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Fibrin-Based Engineered Vascular Tissues as Platforms for Cellular Studies and Disease Modeling

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Chemical and Biochemical Engineering

Abstract

Vascular tissue engineering (VTE) is an emerging alternative therapeutic intervention strategy to treat diseases such as atherosclerosis. While the ultimate goal of VTE is designing tissues to serve as graft substitutes, they can also serve as powerful tools to study tissue and disease development and drug discovery.

In this work, engineered vascular tissues from fibrin gel, mouse embryonic multipotent progenitor cell line (10T1/2 cells), and rat embryonic thoracic artery smooth muscle cells (A-10 cells) were used as models to study the Notch signaling pathway and vascular calcification. The 10T1/2 cells were successfully differentiated into vascular smooth muscle cells with TGFβ1 treatment and compacted the tubular gel significantly owing to the contractile cytoskeletal stress fibers. Notch signaling studies in engineered vascular tissues from A-10 cells demonstrated cis-inhibition while 10T1/2 cells activated Notch and its downstream targets Hes-1 and the smooth muscle α-actin genes.

The results from the calcification studies showed that vascular tissues fabricated from both progenitor and differentiated 10T1/2 cells calcified in response to high inorganic phosphate concentrations and expressed the osteopontin protein. Treatment of the tissues with a model therapeutic agent, Vitamin K, led to the reduction of calcium deposits and osteopontin expression, suggesting its potential protective role. In addition, vitamin K treated engineered tissues resulted in the restoration of smooth muscle cells contractile markers. The effect of elastin degradation on calcification was simulated using exogenous elastin

and showed that while elastin alone did not impact the undifferentiated tissues, it led to an increase in osteogenic markers in the differentiated counterparts.

This work also investigated the role of endothelial cell vimentin in the regulation of Notch signaling and neovascularization in coculture tissue models. The preliminary results showed that vimentin might enhance the Notch signaling strength since the inhibition of vimentin using a chemical inhibitor or siRNA did not completely inhibit the signal. Notwithstanding this, vimentin appeared to be essential for new micro-vasculature formation.

The data collectively presented in this thesis demonstrated the potential of engineered vascular tissues as a novel tool to study cell signaling, vascular calcification, and therapeutic discovery.

Key words: Vascular tissue engineering, vascular disease modeling, Notch signaling, fibrin gel, smooth muscle cells, endothelial cells, progenitor cells, vimentin, vascular calcification.

Summary for Lay Audience

The lack of organ donors has brought about the need for engineering tissues from the lab bench. Whether patients require a blood vessel, bone, a patch of skin, liver, or even a heart, tissue engineering provides a practical alternative to the lack of donated tissues and organs. This research focused on engineering vascular tissues (blood vessels). While the main purpose of tissue engineering is to replace damaged or diseased organs, the use of engineered vascular tissues to study cell communication and diseases that affect the human vasculature is a target application. The clotting protein fibrinogen, which is naturally found in the blood of mammals, was used to entrap precursor cells and to form a tube-shaped tissue that resembles blood vessels.

Communication between cells is called signaling, and it is a defined "dialogue" that carries instructions from one cell to another in a process called signaling pathway. In vascular tissues, a vital Notch signaling occurs between endothelial cells and smooth muscle cells and dictates vital functions such as cell division and survival, among other functions. In this research, the Notch signaling was studied by comparing two different cell types responses to endothelial cells in the engineered tissues. It was found that while one type of cell was responsive, the other type was not. This has implications for the use of certain cell types that are capable of communicating the proper signal for the proper development of engineered tissues. Furthermore, by using the engineered tissues as models, a common problem that occurs in arteries, vascular calcification was studied. The results in this work showed that engineered tissues were capable of calcification just like natural tissues and

that there is a potential of using vitamin K to reduce the negative effects of calcification. This will help us study diseases and discover treatments for vascular diseases.

In conclusion, engineered vascular tissues have the potential to provide insight into human vasculature physiology and pathology. In this research, it has been shown that engineered vascular tissues provide excellent platforms to study cell signaling, vascular diseases, and potential drug discovery.

Co-authorship Statement

Chapters 1 and 2 titled "**Introduction**" and "**Literature Review**", respectively, were written by K. Dayekh. Dr. K. Mequanint reviewed and edited these chapters.

Chapter 3 titled "**Comparative studies of fibrin-based engineered vascular tissues and Notch signaling from progenitor cells**" is a version of the publication: *Khalil Dayekh and Kibret Mequanint. ACS Biomater. Sci. Eng. 5, 2696–2706,* 2020. The manuscript was written by K. Dayekh and Dr. K. Mequanint. Experiments were performed by K. Dayekh in the laboratory of Dr. K. Mequanint.

Chapter 4 titled "**The Effects of Progenitor and Differentiated Cells on Ectopic Calcification of Engineered Vascular Tissues,**" is a version of the publication: *Khalil Dayekh and Kibret Mequanint.* Acta *Biomater. 115, 288-298, 2020*. The publication was written by K. Dayekh and Dr. K. Mequanint. Experiments were performed by K. Dayekh in the laboratory of Dr. K. Mequanint.

Chapter 5 titled "**Exploratory Study on the Role of Vimentin in Notch Signaling,**" was written by K. Dayekh and edited by Dr. K. Mequanint. Experiments were performed by K. Dayekh and Dr. S. Lin in the laboratory of Dr. K. Mequanint.

Chapter 6 titled "**General Discussion,**" was written by K. Dayekh. Dr. K. Mequanint reviewed and edited the chapter.

This thesis is dedicated to the memory of my father,

Raja'i Dayekh

Acknowledgments

I would like to take this opportunity to thank a number of very important people without whom this work would not have been possible. To my supervisor, Dr. Kibret Mequanint, I would like to express my sincerest gratitude for allowing me to be part of your lab group and for your continued support and guidance. You have been an exemplary mentor and a source of inspiration, and I have learned so much from you. I will be forever thankful to you for always believing in me and encouraging me until the very end.

To the members of my advisory committee Drs. Lauren Flynn and Peter Chidiac, I give genuine thanks for your valuable advice and suggestions to put me on the right track to complete this work.

I would also like to thank my lab colleagues who have been supportive throughout my work. I enjoyed our discussions during the group meetings; they helped me look at things from different perspectives.

I am thankful for the financial support I received throughout the period of my studies from the CIHR, HSFC, and the OGS. I am also grateful for the teaching assistantship opportunities, which have been rewarding and educating.

Many thanks to Dr. Andrew Hrymak for allowing me access to the Rheometer and to Dr. Dale Laird for allowing access to the cryostat.

Last but not least, I would like to extend my sincerest gratitude to my family, who have been incredibly supportive. Thank you for all your love and care throughout this journey.

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List of abbreviations

2D	Two dimensional
3D	Three dimensional
ACA	Aminocaproic acid
Acta2	Smooth muscle alpha-actin
Adam	A disintegrin and metalloprotease
Alp1	Alkaline phosphatase
ANOVA	Analysis of variance
AU	Arbitrary unit
bFGF	basic Fibroblast Growth Factor
BSA	Bovine serum albumin
CABG	Coronary artery bypass graft
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CBF	Core binding factor
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary heart disease
Cnn1	Calponin 1
ColI	Collagen type I
ColIII	Collagen type III
CVD	Cardiovascular disease
DAPI	4′,6-diamidino-2-phenylindole
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DEPC	Diethylpyrocarbonate
Dll	Delta-like ligand

DMEM	Dulbecco’s Modified Eagle’s media
DSL	Delta Serrate/jagged Ligand
EC	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELN	Elastin
EndMT	Endothelial to Mesenchymal Transition
ESCRT	Endosomal sorting complexes required for transport
EtOH	Ethanol
EVT	Engineered vascular tissues
FBG	Fibrinogen
FCS	Fetal Calf Serum
FiVe1	FOXCs-inhibiting Vimentin effector 1
GAPDH	Glyceraldehyde phosphate dehydrogenase
Gla	carboxyglutamic acid
H&E	Hematoxylin and eosin
HCAEC	Human coronary artery endothelial cells
HCl	Hydrochloric acid
HDMS	Hexamethyldisilazane
HERT	Hairy-related transcription factor
Hes	Hairy enhancer of split
Hey	Hairy/enhancer-of-split related with YRPW motif protein
HGPS	Hutchinson-Gilford progeria syndrome

IgG	Immunoglobulin G
Jag	Jagged
LDL	Low density lipoproteins
MAPK	Mitogen activated protein kinase
m_i	Initial mass
m_i	Final mass
MLV	Murine leukemia virus
mRNA	Messenger RNA
Myh11	Myosin heavy chain 11
NECD	Notch extracellular domain
NICD	Notch intracellular domain
OCT	Optimal cutting temperature
OPN	osteopontin protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCAM	Platelet endothelial cell adhesion molecule
Pi	Inorganic phosphate
PIM	Proto-oncogene serine/threonine
PiT-1	Sodium-dependent phosphate transporter channel
PMSF	Phenylmethanesulfonyl fluoride
qPCR	Quantitative polymerase chain reaction
RBP-Jκ	Recombination signal binding protein for immunoglobulin kappa J region
RIPA	Radioimmuno precipitation assay
RNA	Ribonucleic acid

RT	Room temperature
Runx2	Runt-related transcription factor
SC-PL	Solvent casting - particulate leaching
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SMAD	Small worm phenotype- Mothers against decapentaplegic
SMC	Smooth muscle cells
Smtn	Smoothelin
Spp1	Osteopontin gene
SRC	Signal receiving cell
SSC	Signal sending cell
TGF	Transforming growth factor
ULF	Unit Length Fiber
VEGF	Vascular Endothelial Growth Factor
Wnt	Wingless and int

Chapter 1

Introduction

1.1 Overview

The role of the vasculature may be seen as a delivery system for blood to transport nutrients, oxygen, waste, and carbon dioxide to and from various tissues and organs. However, the mechanism and regulation of the processes that govern this seemingly simple task are everything but simple. Certainly, regulation of the vascular system to stay in homeostasis is critical, and deviation from homeostasis might lead to cardiovascular diseases that can be detrimental to the individuals' health and quality of life. The vast majority of health-related mortalities can be attributed or linked to cardiovascular diseases, which is a burden not only to the patients but also to the economy [1, 2]. While cardiovascular disease mainly occurs in the elderly population, younger generation cases have been rising due to poor dietary and lifestyle choices[3]. Cardiovascular diseases include atherosclerosis, thrombosis and embolism, peripheral artery disease, stenosis, coronary artery disease, aneurysms, etc. However, the most common is coronary artery disease triggered by atherosclerosis which is the narrowing of arteries due to accumulation of plaque (usually due to fatty streaks) in the lumen [4]. This leads to reduced blood supply to the heart muscles and eventually heart failure.

At early stages of coronary artery disease patients are prescribed medicine, such as lipid regulators like statins, to slow down the progression of the disease. In more advanced stages where a plaque occludes the flow of blood to the heart, surgical intervention might be necessary. Angioplasty and stenting is a common procedure both to reopen and to prevent

the artery from collapsing [5]. However, if there are multiple occlusions, then a coronary artery bypass graft (CABG) surgery is needed[5]. This procedure entails using a graft, usually autologous saphenous vein, or the mammary artery, to flank the blockages establish circulation. While autografts are the safest choices, they are not always available. Allografts and xenografts are other options, but usually, they are avoided due to reasons such as disease transmission and immune rejection.

Research in the field of vascular tissue engineering has been ongoing to provide a reliable source of grafts that can replace the diseased native tissues. However, despite many promising studies, engineered vascular tissues are still not sufficiently developed to be used as graft replacement. One of the main challenges is elastin production and crosslinking which is a process still scarcely understood. Furthermore, the hierarchical organization of cells and their arrangement in the tissue is still hard to control due to poorly understood cell processes and signaling pathways. For these reasons, a recent trend in tissue engineering has been to use engineered tissues to understand cellular processes in a more relevant three-dimensional (3D) environment. While much of our knowledge stems from two-dimensional (2D) cell studies, these studies suffer from many limitations. One obvious limitation is that cells do not exist as a monolayer on a plastic surface but in a complex 3D milieu that involve interactions with multiple extracellular matrix proteins, different cell types, and exposure to dynamic forces that dictate much of the cellular physiological processes. As an example, and in the framework of vascular engineering, the vascular system is the only system that is continuously exposed to cyclic and variable hemodynamic forces. These forces are essential for the proper development and homeostatic maintenance of this unique system [6]. Indeed, studies have shown that cells in a 3D environment will

behave differently when compared to the same cells seeded on a 2D surface[7, 8], and that forces play an important role in tissue development (reviewed in ref [9]).

While the main objective of engineered vascular tissues is to be used as grafts, they have also been used as powerful tools to understand a variety of vascular diseases such as atherosclerosis[10] and calcification [11]. Even though animal and cell models have been extensively used to understand vascular pathology, they each have their shortcomings. in 2D cell culture, the extracellular matrix and other spatial cues are missing. Animal studies are complicated by the interference of other organ systems and are usually costly and time-consuming due to the maintenance of the animals. Furthermore, ethical approvals have to be obtained for such studies. This makes utilizing engineered tissues as tools to study vascular pathology an appealing idea because they can overcome those limitations. That is not to say that animal models will be replaced by engineered tissues. After all, when the systematic effect of a disease is to be studied, engineered vascular tissues will not be sufficient. However, engineered tissue disease models act as a viable complement which narrows down the target of the study and reduces animal studies to focus on a specific objective.

1.2 Thesis outline

The main objective of this research is to showcase the use of engineered vascular tissues as platforms to study cell signaling and calcification. This thesis is composed of 6 chapters as follows: An overview of this work is given in this chapter designated as Chapter 1. A synopsis of the literature that is relevant to this work is presented in Chapter 2. A study of the Notch signaling in engineered tissues follows in Chapter 3[12]. Chapter 4 presented the

use of engineered vascular tissues as a model to study calcification[13]. In Chapter 5, an exploratory study of the role of vimentin in regulating the Notch signaling and neovascularization is presented. And finally, Chapter 6 summarizes the significance and limitations of this research and suggests recommendations.

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Chapter 2

Literature review

Overview. *This chapter provides background information on the vascular system, vascular tissue engineering, and the current status of the field. Furthermore, cell signaling is discussed with emphasis on the Notch signaling pathway and its relevance to vascular tissue formation and development. At the end of the chapter, an outline of the scope of this work and the objectives is provided.*

2.1 The vascular system

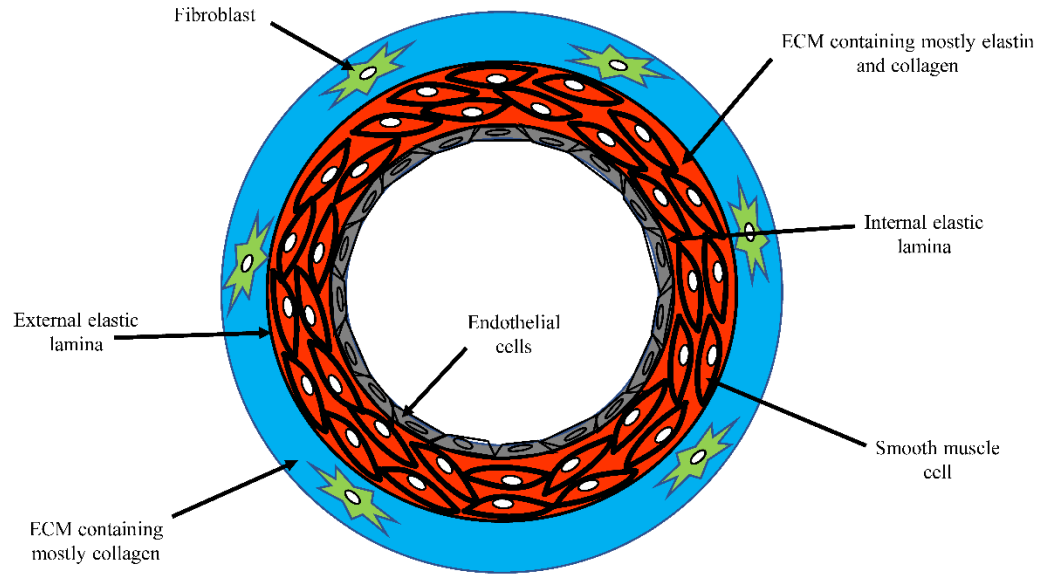
In order to discuss vascular tissue engineering, knowledge about the structure and function of the vascular system is essential. Here the important elements of the anatomy, physiology and pathology of the different components of the mammalian vascular tissues is summarized.

2.1.1 The anatomy of the vascular system

The human vascular system is an integral part of the circulatory system which includes the heart, the vascular system, and the lymphatic system. These vessels are the arteries, arterioles, capillaries, venioles, and veins. With some exceptions (e.g., pulmonary artery), arteries transport oxygenated blood, while the veins transport oxygen-poor blood. While there are differences between these blood vessels, such as their size difference and the presence or absence of valves between veins and arteries, respectively, blood vessels share common anatomical elements. Starting from the lumen and moving outward, blood vessels are composed of the intima, media, and adventitia[1] (**Fig 2.1**). The intima is composed of a single uninterrupted monolayer of squamous endothelial cells. Under physiological

conditions, these cells are oriented parallel to the flow of blood. The medial layer is a muscular layer that is composed of smooth muscle cells (SMCs) and an extracellular matrix (ECM) mainly of elastin and collagen types I and III, but also small amounts of other proteins and proteoglycans[1]. The SMCs and elastic fibers are normally aligned concentrically with respect to the central axis of the artery, while the collagen fibers run parallel and circumferential to it. In the adventitia, fibroblasts are the abundant type of cells, and collagen is the abundant ECM protein. There is no particular alignment of the cells or collagen fibers in the adventitia (**Fig. 2.1**).

It is important to note that while these layers are common between arteries and veins there are also variations along with the branching of arteries and veins. For example, there are notable differences between the thickness and composition of the media of arteries depending on the anatomical location. On that basis, arteries are further divided into elastic and muscular arteries. Whereas elastic arteries comprise a large amount of ECM and SMCs, muscular arteries contain much less ECM and are typically smaller than elastic arteries. These differences hint at their functionality which will be discussed in the following section.



	Cellular component	Major ECM components	Primary function	Common pathology
Intima	Endothelial cells	Elastin basal layer	Anti-thrombotic surface, regulate SMC contractility	Atherosclerotic plaque formation, intimal calcification
Media	Smooth muscle cells	Organized elastin/collagen fibers	Blood vessel diameter control, elastic recoil	Medial calcification, elastin degradation
Adventitia	Fibroblasts	Random collagen fibers	Collagen deposition, structural integrity	Proinflammatory fibroblasts media migration, altered metalloproteinase production

Figure 2.1. Schematic representation of blood vessel cross-section and its different layers.

2.1.2 Physiology of the blood vessels

The heart is the blood pumping station for the body, which makes the blood vessels the pipes. While this might seem a simple task for an organ system, it is a complicated process given the nature of blood as the only fluid tissue and the fact that the body is not always at steady-state and is affected by many external and internal factors such as physical activity,

stress, disease and others. Hence, the anatomy of the vasculature is explained in part by the task to be performed. Starting with the endothelium, it provides a smooth surface layer so the blood can pass through with minimal friction. The blood is a non-Newtonian fluid which is affected by the level of shear stress it is exposed to, and thus it is critical that blood flow is laminar since the turbulent flow has been shown to cause complications, which lead to cardiovascular diseases. It also provides a non-thrombogenic surface to keep in mind for vascular tissue engineering. This was a common cause of failure of the earlier engineered vascular tissues (EVTs). Furthermore, the endothelium acts as a barrier which controls the passage of certain molecules to and from the blood stream (blood brain barrier for example)[1, 2]. The endothelial cells also signal SMCs to contract or relax to control blood flow using soluble factors such as endothelin and nitric oxide. The media which contains the SMCs and ECM proteins has two main functions. The first is to absorb the energy from systolic blood and resisting the burst pressure and then returns the energy to the blood to keep it moving during the diastolic cycle to keep the blood moving even when the heart is resting[2]. This task is collectively performed by the ECM proteins where collagen mainly takes the support function, preventing bursting of the vessel, and elastin is the protein that provides elasticity and recoil. The second function of the media is to control the blood flow. This is done by the SMCs that are sensitive to vasoconstrictors and vasodilators[2]. At certain locations, the media also contains nerve endings, further regulating SMCs by the sympathetic and parasympathetic nervous systems via neurotransmitters[1]. Arteries usually have thicker media than veins because they are exposed to higher pressures. The further division of arteries into elastic and muscular is due to their proximity from the heart. Elastic arteries are usually closer to the heart, where the pressure is highest, and the muscular

arteries are found closer to organs where they regulate the flow of blood. Finally, the adventitia plays mostly a structural support function and attaches the blood vessels to the surrounding tissues. In larger vessels the adventitia contains blood vessels, called the vasa vasorum, and nerve endings.

2.1.3 Pathology

The vascular system is subjected to multiple levels of regulations that keep it in check; however, under certain conditions, the vascular system may deviate from physiological function, which may lead to cardiovascular diseases (CVD). Statistics show that CVDs are the primary cause of death worldwide, claiming nearly 18 million people each year[3]. Diseases affecting the vascular system are among the top causes of death in Canada and other developed nations. In the United States, almost half the population will be expected to suffer from a form of CVD by the year 2035[4]. CVDs primarily target adults and the elderly; however, in recent years, more cases of CVDs are occurring in younger adults due to the increased prevalence of obesity and poor dietary choices[5-7], and reduced physical activities[6, 8]. Furthermore, it can also affect the pediatric population in the form of congenital defects.

Diseases affecting the vascular system are usually caused when one or more of the functions of the three layers mentioned earlier are affected. Coronary heart disease (CHD), which is the most prevalent form of cardiovascular diseases, is triggered by a plaque that builds up in the coronary arteries causing a blockage of the blood flow followed by failure of the heart muscles. Plaque buildup in the coronary artery is caused by many reasons, including, but not limited to, lipid deposition, a defect in the endothelium or calcification of the intima

or the media. In the early stages of a coronary artery blockage, angioplasty is performed, and a permanent stent is often placed 'to keep the artery from collapsing. However, in advanced stages, where a large section of the artery is blocked or multiple blockages, a coronary artery bypass graft (CABG) is required. These strategies have greatly helped in reducing the burden of CHD on the lives of many patients, although they are far from the ideal treatment. Stenting often causes chronic inflammatory response, which has the potential to cause restenosis, eventually requiring a follow-up surgical intervention. Moreover, one of the most common shortcomings of CABG is the sourcing of the graft. There are three general categories for graft sources: xenografts, allografts, and autografts. Xenografts are usually used as a last resort since their use will require administrating life-long immunosuppressants', and there is a chance of disease transmission that is amplified by the immunosuppressants.

The gold standards for autografts are the saphenous vein and the mammary artery having a similar diameter to the coronary artery, and they are accessible with minimal surgical intervention. One of the problems with using those grafts is compliance mismatch, which is a well-known cause of graft failure[9, 10]. Due to the difference in mechanical properties and because of the inherent pressure differences that these blood vessels are exposed to in their native settings, compliance mismatch is usually an issue that leads to turbulent flow at the anastomoses site and causes thrombosis, hyperplasia and low patency[11]. The age of patients and/or their overall health status are also constraints that might hinder the use of autografts. Neointimal hyperplasia is a problem in both angioplasty and CABG, which is caused by the damage these procedures inflict on the endothelial cell (EC) lining of the vasculature. In angioplasty, the catheter inserted into the artery and inflating the balloon

will lead to sloughing off the ECs. This is an issue since the regulation of smooth muscle cells (SMCs) proliferation by ECs has been well documented in the literature[12-15]. There is increasing evidence that the Notch signaling pathway plays an important role in that process. The Mequanint Lab and others have documented the importance of ECs interaction with SMCs via the Notch pathway and the effect it has on regulating SMC gene production[13, 14, 16], and consequently inhibiting smooth muscle cell phenotypic switching to a proliferative state. Therefore, there is a need to find an alternative to these treatment modalities that would overcome the shortcomings of current grafts. The work in this thesis proposes that targeting the Notch signaling pathway is a pivotal step not only to help control the phenotypic switching of SMC, but also to fabricate fibrin-based vascular constructs that closely resemble small diameter arteries for applications like preclinical test models or as graft replacements. Furthermore, this might enable the development of strategies to overcome the shortcomings of angioplasty for example, by modifying stent surfaces.

Vascular calcification is another common disease that not only affects the coronary artery, but also in other parts of the vascular system and is especially prevalent in patients with renal dysfunction. Calcification in the vascular tissues is caused by multiple factors such as cell apoptosis/necrosis, calcium/phosphate ions imbalance (renal dysfunction), bone

degeneration, degradation of the ECM proteins, and inflammation. Calcification will be discussed further in later sections. In summary, whether the problem is finding replacement vascular grafts, studying disease or understanding signaling pathways, vascular tissue engineering is a viable solution to produce tailor-made tissue constructs to meet the requirements.

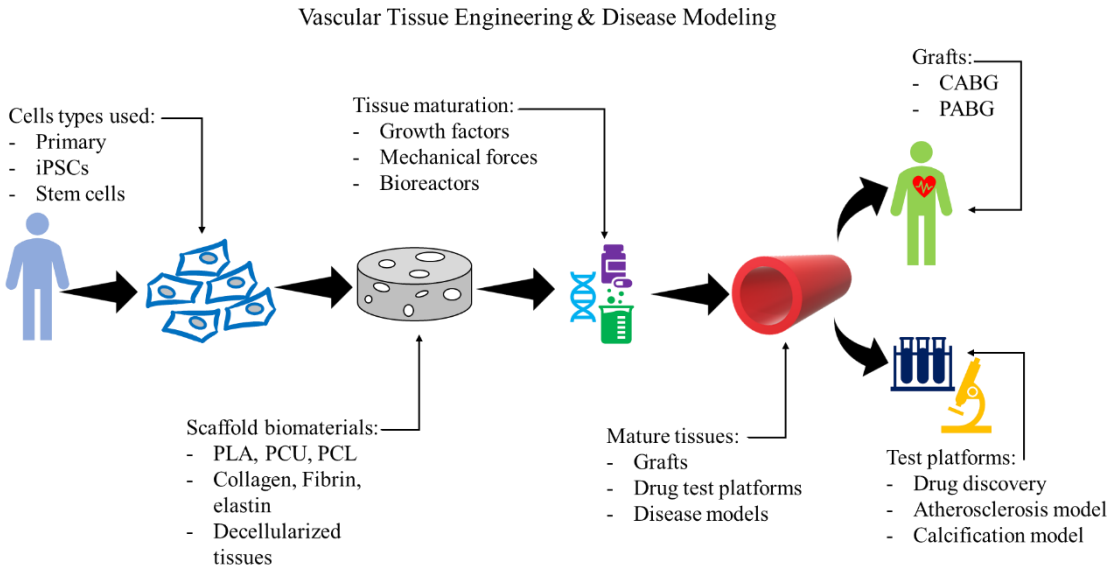


Figure 2.2. Graphical abstract of section 2.2. Schematic representation of the basics of vascular tissue engineering and their use as disease models

2.2 Vascular tissue engineering

Tissue engineering has emerged as a subdivision of a broader field of regenerative medicine. It is a multidisciplinary field of research that includes material science and engineering, biology and biochemistry for the purpose of generating bioartificial tissues.

Research in tissue engineering was initiated as a viable solution to the shortage of organ donations. There are many promising studies that have demonstrated the feasibility of fabricating tissues such as bone[17-19], skin[20, 21], blood vessels[22-24], and others[25, 26] to produce functional tissues that will treat or replace diseased or injured organs. Vascular tissue engineering is an active field of research producing a vast number of studies at a fast pace. Therefore, it is important to mention some distinct milestone studies that have shaped this field into its current state.

2.2.1 Important milestones in vascular tissue engineering

Vascular tissue engineering started in 1986 when Weinberg and Bell made a small diameter vascular tissue-equivalent out of collagen and bovine cells[27]. While this was a promising attempt at engineering a vascular graft, the Dacron sleeve was needed to support it, and this limited its success due to its inert vasoactivity. Thus, there were attempts to strengthen collagen, as a natural polymer, to withstand hemodynamic pressures. In a different study, Wilson et al. developed a protocol to decellularized arteries using detergents and enzymes, creating a cell-free matrix that preserved the structural integrity of those arteries while allowing the host cells to infiltrate and populate the scaffold[28]. A different approach to engineer vascular tissue was employed by L'Heureux et al. using confluent cells to produce their own ECM forming tissue sheets and then rolling them on a mandrel to make a tubular tissue resembling a blood vessel[29]. While these tissues had promising mechanical properties, the culture period to obtain such tissue sheets was considerably long. Niklason et al. made vascular tissues using polyglycolic acid as a scaffolding material and were transplanted into a bovine animal model where they stayed patent for 24 days[30]. This study

highlighted the importance of the use of synthetic materials; however, it also brought into light a critical disadvantage represented by harmful degradation byproducts. While research groups used *in vitro* culture systems to mature their EVTs, an interesting idea that suggested an *in vivo* culture system was introduced[31]. The presented idea was to insert the bare scaffold into an animal model, and the animal itself provides the resident cells, the right environment, and growth factors. On the materials front, the main natural polymer used in vascular tissue engineering was collagen. This is because collagen is one of the main components of vascular tissues. However, several studies reported the use of fibrin as another viable scaffold for EVTs, because it is easily available from a patient's blood[32]. These studies laid the foundation of vascular tissue engineering and are the basis for our current understanding.

The focus of this thesis work is the arteries, and in particular the small diameter coronary and peripheral arteries. Previously the relationship between the anatomy and the physiology of blood vessels it was mentioned. To match the functional and biological roles of the native artery, the engineered counterparts must exhibit similar features. In small diameter arteries such as the coronary artery, the two functional layers are the intima and the media. The average wall thickness is around 1.1 mm, a luminal diameter of about 2.2 mm and an external diameter of 4.5 mm[33].

The intima is a single layer of endothelial cells which can be achieved by seeding the lumen of the engineered vessel with a high-density endothelial cell suspension. On the other hand, the media comprises multiple layers of smooth muscle cells and ECM proteins and has an average thickness of 200 μm[34]. Different research groups have devised various techniques

and materials and have used cells from different sources to fabricate engineered vascular tissues with a varying degree of success [23, 30, 35-37]. The two main components of EVTs are the material to be used as a scaffold to hold the cells and the cells themselves. There have been different materials used as scaffolds for vascular tissues, which are divided into two main categories: synthetic or natural polymers. Moreover, there are many cell types that can be potentially used in vascular tissue engineering, and they are also divided into two general categories: differentiated (non-stem) cells, namely endothelial cells and smooth muscle cells, and stem cells, which can be differentiated via soluble factors, mechanical stimulations, etc. into the desired type of cells. These will be discussed in more detail in the following sections.

2.2.2 Cell types and sources used for vascular tissue engineering

In the context of vascular tissue engineering, cells are responsible for the major functionality of the tissues. After all, endothelial cells constitute the anti-thrombogenic surface, and SMCs produce the ECM and regulate the diameter of blood vessels, among other functions. Thus, the sources of cells to be used to engineer the vascular tissue must be chosen with intent, and for that reason, the cell sources for vascular tissue engineering have been a cause of extensive debate[38, 39].

The first logical cell source is native primary endothelial and SMCs as they are the functional cells of the vasculature and are available from a biopsy. However, the use of primary cells has proven to be challenging because of their limited proliferation rate, which depends on multiple factors, the most important of which is the donor age and health status[40, 41]. Thus, stem cells have been suggested as an alternative due to their proliferative

potential. Sources of stem cells include adult stem cells (e.g. mesenchymal stem cells and adipose tissue-derived stem cells), embryonic stem cells, and induced pluripotent stem cells. Using stem cells has shown potential in vascular tissue engineering not only due to their high proliferation capabilities but also the capacity to be differentiated into both ECs[42-44] and SMCs[44, 45]. Stem cells have been extensively studied due to their multipotency; however, this property of stem cells seems to be a doubled-edged sword. The fact that these cells can be differentiated into many lineages makes it difficult to control the desired cell lineage for a specific tissue, often leading to a mixed population with other undesired types[46]. The fact remains that there are many variables that have to be controlled in order to successfully differentiated stem cells to functional SMCs and/or ECs. Furthermore, stem cells in adult individuals are scarce in comparison to differentiated primary cells, and the procedure of getting certain stem cells, such as bone marrow stem cells, is invasive and painful. Indeed, more research is needed to understand the differentiation pathways that will lead to the specific cell lineage and therefore produce functional engineered vascular tissues.

2.2.3 Vascular tissue engineering strategies

Many strategies have been implemented in vascular tissue engineering to ensure structural and cellular organization and mechanical integrity that resemble those of native human blood vessels. As mentioned in previous sections, blood vessels possess important structural and cellular organizations (such as the concentric organization of the layers of SMCs, the longitudinal assembly of collagen fibers, the centripetal formation of elastic fibers, etc.) that impart crucial physiological functions such as the antithrombotic lumen

and the vasoactivity of the medial layer. These purposeful and organized structural elements have been among the most challenging aspects of tissue engineering in general but especially in vascular tissue engineering. This is due to the sensitive function of each of those cellular and structural components because of the exposure of these constructs to a continual dynamic environment carried out by the systolic and diastolic cycles of the circulatory system. Therefore, there has been a great effort to develop various strategies and methods to give engineered tissues the structural and functional properties of their native counterparts.

2.2.3.1 Cell sheet- based EVTs

This is one of the earliest methods used in the fabrication of engineered vascular tissues. It was developed by L'Heureux et al. in 1998[29] where vascular tissues are formed by growing high-density monolayers of smooth muscle cells that are coaxed to produce extracellular matrix proteins by treatment with ascorbic acid. After a period of time in culture, the cells form a cohesive sheet that can be removed and rolled into a cylindrical mandrel to form the signature tubular structure of vascular tissues. Even though the EVTs formed using this method usually exhibit favorable mechanical properties and have even been grafted in a canine model, their production time is exceedingly long, topping three months of culturing and maturation periods. These prolonged periods of culturing and maturing will inherently increase risks of contamination and will also prevent the use of these tissues as ready-made off-the-shelf constructs. The main reasons for this prolonged period to fabricate such tissues is due to the slow production of the ECM by SMCs and their slow proliferation rate. Therefore, an attempt was made to reduce that time by utilizing skin or saphenous vein

fibroblasts[47]. Fibroblasts are known for their fast production of ECM. After the fibroblast cell sheets were produced, the sheets were decellularized, and SMCs were seeded on the sheets and wrapped around a mandrel in a similar fashion as that employed by L'Heureux and co-workers. These EVTs were comparable to the ones produced by SMCs; however, their production time was still relatively long even though they were able to improve the maturation period by two weeks[47].

2.2.3.2 Decellularized scaffold-based EVTs

Production of an organized and well-structured ECM by cultured cells has proven to be a long and complicated process that scientists are yet to understand completely. In the context of vascular tissues, the ECM plays a critical role in the physiology of the tissues. Therefore, it is essential to replicate the fibrillar organization and content of the different layers of the blood vessels to produce a functional tissue. The ECM of a typical artery is composed mainly of collagen and elastin with other less abundant proteins and glycosaminoglycans. Collagen is relatively easily produced by SMCs and fibroblasts by treatment with ascorbic acid. Elastin fiber production, on the other hand, has been elusive and has proven to be a challenging task in vascular tissue engineering that might require not only soluble factors but also mechanical stimulation of the tissues[48]. While the production of these ECM proteins is essential, the organization of their fibers is equally important. It is not enough to have random fiber arrangement in vascular tissues; they have to be aligned properly for the EVTs to have meaningful structural integrity. Hence, researchers have resorted to taking advantage of already existing blood vessels to circumvent this particular problem. This method involves obtaining a blood vessel either from the same individual that requires

the graft (autologous graft), from a different individual (allogenic graft) or a different species (xenogeneic graft) and decellularizing the blood vessel. After decellularization, the scaffold could be utilized as is and allow the host cells to infiltrate it, or cells will be seeded onto the preserved ECM scaffold and matured in culture or bioreactor before using it as a graft. Both autologous and allogenic grafts exhibit minimal immunogenicity; however, xenogeneic grafts might still show a degree of immune reaction by the host.

The role the extracellular matrix plays in cellular motility[49], differentiation[50, 51], and proliferation[52] is well documented in the literature. The advantage of this strategy is that it preserves the ECM structure, topology, and even certain growth factors embedded in the ECM, all of which have been shown to affect cell function one way or another. Traditionally, vascular tissues from a host were used to make decellularized scaffolds; however, some groups have used decellularized scaffolds from engineered tissues[47, 53]. Decellularization of tissues is accomplished through different approaches, including chemical, biochemical, and physical. Of course, the effectiveness of the approach will depend on factors such as tissue composition, dimensions, and cellular density, among others. Chemical agents include, but are not limited to, acids/basis[54], hypertonic solutions[55], detergents (ionic and non-ionic)[55, 56], some alcohols[54, 56], etc. Biochemical reagents are enzymes such as nucleases, trypsin or dispase[54, 57], and physical decellularization includes temperature change[51], shear force/pressure [58], and electroporation [59]. Each of these agents will affect the ECM in one way or another, and thus, oftentimes, a combination of those strategies is employed to achieve optimal decellularization while preserving the ECM as much as possible [54].

Decellularized tissues have shown promising results; however, they still suffer from limitations such as thrombosis, infection, inflammation, aneurysm, and poor patency. A clinical trial that compared a decellularized bovine ureter against polytetrafluoroethylene in arteriovenous vascular access grafts for dialysis patients showed no advantage of the decellularized tissue over the synthetic graft [60]. Therefore, while the idea of using decellularized conduits for vascular tissue engineering is promising, more research is required to address the previously mentioned shortcomings.

2.2.3.3 Scaffold-based EVTs

Scaffold-based engineered vascular tissues are arguably the most widely used strategy to fabricate vascular prostheses. This strategy entails forming a scaffold onto which the desired cells will be seeded. The scaffold provides structural support and a suitable 3D environment for the cells to deposit their ECM. Ideally, the synthetic scaffold should be remodeled and replaced by the resident cells. However, certain materials have been used that are not biodegradable and the current state of tissue engineering is focusing more on biodegradable materials. As a general guideline, a scaffold should have the following properties: **a)** acceptable surface properties to allow cell adhesion and viability, **b)** sufficient porosity, interconnectivity, and proper pore size permitting cell infiltration as well as nutrient and oxygen exchange, **c)** a degradation rate that matches ECM formation by cells, and **d)** be mechanically competent to withstand the hemodynamic forces. Materials including synthetic and natural polymers, which are also subdivided into biodegradable and non-biodegradable polymers, have all been used in vascular tissue engineering.

Synthetic materials

Both biodegradable and non-biodegradable synthetic materials have been explored in vascular tissue engineering. However, non-biodegradable materials are being phased out due to reasons such as low-grade chronic inflammation, compliance mismatch, and the fact that they are permanently present in the patient's body. Although synthetic materials, such as polyurethane, Dacron, and Teflon have been successfully used as prosthetics to replace blood vessels that are greater than 6mm in diameter[61], their use to replace blood vessels with diameters of less than 6mm has been met with limited success due to thrombus formation[62]. The advantage of biodegradable materials is that they act as a temporary scaffold. The degradation rate can usually be tuned, and thus the cells can degrade the material while producing their own ECM until the whole scaffold is replaced by ECM produced by the engineered tissues and recipient's body. Biodegradable synthetic polymers are sometimes prepared from lactic acid, glycolic acid, caprolactone, or a copolymer made of different ratios of these monomers [35, 63-65]. Synthetic materials exhibit many advantages for their use in vascular tissue engineering, such as: **(i)** minimal batch-to-batch variations which is important for upscaling, **(ii)** relative ease of functionalization with biomolecules, allowing them to have better interaction with cells and host body, and **(iii)** tailorable mechanical properties to withstand hemodynamic forces[66]. On the other hand, synthetic biomaterials usually suffer from poor cell infiltration, the restricted capacity of remodeling by cells, foreign degradation byproducts, and limited bioactivity, especially in the absence of functionalization.

Natural materials

These materials are usually protein-based polymers such as collagen[67, 68], elastin[69, 70], fibrinogen[24, 32, 36] or a combination[71-73]. However, other natural polymers in the form of polysaccharides which do not normally biodegrade in humans have also been used, such as cellulose[74]. One of the advantages of using these polymers is that they are native body components and are usually found in the cell physiological niche. Thus, cells possess the enzymes and surface receptors that will interact with these polymers making remodeling a continuous process. Another advantage is the ability to embed cells while crosslinking these polymers which is not feasible with synthetic polymers due to the harsh conditions need to crosslink them (solvents, UV, etc.). Furthermore, protein-based polymers contain domains that enable cell interaction and impart important physiological responses that guide the healing process. On the other hand, while these materials are advantageous on the biological side, they usually lack mechanical strength to withstand hemodynamic forces. Nevertheless, these polymers have shown promising results as graft substitutes [24, 75].

Methods of scaffold fabrication

Ever since the foundation of tissue engineering, there have been a myriad of methods developed to fabricate scaffolds which is one of the main building blocks of engineered tissues. Methods such as freeze-drying, gas foaming, phase separation, electrospinning, and solvent casting-particulate leaching (SC-PL) have all been used, and each has its pros and cons. In this section, a brief description is provided on the most commonly used scaffold fabrication methods in vascular tissue engineering.

Electrospinning: this is a common technique used for the production of nano- and microfibers. In this method, a polymer is dissolved in a solvent at an optimized ratio and loaded into a syringe placed in a syringe pump. During electrospinning, a high voltage is applied to the tip of the syringe while the syringe pump, at a very slow rate, ejects the polymer solution forming a droplet at the tip of the syringe due to surface tension. The high voltage then counteracts the surface tension of the droplet, causing an eruption of electrically charged get of a polymer solution. As the polymer jet shoots towards the collector, the solvent evaporates, forming fibers. The structural properties of those fibers can be modified by adjusting parameters such as voltage, solvent used and ratio of polymer to solvent, the molecular weight of the polymers, syringe pump flow rate, etc. The advantage of electrospinning is that it produces fibers similar to those found in the ECM of tissues. On the other hand, scaffolds produced by electrospinning are usually difficult to penetrate by cells due to low pore interconnectedness.

Solvent casting-Particulate leaching: SC-PL is a widely used method due to its simplicity. This method involves a mold that determines the bulk shape of the scaffold, which in vascular tissue engineering is usually an outer cylindrical tube and an inner cylindrical mandrel creating an annular space between the two. To fabricate the scaffold, a porogen (usually a salt) is packed into mold, followed by the dissolved polymer. Once the polymer is cross-linked the mold is disassembled, and the scaffold is placed in a solvent that dissolves the porogen resulting in a porous scaffold. The pore-size and shape, which are inversely related to the structural strength of the scaffold, can be controlled by varying the size and shape of the porogen which helps the interconnectivity of the pores and improves surface area leading to better cell growth, infiltration and nutrient/gas exchange.

While SC-PL is a simple and relatively fast method, the scaffolds produced by this method do not resemble the extracellular matrix environment of vascular tissue. Furthermore, the solvents used in this method are toxic to cells, which requires many washing steps to get rid of any residual solvent.

3-Dimensional (3D) printing: with the rapid advancement in computers and 3D printers, making scaffolds using computer-designed/3D printed scaffolds has become an increasingly appealing application for tissue engineers. This method starts with a 3D design using computer software. Then the digital blueprint is sent to a 3D printer that prints the scaffold using thermoplastics, for example. This method is solvent-free, which is beneficial for cell seeding in downstream applications. A more recent development in 3D printer technology has brought about the use of bioprinting. This method utilizes what is known as bioinks, which are composed of cells encapsulated in hydrogels. The advantage of this method is that it can replicate fine topographical details of native tissues that and circumvent issues such as cell infiltration since the cells are part of the printed materials. Furthermore, this method has the potential of scaling up for mass production. On the other hand, the limitations of this method include cost and resolution of the printed materials.

2.3 Cell signaling

It was traditionally believed that cells communicated solely via soluble factors or cell-cell interactions; however, emerging evidence has shown that cells can respond to many other forms of signals such as magnetic forces, electromagnetic fields, sonic vibrations and interactions with surrounding cells and matrix proteins. We have reviewed these atypical cell stimulations in the context of stem cell differentiation[76]; however, other cell types also

do respond to unconventional signaling. In the vascular system, resident cells like the ECs and SMCs have been shown to respond to hemodynamic forces such as shear stress[77] and pulsatile strain[78] in addition to the traditional signaling. While there are many signaling pathways that are considered essential and are involved in the developing and maintenance of blood vessels, Notch signaling is one of the major pathways and is indeed relevant in the context of cellular organization and regulation in engineered vascular tissues.

2.3.1 Notch signaling pathway

There is a myriad of stimuli that dictate the development of the anatomy and physiology of the vasculature. These include chemical, biochemical, and mechanical stimuli that work individually or in tandem to guide the development and organization of the cell layers in a blood vessel. While the general trend in vascular tissue engineering is to treat tissues with bioactive molecules or mechanical stimuli and expect to produce functional tissues, taking advantage of cell signaling to instruct cells to produce vascular tissues with desirable features is proposed. One of the central signaling pathways that govern the development, physiology and pathology of the vasculature is the Notch pathway[79]. There are four Notch receptor variants in mammals, Notch 1 to 4, all of which are type I transmembrane receptors and range from ~120 to 280 kDa in molecular weight. All the Notch receptors share similar structures and domains. They are single-pass membrane proteins with an extracellular domain (N-terminus), a membrane-spanning domain, and an intracellular domain (C-terminus). Notch receptors interact with five ligands: Delta-like ligand (Dll) 1, 3, 4 and Jagged (Jag) 1 and 2. These ligands are transmembrane proteins with structural and domain similarities. Canonical Notch signaling occurs when the ligand of the signal sending cell

(SSC) interacts with the receptor from the signal receiving cell (SRC) (**Fig 2.3** step 1). This triggers an enzymatic cleavage of the receptor ectodomain by the enzyme ADAM (**A** **D**isintegrin **A**nd **M**etalloprotease) (step 2), leading to endocytosis of the ligand and the Notch Extracellular domain (NECD) (step 3).

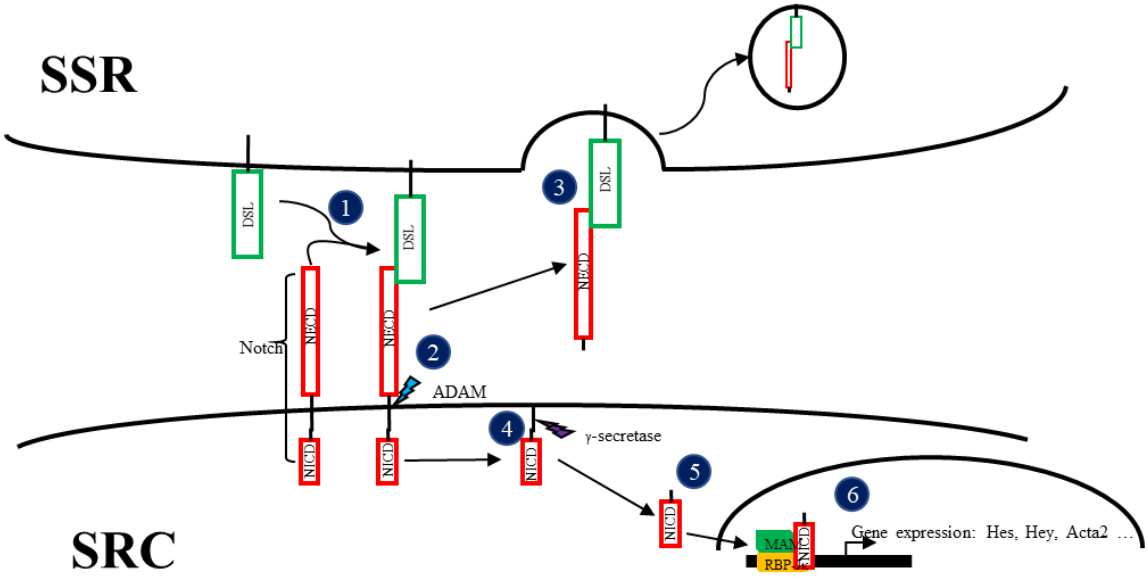


Figure 2.3. Schematic representation of the canonical Notch signal transduction.

This is followed by another enzymatic cleavage by γ-secretase (step 4), which releases the Notch intracellular domain (NICD) into the cytoplasm (step 5). Once in the cytoplasm, the NICD translocates to the nucleus, where it forms a transcriptional complex with RBP-Jκ transcription factor leading to transcription of target genes including Hes, Hey and smooth muscle alpha-actin (Acta2) (step 6). On the other hand, non-canonical Notch signaling has been reported and involves cross-talk between the Notch receptors with members of other signaling pathways like Wnt, MAPK and TGFβ[80-82]. Furthermore, Notch signaling has also

been shown to occur at a distance via cell protrusions[83] and filopodia[84] which can explain the signaling between ECs and SMCs across the internal elastic lamina. In the adult vasculature, endothelial cells predominantly express the Notch1 and 4 receptors, while SMCs mainly express Notch3 and, to a lesser extent, Notch1 and 2[85, 86]. On the other hand, Dll1, Dll4 and Jag1 and 2 are the main ligands expressed on ECs, whereas SMCs express Dll1, Jag1, and Jag2[85, 87]. The expression of these ligands and receptors on the surface of these cells tend to change with development and disease and even in the different branches of the vasculature[88]. There does not seem to be a particular affinity of one of the receptors to any of the ligands, meaning that any receptor is equally capable of interacting with any of the ligands. However, not all receptor-ligand contacts produce a signal[89]. This might indicate that these interactions play a role in signal regulation.

Notch signaling has long been known to be a major regulator and an essential element in the homeostasis of the cardiovascular system [88]. Animal studies demonstrated that dysregulation of the Notch receptors or their ligands has detrimental effects. For example, the Dll4 ligand was determined to be essential for the normal development of the vasculature since haploinsufficiency in the gene will lead to embryonic lethality resulting from defects in the development of arteries[90]. Conversely, expression of a constitutively active form of Notch4 in the endothelium of embryonic mice also shows abnormalities in the development of the vasculature and is also embryonic lethal[91]. Notch3 knock-out in adult mice exhibit structural immaturity and are not responsive to vasoconstrictors such as angiotensin II or phenylephrine[92]. In humans, Notch3 mutations cause Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) due to increased multimerization of the receptor at the surface of the cell and its subsequent

degradation[93, 94]. These studies point to the importance of the Notch signaling and its regulation, warranting the significance of this receptor in the development of the vasculature system.

The expression of the Notch receptors and their ligands vary between the endothelial cells and SMCs and even between the same cell type in different branches of the vasculature. However, the Notch3 receptor and the Jag1 ligand is more relevant for this thesis since they are the major proteins expressed by the SMCs and ECs, respectively[85-87]. The activation of the Notch signaling pathway within the vascular context has been implicated in the development and regulation of the SMC phenotype by modulating the expression of certain SMC genes[14, 16, 95] which is the reason it is the focus of this study.

2.3.2 Notch regulation

At first glance, the canonical Notch signaling pathway seems to be a simple, straightforward signaling mechanism between the SSC and SRC. Compared to other signaling pathways, Notch signaling has no secondary messengers involved and no signal amplification. How can a seemingly simple pathway be conserved among many species and regulate such vital processes in developing animals from development to adult life? The short answer is that it is not a simple pathway. Even though it has been over a century since the discovery of Notch, it is still not a well-understood pathway. It is regulated by various mechanisms from the moment the receptors and ligands are translated in the endoplasmic reticulum to the moment they are degraded/recycled. After Notch is synthesized, it is modified by the addition of O-glycans (O-fucose,-glucose,-GlcNAc and -xylose), which appears to play a role in ligand sensing[96]. After the interaction with the

ligand and the intracellular domain of Notch is cleaved, the NICD translates to the nucleus. The NICD itself has been shown to be affected by various post-translational modifications to further regulate this pathway. Observations have shown that the NICD can be methylated[97], acetylated[98] and ubiquitinated[99]. These modifications will modulate the extent of activation or inhibition of downstream targets of the notch pathway. While the NICD is thought to directly translocate to the nucleus, evidence has shown that Rab5 regulates an alternate route of NICD transport via endosomes[100]. This route then forks into two different paths, either to the nucleus and signaling activation or to the lysosome and signal attenuation. Not surprisingly, these routes are also regulated. PIM (Proto-oncogene serine/threonine) kinase has been shown to phosphorylate a sequence of the Notch nuclear localization domains enhancing the signal[101], while ESCRT proteins control its translocation to lysosomes leading to signal attenuation[102].

A defining feature of the Notch signaling is that both the receptor and the ligand are cell-surface proteins. While the mentioned regulating steps occur at the receptor end of the signaling, the ligands are also subjected to complex cellular regulations. For example, ligand recycling to the surface of the SSC which ensures continual signaling, is regulated by E3 ubiquitin ligases[103, 104]. Furthermore, the number of ligands on both SSC and SRC will dictate whether the signal will be activated or repressed via a mechanism known as cis-inhibition[105]. Studies have shown that whether the cell will behave as an SSC or SRC is contingent upon the relative numbers of the receptors and ligands on that cell. If a particular cell expresses more ligands than receptors on its surface, it will be an SSC; however, if it expresses more receptors than ligands, it is an SRC. Alternatively, if the ratio of receptor to ligand approaches 1, then the cell will be neither SSC or SRC and thus will

be cis-inhibited[105, 106]. However, a recent publication has shown that this is not always the case as the interaction of the receptor with the ligand on the same cell might activate the signaling transduction via cis-activation [107]. This level of regulation at multiple steps of the seemingly simple pathway shows the complexity that allows this pathway to regulate many vital cellular and developmental processes. Furthermore, the fact that there are many conflicting functions of this pathway hints at its dependence on the context in which the signaling is occurring.

2.4 Engineered tissues as disease models

The advent of tissue engineering has been aimed to fabricate functional tissue substitutes to repair or replace damaged tissues or organs. There has been promising progress as evidenced by different clinical studies that use various types of engineered tissues[65, 108-111]. However, even with the substantial progression of cell and molecular biology (especially cell isolation, differentiation, stemness restoration, etc.); and biomaterial science and engineering enabling the preparation of tailor-made scaffolds with tuned molecular and chemical properties, only a few engineered tissues that are comparable to the native counterparts on structural and functional levels have emerged. If anything, this fact emphasizes the challenges to develop functional tissues. The major challenges are the lack of knowledge in culture parameters and conditions (especially cell differentiation), delivery of biochemical and mechanical stimulations, structural organization of the tissues and direction of cellular orientation. Nevertheless, the purpose of engineered tissues has expanded to include an equally significant application as in vitro model platforms to study diseases and for the development of treatment strategies. Even though preclinical platforms

such as cell culture and animal models have contributed significantly to the current understanding of pathology and physiology, they have their shortcomings. On the one hand, the 2D culture systems exclude the vital biochemical and biomechanical cues of the extracellular matrix (ECM), which is known to affect molecular and cellular processes and thus might lead to results that may not be relevant physiologically. On the other hand, animal models do not reflect genetic, physiological, or anatomical elements of the human species and, again, lead to irrelevant results. Not to mention the high cost of maintenance and the ethical concerns that accompany animal studies. Conversely, engineered tissues can be fabricated using relevant human-derived cells and include or intrinsically produce their own ECM components, thereby overcoming the shortcomings of current preclinical models. Another important advantage of engineered tissues is the ability to control the manufacturing procedure and, consequently, highly reproducible results. Additionally, engineered tissues have the advantage of being an isolated system that can be used to systematically study disease without the effect of other organs. On the other hand, if the impact of one organ/tissue on another has to be established (such as the effect of bone resorption on the calcification of vascular tissues), different engineered tissues can still be coculture to study that correlation. Of course, this does not totally exclude the use of animal studies since there is a certain level of systemic complexity that the engineered tissues lack and is important and required before the transition to human trials.

2.4.1 Engineered vascular tissue disease models and test platforms

Vascular tissue engineering is a relatively new field of research. The focus of published studies revolved around exploring different cell types[24, 38, 112], bioreactor parameters and

culture settings[113, 114], and different materials[30, 115] to find the right combination of parameters that produce an engineered tissue that best resembles the native vascular tissues in both structure and function. That being said, a few numbers of studies have started emerging that use engineered vascular tissues as disease models or as platforms to test medical devices or treatments.

Atherosclerosis is a prevalent cardiovascular disease and is one of the major causes of morbidity around the world. As such, many of the studies that emerged to model and understand cardiovascular disease are aimed at different aspects of the pathophysiology of atherosclerosis. For example, one study used an engineered vascular tissue as an in vitro model to study monocyte attachment and insudation as well as LDL insudation into the media of the fabricated tissues[116]. Another similar study but with fibrin gels explored the insudation of lipoproteins and monocytes at hypo-, normo- and hyper-cholesterolemia[117]. A follow-up study by the same group utilized the same fibrin gel system to study neutrophil granulocyte penetration and production of matrix metalloproteinases of a neointimal model[118].

A more recent study modeled a less common disease, the Hutchinson-Gilford Progeria Syndrome (HGPS), which is characterized by accelerated aging and loss of functionality in the SMC of vascular tissues. Engineered vascular tissue was made from type-I collagen and iPSC-derived SMCs obtained from patients with HGPS[119]. These tissue models showed a lower response to vasoconstrictors and lower expression of SMC contractile proteins compared to tissues where the cells were sourced from healthy individuals. Furthermore, this study explored a treatment for the HGPS using a rapamycin analog compound and

showed that some functionality of the diseased engineered constructs was restored within one week of treatment[119].

Vascular calcification is yet another disease that has lately been modeled in vascular tissue engineering. In this first of its kind research study, acellular engineered vascular tissues were used to study the physical characteristics of vascular calcification and the effect of high calcium and phosphate ions in the culture media has on the histochemical, structural, thermal and mechanical properties of these models[120]. However, a major weakness of this study was that it did not explore the cellular response to calcification which are active contributors to the calcification of vascular tissues. Coincidently, the Mequanint lab was working on that specific topic around the same time, aimed specifically at the role progenitor and differentiated cells play during the calcification of engineered vascular arteries[121] which is discussed in more detail in chapter 4.

The potential of EVTs extends even beyond modeling diseases and has also encompassed developing new medical devices as well as studying the effect of medical devices on tissues. Stents are commonly involved in the treatment of coronary artery occlusion due to atherosclerosis, and thus, they are a relevant device to test in engineered tissues. A protein-loaded stent was used to evaluate the behavior of SMCs in the medial layer of engineered vascular tissues[122]. In a different study, a group of researchers employed EVTs to study the effect of stent deployment on the endothelial cells in a bioreactor under physiologically relevant conditions[123].

These studies emphasize the potential and feasibility of engineered vascular tissues to study different aspects of the same phenomenon by systemically introducing different variables

at a time. This allows an understanding of the separate elements involved in a process and then putting all the elements together to look at the bigger picture. Furthermore, these studies show the feasibility of using EVTs as drug and medical device test platforms.

2.5 Motivation and significance of this work

This work was inspired after identifying a novel use of engineered vascular tissue as templates to study cell signaling and disease modeling. Herein, the focus is on the notch signaling pathway which is an essential pathway in vascular development. Furthermore, this research presents novel studies that were amongst the first that used engineered vascular tissues as a disease model for vascular calcification producing only the second study ever to be published on this subject[121].

2.6 Research scope and objectives

The scope of this research is to showcase the use of engineered vascular tissues as platforms to study cell signaling and disease models. As a pivotal signaling pathway that is implicated in the development and disease of the vascular system, the Notch signaling pathway was chosen for this research. Furthermore, calcification of the vascular tissue has never been studied in an engineered tissue model, and thus EVTs were chosen as tools to study the development of medial calcification and explore a potential adjuvant treatment for it using vitamin K. To lay the grounds for this research scope, the following objectives were identified:

1- Comparison of the response of differentiated vs. progenitor cells in Notch signaling activation via a 3D coculture system with endothelial cells (Chapter 3).

2- Study the role of progenitor and differentiated cells in engineered vascular tissue calcification and the role of vitamin K as a calcification inhibitor (Chapter 4).

3- Explore the role of vimentin, as a mechanosensory fiber, in the activation of the Notch signaling and neovascularization (Chapter 5).

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Chapter 3

Comparative studies of fibrin-based engineered vascular tissues and Notch signaling from progenitor cells*

Overview: *The purpose of the study herein is to evaluate the potential of engineered vascular tissues as a platform to study the Notch signaling pathway in a 3D co-culture system. Smooth muscle cell lines from rats (A-10) and a multipotent cell line from mice (10T1/2) were used to compare their interaction with the human coronary artery endothelial cells (HCAEC) in a Notch signaling context. Notch activation was characterized by both gene and protein expression of known Notch downstream targets using qPCR and immunofluorescence microscopy. Physical characterization of the fibrin gel was also reported.*

3.1 Summary

The main impetus of vascular tissue engineering is clinical translation; but an equally appealing and impactful use of engineered vascular tissues is as preclinical testing platforms for studying vascular disease and developing therapeutic drugs and understanding of physiologically relevant vascular biology. Developing model engineered tissues will aid in narrowing the significant knowledge gaps in functional tissue formation, which is regulated by intricate cell signaling in a three-dimensional space. In this study, tubular engineered vascular tissues were fabricated using crosslinked fibrinogen as a scaffold and non-differentiated embryonic rat vascular smooth muscle cell line (A10 cells) and mouse embryonic multipotent mesenchymal progenitor cell line (10T1/2 cells) as cells

*A version of this chapter has been published: K. Dayekh & K. Mequanint, *ACS Biomater. Sci. Eng.* 2020, 6, 5, 2696–2706

embedded in the gel were unable to significantly contract the tissue compared to fibrin-only gels due to their undifferentiated state. In contrast, 10T1/2 cells differentiated with TGFβ1 to a vascular lineage were able to contract the tubular gel significantly owing to the contractile cytoskeletal stress fibers. Owing to its vital role in vascular morphogenesis, tissue specification and maturation, Notch signaling studies in engineered vascular tissues from A10 cells demonstrated cis-inhibition while 10T1/2 cells activated Notch and its downstream targets Hes-1 and the smooth muscle α-actin genes. Taken together, this study showed that: (i) contrary to the previously accepted notion, cell-type is important to gel contractions, (ii) In engineered vascular tissues, Notch signaling is highly context-dependent where cis-inhibition muted signal activation in A10 vascular cells while Notch was fully activated in 10T1/2 cells. These findings may provide insights to fabricate functional vascular tissues.

3.2 Introduction

In vascular tissue engineering strategies, scaffolds and cells are the two critical components. An ideal vascular tissue engineering scaffold is expected to provide both the mechanical support and the microenvironment that is conducive for seeded cells[1]. The mechanical support, which is a temporary one until cells produce their own structural matrix, is readily provided by choosing a scaffold material of synthetic origin (e.g. biodegradable polyesters[2], poly(ester) amides[3] and polyurethanes[4]). The benefit in selecting synthetic scaffolds is to maximize flexibility on the material selection with acceptable initial mechanical properties. The scaffold microenvironment, on the other hand, provides appropriate cues for cells to differentiate and maintain a differentiated

phenotype of vascular smooth muscle cell (SMC) which are often regulated by several signaling processes[3, 5]. Protein-based scaffolds (e.g., decellularized tissues, collagen, fibrin, and elastin) are best suited for providing a better microenvironment than synthetic materials since they provide potential binding sites for receptors. By selecting different synthetic and protein-based biomaterials, several investigators have reported top-down design of engineered vascular tissues (recently reviewed in[6]). Although mechanical properties and matrix deposition in engineered vascular tissues have been studied[7-9], the role of scaffolds and the source of cells to activate important signaling processes are notably lacking.

Fibrin gel is an important naturally occurring vascular tissue engineering scaffold that has been studied with respect to entrapped smooth muscle cells migration and proliferation and to a lesser extent extracellular matrix deposition (abundant collagen and detectable elastin). Unlike many vascular scaffold materials, fibrin is highly stretchable, exhibits non-linear mechanics, and displays unusual effect since there is a dramatic decrease in volume upon stretching thus displaying negative compressibility[10]. Highly compacted fibrin matrix has also acceptable mechanical properties[11]. These features of fibrin are beneficial for vascular tissue engineering scaffolds that are routinely subjected to circumferential stretching forces during the maturation process. Similarly, unlike synthetic hydrogels, fibrin provides binding sites for many growth factors such as fibronectin, hyaluronic acid, and von Willebrand factor[12]. Furthermore, fibrin has an advantage since it is biocompatible, biodegradable, and the fact that the precursor fibrinogen can easily be obtained from autologous blood. Previous studies have utilized fibrin gel to fabricate vascular tissues with some success in animal models[11, 13, 14].

In light of the many benefits (both mechanically and biologically) of fibrin, it will be beneficial to evaluate fibrin scaffold with regards to maintaining a differentiated vascular SMC phenotype and Notch signaling. The vascular SMC is the main cellular component to fabricate an engineered vascular tissue. The limited proliferative capacity of human adult vascular cells and their slow rate of extracellular matrix (ECM) proteins production are challenges to fabricate a biologically functional engineered tissue. However, other sources (embryonic stem cells, induced pluripotent stem cells, or adult stem cells) can be differentiated into vascular SMC[15]. Regardless of the source, phenotype modulation of SMC is an important design goal since these cells switch between the contractile phenotype characterized by well-organized cytoskeletal and contractile marker proteins and the synthetic phenotype characterized by increased proliferation rate and matrix production[16, 17]. The transition between the two states is not well-defined but is a continuum and several signaling processes may be in involved. Notch signaling is a dominant process in vascular specification and cell-fate determination. The expression pattern of the Notch receptors and their ligands differ with the type of tissue. In the context of vascular tissue, Notch3 is the major receptor found on arterial SMCs, and Jag1 is the major ligand expressed by endothelial cells (EC) [18, 19]; however the expression of these proteins can differ throughout development and disease. Notch is recognized as an important regulator of vascular SMC phenotype. Our lab and others have previously shown that Notch signaling leads to expression of contractile vascular SMC markers[20-23]. On the other hand, other studies have shown that this pathway inhibits SMC differentiation to a contractile phenotype via upregulating the expression of Hairy-related transcription factors (HERT) (reviewed in ref[24]) which is a downstream target of notch signaling. These studies certainly indicated

that the outcome of the Notch pathway activation is heavily dependent on cellular and molecular contexts. Identifying these contexts will advance our understanding of this pathway and how it regulates vascular SMC phenotype and will improve our chances in producing better engineered vascular tissues. In view of all the above, the aim of the current study was to compare two different cell types (non-differentiated embryonic vascular smooth muscle cell line (A10 cells) and embryonic multipotent mesenchymal progenitor cell line (10T1/2 cells) in fibrin-based vascular tissue for their gel contraction and their Notch signaling behaviors.

3.3 Materials and Methods

3.3.1 Cell culture and tissue fabrication.

Non-differentiated embryonic vascular smooth muscle cell line (A10 cells) and embryonic multipotent mesenchymal progenitor cell line (10T1/2 cells) were purchased from ATCC and maintained in Dulbeco's Modified Eagle's media (DMEM) (Thermofisher) containing 5% Fetal Calf Serum (FCS) (Thermofisher) and 1% penicillin/streptomycin by volume. Human coronary artery endothelial cells (HCAEC) were obtained from Lonza and maintained in EGM^{TM}-2 Endothelial Cell Growth Medium-2 BulletKit^{TM}. Cells were passaged when they reached 80% confluency and media changes were regularly performed according to supplier's recommendations. To fabricate the fibrin-based tissues, cells were trypsinized with trypsin-EDTA 0.05% (Thermofisher) for 2 min and then suspended in DMEM. The cells were counted, and the appropriate volume was taken from the cell suspension to give a final cell content that varied between 0.5 to 1 million cells/mL of construct. The cells were then centrifuged at 1200 rpm for 5 min, the supernatant aspirated

and cells resuspended in 450 µL of media. To that, 10 µL of 2 M $CaCl_2$, 40 µL of 50 mg/mL ε-aminocaproic acid (ε-ACA) (Sigma-Aldrich), and 2 µL of 1U/µL Thrombin (MP biomedicals) were added. This cell suspension was kept on ice until it was mixed with ice-cold 500 µL solutions of 4, 6 or 8 mg/mL bovine fibrinogen (MP Biomedicals). Right after mixing the two solutions, the mixture was transferred into a clean glass mold that had been disinfected with 70% EtOH, dried and then incubated in a 5% solution of Pluronic F-127 (Sigma-Aldrich) for an hour at room temperature. The mold is composed of a glass shell with an inner diameter of 1cm, and a glass mandrel with an outer diameter of 4mm. Two Teflon plugs were used to seal the ends of the glass shell and position the mandrel in the center. The mold was then transferred to an incubator at 37 ºC for 1.5 h for crosslinking. Initial dimensions of the constructs reflected the dimensions of the mold; however, they changed as the constructs shrunk in culture. Tissue disks to measure water mass loss were made the same way as mentioned previously; however, a cylindrical tube was used instead of the mold.

For co-culture experiments with ECs, A-10 or 10T1/2 cells were seeded in 6 mm plates (Corning) at a density of 0.3×10^6 or 0.5×10^6 cells/plate, respectively, and left overnight in the incubator to attach. The following day ECs were seeded on top of the A-10 and 10T1/2 cells at a density of 0.5×10^6 cells/plate. The co-cultured plates were maintained in a mixture of DMEM and EGM2 at a ratio of 1:1. For Notch signaling studies, the Notch inhibitor DAPT was used at a concentration of 15 µM for 3 days cultures. For seeding ECs in the lumen of tissues, small pieces of sterile gauze were incorporated into the fabrication of the tissues on either end to facilitate tying the ends of the tubes closed with sterile dental floss

without breaking. Prior to seeding ECs in the lumen, one end of the tissue was tied with a sterile dental floss, then 0.5 mL of media containing 1×10^6 cells/mL was pipetted into the lumen and the other end of the tissue was tied with another piece of sterile dental floss. The tissue was given 4 hours in the incubator and then turned 180º to allow the cells to attach all around the lumen. Then the tissue was left overnight in the incubator after which the ties were cut to allow the culture media into the lumen.

3.3.2 Tissue water loss and vitality assays.

Tissue disks were cultured in media for a period of 12 days. During the culture period, the tissues were taken out of culture, blotted on a sterile paper towel, placed in a sterile tube and weighed every 3 days. To assess the vitality of the tissues, tissue disks were cultured in 12 well plates for a period of 12 days and their viability was measured every 3 days using resazurin blue (Sigma-Aldrich) assay. Culture medium was aspirated and replaced with 1 mL of sterile PBS containing 1.75 µg/mL resazurin blue and incubated at 37 ºC for 5 h. After that, 100 µL from each well was taken and placed in a 96 well plate and the absorbance was measured at 570 and 600 nm against a blank.

3.3.3 Scanning electron microscopy (SEM) imaging of fibrin gels.

Fibrin gels were prepared by mixing 50 µL of ice-cold fibrinogen solution (2mg/mL) with 50 µL of ice-cold DMEM containing 1 U thrombin. The 100 µL mixtures were quickly pipetted onto aluminum foils in a 24-well plate culture wells and then transferred to a 37°C incubator to cross-link for 1.5 h. After incubation, 0.5 mL of PBS was added to each well to prevent the gels from drying. The gels were incubated at 37 °C for 3 days after which they were fixed with 4% paraformaldehyde for 30 min. Following fixation, the gels were

washed 3 times with distilled water, and serially dehydrated with EtOH in water (50%, 75%, 95% and 100%) for 15 min each. After dehydration with EtOH, the gels were serially dehydrated with hexamethyldisialazane (HDMS) (Sigma) in EtOH (50%, 75%, and 100% twice) for 15 min each. After removing the HDMS, the gels were allowed to dry overnight at room temperature, sputtered with gold/palladium, and then imaged with an SEM (LEO 1530; Zeiss, Oberkochen, Germany).

3.3.4 Western blotting.

A-10 cells treated with 2 ng/mL TGF-β1 for 3 days were washed 3 × with ice-cold phosphate-buffered saline (PBS) then harvested in ice-cold RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.25% (w/v) sodium deoxycholate and 0.1% (w/v) SDS pH: 7.5) containing protease inhibitor cocktail (Roche) and 1 µM Phenylmethanesulfonyl fluoride (PMSF). The cells were kept on ice for 15 min to allow for lysis to complete. Lysates were centrifuged at 12000 rpm for 15 min. The pellets were discarded, and the supernatants' protein contents were quantified using the Pierce BCA protein assay protocol (Pierce). Protein samples were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (Pall life sciences). Blocking the membrane was performed with 5% BSA (Sigma-Aldrich) in PBS with 0.1% Tween-20 (PBS-T) and Western blotted with the Acta2 primary antibody (1:1000) (Santa Cruz Biotech) diluted in 5% BSA in PBS-T overnight at 4º C. The blots were then washed 2 × with PBS-T for 5 min each, and 1 × with PBS for 5 min, followed by incubation with goat anti-mouse secondary antibody (1:5000) diluted in 5% BSA in PBS-T for 1 h at room temperature.

Finally, the blots were washed as before and incubated with Supersignal west pico chemiluminescence substrate (Pierce) and developed using ChemiDoc XRS+ (BioRad).

3.3.5 Immunofluorescence microscopy and histology.

For 2D studies, cells were seeded in 6-well plates containing a coverslip at a density of 2.5 × 10^5 cells per well in complete media. The next day, the cells were treated with 2 ng/mL TGF-β1 for a period of 3 days. After that, the cells were washed with 37ºC PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The paraformaldehyde solution was aspirated, and the cells washed 3 × with PBS at room temperature. For tissue studies, the tissues where washed with 37º C PBS and then fixed with 4% Paraformaldehyde at RT for 1 h. The tissues were then washed with PBS 3 times and incubated at RT in a 15% and then in a 30% solution of sucrose for 30 mins each, washed with PBS and then dabbed on a tissue to remove excess liquids. After that, the tissues were immersed with OCT compound (Fisher) and transferred into -80º C isopropanol. Tissue sections were obtained by using a Leica cryostat (Leica) and then were washed with PBS 3 times for 5 mins each to remove OCT compound. Cells/tissue were permeabilized with a 0.2% (v/v) Triton x-100 in PBS for 15 min at room temperature and then blocked with 5% BSA in PBS-T for 1 h. The blocking solution was then aspirated, and 100 µL of primary antibodies of Hes1, Acta2, Smtn, Notch3, Myh11, ColI, ColIII, Cnn1, or Jag1 (Santa Cruz Biotech) (1:100) in 5% BSA PBS-T were placed on the coverslip and covered with a piece of parafilm and placed in a humid environment at 4º C overnight. In order to rule out a nonspecific signal, IgG antibodies against rabbit and mouse species have been used as controls at the same dilution ratio. The next day the cells/tissues were washed 2 × with

PBS-T and once with PBS for 5 min each, then incubated with the corresponding secondary antibody (1:250) for 1 h at RT. The coverslips were then washed 2 × with PBS-T and once with PBS and incubated with 2 µg/mL DAPI for 5 min, washed with PBS 3 times and mounted with mounting media. The images were taken by a compound fluorescent ZeissZ1 microscope (Zeiss).

Histological microscopy was performed by taking tissue sections and staining them with hematoxylin for 3 min, and then incubating with tap water for 5 min, followed by washing with acid ethanol (0.3% HCl in 70% EtOH). Excess fluids were removed by blotting the slides with a paper towel before staining with eosin. The tissues were incubated in Eosin for 1 min, and then 3 times in 95% ethanol for 5 min followed by 3 times in 100% ethanol for 5 min each. Slides were allowed to dry and then visualized using a Nikon Eclipse TS100 microscope (Nikon).

3.3.6 RNA isolation and qPCR.

For the engineered tissues, approximately 50 mg of tissue was placed in each Eppendorf tubes and frozen at -80 ºC for at least an hour, crushed by a pestle, and 750 µL of Trizol were added and then homogenized with a tissue homogenizer (BioBasic). The seeded endothelial cells (where applicable) in the engineered tissues were removed using a surgical scalpel, which is a well-established procedure. For 2D cultures, spent media was first aspirated and 500 µL of Trizol (Life Technologies) were pipetted for each 6 cm culture plate. The cells were then scraped and transferred to Eppendorf tubes. To separate endothelial cells from cocultures, cells were trypsinized using 1 mL of trypsin-EDTA 0.05%, and then collected in a conical tube and centrifuged at 1200 rpm for 5 min. The cell

pellet was collected and resuspended in 1 mL of PBS containing 0.5% BSA and 1×10^7 PCAM magnetic beads for 30 min on a tumbler at 4 ºC to separate the HCAECs from the 10T1/2 cells. The magnetic beads were pulled down with a magnet and the 10T1/2 cells were transferred into a separate tube and centrifuged at 1200 rpm for 5 min. The supernatant was aspirated, and the cells were resuspended in 500 µL of Trizol to extract RNA. Lysates were left to incubate at room temperature for about 5 min. After that, chloroform was added at a ratio of 1:5 (chloroform:Trizol) and the samples were vortexed for 15 seconds then incubated at room temperature for 15 min. Samples were then centrifuged at 12000 g for 15 min at 4 ºC. The aqueous phase was transferred into another Eppendorf tube and Isopropanol was added at a ratio of 1:2 (isopropanol:Trizol) and incubated at RT for 10 min followed by centrifugation at 12000 g for another 10 min at 4 ºC. The isopropanol was then aspirated, and the pellet was resuspended in 75% EtOH at a ratio of 1:2 (EtOH:Trizol) and centrifuged at 7500 g for 5 min at 4 ºC. This last step was done twice to wash excess salts. The pellet was then air-dried after the EtOh was removed and the pellet was dissolved in 25 µL of DEPC water and quantified with nanodrop (Thermo scientific). 1 ug of total RNA was used to synthesize cDNA using M-MLV reverse transcriptase kit (Promega) using the supplier's protocol. For qPCR reactions, 1 µL of the formed cDNA was used in 10 µL reactions using the SsoAdvanced universal SYBR green supermix (Biorad) according to the manufacturer's protocol. The qPCR reactions were carried out in a CFX96 Real-Time thermal cycler (BioRad) and GAPDH was used as a reference gene.

3.3.7 Gel swelling, water contact angle, and rheological properties.

For fibrin gel swelling ratio determination, acellular fibrin discs were prepared from 3 mg/mL fibrinogen as previously mentioned in the "Cell culture and tissue fabrication" section. The discs were then dabbed with a paper towel to remove excess water and then placed in a vacuum desiccator 16h to dry the gels. The specimens were weighed to obtain the initial mass (m_i) for each gel and were placed in 1mL PBS and placed in an incubator at 37º C for 2 h. After that, the gels were removed from the PBS and dabbed on a paper towel and weighed to measure the final mass (m_t). The % swelling ratio was calculated using the following formula:

$$\left(\frac{m_t - m_i}{m_i} \right) \times 100$$

Water contact angles were measured by a Kruss DSA100 Drop Shape Analyzer. A fibrin gel was prepared using 3 mg/mL fibrinogen in ice-cold PBS and 2 mM $CaCl_2$. To that, 2 µL of 1U/mL Thrombin was added and the solution was quickly transferred onto a microscope slide and spread on its surface before gelling. The slide was incubated at 37 ºC to accelerate crosslinking. After 1 h, the glass slide was dabbed on a paper towel and placed in a desiccator overnight to dehydrate the gel. The contact angles were measured in triplicate after allowing the water droplet to equilibrate on the gel for 15 sec. For rheological testing, a parallel plate rheometer operating at 37 ºC was used. The plates (25 mm diameter) were set up at a 1mm distance and 500 µL ice-cold solution of 3 mg/mL fibrinogen in PBS, 2 mM $CaCl_2$, and 2 U Thrombin was injected between the plates. After the sample gelled for 30 min, amplitude sweep tests were performed.

3.3.8 Statistical analysis.

Where applicable, data are presented as the means of at least three independent experiments and the error bars are the standard deviation from the means. Statistical significance was calculated either using t-tests or ANOVA depending on experimental design. Tukey's multiple comparison tests were used for post-hoc statistical analysis. Differences were considered significant if $p < 0.05$.

3.4 Results and Discussion

3.4.1 Fibrin gel swelling ratio, water contact angle, and rheological properties.

Since fibrin gel is used as bioadhesive in surgeries for hemostasis, wound closure, sealant, and scaffold for tissue engineering, its characterization as a biomaterial is well-reported (reviewed in Ref [25]). However, property variability may still exist depending on the sources, the fibrinogen concentration used to prepare the gel, and the presence of crosslinking agents other than thrombin [26, 27]. Therefore, the swelling ratio, wettability, and rheological properties were determined for fibrin gels prepared from 3mg/mL solution. While direct comparison with literature was not straightforward due to variations in fibrinogen concentration, additional crosslinking agent, crosslinking time etc, data collectively presented in **Tables 3.1** and **3.2** and **Figure 3.1** indicated that the properties of the fibrin gels were, in general, consistent with reported values in the literature[27-29]. For instance, the percent swelling ratio of fibrin gel is reported to be between 100% to 250% depending on the method of preparation [30, 31], which is in the same range as **Table 3.1**.

Table 3.1. Swelling ratio of five fibrin gel disks prepared from 3 mg/mL fibrinogen.

	Gel 1	**Gel 2**	**Gel 3**	**Gel 4**	**Gel 5**
Initial fibrin gel weight (mg)	14.4	14.2	13.9	10.6	10
Final swollen fibrin gel weight (mg)	31.9	32	35	22.5	23.2
Swell ratio (%)	121.5	125.4	151.8	112.3	132.0
Mean swelling ratio (%)	128.6±14.8				

Fibrin is more hydrophobic than its precursor fibrinogen and its water contact angle is reported to be between 63º and 74º when measured on a dry film[28]. Contact angle is between 44º and 48º likely contributed to by the 15 sec that the water droplet remained on the fibrin film before taking the contact angle reading as suggested by the operating manual (pre-wetting step). On a hydrophilic polymer, such a delay seemingly minor could make a significant difference (**Table 3.2)**.

Table 3.2. Fibrin water contact angle.

	Contact angle (º)
Test #1	43.6
Test #2	47.5
Test #3	47.6
Mean	46.3 ± 2.3

The storage modulus (G') vs. strain amplitude data obtained from rheological measurements of a fibrin gel prepared from 3mg/mL fibrinogen concentration indicated that the storage

modulus ranged from 85Pa to 110Pa without significant strain hardening effect (**Figure 3.1**). This was similar to reported values for pristine fibrin gels [27].

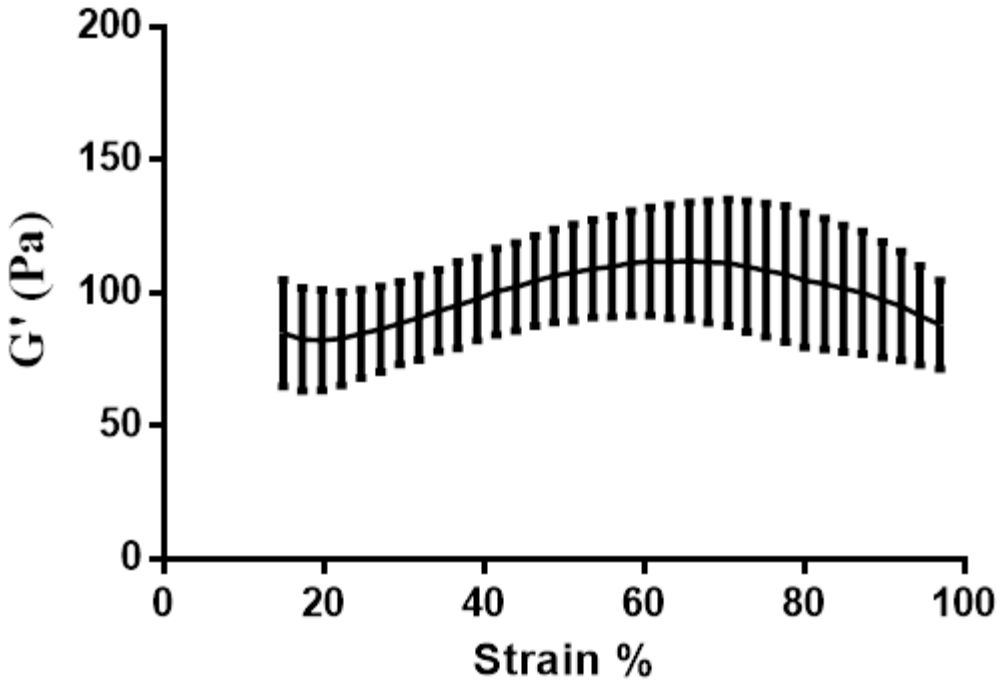

Figure 3.1. Storage modulus vs. amplitude sweep results for a fibrin gel prepared from 3 mg/mL fibrinogen concentration (measurements were taken at 37 ºC)

3.4.2 Smooth muscle marker expression, tissue viability and collagenous matrix synthesis.

In this study, non-differentiated embryonic rat vascular smooth muscle cell line (A10 cells) and mouse embryonic multipotent mesenchymal progenitor cell line (10T1/2 cells) were compared as model vascular cells. Since A10 cells are neointimal stem-derived smooth muscle cells (SMC), SM-α actin expression with and without the presence of TGF-β1 – a

growth factor that differentiates vascular SMC into a contractile phenotype[32] – was tested. Protein analysis for SM-α actin (Acta2) using Western blot showed that treating A10 cells with 2 ng/mL TGF-β1 for 3 days did not affect the expression level of Acta2 **(Figure 3.2A).** However, immunofluorescence microscopy showed increased fiber redistribution, alignment, and reorganization of Acta2 and smoothelin (Smtn) filaments suggesting a more mature contractile phenotype in response to TGF-β1 **(Figure 3.2B)**. However, the role of TGF-β1 in A10 cells appeared to be post-translational since the expression at the protein level of Acta2 did not change. To assess the viability of A-10 cells in the engineered tissues, the absorbance of reduced resazurin for tissue cultures up to 12 days was measured (**Figure 3.2C, D**). **Figure 3.2C** compares the differences in absorbance of cell-embedded fibrin gels prepared from 2, 3 and 4 mg/mL FBG and 0.5×10^6 cells, while **Figure 3.2D** compares fibrin constructs with the same FBG concentrations but with 10^6 cells. While tissue constructs containing 0.5×10^6 cells showed little differences in absorbance, those with 10^6 cells showed an increased absorbance at day 3 and then a reduction in the absorbance for the rest of the duration. Overall, the metabolic activity of these tissues was stable over time. Resazurin is a widely used viability assay; however, its use in tissue viability assays is not very common as the diffusion of the dye into and out of the tissue is limited and therefore, the absorbance is mostly influenced by the cells on the surface of the tissues rather than those embedded in them. Despite this, the benefit of Resazurin is that due to its cytocompatibility, the tissue can be treated multiples times; thus, the metabolic activity of the same tissue can be studied over an extended culture period.

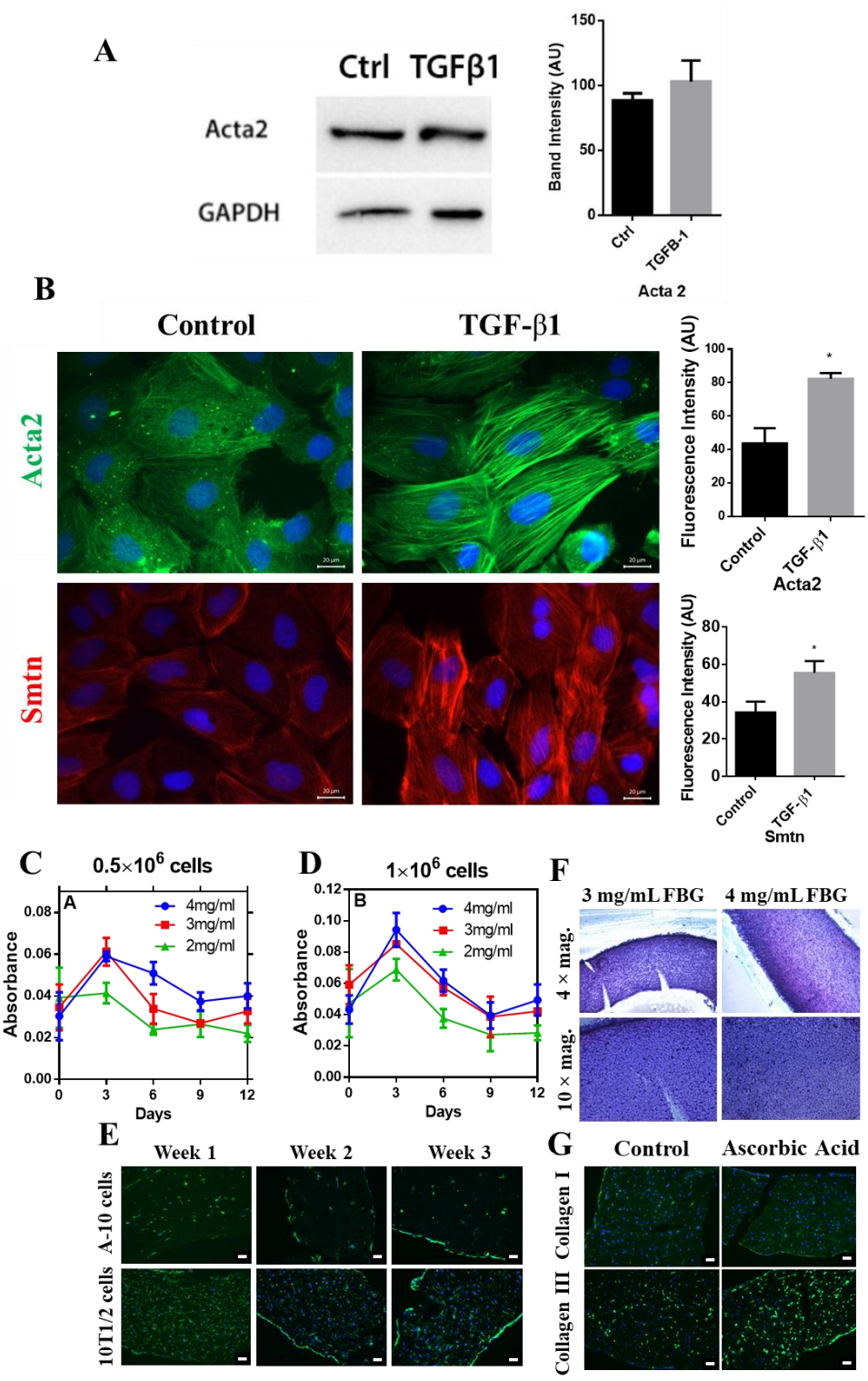

Figure 3.2. SMC marker and matrix protein expression, and tissue vitality and cell distribution. (**A)** Western blot analysis showing the level of expression of smooth muscle

alpha-actin (Acta2) with or without 2 ng/mL TGF-β1 for 3 days. (**B)** Immunofluorescence comparing control A-10 cultures and TGF-β1 treated A-10 cultures of cytoskeletal proteins Acta2 and Smtn. The bar diagrams on the right are the corresponding quantification of the images. Scale bar = 20 μm. (**C)** Resazurin cell viability assay of fibrin constructs with variable concentrations (2, 3, and 4 mg/mL) containing 0.5 million cells/m. (**D)** Resazurin cell viability assay of fibrin constructs with variable concentrations (2, 3, and 4 mg/mL) containing 1.0 million cells/mL. These tissues were cultured for 12 days. (**E)** Fluorescence staining of A10- and 10T1/2- containing tissues cultured for 1, 2 or 3 weeks. Nuclei were stained with DAPI and f-actin with Alexa-488 conjugated phalloidin in green. Sale bar = 50 um. (**F)** Histochemical staining of fibrin-based tissue containing 10T1/2 cells cultured for one week in 3 or 4 mg/mL displayed at two different magnifications 4 and 10 ×. (**G)** 10T1/2 in 3 mg/mL fibrin constructs treated with 50 μg/mL ascorbic acid and probed for collagen I and collagen III. Scale bar = 50 um.

A cell viability test via absorbance measurement provides a global context for the activity of cells within the gels but is not instructive in terms of cell distribution which is important for remodeling and matrix deposition. Fluorescence microscopy images of tissue constructs containing A-10 cells and 10T1/2 cells taken at 1, 2, and 3 weeks during the maturation process are presented in **Figure 3.2 E**. Both cell types were uniformly distributed throughout the cross-section; however, the density of 10T1/2 cells within the construct remained to be higher than A-10 cells. Furthermore, it appeared that cells were forming bundles at the lumen and ablumen of the tubular construct, as evidenced by the intense staining in those regions. H&E staining of tissues containing 10T1/2 cells and matured for 1 week agreed with the fluorescence microscopy images showing the distribution of cells throughout the cross-section (**Figure 3.2 F**). 10T1/2 cells have been studied [3] as SMC progenitor cells and are believed to be a good model to study SMC phenotypic switching. Engineered tissues started to produce collagenous matrix proteins (Collagens I and III) which was further enhanced with 50 μg/mL ascorbic acid treatment (**Figure 3.2 G**). While collagen I is the main type of collagen in the vasculature, collagen III is also important as

its absence or imbalance has been shown to be linked to Vascular Ehlers-Danlos syndrome of arteries [33, 34].

3.4.3 Gel contraction kinetics and fibrous gel stability.

When fibrin and collagen gels are used as a scaffold for tissue engineering, dimensional contraction is often observed in the presence of fully differentiated primary cells[35-38]. However, it is unknown if non-differentiated embryonic and mesenchymal progenitor cells can exert sufficient contraction forces on the gels to bring macroscopic dimensional changes. When non-differentiated embryonic vascular smooth muscle cells were embedded into the gels prepared at different concentrations, a reduction was observed in the volume of the fibrin constructs with 2mg/mL concentration which was more noticable in comparison to the other concentrations after 7 days in culture **(Figure 3.3A).** The effect of fibrinogen concentration with embedded A-10 cells on compaction kinetics was studied at 500, 000 cells/mL and 10^6 cells/mL **(Figures 3.3B, C).** For both cell densities and at all fibrinogen concentrations, the cylindrical disk gels contracted drastically, and the fractional mass of the gels decreased significantly during the first 3 days of culture ($p<0.05$). From day 3 to 12, all tested fibrinogen concentrations with 500, 000 cells/mL showed significant differences amongst them at each time point ($p <0.05$). However, when each concentration was compared at different time points from day 3 to 12, there was no statistical significance on gel contraction kinetics ($p>0.05$). When the cell concentration in the gel was increased to 10^6 cells/mL **(Figure 3.3C)**, a similar behavior was observed except that significance only existed between 4mg/mL and 2 mg/mL fibrinogen concentration at each time point between 3 days and 12 days ($p<0.05$). The role of cell numbers at a fixed fibrinogen

concentration was also studied **(Figures 3.3D, E).** The dimensional contraction was significant until day 3 and remained unchanged for the subsequent 9 days ($p<0.05$). It is worth mentioning that the presence of cells did not change the dimensional contractions of the fibrin gels compared to the fibrin-only gels. While cell-induced contractions of fibrin gels were expected due to the anticipated force exerted by cells, the fact that these gels contract to the same level even without cells implies that other factors are in play. The gels have a large amount of water and were assumed to be incompressible; thus, the observed contraction is primarily caused by water expulsion (syneresis) from the gel rather than fibrin degradation since a plasmin inhibitor (ε-aminocaproic acid) was present in the media throughout these experiments. As additional evidence, fibrin gels were cultured for 3 days and SEM images were taken to visualize fiber morphology (**Figures 3.3F, G**). Clearly, the fibers were morphologically intact, suggesting the absence of degradation notwithstanding the volume change. Although one previous research suggested the absence of compaction in a cell-free fibrin gel[39], this data suggests the contrary. It is likely that crosslinking of fibrinogen into fibrin causes internal stress leading to the unfolding of α helices into β sheets, thus compacting the network [40-42]. Although fibrin gel compaction at a fixed time is previously studied[35], gel compaction kinetics is rarely investigated [43]. In fact, most studies investigated gel compaction after weeks of culturing in the presence of fully differentiated cells; however, the time required to reach constant compaction has not been studied. Data in this study suggested that for all the fibrinogen concentrations and the cell numbers embedded in the gels, the gels reached a steady-state mass after 3 days of culture. Given the current understanding that fibrin gels have accelerated compaction in the presence of cells, significant differences on both the compaction kinetics and in response

to cell number variations was expected. Since the gels without cells compacted similar to the gels with cells, this was attributed to the nature of the undifferentiated A-10 cells. Compared to fully differentiated cells used in previous studies[35-38], A10 cells are immature cells that may not have sufficiently organized cytoskeletal protein fibers to bring significant changes to local and global reorganization and realignment of the gel microstructure.

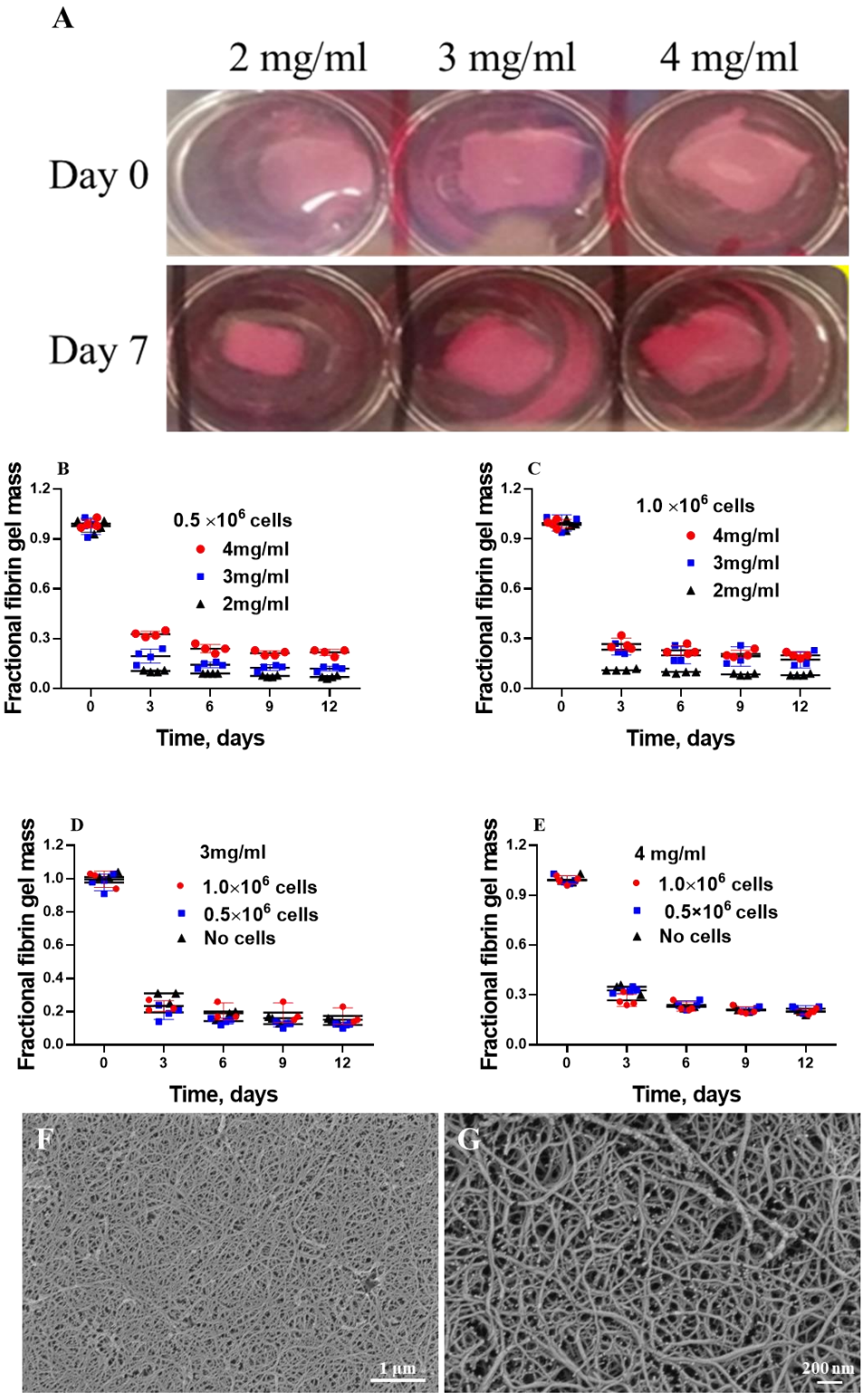

Figure 3.3. Tissue contraction, mass loss and SEM analysis of fibrin fibers. **(A)** macroscopic comparison of compacted fibrin gels of different concentrations (2, 3, and 4

mg/mL) at day 0 and after 7 days in culture. **(B)** mass loss in constructs containing 0.5 million cells in different concentration of fibrinogen (2, 3, and 4 mg/mL) and **(C)** mass loss of constructs containing 1 million cells in different concentrations of fibrinogen (2, 3, and 4 mg/mL) as a function of time. **(D)** mass loss of 3mg/mL FBG and **(E)** 4mg/mL FBG containing 0.5 million and 1 million cells compared to constructs without cells as a function of time. (F) and (G) show the fibrous structure of 1 mg/mL fibrinogen after 3 days in culture at 15,000 and 35,000 magnifications, respectively. Scale bar in **(F)** = 1 μm. Scale bar in **(G)** = 0.2 μm.

3.4.4 SMC differentiation of embryonic multipotent mesenchymal progenitor cells and gel contraction.

The preceding gel contraction kinetics was conducted using A-10 cells. The A10 cells derived from the thoracic aorta of embryonic rat are non-differentiated, neonatal and neointimal vascular smooth muscle cells while 10T1/2 cells are mouse embryonic multipotent mesenchymal progenitor cells. 10T1/2 cells share similar differentiation characteristics with mesenchymal stem cells and have been shown to differentiate into mature vascular smooth muscle cell lineage under appropriate conditions[44, 45]. For this purpose, 10T1/2 cells were embedded into tubular fibrin gels with and without 2ng/mL TGF-β1, matured them for one week and monitored tissue contraction and differentiation into a mature vascular SMC. The rationale for choosing 10T1/2 cells instead of A10 cells is based on the previous findings that TGF-β1 upregulated SMC contractile protein in these cells[3, 45], though the induction of these markers in fibrin gel has not been demonstrated before. As presented in **Figures 3.4A, B** TGF-β1 induced significant contraction of the gels ($p<0.001$) where both the inner and outer diameters were reduced by 33%. As both gels were prepared from the same fibrinogen concentration and had the same cell numbers, contraction is attributed to cytoskeletal traction forces on the fibrin fibers caused by the

differentiation of the 10T1/2 cells towards a mature SMC. To ascertain differentiation, immunofluorescence microscopy images of smooth muscle α-actin (Acta2) and smoothelin (Smtn) were taken (**Figure 3.4C**). Although the expression of smooth muscle α-actin in the differentiated cells was similar to the human coronary artery smooth muscle cells, this marker is not specific to SMC as it is also expressed in other cells (e.g. myofibroblasts)[46]. The only marker unique to vascular SMC that is absent in myofibroblasts is smoothelin[47, 48], and hence this marker was selected in this study. As can be seen, these tissues expressed Acta2 and Smtn, suggesting a SMC lineage commitment in response to TGF-β1 treatment. On the other hand, SM-myosin heavy chain (Myh11) was undetectable (data not shown) and this was not surprising since it is a late-stage SMC differentiation marker and the culture time was only one week. This observation is also consistent with previous findings that longer-term cultures and biomechanical forces were required for Myh11 expression of 10T1/2 cells[45].

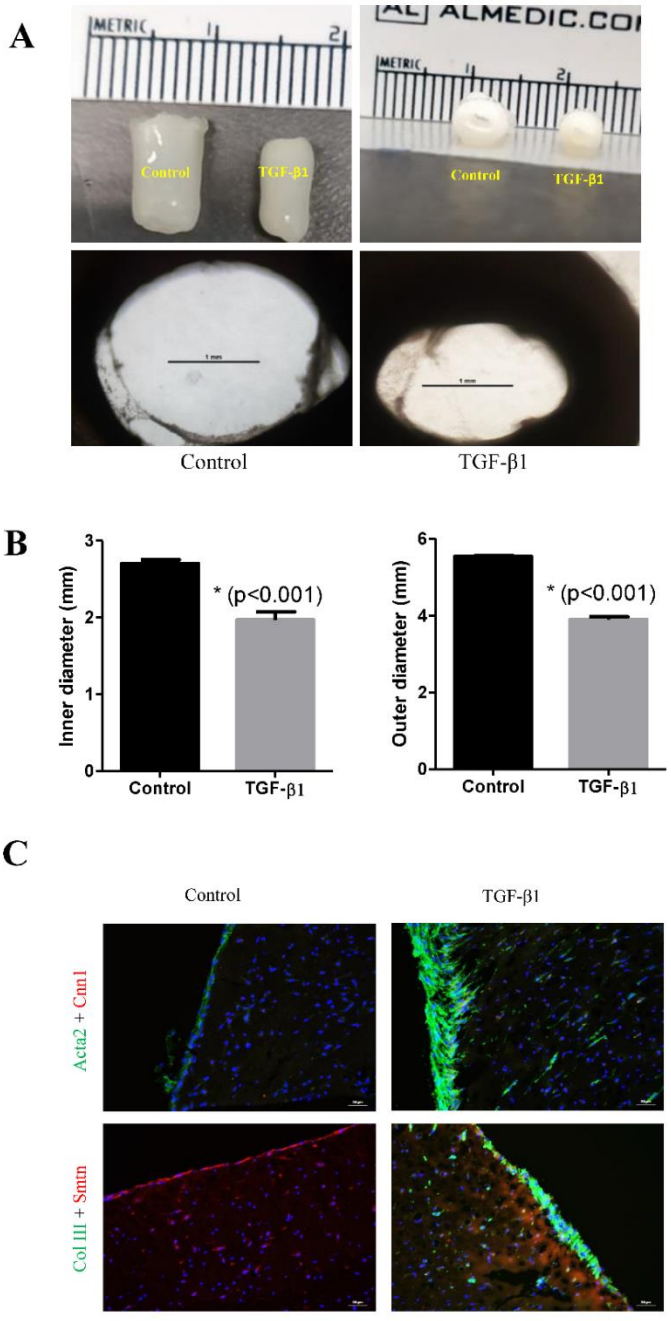

Figure 3.4. Tissue contraction of fibrin-based 10T1/2 constructs treated with TGF-β1 and protein markers expression. **(A)** Macroscopic and microscopic images comparing control tissue with that treated with TGF-β1 showing the contraction of the tissue in response to TGF-β1. Contrast microscopy scale bar = 1 mm. **(B)** inner and outer diameter measurements of control vs TGF-β1 treated tissues. **(C)** Immunofluorescence staining of Acta2, Cnn1, Smtn, and ColIII in 10T1/2 tissue treated with 2 ng/mL TGF-β1 for 1 week. Scale bar = 50 um.

3.4.5 Activation of Notch signaling in engineered vascular tissues.

Although TGF-β1 signaling pathway plays an important role in the SMC differentiation and maturation, the Notch signaling is one pathway that plays an important role in the differentiation, maturation, and function of vascular SMC[20]. The Notch family of receptors, Notch1 to Notch4, are single-pass transmembrane proteins consisting of both an extracellular domain (ECD) and an intracellular domain (ICD)[49]. Upon interaction with the Delta, Serrate/Jagged, Lag-2 (DSL) family of single-pass transmembrane ligands (Jagged1, -2 and Delta1, -3, and -4) expressed on neighboring cells, Notch undergoes proteolytic cleavage, which frees the ICD from the plasma membrane. This results in translocation of the ICD into the nucleus, where it forms a complex with the DSL family of transcriptional repressors (CBF1/RBP-Jk), removing the repression and allowing transcription of downstream target genes (Hes/Hey)[50]. Since the distribution of the Notch receptors and their ligands vary considerably in different tissues, it plays diverse roles in cardiovascular and cerebrovascular development and physiology[24]. The Notch pathway components relevant to vascular tissue engineering of arteries are Notch3 receptor and the ligand Jag1 highly expressed in arterial SMC and EC, respectively [19]. In the present study, the effect of TGF-β1 and Jag1 on the activation of the Notch pathway in A-10 cells was studied via the transcription factor Hes-1, the downstream SMC-specific Myh11, and the matrix protein elastin gene expressions. In addition to a possible signal convergence between TGF-β1 and Notch signaling pathways[51], the addition of TGF-β1 was to evaluate if these cells are able to produce an essential vascular ECM elastin based on previous finding that this growth factor was able to enhance elastin synthesis of human coronary artery smooth

muscle cells[52]. **Figure 3.5A** showed that treatment of A-10 cells by culturing them on either surface adsorbed Jag1 or by adding soluble TGF-β1 to the culture did not impact expressions of the tested genes in comparison to untreated control cultures ($p>0.05$). To investigate the role of the microenvironments (2D vs. 3D), A-10 cells and Jag1 were entrapped into the fibrin gel. Expression of the Notch ligand Jag1, the Notch signaling downstream transcription factor Hes-1 and SMC-specific marker Myh11 were either down-regulated or remained unchanged (**Figure 3.5B**), suggesting that Jag1 entrapment did not activate Notch in A10 cells. Studies have shown that Notch ligands are ubiquitinated by multiple ubiquitin ligases in order to properly activate the Notch receptor [53] suggesting the importance of ubiquitination in the activation of the Notch signaling. Along with ligand ubiquitination, a mechanical pull force plays a critical role in ligand endocytosis and Notch signal activation. During endocytosis of the ligand by the signal sending cell, a pull force is generated by the ligand on the receptor of the signal receiving cell causing a conformational change. This then allows for the enzymatic cleavage of the Notch extracellular domain, and consequently to the propagation of the signal [54]. Since the entrapped recombinant Jag1 ligand was not ubiquitinated, and there were no pulling forces subjected on the fibrin gel, the entrapped Jag1 ligand on its own is insufficient to activate the Notch signaling in this system. Moreover, the downregulation of Jag1 and Myh11 in the Jag1 treated group is occurring independently of the Notch signaling since Hes-1 that is a direct target of the Notch pathway was not activated. In Notch signaling, the ECD of the ligand expressed by signal-sending cell binds to the ECD of the Notch receptor expressed on the surface of adjacent vascular SMC (signal-receiving cell), leading to signal activation and subsequent expression of SMC contractile genes in a heterotypic cell-cell

contact manner [21-23]. Since endothelial cells are the Notch signal sending cell in the vasculature, the effect of A10 cells and ECs coculture on the Notch pathway and contractile marker expression was also investigated. Gene expression data for Hes-1, Jag1, and Myh11 for A-10, and EC in coculture shown in **Figure 3.5C** demonstrated that the expression of Hes-1 and Jag1 was reduced by half in the cocultured A10 cells compared to those cultured without EC. However, the expression of Myh11 did not change. In addition to qPCR, immunofluorescence microscopy results also showed that co-culturing A10 cells with EC did not affect the expression of Acta2, or Myh11 proteins (**Figure 3.5D**).

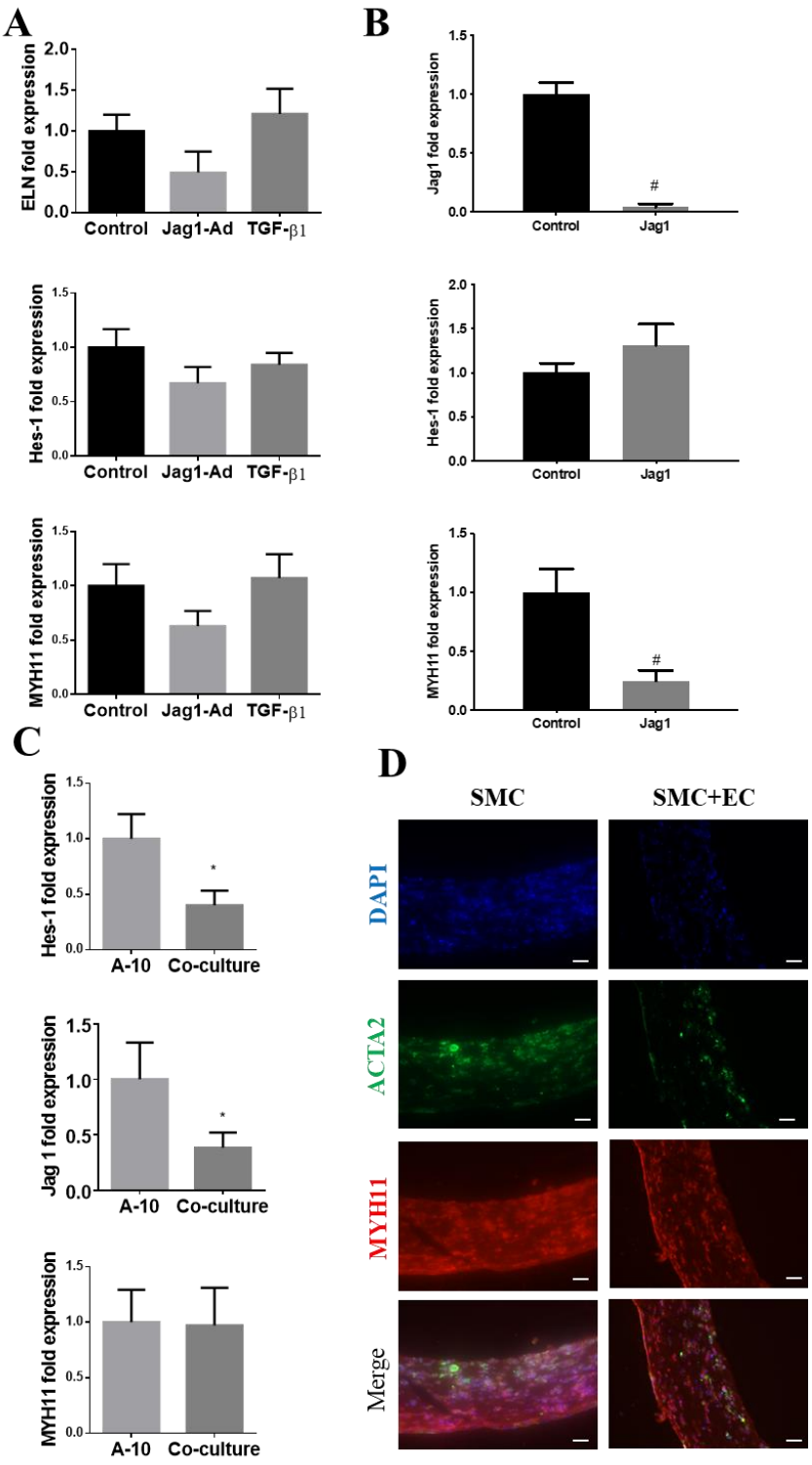

Figure 3.5. Effect of TGF-β1 in 2D, Notch in 2D and 3D, and co-culture on gene and protein expression in A-10 cells. **(A)** Treatment of A-10 cells with Jag1 that is adsorbed to

the culture-ware (Ad) or treated with TGF-β1. **(B)** qRT-PCR of fibrin-based tissue containing A-10 cells with or without entrapped Jag1. (**C)** coculture of A-10 cells with Human coronary artery endothelial cells (EC). (**D)** Immunofluorescence of monoculture of A-10 cells or coculture of A-10 and HCAEC in fibrin-based tissue showing expression of Acta2 and Myh11 after 1 week. Scale bar = 50 μm.

Data collectively presented in **Figure 3.5** showed that (i) adsorbed and entrapped Jag1 did not activate Notch signaling in A10 cells, (ii) co-culturing A10 cells with coronary artery Jag1-expressing ECs also did not active Notch signaling in A10 cells, and (iii) extending the earlier observation in **Figure 3.2A**, neither TGF-β1 nor co-culturing with EC could up-regulate smooth muscle differentiation markers. The first two findings, seemingly counterintuitive point to an important yet less known feature of Notch signaling, namely cis-inhibition[55-57]. In the Notch signaling context, a cell can be either a signal sending (ligand presenting) or signal-receiving cell depending on the relative abundance of receptor to its ligand on the same cell surface. However, when the Notch receptor binds to a ligand present on the same cell, signaling is inhibited. This phenomenon of cis-inhibition is an emerging hallmark of Notch signaling and relies on the relative abundance of the ligand and receptor. To ascertain if the ligand and receptor expression on the cell surface is of equal abundance causing signal inhibition, immunostaining of Notch3 and Jag1 was carried out. **Figure 3.6A** showed similar levels of expression of these proteins with and without TGF-β1 treatment. The rationale for TGF-β1 treatment is to evaluate the potential crosstalk that may exist between Notch and TGF-β through their downstream effectors CBF1 and SMAD2/3[51]. Further quantification of the mRNA showed the levels of gene expression of these two markers are essentially the same **(Figure 3.6B)**. Taken together, these are strong

evidence that Notch signaling is cis-inhibited in A10 cells and that these smooth muscle cells are not a suitable model to study Notch-induced differentiation and tissue maturation.

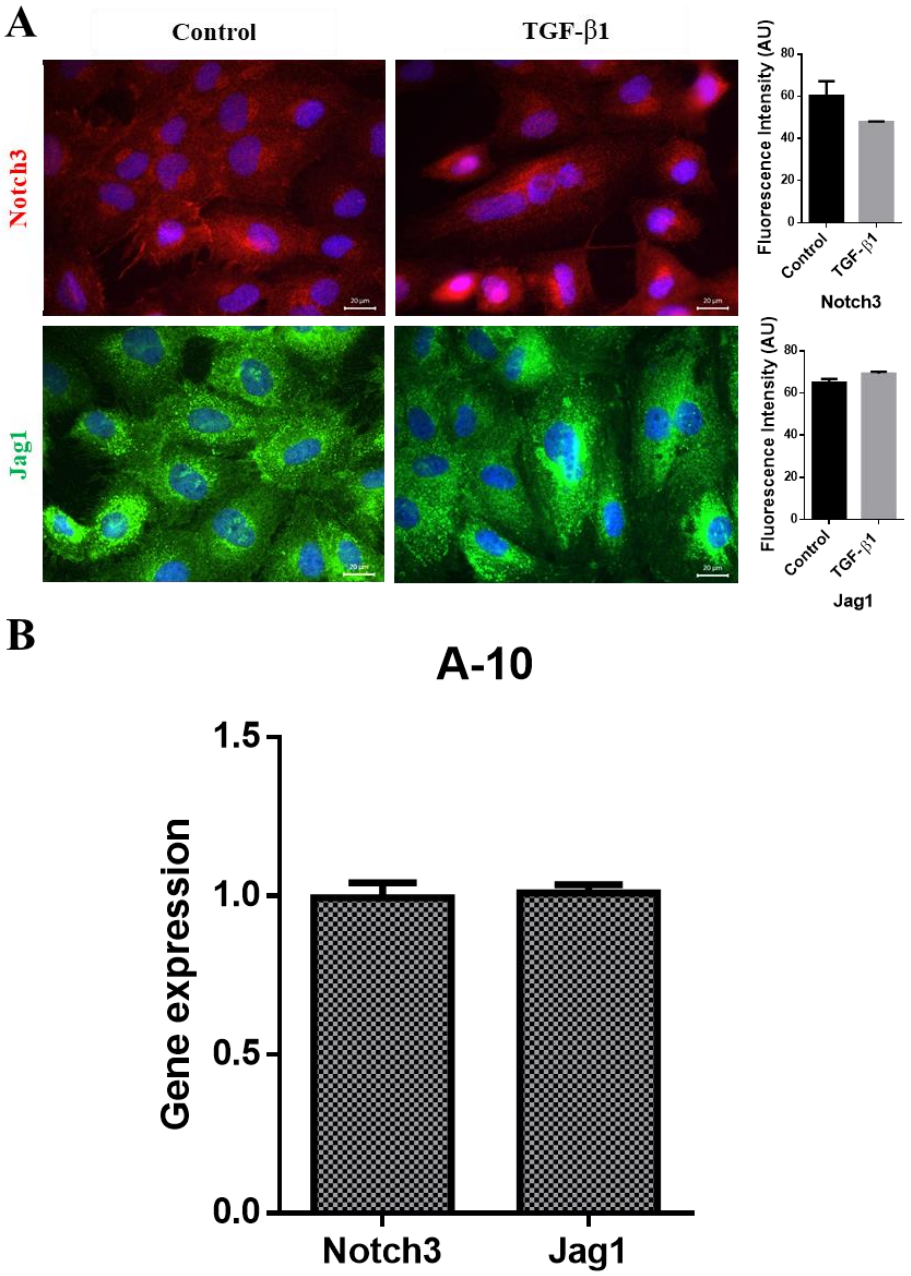

Figure 3.6. Notch3 and Jag1 expression levels in A-10 cells. (**A)** Immunofluorescence microscopy images showing the levels of expression of the Notch3 receptor and its ligand

Jag1 in control cells versus TGF-β1 treated cells. Scale bar = 20 μm. Bar diagrams show the quantification of the red or green channel (AU = Arbitrary Units). (**B)** PCR results showing the gene expression levels of Notch3 and Jag1 in A-10 cells.

Because of the cis-inhibition in non-differentiated embryonic rat vascular smooth muscle cell (A10 cells), the mouse embryonic multipotent mesenchymal progenitor cell (10T1/2 cells) was chosen as an alternative model vascular cells for trans-activating Notch signaling. First, a co-culture system of 10T1/2 cells with human coronary artery EC was established in 2D using a 1:1 ratio of the respective cells growth medium. After EC separation using PECAM-incubated magnetic beads, the differentiated 10T1/2 cells were probed for Notch signaling components and its downstream SMC target genes. Data collectively presented in **Figure 3.7A** demonstrated that Notch3 expression was significantly upregulated in the co-culture system with an accompanying Acta2 ($p<0.05$) while calponin remained unchanged ($p>0.05$). It is worth noting that Acta2 is a direct target of Notch3 signaling [24, 58] while calponin did not demonstrate that Notch3 was activated in differentiated 10T1/2 cells. In addition to the mono-culture controls, 10T1/2 cells were cultured in 1:1 ratio of DMEM:EC media to rule out the effect of EC media on Notch signaling (Data not shown). To establish a cause and effect relationship that links the activation of the Notch pathway in the 10T1/2 cells and the cocultured HCAECs, DAPT, which is a γ-secretase inhibitor of Notch signaling, was used **(Figure 3.7B)**. The data shows that inhibition of the Notch signaling pathway prevented the upregulation of Hes1, Notch3 and Acta2. These results confirm that HCAEC activated the Notch signaling in 10T1/2 cells since in the absence of the inhibitor, the direct target of Notch activation Hes1 was upregulated and was, in turn, downregulated when the cells were treated with DAPT

(Figure 3.7B). Furthermore, the qPCR results were paired with immunofluorescence staining to see if the gene upregulation is reflected on protein levels. Probing for Hes1, Notch3, Acta2 and Smtn proteins showed increased expression of these proteins in the coculture, and downregulation is the presence of DAPT which is consistent with the PCR results **(Figure 3.8, 3.9 & 3.10)**. Extending the 2D culture findings, a fibrin-based tubular engineered vascular tissue was fabricated and ECs were seeded in the lumen **(Figure 3.11)**. After one week in coculture, the ECs were scraped from the lumen using a scalpel and 10T1/2 RNA was extracted from the tissue for qPCR **(Figure 3.7C)**. Consistent with the 2D qPCR data, the levels of Notch3 and its downstream target Acta2 gene expressions increased significantly ($p<0.05$); however, in contrast to the 2D results, calponin gene expression decreased. A fully differentiated and mature vascular SMC is characterized by expressions of the contractile marker proteins smooth muscle α-actin (Acta2), calponin (Cnn1), smooth muscle myosin heavy chain (Myh11) and smoothelin (Smtn). Most of these contractile proteins are myocardin-induced expressions but Notch signaling has also been reported in response to contact-induced signaling with endothelial cells[24]. This study demonstrated that Notch3 signaling was activated when 10T1/2 cells are co-cultured with endothelial cells; however, due to cis-inhibition in A10 cells, Notch signaling was not activated.

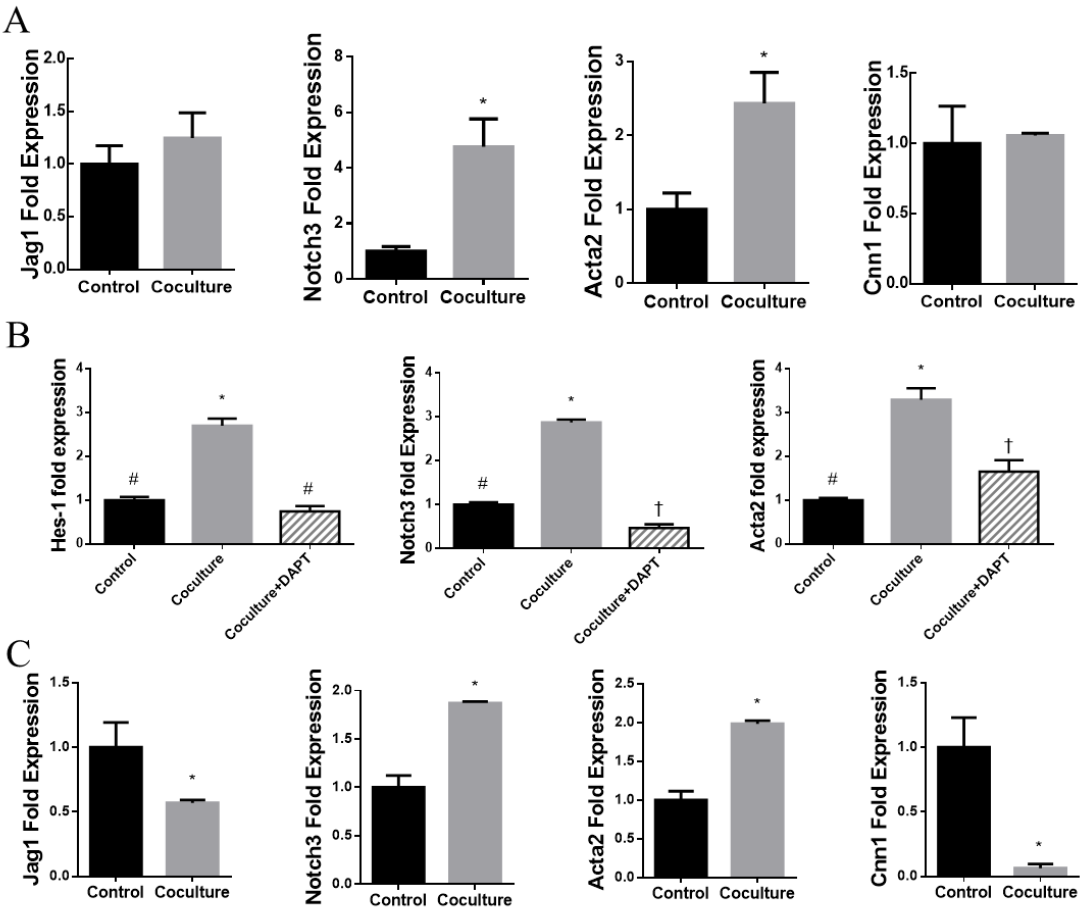

Figure 3.7. Notch activation and inhibition in coculture of HCAEC and 10T1/2 in 2D and 3D environments. **(A)** 2D qPCR of cocultured 10T1/2 and HCAEC after separation of the two cell types using magnetic beads coated with PCAM antibody. **(B)** qPCR of cocultured 10T1/2 and HCAEC treated with 15 μM DAPT followed by separation of the two cell types using magnetic beads. **(C)** qPCR of cocultured 10T1/2 and HCAEC after mechanically removing the ECs by gently scrubbing the lumen with a scalpel. Groups with the different symbols denote statistical significance ($p<0.05$) while those with the same symbol have no significant difference.

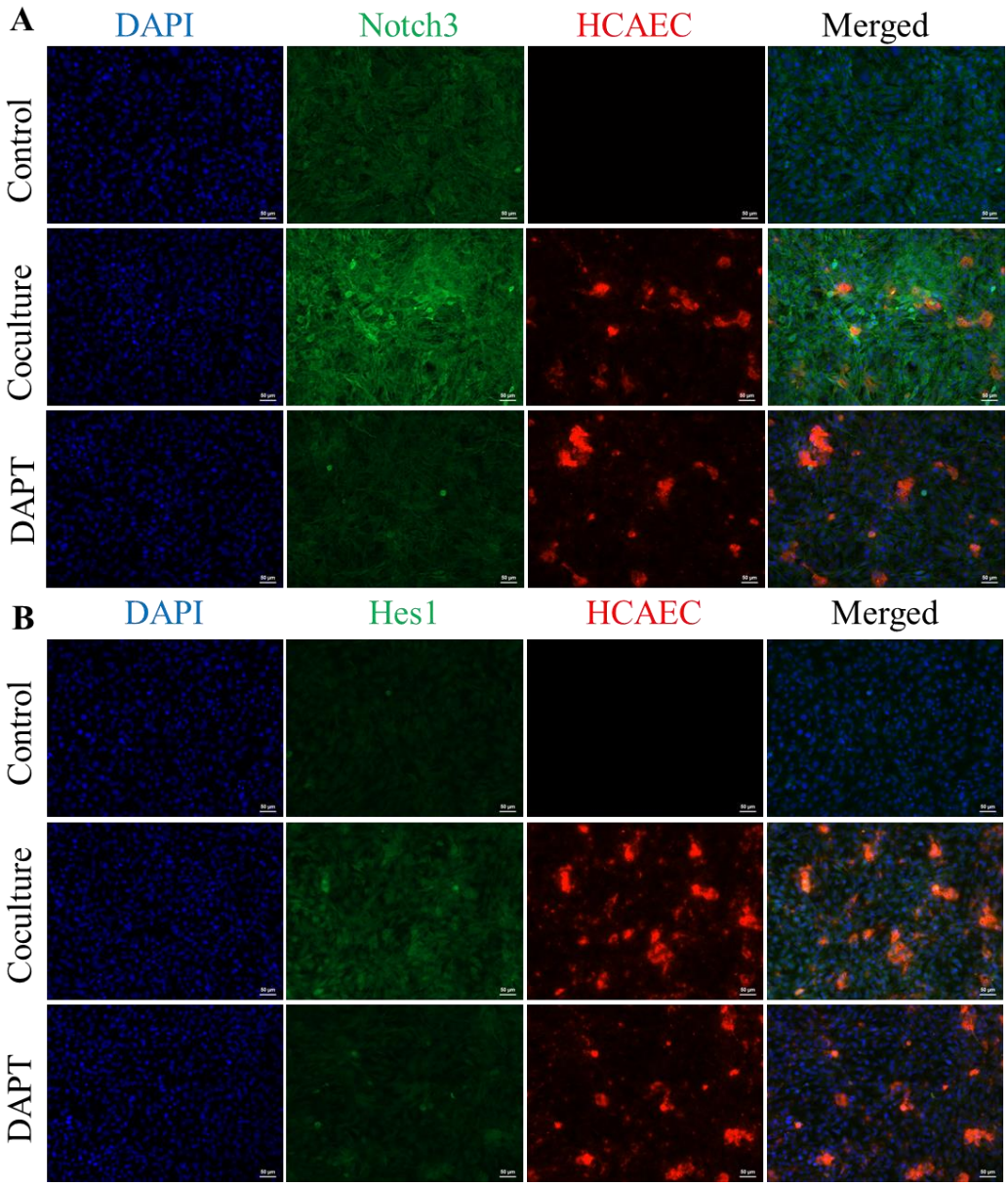

Figure 3.8. Coculture of 10T1/2 cells with endothelial cells upregulated Notch3 and its downstream transcription factor Hes1. **(A)** Immunofluorescence staining showing the expression level of Notch3 in cocultured cells and in cocultured cells treated with 15 μM of the Notch inhibitor DAPT for 3 days. **(B)** expression level of Hes1 in cocultured cells and in cocultured cells treated with 15 μM of the Notch inhibitor DAPT for 3 days. A monoculture of 10T1/2 cells was used as a control to assess the level of the target proteins in the absence of HCECs. Scale bar = 50 μm.

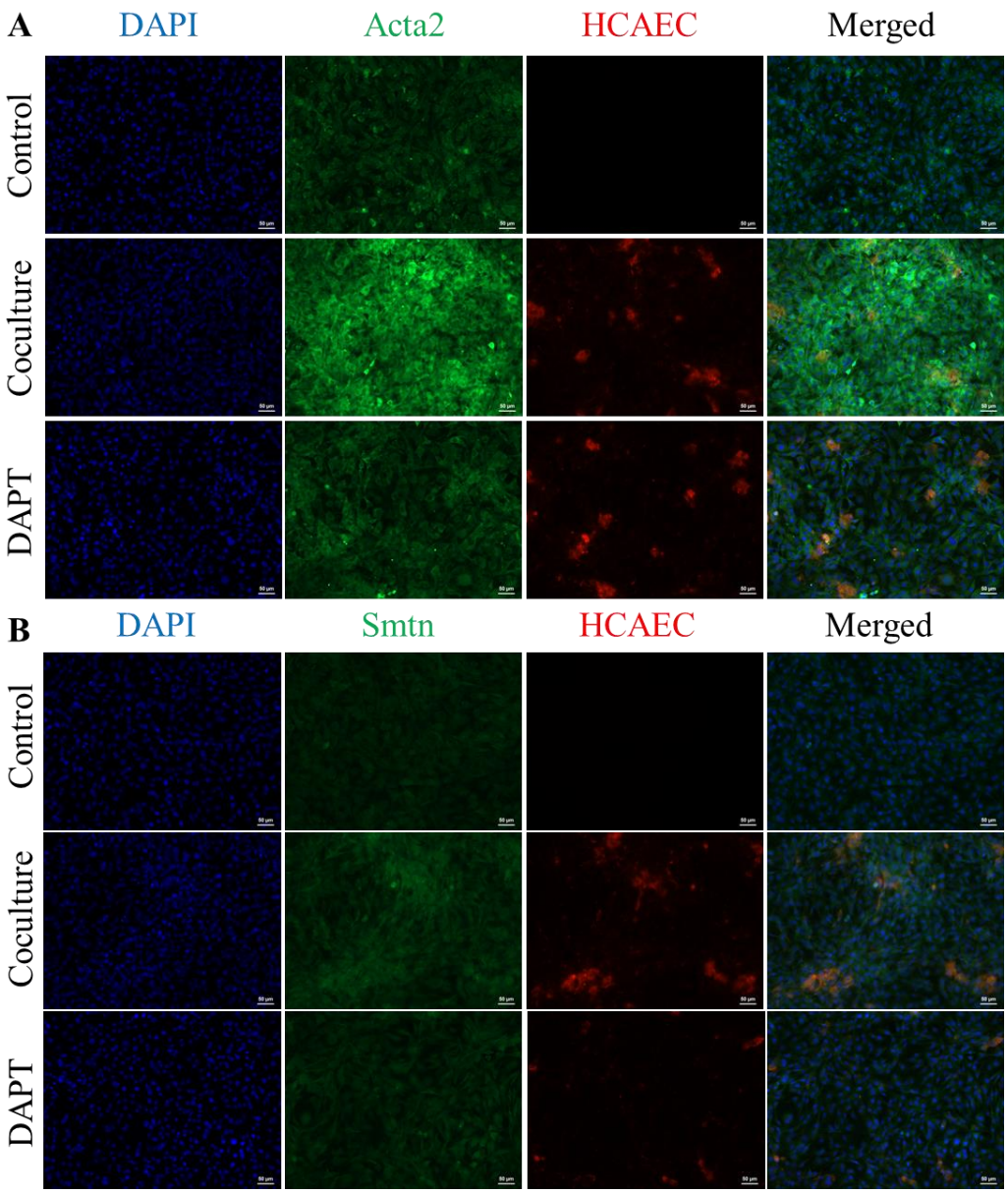

Figure 3.9. Coculture of 10T1/2 cells with endothelial cells upregulated the smooth muscle cytoskeletal proteins Acta2 and Smtn. **(A)** Immunofluorescence staining showing the expression level of Acta2 in cocultured cells and in cocultured cells treated with 15 μM of the Notch inhibitor DAPT for 3 days. **(B)** expression level of Smtn in cocultured cells and in cocultured cells treated with 15 μM of the Notch inhibitor DAPT for 3 days. A monoculture of 10T1/2 cells was used as a control to assess the level of the target proteins in the absence of HCECs. Scale bar = 50 μm.

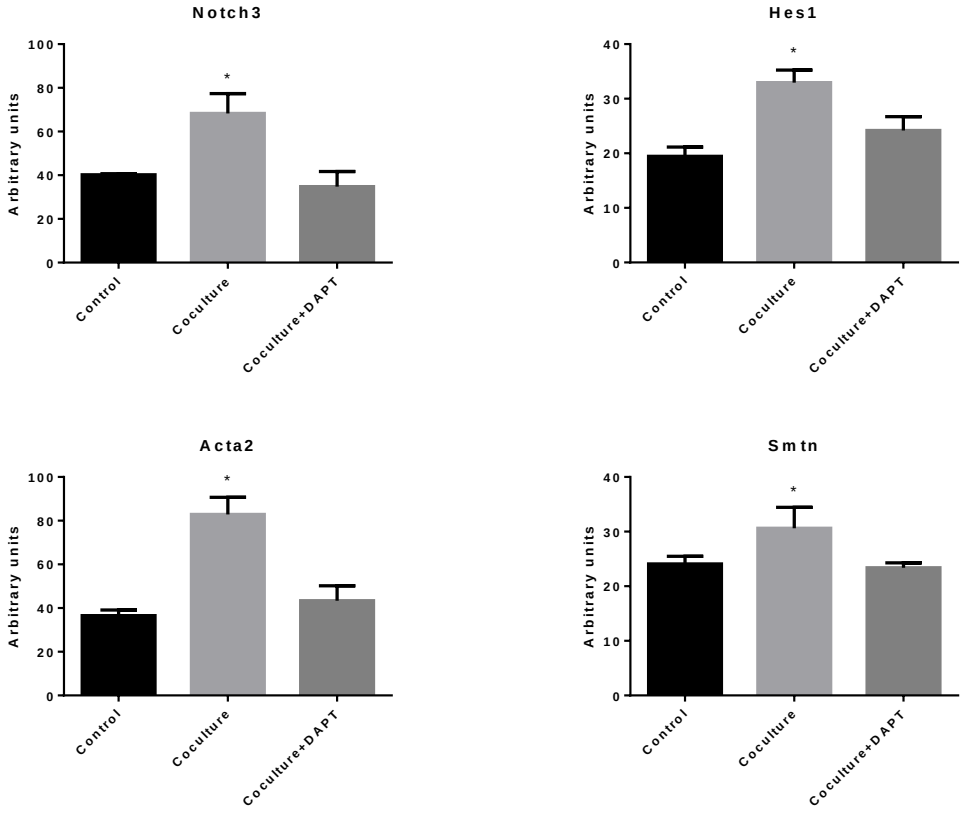

Figure 3.10. Quantification of the fluorescence intensity from green channels of the immunostaining which correspond to the level of expression of the proteins from Figures 3.8 and 3.9 using ImageJ software.

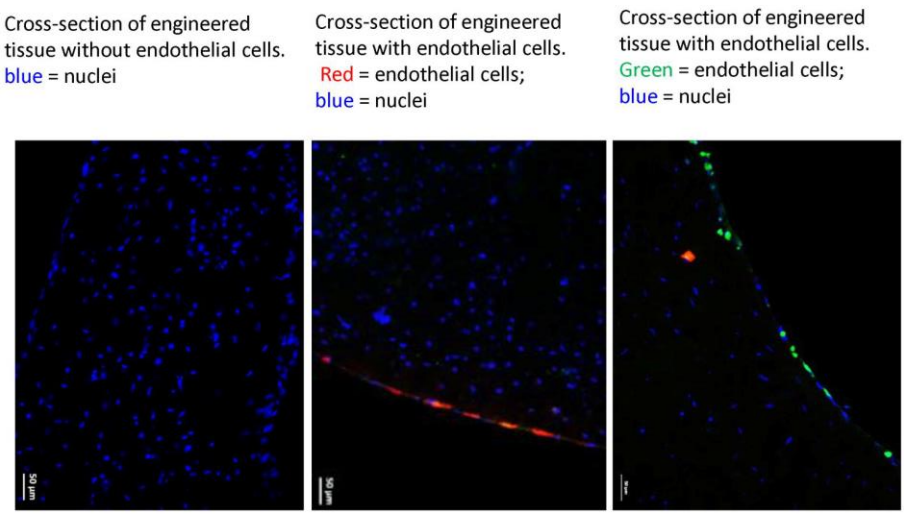

Figure 3.11. Endothelial cell seeding on the luminal side of engineered fibrin-based tissues. The figure shows the absence of endothelial cells at the lumen (left image) compared to the presence of these cells stained with Cell Tracker Green and Cell Tracker Red (middle and right images). Scale bar = 50 µm.

3.5 Conclusion.

In this study, the use of fibrin-based vascular tissues to study cell behavior and signaling in two different cell lines, A10 and 10T1/2 cells was evaluated. Fibrin gel experiments showed that mass loss occurred in the first 3 days after tissue fabrication regardless of fibrinogen concentration or cell number, but the mass remained stable after 3 days. Comparing the two cell lines, 10T1/2 cells tend to persist longer in the wall of the engineered tissues; however, both cell lines showed better growth on the albumen and the lumen. Furthermore, the results show that A10 cells were irresponsive to the presence of the Jag1 ligand or to endothelial cells in the environment likely due to cis-inhibition of the Notch signaling. On the other hand, 10T1/2 cells responded to endothelial cell co-cultures by upregulating the Notch3 and Acta2 gene expression. In conclusion, fibrin-based tissues

are a good platform to study cell signaling in a physiologically relevant 3D environment; however, 10T1/2 cells appear to be better suited for evaluating the role of Notch signaling.

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Chapter 4

The effects of progenitor and differentiated cells on ectopic calcification of engineered vascular tissues*

Overview *this study describes the use of engineered vascular tissues as a model to study vascular calcification in an in vitro 3D environment. A multipotent mouse cell line (10T1/2) was used in its naïve and differentiated states to shed some light on the mode of calcification between the two phenotypes. Calcification was induced using an inorganic phosphate salt and was evaluated by using histology, qPCR, and immunofluorescence of calcification markers. Furthermore, the effect of Vitamin K and simulated elastin degradation on the engineered tissues was shown.*

4.1 Summary

Ectopic vascular calcification associated with aging, diabetes mellitus, atherosclerosis, and chronic kidney disease is a considerable risk factor for cardiovascular events and death. Although vascular smooth muscle cells are primarily implicated in calcification, the role of progenitor cells is less known. In this study, engineered tubular vascular tissues from embryonic multipotent mesenchymal progenitor cells either without differentiating or after differentiating them into smooth muscle cells were fabricated and ectopic calcification through targeted gene analysis were studied. Tissues derived from both differentiated and undifferentiated cells calcified in response to hyperphosphatemic inorganic phosphate (Pi) treatment suggesting that a single cell-type (progenitor cells and differentiated cells) may

* A version of this chapter has been published: K. Dayekh & K. Mequanint. *Acta Biomater*. 2020,115, 288–298.

not be the sole cause of the process. Furthermore, Vitamin K, which is the matrix gla protein activator, was shown to have a protective role against calcification in engineered vascular tissues. The addition of partially-soluble elastin upregulated osteogenic marker genes suggesting a calcification process. Furthermore, partially-soluble elastin downregulated smooth muscle myosin heavy chain (Myh11) gene which is a late-stage differentiation marker. This latter point, in turn, suggests that SMC may be switching into a synthetic phenotype which is one feature of vascular calcification. Taken together, this approach presents a valuable tool to study ectopic calcification and associated gene expressions relevant to clinical therapeutic targets.

4.2 Introduction

Ectopic vascular calcification is a considerable problem in the elderly population, patients with type 2 diabetes, and chronic kidney disease[1], and is linked to the risk of cardiovascular disease and mortality[2-4]. Calcification causes the hardening of blood vessels due to the accumulation of calcium-containing deposits in the lumen (intimal calcification) or the wall of the blood vessel (medial calcification). When calcification occurs in the wall of the vessel, especially in arteries, it causes the blood vessel to lose its recoil (loss of Windkessel effect) and leads to dysfunctional vascular tone, compliance mismatch, and increased blood pressure[2, 5] whereas intimal calcification leads to narrowing of the blood vessel. Factors such as the absence of calcification inhibitors, apoptosis, bone demineralization, renal dysfunction, etc. that lead to an increase in the concentration of calcium ion (Ca^{2+}) in the cytoplasm of vascular smooth muscle cells (SMC). Vascular calcification is a complex process that has been thought to occur passively with age as unregulated mineral

precipitation; however, it is now known to be a regulated process[6-9]. This process involves osteogenic gene marker expression and downregulation of contractile proteins by SMC and secretion of calcium-containing deposits onto the extracellular matrix.

Prior studies[10-13] have investigated the cell types within the vasculature that influence calcification. Some evidence pointed to resident progenitor mesenchymal cells that differentiated into osteogenic lineage [14-16] while others suggested de-differentiation and/or transdifferentiation of SMC into an osteogenic lineage[17]. In response to increased inorganic phosphate (P_i) concentration, SMC calcification proceeds by sodium-dependent phosphate transporter channel (PiT-1) and is accompanied by downregulation of SMC- and upregulation of osteogenic-gene markers [18]. Clearly, the roles of differentiated and progenitor cells in vascular calcification is not well-understood. To date, vascular calcification studies have been predominantly conducted on cell culture and animal models and have been fundamental for the current understanding. However, conventional two-dimensional cell cultures do not accurately replicate the intricate microenvironment of three-dimensional (3D) tissues, since tissue-specific extracellular architecture, mechanical and biochemical cues, and cell-cell communication are disrupted. An alternative approach is the use of engineered vascular tissues that capture both the 3D organization and multi-cellular complexity of the native system to fill a critical gap in the preclinical model tool chest between traditional cell culture and whole animal experiments and has the potential to accelerate the pace of basic biomedical research[19]. A number of important physiological characteristics of native tissues are preserved in an engineered vascular tissue; thus, providing models for specific disease conditions such as vascular calcification. While there

are reported studies to model atherosclerosis using engineered vascular tissues[19, 20], studies on the calcification of the engineered tissue model are very limited[21]. Therefore, the aim of the present work was to investigate the role of progenitor and differentiated cells in vascular calcification using an engineered tissue model.

4.3 Materials and Methods

4.3.1 Cell culture and model tissue fabrication.

Embryonic multipotent mesenchymal progenitor cells (10T1/2 cells) (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Thermofisher) containing 5% v/v Fetal Calf Serum (Thermofisher) and 1% v/v penicillin/streptomycin (Thermofisher). Media was changed every three days and cells were passaged when confluency reached 80%. To fabricate engineered vascular tissues, two cell types were used: (i) undifferentiated progenitor 10T1/2 cells and (ii) 10T1/2 cells treated with 2ng/mL TGF-β1 for three days to differentiate them to a vascular lineage prior to tissue fabrication. Cultured cells were trypsinized with trypsin-EDTA 0.05% (Thermofisher) for 2 min and then suspended in DMEM. The cells were counted, and the appropriate volume was taken from the cell suspension to give a final cell count of 2 million cells/mL of tissue construct. The cells were then centrifuged at 1200 rpm for 5 min, the supernatant aspirated and cells resuspended in 450 µL of media. To that, 10 µL of 2 M $CaCl_2$ and 40 µL of 50 mg/mL ε-aminocaproic acid (ε-ACA) (Sigma-Aldrich), and 2 µL of 1U/µL Thrombin (MP Biomedicals) were added. This cell suspension was kept on ice until it was mixed with ice-cold 500 µL solutions of 20 mg/mL bovine fibrinogen (MP Biomedicals) to give a final concentration of 10 mg/mL fibrinogen per construct. To study the effect of elastin on

calcification, 200 µg/mL partially-soluble bovine elastin (MP Biomedicals) was added to the cell suspension before mixing with fibrinogen solution. Right after mixing the two solutions, the mixture was transferred into a clean glass annulus that had been disinfected with 70% EtOH, dried and then incubated in a 5% solution of Pluronic F-127 (Sigma-Aldrich) for 1 h at room temperature. The annulus was formed from 6 cm long glass shell with 6 mm outer diameter and a mandrel with 4 mm diameter. Two Teflon plugs were used to seal the ends of the glass shell and position the mandrel in the center. The assembly was then transferred to an incubator at 37 ºC for 1.5 h for crosslinking, taken out of the mold and cultured in DMEM for different tissue maturation times of 7 days to 14 days as per a previous study[22]. For calcification, the tissues were treated with 2.5 mM of inorganic phosphate in the form of sodium phosphate monobasic (NaH_2PO_4) after tissue maturation and continued for another week. Some tissues were further treated with 20 µg/mL vitamin K.

4.3.2 Histology and Immunofluorescence microscopy.

For 2D studies, cells were seeded in 6-well plates containing a coverslip at a density of 2.5 × 10^5 cells per well. After 24 h, cells were either left untreated or were treated with 2 ng/mL TGF-β1 for 3 days before washing them with PBS and fixing them with 4% paraformaldehyde for 15 min at room temperature (RT). Engineered tissues were fixed for 1 h, washed with PBS 3 times, and incubated first in a 15% and then in a 30% solution of sucrose for 30 min each at RT. The fixed tissues were then washed with PBS and dabbed using a paper towel to remove the excess liquid. After that, the tissues were immersed with OCT compound (Fisher) and transferred into -80 ºC isopropanol. Tissue sections (10 µm

thickness) were obtained by using a Leica cryostat (Leica) and placed on microscope slides. The slides were washed with PBS 3 times for 5 min each to remove the OCT compound. Cells/tissue were permeabilized with a 0.2% (v/v) Triton x-100 in PBS for 15 min at room temperature and then blocked with 5% BSA in PBS-T for 1 h at RT. The blocking solution was then aspirated, and 100 µL of the appropriate primary antibodies (anti-ACTA2, -CNN1, -MYH11, -COLI, -ELN, -OPN antibodies from mouse, and anti-SMTN antibody from rabbit (Santa Cruz Biotech)) (1:100) in 5% BSA PBS-T were placed on the coverslip and covered with a piece of parafilm and placed in a humid environment at 4º C overnight. The cells/tissues were washed 2 × with PBS-T and once with PBS for 5 min each, then incubated with the corresponding secondary antibody (Alexa-488 conjugated goat anti-mouse and Alexa-594 conjugated goat anti-rabbit (ThermoFisher)) (1:150) in 5% BSA PBS-T for 1 h at RT. The coverslips were then washed 2 × with PBS-T and once with PBS and incubated with 2 µg/mL DAPI for 5 min, washed with PBS 3 times and mounted with anti-fade mounting media. The images were taken by Zeiss Z1 fluorescent microscope. Quantification of the fluorescence intensity was performed using the ImageJ software. The green channel (target protein) and blue (DNA) channels were quantified separately, and then the relative quantification was obtained by dividing the intensity of the green channel by that of the blue channel. The treatments were done in triplicates, and an average of 3 representative images was taken for quantification of figure 1A. Histological staining was performed by staining with Alizarin red S (40mM, pH 4.1) for 30 min. Tissue sections were then washed with diH_2O 5 times to remove the excess and unprecipitated dye. The excess liquid was removed by blotting the slides with a paper towel, allowed to dry, and then visualized using a Nikon Eclipse TS100 microscope.

4.3.3 RNA isolation and qPCR.

For 2D cultures, spent media was aspirated and 500 µL of Trizol (Life Technologies) and cells were scraped and transferred to Eppendorf tubes. For engineered tissues, approximately 50 mg of tissue was placed in each Eppendorf tubes and frozen at –80 °C for at least an hour before crushing using a pestle and addition of 750 µL Trizol. The tissues were then homogenized with a tissue homogenizer (Bio Basic). Cells from 2D cultures and engineered tissues were lysed for 10 min, and chloroform was added at a ratio of 1:5 (chloroform:Trizol) and the samples were vortexed for 15 sec then incubated at RT for 15 min. Samples were then centrifuged at 4 ºC and 12000×g for 15 min. The organic phase was discarded, and the aqueous phase was transferred into another Eppendorf tube. Isopropanol was added at a ratio of 1:2 (isopropanol:Trizol) and incubated at RT for 10 min followed by centrifugation at 12000×g for another 10 min at 4 ºC. The isopropanol was then aspirated, and the pellet was resuspended in 75% EtOH at a ratio of 1:2 (EtOH:Trizol) and centrifuged at 7500×g for 5 min at 4º C. This last step was repeated twice to wash excess salts. The pellet was air-dried after the EtOH was removed, dissolved in 25 µL of DEPC water and quantified with nanodrop (Thermo Scientific). 1 µg of total RNA was used to synthesize cDNA using M-MLV reverse transcriptase kit (Promega) using the supplier's protocol. For qPCR reactions, 1 µL of the formed cDNA was used in 10 µL reactions using the SsoAdvanced universal SYBR green supermix (Bio-rad) according to the manufacturer's protocol. The qPCR reactions were carried out in a CFX96 Real-Time thermal cycler (BioRad) and *Gapdh* was used as a reference gene (**Table 4.1**).

Table 4. 1. Primers for mouse-specific mRNA amplification

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Spp1	ATCTCACCATTCGGATGAGTCT	TGTAGGGACGATTGGAGTGAAA
Alp1	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT
Runx2	CCAACCGAGTCATTTAAGGCT	GCTCACGTCGCTCATCTTG
Acta2	GGGCTATATAACCCTTCAGCG	GCTGTCTTCCTCTTCACACAT
Myh11	CTGGTTACATTGTAGGTGCCA	GCGAGCAGGTAGTAGAAGATG
Gapdh	AAGGGCTCATGACCACAGTC	GTGAGCTTCCCGTTCAGCTC

Spp1- Osteopontin; *Alp1*- Alkaline phosphatase; *Runx2* - Runt-related transcription factor 2; *Acta2*- Smooth muscle-α-actin; *Myh11*- Smooth muscle myosin heavy chain 11.

4.3.4 Statistical analysis.

Data are presented as the means of at least three independent experiments, and the error bars are the standard deviation from the means. Statistical significance was calculated either using Student's t-test or one-way ANOVA depending on experimental design. Tukey's multiple comparison tests were used for post-hoc statistical analysis for statistical significance, p-value of <0.05 was used.

4.4 Results

4.4.1 Cell and tissue differentiation.

In this study, the potential of these cells to differentiate and express smooth muscle cell markers and associated extracellular matrix molecules in response to TGF-β1 treatment was tested. **Figure 4.1A,B** showed that during the 3-day treatment in culture plates, the cells differentiated as shown by the expression of ACTA2 and CNN1, but there was no

difference in the expression of MYH11, collagen type I (COLI), and elastin (ELN) likely due to the short treatment time.

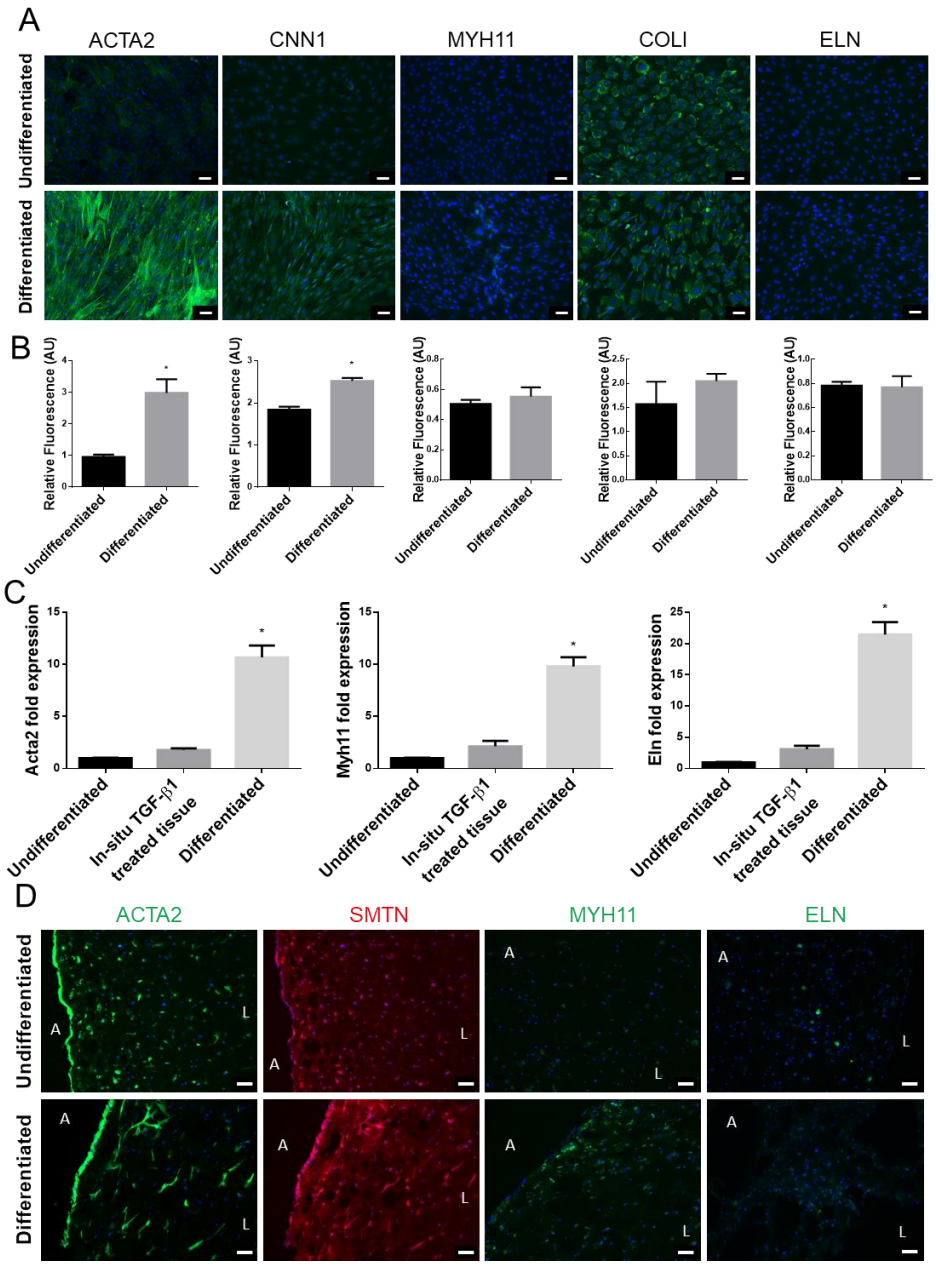

Figure 4.1. Differentiation of embryonic multipotent mesenchymal progenitor cell (10T1/2 cells) into a smooth muscle cell lineage. **(A)** 10T1/2 cells in 2D culture plates were treated with 2ng/mL TGF-β1 for 3 days then stained with antibodies against ACTA2, CNN1, MYH11, COLI and ELN. The figure shows an increase in the expression of ACTA2 and

CNN1 but not MYH11, COLI or ELN. Scale bar = 50µm. **(B)** Quantification of the green channel relative to the blue channel of the immunostaining shown in Fig **(A)** above. **(C)** Comparison in gene expression of undifferentiated tissues, in situ TGF-β1-treated tissues, and tissues fabricated from differentiated cells after tissue maturation. The levels of expression of *Acta2*, *Myh11* and *Eln* are shown. **(D)** Immunofluorescent staining of ACTA2, SMTN, MYH11 and ELN in differentiated and undifferentiated tissues. ACTA2 staining shows that cell morphology changed to spindle-like structures in differentiated tissues, and expression of SMTN and MYH11 increased in the differentiated tissues. The expression of ELN does not appear to change. A = albumen; L = Lumen of the tubular tissues. Scale bar = 50 µm. For Figs. (**B)** and **(C)**, data are means ± SD (n=3) and one-way ANOVA and Tukey's multiple comparison tests were used for statistical analysis. * indicate statistical significance at $p<0.05$.

Furthermore, differentiated cells exhibited a spindle-like morphology which is evident in the ACTA2 and CNN1 stained cells. This is a further indication that multipotent progenitor cells have differentiated into an SMC-like phenotype. For the fabrication of engineered vascular tissues, progenitor cells were either pre-differentiated and incorporated into the fibrinogen and matured to form the tissue or cells were mixed with fibrinogen and expected to differentiate in situ to SMC in the gel. Therefore, three different types of tissues were fabricated: (i) tissues containing undifferentiated progenitor cells which served as control, (ii) tissues that contained undifferentiated cells that were then treated with TGF-β1 in situ, and (iii) tissues with cells that were pre-differentiated prior to fabricating the tissues. **Figure 4.1C** shows the gene expression results of *Acta2*, *Myh11*, and *Eln* from these tissues. While pre-differentiating progenitor cells had the highest level of gene expression in the tissue, an attempt to differentiate progenitor cells in the tubular gel in situ by TGF-β1 treatment did not lead to a significant increase in the expression of these genes. Even though multipotent progenitor 10T1/2 cells have been shown to differentiate into SMC in the presence of TGF-β1[22-25], the entrapment of these cells in the fibrin gel seems to prevent them from interacting with the ligand likely due to poor diffusion of the ligand into the

tubular tissue cross-section. On the other hand, differentiating the cells before entrapping them into the tissues ensures maximum interaction of the cells with the ligand, thereby producing vascular tissues with differentiated cells. Furthermore, immunofluorescence staining of tissue sections showed that the differentiated tissues had elevated levels of ACTA2, SMTN, and MYH11 but not ELN (**Figure 4.1D**). However, the significantly higher *Eln* gene expression in the differentiated tissues does not seem to translate to the protein level. Although this appears to be contrary to two-dimensional cell culture observations, it is not surprising in three-dimensional cell cultures where a similar behavior has been shown before [26] and mechanical stimulation via a bioreactor was needed for elastin protein synthesis [27]. Consistent with **Figure 4.1A**, the cells in the fibrin gel exhibited elongated spindle-like morphology in the ACTA2 and SMTN stained cross-sections (**Figure 4.1D)**, indicating a differentiated state of the tissues. Since in situ differentiation attempts of the cells in the engineered tissues were not effective, it was not further investigated. In this study, qPCR data was complemented with immunofluorescence staining instead of Western blots. The reason is that the target protein extracts from cells in the fibrin gels were masked by RIPA buffer induced fibrin degradation during protein extraction. RIPA buffer contains sodium dodecyl sulfate which has long been known to be a potent anionic fibrinolytic detergent [28]. Increasing the total protein loading from 50 µg to 200 µg for Western blots did not make any difference and protein bands were still not detected (data not shown).

4.4.2 Calcification in engineered vascular tissues.

It is well-known that high concentrations of inorganic phosphates (P_i) lead to calcification in vivo and in vitro [18, 29]. Thus, engineered tissues were treated with hyperphosphatemic concentrations (2.5 mM) of inorganic phosphates for one week to evaluate if the engineered tissues can serve as a model for vascular calcification. As shown in **Figure 4.2A**, P_i-treated tissues were stained with Alizarin Red S which precipitates in the presence of calcium. The choice of Alizarin Red S staining over von Kossa staining is due to the specificity of the former to calcium cations to form a chelate while the latter is non-specific and binds anions (phosphates, sulfates, or carbonates)[30]. It is evident that the P_i-treated engineered tissues contain calcium deposits regardless of whether the cells in the tissues were differentiated or not. Further evaluation of the tissues by immunostaining for the osteogenic marker osteopontin (OPN) confirmed that these tissues calcified in response to hyperphosphatemic conditions (**Figure 4.2A**).

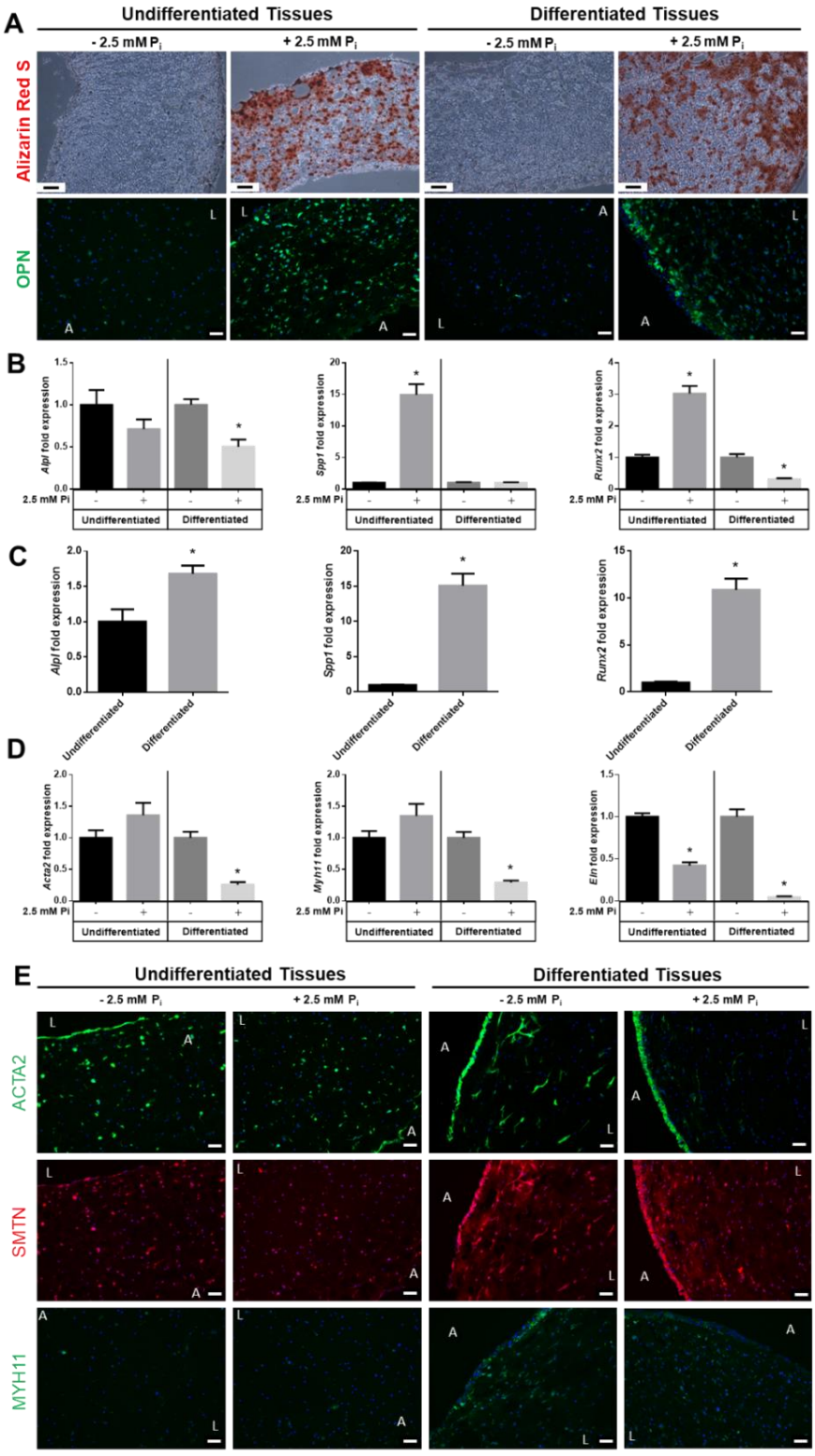

Figure 4.2. Histological staining, gene, and protein expression of calcified engineered tissues. **(A)** Alizarin Red S staining (Scale bar = 0.1 mm) and osteopontin (OPN)

immunofluorescence (Scale bar = 50 µm) in both differentiated and undifferentiated tissues treated with hyperphosphatemic concentrations (2.5 mM Pi) for 7 days. Higher Alizarin Red S staining and Opn expression were observed in the presence of 2.5 mM Pi. The top and bottom panels are different areas of the tissues from repeated experiments. **(B)** Osteogenic- (*Alpl*, *Spp1* and *Runx2*) gene expression of undifferentiated and differentiated tissues treated with 2.5 mM P_i for 2 weeks. **(C)** Osteogenic gene expression comparison of undifferentiated vs. differentiated tissues. **(D)** vascular smooth muscle cell-markers (*Acta2*, *Myh11*) and elastin (*Eln*) genes expression in undifferentiated and differentiated tissues that have been treated with 2.5 mM P_i for 2 weeks. **(E)** Protein expression of SMC markers in undifferentiated and differentiated tissues treated with 2.5 mM Pi for a period of 2 weeks. Protein levels remain unchanged in the undifferentiated tissues; however, elevated levels of staining were observed in the differentiated tissues that were relatively reduced in the presence of P_i. A = ablumen; L = Lumen. Scale bar = 50 µm. For Figs. **B**, **C** and **D**, data are means ± SD (n=3) and t-tests were used for statistical analysis. * indicates the significance at $p<0.05$.

These results showed that both differentiated and non-differentiated engineered tissues are capable of calcifying, suggesting that a single cell-type (undifferentiated progenitor cells or differentiated cells) may not be the sole cause of the process. Given the Alizarin Red positive staining, the gene expression levels of osteogenic (*Alp1*, *Spp1*, *Runx2*), SMC (*Acta2* and *Myh11*) markers, and SMC-derived matrix protein *Eln* in calcified engineered tissue models were explored (**Figure 4.2B**). In the undifferentiated tissues, *Spp1* and *Runx2* genes were upregulated in response to Pi treatment ($p<0.05$) while *Alpl* was not responsive, suggesting a possible temporal effect. In the differentiated tissues, both *Alpl* and *Runx2* were significantly downregulated in response to Pi treatment ($p<0.05$) whereas *Spp1* remained unaffected. A comparison between undifferentiated tissues with differentiated tissues indicated that all the tested osteogenic genes were upregulated in the differentiated tissues (**Fig 4.2C**; $p<0.05$). Since TGF-β1 was used to differentiate the progenitor cells into the vascular lineage, it suggests that these cells were able to express osteogenic genes as TGF-β1 is reported to recruit mesenchymal stem cells to a vascular injury site where these cells upregulate some of the osteogenic lineage genes[15]. However, the observed

osteogenic gene expression was not accompanied by a corresponding calcification since only tissues treated with Pi calcified regardless of the differentiation state (**Figure 4.2A,C**) suggesting that bone-related gene expression by progenitor cells may be a necessary but not a sufficient condition for tissue calcification. Regarding the SMC markers *Acta2* and *Myh11*, Pi treatment had no effect on their expression level in the undifferentiated tissue ($p>0.05$). However, both *Acta2* and *Myh11* were significantly downregulated in response to hyperphosphatemic Pi treatment ($p<0.05$; **Fig 4.2D**). The elastin gene expression was downregulated by Pi treatment in both differentiated and undifferentiated tissues. The downregulation of SMC marker genes was consistent with previous observations that a calcified vascular tissue is accompanied by SMC de-differentiation (phenotype switching to a less contractile state). The switch to a de-differentiated phenotype is known to lead to the loss of contractile SMCs and dysregulation of vascular tone[31]. The immunostaining data presented in **Fig 4.2E** were consistent with the gene expression trend suggesting protein translation. These data collectively demonstrated the benefit of using progenitor (or stem) cells to study calcification in an engineered tissue model. On the one hand, undifferentiated progenitor cells in the presence of hyperphosphatemic Pi calcified by upregulating osteogenic markers; and on the other hand, differentiated SMC cells decreased the expression of SMC markers and elastin in the presence of hyperphosphatemic Pi and calcified. Gaining such information is one of the advantages of using engineered vascular tissues as isolated systems to systematically evaluate physiological processes that might not be feasible to do in vivo. The results of **Figure 4.2A**, while demonstrating that hyperphosphatemic Pi induces calcification, it does not prove whether this is due to passive precipitation in the tissue space or a cell-mediated

process. Thus, acellular scaffolds (cell-free fibrin gel tubes) were tested for potential calcification. As presented in **Figure 4.3A**, the cell-free tubes did not calcify, whereas the corresponding engineered vascular tissues were calcified, demonstrating that the process is mediated by cells (**Figure 4.3A** bottom images and **Figure 4.3B** top images).

4.4.3 Protective role of vitamin K for calcified engineered tissues.

Recent evidence suggests a protective role of vitamin K against calcification by exhibiting anti-inflammatory effects and by activating matrix Gla protein (MGP) which is considered a potent calcification inhibitor[32]. Based on this information, the potential protective effect of vitamin K treatment in both undifferentiated and differentiated tissues exposed to hyperphosphatemic Pi was investigated. Both undifferentiated and differentiated tissues were treated with 2.5 mM Pi for one week followed by another week with a combination of 2.5 mM Pi and 20 µg/mL vitamin K. Untreated tissues, 2.5 mM Pi treated tissues, and tissues treated with 20 µg/mL vitamin K served as controls. The tissues were then cryo-sectioned and stained with Alizarin Red S. The results showed that while Pi-treated tissues (both differentiated and undifferentiated) had marked calcium deposition, the level of staining was reduced after vitamin K treatment (**Figure 4.3B**).

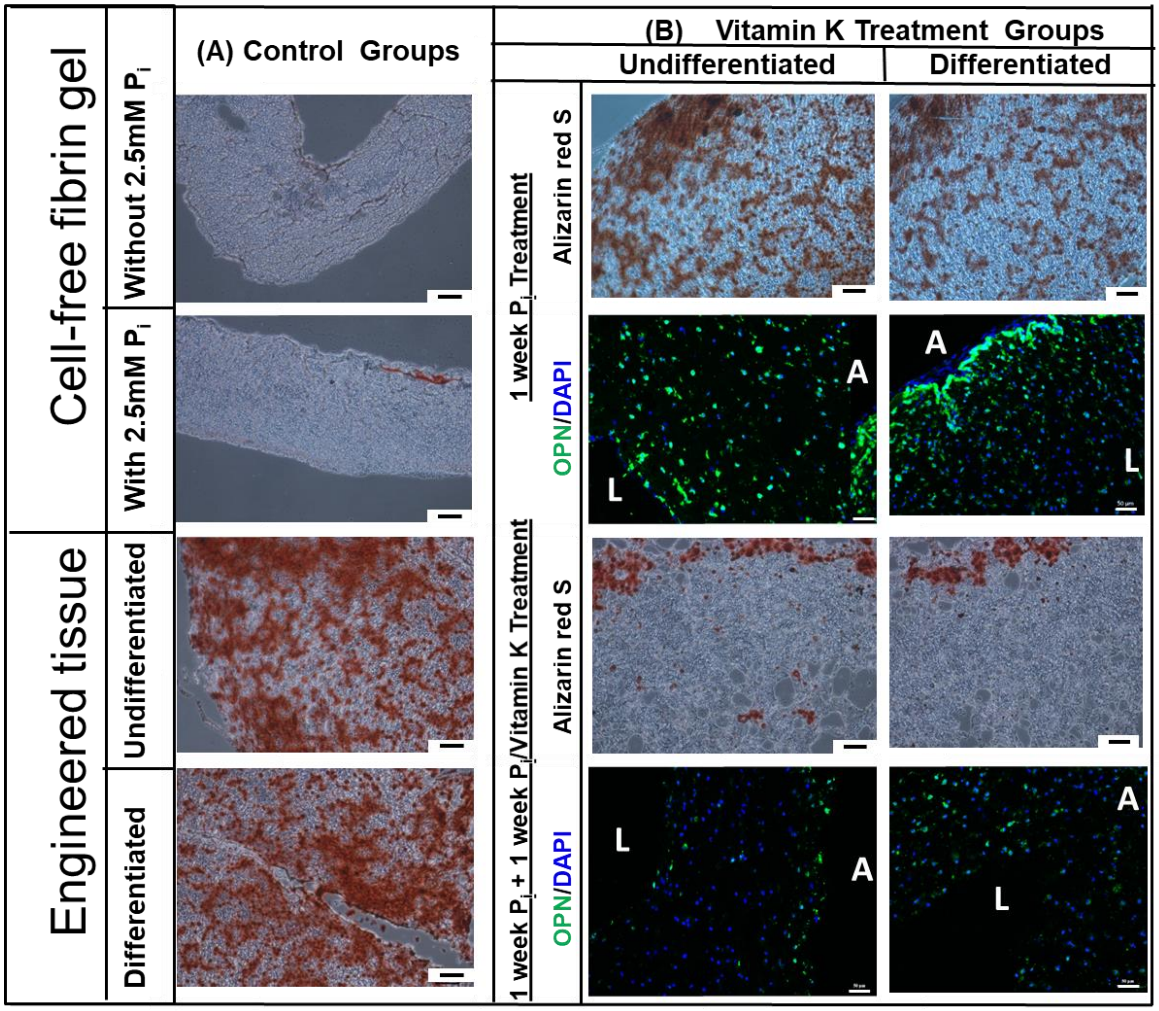

Figure 4.3. Cell-mediated calcification in differentiated and undifferentiated engineered vascular tissues and the protective role of vitamin K. **(A)** cell-free (acellular) fibrin gels were incubated with or without 2.5 mM of inorganic phosphate (P_i) for 7 days and stained using Alizarin Red S to rule out passive precipitation. In contrast, cell-based engineered tissues from undifferentiated and differentiated cells actively calcified over two weeks, as shown by the intense Alizarin Red S staining. **(B)** Alizarin Red S for calcium deposits and immunofluorescence staining for osteogenic marker osteopontin (OPN) in differentiated and undifferentiated tissues are shown for the one-week calcification period. Positive Alizarin Red S staining was accompanied by the expression of OPN (top four images). Following another week in the presence of 2.5mM P_i and 20 µg/mL vitamin K, Alizarin Red S staining intensity was diminished along with OPN due to the protective role of vitamin K (bottom four images). Scale bar = 50 µm.

Furthermore, OPN immunofluorescence staining showed a similar trend (**Figure 4.3B**), corroborating the Alizarin Red S staining data. Comparing the one week with two weeks

of Pi-treatment histological images (**Figure 4.3B** top images vs. **Figure 4.3A** bottom images), it is clear that calcification increased, as shown by the intense staining at two weeks. Remarkably, the presence of vitamin K not only protected the tissue against further calcification but also considerably abrogated it. The effect of vitamin K treatment on gene and protein expression in these tissues was also examined. **Figure 4.4A** shows the level of osteogenic and SMC contractile marker expressions from the undifferentiated tissues treated with vitamin K and Pi. While Spp1 was downregulated in response to vitamin K treatment following calcification, none of the other osteogenic and SMC contractile genes were affected.

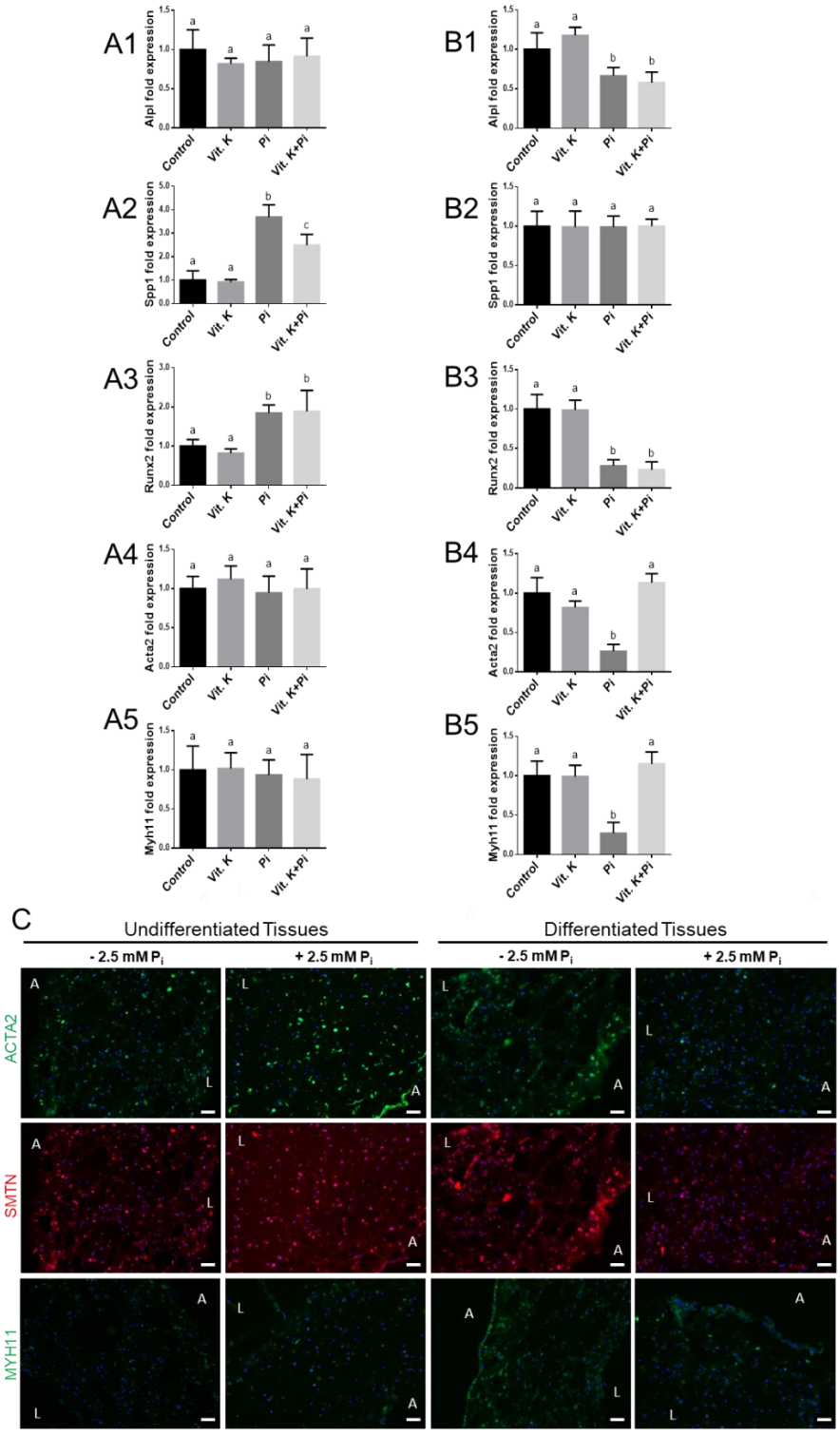

Figure 4. 4. The effect of vitamin K on undifferentiated and differentiated tissues gene and protein expression. **(A)** Osteogenic and contractile markers gene expression in

undifferentiated tissues treated with Pi, Vitamin K, or a combination. **(B)** Osteogenic and contractile markers gene expression in differentiated tissues treated with Pi, Vitamin K or a combination.(C) Immunofluorescence microscopy of contractile markers in undifferentiated and differentiated tissues treated with 2.5 mM Pi and in the presence of vitamin K. Scale bare = 50 µm. For Figs. **A** and **B**, data are means ± SD (n=3) and one-way ANOVA and Tukey's multiple comparison tests were used for statistical analysis. Different letters indicate the significance at $p<0.05$, while similar letters indicate no significance ($p>0.05$).

Gene expression of differentiated tissues treated with vitamin K and Pi is shown in **Figure 4.4B**. Osteogenic markers did not change after treating the calcified tissues with Vitamin K (Figure 4B1-B3); however, Vitamin K treatment was able to rescue the gene expression of contractile markers *Acta2* and *Myh11* (Figure 4B4 and B5). Similar results were observed from immunostaining (**Figure 4.4C**).

4.4.4 The effect of exogenous partially-soluble elastin on the calcification of engineered vascular tissues.

The extracellular matrix (ECM) is an integral component of tissues that not only acts as a structural support of its cellular components but also affects cellular processes such as proliferation, differentiation, survival, and apoptosis. Elastin is a major protein that is found in the ECM of arteries and studies have implicated elastin degradation in causing vascular calcification[33]. Thus, the effect of elastin and inorganic phosphate on the expression of osteogenic and SMC markers in these engineered vascular tissues was investigated. To that end, partially-soluble elastin was used as a surrogate to degraded elastin and its effect on calcification in both undifferentiated and differentiated engineered tissues was tested. **Figure 4.5** showed the gene expression levels of *Alpl*, *Spp1*, *Runx2*, *Acta2*, and *Myh11*.

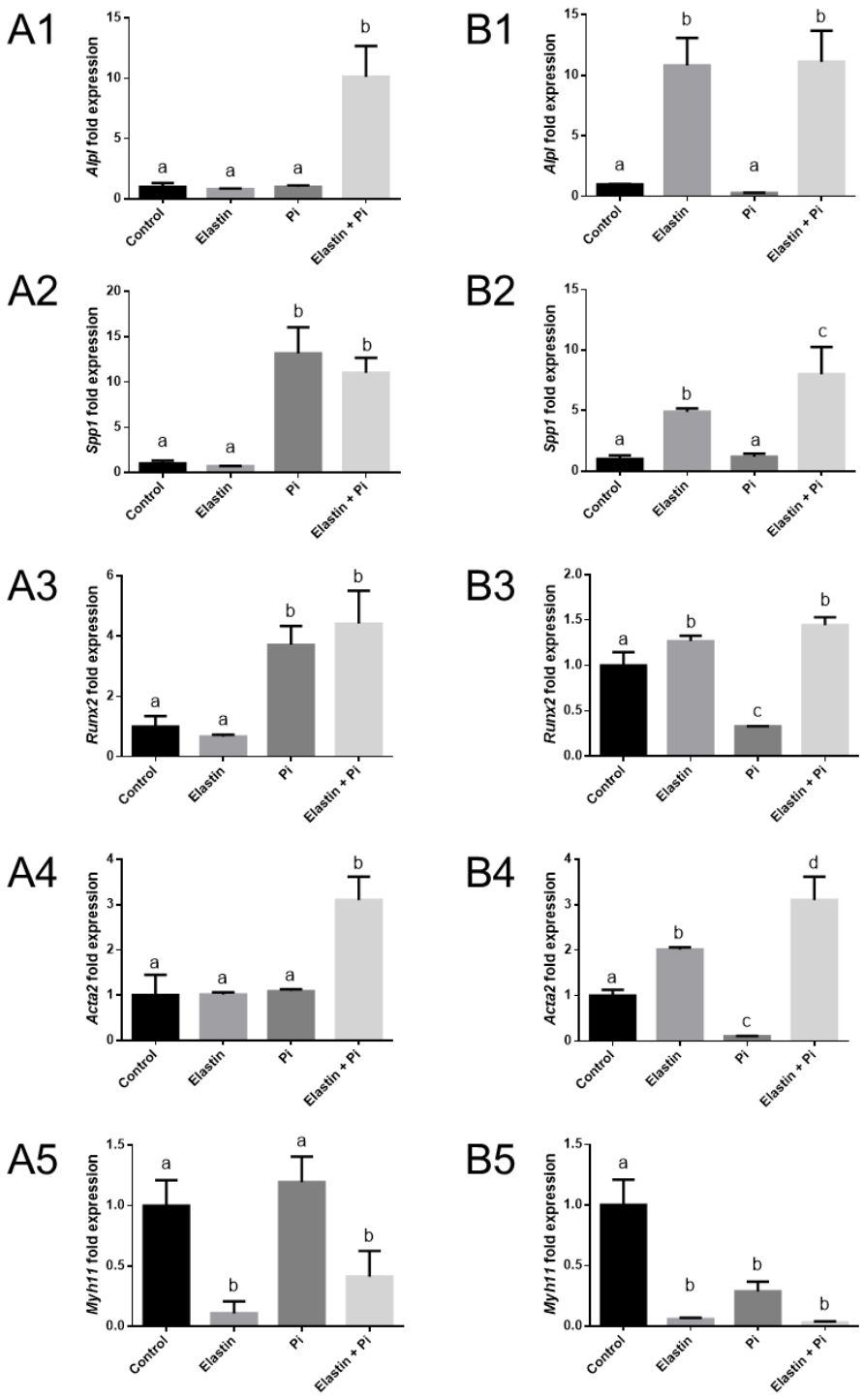

Figure 4.5. The effect of partially-soluble elastin (simulating degraded elastin) on calcification and de-differentiation gene expression in engineered vascular tissues. **(A)**

Contractile and osteogenic gene expression in elastin-containing non-differentiated tissues with or without 2.5 mM inorganic phosphate treatment. Undifferentiated tissues that do not contain elastin were used as controls. **(B)** Contractile and osteogenic gene expression in elastin-containing differentiated tissues with or without 2.5 mM inorganic phosphate treatment. Differentiated tissues that do not contain elastin were used as controls. Data are means ± SD (n=3) and one-way ANOVA and Tukey's multiple comparison tests were used for statistical analysis. Different letters indicate the significance at $p<0.05$, while similar letters indicate no significance ($p>0.05$).

In the undifferentiated tissues, the presence of elastin alone did not affect the expression of any of the tested genes with the exception of *Myh11* which was significantly downregulated (**Figure 4.5A**; $p<0.05$). The addition of Pi with or without elastin did not change the expression of *Spp1* or *Runx2*; however, *Alp1* and *Acta2* expression increased in the elastin+Pi group. Conversely, the expression of *Myh11* was downregulated in the elastin+Pi group (**Figure 4.5A**). While *Alp1* and *Myh11* behavior was consistent with a calcifying tissue, the overexpression of *Acta2* was not expected as SMC contractile markers were predicted to be downregulated (which is the case with *Myh11*).

Differentiated tissues exhibited a different gene expression profile than undifferentiated tissues (**Figure 4.5B**). Unlike the undifferentiated tissues, partially-soluble elastin led to an increase in the expression of *Alp1*, *Spp1*, *Runx2*, and *Acta2*; however, *Myh11* expression decreased. On the other hand, Pi treatment of tissues did not change the levels of *Alpl* or *Spp1* with respect to the control; but, the expression levels of *Runx2*, *Acta2* and *Myh11* decreased. Interestingly, the effect of combined elastin + Pi treatment on the differentiated tissues followed a similar trend as the undifferentiated tissues. The data seems to suggest that while elastin on its own might not affect progenitor cells, it had an effect on the differentiated cells by inducing them to express higher levels of osteogenic markers while downregulating the late SMC differentiation marker (*Myh11*) which indicates SMC de-

differentiation. In addition to partially-soluble elastin, similar experiments using tropoelastin were conducted, and the same trend was observed for both differentiated and undifferentiated engineered tissues (data not shown).

4.5 Discussion

In this study (schematically summarized in **Figure 4.6**), embryonic multipotent mesenchymal progenitor cells (10T1/2 cells) were used to engineer vascular tissue models and the role of their differentiation state on calcification and associated gene and protein expression was studied.

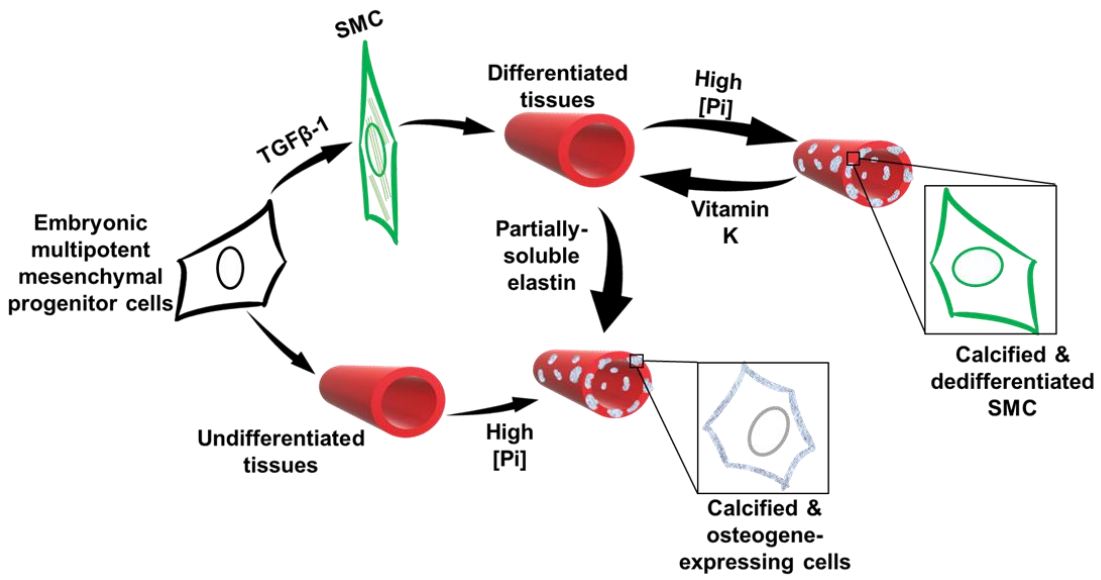

Figure 4.6. Schematic representation of the calcification process in differentiated and undifferentiated engineered vascular tissues. Progenitor cells can either be used to fabricate undifferentiated tissues that calcified in the presence of Pi. Alternatively, they can be differentiated to SMC to fabricate tissues that can calcify in the presence of Pi. Treating differentiated tissues with hyperphosphatemic Pi concentration leads to the de-differentiation of SMC by downregulating SMC-markers. The addition of vitamin K restores SMC-marker expression signifying a protective and rescuing role. On the other hand, the presence of partially-soluble elastin in the differentiated tissues led to the downregulation of late SMC-markers and upregulation of osteogenic markers.

Additionally, the treatment of undifferentiated tissues with a high concentration of Pi directly leads to overexpression of osteogenic markers without affecting SMC genes.

Furthermore, the role of vitamin K as a protective agent of calcification was shown. Finally, the addition of partially-soluble elastin could lead to the de-differentiation of SMC and upregulation of osteogenic markers. 10T1/2 cells have emerged as a model cell in vascular tissue engineering and could be differentiated to SMC by expressing smooth muscle α-actin (ACTA2), smooth muscle myosin heavy chain (MYH11) and smoothelin (SMTN) prior to their incorporation to the fibrin gel. While both smooth muscle α-actin and smooth muscle myosin heavy chain are also expressed in myofibroblasts derived from progenitor cells, including 10T1/2 [34, 35], smoothelin is the only definitive marker for a smooth muscle cell differentiation[36, 37] (**Figure 4.1D and Figure 4.2E**). Both 2D (pre-differentiation) and 3D (*in situ*) differentiation strategies have been evaluated but pre-differentiation was found to be more effective (**Figure 4.1C**). The lack of *in situ* differentiation was attributed to diffusion, where the compacted gel provided the barrier for TGFβ1 transport to induce its action on 10T1/2 cells. Diffusion gradients of signaling factors having typical diffusion coefficients in the order of 10^{-8} to 10^{-11} m^2/s are known to slow stem cell differentiation in 3D [38, 39].

In this study, 2.5mM Pi was specified as hyperphosphatemic concentration compared to 1.4mM which is considered as physiologic[40]. In response to hyperphosphatemic concentration, tissues engineered from undifferentiated and differentiated cells calcified, and such a comparative study is the first to be reported, supporting the emerging theory that progenitor cells to be involved in vascular calcification[41]. Although both aortic organ culture[40] and engineered tissues[21] have been reported, neither investigated the role of

progenitor cells. Both *Alpl* and *Runx2* were significantly downregulated in response to 7 days hyperphosphatemic treatment and indicated that degradation of mRNA or protein occurred as the vascular calcification developed[40]. One of the findings of the current study is the protective action of Vitamin K against calcification in engineered vascular tissues. In vivo, Vitamin K activates matrix gla protein (MGP) that is a potent binder of hydroxyapatite and has been shown to inhibit vascular calcification by scavenging hydroxyapatite and calcium oxalate monohydrate crystals[42, 43]. In clinical observations, patients prescribed with the anticoagulant drug warfarin for the prevention and treatment of thrombotic and thromboembolic diseases had increased risk of vascular calcification since MGP synthesis and activity are blocked by warfarin[44]. Because Warfarin is a common therapy for dialysis patients, vascular calcification is accelerated in chronic kidney disease (CKD)[45, 46] due to vitamin K antagonism, among other factors. In view of these prior studies, the engineered vascular tissues were subjected to vitamin K treatment following 7-days of calcification to scavenge the deposited calcium phosphate deposits. To this end, data in (**Figure 4.3**) showed that vitamin K blunted further calcification and scavenged the deposits likely by activating matrix gla protein, thus demonstrating the ability of engineered vascular tissue to recapitulate in vivo studies.

The engineered tissues did not produce detectable elastin for the studied culture time. However, degradation of the extracellular matrix elastin is implicated in vascular calcification. An in vitro study using rat aortic SMCs showed that elastin-derived peptides induced osteogenic transformation of cells, and this effect was exacerbated by TGF-β1[47]. In an abdominal aorta injury rat model, it is reported that calcification was the result of

elastin degradation induced by matrix metalloproteinases[48]. Furthermore, a recent study showed that degradation of elastin using cathepsins resulted in an 8-fold increase in elemental phosphate and calcium, and that elastin peptides generated by cathepsin degradation led to increased calcification in MOVAS-1 cells and *ex vivo* mouse aorta [49]. In view of the above, partially-soluble elastin as a surrogate for degraded elastin was added to investigate its role in calcification-related and SMC-related gene expression in both undifferentiated and differentiated tissues. While partially-soluble elastin had no clear effect on the undifferentiated tissues, the data collectively presented in **Figure 4.5B** demonstrated that differentiated tissues upregulated osteogenic marker genes suggesting a calcification process. Furthermore, partially-soluble elastin downregulated smooth muscle myosin heavy chain (*Myh11*) which is a late-stage differentiation marker. This latter point, in turn, suggests that SMC may be switching into a synthetic phenotype which is one feature of vascular calcification[50, 51]. Engineered vascular tissues have potential applications to model vascular disease and to develop therapeutic targets. While there are several reported engineered tissue models to study disease and develop therapeutic agents[52], engineered vascular tissues for modeling disease are notably absent except for two prior studies[20, 21]. In the first of these two studies, engineered vascular tissues were investigated for lipoprotein infiltration, endothelial layer activation, and monocyte adhesion to model atherosclerosis[20]. In the most recent report[21] which is related to this study, engineered and decellularized grafts were investigated for mineral aggregates and thermal and mechanical properties. Cell-containing engineered vascular tissues were excluded from this cited study[21] to avoid secretion of mineralization inhibitors. Using targeted gene analysis, the present study focused on the role of progenitor and differentiated

cells on calcification, the effect of vitamin K to protect and rescue calcified tissues, and the role of partially-soluble elastin on calcification – all of which are implicated in clinical ectopic calcification studies.

4.6 Conclusion

In this study, the potential of engineered vascular tissue models to study calcification was reported. While tissues fabricated both from differentiated and progenitor cells can calcify, they do so via different gene expression profiles. Undifferentiated tissues upregulated osteogenic markers while differentiated tissues downregulated contractile markers under hyperphosphatemic conditions. Furthermore, the role of vitamin K in reducing the burden of hyperphosphatemic conditions on the engineered tissues was demonstrated suggesting a potential adjuvant therapy for patients with vascular calcification. Finally, matrix degradation was simulated by using partially-soluble elastin and showed that while elastin on its own does not affect osteogenic markers in undifferentiated tissues, it leads to SMC de-differentiation and switching to an osteogenic marker expressing tissue. Collectively, this study demonstrated these tissue models are promising platforms to study disease and potential therapies.

4.7 References

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Chapter 5

Vimentin involvement in Notch signaling and neovascularization: an exploratory study

***Overview**: Notch is a tightly regulated pathway central to the development and pathology of vascular tissues. Recent studies suggested that the intermediate filament vimentin may be needed for efficient Notch signaling to occur. In this chapter, the role of vimentin in the regulation of the Notch pathway, and the formation of micro-vessels is explored. The data reported are exploratory studies.*

5.1 Summary

The Notch signaling pathway is a conserved pathway that is central in vascular tissue development and pathology. Because this pathway controls such important events, it is regulated at multiple steps of its cascade, such as post-translational modification of its ligand and receptor. Recent studies have suggested regulation of the Notch signaling by a pulling force required for the activation of the Notch signaling to take place. In this exploratory study, 3D fibrin gels were used as a coculture system of endothelial cells and 10T1/2 cells to assess whether vimentin is implicated in the regulation of Notch signaling and neovascularization. The results show that 10T1/2 cells increase the expression of Hes-1, Hes-5 and Acta2 during coculture with human coronary artery endothelial cells (HCAECs) and that vimentin knock-down using siRNA partially reduced the expression under static conditions. On the other hand, while the same trend was observed for Hes-5 under dynamic conditions, Acta2 was overexpressed, and vimentin knock-down did not affect its expression levels. Moreover, the development of newly formed micro-vessels is

observed in 3D fibrin gels in the presence of VEGF but could not be formed when vimentin expression was knocked down. These results suggest that vimentin plays a secondary role in Notch signaling; however, it is essential for neovascularization.

5.2 Introduction

Vimentin is a type of intermediate filament protein and is a major component of the cytoskeleton. It is widely expressed in many tissues, including the brain, lung, liver, gastrointestinal tract, kidneys etc. The vimentin monomer is a 466 amino acid protein and has a molecular weight of about 53 kDa (NCBI Reference Sequence: NP_003371.2). During assembly, two vimentin polypeptides align and bind, forming a dimer. This step is followed by the lateral binding of two dimers to form a tetramer, then eight tetramers bind side-by-side, forming the unit length fiber (ULF). The ULFs are the basic building blocks of the vimentin intermediate fibers that join end to end[1] (**Figure 5.1**). The assembly of vimentin fibers is regulated by phosphorylation of serine residues, which has been shown to disassemble the fibrillar structure of vimentin[2]. Vimentin mainly functions as a structural support protein, and earlier studies in vimentin knock-out mice showed that its absence has no effect on the survival of mice which showed no obvious abnormalities[3]. On the other hand, more recent studies have shown that it is involved in important cellular processes.

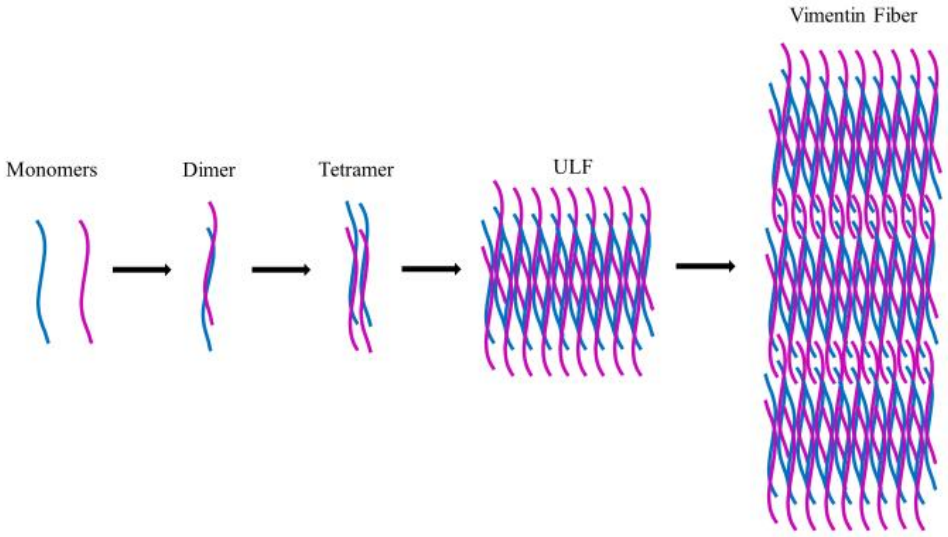

Figure 5. 1. Schematic representation of the assembly of vimentin intermediate fibers.

As an example, vimentin plays a role in cell adhesion due to its ability to interact with integrin at focal adhesion sites[4]. Furthermore, vimentin knock-down in alveolar epithelial cells using shRNA has been shown to reduce their mobility and consequently affect wound-closure rates in an in vitro model of lung injury, and ectopic expression of vimentin reversed those effects[5]. Vimentin has also been implicated in other important processes such as proliferation[6] and differentiation[7].

Interestingly, vimentin has been shown to play a role in regulating signaling pathways, for example, by transporting kinases from one part of a cell to another[8], or localizing receptors to the cell surface[9]. Of importance to this research, new evidence suggests that the Notch signaling pathway requires a force pulling on the ligand-bound receptor to activate the signal[10, 11]. Naturally, the pulling force has to be exerted by the cytoskeleton component, and while some studies suggest that the actin fibers are involved[11], other studies have

pointed to vimentin[12, 13]. Although the Notch signaling pathway is known to be a prominent regulator of vascular development and homeostasis[14-16], it is still not a well-understood pathway due to its complex regulation and context-dependence. In this exploratory study, the role of vimentin in regulating the Notch pathway signaling and its role in neovascularization is inspected.

5.3 Materials and Methods

5.3.1 Cell culture and tissue fabrication.

Embryonic multipotent mesenchymal progenitor cells (10T1/2 cells) (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 5% v/v Fetal Calf Serum (Gibco) and 1% v/v penicillin/streptomycin (Life Technologies). Human coronary artery endothelial cells (HCAEC) (Lonza) were maintained in EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ (Lonza). Spent media was exchanged with fresh media every three days for 10T1/2 cells and two days for HCAECs. Cells were passaged when confluency reached around 80%. For certain experiments, HCAECs were treated with 3 ng/mL VEGF, EGF, or bFGF or transfected with vimentin siRNA for 3 days prior to coculture with 10T1/2 cells. Some experiments were performed under dynamic conditions indicating that the culture plates were incubated on an orbital shaker for 30 minutes at a speed of 120 rpm for a period of 3 days.

To fabricate engineered vascular tissues, cultured cells were trypsinized with trypsin-EDTA 0.05% (Thermofisher) for 2 min and then suspended in DMEM. The cells were counted, and the appropriate volume was taken from the cell suspension to give a final cell count of 10 million cells/mL of tissue construct. The cells were then centrifuged at 1200

rpm for 5 min, the supernatant aspirated and cells resuspended in 150 µL of media. To that, 3 µL of 2 M $CaCl_2$ and 5 µL of 10 mg/mL ε-aminocaproic acid (ε-ACA) (Sigma-Aldrich), and 1 µL of 1U/µL Thrombin (MP Biomedicals) were added. The cell suspension was kept on ice until it was mixed with ice-cold 150 µL solutions of 6 mg/mL bovine fibrinogen (MP Biomedicals) to give a final concentration of 3 mg/mL fibrinogen per construct. Right after mixing the two solutions, the mixture was transferred into a 5 mL round-bottom tube. The tubes were then transferred to an incubator at 37 ºC for 1.5 h for crosslinking. A 3 mL volume of prewarmed DMEM was then added to the tube. The next day, HCAECs were trypsinized using the same procedure, and 1.0×10^4 cells were added to the cultured tissues to form an endothelial cell layer, followed by overnight incubation of the tissues to allow the endothelial cells to adhere. After that, the tissues were taken out of the tubes and cultured in a 50:50 mixture of DMEM: EGM in a culture plate.

5.3.2 Capillary formation assay in fibrin gel.

Tissue plugs made of 3 mg/mL fibrinogen and containing a mixture of 10T1/2 (10 million cells/mL) and HCAECs or siRNA transfected HCAECs (3 million cells/mL) were made in a similar fashion to the tissue preparation protocol above. These plugs were then embedded in 1 mg/mL fibrinogen gels containing 3 ng/mL VEGF or a mixture of 3 ng/mL EGF and bFGF and incubated for a period of 10 days in the presence or absence of 10 µM FOXC2-inhibiting Vimentin effector 1 (FiVe1). The HCAECs cells were stained with cell tracker red (Life Technologies).

5.3.3 Vimentin knock-down with siRNA.

To knock down the expression of vimentin in HCAECs, siRNA reverse transfection protocol was used as described by the manufacturer (Life Technologies) in 24 well plates. The following protocol is based on 1 well of a 24 well plate. In brief, the siRNA-lipofectamine complex was prepared by diluting 6 pmol siRNA in 100 µL of serum and antibiotic-free DMEM. To that, 2 µL lipofectamine RNAiMAX (Life Technologies) was added and mixed gently and then transferred to a plate well. The mixture was incubated at room temperature for 30 minutes. A cell suspension containing 5×10^4 cells /mL was prepared, and 0.5 mL of this suspension was added to each well containing the siRNA-Lipofectamine complex. The plate was incubated for 3 days at 37°C in a CO_2 incubator before using the cells to allow sufficient time for knock-down. Scrambled siRNA and non-transfected cells were used as control.

5.3.4 Immunofluorescence microscopy.

For 2D studies, cells were seeded in 6-well plates containing a coverslip at a density of 2.5×10^5 cells per well. After 24 h, cells were either left untreated or were treated with control, 3 ng/mL bFGF, EGF, VEGF, or treated with 5 µM of FiVe1 for 3 days. After the culture period, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature (RT).

Engineered tissues were fixed overnight in 5 mL tubes, washed with PBS 3 times, and incubated first in a 15% and then in a 30% solution of sucrose at RT until the tissues sunk to the bottom of the tube. The fixed tissues were dabbed using a paper towel to remove the excess liquid. After that, the tissues were immersed with OCT compound (Fisher) and

transferred into -80 ºC isopropanol. Tissue sections (30 µm thickness) were obtained by using a Leica cryostat (Leica) and placed on microscope slides. The slides were washed with PBS 3 times for 5 min each to remove the OCT compound. Cells/tissue sections were permeabilized with a 0.2% (v/v) Triton x-100 in PBS for 15 min at room temperature and then blocked with 5% BSA in PBS-T for 1 h at RT. The blocking solution was aspirated, and 100 µL of the appropriate primary antibodies anti-Acta2 and Hes-5 antibodies from mouse (Santa Cruz Biotech)) (1:100) in 5% BSA PBS-T were placed on the coverslip and covered with a piece of parafilm, and placed in a humid environment at 4º C overnight. The cells/tissues were washed 2 × with PBS-T and once with PBS for 5 min each, then incubated with the corresponding secondary antibody (Alexa-488 conjugated goat anti-mouse and Alexa-594 conjugated goat anti-rabbit (Life Technologies)) (1:150) in 5% BSA PBS-T for 1 h at RT in the dark. The coverslips were then washed 2 × with PBS-T and once with PBS and incubated with 2 µg/mL DAPI for 5 min, washed with PBS 3 times and mounted with anti-fade mounting media. The images were taken by Zeiss Z1 fluorescent microscope.

5.3.5 Endothelial cell separation with PECAM beads.

For experiments where HCAECs were cocultured with 10T1/2 cells, the two cell types were separated using PCAM-conjugated magnetic beads[17, 18]. Briefly, the cocultured cells were washed with HBSS and trypsinized as above. After trypsinization, the cells were suspended in DMEM containing 5% FBS then centrifuged at 1200 rpm at room temperature for 5 minutes. The supernatant was discarded, and the pellet was resuspended in PBS containing 0.1% BSA. PECAM conjugated magnetic beads were used to separate the two cell types by using 100:1 bead to HCAECs ratio. After adding the beads to the cell

suspension, the tubes were tumbled end-over-end for 30 minutes at 4 ºC. The beads-bound cells were then captured using a magnetic rack and the rest of the suspension containing the 10T1/2 cells were collected and centrifuged at 1200 rpm at 4 ºC for 5 minutes. The 10T1/2 cells pellet was collected and washed with ice-cold phosphate-buffered saline (PBS) until further use.

5.3.6 RNA isolation and qPCR.

Pelleted 10T1/2 cells that were separated from HCAECs were collected by centrifugation and 500 µL of Trizol (Life Technologies), and cells were lysed by repeated pipetting. Cells were lysed for 10 min at RT, and chloroform was added at a ratio of 1:5 (chloroform:Trizol), and the samples were vortexed for 15 sec then incubated at RT for 15 min. Samples were then centrifuged at 4 ºC and 12000×g for 15 min. The organic phase was discarded, and the aqueous phase was transferred to another Eppendorf tube. Isopropanol was added at a ratio of 1:2 (isopropanol:Trizol) and incubated at RT for 10 min followed by centrifugation at 12000×g for another 10 min at 4 ºC. The isopropanol was then aspirated, and the pellet was resuspended in 75% EtOH at a ratio of 1:2 (EtOH:Trizol) and centrifuged at 7500×g for 5 min at 4º C. This last step was repeated twice to wash excess salts. The pellet was air-dried after the EtOH was removed, dissolved in 25 µL of DEPC water, and quantified with nanodrop (Thermo Scientific). 1 µg of total RNA was used to synthesize cDNA using M-MLV reverse transcriptase kit (Promega) using the supplier's protocol. For qPCR reactions, 1 µL of the formed cDNA was used in 10 µL reactions using the SsoAdvanced universal SYBR green supermix (Bio-rad) according to the manufacturer's protocol. The qPCR reactions were carried out in a CFX96 Real-Time thermal cycler (BioRad), and GAPDH was used as a reference gene.

5.3.6 Western blotting.

The PECAM separated 10T1/2 cells were harvested using centrifugation and lysed in ice-cold RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.25% (w/v) sodium deoxycholate and 0.1% (w/v) SDS pH: 7.5) containing protease inhibitor cocktail (Roche) and 1 µM Phenylmethanesulfonyl fluoride (PMSF). The cells were kept on ice for 15 min to allow for lysis to complete. Lysates were centrifuged at 12000 rpm for 15 min. The pellets were discarded, and the supernatants' protein contents were quantified using the Pierce BCA protein assay protocol (Pierce). Protein samples were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (Pall life sciences). Blocking the membrane was performed with 5% BSA (Sigma-Aldrich) in PBS with 0.1% Tween-20 (PBS-T) and Western blotted with the Acta2 (1:1000), Hes5 (1:500), and GAPDH (1:1000) primary antibodies (Santa Cruz Biotech) diluted in 5% BSA in PBS-T overnight at 4º C. The blots were then washed 2 × with PBS-T for 5 min each, and 1 × with PBS for 5 min, followed by incubation with goat anti-mouse secondary antibody (1:5000) diluted in 5% BSA in PBS-T for 1 h at room temperature. Finally, the blots were washed as before and incubated with Supersignal west pico chemiluminescence substrate (Pierce) and developed using ChemiDoc XRS+ (BioRad).

5.3.7 Statistical analysis.

Data are presented as the means of at least three independent experiments, and the error bars represent the standard deviation from the means. Statistical significance was calculated using one-way ANOVA. Tukey's multiple comparison tests were used for post-hoc statistical analysis. For statistical significance, p-value of <0.05 was used.

5.4 Results and Discussion

5.4.1 Endothelial cell vimentin expression and knock-down.

The expression of vimentin in endothelial cells is shown in **Figure 5.2.** Endothelial cells were treated with different growth factors bFGF, EGF and VEGF at a 3 ng/mL concentration. Inhibition of vimentin filament formation is accomplished by adding 5 μM FiVe1 or transfecting endothelial cells with vimentin siRNA. The data showed that the ECs cultured in the presence of bFGF grow in close contact with each other similar to the control. On the other hand, cells treated with EGF or VEGF were separated, which may indicate endothelial-to-mesenchymal (EndMT) transition due to loss of cell-cell contact[19]. This transition is important for the formation of new microvasculature[20]. Furthermore, ECs treated with VEGF and EGF exhibit an elongated shape of the vimentin filament network which might also be an indication of the EndMT transition[19]. The addition of Five1 into the culture or transfection of ECs with vimentin siRNA disrupts the vimentin fibers assembly. As expected, the level of expression of vimentin is also reduced in the transfected ECs.

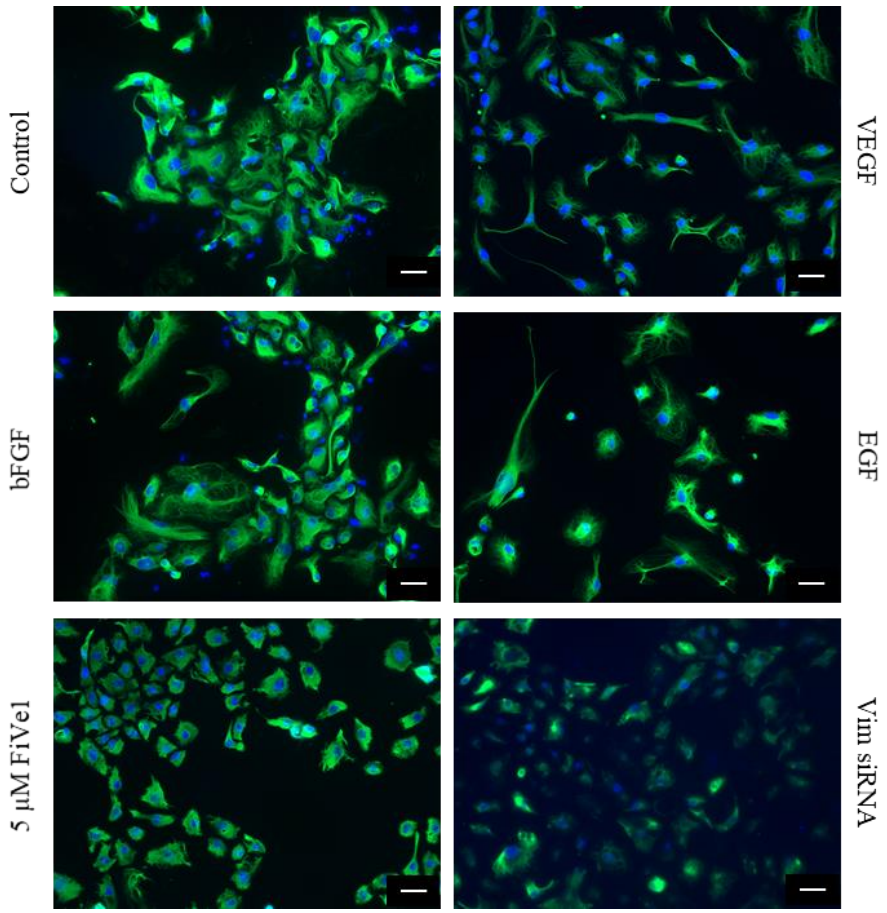

Figure 5.2. Fluorescence microscopy of expression of vimentin in HCAEC. HCAEC were treated with 3 ng/mL of each bFGF, EGF, and VEGF. Vimentin filament disruption is shown by the addition of 5 µM FiVe1 or vimentin knock-down using vimentin siRNA. Scale bar = 50 µm.

5.4.2 Role of vimentin in Notch signaling in static and dynamic cultures.

To assess the role of vimentin in the regulation of the Notch signaling pathway, 10T1/2 cells were cocultured with endothelial cells (EC), or ECs treated with VEGF, or ECs transfected with the vimentin siRNA. **Figure 5.3. A** shows the expression of three Notch target genes, Hes-1; Hes-5 and Acta2, under static conditions.

Hes-1 was upregulated in the presence of ECs regardless of EC treatment conditions. Hes-5 expression was also upregulated in the presence of ECs; however, its expression was reduced slightly when vimentin expression was knocked down with siRNA; however, it was still higher than control levels. The expression of Acta2 increased in the presence of ECs and EC (siRNA) and it was further increased in the VEGF treated ECs. A similar trend is observed for the protein expression of Hes-5 and Acta in **figure 5.3.C**. These results show that in static conditions, only the expression of Hes-5 was affected by the knock-down of vimentin expression.

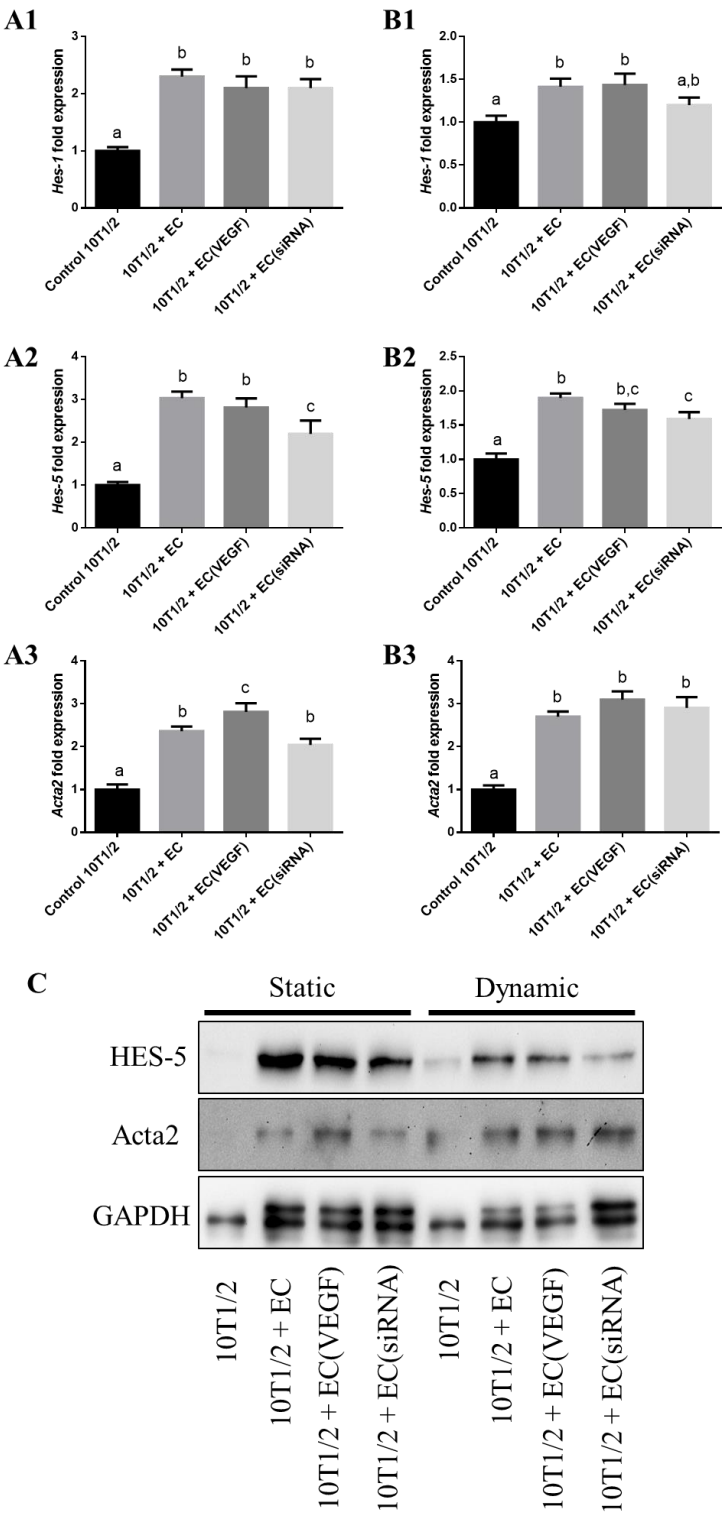

Figure 5.3. Coculture of 10T1/2 and HCAEC in static and dynamic conditions. **(A)** Hes-1, Hes-5 and Acta2 gene expression of 10T1/2 cells cocultured with EC, EC treated with

VEGF (EC(VEGF)) or EC transfected with vimentin siRNA (EC (siRNA)) in static culture conditions. **(B)** Hes-1, Hes-5, and Acta2 gene expression of 10T1/2 cells cocultured with EC, EC(VEGF), and EC (siRNA) in dynamic culture conditions. Letters on top of the columns are used for statistical analysis. Different letters indicate a statistically significant difference ($p<0.05$), whereas the same letters indicate no statistical difference ($p>0.05$). **(C)** Western blot analysis of Acta2 and Hes5 proteins in 10T1/2 cells cocultured with EC, EC(VEGF), or EC (siRNA); GAPDH was used as a loading control.

Due to its tight interaction with integrins, vimentin functions as a mechanosensor of external forces from outside the cell and relays them to the nucleus allowing the cells to respond to such forces[21]. Due to this, the static culture conditions were repeated in dynamic conditions to assess whether shear forces might have an effect on the role of vimentin in regulating the Notch signaling. Under dynamic conditions, the expression of the Hes-1 gene increased in the presence of EC and is slightly reduced when vimentin is knock-down by siRNA (**Figure 5.3.B1**). A similar pattern is observed for the Hes-5 gene in **Figure 5.3.B2.** On the other hand, the expression of Acta2 is upregulated in the presence of EC regardless of EC treatment **(Figure 5.3.B3)**. These patterns are reflected at the protein level shown by the Western blot (**Figure 5.3.C**.). These results show that vimentin might play a partial role in the Notch signal regulation because there was a slight decrease in the expression of Hes-5 when vimentin was knocked down even though the other genes were not downregulated. Interestingly, **Figure 5.3.C** shows that while the expression of Hes-5 was generally lower in dynamic conditions, a comparison between the control groups of static vs. dynamic cultures shows a slight upregulation of the Hes-5 gene. On the other hand, the expression of Acta2 appears to be generally upregulated in dynamic conditions. This might suggest that shear force alone plays a role in regulating Notch even though it might not be through vimentin. The cytoskeleton is a complex network of interconnected

microfilaments, intermediate filaments, and microtubules and therefore, there might be a redundancy in the role of the cytoskeleton in regulating the Notch signal. Thus, despite the vimentin expression was knocked down, other cytoskeletal components might compensate to keep vital signaling pathways operational.

5.4.3 Vimentin filament disruption partially affects Notch signaling.

The effect of vimentin filament disruption was tested on the Hes-1, Hes-5 and Acta2 gene expression levels in 10T1/2 cells cocultured with HCAECs in 2D cultures. **Figure 5.4.A** shows that disruption of vimentin filament networks did not significantly affect the gene expression of Hes-1 or Hes-5 Notch target genes even though there was a downward trend in expression of both these genes in the FiVe1 treated group. On the other hand, while Five1 treatment reduced the gene expression of the Acta2 gene, its expression was still higher than the control. Conversely, Acta2 protein expression levels remained high in 3D coculture even in the presence of FiVe1 treated HCAECs, as shown in **Figure 5.4.B**. This data shows that while vimentin filament disruption slightly decreased the gene expression of Notch downstream targets, its effect is minimal and does not have a noticeable effect on the protein expression level (**Figure 5.4.B**). This could be due to the redundant role of intermediate filaments such as vimentin, where the disruption of one filament network is compensated by other elements in the cytoskeleton.

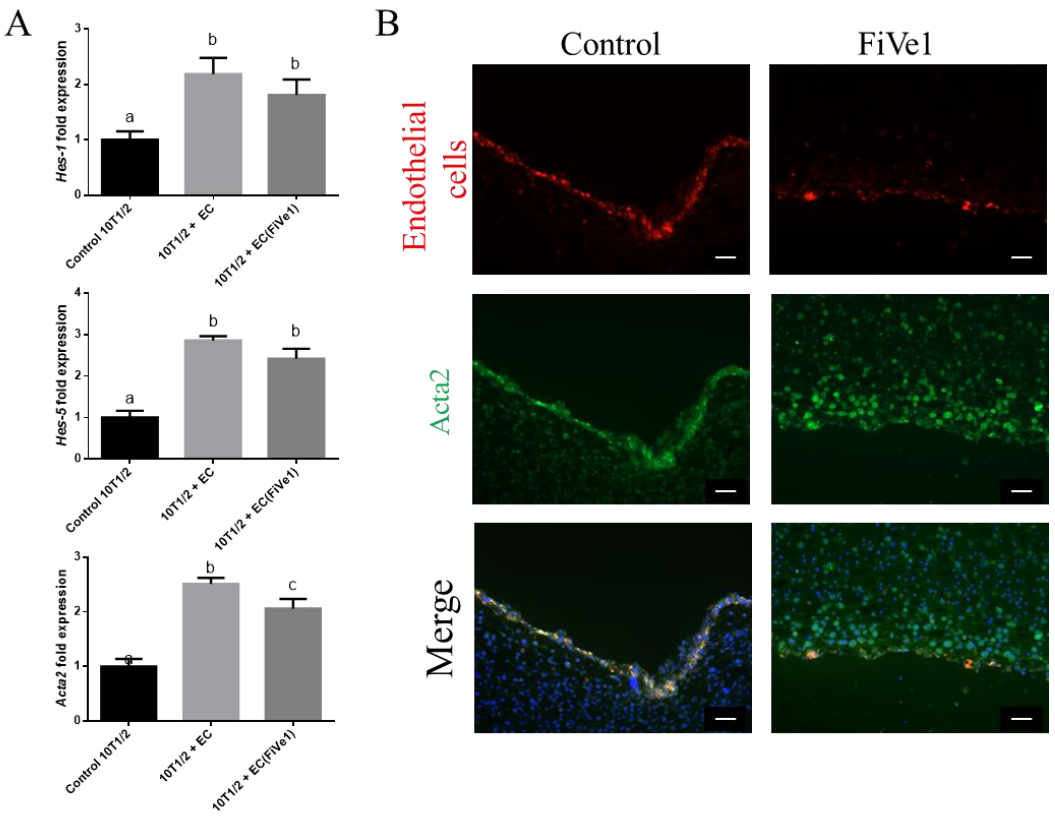

Figure 5.4. Gene and protein expression of 10T1/2 cocultured with HCAEC in 2D and 3D, respectively. **(A)** Hes-1, Hes-5 and Acta2 gene expression in 10T1/2 cells cocultured with HCAECs on 2D. **(B)** Immunofluorescence microscopy showing expression of Acta2 in 3D coculture of 10T1/2 and HCAEC in control vs FiVe1 treated tissues. Scale bar = 50 µm.

5.4.4 Vimentin plays a role in endothelial cell migration and micro-vessel formation.

The ability of endothelial cells to migrate in a 3D environment was tested in response to angiogenic growth factors (bFGF + EGF or VEGF). Furthermore, the effect of vimentin disruption on this process was tested using a small molecule inhibitor, FiVe1, or gene expression knock-down using vimentin siRNA (**Figure 5.5**).

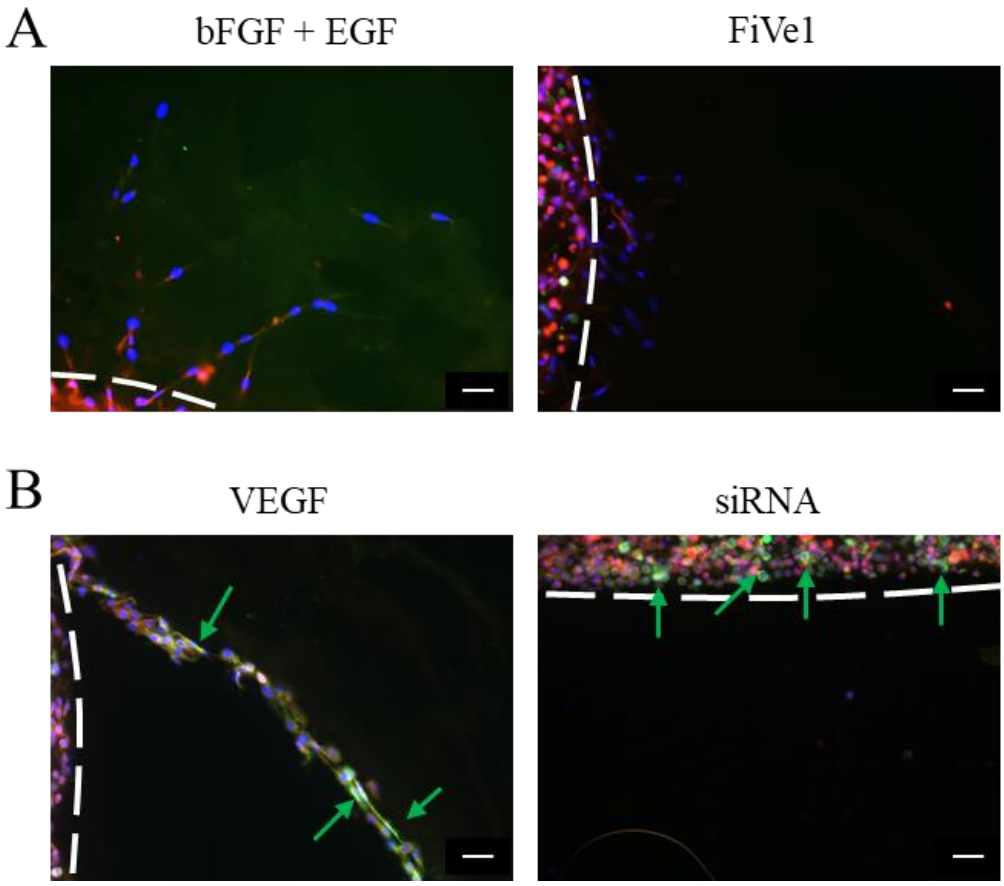

Figure 5.5. Endothelial cell migration and neovascularization in 3D fibrin gel. (**A)** Migration of endothelial cells into low concentration fibrin gel in the presence of bFGF and EGF and FiVe1 treated gels. **(B)** Micro-vessel formation in 3D fibrin gels containing VEGF, or HCAECs that were previously transfected with vimentin siRNA. Scale bar = 50 µm. Dashed white lines indicate tissue outline. The green arrows indicate Acta2 positive cells.

The results showed that angiogenic growth factors were able to induce migration of endothelial cells out of the 3 mg/mL fibrin gel and into the less concentrated 1 mg/mL fibrin gel, which contained the growth factors (**Figure 5.5**). On the other hand, when vimentin filament fiber formation was inhibited, migration was greatly affected. Furthermore, while bFGF and EGF combination has been shown to induce growth and proliferation in endothelial cells[22], VEGF is a notably more potent angiogenic factor since

the sprouting micro-vessels are better organized (**Figure 5.5.B**). Moreover, these micro-vessels were able to recruit 10T1/2 cells that expressed Acta2 as shown by the green staining indicated by the arrows (**Figure 5.5.B**). Contrarily, siRNA transfected HCAECs were not able to migrate out of the 3 mg/mL fibrin; however, they were able to induce expression of Acta2 in 10T1/2 cells (**Figure 5.5.B**), which is in agreement with **Figure 5.4.B**. Taken together, these exploratory study results showed that vimentin plays an important role in the migration of endothelial cells and recruit 10T1/2 cells to form micro-vessels.

5.5 Conclusion

In this exploratory study, the role of vimentin in the regulation of Notch signaling and neovascularization was explored. The results demonstrated that vimentin plays a limited role in Notch signaling, as evidenced by a slight reduction of certain Notch signaling targets (Hes-5 and Acta2) when vimentin filaments were inhibited. However, vimentin plays a much important role in micro-vessel formation, which allows endothelial cells to migrate and recruit 10T1/2cells in response to VEGF. While these pilot results shed some light on the role of vimentin in the Notch signaling and neovascularization, more research is required to fully elucidate the role of this intermediate fiber in the context of vascular development.

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Chapter 6

General discussion

Overview: *In this chapter, a general summary of the work done during this research project is provided, highlighting important findings. Furthermore, some limitations of this work are mentioned, and recommendations are suggested.*

6.1 Summary and conclusions

In this research project, engineered vascular tissues were used to study the Notch signaling pathway and vascular calcification in a physiologically relevant 3D culture system. These are novel and useful tools to improve the current understanding of the physiology and pathology of the vascular system. Fibrin gel-based vascular tissues were prepared with two types of cells to study Notch signaling. Fibrin is known for its biocompatible properties, which has been shown to support important cellular processes and has been widely used as a biomaterial in vascular tissue engineering[1, 2]. Physical properties of the fibrin gels were tested using rheology, water contact angle, and water mass loss studies. Initially, human coronary artery SMCs were selected due to their relevance to vascular tissue engineering; however, their extremely limited proliferation potential was an obstacle to obtain enough cells to fabricate the tissues. The reason behind the slow proliferation rate is that it heavily depends on the age and health of the donor. Therefore, A-10 and 10T1/2 cells were chosen as alternatives to the human coronary artery SMCs due to their rapid proliferation potential. A-10, 10T1/2 and HCAEC have been coculture in these tissues in various combinations and the Notch pathway activation was assessed. In the framework of this study, endothelial

cells were used as signal sending cells due to their expression of Jag1 ligand. The A-10 cells and 10T1/2 cells were used as signal-receiving cells. The former of these two cell lines is an embryonic smooth muscle cell from a rat and the latter is a mouse progenitor cell line. In this study, the activation of the Notch signaling in these two cell lines were evaluated by assessing downstream targets of Notch such as Hes1, Acta2[3].

Fibrin gels were found to lose around 70 % of their mass during culture conditions over a 3-day period. This was attributed to water expulsion rather than degradation since the plasmin inhibitor ACA was present throughout the culture period. SEM images also confirmed that fibrin fibers did not seem to be degraded after 3 days of culture. Moreover, it was expected that the presence of cells might affect the contraction of these gels; however, the presence of cells up to a concentration of 1 million cells had no effect, and the concentration of fibrinogen had a bigger effect on contraction. Additionally, cell studies showed that while 10T1/2 cells were shown to be responsive to the activation of the Notch signaling by endothelial cells, A-10 cells failed to do so likely due to cis-inhibition. It is well established that Notch signaling is extremely context-dependent despite its simple transduction mechanism[4]. Furthermore, Notch is implicated in key processes during vascular development since disruption of this pathway has been shown to cause underdeveloped vasculature as well as the progression of certain diseases[5-8]. Since this pathway was activated in 10T1/2 cells, which are considered multipotent cells that are not terminally differentiated, it can be concluded that the Notch signaling plays an active role in cell commitment. On the other hand, because Notch signaling was not activated in A-10 cells, which are committed but immature SMCs, it might be possible that other signaling

pathways that crosstalk with Notch must be regulated to drive differentiation of these SMCs into a contractile phenotype. Indeed, other pathways are involved in the development and maintenance of the vasculature do cross-talk with the Notch pathway[9]. The results in chapter 3 demonstrate the context-dependence of the Notch signaling pathway and introduce fibrin-based tissues as a relevant tool that can be used to further understand this important pathway and, consequently, the development of better-engineered tissues.

Vascular calcification is a common occurrence in the elderly population and in patients suffering from chronic kidney disease. While much of the current understanding of vascular calcification comes from studies in 2D cell culture and animal models, EVTs can be a powerful tool in the researchers' repertoire that can accelerate the understanding of this disease and aid in devising strategies to reduce its impact on the affected populations. In chapter 4, 10T1/2 cells were used in both their undifferentiated and differentiated forms to shed light on the progression of calcification in an isolated culture system. There is a debate in the literature about the source of cells that contribute to the vascular calcification. Some research points towards SMCs[10] while others attribute it to circulating stem cells[11, 12]. Results in chapter 4 showed that tissues made from both undifferentiated and differentiated 10T1/2 cells calcified, albeit via different mechanisms. Undifferentiated 10T1/2 cells upregulated certain osteogenic markers, while differentiated cells down-regulated SMCs markers in response to the calcification stimulus. However, both tissues expressed osteopontin. This commonality between the two types of tissues might be a

coping mechanism of cells to reduce the concentration of cytoplasmic calcium by sequestering it using osteopontin and then exporting it outside the cell.

Furthermore, elastin degradation has been linked to vascular calcification[13]. In the elderly population, elastin degradation is a natural aging process and might contribute to vascular calcification. The degradation of elastin was simulated by incorporating a partially soluble form of elastin into the tissues. Interestingly, the two tissue types responded differently to this stimulus on the genetic level. While undifferentiated tissues were largely unaffected, differentiated tissues downregulated late SMC markers and upregulated osteogenic markers in the presence of elastin. Moreover, the engineered tissues were used to test the effectiveness of vitamin K in reducing the burden of calcification. Vitamin K is a lipid-soluble molecule that plays a role in coagulation; however, it has also been shown to help reduce calcification[14]. Vitamin K activates the calcium-binding protein matrix gla protein, by carboxylation leading to attenuated calcification. Therefore, the calcified engineered tissues were used to test the efficacy of vitamin K to diminish the extent of calcification. Both types of tissue responded to vitamin K treatment, which reduced the level of calcification and downregulated osteopontin protein expression. Data collectively presented in Chapter 4 revealed the potential of engineered tissues to study and model diseases and test drugs. This strategy can narrow the focus of research work before moving into animal models to study the problem on a systemic level.

Chapter 5 is an extension of chapter 3. Notch signaling has been shown to require a pulling force to be activated. While this concept is still debatable, it might also be context dependent. Unsurprisingly, the involvement of a mechanical force suggests that the

cytoskeletal components are at play. While some studies showed that the actin-myosin might be responsible for that force[15], other studies suspect vimentin[16, 17]. The preliminary results in chapter 5 showed that while inhibition of vimentin fiber formation may partially reduce the strength of the Notch signaling, it does not completely inhibit it. This may indicate that the cytoskeleton as a whole, including the microfilament, intermediate filaments, and microtubules, contribute to a certain extent in generating that force. Moreover, the results show that vimentin plays a key role in forming new vessels since knocking its expression down or inhibiting its polymerization will prevent neovascularization. However, more research is needed to explore the role of intermediate filaments in Notch signal regulation.

6.2 Contribution to the research field

In this thesis, engineered vascular tissues fabricated from fibrin hydrogels were used in a novel approach to study the Notch pathway. Notch signaling is a central pathway for vascular development and homeostasis. While this pathway was discovered over a century ago, there is still much to uncover due to its context-dependence. Much of the knowledge we have of Notch signaling comes from 2D cell culture and animal models; therefore, utilizing fibrin-based tissues sheds a different light on this pathway. One of the first steps of this research was to characterize the properties of this cell culture system. While previous studies have shown that fibrin gels compact[18], kinetic changes in the mass of these gels were shown here. Furthermore, these results also show acellular fibrin gel compaction.

On the signaling front, the two cell lines used in these studies responded oppositely, which highlights context-dependence. Differentiated but immature SMCs cell line A-10 failed to

activate Notch signaling, while multipotent 10T1/2 cells upregulated Hes- and SMC markers in response to coculture with endothelial cells. This upregulation was abolished when treated with DAPT, signifying that the upregulation of SMC markers was via the Notch pathway. The dichotomy in the response of these two cell lines suggests that Notch signaling is not as simple as cell-cell interaction, but other factors affect the activation of this pathway. Furthermore, preliminary results on the role of vimentin in Notch signaling was also explored. These results indicate that the cytoskeleton is not merely for structural support, but different cytoskeletal components might play a complementary part to control vital processes such as signaling mechanisms.

Additionally, the use of engineered vascular tissues to study the effect of calcification on progenitor and differentiated cells was the first of its kind. The source of calcifying cells in the vasculature has never been studied in engineered tissues. In this work, 10T1/2 were used as model cells because it serves the role of both progenitor and differentiated SMCs. There is debate as to what are the source of calcifying cells in the literature. Results in this work have shown that both progenitor and differentiated cells are capable of calcifying in response to high concentrations of inorganic phosphates. However, the two phenotypes calcify through different processes, which were ameliorated with the treatment of vitamin K. Moreover, elastin degradation was simulated by adding elastin to the engineered tissues and also showed different gene expression profiles between the two types of tissues. This work underscores the practicality of engineered tissues to study vascular calcification and lays the foundation for further studies.

6.3 Limitations

Even though fibrin is a promising hydrogel commonly used in engineered vascular tissues, it is not a protein found in the ECM of vascular tissues. Fibrin gel was chosen as a biomaterial for its well-established biocompatibility and the ability to incorporate cells in the process of fabrication, allowing for high cell density tissues. However, it is only meant as a temporary scaffold that would be remodeled and replaced by ECM proteins secreted by the resident cells. Collagen and elastin are the main proteins found in the ECM of vascular tissues. While elastin is challenging to incorporate into the hydrogel due to low solubility, collagen would be a good alternative for fibrin, or maybe a mixture of fibrin and collagen could be used for further studies.

Another limitation of this research is the use of non-human A-10 and 10T1/2 cells which are sourced from rats and mice. The use of these cells was due to the slow proliferation rate of human smooth muscle cell types, which often depends on the donor's age and health status. Nevertheless, the use of A-10 and 10T1/2 cells has provided valuable information that can be used to design future studies using human cells.

Vascular tissues are continuously exposed to pulsatile and shear forces. The work in this thesis was done mostly in static conditions, yet another limitation that can be addressed to reflect a more realistic environment.

6.4 Recommendations

This research project has laid the foundation for the use of engineered vascular tissues to study cell signaling and disease. However, it also warrants further research to advance the understanding of vascular development and disease which will ultimately lead to a better

engineered tissues. For that purpose, the following are some suggestions that can build on this work:

1- **Incorporation of relevant ECM proteins:** Collagen can be incorporated or produced by cells by the addition of ascorbic acid into the media. Collagen is a major component of the vasculature and might provide a more relevant microenvironment for cells.

2- **Mechanical stimulation:** Pulsatile and shear forces are known to be important for the development of tissues and the progression of diseases. Furthermore, these forces might play a role in the activation of signaling pathways such as Notch signaling. Therefore, it would be beneficial to expose those tissues to dynamic forces and study the effect they have on either the signaling or progression of calcification.

3- **Human sourced cells:** Ultimately, these studies are geared towards an understanding of human physiology and pathology. Therefore, it would be useful to utilize human-sourced cells that can give more relevant results. Cells like mesenchymal stem cells or induce pluripotent stem cells are good candidates due to their relatively high proliferation rate prior to differentiation.

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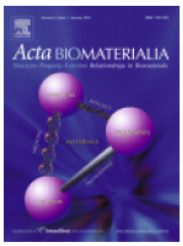
Appendices

Appendix A: Copyright Clearances



The effects of progenitor and differentiated cells on ectopic calcification of engineered vascular tissues

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Publication: Acta Biomaterialia

Publisher: Elsevier

Date: 1 October 2020

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- Volunteered as a member of the student Health Plan Committee, University of Western Ontario (2015).
- Volunteered in the Let's Talk Science program at the University of Ottawa (2012-2013)
- Cooperative education program volunteer, Hillcrest high school and Ottawa Hospital Research Institute (2012).

Awards

- Awarded the OGS at the University of Western Ontario (2015-2016) & (2016-2017)
- Awarded the Dean's scholarship during master's studies (2012).
- Awarded the Cooperative Education Certificate of Appreciation for active participation and support of the cooperative education program (2012).
- Placed on the Dean's Honor List for the 2009-2010 academic year.

Publications & Conferences

- Laurie Ma, Nima Niknejad, Ivan Gorn-Hondermann, **Khalil Dayekh**, Jim Dimitroulakos. Lovastatin induces multiple stress pathways including LKB1/AMPK activation that regulate its cytotoxic effects in squamous cell carcinoma cells. PLoS One. **2012**;7(9):e46055. doi: 10.1371/journal.pone.0046055. Epub **2012** Sep 28. PMID: 23029387; PMCID: PMC3460930.
- **Khalil Dayekh**, Stephanie Johnson-Obaseki, Martin Corsten, Patrick J. Villeneuve, Harmanjatinder S. Sekhon, Johanne I. Weberpals and Jim Dimitroulakos. Monensin Inhibits Epidermal Growth Factor Receptor Trafficking and Activation: Synergistic Cytotoxicity in Combination with EGFR Inhibitors. Mol Cancer Ther. **2014**;13(11):2559-71. doi: 10.1158/1535-7163.MCT-13-1086. Epub **2014** Sep 4. PMID: 25189541.

- **Khalil Dayekh** and Kibret Mequanint. Convergence of Notch Signaling Pathway and the Elastin Receptor (ERC) in Fibrin-based Vascular Tissue Constructs. European Chapter Meeting of the Tissue Engineering and Regenerative Medicine International Society June 26-30 **2017,** Davos, Switzerland.

- Carley Ort, **Khalil Dayekh**, Malcolm Xing, and Kibret Mequanint. Emerging Strategies for Stem Cell Lineage Commitment in Tissue Engineering and Regenerative Medicine. ACS Biomater Sci Eng. **2018**;4(11):3644-3657. doi: 10.1021/acsbiomaterials.8b00532. Epub **2018** Aug 20. PMID: 33429592.

- **Khalil Dayekh**, and Kibret Mequanint. Engineered Vascular Tissues to Model and Study Disease. 35th Annual Meeting of the Canadian Biomaterials Society May 21-24 **2019,** Quebec City, Canada.

- **Khalil Dayekh**, and Kibret Mequanint. Comparative Studies of Fibrin-Based Engineered Vascular Tissues and Notch Signaling from Progenitor Cells. ACS Biomater Sci Eng. **2020**;6(5):2696-2706. doi: 10.1021/acsbiomaterials.0c00255. Epub **2020** Mar 30. PMID: 33463301.

- **Khalil Dayekh**, and Kibret Mequanint. The Effects of Progenitor and Differentiated Cells on Ectopic Calcification of Engineered Vascular Tissues. Acta Biomater. Acta Biomater. **2020**;115:288-298. doi: 10.1016/j.actbio.2020.08.019. Epub **2020** Aug 25. PMID: 32853805.