DEVELOPMENT OF THE RAT MESENTERY CULTURE MODEL FOR THE
MULTI-SYSTEM INVESTIGATION OF MICROVASCULAR NETWORK GROWTH
AN ABSTRACT

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BY

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ABSTRACT

Models that mimic angiogenesis are extremely valuable for elucidating underlining mechanisms and pre-clinical development of therapies. Angiogenesis, defined as the growth of new blood vessels from preexisting vessels, is a multi-cellular process that involves the temporal and spatial coordination between endothelial cells, pericytes, nerves, growth factors, and macrophages. A need exists for biomimetic models that bridge the gap between the mechanistic control of in vitro constructs and the multi-system physiological environment of in vivo models. To meet this need our lab has introduced the rat mesentery culture model as a top down approach with intact microvascular networks and a nearly two-dimensional view. Previous development of the model has proven its time-lapse, angiogenic, and lymphangiogenic capabilities. The goal of this work is to advance the model to include the maintenance of peripheral nerves in culture and develop it as a platform for aging and cell therapy studies.

The first aim of this study was to expand the rat mesentery culture model to maintain nerves and the spatiotemporal relationship between nerves and blood vessels in culture. We developed a nerve culture media to prevent regression of nerves. Nerve alignment was maintained at the network feeding arteriole and capillary plexus levels. We further demonstrated the presence of neurotransmitter precursors was preserved. We
demonstrate for the first time the ability to maintain adult peripheral nerves in an ex vivo model.

For the second aim of this study, we developed an aging rat mesentery culture model as a basis for investigating differences in angiogenesis across age groups. We demonstrated that impaired angiogenesis associated with advanced age could be recovered to adult-like levels with serum and individual growth factor stimulation. The discovery of increased vascular island frequency in aged tissues reveals that the method of angiogenesis for older networks can differ. These results establish the rat mesentery culture model as a method for studying aging effect on angiogenesis.

The objective of the third aim was to demonstrate the capability of the rat mesentery culture model to study stromal vascular fraction therapy. We developed a protocol to isolate the SVF from adipose tissue and transplant onto the mesentery. We identified unique patterns of vasculogenesis and increased vascular coverage. We confirmed that this increase in vascular area was a combination of the vasculogenesis of SVF, proangiogenic effect on host vessels, and incorporation of SVF into the growing host vessels. We used the aging model in developed in the second aim to show that adult SVF on adult tissue has the greatest therapeutic potential. These results display the investigative potential of the rat mesentery culture model in cell therapy research.

This work establishes for the first time, to the best of our knowledge, an ex vivo model capable of maintaining adult peripheral nerves. We demonstrate that angiogenesis can be rescued in aging scenarios. The results, for the first time, reveal the effect that SVF therapy has on preexisting networks as well as how it integrates during microvascular remodeling.
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CHAPTER 1: BACKGROUND

1.1. INTRODUCTION

Microvascular networks consist of multiple systems that are essential to tissue function, transport of nutrients, and injury response. Given the importance of microvascular networks and their ability to remodel in normal and most pathological scenarios, a need exists to understand how the multiple players including various cell types, extracellular matrix proteins, and local molecular cues interact across systems. The remodeling of microvascular networks is most often thought of as angiogenesis, defined as the growth of new vessels from pre-existing capillaries. In attempt to better understand interactions involved in angiogenesis, angiogenic in vitro and ex vivo models are used to probe specific mechanistic interactions between sub-sets of players. These models, however, face the challenge of not recapitulating the complexity associated with a real tissue. For example, common models usually do not incorporate other systems such as lymphatic vessels or nerves and do not include intact microvascular networks. The lack of complexity limits their physiological relevance. Hence, there remain gaps between current models that build complexity from the bottom up and in vivo.

In recent years our laboratory has attempted to fill this gap by developing the rat mesentery culture model based on a top down approach that maintains the complexity of an intact tissue. We have previously utilized the model to demonstrate cultured smooth
muscles maintain contractile ability, time lapse imaging, lymphangiogenesis and angiogenesis inductions, and cell printing capabilities. The overall objective of this thesis work is to further development of this model and demonstrate its application for neurovascular congruence, aging, and stem cell studies.

In this work we will further expand upon the combined results of this study and its contribution. In this first chapter, we will address the multisystem nature of microvascular networks, aging, cell therapies, and the current models of angiogenesis. For the second chapter, we will go into further detail on the rat mesentery culture model and our experiments to expand the multisystem capabilities of the model to include the maintenance of the spatiotemporal structure of the neurovasculature in culture. In chapter three we will convey the potential of altering the environment of the model as a test bed for aging research. For the fourth chapter, we will present our model as a unique investigative tool for evaluating cell therapy effects on angiogenesis.
1.2. **Specific Aims**

The aims of this study endeavor to develop the model for investigating the spatiotemporal structure of the neurovasculature in culture, investigate the efficacy of growth factors to induce angiogenesis in aging, and investigate the dynamics of stromal vascular fraction therapies by utilizing the time lapse and environmental control capabilities of the rat mesentery culture model.

**Specific Aim 1: Develop the rat mesentery culture model as an ex vivo platform for maintaining spatiotemporal relationship with blood vessels and peripheral nerves during angiogenesis.**

*Hypothesis: The spatiotemporal relationship between the microvasculature and nerves can be maintained in the rat mesentery culture model with proper media supplementation.*

In the first study, we develop the rat mesentery culture model as a platform to preserve the spatial and temporal relationship between the nervous and microvascular system in culture. Unstimulated tissues were immunolabeled to characterize this relationship as a base line control. We developed a nerve media to maintain the nerves during angiogenesis and subsequently evaluated the nerve presence at the arteriole and capillary level to determine maintenance. We demonstrated that nerves remain viable and aligned with blood vessels. Further characterization of post-culture nerves revealed the presence of multiple nerve types. We confirmed that Schwann cell presence is maintained along nerves after culture and identify nerves that do not have Schwann cells present.
Results from this aim validate the maintenance of cultured peripheral nerves and their alignment with microvasculature in the rat mesentery culture model.

Specific Aim 2: Evaluate the influence of age on growth factor induced angiogenesis in cultured adult and aged rat mesenteric tissues.

Hypothesis: Impaired angiogenesis in aged tissues can be recovered to adult levels through sufficient angiogenic stimulation.

For the second aim, we utilized the rat mesentery culture model to determine the effects of age on the angiogenic response of tissues treated with growth factors. Adult (9 month) and Aged (24 month) male Fisher-344 rat mesenteric tissue were harvested and cultured for 3 days per media group: 1) minimum essential media (MEM), 2) MEM with 10% fetal bovine serum (FBS), 3) MEM with basic fibroblast growth factor (bFGF), and 4) MEM with vascular endothelial growth factor (minimum of n = 8 tissues from 4 rats per group). We determined no significant difference from aging on the density of microvascular segments and capillary sprouts across media groups although we identified an increase in the number of vascular islands with age.

Specific Aim 3: Assess the rat mesentery culture model as a tool for investigating stromal vascular fraction effect on angiogenesis.

Hypothesis: The rat mesentery culture model can be used to track stromal vascular fraction effects on microvascular remodeling.

The third aim of this study was to leverage the rat mesentery culture model to investigate the effects of stromal vascular fraction transplantation. We first developed a
protocol for isolating the stromal vascular fraction from the inguinal adipose tissue of rats and then seeding them onto the rat mesentery. Using the time lapse capabilities of the rat mesentery culture model, we tracked SVF from GFP rats on the mesentery from seeding to the formation networks characterized by a central aggregate with radially extended vessels. These new SVF-derived networks contained both endothelial cells and pericytes. In addition to forming new vessels, cell tracking labels identified SVF incorporation into the vessel walls of the preexisting vessels. The percentage of the tissue that became vascularized after culture increased in comparison to tissues without SVF. This increased vascularized area was due to both vasculogenesis of the SVF and an increased angiogenic response from the indigenous vasculature. Using adult and aged rats, we then investigated the effects of age on donor cells and the mesenteric windows after transplantation. We reported an increase in the vascularized area of the adult SVF and adult tissue pairing relative to the other combinations.
1.3. **Motivation: Microvascular Networks Structure and Function**

1.3.1. **Microvascular Networks**

Microvascular networks are responsible for the transport of nutrients, oxygen, hormones, drugs, and immune cells throughout the body. This extensive network of microcirculation can be divided into two classifications: the cardiovascular system and the lymphatic system. The blood vessel networks in microcirculation are organized in a closed loop arriving with the arterioles and leaving with the venules. The arterioles with their tightly wrapped smooth muscle layer are responsible for pumping blood to the target capillaries. The capillaries are comprised entirely of endothelial cells with pericytes elongated at certain points to stabilize the vessels. These thin vessels with lumens as small as 5 µm are permeable to allow the passage of nutrients and diffusion of oxygen with the flow of liquid due to pressure differences. The venules then receive the deoxygenated blood so that it may eventually be returned to larger veins and finally the heart to repeat the cycle again. The venules have a thinner, looser smooth muscle cell layer making them much larger than arterioles so that they may act as storage vessels for as much as 64% of the body’s blood. Not all the plasma that passes through the capillary wall is reabsorbed through the capillary wall and deposited in the venules. As much as 15% of this interstitial fluid is suctioned by a system of lymphatic vessels so that it may be returned to the venules [1–4]. These lymphatic vessels are composed of a highly permeable wall of endothelial cells with a system of valves in the lymphatic lumen to allow for peristaltic flow away from the target area. This maintenance of interstitial fluid helps maintain the hydrostatic pressure difference that drives the transport of all the oxygen and nutrients that the capillaries distribute [5]. Thus, both systems as well as the
perivascular and immune cells are pivotal when microvascular remodeling is called for in response to wound healing or pathologies. This means that in order to investigate angiogenesis, a multisystem approach must be taken to accurately represent the cellular dynamics involved.
1.3.2. Microvascular Network Remodeling and Angiogenesis

The importance of the microvasculature in supporting the metabolic needs of the entire body underlies the potential for therapies controlling its formation. De novo blood vessel formation is termed vasculogenesis and involves the assembly of a vascular plexus from endothelial progenitor cells [6]. While endothelial precursor cells continue to circulate in adulthood, the primary method of microvascular growth is angiogenesis not vasculogenesis. Angiogenesis is the growth of new blood vessels from preexisting ones and is divided into two methods. Intussusception is the splitting of an existing vessel into two by the merging of two sides of the vessel wall to create two new lumens. This is particularly useful in adjusting the angle of bifurcation during microvascular remodeling [7]. Alternatively, under hypoxic conditions endothelial cells, pericytes, and immune cells secrete cytokines to recruit new capillaries to a target area. The pro-angiogenic signals prompts endothelial cells to form sprouts from nearby vessels as matrix metalloproteinases help restructure the extracellular matrix to support migration [8,9]. A guiding tip cell with filopodial extensions branching towards the chemical gradient leads the proliferative stalk cells that simultaneously recruit pericytes to stabilize the nascent capillary [10]. The growing endothelial cells secrete PDGF-BB to recruit pericytes, that in turn provide stability to the new vessel [11]. Angiogenic cues like VEGF-C that promote new blood vessels also facilitate the likewise recruitment of new lymphatic vessels to maintain interstitial fluid equilibrium (Fig. 1.1) [12]. Similarly, shared growth cues between nerves and blood vessels influences the pattern that this growth takes [13,14]. Controlling these mechanisms is paramount in pathological intervention. Overabundant angiogenesis drives cancer and diabetic retinopathy, yet attenuation of this
growth is symptomatic of hypertension and atherosclerosis. Moreover, induction of vasculogenesis and angiogenesis represents a therapeutic goal for wound healing and ischemia. Therefore, a need exists for a platform containing the multisystem interactions to study the dynamics of angiogenesis and therapies to control it.
Figure 1.1 Microvascular remodeling schematic. Angiogenesis is a multisystem affair involving growth factor gradients, recruitment of pericytes, and extracellular matrix restructuring. These elements can affect related systems such as lymphatic capillaries.
1.4. **Current Angiogenesis Models**

The development of angiogenic models to research cellular dynamics across systems in different states is profoundly important to the study and treatment of pathological scenarios. In vivo models have the full complexity of the microvascular networks with all the physiological factors. In pursuit of controlling the environment of in vivo models, specific animal strains have been bred to develop hypertension [15] and diabetes [16]. Having the entire organism provides critical information like tracking metastasis in cancer models or determining time scale and dosing of drug and cell therapies. However, it is difficult to isolate variables or to analyze individual dynamics in the system. In vitro models such as the two-dimensional cell co-culture allow for fine-tuned control over environment and direct observation that are difficult to maintain in in vivo scenarios, yet they are limited in their ability to emulate somatic microvascular structure and function. An emerging area of research, biomimetic models attempt to mimic physiologic systems by recapitulating the cellular and molecular players of physiological scenarios. In microvascular research they attempt to reenact the endothelial cell, vascular smooth muscle cell, pericyte, nerve, and lymphatic dynamics. Microfluidic devices build on the complexity of basic 2D models by implementing microcirculatory network arrangement and flow. Recent advances in the design of microfluidic devices have added cell cocultures and simulate physiological oxygen levels. Microfluidic devices utilize the same primary cells that 2D and 3D culture models making them useful for studying development but less effective in studying pathological scenarios and aging research. Ex vivo models offer an approach with preexisting physiological systems allowing response to changes in a controlled environment to be monitored (Fig 1.2).
Examples include the retina explant model and the aortic ring which are common angiogenic models. However, the retina explant requires incisions between the networks and the aortic ring produces a microvascular pattern that is not representative of physiological networks [17,18]. We do not know of a model that incorporates nerves and microvascular networks, an appropriate model for aging research and a model to investigate stem cell-network incorporation dynamics. An ideal biomimetic model of angiogenesis would have multiple cell types—endothelial cells, pericytes, nerves, and lymphatics—for physiological complexity and capabilities for real time tracking of cell migration and proliferation.
Figure 1.2 The need for ex vivo models. Ex vivo models of angiogenesis offer a bridge between the control of in vitro models and the physiological complexity of in vivo models that would be valuable for preclinical drug testing.
1.5. **Development of the Rat Mesentery Culture Model**

Our laboratory has recently introduced the rat mesentery culture model as an ex vivo model to bridge the gap between the physiological relevance of in vivo and the investigative control of in vitro scenarios. Mesenteric windows are harvested from anesthetized rats and then cultured in either minimum essential media or media supplemented with serum or growth factors to stimulate network growth (Fig 1.3). At 40 microns thick, the almost two-dimensional window allows for easy viewing of an intact vascular network without additional embedding. This whole network provides pericytes, endothelial cells, and vascular smooth muscle cells in their physiological configuration. In addition to vasculature this tissue also contains whole networks of nerves, lymphatics, and interstitial cells such as resident macrophages. The tissues are only cut at the periphery to maintain an undamaged window of these systems giving it an advantage over the damage done by organotypic slice culture models. The rat mesentery culture is a promising model for the investigation of neurovascular interactions because it contains whole intact networks of both systems [19]. During culture, the tissue allows for time-lapse imaging of mechanistic intercellular interactions at precise locations and time points [20]. Previous work has shown that the model can undergo angiogenesis and lymphangiogenesis under stimulation [21,22]. Additionally, the cultured smooth muscle cells at the arteriole level maintain their ability to undergo vasoconstriction [23]. The mesentery culture model has previously been utilized with tumor cell printing to test the efficacy of antiangiogenic therapies [24,25]. The same cell printing technique can be used to track the differentiation and migration of adipose derived and bone marrow derived stem cells from adult and aged donors. The rat mesentery culture model is a promising
model for testing cell therapies particularly because it can recapitulate aged microvascular structure and their associated pathologies. For this study, the rat mesentery culture model will be expanded to add applications for neurovascular congruence, aging, and stem cell studies (Fig. 1.3).
Figure 1.3 Contributions to rat mesentery culture model development. The rat mesentery culture model makes is an innovative tool for real time investigation of intact physiological microvascular networks. This works seeks to further develop this model as a platform to maintain peripheral nerves in culture, investigate the relationship between aging and angiogenesis, and evaluate the therapeutic potential of stromal vascular fraction.
1.6. **Need for Models to Study Neurovascular Relationship During Angiogenesis**

The spatiotemporal coordination of nerves and microvessels is critical for microvascular function and tissue remodeling. The microcirculation supplies the nervous system with essential oxygen, macromolecules, and hormones, and in return sympathetic neurons secrete norepinephrine to stimulate vasoconstriction of arterioles. When this relationship is disrupted, as with diabetic neuropathy, attenuation of nerve signaling and apoptosis result. Regarding remodeling, the growth cone of the axon and tip cell of the capillary sprout are the guiding factors for neural and vascular outgrowth respectively. Chemically, nerves and blood vessels share growth cues such as semaphorins, ephrins, growth factors, Slits and netrins [14]. These shared growth cues generate the paradigms of independent and dependent patterning of the two systems (Fig. 1.4) [26]. For example, nerves can secrete VEGF to direct angiogenesis and arterial differentiation in the embryonic mouse forebrain [27]. Vessels have also been shown pattern for nerves via smooth muscle cell paracrine signaling [28]. The neural and vascular patterning is not always dependent as is the case of the mouse whisker model where nerves and blood vessels form complimentary patterns in response to Sema3E [29]. After formation of networks, both systems have supportive cells. Pericytes contribute vascular stability and permeability while Schwann cells improve signal propagation and guide repair of nerves. The shared responsibility of the nervous and the cardiovascular system for chemical communication emphasizes the need for these systems to work in parallel.

Translating these systems into an experimental model comes with significant challenges. In vitro techniques like two dimensional cocultures of neurons and
endothelial cells fail to recapitulate the region-specific growth patterns of the body. The
use of microfluidic devices to increase the complexity and further mimic the
physiological conditions has made promising advancements in blood brain barrier models
[30,31]. Organotypic organ slice models such as the hippocampal brain and spinal cord
slices are effective for investigating the dynamics in neonatal conditions [32–34]. The
nature of the slicing method limits these models to ex vivo representations of
neurodegeneration but may be culture media dependent [35–37]. This is because slicing
creates an apoptotic cascade via intercellular connexin 43 gap junction channels [38]. The
establishment of an adult biomimetic model that could investigate the spatiotemporal
coordination and intercellular interactions of the peripheral nervous and cardiovascular
systems could provide insight into how the two systems influence each other during
microvascular remodeling.
Figure 1.4 Neurovascular congruency in growth patterns. The alignment between blood vessels and nerves can be dependent when one system influences another or independent when they each respond the same growth cues [26].
1.7. **NEED FOR MODELS FOR AGING RESEARCH**

Most investigation of microvascular remodeling and network growth as it pertains to pathologies such as cancer, heart disease, and diabetic retinopathy uses adult models of angiogenesis; however, the incidence of these pathologies is vastly increased in aged individuals. Furthermore, how aged networks respond to intervening therapies given their impaired angiogenesis and structural differences may differ from the adult case. Aged vasculature is associated with endothelial senescence defined as the inability of a cell to reproduce which would limit the amount endothelial cells that could be involved in network growth [39–41]. Additionally, growth factors such as VEGF and PDGF are decreased and the extracellular matrix becomes disorganized in aged networks [42–44]. These factors are indicative of reduced angiogenesis, yet vascular density has been found to be increased or not affected in some studies [45–47].

Models that mimic angiogenesis are extremely valuable for elucidating underlining mechanisms and pre-clinical development of therapies. Historically, in vitro two-dimensional and three-dimensional culture systems have proven crucial for mechanistic investigation of intra-cellular signaling and cell-cell interactions [48–50]. These “bottom-up” approaches add individual players (i.e. ECM, endothelial cells, pericytes, stem cells) to incrementally increase complexity. Culture or co-culture systems, however, are limited in their complexity and the physiological relevance can be unclear. Recognition of the need to incorporate the multi-scale complexity of a real microvascular scenario (i.e. cells, vessels and network) has motivated the application of microfluidic devices [51–53], and ex vivo tissue explant models such as aortic ring, retina, choroid and skin explants [54–57]. Specific to aging research, “top-down” tissue
culture explant models offer valuable methods for comparing aged versus adult tissues harvested from pathological animal strains. “Top-down” importantly refers to the goal of maintaining complexity of intact tissues rather than building complexity up player by player. Specific to aging research, the top down tissue culture explant models offer valuable methods for comparing aged versus adult tissues harvested from pathological animal strains. As examples, Shimada et al. used an aortic ring model to show that the lengths of radial capillary sprouts were decreased in a mutant mouse model of aging versus wild type mice [58]. Reed et al. reported that aortic rings from 20-22 month aged mice exhibited reduced sprouting [59]. Additionally, Shao et al. found that mouse choroid explants have reduced sprouting with increase in age (P8, P19, P90, P240) with and without retinal pigment epithelium [56].

Since angiogenesis involves multiple cell types and is related to the growth of other systems, like lymphatic networks [21,22,60], a need still exists for a model of angiogenesis from intact microvascular networks that more closely reflects an in vivo scenario (Fig 1.5). No definitive standard exists for angiogenic models in aging research. The closest would be the aortic ring model, but in this model sprouting occurs radially from an aortic slice and not a microvascular network where angiogenesis normally occurs. To this end, an opportunity exists to introduce biomimetic platforms that bridge this gap between in vivo and in vitro models, as no model exists that mimics this complexity and enables real-time investigation of cell migration, fate, and function during microvascular network growth, during both angiogenesis and lymphangiogenesis.
Figure 1.5 Angiogenesis research in aging. The discoveries of impaired angiogenesis motivate the need to develop new models for investigation and discover the cellular changes that occur.
1.8. **Need for Models to Investigate Stem Cell Dynamics**

Cell therapies commonly entail the transplantation of a primary cell population into a host to add healthy cells to support, repair, or replace ineffective tissue. Mature cell therapies often take the role of support. For example, Schwann cell transplantation has emerged as a promising treatment for traumatic spinal cord injury. Stem cell populations such as adipose derived stem cells (ASCs) or bone marrow derived stem cells (BMSCs) are utilized to improve recovery in wound healing, counteract ischemia, and treat inflammatory diseases. Similar interest in the therapeutic effects of the stromal vascular fraction (SVF), the heterogenous cell population surrounding the ASCs, has increased. The SVF is the entire assortment of cells isolated from fat after digestion with collagenase that includes ASCs, progenitor cells, and differentiated cells like capillary fragments. This heterogenous mixture of cells may have a more robust effect upon the treatment site. Both in vitro and in vivo, it is known to react differently based on environment and time in culture before transplantation [61–63]. SVF transplantation onto basement membrane proteins results in early vessel formation and extension, but seeding onto stromal sections results in migration and proliferation before forming clusters and later vessels [64]. Moreover, the cells have been found to differentiate to cell phenotypes not present in the initial SVF population [65]. In clinical use, fat transplantation for reconstructive surgeries such as breast augmentation have been plagued by necrosis of central adipocytes while the ASC rich and well vascularized periphery remains viable [66]. Transplantation of fat with SVF in mice ameliorated this cell loss and instead promoted neovascularization of the tissue [67]. The vasculogenic and angiogenic effects of SVF could be of further aid to treatment of myocardial infarction. 7 days after an acute
myocardial infarction, SVF injection into the infarct site were found to reduce infarct size in rats with no adverse effects, in contrast to ASC injection that resulted in one death and three cases of difficulty breathing [68]. Additionally, SVF cell transplantation into a model of peripheral nerve injury found axons regenerated a greater distance over time compared to untreated groups. This was accompanied with a greater number of axons, increased diameter of axons, and increased muscle mass [69]. While these results are promising, further research is needed to understand how the different cell types react to different environments. SVF fate and therapeutic potential can be evaluated by altering the environment to reflect pathological or aged scenarios to better understand the dynamic interactions during microvascular remodeling.
CHAPTER 2: AN EX VIVO TISSUE CULTURE MODEL CAPABLE OF MAINTAINING SPATIOTEMPORAL RELATIONSHIP OF MICROVASCULATURE AND NERVES

2.1. INTRODUCTION

A major challenge in biomimetic model development is the incorporation of multiple cell types and systems. This can be attributed to bottom-up approaches characterized by the addition of one to three cell types via co-culture methods. This challenge is exemplified by the lack of models to investigate the spatiotemporal coordination in neurovascular patterning, which represents an emerging area of research and is critical for tissue remodeling. The paracrine signaling between the neural and vascular systems is location specific and affects patterning during development, disease, and recovery [70–72] and thus is important for growing and therapeutically manipulating functional tissues. The importance of neurovascular interactions as a vital component of the microvascular function in vivo is highlighted by their focus in Alzheimer’s [73] and cancer research [74].

Cocultures of neurons and endothelial cells can be employed to investigate neurovascular interactions and have recently shown that brain microvascular endothelial cells deter hypoxic injury to neurons through nitric oxide production [75]. Other models
have imitated more complex neurovascular interactions involving more cell types such as
the blood brain barrier model used to culture nonfenestrated endothelial cells with
pericytes and astrocytes. These models are often limited to 2D cocultures of astrocytes
and endothelial cells but have utilized microfluidic devices to provide a 3D architecture
for drug testing [30,31]. Additionally, microfluidic devices provide a means to control
fluid flow between cell populations. In nerve culture models this allows for control of
perfusion between presynaptic and postsynaptic regions for drug testing applications
[76]. As for vessels, microfluidics can evaluate the effect of flow on endothelial cell
phenotype [77], control diffusion of oxygen [78], and model disrupted permeability [79].
All together in vitro methods have been successful in integrating neural and vascular cells
in spatially relevant patterns with and without flow, yet still models that “look” like a real
tissue and include vessels and nerves are lacking.

Alternative approaches to incorporate the complexity of a real tissue include ex
vivo models. One of the most common models is the brain slice that employs a vibratome
to cut thin slices (100- 400 μm) of the rodent hippocampus for use as a CNS model with
corresponding vasculature [80]. Variants include the spinal cord slice which when
harvested from adult mice, provides a model of neurotrauma [35]. While brain slice
studies have induced neurogenesis and angiogenesis [81], culturing the adult brain slice
makes a representative model of neurodegeneration [36]. The slicing method and the
culture maintenance start an apoptotic cascade via intercellular connexin 43 gap junction
channels [38,82]. The absence of adult peripheral neural culture models could be
explained by Wallerian regression in peripheral nerve injury [83]. So, a need exists to
develop to develop tools that incorporate both intact microvascular networks and nerves – especially peripheral nerves.

The objective of Aim 1 was to develop the rat mesentery culture model as an ex vivo platform for maintaining spatiotemporal relationship with blood vessels and peripheral nerves during angiogenesis. As detailed in the Introduction, the rat mesentery culture model developed in our laboratory represents a novel assay. We have previously shown that tissues can be cultured and used to investigate angiogenesis and lymphangiogenesis. We have also used chronic animal experiments to investigate and neurogenesis [84–86]. While immunolabeling of uncultered networks revealed nerve wrapping at arterioles, alignment at capillaries, and nuclei presence at nerve locations, the ability to keep alive nerves in cultured mesentery tissues has not been developed. For this aim, we developed a nerve culture media based on the most prevalent medium components from 103 papers that endeavored to culture postnatal and adult brain slices. From these components, 12 combinations were tested until a successful nerve media was identified (Table 2.1). We confirmed neural maintenance by 1) comparison of nerve presence near arterioles and capillaries over time course of 3 days, and 2) lectin identification of nerves did not colabel with dead marker. A subset of the surviving axons coexpressed sensory and sympathetic nerve markers. To our knowledge this is the first ex vivo model to maintain peripheral nerves in culture within a microvascular network.
Table 2.1 Iterations of nerve media compositions

<table>
<thead>
<tr>
<th>Component 1</th>
<th>Component 2</th>
<th>Component 3</th>
<th>Component 4</th>
<th>Component 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum Essential Medium</td>
<td>10% Fetal Bovine Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum Essential Medium</td>
<td>20% Fetal Bovine Serum</td>
<td></td>
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<tr>
<td>Neurobasal Medium</td>
<td>20% Fetal Bovine Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurobasal Medium</td>
<td>20 ng/mL Nerve Growth Factor</td>
<td></td>
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<tr>
<td>Neurobasal Medium</td>
<td>40 ng/mL Nerve Growth Factor</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Neurobasal Medium</td>
<td>25% Hank’s Balanced Salt Solution</td>
<td>25% Horse Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal Medium Eagle</td>
<td>25% Hank’s Balanced Salt Solution</td>
<td>25% Horse Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurobasal Medium</td>
<td>25% Hank’s Balanced Salt Solution</td>
<td>25% Horse Serum</td>
<td>40 ng/mL Nerve Growth Factor</td>
<td></td>
</tr>
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<td>10% Fetal Bovine Serum</td>
<td>40 ng/mL Nerve Growth Factor</td>
<td></td>
</tr>
<tr>
<td>Neurobasal Medium</td>
<td>20% Horse Serum</td>
<td>40 ng/mL Nerve Growth Factor</td>
<td>2% Insulin-Transferrin-Selenium</td>
<td>2% B27 Supplement</td>
</tr>
<tr>
<td>Neurobasal Medium</td>
<td>20% Fetal Bovine Serum</td>
<td>40 ng/mL Nerve Growth Factor</td>
<td>2% Insulin-Transferrin-Selenium</td>
<td>2% B27 Supplement</td>
</tr>
</tbody>
</table>
2.2. MATERIALS AND METHODS

2.2.1. Rat Mesentery Culture Model

All animal experiments were approved by Tulane University’s Institutional Animal and Care Use Committee and performed in accordance with the U.S. Animal Welfare Act, U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and the NIH Guide for the Care and Use of Laboratory Animals. Rat mesenteric tissues were harvested and cultured according to a previously established protocol [85,87]. Adult male Wistar rats (350 – 400 g) were anesthetized with an intramuscular injection of ketamine (80 mg/kg body weight) and xylazine (8 mg/kg body weight). The abdominal region was shaved then sterilized with 70% isopropyl alcohol before an incision was made along the linea alba. The mesentery was exteriorized onto a sterile plastic stage using cotton tip applicators to first remove the cecum and subsequently the ileum and jejunum. The rat was euthanized with an intracardiac injection of 0.2 ml Beuthanasia. Vascularized mesenteric tissues were excised and rinsed once in sterile saline with 0.9% NaCl (Baxter; Deerfield, IL) and immersed in minimum essential media (MEM; Gibco; Grand Island, NY) containing 1% Penicillin-Streptomycin (PenStrep; Gibco; Grand Island, NY) at 37°C. After 1 hour, tissues were transferred randomly to petri dishes containing respective media types either 1) MEM containing 1% PenStrep (Gibco; Grand Island, NY), or 2) Nerve culture media. Tissues were cultured in standard incubation conditions (5% CO2, 37°C) for three days (Fig. 2.1).
Figure 2.1 Schematic of the rat mesentery culture model. Mesenteric windows are surgically removed from the small intestine, rinsed in phosphate buffered saline, and placed in media.
2.2.2. Nerve Media Composition

Nerve culture media was composed of neurobasal medium (NBM; Gibco; Grand Island, NY), supplemented with 20 ng/ml nerve growth factor (NGF; Invitrogen; Carlsbad, CA), 20% FBS (Gibco; Grand Island, NY), 2% B27 supplement (Invitrogen; Carlsbad, CA), 2% Insulin-Transferrin-Selenium (ITS) supplement (Invitrogen; Carlsbad, CA), and 1% PenStrep.

2.2.3. Immunohistochemistry

After methanol fixation for thirty minutes at -20°C, tissues were labeled according to the following protocols.

PECAM/Class III β-tubulin/DAPI: 1) 1:100 mouse monoclonal anti-Class III β-tubulin antibody (2G10; Abcam, Cambridge, MA) for 1 hour at room temperature followed by 24 hours at 4°C; 2) 1:100 goat anti-mouse CY2-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hours at room temperature; 3) 1:20 normal mouse serum (Jackson ImmunoResearch Laboratories) for 1 hour; 4) 1:200 mouse monoclonal biotinylated anti-PECAM antibody (CD31 antibody, BD Pharmigen; San Diego, CA) for 1 hour at room temperature; 5) 1:500 CY3-conjugated streptavidin antibody (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature; 6) 1:3000 4',6-diamidino-2-phenylindole (DAPI) Nucleic Acid Stain for 10 minutes at room temperature.

PECAM/NG2: 1) 1:200 mouse monoclonal biotinylated anti-PECAM antibody with 1:100 rabbit polyclonal anti-NG2 antibody (Millipore/Chemicon, Billerica, MA) for
1 hour; 2) 1:500 CY3-conjugated Streptavidin secondary and 1:100 goat anti-rabbit CY2-conjugated antibody for 1 hour at room temperature.

**Tyrosine Hydroxylase/CGRP:** 1) 1:100 mouse monoclonal anti-CGRP (Abcam, Cambridge, MA) antibody with 1:100 rabbit polyclonal anti-tyrosine hydrolase antibody (Millipore/Chemicon, Billerica, MA, USA) for 1 hour; 2) 1:100 goat anti-mouse CY3-conjugated secondary antibody and 1:100 goat anti-rabbit CY2-conjugated antibody for 1 hour at room temperature.

**Class III β-tubulin /S100:** 1) 1:100 mouse monoclonal anti-Class III β-tubulin antibody with 1:500 rabbit monoclonal anti-S100 antibody (Abcam, Cambridge, MA) for 1 hour at room temperature followed by 24 hours at 4°C; 2) 1:100 goat anti-mouse CY2-conjugated secondary antibody and 1:100 goat anti-rabbit CY3-conjugated antibody for 2 hours at room temperature.

All antibodies were diluted in antibody buffer solution comprised of phosphate-buffered solution (PBS; Sigma-Aldrich) + 0.1% saponin + 2% bovine serum albumin (BSA; Jackson Immunoresearch Laboratories) + 5% normal goat serum. After each step, the tissues were submerged in PBS + 0.1% saponin for 10 minutes 3 times.

### 2.2.4. Quantification of Arteriole Nerve Presence

To determine arteriole nerve maintenance, tissues were harvested from adult male Wistar rats and divided into three experimental groups: 1) control group of unstimulated tissues that were immediately mounted on slides, fixed, and immunolabeled for neural and endothelial cell markers (n = 8 tissues from 2 rats), 2) tissues were cultured in media with no supplementation for 3 days as previously described without imaging during
culture \((n = 8 \text{ tissues from 2 rats})\), and 3) tissues were cultured in media. Following culture, tissues were mounted on slides, fixed, and labeled for neural and endothelial cell markers. Images were taken of each network feeding arteriole at the periphery of the tissue.

Percentage of network feeding arterioles with corresponding nerve alignment were measured per tissue \((n = 8 \text{ tissues; 2 tissues per rat})\) after staining with 1) class III β-tubulin and PECAM and 2) NG2 and PECAM. Network feeding arterioles were identified based on size (>20 μm diameter) and morphology. Nerve alignment was determined by direction and distance from arteriole (<10μm).

### 2.2.5. Quantification of Capillary Nerve Alignment

To determine capillary nerve alignment, tissues were harvested from adult male Wistar rats and divided into three experimental groups: 1) control group of unstimulated tissues that were immediately mounted on slides, fixed, and immunolabeled for neural and endothelial cell markers \((n = 8 \text{ tissues from 2 rats})\), 2) tissues were cultured in media with no supplementation for 3 days as previously described without imaging during culture \((n = 8 \text{ tissues from 2 rats})\), and 3) tissues were cultured in media. Following culture, tissues were mounted on slides, fixed, and labeled for neural and endothelial cell markers. 4 4X images were taken per tissue at capillary regions.

Length of aligned nerves in capillary regions of networks were measured per tissue \((n = 8 \text{ tissues; 2 tissues per rat})\) after staining with 1) class III β-tubulin and PECAM and 2) NG2 and PECAM. Nerve alignment was determined by direction and distance from capillaries (<10μm) in 4X images.
2.2.6. Nerve Viability Assay

To assess viability of nerves after culture, tissues were cultured in nerve media for 3 days. BSI-Lectin conjugated to FITC (Sigma-Aldrich; St. Louis, MO) was added to media containing tissues in a 1:40 ratio and incubated for 30 minutes at 37°C. After incubation, the supplemented media was removed, and the tissues were subsequently rinsed with label free media. Dead staining was applied with 1:200 Ethidium Homodimer-1 (EthD-1; Molecular Probes; Grand Island, NY) supplemented media for 10 min at 37°C. After staining, tissues were spread on a microscope slide and immediately imaged using an epifluorescent microscope. The lectin was used to identify nerves before assessing dead coverage.

2.2.7. Image Acquisition

Images were acquired using an epifluorescent inverted microscope (Olympus IX70) combined with a Photometrics CoolSNAP EZ camera with 4x (dry, NA = 0.1), 10x (dry, NA = 0.3), 20x (oil, NA = 0.8), 40x (oil, NA = 1.0) and 60x (oil, NA = 1.4) objectives. Image analysis and quantification were done using ImageJ 2.0.0-rc-54 (U.S. National Institutes of Health, Bethesda, MD).

2.2.8. Statistical Analysis

Percentage of network feeding arterioles with nerve alignment and nerve length along capillaries were compared among media groups using one-way ANOVA followed by Student-Newman-Keuls pairwise comparison test using SigmaStat ver. 3.5 (Systat
Software, San Jose, CA, USA). Statistical significance was accepted for p < 0.05. Values are presented as means ± SEM.
2.3. **RESULTS**

2.3.1. **Nerves within Rat Mesenteric Tissues Contain Nuclei**

Class III β-tubulin and PECAM labeling of tissues at day 0 identified nerves aligned with arterioles, venules, and capillaries. The neurovascular congruency was most pronounced at the periphery of the tissue along network feeding arterioles and draining venules. Nerves at arterioles and venules had extensions that tightly wrapped around the vessel. In addition, large bundles of class III β-tubulin positive nerves not associated with vasculature were identified throughout the tissues. DAPI staining of these bundles revealed nuclei colabeling aligned with the direction of the tubulin filaments (Fig 2.2 A). These nuclei were similarly arranged within class III β-tubulin positive nerves aligned with arterioles (Fig 2.2 B).
Figure 2.2 Nuclei location along axons. A) DAPI (arrowheads) arranged in line with class III β-tubulin nerve labeling in control tissues at day 0. Nerves along arterioles have nuclei presence similarly arranged in line with bulges in class III β-tubulin bundles. Scale bar = 50 µm.
2.3.2. Spatiotemporal Arrangement of Nerves Can be Maintained During Microvascular Growth During Tissue Culture

Class III β-tubulin and NG2 labeling identified nerves and was analyzed in relation to alignment to PECAM labeled vessels in unstimulated and cultured conditions. After three days in culture with MEM, the microvasculature was maintained but nerves were significantly less present along arterioles (p < 0.001). There was no significant difference in nerve alignment with network feeding arterioles in the day 3 nerve media culture group compared to unstimulated controls although the class III β-tubulin nerve wrapping appeared less constricted (Fig 2.3). Nerve media preserved neural alignment at capillary plexuses undergoing angiogenesis, characterized by high vessel density regions and increased capillary sprouting. In some cases, nerve density was increased at capillary plexuses undergoing angiogenesis (Fig 2.4 B). Day 3 MEM cultured capillary networks had significantly less NG2 nerves and were almost absent of the class III β-tubulin nerves (Fig 2.4; p < 0.001).
Figure 2.3 Major nerves remain at network feeding arterioles. A-B) Comparison of class III β-tubulin nerve presence (arrowheads) at day 0 and day 3 at the arteriole level. Nerves are more closely wrapped initially. C) Nerve media maintained class III β-tubulin nerve presence compared to MEM alone. D-E) Comparison of NG2 nerve presence (arrowheads) at day 0 and day 3 at the arteriole level. NG2 labeling of vascular smooth muscle cells obscures observation of wrapping nerves. F) Nerve media maintained NG2 nerve presence compared to MEM alone. P < 0.001. Scale bar = 100 µm.
Figure 2.4 Nerve presence and alignment is maintained in culture at angiogenic capillaries. A-B) Comparison of class III β-tubulin nerve alignment (arrowheads) at day 0 and day 3 at the capillary level. Nerves are more closely wrapped initially. C) Quantification of alignment of class III β-tubulin nerves found significantly more nerves in unstimulated and nerve media groups compared to the MEM alone group. D-E) Comparison of NG2 nerve alignment (arrowheads) at day 0 and day 3 at the capillary level. NG2 labeling of vascular smooth muscle cells obscures observation of wrapping nerves. F) Quantification of alignment of NG2 found significantly more nerves in unstimulated and nerve media groups compared to the MEM alone group. P < 0.001; values are shown as mean ± SEM. Scale bar = 100 µm.
2.3.3. Mesenteric Nerves Remain Viable After Culture

Lectin labeling identified nerves and vessels that were cultured in nerve media (Fig. 2.5 A). Following lectin, EthD-1 application labeled the DNA of cells without an intact cell membrane, an indicator of death (Fig. 2.5 B). Lectin and dead cell labeling did not overlap at identified nerve locations indicating cultured nerves remained viable (Fig. 2.5 C).
Figure 2.5 Assessment of nerve viability after three days in nerve media culture. A-C) Lectin and EthD-1 do not overlap at nerve location (arrowheads) after culture. Scale bars = 100 µm.
2.3.4. Cultured Nerves Are Heterogeneous and Maintain Components of Multiple Neurotransmitters

Class III β-tubulin labeling with neural markers at day 0 established the presence of both sympathetic and sensory neurotransmitters via tyrosine hydroxylase and CGRP respectively (data not shown). Labeling after 3 days in culture with nerve media confirmed that tyrosine hydroxylase was maintained (Fig 2.6 A-C). As tyrosine hydrolase is a precursor for epinephrine and norepinephrine, this indicates that sympathetic nerves maintain the ability to produce neurotransmitters. CGRP had reduced expression but still present after culture. These labels overlap in places indicating that the same axons can possess multiple neurotransmitters (Fig 2.6 D-F).
Figure 2.6 Sympathetic and sensory nerve labeling after three days. A-C) Tyrosine hydroxylase labels a subset of class III β-tubulin positive nerves. D-F) CGRP and tyrosine hydroxylase expression overlap at some locations within the nerve. White arrowheads indicate colabel. Yellow arrowheads indicate singular label. Scale bars = 100 µm.
2.4. **DISCUSSION**

Neurovascular patterning is critical in understanding not only development, but how the two systems interact in models of disease and regeneration. While neurovascular patterning remains an emerging area of research, there remains a paucity of adult peripheral culture models. The results of this aim establish rat mesentery culture model as the first to maintain peripheral nerves *ex vivo* in intact microvascular networks. This expands on previous work in our lab regarding the establishment of the model for investigating angiogenesis, lymphangiogenesis, time-lapse, and drug testing studies [85,87]. This study advances our lab’s goal to develop a tool capable of investigating these fields in tandem and establishes a foundation platform for future studies focused on investigating the simultaneous remodeling of peripheral nerves and blood vessels.

A major contribution of this study is the identification and development of a culture protocol to maintain the presence of neural cells during tissue culture. Not surprisingly based on the literature, the maintenance of neural cells required a specific media cocktail compared to other cell types in the tissue. To determine the components for the nerve culture media, a literature search of an analogous CNS *ex vivo* model, the brain slice, was undertaken. A comprehensive list of every media component and combination of components was compiled from 103 papers that attempted to culture adult or postnatal brain slices *ex vivo*. After trial and error rounds of culturing for 3 days with combinations, we selected the final “nerve media” cocktail of NBM, 20 ng/ml NGF, 20% FBS, 2% B27, and 2% ITS used for our analysis in this aim. This was combined with 1% PenStrep to prevent bacterial contamination.
To characterize the nerves in the culture model, we labeled for a variety of nerve markers to different effect. A specific challenge for this study was that common nerve markers have not been characterized in enteric tissues. Hence, we found specific nerve markers were not nerve or nerve type specific. In culture, our lab has previously shown that class III β-tubulin, a neural cytoskeletal marker, is a standard neural marker but can also label angiogenic perivascular cells [88]. DAPI colabeling and alignment with class III β-tubulin fibers identified patterns of nuclei at bulges within bundles of fibers. NG2, a common pericyte and smooth muscle cell marker, was also shown to label the surface of neural networks extensively. After confidently establishing maintenance by these general nervous system labels, we sought to identify specific types of nerves. PGP 9.5 was selected as a potential enteric nervous system label but overlapped completely with class III β-tubulin and marked interstitial cells [89,90]. Tyrosine hydroxylase, an enzyme involved in the production of epinephrine and norepinephrine, was used to identify sympathetic nerves in the mesentery. Conservation in tyrosine hydroxylase expression suggests that the nerves are still capable of producing norepinephrine. The maintenance of class III β-tubulin and NG2 indicates that the reduction of TH expression relative to day 0 controls is not due to general neuronal loss. Calcitonin gene-related peptide (CGRP) labeled sensory neurons in the mesentery, however, there was less coverage in comparison to sympathetic nerves. The maintenance of CGRP, supports the ability of cultured sensory neurons maintain functionality. These results establish that a heterogenous population of nerves and their neurotransmitters can be maintained in the rat mesentery culture model.
Though glial cells were not the focus of this study, we determined through S100 labeling of Schwann cells with class III β-tubulin, and DAPI that nerve fibers with and without Schwann cells had nuclei present. The previous finding that class III β-tubulin labels angiogenic perivascular cells leaves the foundation for the investigation of pericyte/Schwann cell plasticity. Examples of nervous structures leading into pericytes in this current study support the plausibility that pericytes could act as glial cells. In the brain, the role of pericytes is vital to blood brain barrier integrity, microvascular remodeling, phagocytosis of toxins, cerebral blood flow, neuroinflammation, and stem cell activation [91]. The pericytes of the brain have shown mesenchymal and neural stem cell characteristics supporting their plasticity [92,93]. Similarly in the periphery, pericytes have similarly shown to be integral in microvascular remodeling, phagocytosis, blood flow, and inflammation [94]. Furthermore, mesenchymal stem cells and pericytes share expression profiles which has fostered debate about their connection [95]. This leaves a critical question: are pericytes capable of acting as glial cells in the periphery?

Along with sympathetic and sensory nerves, a subclass of nerves in the gut responsible for mechanical reflexes without CNS communication is the enteric nervous system. Enteric nerves are unique in their ability to respond to stimuli independent from the CNS and in their ability to express a multitude of neurotransmitters. This is important because the nerve bundles in the rat mesentery model may contain enteric nerves in addition to the sympathetic and sensory neurons which can affect the expression of certain neural markers such as colabeling of sympathetic and sensory markers. In addition, the enteric nervous system has unique responses to the growth cues like proliferation of glia [96].
At present, the rat mesentery culture model is limited by lack of perfusion and the effects that the shear stress and interstitial pressure provides for the maintenance of microvascular function and structure. Without such circulation, the network is deprived of nonresident circulating cells after excision. This limitation, however, is shared by \textit{in vitro} co-cultures, aortic ring assay, and brain slice models making it not specific to our rat tissue culture model. Another limitation to our approach is that the scarcity of transgenic rats compared to murine stains represents a barrier to genetic engineering applications. For unknown reasons the mouse mesentery is avascular making it difficult to translate our method to murine tissues.

The biggest limitation of our study that influences the interpretation of our results is the lack of functional data. While we demonstrate the maintenance of nerve presence based on immunolabelling and neurovascular alignment metrics, the functionality of these vessels over time remains unclear. Attempts to apply a patch clamp were met with difficulty getting through the mesothelial layer at the surface of the tissue, an issue not shared by slice culture models with exposed nerves. Later collagenase application proved ineffective in digesting the mesothelial layer. Without direct contact with nerves, microelectrode measurements could get interference from smooth muscle and endothelial cells in the mesentery as they generate their own action potentials [97]. Future experiments will be needed to determine how long the nerves remain present and whether nerves can directly influence microvascular function, which would be an alternative indicator of function.

While our results provoke critical questions and further characterization, our results suggest that the rat mesentery culture model could be used investigation of neural
and vascular interactions and this represents a major advance in the field of biomimetic model development. Nerves and their alignment with the vasculature are maintained in culture in the rat mesentery model. These nerves are found both at the arteriole level and the angiogenic capillary plexus levels. The larger nerve bundles contain nuclei and Schwann cells. Evidence from this study further support the heterogenous nature of the cultured nerves and their ability to produce neurotransmitters. To our knowledge, the results support the first successful attempt to maintain peripheral nerves in an \textit{ex vivo} culture model.
2.5. **Future Studies**

While the results of this study do not explicitly prove the neural network is undergoing neurogenesis, they do show instances of increased neural density in class III β-tubulin labeled nerves in capillary plexuses. Further investigations will target these examples to determine whether the increased density is attributable to the unraveling of the nervous structure, axonal elongation, or neurogenesis. The unraveling of the nervous structure would be a disassociation of individual nerve fibers from a larger nerve bundle. Axonal elongation would indicate that the nerves present can produce growth cones in response to stimulation. Neurogenesis would provide more evidence in a long-standing debate on the possibility of neurogenesis in the peripheral nervous system ex vivo. There has been research to suggest that the formation of new nerves is possible from satellite glial cells turned progenitor during peripheral nerve crush injury [98]. Particularly nerves in the gut have been found to undergo neurogenesis by this method [96,99,100]. Future applications of the rat mesentery model could elucidate whether peripheral neurogenesis occurs by proliferation or differentiation.
CHAPTER 3: UTILIZATION OF AN AGING RAT MESENTERY CULTURE MODEL TO INVESTIGATE ANGIOGENIC RESPONSE IN AGING

3.1. INTRODUCTION

A common characteristic of aging is impaired angiogenesis, most often defined as the sprouting of new capillaries from existing vessels. Intriguingly, our understanding of how and why angiogenesis is impaired remains under-investigated. Angiogenesis is a multi-cellular process that involves the temporal and spatial coordination between endothelial cells, pericytes, growth factors, extracellular matrix, and macrophages [60,101–103]. Further complicating our understanding are the cellular dynamics that vary at different locations within a microvascular network and vary across different types of tissues.

While the influence of aging might seem logical given its relationship with common pathologies such as myocardial infarction, diabetes, and cancer, these same pathologies are often investigated using adult animal models. Hence, fundamental questions remain regarding the role of aging in the initiation of disease states and, more specific to this aim, the role of aging in microvascular growth. The lack of consistency in the literature motivates the need for additional studies focused on understanding aging related angiogenic effects. For example, endothelial cells that have become senescent in
aged tissues no longer proliferate, yet a lack of proliferation can also be characteristic of younger tissues [39,104]. Angiogenesis is commonly considered to be decreased in aged tissues, yet age related diseases can be both associated with a loss of blood vessels as with hypertension and an increase as with cancer. For impaired angiogenesis scenarios, questions remain as to why. The lack of angiogenesis may be the result of altered cell function or attenuated growth factor production in the local environment [105]. Considering the prevailing notion that angiogenesis is impaired with age, a need exists to identify the causes and mechanisms of angiogenesis in aged scenarios, and for new tools to enable comparison of aged versus adult responses to therapy.

A challenge is that cell based in vitro models do not recapitulate the real tissue environment. For example aged endothelial progenitor cells (EPCs) have been shown to have decreased migration proliferation and survival in 2D cell culture [106,107]. However, questions remain as to what physiological relevance the EPC studies have for therapies in real tissue scenarios. In comparison, chronic in vivo models do take advantage of aged mouse or rat tissue environments. Examples include the dorsal skin chamber, retinal assay and Matrigel plug assay, yet these are limited in imaging methods to reveal individual vessel dynamics [10,108,109]. Again, a gap between common in vitro assays and in vivo models is highlighted.

Since angiogenesis involves multiple cell types and is related to the growth of other systems, like lymphatic networks [21,60,110], a need still exists for a model of angiogenesis from intact microvascular networks that more closely reflects an in vivo scenario. No definitive standard exists for angiogenic models in aging research. The closest would be the aortic ring model [18], but in this model sprouting occurs radially
from an aortic slice and not a microvascular network where angiogenesis normally occurs. To this end, an opportunity exists to introduce biomimetic platforms that bridge this gap between in vivo and in vitro models, as no model exists that mimics this complexity and enables real-time investigation of cell migration, fate, and function during microvascular network growth, during both angiogenesis and lymphangiogenesis.

The objective of Aim 2 was to demonstrate the usefulness of the rat mesentery culture model for aging studies by evaluating the influence of age on growth factor induced angiogenesis in cultured adult and aged rat mesenteric tissues. To accomplish this, we excised mesenteric windows from Adult (9 month) and Aged (24 month) Fisher-344 rats. To establish base level angiogenesis, we used serum stimulation to illicit a general angiogenic response. In order to test aging differences in response to intervening therapies, we used rat recombinant vascular endothelial growth factor (rrVEGF) and basic fibroblast growth factor (bFGF). A heparin-binding glycoprotein, VEGF is considered the foremost promoter of angiogenesis through paracrine signaling from a multitude of cell types including pericytes and macrophages [111,112]. bFGF acts as a potent angiogenic factor by altering the expression of integrins increasing vascular permeability, an important step in angiogenesis [113]. We evaluated angiogenic response to each of these treatment groups by 1) vascular density, 2) capillary sprout density, and 3) vascular island density. Our results suggest that the rat mesentery culture model offers an aging platform that enables investigation of angiogenic response, and that angiogenesis can be rescued in an aged scenario through stimulation.
3.2. MATERIALS AND METHODS

3.2.1 Rat Mesentery Culture Model

All animal experiments were approved by Tulane University’s Institutional Animal and Care Use Committee and performed in accordance with the U.S. Animal Welfare Act, U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and the NIH Guide for the Care and Use of Laboratory Animals. Rat mesenteric tissues were harvested and cultured according to a previously established protocol [22,87]. Adult (9 months) and Aged (24 months) male Fisher-344 rats were anesthetized with an intramuscular injection of ketamine (80 mg/kg body weight) and xylazine (8 mg/kg body weight). The abdominal region was shaved then sterilized with 70% isopropyl alcohol before an incision was made along the linea alba. The mesentery was exteriorized onto a sterile plastic stage using cotton tip applicators to first remove the cecum and subsequently the ileum and jejunum. The rat was euthanized with an intracardiac injection of 0.2 ml Beuthanasia. Vascularized mesenteric tissues were excised and rinsed once in sterile saline with 0.9% NaCl (Baxter; Deerfield, IL) and immersed in minimum essential media (MEM; Gibco; Grand Island, NY) containing 1% Penicillin-Streptomycin (PenStrep; Gibco; Grand Island, NY) at 37°C. 1 mL of culture media was used to cover the surface of each well of a 6-well plate. Tissues were the spread onto a polycarbonate filter membrane (pore size = 5µm) on a cell crown before inversion into the 6-well with the fat of the tissue pressing against the surface. 2 mL of culture media were then added to each well on top of the filter. Tissues were cultured in standard incubation conditions (5% CO2, 37°C) for three days (Fig. 3.1).
Figure 3.1 Schematic of the rat mesentery culture model used for aging study. The mesenteric tissues are surgically removed from adult and aged Fisher-344 rats, rinsed in buffered saline, and placed in media. Tissues are later spread onto filter membrane, inverted into the well, and covered with media.
3.2.2 Immunohistochemistry

Cultured tissues were fixed in methanol for thirty minutes at -20°C. Tissues were spread on glass slides before being rinsed three times for 10 minutes in PBS +0.1% saponin. Fat around window was removed and a hydrophobic marker was applied around the tissue before being labeled according to the following protocol.

PECAM: 1) 1:200 mouse monoclonal biotinylated anti-PECAM antibody (CD31 antibody, BD Pharmigen; San Diego, CA) for 1 hour at room temperature; 2) 1:500 CY3-conjugated streptavidin antibody (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature.

Antibodies were diluted in antibody buffer solution comprised of phosphate-buffered solution (PBS; Sigma-Aldrich) + 0.1% saponin + 2% bovine serum albumin (BSA; Jackson Immunoresearch Laboratories) + 5% normal goat serum. After each step, the tissues were submerged in PBS + 0.1% saponin for 10 minutes 3 times.

3.2.3 Quantification of Angiogenesis

In addition to unstimulated controls (n= 8 tissues from 4 rats in each age group), we performed two studies of media groups to evaluate the differences in angiogenic response from adult (9 month) and aged (24 month) microvascular networks. The first study used 10% FBS (Gibco; Grand Island, NY) in MEM to illicit a general angiogenic response (n= 8 tissues from 4 rats in each age group). The second study utilized specific growth factors as an angiogenic therapy in two experimental groups: 1) 200 ng/ml of rrVEGF (R&D Systems; Minneapolis, MN) in MEM, and 2) 60 ng/ml of recombinant
human bFGF (Invitrogen; Waltham, MA) in MEM (n= 8 tissues from 4 rats in each age group). The concentration for rrVEGF and bFGF was selected based on previously published studies [19].

Quantification of angiogenesis was measured across the 3 experimental groups listed above and unstimulated controls. The number of vessel segments per vascular area and the number of capillary sprouts per vascular area were quantified per tissue from 4x images of randomly selected network regions per tissue. In the analysis a node was defined as the end of any vessel, the connections between any two vessels, and any point along the boundary where the vessel ends at the edge of the image. Vessel segments were defined as any segment between two nodes and capillary sprouts were defined as a segment with one unconnected end. Vascular island density was also analyzed from 4x images that were randomly selected from the periphery of the vascular networks as they are rarely observed within the network. Vascular islands were defined as a vessel segment with two unconnected ends.

3.2.4 Image Acquisition

Images were acquired using an epifluorescent inverted microscope (Olympus IX70) combined with a Photometrics CoolSNAP EZ camera with 4x (dry, NA = 0.1), 10x (dry, NA = 0.3), and 20x (oil, NA = 0.8) objectives. Image analysis and quantification were done using ImageJ 2.0.0-rc-54 (U.S. National Institutes of Health, Bethesda, MD).
3.2.5 Statistical Analysis

Data was analyzed in terms of change in number of vessel segments and number of capillary sprouts between adult and aged groups at day 3 and unstimulated controls at day 0. These densities were compared within each treatment group with a two-way ANOVA followed by Bonferonni pair-wise comparison test. Vascular island frequency was similarly analyzed between age groups at day 0 and day 3. Vascular island age groups were compared using Student’s t-test. All analyses were using SigmaStat ver. 3.5 (Systat Software, San Jose, CA, USA). Statistical significance was accepted for p < 0.05. Values are presented as means ± SEM.
3.3. RESULTS

3.3.1 Angiogenesis in Aging Tissues Can Be Stimulated in an Adult-like Response

Adult tissues in MEM supplemented with 10% FBS underwent characteristic angiogenesis as defined by the increase in both vascular density and sprout density relative to the unstimulated day 0 tissues. Interestingly, Aged tissues also underwent angiogenesis at comparable rates to the adult scenario in culture with serum (Fig. 3.2). This angiogenesis is characterized by “hot spots” of increased angiogenesis relative to the surrounding network (Fig 3.3).
Figure 3.2 Quantification of angiogenesis following serum stimulation. Comparison of vessel density (A) and number of vascular sprouts (B) per area in mesenteric tissues from adult (black bars) and aged (white bars) Fisher-344 rats in unstimulated (UN) and serum stimulated (FBS) conditions. n.s. indicates no significant difference between adult and aged networks. * indicates significant difference between unstimulated and FBS stimulated conditions (p < 0.05; two-way ANOVA followed by a Bonferroni pair-wise comparison test). Values are shown as mean ± SEM.
Figure 3.3 Angiogenic response in aged tissues. Representative images of an aged mesenteric network from an aged 24 month-old Fisher-344 rat immediately after harvesting and after culturing for 3 days in MEM supplemented with 10% FBS. PECAM labeling identifies the hierarchy of intact networks including arterioles, “A,” venules, “V,” and capillaries. Angiogenesis in the cultured tissues is supported by the observation of regions with high vascular density “*” and capillary sprouting (arrows). Scale bar = 100 µm.
3.3.2. Growth Factor Can Stimulate Angiogenesis in Aging Tissues in an Adult-like Response

rrVEGF and bFGF were selected for this study as they are common promoters of angiogenesis and have previously been used by our lab in 8-12 week old male Wistar rats [19]. rrVEGF stimulation of Aged tissues yielded vascular density and sprout density increases comparable to Adult. There was no significant difference found between age groups after culture with rrVEGF stimulation (Fig 3.4 A-B). bFGF stimulation also produced higher rates of stimulation in Adult and Aged tissues relative to unstimulated tissues (Fig 3.4 C-D).
Figure 3.4 Quantification of angiogenesis following growth factor stimulation. Comparison of vascular and capillary sprout densities in mesenteric tissues from adult (black bars) and aged (white bars) Fisher-344 rats in response to bFGF (A-B) and rrVEGF (C-D) stimulation. n.s. indicates no significant difference between adult and aged networks. * indicates significant difference between unstimulated and FBS stimulated conditions (p < 0.05; two-way ANOVA followed by a Bonferroni pair-wise comparison test). Values are shown as mean ± SEM.
3.3.3. bFGF Stimulation of Aged Tissues Exhibited Increased Number of Vascular Islands

For an alternate metric of microvascular remodeling, we compared the frequency of vascular islands Adult and Aged groups. Vascular islands are unconnected endothelial cell segments capable of contributing to angiogenesis by proliferation or linking to vascular networks [50,114]. Although no significant differences were discovered between Adult and Aged tissues in serum stimulated or sprout rrVEGF stimulated tissues, vascular island density was increased in the aged bFGF tissues relative to the Adult scenario (Fig 3.5).
Figure 3.5 Vascular island distribution after angiogenic response to bFGF stimulation. A-B) Representative images of vascular island frequency in adult and aged mesenteric tissues after 3 days of bFGF stimulation. Arrowheads indicate vascular islands. Quantification of vascular island frequency found significantly more islands in aged bFGF cultured tissues compared to adult. P < 0.05; values are shown as mean ± SEM. Scale bars = 100 µm.
3.4 Discussion

The main contribution of this aim is the demonstration that the rat mesentery culture model can be used to evaluate aged versus adult angiogenesis. Angiogenesis defined as the growth of new blood vessels from preexisting ones is the target of therapies aiming to treat cancer, hypertension, and diabetes. However, research of these pathologies is often conducted in younger animals while aging is associated with increased incidence. Our lab has previously established the rat mesentery culture model as a platform for testing anti-angiogenic therapies [87], and the goal of Aim 2 was to further establish the application of the model by testing angiogenic therapies on physiologically relevant aged networks.

Angiogenesis is most commonly assumed to be impaired in aged scenarios, but our results support that aged tissues can undergo adult-like angiogenesis. Vascular density and capillary sprouting were recovered in the aged group under serum stimulation. Further, comparable angiogenic responses observed in rrVEGF and bFGF groups demonstrate that specific growth factor responses are sufficient to also induce adult-like angiogenesis in aged tissues. The supplementation of the media with serum and specific growth factors represents changes in the environment, hence, any impaired angiogenesis in the in vivo scenario might be also due to environment changes rather than the ability of microvessels to grow.

These results also raise an important question: Why is angiogenesis impaired in vitro but not in the rat mesentery culture model? 2D cultures have previously made use of aged endothelial cells to link cell senescence to impaired angiogenic potential [115–117]. These cultures frequently utilize human umbilical vein endothelial cells or circulating...
endothelial progenitor cells, but a subpopulation of endothelial progenitor cells have been identified in the vessel walls of mature tissue [118,119]. Manavaski et al. has shown that specific cells within the microvascular network are more disposed to dominate microvascular remodeling during hypoxic conditions in a confetti mouse model [104]. Our results support that having a whole tissue with complete microvascular networks may minimize the effect of endothelial senescent cells.

“Bottom-up” approaches like in \textit{in vitro} 2D cell culture systems and microfluidic devices attempt to build complexity by adding cell types. These models have made significant discoveries in the mechanistic interactions during angiogenesis in aging [120,121]. For aging research, “top-down” tissue culture explant models offer valuable methods for comparing pathological conditions and angiogenic response in aged versus adult tissues. The “top-down” approach refers to the goal of maintaining complexity of intact tissues rather than develop complexity with each cell added. The so called “top-down” approach of the rat mesentery culture model enables the investigation of real network response and the ability to characterize responses across a network. This capability and importance is highlighted by our identification of the increase in vascular islands in bFGF, which acts as a potent angiogenic factor by altering the expression of integrins [113] and has also been implicated in leukocyte recruitment during inflammation [122], stimulated aged tissues compared to bFGF stimulated adult tissues. Our lab has previously identified vascular islands, unconnected endothelial cell segments, as a method microvascular remodeling [50,114], and they represent a mode of growth not captured in other in vitro assays or even by analysis of tissue section derived from in vivo studies. While we do not the reasons of this different in island formation, our results raise
caution of interpretation of our other results and the evidence from the literature based on vessel counts. Vessel density metrics do not tell the whole story. Aged tissues might be able to undergo angiogenesis, but the new question is whether aged angiogenesis is the same as adult angiogenesis.

A limitation of applying the rat mesentery culture model tissue for aging research is the few number of transgenic rat options to further probe aging research. As presented for Aim 1, the use of mouse tissues would potentially increase the usefulness of the model for mechanistic studies. Another limitation of the model, potentially influencing long-term viability is that it lacks shear stress from fluid flow, which has been implicated in the regulation of angiogenesis [123], endothelial cell differentiation [124], and the absence of circulating cell populations. Nonetheless, we have confirmed that non-perfused cultured tissues remain viable out to at least seven days [19]. So while the lack of flow remains an issue, our results suggest that the model can still be valuable for specific questions.

Finally, a noteworthy observation made in our studies was that not all aged rats looked the same. This general observation is consistent with the phenomenon of “healthy aging” and the explanation for increased heterogeneity between aged tissue responses. For example, two of the aged rats had noticeably enlarged livers of ~5 inches in length which may have affected the quality of the vasculature harvested from the abdominal region. The afflicted rats had denser vasculature compared to the healthier aged rats which could be explained by greater VEGF production from the liver [125,126]. These samples were included in our analysis but suggest that sub-population responses within aged groups maybe should be considered for future studies. Differences in responses and
baseline levels could alone help explain discrepancies in the literature regarding whether angiogenesis is impaired.

In summary, this study takes advantage of the real network view and modifiable environment of the rat mesentery culture model to investigate aging microvascular response to angiogenic stimuli. Aging research has been limited in options for models with intact microvascular networks and modifiable physiological environments. These results promote the rat mesentery culture model to meet this need. The comparable increase in vascular density and capillary sprouting between Aged and Adult tissues demonstrates the importance of real networks in evaluating the angiogenic response. Similar results for serum, VEGF, and bFGF in stimulating angiogenesis across age groups suggests that the source of growth cues may be important in modeling pathologies in aging. The discovery of increased vascular island frequency under bFGF stimulation of aged tissues also supports the unique view of the rat mesentery culture model that might be able to add more to our understanding of whether aged angiogenesis is really the same or different than adult angiogenesis. This supports the use of the rat mesentery culture model as a new tool for mechanistic aging research and applied pre-clinical therapy evaluation.
3.5 **FUTURE STUDIES**

The results of the second aim highlight the importance of altering the environment to reflect the physiology being investigated. In the future, we can combine our previous work of cancer cell printing with our newly developed view of aged microvascular networks to create an age specific disease model [24]. This would further open possibilities of testing therapeutic interventions. Future studies will be needed to investigate how other aging effects alter the response of different cell types in our model such as pericytes, lymphatics, or nerves. By utilizing the multi-system approach of our model, we could decipher the effect that age-related thinning of basement membranes has on lymphangiogenesis and angiogenesis simultaneously.
CHAPTER 4: INVESTIGATING STROMAL VASCULAR FRACTION FATE AND THERAPEUTIC EFFECT USING RAT MESENTERY CULTURE MODEL

4.1. **Introduction**

Adipose derived stromal vascular fraction (SVF) transplantation is a promising new therapy used in clinical trials for multiple sclerosis, facial reconstruction, cardiac ischemia, degenerative disc disease, etc. [127–131]. The SVF is isolated from adipose tissue to yield a heterogenous population consisting of endothelial cells, endothelial progenitor cells, pericytes, stem cells, fibroblasts and immune cells. Transplanted SVF has been shown to adapt based on its environment. For example regarding SVF potential to influence angiogenesis, Nunes et al. demonstrated that SVF could form into fenestrated vessels in the presence of human liver cells [65]. In vitro studies have demonstrated that the activity of these cells after transplantation is both concentration dependent and matrix dependent [61,132], yet the way in which the SVF interacts with host microvascular networks remains unclear. SVF has indeed emerged as a potential source for new vessels for tissue engineering or therapeutic in vivo applications. However, the spatiotemporal incorporation of the SVF within living tissues remains largely unknown. As such, understanding where and how SVF cells contribute to microvascular growth and remodeling will help guide their therapeutic use. Big knowledge gaps remain related to
the time course of the de novo vessel growth (i.e. vasculogenesis), the integration of SVF derived vessels with nearby microvascular networks, and the cell makeup of the new vessels. Also, questions remain whether SVF efficacy is age dependent as well. In regard to aging research, ASC and BMSC cell therapies sourced from aged mice have been shown to have stimulated a reduced angiogenic response compared to younger cells [133]. Young and old SVF, however, shows similar rates of angiogenesis in vivo initially but eventually the old SVF exhibits reduced vascular density and perfusion. Modulation of the old SVF was able to overcome the long-term effects of the aged cell population [134]. Continued investigation into aged host response is needed to provide greater understanding of how aged related pathologies affect SVF fate.

The objective of this aim was to validate the rat mesentery culture model as a platform to investigate the interactions between seeded SVF cells and the host vasculature. The results confirmed that SVF can be translated onto the mesentery tissue and integrated into the networks when cultured for 3 days. The SVF formed unique networks unlike the typical branching hierarchy of mesenteric vessels. These SVF derived vessels integrated into the preexisting networks and induced angiogenesis, and individual cells assimilated into the native vessels. The effects of aging were found to be generally negative in donor/host relationships. This study exhibits the therapeutic potential of SVF, the importance of SVF interaction with the native microvascular network and substantiates the use of the rat mesentery culture model for providing a dynamic view for characterizing SVF effects and fate in a real tissue.
4.2. MATERIALS AND METHODS

4.2.1. Rat Mesentery Culture Model

All animal experiments were approved by University of Florida’s Institutional
Animal and Care Use Committee and performed in accordance with the U.S. Animal
Welfare Act, U.S. Public Health Service Policy on the Humane Care and Use of
Laboratory Animals, and the NIH Guide for the Care and Use of Laboratory Animals.
Rat mesenteric tissues were harvested and cultured according to a previously established
protocol [22,87]. Adult male Wistar, Adult (9 month) Fisher-344, and Aged (24 month)
Fisher-344 rats were anesthetized with an intramuscular injection of ketamine (80 mg/kg
body weight) and xylazine (8 mg/kg body weight). In addition, transgenic eGFP rats were
utilized for the culture model. Anesthetization was conducted using isoflurane.

The abdominal region was shaved then sterilized with 70% isopropyl alcohol
before an incision was made along the linea alba. The mesentery was exteriorized onto a
sterile plastic stage using cotton tip applicators to first remove the cecum and
subsequently the ileum and jejunum. The rat was euthanized with an intracardiac
injection of 0.2 ml Beuthanasia. Vascularized mesenteric tissues were excised and rinsed
once in sterile saline with 0.9% NaCl (Baxter; Deerfield, IL) and immersed in minimum
essential media (MEM; Gibco; Grand Island, NY) containing 1% Penicillin-Streptomycin
(PenStrep; Gibco; Grand Island, NY) at 37°C.

4.2.2. Adipose Tissue Harvest

Following the mesentery tissue harvest, a scalpel was used to make a Y shaped
incision into the skin from the bottom of the previous incision of the linea alba to each of
the inner thighs along the midline stopping above the knee. The skin was carefully
removed from the subcutaneous adipose and muscle layers using the scalpel and
microscissors. Hemostats were used to hold the skin back as the separation was advanced
to the coxal region. The center skin flap of pelvic region was removed in a similar
fashion. The inguinal fat pad was removed in a continuous strip from one hip and leg to
the other and placed in a 50 mL conical tube containing sterile saline at 37°C.

4.2.3. **Stromal Vascular Fraction Isolation**

Fat was placed onto a large petri dish and diced into fine pieces before transfer
into an empty 50 mL conical tube for weighing. Once weight was measured a 0.15%
collagenase type 1 (Thermo Fisher; Waltham, MA) and DPBS solution heated to 37°C
was prepared. Collagenase solution was pushed through 0.22 μm filter with a syringe for
sterilization at a ratio of 2 mL per 1 gram of fat. In the event that the fat weighed less
than 3 grams, 5 mL was used to ensure enough volume was in the conical tube containing
the diced adipose. The collagenase/adipose mixture was then transferred to a shaker set to
150 RPM in an incubator for a 45 min digest at 37°C. After digestion, the mixture was
centrifuged for 10 min at 600 G separate the cells from the collagenase and the
undigested fat. The top layer of undigested fat was transferred to a new conical tube
containing the same volume of fresh filtered collagenase and transferred back to the
incubator for a second digest. The collagenase solution was then removed from the
centrifuged tube and the bottom layer of cells was resuspended with 5 mL of 5% FBS +
DPBS. The resuspended mixture was filtered through a 250 μm cytostrainer into a new
50 mL conical tube and centrifuged at 600 G for 5 min. Media was removed and 5 mL of
ACK lysing buffer (Thermo Fisher; Waltham, MA) was added to the cells through a 0.22 µm filter for sterilization. The solution was then gently shaken in the dark at room temperature for 3 min. Immediately afterwards 5 mL of 5% FBS + DPBS was added to the solution to neutralize the lysing buffer. This solution was filtered through a 70 µm filter into a new conical tube and the centrifuged for 5 min at 600 G. The media was removed, and the remaining stromal vascular fraction was resuspended with 1 mL of 5% FBS + DPBS. 10 µL of the SVF solution was added to 10 µL of trypan blue, mixed, and then added to disposable slide for cell counting with a Countess™ II Automated Cell Counter (Thermo Fisher; Waltham, MA).

4.2.4. Stromal Vascular Fraction Seeding on Mesenteric Tissues

Isolated SVF cells were suspended in 10% FBS + MEM solution at a concentration of 10 million cells/mL. Tissues were the spread onto a polycarbonate filter membrane (pore size = 5µm) on a cell crown. 100 µL of SVF solution was transferred to the surface of each mesenteric window before incubation for 20 min so the SVF cells was attach to the tissue. Cell crowns were then inverted into the 6-well with the fat of the tissue pressing against the surface. 3 mL of culture media composed of 10% FBS + MEM were then added to each well on top of the filter. Tissues were cultured in standard incubation conditions (5% CO2, 37°C) for three days (Fig. 4.1).
Figure 4.1 Schematic of rat mesentery culture model adapted for cell seeding. The mesenteric tissues are surgically removed, rinsed in buffered saline, and placed in media. Tissues undergo cell seeding according to protocol detailed in 4.2.4, inverted into the well, and covered with media.
4.2.5. Immunohistochemistry

Cultured tissues were fixed in methanol for thirty minutes at -20°C. Tissues were spread on glass slides before being rinsed three times for 10 minutes in PBS +0.1% saponin. Fat around window was removed and a hydrophobic marker was applied around the tissue before being labeled according to the following protocol.

PECAM/NG2: 1) 1:200 mouse monoclonal biotinylated anti-PECAM antibody (CD31 antibody, BD Pharmigen; San Diego, CA) with 1:100 rabbit polyclonal anti-NG2 antibody (Millipore/Chemicon, Billerica, MA) for 1 hour; 2) 1:500 CY3-conjugated Streptavidin secondary and 1:100 goat anti-rabbit CY2-conjugated antibody for 1 hour at room temperature.

Antibodies were diluted in antibody buffer solution comprised of phosphate-buffered solution (PBS; Sigma-Aldrich) + 0.1% saponin + 2% bovine serum albumin (BSA; Jackson Immunoresearch Laboratories) + 5% normal goat serum. After each step, the tissues were submerged in PBS + 0.1% saponin for 10 minutes 3 times.

4.2.6. Cell and Vascular Network Tracking

In order to investigate the fate of the stromal vascular fraction and the microvascular networks to which they were transplanted cell tracking models were implemented. For the first experimental model DiI solution was mixed with MEM to make a 5 µL/mL labeling solution. Isolated Adult male Wistar SVF cell suspension was centrifuged, aspirated, and resuspended in the DiI solution. The SVF was then incubated
for 5 minutes at 37°C followed by 10 minutes at 4°C. The SVF suspension was centrifuged at 600 G for 5 min and the labeling media was replaced with suspension media. Cell were then centrifuged again and washed for two more times before seeding onto Wistar tissues according to the protocol in 4.2.4. After 3 days, BSI-Lectin conjugated to FITC (Sigma-Aldrich; St. Louis, MO) was added to media in a 1:40 ratio, transferred to the well containing the seeded tissue, and incubated for 30 minutes at 37°C. After incubation, the supplemented media was removed, and the tissues were subsequently rinsed with label free media. To preserve DiI labeling, tissues were fixed in methanol-free 4% formaldehyde solution for 10 min at room temperature.

Another group used SVF from GFP rats transplanted onto unlabeled Adult Wistar tissues. The 6-well plates containing these tissues were then placed into a culture chamber mounted on the microscope stage to ensure maintaining the temperature at 37 °C. Two to four microvascular networks were imaged per tissue every 24 hours using an inverted epifluorescent microscope. After 3 days, tissues were labeled with TRITC lectin according to the same protocol above and imaged again.

The third tracking experimental group used adult male Wistar SVF on GFP tissues. The time lapse was performed as described above. Following 3 day culture, TRITC lectin labeling of the whole tissue was performed as described above and imaged again.

4.2.7. Aging Experimental Models

To determine the effects of age on SVF transplantation therapy, tissues were harvested and SVF was isolated from Adult and Aged Fisher-344 rats and combined in four experimental groups: 1) Adult SVF and Adult tissue, 2) Adult SVF and Aged tissue,
3) Aged SVF and Adult tissue, and 4) Aged SVF and Aged tissue (n = 12 tissues from 3 rats). These were cultured in 10% FBS + MEM for 3 days.

4.2.8. Vascularized Area Quantification

Percent vascularized area for each tissue was measured to evaluate angiogenic effect of SVF transplantation on networks. This metric was chosen because the SVF transplantation created highly vascularized tissues with structures that made analysis of vascular segment and capillary sprout density difficult to determine. Vascularized areas were measured by drawing the perimeter along the edge of the vascular networks. Avascular regions within the network smaller than 130 µm² were subtracted from the vascularized area metric. The final vascularized area was divided by the total tissue area.

4.2.9. Image Acquisition

Images were acquired using 4x (dry, NA = 0.1), 10x (dry, NA = 0.3), and 20x (oil, NA = 0.8) objectives on an inverted microscope (Nikon Eclipse Ti2) coupled with an Andor Zyla sCMOS camera. Image analysis and quantification were done using ImageJ 2.0.0-rc-54 (U.S. National Institutes of Health, Bethesda, MD).

4.2.10. Statistical Analysis

Comparison between FBS and SVF + FBS experimental groups were made using two-tailed Student’s t-test. The comparison between the vascularized area’s of native vessels in GFP tissues at day 0 and day 3 were made using a paired t-test. Two-way ANOVA with pairwise Tukey’s multiple comparisons post-hoc tests were used for the
aging experimental groups. Tukey test compared both factors and their interactions. Results were considered statistically significant when \( P < 0.05 \). Statistical analysis was performed using SigmaStat ver. 3.5 (Systat Software, San Jose, CA, USA). Values are presented as mean ± SEM.
4.3. **RESULTS**

4.3.1. **SVF Forms Unique Vascular Structures with Multiple Cell Types**

Lectin labeling of tissues after SVF transplantation and culture for 3 days revealed atypical clusters of vessels with a central nexus and radial outgrowth (Fig. 4.2 A). DiI cell tracking of the SVF colabeled with these structures (Fig. 4.2 B-C). In addition to lectin, PECAM labeling confirmed these aggregates of vessels were endothelial in nature (Fig. 4.2 D). NG2 labeling revealed pericytes supporting these vessels both at the center and along the extensions (Fig 4.2 E-F).
Figure 4.2 Cluster and outgrowth pattern of SVF derived cells. Dil labeled SVF cells form central hubs with spoked vascular branching (A-C). These formations consist of endothelial cells and supportive pericytes (D-F). Scale bar = 100 µm.
4.3.2. Rat Mesentery Culture Model Enables Tracking of SVF

Time-lapse imaging of SVF from transgenic GFP rat was used to track vasculogenesis over time. SVF tracking revealed cell extension at 24 hours, vessel formation beginning at 48 hours, and atypical vascular network patterning identified in 4.3.1. at 72 hours (Fig. 4.3).
Figure 4.3 Time lapse of GFP SVF vessel development. GFP sourced SVF was adhered to tissue at 24 hours (A), forming vessels at 48 hours (B), and creating the central nexus with outward growth at 72 hours (C). Scale bar = 250 µm.
4.3.3. SVF Generates Large Vascular Networks Covering the Mesentery

After 3 days of culture, the SVF treated tissues displayed a drastic increase in percentage of vascularized area. The vascular coverage increased nearly two-fold compared to serum stimulation alone (Fig. 4.4). The hierarchical branching structure observed in serum stimulated tissues was obscured by the high levels of growth.
Figure 4.4 SVF treatment significantly increases vascularized area. (A) Serum stimulated tissues presented typical branching patterns of microvascular structure. (B) SVF treated tissues displayed large networks with unusual patterns. Green outline indicates vascularized area. Red outline indicates avascular area. (C) Quantitative analysis of percent vascularized area was significantly higher in the SVF treated tissues. P < 0.05; values are shown as mean ± SEM. Scale bar = 2 mm.
4.3.4. SVF Increases Vascular Area Through Vasculogenesis, Promoting Angiogenesis, and Incorporating into Growing Resident Vessels

GFP tissues were utilized to track changes in the non-SVF derived vascular networks. After 3 days in culture, SVF networks formed between native vascular networks and made integrative connections (Fig. 4.5). Vascularized area measurements of GFP tissues at day 0 and day 3 showed a significant increase in vascular coverage from native vessels in tissues treated with SVF compared to serum stimulation alone indicating a proangiogenic effect (Fig. 4.6). DiI tracking of SVF revealed incorporation of cells with endothelial morphology incorporated in the vessel wall growing native vessels labeled with lectin (Fig. 4.7).
Figure 4.5 SVF vascular networks integrate with native vessels. (A-C) GFP resident vascular networks are connected by nonresident lectin networks 3 days after SVF transplantation. Scale bar = 250 µm.
**Figure 4.6** SVF induces angiogenesis. Quantitative analysis of percent increase in the vascularized area of native GFP vessels after 3 days in culture reflects a significantly higher increase of vascularized area in SVF treated tissues compared to serum stimulation alone. P < 0.05; values are shown as mean ± SEM.
Figure 4.7 Incorporation of SVF into native vessel wall. DiI labeled SVF with endothelial cell morphology (white arrows) are embedded in the vessel wall of native vasculature. Scale bar = 50 µm.
4.3.5. **Adult SVF Treatment on Adult Host Has Greatest Therapeutic Effect**

Our age combinations of host and donor showed a significant increase between adult SVF on adult tissue compared to all other combinations (Fig. 4.8). There was a generally reduced level of vascularized area in both aged SVF and aged tissue groups that did not compound to make aged SVF on aged tissue significantly worse than other two groups (Fig. 4.9).
Figure 4.8 Adult SVF treatment on adult tissue has greatest vascular area coverage. Vascular coverage is greatest in adult SVF/adult tissue pairing (A) compared to aged SVF on adult tissue (B), adult SVF on aged tissue (C), and aged SVF on aged tissue (D). Green outline indicates vascularized area. Red outline indicates avascular area. Scale bar = 2 mm.
Figure 4.9 Aging effects between donor SVF and host tissue. Quantitative analysis of percent vascularized area of adult and aged tissues seeded with either adult or aged SVF after 3 days in culture reflects a significantly higher increase of vascularized area in adult SVF treated adult tissues compared to all other combinations. $P < 0.05$; values are shown as mean ± SEM.
4.4. **DISCUSSION**

Our third aim establishes the rat mesentery culture model as a new platform for characterizing the vasculogenic potential of SVF in real microvascular networks. We utilize the real network interaction of the rat mesentery culture model to more accurately model the SVF actions in the presence of many physiologically relevant cell types including microvessels, pericytes and lymphatics. This is of particular importance for understanding SVF interaction because it is composed of many of these cell types. We utilize the time-lapse capabilities of the model to get real time information on changes in the SVF. This study expands the aging model outlined in aim two by using the ability to investigate recipient age specific microvascular environment reaction to donor age specific SVF transplantation for the first time. Additionally, switching rat strains to a transgenic GFP rat track allowed us to track SVF movement, proliferation, and reorganization. This contributed novel findings for the field regarding the SVF’s proangiogenic effect on indigenous vessels.

In our study, we used the rat mesentery tissue culture model to facilitate the differentiation and integration of SVF into vascular networks. Previous studies utilizing SVF therapy to improve tendon, cardiac and nerve regeneration emphasize the ability of SVF to interact with multiple systems [130,135,136]. As a thin (40 µm) translucent tissue, the mesentery provides an intact microvascular network ideal for observation of cellular dynamics between the SVF and multiple systems during remodeling. Initial investigation via DiI labeled SVF identified irregular vascular structures consisting of a central nexus with vessels projecting radially. These central aggregates of cells and their projections labeled positively for endothelial marker and pericytes were identified along
them. We then used the time lapse imaging capabilities of our model with GFP rat derived SVF to examine the development of vasculogenesis over time. The SVF cells form extensions at day 1, cluster and form vessels at day 2, and inosculate with each other at day 3.

After confirmation of successful transplantation, the rat mesentery culture model was used to validate the angiogenic and vasculogenic potential of SVF. SVF transplantation with serum nearly doubled the percentage of vascularized area of the Wistar tissues in comparison to serum stimulation alone proving the therapeutic potential. The SVF treated tissues possessed typical branching vascular hierarchy in addition to patterns of central clusters with radial vessel outgrowth. To determine if the increase in vascular area was SVF derived we utilized GFP rat tissues to observe distinguish the indigenous vessels from the lectin labeling of the entire network after 3 days in culture. The results revealed that SVF was forming new vascular networks exhibiting the atypical patterns that were making integrative connections to the native vessels. Combining these experiments, SVF treated and untreated GFP tissues were cultured for 3 day with serum to determine if SVF has greater angiogenic effect of the native vessels. Percent vascularized area of only indigenous vascular networks was performed on time-lapse imaging of the tissues at day 0 and day 3. The SVF transplantation induced a greater increase in vascularized area relative to untreated GFP tissues confirming that SVF promotes angiogenesis in native vessels. Additional studies utilized DiI to determine cell fate during angiogenesis. DiI tracking of SVF on lectin labeled tissues revealed that the SVF can integrate into the vessel wall of growing native vessels. These results provide novel insight into the therapeutic potential of SVF and the active role SVF takes in
promoting angiogenesis. Intriguingly, similar types of chronic cell therapy studies in vivo with single stem cell populations (mesenchymal stem cells or circulating stem cell types) have suggested cells incorporate into the existing vasculature at rates of ~ 0% – 10% [137,138]. In comparison, the SVF results are dramatically obvious where previously avascular tissue regions become covered with new SVF vessels. Additionally, these results are the first to show that SVF promotes angiogenesis of native vessels.

Taking advantage of the capabilities of the rat mesentery culture model for cell population lineage studies, a variety of methods were utilized to investigate the microvascular dynamics of SVF transplantation. However, specific to the characterization of SVF dynamics, these methods were associated with issues. DiI labeling of cells is valuable for tracking the original transplanted cells but as a membrane marker, each new generation of cell has less vibrant labeling making long-term observation difficult. SVF from GFP rats was implemented to track cell dynamics, however NG2 labeling of these cells revealed that pericytes do not express GFP, limiting the scope of multisystem observations. Though we employ a GFP rat in this study, options for transgenic rats remain limited. Another limitation is the lack of perfusion that provides shear stress and interstitial pressure for the maintenance of microvascular structure.

In this study, we leveraged the aging rat mesentery culture model previously used in chapter 3 to investigate aging effects on donor cells and host tissue. Previous work in stem cells has shown that while age has a negative effective on number, migration, and proliferative effect, intervening therapies can delay or counteract aging [133,139,140]. How age effects SVF is still a nascent topic of research. The main results from our aging
studies were that adult SVF transplanted on adult tissue has a significantly great therapeutic effect than any other SVF/host combination. Interestingly, a decreased vasculogenic effect was seen for both the aged SVF + adult tissue and adult SVF + aged tissue groups suggesting that effects due to both cell source and environment are possible. The lack of a difference between adult SVF + adult tissues and aged SVF + aged tissues was surprising and suggests that evidence in the literature reported for SVF aging studies should be interpreted with caution and should include the necessary controls to include environmental effects.

In addition to the limitations of the model, several challenges were encountered when executing the study. When seeding the cells onto the tissue, it was sometimes difficult to know if the SVF had integrated into the tissue, adhered to the surface of the tissue, or adhered to the bottom of the six-well plate. This created three possibly visible planes of cells that obfuscated our ability to track the appropriate cells. To combat this, lectin staining and GFP tissues were both used to identify the correct plane. GFP tissues themselves were sensitive and would lose expression of GFP at any point of contact when spreading onto the filter membrane. Fortunately, this expression came back over time. Further obstacles in SVF yield was experienced in GFP and aged rats. The lower yield in SVF of aged individuals has been documented [141], and the GFP yield could be explained by the obese stature of the rats. The GFP rats weighed as much as 1 kg, and while more fat was harvested, the yield of viable cells was low.

In summary, this study presents the rat mesentery culture model as a tool to make new discoveries into the relationship between angiogenesis and SVF integration into microvascular networks. In addition to time-lapse imaging of SVF vasculogenesis, we
were able to probe structure of the new networks. Vascularized area increased by nearly two-fold in stromal vascular fraction treated tissues and was determined to be largely induced by an SVF derived vasculogenesis that links to preexisting vessels. For the first time in the research, we determined that the angiogenic relationship between SVF and native vessels was caused by proangiogenic support of SVF networks. Individual cells of the SVF were also found to incorporate into the walls of resident vessels. Taking advantage of the aging model development in aim 2, we investigated the relationship of aging and the efficacy of SVF transplantation for both donor cells and recipient tissues.
4.5. Future Studies

While the results of this study provide novel results regarding the angiogenic effect of SVF and the role of aging in the efficacy of SVF therapy, future work can adapt the model to investigate pathological scenarios. The model could accommodate hypertension and diabetic research by switching rat strains to spontaneously hypertensive or Zucker diabetic fatty rats. Further research into the cellular players involved could be done by harvesting fat from transgenic mice since the model is no longer limited to one species. SVF from NG2 knockout mice could determine if the unique pattern displayed in SVF vasculogenesis is pericyte dependent.
CHAPTER 5: CONCLUSIONS

Models that mimic angiogenesis are extremely valuable for elucidating underlining mechanisms and pre-clinical development of therapies. Angiogenesis, defined as the growth of new blood vessels from preexisting vessels, is a multi-cellular process that involves the temporal and spatial coordination between endothelial cells, pericytes, nerves, growth factors, and macrophages. A need exists for biomimetic models that bridge the gap between the mechanistic control of in vitro constructs and the multi-system physiological environment of in vivo models. To meet this need our lab has introduced the rat mesentery culture model as top down approach with intact microvascular networks and a nearly two-dimensional view. Previous development of the model has proven its time-lapse, angiogenic, and lymphangiogenic capabilities. The goal of this work was to advance the model to include the maintenance of peripheral nerves in culture and develop it as a platform for aging and cell therapy studies.

The first aim of this study was to expand the rat mesentery culture model to maintain nerves and the spatiotemporal relationship between nerves and blood vessels in culture. We developed a nerve culture media to prevent regression of nerves. Nerve alignment was maintained at the network feeding arteriole and capillary plexus levels and these nerves possessed neurotransmitter precursors was. We demonstrate for the first time
the ability to maintain adult peripheral nerves in an ex vivo model. In the second aim of this study, we developed an aging rat mesentery culture model as a basis for investigating differences in angiogenesis across age groups. We demonstrated that impaired angiogenesis associated with advanced age could be recovered to adult-like levels with serum and individual growth factor stimulation. The discovery of increased vascular island frequency in aged tissues reveals that the method of angiogenesis for older networks can differ. Our third aim, for the first time, determined that the angiogenic relationship between SVF and native vessels is caused by vasculogenesis and proangiogenic support of SVF networks. We used the aging model in developed in the second aim to show that adult SVF on adult tissue has the greatest therapeutic potential. The combined results of this study have advanced the rat mesentery culture model as a platform to maintain peripheral nerves in culture, investigate the relationship between aging and angiogenesis, and evaluate the therapeutic potential of stromal vascular fraction.
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BIOGRAPHY

Nicholas Aaron Hodges was born on January 8th, 1991 in El Dorado, Arkansas, to Eddie and Robin Hodges and raised with his sister Kassie. He earned his Bachelor of Science in Biomedical Engineering from Mississippi State University in 2013. He joined the Microvascular Dynamics Laboratory at Tulane University in the fall of 2014 to pursue a Ph.D. in Biomedical Engineering under the advisement of Dr. Walter Lee Murfee. His research focused on microvascular model development to maintain peripheral nerves in culture, investigate the relationship between aging and angiogenesis, and evaluate the therapeutic potential of stromal vascular fraction.