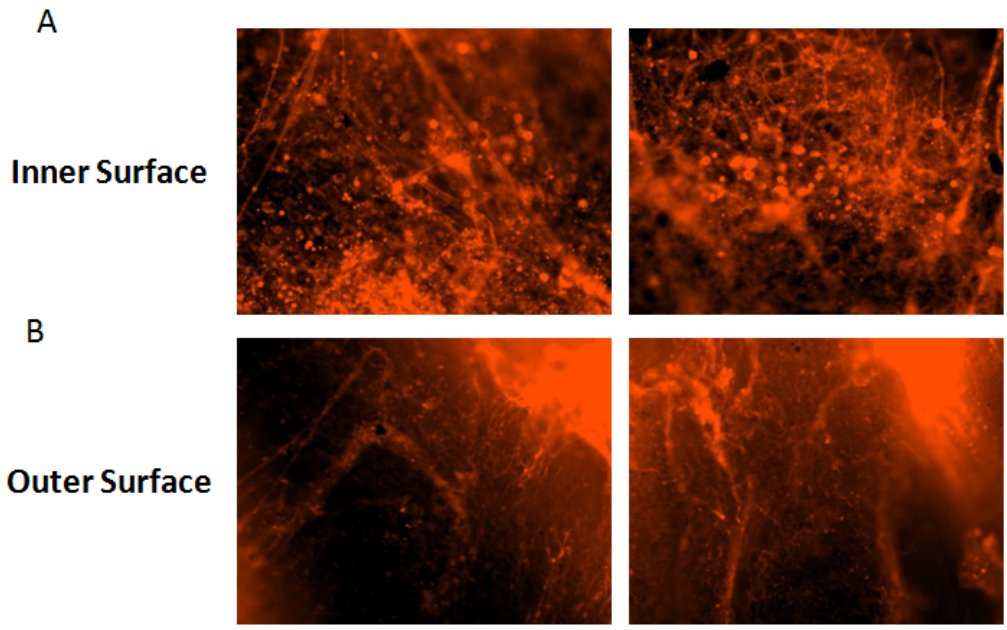

Figure 37: Fluorescent imaging of aorta. ECs are membrane labeled with PKH26 and imaged using the TRITC filter.

5.3.3 Fibrin Constructs in Polyethylene Boxes

To study the growth of ECs from a central vessel in a 3D culture, a polyethylene (PE) box was created and fibrin constructs were injected into them with a vessel attached to two syringes at the ends of the boxes. Figure 38A is a representative image of the fibrin hydrogel in the polyethylene box with Velcro attached to the ends. Figure 38B-D are schematic images of the design for the polyethylene boxes. The first study involved the culture of fibrin + endothelial cells and cardiac fibroblasts in the polyethylene boxes without a central vessel (Fig. 38B), then Velcro was added to increase the surface area for the fibrin construct to adhere to (Fig 38C) and finally, the central vessel was added to the polyethylene box with the Velcro and fibrin was injected into the box.

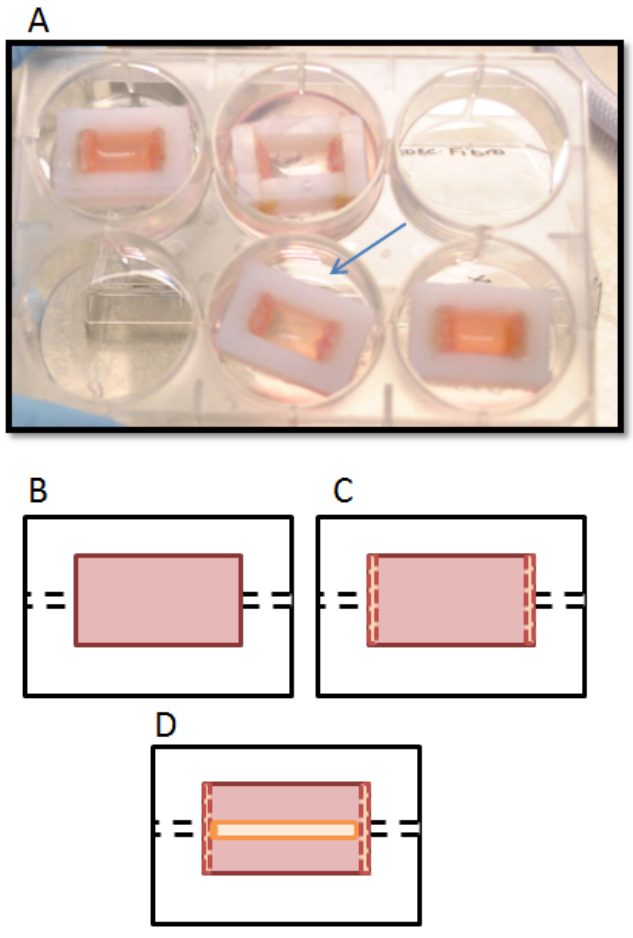

Figure 38: Design for polyethylene box studies. A) Experimental design of fibrin hydrogel in polyethylene box. B) fibrin + cells only in box C) addition of Velcro to PE box D) central vessel suspended in fibrin construct

5.3.4 Fluorescent and Confocal Imaging of Fibrin hydrogel

In order to study the growth of ECs in the central vessel, cells were pre-labeled with PKH26 (red) or CellTracker Green. Figure 39 below is a representative fluorescent image of ECs seeded inside a carotid artery at 10X, in both the GFP and Transmitted fields. Confocal imaging was also conducted with the attempt to create a 3D z-stack projection of the central vessel in the hydrogel, although no successful images were acquired.

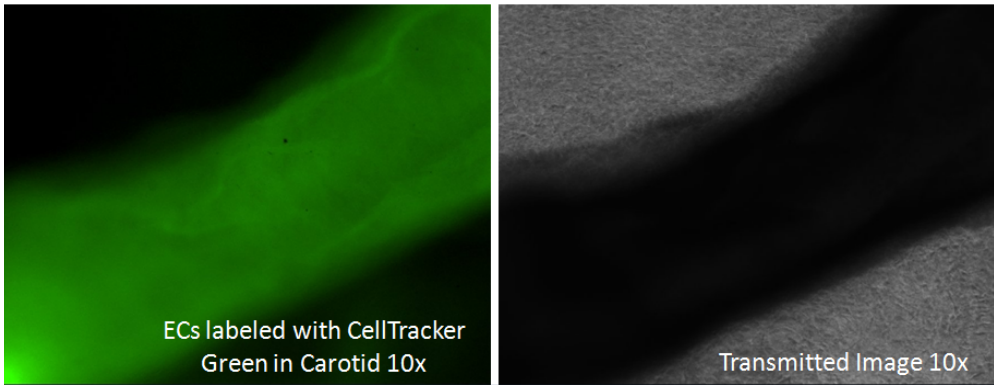

Figure 39: Endothelial cells seeded inside a carotid artery, in a fibrin hydrogel membrane labeled with CellTracker Green.

5.4 Discussion

Significant progress has been made to achieve the development of tissue engineered organs as a replacement for the shortage of donated organs and tissues to treat disease. Achieving sufficient vascularization inside tissue engineered constructs to ensure the proper transfer of oxygen and nutrients is paramount to the success of the tissue engineered construct. By creating a construct with a central vessel in a fibrin hydrogel, the patient can serve as the source for all

biological component so the construct. This would greatly reduce the instances of host immune rejection due to the implantation of a foreign body.

In this study, we demonstrate the ability to adequately decellularize an excised vessel, recellularize it with endothelial cells and implant it into a fibrin hydrogel. Although not a complete study, these experiments demonstrate the feasibility of creating a functional tissue construct with a central vessel as the starter conduit for angiogenesis.

Chapter 6: Conclusions and Future Directions

It is apparent, through these studies and comprehensive background research, that there is a need for more experimentation regarding vascularization and cardiac tissue scaffolds. This thesis presents a novel approach to fabricating a vascularized tissue scaffold, utilizing cardiac fibroblasts as a support for endothelial cell sprout formation, for implantation on the left ventricle of the heart. Although the primary aim of this thesis is to create a left ventricular tissue graft, these studies can be carried through for implantation in almost any area of the heart. Furthermore, the results of these studies can be applied to tissue engineered scaffolds for other organs and tissues of the body, for example, liver tissue scaffolds, kidney grafts, lung scaffolds, and even the formation and implantation of individual blood vessels.

The work outlined in this thesis demonstrates the feasibility of fabricating a cardiac tissue scaffold utilizing cardiac fibroblasts as a support cell source for endothelial cell sprout formation. However, these studies are not complete. Further work should be done to characterize the different endothelial cell types in culture. For example, quantitative studies like western blotting should be completed to look at the presence of different paracrine signaling factors and the relative amounts of growth factors in the constructs. These studies should be aimed at determining if one support cell type allows for the formation of more tip or stalk cells.

In addition, future work should focus on the factors that lead to the multi-cellularity of the sprouts. What causes endothelial cells to align and form

multicellular sprouts? Do MSC secrete different growth factors, or different levels of the same growth factors to allow ECs to form more multicellular sprouts than they do in culture with CFs? Further analysis of the constructs should focus on the quantification of the growth of EC sprouts in co-culture with fetal and adult cardiac fibroblasts and whether patent lumens form when ECs are in culture with one support cell type versus another. In addition, a key component that should be studied is the formation of tip and stalk cells. It should be determined whether one condition fosters the formation of more tip or stalk cells and whether the appropriate ratios of these cell types are being produced. Furthermore, although MSCs aid in the formation of more multicellular EC sprouts, it must be considered whether there is the correct ratio of tip and stalk cells. This can be done by analyzing the growth factors present in the construct to see if the amounts of VEGF, Dll4/Notch and other EC sprout stabilization factors are similar to those published in literature for stable EC sprout formation.

Additionally, other studies of interest include: the creation of a perfusion system to perfusion media through a central vessel in a fibrin construct and the tri-culture of endothelial cells with cardiomyocytes and cardiac fibroblasts.

One key limitation of these studies is the time course. It would be interesting to document the stability of the formation of EC networks over time and to carry the studies out for greater than 2 weeks. An example would be to culture constructs at 3 different time points: 1 week, 2 weeks and 1 month to demonstrate whether the EC sprouts begin to recede over time in one culture or whether they form more mature stable sprouts over time.

Furthermore, the creation of thicker constructs greater than 500μm-1mm would be interesting. This would allow for better visibility of EC vascularization with the tissue construct. Additionally, creating a thicker tissue construct would make it easier to assess how quickly the fibroblasts break down the fibrin and replace the scaffold with their own ECM.

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