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LOYOLA UNIVERSITY CHICAGO

THE EFFECTS OF SMOOTH MUSCLE CELLS ON ENGINEERED MICROVASCULATURES IN FIBRIN HYDROGELS

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CELL BIOLOGY, NEUROBIOLOGY, AND ANATOMY

BY
ARECK A. UCUZIAN

CHICAGO, IL

AUGUST 2012
To Dr. Howard Greisler, without whose encouragement I wouldn’t have started graduate training, and Dr. Richard Gamelli, without whose support I probably couldn’t have finished.
TABLE OF CONTENTS

LIST OF TABLES ............................................................................................................... vi
LIST OF FIGURES ........................................................................................................... vii
LIST OF ABBREVIATIONS ............................................................................................... xii

CHAPTER ONE: INTRODUCTION TO TISSUE ENGINEERED BLOOD VESSELS ........................................... ................................................... .................... 1
Introduction to Tissue Engineering ................................................................................. 1
Introduction to Tissue Engineered Blood Vessels .......................................................... 2

CHAPTER TWO: ENGINEERING ANGIOGENESIS .......................................................... 14
Angiogenic Vascularization of Tissue Engineered Organs ............................................. 14
The Process of Angiogenesis ......................................................................................... 18
Tissue Engineered Blood Vessel and Vasa Vasorum Construction ............................... 31

CHAPTER THREE: AIMS ............................................................................................... 34
Aims ................................................................................................................................ 36

CHAPTER FOUR: THE EFFECTS OF SMCS ON ANGIOGENESIS IN FIBRIN HYDROGELS .......................................................... 39
Introduction .................................................................................................................. 39
Materials and Methods ................................................................................................. 41
Results .......................................................................................................................... 48
Discussion ..................................................................................................................... 58

CHAPTER FIVE: THE EFFECTS OF CO-CULTURE ON EC SIGNALING ...................... 63
Introduction .................................................................................................................. 63
Methods ......................................................................................................................... 64
Results .......................................................................................................................... 74
Discussion ..................................................................................................................... 79

CHAPTER SIX: THE ROLE OF NOTCH IN MEDIATING SMC/EC INTERACTIONS ................................................... 84
Introduction .................................................................................................................. 84
Materials and Methods ................................................................................................. 86
Results .......................................................................................................................... 93
Discussion ..................................................................................................................... 104

CHAPTER SEVEN: SUMMARY OF RESULTS AND CONCLUSIONS .................... 110
Aim 1 ............................................................................................................................. 110
Aim 2 ............................................................................................................................. 111
Aim 3 ............................................................................................................................. 111
Overall Summary of Major Findings ............................................................................ 112
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Summary of Conditioned Media and Controls Tested in EC Assays in Aim 2.</td>
<td>74</td>
</tr>
<tr>
<td>2. Summary of Experiments in Aim 3.</td>
<td>92</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Components of the vessel wall.</td>
<td>6</td>
</tr>
<tr>
<td>2.</td>
<td>EC coverage and SMC proliferation. SMC proliferation decreases with increased percent re-endothelialization in vivo using a canine carotid artery balloon angioplasty model.</td>
<td>17</td>
</tr>
<tr>
<td>3.</td>
<td>Diagrammatic description of the steps involved in angiogenesis. A) A stable vascular structure comprised of an endothelial monolayer surrounded by mural tissue (i.e. basement membrane, pericytes, and adventitia); B) Destabilized vessel demonstrating the disruption of mural tissue by endothelial cells; C) Endothelial cell invasion into perivascular tissue and subsequent sprout formation; D) Endothelial cell lumenogenesis and tube formation with inosculcation (not shown) to other pre-existing vascular structures; E) Stabilization of the capillary sprout by recruitment of pericytes and deposition of basement membrane.</td>
<td>19</td>
</tr>
<tr>
<td>4.</td>
<td>TEBV Construction. (A) A pre-polymerized fibrin hydrogel containing SMCs (not shown) are poured around a mandrel. B) EC aggregates polymerized within the hydrogel. C) ECs form microvasculatures. D) Final structure.</td>
<td>32</td>
</tr>
<tr>
<td>5.</td>
<td>Creation of TEBV. By 4 days of static culture, compaction of the hydrogel around the central mandrel is noted. On Day 7, the TEBVs are placed within a bioreactor chamber.</td>
<td>32</td>
</tr>
<tr>
<td>6.</td>
<td>Pericytes in capillaries. Left) Pericytes are seen associated with the abluminal surface of a capillary comprised of 1-2 ECs. Right) Depiction of pericyte recruitment by an angiogenic EC sprout. Modified from Armulik, et al.</td>
<td>34</td>
</tr>
<tr>
<td>7.</td>
<td>Technique of co-culture assay #1. SMC (green with blue outline) containing fibrin hydrogel layer is plated on a 96 well flat bottom plate. Fluorescently labeled aggregate of ECs (red) are placed on top of gel and covered with second fibrin hydrogel containing SMCs and then cultured in angiogenesis assay media.</td>
<td>44</td>
</tr>
<tr>
<td>8.</td>
<td>Quantification of angiogenesis. ALS=Average length of sprouts; DSF=Density of sprout formation; L=Length; R=Radius of EC pellet.</td>
<td>46</td>
</tr>
<tr>
<td>9.</td>
<td>Coculture assay #2. SMCs green are aggregated in a methylcellulose containing solution and seeded with ECs (red). The co-culture aggregated is then embedded between two layers of fibrin and cultured in angiogenesis assay media.</td>
<td>47</td>
</tr>
</tbody>
</table>
10. Co-culture aggregate assay. Left) Fluorescently green labeled SMC aggregate. Middle) Fluorescently labeled red ECs coating SMC aggregate. Right) Merged image of red ECs covering the surface of the green labeled SMC aggregate. Mag.10x..........................................................................................................................48

11. Sprouting from co-culture assay #1. Left) Fluorescently labeled green SMCs are homogenously suspended within the fibrin hydrogel. Middle) Fluorescently red labeled ECs are seen sprouting from a central aggregate of ECs into the matrix. Right) Merged image of ECs sprouting into the ECM containing SMCs which associate with EC sprouts. Mag 10x. (Lower image) Digital zoom of merged image..................................................................................49

12. Cross sectional morphology of co-culture sprouts by transmission electron microscopy. Selected images demonstrate EC lined luminal structures with SMCs apposed to the abluminal surface of the capillary. The structure is surrounded by the fibrin matrix. ECM = extracellular matrix; L = Lumen; Arrows = SMCs; Arrowheads = ECs..................................................................................................................50

13. Average length of EC sprouts cultured in the presence of absence of SMCs in media containing VEGF and FGF-2 (10 ng/mL for each growth factor). ..........51

14. Average length of EC sprouts cultured in the presence of absence of 22,500 SMCs in media containing VEGF and FGF-2 (10/ng/mL for each growth factor). After 3 days, the media was replaced with media that did not contain exogenous growth factors. ........................................................................................................................51

15. Angiogenesis after VEGF, FGF-2 withdrawal in the presence and absence of SMCs (only ECs shown for simplification). (Mag. 4x).........................................................52

16. Quantification of angiogenesis in the presence and absence of SMCs (400,000 SMCs/mL) in the absence of exogenous growth factors. ..................................................53

17. Angiogenesis in the presence and absence of SMCs (400,000 SMCs/mL) in the absence of exogenous growth factors. Only ECs shown for simplification (Mag. 4x).........................................................................................54

18. Invasion of ECs and SMCs into fibrin from co-culture aggregates. Left) ECs (red) co-invade with SMCs (green) into fibrin hydrogel (Mag. 10x). Right) Average length of EC and SMC invasion in co-culture aggregate model. ..........55
19. Treatment of SMCs with MMC inhibits SMC (a) proliferation and (b) matrix invasion. The effects of MMC treatment on SMC proliferation and matrix invasion in the absence of growth factors. (a) SMC proliferation. SMCs either treated or untreated with MMC were seeded onto 96 well plates and cultured in indicated media. SMC proliferation was determined by quantifying tritiated thymidine incorporation. Results are mean CPM +/- SEM from a representative experiment (n=4). (b) SMCs either treated or untreated with MMC were embedded within fibrin hydrogels and matrix invasion was quantified. Results represent the mean distance of invasion +/- SEM from a representative experiment (n=4-5). * p < 0.05 vs. MMC untreated control.

20. Inhibiting SMC proliferation with MMC inhibits both SMC and EC matrix invasion into fibrin hydrogels in the co-culture aggregate model.

21. EC sprouts in co-culture aggregate model in the presence and absence of SMCs. Only EC sprouts are shown for simplification. (Mag. 4x)

22. Quantification of EC invasion in co-culture aggregate model. ECs were cultured in the presence and absence of SMCs in the co-culture aggregate model and average length of sprouts (left) and density of sprout formation was quantified (right).

23. PI3-K/Akt pathway.

24. The Ras/Raf/ERK 1/2 pathway.

25. Schematic depiction of conditioned media collection utilized as a stimulant for EC proliferation, migration, angiogenesis and signaling. (Left) 2-D SMC-conditioned media. SMCs are cultured on Petri dishes and the media is collected after 72 hours. (Bottom left) Unconditioned media is used as a control for experiments utilizing 2-D SMC-conditioned media. (Right) 3-D Fibrin-SMC-conditioned media. SMCs are cultured in fibrin hydrogels and the culture media is collected after 72 hours. (Bottom right) Media conditioned by acellular fibrin gels are used as controls for experiments utilizing 3-D SMC-Fibrin-conditioned media.

26. Directional invasion for co-culture aggregates. (Left) Individual SMC/EC co-culture pellets demonstrate random invasion into hydrogel. (Right panels) Co-culture pellets demonstrate directional invasion towards one another in hydrogels.

27. SMC-conditioned media induces EC proliferation. EC were cultured in the presence SMC-conditioned or unconditioned media and EC mitogenicity was determined by tritiated thymidine incorporation. (Left) ECs stimulated by media conditioned by SMCs in 2-D culture. (Right) ECs stimulated by media conditioned by SMCs in 3-D culture.
28. SMC-conditioned media induces EC migration. (Top panels) Migratory cells in a representative quadrant (Mag. 4x) in a modified Boyden chamber assay in response to unconditioned (left) and SMC-conditioned (right) media. ..............76

29. SMC-conditioned media induces angiogenesis. EC cultured in unconditioned media (top left) vs. SMC-conditioned media (top right). Bottom) Average length of EC sprouts cultured in SMC-conditioned vs. unconditioned media. .....77

30. Levels of phosphorylated endothelial cell ERK after stimulation with SMC-conditioned or unconditioned media. Cell lysates were collected and pERK levels were quantified by Western blot analysis..................................................78

31. Levels of phosphorylated endothelial cell Akt after stimulation with SMC-conditioned or unconditioned media. Cell lysates were collected and pAkt levels were quantified by Western blot analysis..................................................78

32. Levels of phosphorylated Akt in ECs after stimulation with fetal bovine serum. ECs were plated on 30mm Petri dishes and stimulated by angiogenesis assay media with or without serum for 20 minutes. Cell lysates were collected and pAkt levels were assayed with Western blot analysis........................................79

33. Summary of results of Aims 1 and 2. ...............................................................83

34. Ligand-mediated Notch signaling. .................................................................85

35. ECs treated with DAPT and stimulated with growth factors for 20 minutes demonstrate increased levels of phosphorylated Akt compared to DAPT untreated cells stimulated with growth factors. *p < .05. ........................................93

36. Notch inhibition and growth factor induced ERK1/2 phosphorylation in ECs. Notch inhibition with DAPT does not affect levels of phosphorylated ERK 1/2 in growth factor stimulated ECs (20 minutes of stimulation) treated with DAPT compared to DAPT untreated cells. ........................................94

37. Notch inhibition has no effect on levels of phosphorylated Akt or total Akt levels in response to VEGF and FGF-2 after 48 hours of stimulation.................96

38. Notch inhibition has no effect on levels of phosphorylated or total ERK 1/2 levels in response to VEGF and FGF-2 after 48 hours of stimulation. * < .05.................................................................98

39. Dll-4 expression on ECs and SMCs in the presence and absence of VEGF + FGF-2 (10ng/mL for each growth factor). ..........................................................99

40. Notch receptors on ECs and SMCs in the presence and absence of VEGF + FGF-2 (10ng/mL for each growth factor). ..........................................................99
41. Notch inhibition on levels of phosphorylated Akt in ECs stimulated with SMC derived soluble factors. ECs were plated on Petri dishes and stimulated with SMC-conditioned media either in the presence or absence of DAPT for 20 minutes. ................................................................. 100

42. Effect of Notch inhibition on levels of phosphorylated ERK in response to SMC derived soluble factors. ECs were plated on Petri dishes and stimulated with SMC-conditioned media either in the presence or absence of DAPT. ................. 101

43. Eosin uptake in fibrin hydrogels. Fibrin hydrogels were cultured in media containing the fluorophore eosin. Fluorescence in the gel and the media was measured. * < .05. ........................................................................ 102

44. Effect of Notch inhibition on angiogenesis in response to SMC derived soluble factors. ECs were aggregated and embedded within fibrin hydrogels. They were cultured in SMC-conditioned media either in the presence of absence of DAPT. ................................................................. 103

45. Summary of results. Aggregated ECs in fibrin form sprouts containing lumens which are surrounded by SMCs, resembling native capillaries histologically. SMCs promote the formation and persistence of the engineered 3-D capillary structures, in part by the release of soluble factors into the extracellular environment. These SMC-released products have the effect of increasing phosphorylated ERK 1/2 and unphosphorylated Akt in ECs. The increase in unphosphorylated Akt in ECs in response to SMC-released factors is EC Notch dependent. ................................................................. 110
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3-D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>Akt</td>
<td>aka Protein Kinase B</td>
</tr>
<tr>
<td>ALS</td>
<td>Average length of sprouts</td>
</tr>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>Bcl</td>
<td>B-cell CII/Lymphoma</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>DAPT</td>
<td>$N\cdot[N\cdot(3,5\text{-difluorophenacetyl})\cdot l\text{-alanyl}]\cdot S\cdot \text{phenylglycine}\ t\text{-butyl ester}$</td>
</tr>
<tr>
<td>Dll</td>
<td>Delta like ligand</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSF</td>
<td>Density of sprout formation</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cells</td>
</tr>
<tr>
<td>ePTFE</td>
<td>Expanded polytetrafluoroethylene</td>
</tr>
</tbody>
</table>
ERK  Extracellular signal related kinase
FBS  Fetal bovine serum
FGF  Fibroblast growth factor
FITC Fluorescein Isothiocyanate
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
HBSS Hank's buffered salt solution
HRP  Horseradish peroxidase
M199 Medium 199
MAPK Mitogen activated protein kinase
MMC Mitomycin C
MT-MMP Membrane type - Matrix metalloproteinase
NG2 Nerve/glial antigen 2
NICD Notch intracellular domain
P4HB Poly-4-hydroxybutyrate
PAI  Plasminogen activator inhibitor
PBS  Phosphate buffered saline
PCLA Polycaprolactone
PCN  Penicillin
PDGF Platelet derived growth factor
PEG  Polyethylene glycol
PET  Polyethylene terephthalate
PGA  Polyglycolic acid
PI3-K Phosphoinositide 3-Kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEBV</td>
<td>Tissue engineered blood vessel</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Tie</td>
<td>Tyrosine kinase with immunoglobulin-like and EGF-like domains</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethyl Rhodamine Iso-Thiocyanate</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula occludens</td>
</tr>
<tr>
<td>α-SMA</td>
<td>alpha smooth muscle cell actin</td>
</tr>
</tbody>
</table>
CHAPTER ONE
INTRODUCTION TO TISSUE ENGINEERED BLOOD VESSELS

Introduction to Tissue Engineering

“Tissue engineering,” as originally described by Robert Langer and Joseph Vacanti, is “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function.” In practice, the potential applications for tissue engineering are as broad and inclusive as the methods and modalities employed to achieve the above described goal. The somewhat amorphous field includes endeavors which range from the decellularization of existing tissues for implantation in the body, as in many commercially available abdominal wall hernia meshes and biologic heart valves, to the yet unrealized goal of organ creation de novo for complex applications such as organ transplantation.

The intrinsic multidisciplinary nature of tissue engineering, while allowing for exciting, unusual, and often “out of the box” project development, nevertheless dictates that optimal results will be achieved at the delicate intersection of stringent hypothesis-based research and the almost alchemic creativity of bioengineering. The following project attempts to approach this intersection by describing a strategy for the development of a microvascular network in a commonly used tissue engineering scaffold, while investigating the basic biologic processes which affect their creation. The
microvascularization of tissue engineered organs, including tissue engineered blood vessels large enough for use as vascular conduits for bypass or for organ perfusion, is thought to be a critical need in tissue engineering. The ultimate goal of this project, hopefully, is to both elucidate the basic mechanisms in the angiogenic process which can be exploited and altered for the practical purpose of tissue construction, in the case of this project a tissue engineered blood vessel (TEBV), and demonstrate a modality for the creation of more complex and precise 3-D *in vitro* models which may be used to model *in vivo* biologic processes in the laboratory.

**Introduction to Tissue Engineered Blood Vessels**

The Clinical Need for Biologic Alternatives to Synthetic Vascular Bypass Grafts

Since its first use in 1906 by Goyanes to repair a popliteal arterial aneurysm, native autogenous vessels have served as the gold standard material in the treatment of many vascular diseases. They function as bypass conduits for aneurysmal defects and stenoses in the peripheral or coronary vascular system, as patches for the closure of arteriotomies, or as conduits for the creation of arteriovenous fistulae for patients receiving hemodialysis. However, a significant percentage of patients lack suitable autogenous vessels for use in vascular interventions either due to intrinsic vascular disease or due to their use in previous vascular procedures. For these patients, alternative synthetic grafts comprised most commonly of expanded polytetrafluoroethylene (ePTFE) and polyethylene terephthalate (PET) are the most commonly used when suitable autogenous vessels are lacking.
While these synthetic alternatives have generally been suitable for the bypass of larger vessels, their long-term success in bypasses of arteries of smaller caliber (<6 mm) has been limited. The inferiority of ePTFE in comparison to native vessel conduits such as saphenous vein is most pronounced for the bypass of more distal targets. In addition, while bypass of the femoral artery to the more proximal target of the popliteal artery above the knee with ePTFE has demonstrated comparable rates of patency in comparison to saphenous vein within the first year or two, it is apparent that the intermediate to longer term results of these more proximal bypasses are also less successful. The primary 5 year patency (the duration of follow-up in which there is an absence of occlusion or clinically significant restenosis within the treated segment) of bypasses from the femoral artery to the popliteal artery above the knee utilizing saphenous vein range from 73%-77.2% versus 39%-57% for bypasses utilizing ePTFE.\textsuperscript{11,12} The patency of bypasses to the popliteal artery below the knee are significantly worse than the patency of bypasses to the above-knee popliteal artery. Long term results of infrapopliteal bypasses (bypasses to the tibial or peroneal arteries) with ePTFE are even less successful. In a meta-analysis of 43 studies, pooled estimates for the 5 year primary patency, secondary patency (patency that has been restored by the treating physician after occlusion of the treated arterial segment), and limb salvage rates of infrapopliteal bypasses with ePTFE were 30.5%, 39.7%, and 55.7%, respectively.\textsuperscript{13} Although not commonly used for infrainguinal bypasses, PET (tradename Dacron) demonstrates comparable patency rates to ePTFE when used for femoro-femoral, axillo-femoral, or femoro-popliteal bypasses.\textsuperscript{14-16}
These poor intermediate and long-term results of available synthetic conduits used for bypasses in the peripheral vasculature in comparison to those of native vessels have led researchers to investigate the creation of wholly tissue engineered biologic alternative for patients lacking suitable autogenous vessels. Among the strategies detailed below for creating these biologic alternatives include constructing conduits comprised of hydrogels such as fibrin, collagen, polyethylene glycol (PEG), and others. It is our overall hypothesis that biologic alternatives with sufficient thickness to withstand the pulsatile pressures of the circulatory system will require a vasa vasorum to support smooth muscle cells within the outer 2/3’s of the blood vessel tunica media. The studies described in the Aims of this study are meant to model both the technical creation of these hydrogel based blood vessel alternatives as well as the basic biologic processes which occur during the angiogenic development of the vasa vasorum within the wall of these constructs.

Strategies for Engineering Blood Vessels

The studies within this project are meant to model angiogenic processes within the tissue engineered blood vessel tunica media. For clarification purposes, the term “tissue engineered blood vessel (TEBV)” refers to the muscular arteries utilized as conduit alternatives, while the term “engineered microvasculature” refers to the engineered capillaries which will serve as the source of the TEBV vasa vasorum. The following is a brief introduction to major strategies for the creation of tissue engineered blood vessels in order to provide context to the studies within this dissertation.
General Concepts

While methodologies described in the literature for the development of a TEBV can seem infinitely variable, they are founded on one ultimate goal. That is to organize a structure comprised of cells and extracellular matrix (ECM) which mimics the biologic functionalities of a native blood vessel, including mechanotransduction and vasoreactivity for the maintenance of vessel wall and hemodynamic homeostasis.

Specifically, a TEBV should be comprised of a cellular intimal component containing a quiescent, anti-thrombogenic endothelial monolayer that transduces signals from shear and cyclic stresses created by blood flow and a TEBV *media* comprised of organized quiescent and vasoactive smooth muscle cells (SMCs). The extracellular matrix component should be organized circumferentially by an internal and external elastic lamina, and an arterial *media* organized as fibers of collagen and elastin oriented perpendicular to the direction of blood flow to allow for proper compliance and elastic mechanical compatibility. Additionally, a *vasa vasorum* which anastomoses with the host vasculature, hypothetically required for the long-term viability of cells within the outer 1/3 to 2/3 of the arterial *media* and *adventitia* incapable of receiving nutritional and oxygen support from the circulating blood flow (discussed in the following chapter). The engineering of an *adventitia* which provides mechanical support as well as vascular support for the TEBV is a less common focus, but is likely to be of some importance in the construction of a TEBV (Figure 1).
Investigating optimal methodologies and materials for organ cellularization, the construction or direction of ECM deposition, and the allowance for proper cell-matrix interactions requisite to the aforementioned biologic functionalities are theoretically endless and among the widely studied topics. The broad strategic categories in tissue engineering include 1) the implantation of cells, materials, and bioactive molecules and drugs \textit{in vivo}, utilizing the host body as a living bioreactor which responds to the implanted agents and formulates the final structure after implantation; 2) the complete creation of a tissue or organ \textit{ex vivo} prior to implantation; or 3) a combination of both strategies.

The sourcing of the cellular component, the materials utilized as scaffolds or drug delivery vehicles, and the nature of the genetic and protein level manipulations are fundamental and exciting territories in the field of tissue engineering. In addition, due to the advent of bioresorbable materials, the engineering of blood vessels from bioresorbable polymers which are replaced by the host tissue as a result of normal
vascular healing has become a possibility. The following discussion will focus on hydrogel-based TEBVs, a widely supported approach for TEBV construction, and the strategy which the studies of this project are intended to model. It will also briefly describe the concept of self-assembled TEBVs, bioresorbable TEBVs, and TEBVs constructed from decellularized vessels.

*Hydrogel-Based TEBVs*

Natural hydrogel polymers such as fibrin, collagen, alginate, hyaluronan, as well as synthetic hydrogels such as poly-ethylene glycol (PEG) and polyvinyl alcohol are commonly used in tissue engineering as scaffolds or components of scaffolds for a variety of engineered tissues including TEBVs, and are the strategy which is modeled within this dissertation.\(^{17}\) The first credited attempt at the creation of a TEBV by Weinberg and Bell in 1986 utilized a collagen hydrogel scaffold constructed as a tubular structure with concentric layers of endothelial cells, smooth muscle cells and fibroblasts.\(^{18}\) While poor burst strengths, a common limitation to hydrogel based TEBVs, necessitated the use of supporting sleeves, comprised of PET in the Weinberg and Bell studies, for mechanical support, these investigators demonstrated the feasibility of TEBV creation utilizing hydrogels as scaffolds. In addition, while early and most current studies focus on early mechanical properties *ex vivo*, a full understanding of the short and long-term mechanical and cell functional properties will require *in vivo* studies in animal models.

Several modifications to hydrogel based TEBVs have been developed in order to improve the mechanical strength of the constructs. These include chemical modifications...
which regulate the degradation of the hydrogel matrix, induce the cellular deposition of endogenously produced matrix proteins like collagens and elastins, and promote covalent cross linking among ECM fibers. For example, mechanical properties of fibrin-based TEBVs can be modified by the addition of plasmin inhibitors such as aprotinin, cross-linking agents, and media supplements such as insulin and TGF-β which induce endogenous matrix deposition by embedded cells.\textsuperscript{19,20} The use of media supplements such as retinoic acid and ascorbic acid has been shown to promote SMC elastin and collagen mRNA and protein expression within type I collagen tubular constructs.\textsuperscript{21} This was associated with increased TEBV tensile strength and decreased stiffness compared to untreated TEBVs. Finally, TEBVs can be constructed in layers varied by the composition of the ECM and cellular component, with each layer performing separate functions. For example, fibrin-based TEBVs with an inner layer containing a lower concentration of fibrinogen to support SMC vasoactivity, and an outer cell-free layer comprised of a higher concentration of fibrinogen for mechanical stability demonstrated significantly improved burst strengths compared to single layer constructs while maintaining vasoreactivity.\textsuperscript{22}

Mechanical preconditioning with cyclic strain bioreactors has also been efficacious in improving the structural integrity of hydrogel based TEBVs. In collagen based constructs, this has been shown to be attributable in part by the modulation of cell and matrix organization, as well as by the alteration of the expression of matrix metalloproteases (MMPs).\textsuperscript{23,24} Recent studies also demonstrated that the application of incremental cyclic distention from 5% to 15% significantly improved tensile strength,
tensile modulus, and collagen deposition per cell in fibrin-based TEBVs. In addition, a direct comparison of the mechanics of fibrin, collagen, and fibrin-collagen TEBVs showed that the mechanical properties of the TEBV were not only dependent on the application of mechanical strain, but also on the composition of the hydrogel scaffold. Linear modulus was directly associated with collagen content, and gel compaction (a process resulting in a more compact matrix secondary to cellular behavior or biomechanical stimuli), which was associated with improved tensile strength, was augmented by the addition of fibrin. This study also found that cyclic mechanical strain not only augmented the tensile strength and gel compaction of collagen-fibrin TEBVs, but also decreased cellular proliferation. The effects of mechanical strain on cellular phenotype is further exemplified by increased levels of vWF, nitric oxide, SMC α-actin, and SMC myosin heavy chain in mechanically conditioned collagen/PLGA (poly (lactic-co-glycolic acid)) TEBVs containing ECs and SMCs compared with statically cultured TEBVs.

In addition, this study demonstrated that mechanical strain affected cellular orientation, with embedded cells aligning radially in mechanically conditioned TEBVs in contrast to the random alignment of statically cultured TEBVs.

Bioresorbable TEBVs

Bioresorbable materials can serve as temporary scaffolds for vascular prostheses which are replaced by the native tissue or as polymeric vehicles from which drugs can be eluted with controlled kinetics. Implanted devices comprised of these materials, such as drug eluting stents, can serve their functional purpose for the time it is required and then histologically “disappear,” theoretically minimizing the long-term inflammatory and
injury responses and the potential for graft infection. As a scaffold for a tissue engineered blood vessel, bioresorbable materials can serve as “smart” materials that are modified to promote native tissue infiltration after implantation. Knowledge of the resorption kinetics of specific bioresorbable materials based on the intrinsic properties of the materials being used are critical to controlling host-material interactions which lead to tissue incorporation.\textsuperscript{28-31}

Some clinical experiences with bioresorbable grafts have provided encouraging results. Because current synthetic prosthetic materials cannot grow proportional to the growth of the native tissue during child development, bioresorbable TEBVs are especially useful in the pediatric population. TEBVs comprised of a composite PGA (\textit{polyglycolic acid})/poly-4-hydroxybutyrate (P4HB)) scaffold were seeded with vascular cells and implanted in the pulmonary arteries of a growing lamb model in which body size doubled over a two year period.\textsuperscript{32} Similar to the growth of native vessels, the TEBVs demonstrated a 30\% increase in diameter and a 45\% increase in length over time, with concomitant increases in collagen, proteoglycan, and cellular content. In addition, there was no evidence of thrombus formation, calcification, stenosis, suture dehiscence, or aneurysm in any of the implanted grafts. Decreased elasticity and an absence of elastin in the TEBVs in comparison to native pulmonary arteries was noted, however.

In 2001, a TEBV comprised of a bioresorbable polycaprolactone (PCL) / polylactic acid (PLA) tubular scaffold seeded with autogenous cells were implanted into the pulmonary artery of a 4 year old child with pulmonary artery atresia.\textsuperscript{33} At seven month follow up, the patient was doing well, with no evidence of aneurysm formation or
graft occlusion. Subsequent efforts utilized autologous bone marrow cells as a cell source to seed hybrid scaffolds of a copolymer of PLA and PCLA with a PGA reinforcement. These were implanted as grafts for extracardiac total cavopulmonary connections in 23 patients (ages 1-24) with congenital heart defects with a 6 year follow-up.\textsuperscript{33,34} Computed tomography studies did not demonstrate evidence of aneurysmal dilation and there was only one case of graft stenosis which was successfully treated with angioplasty. With evidence of growth of the vessel conduit over the time period, these results provided encouraging initial clinical experiences. The investigators have received IRB approval and are hoping to begin clinical trials in the United States in the near future.

\textit{Self-Assembled TEBVs}

A promising strategy for TEBV construction which has been tested in human subjects is the self-assembly strategy described by L’Heureux and Auger et al.\textsuperscript{35} Human SMCs were cultured with ascorbic acid and served as a cellular sheet which was wrapped around a tubular support to produce the \textit{media} of the vessel. A second sheet generated by cultured human fibroblasts was then wrapped around the media to provide the vessel \textit{adventitia}. After a period of culture, the tubular support was removed and ECs were seeded onto the lumen of the TEBVs. This methodology is advantageous as it avoids the use of any foreign materials, and relies on the cellular components to provide for the ECM scaffold. Implantation of these cell-assembled TEBVs in a canine model demonstrated tissue incorporation with intact endothelium, \textit{vasa vasorum}, and an arterial \textit{media} containing \(\alpha\)-SMA positive SMCs as well as elastin and collagen.\textsuperscript{36} Mechanical
stability was demonstrated out to 8 months after implantation. Implantation in a limited number of human subjects for hemodialysis access has demonstrated encouraging early results, although long-term aneurysmal dilatation has appeared to be a limitation of this strategy.\textsuperscript{37,38} Additionally, the significant time required for construction of these TEBVs, a common criticism for most TEBV construction strategies, have brought the real-world cost-effectiveness of this approach in question.

\textit{Decellularized Vessel Scaffolds}

Decellularized veins and arteries are a potential scaffold for TEBVs, and have the obvious advantage of possessing intact native vascular ECM structures.\textsuperscript{39,40} Animal experiments have demonstrated some success with this methodology. Explanted decellularized grafts seeded with autologous ECs and implanted in animal models have demonstrated EC lined lumens at the time of explant with repopulating SMCs after four weeks.\textsuperscript{41,42} Of note, 89\% of grafts seeded with ECs prior to implantation remained patent at four weeks, with only 29\% of unseeded grafts remaining patent over the same time period. A decellularized cryopreserved human vein allograft which was seeded with autologous ECs in a bioreactor was implanted in a human patient for iliac vein reconstruction. This demonstrated the feasibility of this approach, although the tissue engineered graft failed after 4 months, reportedly secondary to progression of disease.\textsuperscript{43}

Each of these strategies is associated with their own unique set of advantages and disadvantages. The interest of our lab is that of promoting the growth of a microvasculature within the wall of a hydrogel-based TEBV as a functional \textit{vasa vasorum}. Our overall project hypothesis is that TEBVs of sufficient thickness
withstand arterial pressures, as in native vessels, will require a *vasa vasorum* to provide nutritional and oxygen support to cells in the outer 1/3-2/3 of the blood vessel wall. The studies described are intended to model this process utilizing fibrin, one scaffold of interest for our lab.
CHAPTER TWO

ENGINEERING ANGIOGENESIS

Angiogenic Vascularization of Tissue Engineered Organs

The study of angiogenesis has clinical applications in numerous fields including peripheral and coronary vascular disease, oncology, hematology, wound healing, dermatology, ophthalmology, and many others. Experimental evidence demonstrates that the promotion of angiogenic processes can have potential therapeutic benefit in ischemic tissue, while the study of anti-angiogenic therapies have yielded several FDA approved additions to chemotherapeutic regimens in the field of oncology.  

In tissue engineering, the survival of thicker constructs will likely require either a microvascular network during development or the ability to rapidly foster neovascular ingrowth from a pre-existing vasculature after implantation. (Black et al., 1998) The observation that metabolically active cells cannot survive >100-200µm (the distance depending primarily on cell type) from their nutrient source is consistent with this hypothesis. (Folkman and Hochberg 1973) As a result of this critical limitation, successes in tissue engineering have resulted in relatively thin and simply organized implants. In addition, more complex organs containing segmental specialized native vascular supplies such as the liver, for example, will in turn require increasingly sophisticated vascularization methods during organ construction. Thus, the study of angiogenesis and
the development of strategies which allow for precise control of angiogenic mechanisms within tissue engineered constructs has broad applications in the field of tissue engineering. The studies in this dissertation are meant to model angiogenic processes which occur within the wall of a fibrin hydrogel based tissue engineered blood vessel during the engineering of a TEBV vasa vasorum.

Vascularization for Tissue Engineered Blood Vessel Viability

The ex vivo development of a 3-D vascular conduit resembling a native vessel which can be utilized as an alternative for synthetic bypass grafts in vasculopathic patients, as a model for studying vascular biology, or as a conduit for other more complex tissue engineered organs is of great interest in tissue engineering. Although progress has been made, this endeavor continues to present significant challenges. Chief among these is that TEBVs thick enough to withstand arterial physiologic pressure will likely need a mature and functional microvasculature akin to a vasa vasorum in order to meet the metabolic and gas exchange requirements of SMCs in the outer two thirds of the arterial media. A normal artery, usually greater than 1mm in thickness, requires the presence of a vasa vasorum through the outer two-thirds of the arterial media because diffusion of oxygen and nutrients from intraluminal circulating blood can only efficiently support the inner 100-200 µm of the arterial media. It is logical, then, that for the development of a sufficiently thick conduit able to withstand arterial pressures (at least 1mm wall thickness in the published reports), the growth and maturation of a vasa vasorum with subsequent inosculation with the host capillary system will be essential to long-term functionality and viability of a TEBV. This is further supported by several observations: 1) with the
exception of a handful of tissues, most cells within the body are no further than 200µm from capillary support; 2) removal of peri-aortic fat (with vasa vasorum) decreases aortic distensability, increases medial necrosis, and causes alterations in elastic fibers and the collagen/elastin ratio;\textsuperscript{47} and 3) arterialized vein grafts without vasa vasora support have documented hypoxia in the middle portion of the vessel’s cross-sectional thickness.\textsuperscript{48}

These observations and others have led us to develop the overall hypothesis that the presence of a prevascularized structure will have the benefit of promoting the viability of TEBV SMCs after implantation with earlier inosculation within the host circulation.

Engineered Vasa Vasorum as a Source of Intimal Development

In addition to its benefits on TEBV viability, we hypothesize that the presence of microvascular networks within the wall of a TEBV would have the secondary benefit of promoting a more durable construct by serving as a source of a viable endothelium at the luminal surface. A quiescent endothelial monolayer provides a non-thrombogenic and mechano-transductive surface which is thought to be critical in the prevention of synthetic SMC activity which leads to the intimal hyperplastic response after vascular injury (Figure 2).\textsuperscript{1} This effect is partially dependent on the phenotypic characterization of the endothelium, however, as an endothelium activated by injury may function to promote thrombosis or intimal hyperplasia by the upregulation and release of adhesion receptors, vWF, tissue factor, endothelin-1, PDGF, and other EC products.\textsuperscript{49,50}

Thus, it is believed that the induction of endothelialization on biomaterial blood-contacting surfaces is critical in establishing the long-term biocompatibility of both vascular grafts and tissue engineered blood vessels, and it is likely that the inability of
Figure 2. EC coverage and SMC proliferation. SMC proliferation decreases with increased percent re-endothelialization in vivo using a canine carotid artery balloon angioplasty model.\textsuperscript{1}

humans to promptly generate a quiescent functional endothelium and establish normal histological archeology in the coronary and peripheral vascular system after graft implantation or intravascular intervention impacts the long-term patency of synthetic vascular prostheses.\textsuperscript{51}

This phenomenon is not simply a function of insufficient EC proliferation, as the cessation of EC ingrowth approximately 1-3 cm from the edges of vascular anastomoses is seen even in the presence of elevated EC mitotic activity at these points on standard ePTFE grafts (30µm internodal distance) implanted clinically.\textsuperscript{52-54} Early observations in animal models suggested that ePTFE grafts with larger void spaces (60µm internodal distance) compared with standard ePTFE (30µm internodal distance) demonstrated increased EC coverage on the luminal surface.\textsuperscript{52} This has led to the general belief that a significant component of vascular endothelialization may result from transmural angiogenic mechanisms which consequently seed the luminal surface of the graft, in
addition to 1) the relatively limited spontaneous transanastomotic endothelial pannus ingrowth, and likely, 2) the deposition and differentiation of endothelial progenitor cells onto the de-endothelialized surface within the circulation.\textsuperscript{52,55-58}

Strategies which can augment more rapid graft endothelialization in available and investigational prostheses will likely prove useful.\textsuperscript{59,60} These strategies can be broadly separated into two categories: 1) the \textit{in vivo} augmentation of transmural angiogenesis, spontaneous anastomotic endothelialization, and endothelial progenitor cell (EPC) incorporation utilizing locally or systemically delivered drugs like angiogenic growth factors,\textsuperscript{61-64} and 2) the seeding of ECs or EPCs onto the graft surface via natural and synthetic polymeric coatings with or without adjunctive angiogenic growth factors prior to implantation.\textsuperscript{65-68}

Thus, it is our belief that the prevascularization of tissue engineered blood vessels will serve to improve construct cellular viability within the arterial \textit{tunica media}, provide for earlier inosculation with the host vasculature, and source an endothelial-lined \textit{intima}.

\textbf{The Process of Angiogenesis}

In order to construct a TEBV \textit{vasa vasorum} (or a microvasculature of any tissue engineered organ), a major interest in many tissue engineering labs, including ours, is the modulation of angiogenic mechanisms within the scaffolding of tissue engineered constructs. The delivery of natural and synthetic growth factors, modifications of genes and cell signaling pathways, and alterations in the extracellular matrix composition have been some of the strategies used in order to promote microvascular network formation. A discussion of the process of angiogenesis is thus critical in understanding the
fundamental strategies utilized in tissue engineering in order to optimize microvascular development (Figure 3).

Figure 3. Diagrammatic description of the steps involved in angiogenesis. A) A stable vascular structure comprised of an endothelial monolayer surrounded by mural tissue (i.e. basement membrane, pericytes, and adventitia); B) Destabilized vessel demonstrating the disruption of mural tissue by endothelial cells; C) Endothelial cell invasion into perivascular tissue and subsequent sprout formation; D) Endothelial cell lumenogenesis and tube formation with inosculcation (not shown) to other pre-existing vascular structures; E) Stabilization of the capillary sprout by recruitment of pericytes and deposition of basement membrane.

New blood vessel formation, or neovascularization, occurs either by angiogenesis or by vasculogenesis. Vasculogenesis occurs primarily in utero and in select situations in adults such as during tumor growth, myocardial revascularization, and endometriosis. It is the creation of new vessels de novo from precursor cells, such as angioblasts, which differentiate into endothelial cells (ECs), form lumens, and create primitive blood vessels. In contrast, angiogenesis is the formation of new capillaries from the preexisting vasculature. Arteriogenesis, or collateralization, results from the hypertrophy and luminal distention of preexisting vessels in response to mechanical stresses caused by redirected blood flow from occluded or stenosed distal vessels, mediated in part by
mechanosensitive signaling in vascular wall cells and by macrophage-derived biomolecular signals.\textsuperscript{69}

The process of angiogenesis involves a complex and dynamic interaction between ECs and the corresponding extracellular environment. \textit{In vivo}, angiogenesis occurs either by the sprouting of vascular ECs from pre-existing capillary endothelia into the surrounding tissues, or by intussusception (aka non-sprouting angiogenesis), which involves the division of capillaries by tissue pillars into two or more daughter vessels.\textsuperscript{70,71} The distinctions made between sprouting and non-sprouting angiogenesis, and the individual processes described below, may be somewhat arbitrary, as they all likely occur in concert and are regulated by related cellular and molecular mechanisms.

\textbf{Endothelial Cell Sprouting}

In a normal blood vessel, a 100-200 $\text{\textmu m}$ thick basement membrane (BM) lies immediately deep to the EC monolayer in the arterial \textit{intima}. Composed mainly of laminins, type-IV collagen, type-VIII collagen, and proteoglycans, the BM must be degraded prior to EC invasion into the surrounding ECM, which itself is comprised of fibrillar collagens, elastins, and various other ECM proteins.\textsuperscript{72} ECs, in response to angiogenic stimuli, convert from a quiescent to a synthetically active phenotype characterized by a high mitotic index and increased capacity for migration and matrix proteolysis. These activated ECs are capable of disrupting the tight junctions, adherens junctions and gap junctions which exist between neighboring \textit{intimal} ECs and perivascular cells, and invade into the BM and surrounding ECM.\textsuperscript{70,73} Once freed from the capillary \textit{intima} and in the extravascular space, ECs proliferate and migrate towards
chemotactic and angiogenic stimuli in a 3-D extracellular environment and form new angiogenic sprouts.\cite{74}

Lumenogenesis and Tubulogenesis

Lumen formation (and subsequent tube formation) can be considered a hallmark of angiogenesis as it is a relatively specific behavior attributed mainly to cells of the epithelium and endothelium. It is the genetically programmed capacity of ECs to create luminal compartments within multicellular chains which allows for the flow of blood from the pre-existing vasculature to the neovasculature, without which new capillary networks would be unable to perform their central function of oxygen and nutrient transport to normal or pathologic tissues. Indeed, one of the earliest demonstrations of angiogenesis \textit{in vitro} by Folkman and Haudenschild provided evidence that, in the presence of “direct” angiogens, ECs in culture form capillary-like structures with rudimentary luminal compartments.\cite{75}

While several mechanisms for lumen formation have been suggested, the most widely investigated mechanism is the process of intracellular vacuolization (or intracellular canalization). The earliest observations of angiogenesis described the presence of “seamless” EC lined capillary lumens \textit{in vivo} which were lacking in apparent cross-sectional EC-EC junctions.\cite{76,77} Since then, several \textit{in vitro} and \textit{in vivo} studies have provided for a mechanistic model of lumen formation consistent with this observation. Mediated by the $\alpha_\text{v}\beta_1$ integrin and members of the Rho GTPase family, ECs undergo pinocytosis, leading to the formation of intracellular vacuoles which coalesce and form one larger intracellular lumen.\cite{78-83} This would explain the appearance of “seamless” EC
lined capillary lumens lacking apparent junctional contacts among several lumen lining cells. The association of caveolin-1 expression, a protein involved in receptor mediated endocytosis and the formation of caveolae (cellular invaginations which often precede vacuole formation), with EC lumenogenesis is an interesting observation in light of this mechanistic hypothesis.\textsuperscript{84} Concurrently, cytoplasmic projections have been suggested to sense and form junctional contacts with neighboring ECs to thereby form more complex multicellular capillary tubes.\textsuperscript{82}

While intracellular vacuolization is the most widely studied model of lumen formation in ECs, there are descriptions of other mechanisms. These include intussusception, or the insertion of tissue pillars into the newly forming capillaries,\textsuperscript{85} autophagy by lysosomes within individual ECs which leave behind luminal structures,\textsuperscript{86} trans-luminal ingrowth of cytoplasmic filopodial projections which create a network of luminal structures,\textsuperscript{83} as well as the exocytosis of vacuoles between two (or more) ECs which are sprouting in close apposition to one another to form intercellular lumens (intercellular canalization).\textsuperscript{78,80} Other groups have proposed that apoptosis of centrally located ECs within a cluster of many ECs can lead to tubular neovascular structures.\textsuperscript{87,88} This is consistent with data that demonstrates deficiencies in embryologic lumen formation and microvascular development in transgenic mice which conditionally express the anti-apoptotic protein Bcl-2 in ECs.\textsuperscript{89}

It is important to note that these processes are often described or observed using \textit{in vitro} assays of angiogenesis, and that the translation of these finding to the modeling of physiologic lumen formation \textit{in vivo} is challenging.\textsuperscript{90} Nonetheless, these models of
angiogenesis are invaluable in attempting to understand the basic cellular and molecular mechanisms regulating lumenogenesis and angiogenesis in general. It is also important to note that while the above mechanisms for lumen formation have been proposed as separate entities, they may in fact all be contributing to EC lumen formation concurrently, and the relative importance of any one particular mechanism may be dependent on factors such as the location of the cells undergoing lumenogenesis (i.e. the sprouting tip vs. the trunk/stalk of the sprout). It has also been proposed that heterogeneous populations of ECs with different functional characteristics can contribute to lumen and tube formation simultaneously. Meyer et al. suggested a model of angiogenic tube formation which relies on three different populations of ECs: 1) those with phagocytic functions which create matrix channels and later undergo apoptosis, 2) those which form vacuoles which coalesce and get exocytosed to the intercellular space leading to a primitive lumen which is subsequently remodeled, and 3) those which are added into the capillary tube at the final stage of angiogenesis and aid in lumen growth and remodeling.

A further consideration is the temporal relationship of lumen formation to sprouting. Whether one precedes the other, or, as suggested above, these processes occur simultaneously, is not entirely clear. There is experimental evidence to suggest both possibilities. Davis and Camarillo have demonstrated that vacuoles can be visible in ECs which are actively sprouting, while other studies demonstrate that branching and new sprout formation can occur in the absence of lumen formation.
Cell-Cell Interactions

Perivascular cells such as pericytes (aka mural cells), SMCs, and adventitial fibroblasts, are associated with the endothelium of capillaries and are known to be involved in the later stages of angiogenesis during vessel maturation and stabilization (discussed below). However, these primarily mesenchymally derived cells surrounding the endothelium also appear to have a significant role in mediating the early events of active angiogenesis.\(^{95-98}\) Secreted VEGF or deposited ECM proteins such as fibronectin by SMCs and fibroblasts \textit{in vitro} have been demonstrated to elicit angiogenic activities such as increased plasminogen activity, proliferation, motility, and cordlike structure formation in co-cultured ECs.\(^{99-104}\) Evidence that these paracrine interactions may be regulated by the local extracellular environment is provided by the observed endothelin-1-mediated activation of EC mitogenesis by fibroblasts,\(^{100}\) and the production of Ang-2 and VEGF in mesangial cells (renal pericytes) under hypoxic conditions.\(^{105}\) \textit{In vivo} observations of pericytes found “leading” angiogenic sprout tips and lining vascular tubes connected to tumor vasculatures which lack ECs \textit{in vivo} suggest a more direct role for pericytes as guiding cells during angiogenesis.\(^{106,107}\) These data are consistent with observations that under hypoxic stimulation, VEGF-secreting perivascular cells appear to be the first vascular cells to invade the \textit{corpus luteum} parenchyma during ovulatory angiogenesis.\(^{108}\) Thus, while the later stabilization and maturation of the microvasculature by pericytes is well established, the likelihood that they promote angiogenesis given the proper pro-angiogenic environmental context suggests a more broad role for these cells which warrants further study.\(^{109}\)
Inosculation

Inosculation is the fusion or anastomosis of two vascular lumens or luminal segments to form one continuous lumen. Although an understudied process in angiogenesis, some literature from the field of skin graft vascularization can be extrapolated to provide some insights into this later step of angiogenesis. In 1980, Tsukada demonstrated that the preservation of the subcutaneous vascular network of a skin graft facilitated free tissue transfer, and that this was associated with both the formation of a neovasculature, as well as the inosculation between the preexisting host and donor vasculature.\textsuperscript{110} By analyzing the establishment of donor/host vascular continuity in grafted skin, studies have demonstrated that skin neovascularization with complete inosculation occurs between three to seven post-operative days following skin grafting.\textsuperscript{111,112} Immunohistochemical staining of human skin transplanted onto athymic mice has documented recipient EC invasion into the graft between days 3 and 21 via neovascularization with blood flow commencing on days 3-7. Of note, recipient beds were not seen to have donor EC ingrowth.\textsuperscript{113} The molecular mechanisms involved in inosculation, however, are still relatively unknown, and further study of this phase of angiogenesis is required.

Pericytes

The recruitment and incorporation of pericytes are key events which mediate the final steps required for the stabilization and maturation of the neovasculature.\textsuperscript{73} Pericytes are primarily thought to be derived from cells of mesenchymal origins such as SMCs, fibroblasts or other progenitor cells and possess markers such as $\alpha$-actin, desmin, PDGF-
β receptor, and NG2 which are common to those cells. Several lines of evidence support the role of pericyte incorporation for the stabilization of neovessels and the regulation of uncontrolled angiogenic growth. Early studies showed that mice lacking the ability to recruit pericytes during embryonic vascular development demonstrated increased vessel microaneurysm formation and rupture. Generally speaking, abnormal pericyte coverage and function in the microvasculatures of certain types of tumors is associated with increased EC sensitivity to VEGF withdrawal and microvasculatures with disrupted EC/pericyte interactions are associated with more dynamic remodeling.

Direct contact via gap junctions, adhesion plaques, and adherens junctions, as well as indirect paracrine mechanisms between ECs and pericytes, allow for significant interactions which impact cellular differentiation, migration, proliferation, and quiescence. Among the paracrine mediators, PDGF is thought to be critical in the recruitment and proliferation of pericytes during angiogenesis. PDGF is a mitogen and chemoattractant for fibroblasts, SMCs, and other mesenchymal cells which is present in four known isoforms A, B, C, and D (with the A and B isoforms most notably studied in the literature in relation to the recruitment of pericytes during angiogenesis, and to the migratory and proliferative behavior of smooth muscle cells). Two known PDGF transmembrane receptor tyrosine kinases known as the α and β subunit bind each chain with relative specificity (the α receptor binds the A, B, and C chains primarily while the β receptor binds the B and D chains). Because PDGF exists as a hetero- or homodimer, ligand binding causes receptor hetero- or homodimerization (i.e. PDGF-BB and PDGF-
AB can bind α/α, α/β, or β/β, while PDGF AA binds α/α exclusively) and subsequent cell signaling.\textsuperscript{126-128} As PDGF-β is the primary receptor present on SMCs and other pericytes, PDGF-B/β signaling is especially critical in the proper recruitment of pericytes to capillaries. Disrupted PDGF-B/β signaling is associated with the attenuated ability to recruit PDGF-β receptor positive pericytes and precursors from the perivascular tissue and the bone marrow and is associated with significant vascular abnormalities in physiologic and pathologic angiogenesis.\textsuperscript{115,118,129-134}

The source of PDGF during this process is likely from actively angiogenic ECs themselves, which suggests an inherent negative feedback mechanism to prevent uncontrolled neovascularization.\textsuperscript{73} Proliferative ECs \textit{in vitro} are potent stimulators of pericyte/SMC growth and migration, in part mediated by PDGF-BB, although this effect is dependent on the time in co-culture, the presence or absence of direct contact, and the deposition of endothelial-specific ECM proteins.\textsuperscript{135-143} The importance of EC-specific PDGF-B production in pericycle recruitment \textit{in vivo} was demonstrated in studies which showed that EC-specific knockout of PDGF-B led to significant vascular abnormalities with pericycle loss in mice.\textsuperscript{144-146}

Clinically, the simultaneous targeting of pericytes and ECs by pro- or anti-PDGF and VEGF therapy, has been investigated in tissue engineering and oncologic research. Combination therapy (manipulating both PDGF and VEGF) has been shown in some studies to be more effective in disrupting tumor vascularity than either agent alone and, as such, combinatorial antitumor therapy is under investigation.\textsuperscript{147-150} It is unclear, however, if the apparent improved anti-angiogenic effect observed in combination
therapy is specifically attributable to PDGF blockade or to the broad non-specific effects
of the receptor tyrosine kinase inhibitors used in most studies of combination therapy.
Recently, it was shown that adenoviral expression of soluble anti-PDGF-β receptor
provided added inhibition of tumor growth and angiogenesis only when sub-optimal
levels of VEGF blockade were present.\textsuperscript{151} Clearly, however, PDGF signaling does
impact angiogenesis \textit{in vivo}, as the spatiotemporal delivery of PDGF with VEGF has
been shown to induce denser and more mature appearing vascular networks within tissue
engineered constructs, which is in part associated with the recruitment of α-actin positive
(indicative of pericytes) cells into the neovascular networks.\textsuperscript{152,153}

Although the expansion, spreading, and migration of the SMC/pericyte population
on newly formed capillaries may be dependent on PDGF-B/β signaling, it does not
appear to be sufficient or necessary for the phenotypic differentiation of undifferentiated
mesenchymal cells to pericytes. Instead, this appears to be significantly regulated by
TGF-β,\textsuperscript{131,154} as evidenced by observations that ECs direct 10T1/2 cells (a cell line
commonly used to model pericyte precursor cells \textit{in vitro}) to an SMC/pericyte phenotype
via a TGF-β-mediated mechanism,\textsuperscript{141,155} and that the phenotypic differentiation of SMCs
in co-culture with ECs involves activation of the SMC PI3-K/Akt pathway, which may be
mediated by TGF-β.\textsuperscript{156,157}

With these above observation taken together, it has been proposed that two modes
of pericyte incorporation occur simultaneously: 1) the differentiation of undifferentiated
mesenchymal and progenitor cells into pericytes; and 2) the recruitment and co-migration
of differentiated mesenchymal cells such as SMCs from a pool of available cells in the perivascular tissue into neovessels.\(^{131}\)

It is believed that once incorporated, a major function of pericytes is to mediate the quiescence of ECs. \textit{In vitro}, ECs in direct contact with pericytes have lower levels of mitosis compared to ECs cultured alone, a process which may require Rho GTPase activity in pericytes.\(^{142,158,159}\) Similarly, ECs in close contact with SMCs in a spherical aggregate of SMCs in collagen gels demonstrated decreased responsiveness to VEGF (in the absence of Ang-2), increased inter-endothelial junctions, decreased PDGF-B growth factor chain expression, and reduced apoptosis.\(^{160}\) ECs in co-culture with 10T1/2 cells also displayed a significantly higher resistance to the permeability of a tracer molecule biotin-dextran which was associated with tightening of EC-EC tight junctions and with the localization of tight junction proteins plakoglobin, ZO-1, ZO-2, and occludin.\(^{161}\)

It has also been suggested that pericytes induce vessel stabilization by the production of survival factors. This is supported by several lines of evidence: 1) neonatal vessels which are resistant to oxygen-induced degeneration are associated with pericytes which express TGF-\(\beta_1\), which induces VEGFR-1 expression in ECs;\(^{162}\) 2) 10T1/2 precursor cells produce VEGF upon TGF-\(\beta\)-mediated conversion to pericytes when in direct contact with ECs;\(^{163}\) 3) pericytes express Ang-1 \textit{in vitro} and \textit{in vivo}, a growth factor which is associated with basement membrane formation, EC quiescence and endothelial leak resistance, and which can mature vessels lacking pericytes;\(^{164-167}\) and 4) pericyte loss caused by PDGF inhibition in a rat model of retinopathy results in overexpression of VEGF/VEGFR2 in ganglion cells and other supporting cells.\(^{168}\)
A final mechanistic hypothesis for the pericyte-mediated regulation of angiogenesis is that pericytes significantly modulate the ECM remodeling capacity of ECs by directly influencing the local ECM composition. Pericyte-conditioned media has been shown to limit both EC migration and branching *in vitro* by inducing the upregulation of PAI-1 in ECs. In addition, pericytes have been shown to produce TIMP-3 and induce TIMP-2 production in ECs, which specifically targets MT1-MMP, MT2-MMP and ADAM (a disintegrin and metalloprotease) proteinases, with the net effect of decreasing angiogenic potential and tube regression. In addition, pericyte-mediated ECM deposition, specifically the deposition and synthesis of basement membrane, has also been proposed to promote EC quiescence and vessel stability. The importance of the basement membrane in vascular stability is demonstrated in laminin-8 knockout animals which demonstrate a poorly developed microvasculature with defective basement membranes, noticeable deformities, and increased responsiveness to pro-angiogenic stimuli.

While the mechanisms discussed above in the maturation and stabilization of existing and newly formed vessels are still topics of current study, it is becoming clear that this final stage of angiogenesis is a significant contributor to the persistence and function of pathologic and physiologic neovasculatures, and will likely provide for future therapeutic strategies.

**Cell Signaling**

The Aims of this proposal will address cell signaling processes, specifically those mediated by Notch, ERK, and Akt, as it relates to SMC/pericyte and EC interactions
during angiogenesis. These pathways were chosen for their known importance in cell proliferation, migration, and survival, as well as in the mediation of inter- and intracellular signaling processes during angiogenesis. More detailed discussions of these pathways and mechanisms will be described within the Aims of the proposal.

The remainder of the dissertation will focus specifically on the interaction between the SMCs and ECs within the wall of the TEBV during microvascular growth, hypothesizing that SMCs will function as pericytes in the engineered neovasculature. The studies and aims outlined below are intended to model these processes within a fibrin-based, or fibrin-containing TEBV, but can be applied more broadly to any hydrogel-based tissue engineered construct.

**Tissue Engineered Blood Vessel and Vasa Vasorum Construction**

For the reasons discussed above, the studies and objectives detailed in this project are intended to model angiogenic processes occurring within the wall of a tissue engineered blood vessel during *vasa vasorum* development, and more broadly, during microvascular development in any hydrogel based or hydrogel-containing tissue engineered construct. Specifically for the purposes of our lab, TEBV construction is that of a fibrin hydrogel-based strategy (Figures 4 and 5).

Previously in our lab, the combination of VEGF-165 and FGF-1 was shown to synergistically augment angiogenic sprout formation from an aggregate of bovine aortic endothelial cells placed within fibrin hydrogels in a 3-D *in vitro* angiogenesis assay. We utilize this strategy in order to increase EC sprouting potential during the construction of our TEBVs. SMCs are first embedded within a solution of fibrinogen,
Figure 4. TEBV Construction. (A) A pre-polymerized fibrin hydrogel containing SMCs (not shown) are poured around a mandrel. B) EC aggregates polymerized within the hydrogel. C) ECs form microvasculatures. D) Final structure.

Figure 5. Creation of TEBV. By 4 days of static culture, compaction of the hydrogel around the central mandrel is noted. On Day 7, the TEBVs are placed within a bioreactor chamber.

heparin, VEGF-165 and FGF-2 (FGF-2, a more commonly studied FGF isoform, demonstrates grossly similar mitogenic and chemotactic stimulus for ECs and SMCs, and was chosen for these studies due to availability from commercial vendors), smooth muscle cells and culture media and poured into a tubular construct mold. This serves as the foundation of the arterial tunica media. Aggregated endothelial cells are mixed in the
fibrinogen based solution as a source of the microvasculature. Finally, thrombin is added in order to polymerize the fibrinogen solution, leading to a tubular structure containing aggregated ECs with a fibrin-based scaffold containing homogenously distributed SMCs. A period of preconditioning in a bioreactor exposing the TEBV to 7 days of 10% cyclic volumetric distension, mimicking the pulsatile forces of the vascular system, allows for the improvement of mechanical properties prior to use in experiments or as a potential conduit.\textsuperscript{3,26,178}

The specific Aims of this proposal focus on the interactions between SMCs and ECs during microvascular development in the TEBV \textit{tunica media}. The experiments described below utilize fibrin-based disks, instead of tubes, in order to simplify the model to focus specifically on the angiogenic process within the construct wall.
CHAPTER THREE

AIMS

Capillaries are comprised of two cell types: the endothelial cells which line the lumen of the capillary, and the supporting pericytes which are closely apposed to the abluminal surface of the capillary wall (sometimes called mural cells; Figure 6). These pericytes are phenotypic derivations of mesenchymal organ stromal cells (from smooth muscle cells and fibroblasts in blood vessels, for example), are recruited and incorporate into the neocapillaries, and are known to mediate reciprocal interactions with ECs during angiogenesis. They are not only considered necessary for the stabilization of the microvasculature in vivo but are also thought to be involved in the early formation of angiogenic networks.

Figure 6. Pericytes in capillaries. Left) Pericytes are seen associated with the abluminal surface of a capillary comprised of 1-2 ECs. Right) Depiction of pericyte recruitment by an angiogenic EC sprout. Modified from Armulik, et al.2
The creation of microvasculatures to feed the stromal cells of tissue engineered organs (i.e. SMC in TEBVs) is thought to be a critical need. How these stromal cells in tissue engineered organs affect angiogenesis during organ and microvasculature creation, and the mechanisms underlying EC and pericyte interactions, are still a topic of considerable study. In the following Aims and studies, we utilize SMCs as both the primary cellular component of the TEBV stroma, as well as the source for incorporated pericytes in our engineered TEBV microvasculatures. Several lines of evidence support the role of pericyte incorporation for the stabilization of neovessels and the regulation of uncontrolled angiogenic growth. Early studies showed that mice lacking the ability to recruit pericytes during embryonic vascular development demonstrated increased vessel microaneurysm formation and rupture. Generally speaking, abnormal pericyte coverage and function in the microvasculatures of certain types of tumors is associated with increased EC sensitivity to VEGF withdrawal and microvasculatures with disrupted EC/pericyte interactions are associated with more dynamic remodeling. In addition, pericytes appear to have a significant role in mediating the early events of active angiogenesis. Secreted VEGF or deposited ECM proteins such as fibronectin by SMCs and fibroblasts in vitro have been demonstrated to elicit angiogenic activities such as increased plasminogen activity, proliferation, motility, and cordlike structure formation in co-cultured ECs. In vivo observations of pericytes found “leading” angiogenic sprout tips and lining vascular tubes connected to tumor vasculatures which lack ECs in vivo suggest a more direct role for pericytes as guiding cells during angiogenesis. These data are consistent with observations that under
hypoxic stimulation, VEGF-secreting perivascular cells appear to be the first vascular cells to invade the corpus luteum parenchyma during ovulatory angiogenesis.\textsuperscript{108}

The overall purpose of this project is to determine how SMCs affect angiogenesis during microvascular development in fibrin hydrogels using 3-dimensional co-culture assays of angiogenesis. These studies are meant to model angiogenic processes in the wall of a fibrin hydrogel based TEBV containing ECs and SMCs described above. The overall hypothesis is that SMCs promote angiogenic network formation and persistence of microvasculatures in fibrin hydrogels and that this effect is modulated in part by a Notch dependent pathway.

\textbf{Aims}

\textbf{Aim 1}

Quantify the effect of SMCs on the formation and persistence of engineered microvascular networks in fibrin hydrogels.

The hypotheses are that: 1) Endothelial cells (ECs) will sprout from an aggregated spheroid of ECs and form angiogenic networks in the presence of SMCs in fibrin hydrogels; 2) SMCs will co-localize with angiogenic sprouts indicating incorporation into the neovascularure as pericytes; and 3) SMCs will promote both angiogenic network formation and persistence during microvascularization of fibrin hydrogels.

\textit{Rationale}

The microvascularization of tissue engineered organs is thought to be a critical need in tissue engineering. Growth factors such as VEGF and FGF-2 are commonly utilized to augment the angiogenic processes required for construct microvascularization,
and are known to be important pro-angiogenic growth factors during angiogenesis in vivo. Mesenchymally derived pericytes, such as those derived from SMCs (the stromal cells of tissue engineered blood vessels) may modulate the initiation and persistence of angiogenic networks by several potential mechanisms: 1) the production of soluble angiogenic factors, 2) the activation of cell-cell signaling pathways, and 3) the deposition and remodeling of the extracellular matrix surrounding neovasculatures. The purpose of this Aim is to investigate how SMCs affect angiogenesis in the presence and absence of exogenous growth factors during microvascular development in fibrin hydrogels.

Aim 2

Determine how EC signaling pathways involved in angiogenic network formation (e.g. proliferation) and persistence (e.g. survival) are affected by co-culture with SMCs.

Hypothesis: Co-culture with SMCs will activate ERK and Akt in ECs by the release of soluble angiogenic products.

Rationale

The co-culture of ECs with SMCs has the potential to alter EC proliferation and survival signaling pathways via the release of soluble factors, cell-cell signaling, or other mechanisms. Previous work has demonstrated that SMCs can produce several growth factors including VEGF, angiopoietins, and others. This Aim will investigate how soluble products released by SMCs affect the phosphorylation of Akt and ERK cell signaling pathways in ECs.
Aim 3

Determine if Notch signaling mediates the SMC effects on angiogenesis in fibrin hydrogels.

Hypothesis: Notch in ECs will mediate the angiogenic and signaling responses to SMC derived soluble factors.

Rationale

Notch is a known mediator of EC signaling and angiogenesis in response to growth factors such as VEGF. In addition, Notch signaling affects pericyte recruitment and SMC migration, modulates pericyte and EC gene expression in co-culture systems, and may also be directly involved in cell-cell signaling pathways at points of EC and SMC contact. This Aim will determine if Notch signaling is involved in the angiogenic processes during engineering of co-culture microvascular networks in fibrin hydrogels. Based on the results of the previous two Aims, these studies will specifically address the role of EC Notch activation in response to exogenous growth factors and to soluble products produced by SMCs.
CHAPTER FOUR
THE EFFECTS OF SMCS ON ANGIOGENESIS
IN FIBRIN HYDROGELS

Introduction

Pericyte-EC Interactions during Angiogenesis

Perivascular cells such as pericytes (aka mural cells), SMCs, and adventitial fibroblasts, are associated with the endothelium of capillaries and are known to be involved in the later stages of angiogenesis during vessel maturation and stabilization (discussed below). However, these primarily mesenchymally derived cells surrounding the endothelium also appear to have a significant role in mediating the early events of active angiogenesis. Secreted VEGF or deposited ECM proteins such as fibronectin by SMCs and fibroblasts in vitro have been demonstrated to elicit angiogenic activities such as increased plasminogen activity, proliferation, motility, and cordlike structure formation in co-cultured ECs. Evidence that these paracrine interactions may be regulated by the local extracellular environment is provided by the observed endothelin-1-mediated activation of EC mitogenesis by fibroblasts, and the production of Ang-2 and VEGF in mesangial cells (renal pericytes) under hypoxic conditions. In vivo observations of pericytes found “leading” angiogenic sprout tips and lining vascular tubes connected to tumor vasculatures which lack ECs in vivo suggest a more direct role for pericytes as guiding cells during angiogenesis.
These data are consistent with observations that under hypoxic stimulation, VEGF-secreting perivascular cells appear to be the first vascular cells to invade the corpus luteum parenchyma during ovulatory angiogenesis. Thus, while the later stabilization and maturation of the microvasculature by pericytes is well established, the likelihood that they promote angiogenesis given the proper pro-angiogenic environmental context suggests a more broad role for these cells which warrants further study.

Pericytes and Vascular Maturation

The recruitment and incorporation of pericytes are key events which mediate the final steps required for the stabilization and maturation of the neovasculature. Pericytes are primarily thought to be derived from cells of mesenchymal origins such as SMCs, fibroblasts or other progenitor cells and possess markers such as α-actin, desmin, PDGF-β receptor, and NG2 which are common to those cells. Several lines of evidence support the role of pericyte incorporation for the stabilization of neovessels and the regulation of uncontrolled angiogenic growth. Early studies showed that mice lacking the ability to recruit pericytes during embryonic vascular development demonstrated increased vessel microaneurysm formation and rupture. Generally speaking, abnormal pericyte coverage and function in the microvasculatures of certain types of tumors is associated with increased EC sensitivity to VEGF withdrawal and microvasculatures with disrupted EC/pericyte interactions are associated with more dynamic remodeling.

The purpose of these studies is to investigate EC and SMC interactions utilizing novel 3-D co-culture assays of angiogenesis. The SMCs in these experiments are
intended to act as both the stromal cells of a fibrin-based tissue engineered blood vessel as well as the source of pericytes of engineered neovasculatures.

**Materials and Methods**

**Materials**

Chemicals, biological reagents, and experimental supplies were obtained as follows: collagenase (Invitrogen); human thrombin (American Red Cross; Rockville, MD); FGF-2, VEGF (R&D Biosciences); Mitomycin C, L-ascorbic acid, methylcellulose, fibrinogen, transferrin, insulin, anti-α-actin antibody and aprotinin, anti-VWF antibody, PKH-27, PKH67 (Sigma Chemical Co.; St. Louis, MO); tritiated thymidine (NEN Life Science Products; Boston, MA); methanol, trichloroacetic acid, acetic acid, and scintillation fluid (Fisher Scientific; Fair Lawn, NJ); bovine lung heparin (Upjohn; Kalamazoo, MI); Calcein AM (Molecular Probes, Eugene, Ore); 0.05% trypsin/EDTA, HBSS, M199, DMEM, L-nonessential amino acids, sodium pyruvate, penicillin, streptomycin, DMEM-F12 (Gibco, Grand Island, NY); fetal bovine serum (FBS) (Hyclone, Logan, UY); woven nylon mesh rings (ID = 7.5 mm, OD = 13 mm) (Sefar America Inc; Kansas City, MO); Parafilm M (American National Can, Greenwich, CT); 100-mm and 60mm Petri dishes (Fisher Scientific; Pittsburgh, PA); 24 wells plates and tissue culture flasks, Costar Transwell polystyrene plates (Corning Costar Corp; Cambridge, MA); round bottom 96 well plates (Greiner Bio-one; North Carolina); 96-well polystyrene plastic plates (Beckton Dickinson; Lincoln Park, NJ).
Animal Care

All animal procedures complied with The Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, 1996) and The Principles of Laboratory Animal Care (National Institutes of Health publication no. 85-23, revised 1985) and were approved by our institutional IACUC.

Cell Isolation

SMCs and ECs were harvested from canine carotid arteries and jugular veins, respectively per previously published protocols. Briefly, adult mongrel dogs were anesthetized with thiopental sodium, intubated, and ventilated. Anesthesia was maintained with nitrous oxide and methoxyflurane. Bilateral neck incisions were made.

For ECs, bilateral external jugular veins were removed, inverted, and processed per our previously reported protocol beginning with the veins being sequentially placed into 0.05% trypsin/EDTA 0.53 mmol/L and collagenase 100 U/mL at 37°C for 10 minutes each. Next, the veins were discarded, and the trypsin and collagenase solutions were centrifuged. The subsequent pellets were resuspended in EC complete medium supplemented with FGF-1 (5 ng/mL), and the ECs were plated onto a fibronectin-coated (2.5 µg/cm²) T-25 culture flask that was incubated in a 37°C, 5% carbon dioxide chamber. The media was changed every 2 to 3 days, and confluent cells were passaged with trypsin-EDTA. EC identity was confirmed using immunofluorescent dual staining with anti-vWF and anti-α-actin antibodies. Only EC exhibiting 95% positive vWF
staining and 2% or less α-actin staining were used for the proliferation assays. ECs were used within passages 1 to 5.

For SMCs, the carotid arteries were opened longitudinally, and the intima and adventitia were removed by sharp dissection. The medial layer was minced and placed into SMC growth media. Primary SMCs migrating from the explants were used in all experiments. SMC identity was confirmed by immunofluorescent staining with anti-α-actin antibodies, and only cultures exhibiting 95% positive staining were used in the assays. Individual carotid artery explants were used as the source of cells for assays no more than 3 times.

Mitomycin C Treatment

In experiments using Mitomycin C (MMC) treatment to inhibit cell proliferation, 500,000 SMCs were plated onto 60mm Petri dishes and treated with 10 µg/mL of MMC for one hour prior to use in experiments. SMCs which were plated, but not treated with MMC, served as controls.

Methocel Preparation

Methylcellulose reagent (1.2 g) was autoclaved in a 250-mL beaker at 120°C for 20 minutes. The autoclaved powder was dissolved in 50 mL of preheated (60°C) M199, stirred at 60°C for 20 minutes, and then 50 mL of M199 at room temperature was added. The solution was mixed for 2 hours at 4°C, centrifuged for 2 hours at 4,000 rpm, and the supernatant was collected after centrifugation.
Angiogenesis Assays

Co-Culture Assay 1: Angiogenesis in the Presence of Homogenously Distributed SMCs

SMCs were fluorescently labeled green with PKH-67 (10 µM) and ECs were fluorescently labeled red with PKH-26 (10 µM) per manufacturer instructions. PKH is a cell membrane label which incorporates into the plasma membrane of cells and is divided equally into subsequent progeny cells. Fibrin hydrogels were constructed by preparing a fibrinogen solution consisting of fibrinogen (2.5 mg/mL), M199, heparin (5U/mL), and green fluorescently labeled SMC. The solution was polymerized by the addition of thrombin (0.32U/mL) (Figure 7).

![Figure 7](image)

Figure 7. Technique of co-culture assay #1. SMC (green with blue outline) containing fibrin hydrogel layer is plated on a 96 well flat bottom plate. Fluorescently labeled aggregate of ECs (red) are placed on top of gel and covered with second fibrin hydrogel containing SMCs and then cultured in angiogenesis assay media.

Red fluorescently labeled ECs, 10,000-20,000, were suspended in an aggregating solution consisting of angiogenesis assay media (see appendix) and a 20% methocel solution into each well of a round bottom 96-well plate and incubated at 37°C for 24 hours until cell aggregates formed. ECs were embedded between two layers of fibrin
gels in the wells of flat-bottom 96 well plates. In experiments with higher concentrations of SMCs followed for longer periods, the hydrogels were polymerized onto nylon mesh rings to minimize the impact of SMC-mediated gel contraction and then cultured in 24 well plates. EC aggregates embedded within hydrogels that did not contain SMCs served as controls. Angiogenesis assay media was added to the top of the hydrogels containing the cells, and the assays were incubated at 37°C. In experiments testing the effects of growth factor addition and withdrawal, VEGF and FGF-2 were added to the prepolymerized hydrogel as well as the assay media to achieve a final concentration of 10ng/mL for each growth factor in both the media and the hydrogel. After a period of time, ECs invade from the central aggregate into the surrounding hydrogel. Digital images were taken daily for up to 13-17 days at a magnification of 4x using a Zeiss Axiovert 200M microscope (Carl Zeiss, Inc, Oberkochen, Germany) and Axiovision software. The digital photographs were aligned with a grid evenly divided in 36 radial intervals using Adobe Photoshop for quantification using both TRITC and FITC filters. For each assay, the average length of sprouts (ALS) was determined by measuring the furthest point on each grid line with an invading chain of cells. A secondary data point quantifying the density of sprout formation (DSF) was also measured by calculating the number of grid lines with crossing sprouts/36 total grid lines (Figure 8). The presence of cellular debris and discontinuity of previously continuous chains of invading cells is indicative of cell death. For this reason, invasion was quantified by measuring only out to the final point of a continuous chain of cells. Data from three individual experiments were pooled and presented +/- SEM.
Histology

At the end of selected assays, representative angiogenesis assay hydrogels were processed for cross-sectional morphologic analysis with transmission electron microscopy. The assay disks were fixed with a 1% glutaraldehyde/4% paraformaldehyde solution, postfixed in 1% osmium tetroxide, dehydrated in a graded series of acetone, and embedded in epoxy resin. Ultrathin sections were cut, collected on copper grids, and stained with uranyl acetate and lead citrate. The sections were viewed on a transmission electron microscope (H-600; Hitachi, Tokyo, Japan) at 75 kV.

Co-Culture Assay 2: Direct Contact Co-Culture Angiogenesis Assay

2250 green fluorescently labeled SMCs were suspended in an aggregating solution consisting of angiogenesis assay media and a 20% Methocel solution\textsuperscript{182} and added into each well of a round bottom 96-well plate and incubated at 37°C for 24 hours until cell aggregates formed (Figure 9). After 24 hours, 350 red fluorescently labeled ECs were added to each well and allowed to seed the surface of the SMC aggregate. In
experiments which tested the effects of inhibiting SMC proliferation on EC matrix invasion, ECs were seeded onto aggregates of SMCs which were either treated or untreated with MMC as described above. The co-culture aggregates were then embedded between two 150 µL layers of fibrin that were polymerized on a nylon mesh ring such that the aggregates were completely surrounded by the hydrogel. Once completely polymerized, the disks were transferred into 24 wells plates and cultured in angiogenesis assay media at 37°C. After a period of time, SMCs and ECs invade from the central aggregate into the surrounding hydrogel (Figure 10). Digital images were taken daily for up to 19 days at a magnification of 4x and quantified as described above. Each individual experiment was analyzed by Student’s T-test with an α of 0.05 with statistically consistent results. Data from a representative experiment is presented in the results.

![Figure 9](image)

**Figure 9.** Coculture assay #2. SMCs green are aggregated in a methylcellulose containing solution and seeded with ECs (red). The co-culture aggregated is then embedded between two layers of fibrin and cultured in angiogenesis assay media.

**Statistical Analysis**

We used analysis of variance with Tukey posttests or Student’s T-tests using SigmaStat (Systat Software Inc, San Jose, CA) at an α of 0.05 to determine statistical significance.
Results

Coculture Assay 1

Morphology

Fluorescent microscopic evaluation of angiogenic sprouts demonstrated endothelial cell sprouts radiating from the central aggregate into the hydrogel matrix. Fluorescently labeled SMCs were found to closely associate with both the EC aggregate as well as individual endothelial cell sprouts (Figure 11).
Figure 11. Sprouting from co-culture assay #1. Left) Fluorescently labeled green SMCs are homogenously suspended within the fibrin hydrogel. Middle) Fluorescently red labeled ECs are seen sprouting from a central aggregate of ECs into the matrix. Right) Merged image of ECs sprouting into the ECM containing SMCs which associate with EC sprouts. Mag 10x. (Lower image) Digital zoom of merged image.

Cross-sectional morphology of angiogenic sprouts examined under transmission electron microscopy after the 13 days demonstrated luminal structures surrounded by 2-3 endothelial cells which were closely associated by smooth muscle cells on the abluminal surface (Figure 12). The sprouts and associated cells were completely surrounded by the fibrin extracellular matrix. Debris was present within the lumen of the sprouts in each of the sections. SMCs within the matrix seen in sections lacking angiogenic sprouts did not form luminal structures (not shown).
Figure 12. Cross sectional morphology of co-culture sprouts by transmission electron microscopy. Selected images demonstrate EC lined luminal structures with SMCs apposed to the abluminal surface of the capillary. The structure is surrounded by the fibrin matrix. ECM = extracellular matrix; L = Lumen; Arrows = SMCs; Arrowheads = ECs.

**Angiogenesis in Response to Growth Factors**

The effects of SMCs on the angiogenic response to VEGF and FGF-2 in combination was determined (Figure 13). Angiogenesis was quantified in the presence and absence of SMCs at concentrations of 0, 2500, 7500, 22,500, 67,500 SMCs/mL. At Day 3, the average length of sprouts (ALS) in ECs cultured in the presence of VEGF and FGF-2 was 331 +/- 10 µm vs. 209 +/- 10 in ECs cultured in the absence of growth factors (p < 0.001). At Day 3 in the presence of growth factors, the ALS of EC sprouts co-cultured in the presence of any concentration of SMCs did not significantly differ from the ALS of EC sprouts cultured in the absence of SMCs (p=.19).

**Angiogenesis after Growth Factor Withdrawal**

To model the effects of SMCs on angiogenesis after the withdrawal of angiogenic growth factors, angiogenesis was quantified after 3 days of culture in the presence of
VEGF and FGF-2, and then quantified up to 13 days after withdrawal of growth factors from the culture media (Figures 14 and 15).

Figure 13. Average length of EC sprouts cultured in the presence of absence of SMCs in media containing VEGF and FGF-2 (10 ng/mL for each growth factor).

Figure 14. Average length of EC sprouts cultured in the presence of absence of 22,500 SMCs in media containing VEGF and FGF-2 (10 ng/mL for each growth factor). After 3 days, the media was replaced with media that did not contain exogenous growth factors.
In the presence of growth factors after 3 days, the ALS of ECs cultured in absence of SMCs was 133 +/- 17µm, and the ALS of ECs co-cultured in the presence of SMCs (22,000 SMCs/mL) was 128 +/- 13 µm (p = .70). After withdrawal of growth factors, the ALS of ECs cultured in the absence of SMCs was 112 +/- 12 µm versus 211 +/- 16 in the presence of SMCs at Day 9 (p < .001). Similarly at Day 13, ALS of ECs cultured in the absence of SMCs was 97 +/- 12 µm versus 222 +/- 26 in ECs cultured in the presence of SMCs (p < .001).

At 13 Days, the average length of EC sprouts cultured in the absence of SMCs were 27.2 +/- 8.7% less than the length of EC sprouts at Day 3, while the average length of EC sprouts cultured in the presence of SMCs were 73.1 +/- 20.2% greater than the length of EC sprouts at Day 3 (p < 0.001).

No differences in the density of sprout network formation were found in groups which had been treated by exogenous growth factors.
**SMCs Effects on Angiogenesis in Absence of Exogenous Growth Factors**

To determine the effects of SMCs in the absence of exogenous growth factors, angiogenesis from EC aggregates embedded within fibrin hydrogels that contained SMCs (400,000 cells/mL) were compared to angiogenesis from EC aggregates embedded in fibrin hydrogels which did not contain SMCs (Figures 16 and 17). The assays were cultured in angiogenesis assay media without exogenous growth factors for up to 17 days. By Day 3, the ALS of ECs cultured in the presence of SMCs was significantly greater than those cultured in the absence of SMCs (320 +/- 21 µm versus 187 +/- 16 µm; p < .005). This significant difference persisted throughout the course of the entire experiment.

![Graphs showing average length of sprouts (ALS) and density of sprout formation (DSF) from EC aggregates with and without SMCs in fibrin](image)

**Figure 16.** Quantification of angiogenesis in the presence and absence of SMCs (400,000 SMCs/mL) in the absence of exogenous growth factors.

Angiogenic networks in coculture continued to grow throughout the course of the experiment with the peak average length of co-cultured sprouts being 462 +/- 13 µm at Day 17. The ALS of ECs cultured in the absence of SMCs at Day 17 was 202 +/- 27 µm, with a peak ALS of 233 +/- 8 µm at Day 13.
Figure 17. Angiogenesis in the presence and absence of SMCs (400,000 SMCs/mL) in the absence of exogenous growth factors. Only ECs shown for simplification (Mag. 4x).

Similarly, ECs in coculture formed more robust sprout networks, with the density of sprout formation (DSF) of co-cultured ECs being 42 +/- 8% versus 12 +/- 2% in ECs cultured in the absence of SMCs at Day 3 (p=.01). At Day 17, the DSF of co-cultured ECs was 69 +/- 25% versus 7 +/- 3% in ECs not in co-culture (p= .02). The peak DSF in co-cultured ECs was 76 +/- 7% at Day 13 versus 31 +/- 4% at Day 9 in ECs not in co-culture.

The Effects of SMCs on Angiogenesis with Direct SMC-EC Contact

The modulation the pro-angiogenic effects of SMCs by direct cell-cell contact was tested utilizing a direct cell contact angiogenesis assay modified from the methods of Korff and Augustin. ECs seeded on the surface of an aggregate of SMCs were embedded in fibrin hydrogels and cellular invasion of each cell type was imaged and quantified over a 19 day period. ECs and SMCs were found to co-invade into the extracellular matrix in close approximation to one another. (Figure 18) At Day 7, the average length of EC sprouts was 205 +/- 32 µm and the average length of SMC invasion was 229 +/- 19 µm (p=.54). Similarly, the ALS of EC and SMC sprouts did not
significantly differ at Day 13 (564 +/- 79 µm for SMCs versus 378 +/- 55 µm for ECs; p=.23). At Day 19, SMC sprout lengths were significantly greater than EC sprout lengths (551 +/- 39 µm versus 327 +/- 58 µm; p=.03).

Figure 18. Invasion of ECs and SMCs into fibrin from co-culture aggregates. Left) ECs (red) co-invade with SMCs (green) into fibrin hydrogel (Mag. 10x). Right) Average length of EC and SMC invasion in co-culture aggregate model.

To quantify the effects of SMC proliferation and invasion on EC invasion, ECs were seeded onto aggregates of SMCs that were either treated or untreated with MMC. MMC treatment had the effect of both inhibiting SMC proliferation and attenuating SMC matrix invasion to a maximal reduction of 40.0 +/- 2.2% of MMC untreated controls (p<.001), with significant reductions in SMC invasion at all time points (Figure 19).

ECs in co-culture with SMCs treated with MMC demonstrated significantly attenuated matrix invasion compared to ECs co-cultured with SMCs that were untreated with MMC (82 +/- 14 µm versus 205 +/- 32; p < .05) (Figure 20).
Figure 19. Treatment of SMCs with MMC inhibits SMC (a) proliferation and (b) matrix invasion. The effects of MMC treatment on SMC proliferation and matrix invasion in the absence of growth factors. (a) SMC proliferation. SMCs either treated or untreated with MMC were seeded onto 96 well plates and cultured in indicated media. SMC proliferation was determined by quantifying tritiated thymidine incorporation. Results are mean CPM +/- SEM from a representative experiment (n=4). (b) SMCs either treated or untreated with MMC were embedded within fibrin hydrogels and matrix invasion was quantified. Results represent the mean distance of invasion +/- SEM from a representative experiment (n=4-5). * p < 0.05 vs. MMC untreated control.

Figure 20. Inhibiting SMC proliferation with MMC inhibits both SMC and EC matrix invasion into fibrin hydrogels in the co-culture aggregate model.
In the direct contact co-culture model, ECs that were co-cultured with SMCs demonstrated increased ALS compared to ECs that were cultured alone at all time points (Figures 21 and 22). At Day 7 the ALS of ECs cultured alone was 103 +/- 31 µm versus 205 +/- 32 µm of ECs in co-culture (p=.08). At Day 19, ALS of ECs cultured alone was 70 +/- 11 µm versus 327 +/- 58 of ECs in co-culture (p=.01).

![EC sprouts from EC aggregates](image1.png) ![EC sprouts from co-culture aggregates](image2.png)

Figure 21. EC sprouts in co-culture aggregate model in the presence and absence of SMCs. Only EC sprouts are shown for simplification. (Mag. 4x)

![Effects of SMCs in direct contact model](image3.png) ![Effects of SMCs in direct contact model](image4.png)

Figure 22. Quantification of EC invasion in co-culture aggregate model. ECs were cultured in the presence and absence of SMCs in the co-culture aggregate model and average length of sprouts (left) and density of sprout formation was quantified (right).

The DSF of co-cultured ECs was also significantly greater than that of ECs not in co-culture at Day 13 and Day 19 (p=.014 and .007, respectively).
Discussion

We have demonstrated a novel method for creating structures resembling capillaries in a 3-dimensional extracellular matrix incorporating both the endothelial cells and SMC-derived pericytes. The presence of SMCs in the extracellular matrix did not alter the angiogenic response to angiogenic growth factors commonly used in tissue engineering to promote construct microvascularization. In fact, the presence of SMCs in the extracellular matrix promoted the formation of microvascular networks in the absence of exogenous growth factors and promoted the persistence of the microvascular networks by blocking the regression of microvasculatures after growth factor removal over the length of the experiments carried out in these studies.

Several mechanisms for the promotion of capillary persistence by pericytes have been set forth. One is that pericytes induce vessel stabilization by the production of survival factors. This is supported by several lines of evidence: 1) Neonatal vessels which are resistant to oxygen-induced degeneration are associated with pericytes which express TGF-β1, which induces VEGFR-1 expression in ECs;162 2) 10T1/2 precursor cells produce VEGF upon TGF-β-mediated conversion to pericytes when in direct contact with ECs;163 3) Pericytes express Ang-1 in vitro and in vivo, a growth factor which is associated with basement membrane formation, EC quiescence and endothelial leak resistance, and capillary maturation;164-167 and 4) Pericyte loss caused by PDGF inhibition in a rat model of retinopathy results in overexpression of VEGF/VEGFR2 in ganglion cells and other supporting cells.168
Another is that pericytes significantly modulate the ECM remodeling capacity of ECs by directly influencing the local ECM composition. Pericyte-conditioned media has been shown to limit both EC migration and branching *in vitro* by inducing the upregulation of PAI-1 in ECs.\(^ {169}\) In addition, pericytes have been shown to produce TIMP-3 and induce TIMP-2 production in ECs, which specifically targets MT1-MMP, MT2-MMP and ADAM (a disintegrin and metalloprotease) proteinases, with the net effect of stabilizing microvasculatures by blocking both tube formation and regression.\(^ {170-172}\) In addition, pericyte-mediated ECM deposition, specifically the deposition and synthesis of basement membrane, has also been proposed to promote EC quiescence and vessel stability.\(^ {173-175}\) The importance of the basement membrane in vascular stability is demonstrated in laminin-8 knockout animals which demonstrate poorly developed microvasculature with defective basement membranes, noticeable deformities, and increased responsiveness to pro-angiogenic stimuli.\(^ {176}\)

In our studies, the presence of cell-cell contact, as demonstrated by experiments utilizing co-culture aggregates (co-culture model #2) composed of both ECs and SMCs was not sufficient in inhibiting the pro-angiogenic effects of SMCs during the time-course of these studies. We showed that even in the presence of direct SMC-EC contact, SMCs appeared to promote the invasion ECs into the fibrin matrix, with ECs appearing to co-invade in close approximation to SMCs. Indeed, the inhibition of SMC proliferation and invasion alone inhibited both SMC and EC matrix invasion, suggesting that SMCs can direct the growth of ECs into the extracellular matrix. This appears contradictory to the generally held belief that once incorporated into capillaries, pericytes
mediate the quiescence of ECs. Direct contact via gap junctions, adhesion plaques, and adherens junctions, as well as indirect paracrine mechanisms between ECs and pericytes, allow for significant interactions which impact EC differentiation, migration, proliferation, and quiescence. In vitro, ECs in direct contact with pericytes have lower levels mitosis compared to ECs cultured alone, a process which may require Rho GTPase activity in pericytes. Similarly, ECs in close contact with SMCs in a spherical aggregate of SMCs in collagen gels demonstrated decreased responsiveness to VEGF (in the absence of Ang-2), increased inter-endothelial junctions, decreased PDGF-B growth factor chain expression, and reduced apoptosis. ECs in co-culture with 10T1/2 cells also display a significantly higher resistance to the permeability of a tracer molecule biotin-dextran which was associated with tightening of EC-EC tight junctions and with the localization of tight junction proteins plakoglobin, ZO-1, ZO-2, and occludin.

The observations in our experiments, however, clearly show that SMCs in close contact with ECs can promote matrix invasion of ECs central to angiogenic processes. Thus, we conclude that the presence of cell-cell contact alone is not sufficient in promoting EC quiescence within the examined time course in this model. Above discussed factors such as the components and structure of the extracellular matrix and culture media, EC and pericyte differentiation, the time course of pericyte-mediated effects (early vs. late in angiogenesis) and specific paracrine cytokines and growth factors released by either ECs or SMC all likely contribute to cell behavior in co-culture systems.
In addition, the observation that SMCs promote the early formation of microvascular networks within the first 3 days prior to significant SMC incorporation into capillaries in co-culture experiments with SMCs homogenously distributed around EC aggregates (co-culture model #1) suggests that cell-cell contact is not required for the pro-angiogenic effects of SMCs. The release of pro-angiogenic growth factors in this early period is a hypothetical explanation for this observed effect. It is conceivable that the identity of the growth factors released by SMCs prior to significant incorporation into microvasculatures, differ from those released later in angiogenesis or after pericyte incorporation. This could be an explanation for the apparent pro-angiogenic effects of SMCs early during our co-culture assays, and the apparent contradiction to the above described studies which demonstrated the stabilizing effects of pericyte-conditioned media.

Clinically, the simultaneous targeting of pericytes and ECs by pro- or anti-PDGF and VEGF therapy has been investigated in tissue engineering and oncologic research. Combination therapy has been shown in some studies to be more effective in disrupting tumor vascularity than either agent alone and, as such, combinatorial antitumor therapy is under investigation.\textsuperscript{147-150} It is unclear, however, if the apparent improved anti-angiogenic effect observed in combination therapy is specifically attributable to PDGF blockade or to the broad non-specific effects of the receptor tyrosine kinase inhibitors used in most studies of combination therapy. Recently, it was shown that adenoviral expression of soluble anti-PDGF-β receptor provided added inhibition of tumor growth and angiogenesis only when sub-optimal levels of VEGF blockade were present.\textsuperscript{151}
Clearly, however, PDGF signaling does impact angiogenesis in vivo, as the spatiotemporal delivery of PDGF with VEGF has been shown to induce denser and more mature appearing vascular networks within tissue engineered constructs, which is in part associated with the recruitment of α-actin positive (indicative of pericytes) cells into the neovascular networks.152,153

The structures formed in our model resemble capillaries, with 2-3 ECs lining lumens within the matrix surrounded by several pericyte-like appearing structures derived from the SMCs in the matrix. Histologically, the engineered microvasculatures appear to recapitulate the native structure of capillaries in vivo. The functionality of these structures remains unclear. Responsiveness to the angiogenic growth factors VEGF and FGF-2 suggest their viability and functionality. Further studies studying nitric oxide pathways, prostacyclin synthesis, and other EC functional assays could be performed as potential future studies to determine the potential functionality of these capillaries in tissue engineered constructs.

In summary, we have demonstrated that SMCs promote both the formation and persistence of microvascular networks engineered in fibrin hydrogels, and that the presence of SMCs in the extracellular matrix does not alter the angiogenic response to exogenous growth factors commonly used in tissue engineering.
CHAPTER FIVE

THE EFFECTS OF CO-CULTURE ON EC SIGNALING

Introduction

The results of the previous Aim demonstrate that capillary networks formed in co-culture with SMCs display both increased early formation and later persistence compared to ECs cultured in the absence of SMCs. Our in vitro co-culture model of angiogenesis was devised both to test basic biologic processes within a 3-D microenvironment, and also to model angiogenic processes during vasa vasorum development in the wall of a fibrin-based tissue engineered blood vessel. The goals of this Aim are 1) to test the hypothesis that the pro-angiogenic effects of SMCs are due to the release of SMC-derived angiogens into the extracellular environment; and 2) to test the effects of these released products on EC signaling pathways involved in cell proliferation and survival.

The possibility that the demonstrated pro-angiogenic effects of SMCs in our fibrin-based angiogenesis model is accounted for, at least in part, by the release or deposition of products into the extracellular environment is supported by several observations in the literature. Secreted VEGF or deposited ECM proteins such as fibronectin by SMCs and fibroblasts in vitro have been demonstrated to elicit angiogenic activities such as increased plasminogen activity, proliferation, motility, and cordlike structure formation in co-cultured ECs.99-104
The observed endothelin-1-mediated activation of EC mitogenesis by fibroblasts and the production of Ang-2 and VEGF in mesangial cells (renal pericytes) under hypoxic conditions provide evidence that these paracrine interactions may be regulated by the local extracellular environment. These data are consistent with observations that under hypoxic stimulation, VEGF-secreting perivascular cells appear to be the first vascular cells to invade the corpus luteum parenchyma during ovulatory angiogenesis. It is likely that SMC-derived angiogens activate pathways mediated by the intracellular kinases ERK and Akt. Capillary thickness as a result of cell proliferation and migration has been shown to be dependent on ERK 1/2 signaling via VEGF/VEGFR-2 signaling, while PI3-K, an upstream regulator of Akt activity has been shown to be required for cell survival. We have chosen to quantify the extent of Akt and ERK phosphorylation in response to SMC-derived soluble factors as a proxy for cell signaling processes involved in cell survival and proliferation. A detailed and extensive review of the intracellular signaling pathways involved in angiogenesis is well beyond the scope of this dissertation. Reviews for further study are cited, and figures summarizing the basic PI3-K/Akt and ERK MAP Kinase pathways (Figures 23 and 24).

This aim is intended to investigate these interactions in a 3-D fibrin-based extracellular environment.

**Methods**

**Materials**

Chemicals, biological reagents, and experimental supplies were obtained as follows: collagenase (Invitrogen); human thrombin (American Red Cross; Rockville, MD); FGF-2, VEGF (R&D Biosciences); L-ascorbic acid, methylcellulose, fibrinogen,
transferrin, insulin, anti-α-actin antibody and aprotinin, anti-VWF antibody, PKH-27,
PKH67 (Sigma Chemical Co.; St. Louis, MO); tritiated thymidine (NEN Life Science
Products; Boston, MA); methanol, trichloroacetic acid, acetic acid, and scintillation fluid
(Fisher Scientific; Fair Lawn, NJ); bovine lung heparin (Upjohn; Kalamazoo, MI);
Calcein AM (Molecular Probes, Eugene, Ore); 0.05% trypsin/EDTA, HBSS, M199,
DMEM, L-nonessential amino acids, sodium pyruvate, penicillin, streptomycin, DMEM-
F12 (Gibco, Grand Island, NY); fetal bovine serum (FBS) (Hyclone, Logan, UT); 100-
mm and 60mm Petri dishes (Fisher Scientific; Pittsburgh, PA); 24 well plates and tissue culture flasks, Costar Transwell polystyrene plates (Corning Costar Corp; Cambridge, MA); round bottom 96 well plates (Greiner Bio-one; North Carolina); 96 well polystyrene plastic plates (Beckton Dickinson; Lincoln Park, NJ).

Figure 24. The Ras/Raf/ERK 1/2 pathway.⁷

Animal Care

All animal procedures complied with The Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, 1996) and The Principles of Laboratory Animal Care (National Institutes of Health publication no. 85-23, revised 1985) and were approved by our institutional IACUC.
Cell Isolation

SMCs and ECs were harvested from the canine carotid artery and jugular veins respectively per previously published protocols. Briefly, adult mongrel dogs were anesthetized with thiopental sodium, intubated, and ventilated. Anesthesia was maintained with nitrous oxide and methoxyflurane. Bilateral neck incisions were made.

For ECs, bilateral external jugular veins were removed, inverted, and processed per our previously reported protocol beginning with the veins being sequentially placed into 0.05% trypsin/EDTA 0.53 mmol/L and collagenase 100 U/mL at 37°C for 10 minutes each. Next, the veins were discarded, and the trypsin and collagenase solutions were centrifuged. The subsequent pellets were resuspended in EC complete medium supplemented with FGF-1 (5 ng/mL), and the ECs were plated onto a fibronectin-coated (2.5 µg/cm²) T-25 culture flask that was incubated in a 37°C, 5% carbon dioxide chamber. The media was changed every 2 to 3 days, and confluent cells were passaged with trypsin-EDTA. EC identity was confirmed using immunofluorescent dual staining with anti-vWF and anti-α-actin antibodies. Only ECs exhibiting 95% positive vWF staining and 2% or less α-actin staining were used for the proliferation assays. EC were used within passages 1 to 5.

For SMCs, the carotid arteries were opened longitudinally, and the intima and adventitia were removed by sharp dissection. The medial layer was minced and placed into SMC growth media. Primary SMCs migrating from the explants were used in all experiments. SMC identity was confirmed by immunofluorescent staining with α-actin antibodies, and only cultures exhibiting 95% positive staining were used in the assays.
Individual carotid artery explants were used as the source of cells for assays no more than 3 times.

Methocel Preparation

Methylcellulose reagent (1.2 g) was autoclaved in a 250-mL beaker at 120°C for 20 minutes. The autoclaved powder was dissolved in 50 mL of preheated (60°C) M199, stirred at 60°C for 20 minutes, and then 50 mL of M199 at room temperature was added. The solution was mixed for 2 hours at 4°C, centrifuged for 2 hours at 4,000 rpm, and the supernatant was collected after centrifugation.

Multiple Aggregate Co-culture Assay

Multiple EC and SMC co-culture aggregates were embedded within the same hydrogel to test for directional invasion towards the other aggregates. Co-culture aggregates were created as described in Aim #1 by suspending 2250 SMCs in an aggregating solution consisting of angiogenesis assay media and a 20% Methocel solution into each well of a round bottom 96-well plate. The cells were incubated at 37°C for 24 hours until smooth muscle cell aggregates formed. After 24 hours, 350 ECs were added to each well and allowed to seed the surface of the SMC aggregates. The aggregates of SMCs and ECs were incubated at 37°C for another 24 hours until co-culture cell aggregates formed comprised of a smooth muscle cell core seeded by endothelial cells on the surface. Multiple aggregates were embedded between two layers of fibrin gels in the wells of flat-bottom 96 well plates and cultured in angiogenesis assay media containing at 37°C. After a period of time, SMCs and ECs invade from the central aggregate into the surrounding hydrogel. Digital images were taken at a magnification of
4x in order to visually demonstrate the direction of radial invasion by co-culture sprouts. The experiment was performed 5 times, and representative images are reported.

**Conditioned Media Collection**

To test if SMCs in culture produce soluble factors which affect EC angiogenic behavior, media conditioned by SMCs in culture was collected and used to stimulate EC proliferation, migration, angiogenic sprouting, and signaling responses (Figure 25). Proliferation and migration assays were chosen as they are generally considered requisite facets of angiogenic induction. These data are meant to complement the results of the 3-D angiogenesis assays, which hypothetically include proliferation, migration, matrix remodeling, and other aspects of angiogenesis and matrix invasion. The presence of extracellular matrix can have the effects of altering cellular behavior. For this reason, select experiments were carried out in which media conditioned by SMCs in standard 2-D cell culture in 24-well polystyrene plates were collected in addition to media conditioned by SMCs embedded in a 3-D environment within fibrin hydrogels.

To collect 2-D SMC-conditioned media, SMCs were cultured onto 24 well polystyrene plates (240,000 SMCs per well) in angiogenesis assay media. After 72 hours the media was collected and the cells were discarded. Angiogenesis assay media incubated in 24 well plates for 72 hours without cells served as the unconditioned media controls. To collect 3-D SMC-fibrin-conditioned media, fibrin hydrogels with or without homogenously distributed SMCs as previously described (400,000 SMCs/mL of gel or 240,000 cells when calculated by volume) were cultured in invasion assay media. After 72 hours of culture, the media in each group was collected and the hydrogels were
Figure 25. Schematic depiction of conditioned media collection utilized as a stimulant for EC proliferation, migration, angiogenesis and signaling. (Left) 2-D SMC-conditioned media. SMCs are cultured on Petri dishes and the media is collected after 72 hours. (Bottom left) Unconditioned media is used as a control for experiments utilizing 2-D SMC-conditioned media. (Right) 3-D Fibrin-SMC-conditioned media. SMCs are cultured in fibrin hydrogels and the culture media is collected after 72 hours. (Bottom right) Media conditioned by acellular fibrin gels are used as controls for experiments utilizing 3-D SMC-Fibrin-conditioned media.

discarded. A bicinchoninic acid (BCA) protein assay was used to calculate the total protein content in each group. This media was used to test for EC mitogenic, chemotactic, angiogenic, and signaling responses as described below.

EC 2-D Proliferation Assay

Tritiated thymidine incorporation for evaluation of DNA synthesis was used to assay cell proliferation in 2-D culture as previously described. ECs were plated onto fibronectin-coated (2.5 µg/cm²) 96-well plates at an initial concentration of 10,000 cells per well with 200 µL of EC complete media and cultured for three days at 37 °C until they reached 80-90% confluence by estimation of cellular coverage over the area of the bottom of the well. At that point, the medium was then removed; the cells were washed
with phosphate-buffered saline (PBS), and 200 µL of EC quiescent medium was placed in each test well to synchronize cells in G₀ for 24 hours for ECs. Cells were presumed to be viable if they did not wash away with washings, appeared morphologically normal, and proliferated in response to positive control wells stimulated by FBS. The cells were then stimulated with unconditioned media, SMC-conditioned media, fibrin-conditioned media, or 3-D SMC-fibrin-conditioned media. A BCA protein assay determined the total protein content in each conditioned media group, and the total volume was normalized to stimulate each group with equivalent amounts of total protein (total volume of 50 µL per well). Fifty microliters of PBS was the negative control, and 50 µL of FBS (20% of total volume in the well) was the positive control. The next day, 1 µCi of tritiated thymidine was placed in each well for 24 hours. The wells were processed by removal of the media and washing with PBS. The cells were fixed in 100% methanol for 10 minutes, lysed with distilled water, and precipitated with 5% trichloroacetic acid. The cell lysates were washed with distilled water, and the DNA was solubilized with 0.3 mol/L sodium hydroxide. This solution was placed into 10 mL of scintillation fluid that contained 20 µL of acetic acid to avoid opacification of the scintillation fluid. DNA synthesis was quantified by the amount of tritiated thymidine incorporated into cellular DNA over a 24 hour incubation period and radioactivity in TCA precipitable material was measured using a scintillation counter units of counts per minute (CPM). Each individual experiment was analyzed statistically with Student’s T-test with statistically consistent results. The data represents the mean CPM +/- SEM from 3 independent experiments normalized to unconditioned or fibrin-conditioned controls.
EC 2-D Migration Assay

ECs were seeded on the inner membrane of a fibronectin-coated Costar Transwell polystyrene plate (8.0 µm pore size in the polycarbonate membrane) in angiogenesis assay media with 1% FBS. After allowing the cells to attach for four hours, a test solution containing fibrin-conditioned media or 3-D SMC-fibrin-conditioned media was placed in the lower chamber in a total volume of 600 µL, normalized to total protein. After 6 hours of migration, the media in the upper and lower chambers were gently suctioned off and the non-migratory cells were scraped from the inner membrane with a cotton-tipped applicator. The remaining migratory cells were fluorescently labeled with Calcein AM. Four quadrants of the membrane were visualized under FITC at a magnification of 4x and fluorescent cells were counted and averaged for each well. Each group was tested in replicates of four and the experiment was performed twice. Mean results from each individual well are presented as the mean migratory cells per visual field +/- SEM (n=8).

Angiogenesis Assay

ECs were aggregated and embedded within fibrin hydrogels as previously described in Aim#1. They were cultured in media conditioned either by SMC-fibrin gels or by acellular fibrin gels. The average lengths of sprouts were quantified as described in Aim #1. The experiment was performed in replicates of four and the experiment was duplicated. The pooled data from the two independent experiments are presented as mean length of sprouts +/- SEM (n= 8).
Signaling Assays

ECs were plated on fibronectin-coated (2.5 µg/cm²) polystyrene 30mm Petri dishes. After a 4 hour quiescence period in serum free M199, the cells were stimulated with either 2-D SMC or 3-D SMC-fibrin-conditioned media, or 2-D unconditioned or 3-D fibrin-conditioned media for 20 minutes. Protein content was determined in each of the conditioned or unconditioned medias prior to use as stimulants. Volumes were adjusted in order to stimulate with equal amounts of protein (from serum, SMC released products, etc.) in each conditioned and unconditioned media pair. After 20 minutes, the cells were lysed with buffer, scraped, and centrifuged for 20 minutes at 10,000 g. The supernatant was collected and a BCA protein assay was performed in order to determine the protein content in each group. Western blot analysis was used to determine the amount of total ERK, phosphorylated ERK, total Akt, and phosphorylated Akt. Blots were probed with anti-active MAPK (1:5000), anti-total ERK 2(1:3300), anti-pAkt (Ser 471; 1:1000), and anti-pan Akt (1:1000) overnight at 4°C. The blots were washed and probed by HRP-conjugated IgG (1:5000) for 2 hours, washed, and exposed on ECL hyperfilm. Western blots were quantified by densitometry (Un-Scan-it Gel 6.1, Silk Scientific, Inc; Orem, Utah), and phosphorylated ERK and Akt was normalized to their respective total protein amounts. The experiment was performed 3 times. The data are the pooled means of each independent experiment +/- SEM. Table 1 summarizes the conditioned media tested for each assay.
Table 1. Summary of Conditioned Media and Controls Tested in EC Assays in Aim 2.

<table>
<thead>
<tr>
<th></th>
<th>2-D SMC-Conditioned media</th>
<th>Unconditioned media (control)</th>
<th>3-D SMC-Fibrin-conditioned media</th>
<th>3-D Fibrin-conditioned media (control)</th>
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<tbody>
<tr>
<td>EC Proliferation</td>
<td>X</td>
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<tr>
<td>EC Migration</td>
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<tr>
<td>EC Angiogenesis</td>
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<tr>
<td>EC Signaling</td>
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Results

Directional Invasion from Multiple Co-Culture Aggregates

Multiple co-culture aggregates were embedded within individual fibrin hydrogels (Figure 26). Digital images were taken which included the embedded aggregates and the sprouts radiating from the aggregates. Sprouts radiating from the co-culture aggregates qualitatively demonstrated directional invasion towards other sprouts within the same hydrogel. Single aggregates demonstrated random, non-directional sprouting by visual estimation. Assays were performed 5 times with representative images demonstrated in the figure.

Figure 26. Directional invasion for co-culture aggregates. (Left) Individual SMC/EC co-culture pellets demonstrate random invasion into hydrogel. (Right panels) Co-culture pellets demonstrate directional invasion towards one another in hydrogels.
Proliferation Assay

Media conditioned by SMCs in either 2-D culture or in 3-D fibrin culture was used to stimulate quiescent ECs cultured in a monolayer on 96 well plates (Figure 27). ECs stimulated with media conditioned by SMCs in 2-D culture demonstrated thymidine incorporation that was 30.7% greater than ECs stimulated by media unconditioned by SMCs (100 +/- 5.0% vs. 131 +/- 15.0%; p=.066). ECs stimulated by media conditioned by SMCs cultured in 3-D fibrin hydrogels demonstrated thymidine incorporation that was 35.9% greater than ECs stimulated by media conditioned by acellular fibrin hydrogels (100 +/- 4.0 vs. 136 +/- 4.2%; p < .001).

![Image of ECs stimulated by conditioned media](image)

Figure 27. SMC-conditioned media induces EC proliferation. EC were cultured in the presence SMC-conditioned or unconditioned media and EC mitogenicity was determined by tritiated thymidine incorporation. (Left) ECs stimulated by media conditioned by SMCs in 2-D culture. (Right) ECs stimulated by media conditioned by SMCs in 3-D culture.
Migration assay

EC chemotaxis towards media conditioned by SMCs in 3-D fibrin hydrogel culture was tested with a modified Boyden chamber assay (Figure 28). Migratory cells in groups migrating towards SMC-conditioned media were 4.7–fold greater than control groups migrating toward media conditioned by acellular fibrin hydrogels (3.6 +/- 1.0 vs. 17.0 +/- 1.9 cells/visual field; p < .001).

Angiogenesis assay

EC angiogenesis in response to media conditioned by SMCs in 3-D fibrin hydrogel culture was tested (Figure 29). The average length of sprouts in groups cultured in 3-D SMC-fibrin-conditioned media was 488% of controls containing media conditioned by acellular fibrin hydrogels (63.1 +/- 7.1 vs. 12.9 +/- 7.5 µm; p < .01).

Signaling

Media conditioned by SMCs in either 2-D culture or in 3-D fibrin culture was used to stimulate quiescent ECs cultured in 30 mm polystyrene Petri dishes. The phosphorylation of ERK and Akt was tested with Western blot analysis (Figures 30 and
31) pERK/total ERK levels in ECs stimulated by 2-D SMC-conditioned media was 7.7X greater than controls (771.1 +/- 182% vs. 100.0 +/- 12.6%; p=0.02) and 3.2X greater than controls in ECs stimulated with 3-D SMC-Fibrin-conditioned media (324.2 +/- 60.8% vs. 100.0 +/- 17.0%; p=0.02). pAkt/total Akt levels in ECs stimulated by 2-D SMC-conditioned media was significantly less than controls (100.0 +/- 14.2% vs. 27.3 +/- 10.7%; p=0.006). This was also seen in ECs stimulated with 3-D SMC-Fibrin-conditioned media (100.0 +/- 34.3% vs. 26.6 +/- 4.4%; p=0.03).

In the absence of fetal bovine serum, no Akt phosphorylation was demonstrated in control unconditioned media groups (Figure 32). ERK phosphorylation was unaffected (not shown).
Figure 30. Levels of phosphorylated endothelial cell ERK after stimulation with SMC-conditioned or unconditioned media. Cell lysates were collected and pERK levels were quantified by Western blot analysis.

Figure 31. Levels of phosphorylated endothelial cell Akt after stimulation with SMC-conditioned or unconditioned media. Cell lysates were collected and pAkt levels were quantified by Western blot analysis.
Discussion

The results of this Aim provide a mechanistic explanation for the pro-angiogenic effects of SMCs in our co-culture model system. SMCs cultured both in conventional 2-D culture and in 3-D fibrin hydrogels release products into the extracellular environment that promote angiogenic processes in ECs. This is supported by the observation that sprouts from multiple SMC/EC co-culture aggregates placed within the same fibrin hydrogel demonstrate preferential invasion towards sprouts from adjacent co-culture aggregates. We hypothesize that this is at least partly a function of the release of chemotactic agents that are released and deposited as a gradient onto the extracellular matrix. In addition, remodeling of the ECM by proteases from the SMCs, ECs, or both may also create microscopic channels in the hydrogel that provide a path of least resistance for sprouts within the vicinity. The results of the previous Aim, which demonstrated that the inhibition of SMC matrix invasion and proliferation inhibited both SMC and EC matrix invasion, provide support for this mechanistic hypothesis.
It is clear, however, that the release of pro-angiogenic products into the extracellular environment is a key component to the pro-angiogenic effects of SMCs. ECs cultured in SMC-conditioned media (but in the absence of SMCs) demonstrate increased proliferation, migration and angiogenesis compared to ECs cultured in media unconditioned by SMCs. We have not identified these products. However, we hypothesize that these include growth factors and other cytokines such as VEGF, angiopoietin, endothelin-1, and others, which are known to be released by perivascular cells such as SMCs.  

In addition, the degradation of the fibrin products by SMC-derived proteases may release fibrin degradation products into the media, which are known to affect angiogenesis.  

In fact, the modulation of the ECM by pericytes is thought to be a critical component for the function of pericytes during angiogenesis, most commonly studied in the context of the later stabilization of microvasculatures. Pericyte-conditioned media has been shown to limit both EC migration and branching in vitro by inducing the upregulation of protease inhibitors in ECs. In addition, pericytes have been shown to produce various tissue inhibitors of metalloproteinases (TIMP) and induce specific TIMP production in ECs, which have been shown to decrease the angiogenic potential of ECs. In addition, pericyte-mediated ECM deposition, specifically the synthesis and deposition of basement membrane, has also been proposed to promote EC quiescence and vessel stability. The results of these studies demonstrate that perivascular cells such as SMCs can not only provide the stabilizing stimuli, but also have functions which promote the early formation of angiogenic networks.
In our studies, media conditioned by SMCs in 2-D culture absent of fibrin induced similar EC cell signaling responses to those seen in ECs stimulated by media conditioned by SMCs in 3-D fibrin culture. This suggests that the extracellular matrix is unnecessary for the pro-angiogenic effect of SMCs in our model. Similar results in cell proliferation also support this conclusion. This does not, however, discount the importance of the ECM in modifying the cell-cell interactions either by modifying the identity of the angiogenic products released by SMCs, affecting the functionality of those products, or modulating the specific EC responses to those products.\(^{189}\) The effects of the fibrin ECM are not specifically investigated in these studies. However, these are an important component to the cell-cell and cell-matrix interactions present during angiogenesis and are worthy of further inquiry.

We also have demonstrated that the stimulation of ECs with media conditioned by SMCs cultured in both 2-D and 3D culture environments affects cell signaling responses involved in cell proliferation and survival. The activation of the intracellular signaling kinase ERK 1/2 is consistent with the demonstrated pro-mitogenic effects of SMC-conditioned media seen at the cellular level. This suggests that the pro-angiogenic response of ECs to SMC-conditioned media is likely in part due to either growth factors directly secreted by SMCs or to induced autocrine growth factor effects in ECs.

Results of the previous Aim demonstrated that the presence of SMCs induced both the early formation of angiogenic networks and the later persistence of the networks in comparison to ECs cultured alone. This appears to be in contrast to the observed suppression of basal levels of EC Akt by SMC-conditioned media, considering the
known pro-survival effects of the PI3-K/Akt pathway. Serum factors in experimental media were required for the activation of Akt. The possibility that these serum factors were present in lower concentrations in SMC-conditioned media (hypothetically, cultured SMCs could have utilized these factors during the conditioning culture period) was controlled for by normalizing the conditioned and control media to total protein levels prior to stimulating ECs.

Explanations for these are speculative at this point. One hypothesis is that the identity of the secreted growth factors in our system, and/or the EC signaling responses to these growth factors, change over time in culture. This could be due to potential phenotypic changes in SMCs and/or ECs induced by direct cell-cell contact or close cellular approximation after incorporation of SMCs as capillary pericytes. PDGF, known to be secreted by ECs, and crucial in the recruitment of pericytes to neocapillaries, has been shown to increase expression of pro-angiogenic mediators including angiopoietin-1 (Ang-1) in SMCs. Ang-1 acts as an EC Tie-2 receptor agonist, causing rapid receptor transphosphorylation, leading to subsequent downstream activation of the protein kinase B/Akt/FKHR (FOX01) pathway to promote EC survival, a potential mechanism of pericyte-induced neocapillary stability. In these studies, SMC-conditioned media was collected after 72 hours of culture without any reciprocal interaction with ECs. Therefore, it is possible that the signaling events that are being tested in these experiments more precisely model the early events in our co-culture system described in Aim #1, prior to the events which lead to the activation of EC pro-survival and stability pathways. Indeed, the deactivation of Akt, may reflect processes...
early in angiogenesis which destabilize the endothelium, and thus promote a more synthetic and dynamic EC phenotype required for initial capillary formation.

In summary, we have demonstrated that SMCs promote the early formation and later persistence of capillary networks engineered in fibrin hydrogels (Figure 33). Our fibrin-based co-culture system results in capillary networks comprised of SMCs acting as pericytes around EC lined tubules in a 3-D environment. SMCs release angiogens that promote EC proliferation, migration, and angiogenesis. These angiogens activate ERK 1/2 while deactivating basal levels of Akt in ECs. Alternatively, SMCs may release products in the extracellular environment that induce the release of angiogens from ECs which act in autocrine fashion to promote angiogenesis.

Figure 33. Summary of results of Aims 1 and 2.
CHAPTER SIX
THE ROLE OF NOTCH IN MEDIATING SMC/EC INTERACTIONS

Introduction

In the previous two Aims, we have demonstrated that SMCs promote angiogenic network formation and persistence in fibrin hydrogels, at least in part due to the release of products that are mitogenic, chemotactic, and angiogenic for ECs. These products also activate ERK 1/2 by inducing its phosphorylation, while deactivating Akt by affecting its dephosphorylation. The current Aim intends to identify the role of Notch signaling in mediating these molecular and cellular events.

Signaling through Notch, a cell membrane receptor, is an evolutionarily conserved pathway which impacts cell fate and differentiation determination (Figure 34). Notch is a known mediator of EC-EC cell signaling during angiogenesis which affects EC quiescence, apoptosis, differentiation, and branching behavior. Notch receptors and their Delta-like-ligand 4 (Dll4) appear to be central components in restricting the formation of tip cells in response to VEGF within angiogenic sprouts, which limits the branching and sprouting patterns of angiogenic networks and limits tube diameter by inhibiting EC proliferation. Consistent with these results, the Notch intracellular domain (NICD), which is the cleaved active domain of the Notch receptor, has been shown to decrease VEGF-induced ERK 1/2 phosphorylation in ECs, and Notch has been shown to activate expression of VEGFR-3 and the anti-
apoptotic protein Bcl-2. Blockade of delta-like ligand 4 in tumor-associated ECs leads to the increased vascularity and branching of tumors, although these microvasculatures demonstrate poor function and are associated with decreased tumor growth.

Figure 34. Ligand-mediated Notch signaling.

In addition to its role in modulating EC-EC cell-cell signaling, several studies have suggested the role of Notch in EC and pericyte interactions during angiogenesis: 1) SMC Notch mediates post-ischemic neovascular patterning, morphology and collateralization in the cerebral arterial circulation after carotid artery ligation; 2) Disruptions in Notch signaling are associated with dysfunction in pericyte coverage in angiogenic networks; 3) Notch-3 mediates PDGF-β expression in SMCs and the SMC response to PDGF-BB; 4) Notch is required for differential gene expression seen in fibroblast/EC co-culture systems; and 5) Pericyte Notch-3 is required for EC-induced
phenotypic differentiation of pericytes, and mediates subsequent autoregulatory 
upregulation of pericyte Notch-3 and Jagged-1 expression.\textsuperscript{208} These observations suggest 
that Notch signaling mediates the recruitment of pericytes to neovasculatures, as well as 
their subsequent genetic expression and phenotype, including the induced expression of 
pericyte Notch ligands. However, while Notch signaling within ECs is known to 
modulate their angiogenic behavior, and while the active intracellular cleavage domain, 
NICD, can directly affect intracellular phosphorylation events, the modulation of pericyte 
effects on ECs by Notch is unknown.

This Aim will investigate the role of EC notch signaling on the pro-angiogenic 
effects of SMCs on ECs in our fibrin-based co-culture model system.

\textbf{Materials and Methods}

\textbf{Materials}

Chemicals, biological reagents, and experimental supplies were obtained as 
follows: collagenase (Invitrogen); human thrombin (American Red Cross; Rockville, 
MD); FGF-2, VEGF (R&D Biosciences); DAPT, methylcellulose, fibrinogen, insulin, 
anti–α-actin antibody and aprotinin, Anti-VWF antibody (Sigma Chemical Co.; St. Louis, 
MO); bovine lung heparin (Upjohn; Kalamazoo, MI); 0.05% trypsin/EDTA, HBSS, 
M199, DMEM, L-nonessential amino acids, sodium pyruvate, penicillin, streptomycin, 
DMEM-F12 (Gibco, Grand Island, NY); fetal bovine serum (FBS) (Hyclone, Logan, 
UY); 100-mm and 60mm Petri dishes (Fisher Scientific; Pittsburgh, PA); 24 well plates 
and tissue culture flasks, Costar Transwell polystyrene plate (Corning Costar Corp;
Cambridge, MA); round bottom 96 well plates (Greiner Bio-one; North Carolina); 96-well polystyrene plastic plates (Beckton Dickinson; Lincoln Park, NJ).

Animal Care

All animal procedures complied with The Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, 1996) and The Principles of Laboratory Animal Care (National Institutes of Health publication no. 85-23, revised 1985) and were approved by our institutional IACUC.

Cell Isolation

SMCs and ECs were harvested from the canine carotid artery and jugular veins, respectively per previously published protocols. Briefly, adult mongrel dogs were anesthetized with thiopental sodium, intubated, and ventilated. Anesthesia was maintained with nitrous oxide and methoxyflurane. Bilateral neck incisions were made.

For ECs, bilateral external jugular veins were removed, inverted, and processed per our previously reported protocol beginning with the veins being sequentially placed into 0.05% trypsin/EDTA 0.53 mmol/L and collagenase 100 U/mL at 37°C for 10 minutes each. Next, the veins were discarded, and the trypsin and collagenase solutions were centrifuged. The subsequent pellets were resuspended in EC complete medium supplemented with FGF-1 (5 ng/mL), and the EC were plated onto a fibronectin-coated (2.5 µg/cm²) T-25 culture flask that was incubated in a 37°C, 5% carbon dioxide chamber. The media was changed every 2 to 3 days, and confluent cells were passaged with trypsin-EDTA. EC identity was confirmed using immunofluorescent dual staining
with anti-vWF and anti-α-actin antibodies. Only EC exhibiting 95% positive vWF staining and 2% or less α-actin staining were used for the proliferation assays. EC were used within passages 1 to 5.

For SMCs, the carotid arteries were opened longitudinally, and the intima and adventitia were removed by sharp dissection. The medial layer was minced and placed into SMC growth media. Primary SMCs migrating from the explants were used in all experiments. SMC identity was confirmed by immunofluorescent staining with α-actin antibodies, and only cultures exhibiting 95% positive staining were used in the assays. Individual carotid artery explants were used as the source of cells for assays no more than 3 times.

**Methocel Preparation**

Methylcellulose reagent (1.2 g) was autoclaved in a 250-mL beaker at 120°C for 20 minutes. The autoclaved powder was dissolved in 50 mL of preheated (60°C) M199, stirred at 60°C for 20 minutes, and then 50 mL of M199 at room temperature was added. The solution was mixed for 2 hours at 4°C, centrifuged for 2 hours at 4,000 rpm, and the supernatant was collected after centrifugation.

**Conditioned Media Collection**

To collect SMC conditioned media, 240,000 SMCs per well were cultured in 24 well plates in angiogenesis assay media as previously described in Aim #2. After 72 hours of culture, the media was collected. Angiogenesis assay media incubated in the absence of SMCs for 72 hours serve as the unconditioned media controls. A BCA protein assay was used to calculate the total protein content in each group and
experimental and control groups in experiments were normalized to total protein. This media was used to test for EC angiogenic and signaling responses as described below.

**Notch Inhibition and Signaling**

In experiments that tested the effects of Notch inhibition on EC signaling pathways, ECs were plated on fibronectin-coated (2.5 µg/cm²) 30 mm polystyrene Petri dishes. After a 3 hour quiescence period in serum free M199, DAPT (25µM) was added to the media and cells were incubated for an additional hour.

**Effects of Notch Inhibition on Growth Factor Induced Signaling**

After the period of quiescence and DAPT pretreatment, the media was removed, and the cells were stimulated with angiogenesis assay media with 2.5% FBS containing VEGF + FGF-2 (10ng/mL for each growth factor in combination) and DAPT (25µM) for either 20 minutes or 48 hours in fresh serum free media containing 25µM DAPT. In experiments carried out for 48 hours, media, growth factors and DAPT were replenished after the first 24 hours. Controls in all DAPT experiments were pretreated with the DMSO carrier in equimolar amounts.

**Effects of Notch Inhibition on SMC-Conditioned Media Induced Signaling**

In experiments testing SMC-conditioned media, after the period of quiescence and DAPT pretreatment, the media was removed, and 2-D SMC-conditioned or unconditioned media was added with or without DAPT to stimulate ECs for 20 minutes. Controls in all DAPT experiments were pretreated with the DMSO carrier in equimolar amounts. Protein content was determined in each of the conditioned or unconditioned
media prior to use as stimulants. Volumes were adjusted in order to stimulate with equal amounts of protein in each conditioned and unconditioned media pair.

Western Blots

Cells were lysed with buffer, scraped, and centrifuged for 20 minutes at 10,000 g. The supernatants were collected and a BCA protein assay was performed in order to determine the protein content in each group. Western blot analysis was used to determine the amount of total ERK, phosphorylated ERK, total Akt, and phosphorylated Akt. Blots were probed with anti-active MAPK (1:5000), anti-total ERK 2(1:3300), anti-pAkt (Ser 471; 1:1000), anti-pan Akt (1:1000), or anti-GAPDH (1:1000) overnight at 4°C. The blots were then washed and probed by HRP-conjugated IgG (1:5000) for 2 hours, washed, and exposed on ECL hyperfilm. Western blots were quantified by densitometry (Un-Scan-it Gel 6.1, Silk Scientific, Inc; Orem, Utah), and phosphorylated ERK and Akt was normalized to their respective total protein amounts. Experiments were performed 3-5 times, and the pooled means +/- SEM from each independent experiment is presented. For experiments testing the effects of DAPT pretreatment on EC signaling responses to VEGF + FGF-2 the results were normalized to ECs unstimulated by growth factors and untreated with DAPT. In experiments testing the effects of DAPT pretreatment on EC signaling responses to SMC-conditioned media, the results were normalized to the mean densitometric measurements of ECs stimulated by SMC-conditioned media untreated with DAPT.
Identifying Notch Receptor and Ligand

ECs or SMCs were plated on fibronectin-coated plates in angiogenesis assay media with 2.5% FBS and treated with VEGF and FGF-2 in combination (10ng/mL for each growth factor) for 48 hours. The growth factors and media were replenished after 48 hours. The presence of Notch 1, 2, 3, and 4 as well as Jagged-1 and Dll-4 was tested by Western blot analysis as previously described. Primary antibodies (Santa Cruz) were added at a dilution of 1:250 and incubated overnight at 4°C. The blots were washed and probed by HRP-conjugated -anti rabbit IgG (1:5000) for 2 hours, washed, and exposed on ECL hyperfilm.

Notch Inhibition and Angiogenesis

ECs were aggregated as previously described and embedded in fibrin hydrogels which contained 25µM of DAPT or an equal volume of DMSO. The assays were then cultured in 2-D SMC-conditioned media or unconditioned media. To test the effects of Notch signaling, either 25µM of DAPT or an equal volume of DMSO (DAPT carrier) was added to the culture media. The media was changed and the DAPT or DMSO was replenished daily. After 72 hours, the assays were digitally imaged and angiogenesis was quantified by calculating the average length of sprouts as described in Aim #1. The experiment was performed in duplicate and the pooled results are presented as mean length of sprouts +/- SEM (n=9-10).

Eosin Uptake

In order to approximate the kinetics of uptake of DAPT into fibrin hydrogels, the fluorophore eosin (620 Da) was utilized due to its similar molecular weight to DAPT.
Acellular fibrin hydrogels were created in 96 well plates. Angiogenesis assay media containing eosin (1:100) was added to each well. Gels and media were collected at time 0 and then at 1, 2, 4, 24, and 48 hours of incubation with eosin containing media. The fluorescence of both the gel and media component was measured with a fluorescence plate reader (Perkin Elmer, Waltham, MA) at 560 nm. A group of gels were also analyzed further by counting the fluorescence of gel surfaces and deep layers separately in order to demonstrate penetration of eosin into the gel. The predicted amount of fluorescence in the gel and media components based on volume distribution was calculated by multiplying the total fluorescence in the well (fluorescence in media + fluorescence in gel) by the % of the volume occupied by the gel or the media, respectively. The data was normalized to this predicted amount. Positive controls were wells that contained eosin-containing media without gels that was collected after periods of incubations at time points described above. The experiment was duplicated and a representative set of results are presented as mean fluorescence +/- SEM (n=4-5). Table 2 summarizes the groups in the major above described experiments.

Table 2. Summary of Experiments in Aim 3.

<table>
<thead>
<tr>
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<th>VEGF + FGF-2</th>
<th>VEGF + FGF-2 + DAPT</th>
<th>Unconditioned Media</th>
<th>2-D SMC-condition Media</th>
<th>2-D SMC-condition Media + DAPT</th>
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<td>EC signaling</td>
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<td>Notch Receptor/Ligand</td>
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<td>Angiogenesis</td>
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**Results**

Effects of Notch Inhibition on EC Akt and ERK 1/2

Phosphorylation in Response to Growth Factors:

ECs stimulated by VEGF and FGF-2 in combination (10ng/mL for each growth factor) demonstrated a 2.25-fold increase in phosphorylated Akt compared with unstimulated controls (225 +/- 43.2% vs. 100.0 +/- 36.1%; p =.04; Figure 35). Increases in phosphorylated Akt in growth factor stimulated ECs pretreated with DAPT was significantly higher compared with growth factor stimulated ECs that were not pre-treated with DAPT (373.7 +/- 51.5% vs. 225 +/- 43.2% of unstimulated, non-DAPT treated controls; p< .05). DAPT pre-treatment had no effect on levels of phosphorylated Akt in ECs that were not stimulated with growth factors.

![Akt](image)

Figure 35. ECs treated with DAPT and stimulated with growth factors for 20 minutes demonstrate increased levels of phosphorylated Akt compared to DAPT untreated cells stimulated with growth factors. *p < .05.

Levels of phosphorylated ERK was 12.3-fold higher in ECs that were stimulated with growth factors compared with ECs that were unstimulated with growth factors
(1230.0 +/- 112% vs. 100.0 +/- 36.8%; p < .001; Figure 36). The pretreatment of ECs with DAPT had no effect on the levels of phosphorylated ERK in response to growth factors compared with cells that were not stimulated with growth factors (p=.46). DAPT pretreatment had no effect on levels of phosphorylated ERK in cells that were not stimulated with growth factors.

Figure 36. Notch inhibition and growth factor induced ERK1/2 phosphorylation in ECs. Notch inhibition with DAPT does not affect levels of phosphorylated ERK 1/2 in growth factor stimulated ECs (20 minutes of stimulation) treated with DAPT compared to DAPT untreated cells.

ECs were stimulated with VEGF + FGF-2 in the presence and absence of DAPT for 48 continuous hours to determine if protein levels were affected by growth factors (Figures 37 and 38). After 48 hours, levels of phosphorylated Akt did not differ in growth factor-treated groups in the presence or absence of DAPT compared with
unstimulated cells untreated with DAPT (p = 0.72). Total Akt also did not differ among any of the groups (p = 0.46; Figure 37).
Figure 37. Notch inhibition has no effect on levels of phosphorylated Akt or total Akt levels in response to VEGF and FGF-2 after 48 hours of stimulation.
After 48 hours of continuous growth factor stimulation, levels of phosphorylated ERK were not significantly increased compared with unstimulated ECs in DAPT untreated cells (p = .19; Figure 38). DAPT pretreatment did not affect levels of phosphorylated ERK in growth factor-stimulated cells compared with unstimulated ECs that were untreated with DAPT. Total levels of ERK were not significantly altered by growth factor stimulation or DAPT treatment (p = 0.75).

**Notch Ligands and Receptors on ECs and SMCs**

Notch 1, 3 and 4 was detectable on ECs and Notch 1 and 3 was detectable on SMCs by Western blot analysis. This was consistent in cells that were either stimulated or unstimulated with VEGF + FGF-2 for 48 hours. Dll-4 was detectable in both cell types in the presence and absence of growth factors in ECs and SMCs. Neither Notch-2 nor Jagged-1 was detectable in either cell type both in the presence and absence of growth factors (Figures 39 and 40).

**Effect of Notch Inhibition on EC Signaling Responses to SMC-Derived Soluble Factors**

Phosphorylated Akt levels in ECs stimulated by media unconditioned by SMCs in 2-D culture were significantly greater than cells which were stimulated by conditioned media (366.9 +/- 52.1% vs. 100.0 +/- 39.3% for unconditioned vs. conditioned media groups, respectively; p < 0.01; Figure 41). pAkt levels in DAPT-pretreated ECs stimulated with SMC-conditioned media was 5.6X greater than in non-pretreated ECs stimulated with SMC-conditioned media (558.1 +/- 104.4% vs. 100.0 +/- 39.3%; p = .003), not significantly different than ECs untreated with DAPT and stimulated with
Figure 38. Notch inhibition has no effect on levels of phosphorylated or total ERK 1/2 levels in response to VEGF and FGF-2 after 48 hours of stimulation. * < .05.
Figure 39. Dll-4 expression on ECs and SMCs in the presence and absence of VEGF + FGF-2 (10ng/mL for each growth factor).

Figure 40. Notch receptors on ECs and SMCs in the presence and absence of VEGF + FGF-2 (10ng/mL for each growth factor).
Figure 41. Notch inhibition on levels of phosphorylated Akt in ECs stimulated with SMC derived soluble factors. ECs were plated on Petri dishes and stimulated with SMC-conditioned media either in the presence or absence of DAPT for 20 minutes. Unconditioned media (558.1 +/- 104.4% vs. 366.9 +/- 52.1% for DAPT/SMC-conditioned vs. no DAPT/unconditioned groups, respectively; p= .18).

Phosphorylated ERK 1/2 levels in ECs stimulated by media conditioned by SMCs in 2-D culture was significantly greater than ERK 1/2 levels in ECs stimulated by unconditioned culture media (12.9 +/- 1.6% vs. 100.0 +/- 23.6% for unconditioned vs. conditioned groups, respectively, p=0.02; Figure 42). DAPT treatment did not affect levels of phosphorylated ERK in response to SMC-conditioned media (p=0.3).
Effects of Notch on Angiogenesis in Response to SMC-Derived Factors

In order to test the effects of Notch inhibition on angiogenesis in a 3-D environment, preliminary studies were done testing the uptake of eosin, a molecule of similar molecular weight to DAPT, to demonstrate penetration of drug into the hydrogel. (Figure 43) Positive control groups demonstrated that the fluorescence of eosin persisted throughout the 48 hour time course when cultured in media. In experimental groups, uptake of eosin into hydrogels from the media approached a steady state level by 48 hours, with 55% of eosin uptake occurring within the first 4 hours of incubation, and 89% by 24 hours. The final steady state values did not significantly differ from the predicted amounts based on the volume occupied by the hydrogel and media.
Figure 43. Eosin uptake in fibrin hydrogels. Fibrin hydrogels were cultured in media containing the fluorophore eosin. Fluorescence in the gel and the media was measured. * p < 0.05.
The effect of EC Notch inhibition with DAPT on the angiogenic response to SMC-conditioned media was determined. After 72 hours, the average length of EC sprouts in groups that were cultured in the presence of SMC-conditioned media was 289% of controls (289.1 +/- 32.0 vs. 100.0 +/- 43.5%; p=.003). The length of EC sprouts in groups treated with DAPT cultured in the presence of SMC-conditioned media was significantly less than ECs untreated with DAPT and cultured in the presence of SMC-conditioned media (172.6 +/- 20.4% vs. 289.1 +/- 32.0% of controls for DAPT treated and untreated ECs, respectively; p=.01) (Figure 44). The length of EC sprouts in groups treated with DAPT cultured in the presence of SMC-conditioned media did not significantly differ from DAPT-untreated controls cultured in the presence of unconditioned media (172 +/- 20.4% vs. 100 +/- 43.5%; p = .15).

Figure 44. Effect of Notch inhibition on angiogenesis in response to SMC derived soluble factors. ECs were aggregated and embedded within fibrin hydrogels. They were cultured in SMC-conditioned media either in the presence of absence of DAPT.
**Discussion**

Notch is an evolutionarily conserved cell signaling pathway which, among a diverse set of functions, plays a critical role in angiogenesis regulation. The specific effects of Notch on angiogenesis, and the specific factors which modulate those effects, are still somewhat unclear. Further, the effects of Notch signaling in ECs in response to pericyte-mediated effects on angiogenesis are largely unknown. In the studies presented in this Aim, we demonstrate that Notch inhibition with DAPT, a known inhibitor of gamma-secretase, inhibits the decrease in endothelial cell phosphorylated Akt seen after treatment with SMC-conditioned media. Thus, we show that active Notch signaling in ECs is required for the reduction of active Akt in response to soluble factors released by SMCs. Whether the effects of SMC conditioned media on levels of phosphorylated Akt are directly attributable to the modulation of kinase phosphorylation or phosphatase dephosphorylation of Akt, or both, is not known. Further we show that while SMC-conditioned media promotes the sprouting of endothelial cells in an *in vitro* angiogenesis assay, Notch inhibition attenuates this response, suggesting that active Notch in endothelial cells is required for the increased EC sprouting in response to SMC-derived soluble factors.

Notch has become an increasingly attractive target for strategies that aim to modulate angiogenesis. The bulk of this research is directed toward the treatment of oncologic disease, with the purpose of modulating Notch-mediated tumor angiogenesis and invasiveness. Inhibitors of gamma secretase, a proteolytic enzyme required for Notch activation, have been studied for this purpose. The mechanism of Notch inhibition
by DAPT is attributable to the inhibition of the second of two proteolytic cleavages of the Notch receptor after binding to Notch ligand. DAPT inhibits this intramembrane cleavage step catalyzed by the integral membrane multi-subunit gamma-secretase complex. This prevents the release of NICD (Notch intracellular domain), which is the intracellular domain that translocates to the nucleus and leads to gene transcription.\textsuperscript{209}

While commonly used as an inhibitor of this key proteolytic step, DAPT may also affect other pathways. Substrates of gamma-secretase which have been identified to date include the β-amyloid precursor protein, Erb-B4, E- and N-cadherins, CD44, the low density lipoprotein receptor, Nectin-1, and the Notch ligands Delta and Jagged.\textsuperscript{210} In addition to inhibition of the Notch pathway, DAPT has been evaluated as a potential pharmacologic option for the treatment of Alzheimer’s disease, but how DAPT selectively modulates the cleavage of each of the above listed substrates and the subsequent intracellular events is not fully clear, is likely quite complex, and is beyond the scope of this discussion. Certainly non-specific proteolytic cleavages may affect EC surface proteolytic enzymes which may affect angiogenesis, although this is speculation. Proteolytic events related to Notch ligands may affect endothelial cell signaling responses mediated by these ligands. To the knowledge of the author, there is no clear connection between these above described pathways and Akt or Erk activity in ECs or angiogenesis in response to SMCs. However, while DAPT is commonly used as a gamma-secretase inhibitor for the purposes of Notch inhibition, possible non-Notch related responses in ECs must lead one to use caution in interpreting the results of the experiments in these Aims.
The action of various gamma secretase inhibitors on angiogenesis in vitro and in vivo are still unclear, with both increased and decreased angiogenesis being demonstrated in response to gamma secretase inhibition. Several studies, however, have suggested that the resulting microvasculatures tend to demonstrate abnormal function and structure. Kalen et. al., for example, demonstrated that while Notch inhibition led to increased vascular density of tumors, vasculatures in animal models treated with gamma secretase inhibitors form abnormal blood vessels characterized by vessel occlusion, disrupted blood flow, and increased vascular leakage.\(^{211}\)

In our studies, we show the apparent pro-angiogenic function of active Notch signaling, as the inhibition of Notch with DAPT inhibited the sprouting response of ECs to SMC-conditioned media. This is in apparent contrast to other studies that demonstrate the function of Notch in downregulating pro-angiogenic behavior.\(^{200-204}\) Based on Western blot studies, the Dll-4 is the dominant Notch ligand present on ECs, while Jagged-1 was undetectable. Dll-4 is thought to a negative regulator of angiogenesis,\(^{212}\) which further complicates the response of ECs to SMC-conditioned media, as one would expect that inhibiting Notch signaling in ECs would primarily inhibit Dll-4 mediated Notch signaling in ECs, which should theoretically lead to increased angiogenesis in Notch inhibited cells. These studies suggest that the identity of the Notch ligand cannot be the sole factor for the pro- or anti-angiogenic responses, that these responses are at least in part modulated by other factors. We suggest that the apparent contradiction of our results to results of studies which investigate the role of Notch signaling in ECs primarily in the context of VEGF stimulation, is likely due to the identity and
combination of cytokines and growth factors released by SMCs in our model. Future studies are required to examine these issues. However, the demonstration that ERK phosphorylation occurs in a Notch independent manner when ECs are stimulated with FGF-2 in combination with VEGF is consistent with this hypothesis, as ERK phosphorylation in response to VEGF alone has been shown by others to be Notch dependent. In addition, the concentration of DAPT (25µM) utilized in these studies have effectively inhibited endothelial cell proliferation and migration in response to VEGF,\textsuperscript{213} while dose response curves have demonstrated potent inhibition of Notch signaling at concentrations as low as 1µM.\textsuperscript{214} Dose-specific effects demonstrated in these and other studies may account for the potential discrepancy of the results seen in our experiments with other studies.

It would be interesting if the demonstrated increased Akt phosphorylation, thought to be a survival signal, in endothelial cells treated with DAPT reflected Notch-associated conversion to a less stable EC phenotype responsible for a more dynamic developing microvasculature. The Ang1/Tie-2 signaling system, thought to be a key signal for microvascular quiescence and stability, is an upstream regulator of Akt phosphorylation.\textsuperscript{196} The demonstration that Ang-1 potentiates vascular quiescence by up-regulating Dll4 through Akt-mediated activation of β-catenin suggests a link among Ang-1/Tie-2, Akt, and Notch signaling.\textsuperscript{215} Ang-2, a competitive antagonist of Ang-1 that prevents Tie-2 receptor phosphorylation and signaling, is involved in the disruption of the endothelial monolayer, and promotes EC survival, vessel sprouting and angiogenesis.\textsuperscript{216-219} The potential secretion of Ang-2 by SMCs could hypothetically disrupt basal Ang-1
signaling and lead to the decrease in basal levels of phosphorylated Akt in ECs. As previously noted, the requirement for active EC Notch signaling in the demonstrated SMC-induced decrease in phospho-Akt suggests that EC Notch is an upstream regulator of Akt dephosphorylation. Thus, a hypothetical pathway is that SMCs release Ang-2 or induce the release of Ang-2 from EC Weibel-Palade bodies by secreting other factors such as VEGF and FGFs. This release of Ang-2 then inhibits Tie-2-mediated Akt phosphorylation in a Notch dependent manner, and converts ECs from a quiescent phenotype to a synthetic phenotype leading to the pro-angiogenic response to SMC-conditioned media. The demonstration that Notch signaling is required for the induced expression and activation of specific proteases in response to VEGF supports the hypothesis that Notch can regulate pathways which lead to the “synthetic” behavior of activated endothelial cells. This is consistent with the observation that Notch is required for the pro-angiogenic response to SMC-conditioned media. The specific role of Dll-4, the expression of it or other Notch ligands and receptors, and specific Notch receptor and ligand combinations on mediating this pathway is also unknown.

It would be of interest to study the effects of delivery of a constitutively active Akt to determine if the decrease in active Akt was a direct upstream regulator in the induction of angiogenesis in response to SMC-conditioned media. In this case, one would not expect ECs cultured in SMC-conditioned media to demonstrate any increase in angiogenesis compared with controls. The role of Akt activity as a mediator of Notch dependent angiogenic induction in response to SMC-conditioned media could also be tested in cells expressing constitutively active Akt. The use of selective Akt inhibitors
such as LY compound could be used to corroborate these experiments. The reduction of active Akt at basal levels would be expected to increase angiogenesis if inactive Akt is associated with the induction of angiogenesis. Future studies using Notch-overexpressing ECs cultured in the presence and absence of SMC-conditioned would be required to determine whether the Notch mediated response to SMC-conditioned leads to functional or abnormal EC microvasculatures. Further, the interaction between Notch receptor and ligand activity or expression in response to SMC-conditioned media has not been quantified in these studies and would be a subject of future investigations.
CHAPTER SEVEN

SUMMARY OF RESULTS AND CONCLUSIONS

The following are the major findings of the above discussed studies (Figure 45).

Figure 45. Summary of results. Aggregated ECs in fibrin form sprouts containing lumens which are surrounded by SMCs, resembling native capillaries histologically. SMCs promote the formation and persistence of the engineered 3-D capillary structures, in part by the release of soluble factors into the extracellular environment. These SMC-released products have the effect of increasing phosphorylated ERK 1/2 and unphosphorylated Akt in ECs. The increase in unphosphorylated Akt in ECs in response to SMC-released factors is EC Notch dependent.

Aim 1

ECs co-cultured with SMCs form angiogenic sprouts in 3-D fibrin hydrogels.

Transmission electron microscopy and fluorescence microscopy demonstrate lumen formation and SMC/EC colocalization in capillaries.

VEGF delivered in combination with FGF-2 induces angiogenic network formation in fibrin hydrogels, and SMCs do not inhibit the angiogenic response of ECs to
these growth factors. After the withdrawal of VEGF and FGF-2, SMCs promote both the formation and persistence of fibrin-based microvasculatures. In the absence of SMCs, growth factor withdrawal led to microvascular regression.

In the absence of exogenous angiogenic factors, SMCs promote the formation and persistence of angiogenic networks in fibrin hydrogels. The presence of direct contact between the two cell types does not inhibit this effect. In fact, ECs and SMCs co-invade into the ECM in a direct contact angiogenesis model, and the inhibition of SMC proliferation/matrix invasion alone inhibits both EC and SMC invasion.

Aim 2

Sprouts from co-cultured aggregates demonstrate directed invasion towards one another. Media conditioned by SMCs is mitogenic, chemotactic, and angiogenic for ECs and induces ERK phosphorylation in ECs. SMC-conditioned media suppresses serum induced basal Akt phosphorylation in ECs.

Aim 3

VEGF in combination with FGF-2 induces both ERK and Akt phosphorylation in ECs after 20 minutes of stimulation. This returns to unstimulated levels after 48 hours of continuous growth factor stimulation, and ERK and Akt protein levels are not altered by 48 hours of stimulation with VEGF + FGF-2.

Inhibition of EC Notch signaling by DAPT increases Akt phosphorylation in response to 20 minutes of VEGF + FGF-2 stimulation. This returns to unstimulated levels after 48 hours of continuous growth factor and DAPT treatment. Notch inhibition with DAPT does not alter Akt protein levels. Neither ERK phosphorylation nor protein
levels after 20 minutes and 48 hours of stimulation, respectively, with VEGF + FGF-2 is affected by Notch inhibition with DAPT.

Dll-4 is the predominant Notch ligand in ECs; Notch 1 is the predominant Notch receptor in ECs. Stimulation with VEGF + FGF-2 for 48 hours did not alter the identity of Notch receptors or ligands in ECs or SMCs.

Inhibiting Notch signaling with DAPT reverses the dephosphorylation of Akt by SMC-conditioned media. Notch inhibition does not affect the phosphorylation of ERK in response to SMC-conditioned media. Inhibiting Notch signaling with DAPT reverses the induction of angiogenesis by SMC-conditioned media.

**Overall Summary of Major Findings**

An *ex vivo* SMC/EC co-culture strategy is capable of engineering microvascular networks that resemble native capillaries in fibrin hydrogels. SMCs do not attenuate the early pro-angiogenic effects of exogenously delivered VEGF + FGF-2 on fibrin-based microvascular networks. SMCs promote the early formation and later persistence of fibrin-based microvascular networks in part by release of soluble factors and can do so in the absence of any exogenous growth factor delivery.

VEGF + FGF-2 induces the phosphorylation of ERK and Akt in endothelial cells. Active endothelial cell Notch signaling suppresses the phosphorylation of Akt in response to VEGF + FGF-2. The abundance of Dll-4 on ECs suggests that it may be the ligand through which these effects occur. The phosphorylation of ERK in response to VEGF + FGF-2 is independent of Notch signaling.
SMC-released factors promote phosphorylation of ERK and dephosphorylation of Akt in ECs, and promote the proliferation, migration, and early sprouting of ECs. Active endothelial cell Notch signaling is necessary for the dephosphorylation of Akt in response to SMC-released factors. Active endothelial cell Notch signaling is necessary for the induction of angiogenesis in response to SMC-released factors. The abundance of Dll-4 on ECs suggests that it may be the ligand through which these effects occur.
CHAPTER EIGHT
CONCLUDING REMARKS

Overall, we have demonstrated a method for engineering microvasculatures in fibrin, a hydrogel commonly used in tissue engineering. We hypothesize that the prevascularization of the walls of tissue engineered blood vessels will provide benefits on medial cell viability, reduce intimal hyperplasia, promote graft endothelialization, and improve the mechanical biocompatibility of tissue engineered blood vessels. We believe that the delineation of these potential effects would be important studies for the advancement of the creation of a clinically efficacious tissue engineered blood vessel. Additionally, we have attempted to delineate some of the basic cellular and molecular processes that modulate the development of these microvasculatures in a 3-D extracellular environment. The Notch pathway is a known mediator of angiogenesis and has implications both in oncologic therapies as well as for the regulation of angiogenesis in tissue engineering.\textsuperscript{213,221} These studies may offer insights into potential protein and genetic targets for the manipulation of angiogenesis, which may be beneficial in both selectively promoting and inhibiting angiogenesis. Further studies to more clearly delineate how these processes mediate angiogenic activity during specific stages of microvascular development would provide for more precise therapeutic interventions.

In addition, it is important to recognize some of the major limitations of these studies. The inherent inaccuracies of \textit{in vitro} models of basic biologic processes in
relation to the *in vivo* environment may be a contributing factor to the results of these studies. These studies utilize several cell types (both ECs and SMCs), signaling pathways, delivered proteins, culture media, and extracellular matrix proteins. Each of these factors can affect the results of these studies, with specific cellular and molecular responses contingent on the doses, reagents, concentrations, etc. used in these experiments. Also, as alluded to in previous discussions, the processes investigated, specifically in the second and third Aims, likely represent events which are occurring early in the process of angiogenesis. The SMC-mediated induction of angiogenesis, the activation and deactivation of ERK 1/2 and Akt, respectively, and the regulation of these events by EC Notch signaling are likely occurring within the first 72 hours of angiogenesis. This is not to say, however, that the later events occurring after pericyte incorporation or microvascular stabilization are occurring independent of early events. It is highly likely that these early processes, by the secretion of growth factors and cytokines, the deposition of extracellular matrix, or the regulation of signaling pathways affect cellular behavior via negative (or positive) feedback loops which may serve to counteract and “turn off” the early angiogenic events as the angiogenic process progresses. This discussion is mostly speculative at this point, however, and further investigations into how early events in angiogenesis affect the later events would be interesting to perform.

A final, and especially important consideration related to the above-described studies is the choice of fibrin as the extracellular matrix. The ECM is a highly important modulator of angiogenesis during oncogenesis, wound healing, and development. For example, the differentiation of ECs grown as a monolayer *in vitro* into branching tubule
structures has been shown to require the coverage of cells with collagen and the establishment of appropriate cell-matrix interactions. Angiostatic molecules like thrombospondin-1 (TSP-1) and interferon-inducible protein-10 have been shown to be less effective in inhibiting VEGF-induced EC proliferation when cultured on type-I collagen, an observation that has intuitive implications in the clinical use of pro- or anti-angiogenesis drugs. Laminin, a non-collagen hetero-trimeric (3 chains: $\alpha$, $\beta$, and $\gamma$) glycoprotein found in basement membrane and granulation tissue, has been shown to increase EC-ECM attachment and migration in vitro, is produced throughout the length of EC sprouts and has been shown to limit proliferation and lumen size, and is associated with breast and brain cancer neovascularization and membrane invasion (i.e. laminin-8 and laminin-10). Fibronectin, often an injury response protein, is actively unfolded by ECs and serves as an ECM scaffold for cellular matrix deposition and neovascularization, while combinations of other proteins active in wound healing, like hyaluronic acid, type I collagen and fibrin, can have differential angiogenic effects compared to when they are present alone.

While the composition of the ECM is clearly an important factor in the regulation of angiogenesis, the organizational structure of the ECM is also key. Studies have demonstrated in numerous cell types that cell culture in 3-D environments vs. 2-D environments can significantly impact the expression of differentiation markers, diminish cell proliferation, and affect the expression of proteins like MMP-2. Physical characteristics such as fiber thickness, density, orientation, cross-linking, and pore size have all been shown to affect the number, length, and cross-sectional area of angiogenic sprouts. In vitro, ECs have been shown to demonstrate decreased cell migration
and invasion with increased capillary morphogenesis in fibrin as the rigidity is decreased by altering pH, NaCl concentration, and thrombin content. \textsuperscript{235-237} In chick chorioallantoic membrane (CAM) angiogenesis assays, significantly more sprout invasion is associated with a reduction in fibrin substrate concentration, and the physical characteristics of the ECM were found to be as important as the presence or absence of growth factors such as FGF and VEGF.\textsuperscript{229}

In tissue engineering, fibrin has been utilized as a scaffold for TEBVs with its own distinct set of advantages and disadvantages. In comparison to collagen hydrogels, for example, fibrin has been found to promote increased native collagen production by entrapped fibroblasts and SMCs, suggesting its ability to support the matrix remodeling processes involved in host tissue incorporation after implantation.\textsuperscript{238} The presence of deposited elastin in fibrin hydrogels as compared to collagen hydrogels also suggests potentially beneficial effects on cell mediated matrix remodeling processes affecting construct biomechanics.\textsuperscript{239,240} Implantation of fibrin-based TEBVs into lamb external jugular veins demonstrated endogenous collagen and elastin deposition by SMCs in the arterial media which orientated circumferentially in a direction which was perpendicular to circulatory blood flow.\textsuperscript{241} Interestingly, these TEBVs gained mechanical strength and vasoactivity which were comparable to that of native blood vessels after 15 weeks \textit{in vivo}. Fibrin-coated vascular prostheses have been evaluated in animal models for coronary artery bypass.\textsuperscript{242} While early results were encouraging with patency up to 12-2 days, thrombus formation and platelet deposition as well as degradation of the hydrogel after the first two weeks limited the efficacy of these prostheses. As in collagen-based constructs, the mechanical strength of fibrin-based TEBVs in general remains a
significant limitation and often requires the use of synthetic polymers such as polyvinylidene fluoride mesh to improve the mechanical stability of fibrin-based TEBVs.\textsuperscript{243} However, given the benefits of fibrin as a scaffold for tissue engineered constructs, or at least as a component of the scaffold, in addition to its utility as a drug and cell delivery system, we have adopted a fibrin based \textit{in vitro} angiogenesis model. Investigating the specific cell, molecular, and fibrin interactions in mediating the effects seen in the results of these Aims would be an interesting and worthwhile pursuit in the future.
APPENDIX A:

LIST OF MEDIAS USED IN EXPERIMENTS
EC Complete Media

M199, 10% FBS, PCN/Strep (100 U/mL), Amphotericin (.25 µg/mL), FGF-1 (5 ng/mL), heparan sulfate (5U/mL)

SMC Growth Media

DMEM, 10% FBS, PCN/Strep (100 U/mL), Gentamicin (.05 µg/mL), Amphotericin (.25 µg/mL), non-essential amino acids, MEN, sodium pyruvate

Angiogenesis Assay Media

M199, 10% FBS, PCN/Strep (100 U/mL), Amphotericin (.25 µg/mL), aprotinin (100 KIU/mL), heparan sulfate (5U/mL)

Migration Assay Media

M199, 1% FBS, PCN/Strep (100 U/mL), Amphotericin (.25 µg/mL), aprotinin (100 KIU/mL), heparan sulfate (5U/mL)

Proliferation Quiescence Media

DMEM-F12, PCN/Strep (100 U/mL), L-ascorbic acid, insulin, transferrin

Notch Signaling Assay Stimulation Media

M199, 2.5% FBS, PCN/Strep (100 U/mL), Amphotericin (.25 µg/mL), aprotinin (100 KIU/mL), heparan sulfate (5U/mL), +/- VEGF + FGF-2 (10 ng/mL for each growth factor), +/- DAPT (25µM) or DMSO
REFERENCES


86. Stromhaug PE, Klionsky DJ. Approaching the molecular mechanism of autophagy. Traffic (Copenhagen, Denmark) 2001;2(8):524.


121. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development (Cambridge, England) 1998;125(9):1591-8.


148. Potapova O, Laird AD, Nannini MA, Barone A, Li G, Moss KG, Cherrington JM, Mendel DB. Contribution of individual targets to the antitumor efficacy of the


158. Kutcher ME, Kolyada AY, Surks HK, Herman IM. Pericyte Rho GTPase mediates both pericyte contractile phenotype and capillary endothelial growth state. The American journal of pathology 2007;171(2):693.


179. Brewster LP, Brey EM, Tassiopoulos AK, Xue L, Maddox E, Armistead D, Burgess WH, Greisler HP. Heparin-independent mitogenicity in an endothelial


229. Smith JD, Melhem ME, Magge KT, Waggoner AS, Campbell PG. Improved growth factor directed vascularization into fibrin constructs through inclusion of additional extracellular molecules. Microvascular research 2007;73(2):84.


VITA

The author, Areck Ucuzian was born in Boston, MA on January 18, 1978 to Hrach and Anoush Ucuzian. He received a Bachelor of Arts in Cell/Molecular Biology from Cornell University, College of Arts and Sciences (Ithaca, NY) in May of 2000, graduating with Distinction in All Subjects. He received his medical degree from Tufts University School of Medicine in May of 2004 (Boston, MA), and was accepted into the Alpha Omega Alpha medical honor society. In July of 2004, he entered the general surgery residency at Loyola University Medical Center.

In July of 2006, Areck joined the lab of Dr. Howard P. Greisler at Edward Hines, Jr. VA Hospital (Hines, IL) as part of a planned two year research fellowship through the Burn and Shock Trauma Institute and the Department of Surgery at Loyola University Medical Center. In Dr. Greisler’s lab, Areck conducted research related to vascular smooth muscle cell and endothelial cell biology, vascular healing, intimal hyperplasia, and tissue engineering. After his first year in the lab, Areck applied and was accepted to the Ph.D. program in the department of Cell Biology, Neurobiology, and Anatomy at Loyola University Chicago (Maywood, IL). He ultimately focused his work on investigating the effects of smooth muscle cells on endothelial cell angiogenesis in fibrin-based tissue engineered constructs. While at Loyola, Areck received a pre-doctoral training grant from the American Heart Association. He has also published several book
chapters, reviews, original articles, and abstracts, and has given poster and oral presentations in national and international conferences, receiving an award for “Best Basic Science Presentation” at the The Association of VA Surgeons Annual Meeting in May of 2007.

After completing his Ph.D., Areck will complete his general surgery residency at Loyola University Medical Center (anticipated June 2013). He then plans to enter a two year subspecialty fellowship in peripheral vascular surgery, after which he intends on pursuing a career as an academic vascular surgeon.