EVALUATION OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS IN
BLOOD VESSEL MIMICS THROUGH CHANGES IN GENE
EXPRESSION AND CASPASE ACTIVITY

A thesis
presented to
the Faculty of California Polytechnic State University,
San Luis Obispo

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Biomedical Engineering

by
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June 2019

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Endothelial Cells in Blood Vessel Mimics
through Changes in Gene Expression and
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ABSTRACT

Evaluation of Human Umbilical Vein Endothelial Cells in Blood Vessel Mimics through Changes in Gene Expression and Caspase Activity

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Blood vessel mimics (BVMs) are simple tissue engineered blood vessel constructs intended for preclinical testing of vascular devices. This thesis developed and implemented methods to characterize two of these components. The first aim of this thesis investigated the effect of cell culture duration and flow conditions on endothelial cell gene expression, especially regarding endothelial-to-mesenchymal transition (EndMT). A trend of decreased endothelial marker gene expression and increased mesenchymal marker gene expression would indicate EndMT. qPCR analysis revealed that increased cell culture duration did not result in EndMT, and in fact increased endothelial marker expression as cell culture duration increased. Disturbed flow conditions decreased endothelial marker and increased mesenchymal marker expression relative to static culture.

The second aim of this thesis developed methods to determine cytotoxicity of, and endothelial cell adhesion to, novel BTEAC salt scaffolds. Immunostaining was used to visualize these scaffold effects. The cytotoxicity elution assay showed that BTEAC salt scaffolds were not more cytotoxic than the standard PLGA scaffold. Direct contact assays spanning several timepoints also found that BTEAC salt scaffolds were not more cytotoxic than standard scaffolds but had higher endothelial cell adhesion and coverage than standard scaffolds. Overall, this thesis developed and implemented methods to characterize the endothelial cells used in the BVM model.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Kristen Cardinal, for her support throughout my undergraduate and graduate studies. The investment she makes in each student, including myself, is remarkable and unprecedented. I cannot imagine my college experience without the knowledge and passion for innovation that she imparts in the laboratory and classroom. Furthermore, I would like to thank her for her dedication to her role as a thesis advisor, with which her countless hours of guidance did not go unnoticed and I will always be appreciative of.

I would like to thank my committee members, Dr. Trevor Cardinal and Dr. Lily Laiho, for their time and effort to enhance my thesis. Fellow laboratory research members contributed to the efforts of this thesis as well, and for that I would like to acknowledge and thank Ryan Murphy, Ali Flesch, and Cami Dozois. I would also like to thank Ella Sugerman and Ben Dennis for their assistance in the laboratory and willingness to share their knowledge to promote my success.

Finally, I would like to thank my family and friends for their support throughout my years in San Luis Obispo.

TABLE OF CONTENTS

	Page
LIST OF TABLES	xiv
LIST OF FIGURES.....	xv
LIST OF EQUATIONS	xxi
CHAPTER	
1. INTRODUCTION.....	1
1.1 Blood Vessel Mimic Background	1
1.1.1 Relevance.....	1
1.1.1.1 Cardiovascular Disease and Intravascular Devices	1
1.1.1.2 Preclinical Cardiovascular Device Testing in Blood Vessel Mimics	2
1.1.2 Blood Vessel Mimic Design	3
1.1.2.1 Native Blood Vessel Anatomy.....	3
1.1.2.2 Blood Vessel Mimic Fabrication	4
1.1.2.3 Blood Vessel Mimic Anatomy.....	5
1.1.3 Types of Blood Vessel Mimics.....	6
1.1.4 Importance of Endothelial Cell Characterization	7
1.2 BVM Characterization	8
1.2.1 Previous Characterization.....	9
1.2.2 Need for Assessment.....	10

	Page
1.3 Summary and Aims of the Thesis	12
1.3.1 Aim 1: EC Characterization through Changes in Gene Expression	12
1.3.2 Aim 2: Characterization of Endothelial Cell and Scaffold Interaction	13
2. AIM 1: GENE EXPRESSION CHANGES OF ENDOTHELIAL CELLS IN CULTURE AS A FACTOR OF TIME AND FLOW CONDITION.....	14
2.1 Introduction	14
2.1.1 Flow Environment.....	14
2.1.1.1 Native Flow Environment.....	14
2.1.1.2 Previously Determined Flow Conditions in Blood Vessel Mimics	17
2.1.2 BVM Cell Phenotype	19
2.1.2.1 Immunostaining of HUVECs in the Tissue Engineering Lab	19
2.1.2.2 Endothelial-to-Mesenchymal Transition	20
2.1.2.2.1 EndMT in Heart Development.....	21
2.1.2.2.2 EndMT in Disease Progression	21
2.1.2.2.3 EndMT in Tissue Regeneration	22
2.1.2.3 EndMT Activation Pathways.....	24
2.1.2.3.1 Transforming Growth Factor Beta	24
2.1.2.3.2 Notch Signaling.....	25
2.1.2.3.3 Wnt/ β -catenin Signaling.....	26

	Page
2.1.2.3.4 Pro-inflammatory Cytokines	26
2.1.2.3.5 Oxidative Stress.....	26
2.1.2.3.6 Disturbed Flow	27
2.1.2.4 Inhibitors of EndMT	27
2.1.3 The Mechanosensory Complex	29
2.1.3.1 Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1)	30
2.1.3.2 Vascular Endothelial Cadherin (VE-Cad)	32
2.1.3.3 Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)	33
2.1.3.4 VE-Cadherin Regulation of VEGFR2	34
2.1.3.5 Mechanosensory Complex and EndMT.....	36
2.1.4 Mesenchymal Markers.....	37
2.1.5 Using EC and Mesenchymal Markers to Determine EndMT Progression	39
2.1.6 RT-qPCR Background.....	40
2.1.7 Summary.....	45
2.2 Materials and Methods	46
2.2.1 Gene Selection and Primer Acquisition.....	46
2.2.2 RNA Isolation	47
2.2.3 Reverse Transcription	48

	Page
2.2.4 Quantitative Polymerase Chain Reaction.....	49
2.2.5 Experiment 1 Pilot: Change in EC Expression with Increasing Passage	50
2.2.6 Experiment 1: Change in EC Expression with Increasing Passage Number	51
2.2.7 Experiment 2: Changes in EC Expression from Disturbed Flow Conditions ...	51
2.2.8 Experiment 3 Preparation: RNA Isolation Protocol for BVMs.....	52
2.2.9 Experiment 3 Pilot: Changes in Gene Expression in BVMs	53
2.3 Results	54
2.3.1 Primer Efficiency.....	54
2.3.2 Experiment 1 Pilot Results	55
2.3.3 Experiment 1 Results	59
2.3.4 Experiment 2 Results	64
2.3.5 Verification of RNA Isolation Protocol for BVMs.....	67
2.3.6 Experiment 3 Pilot Results	68
2.4 Discussion and Conclusion	73
2.4.1 Experiment 1 Pilot and Experiment 1	73
2.4.2 Experiment 2	77
2.4.3 Experiment 3 Pilot.....	79
2.4.4 Aim 1: Limitations and Future Work.....	83

	Page
3. AIM 2: CHARACTERIZATION OF NOVEL SCAFFOLDS THROUGH HUVES CASPASE ACTIVITY AND SCAFFOLD COVERAGE	86
3.1 Introduction	86
3.1.1 Scaffold Effects on Cells	86
3.1.1.1 Fiber Diameter	87
3.1.1.2 Fiber Alignment	88
3.1.1.3 Porosity	88
3.1.1.4 Stiffness.....	89
3.1.1.5 Topography	90
3.1.1.6 Scaffold Degradation	90
3.1.2 Measurement of Cell Responses	91
3.1.2.1 Apoptosis	91
3.1.2.1.1 Apoptosis Pathways	92
3.1.2.1.2 Caspases	94
3.1.2.1.3 Apoptosis Detection and Quantification	94
3.1.2.2 Cell Adhesion.....	95
3.1.2.2.1 Passive In Vitro Cell Adhesion	95
3.1.2.2.2 Cell Adhesion Tests	97

	Page
3.1.3 Addition of BTEAC Salt in Electrospinning of Scaffolds	98
3.1.4 Summary.....	102
3.2 Materials and Methods	102
3.2.1 Caspase Stain Implementation and Verification	103
3.2.2 Cytotoxicity Assay	105
3.2.3 Cell Adhesion and Viability Assay	109
3.3 Results	112
3.3.1 Stain Verification.....	113
3.3.2 Cytotoxicity Elution Assay	118
3.3.3 Cell Adhesion and Viability Assay	126
3.3.4 BVM Cell Coverage and Viability Assay	133
3.4 Discussion and Conclusion	143
3.4.1 Cytotoxicity Elution Assay	143
3.4.2 Cell Adhesion and Viability Assay	145
3.4.3 BVM Cell Coverage and Viability Assay	146
3.4.4 Limitations and Future Work.....	148
3.4.5 Summary.....	152
4: DISCUSSION AND CONCLUSION.....	153
4.1 Summary and Aims of the Thesis	153

	Page
4.2 Results and Discussion.....	154
4.3 Challenges and Limitations.....	157
4.4 Future Work	158
4.5 Conclusion.....	163
REFERENCES	164
APPENDICES	186
Appendix A: Preverified Primer Sequences and Characteristics	186
Appendix B: Primer Efficiency Testing SOP	188
Appendix C: RNA Isolation and Evaluation SOP	188
Appendix D: cDNA through Reverse Transcription SOP	188
Appendix E: qPCR SOP.....	188
Appendix F: qPCR Data Analysis SOP	188
Appendix G: Primer Efficiency Plots.....	189
Appendix H: BVM Conditioning SOP	191
Appendix I: Endothelial Cell-only BVM Sodding SOP	191
Appendix J: Apoptosis Kit Staining SOP	191
Appendix K: Cytotoxicity Elution Assay SOP	191
Appendix L: Scaffold Adhesion Assay SOP.....	192

	Page
Appendix M: Positive Control Test Representative Images	192
Appendix N: Cytotoxicity Elution Assay Representative Images	197
Appendix O: Scaffold Adhesion Assay Representative Images	203
Appendix P: BVM Study Representative Images	207
Appendix Q: Magnification Justification Raw Data	213
Appendix R: Pixel Intensity Reduction Raw Data.....	215
Appendix S: Nuclei Area Multiplication Factor Raw Data	218
Appendix T: Statistical Tests for Cytotoxicity Elution Assay	219
Appendix U: Statistical Tests for Scaffold Adhesion Assay.....	223
Appendix V: Statistical Tests for BVM Study.....	225

LIST OF TABLES

Table	Page
1: Drugs that Inhibit or Reduce EndMT	28
2: Primer Efficiency	55
3: Comparison of RNA isolation from the RNeasy Mini Kit and Trizol extraction.....	68
4: Confocal Microscope Lasers and Settings.....	104
5: Apoptosis Staining Kit Verification using HUVECs	113
6: pH of Elutions from Treatment Methods.....	120
7: Short-term Adhesion Assay Cell Sodding and Collection Data	127

LIST OF FIGURES

Figure	Page
1: The Effects of Plaque on Blood Flow.....	2
2: Testing Methods before FDA Approval	3
3: The anatomy of native arteries, veins, and capillaries ⁸	4
4: Blood Vessel Mimic Anatomy	5
5: Forces Acting on Blood Vessels	15
6: Laminar Flow versus Shear Flow	16
7: Flow in a Bifurcation	17
8: Endothelial Cells Stain for α SMA.....	20
9: EndMT in Tissue Engineering.....	23
10: Types of Endothelial Mechanosensors	30
11: Mechanisms of VE-Cad and VEGFR2 on Cell Seeding Density.....	35
12: VE-cad and Transgelin Expression in a Simple Flow Model.....	40
13: qPCR Amplification Curve.....	42
14: Types of Primers.....	43
15: Example Dissociation Curves.....	44

16: Primer Efficiency Plot	55
17: Experiment 1 Pilot Relative Abundance Relative to β -Actin.....	57
18: Experiment 1 Pilot Relative Abundance Relative to β Galactosidase.....	58
19: Experiment 1 Pilot Housekeeping Gene Stability	59
20: Experiment 1 Housekeeping Gene Stability	60
21: Experiment 1 Relative Abundance	61
22: Experiment 1 Pilot Relative Abundance of Mesenchymal Markers in SMCs	62
23: Representative dissociation curves of all GOI.....	63
24: Experiment 2 Relative Abundance.	66
25: Experiment 2 Housekeeping Gene Stability	67
26: HUVEC Morphology in Turbulent Flow Condition.....	69
27: Experiment 3 Housekeeping Gene Stability.....	70
28: Disturbed Flow Dissociation Curves	71
29: Experiment 3 Relative Abundance	72
30: Dissociation Curves for GOI for EC-only BVMs	81
31: Cell Interactions with Fibers of Varying Sizes.....	87

32: The Apoptosis Pathway	93
33: Cell Adhesion Phases.....	96
34: SEM of Standard Scaffold	99
35: SEM of Salt Scaffold	99
36: Fiber Diameter Reduction with BTEAC Percentage.....	100
37: Hoechst Channel Automatic Cell Counting.....	106
38: Pixel Reduction in Caspase Channel	108
39: Pixel Reduction in Propidium Iodide Channel	108
40: Hoechst Channel Coverage Quantification.....	111
41: Ineffective Positive Control DMSO 15% 30 Minutes	116
42: Ineffective Positive Control Hydrogen Peroxide 1% 2 Minutes	116
43: Effective Positive Control Hydrogen Peroxide 0.5% 5 Minutes	117
44: Effective Positive Control Hydrogen Peroxide 1% 5 Minutes	117
45: Scaffolds After Elution Treatment.....	119
46: Media After Elution Treatment.....	119
47: Cell Count Reduction Curves	121
48: Cytotoxicity Elution Assay Percent Caspase Positive Cells.....	123

49: Normalized Cytotoxicity Elution Assay Percent Caspase Positive Cells.....	124
50: Cytotoxicity Elution Assay Percent Propidium Iodide Positive Cells.....	125
51: Normalized Cytotoxicity Elution Assay Percent Caspase Positive Cells.....	126
52: Sample 20x HUVECs	128
53: Standard and Salt Scaffolds Hoechst Channel.....	129
54: Short-term Adhesion Assay Percent Cell Coverage	130
55: Caspase and Propidium Iodide Channels Short-term Adhesion Assay	131
56: Short-term Adhesion Assay Percent Apoptotic Cells.....	132
57: Short-term Adhesion Assay Percent Dead Cells	132
58: Salt Scaffold Shortening Before and After BVM Cultivation.....	134
59: Standard and Salt Scaffold Shortening After BVM Cultivation.....	134
60: Wall Thicknesses of Scaffolds in BVMs.....	135
61: Representative 3-day BVM Hoechst Images.....	136
62: Representative 7-day BVM Hoechst Images.....	137
63: BVM Cell Coverage in 3 and 7-Day BVMs.....	138
64: Images of 3-day BVM Caspase and Propidium Iodide Channels	140
65: Images of 7-day BVM Caspase and Propidium Iodide Channels	141

66: Percent Apoptotic Cells in 3 and 7-Day BVMs.....	142
67: Percent Dead Cells in 3 and 7-Day BVMs	143
68: Calponin immunostaining of HUVECs and HUASMCs.....	160

List of Equations

Equation	Page
1: Efficiency (%) = $(10^{(-1/\text{Slope})} - 1) * 100$	47
2: $(1000 \text{ ng RNA}) * (1 \text{ mL} / [\text{RNA concentration}] \text{ ng/nL}) = [\text{Volume RNA}] \mu\text{L}$	49
3: $\Delta\text{Ct} = \text{Ct}(\text{GOI}) - \text{Ct}(\text{reference gene})$	50

CHAPTER 1. INTRODUCTION

1.1 Blood Vessel Mimic Background

Cal Poly's Tissue Engineering Laboratory creates blood vessel mimics (BVMs) to test intravascular devices in an environment that simplistically mimics the anatomy and physiology of native blood vessels. The BVMs are fabricated using endothelial cells (ECs), smooth muscle cells (SMCs), and a polymer scaffold tube assembled in a bioreactor. While this design is simple, it offers a three-dimensional environment with some physiologic relevance, which can be used to determine the interaction between cells and vascular devices. This model serves as an intermediate step between basic *in vitro* testing and *in vivo* testing of devices. A full explanation of BVM relevance for cardiovascular device testing will be explained in the next section.

1.1.1 Relevance

1.1.1.1 Cardiovascular Disease and Intravascular Devices

Heart disease is responsible for one of every four deaths per year in the United States, with coronary artery disease (CAD) being the most common type of heart disease¹. Coronary heart disease results in the hardening and thickening of coronary arteries and the formation of atherosclerotic plaques. These plaques are composed of immune cells, SMCs, myofibroblasts, lipids, and extracellular matrix, and develop preferentially at areas of disturbed flow in the vessels². These plaques, impede blood delivery to the heart and are prone to rupturing, which can result in a myocardial

infarction or stroke¹. Figure 1 displays the progression of atherosclerotic plaque building and the resulting flow.

Figure 1: The Effects of Plaque on Blood Flow. Blood flow is impeded as atherosclerotic plaque buildup progresses¹.

Intervention is often necessary to reduce the area of plaque blockage and increase blood flow to downstream tissue. Coronary artery bypass grafting (CABG) is a procedure commonly used to bypass the occluded vessel and restore blood flow, but the procedure is invasive. Minimally invasive procedures include coronary balloon angioplasty and stenting. Coronary balloon angioplasty uses a balloon to push the plaque against the wall of the vessel, widening the lumen of the vessel³. Stenting restores blood flow, similar to coronary balloon angioplasty, but leaves a stent in the artery.

1.1.1.2 Preclinical Cardiovascular Device Testing in Blood Vessel Mimics

The BVM model is intended as an intermediate testing environment for intravascular devices, such as coronary stents, to support safety of the device. These types of devices must show safety and efficacy *in vitro*, *in vivo*, and during clinical trials. Most *in vitro* testing simply uses cells cultured in a two-dimensional plate to determine cell

interaction with the device material. Testing then progresses to *in vivo* studies, where devices are tested in animal models. There is a disparity between how cells interact in a plate with a device and how a living organism interacts with that device. *In vivo* studies are also expensive, so alternative testing environments can be useful in determining safety as well as reducing testing costs. BVMs can be used as a testing environment to bridge current *in vitro* and *in vivo* testing, which is shown in Figure 2.

Figure 2: Testing Methods before FDA Approval. BVMs can provide valuable data on device safety and efficacy between *in vitro* and *in vivo* testing⁴.

1.1.2 Blood Vessel Mimic Design

1.1.2.1 Native Blood Vessel Anatomy

The BVM model is designed to have a simpler anatomy than a native vessel. Different types of blood vessels vary slightly in anatomy but share three main tunica layers and a lumen. The lumen is an open tube at the center of the vessel and is the site of blood flow. The tunica intima is the innermost layer which interacts with blood in the lumen and is composed of the endothelium and basement membrane⁵. The endothelium is composed of ECs and acts as a barrier between the body and blood while being selectively permeable to chemicals, white blood cell, and waste movement⁶. The basement membrane anchors the endothelium to the connective tissue of the middle layer

of blood vessel tissue, the tunica media. The tunica media contains smooth muscle cells and connective tissue and is responsible for vasoconstriction and vasodilation⁵. The outermost layer, the tunica adventitia, consists of collagenous connective tissue, fibroblasts, and nerves⁷. A diagram of these layers is shown in Figure 3. BVMs attempt to include the lumen, tunica intima, tunica media, and tunica adventitia.

Figure 3: The anatomy of native arteries, veins, and capillaries⁸.

1.1.2.2 Blood Vessel Mimic Fabrication

BVMs are fabricated using three components: an exterior polymer scaffold tube, a middle layer of SMCs, and an interior layer of ECs⁹. The scaffold is composed of poly-lactic-co-glycolic-acid (PLGA) fibers, in which fibers are created through electrospinning. During the electrospinning process, a solution of chloroform and PLGA is charged and pulled to a grounded metal mandrel. The mandrel translates and rotates to collect the fibers, which typically range between 4 μm to 5 μm in fiber diameter.

The scaffold is then conditioned in high serum media within a bioreactor attached to a peristaltic pump, where proteins from the media intercalate the scaffold. These proteins enhance cell adhesion and proliferation once cells are seeded on the scaffold.

With the current model, human umbilical artery smooth muscle cells (HUASMCs) are pressure seeded to the interior of the polymer tube, followed by human umbilical vein endothelial cells (HUVECs) after conditioning. The fully fabricated BVM is cultured inside of the bioreactor, which is connected to an 8-roller peristaltic pump that pushes media through the lumen of the BVM. After reaching the appropriate time point, the BVM is removed from the bioreactor and prepared for one of several techniques to analyze the resulting construct, including confocal imaging, scanning electron microscope (SEM) imaging, and gene expression analysis.

1.1.2.3 Blood Vessel Mimic Anatomy

Due to the fabrication technique, and as introduced above, the BVM has an anatomy mimicking the three basic layers of a native vessel. The polymer scaffold acts as the tunica adventitia of the BVM, supporting the geometry and providing a matrix for SMC growth. Figure 4 displays the layer of HUASMCs acting as the tunica media, and the layer of HUVECs acting as the tunica intima.

Figure 4: Blood Vessel Mimic Anatomy.

1.1.3 Types of Blood Vessel Mimics

Cal Poly's Tissue Engineering Lab currently creates three different types of BVMs for use with different devices or diseases. The straight BVM is the standard model used to mimic a healthy blood vessel and has primarily been used for stent and intravascular imaging testing⁹. Aneurysm BVMs are fabricated to mimic the geometry of an aneurysm in a vessel¹⁰. Several aneurysm geometries have been created, including blister, fusiform, and saccular, which are useful for testing neurovascular devices such as flow diverters¹⁰. A diabetic BVM has also been developed, which uses diabetic cells instead of non-diseased ECs and SMCs^{11,12}. Stenting in diabetic patients has a lower rate of success than in non-diabetic patients, so further testing of devices that will be used in diabetics may be important to understand safety concerns.

Although the examples described above incorporated both ECs and SMCs, BVMs using only endothelial cells have also been developed and implemented in the past. Cardinal et al. initially developed these BVMs using ePTFE scaffolds that were seeded with only human microvessel endothelial cells⁴. It was observed that cell coverage was significantly reduced upon the implementation of PLGA scaffolds in the BVM model, so SMCs were introduced to promote EC adhesion. While the addition of SMCs makes the model anatomically more similar, it also adds an increased amount of additional resources and time to create the model. The addition of SMCs also complicates analysis of the BVMs, requiring the ability to distinguish between cell types and understand the interactions of the two cell types. Therefore, it would be beneficial for the Tissue Engineering Lab to have the capability to create EC only BVMs. Analysis of cell-cell

interactions, cell-scaffold interactions, and cell-device interactions in these BVMs would be simplified.

1.1.4 Importance of Endothelial Cell Characterization

Device testing has always been the goal for the BVM model. The interaction between the device and endothelial cells is the primary consideration for analysis since this cell-device interaction is the dominant interaction *in vivo*; therefore, the endothelial cells used in the model are of utmost importance. These considerations lead to the necessity of understanding how the ECs are reacting during culture, in interaction with scaffolds, and when exposed to shear flow. Efforts need to be made to characterize endothelial cell behavior, especially since endothelial cells commonly undergo morphological changes *in vitro*. These changes have been observed in the Tissue Engineering Lab and documented in literature^{13–16}. Analyzing these changes enhances the legitimacy of the model by ensuring that the endothelial cells retain their endothelial-like phenotype and function throughout culture and sodding.

In order to develop EC-only BVMs, new scaffolds would likely need to be implemented. While several different scaffolds have been proposed and manufactured in the past, use of these scaffolds could not be justified over the standard 75:25 PLGA scaffolds. Current models with PLGA have been working, and no methods have been established to investigate scaffold cytotoxicity, cell adhesion, or cell proliferation between scaffold types. These methods must be developed in order to propose novel scaffolds that can be used for EC only BVMs. Important considerations include the ability to sterilize the scaffold, retention of scaffold geometry, scaffold cytotoxicity, EC

adhesion at short timepoints, and EC coverage at longer timepoints. The BVM model must be broken down component by component to thoroughly assess the completed model.

1.2 BVM Characterization

BVMs must be characterized by the individual components of cells and scaffolds, both separately and together. qPCR and immunostaining are useful techniques to analyze cells. qPCR collects quantitative measurements of gene expression, which indicate changes to specific genetic markers. This technique is used in molecular diagnostics to detect and quantify bacteria, as well as in disease diagnostics to understand how genetic markers change with disease onset and progression¹⁷. Immunostaining uses antibodies to label proteins in cells, which can be visualized by confocal microscopy to identify protein presence or intensity¹¹. Both techniques can be applied to BVM characterization through analyzing gene expression and protein changes in ECs^{11,12,18}. Gene expression analysis will be extensively covered in Chapter 2 of this thesis.

Methods are also necessary to characterize scaffolds before cells are seeded. Scanning electron microscopy (SEM) focuses an electron beam on a sample and collects the scattered electrons to generate an image. These high-resolution images work well for imaging electrospun scaffolds, where the images can be used to quantify data regarding fiber diameter and porosity. This quantification processing can be done using software like ImageJ or MATLAB¹⁹.

Once the individual components have been characterized, the interaction between cells and scaffold can be evaluated. The previously identified methods are useful for this

process. SEM can visualize cell coverage, either in the lumen of scaffolds or on deployed stents. qPCR can quantify cell changes as they interact with different scaffold types, flow conditions, or lengths of time cultured in bioreactors. Immunostaining can be used for similar purposes, characterizing the cell interaction with the scaffold through investigations of adhesion, proliferation, or cell identification.

1.2.1 Previous Characterization

Several techniques have been investigated and implemented over the years in the Tissue Engineering Lab to characterize cells in the BVMs. Multiple previous projects have attempted to characterize BVMs through qPCR. Tristin McGuffick evaluated gene expression changes in stented and unstented vessels. PECAM, ICAM, SMA, Ki67, and BAX were chosen as endothelial cell, inflammation, smooth muscle, proliferation, and apoptosis markers, respectively. She found downregulation of PECAM after stent deployment in umbilical BVMs, while coronary BVMs downregulated SMA and upregulated Ki67 and BAX¹⁸. Shelby Kunz and Gabriella Sugerman used gene expression to characterize ECs cultured in high glucose conditions. Kunz used the markers eNOS, PECAM, and RAGE to characterize and compare HUVECs and diabetic human coronary artery ECs (DHCAECs). DHCAECs had higher expression of RAGE and eNOS compared to HUVECs. HUVECs treated with high glucose media had increased expression of RAGE compared to normal media. These findings set baseline gene expression levels and showed how BVMs using HUVECs differed from those using DHCAECs¹². Sugerman continued work with high glucose treatments of ECs, using IL-6 and CD36 as markers for oxidative stress. She found that IL-6 expression increased with

glucose treatment¹¹. These three theses struggled to identify a consistent housekeeping gene, making conclusions regarding treatment trends more difficult to confirm.

Imaging techniques have been implemented in many projects, mostly through SEM imaging or immunostaining. SEM images are routinely used to verify a confluent cell lining in the lumen of the BVM. While useful, this technique cannot easily quantify cell presence. Immunostaining techniques have also been used for various purposes. Most common have been nuclear stains to identify and quantify cell presence. Another important project has been to implement staining protocols to distinguish ECs versus SMCs in BVMs²⁰. PECAM has been used as a marker for ECs. Myosin heavy chain, α -smooth muscle actin (α SMA), and, most recently, calponin have been used to identify SMCs.

1.2.2 Need for Assessment

Despite the past work done, there is still need for additional characterization of cells, scaffolds, and BVMs. It has been observed that endothelial cells undergo morphological and genetic changes during culture *in vitro*²¹. The endothelial lining of the BVMs is, arguably, the most important component of the model. Intravascular devices will theoretically primarily interact with the endothelial lining of the BVMs, and that interaction will provide useful preclinical data. Observations of interest, such as a change in proliferation, migration over the stent, or death, are all observations that have to do with changes in ECs. Therefore, further characterization of ECs specifically is of great importance.

There are several potential areas where better characterization of endothelial cells would progress our research. The first is to better understand how ECs change at different timepoints in the BVM fabrication process. The first interval is the time between when ECs begin culture in flasks until just before sodding into scaffolds. This information would allow us to track the utility of our cells as they age and identify an optimal passage number to use in BVMs. Depending on how, or if, the cells change, it would also suggest how culture procedures could be changed to optimize EC cell growth. The second interval is the time between when cells are sodded into scaffolds and the resultant BVM is taken down for assessment. There is a need to assess the effect of shear stress on endothelial cells in the BVM model, and to compare how those cells have changed relative to the initial cells that were sodded. A third time interval, when a device is implanted in the BVM, could be included in this section; however, the first two intervals were the focus of this thesis.

The second area for better characterization involves the cell-scaffold interaction. This includes both the cell interaction with the scaffold and the scaffold interaction with the cells. The Tissue Engineering Lab has no current methods to assess these interactions, and without these protocols there is no way to justify or quantify a change from one type of scaffold to another. This must be assessed through initial adhesion data at short timepoints, as well as multi-day time points to address cell proliferation on the scaffold. Scaffold cytotoxicity must also be investigated, especially regarding changes in polymer type or the addition of supplements to the electrospinning solution.

1.3 Summary and Aims of the Thesis

BVMs have proven to be a useful model for testing vascular devices. This utility warrants more exploration into the details of the individual components being used in the model. Before the components can be investigated, methods must be established and verified. These methods will be applied to current problems encountered by the Tissue Engineering Lab to determine the effectiveness of the methods. The overall goal of this thesis was to establish methods that could be used to understand endothelial cell changes in the BVM model. In order to accomplish this goal, two aims were pursued: 1) to characterize ECs alone through changes in gene expression, and 2) to characterize EC-scaffold interactions. Each aim is further summarized below.

1.3.1 Aim 1: Endothelial Cell Characterization through Changes in Gene Expression

The goal of the first aim of this thesis was to analyze gene expression changes in endothelial cells with a focus on endothelial cell dedifferentiation. The markers explored can also be applied to a broader understanding of cell-cell interactions between ECs. This first aim can be divided into two components relating to changes in HUVEC gene expression. The first component studied the effect of culture time on EC expression, where the treatment groups were defined by the passage number of the cells. The hypothesis was that increasing cell culture duration would decrease endothelial marker gene expression and increase mesenchymal marker gene expression. The second investigation sought to examine the effect of three flow conditions on gene expression. The flow conditions in question were static culture, disturbed flow, and laminar flow. The

hypothesis here was that static and disturbed flow would decrease endothelial marker expression and increase mesenchymal marker expression, while laminar flow would have the opposite effect on endothelial and mesenchymal markers.

1.3.2 Aim 2: Characterization of Endothelial Cell and Scaffold Interaction

The second aim of this thesis sought to establish methods for investigating and quantifying cell-scaffold interactions. This was achieved through designing two assays. The first assay investigated scaffold cytotoxicity through exposure of ECs to scaffold elution products. The second assay was developed to understand cell adhesion on scaffolds at shorter timepoints and cell coverage in BVMs at longer timepoints. Cell apoptosis and dead cell presence were also quantified, respectively, through Caspase and Propidium Iodide activity. These assays were developed and applied to an initial investigation of novel nanofiber scaffolds, with the potential of replacing the current scaffolds used by the Tissue Engineering Lab with the novel scaffolds.

CHAPTER 2. AIM 1: GENE EXPRESSION CHANGES OF ENDOTHELIAL CELLS IN CULTURE AS A FACTOR OF TIME AND FLOW CONDITION

2.1 Introduction

The first aim of this thesis explored the gene expression changes experienced by endothelial cells in culture as a factor of time and flow condition. In order to understand these changes, several topics must be prefaced, including flow environment, cell phenotype, endothelial-mesenchymal transition, the mechanosensory complex, and qPCR. These topics will provide background and justification for the methods chosen in this aim and will help with explanation of the resulting data.

2.1.1 Flow Environment

Endothelial cells are highly reactive to flow conditions of fluid across the cells. Native flow environments within blood vessels are dynamic; vasodilation and constriction, changes in blood pressure and heart rate, and bifurcations all effect blood shear forces across the endothelium. BVMs have consistent and controlled flow, but the forces within BVM are not representative of physiological conditions. Flow within native vessels and BVMs will be described in this section.

2.1.1.1 Native Flow Environment

A baseline set of *in vivo* flow conditions must be established to compare the BVM model against. The lumen diameter of BVMs range from 3 mm to 4 mm depending on the fabrication technique. Therefore, comparable arteries should have similar lumen sizes and previously measured hemodynamic force values for shear stress and cyclic strain.

Shear stress is the tangential force of blood flowing over the endothelial cells of the tunica intima, and has values ranging from 10 to 70 dynes/cm²²². Vasoregulatory mechanisms attempt to maintain a shear stress around 15 dynes/cm²²³. Cyclic strain is derived from blood pressure and produces forces that stretch the vessel circumferentially²⁴. While shear stress mostly affects the tunica intima, cyclic strain affects all three layers of the vessel²². For the purpose of this thesis, shear stress will be the primary focus of hemodynamic force, which is indicated in Figure 5.

Figure 5: Forces Acting on Blood Vessels. Hemodynamic forces of shear stress (SS), pressure (P), and tensile stress on the endothelium²⁴.

One such artery that is a suitable comparison for the BVM model is the left coronary artery, which is on average 4.0 mm in diameter and has wall shear stress forces between 15 and 40 dynes/cm² in areas of undisturbed flow^{25,26}. The coronaries are also a common sight of device use, with 91% of percutaneous artery interventions involving stenting in 2006²⁷. The carotid artery is also useful for comparing to BVMs. While larger than BVMs at 4.3 to 7.7 mm in lumen diameter, the mean wall shear stress is lower than that of the left coronary artery at 12 to 15 dynes/cm²^{28,29}. The carotids are also a site where stenting is a common treatment³⁰. The HUVECs used in the BVMs are sourced

from umbilical veins, which have an average wall shear stress of 5.2 dynes/cm² and a lumen diameter of between 600 and 800 μm ^{31,32}.

Native vessels have areas of laminar and disturbed flow, shown in Figure 6, so wall shear stress is not consistent³³. Disturbed flow is common near bifurcations and bending in the vessel path where blood flow enters low pressure regions, slows the blood velocity, and causes circulation²⁴. These areas, like the one shown in Figure 7, experience less shear stress than under laminar flow, inducing an active phenotype in the ECs that have proinflammatory, pro-adhesive, procoagulant, and pro-proliferative properties^{34,35}. Laminar flow and high shear stress, on the other hand, induces a quiescent phenotype that promotes cell alignment and survival while reducing proliferation^{22,34}.

Figure 6: Laminar Flow versus Shear Flow²².

Figure 7: Flow in a Bifurcation. Disturbed flow results at areas of bifurcation, which results in EC activation ³⁶.

2.1.1.2 Previously Determined Flow Conditions in Blood Vessel Mimics

Previous Biomedical Engineering graduate student Marc Dawson explored physiological flow conditions in BVMs³⁷. He completed his thesis in 2009, so almost 10 years have passed since his explorations of BVM flow conditions. Dawson explored two main facets of flow: pressure and wall shear stress (WSS). *In vivo* changes in pressure only significantly affect the tunica media, which corresponds to changes in the layer of SMCs in BVMs. Therefore, changes in pressure may not significantly affect the EC layer in the BVM, which is the primary concern of this thesis. In his pressure tests, Dawson did determine that the 8-roller pump produced a pressure profile with only small pressure fluctuations at high RPMs. At 100 RPMs, or 10 mL/min, the 8-roller pump produced pressures of 16 mmHg at maximum and 8 mmHg at minimum. This testing lead to the implementation of an 8-roller peristaltic pump over a 3-roller, so all shear stress measurements were made using the 8-roller pump.

Dawson determined that his BVM systems had shear stress values of about 0.35 dyne/cm², which is significantly lower than physiological shear stress of around 10 dyne/cm². Since previous studies determined that EC alignment occurred between 6-10 dyne/cm², Dawson attempted to produce WSS values of between 6-8 dyne/cm². He initially used Dextran and Methylcellulose as additives to cell media to increase the viscosity, and therefore shear stress, in the BVMs. However, Methylcellulose was determined to be too difficult to implement so only Dextran was pursued as an additive. The lowest flow rate used in calculating WSS was 15 mL/min, or 140 RPMs, which produced WSS of 6.04 dyne/cm² for a 4 mm vessel and 14.4 dyne/cm² for a 3 mm vessel. To produce this WSS the viscosity of the media would need to be 15.2 cP, or almost 30% Dextran. That percentage of Dextran could negatively affect cell growth, so a lower percentage was implemented in the BVMs. He used 14% Dextran at 28 mL/min (270 RPM) to produce a WSS of 6.4 dyne/cm². This concentration of Dextran was not found to significantly disrupt cell growth, but the desired cell morphology was not produced.

Several important changes have been made to the model since Dawson's thesis. Scaffolds constructed of ePTFE used to be used in the BVMs, and the standard size of these scaffolds were 4 mm. The current model uses PLGA scaffolds, which have significantly different porosity and cellular interactions, and a standard vessel size of 3 mm. The flow tested by Dawson, especially regarding his exploration of increasing WSS by increasing media viscosity, resulted in higher shear values than in the current model. The lowest flow rate he investigated at standard media viscosity was 10 mL/min, which corresponds with 100 RPMs on the 8-roller peristaltic pump. The current model uses a maximum flow rate of 90 RPMs. These differences have significant impacts on fluid

flow, especially regarding shear stress. The use of a 3 mm vessel instead of 4 mm could make the implementation of dextran in cell media more feasible since a lower percentage of dextran would produce the same shear stress. However, the lower RPMs of the pump could counteract any difference that the smaller diameter increases the WSS. Exploration of these differences may be necessary in future work, as adjustments to the flow conditions currently used in BVMs may benefit cellular responses like cell adhesion, growth, and phenotype. Nevertheless, this information is just worth noting for this current thesis as background on the model.

2.1.2 BVM Cell Phenotype

Phenotype refers to the appearance of a cell as a result of genetic and environmental interactions. In the BVM model, culture conditions and flow conditions contribute to a distinct EC phenotype. Whether this phenotype is similar to a physiologic phenotype has yet to be determined, but this section will elucidate the current understanding of EC phenotype in BVMs and offer a probable explanation for this observed phenotype as background for the methods and experiments in this chapter.

2.1.2.1 Immunostaining of HUVECs in the Tissue Engineering Lab

A previous project in the Tissue Engineering Lab worked to implement immunostaining for the purpose of discerning between ECs and SMCs in BVMs. During the process of developing this protocol, it was noticed that the HUVECs were staining positive for alpha smooth muscle actin (α SMA), which was the marker that was being used to identify SMCs. This finding is shown in Figure 8. After testing for bleed through

from other channels and testing controls for the primary and secondary probes, it was concluded that the HUVECs were expressing α SMA, which was hypothesized to potentially be due to the cells undergoing EndMT.

Figure 8: Endothelial Cells Stain for α SMA. HUVECs stained for DAPI, left, and α SMA, right, show expression of α SMA in HUVEC cytoplasm and nucleus.

2.1.2.2 Endothelial-to-Mesenchymal Transition

Endothelial-to-mesenchymal transition (EndMT) is a process where endothelial cells lose endothelial features and gain fibrotic features^{13,38}. During this process, the expression of EC markers, such as VE-Cadherin (VE-cad), platelet endothelial cell adhesion molecule (PECAM), and vascular endothelial growth factor receptor 2 (VEGFR2), decrease while the cells increasingly begin to express fibrotic and smooth muscle markers like α SMA, calponin, and transgelin (SM22 α)^{21,39–41}. The mesenchymal phenotype is also characterized by the loss of cell-cell junctions and the gain of migratory abilities⁴². This process may be the reason that HUVECs in the Tissue Engineering Lab express α SMA. Context behind EndMT is necessary to understanding why the phenomenon occurs, and the utility and hinderance of EndMT *in vitro* and *in vivo*. The following sections will summarize these topics.

2.1.2.2.1 EndMT in Heart Development

EndMT was first discovered as a mechanism of heart development *in utero*^{2,42,43}.

In the early stages of heart development, no valves exist to prevent backflow⁴⁴.

Endocardial cushions are thick areas of tissue in the forming heart that eventually develop into the septum, mitral valve, and tricuspid valve^{45,46}. During endocardial cushion formation, ECs undergo EndMT and the mesenchymal cells migrate to the endocardial cushions^{42,45}. The endocardial cushions elongate to form the septum and valves at different stages of heart formation⁴⁵.

2.1.2.2.2 EndMT in Disease Progression

EndMT is involved in various diseases, including fibrosis (cardiac, pulmonary, kidney, liver)^{41,47,48}, cancer tumor growth^{16,41,42,47}, fibrodysplasia ossificans progressiva (FOP)^{40,47}, atherosclerosis and coronary artery disease^{2,49–52}, and cerebral cavernous malformation^{40,53}. The involvement of EndMT continues to be discovered in increasing types of diseases.

As previously discussed, blood vessels have areas of laminar and disturbed flow; these areas of disturbed flow are prone to the formation of atherosclerotic lesions^{2,49,50}. Specific areas of disturbed flow include carotid bifurcations, the aortic arch, and branching points of the coronary arteries⁵¹. EndMT-derived fibroblast-like cells have been identified in human atherosclerotic plaque through the co-expression of endothelial and smooth muscle markers^{49,50}. This process is caused by TGF- β signaling, oxidative stress, and hypoxia. Fibroblast growth factor (FGF) signaling is an inhibitor of EndMT, but disturbed flow inhibits FGF signaling by blocking expression of FGF receptors.

Hypertension, hyperglycemia, and obesity increase endothelial oxidative stress, making those conditions risk factors of EndMT and atherosclerosis^{2,49,54}.

Solid tumors are composed of multiple cell types, including cancer cells, endothelial cells, inflammatory cells, and fibroblasts^{16,40,47}. Cancer-associated-fibroblasts (CAFs) are a subpopulation of fibroblasts in tumors and have been identified as promoters of tumor growth and progression^{41,47}. Up to 40% of CAFs in tumors may be derived through EndMT, but the underlying mechanisms for CAF activation and development from EndMT must be further studied^{42,47}.

It is still debated as to the extent that EndMT causes fibrosis; other sources of activated fibroblasts arise from local fibroblasts, epithelial-to-mesenchymal transition (EMT), and from the bone marrow¹⁵. All fibrotic conditions display elevated expression of genes encoding for collagens and ECM and a reduction in ECM degrading enzymes⁴⁸. In three mouse models of kidney fibrosis, 30% to 50% of fibroblasts originated from endothelial origin¹⁵. A mouse model for cardiac fibrosis demonstrated that 27% to 35% of fibroblasts in the fibrosis tissue originated from EndMT⁵⁵. The contribution of EndMT in human tissue has less robust evidence. However, a study analyzing myocardial tissue from patients with cardiac fibrosis showed that about 17% of fibroblasts in the fibrotic myocardium were derived from EndMT⁴⁸. This suggests that dedifferentiated endothelial cells are a significant component of atherosclerotic plaque in humans.

2.1.2.2.3 EndMT in Tissue Regeneration

While EndMT is highly involved in disease progressions, it also has the potential to assist in tissue regeneration *in vitro* and *in vivo*. Since EndMT differentiates ECs into a

mesenchymal stem cell-like phenotype, those mesenchymal cells can be further differentiated into other tissue types, as shown in Figure 9^{53,56}. One potential application for EndMT in tissue regeneration is for the treatment of osteoarthritis and temporomandibular joint disorder (TMJD)⁵³. Chondrogenesis is the process by which cartilage is formed, where chondrogenic mesenchymal cells proliferate into precartilaginous nodules before differentiation into chondrocytes producing cartilage ECM⁵⁷. Cartilage grafts could be produced using EndMT derived chondrocytes. A similar concept was shown with osteogenesis in mice, where endothelial cells labeled with fluorescent quantum dots and treated with TGF β 2 were seeded onto scaffolds, implanted, and locally treated with differentiation media. The scaffolds were explanted and stained for bone, in which it was found that the scaffolds stained for bone in the same region that the quantum dot fluorescence occurred⁵⁸. This displays the utility of endothelial cells and the ability of the cells to readily differentiate in proper conditions. The conditions of static culture and of BMVs may be appropriate conditions to cause EndMT.

Figure 9: EndMT in Tissue Engineering. Potential use of EndMT in tissue engineering through dedifferentiating ECs into multiple other cell types ⁵⁶.

2.1.2.3 EndMT Activation Pathways

The progression of EndMT in ECs is most easily explained through the pathways of the different inducers, which includes transforming growth factor beta (TGF- β), the pro-inflammatory cytokines interleukin-1 beta (IL1- β) and tumor necrosis factor-alpha (TNF α), transcription factor NF-kB, and oxidative stress³⁸. Each of these inducers will be introduced below.

2.1.2.3.1 Transforming Growth Factor Beta

The most well studied EndMT inducer is TGF- β , a growth factor that has roles in cell proliferation, differentiation, apoptosis, and migration. It has three isoforms (TGF- β 1, TGF- β 2, TGF- β 3) that bind to TGF- β transmembrane receptor type II (TGF β -RII). This phosphorylates and activates one of two TGF- β type I receptors, either activin receptor-like kinase 5 (ALK5) or ALK1. ALK5 phosphorylation activates SMAD2/3, while ALK1 activates SMAD1/5. Both SMAD2/3 and SMAD1/5 form complexes with co-SMAD (SMAD4) and move into the nucleus. High levels of TGF- β will signal mainly through ALK5, while low levels of TGF- β will signal through ALK5 and ALK1. Endoglin (END) acts as an auxiliary receptor that inhibits ALK5 signaling and promotes ALK1 signaling^{13,38,48,59}.

This point in the signaling is important, as ALK1 and ALK5 cause opposite effects. ALK1 signaling promotes EC proliferation, migration, and organization; ALK5 signaling inhibits EC proliferation, migration, organization, and continues the EndMT pathway. SMAD2/3 induces expression of Snail1, Slug (Snail2), Twist1, and GATA4. These are the transcriptional regulators of EndMT in the nucleus of ECs. GATA4

regulates Twist1 expression, which induces Snail pathway activation in ECs². The Snail pathways downregulate VE-cadherin, an important protein in cell-cell interactions and an indicator of EndMT progression^{60–62}. However, the Snail pathway alone is insufficient in inducing EndMT: GSK-3 β , the inhibitor of Snail, needs to be inhibited through phosphorylation for EndMT to occur⁶².

TGF- β also activates mitogen-activated protein kinase (MAPK) pathways that activate the Snail pathway downstream. This includes extracellular signal-regulated kinase (ERK)1/2, p38 MAPK, and c-Jun-N-terminal kinase (JNK) [50]. The TGF- β provides a pathway for positive feedback that sustains EndMT progression. TGF- β activates TGF- β -activated kinase 1 (TAK1), which activates p38 MAPK expression. p38 MAPK increases the expression of TGF- β , creating a positive feedback loop for EndMT². Therefore, EndMT can occur without constant stimulation by TGF- β , making this pathway a possible source of EndMT *in vitro*.

2.1.2.3.2 Notch Signaling

While TGF- β is considered the main inducer of EndMT, several other pathways contribute by aiding the TGF- β pathway⁶². Notch can activate Twist1 expression, contributing to the activation of the Snail pathway². Notch also upregulated mesenchymal genes by recruiting Smad3 to Smad binding sites⁴⁸. Snail can also be directly upregulated by Notch signaling; however, this role of is more robust during development of the cardiac cushion⁶⁰.

2.1.2.3.3 Wnt/ β -catenin Signaling

Wnt/ β -catenin signaling is necessary for heart valve development *in utero*, but also plays a role in tissue fibrosis⁴⁸. It contributes indirectly to EndMT by stabilizing Snail, while contributing directly by inducing expression of transgelin (SM22- α)². The Wnt pathway also plays a role in EndMT caused by hyperglycemic conditions⁶³.

2.1.2.3.4 Pro-inflammatory Cytokines

Several cytokines associated with the inflammatory response have been shown to induce EndMT. Interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF- α) activate NF- κ B through the Akt pathway^{38,64}. NF- κ B translocates to the nucleus where it affects gene expression. It is not apparent exactly how NF- κ B changes gene expression, but NF- κ B is required for EndMT to occur. When the NF- κ B inhibitor I κ B α remained active in HUVECs, mesenchymal markers were not expressed⁶⁵.

Interestingly, the treatment of these cytokines themselves does not seem to create enough stimulus to cause EndMT. IL-1 β treated ECs only increased expression of SM22- α when they were also treated with TGF β , suggesting that IL-1 β alone does not cause EndMT⁶⁶. These cytokines also have varying strengths of inducing EndMT, with TNF- α being a more potent inducer than IL-6⁶⁴.

2.1.2.3.5 Oxidative Stress

Reactive oxygen species (ROS) include hydroxyl radicals, superoxide radicals, hydrogen peroxide, and peroxynitrite. Most ROS production is the result of NADPH oxidase system, specifically NOX4, with which the production of ROS activates NF- κ B

and leads to EndMT^{38,55}. ROS production also activates p38, which leads to the production of TGF- β and continues the TGF- β signaling of EndMT³⁸. In normal cell functioning, TGF- β stimulates the generation of ROS, so the TGF- β pathway of inducing EndMT will inherently cause ROS production, adding another source of EndMT stimulus⁶⁷.

2.1.2.3.6 Disturbed Flow

Areas of disturbed flow, where it was previously established that EndMT occurs, are prone to the activation of the pathways that cause EndMT. Disturbed flow activates inflammatory signaling, ROS production, and TGF- β ². Areas of disturbed flow are also prone to high blood pressure and stiffening of the vascular walls, which causes high cyclic strain. Cyclic strain is the stretch force circumferentially experienced by ECs. This strain further activates TGF- β , while additionally activating Wnt/ β -catenin signaling. While BVM should experience low levels of circumferential strain, it is possible that the flow through BVMs results in inflammatory pathway activation.

2.1.2.4 Inhibitors of EndMT

Although many factors lead to EndMT onset, there are treatments to inhibit this transition, which may be useful as cell treatments during culture. Common media additives like FGF2 induce the expression of microRNA-20a (miR-20a). MiR-20a blocks ALK5 (TGF β RI) and TGF β RII, inhibiting TGF β activation of Smad2⁶⁸. FGF also increases the expression of VEGF in ECs⁵⁴; the combination of reduced TGF β activity

and increased VEGF expression results in reduction of EndMT. Other proteins that, when added to cell media, reduce EndMT include VEGF-A and BMP7⁵³.

Several drugs reduce the effects of EndMT, which are briefly outlined in table I. However, some have only proven efficacy in animal models, such as Relaxin.

Table 1: Drugs that Inhibit or Reduce EndMT⁵³.

Drug	Effect
Linagliptin	DPP-4 inhibitor, blocks TGF β 2
Rapamycin	Blocks mTOR signaling
Relaxin	Disrupts Notch signaling
Macitentan	Endothelin-1 receptor inhibitor
Kallistatin	MMP inhibitor
Spironolactone	Aldosterone receptor blocker, inhibit Notch1 expression
Scutellarin	Reduces Notch1 expression
Losartan	Inhibits angiotensin II type 1 receptor
Cinacalcet	Suppresses hormone levels
Hydrocortisone	Enhances EC adhesion

Alterations in mechanical stimuli can also reduce EndMT. Opposite to how disturbed flow can induce dedifferentiation, laminar shear stress can protect endothelial cells from EndMT. The activation of Kruppel-like factor (KLF) 2 and 4 by extracellular signal-related kinase 5 (Erk5) are responsible; Erk5 is continuously activated by laminar shear stress, and KLF2/4 are transcription factors activated downstream that have a wide range of effects, but mainly target the NF-kB and TGF β pathways^{69,70}. KLF2/4 directly inhibits NF-kB activation while suppressing activator protein 1 (AP-1) and Smad2

nuclear translocation, both important proteins in the TGF β pathway^{70,71}. Furthermore, KLF4 binds to and activates the VE-cadherin promoter, leading to the stabilization of adherens junctions⁷².

The investigation of laminar shear stress offers a plausible and logical starting point to impacting and potentially reducing EndMT in the BVM model. These alterations would not require media supplements or drugs which may be expensive, difficult to obtain, and whose effect may have unanticipated consequences such as toxicity or decreased cell viability. In order to determine if any treatments affect EndMT, gene markers must be identified that correlate with EndMT progression as a result of flow changes.

2.1.3 The Mechanosensory Complex

Mechanical connections between endothelial cells are generally known as adherens junctions, which in ECs are important for monolayer integrity, contact inhibition of growth, and apoptosis⁷³. One type of adherens junction specific to ECs, known as the mechanosensory complex, is composed of PECAM, VE-cadherin, and VEGFR2. This set of proteins is commonly used to identify and quantify EndMT^{14,21,49,50,64,69,74,75}, which may also be applicable for use in characterizing EndMT in HUVECs used for BVMs. The mechanosensory complex is not the only mechanotransducer that causes cellular changes after exposure to flow, as shown in Figure 10. G-proteins, primary cilia, integrins, intermediate filaments, glycocalyx, caveolae, ion channels, and the actin cytoskeleton all have proposed roles in flow transduction^{36,76}. However, the mechanosensory complex is primarily responsible for

maintaining the EC monolayer, and its functions have been well researched in relation to EndMT. This section will discuss the proteins in this complex, how they interact, and how they are stimulated in response to different flow conditions.

Figure 10: Types of Endothelial Mechanosensors. A depiction of the many mechanotransducers present on endothelial cells³⁶.

2.1.3.1 Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1)

PECAM-1 primarily functions as a mechanosensor and transmitter of mechanical force from shear stress to the cytoskeleton^{70,73,77}. Shear stress induces rapid phosphorylation of PECAM and eventual downstream phosphorylation and activation of VEGFR2, making it the principal initiator of the mechanosensory complex when exposed to stresses^{73,77}. The expression of PECAM-1, as well as the phosphorylation of PECAM, is highly dependent on cell confluence. 100% confluent HUVEC cultures showed significantly more PECAM-1 and phosphorylated PECAM-1 expression than 50% confluent cultures⁷⁸. The Cal Poly Tissue Engineering Lab has verified through

immunostaining that PECAM-1 expression is reduced in areas where cell confluency is reduced, as cell-cell contact is necessary for PECAM-1 expression²⁰. Furthermore, for confluent HUVEC cultures, less phosphorylated PECAM-1 was expressed after exposure to disturbed flow versus laminar flow⁷⁸. This is an important consideration, as PECAM-1 phosphorylation is necessary for mechanosensory complex function.

Several studies have investigated the role of PECAM under direct exposure of mechanical stimuli. Ferromagnetic beads coated with PECAM-1 antibody were used to stimulate the protein with direct mechanical force. After 10 minutes of magnetic exposure, Western blots showed the phosphorylation of both PECAM-1 and ERK 2⁷⁹. As previously discussed, ERK 2 is implicated in the activation of Snail, a major contributor of EndMT, and this activation of ERK 2 was verified in ECs exposed to shear stress. Fluorescence resonance energy transfer (FRET) based tension sensors were designed to quantify changes in direct force across PECAM-1 during shear stress exposure⁸⁰. Shear stress on bovine aortic endothelial cells (BAECs) resulted in an increase in tension on PECAM-1 and an association between PECAM-1 and vimentin, an intermediate filament⁸¹. In the same study, the role of vimentin was explored with a vimentin knockdown, which resulted in blocking the tension increase on PECAM-1. It was concluded that vimentin transmits the mechanical force from flow shear stress to PECAM-1 in order to initiate downstream effects⁸⁰.

Not all literature agrees on the role of PECAM-1 during EC exposure to shear stress, as some studies implicate it in inducing atherosclerosis instead of protecting from or reducing atherosclerosis⁸². PECAM knockout mice showed reduced atherosclerotic lesions in the aortic arch, as well as a decrease in NF- κ B⁸³. This may indicate that

PECAM-1 is associated with NF- κ B-induced EndMT and atherosclerosis. It may also indicate that PECAM-1 knockout simply dampens general inflammatory signaling in ECs³⁶. This is most likely the case: since ENOS is deregulated in the absence of PECAM-1, PECAM knockout mice have high NO production which suppresses inflammation⁵⁴. Based on this information, the assumed role of PECAM-1 as atheroprotective remains valid; regardless, the necessity of PECAM for transmitting fluid forces and retaining the function of the mechanosensory complex makes it a marker of interest for this aim.

2.1.3.2 Vascular Endothelial Cadherin (VE-Cad)

While the application of force directly to PECAM-1 triggers the same responses as flow shear stress, these effects are not seen when force is applied to VE-cad^{76,80}. This demonstrates the functional difference between the two proteins; VE-cad acts as an adaptor molecule that links the mechanical force transduced by PECAM-1 to VEGFR2^{70,73,76,84}. This is achieved through the formation of complexes that bind to the actin cytoskeleton that then transfer signals within ECs^{70,77,85}. These complexes are formed with alpha and beta catenin, plakoglobin, vinculin, and cingulin⁷⁷. VE-cad binds directly to β -catenin or plakoglobin, which then binds the actin binding protein α -catenin⁸⁶.

VE-cad is the major determinant of EC contact integrity, so remodeling of VE-cad is important to adapt to changes in stimulus^{77,86}. During angiogenesis the interactions formed between VE-cad and neighboring cells, as well as the interactions between VE-cad and cytoplasmic proteins, must be broken to allow cell migration and for new cell-cell adhesions to form⁸⁷. VE-cad interactions are also disrupted with increased matrix

stiffness due to changes in cytoskeletal mechanics, resulting in increased EC monolayer permeability⁷³. Arterial fibrosis associated with EndMT results in positive feedback where increasing matrix stiffness disrupts EC monolayers and encourages uncontrolled cell migration. This relaxation of the EC monolayer was demonstrated with FRET-based tension sensors which saw a rapid 25% decrease in tension across VE-cad with the onset of flow⁸⁰.

VE-cad plays other important role in response to flow *in vitro*. VE-cad depleted ECs aligned at lower rates than depleted cells transfected with VE-cad after exposure to 12 dynes/cm² of shear stress for 18 hours⁸⁸. VE-cad expression does not change in VE-cad depleted cells exposed to static or shear flow, but expression doubled in VE-cad positive cells exposed to shear stress versus a static condition¹⁴. Therefore, VE-cad expression is necessary for HUVECs used in BVMs to align in the direction of flow.

VE-cad also has an important relationship with KLF4, which was previously discussed as an inhibitor of EndMT. VE-cad is transcriptionally regulated by KLF4, in which KLF4 binds the promoter region of VE-cad and induces VE-cad transcription⁷². This promotes VE-cad interactions to sustain the EC monolayer, helping to reduce cell migration and EndMT. VE-cad may be a useful marker to study in HUVECs intended for BVMs, as high expression of VE-cad would suggest EndMT inhibition.

2.1.3.3 Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)

VEGFR2 is the component of the mechanosensory complex that activates biochemical signaling and is necessary for EC migration, proliferation, angiogenesis, and regulation of cell death^{73,85}. VEGFR2 is the main RTK that mediates VEGF signaling,

and thus will be focused on even though VEGFR3 has recently been recognized as a member of the mechanosensory complex⁷³. Binding of VEGF to VEGFR2 initiates activation of PI3K and MEKK3, causing downstream activation of Akt and MEK5/Erk5⁷⁰. Erk5 is an inhibitor of EndMT and activates KLF2/4 which promotes VE-cad transcription, demonstrating only one of many interactions between VE-cad and VEGFR2. The role of VEGFR2 is highly dependent on its interaction with VE-cad and should therefore be discussed together.

2.1.3.4 VE-Cadherin Regulation of VEGFR2

VE-cad alters cell proliferation and apoptosis through modulating VEGFR2 function. Cell confluence is one critical component on which VE-cad acts to inhibit VEGFR2 function *in vitro*. Cadherins in general, including VE-cad, transfer negative intracellular signals upon the establishment of cell-cell contact such as when confluence is reached. This results in a decreased response to growth factors like VEGF.

Lampugnani et al. investigated the relationship between confluent or sparse VE-cad-null and VE-cad-positive cells stimulated with or without VEGF⁸⁵. VE-cad-null cells continued growth into over-confluence, while VE-cad-positive cells arrested growth at confluence, demonstrating the growth restricting role of VE-cad. VE-cad-null cells were responsive to VEGF regardless of confluence, while VE-cad-positive cells were only responsive in the sparse condition. The lack of response in the VE-cad-positive confluent condition was associated with a reduction of VEGFR2 phosphorylation. The same trend was verified in HUVECs, where an upregulation of VEGFR2 phosphorylation in VEGF

occurred in sparsely distributed cells. The role of cell density on expression will be important to control during experiments studying VE-cad and VEGFR2.

That study, as well as others, ultimately concluded these interactions were a result of VE-cad forming a complex with VEGFR2 and density-enhanced receptor-like tyrosine phosphatase (DEP-1)^{14,85,88}. In sparse ECs *in vitro*, VEGF binds VEGFR2 to activate the Erk1/2 pathway. Erk1/2 phosphorylates VE-cad, which causes the detachment of β -catenin from VE-cad. β -catenin translocates to the nucleus where it initiates transcription that results in cell proliferation. In confluent ECs, DEP-1 congregates at cell-cell junctions and inhibits Erk1/2 phosphorylation of VEGFR2, blocking β -catenin detachment and nuclear translocation^{89,90}. This mechanism is depicted in Figure 11.

Figure 11: Mechanisms of VE-Cad and VEGFR2 on Cell Seeding Density⁸⁹.

While those interactions are important for understanding the mechanosensory complex in quiescent EC monolayers *in vivo* and *in vitro*, it lacks the context of how these interactions fare with alternative, non-physiologic flow conditions such as in

BVMs. Parallel plate flow models showed that, upon exposure to shear stress, VEGFR2 expression increased and VE-cad promoted VEGFR2 activation instead of inhibiting it^{91,92}. β -catenin phosphorylation increased in HUVECs exposed to shear stress, indicating that cells were being induced into a proliferative state⁹³. The same study found that fluid shear stress causes the dissociation of VE-cad and β -catenin. While the mechanism isn't entirely elucidated, shear stress ultimately contributes to EC survival by VEGFR2⁹⁴. Interestingly, the type of laminar flow does not affect cell survival *in vitro*. A TUNEL assay of cells exposed to static media, steady laminar flow, pulsatile laminar flow, and to-and-fro laminar flow showed no difference in percent of apoptotic cells⁹⁵. The length of time in which cells are exposed to these conditions plays a key role in VE-cad and VEGFR2 activation, and this duration is key for the induction of EndMT.

2.1.3.5 Mechanosensory Complex and EndMT

The important roles of the mechanosensory complex in maintaining EC monolayers and in flow signaling is undisputed, making it an ideal candidate for understanding and quantifying EndMT in ECs in culture or in BVMs. For this, and to better understand the second component of Aim 1, the focus must shift back to the effects of laminar versus disturbed flow on ECs and how the mechanosensory complex changes. The general difference between these two flow conditions that should be emphasized are as follows: ECs adapt to flow under laminar flow so that EndMT events are downregulated after the initial stimulation, while these events are sustained in disturbed flow¹⁴. An important example of this involves the activation of Erk1/2 by both PECAM and VEGFR2. Erk1/2 activation stimulates the TGF β pathway, activates Snail, and

eventually leads to EndMT. However, this activation comes with the same caveat, where type of flow is the major consideration in adaptive expression versus sustained expression. While expression of Erk1/2 is sustained over time, the phosphorylated form is not sustained in laminar flow⁵⁰.

The general characteristics of EndMT, identified by expression of mechanosensory complex markers, is the downregulation of PECAM-1, VE-cad, and VEGFR2. Therefore, if EndMT was occurring during HUVEC culture or BVM creation, all three of those markers would be downregulated. In the case of partial EndMT or partial laminar flow, the expression profile of the mechanosensory complex would not be as straightforward. While stimulation of the mechanosensory complex changes expression of the endothelial markers of EndMT, it does not capture fibrotic changes to the endothelial cells. These changes are evaluated through upregulation of mesenchymal markers.

2.1.4 Mesenchymal Markers

EC markers indicate the extent of EndMT away from endothelial characteristics, but mesenchymal markers (also referred to as smooth muscle or fibroblast characteristics in the literature) indicate the extent of EndMT towards the fibrotic state. Common markers include α -smooth muscle actin (α SMA), transgelin (SM22 α), fibroblast specific protein 1 (FSP-1), calponin, and smooth muscle-myosin heavy chain 2 (SM-MHC2)^{21,49,50,62,64,69,74,75}. These markers will generally be associated with cells double positive for EC and mesenchymal/SMC markers, which identify intermediate stages of EndMT.

Smooth muscle cell differentiation can give insight into mesenchymal marker onset in EndMT. Five molecular markers are commonly used to assess SMC differentiation, which are also markers used to assess EndMT. These include α SMA, transgelin, calponin, caldesmon, and myosin heavy chain (MHC), where α SMA and transgelin are early markers of SMCs and calponin, caldesmon, and MHC are intermediate to late markers⁹⁶. The early expression of α SMA and its abundance in differentiated SMCs makes α SMA the most widely used marker for EndMT identification⁷⁴. Therefore, α SMA is a candidate to include in EndMT evaluation for this aim, especially because α SMA expression has already been observed in HUVECs used in BVMs.

The pathways of EndMT previously discussed stimulate the onset of these mesenchymal markers, especially through the TGF β pathway. TGF β stimulates the expression of many genes associated with fibrosis, including varieties of collagen and fibronectin, through Smad2/3 and p38 MAPK activation. This pathway also induces collagen cross-linking, which increases stability, and stimulates the synthesis of protease inhibitors, increasing ECM production⁶². *In vitro* analysis reveals the onset of mesenchymal markers through TGF β stimulation. HUVECs in static media stimulated with TGF β were found to increase expression of calponin and transgelin, while the same cells in laminar flow did not increase expression of mesenchymal markers⁵⁰. This indicates that EndMT can be induced without flow induction. Erk5, which protects EC monolayers from EndMT, was then silenced and resulted in increased calponin and transgelin expression regardless of the flow condition with TGF β stimulation. A different study used orbital shakers without TGF β stimulation to induce disturbed flow. They

found that HUVECs in these conditions expressed Snail, Slug, and α SMA, markers which indicate that disturbed flow alone was enough to activate EndMT pathways⁶⁹.

Mesenchymal markers are also found *in vivo* in EC monolayers. ECs positive for α SMA have been found in the aorta of rats, mice, and humans; these cells were determined to be of EC origin based on their co-expression of VE-cad with α SMA⁷⁴. While these cells were sparsely distributed, the presence of these cells displays EndMT progression in an atheroprone region. Mesenchymal markers are also abundant in atherosclerotic plaques, where EndMT contributes to cell migration and transdifferentiation in these plaques. Atherosclerotic lesions on a porcine bifurcation expressed α SMA, transgelin, and calponin; similarly, human atherosclerotic plaque was shown to co-expresses PECAM-1 and α SMA¹³.

2.1.5 Using Endothelial and Mesenchymal Markers to Determine EndMT Progression

The Tissue Engineering Lab should be concerned with EndMT at all stages of BVM fabrication. EndMT can result from both static culture and disturbed flow. Therefore, the first consideration for this aim was whether the seeded ECs are sustaining an EC phenotype after their time in culture, defined by a cobblestone appearance and the expression of endothelial markers without mesenchymal markers. It has previously been noted that ECs cultured in the Tissue Engineering Lab develop a spindle-like phenotype over time in culture. This consideration has been verified in literature. Freshly isolated adult bovine ECs positive for VE-cad and PECAM-1 displayed EndMT over time in static culture²¹. At day one of culture, cells displayed a cobblestone appearance and were

negative for SMC marker expression. By the second day of culture, cell-cell contact was disrupted and α SMA was lightly expressed. By day 5 ECs acquired an elongated morphology, increased α SMA expression, and no longer expressed VE-cad. Flow models have also displayed EndMT onset. In a bifurcation model of flow, in which areas downstream of the bifurcation were characterized by disturbed flow, shown in Figure 12, VE-cad expression decreased while transgelin expression increased⁴⁹. Collagen I was also prevalent in the area of disturbed flow, which is the most prevalent collagen type in atherosclerotic plaques. The onset and co-expression of EC and mesenchymal markers allow for EndMT assessment *in vitro*. However, to understand the progression of EndMT, changes in mesenchymal and endothelial markers must be quantified.

Figure 12: VE-cad and Transgelin Expression in a Simple Flow Model. (1) VE-cad is expressed upstream. (2) Upon turbulent flow induction transgelin expression is present ⁵⁰.

2.1.6 RT-qPCR Background

Studies of gene expression have previously been implemented in the Tissue Engineering Lab^{11,18}, and the technique of reverse transcription quantitative polymerase chain reaction (RT-qPCR) has been used throughout literature to identify changes in

EndMT^{49,50,59,69,97–99}. For the characterization of ECs, this technique is a powerful and efficient method of determining alterations in genes that are associated with a mesenchymal phenotype over an endothelial phenotype. This technique quantifies the amount of mRNA present in a cell with the assumption that these mRNA will become proteins and effect cell function and behavior. However, total RNA, not specifically mRNA, is often used; total RNA involves fewer purification steps, a higher yield, and less chance of skewed results from different recovery yields. Therefore, total RNA is more often used since relative quantification of the genes of interest (GOI) is more important than the sensitivity of detection of mRNA¹⁰⁰. Paired with properly designed experiments, qPCR data can convey changes in gene expression across many treatments groups and communicate trends within these groups.

Before beginning qPCR, the RNA of cells must be isolated. Kits for RNA isolation are readily available from manufacturers, but there are methods that do not require kits which yield high purity RNA. Next, the single stranded RNA must be diluted, to normalize the amount of RNA per treatment group, and made into double stranded complementary DNA (cDNA) for use as template DNA. cDNA is produced by adding RNA, unpaired nucleotides (dNTPs), DNA polymerase, and reverse transcriptase to a thermocycler to create one cDNA molecule for every RNA strand added. This cDNA can then be used for qPCR, where the cDNA, primers specific for each GOI, probes, dNTPs, and DNA polymerase are combined and added to a thermal cycler that heats and cools the samples repeatedly, replicating the template strand. Primers that bind to each replication fluoresce to indicate a new replicate of the GOI, and this fluorescence is

counted by a camera. The thermocycler repeats this for forty cycles, generating an amplification curve with the amount of fluorescence at each cycle for every sample^{11,101}.

As the cycles increase during qPCR, fluorescence linearly increases until a plateau is reached indicating that all dNTPs have been depleted. A common threshold of fluorescent intensity is established to compare the amount of cycles necessary for each sample to achieve that threshold, in which this cycle number is known as the cycles to threshold (Ct)¹⁷. This Ct value is the numerical value used in the analysis of the data. The Ct value is shown in Figure 13 at the intersection of the amplification curve and threshold line.

Figure 13: qPCR Amplification Curve. An example amplification curve with the three phases and threshold labeled¹⁷.

The fluorescence measurement is the “quantitative” portion of qPCR and results from the type of probe used in the primers. Therefore, the function of these probes must be expanded on. Primers are a short single strand of nucleotides that serves as the starting point for DNA synthesis. During each cooling cycle by the thermocycler primers initiate

the extension of new double stranded DNA (dsDNA) from the single stranded DNA (ssDNA). During this extension probes are utilized to indicate the replication of the template DNA, in which two main types of probes are commonly used: SYBR Green and TaqMan. TaqMan probes, also known as hydrolysis probes, are composed of a fluorescent reporter and quencher attached to a specific nucleotide sequence. During annealing the probe binds to the specific target sequence. As extension occurs, the quencher is cleaved from the probe. The change in proximity of the quencher allows the reporter to fluoresce. These probes, while more specific, are also far more expensive than SYBR Green probes. Therefore, SYBR Green probes were used in all experiments in this thesis. SYBR Green is a DNA-binding dye which binds nonspecifically to any dsDNA. Figure 14 depicts how SYBR Green intercalates within the nucleotides of the extending dsDNA, and this binding event causes fluorescence of the dye¹⁰².

Figure 14: Types of Primers. Depictions of SYBR Green intercalation in double stranded DNA versus TaqMan labeling¹⁰³.

Since SYBR Green binds to any dsDNA, it is possible to have fluorescence measured when non-template DNA intercalates SYBR Green. One example of this is primer-dimer formation, when two primers hybridize to each other, which leads to false fluorescence measurements. Therefore, a dissociation (or melt) curve must be run when using SYBR Green after the amplification cycle to test that the qPCR assay has yielded single, specific products¹⁰⁴. The dissociation curve measures the change in fluorescence when the SYBR Green dissociates from the dsDNA as temperature increases¹⁰¹. Ideally, qPCR products will melt at the same time, so the resulting change in fluorescence over time will result in a similar curve between samples. These ideal curves are labelled “specific products” in Figure 15.

Figure 15: Example Dissociation Curves. Dissociation curves can be used to distinguish PCR products from false signals caused by primer dimers¹⁰⁵.

Housekeeping genes are necessary for any qPCR experiment in order to analyze the Ct values. These genes are selected to have stable expression regardless of treatment

group, which is then used to normalize the Ct values between experimental groups. While many genes theoretically fit this requirement, there is no agreed upon, consistently stable housekeeping gene. Housekeeping genes must be selected and verified based on the experiment type in which they will be used. Two housekeeping genes were initially selected for Aim 1. The first, β -actin (β ACT), is one of several types of actin that are a part of the cytoskeleton microfilaments. It is commonly used in experiments regarding EndMT^{106–109}. The second, β glucuronidase ($\text{GUS}\beta$), is an enzyme involved in the breakdown of glycosaminoglycans (GAGs) in lysosomes¹¹⁰. Both $\text{GUS}\beta$ and β ACT were shown to be a stable reference gene for experiments using HUVECs when challenged against many other common housekeeping genes^{111,112}.

2.1.7 Summary

This introduction to Chapter 2 has discussed a variety of topics in order to gain an understanding of all components that apply to this aim. A stark difference between physiologic and BVM flow conditions was established. Normal EC phenotype was defined and compared against the phenotype of ECs undergoing EndMT. A general, yet comprehensive, review of EndMT was provided in order to understand the complexity of EC dedifferentiation *in vitro* and *in vivo*. This introduction identified EC markers and mesenchymal markers that can be useful in identifying gene expression changes. Finally, an introduction to qPCR was presented, which will be the primary analysis method of EC characterization in the following section. The following section will describe the methods that were used in this aim to culture cells, implement a disturbed flow condition, create BVMs, and perform qPCR to evaluate phenotype and EndMT markers in HUVECs.

2.2 Materials and Methods

2.2.1 Gene Selection and Primer Acquisition

Many relevant genes were identified and discussed in Section 2.1. This list needed to be narrowed down to identify only a few genes of interest (GOI). To track EndMT progression representative endothelial and mesenchymal markers had to be selected. It was decided that genes coding for the three main proteins of the mechanosensory complex would be used as endothelial markers: PECAM-1, VE-Cadherin, VEGFR2. The genes α SMA and calponin were selected as mesenchymal markers, with α SMA as an early indicator of mesenchymal acquisition and calponin as a late transition marker. The markers β ACT and GUS β were chosen as housekeeping genes.

With GOI selected, primers for each gene needed to be acquired. Previous projects attempted to design primers, resulting in poor primer selection. Therefore, preverified primers were used throughout all experiments. PrimeTime qPCR primers (IDT, Coralville, IA) were ordered, which include forward and reverse primers that have already been tested for greater than 90% efficiency. Sequences of the primers, as well as other primer characteristics, can be found in Appendix A.

Primer assays, which include both the forward and reverse primer, were resuspended in 500 μ L of IDTE buffer for a final desired concentration of 20x. While these preverified primers are guaranteed to have an efficiency of greater than 90%, it was still necessary to test primer efficiency once resuspended to ensure that storage and resuspension had not affected the quality of the primers. Primer efficiency shows how well the target sequence doubles during each replication cycle. If each molecule of target sequence doubles during each cycle, the primer has an efficiency of 100%. It is necessary

to verify that efficiencies are between 90% and 110% to correctly analyze results.

Calculations of gene expression assumes that primer efficiencies are comparable between primers¹¹³.

Primer efficiency was calculated by running a logarithmic dilution of cDNA with each primer, then evaluating the slope of the Ct values for the dilutions. The efficiency (E) percentage was calculated using this slope, which can be seen in Equation 1.

$$\text{Equation 1} \quad \text{Efficiency (\%)} = (10^{(-1/\text{Slope})} - 1) * 100$$

The protocol for primer efficiency testing can be found in Appendix B. PECAM1, VEGFR2, VE-cadherin, and GUS β used the same HUVEC cDNA to test for efficiency, while ACTA2 and CNN1 used SMC cDNA. The use of SMC cDNA was necessary since there was not a high enough expression of ACTA2 and CNN1 in the HUVEC cDNA to accurately dilute cDNA and observe a linear decrease in cycles to threshold.

2.2.2 RNA Isolation

Several commercial kits are available for RNA isolation. The RNeasy Mini Kit (QIAGEN) has previously been used to yield high purity RNA, so the use of this kit was continued for experiments with cells only (the kit cannot be used for RNA isolation from BVMs). The RNeasy kit uses spin columns to isolate up to 100 μ g of RNA longer than 200 bases. A high-salt buffer system binds RNA to silica membranes¹¹⁴. The samples were first snap-frozen in liquid nitrogen to preserve the RNA and disrupt the cell membranes. The samples were then lysed and homogenized before binding to the

RNeasy spin column, where multiple wash steps purify the RNases. The final RNA product was eluted from the membranes using water.

Once RNA was isolated, the purity and concentration of the RNA product was assessed. The NanoDrop 2000 Spectrophotometer (Thermo Scientific) was used to measure 2 μ L RNA samples. The spectrophotometer measures the absorbance of a solution and breaks the signal into functions of wavelength. Measurements on nucleic acid content include concentration, A260, A280, 260/280, and 260/230. The concentration is measured as ng/nL based on the absorbance at 260 nm; this measurement is important in order to normalize the amount of RNA added to each reverse transcription reaction. A260 and A280 are the absorbance at 260 and 280 nm, respectively. The 260/280 and 260/230 measurements are ratios of absorbance that indicate the purity of the RNA. A 260/280 ratio of approximately 2.0 is accepted as pure, and values above or below 2.0 indicate a contaminant near 280 nm. The 260/230 ratio is a secondary measurement of purity with an accepted ratio between 1.8 and 2.2¹¹⁵. A full protocol for RNA isolation using the RNeasy Mini Kit and purity assessment using the NanoDrop 2000 can be found in Appendix C.

2.2.3 Reverse Transcription

After verifying that the purity of isolated RNA was acceptable, cDNA was synthesized through reverse transcription. The iScript cDNA Synthesis Kit (Bio-Rad) contains only three components: 5x iScript Reaction Mix, iScript Reverse Transcriptase, nuclease-free water. The reaction mix contains a blend of oligo(dT) and random hexamer primers. The reverse transcriptase is RNase H⁺, which improves the amplification

sensitivity, and contains RNase inhibitors to prevent sample degradation^{18,116}. The RNase free water was used to dilute RNA concentrations to maintain constant concentrations across all samples. That way, the same amount of cDNA product will be created, and amplification during qPCR will not be a factor of cDNA concentration. The equation for calculating the volume of RNA to add per sample is shown in Equation 2 for 1000 ng of RNA per sample. The calculated volume of water was subtracted from 15 μ L to determine the volume of water needed for the reaction.

$$\text{Equation 2} \quad (1000 \text{ ng RNA}) * (1 \text{ mL} / [\text{RNA concentration}] \text{ ng/nL}) = [\text{Volume RNA}] \mu\text{L}$$

The 5x iScript Reaction Mix, reverse transcriptase, nuclease-free water, and RNA were added to a nuclease free tube. Each reaction contains a total of 20 μ L per well. After loading the samples in a thermocycler, the samples were heated and cooled for one cycle to produce the double-stranded cDNA. The samples were then diluted with 80 μ L of nuclease-free water and stored at -20 C. The full protocol for cDNA synthesis can be found in Appendix D.

2.2.4 Quantitative Polymerase Chain Reaction

Once ds cDNA was synthesized and diluted, qPCR was performed. SYBR Green dye was used for the reactions, in which the Fast SYBR Green Master Mix (Life Technologies) was used. This master mix contains SYBR Green dye, DNA polymerase, uracil-DNA glycosylase, ROX passive reference dye, and dNTPs¹¹⁷. In a 96-well plate, 4 μ L SYBR Green Master Mix, 3.2 μ L cDNA, 1 μ L primers, and 11.8 μ L of water was loaded in each well for a total reaction volume of 20 μ L. Triplicates of each sample were

run, as well as non-template controls (NTCs) for each primer. NTCs contain no cDNA (the volume of cDNA is replaced by water) and are included to monitor for contamination and primer-dimer formation, both of which could produce false positive results¹¹⁸.

Once loaded into the well plate, the plate was placed into the qPCR 7500 Fast System (Applied Biosystems). A standard run time was selected, to which the cycle settings can be found in Appendix E, and a dissociation cycle was added. After the cycles were complete, the Ct values were exported for analysis. Several assumptions must be verified for different qPCR analysis methods. The method selected for this thesis, relative abundance, assumes a primer efficiency with an efficiency of 2 and a stable reference gene were used. The assumption of primer efficiency was checked and verified as described in Section 2.2.1, and the assumption of using a stable reference gene was checked after each experiment. Relative abundance was measured by the $2^{-\Delta Ct}$ value, where ΔCt is calculated as shown by Equation 3.

$$\text{Equation 3} \quad \Delta Ct = Ct(GOI) - Ct(\text{reference gene})$$

2.2.5 Experiment 1 Pilot: Change in EC Expression with Increasing Passage Number

As previously established, the goal of Aim 1 was to determine how endothelial cell-gene expression changes in respect to passage number and flow conditions, with the understanding that increased time in culture and disturbed flow should contribute to EndMT. The effect of increasing time in culture was initially investigated. This preliminary pilot served to establish an understanding of how gene expression changed

during just a few passage numbers, as well as to check the stability of the housekeeping genes as passage number increased. RNA was isolated from HUVECs at passages 4, 6, 7, 8, 9, and 10, cDNA synthesized, and qPCR run. Relative abundance was calculated, and this data, along with the results of the dissociation cycle, were analyzed. Ct values for the housekeeping genes β ACT and GUS β were also examined in order to determine the viability of the genes as housekeepers.

2.2.6 Experiment 1: Change in EC Expression with Increasing Passage Number

Experiment 1 served as a more thorough investigation of the goals established by the Experiment 1 Pilot. The goal of the experiment was still to examine changes in gene expression as culture time increased as well as to add more passage number conditions for the HUVECs. This experiment also added SMCs at early and late passages to compare mesenchymal marker expression to endothelial expression of the same markers. RNA was isolated from HUVECs at passage 3, 5, 7, 9, 11, and 13, whole RNA was isolated from SMCs at passage 6 and 11.

2.2.7 Experiment 2: Changes in EC Expression from Disturbed Flow Conditions

Experiment 2 investigated the effect of disturbed flow on EC gene expression; disturbed flow should induce changes in gene expression towards a mesenchymal state. In order to mimic disturbed flow conditions, 6-well plates of HUVECs were secured to an orbital shaker in an incubator and allowed to run at 210 RPM for 24 hours. This technique was borrowed from Mahmoud et al., which found that the orbital shaker caused low shear stress (about 5 dyne/cm²) with variations in direction in the center of the 6-

well, and high shear stress (about 15 dyne/cm²) at the edge of the 6-well with uniform flow direction⁶⁹. However, since it was unknown to what extent these conditions would cause changes in gene expression towards a mesenchymal phenotype, a positive control was necessary. Therefore, HUVECs were cultured in SMC specific media for 48 hours, which will induce phenotypic changes in HUVECs towards a mesenchymal phenotype. This would serve as the positive control for which to compare the HUVECs exposed to disturbed flow. HUVECs cultured in static conditions were used as a no-treatment group. The static, disturbed flow, and SMC-media conditions included passage numbers 6, 10, and 14.

2.2.8 Experiment 3 Preparation: Development of RNA Isolation Protocol for BVMs

The previous experiments used only cells cultured in dishes. In order to investigate the changes in gene expression of cells in a BVM, the RNeasy Mini Kit for RNA isolation could not be used. Therefore, a new isolation protocol needed to be developed and verified for high purity and appropriate concentration RNA. One method to isolate RNA from BVMs was to homogenize the BVM, scaffold and cells, into a solution and then isolate the RNA. Trizol (Life Technologies) isolation is a common procedure in which tissue is homogenized in Trizol and then RNA is extracted from that solution. This method was pursued for RNA isolation from BVMs.

Many protocols for Trizol isolation are available, and most have only minor differences. A reliable protocol that had been used previously at an internship was employed for the isolation in this thesis, and can be found in Appendix C. The BVMs were frozen in liquid nitrogen directly after removal from bioreactors and stored at -80 C

until use. The BVMs were then cut into small pieces using scissors, and 5 mL of Trizol was added before homogenizing the BVM in the Trizol using a TissueRuptor (Qiagen). Chloroform was added to the homogenate, and the solution was centrifuged to separate a Trizol and aqueous phase. The aqueous phase, which contains the RNA, was removed and added to 99% IPA. GlycoBlue (Thermo Fisher Scientific) was also added to the solution before centrifugation. The supernatant was removed, leaving only a blue pellet containing RNA in the bottom of the centrifuge tube. The pellet was resuspended in 75% EtOH and stored at -80 C overnight. The following day the tube was centrifuged, and the supernatant removed. The pellet dried completely before resuspending in nuclease-free water. The RNA purity and concentration were checked as described in Section 2.2.2.

In order to compare the product of this isolation protocol against the RNA isolation from the RNeasy Mini Kit, both protocols were used for the isolation of RNA from HUVECs. The purity and concentrations were then compared to determine whether the Trizol isolation was comparable to the RNeasy isolation.

2.2.9 Experiment 3 Pilot: Changes in Gene Expression in BVMs

Having already investigated HUVECs in static culture and in disturbed flow, as well as having developed an RNA isolation protocol for use on BVMs, the proper criteria had been met to investigate gene expression changes in BVMs. This pilot experiment used only two BVMs that ran for 24 hours. A static condition and a disturbed flow condition were also used to compare all three flow types. The passage number was kept constant in this pilot to remove the variability of expression as cells remain in culture for

different lengths of time. The three flow condition groups (BVM, static, and disturbed flow) used HUVECs at passage number 6.

The disturbed flow condition with HUVECs in 6-well plates underwent a ramp-up period on the orbital shaker. This change was implemented as a result of a high percent of cell death in the disturbed flow groups in Experiment 2. The ramp-up period increased the orbital shaker from 50 RPM to 200 RPM at intervals of 50 RPM every 2 hours. The plates remained at 200 RPM for 24 hours.

2.3 Results

2.3.1 Primer Efficiency

Preverified primers should not need to be checked for efficiency; however, appropriate efficiencies are necessary to meet the assumptions for relative abundance calculations. A representative graph of primer efficiency is shown in Figure 16, and all efficiency plots can be found in Appendix G. Slope, R^2 , and efficiency percentage values for all GOI can be found in Table 2. Efficiency percentages ranged from 86.5% to 99.5%, with *GUS β* having the lowest efficiency. An efficiency range between 90% and 110% is adequate to meet the assumptions for relative abundance, of which *VEGFR2* and *GUS β* fell outside of this range at 86.5% and 88.6%, respectively. All R^2 values were above 0.97, in which this linearity indicates pipetting accuracy for the dilutions. The efficiency for *β ACT* was not calculated, as this gene had already been eliminated as a viable housekeeping gene at the time of efficiency testing. This decision will be discussed in Section 2.4.

Figure 16: Primer Efficiency Plot. Representative primer efficiency graph of the log of the cDNA dilution factor versus the average Ct value. The slope of the trendline is used for efficiency calculations.

Table 2: Primer Efficiency. Efficiency data from GOI, which includes the slope, R^2 , and efficiency percentage.

	Slope	R^2	Efficiency (%)
PECAM	-3.3333	0.9714	99.52761
VEGFR2	-3.6301	0.999	88.57083
VE-Cadherin	-3.4575	0.9988	94.63741
ACTA2	-3.3482	0.9943	98.91519
CNN1	-3.4528	0.9848	94.81393
GUSβ	-3.6939	0.9979	86.51621

2.3.2 Experiment 1 Pilot Results

This pilot served to investigate the effect of static culture time on gene expression in HUVECs, evaluate β ACT and GUS β as housekeeping genes, and accumulate data in order to better design Experiment 1. Figure 17A shows relative abundance of all GOI as

compared to β ACT. VE-cadherin had the highest abundance among the GOI, followed by VEGFR2. A slight trend can be seen for both VE-cad and VEGFR2, with increasing abundance as passage number increased. VEGFR2 followed this trend except at passage 6. VE-cad had a maximum abundance at passage 8, where abundance slightly decreased at passages 9 and 10. The high abundance of VE-cad made identifying trends in PECAM, ACTA2 and CNN1 expression difficult, so Figure 17B shows the same data as Figure 17A but excluded VE-cad and VEGFR2 data. Interestingly, PECAM and ACTA2 had similar abundance in HUVECs regardless of the passage number. Calponin had almost no expression.

Figure 17: Experiment 1 Pilot Relative Abundance Relative to β -Actin. (A) Relative abundance of all genes of interest using β -Actin (β ACT) as the housekeeping gene. (B) Platelet Endothelial Cell Adhesion Molecule (PECAM), Alpha Actin (ACTA2), and Calponin (CNN1) relative abundance using β ACT as the housekeeping gene.

Figure 18A shows relative abundance levels of all GOI compared to $GUS\beta$ as the housekeeping gene. Like the previous figure, Figure 18B shows the same data as Figure 18A but excludes VE-cad and VEGFR2. The trends of VE-cad and VEGFR2 relative to $GUS\beta$ followed closely with the trends compared to β ACT. However, the abundance

levels compared to $GUS\beta$ were about half that as when compared to βACT . PECAM and ACTA2 also had lower abundance levels when compared to $GUS\beta$ than βACT . No trend persisted for PECAM or ACTA2 as passage number increases. Furthermore, calponin had almost no expression.

Figure 18: Experiment 1 Pilot Relative Abundance Relative to β Galactosidase. (A) HUVEC relative abundance of all genes of interest using β Galactosidase ($GUS\beta$) as the housekeeping gene. (B) HUVEC relative abundance of PECAM, ACTA2, and CNN1 using $GUS\beta$ as the housekeeping gene.

It is important to check for the stable expression of housekeeping genes during every experiment to determine whether new treatment groups affect the genes viability as a housekeeper. Figure 19 shows plots of the average Ct for β ACT and GUS β at each passage number. The Ct average for β ACT was variable between groups, ranging from 20.87 and 24.10 cycles. GUS β displayed more stable Ct values, which range between 20.77 and 21.26 cycles.

Figure 19: Experiment 1 Pilot Housekeeping Gene Stability. Average Ct values of (A) β ACT and (B) GUS β from each treatment condition.

2.3.3 Experiment 1 Results

Based on the results of the previous pilot study, Experiment 1 increased the range of passage numbers in order to accentuate any trends that were previously identified and to reveal other trends that may exist in HUVECs in static culture. Experiment 1 also added an early and late passage of HUASMCs to compare the expression of mesenchymal markers between HUVECs and HUASMCs. Since β ACT Ct values were more variable than GUS β in the pilot, expression of the housekeeping genes were analyzed first before analyzing relative abundance data. Figure 20 displays how β ACT Ct values fluctuated between 16.83 and 25.46 cycles, while GUS β Ct values remained relatively stable between 17.44 and 20.37 cycles. As a result of the instability of β ACT

identified in the pilot and this experiment, β ACT was eliminated as a housekeeping gene. From this point forward, only $GUS\beta$ was used while running qPCR and during data analysis.

Figure 20: Experiment 1 Housekeeping Gene Stability. Average Ct values of (A) β ACT and (B) $GUS\beta$ from each treatment condition.

Figure 21A shows the relative abundance of all GOI in HUVECs of increasing passage number in static culture. VE-cad and VEGFR2 had a positive trend between relative abundance and passage number. Consistent with the results of the pilot study, these two EC markers were more highly expressed than PECAM. No trends were identified in expression of PECAM and ACTA2, as seen in Figure 21B. Calponin abundance was negligible in HUVECs.

Figure 21: Experiment 1 Relative Abundance. HUVEC relative abundance of (A) all GOI and (B) PECAM, ACTA2, and CNN1 using GUS β as the housekeeping gene.

Relative abundance of mesenchymal markers ACTA2 and calponin in early passage (passage 6) and late passage (passage 11) HUASMCs in static culture are shown in Figure 22. ACTA2 was highly expressed compared to calponin in HUASMCs. Expression of ACTA2 and calponin in HUVECs was almost negligible when compared to expression in HUASMCs.

Figure 22: Experiment 1 Pilot Relative Abundance of Mesenchymal Markers in SMCs. (A) HUASMCs relative abundance of ACTA2 and CNN1 using GUSB as the housekeeping gene. (B) HUASMC relative abundance of CNN1 using GUSB as the housekeeping gene.

SYBR Green dye will intercalate any double stranded DNA, including primer-dimers and contaminating DNA. Dissociation curve analysis will detect whether primer-dimers or contaminating DNA are present in the qPCR product, and in this way is a check of quality for a single product. A dissociation curve for a product will have a single peak at the same temperature for genes of the same type regardless of treatment. Figure 23 displays examples dissociation curves for each GOI. Dissociation curves were analyzed prior to the analysis of each data set to identify individual samples or entire groups that had abnormal dissociation curves.

Figure 23: Representative dissociation curves of all GOI. (A) β ACT, (B) GUS β , (C) VEGFR2, (D) VE-cad, (E) PECAM, (F) ACTA2, (G) calponin from ECs, (H) calponin from SMCs.

Dissociation curves appeared acceptable for all except one GOI. Figure 23G shows the dissociation curve from PCR products for calponin from ECs. These curves had several peaks and low derivative values, indicating poor quality of PCR product. However, this most likely occurred because not enough product could be produced since the abundance of calponin was so low in ECs. The dissociation curve for calponin from SMCs, shown in Figure 23H, showed a single peak. Therefore, the primer was still verified to be working properly and producing high quality PCR products. Figure 23A-F shows single peaks for all other GOI.

2.3.4 Experiment 2 Results

While some trends were identified between EC marker expression and passage number in Experiment 1, these results are not as meaningful unless compared against conditions that are known to induce EndMT in ECs. Two treatment groups were added in Experiment 2 in which to compare the non-treatment group of HUVECs cultured in static EC media: HUVECs cultured in disturbed flow, and HUVECs cultured in static SMC media. In each group three passage numbers were chosen to investigate the effect of culture time.

The relative abundance of all GOI are shown in Figure 24A. Unfortunately, due to high amounts of cell death in the disturbed flow treatment groups, a high enough concentration of RNA was only isolated from HUVECs at passage 14. The RNA concentrations at passages 6 and 10 were too low to continue those groups in the experiment.

VE-cad and VEGFR2 expression increased with passage number in the non-treatment group, while the expression of these two markers decreased with passage number in the SMC media treated group. VE-cad expression of the disturbed flow treatment was lower than that of any non-treatment groups but was higher than the passage 14 SMC media treated group. VEGFR2 expression showed a slight positive trend between culture time and relative abundance in the non-treatment group, and a slight negative trend in the SMC media treatment group. However, the SMC media treatment group had a higher relative abundance at all passages than the non-treatment passage 6 and 10. The disturbed flow treatment had a lower relative abundance of VEGFR2 than all other groups.

PECAM expression was slightly lower in the disturbed flow and SMC media-treated groups, but not statistically. There was no difference in ACTA2 expression between the non-treatment group and the disturbed flow group. ACTA2 expression trended towards increasing with passage time in the SMC media treated group. Calponin expression was negligible in all groups.

Figure 24: Experiment 2 Relative Abundance. HUVEC relative abundance of (A) all GOI and (B) PECAM, ACTA2, and CNN1 using GUS β as the housekeeping gene.

Since two treatment groups were included in Experiment 2, analysis of the stability of GUS β was important to determine whether the treatments affected the viability of GUS β as a housekeeper. Figure 25 shows that GUS β expression remained relatively constant through all treatments, ranging between 19.62 and 20.45 cycles.

Figure 25: Experiment 2 Housekeeping Gene Stability. Average Ct values of *GUS β* from each treatment condition.

2.3.5 Verification of RNA Isolation Protocol for BVMs

A new method had to be developed to be able to isolate RNA from BVMs. It was necessary to verify that this extraction protocol rendered equivalent RNA as the RNeasy Mini Kit. The resulting concentrations, 260/280, and 260/230 values from both isolation processes are shown in Table 3. The concentration of RNA from the Trizol extraction did not differ from the Mini Kit ($p > 0.05$). The 260/280 and 260/230 ratios did not differ between the isolation methods ($p > 0.05$), and the purity average of both methods was within the accepted range. Based on this, the Trizol extraction method was deemed acceptable for BVM RNA isolation.

Table 3: Comparison of RNA isolation from the RNeasy Mini Kit and Trizol extraction.

	RNeasy Mini Kit 1	RNeasy Mini Kit 2	RNeasy Mini Kit Average	Trizol Extraction 1	Trizol Extraction 2	Trizol Extraction Average
Concentration (ng/nL)	2682.9	2593.9	2638.4	2604.3	2655.7	2630.0
260/280	2.03	2.06	2.045	1.97	2.01	1.99
260/230	2.18	2.24	2.21	2.07	2.19	2.13

2.3.6 Experiment 3 Pilot Results

Experiments 1 and 2 worked to investigate gene expression of HUVECs in static and disturbed flow conditions. With those conditions established, Experiment 3 served to explore gene expression in the laminar flow conditions of BVMs in bioreactors. Before performing an entire study, a pilot was completed which included the three flow conditions but controlled for passage number. Gene expression was analyzed for passage 6 HUVECs in static culture, disturbed flow, and laminar flow. Since a high amount of cell death occurred in Experiment 2 for the disturbed flow condition, a ramp-up period was employed to reduce the shock of the flow on the cells. Figure 26 displays white light images of HUVECs after the ramp-up period and culture for 24 hours at 200 RPM. The well plate remained confluent at the center and edges, indicating that the ramp-up period reduced cell death. Cells at the center of the well were oriented randomly, while cells at the edge of the well were highly aligned and stretched in the direction of flow.

Figure 26: HUVEC Morphology in Turbulent Flow Condition. HUVECs in 6-well plates after 24 hours on a rotator plate at 200 RPM. (A) HUVECs in the center of the well are not aligned. (B) HUVECs on the edge of the well plate align in the direction of flow.

Since a new treatment condition was added to this experiment, Ct data had to be analyzed to determine whether the treatment affected the utility of $GUS\beta$ as a housekeeper. $GUS\beta$ Ct values are plotted for each treatment group in Figure 27, which makes clear that the Ct values varied based on treatment. The non-treated, static culture condition had a Ct average of 19.9, which was similar to the results seen in previous experiments. The Ct values for both BVMs differed, with vessel 8 and 9 having Ct values of 27.72 and 23.57, respectively. Surprisingly, the disturbed flow treatment had a Ct of 39.49, even though the Ct average for the same condition in Experiment 2 was 20.45.

Figure 27: Experiment 3 Housekeeping Gene Stability. Average Ct values of $GUS\beta$ from each treatment condition.

Upon consulting the dissociation curves, it was observed that all GOI in the turbulent flow condition, excluding PECAM, had multiple peaks. Therefore, these samples were excluded from the study. Only PECAM had a melt curve with a single peak, which can be seen by the peak on the right side of Figure 28. However, the abundance value was over 1700 for PECAM, making it an obvious outlier. Therefore, this sample was also excluded from the study, so the entire disturbed flow condition was not included in the results. In doing so, the Ct values of $GUS\beta$ were more stable. An average between $GUS\beta$ values for the static condition and vessels was used for normalizing the data.

Figure 28: Disturbed Flow Dissociation Curves. Dissociation curve of all GOI for the disturbed flow condition in Experiment 3.

The static condition had higher expression of both VEGFR2, VE-cad, and PECAM than the two BVMs, as shown in Figure 29. The BVMs had comparable expression of VEGFR2, PECAM, and ACTA2. Only VE-cad was expressed higher in Vessel B than Vessel A. ACTA2 had higher expression in the static condition than either of the BVMs.

Figure 29: Experiment 3 Relative Abundance. HUVEC relative abundance of all GOI using $GUS\beta$ as the housekeeping gene. (A) all GOI and (B) PECAM, ACTA2, and CNN1.

Based on the results of this pilot, a full Experiment 3 was not explored. This study would have included more BVM replicates and multiple cell passage numbers. However, these three experiments did not indicate EndMT with the ECs during static culture or during culture in BVMs, which will be discussed in detail in the following section.

2.4 Discussion and Conclusion

2.4.1 Experiment 1 Pilot and Experiment 1

The purpose of Aim 1 was to investigate and characterize the gene expression changes experienced by HUVECs *in vitro* with respect to increasing culture time and changing flow conditions during culture. The effect of cell culture time on HUVEC gene expression was established first through analyzing three endothelial markers and two mesenchymal markers. The primers for these markers were verified by calculating the efficiency of each primer assay. PECAM, VE-cad, ACTA2, and calponin had efficiency values between 90 and 100%. Even though VEGFR2 and GUS β had efficiency values of 88.57 and 86.5, respectively, it was decided that these efficiency values were still acceptable, especially considering the primers were already preverified. Therefore, all six primers were concluded to be acceptable for use in qPCR experiments.

The pilot for Experiment 1 used HUVECs at passages 4, 6, 7, 8, 9, and 10 to study the effect of culture time on gene expression. The qPCR results were then analyzed using one of two housekeeping genes, β ACT or GUS β . The use of either housekeeper in analyzing relative abundance revealed similar trends. VE-cad had the highest abundance, followed by VEGFR2. Passage 4 HUVECs had significantly lower expression of VE-cad than passages 6 through 10 cells. Passages 6 and 7 had significantly lower expression than passages 8 through 10, creating a slight positive correlation between passage number and relative abundance. A slight positive correlation was also shown between passage number and VEGFR2 abundance. This trend is unexpected, as literature suggests that

expression of endothelial markers like VE-cad and VEGFR2 decreases as passage number increases. However, the opposite trend was seen with this data.

The third EC marker, PECAM, did not have any expression differences between passage numbers when compared to β ACT or GUS β . Like with the other EC markers, it would be expected that PECAM abundance would decrease with increased culture time. PECAM abundance was also much lower than the other EC markers, and had an abundance closer to ACTA2. This observation was interesting, especially considering the expression of ACTA2 in HUVECs seen by immunostaining. The mesenchymal markers, ACTA2 and calponin, had no correlation between passage time and abundance. While literature suggests that these genes should increase with culture time, this was not observed.

Another important consideration of this pilot was the viability of β ACT and GUS β as housekeepers. The Ct data for β ACT showed variability between passage time, making the gene non-ideal as a housekeeper. GUS β expression, on the other hand, was stable over culture time. With this in mind, β ACT was still included as a housekeeper in Experiment 1 to determine whether the inconsistent stability would be observed again.

Based on the results of the pilot, it was decided to expand the passage numbers used for Experiment 1; the culture times of the pilot may have not included early and late enough passages to elucidate all trends in gene expression. Therefore, passage times were increased to include passages 3, 5, 7, 9, and 11. The results of the Ct values for β ACT and GUS β in Experiment 1 confirmed the results seen in the pilot. β ACT was not stably expressed, while GUS β expression remained relatively constant. Therefore, β ACT was

eliminated as a housekeeping gene, and GUS β was used for data analysis in Experiment 1 and in all other experiments.

Experiment 1 result trends resembled those of the pilot. The later passages (9, 11, 13) had higher expression of VE-cad than the early passages (3, 5, 7). VEGFR2 expression increased with increased culture time. PECAM, ACTA2, and calponin expression did not change with increasing culture time. These data do not support the trends identified in literature, where HUVECs decrease expression of EC markers and increase expression of mesenchymal markers as EndMT occurs. In fact, this data suggests that the HUVECs are sustaining, and even forming, stronger cell-cell interactions as culture time increases. Two of the three mechanosensory complex markers, VE-cad and VEGFR2, increased abundance between early and late passages. The stability of PECAM expression with respect to culture time also supports the idea that culture time does not result in dedifferentiation.

The relationships seen in these data are difficult to explain. PECAM stability over culture time can be attributed to its function as a transmitter of mechanical force. Shear stress phosphorylates and activates PECAM; in the static culture condition, no shear forces was present to activate PECAM. PECAM RNA transcription should change as a result of mechanical force stimulation, so static culture should not increase PECAM expression. While this explanation seems plausible for PECAM, it contradicts VE-cad and VEGFR2 expression data. PECAM transmits mechanical force to VE-cad, which responds by modulating VEGFR2. Since no mechanical force is being transmitted by PECAM, VE-cad expression would be expected to remain constant. If VE-cad expression

was constant, there should be no modulation of VEGFR2. However, both VE-cad and VEGFR2 were upregulated despite the lack of mechanical force.

One explanation for increased VE-cad expression could be the confluency of HUVECs used in the study. HUVECs were cultured to between 90 and 100% confluency, meaning high amounts of cell-cell interactions and establishment of contact integrity by VE-cad. However, by this explanation VEGFR2 expression would decrease, which was not the observed response. A simpler explanation is perhaps the result of a response initiated by VEGFR2 and not PECAM or VE-cad. HUVEC media contains VEGF, which binds VEGFR2 and causes downstream increases in VE-cad transcription. This explains the increase in expression over culture time, since an increase in culture time means more time spent in media containing VEGF. This pathway is also independent of PECAM, explaining the stability of PECAM expression over culture time. Regardless of any of these explanations, this endothelial marker data did not indicate any dedifferentiation occurring within the ECs.

The stability of the mesenchymal markers adds to the conclusion that culture time does not contribute to dedifferentiation, or at least suggests that a mesenchymal genetic change does not occur in the culture period tested here. Furthermore, the expression of mesenchymal markers in HUVECs was almost negligible when compared to the same markers in HUASMCs. It would not be expected that HUVECs would express those markers at an abundance even close to that of HUASMCs. However, one study showed that mesenchymal-progenitor cells produced between 2 and 9 times less ACTA2 and between 5 and 50 times less calponin than SMC¹¹⁹. EndMT would result in higher ACTA2 and calponin abundance than those found in this experiment.

This experiment found that static culture time was a variable contributing to changes in gene expression. However, dedifferentiation towards a mesenchymal state was not observed over culture time. Since culture duration influences gene expression, several passage numbers were included in experiments which tested multiple flow conditions.

2.4.2 Experiment 2

With the effect of static culture time determined by Experiment 1, the second part of Aim 1 could be pursued: to investigate the effect of flow conditions on HUVEC gene expression. Experiment 2 served to establish a disturbed flow condition; this disturbed flow, as well as the static culture, was used for comparing the effect of laminar flow on HUVECs in the BVMs. The SMC media treatment group served as a positive control for gene expression change towards a mesenchymal state. $GUS\beta$ expression remained stable despite the addition of two new treatments, meaning that $GUS\beta$ retained its viability as a housekeeper.

The non-treatment, or static culture in HUVEC media, group resulted in similar trends as seen in Experiment 1. Gene expression in the SMC media treatment group trended in a direction that aligns with a mesenchymal shift; VE-cad and VEGFR2 expression decreased as passage number increased, and ACTA2 expression had an increasing trend with passage number. While PECAM expression was stable with passage number for the SMC media treatment group, expression was lower than that of the non-treatment group. These results confirmed that the treatment of HUVECs with SMC media served as a positive control for EndMT. It is important to note that SMC media treatment length was not a variable, as all passages of HUVECs were treated with

SMC media for the same length of time; therefore, any trends in gene expression can be attributed to the culture duration variable.

Since a high enough concentration of RNA was only isolated from the disturbed flow treatment at passage 14, passage number did not serve as a variable in this case. However, the data collected at the singular passage number was still interesting. The disturbed flow HUVECs had significantly lower expression of VE-cad and VEGFR2 than all non-treatment group passages. The SMC media treated passage 10 HUVECs had similar expression to the passage 14 disturbed flow cells. Passage 6 SMC-media treated cells had higher expression of VE-cad than the disturbed flow cells, while the passage 14 cells had lower expression. The disturbed flow condition had lower expression of VEGFR2 compared to all SMC media treated passages. PECAM expression was not different between disturbed flow and SMC media treatments. Surprisingly, ACTA2 expression of the disturbed flow condition resembled non-treatment expression, which was lower than that of SMC media treatment. Taken together, these comparisons indicated that the SMC media-treated group succeeded as a positive control for EndMT while the disturbed flow condition achieved some loss in endothelial markers and some gain of mesenchymal markers.

PECAM expression in the disturbed flow group was not different than the non-treatment group, so the effects of disturbed flow on VE-cad and VEGFR2 expression worked independent of PECAM expression. It is not necessarily surprising that PECAM expression remained constant regardless of treatment; changes in PECAM expression are more heavily connected to cell-cell contact than to shear stress. Balaguru et al. found that PECAM expression remained stable between static and disturbed flow conditions¹²⁰.

Changes in VE-cad and VEGFR2 expression are more correlated with flow changes, which was observed by the results of this experiment. The downregulation of VE-cad and VEGFR2 compared to the non-treatment group demonstrated the utility of the disturbed flow condition in being an inducer of EndMT.

While EC marker expression indicated induction of EndMT by the disturbed flow protocol, it did not align with the expression of mesenchymal markers. The trends that would be expected from EndMT induction were observed by the positive control treatment of SMC media, supporting the treatment's use as a positive control for EndMT. This treatment also aligned well with the result of the disturbed flow condition, except with regards to ACTA2. This could simply be a limitation of the time point at which HUVECs were exposed to disturbed flow. Despite this, Experiment 2 succeeded in validating the method of inducing disturbed flow and establishing gene expression changes of a single passage of HUVECs exposed to this flow.

2.4.3 Experiment 3 Pilot

Experiment 3 was intended to combine previously investigated flow conditions with BVMs to provide an encompassing set of data on how gene expression changed between static culture, disturbed flow, and laminar flow as culture time increased. The pilot for this study eliminated the factor of culture time, and only investigated flow conditions at a single passage of HUVECs.

As with previous experiments, the stability of the reference gene $GUS\beta$ had to be determined. Upon plotting the Ct values, it appeared that $GUS\beta$ was not stable with the addition of new flow conditions. The two BVMs had Ct averages of 27.72 and 23.56

cycles, which was higher than the Ct average of the static culture at 19.9 cycles. While the static culture maintained a Ct value similar to previous experiments, the disturbed flow condition did not. Experiment 2 resulted in a Ct average of 20.45 cycles, while this pilot resulted in a Ct value of 39.49 cycles. This discrepancy between Ct values for the disturbed flow condition between the two experiments was alarming. It indicated that the gene was barely expressed at all and took almost all the cycles for a signal to be detected. Since this was not observed in Experiment 2 for the same treatment condition, other factors were looked at to explain this. Only one of the three wells ran with this treatment resulted in a signal, indicating a potential issue with either the RNA quality or cDNA concentration. The disturbed flow RNA had 260/280 and 260/230 ratios of 2.01 and 2.18, respectively. These values were within the accepted range for RNA purity, so the dissociation curves were analyzed to determine whether the reaction products caused an issue.

It was found that all GOI besides PECAM had multiple peaks in the disturbed flow condition, and therefore were omitted from analysis in this experiment only. Even though PECAM had a single peak in its dissociation curve, it was still omitted as an outlier because of the extremity of the resulting abundance value. There was most likely an issue with this individual PECAM sample, and the disturbed flow condition itself did not cause the high Ct value observed here. However, the exact cause of this was unclear. More repetitions of the flow condition are required to identify the source of this issue, or whether this issue will persist. The issues found by the dissociation curve with all other GOI in this group was also surprising, since the same issues were not found in a similar treatment group in Experiment 2.

The BVM RNA values were also concerning. The 260/280 ratios were 1.63 and 1.81 and the 260/230 ratios were 0.35 and 0.60. While the 260/280 ratios were only slightly below an ideal ratio, the 260/230 ratios were excessively low. A cause for both low 260/280 and 260/230 ratios is contaminating phenol from the isolation protocol¹²¹. Phenol is a main component of Trizol, and if the supernatant is not carefully separated during the isolation phenol could remain in the final product. This explanation is plausible, but unlikely considering the RNA purity of the static and disturbed flow samples were isolated using the same protocol and resulted in appropriate purity ratios. The BVMs, unlike the other two conditions, had scaffolds that were homogenized. It is unknown how the scaffolds affect the purity of the isolated RNA but could potentially cause contamination. The dissociation curve for each gene had a singular peak, shown in Figure 30, so the low RNA purity ratios may have not affected the quality of the PCR products.

Figure 30: Dissociation Curves for GOI for EC-only BVMs.

The results of this experiment were not expected. Shear stress has been shown to increase VE-cad and VEGFR2 expression in ECs compared to ECs in static culture^{92,122}. The opposite was found in this experiment; VE-cad and VEGFR2 expression decreased relative to static culture. Two theories persist to explain these results. First, the amount of shear stress in the BVMs may be too low to induce EC activation. The shear stress in BVMs is approximately 0.35 dyne/cm², which is significantly lower than shear stresses used in literature that showed upregulation of VE-cad and VEGFR2 which were between 10 and 20 dyne/cm².

A more likely explanation is that the 24-hour time point used for the BVMs was too short a duration to observe changes in EC expression. A study using a similar shear stress to the one in BVMs found that VE-cad was significantly downregulated 24 hours after flow induction¹²³. VE-cad interactions must be broken to adapt to changes in stimulus, such as during the induction of flow. The studies that found an increase in VE-cad and VEGFR2 expression used between 3 and 7 days of flow exposure. This explanation also gains merit from the expression of PECAM in this study. The previous experiments in this aim saw relatively stable expression of PECAM. However, this study saw lower expression of PECAM in the BVMs than in the static culture. This indicates that the cell-cell contact, which is necessary for PECAM expression, may have not been reestablished at this time point.

While it is disappointing that the disturbed flow condition was emitted, it may not have contributed to an explanation of these findings because of the short duration. Expression levels of mesenchymal markers were also not meaningful if the time point

was too short. ACTA2 abundance was higher in static culture than in BVMs. It would be interesting to see if this trend persists at longer time point BVM, such as at 3 or 7 days.

The data collected by the pilot stemmed the decision to not pursue a full study. The experiments did not indicate EndMT occurring during culture or within the two BVMs of this pilot. The exploration of EndMT was the main topic of interest, and while the results gathered by these experiments were useful, they did not support any dedifferentiation occurring.

2.4.4 Aim 1: Limitations and Future Work

Several limitations became clear from these experiments. The stability of GUS β in BVMs was a limitation, but this was worked around by averaging GUS β Ct values in experiment 3. Another limitation was the quality of RNA from the BVMs. This could have been caused by the isolation protocol, but more likely was a result of introducing scaffolds in the RNA isolation. How, and why this would occur is unclear. However, the greatest limitation was not completing a full Experiment 3 study. Even if passage number was kept constant, a full experiment that included many replicates of vessels would have helped discern the consistency from vessel to vessel, both regarding genes of interest variability and housekeeping gene stability. It is disappointing to have established the methods and completed the prerequisite work for a full experiment to compare the three flow conditions, only to not complete the full study; however, this decision was logical. Since the results previously discussed did not indicate a shift towards a mesenchymal phenotype, it did not seem productive to continue this exploration.

This area of the project still has areas that can be explored and improved. A full experiment could be conducted to compare the three flow conditions established here, not as a means to determine dedifferentiation but to better understand how the laminar flow in our model affects the cell-cell interactions. This experiment could be set up to study the effect of increasing RPMs on our model; increasing the flow rate would improve the physiological relevance of the model by increasing the shear stress. Shear stress would not be able to reach the conditions of the native vessel without changes to the viscosity of the media, but the methods explored here could be utilized for this exploration³⁷. A different experiment could also include BVMs at different timepoints to verify if trends from longer timepoints agree with those from literature. A disturbed flow condition would be beneficial if that study was to be performed. Hui et al. found that VE-cad distribution was intermittent after exposure to disturbed flow, whereas a continuous distribution of VE-cad was observed after exposure to laminar flow¹²⁴. This study could compare the effects of disturbed flow to laminar flow since the extent of mechanosensory complex reestablishment differs depending on flow type.

The methods used in these experiments could also be improved in the future. By using 6-well plates, two flow conditions are present within that flow treatment. The edges of the well plate are exposed to disturbed flow at a higher shear than the center. Well plates shaped like a ring are available, which eliminates the center of the well plate so the shear on all cells would be similar. More time points could be explored for the disturbed flow condition to observe how expression changes after multiple days of exposure to disturbed flow. This could be used to compare against 3, 7 or 14-day BVMs.

These methods can also be used to generate data in support of other projects. One possible application is in the exploration of novel scaffolds to understand how the cell-cell interaction changes between scaffold types. A scaffold that retains cells better within the lumen should have higher expression of the three mechanosensory complex markers used in the previous experiments. Specifically, this scaffold material would result in EC-only BVMs with elevated expression of PECAM, VE-cad, and VEGFR2 because more cell-cell junctions exist. However, for the current thesis, five markers were used to assess EndMT for increasing cell passages and for static, disturbed, and laminar flow conditions through changes in gene expression.

CHAPTER 3. AIM 2: CHARACTERIZATION OF NOVEL SCAFFOLDS THROUGH HUVEC CASPASE ACTIVITY AND SCAFFOLD COVERAGE

3.1 Introduction

After evaluating gene expression changes in Aim 1, Aim 2 sought to establish methods to better understand how cells and scaffolds interact in the BVM model. Methods for quantifying cytotoxicity, short-term cell adhesion, and long-term cell coverage were investigated. These methods were applied by comparing the current scaffolds with novel, nanofiber scaffolds. The following sections will overview how cells interact with and respond to scaffolds in tissue engineering applications. It will also introduce the novelty of adding BTEAC salt to scaffolds for nanofiber production.

3.1.1 Scaffold Effects on Cells

Tissue engineers attempt to create biomaterial scaffolds that resemble the native ECM of the tissue that is being mimicked. Electrospinning is a useful technique for creating scaffolds because scaffolds can be customized during fabrication by adjusting a variety of parameters, like voltage, percent polymer, polymer solution flow rate, and collector speed. Regardless of the fabrication process and material used, there are some general trends between scaffold characteristics and cell interactions. These scaffold characteristics include fiber diameter, fiber alignment, porosity, stiffness, topography, and scaffold degradation. Understanding the possible effects of these characteristics on cells is important when fabricating a scaffold using new solvents or additives.

3.1.1.1 Fiber Diameter

Figure 31 demonstrates how fiber size affects cell attachment. Microfiber scaffolds encourage cell attachment to single fibers, which does not resemble a normal cellular interaction with native ECM. Nanofiber scaffolds encourage cells to form attachments with multiple fibers, which is closer to resembling native ECM where cells have many attachment sites¹²⁵. As a result, fiber size effects cell morphology. This was demonstrated by Hodgkinson et al., where they found that primary human dermal fibroblasts became more elongated on microfiber scaffolds whereas cells on nanofiber scaffolds had a spread morphology¹²⁶. These smaller fibers enhance cell attachment through increasing the available attachment sites, and the likelihood of these attachment forming is increased since the specific surface area of small fiber scaffolds is larger than that of scaffolds with larger fiber sizes. A higher surface area is also useful during the conditioning processes, as smaller fibers have increased protein adsorption¹²⁷. As a result of increased protein adsorption and cell attachment, it is unsurprising that a decreased fiber diameter is associated with increased cell proliferation and viability^{126–128}.

Figure 31: Cell Interactions with Fibers of Varying Sizes ¹²⁵.

3.1.1.2 Fiber Alignment

Fiber orientation can be controlled during electrospinning but depends on the collector type and electrospinning parameters. The application of the scaffold dictates the benefits of aligned, semi-aligned, or random fibers. The Tissue Engineering Lab's PLGA scaffolds are fabricated such that the fiber orientation is random. However, it has been found that HUVECs adhere better to aligned fibers. Whited et al. found that aligned fibers led to increased HUVEC attachment to collagen-I/PCL scaffolds. Cells were seeded onto aligned, semi-aligned, or random fiber orientations and exposed to 20 dyne/cm² of shear stress. The aligned fiber scaffold had 95% cell adherence, while the semi-aligned and random fiber scaffolds had 80% and 60% adherence¹²⁹. The aligned fibers in this study were oriented in the direction of flow, which is not possible to fabricate using the current electrospinning system in the Tissue Engineering Lab. Aligned fibers on a cylindrical mandrel would be perpendicular to flow, and it is unclear how this would affect adhesion.

3.1.1.3 Porosity

The electrospinning process results in a porous scaffolds, and random fibers have a higher porosity than aligned fibers¹²⁷. Porosity mediates cell infiltration into scaffolds; if pores are too small cells cannot migrate into the scaffold, but if pores are too large the specific surface area is decreased and limits the area for cell attachment^{130,131}. The migration or infiltration of cells in the scaffold is also dependent on the model or tissue being mimicked. For BVMs, a confluent monolayer of HUVECs should line the lumen, so cell infiltration would ideally be avoided. The fiber packing density is inversely

correlated with porosity, where high fiber packing density results in low porosity¹³². Therefore, BVMs should have a high fiber packing density that encourages cells to proliferate along the lumen wall. For co-cultures of ECs and SMCs, like in the case of standard BVMs, it was found that pore diameters of 0.02, 0.4, 0.6, and 0.8 μm were optimal, whereas pore sizes greater than 2 μm resulted in SMC migration into the scaffold¹³³.

Pore sizes can be divided into three categories: nano ($>100\text{ nm}$), micro (100 nm - 100 μm), macro (100 μm - millimeters)¹³⁴. *In vivo*, macropores are useful for cell migration and vascularization, while micropores are effective for nutrient, oxygen, and chemical delivery¹³⁵. Higher porosity in scaffolds is associated with greater cell deposition and adhesion. Murphy et al. used collagen-glycosaminoglycan scaffolds seeded with osteoblasts and found that the largest pore size resulted in the highest cell count at all time points. It was assumed that this was a result of cell migratory capacity, where at late time points small pores inhibited migration and proliferation¹³⁰. The higher cell number could have also been a result of better cell adhesion due to increased protein deposition on the scaffolds, since scaffolds with higher porosity have been found to have higher protein adsorption than those with smaller pores¹³⁵.

3.1.1.4 Stiffness

ECM stiffness can fluctuate drastically across tissue *in vivo*, as cells respond to the stiffness through changes in actomyosin interaction with the ECM¹³⁶. A similar response with adhesion proteins occurs *in vitro*¹³⁷. Stiffness is measured in terms of elastic modulus, where a stiff material has a high elastic modulus. Cell adhesion,

spreading, proliferation, and even cell lineage is affected by scaffold elasticity, but how those characteristics are affected depend on the cell type^{132,138}. For HUVECs, it was found that cells spread better and had a higher metabolic activity on stiffer surfaces^{137,139}. Stiffness also effects the shear level necessary to affect morphology. Stiffer surfaces required less shear stress to align in bovine aorta endothelial cells (BAECs) than soft surfaces. These stiffer surfaces also showed less of an inflammatory response¹⁴⁰.

3.1.1.5 Topography

The surface roughness of the PLGA scaffolds used in BVMS should be low upon removal from mandrels but may increase during ethanol sterilization and conditioning. Surface roughness can be divided into macroroughness (100 μm – millimeters), microroughness (100 nm – 100 μm), and nanoroughness (less than 100 nm)¹³². Increasing surface microroughness is associated with an increase in cell adhesion and proliferation. Osteoblasts attached and proliferated most effectively on a surface with a roughness of 0.81 μm than a smooth surface¹³⁴. HUVECs showed increased cell adhesion and growth with increasing microroughness between 10 nm and 102 nm¹³². This trend could be a result of increased attachment sites on more rough surfaces.

3.1.1.6 Scaffold Degradation

PLGA degrades faster than either of its components, hydrophilic PGA and hydrophobic PLA, so PLGA composition percentages heavily effects degradation through changes in hydrophobicity. PLGA degradation occurs mainly as a result of ester

bond hydrolysis, which forms acidic monomers than can reduce pH¹⁴¹. Degradation of PLGA in BVMs is reduced using 75:25 PLGA, while acidic byproducts are distributed throughout the system by dynamic flow so local pH around the scaffold does not increase. Despite this, scaffold degradation can have unintended byproducts that affect cell health and result in apoptosis.

3.1.2 Measurement of Cell Responses

Scaffold characteristics have diverse effects on cellular response, including apoptosis and adhesion. These are two of the most important cellular responses in relation to the BVM model and must be evaluated when proposing the use of a new scaffold. Assessment of apoptosis and adhesion will provide evidence for the benefits, or detriments, of introducing scaffold changes.

3.1.2.1 Apoptosis

Apoptosis is the mechanism by which cells are killed and removed by the body in a controlled procedure that includes membrane dissociation, cell shrinkage, condensation of chromatin, DNA fragmentation, engulfment, and removal¹⁴². *In vivo*, physiologic apoptosis occurs in natural and controlled processes, and is often associated with developmental biology where overproduction of cells is necessary to develop the nervous and immune systems. Pathologic apoptosis results from abnormal and uncontrolled levels of cell death, which is associated with cancer, AIDS, and Parkinson's disease¹⁴³.

Apoptosis also occurs *in vitro*, except for events mediated by lymphocytes such as engulfment and removal. The inability to remove dead cells enhances cell apoptosis if

cell debris is not cleared¹⁴⁴. In the case of BVMs, apoptosis can occur through several factors. Scaffold degradation and improper CO₂ conditions can lead to increased pH and apoptosis. Cell over-confluence or reduced cell adhesion also increases apoptosis. Therefore, induction of apoptosis must be understood.

3.1.2.1.1 Apoptosis Pathways

Apoptosis can be induced by either the extrinsic, intrinsic, or perforin/granzyme pathway, which converge on the execution pathway to end the cascade¹⁴³. The extrinsic pathway initiates through tumor necrosis factor (TNF) transmembrane receptor-mediated interactions. These receptors transmit the signals through the membrane to initiate further intracellular signals. One common death ligand and receptor is Fas. The Fas ligand binds its receptor on the surface of the cells, which recruits Fas-associated protein with death domain (FADD) intracellularly. FADD associates and activates procaspase-8, activating caspase-8 and the execution pathway^{145,146}.

The intrinsic pathway is stimulated by positive or negative intracellular signals that cause changes to the mitochondrial membrane. Negative signals include the lack of growth factors, hormones, or cytokines. Positive signals include radiation, toxins, hypoxia, free radicals, and viral infections. These signals result in the opening of the mitochondrial permeability transition (MPT) pore that releases pro-apoptotic proteins, such as the intermembrane mitochondrial protein cytochrome C. Cytochrome C binds Apaf-1 and procaspase-9 upon being released into the cytosol, forming an apoptosome. Apaf-1 contains a caspase recruitment domain, and the clustering of procaspase-9 to cytochrome C and apaf-1 results in caspase-9 activation^{145,146}.

The perforin/granzyme pathway is activated by cytotoxic T lymphocytes (CTLs), which mainly targets tumor and virus-infected cells. CTLs secrete perforin, a pore-forming transmembrane molecule, and then secrete cytoplasmic granules into the cell. Granzyme A and B are included in these granules; granzyme A activates DNA nicking, while granzyme B activates procaspase-10^{145,146}.

Caspase 8 in the extrinsic pathway, caspase 9 in the intrinsic pathway, and caspase 10 in the perforin/granzyme pathway all activate caspase-3, the initiator of the execution pathway. While caspase-3 is the initiator, caspase-6 and caspase 7 are also considered execution caspases. The three caspases activate cytoplasmic endonuclease to degrade nuclear material and proteases to degrade cytoskeletal proteins, ultimately resulting in cell death^{145,146}.

Figure 32: The Apoptosis Pathway. The three arms of the apoptosis pathways, converging on the execution pathway and cell death¹⁴³.

3.1.2.1.2 Caspases

Caspases, which stands for cysteine aspartases, are heavily involved in the apoptosis cascade¹⁴⁷. There are at least 14 identified caspases, with 10 being major caspases. These include initiators (caspases 2, 8, 9, 10), proinflammatory cytokine activators (caspases 1, 4, 5), and executioners (caspases 3, 6, 7)¹⁴⁸. Caspases remain in an inactive proenzyme form until activation, referred to as procaspases¹⁴³. Once active, caspases have two main functions: to breakdown proteins into smaller peptides and to activate other caspases to amplify the apoptotic signal. The proteins targeted include mediators and regulators of apoptosis, structural proteins, cellular DNA repair proteins, and cell cycle-related proteins¹⁴⁵. Since caspases are almost always associated with apoptosis, measurement of caspase activity can be used to assess apoptosis positive cells¹⁴⁷.

3.1.2.1.3 Apoptosis Detection and Quantification

Methods exist to detect apoptosis *in vitro*, mostly through staining or collecting absorbance values. Toluidine and methylene blue are dyes that reveal intensely stained apoptotic cells. This method preserves the tissue or cells well but can underestimate apoptosis because it lacks the ability to detect smaller apoptotic proteins. Annexin V is a protein that binds phosphatidylserine present only on cell membrane surfaces of cells undergoing apoptosis. Annexins are proteins that bind phospholipids in the presence of calcium, which occurs on phosphatidylserine¹⁴⁹. Since mitochondrial membrane potential changes occur early in apoptosis, cationic fluorescent dyes can be used to identify apoptotic cells. Healthy cells accumulate the dye in the mitochondria, while the stain remains in the cytosol of apoptotic cells¹⁵⁰.

Methods to label fragmented DNA are also used to detect apoptosis. DNA laddering through gel electrophoresis can be used in cases where high numbers of apoptotic cells are present. Since DNA is cut in relatively consistent sizes during apoptosis, regularly spaced bands can be identified on agarose gel. Terminal dUTP Nick End-Labeling (TUNEL) assays label DNA fragments with fluorescent nucleotides and use fluorescent intensity to identify apoptotic activity. However, this assay is known to falsely identify cells undergoing DNA repair or are active in gene transcription¹⁵¹.

As mentioned previously, caspase activity can be used to detect and quantify apoptosis, which can be achieved through fluorescent microscopy or measuring absorbance. One common caspase 3 assay, which indicates the initiation of the execution pathway, requires tissue homogenization to expose the active caspases¹⁵¹. Immunostaining kits are also available which target the execution pathway, staining caspase 3 and 7, while others more broadly stain for caspase activity and label caspases 1 through 10¹⁵².

3.1.2.2 Cell Adhesion

3.1.2.2.1 Passive In Vitro Cell Adhesion

If endothelial cells are unable to form interactions with the surrounding scaffold within 24 to 48 hours after seeding, they will undergo apoptosis¹⁵³. The adhesion process that occurs in culture flasks, petri dishes, and scaffolds of BVMs is known as static cell adhesion. This adhesion process differs greatly from the events that occur *in vivo*, where endothelium adhesion processes occur in dynamic conditions defined by tethering, rolling, adhesion, and transmigration¹⁵⁴. *In vitro* adhesion can be divided into non-

receptor and receptor-mediated binding. Non-receptor-mediated cell adhesion includes weak chemical bonds between cell membranes and substrates, such as electrostatic, polar, or ionic interactions. Receptor-mediated adhesion creates far stronger and specific interactions, in which interactions occur between matrix molecules and cell adhesion proteins¹⁵³.

Static adhesion has three main phases, shown in Figure 33: cell attachment, flattening and spreading, and reorganization of the cytoskeleton with the development of focal adhesion complexes (FAC). Integrin interactions with the substrate are responsible for the initial cell attachment, and the strength of the interaction increases over time as more receptor-ligand bonds are formed¹⁵⁴. As more interactions form, cell spreading begins where the cell body decreases in height and increases contact area with the substrate. During spreading the actin cytoskeleton of the cell begins to reorganize. With this reorganization comes the formation of FAC, which connect integrins and other adhesion molecules to the cytoskeleton¹⁵⁵.

Cell Adhesion Phases	Phase I	Phase II	Phase III
Schematic diagram of cell adhesion
Schematic diagram of the transformation of cell shape
Cell adhesion intervention	Electrostatic interaction	Integrin bonding	Focal adhesion
Adhesion stages	Sedimentation	Cell attachment	Cell spreading and stable adhesion

Figure 33: Cell Adhesion Phases. The progression of cell adhesion in vitro over three phases¹⁵⁴.

Fully developed focal adhesions are formed from over 150 proteins and researchers are still discovering exactly how these interactions develop; however, two kinases in particular are well researched and essential for FAC development¹⁵⁶. Focal adhesion kinase (FAK) interacts with β 1 integrin cytoplasmic tails, as well as cytoskeletal proteins like paxillin. FAK is important for phosphorylation and assembly of focal adhesion components, where it functions mainly as a scaffold for other proteins to interact with. This function was determined through studies of kinase-dead FAK, where it was found that most of FAK's function was retained¹⁵⁵.

Another essential kinase is Src, which is activated through its binding to FAK. While Src binds directly to β integrins, the complex formed by FAK and Src plays important roles in cell spreading by relieving cytoskeletal tension during spreading and inhibiting cytoskeletal contractility¹⁵⁶. These events are important for creation of stable focal adhesions and strong cell adhesion interactions.

3.1.2.2.2 Cell Adhesion Tests

Many tests exist to test cell adhesion to polymer surfaces, which can be divided broadly into tests for strength of adhesion and tests for percent adhesion. Some strength of adhesion test methods, like polyacrylamide-traction force microscopy (PA-TFM) and biomembrane force probes (BFPs), study single cell events. In PA-TFM, fluorescent beads are embedded near the surface of a polyacrylamide gel. When cell adhesion occurs, traction forces on the gel displace the beads so that bead movement can be quantified and related to adhesion force¹⁵⁷. BFPs use a force transducer composed of two micropipettes, one holding a cell and the other holding an antibody-coated bead attached to the cell.

Adhesion forces are quantified by cell displacement as a pulling force is exerted between the cell and bead¹⁵⁸. Other tests for adhesion strength quantify the cell response through detachment. Centrifugation assays measure the number of cells that detach during centrifugation, which is reflective of cell adhesion strength¹⁵⁴.

Tests for the percent of cell adhesion require knowledge of the initial number of cells seeded to a surface and are limited to short time points so that cell proliferation does not occur. Wash assays estimate cells remaining on a surface through counting removed cells. After performing wash steps on a surface with seeded cells, the cells from the wash solution are counted and subtracted from the known number of cells seeded to the surface¹⁵⁴. Cell counting techniques have also been implemented that involve immunostaining and counting cells on a small fraction of the polymer surface, which can then be used to estimate cell adhesion density. These tests can be useful for determining cell adhesion potential, such as when investigating cell interactions with a novel scaffold.

3.1.3 Addition of BTEAC Salt in Electrospinning of Scaffolds

It has already been established that decreasing fiber diameter and porosity will increase EC adhesion to polymer scaffolds, making fiber diameter and porosity reduction a plausible solution to successfully creating BVMs that only use ECs. Therefore, methods for decreasing these scaffold characteristics were pursued in our laboratory by a different student researcher. One method investigated was to add benzyl triethyl ammonium chloride (BTEAC) salt to the PLGA and chloroform mixture before electrospinning. Figure 34 and Figure 35 compare standard electrospun scaffold versus a scaffold spun with 3% BTEAC in the Tissue Engineering Lab (unpublished data). The average fiber

diameter of the standard PLGA scaffolds was 5.55 μm , while the 3% BTEAC scaffold had significantly smaller fibers with an average of 0.826 μm ($p < 0.01$).

Figure 34: SEM of Standard Scaffold. Standard scaffold SEM images at 100x (left) and 600x (right) magnification.

Figure 35: SEM of Salt Scaffold. 3% benzyl triethyl ammonium chloride (BTEAC) salt scaffold SEM images at 100x (left) and 600x (right) magnification.

The addition of BTEAC has been shown to decrease fiber diameters of electrospun scaffolds^{159–161}. The addition of the ionic salts like BTEAC increases the electrical conductivity of the solution by increasing the charge density. Within the electric field, the salt ions electrostatically repel each other, increasing the stretch of the

polymer jet and reducing fiber diameter¹⁶². You et al. showed decreasing fiber diameter with increasing BTEAC content in PLGA scaffolds using chloroform as the solvent, seen in Figure 36¹⁶⁰. Fiber diameter reduction is limited at salt concentrations above 3% w/v. The same study also saw a decrease in porosity from 88% to 77.2%. Another study which electrospun poly(hydroxybutrate-co-hydroxyvalerate) (PHBV) dissolved in chloroform saw a reduction in fiber diameter from 1662 ± 490 nm to 603 ± 250 nm after the addition of BTEAC¹⁶³.

Figure 36: Fiber Diameter Reduction with BTEAC Percentage. The average fiber diameters of electrospun PLGA with varying percentages of BTEAC ¹⁶⁰.

The addition of BTEAC does not affect other important characteristics of the electrospinning solution, such as viscosity and surface tension, making it simple to implement in existing protocols^{159,160}. Thorsten et al. electrospun PLA dissolved in either chloroform or dichloromethane and showed that, while electrical conductivity increases as BTEAC weight percentage increases, the surface tension of the solution remains constant¹⁵⁹. While the solution characteristics remain constant, the addition of BTEAC affects the mechanical strength of the resulting scaffolds. Scaffolds spun with BTEAC

had a decreased ultimate tensile strength and Young's modulus as compared to those spun without the salt, but scaffolds with BTEAC had increased elongation at fracture¹⁶³.

Interestingly, electrospun scaffolds with BTEAC have been investigated for antimicrobial properties for use as filters. Quaternary ammonium compounds (QACs), such as BTEAC, target and disrupt the cytoplasmic membrane of bacteria and the viral envelopes of viruses¹⁶¹. Park et al. characterized the antibacterial and antiviral effects of electrospun polyvinyl alcohol (PVA) with BTEAC dissolved in water. They found that the growth of *K. pneumonia*, *E. coli*, and *S. aureus* were inhibited at BTEAC concentrations above 2.6%. Besides inhibiting growth, the scaffolds also reduced live bacteria and viruses over time. There was reduction of *S. aureus* and *K. pneumoniae* from 30 to 120 minutes, as well as reduction of PhiX174 and MS2 from 60 to 300 minutes¹⁶¹. These properties that make the BTEAC a good antimicrobial agent may disqualify it as a candidate for use in biomaterial scaffolds. If the salt has toxic interactions with cells, like it does with bacteria and viruses, cell death would increase while proliferation decreases. However, no current published studies have investigated the interaction of these scaffolds with human cells. Park et al. investigated BTEAC toxicity through leaching scaffolds and observing the effect of the leaching product on daphnia magna, a planktonic crustacean. They found that *D. magna* was not affected by BTEAC concentration between 0.1 mg/L and 5 mg/L. The highest concentration leached from the scaffolds was 0.72 mg/L, far less than a concentration that would be toxic to *D. magna*¹⁶⁴.

While the addition of BTEAC salt to electrospinning solutions is a simple method to create nanofibers in scaffolds, there is potential for toxicity when cells interact with these fibers. For example, BTEAC salts have been shown to elute from the scaffolds in

small amounts, but the effect of these salts has not been tested on cells¹⁶⁴. Therefore, investigation of the cell interactions is necessary to determine the viability of these scaffolds for tissue engineering applications.

3.1.4 Summary

The prospect of implementing a new scaffold for use in BVMs requires an understanding of the interaction between cells and scaffold. This section outlines the effect of scaffold characteristics on cells, cell adhesion *in vitro*, and the apoptosis pathway. The following sections will describe how this knowledge was applied to create assays for testing scaffold cytotoxicity and cell adhesion along with experiments performed to verify the utility of these assays.

3.2 Materials and Methods

In order to implement a novel scaffold in the BVM model, three different assays were developed. The following sections will describe methods establishing these assays to compare BTEAC salt scaffolds against standard PLGA scaffolds. A large portion of developing these methods involved quantifying images that were collected through immunostaining and confocal imaging. The cytotoxicity elution assay tested scaffold viability indirectly, while the short-term adhesion and BVM cultivation assays determined direct utility of scaffolds on HUVECs. These direct toxicity assays provided useful data on HUVEC adhesion to and coverage on different scaffold types at increasing time intervals.

3.2.1 Caspase Stain Implementation and Verification

Caspases are enzymes that are activated throughout the apoptosis cascades, so caspase activity is useful for assessing apoptosis. The Image-iT LIVE Green Poly Caspases Detection Kit (Molecular Probes) was used for immunostaining cells to visualize apoptotic activity. The kit also includes a Hoechst 33342 stain, which stains cell nuclei, and a Propidium Iodide stain, which stains dead cells. Besides the three stains, an Apoptosis wash buffer and Apoptosis fixative solution were included. The fixative solution was a 10% formaldehyde solution.

It was first necessary to establish positive controls to verify if the kit was performing properly; in this case, the positive control was a treatment that induced apoptosis. The caspase activity in this treatment serves as a baseline to compare the caspase activity of treatments in which toxicity is unknown. Hydrogen peroxide (H_2O_2) and dimethylsulfoxide (DMSO) are known to be cytotoxic to cells, so these were chosen as candidates for the positive control treatments¹⁶⁵. HUVECs were grown to confluency in 8-well plates before receiving one of the following 16 treatments: 2%, 5%, 10%, or 15% DMSO in EC media for 30 minutes or 60 minutes, or 0.05%, 0.1%, 0.5%, or 1% H_2O_2 in EC media for 2 minutes or 5 minutes. A negative control that received EC media, and no DMSO or H_2O_2 , was also included.

After treatment, each well was washed with EC media before staining with the caspase kit. Cells were incubated for 90 minutes with 200 μL of caspase detection reagent diluted in EC media. The staining solution was removed, and cells were washed with EC media before separately staining with Hoechst and Propidium Iodide, both at dilutions of 1:500 in EC media for 10 minutes. The cells were then washed twice with

Apoptosis wash buffer before being fixed with Apoptosis fixative solution for at least 12 hours at room temperature before imaging. All samples in this aim were stained using this protocol; therefore, other sections will simply refer to this as “apoptosis staining protocol”. This protocol is provided in Appendix J. Samples were imaged on the Confocal Microscope at a 40x magnification and the laser settings as seen in Table 4. Images were analyzed qualitatively based on caspase signal intensity. Samples with a strong caspase signal in the cytosol and nucleus but with low Propidium Iodide activity were selected as positive control treatments.

Table 4: Confocal Microscope Lasers and Settings.

	Hoechst	Caspase	Propidium Iodide
Laser	405	488	559
Laser Power	15%	30%	30%
High Voltage	700	700	700

Once positive control treatments were selected, each of the three individual stains needed to be checked for bleed-through to ensure that signal from one stain was not being detected by a separate laser. HUVECs cultured in 8-well plates were stained with either no stain, Hoechst stain only, Caspase stain only, Propidium Iodide stain only, or all three stains. These samples were imaged at 20x magnification and analyzed to determine if any bleed-through occurred. With these stains verified and positive controls established, the stain was then used with confidence to assess treatments in the following assays.

3.2.2 Cytotoxicity Assay

The cytotoxicity of scaffolds was first assessed using a standard elution approach. This means that the assay was performed without direct cell-scaffold contact, but rather by treating cells with media that had been eluted with a scaffold. An elution is a liquid that has been incubated with a substance at a certain temperature for a length of time. The theory behind this is that any toxic particles will be drawn into the solution and the effect of this elution on cells will indicate the toxic effects of that original eluted substance¹⁶⁶.

In this assay, standard PLGA scaffolds and 3% BTEAC salt scaffolds were eluted in EC media for 72 hours at 50° C as advised by ISO-10993. To prepare the elution, scaffolds were cut to 43 mm and attached to fittings before being sterilized in 70% ethanol for 20 minutes. Using sterile technique, the scaffolds were then removed from the ethanol and flushed transluminally and transmurally with DPBS and EC media. The scaffolds were then placed in 10 mL of EC media and placed in a water bath heated to 50° C. After 72 hours, the scaffolds were removed from the elution and stored at 4° C until use, approximately five to seven days. pH measurements were also taken of each elution to determine if heat or scaffold degradation had changed the media to be more acidic or basic.

The elution was next applied to HUVECs grown to approximately 80% confluence in 8-well plates. Cells were either treated with pure eluted media or eluted media that had been diluted by 50% for 24 or 48 hours. The dilution group was included because the strength of the original elution was unknown. If the full elution treatment was too cytotoxic there would be no way to discern between treatments. The dilution should emphasize any difference between the salt scaffold and standard scaffold elution, if a

difference exists. After the treatment lengths, cells were stained using the apoptosis staining protocol and imaged at 40x magnification. Positive and negative controls were also included in each time group, where a positive control was treated with hydrogen peroxide just before staining and a negative control was treated with standard EC media.

Image quantification methods had to be established to determine how many cells from each treatment group were positive for caspase or propidium iodide staining. To do this, images were separated by channel and false colored to the respective laser color (blue, green, or red). Nuclei were counted automatically using the Analyze Particle function in ImageJ, which required a maximum pixel area to be set. Therefore, any area below that set value would be excluded in the count, which prevented non-nuclei from being counted. Before counting, a threshold was set, and the image was made binary. An example of this process can be seen in Figure 37. Because of the variability in manually setting the threshold and the possibility of setting the size restriction too high, these settings needed to be verified.

Figure 37: Hoechst Channel Automatic Cell Counting. (A) Raw Hoechst channel image with cell nuclei. (B) Threshold applied, reducing noise and making the image binary. (C) Outlines of automated cell count.

The settings were tested by manually counting a sample of images and comparing the manual count to the automated count. The threshold values used varied but were set between 1000 and 1200, depending on the image and necessity to eliminate signals that were non-nuclei. Two magnifications were tested, 20x and 40x, and the smallest area excluded was set to 20 pixels² and 50 pixels² for the two magnifications, respectively. For the 20x magnification, it was found that there was an average error of 40.03% between the manual and automated counts with an average error of 18.5 nuclei per image. For the 40x magnification, there was an average error of 9.15% with an average error of 5.2 nuclei. With this data, it was clear that the 40x magnification was superior for analyzing.

After automatically counting the Hoechst channel, the Caspase and Propidium Iodide channels needed to be quantified. These channels had to be counted manually because of the variability in signal location; some cells were positive for the stains in only the nucleus, others only in the cytoplasm, while some had signal throughout the entire cell. There was also overlap between cells, so automated counting would not be able to discern between large groups of cells. To aid with manual counting, pixel intensities below a certain value were eliminated to ensure that background noise did not appear to be a signal indicating the cells were positive for apoptosis or cell death. This pixel reduction value had to be set to avoid eliminating cells that were truly positive for the stain versus those that were noise. Examples of these methods can be seen in Figure 38 and Figure 39.

Figure 38: Pixel Reduction in Caspase Channel. (A) Raw image of Caspase channel. (B) Caspase channel after pixel reduction. (C) Overlay of Hoechst and Caspase channels, which was used for counting Caspase positive cells.

Figure 39: Pixel Reduction in Propidium Iodide Channel. (A) Raw image of Propidium Iodide channel. (B) Propidium Iodide channel after pixel reduction. (C) Overlay of Hoechst and Propidium Iodide channels, which was used for counting Propidium Iodide positive cells.

Graphs were created for Caspase and Propidium Iodide channels of pixel reduction values versus number of cells manually counted. Pixel intensity values range from 0 (black) to 255 (white), so the values tested were in the lower range of intensities. For both stains cell count started to decrease rapidly after eliminating pixels with an intensity over 15, so this was set as the pixel elimination quantity for the Caspase and

Propidium Iodide images. After eliminating these pixels, the Hoechst channel was overlaid on both channels separately. Only signals that surrounded or overlaid with nuclei were counted, so signals not associated with a nucleus were not counted as cells. After quantification, the percent Caspase positive cells and percent Propidium Iodide cells were calculated by comparing the number of positive stained cells to the total number of nuclei. Finally, the percent positive cells for both caspase and propidium iodide were normalized to the negative control (non-treatment) group, which were cells with only EC media, essentially subtracting out the natural population of positive cells for both stains that were present in groups that did not receive an elution treatment.

3.2.3 Cell Adhesion and Viability Assay

The nanofibers of the BTEAC salt scaffolds have much smaller fiber diameters than the standard scaffolds, which should improve EC adhesion¹⁶⁷. Therefore, it was valuable to investigate cell adhesion to the scaffold at a short time point. This adhesion assay involved pressure sodding ECs to both types of scaffolds and analyzing the scaffolds after 4 hours. Methods were also determined to quantify cell adhesion and short-term data on Caspase activity and number of dead cells.

Since this time point was short, the assay could be conducted without using sterile technique. Standard scaffolds and 3% BTEAC salt scaffolds were attached to fittings and sterilized in 70% ethanol for 20 minutes. The scaffolds were then flushed transluminally and transmurally in DPBS and EC media. HUVECs were then pressure sodded into the scaffolds by placing a stop-cap on one end of the scaffold, and then using a syringe to push cells resuspended in media into the scaffold. The cell density of the resuspended

cells was counted, and the volume of cell solution added for each scaffold was recorded. The cell-scaffold construct was then incubated at 37°C for 4 hours in a petri dish filled with EC media. After 4 hours, constructs were removed and stained using the apoptosis staining protocol. The media that the constructs were incubated in was collected and spun down, and the cells that had adhered to the petri dish were also trypsinized and spun down. These cells were counted to determine how many cells did not adhere to the scaffold from the volume that was added.

Image quantification methods had to be established to determine cell coverage, caspase activity, and dead cell coverage from the images taken on the confocal microscope. Images were taken at 20x magnification, as the focal plane of the microscope does not reach the curved scaffold at 40x magnification. Due to this, individual cell nuclei could not accurately be counted. Instead, an assumption regarding the size of a cell nuclei compared to the whole area had to be made to estimate the number of cells present. Using sample 20x magnification images from literature of HUVECs stained with Hoechst and cadherin (which outlines the cytosol), the ratio of nucleus size compared to the entire cell size was estimated to be 23.42%. Dividing 100% (the area of the entire cell) by 23.42% gives a value of 4.27, which acts as a multiplication factor for the total stained nuclei area to extrapolate a cell coverage estimation. The Analyze Particle function in ImageJ was used to calculate the total nuclei area of each image in pixels², seen in Figure 40, which was then multiplied by 4.27 to estimate cell area. The cell area estimation was then compared to the whole image area to determine percent of cell coverage.

Figure 40: Hoechst Channel Coverage Quantification. (A) Raw Hoechst image. (B) Hoechst channel with threshold applied. (C) Automated cell area quantification.

The Caspase and Propidium Iodide channel images were quantified in a similar method, although the assumption was made that the whole cell was fluorescing in these channels, so no multiplication factor was necessary to determine signal coverage. The Analyze Particle function was used again to determine area of both Caspase and Propidium Iodide activity, and a percent coverage was calculated. This coverage was then normalized to the Hoechst channel cell coverage percentage of the same image to determine an estimated percent apoptotic or dead cells. Salt scaffold coverage in all three channels was compared against coverage of the standard scaffolds.

3.2.4 BVM Cell Coverage and Viability Assay

The final assay developed in this aim compared the standard scaffolds against two types of BTEAC scaffolds in BVM constructs at 3 and 7-day time points. Seven vessels were included in each time point: two standard PLGA scaffolds, two 0.5% BTEAC salt scaffolds, two 3% BTEAC salt scaffolds, and one ePTFE scaffold. The 0.5% BTEAC salt scaffold served as a low-salt group, while the 3% BTEAC salt was a high-salt group. The

ePTFE served as a negative control because ePTFE is known to be bioinert and nontoxic to cells. Since the standard PLGA scaffolds have not been characterized before, they could not serve as a negative control.

The standard methods for constructing EC-only BVMs were used, which can be found in Appendix H and Appendix I. One additional step had to be used on the salt scaffolds; it had been found that these scaffolds shrink substantially upon ethanol sterilization, both in length and circumferentially. Therefore, 4 mm salt scaffolds were electrospun and were then pre-shrunk on 3 mm mandrels in 70% ethanol for 15 minutes before being cut to the appropriate length and attached to fittings. Standard scaffolds were still electrospun to be 3 mm. Changes in scaffold lengths were tracked throughout the preshrink and cultivation.

At the 3 and 7-day timepoints, BVMs were removed from the bioreactors and cut in half. One half of the scaffold was fixed in 2.5% glutaraldehyde and used for SEM imaging, while the other half was immunostained using the apoptosis staining protocol. Image quantification was performed for all three channels using the methods described in Section 3.2.3.

3.3 Results

Due to the large number of images, only representative images will be included in this section as needed. A larger set of images can be found in Appendix M, Appendix N, Appendix O, and Appendix P.

3.3.1 Stain Verification

Before applying the apoptosis stain kit to any experiment, the three stains included in the kit were verified and optimized on the confocal microscope. Table 5 shows images of each case necessary to verify that no bleed through occurred between channels during imaging. Each row shows the same imaging area taken with a different laser in each channel. The three channels, Hoechst, Caspase, and Propidium Iodide, correspond to the three stains in the apoptosis kit. The “no stain” group in the first row serves to verify that no autofluorescence was present in the sample. The lack of signal in each channel shows that there was no autofluorescence from the HUVECs used in these images.

Table 5: Apoptosis Staining Kit Verification using HUVECs.

	Hoechst	Caspase	Propidium Iodide
No Stain 20x
Hoechst only 20x

The middle three rows in Table 5 show samples that have only been stained with a single staining component from the kit. If bleed-through were occurring from one of the three stains, more than one channel would have signal in it. In this case, each row has a single image with signal in the correct channel. Therefore, no bleed-through was occurring between channels in this kit. The fifth row shows HUVECs that were stained with all three kit components, so each channel has signal in it. However, the Caspase channel has little signal in it, which is expected for untreated HUVECs in culture that should not be undergoing apoptosis. A positive control, in this case a treatment to induce

apoptosis, was necessary in order to verify that the Caspase stain appropriately identifies apoptotic cells.

The two inducers of apoptosis, hydrogen peroxide and DMSO, were applied to HUVECs at increasing concentrations and lengths of time to determine a treatment that resulted in appropriate apoptosis detection. This treatment group should result in nearly all cells being labeled for apoptosis without completely killing the cells. This treatment should also result in cells stained in the cytosol and the nucleus, as the poly caspase stain used here should label caspases present in both areas of the cell. A sufficient apoptosis inducer should result in the labeling of the whole cell. Figure 41 and Figure 42 show treatment groups that were insufficient at inducing apoptosis. The 15% DMSO treatment for 30 minutes in Figure 41 resulted in some signal in the cytosol of the cell. This signal, however, was not as strong as would be expected from a positive control, and no cells were stained in the nuclei. An important clarification is necessary to establish here: while these cells do not have sufficient caspase activity to be positive controls, these cells are still apoptotic. Any cell with caspase signal throughout the cytosol will still be considered apoptotic and quantified as so in the remaining experiments. All DMSO treatment groups were insufficient as the positive control, which can be seen in Appendix M.

Figure 41: Ineffective Positive Control DMSO 15% 30 Minutes. HUVECs treated with 15% DMSO for 30 minutes in Hoechst (left), Caspase (middle) and Propidium Iodide (right) channels. These images indicate that 15% DMSO treatment for 30 minutes was not a strong inducer of apoptosis. High resolution images can be obtained from Dr. Cardinal.

Figure 42: Ineffective Positive Control Hydrogen Peroxide 1% 2 Minutes. HUVECs treated with 1% hydrogen peroxide for 2 minutes in Hoechst (left), Caspase (middle) and Propidium Iodide (right) channels. These images indicate that 1% hydrogen peroxide treatment for 2 minutes was not a strong inducer of apoptosis. Red arrows point to cells that are stained for Caspase in the cytosol but not the nuclei. High resolution images can be obtained from Dr. Cardinal.

The 1% hydrogen peroxide treatment for 2 minutes shown in Figure 42 was insufficient as well; while many of the cells were stained in the cytosol and nucleus, there were some cells that lacked the stain in the nucleus. Many of the other hydrogen peroxide treated groups were also insufficient, which can be seen in Appendix M. However, two

hydrogen peroxide groups were identified as sufficient positive controls, which are displayed in Figure 43 and Figure 44. Both groups had the cytosol and nucleus of all cells intensely labeled for caspase activity without resulting in large amounts of dead cells. These two treatment groups were used as positive controls in the cytotoxicity elution tests.

Figure 43: Effective Positive Control Hydrogen Peroxide 0.5% 5 Minutes. HUVECs treated with 0.5% hydrogen peroxide for 5 minutes in Hoechst (left), Caspase (middle) and Propidium Iodide (right) channels. High resolution images can be obtained from Dr. Cardinal.

Figure 44: Effective Positive Control Hydrogen Peroxide 1% 5 Minutes. HUVECs treated with 1% hydrogen peroxide for 5 minutes in Hoechst (left), Caspase (middle) and Propidium Iodide (right) channels. High resolution images can be obtained from Dr. Cardinal.

3.3.2 Cytotoxicity Elution Assay

The primary purpose of this study was to determine if the eluted contents from the BTEAC salt scaffolds resulted in higher numbers of apoptotic cells than standard scaffolds. Two elution methods were initially investigated, both of which were recommended by ISO 10993, and evaluated based on scaffold degradation and pH. Elutions were conducted at 70°C for 24 hours and 50°C for 72 hours. Figure 45 shows scaffolds after removal from elutions, Figure 46 shows the color of the eluted media, and Table 6 displays the pH of elution from different scaffold types from each protocol. Each of these figures will be described and included below.

The 70°C elution for 24 hours, shown in Figure 45A, resulted in warped and crumbling scaffolds, many of which began to flake into the eluted media upon removal. The 50°C elution for 72 hours resulted in stiffening and brittleness of the scaffolds, but they retained their shape and did not crumble during removal from the elution. There was also a stark difference in media color from the two elution protocols, in which the color was independent of the type of scaffold used. Figure 46B shows a yellow coloration to the eluted media, while the eluted media in Figure 46C is pink in color. The pink color in the 50°C eluted media is like the color of the HUVEC media before the elution protocol.

Figure 45: Scaffolds After Elution Treatment. (A) Scaffolds after 70°C elution for 24 hours. (B) Scaffolds after 50°C elution for 72 hours. In both A and B, two scaffolds on the left are 3% salt scaffolds and the scaffolds on the right are standard scaffolds.

Figure 46: Media After Elution Treatment. (A) Standard media that has not been eluted. (B) Media eluted at 70°C for 24 hours. (C) Media eluted at 50°C for 72 hours.

A change in color from pink to yellow generally indicates a change in pH towards an acid. Based on the change in media color in the 70°C elution, it would be expected that this media would be acidic. This was confirmed after testing the pH, seen in Table 6. For the 70°C elution the standard scaffold resulted in a pH of 7.0, while the 3% salt scaffold had a pH between 7.0 and 7.5. The 50°C elution had consistent pH values of 7.5 for both

scaffold types. These values were similar to the non-eluted media, which had a pH of 7.5. It was unsurprising that some of the elutions were slightly acidic, as PLGA breaks down into acidic byproducts¹⁶⁸. Based on the structural retention of the scaffolds after the elution and no observations of a color or pH change in the eluted media, it was decided to use the 50°C elution for 72 hours during the cytotoxicity study.

Table 6: pH of Elutions from Treatment Methods.

	Non-eluted Media	70°C Elution for 24 hours	50°C Elution for 72 hours
Standard Scaffold Average	7.5	7.0	7.5
3% Salt Scaffold Average		7.0 - 7.5	7.5

The eluted media from each type of scaffold was then used to treat HUVECs for 24 or 48 hours before the samples were stained and imaged. The quantification methods for each channel differed. An automated cell counter for the Hoechst channel was used to count the nuclei; however, it was unknown what magnification was appropriate for automated counting. Two magnifications were investigated, 20x and 40x, to determine the accuracy of the automated counter compared to a manual count. It was found that the automated cell count at a 20x magnification had an error of 40.02%, while the automated cell count at 40x had an error of 9.15%. This data can be found in Appendix Q. With such a high margin of error from the 20x magnification automated counting, images at 40x magnification were used for the cytotoxicity assay.

Unlike the Hoechst channel, the Caspase and Propidium Iodide channels had to be quantified manually. In order to aid the counter, it was decided to eliminate pixels below a certain intensity to emphasize cells that were truly apoptotic or dead. This would help

reduce background noise and increase the accuracy of Caspase or Propidium Iodide-positive cell counts. In order to determine the range of pixel intensities to eliminate, a manual cell count was performed on Caspase and Propidium Iodide channels at increasing pixel reduction values. The cell count after each pixel reduction was then compared to the original number of Caspase or Propidium Iodide-positive cells. The cell count percent change for the Caspase channel and Propidium Iodide channel is presented in Figure 47. It was hoped that a plateau region would be obvious in both graphs, which would indicate an appropriate minimum pixel intensity. However, neither channel had a plateau, so it was decided to limit the loss of signal positive cells to less than 10%. Therefore, a pixel intensity reduction of 15 and 20 were used for the Caspase and Propidium Iodide channels, respectively. The raw data for these graphs can be found in Appendix R.

Figure 47: Cell Count Reduction Curves. (A) Caspase-positive cells percent change as minimum pixel intensity increases. (B) Propidium Iodide-positive cells percent change as minimum pixel intensity increases.

With the image quantification methods established, the raw images of HUVECs treated with either a dilution or non-dilution of eluted media from standard or salt scaffolds could be quantified and analyzed. A negative control of HUVECs treated with normal EC media was included, as well as two positive control groups using 0.5% and 1% hydrogen peroxide for 5 minutes. Representative raw images from this study can be found in Appendix N.

Figure 48 displays the percent of Caspase-positive cells in each treatment group. A three-way ANOVA was run for treatment length, dilution, and scaffold type. At each treatment length there was no difference between the 50% dilution and no-dilution groups regardless of scaffold type. For each treatment length, the negative control, 50% dilution standard scaffold, and no-dilution standard scaffold groups had lower percentages of Caspase-positive cells than the 50% dilution salt scaffold and no-dilution salt scaffold groups. Furthermore, for each treatment length, the 50% dilution salt scaffold and no-dilution salt scaffold groups had lower percentages of Caspase-positive cells than either positive control groups. In summary, the negative control and standard scaffold groups had less caspase activity than the salt scaffold groups, which had less activity than the positive control groups.

Figure 48: Cytotoxicity Elution Assay Percent Caspase Positive Cells. Percentage of Caspase positive cells in each treatment group. The * indicates a difference in the 24-hour timepoint compared to the 24 hour no dilution salt scaffold group and a difference in the 48-hour timepoint compared to the 48-hour no dilution salt scaffold group ($p < 0.001$).

In order to eliminate rates of naturally occurring apoptosis from the data, the normal scaffold and salt scaffold treatment groups were normalized to the negative control. This is shown in Figure 49, and this normalization eliminated significant differences seen previously in Figure 48. No differences in percent caspase-positive cells were found between time points, within timepoints of the same treatment, between scaffold types, nor between dilution groups.

Figure 49: Normalized Cytotoxicity Elution Assay Percent Caspase Positive Cells. Percentage of Caspase positive cells normalized to the negative control group. No differences were observed between treatment groups nor timepoints.

Figure 50 shows the percent of Propidium Iodide positive cells per treatment group, which includes a negative control and two positive controls. All treatment groups had a higher percent of Propidium Iodide positive cells at the 48-hour treatment length than at 24 hours. No differences were seen between treatment groups at 24 hours. For the 48-hour treatment length, there was no difference between the positive control groups and the 50% dilution salt scaffold. However, the no dilution salt scaffold group had a lower percentage of Propidium Iodide positive cells than the 50% dilution salt scaffold group. Similarly, in the 48-hour treatment length, the no dilution standard scaffold had a lower percentage of Propidium Iodide positive cells than both the 50% dilution standard scaffold and the negative control group. These relationships between dilution and

undiluted groups is surprising, as it is opposite of how a dilution group is expected to affect cell death if the elution does have an effect.

Figure 50: Cytotoxicity Elution Assay Percent Propidium Iodide Positive Cells. Percentage of Propidium Iodide positive cells in each treatment group. The * indicates a difference in percentage at 48 hours compared to the no dilution salt scaffold group at 48 hours ($p < 0.05$). The # indicates a difference between the 24 and 48-hour time point within the group ($p < 0.05$).

Like with the Caspase group, the treatment groups for Propidium Iodide positive cells were normalized to the negative control group to eliminate natural cell death rates. Figure 51 displays these results after normalization. This normalization did not eliminate the pattern of dilution effects seen previously in Figure 50. At 48 hours, both the standard scaffold and salt scaffold dilution groups had more Propidium Iodide positive cells than their corresponding undiluted groups. At 24 hours, no difference persisted between all treatment groups.

Figure 51: Normalized Cytotoxicity Elution Assay Percent Caspase Positive Cells. Percentage of Propidium Iodide positive cells normalized to the negative control group. The * indicates a difference compared to all other groups at both timepoints ($p < 0.01$). The # indicates a difference compared to the 48-hour no dilution standard scaffold group ($p < 0.01$).

3.3.3 Cell Adhesion and Viability Assay

The cytotoxicity elution assay assessed cell apoptosis and death without direct scaffold contact. While these toxicity measurements are useful, other metrics are necessary to assess novel scaffolds, especially regarding initial cell interactions.

Therefore, the cell adhesion assay assessed cell coverage and scaffold toxicity 4 hours after HUVECs had been seeded onto the scaffolds. Some data regarding cell deposition could be collected without imaging through cell counts. Estimations of cell seeding numbers are shown in Table 7. While this data does not visually measure cell adhesion, it is an indirect calculation of how many cells remained seeded to the scaffold. The

standard scaffolds were sodded with 9.02 million cells, while the salt scaffolds were only sodded with 3.76 million cells. The difference in the number of cells sodded is because, unlike the standard scaffolds, only a portion of the desired cell solution could be injected into the salt scaffolds due to pressure buildup. This pressure could be a result of cells clogging the smaller pores of the salt scaffolds. Interestingly, a much higher percent of the sodded cells was collected from the petri dishes of salt scaffolds even though less cells were sodded. This would suggest that less of the cells sodded to the salt scaffolds remained in the scaffolds, or that less cells are needed to sod salt scaffolds to create a confluent cell lining.

Table 7: Short-term Adhesion Assay Cell Sodding and Collection Data.

	Number of cells sodded per scaffold (cells/scaffold)	Collected cells from petri dish (cells)	Percent of cells collected (%)
Standard Scaffold	9.02E6	1.17E6	12.97
3% Salt Scaffold	3.76E6	1.59E6	42.29

The immunofluorescent imaging of the scaffolds was a more direct and visual assessment of cell coverage. To estimate cell coverage, an assumption had to be made regarding the size of a cell nucleus compared to the size of the entire cell. The ratio of these measurements could then be used as a factor with which to multiply the area of cell nuclei by to estimate the area of cell coverage. Since images were being taken at 20x magnification, sample images from literature of HUVECs taken at 20x, seen in Figure 52, were measured in ImageJ to determine the average nuclei and whole cell size. It is unknown how these images may differ from the images taken from the 20x setting on the

confocal microscope used in this aim, but this value was only intended to be an estimation, so some difference was expected. Raw area measurements can be found in Appendix S. The average nucleus was found to be 23.42% of the entire cell. Dividing 100% of the cell area by 23.42% results in the factor 4.27, which was used as the multiplication factor to estimate cell coverage.

Figure 52: Sample 20x HUVECs. Images from literature of HUVECs taken at a 20x magnification. These cells were stained with junctional markers, which outline the entire cell¹⁶⁹.

Figure 53 displays representative images of the Hoechst channel for standard and salt scaffolds. Upon visual comparison, the salt scaffolds had a far greater number of cells than the standard scaffolds. It also appeared that the standard scaffolds had decreasing cell coverage from proximal to distal ends, while the salt scaffold had similar coverage along the entire scaffold. Quantification data supported this visual assessment.

Figure 53: Standard and Salt Scaffolds Hoechst Channel. Standard scaffolds proximal (A), middle (B), and distal (C) show less coverage than salt scaffolds at proximal (D), middle (E), and distal (F) locations. High resolution images can be obtained from Dr. Cardinal.

Figure 54 compares estimated cell coverage between scaffold types at different areas of the scaffold. Salt scaffolds had higher coverage than standard scaffolds. There was also a trend of decreasing coverage along the length of the standard scaffold group.

Figure 54: Short-term Adhesion Assay Percent Cell Coverage. Estimated percent cell coverage for standard and salt scaffolds on the proximal, middle, and distal areas of the scaffold. The * indicates a difference in coverage between the scaffold types ($p < 0.001$).

Apoptosis and cell death were also quantified using the Caspase and Propidium Iodide channels, respectively. Figure 55 shows representative images of Caspase and Propidium Iodide channels for the different scaffolds. Figure 56 shows that no differences were seen in the estimated percent of apoptotic cells between the standard scaffolds and salt scaffolds. Even though apoptotic cells showed no differences, there were differences in the percent of dead cells. Figure 57 shows that there was a higher estimated percent of dead cells in the salt scaffold group than the standard scaffold group.

Figure 55: Caspase and Propidium Iodide Channels Short-term Adhesion Assay.

Salt and standard scaffolds at 20x magnification. (A) Caspase channel for standard scaffold. (B) Propidium Iodide channel for standard scaffold. (C) Caspase channel for salt scaffold. (D) Propidium Iodide channel for salt scaffold. High resolution images can be obtained from Dr. Cardinal.

Figure 56: Short-term Adhesion Assay Percent Apoptotic Cells. Estimated percent apoptotic cells for standard and salt scaffolds on the proximal, middle, and distal areas of the scaffold. No differences were observed between scaffold types or areas of the scaffold.

Figure 57: Short-term Adhesion Assay Percent Dead Cells. Estimated percent dead cells for standard and salt scaffolds on the proximal, middle, and distal areas of the scaffold. The * indicates a difference in dead cells between the scaffold types ($p < 0.001$).

3.3.4 BVM Cell Coverage and Viability Assay

The 4-hour time point was useful for assessing immediate cell attachment and interactions with the scaffold. However, to implement novel scaffolds these scaffolds need to prove useful in BVMs for multiple days. This experiment investigated how low and high percentage salt scaffolds compare to standard scaffolds at 3 and 7-day timepoints. Since this assay has not been previously conducted on standard scaffolds, an ePTFE scaffold was added as a negative control with which to compare all electrospun scaffold types. At each time point, 2 vessels of each electrospun scaffold type were fabricated. Unfortunately, one 0.5% vessel at the 7-day time point was lost due to contamination.

Since it was previously noted that the salt scaffolds shrink during sterilization, changes in scaffold size were measured throughout the fabrication of the BVMs. Measurements of salt scaffold lengths were taken before and after preshrinking, and all scaffolds were measured before and after BVM cultivation. Figure 58 shows the changes in salt scaffolds from the preshrink and cultivation. The scaffold groups display a proximal and distal, which is differentiating between the end of the mandrel in which the scaffold was sourced since each mandrel produces two scaffolds. Scaffold length changed more during preshrinking than during cultivation, which suggests that the preshrinking is useful as a method of reducing salt scaffold shrinking during cultivation. The change in length for all scaffolds after BVM cultivation is provided in Figure 59, which shows that both salt scaffold groups shrank more than standard scaffolds. Surprisingly, the standard scaffolds slightly expanded in length during the BVM cultivation.

Figure 58: Salt Scaffold Shortening Before and After BVM Cultivation. Reduction in salt scaffold lengths from preshrinking and from the BVM cultivation. The * indicates a difference of change in length compared to the BVM cultivation group ($p < 0.01$).

Figure 59: Standard and Salt Scaffold Shortening After BVM Cultivation. Reduction in the scaffold length during the 3 or 7-day BVM cultivation. The * indicates a difference for change in scaffold length compared to the standard scaffold group ($p < 0.05$).

The thickness of the scaffold walls was measured after BVM cultivation. There was no difference in scaffold thickness between the 0.5% and 3% salt scaffold groups,

but the 3% salt scaffold group had a greater wall thickness than the standard scaffold group. This difference was expected; the salt scaffolds used 25% more PLGA than the standard scaffolds to accommodate for the 4 mm mandrel.

Figure 60: Wall Thicknesses of Scaffolds in BVMs. The * indicates a difference in thickness compared to the standard scaffold group ($p < 0.05$).

Like with the cell adhesion assay, cell coverage was first quantified for the BVMs to investigate the effect of scaffold type and cultivation duration. Figure 61 shows images of 3-day BVMs from each scaffold group, and Figure 62 shows 7-day BVM images from each scaffold group.

Figure 61: Representative 3-day BVM Hoechst Images. Proximal (left), middle (middle), and distal (right) locations. (A) ePTFE scaffold, (B) standards scaffold, (C) 0.5% salt scaffold, (D) 3% salt scaffold. High resolution images can be obtained from Dr. Cardinal.

Figure 62: Representative 7-day BVM Hoechst Images. Proximal (left), middle (middle), and distal (right) locations. (A) ePTFE scaffold, (B) standards scaffold, (C) 0.5% salt scaffold, (D) 3% salt scaffold. High resolution images can be obtained from Dr. Cardinal.

The quantification results of BVM cell adhesion images are shown in Figure 63. No differences between image locations (proximal, middle, distal) were detected, so these images were averaged in the values seen in this figure. The 0.5% and 3% salt scaffold BVMs had greater cell coverage than the ePTFE and standard scaffold BVMs. Every scaffold type had a difference in coverage between the 3 and 7-day timepoints; however, the 7-day vessels had less coverage than the 3-day vessels for all scaffold types. It would be expected that the longer time point vessels would have a better cell coverage than the shorter time point.

Figure 63: BVM Cell Coverage in 3 and 7-Day BVMs. The * indicates a difference in 3-day cell coverage compared to the 3-day ePTFE group and a difference 7-day cell coverage compared to the 7-day ePTFE group ($p < 0.05$). The # indicates a difference in cell coverage between timepoints within the scaffold type ($p < 0.05$).

Cell viability was also of interest, which was determined through Caspase and Propidium Iodide activity. Figure 64 and Figure 65 show representative images of the Caspase and Propidium Iodide channels from every BVM type at each time point. The

quantification results of these images are shown in terms of estimated percent apoptotic and dead cells, as the Caspase and Propidium Iodide coverage was normalized to the cell coverage. The standard and 3% salt scaffold BVM groups at 3 and 7 days, as well as the 7-day time point of the 0.5% salt scaffold BVM group, had a higher percent of apoptotic cells than the ePTFE BVM group, shown in Figure 66. The 0.5% salt scaffold BVM was the only treatment group to show a difference between the 3 and 7-day timepoints, in which the 7-day BVMs had a higher percentage of apoptotic cells than the 3-day BVMs. For this group the 3-day time point showed levels of apoptosis similar to that of the ePTFE BVMs. Estimated dead cells did not reflect this pattern.

Figure 64: Images of 3-day BVM Caspase and Propidium Iodide Channels. Caspase channel (left) and Propidium Iodide channel (right) for all BVM types: (A) ePTFE scaffold, (B) standards scaffold, (C) 0.5% salt scaffold, (D) 3% salt scaffold. High resolution images can be obtained from Dr. Cardinal.

Figure 65: Images of 7-day BVM Caspase and Propidium Iodide Channels. Caspase channel (left) and Propidium Iodide channel (right) for all BVM types: (A) ePTFE scaffold, (B) standards scaffold, (C) 0.5% salt scaffold, (D) 3% salt scaffold. High resolution images can be obtained from Dr. Cardinal.

Figure 66: Percent Apoptotic Cells in 3 and 7-Day BVMs. The * indicates a difference in 3-day apoptosis percentage compared to the 3-day ePTFE group and a difference 7-day apoptosis percentage compared to the 7-day ePTFE group ($p < 0.05$). The & indicates a difference in apoptosis percentage from the ePTFE group compared only the 7-day time point ($p < 0.05$). The # indicates a difference in percent apoptosis between timepoints within the scaffold type ($p < 0.01$).

Figure 67 shows that there were no differences in cell death between BVM types at 3 days, and at 7 days only the 3% salt group was higher than the other three groups.

Figure 67: Percent Dead Cells in 3 and 7-Day BVMs. The * indicates a difference when compared to all other timepoints and scaffold types ($p < 0.01$).

3.4 Discussion and Conclusion

The purpose of Aim 2 was to establish methods with which to assess novel scaffolds through the cell-scaffold interactions. The primary measurements by which to achieve this were estimates of cell coverage and cell viability through measuring apoptotic and dead cells. It should be noted that it is possible for cells to be positive for both Caspase and Propidium Iodide¹⁷⁰.

3.4.1 Cytotoxicity Elution Assay

The cytotoxicity assay treated cells with diluted or undiluted media that had been eluted with standard or salt scaffolds. Eluted media was applied for 24 and 48-hour timepoints. This study found that the 3% salt treated groups resulted in more apoptotic

cells than the standard scaffold treated groups, either diluted or undiluted, until being normalized to the negative control. Once this data was normalized, this difference was no longer significant. This displays the importance of including appropriate controls within experiments; without removing the natural amount of apoptosis, incorrect conclusions could be drawn. The knowledge that the salt scaffold elutions were comparable to standard scaffolds in inducing apoptosis will be important when considering whether salt scaffolds should be used in BVMs.

After normalization, more dead cells were present at the 48-hour time point than the 24-hour time point for all treatment groups except the no dilution standard scaffold group. An unexpected trend was found between the diluted and undiluted treatments. It would be expected that the undiluted treatments would result in more dead cells because there are more cytotoxic substances than in the dilution group. However, for both types of scaffolds the dilution groups resulted in more Propidium Iodide positive cells than the undiluted treatments. This disconnect between how the elutions are affecting the cells implies an unknown variable affecting cell death.

While conclusions regarding the effectiveness of the dilution groups cannot be drawn, it can be concluded that undiluted eluted media did not cause extensive levels of apoptosis or cell death. The dilution groups were a preemptive measure. If the undiluted elutions, regardless of the scaffold type, were too cytotoxic to the cells and killed all treatment groups, the resulting data would have been largely inconclusive. Since the undiluted treatments did not result in high amounts of apoptosis and dead cells, the dilution groups would serve no purpose in future studies and should therefore not be

included. A control group of cell media heated at the elution settings should be added to future work to ensure that the elution process is not affecting the media characteristics.

Overall, this assay was useful in determining differences in cytotoxicity between scaffolds. It confirmed the results seen in the cytotoxicity study by Park et al in which a small planktonic crustacean was treated with BTEAC scaffold elution product and found to be unaffected¹⁶⁴. Since the scaffolds were not cytotoxic in this assay, further experiments were carried out to address direct scaffold cytotoxicity and cell adhesion.

3.4.2 Cell Adhesion and Viability Assay

Theoretically, the nanofibers of the salt scaffolds should improve cell adhesion compared to the microfibers of the standard scaffolds¹²⁸. The present work found that the 3% salt scaffolds had higher cell coverage than the standard scaffolds. Two to three times the number of cells were adhered to the salt scaffolds, even though half the number of cells had been initially sodded as compared to the standard scaffold. On top of that, a far greater percentage of those sodded cells were collected from the petri dish in which the scaffolds had been sodded. This speaks to the sodding efficiency of these scaffolds and implies that perhaps only $1.58E6$ cells/mL, or about $6.7E5$ cells/cm², would have been needed.

Another important observation was the resistance of the salt scaffolds during pressure sodding. The reason that less cells were sodded on the salt scaffolds as compared with the standard scaffolds was that more cell solution could not be physically forced through the salt scaffolds. It is possible that the cells quickly clogged the small pores in the salt scaffolds, blocking additional cells from being sodded. The standard

scaffolds did not react in this way; little resistance was felt during pressure sodding. One explanation for this is that the cells were either passing through the standard scaffold pores or were populating within the walls of the scaffolds. The latter is more likely, as a low percentage of the sodded cells was collected from the petri dish, indicating that the cells remained in the scaffold. This could be verified through hematoxylin and eosin staining of cross-sections of scaffold wall to determine cell infiltration.

The estimated percent of apoptotic cells in the 3% salt scaffold group was not found to be different than that of the standard scaffolds, continuing to support the utility of salt scaffolds as replacements for standard scaffolds. Even though the percent of apoptotic cells did not differ, the percent of dead cells did, with the salt scaffolds having a higher percentage of dead cells than standard scaffolds. Since this difference was not seen regarding apoptosis, one of two explanations could explain this data. Either cells are dying as a result of pathways outside of apoptosis, or the methods used to remove background noise from the Propidium Iodide channel were insufficient. The second option is more likely than the first because it is unlikely that many cells are dying as a result of necrosis instead of apoptosis. The Propidium Iodide channel had a high amount of background noise in the images, so pre-quantification alterations should be revisited during image analysis of future studies.

3.4.3 BVM Cell Coverage and Viability Assay

Since the salt scaffolds improved cell adhesion without affecting apoptosis in the previous two studies, the scaffolds were tested for efficacy in BVMs. The salt scaffolds required preshrinking, and this method was shown to be effective at eliminating

shrinkage before conditioning scaffolds in the bioreactors. Aside from this step, the salt scaffolds did not react differently than standard scaffolds when preparing them for or securing them in bioreactors. In fact, the salt scaffolds were often easier to secure to fittings because the material was more elastic than standard scaffolds, so the scaffold ends could be widened more easily without risk of tearing the scaffolds.

Cell coverage was greater in the 0.5% and 3% salt scaffold BVMs than the standard scaffold, which reflects the findings of the cell adhesion study. Cell adhesion between the two types of salt scaffolds was comparable. Regarding scaffold effects on apoptosis in BVMs, standard and 3% salt scaffold BVMs at both timepoints had a higher percent of apoptotic cells than the ePTFE BVM. The 7-day time point for the 0.5% salt scaffold also had this difference from the ePTFE scaffold, but the 3-day time point did not. The 0.5% salt scaffold was the only group to have a difference in apoptosis between timepoints. The 0.5% salt scaffold at 7 days only had a single repetition (as a result of contamination), so this difference may not present itself with increasing repetitions of 7-day BVMs. This difference did not translate to estimated dead cells. Only the 7-day 3% salt scaffold had a higher percentage of dead cells than any of the other treatment groups. The 0.5% salt group was comparable to the standard scaffold group at both timepoints.

While these findings support the utility of the salt scaffolds in BVMs, there was one surprising trend in cell coverage. Each treatment group had lower coverage at the 7-day time point than the 3-day time point. Since apoptosis did not increase between timepoints (except for the 0.5% salt scaffold BVM), scaffold cytotoxicity over time is unlikely to be the cause of this decreased coverage. Since this trend is consistent, it is possible that user error was a result. Since the two timepoints were not started on the

same day and not sodded with the same cell solution, variability in the number of sodded cells and how the cells were sodded on that day may exist. Since the same concentration of cells was used on both timepoints, differences in sodding technique each day may be the cause. To eliminate this possible variability in the future, sets of BVMs should include vessels of both timepoints.

This study also dispelled concerns about salt scaffold cytotoxicity through direct contact. Park et al. documented the use of electrospun BTEAC salt scaffolds as antiviral and antibacterial surfaces¹⁶¹. This antiviral and antibacterial characteristic could negatively affect cells in direct contact with the scaffold. However, the work in this thesis did not show that high or low concentrations of BTEAC salt scaffolds caused more cytotoxicity than standard PLGA scaffolds. Furthermore, even though these scaffolds were shown to be antibacterial by Park et al., a 0.5% salt scaffold did become contaminated with what was most likely bacteria. The contamination of this 0.5% salt scaffold BVM was unlike past contaminations of standard scaffold BVMs. Usually, the bioreactor and reservoir media changes color and becomes cloudy. However, this BVM only showed this color change in the reservoir and not in the bioreactor. This indicates that the salt scaffold contained the contamination to only the flow loop from the lumen of the scaffold to the bioreactor. If this is the case, then this suggests that salt scaffolds may allow little perfusion of media through the wall of the scaffolds.

3.4.4 Limitations and Future Work

A substantial limitation in this aim was the use of a poly-Caspase stain, which stained many types of Caspase in the cell. While this stain was useful for initial

investigations, it may have been nonspecific. Other stains are available which only stain for Caspase that are active in the execution pathway of the apoptosis cascade. Future work should investigate and, most likely, implement a more specific Caspase stain, such as the Image-IT LIVE Green Caspase-3 and 7 Detection Kit (Molecular Probes). This would only label cells in the execution pathway which will apoptose, unlike with other Caspases that may be active even though the cell will save itself from apoptosis.

The quantification method for analyzing cells on scaffolds was limited by several assumptions. The first assumption was the factor used to multiply the nucleus area to estimate the cell coverage. This is not necessarily an accurate estimate because it does not reflect coverage in areas where cells are clumped. Clumped cells reflect a higher cell coverage than should be represented. This factor, while limiting, does not change the trends seen by the data because the value is constant. Since it was possible for this factor to be incorrect, conclusions were not made that mentioned specific coverage percentages.

Another concern was the possibility that the Caspase antibody stained both active caspase and procaspases. The fluorochrome-labeled inhibitors of caspase (FLICA) method of labelling, which was utilized by the Caspase stain in these studies, binds the active centers of caspases with a fluoromethyl ketone (FMK)¹⁷¹. The active recognition sequence on which FMK binds is valine-alanine-aspartic acid (VAD); this active site should only be available in active caspases in which the small subunit and large subunit of the procaspase bind. Procaspase to not have this active site^{170,171}. However, a pro-caspase 3 monoclonal antibody (ThermoFisher Scientific) could be compared against an active caspase 3 immunostain (Abcam) to determine if this staining mechanism is nonspecific. Active caspase 3 should only be present in the execution phase of apoptosis,

while procaspase 3 should be present in non-apoptotic and apoptotic cells. Therefore, procaspase would be visible in healthy cells, while a high abundance of active caspase would only be present after an apoptosis-inducing treatment.

The BVM cell coverage and viability assay showed some cell clumping, which was not observed in the short-term cell adhesion and viability assay of cells on scaffolds in static media. The concern with this is that these clumps do not display standard endothelial morphology and have some areas that look like scaffold stained with antibodies. It was verified that salt and standard scaffolds do not autofluorescence using the Hoechst, Caspase, and Propidium Iodide stains, and that these cell clumps contained individual nuclei in the Hoechst channel. Despite this, future work should make note of cell clumping when working with the BTEAC salt scaffolds to ensure that cell clumping does not present as a continued issue.

These three studies did not contain enough repetitions to be statistically significant, which limits the conclusions drawn in this aim. The cost and labor associated with creating BVMs generally make a statistically designed study less plausible. However, power analyses of cell coverage, percent apoptotic cells, and percent dead cells of cell coverage for a power of 0.80 found that five repetitions per treatment is necessary for cell coverage and percent apoptotic cells, while six repetitions per treatment are necessary for percent dead cells. It would be possible to conduct an experiment with 48 vessels, but would require a huge effort by the Tissue Engineering Lab. It is, however, more plausible to design experiments for the cytotoxicity elution assay and short-term cell adhesion assay that include enough repetitions of each treatment group to be statistically significant. Power assessments found that 6 scaffolds of each type are

necessary for a statistically designed cytotoxicity elution study, while 3 scaffolds of each type are necessary for a short-term adhesion study. Future studies of scaffold cytotoxicity and cell adhesion should be planned and executed for both assays to draw conclusions backed by statistical repetitions.

Modifications could be made to the elution protocol in future studies by concentrating the eluted media. This would allow for control of the intensity of the media in which is being delivered to cells. Using a more concentrated product may make evident trends that could not be identified with less concentrated eluted media. Concentration of the media could be achieved through filtration using the Ultrafree15 (Sigma-Aldrich) or through phenol extraction¹⁷².

Other experiments and analysis could be conducted to investigate the use of these scaffolds in BVMs. BVM cross-sections could be stained to compare and determine differences in cell penetration into the scaffold walls. Cryosectioning scaffolds and staining with hematoxylin and eosin would provide this information.

Since the purpose of the BVM model is to test vascular devices, scaffold types should eventually be compared for use as BVMs with stents deployed within them. If HUVEC-only BVMs using salt scaffolds displayed similar efficacy to dual-sodded standard scaffolds with stents deployed in both groups, the salt scaffolds would be superior due to the cell sodding efficiency and simplicity of EC-only BVMs and could be implemented for future BVM studies.

3.4.5 Summary

This aim was successful in implementing methods and comparing scaffold cytotoxicity and cell adhesion between BTEAC salt and standard PLGA scaffolds. No differences were seen in apoptotic activity between the 3% salt and standard scaffolds in the cytotoxicity elution assay or short-term adhesion assay. This trend continued between the 3% salt and standard scaffolds in BVMs, while the 0.5% scaffold had less activity at the shorter time point but caught up by the 7-day time point. Cell coverage was superior in the salt scaffolds. With this knowledge, it can be concluded that the salt scaffolds are comparable to the standard scaffolds regarding cytotoxicity but superior in cell adhesion and coverage. However, further comparisons of the 0.5% and 3% salt scaffolds still must be conducted. This conclusion may rely upon some physical evaluations of 0.5% and 3% salt scaffolds, such as yield strength and fiber diameter. Regardless, testing the interaction of cells on electrospun BTEAC salt scaffolds is novel, as it has not been previously documented in published literature. The results in this aim support the conclusion that standard, 0.5% salt, and 3% salt scaffolds have similar cell viability, as assessed through Caspase and Propidium Iodide activity, and salt scaffolds have higher cell adhesion and coverage than standard scaffolds.

Chapter 4: DISCUSSION AND CONCLUSION

4.1 Summary and Aims of the Thesis

BVMs have proven useful as a preclinical model for testing vascular devices by providing initial data on how the device interacts with cells^{4,9}. This success warrants further investigation into the individual components of the model. Improvements to these components must be justified and reinforced before implementing them as standard procedures. Therefore, after identifying an area of improvement, methods must be established to measure changes in component responses.

The overall goal of this thesis was to establish methods to characterize the endothelial cells used in the BVM model. The endothelial cell component is, arguably, the most important part of the model since the EC interaction with vascular devices is the most important measured response¹⁷³. Two aspects of EC involvement were identified which could be improved but lacked established methods to support any changes. Aim 1 established methods to compare EC gene expression at different passages and in different flow conditions in the context of studying EC dedifferentiation. The use of ECs that respond like endothelial cells and appropriately express endothelial markers is necessary to claim that the cell interaction with the vascular device is reflective of an interaction that would occur *in vivo*. Aim 2 established methods to compare standard PLGA scaffolds to BTEAC salt-PLGA scaffolds through studying scaffold cytotoxicity on ECs and EC cell coverage at varying lengths of time. These methods and the subsequent results contributed to the model in two ways. The Tissue Engineering lab had no previous methods for implementing new scaffolds despite having investigated and designed other

types of scaffolds in the past, so this aim established methods that can be used for future scaffolds. This aim also identified and tested a novel scaffold that could be implemented as an improved scaffold for creating BVMs.

4.2 Results and Discussion

Aim 1 can be divided into two subsections: changes in EC gene expression over culture time, and changes in EC gene expression as a result of various flow conditions. EndMT would result in downregulation of endothelial markers and upregulation of mesenchymal markers, while ECs which retain their phenotype would increase expression of VEGFR2 and VE-cad through VEGF signaling. In the first set of experiments, a trend was identified of increasing expression of the endothelial markers VE-cadherin and VEGFR2 as culture time increased. The EC marker PECAM, as well as the mesenchymal markers ACTA2 and Calponin, remained stable as culture time increased. These trends do not support culture time contributing to EC dedifferentiation; EC dedifferentiation would result in loss of EC markers and an increase in mesenchymal markers.

The second set of experiments in this aim studied gene expression changes in ECs cultured in static, disturbed, or laminar flow conditions. The establishment of a disturbed flow condition was successful in that it showed a decrease in VE-cadherin and VEGFR2 abundance compared to ECs in static culture. Unfortunately, the disturbed flow condition had to be removed from the analysis. The dissociation curves for this group showed untargeted amplification during qPCR, indicating contaminants within the RNA or cDNA. However, the static culture group was still compared to the laminar flow group of

the BVMs. This found that the laminar flow group had lower expression of all three endothelial markers and ACTA2 than the static culture group, which contradicts trends seen in literature. These findings are likely explained by the 24-hour time point used for the BVM, which may have not allowed enough time for ECs to re-establish the cell-cell junctions measured by the EC markers.

The investigations in Aim 1 were largely inconclusive, although the methods established can be applied to ongoing and future work. Some trends were identified, but they did not provide evidence of EC dedifferentiation occurring. These trends are positive for the BVM model, as they suggest cells retaining an EC phenotype. However, all studies in this Aim were investigational for method development and not statistically powered. Properly powered studies would have been conducted had the initial data suggested that further studies would provide useful data. Unfortunately, this was not the case, so further studies were not pursued. Despite this, the methods developed here may prove useful in future studies, which will be discussed in Section 4.4, so gene expression analysis through qPCR should continue to be implemented as an analysis technique by the Tissue Engineering Lab.

Aim 2 was composed of three parts corresponding to each of the three assays developed. In the first part, the cytotoxicity elution assay treated ECs with scaffold-eluted media for 24 or 48 hours. No differences were found in apoptosis between scaffold type, and more dead cells were found in the 48-hour group regardless of scaffold type. Since the change in dead cells was not a factor of scaffold type and apoptotic activity was not different between scaffold types, it was concluded that salt scaffolds did not adversely

affect cell viability. This elution assay did not test viability through direct cell contact, but the two other assays did.

In the second part, the cell adhesion and viability assay looked at initial cell deposition and apoptotic activity at a 4-hour timepoint. The salt scaffold did not differ in Caspase activity compared to standard scaffolds, but cell death was higher in the salt scaffold group than the standard scaffold group. Cell adhesion was higher for salt scaffolds than standard scaffolds. This suggested that, while more HUVECs adhered at a short timepoint, the cells may not persist as time increased.

The final part of this aim, the BVM cell coverage and viability assay, was similar to the previous assay but compared scaffold types in BVMs over multiple days. Cell coverage was higher in the 0.5% and 3% salt scaffolds than standard scaffolds regardless of time point. Apoptosis was not higher in the salt scaffolds than the standard scaffolds. This was also true for cell death, except in the 7-day 3% salt scaffold group which had more cell death than any of the other scaffold types at that time point. These findings suggested that HUVECs had similar rates of survival but better coverage in BTEAC salt scaffolds than standard PLGA scaffolds.

These three parts of Aim 2 found that apoptosis did not differ between standard PLGA scaffolds and salt scaffolds, but cell adhesion and coverage was notably improved for salt scaffolds. The only adverse effect of the salt scaffolds was regarding cell death. This may have been due to the quantification method because it would be expected that apoptotic activity would reflect the trends of cell death, which was not seen. The form in which cells died may have also contributed to this result; if cells died through necrosis, the apoptosis pathway and caspases would not have been activated. A comparison of

apoptosis to necrosis could be evaluated through immunofluorescence using an Apoptosis/Necrosis Assay Kit (Abcam), which identifies all cells, apoptotic cells, and necrotic cells. Nonetheless, it can be concluded from this data that the salt scaffolds are superior to the standard scaffolds. The 0.5% salt scaffold is recommended as the scaffold candidate to pursue if the comparison of fiber diameter and other physical characteristics in the 0.5% and 3% salt scaffolds do not reveal significant differences.

4.3 Challenges and Limitations

The greatest limitation of both Aim 1 and Aim 2 was the lack of statistical significance in any of the studies. Since both aims were investigational and intended for method development, the experiments were not designed with statistical power. The intent of these studies was to properly design experiments if the initial pilots revealed promising information. For Aim 1, this was not the case, which lead to no statistically powered studies being conducted. For Aim 2, the pilot studies did reveal promising information that warranted full studies to be conducted, which will be discussed in the next section.

A similar limitation has to do with designing properly powered experiments that use BVMs. The amount of resources and time necessary to produce a small amount of BVMs makes it difficult to design and complete properly powered experiments. In order to statistically power Aim 2, 48 BVMs would have been necessary. This is not a practical amount of BVMs to fabricate for a single study. Issues with contamination can further reduce the repetitions of treatment groups. Triplicates of each BVM treatment group is a feasible goal that could be used in place of a statistically powered study.

The main challenges in Aim 1 were with the disturbed flow condition. In the second set of experiments, which compared the disturbed flow condition to a negative control (static EC media treated) and positive control (static SMC media treated), there was a high amount of cell death which yielded too low of RNA concentrations to run qPCR on the samples. As a result, only one of the disturbed flow passages could be compared with the other two treatments, so comparisons of expression change over culture time were lost. There were also issues with the disturbed flow treatment group in the third experiment comparing all three flow groups. A ramp up period was introduced in this study, which greatly reduced the cell death in the disturbed flow group and a high enough concentration of RNA was isolated to run qPCR. However, the dissociation curve for this group showed nonspecific targets in the qPCR products, so they were removed from the study. It was unclear at which step this issue occurred.

A main limitation of Aim 2 was having no SEM images of BVMs, as it was initially planned to include these images. This would have provided additional images from which to observe cell coverage. These images may have been able to visualize differences cell morphology on the scaffold types. Unfortunately, the SEM was broken, and images could not be taken in time to include in this thesis.

4.4 Future Work

Although this thesis served to establish methods to characterize endothelial cells for specific applications, these methods are generic and can be implemented for other studies. Many other applications of these methods may exist and will prove to be useful in future studies that have not yet been identified. These sections outline some

applications that could be investigated in the future, and some applications that are already being investigated.

A study that links Aim 1 and Aim 2 would be an investigation of gene expression changes with different scaffold types. This could provide further data to support implementing salt scaffolds for general use by providing evidence of scaffold type promoting an endothelial phenotype or endothelial growth. Since calponin did not prove to be a useful gene in characterizing EC expression changes, that marker could be removed from the GOI and replaced by a proliferation marker like Ki67. This marker would indicate whether the seeded cells were quiescent, or whether the cells were proliferating in the BVM. The seeding density used within BVMs should not promote proliferation, but this marker would provide information to determine scaffold effects on proliferative potential.

Aim 1 has already proven effective at steering current work. The previous immunostaining protocol implemented in the Tissue Engineering Lab used PECAM and ACTA2 as markers to discern between ECs and SMCs, respectively. However, as previously established, HUVECs co-expressed PECAM and ACTA2. Through the gene expression studies in Aim 1, it was clear that Calponin was not expressed in HUVECs. However, Aim 1 did establish that Calponin was abundant in HUASMCs, making Calponin an excellent marker to replace ACTA2 for immunostaining. This recommendation was implemented, and no signal was observed in immunostaining for Calponin in HUVECs. The Calponin stain also proved to be a clear marker for immunostaining HUASMCs, shown in Figure 68.

Figure 68: Calponin immunostaining of HUVECs and HUASMCs. No Calponin signal is seen in HUVECs (top row), while Calponin signal is abundant in HUASMCs (bottom row).

The three EC markers used in Aim 1 could also be applied for studies related to changing flow rates within BVMs. The current maximum pump rate used in BVMs is 90 RPMs, which translates to only 5.9 mL of media being pushed through BVMs each minute. Increasing this pump rate or increasing the viscosity of media would increase shear stress, which is currently far below physiologic levels. Aim 1 showed HUVECs survived under higher levels of shear stress in the disturbed flow condition, which had shear stress values ranging from 5 to 15 dyne/cm². Changes to the pump rate may be reflected by changes in the mechanosensory complex genes.

Regarding Aim 2, and as mentioned in Section 4.3, a major limitation was not running statistically powered studies. The data from the cytotoxicity elution study and short time point adhesion study provided useful data regarding cytotoxicity and cell adhesion, so properly designed studies using both assays have been planned. The cytotoxicity study will include three time points (24, 48, 72 hours) and three scaffold

types (standard, 0.5% salt, 3% salt), but will eliminate the dilution group. The 4-hour adhesion and viability assay will add the 0.5% salt scaffold group as well. These two experiments will continue to build upon conclusions made in Aim 2.

The cytotoxicity elution assay is also already being implemented for testing the cytotoxicity of aneurysm scaffolds. Wax is molded in the shape of the aneurysm on a mandrel before electrospinning the mandrel. After electrospinning, the wax is dissolved in a warm media solution to remove the wax. However, some wax remains in the lumen of the scaffold after dissolving. Multiple types of wax have been used to make these aneurysm geometries. An ongoing study uses the elution assay to determine whether wax remaining in the scaffold is cytotoxic, and whether there is a difference in toxicity between wax types.

Future process adjustments can utilize the cytotoxicity assay to support viability assessments. A possible application is switching from ethanol sterilization of scaffolds to ethylene oxide (EtO) sterilization. Concern of EtO gas residuals in the scaffolds has always precluded this prospect. Current methods for testing EtO residuals involve extraction in water, so the cytotoxicity elution assay would be an appropriate method to determine the effect of residuals on cell viability¹⁷⁴. Most importantly, EtO sterilization would substantially simplify the BVM fabrication process; scaffolds could be secured in bioreactors before sterilization, so only media would need to be added to the bioreactors and the flow circuit assembled before starting conditioning. No sterile gloves would need to be used since the scaffolds would not need to be flushed or secured in the sterile bioreactor. The removal of these steps would considerably decrease the amount of time necessary to create BVMs. Currently, it takes about 10 hours to set up six BVMs.

However, the removal of these steps would decrease that time to approximately five hours, which would allow the Tissue Engineering Lab to substantially increase the amount of BVMs produced. EtO sterilization is also a superior sterilization method. Ethanol sterilization is considered a chemical disinfectant, not a sterilization process, because certain viruses and bacterial spores are resistant to the effects of ethanol¹⁷⁵. An experiment could be run which used the cytotoxicity elution assay or direct contact assays to determine how EtO sterilized scaffolds affect cell viability.

A discussion of the future use of salt scaffolds must also be had within the Tissue Engineering Lab. Based on the literature, nanofiber scaffolds will likely be advantageous to implement because of the superiority to microfibers, and the data in Aim 2 suggests that the BTEAC salt scaffolds are a viable option for fabricating nanofibers. The question is not if, but when, nanofiber scaffolds replace the current microfiber scaffolds. A discussion must be had of what further testing of these scaffolds needs to be completed to justify implementation. Some potential further testing includes mechanical testing of scaffold characteristics like yield strength, fiber diameter assessment in larger data sets to ensure consistent sized nanofibers, and deployment of intravascular devices within BVMs using these scaffolds to determine that these scaffolds are useful for obtaining safety data of intravascular devices. Logistics of salt scaffold use must be explored as well, such as whether one or two cell types will be used, and the appropriate seeding density.

4.5 Conclusion

Aim 1 of this thesis established methods for evaluating endothelial cells and BVMs through gene expression analysis and results supported the conclusion that HUVECs used in BVMs are retaining an endothelial phenotype during culture and not dedifferentiating from EndMT. In fact, longer culture lengths trended towards increased expression of VEGFR2 and VE-cadherin, indicating a stronger endothelial phenotype. Disturbed flow conditions were established as a positive control for dedifferentiation, which decreased VEGFR2 and VE-cadherin expression. Gene expression comparisons of BVMs to static and disturbed flow cultures were inconclusive.

Aim 2 of this thesis successfully developed protocols to evaluate scaffold cytotoxicity, as well as cell viability and coverage. These assays were applied to novel BTEAC salt scaffolds to compare against standard PLGA scaffolds. These studies found that BTEAC salt scaffolds do not affect cytotoxicity and cell viability more than standard PLGA scaffolds. The BTEAC salt scaffolds also have more cell coverage than standard scaffolds at all timepoints. Therefore, the integration of BTEAC salt scaffolds into the BVM model should be considered, replacing the current scaffolds.

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APPENDICES

Appendix A: Preverified Primer Sequences and Characteristics

Primer Name	Primer Sequence	Exon Location	Molecular Weight (Da)	Extinction Coefficient (L/(mol*cm))	GC Content (%)
PECAM 1 Primer 1	5'-ATTGCTCTGGTCACTTCTCC-3'	1b-2b	6,009.9	172,300.0	50.0
PECAM 1 Primer 2	5'-CAGGCCCCATTGTTCCC-3'	1b-2b	5,082.3	146,700	64.7
CDH5 (VE-cadherin) Primer 1	5'-TGCCCACATATTCTCCTTTGAG-3'	2-3	6,636.4	198,100.0	45.5
CDH5 (VE-cadherin) Primer 1	5'-GAACCAGATGCACATTGATGAAG-3'	2-3	7,105.7	237,000.0	43.5
KDR (VEGFR 2) Primer 1	5'-GAGGATCTTGAGTTCAGACATGAG-3'	18-19	7,456.9	244,800.0	45.8

KDR (VEGFR 2) Primer 2	5'- TTGGAATTGACAAGACAGCA AC-3'	18-19	6,776.5	224,000.0	40.9
ACTA2 (α SMA) Primer 1	5'- CTGTTGTAGGTGGTTTCATG GA-3'	8-9	6,827.5	211,700.0	45.5
ACTA2 (α SMA) Primer 2	5'- AGAGTTACGAGTTGCCTGAT G-3'	8-9	6,501.3	209,000.0	47.6
CNN1 Primer 1	5'- CATGAAGTTGTTGCCGATGC -3'	1-2	6,148.0	189,400.0	50.0
CNN1 Primer 2	5'- TCAGCCGAGGTTAAGAACA AG-3'	1-2	6,488.3	216,700.0	47.6
ACTB Primer 1	5'-CCTTGCACATGCCGGAG- 3'	1-2	5,171.4	155,300.0	64.7
ACTB Primer 2	5'-ACAGAGCCTCGCCTTTG-3'	1-2	5,146.4	154,700.0	58.8
GUS β Primer 1	5'- GTTTTTGATCCAGACCCAGA TG-3'	10-11	6,725.4	210,600.0	45.5
GUS β Primer 2	5'- GCCCATATTTCAGAGCGAGT A-3'	10-11	6,430.2	208,900.0	47.6

Appendix B: Primer Efficiency Testing SOP

Appendix C: RNA Isolation and Evaluation SOP

Appendix D: cDNA through Reverse Transcription SOP

Appendix E: qPCR SOP

Appendix F: qPCR Data Analysis SOP

Appendix G: Primer Efficiency Plots

Appendix H: BVM Conditioning SOP

Appendix I: Endothelial Cell-only BVM Sodding SOP

Appendix J: Apoptosis Kit Staining SOP

Appendix K: Cytotoxicity Elution Assay SOP

Appendix L: Scaffold Adhesion Assay SOP

Appendix M: Positive Control Test Representative Images

	Hoechst	Caspase	Propidium Iodide
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DMSO 2% 30 minute s 40x
DMSO 5% 30 minute s 40x
DMSO 10% 30 minute s 40x
DMSO 15% 30 minute s 40x

H2O2 0.05% 2 minute s 40x
H2O2 0.1% 2 minute s 40x
H2O2 0.5% 2 minute s 40x
H2O2 1% 2 minute s 40x

H2O2 0.05% 5 minute s 40x
H2O2 0.1% 5 minute s 40x
H2O2 0.5% 5 minute s 40x
H2O2 1% 5 minute s 40x

Appendix N: Cytotoxicity Elution Assay Representative Images

24 Hours, 40x

	Hoechst	Caspase	Propidium Iodide
Negative Control
Positive Control 0.5%
Positive Control 1%

Standard Scaffold 1 50% Dilution
Standard Scaffold 2 50% Dilution
3% Salt Scaffold 1 50% Dilution
3% Salt Scaffold 2 50% Dilution

48 Hours, 40x

	Hoechst	Caspase	Propidium Iodide
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Negative Control
Positive Control 0.5%
Positive Control 1%
Standard Scaffold 1 No Dilution

Standard Scaffold 2 No Dilution
3% Salt Scaffold 1 No Dilution
3% Salt Scaffold 2 No Dilution
Standard Scaffold 1 50% Dilution

Standard Scaffold 2 50% Dilution
3% Salt Scaffold 1 50% Dilution
3% Salt Scaffold 2 50% Dilution

Appendix O: Scaffold Adhesion Assay Representative Images

	Hoechst	Caspase	Propidium Iodide
Negative Control of Standard Scaffold
Negative Control of Salt Scaffold
Standard Scaffold 1 Proximal

Standard Scaffold 1 Middle
Standard Scaffold 1 Distal
Standard Scaffold 2 Proximal
Standard Scaffold 2 Middle

Standard Scaffold 2 Distal
3% Salt Scaffold 1 Proximal
3% Salt Scaffold 1 Middle
3% Salt Scaffold 1 Distal

3% Salt Scaffold 2 Proximal
3% Salt Scaffold 2 Middle
3% Salt Scaffold 2 Distal

Appendix P: BVM Study Representative Images

	Hoechst	Caspase	Propidium Iodide
ePTFE 3-Day Proximal
ePTFE 3-Day Middle
ePTFE 3-Day Distal

ePTFE 7-Day Proximal
ePTFE 7-Day Middle
ePTFE 7-Day Distal
Standard Scaffold 3-Day Proximal

Standard Scaffold 3-Day Middle
Standard Scaffold 3-Day Distal
Standard Scaffold 7-Day Proximal
Standard Scaffold 7-Day Middle
Standard Scaffold 7-Day Distal

Appendix Q: Magnification Justification Raw Data

Cytotoxicity 24 Hours 40x

Image Name	Manual Cell Count	Automated Cell Count	Difference (Automated-Manual)	Threshold (1st slider)	Smallest Cell Size (pizels^2)	Cell Density	Notes	Percent Error
No Dilution No Salt 1 40x 1	44	34	-10	863	20	Medium		29.41176471
No Dilution No Salt 1 40x 2	57	59	2	1371	30	Low		3.389830508
No Dilution No Salt 2 40x 1	72	64	-8	1776	30	High		12.5
No Dilution No Salt 2 40x 2	46	43	-3	1390	30	Medium		6.976744186
No Dilution No Salt 2 40x 3	16	18	2	1466	30	Low		11.11111111
No Dilution No Salt 2 40x 4	22	24	2	1729	50	Low	Lots of cell debris	8.333333333
No Dilution Salt 1 40x 1	68	53	-15	1070	50	Medium		28.30188679
No Dilution Salt 1 40x 2	68	70	2	1546	30	Low		2.857142857

No Dilution Salt 1 40x 3	30	30	0	1005	50	Low		0
No Dilution Salt 1 40x 4	101	111	10	1070	50	Medium		9.009009009
No Dilution Salt 1 40x 5	105	111	6	1101	50	Medium		5.405405405
No Dilution Salt 2 40x 1	110	123	13	1031	50	Medium		10.56910569
No Dilution Salt 2 40x 2	108	111	3	1014	50	Medium		2.702702703
No Dilution Salt 2 40x 3	28	30	2	1009	50	Low		6.666666667
No Dilution Salt 2 40x 4	40	40	0	1000	50	Low		0
							Average Error	9.148980198

Cytotoxicity 24 hours 20x

Image Name	Manual Cell Count	Automated Cell Count	Difference (Automated-Manual)	Threshold (1st slider)	Smallest Cell Size (pizels^2)	Cell Density	Notes	Error
No Salt 1 20x 1	17	17	0	1009	20	Low		0
No Salt 1 20x 2	42	37	5	1334	20	Medium		13.51351351
No Salt 1 20x 3	31	23	8	1008	20	Low		34.7826087
No Salt 1 20x 4	81	43	38	1009	20	High		88.37209302

No Salt 2 20x 1	127	73	54	1102	20	Medium		73.97260274
No Salt 2 20x 2	22	20	2	1084	20	Low		10
No Salt 2 20x 3	100	101	-1	1101	20	Medium		0.9900990099
No Salt 2 20x 4	101	64	37	1181	20	High		57.8125
No Salt 2 20x 5	120	90	30	1087	20	High		33.33333333
Salt 1 20x 1	49	22	27	1087	20	Medium		122.7272727
Salt 1 20x 2	55	35	20	1060	20	High		57.14285714
Salt 1 20x 4	84	67	17	811	20	Medium		25.37313433
Salt 2 20x 1	96	82	14	1149	20	Low		17.07317073
Salt 2 20x 2	74	79	-5	1027	20	Medium		6.329113924
Salt 2 20x 3	59	51	8	1009	20	Medium		15.68627451
Salt 2 20x 4	66	36	30	1227	20	Low		83.33333333
							Average	40.02761919

Appendix R: Pixel Intensity Reduction Raw Data

Propidium Iodide	Min Intensity	Cell Count	Percent Cell Count Reduction	Average Percent Cell Count Reduction
	0	22	0	0
	5	21	4.545454545	1.515151515
	10	21	4.545454545	4.62701022
	15	21	4.545454545	6.25302648
	20	18	18.18181818	10.79848103
	25	15	31.81818182	20.08181054
	30	13	40.90909091	27.17715422

	35	10	54.54545455	36.79690088
	40	9	59.09090909	42.37709305
	45	5	77.27272727	50.8767235
	50	3	86.36363636	57.15905905
	55	1	95.45454545	64.11422892
	60	1	95.45454545	64.11422892
	65	1	95.45454545	64.11422892
	70	1	95.45454545	65.2636542
	75	1	95.45454545	65.2636542
	80	1	95.45454545	68.51568672
	0	41	0	
	5	41	0	
	10	40	2.43902439	
	15	38	7.317073171	
	20	38	7.317073171	
	25	35	14.63414634	
	30	30	26.82926829	
	35	28	31.70731707	
	40	23	43.90243902	
	45	20	51.2195122	
	50	16	60.97560976	
	55	14	65.85365854	
	60	14	65.85365854	
	65	14	65.85365854	
	70	14	65.85365854	
	75	14	65.85365854	
	80	10	75.6097561	
	0	29	0	
	5	29	0	
	10	27	6.896551724	
	15	27	6.896551724	
	20	27	6.896551724	
	25	25	13.79310345	
	30	25	13.79310345	
	35	22	24.13793103	
	40	22	24.13793103	
	45	22	24.13793103	
	50	22	24.13793103	
	55	20	31.03448276	
	60	20	31.03448276	
	65	20	31.03448276	
	70	19	34.48275862	

	75	19	34.48275862	
	80	19	34.48275862	
Caspase	Min Intensity	Cell Count	Percent Cell Count Reduction	Average Percent Cell Count Reduction
	0	22	0	0
	5	22	0	0
	10	21	4.545454545	2.727272727
	15	18	18.18181818	10.71566731
	20	15	31.81818182	17.38878143
	25	15	31.81818182	17.38878143
	30	12	45.45454545	26.48613798
	35	11	50	28.6073501
	40	11	50	29.81947131
	45	11	50	37.81431335
	50	11	50	37.81431335
	55	8	63.63636364	43.77820761
	60	6	72.72727273	52.88201161
	65	6	72.72727273	55.00967118
	70	6	72.72727273	56.4281109
	75	4	81.81818182	64.61637653
	80	3	86.36363636	67.34364926
	0	55	0	
	5	55	0	
	10	53	3.636363636	
	15	52	5.454545455	
	20	52	5.454545455	
	25	52	5.454545455	
	30	48	12.72727273	
	35	47	14.54545455	
	40	45	18.18181818	
	45	40	27.27272727	
	50	40	27.27272727	
	55	40	27.27272727	
	60	37	32.72727273	
	65	37	32.72727273	
	70	37	32.72727273	
	75	32	41.81818182	
	80	30	45.45454545	
	0	47	0	
	5	47	0	
	10	47	0	
	15	43	8.510638298	

	20	40	14.89361702	
	25	40	14.89361702	
	30	37	21.27659574	
	35	37	21.27659574	
	40	37	21.27659574	
	45	30	36.17021277	
	50	30	36.17021277	
	55	28	40.42553191	
	60	22	53.19148936	
	65	19	59.57446809	
	70	17	63.82978723	
	75	14	70.21276596	
	80	14	70.21276596	

Appendix S: Nuclei Area Multiplication Factor Raw Data

Cell	Nucleus Size	Whole Cell Size	Nucleus %	Multiplication Factor
1	14	84	16.66666667	6
2	29	209	13.87559809	7.206896552
3	48	171	28.07017544	3.5625
4	26	135	19.25925926	5.192307692
5	38	137	27.73722628	3.605263158
6	50	158	31.64556962	3.16
7	44	84	52.38095238	1.909090909

8	22	100	22	4.545454545
9	44	105	41.9047619	2.386363636
10	57	169	33.72781065	2.964912281
11	27	141	19.14893617	5.222222222
12	20	161	12.42236025	8.05
13	24	110	21.81818182	4.583333333
14	40	328	12.19512195	8.2
15	26	173	15.02890173	6.653846154
16	29	161	18.01242236	5.551724138
17	52	245	21.2244898	4.711538462
18	23	91	25.27472527	3.956521739
19	29	158	18.35443038	5.448275862
20	21	119	17.64705882	5.666666667
		Nucleus % Average	23.41973244	
		Multiplication Factor	4.269903606	

Appendix T: Statistical Tests for Cytotoxicity Elution Assay

ANOVA for Caspase Activity without normalizing to negative control

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	13	39209.060	3016.08	7.5080
Error	73	29325.301	401.72	Prob > F
C. Total	86	68534.361		<.0001*

Parameter Estimates				
Term	Estimate	Std Error	t Ratio	Prob> t
Intercept	46.277194	2.263431	20.45	<.0001*
Duration[24 Hours]	-7.074356	2.263431	-3.13	0.0025*
Treatment[Negative Control]	-27.12803	5.917156	-4.58	<.0001*
Treatment[Normal 50% Dilution]	-18.48435	4.801759	-3.85	0.0003*
Treatment[Normal No Dilution]	-21.17104	5.238387	-4.04	0.0001*
Treatment[Positive Control 0.5%]	30.289234	6.402395	4.73	<.0001*
Treatment[Positive Control 1%]	27.596974	6.402395	4.31	<.0001*
Treatment[Salt 50% Dilution]	6.0206489	4.933343	1.22	0.2262
Duration[24 Hours]*Treatment[Negative Control]	3.606543	5.917156	0.61	0.5441
Duration[24 Hours]*Treatment[Normal 50% Dilution]	3.3408742	4.801759	0.70	0.4888
Duration[24 Hours]*Treatment[Normal No Dilution]	-0.312345	5.238387	-0.06	0.9526
Duration[24 Hours]*Treatment[Positive Control 0.5%]	-7.778947	6.402395	-1.22	0.2283
Duration[24 Hours]*Treatment[Positive Control 1%]	10.86819	6.402395	1.70	0.0939
Duration[24 Hours]*Treatment[Salt 50% Dilution]	-9.687699	4.933343	-1.96	0.0534

Level					Least Sq Mean
48 Hours,Positive Control 0.5%	A				91.419731
24 Hours,Positive Control 1%	A B				77.668002
48 Hours,Positive Control 1%	A B C				70.080334
48 Hours,Salt 50% Dilution	A B				69.059898
24 Hours,Positive Control 0.5%	A B C D				61.713125
48 Hours,Salt No Dilution	A B C D				56.264733
24 Hours,Salt No Dilution	B C D E				42.042789
24 Hours,Salt 50% Dilution	B C D E				35.535787
48 Hours,Normal No Dilution	C D E				32.492856
48 Hours,Normal 50% Dilution	C D E				31.526323
24 Hours,Normal 50% Dilution	D E				24.059359
48 Hours,Negative Control	C D E				22.616975
24 Hours,Normal No Dilution	D E				17.719454
24 Hours,Negative Control	E				15.681349

Level				Least Sq Mean
Positive Control 0.5%	A			76.566428
Positive Control 1%	A			73.874168
Salt 50% Dilution	B			51.680554
Salt No Dilution	B			49.202730
Normal 50% Dilution	C			27.792841
Normal No Dilution	C			25.073006
Negative Control	C			19.956144

ANOVA for Caspase Activity after normalizing to negative control

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	19395.360	2770.77	0.7845
Error	8	28254.009	3531.75	Prob > F
C. Total	15	47649.369		0.6190

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob> t
Intercept	37.229336	14.85713	2.51	0.0366*
Duration 2[24 Hours]	-13.64566	14.85713	-0.92	0.3852
Group.[50% Dilution]	2.677638	14.85713	0.18	0.8615
Scaffold Type.[Normal]	-19.41099	14.85713	-1.31	0.2277
Duration 2[24 Hours]*Group.[50% Dilution]	4.302638	14.85713	0.29	0.7795
Duration 2[24 Hours]*Scaffold Type.[Normal]	22.964011	14.85713	1.55	0.1608
Group.[50% Dilution]*Scaffold Type.[Normal]	1.3406457	14.85713	0.09	0.9303
Duration 2[24 Hours]*Group.[50% Dilution]*Scaffold Type.[Normal]	9.7156457	14.85713	0.65	0.5315

ANOVA for Propidium Iodide Activity without normalizing to negative control

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	13	43554.082	3350.31	11.2222
Error	73	21793.654	298.54	Prob > F
C. Total	86	65347.737		<.0001*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob> t	Lower 95%	Upper 95%
Intercept	25.123931	1.951242	12.88	<.0001*	21.235112	29.012751
Duration[24 Hours]	-20.08243	1.951242	-10.29	<.0001*	-23.97125	-16.19361
Treatment[Negative Control]	-5.085755	5.101018	-1.00	0.3221	-15.25207	5.0805589
Treatment[Normal 50% Dilution]	-4.75773	4.139465	-1.15	0.2542	-13.00767	3.492211
Treatment[Normal No Dilution]	-13.82881	4.51587	-3.06	0.0031*	-22.82892	-4.828691
Treatment[Positive Control 0.5%]	10.676368	5.519329	1.93	0.0569	-0.323638	21.676374
Treatment[Positive Control 1%]	11.523457	5.519329	2.09	0.0403*	0.5234507	22.523463
Treatment[Salt 50% Dilution]	9.5481082	4.252899	2.25	0.0278*	1.0720918	18.024125
Duration[24 Hours]*Treatment[Negative Control]	10.155419	5.101018	1.99	0.0502	-0.010895	20.321732
Duration[24 Hours]*Treatment[Normal 50% Dilution]	5.3469688	4.139465	1.29	0.2005	-2.902972	13.59691
Duration[24 Hours]*Treatment[Normal No Dilution]	10.193553	4.51587	2.26	0.0270*	1.1934376	19.193668
Duration[24 Hours]*Treatment[Positive Control 0.5%]	-13.36712	5.519329	-2.42	0.0179*	-24.36713	-2.367118
Duration[24 Hours]*Treatment[Positive Control 1%]	-13.03184	5.519329	-2.36	0.0209*	-24.03184	-2.031829
Duration[24 Hours]*Treatment[Salt 50% Dilution]	-7.451075	4.252899	-1.75	0.0840	-15.92709	1.0249415

Level			Least Sq Mean
48 Hours,Positive Control 1%	A		69.761651
48 Hours,Positive Control 0.5%	A		69.249851
48 Hours,Salt 50% Dilution	A		62.205542
48 Hours,Normal 50% Dilution	A B		35.101660
48 Hours,Negative Control	A B C		29.965186
48 Hours,Salt No Dilution	B C		28.976623
48 Hours,Normal No Dilution	B C		21.184000
24 Hours,Negative Control	B C		10.111167
24 Hours,Salt 50% Dilution	B C		7.138537
24 Hours,Normal 50% Dilution	B C		5.630743
24 Hours,Salt No Dilution	C		5.119955
24 Hours,Positive Control 1%	B C		3.533125
24 Hours,Positive Control 0.5%	B C		2.350748
24 Hours,Normal No Dilution	B C		1.406250

ANOVA for Propidium Iodide Activity after normalizing to negative control

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	2605.0279	372.147	13.2244
Error	8	225.1281	28.141	Prob > F
C. Total	15	2830.1560		0.0008*

Parameter Estimates				
Term	Estimate	Std Error	t Ratio	Prob> t
Intercept	4.7280343	1.326203	3.57	0.0073*
Duration 2[24 Hours]	-3.986104	1.326203	-3.01	0.0169*
Group.[50% Dilution]	7.6524997	1.326203	5.77	0.0004*
Scaffold Type.[Normal]	-5.339385	1.326203	-4.03	0.0038*
Duration 2[24 Hours]*Group.[50% Dilution]	-6.072291	1.326203	-4.58	0.0018*
Duration 2[24 Hours]*Scaffold Type.[Normal]	4.6136641	1.326203	3.48	0.0083*
Group.[50% Dilution]*Scaffold Type.[Normal]	-0.624687	1.326203	-0.47	0.6502
Duration 2[24 Hours]*Group.[50% Dilution]*Scaffold Type.[Normal]	1.1430565	1.326203	0.86	0.4138

Level			Least Sq Mean
48 Hours,50% Dilution,Salt	A		34.15972
48 Hours,50% Dilution,Normal	B		10.71814
48 Hours,No Dilution,Salt	B C		3.17465
24 Hours,50% Dilution,Salt	B C		2.52949
24 Hours,50% Dilution,Normal	B C		2.11479
24 Hours,No Dilution,Salt	B C		0.40581
24 Hours,No Dilution,Normal	B C		-2.08237
48 Hours,No Dilution,Normal	C		-13.19596

Appendix U: Statistical Tests for Scaffold Adhesion Assay

ANOVA for Cell Coverage

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	20465.166	4093.03	17.6905
Error	18	4164.644	231.37	Prob > F
C. Total	23	24629.810		<.0001*

Parameter Estimates						
Term	Estimate	Std Error	t Ratio	Prob> t	Lower 95%	Upper 95%
Intercept	59.736792	3.104896	19.24	<.0001*	53.213648	66.259937
Scaffold Type[Salt]	27.842834	3.104896	8.97	<.0001*	21.319689	34.365978
Location[Distal]	2.3622627	4.390986	0.54	0.5972	-6.862857	11.587382
Location[Middle]	-4.283754	4.390986	-0.98	0.3422	-13.50887	4.9413656
Scaffold Type[Salt]*Location[Distal]	11.206408	4.390986	2.55	0.0200*	1.9812884	20.431527
Scaffold Type[Salt]*Location[Middle]	-2.7329	4.390986	-0.62	0.5415	-11.95802	6.4922196

Level		Least Sq Mean
Salt,Distal	A	101.14830
Salt,Proximal	A	81.02761
Salt,Middle	A	80.56297
Standard,Proximal	B	42.28896
Standard,Middle	B	30.34310
Standard,Distal	B	23.04981

ANOVA for Percent Apoptotic Cells

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	87.89023	17.5780	0.9983
Error	18	316.94525	17.6081	Prob > F
C. Total	23	404.83548		0.4466

Parameter Estimates						
Term	Estimate	Std Error	t Ratio	Prob> t	Lower 95%	Upper 95%
Intercept	14.442176	0.856545	16.86	<.0001*	12.642641	16.24171
Scaffold Type[Salt]	-0.423776	0.856545	-0.49	0.6268	-2.223311	1.3757581
Location[Distal]	1.4972447	1.211338	1.24	0.2323	-1.047681	4.0421708
Location[Middle]	-0.167609	1.211338	-0.14	0.8915	-2.712535	2.3773173
Scaffold Type[Salt]*Location[Distal]	-2.066265	1.211338	-1.71	0.1052	-4.611191	0.4786615
Scaffold Type[Salt]*Location[Middle]	1.0859496	1.211338	0.90	0.3818	-1.458977	3.6308758

Level		Least Sq Mean
Standard,Distal	A	18.429462
Salt,Middle	A	14.936740
Salt,Proximal	A	13.669078
Standard,Middle	A	13.612394
Salt,Distal	A	13.449379
Standard,Proximal	A	12.556001

ANOVA for Percent Dead Cells

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	2097.5920	419.518	70.3957
Error	18	107.2697	5.959	Prob > F
C. Total	23	2204.8617		<.0001*

Parameter Estimates						
Term	Estimate	Std Error	t Ratio	Prob> t	Lower 95%	Upper 95%
Intercept	10.539004	0.498307	21.15	<.0001*	9.4921005	11.585908
Scaffold Type[Salt]	9.2319103	0.498307	18.53	<.0001*	8.1850068	10.278814
Location[Distal]	0.9683072	0.704712	1.37	0.1863	-0.512238	2.4488524
Location[Middle]	-0.182087	0.704712	-0.26	0.7990	-1.662632	1.2984581
Scaffold Type[Salt]*Location[Distal]	1.7496293	0.704712	2.48	0.0231*	0.2690841	3.2301745
Scaffold Type[Salt]*Location[Middle]	-0.466298	0.704712	-0.66	0.5166	-1.946843	1.0142476

Level		Least Sq Mean
Salt,Distal	A	22.488851
Salt,Middle	A	19.122530
Salt,Proximal	A	17.701363
Standard,Proximal	B	1.804205
Standard,Middle	B	1.591304
Standard,Distal	B	0.525772

Appendix V: Statistical Tests for BVM Study

ANOVA for Change in Scaffold Thickness

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	0.37531111	0.075062	4.4709
Error	12	0.20146667	0.016789	Prob > F
C. Total	17	0.57677778		0.0156*

Parameter Estimates				
Term	Estimate	Std Error	t Ratio	Prob> t
Intercept	0.9211111	0.03054	30.16	<.0001*
Scaffold Type[0.5% Salt]	0.0422222	0.043191	0.98	0.3476
Scaffold Type[3% Salt]	0.1388889	0.043191	3.22	0.0074*
Proximal/Distal[Distal]	0.0477778	0.03054	1.56	0.1437
Scaffold Type[0.5% Salt]*Proximal/Distal[Distal]	0.0222222	0.043191	0.51	0.6162
Scaffold Type[3% Salt]*Proximal/Distal[Distal]	0.0122222	0.043191	0.28	0.7820

Level		Least Sq Mean
3% Salt,Distal	A	1.1200000
0.5% Salt,Distal	A	1.0333333
3% Salt,Proximal	A	1.0000000
0.5% Salt,Proximal	A B	0.8933333
Standard,Distal	B	0.7533333
Standard,Proximal	B	0.7266667

ANOVA for Scaffold Shrinkage during pre-shrinking versus during BVM culture

Effect Tests					
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Scaffold Type.	1	1	9.59380	0.7144	0.4225
Proximal/Distal 2	1	1	0.10057	0.0075	0.9332
Preshrink/BVM	1	1	837.92853	62.3981	<.0001*
Scaffold Type.*Proximal/Distal 2	1	1	17.52677	1.3052	0.2863
Scaffold Type.*Preshrink/BVM	1	1	0.01142	0.0009	0.9774
Proximal/Distal 2*Preshrink/BVM	1	1	0.00207	0.0002	0.9904
Scaffold Type.*Proximal/Distal 2*Preshrink/BVM	1	1	19.60393	1.4598	0.2615

ANOVA for Change in BVM Lengths

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	185.48956	37.0979	4.6198
Error	6	48.18135	8.0302	Prob > F
C. Total	11	233.67091		0.0448*

Parameter Estimates				
Term	Estimate	Std Error	t Ratio	Prob> t
Intercept	3.370513	0.818038	4.12	0.0062*
Scaffold Type[0.5% Salt]	1.6876265	1.15688	1.46	0.1949
Scaffold Type[3% Salt]	3.1828865	1.15688	2.75	0.0332*
Proximal/Distal[Distal]	0.2658619	0.818038	0.32	0.7562
Scaffold Type[0.5% Salt]*Proximal/Distal[Distal]	1.9783242	1.15688	1.71	0.1381
Scaffold Type[3% Salt]*Proximal/Distal[Distal]	-2.328741	1.15688	-2.01	0.0908

Level		Least Sq Mean
3% Salt	A	6.553400
0.5% Salt	A	5.058140
Standard	B	-1.500000

ANOVA for Cell Coverage

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	66686.60	9526.66	14.6915
Error	148	95970.40	648.45	Prob > F
C. Total	155	162657.00		<.0001*

Parameter Estimates						
Term	Estimate	Std Error	t Ratio	Prob> t	Lower 95%	Upper 95%
Intercept	41.043738	2.154957	19.05	<.0001*	36.78528	45.302196
Timepoint[3 Day]	9.9654381	2.154957	4.62	<.0001*	5.70698	14.223896
Scaffold Type[0.5% Salt]	9.3992989	3.843936	2.45	0.0156*	1.8032096	16.995388
Scaffold Type[3% Salt]	21.213683	3.376168	6.28	<.0001*	14.541961	27.885404
Scaffold Type[ePTFE]	-9.950619	4.260655	-2.34	0.0209*	-18.3702	-1.531042
Timepoint[3 Day]*Scaffold Type[0.5% Salt]	3.112683	3.843936	0.81	0.4194	-4.483406	10.708772
Timepoint[3 Day]*Scaffold Type[3% Salt]	0.7523346	3.376168	0.22	0.8240	-5.919387	7.4240563
Timepoint[3 Day]*Scaffold Type[ePTFE]	-2.58427	4.260655	-0.61	0.5451	-11.00385	5.8353071

Level				Least Sq Mean
3 Day,3% Salt	A			72.975194
3 Day,0.5% Salt	A B			63.521158
7 Day,3% Salt	A B C			51.539648
3 Day,ePTFE	B C D			38.474288
7 Day,0.5% Salt	B C D			37.364916
3 Day,Standard	C D			29.066066
7 Day,ePTFE	D			23.711951
7 Day,Standard	D			11.696685

ANOVA for Percent Apoptotic Cells

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	23055.601	3293.66	10.3093
Error	148	47283.455	319.48	Prob > F
C. Total	155	70339.056		<.0001*

Parameter Estimates						
Term	Estimate	Std Error	t Ratio	Prob> t	Lower 95%	Upper 95%
Intercept	39.637433	1.512601	26.20	<.0001*	36.648347	42.626518
Timepoint[3 Day]	-7.609559	1.512601	-5.03	<.0001*	-10.59864	-4.620473
Scaffold Type[0.5% Salt]	4.5241166	2.698126	1.68	0.0957	-0.80771	9.8559433
Scaffold Type[3% Salt]	6.2814726	2.369791	2.65	0.0089*	1.5984756	10.96447
Scaffold Type[ePTFE]	-15.39067	2.990628	-5.15	<.0001*	-21.30052	-9.480823
Timepoint[3 Day]*Scaffold Type[0.5% Salt]	-11.18964	2.698126	-4.15	<.0001*	-16.52147	-5.857813
Timepoint[3 Day]*Scaffold Type[3% Salt]	1.605828	2.369791	0.68	0.4991	-3.077169	6.288825
Timepoint[3 Day]*Scaffold Type[ePTFE]	7.3134086	2.990628	2.45	0.0156*	1.4035617	13.223255

Level			Least Sq Mean
7 Day,0.5% Salt	A		62.960748
7 Day,3% Salt	A B		51.922636
7 Day,Standard	A B		49.561668
3 Day,3% Salt	B C		39.915175
3 Day,Standard	B C		38.883358
3 Day,0.5% Salt	C		25.362351
7 Day,ePTFE	C		24.542913
3 Day,ePTFE	C		23.950613

ANOVA for Percent Dead Cells

Analysis of Variance				
Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	7968.511	1138.36	10.8175
Error	148	15574.502	105.23	Prob > F
C. Total	155	23543.013		<.0001*

Parameter Estimates						
Term	Estimate	Std Error	t Ratio	Prob> t	Lower 95%	Upper 95%
Intercept	7.663905	0.868114	8.83	<.0001*	5.9484045	9.3794054
Timepoint[3 Day]	-3.913307	0.868114	-4.51	<.0001*	-5.628807	-2.197807
Scaffold Type[0.5% Salt]	-2.011364	1.548512	-1.30	0.1960	-5.071414	1.0486854
Scaffold Type[3% Salt]	6.403738	1.360074	4.71	<.0001*	3.7160653	9.0914106
Scaffold Type[ePTFE]	-1.055343	1.716386	-0.61	0.5396	-4.447131	2.3364453
Timepoint[3 Day]*Scaffold Type[0.5% Salt]	1.4376381	1.548512	0.93	0.3547	-1.622412	4.497688
Timepoint[3 Day]*Scaffold Type[3% Salt]	-6.049475	1.360074	-4.45	<.0001*	-8.737148	-3.361803
Timepoint[3 Day]*Scaffold Type[ePTFE]	1.1703028	1.716386	0.68	0.4964	-2.221485	4.5620909

Level		Least Sq Mean
7 Day,3% Salt	A	24.030425
7 Day,ePTFE	B	9.351566
7 Day,0.5% Salt	B	8.128209
7 Day,Standard	B	4.798647
3 Day,3% Salt	B	4.104861
3 Day,ePTFE	B	3.865558
3 Day,Standard	B	3.855102
3 Day,0.5% Salt	B	3.176872