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Co-culture of Smooth Muscle Cells and Endothelial Cells on Porous 3D Polyurethane Scaffolds for Vascular Tissue Engineering

Aparna Bhattacharyya, *The University of Western Ontario*

Supervisor: Kibret Mequanint, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Engineering Science degree in Biomedical Engineering

CO-CULTURE OF SMOOTH MUSCLE CELLS AND ENDOTHELIAL CELLS ON POROUS 3D POLYURETHANE SCAFFOLDS FOR VASCULAR TISSUE ENGINEERING

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Aparna Bhattacharyya

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Supervisor	Examiners
_____	_____
Dr. Kibret Mequanint	Dr. Hiran Perinpanayagam
	_____
	Dr. Silvia Mittler
	_____
	Dr. Jin Zhang

The thesis by

Aparna Bhattacharyya

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Abstract

Vascular tissue engineering aims to design more reliable substitutes for diseased or otherwise failed blood vessels. For this approach to be successful, the engineered blood vessel must provide similar structure and integrity to that of the native vascular tissue. Despite extensive studies documented in scientific literature, the designing of clinically-relevant vascular tissue has been largely unsuccessful. One of the challenges is our lack of understanding on how vascular smooth muscle cells (VSMCs) and vascular endothelial cells (VECs) interact in the graft. The objective of this study was to examine the factors that play a role in VSMC and VEC interaction in 3D co-culture so that a compliant graft can be constructed. Highly porous 3D poly(carbonate urethane) (PCU) scaffolds were fabricated using a solvent casting particulate leaching method. Human coronary artery smooth muscle cells (HCASMCs) and human coronary artery endothelial cells (HCAECs) were seeded sequentially on scaffolds containing basal and medial adhesion protein layers. Co-cultures were carried out and stained to allow observation of cell orientation and morphology. Results showed that HCASMCs readily attached to the scaffold and formed dense confluent layers which facilitated the attachment and organization of HCAECs into a monolayer above the HCASMC layer. In addition, the HCAECs showed a greater affinity toward the HCASMCs than to the scaffold. Western blot analysis showed that co-culture induced an up-regulation of the contractile phenotype in HCASMCs as well as the Notch3 receptor and its ligand Jagged1. In order to identify the link between Jagged1 and the expression of contractile proteins a Jagged 1 knockdown study was conducted using small interfering RNA (siRNA). Results showed a reduction in smooth muscle α-actin and calponin in co-cultures treated with Jagged1

siRNA compared with expression levels in co-cultures treated with control siRNA. It can therefore be concluded that the Jagged1-Notch3 pathway is an important regulator of VSMC phenotype and can be taken advantage of when fabricating engineered tissues.

Keywords: tissue engineering, vascular smooth muscle cell, vascular endothelial cell, co-culture, polyurethane scaffolds, phenotype modulation, contractile protein expression, Notch signaling, Jagged1

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List of Abbreviations

NH_4Cl	Ammonium chloride
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CTG	CellTracker™ Green
CABGs	Coronary artery bypass grafts
CAD	Coronary artery disease
DMF	Dimethylformamide
EC	Endothelial cell
EGM	Endothelial growth media
ePTFE	Expanded polytetrafluoroethylene
ECM	Extracellular matrix
FN	Fibronectin
GAPDH	Glyceraldehyde3-phosphate dehydrogenase
HCAEC	Human coronary artery endothelial cell
HCASMC	Human coronary artery smooth muscle cell
IGF-1	Insulin-like growth factor-1
IMA	Internal mammary artery
LN	Laminin
LDL	Low density lipoprotein
MHC	Myosin heavy chain
NO	Nitric oxide
PBS	Phosphate buffered saline

PDGF	Platelet derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PCU	Poly(carbonate urethane)
PGA	Poly(glycolic acid)
PLLA	Poly(L-lactic acid)
PAGE	Polyacrylamide gel electrophoresis
PET	Polyethylene teraphthalate
PCR	Polymerase chain reaction
SV	Saphenous vein
SEM	Scanning electron microscope
siRNA	Small interfering RNA
SM-α-actin	Smooth muscle α-actin
SMC	Smooth muscle cell
SmGM	Smooth muscle growth media
SDS	Sodium dodecyl sulfate
SCPL	Solvent casting particulate leaching
3D	Three-dimensional
TEVG	Tissue engineered vascular graft
TGF- β1	Transforming growth factor-β1
TNF	Tumor necrosis factor
2D	Two-dimensional
VEC	Vascular endothelial cell
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

Chapter 1 - Introduction

1.1 Overview

One of the strongest motivators behind the field of vascular tissue engineering is the ever-present incidence of coronary artery disease (CAD). Cardiovascular diseases- of which CAD comprises the majority- are currently the number one cause of death worldwide[1] and are projected to increase in prevalence with the aging of the baby boomer population and a growing incidence of diabetes[2]. Autologous vessels are the most reliable option for the replacement of diseased coronary arteries and as such are the most frequently used. Graft vessels are generally harvested from the internal mammary artery (IMA), saphenous vein (SV) or the internal thoracic artery[3]. The structure of the IMA matches well with that of the coronary artery and thus it provides a much more patent graft vessel as compared with the SV[4]. In addition to concerns with patency, the source for viable graft vessels may be depleted in many patients[5-6]. These individuals have the option of having a synthetic graft implanted; however, these grafts are accompanied by a high risk of bacterial infection, intimal hyperplasia and thrombus formation. Furthermore, their noncompliance with the native artery contributes to their limited use as coronary artery bypass grafts (CABGs)[7]. The emergence of vascular tissue engineering is triggered by the lack of major advancements in current therapies as a means to overcome the drawbacks that they present. In view of this, tissue engineering aims to produce functional living substitutes. The fundamental stages in the fabrication of a tissue engineered vascular substitute are: (1) expansion of cells and infiltration into a scaffold,

(2) maturation of the cell-scaffold construct to achieve the necessary mechanical and biological properties and (3) surgical implantation into the host[8]. Success with small-diameter tissue engineered vascular grafts (TEVGs) has been limited and is attributed to a mismatch in compliance with the adjacent artery, misalignment of smooth muscle cells (SMCs) and insufficient amounts of elastin, collagen and SMCs[9]. These critical components can in principle be enhanced through strategic manipulation of SMC phenotype and it has been largely acknowledged that phenotype switching at the appropriate maturation stage would produce a TEVG that emulates the native tissue in structure, function and composition[10-11]. Investigators have sought to understand the role of various factors in the modulation of SMC phenotype including: matrix components, growth factors, scaffold geometry, mechanical stimulation and endothelial cell (EC)-SMC interactions[9]. Much of the work completed thus far in the investigation of SMC in co-culture with EC has either relied on models that do not accurately represent the native *in vivo* conditions, has used exogenous stimuli that are inapplicable to tissue engineering applications or has assessed the production of matrix molecules in co-culture. However, a direct dependence of SMC phenotype on the presence of ECs has also been reported in literature[12-13] and some of the mechanisms governing their interactions have started to emerge[14-16]. A deeper understanding of these mechanisms in the context of three-dimensional (3D) engineered vasculature may enable their exploitation in the creation of more physiologically accurate substitutes.

1.2 Thesis Outline

This thesis contains five chapters that detail the work carried out in the investigation of the interactions between vascular cells in 3D polyurethane scaffolds. Chapter 2 introduces the basis for vascular tissue engineering, illustrates the motivation for this study and outlines its specific objectives. The materials and experimental methodologies used are described in Chapter 3 and are followed by the significant findings of this work in Chapter 4. Chapter 5 summarizes the results and provides the significance and future directions of this project.

1.3 References

1. Mendis, S.; Puska, P.; Norrving, B., Global Atlas on Cardiovascular Disease Prevention and Control. In World Health Organization: Geneva, 2011.
2. Murray, C. J.; Lopez, A. D., Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *Lancet* **1997,** 349, (9063), 1436-42.
3. Motwani, J. G.; Topol, E. J., Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. *Circulation* **1998,** 97, (9), 916-31.
4. Rashid, S. T.; Salacinski, H. J.; Fuller, B. J.; Hamilton, G.; Seifalian, A. M., Engineering of bypass conduits to improve patency. *Cell Proliferat* **2004,** 37, (5), 351-66.
5. Kaushal, S.; Amiel, G. E.; Guleserian, K. J.; Shapira, O. M.; Perry, T.; Sutherland, F. W.; Rabkin, E.; Moran, A. M.; Schoen, F. J.; Atala, A.; Soker, S.; Bischoff, J.; Mayer, J. E., Jr., Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. *Nat Med* **2001,** 7, (9), 1035-40.
6. Baguneid, M.; Murray, D.; Salacinski, H. J.; Fuller, B.; Hamilton, G.; Walker, M.; Seifalian, A. M., Shear-stress preconditioning and tissue-engineering-based paradigms for generating arterial substitutes. *Biotechnol Appl Bioc* **2004,** 39, (Pt 2), 151-7.
7. Thomas, A. C.; Campbell, G. R.; Campbell, J. H., Advances in vascular tissue engineering. *Cardiovasc Pathol* **2003,** 12, (5), 271-6.

8. Rabkin, E.; Schoen, F. J., Cardiovascular tissue engineering. *Cardiovasc Pathol* **2002,** 11, (6), 305-17.

9. Chan-Park, M. B.; Shen, J. Y.; Cao, Y.; Xiong, Y.; Liu, Y.; Rayatpisheh, S.; Kang, G. C.; Greisler, H. P., Biomimetic control of vascular smooth muscle cell morphology and phenotype for functional tissue-engineered small-diameter blood vessels. *J Biomed Mater Res A* **2009,** 88, (4), 1104-21.

10. Beamish, J. A.; He, P.; Kottke-Marchant, K.; Marchant, R. E., Molecular regulation of contractile smooth muscle cell phenotype: implications for vascular tissue engineering. *Tissue Eng Pt B-Rev* **2010,** 16, (5), 467-91.

11. Stegemann, J. P.; Hong, H.; Nerem, R. M., Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype. *J Appl Physiol* **2005,** 98, (6), 2321-7.

12. Hirschi, K. K.; Rohovsky, S. A.; D'Amore, P. A., PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J Cell Biol* **1998,** 141, (3), 805-14.

13. Powell, R. J.; Cronenwett, J. L.; Fillinger, M. F.; Wagner, R. J.; Sampson, L. N., Endothelial cell modulation of smooth muscle cell morphology and organizational growth pattern. *Ann Vasc Surg* **1996,** 10, (1), 4-10.

14. Brown, D. J.; Rzucidlo, E. M.; Merenick, B. L.; Wagner, R. J.; Martin, K. A.; Powell, R. J., Endothelial cell activation of the smooth muscle cell phosphoinositide 3-kinase/Akt pathway promotes differentiation. *J Vasc Surg* **2005,** 41, (3), 509-16.

15. Fetalvero, K. M.; Shyu, M.; Nomikos, A. P.; Chiu, Y. F.; Wagner, R. J.; Powell, R. J.; Hwa, J.; Martin, K. A., The prostacyclin receptor induces human vascular smooth muscle cell differentiation via the protein kinase A pathway. *Am J Physiol- Heart C* **2006,** 290, (4), H1337-46.

16. High, F. A.; Lu, M. M.; Pear, W. S.; Loomes, K. M.; Kaestner, K. H.; Epstein, J. A., Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. *P Natl Acad Sci USA* **2008,** 105, (6), 1955-9.

Chapter 2 - Literature Review

2.1 Structure and Function of Blood Vessels

Blood vessels are complex networks of hollow tubes with distinct structures that allow them to transport blood throughout the body. Oxygenated blood is pumped by the heart into the aorta which distributes the blood to large arteries. From here the blood is dispersed to smaller arteries, arterioles and finally capillaries where nutrient, oxygen and waste exchange occurs to and from tissues and organs. Venules then carry the oxygen-depleted blood to larger veins and finally back to the heart[1]. Apart from small arterioles and capillaries, vessels are constructed of three distinct concentric layers that vary in thickness depending on the location of the vessel and its function[1-2]. The inner most layer that contacts the blood is the tunica intima and consists of a lining of ECs attached to a basement membrane of extracellular matrix (ECM) proteins[1]. Adjacent to the intima is the tunica media- a densely packed layer of SMCs. These SMCs are oriented circumferentially into dense layers with fenestrated elastic lamellae interspersed between them[1,3]. Veins do not function in a primarily contractile manner and so have a much thinner tunica media than do arteries. The outermost layer is the tunica adventitia and is composed of fibroblasts and a collagenous ECM[2] that protects and reinforces the blood vessel and anchors it to the surrounding tissue. Nerves and lymphatic vessels infiltrate the adventitia and provide further structural support[3]. In addition, arteries have an internal and external elastic lamina. The medial layer is bound by the elastic laminae where the majority of the elastin is concentrated[4]. The elastic tissue aids the vessel wall in

maintaining its resilience[3] and imparts arterial elasticity under pulsatile flow[5]. The structure of an artery wall is shown in Figure 2.1.

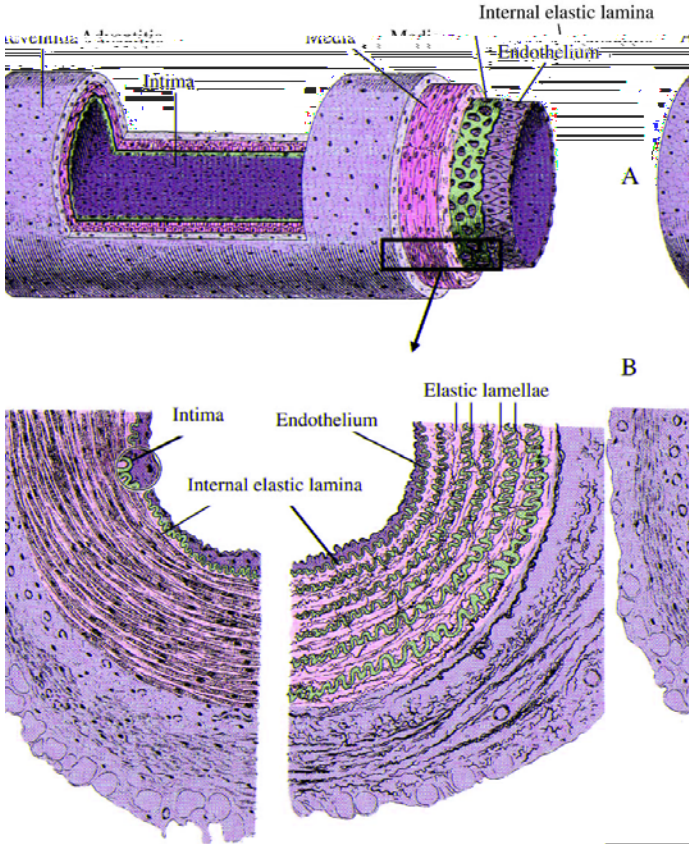

Figure 2.1: Structure of the artery wall[2]. (A) Organization of the intima, media and adventitia. (B) Cross-sectional view showing the organization of elastin in the media of muscular arteries (left) and elastic arteries (right).

The endothelial monolayer was once thought to be a passive lining of the vasculature, but it is now known that it serves several important functions such as the prevention of thrombus formation, coagulation and intimal hyperplasia[6]. Under physiological conditions the glycocalyx layer on the ECs cells in conjunction with nitric oxide and prostacyclin prevent platelet adhesion[7-8]. Additionally, ectonucleotidases on the endothelial cell surface metabolize adenosine diphosphate which inhibits platelet

recruitment. Coagulation is initiated by the exposure of tissue factor to blood and progresses as fibrinogen is converted to fibrin by thrombin. Under normal conditions endothelial cells inhibit the tissue factor expression pathway and produce thrombomodulin which deactivates thrombin, thereby preventing the onset of coagulation[6]. Thrombus formation is also suppressed by the binding of antithrombin III to heparin sulfate proteoglycans. In the event that coagulation does occur, the endothelial cells release tissue type plasminogen activator to break up the fibrin clots[7]. Intimal hyperplasia is averted through the release of molecules that prevent SMC differentiation. The intact EC layer also controls the movement of molecules through the vascular wall, regulates vasomotor tone and maintains homeostatis of the vessel[3].

The function of the SMCs that populate the tunica media is to regulate the flow of blood. These cells are capable of shifting between a contractile phenotype, found in physiological conditions, and a synthetic phenotype which is characteristic of pathological conditions. In the contractile phenotype SMCs dilate and contract in a coordinated manner to increase or decrease the vessel diameter, thereby changing the flow of blood through the lumen[2]. Vasoactivity is modulated primarily by the SMCs and the neighboring ECs. The VSMCs receive neuronal or hormonal signals from the endothelial monolayer and react by generating the appropriate contractile force to meet the necessary blood pressure. Cells in the contractile phenotype have a spindle-shaped morphology, a centrally located nucleus and proliferate at a very low rate. This morphology can quickly transform to that of a fibroblast's upon stimulation by pathological conditions. This state is known as the synthetic phenotype and is characterized by high levels of ECM production and proliferation[7].

The mechanical properties of the blood vessel arise from a network of ECM component, namely: collagen (types I and III), fibrous elastin, proteoglycans (versican, decorin, biglycan, lumican, perlican), hyaluronan, and glycoproteins (laminin, fibronectin, thrombospondin, tenascin)[1]. Properties such as tensile stiffness, elasticity and incompressibility are imparted by collagens, elastin and proteoglycans respectively, while viscoelasticity is provided by the combination of all three[1-2]. The composition and organization of the ECM and the interactions between these proteins and the cells determine the degree of mechanical functioning in the blood vessel. Additionally, these proteins provide anchorage to the cells, and guide the biological functions of the tissue under both physiological and pathological conditions. This is achieved through bioactive domains on ECM molecules that can bind to structural or connecting proteins, signaling molecules (growth factors, cytokines, chemokines, and matrix proteinases) and membrane receptors. Upon binding to cell surface receptors, intracellular signaling pathways can be activated or deactivated via either biochemical cues or mechanotransduction[3].

The internal and external elastic laminae have a convoluted structure that arises during vasculogenesis. A recent study[9] of elastin fiber organization in rabbit aorta has shown that whereas the internal elastic laminae supports loading in the longitudinal direction – that is stretching along the vessel axis- , the external elastic laminae supports circumferential loading – that is stretching in the radial direction. At low strains, the elastin stretches for the duration of the pulse and returns to its original state as the pulse

wanes. These properties are critical since pressure waves arising from pulsatile blood flow act in both the radial and longitudinal directions[4]. The elastin molecules in the laminae also convey signals between the media and intima to regulate the phenotypic modulation, proliferation and organization of VSMCs and so are critical components of the arterial structure[2].

2.2 Coronary Arteries

Coronary arteries have the same distinct structure as depicted in Figure 1; however, because they are considered to be muscular arteries, their medial layer constitutes the bulk of the arterial wall (left side of Figure 2.1B). Typically their internal diameter ranges from 3 to 4 mm with a wall thickness of 1 mm. Muscular arteries transport blood to organs at high pressures (80-120 mmHg) and experience shear stresses in the range of 0.75-2.25 Pa during a single cardiac cycle[3]. The pumping heart undergoes large deformations and so the coronary arteries are subjected to larger longitudinal strains and smaller circumferential wall strains (10-15%)[10-11]. The elastic laminae surrounding the tunica media allow the artery to recoil and prevent vascular dilation that would result from the creep of collagen under high blood pressures. For human coronary arteries, the burst pressure is about 2000 mm Hg (266 kPa)[3]. The coronary arteries originate at the root of the aorta and split into two branches that vascularize the myocardium. The left coronary artery supplies blood to the left atrium and left ventricle of the heart, while the right coronary artery supplies blood to the right atrium and right ventricle of the heart[12].

2.3 Diseases of the Coronary Artery

According to recent data, cardiovascular diseases – of which CAD comprises the majority- are the prevailing cause of death worldwide[13]. Incidences of CAD are expected to rise over the next 15 years due to increasing prevalence in developing countries and Eastern Europe as well as an increasing prevalence of obesity and diabetes[14] which can lead to cardiovascular complications[4]. CAD most commonly arises from atherosclerosis, an inflammatory disease. It is initiated by injury (for example- high cholesterol or glucose, smoking and high blood pressure) to the endothelial lining of the blood vessel and eventually results in the formation of a complex lesion in the artery wall[15]. At physiological conditions, low density lipoprotein (LDL) in the blood infiltrates through the intima and is mostly eliminated on the abluminal side of the artery wall, though some of it is retained. In periods of hypercholestoremia, circulating levels of LDL are elevated. Accumulation of these molecules is facilitated by proteoglycans, of the ECM, which bind the LDL molecules. Subsequent oxidation of LDL particles results in the release of phospholipids that activate the endothelium[16]. ECs then upregulate expression of vascular cell adhesion molecule-1 (and various others) which recruit monocytes, lymphocytes, macrophages and mast cells (immune cells) causing them to adhere to these sites[15]. Chemokines produced in the intima stimulate their migration to the subendothelial space[17-18]. Monocytes then transform into macrophages which engulf the oxidized LDL particles and become foam cells[17,19] while molecules such as platelet derived growth factor (PDGF), tumor necrosis factor (TNF) and interleukins change the qualitative composition of the ECM and induce phenotypic modulation of the VSMCs[15]. As foam cells rupture they release their lipid contents developing a lipid necrotic core which

continues to grow as SMCs migrate to the intima, proliferate and secrete ECM proteins that form a fibrous cap around the core[15, 19-21]. If unabated, this inflammatory response continues resulting in narrowing of the lumen which is compensated for by gradual dilation. However, it reaches a point where the lumen narrows so much that the flow of blood is altered to the succeeding tissues resulting in ischemia (inadequate blood supply to the heart). Furthermore, if the plaque becomes unstable and ruptures, coagulation is initiated resulting in a deep thrombus formation that can occlude the blood vessel leading to acute myocardial damage[19,21].

2.4 Surgical Interventions

CABGs are the gold standard for treatment of diseased coronary arteries and as such are the most frequently used surgical intervention. Grafts of principally autologous tissue from the IMA, SV or internal thoracic artery are used. Although these vessels are considered of highest quality, they present several unresolved concerns. SV grafts from elderly patients are prone to thrombus and neointima formation as well as atherosclerosis and aneurysms upon implantation at high pressure locations[22]. On average, patency rates after ten years tend to be high for IMA grafts but approximately 50% of SV grafts become occluded as a result of neo-intima formation[23]. The IMA is structurally more similar to the coronary artery than the SV (which tends to distend excessively resulting in failure[23-24]) and thus has a higher likelihood of remaining patent. In many patients (30-50%), however, the supply of viable autologous vessels may not be sufficient due to their use in prior surgeries[25-26]. Additionally, the graft vessel may be diseased, rendering it unusable, or may not match the compliance of the target artery[3, 4]. Autologous vessels

commonly become thrombogenic causing SMCs to proliferate and migrate to the intima resulting in restenosis and eventual graft failure[27]. Recent studies have shown that SV graft failure may be reduced up to 70% with the insertion of stent[28-29]. Donor site morbidity is another serious complication of using vein grafts. Although the option of using allografts (same species but different donor) and xenografts (different species) exists, it is accompanied by a high risk of immune rejection and pathogen transmission[15]. Moreover, allografts have been associated with EC sloughing and reaction with leukocytes, poor patency, rejection and a loss of vascular reactivity; thus, they are no longer in use for by-pass surgery[30]. Patients who do not have available autologous tissue may receive a prosthetic graft. Synthetic materials such as expanded polytetrafluoroethylene (ePTFE) , and polyethylene teraphthalate (PET), also known as Dacron, have seen some success in large-diameter vessel replacement but have major flaws that limit their use in small-diameter, low flow vessels, like the coronary artery. These include compliance mismatch between the graft and native artery (due to a lack of viscoelasticity in the synthetic material), intimal hyperplasia especially around the anastomoses (location of suture), risk of bacterial infection (the graft may harbor bacteria) and thrombus formation on the internal surface of the graft[30] (due to the lack of an endothelial lining)[31]. The continuous endothelial lining is an essential component of the graft as it provides a selectively permeable, anti-thrombotic barrier between the blood and artery wall by controlling platelet activation and adhesion[32], and leukocyte adhesion[33]. Additionally, ECs regulate the vascular tone[34] and SMC behaviour[35]. In larger artery replacements the endothelial lining is not as critical for long-term patency because the high blood flow rate does not allow platelets sufficient time to adhere to the graft. In

vessels such as the coronary artery however, the flow is markedly slower allowing immune cells to accumulate on the lumen of the graft[15]. Unfortunately, unlike what is observed in animal models, these grafts do not spontaneously endothelialize in humans[36]. *In vitro* seeding of ECs onto ePTFE grafts prior to use in coronary by-pass surgery has improved its long term patency to 91% at 2.5 years (for 4 mm diameter grafts) and 65% at 9 years (for 5 mm diameter grafts) compared with an average patency of 54% at 4 years for non-endothelialized grafts. However, this approach requires a long culture period (2-4 weeks) prior to implantation[27]. Synthetic grafts also require extensive use of anticoagulant/ antithrombotic therapy to suppress immune rejection, which can have negative side effects[15,30].

Several non-invasive therapeutic options have emerged in recent years for the treatment of atherosclerosis. These include immunosuppressive drugs such as cyclosporine and sirolimus (also used for coating stents that are implanted for the treatment of post-angioplasty restenosis) which block T-cell activation, smooth muscle cell proliferation and inhibit intimal lesions. However, their efficacy in treating coronary syndromes is unknown. Results from the use of vaccination with oxidized LDL, bacteria containing modified phospholipids, or heat-shock protein 60 have been encouraging but additional elucidation is needed before they can be tested in humans[17].

Although significant progress has been made in the quality of therapies available there are still many challenges to overcome. Whilst life expectancy among afflicted individuals has improved and mortality rates have decreased as a result of advancements

in therapeutic treatments, the burden of disease still exists and may actually have increased. CAD can develop into a chronic condition if the implanted graft does not remain patent and repeated failures occur. In addition to the psychological impact of the disease they endure, many of these individuals live a debilitating lifestyle and have difficulty performing menial tasks. Thus the need for an alternative vascular replacement is urgent and indisputable[37]. Tissue engineering is one such alternative with great potential for providing a source of replacement vessels[1].

2.5 Vascular Tissue Engineering

Over the last few decades tissue engineering has emerged to offer an alternative means of addressing the short comings of current therapies such as immune rejection and unavailability. Broadly defined, tissue engineering is "the application of principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue function"[38].

The most widely used paradigm of tissue engineering (shown in Figure 2.2) involves the seeding of cells onto a scaffold (of natural or synthetic composition), maturation of cell-scaffold constructs in a metabolically and mechanically supportive environment (bioreactor) and the subsequent implantation of the engineered tissues to the appropriate anatomical location.

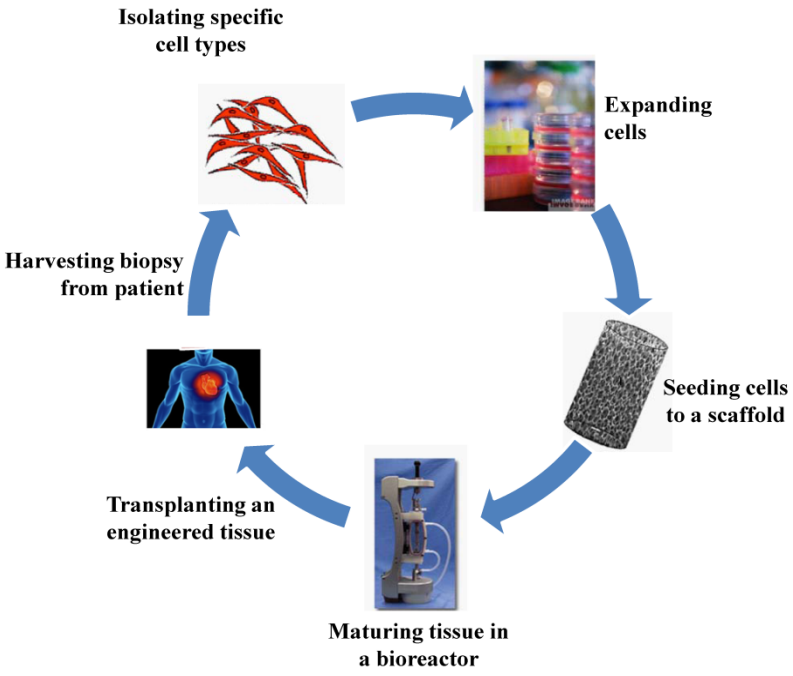

Figure 2.2: Tissue engineering paradigm showing the essential stages. Autologous cells are first harvested and expanded *in vitro*. This is followed by seeding of cells onto a natural or synthetic scaffold and maturation of the construct in a bioreactor. The engineered tissue is then implanted at the appropriate anatomical location.

Once implanted, further remodeling allows tissue integration with the host tissue[39]. The various elements for successful tissue engineering are discussed below.

2.5.1 Cell Source

A variety of primary cell sources can be used for creating engineered tissues. Each has limitations and risks but can also offer unique advantages. Autologous cells are those that are harvested from the same patient into which the final tissue will be implanted. The advantage of using autologous cells is that they minimize the risk of inciting an adverse host immune response as well as the risk of disease transmission[40].

The drawback is that these cells (especially from elderly patients) may be unavailable, have low proliferative capacity, require long expansion periods and have high variability[39]. Allogeneic cells are harvested from the same species but a different donor while xenogeneic cells come from a different species altogether. Both allogeneic and xenogeneic cells offer the advantage of creating engineered tissue banks that eliminate waiting times; however, their use is complicated by the risk of disease transmission and adverse host immune response[40-41]. Other potential sources of cells are adult stem cells, bone marrow stromal cells, bone marrow-dervied circulating stem cells and pluripotent embryonic stem cells[39].

2.5.2 Scaffolds

Scaffolds are used to provide a 3D template to guide tissue generation and mimic the functions of native ECM. Because most primary cells are anchorage-dependant, they require a supporting material for survival and growth[42]. Scaffolds support cell adhesion, migration, proliferation, differentiation and contribute to the initial and/or final mechanical properties of the tissue. It has been shown that scaffold composition, degradation and architecture influence cell behaviour[4,43]. Scaffolds may be composed of natural or synthetic materials. Synthetic materials can be functionalized with bioactive ligands to deliver growth factors and signals or to direct 3D cell orientation. Upon implantation, if the scaffold is biostable, it also facilitates spatial arrangement of cells under aggressive *in vivo* conditions and contributes to the mechanical properties of the graft. Desirable characteristics of a scaffold are biocompatibility and biodegradability. Ideally, the scaffold should degrade at a rate that does not compromise the function of the

tissue while cells simultaneously go through the process of creating their own ECM to replace the scaffold[44]. However, commonly used biodegradable polymers (poly(L-lactic acid) (PLLA) and poly(glycolic acid) (PGA)) for vascular tissue engineering tend to generate acidic degradation products[45-46] that adversely affect VSMC behaviour[47].

2.5.3 The Role of Bioreactors

Bioreactors play an important role in the maturation of engineered tissue. They supply the growing tissue with the necessary biochemical stimuli, such as oxygen and growth factors, and mechanical stimuli, such as tension, compression and shear, to mimic *in vivo* conditions. In the context of vascular tissue engineering this is a critical stage that promotes uniform cell distribution[48], alignment, proliferation and secretion of ECM components. Such stresses have been shown to improve the structural and mechanical properties of engineered tissues[39].

2.5.4 Implantation and Remodeling

Remodeling by the host is often viewed as essential and beneficial stage of tissue engineering[49]. Upon implantation, even autogolous constructs will induce immune reactions and undergo remodeling[50]. This process can affect the functioning of the construct and may facilitate its successful integration into the surrounding tissue[44]. The degree to which a tissue engineered vascular grafts is remodeled is of critical importance. Excessive remodeling is undesirable as it can lead to intimal hyperplasia and restenosis after grafting[4].

2.5.5 Applications of Tissue Engineering

Tissue engineering allows for the fabrication of tailored blood vessels that respond to both mechanical and biological cues allowing them to easily acclimate to the native hemodynamic environment. They also have the ability to remodel, grow and repair, making them appealing for use in pediatric patients[51-52]. Apart from the obvious application of engineered tissues as replacement vessels, they can also serve as tools for studying normal physiological processes and pathogenesis of diseases. Furthermore, they can allow us to study the responses of tissue to injury, the toxicity of drugs on tissues and contractile responses in vascular tissues, enhance cell-based drug discovery and study interactions between cells and between cells and their surrounding matrix. Some examples of tissue engineered products that are currently in use or in development include: replacement skin, biohybrid extracorporeal artificial organs, cartilage regeneration using autolougous chondrocyte transplantation and a replacement thumb[39]. This shows the great potential of tissue engineering and the promise it holds as an alternative treatment modality.

2.6 Missing Elements of Current TEVGs

Despite the rapid progress that has been made in the field of vascular tissue engineering there are still several factors limiting graft function. Unlike other tissue engineering approaches, vascular grafts cannot rely on *in vivo* remodeling to approach functionality with time. They are designed to be implanted into demanding and dynamic environments and so must function immediately upon implantation. Some of the

requirements of engineered vascular grafts include: appropriate burst strength to withstand changes in blood pressure, matching compliance to the adjacent vessel, appropriate elasticity to withstand cyclic loading and a nonthrombogenic lining[1]. Many of these mechanical properties are imparted by the ECM proteins collagen and elastin. Indeed, a critical factor in the failure of current engineered conduits is a lack of these proteins. Furthermore, misalignment of SMCs in the longitudinal direction and low SMC numbers have also been identified as limiting factors of graft performance. These problems can essentially be resolved with proper regulation of SMC phenotype[4]. For this reason, a key research area in the fabrication of small diameter blood vessels is the plasticity of smooth muscle cells[30,53]. Both the synthetic and contractile phenotypes of VSMCs are needed for vascular tissue engineering in order to produce a graft that emulates the *in vivo* composition, morphology and function[4]. The following section elaborates on vascular smooth muscle cell modulation with accompanying literature.

2.7 VSMC Phenotype and Relevant Co-culture Studies

2.7.1 VSMC Phenotype

Despite the urgent need for a tissue engineered blood vessel, there are still several outstanding issues to resolve. Among these is the regulation of VSMC phenotype in 3D culture. VSMCs are known to exhibit remarkable plasticity in their native *in vivo* environment. One of the challenges in understanding the differentiation process that they undergo is that SMCs are present in a distinct synthetic and contractile phenotype as well

as a wide spectrum of phenotypes in between. These phenotypes are reversible and are a function of changing local environmental cues[4,54-55]. For example, during vasculogenesis SMCs are highly proliferative and migratory and lay down an abundance of ECM molecules such as collagen, elastin, proteoglycans, cadherins and integrins which constitute a majority of the blood vessel's composition[54]. A critical step in vasculogenesis is the formation of gap junctions with ECs; these junctions play a major role in vessel maturation and remodeling[56]. Mature SMCs on the other hand acquire a contractile phenotype that is marked by a very low proliferative index, extremely low synthetic activity and expression of an array of contractile proteins, receptors, ion channels, calcium regulatory proteins and signaling molecules that are essential to the contractile activity of the cells[57]. Smooth muscle α-actin (SM-α-actin), smooth muscle myosin heavy chain (MHC), smoothelin-B, h-caldesmon and calponin are among the cytoskeletal proteins expressed by these cells[58]. The principle function of mature SMCs is the regulation of vessel tone-diameter, blood pressure and blood flow distribution[54]. The characteristic morphology of contractile VSMCs is spindle-like. This allows them to pack together into dense, circumferentially aligned layers that can translate individual contractions and dilations into macroscopic ones[4]. A unique characteristic of SMCs is that unlike skeletal or cardiac muscle cells, they are non-terminally differentiated, that is they have the ability to shift between phenotypes. Therefore, although these cells perform predominantly contractile functions they can easily revert to the synthetic phenotype if exposed to certain biochemical or biomechanical stimuli[56,58]. The transition to a synthetic phenotype involves suppression of genes that encode for contractile marker proteins and a pronounced increase in expression of genes that promote proliferation and matrix

turnover such as metalloproteinases and l-caldesmon[54,59]. The synthetic phenotype of mature SMCs is somewhat akin to the embryonic and fetal vascular smooth muscle phenotype[60-61]. Morphologically, cells in the synthetic phenotype resemble fibroblasts, have an extensive endoplasmic reticulum and few myofilaments. Furthermore, actin is present predominantly in the non-muscle p-form[62]. It is thought that the plasticity of SMCs has evolved as a survival mechanism and that mutations that hampered the ability of these cells to participate in vascular repair would have been detrimental and eventually diminished[54-55]. There are however, negative consequences to this, since SMCs will adversely switch phenotypes in the presence of abnormal environmental cues. De-differentiation (shifting to synthetic phenotype) plays a key role in the development and/or progression of several diseases such as cancer, hypertension, and most notably atherosclerosis[54]. The phenotypic characteristics of VSMCs are illustrated in Figure 2.3 below.

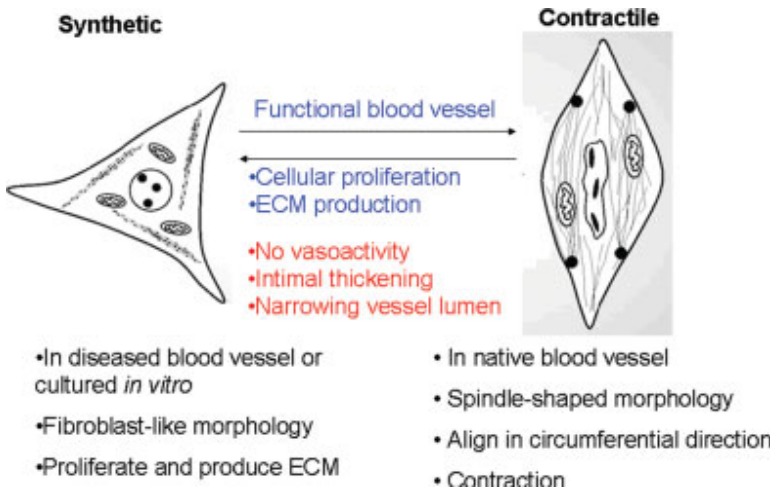

Figure 2.3: Vascular smooth muscle morphology in physiological and pathological states[4].

2.7.2 VSMC Phenotype Modulation in TEVGs

The phenotype shifting property of VSMCs is considered of paramount importance in the construction of a functioning vasculature. Initially, the synthetic phenotype is desired to enable the cells to expand rapidly in culture. This would in turn promote infiltration and population of the scaffold by the VSMCs to create dense cell layers. This proliferation phase would also result in the secretion of appropriate ECM components to provide the tissue engineered vessel with sufficient mechanical properties and to provide a substrate for EC adhesion. Once the desired population of cells is achieved, the cells must then shift to a quiescent contractile phenotype to impart the vasoactive properties found in native blood vessels. Furthermore, the VSMCs must organize themselves circumferentially in order to constrict and dilate the construct in the necessary direction and to improve the mechanical properties[4]. Failure of the cells to differentiate would, upon implantation, result in the onset of intimal hyperplasia and restenosis, resulting in immediate graft failure[58]. It has been noted that significant causes of graft failure are misalignment of SMCs in the longitudinal direction, insufficient amounts of collagen, elastin and SMCs, all of which can be manipulated through the strategic regulation of VSMC phenotype[4]. Premature graft implantation causes SMCs to continually proliferate, thereby thickening the vessel wall and narrowing the lumen. VSMC phenotype switching from synthetic to contractile is considered crucial for the manufacture of a successful TEVG[4]. The tissue engineering strategies that are currently employed do not take advantage of this unique property to fabricate a blood vessel with a vascular media that mimics that of native arteries[30,53]. However, it is thought that manipulation of SMC phenotype at the appropriate maturation stage would be greatly

beneficial in creating a blood vessel substitute that more closely resembles native arteries in both organization and function[63-64]. For this reason, many investigators have studied the various factors that are involved in phenotype modulation of VSMCs.

2.7.3 Factors Influencing VSMC Phenotype

A variety of factors have been shown to influence the behavior of VSMCs including ECM components, growth factors, mechanical conditions and cell-cell interactions. It has also been observed that the initial phenotype of SMCs affects their subsequent response to the above mentioned stimuli[65]. Furthermore, it has been observed that SMCs respond to some of these stimuli differently depending on whether they are grown in a two-dimensional (2D) environment versus a 3D environment.

2.7.3.1 2D Substrates

SMCs in healthy arteries exist in the contractile phenotype, but when harvested and grown on 2D culture dishes, they lose their contractility[55,66]. The loss of myofilaments occurs in parallel with the development of a large Glogi complex, rough endoplasmic reticulum and the secretion of ECM components. The expression of genes that promote α-actin production are downregulated while those that code for growth factors and their receptors are upregulated[67]. Collectively the introduction of SMCs into an *in vitro* culture environment results in a shift from a contractile to a synthetic phenotype over a period of days[62].

2.7.3.1.1 ECM & Growth Factors

As mentioned previously, SMCs in the native artery are embedded in a 3D matrix of fibrous ECM molecules. These proteins participate in the transduction of signals between the cell layers to regulate the adhesion, proliferation, migration and gene expression of the SMCs by way of transmembrane receptors known as integrins[15,68]. Thus, a host of *in vitro* studies have aimed to elucidate the specific effects of some of the major ECM proteins. Several of these studies have shown that the presence of fibronectin (FN) and type I collagen cause smooth muscle cells to shift to a synthetic phenotype[4,69] while other proteins such as laminin (LN), heparin, elastin and type IV collagen have the reverse effect, that is, they cause the cells to shift to a quiescent contractile phenotype[4,70]. It has also been shown that growth factors like basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF- β1) and PDGF have regulatory effects on SMC phenotype; the effects of the latter two being the most profound. TGF-β1 seems to promote the migration of SMCs[71-72] and increases ECM production[73] but generally suppresses proliferation[4]. However, as mentioned previously, the initial phenotype of VSMCs can also dictate their response to certain stimuli. With respect to TFG-β1, rat aortic SMCs grown in its presence become less proliferative when the initial phenotype is a sub-confluent synthetic state. On the other hand, when confluent contractile cells are cultured with TGF-β1 they respond with a transition to the synthetic state, simulating hypertrophy[61]. Basic fibroblast growth factor bound to heparin sulfate proteoglycan has been found to upregulate SMC proliferation[56]; while PDGF has more of a chemotactic effect and indicates a shift to the synthetic phenotype by upregulating migration,

proliferation and ECM production[74]. LN, a basement membrane protein, has been shown to retard the transformation of SMCs to a synthetic phenotype and maintains the contractile phenotype for a long period of time upon initiation of *in vitro* culture (immediately after harvest)[75]. A study conducted by Li et al.[62] tested the effects of Matrigel on smooth muscle cell phenotype. Matrigel is a solubilized basement membrane matrix whose primary component is LN[62]. In the study, rat aortic SMCs were cultured on plastic culture dishes (control) and on Matrigel coated culture dishes. The result was an induction of the contractile morphology in cells grown on Matrigel compared to the control group. Furthermore, cells grown on Matrigel had a low proliferative index, increased expression of SM-α-actin and MHC, and finally, they exhibited increased responsiveness to vasoconstrictors. Several studies have demonstrated a relationship between serum content in growth media and VSMC phenotype[76-82]. In the absence of serum or in very low serum concentrations it seems that SMCs shift from a synthetic to a contractile phenotype. Although this practice may be of use in *in vitro* cell culture studies, it cannot be adopted for tissue engineering procedures. Since the end goal is to introduce the tissue engineered product into the human body, the abundance of serum may undesirably trigger a pathological response (high synthetic activity). One of the challenges in studying the effects is that these various locally produced growth factors and matrix molecules tend to interact with each other to have a coordinated effect on the smooth muscle and their effects tend to be interdependent[4,74,83]. It should also be noted that these studies were conducted in a 2D environment and in serum-free conditions which do not accurately emulate the *in vivo* environment.

2.7.3.2 3D Substrates

2.7.3.2.1 ECM & Growth Factors

Several studies by Mequanint and co-workers have examined the effect of scaffold surface modification with matrix proteins on VSMC behavior. Matrigel-coated scaffolds, compared with bare scaffolds, promoted cell migration throughout the scaffold depth and resulted in cells that were aligned in parallel to each other with rich F-actin bundles, suggesting a differentiated phenotype[84]. A subsequent study comparing FN-coated scaffolds with Matrigel-coated and bare scaffolds revealed that FN promoted cell infiltration to a greater extent than did Matrigel[45]. However, it was found that coating scaffolds with matrix molecules had no effect on contractile marker expression. Another study showed that polyurethane scaffolds conjugated with full length FN molecules displayed favourable interactions with human coronary artery smooth muscle cells (HCASMCs)[85]. Compared with the bare scaffold, the FN-functionalized scaffolds promoted cell infiltration. FN, as discussed earlier, induces the synthetic phenotype in SMCs. Therefore, fibronectin conjugated scaffolds have the potential to maintain SMCs in a de-differentiated phenotype in order to produce the necessary cell numbers and ECM components required of TEVGs. In a recent study, Baker and Southgate showed that LN pre-coating of electrospun polystyrene scaffolds promotes cell differentiation to a contractile phenotype[86]. As part of the same study, they found that the presence of serum proteins in the culture medium prevented differentiation of the cells on bare scaffolds. Upon removal of serum from the growth media, it was found that cells were no longer able to adhere to the scaffold. Therefore, in tissue engineering of blood vessels, which rely on the use of 3D scaffolds, serum withdrawal is not applicable.

2.7.3.2.2 Scaffold Geometry

Another known regulator of SMC phenotype is the geometry of scaffolds used in the engineering of vessels. Scaffolds aid in maintaining the spatial arrangement of cells, provide mechanical strength and can also provide biological cues to guide the development of the tissue. It has been shown that scaffold geometry can be used to regulate various processes in SMCs, including: proliferation, migration, attachment to the scaffold and other cells, differentiation and apoptosis[43]. Micropatterning is one such method used to design the scaffold architecture in such a way as to precisely control the behavior of cells. This is achieved through the addition of biological or chemical molecules and topographical features onto the scaffold surface. Micropatterned surfaces provide the advantage of replicating the dimensions and composition of the native *in vivo* environment. Studies have shown that patterning of substrates has resulted in the organization and alignment of SMCs into the contractile morphology[4]. For instance Goessl et al.[87] used lithography while Ra et al.[88] patterned a substrate with collagen to induce the spindle-like morphology. Problems associated with these studies include a restriction or inhibition of cell proliferation resulting from the narrow microchannels, and a large area of the substrate that is unavailable for cell growth. Furthermore, these studies focused on cell alignment but failed to show a phenotype shift from synthetic to contractile at the appropriate vessel maturation stage[4].

2.7.3.2.3 Mechanical Stimulation

There are a variety of biomechanical stimuli in the native artery that make it an incredibly dynamic environment. For example, longitudinal blood flow through the

vessel exerts shear stress on the wall, while cyclic pressure variations due to blood flow exert radial and longitudinal forces. Furthermore, the cells of the vasculature respond to the constant stresses and strains and produce forces arising from vasoactivity. Of these, it has been shown that cyclic stretching has the strongest correlation to SMC phenotype generally resulting in increased ECM matrix secretion, cell proliferation, growth factor production and contractile activity[4]. In 2D culture, experiments have shown a relationship between cyclic strain and cell alignment. Mills et al.[89] demonstrated the dependence of SMC alignment on the amount of cyclic strain. High strain (7-24%) was shown to align cells perpendicular to the strain gradient whereas low strains (0-7%) failed to organize the randomly aligned cells. Various other studies have demonstrated a mitogenic effect of mechanical strain and shear stress on SMC behavior through expression and activation of PDGF[90-92]. Wilson et al.[93] found that cyclic strain (60 cycles/ min) induced SMC proliferation via production of PDGF. In the fabrication of vascular tissues, it has been demonstrated that the mechanical properties of the engineered vessel as well as cellular organization may be improved through dynamic mechanical conditioning. In a study by Niklason et al.[94], pulsatile flow (5% radial distension at 165 pulses per minute) induced the desirable alignment of SMCs and production of large amounts of ECM proteins. Eight weeks of exposure resulted in high values for burst strength. Mooney and Mikos showed that cyclic strain improves histological organization and increased production of elastin and collagen, both of which contributed to an improvement in mechanical properties[95]. Seliktar et al.[96] showed that engineered vessels consisting of rat aortic SMCs exposed to 10% cyclic strain at 1 Hz for 8 days resulted in even distribution of and circumferential orientation of cells as compared with static cultures. These responses are

further dependant on the particular substrate on which the cells are grown. For example, SMCs cultured (under cyclic mechanical strain) on FN or vitronectin were more proliferative and secreted ECM molecules in comparison to cells cultured on collagen or LN[97]. As promising as these results are, they also have drawbacks including long conditioning periods which makes them impractical for clinical use and the lack of formation of elastic laminae which contributes to irreversible creep in the graft vessel. In addition, mechanical stimulation promotes the expression and activity of matrix metalloproteinase-2 (responsible for the degradation of short collagens) which would compromise the structure of the matrix. Furthermore, if mechanical conditioning is the only stimuli used to maintain the SMC organization up until implantation of the vessel, then risk of EC detachment poses another problem[4].

2.7.3.3 The Role of ECs to Regulate VSMC Phenotype

ECs reside in close proximity to SMCs in the blood vessel and play an extensive role in coordinating SMC behavior through the production of growth factors, stimulators and inhibitors[4]. For instance, during blood vessel formation, ECs secrete PDGF and TGF – β1 to recruit VSMCs or pericytes to the newly formed endothelial tube. In mature arteries, the endothelium maintains homeostasis through the production of vasoactive agents such as eicosanoids. In particular, prostacyclin has been shown to maintain SMCs in a quiescent, contractile state in which they react to vasoactive stimuli but not to mitogens[98]. Heparin is another endothelium derived molecule that maintains the vascular media in a contractile phenotype while inhibiting proliferation and migration[69]. Much of what we currently know about the interactions between ECs and SMCs has been derived

from the study of vascular pathologies. In fact, atherosclerosis is initiated through a mechanical disruption in the endothelial monolayer[15]. The disrupted endothelium is no longer quiescent and becomes immunogenic. The subsequent cascade that is initiated results in the migration and proliferation of SMCs to the intima. Therefore, a quiescent endothelium is of critical importance in tissue engineered vascular grafts so as to not provoke a pathological SMC phenotype upon implantation into the body. Various groups have developed co-culture models to examine the interplay between these vascular cells. These include: conditioned media, co-culture on opposite sides of a porous membrane, microcarrier techniques, direct co-culture, and co-culture on a 3D collagen gel, which are summarized below.

2.7.3.3.1 Conditioned Media

It has been reported that medium conditioned by ECs in various stages of growth has an effect on smooth muscle phenotype, the results have however been mixed[99-100]. Unlike proliferating ECs, cells that are organized in a quiescent confluent layer produce heparin-like substances which have been shown to inhibit SMC proliferation[99]. A study conducted by Campbell and Campbell concluded that conditioned media from quiescent confluent endothelial cells shifted the SMCs into a contractile phenotype, whereas conditioned media from proliferating ECs did not suppress SMC proliferation[99]. On the other hand, Fillinger et al.[100] found that conditioned media from quiescent confluent ECs actually increased SMC proliferation. Other investigators observed no significant changes in VSMC phenotype in culture with EC-conditioned media[101-102]. The results appear to be mixed and the response of the SMCs appears to be dependent on culture duration[103-107].

2.7.3.3.2 Co-culture on Opposite Sides of a Membrane

Fillinger et al.[108] constructed a co-culture model by seeding ECs and SMCs on opposite sides of a 13μm thick PET membrane. They found that a confluent layer of ECs increased SMC proliferation initially but after 4 days seemed to inhibit cell growth. Additionally, the SMCs in co-culture displayed a spindle-like morphology. Van Buul-Wortelboer et al.[107] used collagen gel to separate the two cell layers in culture and found that in the presence of ECs, SMCs had lower proliferative activity. A study by Jacot and Wong showed that endothelium, once injured and allowed to partially re-endothelialize, induced a highly proliferative state in SMCs cultured on the opposite side of the same membrane compared with uninjured endothelium in co-culture[109]. The regulation of SMC behavior was attributed to PDGF-BB. Other studies[110-111] altered serum concentration to induce the synthetic or contractile phenotype in SMCs and then tested its effect on EC or SMC gene expression or growth. Yet others[112-113] used shear stress in a co-culture model on opposite sides of a membrane to elucidate its combined effect (with SMCs) on EC gene expression. In the native artery the internal elastic laminae, which separates the intimal layer (containing ECs) from the medial layer (containing SMCs), has a thickness ranging from 0.2 to 2 μm. Direct contact between the two cell layers is facilitated by fenestrations in the lamina of 1.5 μm depth. Therefore, using porous membranes in *in vitro* culture creates inaccurate models of native arterial structure. The large gap created by these membranes (10 microns or greater) may restrict diffusion of EC-produced molecules and gap junction formation between the ECs and SMCs[114]. Furthermore, the use of a synthetic membrane is not applicable in the engineering of blood vessels[115].

2.7.3.3.3 Microcarrier Techniques

Microcarrier techniques rely on use of small spheres/ spheroids as the adherent surface for anchorage-dependent cells. They are employed in order to achieve high yields of cells as they provide a much larger surface area for monolayer formation compared with standard culture dishes[116]. Korff et al.[117] used a microcarrier system to construct co-cultures of ECs and SMCs. They found that cells spontaneously organized into layers resembling that of an artery wall. However, as in many other co-culture systems, they only assessed the effect of SMCs on EC phenotype (proliferative vs. quiescent). Davies et al.[118] used microcarrier techniques to study the influence of ECs on LDL metabolism by SMCs. These models, though important for investigating vascular cell interactions, are not viable from a tissue engineering perspective. A tissue-engineered blood vessel must have a tubular rather than spherical geometry in order to integrate into the surrounding tissue and mimic the native vessel functions.

2.7.3.3.4 Direct Co-culture

Niwa et al.[119] compared the uptake of LDL and acetylated LDL by ECs in monoculture versus ECs in co-culture with SMCs. Co-culture increased the uptake of LDL but had no effect on uptake of acetylated LDL. Additionally, shear flow had a more pronounced effect on acetylated LDL uptake as compared with LDL uptake. In a study more relevant to this project, Hirschi et al.[120] cultured ECs with multipotent embryonic 10T1/2 cells (mural cell precursors). They demonstrated that co-culture induced a morphological shift from polygonal to spindle-like shape. Additionally, several contractile protein markers (MHC, smooth muscle 22α and calponin) were upregulated in

co-culture compared with monocultured 10T1/2 cells. The differentiation process was shown to be mediated by TGF-β. Similar results were observed in another study with ECs and 10T1/1 cells[121]. It has also been shown that EC attachment onto SMCs can be enhanced through the addition of ascorbic acid to increase collagen production by SMCs[122]. A co-culture study by Chaterji et al.[123] demonstrated the influence of EC seeding density on SMC phenotype; culturing SMCs with near-confluent ECs upregulated the expression of SM-α-actin and calponin. Expression levels were further upregulated by pretreatment of SMCs with heparin and TGF-β. On the other hand, SMCs cultured with sparse amounts of ECs displayed a hyperplastic phenotype. Heydarkhan-Hagvall et al.[124] applied shear stress to a direct co-culture model to determine which genes were differentially expressed. The major finding was that co-culture is a significant factor in modulating EC responses to shear stress. A series of studies conducted by Truskey and colleagues have used direct co-culture models to observe a variety of behaviors. However, in all of these studies they induced a quiescent SMC phenotype through serum withdrawal and examined primarily the behavior of ECs. These include: the adhesiveness of ECs on SMCs[115,125] and molecules mediating adhesion[126], the maintenance of a confluent endothelium under flow conditions, the effect of co-culture on EC response to TNF-α[127] and the expression of tissue factor by ECs in co-culture[128]. It is important to note that all of these studies were performed on a 2D substrate. The major drawbacks of using 2D co-culture models are that they ignore hemodynamic forces by using static culture conditions or they ignore the effect of 3D geometry. A requirement of tissue-engineered vessels is that they must be in the form of a 3D cellular construct. Thus,

although data from 2D cultures can be used as a starting point, they may not truly reflect cell behavior on 3D substrates.

2.7.3.3.5 Co-culture on 3D Collagen Gels

An investigation by Ziegler et al.[129] involved the construction of a co-culture system on 3D collagen gels. Porcine SMCs were embedded in a gel of collagen type 1 over which ECs were seeded. The ECs exhibited an elongated shape and random orientation. Upon exposure to laminar shear stress the ECs aligned to the direction of flow. Additionally, the collagen matrix was significant in maintaining the ECs in a quiescent phenotype (as compared with plastic culture dishes that promoted proliferation). Similarly, Imberti et al.[130] constructed a similar model of SMCs imbedded in a collagen matrix with an overlying layer of ECs. They also noticed that shear stress induced alignment of ECs in the direction of flow, as well as a quiescent endothelium that was attributed to the shear stress and collagen matrix. Neither of these studies, however, observed interactions between ECs and SMCs.

Apart from the aforementioned studies, several investigators have carried out co-cultures on 3D tubular scaffolds under dynamic growth conditions. Crouchley et al.[131] used a tubular silicone scaffold for the sequential seeding of SMCs and ECs. Application of mechanical stimulation resulted in the circumferential alignment of SMCs and was accompanied by an increase in contractile protein expression. Cells were also able to retain this phenotype after 24 hours of mechanical stimulation in co-culture. It should be noted that serum-withdrawal was used to induce contractility in SMCs. Williams and Wick sutured a biodegradable PGA nonwoven felt into a tubular construct that was

subsequently seeded with SMCs and ECs[132]. Long-term culture versus short-term culture in a bioreactor was compared. SMCs from long-term culture exhibited a more contractile phenotype and increased levels of proliferation compared with cells from short-term culture. ECM deposition had also decreased and was more uniform in long-term cultured constructs. Pullens et al.[133] conducted a study in which myofibroblasts and ECs were cultured on 3D rectangular PGA and poly-4-hydroxybutyrate scaffolds for one or two weeks to assess the impact of culture medium composition and ECs on ECM deposition. Results indicated that EC culture medium and the presence of ECs in co-culture decreased collagen content in the scaffolds.

In summary, these models have been important in elucidating the numerous interactions between ECs and SMCs but have notable drawbacks. 2D models fail to take into account the effect of geometry or hemodynamic forces observed *in vivo*. Furthermore, the use of membrane separated co-culture system cannot be applied to tissue engineering. Many of these studies also focused on the EC gene expression in co-culture or the production of ECM proteins in 3D cultures. Recent reports have emerged that document an induction of the contractile phenotype in VSMCs by VECs[120,134-136]. The underlying mechanisms are only now beginning to emerge from 2D co-culture and gene knockout experiments[137-139]. Mechanisms that have been shown to play a role in this modulation are the PI3-kinase/Akt pathway[140], the protein kinase A pathway via prostacyclin receptor[141] and the Notch signaling pathway via Jagged1 ligand[101,138,142-146]. Due to the relevance of Notch signaling to this thesis, a brief review is presented.

The Notch signaling pathway is an evolutionarily conserved pathway that dictates cell fate through local cell-cell interactions. These interactions are critical in the control of cell proliferation, differentiation and apoptosis[143]. Components of the Notch signaling pathway include the Notch family of transmembrane receptors (Notch1 to 4), the Delta, Serrate/Jagged, Lag-2 family of transmembrane ligands (Delta like1, 3 and 4 and Jagged1 and 2), and the various effectors from the hairy enhancer of split (HES) and HES-related repressor protein (HERP) family[143, 144]. The distribution of Notch signaling components within vertebrates varies significantly throughout tissues and during development. However, data suggests that several of these are confined to the vasculature including the ligands Delta like4, Jagged1 and 2, the receptors Notch1, 3 and 4 and the effectors HERP1, 2 and 3[143]. Interaction of the extracellular domain of the Notch receptors with their ligands on neighbouring cells leads to proteolytic cleavage of the receptor thereby freeing the Notch intracellular domain (ICD). Once the ICD translocates to the nucleus, it associates with C-promoter-binding factor-1 to form a multiprotein complex that initiates DNA transcription of the Notch effector genes (HES and HERP) [151]. Notch signaling plays an important role in vascular development and in the pathogenesis of vascular diseases. In VSMCs in particular, Notch activity regulates cell differentiation, proliferation, migration and survival[144, 152-153]. Notch3 is the primary receptor that is expressed by VSMCs and its ligand Jagged1 is predominantly expressed by VECs[143, 101, 154-155]. Impaired activity between Notch3 and Jagged1 has been shown to induce pathologies such as cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and Alagille syndrome in humans[143]. Further data supporting the importance of Notch signaling in vasculature comes from experiments

involving the knockout of Jagged1 in mice which resulted in the absence of smooth muscle gene expression giving rise to an embryonic lethal phenotype[156-157]. Based on this evidence, the role of VEC-induced VSMC phenotype modulation via the Notch signaling pathway will be a focus of this study.

2.8 Study Rationale and Objectives

The shortcomings of CAD interventions have actuated a venture into the emerging field of vascular tissue engineering. More specifically, investigations undertaken by our laboratory have focused on the development of novel materials for use as vascular scaffolds and more recently on the interactions between VSMCs and 3D substrates. The failure of small-diameter vascular substitutes consisting of only a subset of the arterial components has spurred investigations of issues like VSMC phenotype regulation. It has been widely acknowledged that strategic control of VSMC phenotype at the appropriate tissue maturation stage would be greatly beneficial in creating a physiologically compliant vascular graft. Studies have shown that VECs play a key role in the modulation of VSMC phenotype. Investigation of VEC-induced VSMC phenotype modulation is therefore beneficial for the fabrication of TEVGs. In view of this, the purpose of this study was to characterize the interactions between HCASMCs and human coronary artery endothelial cells (HCAECs) on a 3D scaffold.

In particular, we have chosen to study primary human coronary artery vascular smooth muscle and endothelial cells. Given that there are major functional differences

between vascular cells from different vascular beds[147-148] and from different species[149], the use of adult human coronary artery vascular cells best emulates the expected behavior of autologous cells[150].

We hypothesize that HCAECs will modulate the phenotype of HCASMCs from a synthetic to a contractile phenotype via the Notch signaling pathway when cultured on a 3D substrate of PCU. To test this, we have set out the following specific objectives.

i) Co-culture HCASMCs and HCAECs on porous 3D PCU scaffolds in distinct layers mimicking the native artery wall.
ii) Determine if the presence of HCAECs has an effect on HCASMC phenotype through the examination of differentiation markers.
iii) Elucidate whether any observed modulation of phenotype is mediated by Notch 3-Jagged1 interactions.

2.9 References

1. Ratcliffe, A., Tissue engineering of vascular grafts. *Matrix Biol* **2000,** 19, (4), 353-7.
2. Patel, A.; Fine, B.; Sandig, M.; Mequanint, K., Elastin biosynthesis: The missing link in tissue-engineered blood vessels. *Cardiovasc Res* **2006,** 71, (1), 40-9.
3. Bouten, C. V.; Dankers, P. Y.; Driessen-Mol, A.; Pedron, S.; Brizard, A. M.; Baaijens, F. P., Substrates for cardiovascular tissue engineering. *Adv Drug Deliver Rev* **2011,** 63, (4-5), 221-41.
4. Chan-Park, M. B.; Shen, J. Y.; Cao, Y.; Xiong, Y.; Liu, Y.; Rayatpisheh, S.; Kang, G. C.; Greisler, H. P., Biomimetic control of vascular smooth muscle cell morphology and phenotype for functional tissue-engineered small-diameter blood vessels. *J Biomed Mater Res-A* **2009,** 88, (4), 1104-21.

5. Waterhouse, A.; Wise, S. G.; Ng, M. K. C.; Weiss, A. S., Elastin as a Nonthrombogenic Biomaterial. *Tissue Eng Pt B-Rev* **2011,** 17, (2), 93-99.

6. Heyligers, J. M.; Arts, C. H.; Verhagen, H. J.; de Groot, P. G.; Moll, F. L., Improving small-diameter vascular grafts: from the application of an endothelial cell lining to the construction of a tissue-engineered blood vessel. *Ann Vasc Surg* **2005,** 19, (3), 448-56.

7. Mitchell, S. L.; Niklason, L. E., Requirements for growing tissue-engineered vascular grafts. *Cardiovasc Pathol* **2003,** 12, (2), 59-64.

8. Keller, R., Endothelial proteoheparan sulfates as components of the non-adhesive luminal vascular surface and the subendothelial matrix In *Endothelialization of Vascular Grafts*, Zilla P, F. R., Callow A, Ed. 1987; pp 106-111.

9. Farand, P.; Garon, A.; Plante, G. E., Structure of large arteries: orientation of elastin in rabbit aortic internal elastic lamina and in the elastic lamellae of aortic media. *Microvasc Res* **2007,** 73, (2), 95-9.

10. van Andel, C. J.; Pistecky, P. V.; Borst, C., Mechanical properties of porcine and human arteries: implications for coronary anastomotic connectors. *Ann Thorac Surg* **2003,** 76, (1), 58-64; discussion 64-5.

11. Soulis, J. V.; Farmakis, T. M.; Giannoglou, G. D.; Louridas, G. E., Wall shear stress in normal left coronary artery tree. *J Biomech* **2006,** 39, (4), 742-9.

12. Fawcett, D. W., *Bloom and Fawcett: A Textbook of Histology*. A Hodder Arnold Publication: 1997.

13. Mendis, S.; Puska, P.; Norrving, B., Global Atlas on Cardiovascular Disease Prevention and Control. In World Health Organization: Geneva, 2011.

14. Murray, C. J. L.; Lopez, A. D., Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *Lancet* **1997,** 349, (9063), 1436-1442.

15. Parizek, M.; Novotna, K.; Bacakova, L., The Role of Smooth Muscle Cells in Vessel Wall Pathophysiology and Reconstruction Using Bioactive Synthetic Polymers. *Physiological Res* **2011,** 60, (3), 419-437.

16. Hallenbeck, J. M.; Hansson, G. K.; Becker, K. J., Immunology of ischemic vascular disease: plaque to attack. *Trends Immunol* **2005,** 26, (10), 550-556.

17. Hansson, G. K., Inflammation, atherosclerosis, and coronary artery disease. *New Engl J Med* **2005,** 352, (16), 1685-95.

18. Hansson, G. K.; Robertson, A. K.; Soderberg-Naucler, C., Inflammation and atherosclerosis. *Annu Rev Pathol-Mech* **2006,** 1, 297-329.

19. Seneviratne, A. N.; Sivagurunathan, B.; Monaco, C., Toll-like receptors and macrophage activation in atherosclerosis. *Clin Chim Acta* **2012,** 413, (1-2), 3-14.

20. Caplice, N. M.; Bunch, T. J.; Stalboerger, P. G.; Wang, S. H.; Simper, D.; Miller, D. V.; Russell, S. J.; Litzow, M. R.; Edwards, W. D., Smooth muscle cells in

human coronary atherosclerosis can originate from cells administered at marrow transplantation. *P Natl Acad Sci USA* **2003,** 100, (8), 4754-4759.

21. Ross, R., Atherosclerosis--an inflammatory disease. *New Engl J Med* **1999,** 340, (2), 115-26.

22. Motwani, J. G.; Topol, E. J., Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. *Circulation* **1998,** 97, (9), 916-31.

23. Rashid, S. T.; Salacinski, H. J.; Fuller, B. J.; Hamilton, G.; Seifalian, A. M., Engineering of bypass conduits to improve patency. *Cell Proliferat* **2004,** 37, (5), 351-66.

24. Seifalian, A. M.; Tiwari, A.; Hamilton, G.; Salacinski, H. J., Improving the clinical patency of prosthetic vascular and coronary bypass grafts: the role of seeding and tissue engineering. *Artif Organs* **2002,** 26, (4), 307-20.

25. Baguneid, M.; Murray, D.; Salacinski, H. J.; Fuller, B.; Hamilton, G.; Walker, M.; Seifalian, A. M., Shear-stress preconditioning and tissue-engineering-based paradigms for generating arterial substitutes. *Biotechnol Appl Bioc* **2004,** 39, 151-157.

26. Kaushal, S.; Amiel, G. E.; Guleserian, K. J.; Shapira, O. M.; Perry, T.; Sutherland, F. W.; Rabkin, E.; Moran, A. M.; Schoen, F. J.; Atala, A.; Soker, S.; Bischoff, J.; Mayer, J. E., Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. *Nat Med* **2001,** 7, (9), 1035-1040.

27. Sales, K. M.; Salacinski, H. J.; Alobaid, N.; Mikhail, M.; Balakrishnan, V.; Seifalian, A. M., Advancing vascular tissue engineering: the role of stem cell technology. *Trends Biotechnol* **2005,** 23, (9), 461-7.

28. Angelini, G. D.; Lloyd, C.; Bush, R.; Johnson, J.; Newby, A. C., An external, oversized, porous polyester stent reduces vein graft neointima formation, cholesterol concentration, and vascular cell adhesion molecule 1 expression in cholesterol-fed pigs. *J Thorac Cardiov Sur* **2002,** 124, (5), 950-6.

29. Mehta, D.; George, S. J.; Jeremy, J. Y.; Izzat, M. B.; Southgate, K. M.; Bryan, A. J.; Newby, A. C.; Angelini, G. D., External stenting reduces long-term medial and neointimal thickening and platelet derived growth factor expression in a pig model of arteriovenous bypass grafting. *Nat Med* **1998,** 4, (2), 235-9.

30. Thomas, A. C.; Campbell, G. R.; Campbell, J. H., Advances in vascular tissue engineering. *Cardiovasc Pathol* **2003,** 12, (5), 271-6.

31. Berger, K.; Sauvage, L. R.; Rao, A. M.; Wood, S. J., Healing of arterial prostheses in man: its incompleteness. *Ann Surg* **1972,** 175, (1), 118-27.

32. Autio, I.; Malo-Ranta, U.; Kallioniemi, O. P.; Nikkari, T., Cultured bovine aortic endothelial cells secrete factor(s) chemotactic for aortic smooth muscle cells. *Artery* **1989,** 16, (2), 72-83.

33. Cybulsky, M. I.; Gimbrone, M. A., Jr., Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* **1991,** 251, (4995), 788-91.

34. Furchgott, R. F.; Zawadzki, J. V., The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **1980,** 288, (5789), 373-6.

35. Casscells, W., Migration of smooth muscle and endothelial cells. Critical events in restenosis. *Circulation* **1992,** 86, (3), 723-9.

36. Rashid, S. T.; Salacinski, H. J.; Hamilton, G.; Seifalian, A. M., The use of animal models in developing the discipline of cardiovascular tissue engineering: a review. *Biomaterials* **2004,** 25, (9), 1627-1637.

37. Manuel, D. G.; Leung, M.; Nguyen, K.; Tanuseputro, P.; Johansen, H.; R, C. C. O., Burden of cardiovascular disease in Canada. *Can J Cardiol* **2003,** 19, (9), 997-1004.

38. Skalak, R., Fox, C.F. In *Tissue Engineering.* , Proceedings for a Workshop, Granlibakken, Lake Tahoe, 1988; New York: Liss: Granlibakken, Lake Tahoe, 1988; pp 26-29.

39. Rabkin, E.; Schoen, F. J., Cardiovascular tissue engineering. *Cardiovasc Pathol* **2002,** 11, (6), 305-17.

40. Germain, L.; Goulet, F.; Moulin, V.; Berthod, F.; Auger, F. A., Engineering human tissues for in vivo applications. *Ann NY Acad Sci* **2002,** 961, 268-70.

41. Faustman, D. L.; Pedersen, R. L.; Kim, S. K.; Lemischka, I. R.; McKay, R. D., Cells for repair: breakout session summary. *Ann NY Acad Sci* **2002,** 961, 45-7.

42. Yang, S. F.; Leong, K. F.; Du, Z. H.; Chua, C. K., The design of scaffolds for use in tissue engineering. Part 1. Traditional factors. *Tissue Eng* **2001,** 7, (6), 679-689.

43. Curtis, A.; Riehle, M., Tissue engineering: the biophysical background. *Phys Med Biol* **2001,** 46, (4), R47-65.

44. Sipe, J. D., Tissue engineering and reparative medicine. *Ann NY Acad Sci* **2002,** 961, 1-9.

45. Grenier, S.; Sandig, M.; Mequanint, K., Smooth muscle alpha-actin and calponin expression and extracellular matrix production of human coronary artery smooth muscle cells in 3D scaffolds. *Tissue Eng Pt A* **2009,** 15, (10), 3001-11.

46. Pankajakshan, D.; Philipose, L. P.; Palakkal, M.; Krishnan, K.; Krishnan, L. K., Development of a fibrin composite-coated poly(epsilon-caprolactone) scaffold for potential vascular tissue engineering applications. *J Biomed Mater Res B* **2008,** 87, (2), 570-9.

47. Higgins, S. P.; Solan, A. K.; Niklason, L. E., Effects of polyglycolic acid on porcine smooth muscle cell growth and differentiation. *J Biomed Mater Res A* **2003,** 67, (1), 295-302.

48. Lyons, E., Pandit, A., Design of bioreactors for cardiovascular applications. In *Topics in Tissue Engineering*, Ashammakhi, N., Reis, R.I., Ed. 2006; Vol. 2.

49. Badylak, S. F.; Grompe, M.; Caplan, A. I.; Greisler, H. P.; Guldberg, R. E.; Taylor, D. A., In vivo remodeling - Breakout session summary. *Ann NY Acad Sci* **2002,** 961, 319-322.

50. Harlan, D. M.; Karp, C. L.; Matzinger, P.; Munn, D. H.; Ransohoff, R. M.; Metzger, D. W., Immunological concerns with bioengineering approaches. *Ann NY Acad Sci* **2002,** 961, 323-330.

51. Goyal, A.; Wang, Y.; Su, H.; Dobrucki, L. W.; Brennan, M.; Fong, P.; Dardik, A.; Tellides, G.; Sinusas, A.; Pober, J. S.; Saltzman, W. M.; Breuer, C. K., Development of a model system for preliminary evaluation of tissue-engineered vascular conduits. *J Pediatr Surg* **2006,** 41, (4), 787-91.

52. Shin'oka, T.; Imai, Y.; Ikada, Y., Transplantation of a tissue-engineered pulmonary artery. *New Engl J Med* **2001,** 344, (7), 532-3.

53. Isenberg, B. C.; Williams, C.; Tranquillo, R. T., Small-diameter artificial arteries engineered in vitro. *Circ Res* **2006,** 98, (1), 25-35.

54. Owens, G. K.; Kumar, M. S.; Wamhoff, B. R., Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* **2004,** 84, (3), 767-801.

55. Wamhoff, B. R.; Bowles, D. K.; Owens, G. K., Excitation-transcription coupling in arterial smooth muscle. *Circ Res* **2006,** 98, (7), 868-78.

56. Hungerford, J. E.; Little, C. D., Developmental biology of the vascular smooth muscle cell: building a multilayered vessel wall. *J Vasc Res* **1999,** 36, (1), 2-27.

57. Owens, G. K., Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* **1995,** 75, (3), 487-517.

58. Lin, S.; Sandig, M.; Mequanint, K., Three-dimensional topography of synthetic scaffolds induces elastin synthesis by human coronary artery smooth muscle cells. *Tissue Eng Pt A* **2011,** 17, (11-12), 1561-71.

59. Ueki, N.; Sobue, K.; Kanda, K.; Hada, T.; Higashino, K., Expression of high and low molecular weight caldesmons during phenotypic modulation of smooth muscle cells. *P Natl Acad Sci USA* **1987,** 84, (24), 9049-53.

60. Bacakova, L.; Wilhelm, J.; Herget, J.; Novotna, J.; Eckhart, A., Oxidized collagen stimulates proliferation of vascular smooth muscle cells. *Exp Mol Pathol* **1997,** 64, (3), 185-194.

61. Owens, G. K.; Geisterfer, A. A.; Yang, Y. W.; Komoriya, A., Transforming growth factor-beta-induced growth inhibition and cellular hypertrophy in cultured vascular smooth muscle cells. *J Cell Biol* **1988,** 107, (2), 771-80.

62. Li, X.; Tsai, P.; Wieder, E. D.; Kribben, A.; Van Putten, V.; Schrier, R. W.; Nemenoff, R. A., Vascular smooth muscle cells grown on Matrigel. A model of the contractile phenotype with decreased activation of mitogen-activated protein kinase. *J Biol Chem* **1994,** 269, (30), 19653-8.

63. Stegemann, J. P.; Hong, H.; Nerem, R. M., Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype. *J Appl Physiol* **2005,** 98, (6), 2321-7.

64. Beamish, J. A.; He, P.; Kottke-Marchant, K.; Marchant, R. E., Molecular regulation of contractile smooth muscle cell phenotype: implications for vascular tissue engineering. *Tissue Eng Pt B-Rev* **2010,** 16, (5), 467-91.

65. Rensen, S. S.; Doevendans, P. A.; van Eys, G. J., Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. *Neth Heart J* **2007,** 15, (3), 100-8.

66. Chamley-Campbell, J.; Campbell, G. R.; Ross, R., The smooth muscle cell in culture. *Physiol Rev* **1979,** 59, (1), 1-61.

67. Raines, E. W.; Ross, R., Smooth muscle cells and the pathogenesis of the lesions of atherosclerosis. *Brit Heart J* **1993,** 69, (1 Suppl), S30-7.

68. Moiseeva, E. P., Adhesion receptors of vascular smooth muscle cells and their functions. *Cardiovas Res* **2001,** 52, (3), 372-386.

69. Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L., Beyond Cholesterol - Modifications of Low-Density Lipoprotein That Increase Its Atherogenicity. *New Engl J Med* **1989,** 320, (14), 915-924.

70. Hedin, U.; Thyberg, J., Plasma fibronectin promotes modulation of arterial smooth-muscle cells from contractile to synthetic phenotype. *Differentiation* **1987,** 33, (3), 239-46.

71. Hishikawa, K.; Nakaki, T.; Fujii, T., Transforming growth factor-beta(1) induces apoptosis via connective tissue growth factor in human aortic smooth muscle cells. *Eur J Pharmacol* **1999,** 385, (2-3), 287-90.

72. Pepper, M. S., Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth F R* **1997,** 8, (1), 21-43.

73. Mann, B. K.; Schmedlen, R. H.; West, J. L., Tethered-TGF-beta increases extracellular matrix production of vascular smooth muscle cells. *Biomaterials* **2001,** 22, (5), 439-44.

74. Kingsley, K.; Huff, J. L.; Rust, W. L.; Carroll, K.; Martinez, A. M.; Fitchmun, M.; Plopper, G. E., ERK1/2 mediates PDGF-BB stimulated vascular smooth muscle cell proliferation and migration on laminin-5. *Biochem Bioph Res Co* **2002,** 293, (3), 1000-6.

75. Hedin, U.; Bottger, B. A.; Forsberg, E.; Johansson, S.; Thyberg, J., Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. *J Cell Biol* **1988,** 107, (1), 307-19.

76. Fager, G.; Hansson, G. K.; Gown, A. M.; Larson, D. M.; Skalli, O.; Bondjers, G., Human arterial smooth muscle cells in culture: inverse relationship between proliferation and expression of contractile proteins. *In Vitro Cell Dev B* **1989,** 25, (6), 511-20.

77. Han, M.; Wen, J. K.; Zheng, B.; Cheng, Y.; Zhang, C., Serum deprivation results in redifferentiation of human umbilical vascular smooth muscle cells. *Am J Physiol-Cell Ph* **2006,** 291, (1), C50-8.

78. Li, S.; Fan, Y. S.; Chow, L. H.; Van Den Diepstraten, C.; van Der Veer, E.; Sims, S. M.; Pickering, J. G., Innate diversity of adult human arterial smooth muscle cells: cloning of distinct subtypes from the internal thoracic artery. *Circ Res* **2001,** 89, (6), 517-25.

79. Li, S. H.; Sims, S.; Jiao, Y.; Chow, L. H.; Pickering, J. G., Evidence from a novel human cell clone that adult vascular smooth muscle cells can convert reversibly between noncontractile and contractile phenotypes. *Circ Res* **1999,** 85, (4), 338-348.

80. Ma, X. F.; Wang, Y.; Stephens, N. L., Serum deprivation induces a unique hypercontractile phenotype of cultured smooth muscle cells. *Am J Physiol-Cell Ph* **1998,** 274, (5), C1206-C1214.

81. Poliseno, L.; Cecchettini, A.; Mariani, L.; Evangelista, M.; Ricci, F.; Giorgi, F.; Citti, L.; Rainaldi, G., Resting smooth muscle cells as a model for studying vascular cell activation. *Tissue Cell* **2006,** 38, (2), 111-120.

82. Sasaki, Y.; Hayashi, N.; Kamada, T., Heterogeneous Activation of Protein Kinase-C during Rat-Liver Regeneration. *Adv Sec Mess Phosph* **1990,** 24, 345-351.

83. Hedin, U.; Roy, J.; Tran, P. K., Control of smooth muscle cell proliferation in vascular disease. *Curr Opin Lipidol* **2004,** 15, (5), 559-65.

84. Grenier, S.; Sandig, M.; Holdsworth, D. W.; Mequanint, K., Interactions of coronary artery smooth muscle cells with 3D porous polyurethane scaffolds. *J Biomed Mater Res A* **2009,** 89, (2), 293-303.

85. Dubey, G.; Mequanint, K., Conjugation of fibronectin onto three-dimensional porous scaffolds for vascular tissue engineering applications. *Acta Biomater* **2011,** 7, (3), 1114-25.

86. Baker, S. C.; Southgate, J., Towards control of smooth muscle cell differentiation in synthetic 3D scaffolds. *Biomaterials* **2008,** 29, (23), 3357-66.

87. Goessl, A.; Bowen-Pope, D. F.; Hoffman, A. S., Control of shape and size of vascular smooth muscle cells in vitro by plasma lithography. *J Biomed Mater Res* **2001,** 57, (1), 15-24.

88. Ra, H. J.; Picart, C.; Feng, H.; Sweeney, H. L.; Discher, D. E., Muscle cell peeling from micropatterned collagen: direct probing of focal and molecular properties of matrix adhesion. *J Cell Sci* **1999,** 112 (Pt 10), 1425-36.

89. Mills, I.; Cohen, C. R.; Kamal, K.; Li, G.; Shin, T.; Du, W.; Sumpio, B. E., Strain activation of bovine aortic smooth muscle cell proliferation and alignment: study of strain dependency and the role of protein kinase A and C signaling pathways. *J Cell Physiol* **1997,** 170, (3), 228-34.

90. Hu, Y.; Bock, G.; Wick, G.; Xu, Q., Activation of PDGF receptor alpha in vascular smooth muscle cells by mechanical stress. *FASEB J* **1998,** 12, (12), 1135-42.

91. Stringa, E.; Knauper, V.; Murphy, G.; Gavrilovic, J., Collagen degradation and platelet-derived growth factor stimulate the migration of vascular smooth muscle cells. *J Cell Sci* **2000,** 113 (Pt 11), 2055-64.

92. Sudhir, K.; Hashimura, K.; Bobik, A.; Dilley, R. J.; Jennings, G. L.; Little, P. J., Mechanical strain stimulates a mitogenic response in coronary vascular smooth muscle cells via release of basic fibroblast growth factor. *Am J Hypertens* **2001,** 14, (11 Pt 1), 1128-34.

93. Wilson, E.; Mai, Q.; Sudhir, K.; Weiss, R. H.; Ives, H. E., Mechanical strain induces growth of vascular smooth muscle cells via autocrine action of PDGF. *J Cell Biol* **1993,** 123, (3), 741-7.

94. Niklason, L. E.; Gao, J.; Abbott, W. M.; Hirschi, K. K.; Houser, S.; Marini, R.; Langer, R., Functional arteries grown in vitro. *Science* **1999,** 284, (5413), 489-93.

95. Mooney, D. J.; Mikos, A. G., Growing new organs. *Sci Am* **1999,** 280, (4), 60-5.

96. Seliktar, D.; Black, R. A.; Vito, R. P.; Nerem, R. M., Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling in vitro. *Ann Biomed Eng* **2000,** 28, (4), 351-62.

97. Bacakova, L.; Lisa, V.; Kubinova, L.; Wilhelm, J.; Novotna, J.; Eckhart, A.; Herget, J., Ultraviolet light-irradiated collagen III modulates expression of cytoskeletal and surface adhesion molecules in rat aortic smooth muscle cells in vitro. *Virchows Arch* **2002,** 440, (1), 50-62.

98. Kim, B. S.; Nikolovski, J.; Bonadio, J.; Mooney, D. J., Cyclic mechanical strain regulates the development of engineered smooth muscle tissue. *Nat Biotechnol* **1999,** 17, (10), 979-83.

99. Campbell, J. H.; Campbell, G. R., Endothelial cell influences on vascular smooth muscle phenotype. *Annu Rev Physiol* **1986,** 48, 295-306.

100. Fillinger, M. F.; Sampson, L. N.; Cronenwett, J. L.; Powell, R. J.; Wagner, R. J., Coculture of endothelial cells and smooth muscle cells in bilayer and conditioned media models. *J Surg Res* **1997,** 67, (2), 169-78.

101. Liu, H.; Kennard, S.; Lilly, B., NOTCH3 expression is induced in mural cells through an autoregulatory loop that requires endothelial-expressed JAGGED1. *Circ Res* **2009,** 104, (4), 466-75.

102. Xia, Y.; Bhattacharyya, A.; Roszell, E. E.; Sandig, M.; Mequanint, K., The role of endothelial cell-bound Jagged1 in Notch3-induced human coronary artery smooth muscle cell differentiation. *Biomaterials* **2012,** 33, (8), 2462-72.

103. Allen, K. E.; Varty, K.; Jones, L.; Sayers, R. D.; Bell, P. R.; London, N. J., Human venous endothelium can promote intimal hyperplasia in a paracrine manner. *J Vasc Surg* **1994,** 19, (4), 577-84.

104. Castellot, J. J., Jr.; Addonizio, M. L.; Rosenberg, R.; Karnovsky, M. J., Cultured endothelial cells produce a heparinlike inhibitor of smooth muscle cell growth. *J Cell Biol* **1981,** 90, (2), 372-9.

105. Chamley-Campbell, J. H.; Campbell, G. R., What controls smooth muscle phenotype? *Atherosclerosis* **1981,** 40, (3-4), 347-57.

106. Koo, E. W.; Gotlieb, A. I., Endothelial stimulation of intimal cell proliferation in a porcine aortic organ culture. *Am J Pathol* **1989,** 134, (3), 497-503.

107. van Buul-Wortelboer, M. F.; Brinkman, H. J.; Dingemans, K. P.; de Groot, P. G.; van Aken, W. G.; van Mourik, J. A., Reconstitution of the vascular wall in vitro. A novel model to study interactions between endothelial and smooth muscle cells. *Exp Cell Res* **1986,** 162, (1), 151-8.

108. Fillinger, M. F.; O'Connor, S. E.; Wagner, R. J.; Cronenwett, J. L., The effect of endothelial cell coculture on smooth muscle cell proliferation. *J Vasc Surg* **1993,** 17, (6), 1058-67; discussion 1067-8.

109. Jacot, J. G.; Wong, J. Y., Endothelial injury induces vascular smooth muscle cell proliferation in highly localized regions of a direct contact co-culture system. *Cell Biochem Biophys* **2008,** 52, (1), 37-46.

110. Rose, S. L.; Babensee, J. E., Complimentary endothelial cell/smooth muscle cell co-culture systems with alternate smooth muscle cell phenotypes. *Ann Biomed Eng* **2007,** 35, (8), 1382-90.

111. Rose, S. L.; Babensee, J. E., Smooth muscle cell phenotype alters cocultured endothelial cell response to biomaterial-pretreated leukocytes. *J Biomed Mater Res A* **2008,** 84, (3), 661-71.

112. Chiu, J. J.; Chen, L. J.; Chen, C. N.; Lee, P. L.; Lee, C. I., A model for studying the effect of shear stress on interactions between vascular endothelial cells and smooth muscle cells. *J Biomech* **2004,** 37, (4), 531-9.

113. Chiu, J. J.; Chen, L. J.; Lee, P. L.; Lee, C. I.; Lo, L. W.; Usami, S.; Chien, S., Shear stress inhibits adhesion molecule expression in vascular endothelial cells induced by coculture with smooth muscle cells. *Blood* **2003,** 101, (7), 2667-74.

114. Saunders, K. B.; D'Amore, P. A., An in vitro model for cell-cell interactions. *In Vitro Cell Dev B* **1992,** 28A, (7-8), 521-8.

115. Lavender, M. D.; Pang, Z.; Wallace, C. S.; Niklason, L. E.; Truskey, G. A., A system for the direct co-culture of endothelium on smooth muscle cells. *Biomaterials* **2005,** 26, (22), 4642-53.

116. Martin, Y.; Eldardiri, M.; Lawrence-Watt, D. J.; Sharpe, J. R., Microcarriers and their potential in tissue regeneration. *Tissue Eng Pt B-Rev* **2011,** 17, (1), 71-80.

117. Korff, T.; Kimmina, S.; Martiny-Baron, G.; Augustin, H. G., Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J* **2001,** 15, (2), 447-57.

118. Davies, P. F.; Truskey, G. A.; Warren, H. B.; O'Connor, S. E.; Eisenhaure, B. H., Metabolic cooperation between vascular endothelial cells and smooth muscle cells in co-culture: changes in low density lipoprotein metabolism. *J Cell Biol* **1985,** 101, (3), 871-9.

119. Niwa, K.; Kado, T.; Sakai, J.; Karino, T., The effects of a shear flow on the uptake of LDL and acetylated LDL by an EC monoculture and an EC-SMC coculture. *Ann Biomed Eng* **2004,** 32, (4), 537-43.

120. Hirschi, K. K.; Rohovsky, S. A.; D'Amore, P. A., PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J Cell Biol* **1998,** 141, (3), 805-14.

121. Ding, R.; Darland, D. C.; Parmacek, M. S.; D'Amore, P. A., Endothelial-mesenchymal interactions in vitro reveal molecular mechanisms of smooth muscle/pericyte differentiation. *Stem Cells Dev* **2004,** 13, (5), 509-20.

122. Niwa, K.; Sakai, J.; Watanabe, T.; Ohyama, T.; Karino, T., Improved arterial wall model by coculturing vascular endothelial and smooth muscle cells. *In Vitro Cell Dev-An* **2007,** 43, (1), 17-20.

123. Chaterji, S.; Park, K.; Panitch, A., Scaffold-free in vitro arterial mimetics: the importance of smooth muscle-endothelium contact. *Tissue Eng Pt A* **2010,** 16, (6), 1901-12.

124. Heydarkhan-Hagvall, S.; Chien, S.; Nelander, S.; Li, Y. C.; Yuan, S.; Lao, J.; Haga, J. H.; Lian, I.; Nguyen, P.; Risberg, B.; Li, Y. S., DNA microarray study on gene expression profiles in co-cultured endothelial and smooth muscle cells in response to 4- and 24-h shear stress. *Mol Cell Biochem* **2006,** 281, (1-2), 1-15.

125. Wallace, C. S.; Champion, J. C.; Truskey, G. A., Adhesion and function of human endothelial cells co-cultured on smooth muscle cells. *Ann Biomed Eng* **2007,** 35, (3), 375-86.

126. Wallace, C. S.; Strike, S. A.; Truskey, G. A., Smooth muscle cell rigidity and extracellular matrix organization influence endothelial cell spreading and adhesion formation in coculture. *Am J Physiol-Heart C* **2007,** 293, (3), H1978-86.

127. Wallace, C. S.; Truskey, G. A., Direct-contact co-culture between smooth muscle and endothelial cells inhibits TNF-alpha-mediated endothelial cell activation. *Am J Physiol-Heart C* **2010,** 299, (2), H338-46.

128. Pang, Z.; Niklason, L. E.; Truskey, G. A., Porcine endothelial cells cocultured with smooth muscle cells became procoagulant in vitro. *Tissue Eng Pt A* **2010,** 16, (6), 1835-44.

129. Ziegler, T.; Alexander, R. W.; Nerem, R. M., An endothelial cell-smooth muscle cell co-culture model for use in the investigation of flow effects on vascular biology. *Ann Biomed Eng* **1995,** 23, (3), 216-25.

130. Imberti, B.; Seliktar, D.; Nerem, R. M.; Remuzzi, A., The response of endothelial cells to fluid shear stress using a co-culture model of the arterial wall. *Endothelium-J Endoth* **2002,** 9, (1), 11-23.

131. Crouchley, C. M.; Barron, V.; Punchard, M.; O'Cearbhaill, E.; Smith, T., Development of a co-culture system for tissue engineered vascular grafts. *Biomed Mater Eng* **2008,** 18, (4-5), 291-4.

132. Williams, C.; Wick, T. M., Endothelial cell-smooth muscle cell co-culture in a perfusion bioreactor system. *Ann Biomed Eng* **2005,** 33, (7), 920-8.

133. Pullens, R. A.; Stekelenburg, M.; Baaijens, F. P.; Post, M. J., The influence of endothelial cells on the ECM composition of 3D engineered cardiovascular constructs. *J Tissue Eng Regen M* **2009,** 3, (1), 11-8.

134. Powell, R. J.; Bhargava, J.; Basson, M. D.; Sumpio, B. E., Coculture conditions alter endothelial modulation of TGF-beta 1 activation and smooth muscle growth morphology. *Am J Physiol* **1998,** 274, (2 Pt 2), H642-9.

135. Powell, R. J.; Cronenwett, J. L.; Fillinger, M. F.; Wagner, R. J.; Sampson, L. N., Endothelial cell modulation of smooth muscle cell morphology and organizational growth pattern. *Ann Vasc Surg* **1996,** 10, (1), 4-10.

136. Powell, R. J.; Hydowski, J.; Frank, O.; Bhargava, J.; Sumpio, B. E., Endothelial cell effect on smooth muscle cell collagen synthesis. *J Surg Res* **1997,** 69, (1), 113-8.

137. Domenga, V.; Fardoux, P.; Lacombe, P.; Monet, M.; Maciazek, J.; Krebs, L. T.; Klonjkowski, B.; Berrou, E.; Mericskay, M.; Li, Z.; Tournier-Lasserve, E.; Gridley, T.; Joutel, A., Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. *Gene Dev* **2004,** 18, (22), 2730-5.

138. High, F. A.; Lu, M. M.; Pear, W. S.; Loomes, K. M.; Kaestner, K. H.; Epstein, J. A., Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. *P Natl Acad Sci USA* **2008,** 105, (6), 1955-9.

139. Wang, T.; Baron, M.; Trump, D., An overview of Notch3 function in vascular smooth muscle cells. *Prog Biophys Mol Bio* **2008,** 96, (1-3), 499-509.

140. Brown, D. J.; Rzucidlo, E. M.; Merenick, B. L.; Wagner, R. J.; Martin, K. A.; Powell, R. J., Endothelial cell activation of the smooth muscle cell phosphoinositide 3-kinase/Akt pathway promotes differentiation. *J Vasc Surg* **2005,** 41, (3), 509-16.

141. Fetalvero, K. M.; Shyu, M.; Nomikos, A. P.; Chiu, Y. F.; Wagner, R. J.; Powell, R. J.; Hwa, J.; Martin, K. A., The prostacyclin receptor induces human vascular smooth muscle cell differentiation via the protein kinase A pathway. *Am J Physiol-Heart C* **2006,** 290, (4), H1337-46.

142. Caolo, V.; Schulten, H. M.; Zhuang, Z. W.; Murakami, M.; Wagenaar, A.; Verbruggen, S.; Molin, D. G.; Post, M. J., Soluble Jagged-1 inhibits neointima formation by attenuating Notch-Herp2 signaling. *Arterioscl Thromb Vas* **2011,** 31, (5), 1059-65.

143. Iso, T.; Hamamori, Y.; Kedes, L., Notch signaling in vascular development. *Arterioscl Thromb Vas* **2003,** 23, (4), 543-53.

144. Shawber, C. J.; Kitajewski, J., Notch function in the vasculature: insights from zebrafish, mouse and man. *Bioessays* **2004,** 26, (3), 225-34.

145. Tang, Y.; Urs, S.; Boucher, J.; Bernaiche, T.; Venkatesh, D.; Spicer, D. B.; Vary, C. P.; Liaw, L., Notch and transforming growth factor-beta (TGFbeta) signaling pathways cooperatively regulate vascular smooth muscle cell differentiation. *J Biol Chem* **2010,** 285, (23), 17556-63.

146. Wu, X.; Zou, Y.; Zhou, Q.; Huang, L.; Gong, H.; Sun, A.; Tateno, K.; Katsube, K.; Radtke, F.; Ge, J.; Minamino, T.; Komuro, I., Role of Jagged1 in arterial lesions after vascular injury. *Arterioscl Thromb Vas* **2011,** 31, (9), 2000-6.

147. Cines, D. B.; Pollak, E. S.; Buck, C. A.; Loscalzo, J.; Zimmerman, G. A.; McEver, R. P.; Pober, J. S.; Wick, T. M.; Konkle, B. A.; Schwartz, B. S.; Barnathan, E. S.; McCrae, K. R.; Hug, B. A.; Schmidt, A. M.; Stern, D. M., Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* **1998,** 91, (10), 3527-61.

148. Deng, D. X.; Spin, J. M.; Tsalenko, A.; Vailaya, A.; Ben-Dor, A.; Yakhini, Z.; Tsao, P.; Bruhn, L.; Quertermous, T., Molecular signatures determining coronary artery and saphenous vein smooth muscle cell phenotypes: distinct responses to stimuli. *Arterioscl Thromb Vas* **2006,** 26, (5), 1058-65.

149. Imegwu, O. J.; Entersz, I.; Graham, A. M.; Nackman, G. B., Heterotypic smooth muscle cell/endothelial cell interactions differ between species. *J Surg Res* **2001,** 98, (2), 85-8.

150. Gong, Z.; Niklason, L. E., Blood vessels engineered from human cells. *Trends Cardiovas Med* **2006,** 16, (5), 153-6.

151 Tang, Y.; Urs, S.; Liaw, L., Hairy-related transcription factors inhibit Notch-induced smooth muscle alpha-actin expression by interfering with Notch intracellular domain/CBF-1 complex interaction with the CBF-1-binding site. *Circ Res* **2008,** 102, (6), 661-8.

152 Doi, H.; Iso, T.; Shiba, Y.; Sato, H.; Yamazaki, M.; Oyama, Y.; Akiyama, H.; Tanaka, T.; Tomita, T.; Arai, M.; Takahashi, M.; Ikeda, U.; Kurabayashi, M., Notch signaling regulates the differentiation of bone marrow-derived cells into smooth muscle-like cells during arterial lesion formation. *Biochem Bioph Res Co* **2009,** 381, (4), 654-9.

153 Weber, D. S., A novel mechanism of vascular smooth muscle cell regulation by Notch: platelet-derived growth factor receptor-beta expression? *Circ Res* **2008,** 102, (12), 1448-50.

154 Ghosh, S.; Paez-Cortez, J. R.; Boppidi, K.; Vasconcelos, M.; Roy, M.; Cardoso, W.; Ai, X.; Fine, A., Activation dynamics and signaling properties of Notch3 receptor in the developing pulmonary artery. *J Biol Chem* **2011,** 286, (25), 22678-87.

155 Villa, N.; Walker, L.; Lindsell, C. E.; Gasson, J.; Iruela-Arispe, M. L.; Weinmaster, G., Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mech Develop* **2001,** 108, (1-2), 161-4.

156 High, F. A.; Lu, M. M.; Pear, W. S.; Loomes, K. M.; Kaestner, K. H.; Epstein, J. A., Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. *P Natl Acad Sci USA* **2008,** 105, (6), 1955-9.

157 Xue, Y.; Gao, X.; Lindsell, C. E.; Norton, C. R.; Chang, B.; Hicks, C.; Gendron-Maguire, M.; Rand, E. B.; Weinmaster, G.; Gridley, T., Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet* **1999,** 8, (5), 723-30.

Chapter 3 - Materials and Methods

3.1 Materials

A poly(carbonate urethane) (PCU; Bionate® 55D) was used to fabricate the 3D scaffolds in this study and was supplied by Polymer Technology Group (Berkeley, CA). Ammonium chloride (NH_4Cl) supplied by Caledon Laboratories Ltd (Georgetown, ON, Canada) was used to create the porous structure of the scaffolds. Dimethylformamide (DMF) from Caledon Laboratories was used as a solvent for PCU. FN and Matrigel were supplied by Santa Cruz Biotechnology (Santa Cruz, CA) and Becton Dickenson (Franklin Lakes, NJ) respectively. Hank's balanced salt solution (HBSS), used for solubilizing FN was supplied by Invitrogen (Burlington, ON, Canada). Penicillin G and streptomycin sulfate were purchased from Invitrogen. Cell culture studies were conducted utilizing primary HCASMCs and primary HCAECs in smooth muscle growth media (SmGM®-2 BulletKit) (SmGM) and endothelial cell growth media (EGM®-2 Bullet Kit) (EGM), respectively, all of which was supplied by Lonza Walkersville Inc. (Walkersville, MD). For cell fixation purposes paraformaldehyde was purchased from EMD Chemicals (Gibbstown, NJ). Saponin, purchased from Sigma Chemical Co. (Germany), was used for cell permeabilization in some experiments. For immunostaining: Alexa Fluor® 488 Phalloidin and CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) (CTG) were purchased from Invitrogen (Eugene, OR), anti-VE-cadherin antibody was purchased from Santa Cruz Biotechnology and lastly Hoechst 33342 was purchased from

Sigma-Aldrich (Oakville, ON, Canada). Bovine serum albumin (BSA) was supplied by Sigma-Aldrich and Vectashield mounting medium was supplied by Vector Laboratories (Burlington, ON, Canada). Anti-PECAM-conjugated Dynabeads (Invitrogen Dynal AS, Oslo, Norway) were employed for cell separation in co-culture. Control and Jagged1 siRNA ON-TARGET*plus* SMARTpool duplexes from Dharmacon were used for gene knockdown studies. siRNA reagents Opti-MEM reduced serum medium and Lipofectamine™ RNAiMAX Reagent were purchased from Invitrogen (Grand Island, NY) and Invitrogen (Carlsbad, CA) respectively. Antibodies for use in Western blot (anti-SM α-actin, anti-calponin, anti-Notch 3, anti-Jagged1) were purchased from Santa Cruz Biotechnology with the exception of GAPDH which was purchased from Millipore (Temecula, CA). Protein concentrations were measured using 660 nm Protein Assay supplied by Thermo Scientific (Ottawa, Canada). SuperSignal® West Pico Chemiluminescent Substrate was supplied by Thermo Scientific (Rockford, IL). Protein G Dynabeads and recombinant human Jagged1/Fc chimera protein (1277-JG) were supplied by Invitrogen (Burlington, ON, Canada) and R&D Systems (Minneapolis, MN) respectively. RNA analysis of HCASMCs was achieved using TRIzol® Reagent and SuperScript™ from Invitrogen and a Chromo4 Real-time Thermal Cycler, iQ™ SYBR® Green Supermix and Gene Expression Macro analysis software from Bio-Rad (Mississauga, ON, Canada).

3.2 Methods

3.2.1 Scaffold Fabrication

PCU scaffolds were fabricated using a solvent casting and particulate leaching (SCPL) method as established in our laboratory[1]. In brief, NH_4Cl porogen particles were ground to the diameter range of 180-210 μm using a mortar and pestle. Particles were then passed through graded sieves and those falling into the desired range of 180-212μm were collected. The collected particles were re-sieved to ensure they fell into narrow size distributions. Using the set-up shown below (Figure 3.1) the porogen was poured into the glass mold and compressed using air pressure to achieve high pore density and uniformity. A polymer solution consisting of 20% (w/v) PCU in DMF was added to the steel chamber and infiltration through the porogen bed was aided by the application of a pressure differential. The scaffold was then left for a period of three days to evaporate the solvent. The NH_4Cl particles were then leached out using water (a non-solvent for PCU) after which the scaffold was dried (Figure 3.1B) and sectioned into 0.5mm thick discs (Figure 3.1C) prior to use in cell culture studies.

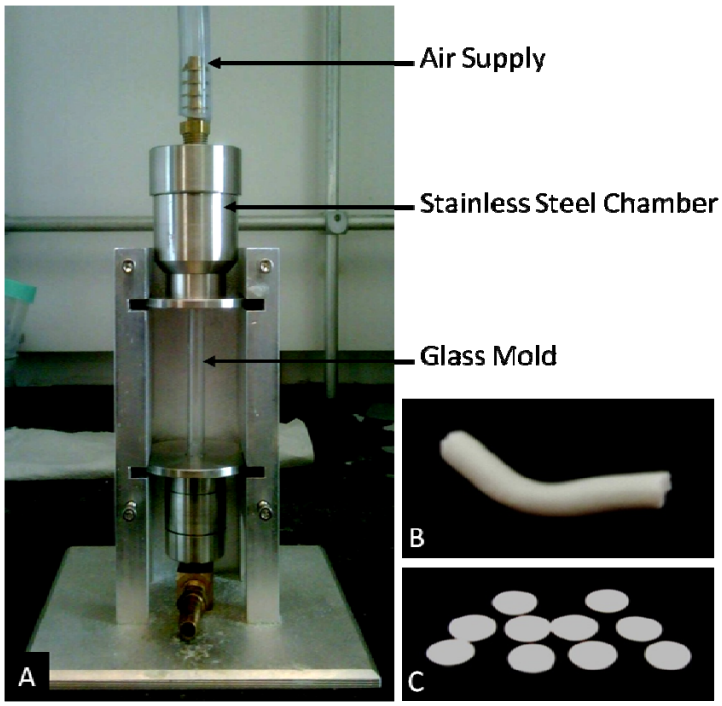

Figure 3. 1: Scaffold fabrication apparatus. Apparatus used for fabricating PCU scaffolds using SCLP technique (A); Resulting scaffold after porogen leaching and drying (B); Scaffold discs used for cell culture (C).

3.2.2 Scaffold Characterization

To verify uniform scaffold structure and pore size, the fabricated scaffolds were imaged using a scanning electron microscope (SEM) (S-2600N, Hitachi, Japan). Scaffolds were sectioned into cylindrical disks of 5.5 mm diameter and 0.5 mm thickness using a rotary blade cutter, then sputter coated with gold and imaged.

3.2.3 Scaffold Preparation for Cell Culture

Cylindrical scaffold disks were affixed to the bottom of a 96-well culture plate using silicone grease. A solution of FN diluted in HBSS was adsorbed onto the scaffold surfaces overnight in the dark at a density of 5μg/scaffold. Scaffolds were kept hydrated in HBSS prior to cell seeding.

3.2.4 Cell Culture

Primary HCASMCs and primary HCAECs were cultured in SmGM and EGM respectively. Both media were supplemented with 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. Cell cultures were maintained in a humidified incubator at 5% CO_2 and 37°C and were used between passages 5 and 9.

3.2.5 2D Monoculture

Experiments that were performed for optimizing FN concentration and media composition were carried out on glass coverslips. FN was diluted in distilled water to desired concentrations of 10 μg/cm^2, 5 μg/cm^2, and 2 μg/cm^2 and adsorbed onto coverslips for 1 hour. HCASMCs were seeded onto coverslips of different FN concentrations at a density of 25,000 cells/coverslip and cultured in SmGM for 2, 4 or 7 days. For growth media experiments, HCASMCs and HCAECs were seeded at densities of 25,000 cells/coverslip and 20,000 cells/coverslip, respectively, onto substrates of 5μg

FN/cm^2. HCASMCs were cultured for 4 days while HCAECs were cultured for 2 days in media compositions of: pure EGM, pure SmGM, or 1 part EGM and 1 part SmGM.

3.2.6 2D Culture for RT-PCR

For RT-PCR work, cultures were carried out on 24-well treated polystyrene tissue culture dishes. Culture wells were first coated with 5 μg/cm^2 of FN diluted in HBSS. For monocultures, HCASMCs were seeded at a density of 1.7x10^4 cells/well and cultured for 48 hours with 5 μg/ml of Jagged1 protein or 10 μL/well of Jagged1/IgG-immobilized Dynabeads. For co-cultures, HCASMCs were seeded at a density of 1.7x10^4 cells/well and cultured for 48 hours in SmGM. HCAECs were then seeded at a density of 1.7x10^4 cells/well over the HCASMC layer and cultured for an additional 48 hours in co-culture media. Co-culture media consisted of SmGM and EGM at a 1:1 ratio.

3.2.7 3D Co-culture of HCASMCs and HCAECs on PCU scaffolds

HCASMCs were seeded onto the scaffold discs at varying initial densities depending on the experiment and allowed sufficient time for attachment in a 37°C, 5% CO_2 incubator. After attachment, scaffolds were transferred to a 24-well culture plate with 2 ml of SmGM and cultured at 37°C, 5% CO_2 for 48 hours. Matrigel was solubilized in serum-free growth media and added over the HCASMC layer (for co-cultures) or over bare scaffolds (for HCAEC monocultures) to promote HCAEC attachment. The Matrigel solution was adsorbed for one hour at 37°C just prior to HCAEC seeding in a 96-well plate. HCAECs were seeded onto scaffolds containing HCASMCs or onto Matrigel-

adsorbed scaffolds and incubated at 37°C and 5% CO_2 for three hours to allow cell attachment. Scaffolds were then transferred back to a 24-well dish with 2 ml of growth media (EGM for EC monocultures or SmGM and EGM at a 1:1 ratio for co-cultures) and cultured for an additional 48 hours.

3.2.8 Immunofluorescence Staining and Fluorescence Microscopy of 2D cultures

Cell cultures from FN concentration and growth media experiments were stained for F-actin filaments prior to image analysis. Cells were fixed to their coverslips for 10 minutes using freshly prepared 2% paraformaldehyde and washed 3 times with 1x phosphate buffered saline (PBS). A solution of 0.1% Saponin was applied for 5 minutes to permeabilize the cell membranes. Following 3 washes in 1 x PBS, samples were incubated in Alexa Fluor® 488 Phalloidin diluted (1:50) in 1% BSA/PBS for 1 hour. Finally, cells were washed 3 times using 1 x PBS on the first and third washes and 1 x Hoechst 33342 (10ug/ml) on the second wash. Coverslips were mounted onto microscope slides using Vectashield as a mounting medium and were sealed using nail enamel. Fluorescence images were captured using a Leica CTR Mic fluorescence microscope (Hg bulb) and Openlab software.

3.2.9 Immunofluorescence Staining and Laser Scanning Confocal Microscopy of 3D cultures

Post-culture immunostaining was performed to allow for visualization of cell organization and morphology on 3D scaffolds. SMCs were live-stained using CTG at a concentration of 10 μM in serum-free media for 45 minutes. Following two 30-minute incubations in SmGM (to rinse out the unincorporated dye and by-products), a thin coating of Matrigel was applied and HCAECs were seeded and cultured for 48 hours as described above. Cells were fixed using a 4% solution of paraformaldehyde, permeabilized in 0.5 % Triton X-100 and washed three times with 1 x PBS. Long-term SMC monocultures were stained as described for 2D cultures above. Co-cultured cell-scaffold constructs and HCAEC-scaffold constructs were incubated in 1% BSA/PBS with VE-cadherin antibody (1:50 dilution) for one hour followed by three washes in 1 x PBS. Scaffolds were then incubated in Alexa Fluor® 568-conjugated secondary antibody for one hour, washed three times with 1 x PBS and incubated with Hoechst 33342 (10 μg/ml) for 5 minutes to label the nuclei of all cells. Samples were mounted on glass microscope slides in a mounting medium composed of glycerol and water (glycerol:water, 9:1v/v). Spacers were used to construct elevated wells in which the scaffolds were contained; wells were sealed using coverslips and nail enamel. A Zeiss LSM 410 confocal microscope (Carl Zeiss, Germany) equipped with argon/ neon and UV lasers was used for imaging the samples. 3D image stacks were created by taking serial optical slices at regular increments through the samples.

3.2.10 Separation of HCAEC from Co-culture

In order to examine the individual contributions of each cell type toward protein expression in co-culture, anti-PECAM conjugated Dynabeads were employed to separate the HCAECs from the HCASMCs. First, cells were recovered from scaffolds or culture plates by incubating in a 0.25% Trypsin/EDTA solution at 37°C for 5 minutes. This method has proved effective in the past for cell recovery from PCU scaffolds[2]. Scaffolds or culture plates were then rinsed several times with a low serum content-buffer (5% fetal bovine serum in 1 x PBS) to neutralize the trypsin activity. The trypsinized cell suspension was centrifuged for 5 minutes at 21°C and the pellet was re-suspended in 0.1% BSA/PBS. Washed Dynabeads (25 μL/10^8 HCAECs) were mixed with the cell suspension and rotated at 4°C for 20 minutes to facilitate attachment to HCAECs. Following incubation, samples were placed in a magnet to separate the bead-bound HCAECs from the supernatant (HCASMC). The supernatant was collected and the bead-bound cells were rinsed and magnetized three more times to increase the separation efficiency. For protein extraction, the bead-bound HCAECs and supernatant (HCASMCs) were immersed in sodium dodecyl sulfate (SDS) sample buffer after centrifugation. For RNA extraction, the bead-bound HCAECs and the supernatant (HCASMCs) were centrifuged and resuspended in TRIzol® Reagent.

3.2.11 Jagged1/Fc Protein Immobilization to Dynabeads

Protein G Dynabeads were washed 3 times with PBS (pH 7.4, 0.02% Tween) and mixed with 5 μg of human Jagged1/Fc chimera protein in the original bead volume. The

mixture was incubated for 10 min at room temperature on a rotating device and the Jagged1-immobilized beads were washed 3 times with PBS. As a control of Jagged1/Fc chimeric protein, beads were incubated with human IgG solution (5 μg/ml) at the same conditions with those used for Jagged1 binding. This control addresses the effect of the Fc fragment of Jagged1 for any possible non-specific effects of the Fc protein. Beads were added to cell cultures at a concentration of 10 μL/well.

3.2.12 Transfection of HCAEC with Jagged1 siRNA

Prior to transfection, HCAECs were passaged in antibiotics-free growth media such that they would be at 50% confluence at the time of transfection. 200 pmol of control siRNA or Jagged1 siRNA was diluted in 1 ml of Opti-MEM reduced serum medium. Each of these solutions was then mixed with another 1 ml of Opti-MEM reduced serum medium containing 20 μL of Lipofectamine™ RNAiMAX. Solutions were incubated at room temperature for 20 minutes before being added to a culture dish of 50% confluent HCAECs. Following 24 hours in a 37°C, 5% CO_2 incubator, cells were trypsinized and seeded onto either bare scaffolds or scaffolds containing HCASMCs. Following the 48 hour co-culture period, cells were harvested and lysed to test the transfection efficiency and protein expression levels.

3.2.13 RNA Isolation and Quantitative Real-time PCR Analysis

Real-time polymerase chain reaction (PCR) combined with reverse transcription was used to quantify messenger RNA of Notch3, SM-α-actin and calponin in HCASMCs grown on 2D surfaces. Total RNA from HCASMCs was isolated using TRIzol® Reagent following the manufacturer's protocol. Complementary DNA was synthesized using 1 μg of total RNA primed with oligo$(dT)_{12-18}$ as described in SuperScript™. Conventional reverse transcription PCR was used to test primer specificity by running PCR for 40 cycles at 95°C for 20 s and 52°C for 1 min. Quantitative real-time PCR was conducted in 10 μL reaction volumes, using a Chromo4 Real-time Thermal Cycler and gene expression of human Notch3, SM-α-actin, calponin and glyceraldehyde3-phosphate dehydrogenase (GAPDH) were then determined with iQ™ SYBR® Green Supermix according to the recommended protocol of the manufacturer. Notch3 forward primer, 5′-CCT AGA CCT GGT GGA CAA G -3′, and reverse primer, 5′-ACA CAG TCG TAG CGG TTG -3′; SM-α-actin forward primer, 5′-CAA GTG ATC ACC ATC GGA AAT G-3′, and reverse primer, 5′-GAC TCC ATC CCG ATG AAG GA-3′; calponin forward primer, 5′-TGA AGC CCC ACG ACA TTT TT-3′, and reverse primer, 5′-GGG TGG ACT GCA CCT GTG TA-3′; GAPDH forward primer, GGT GGT CTC CTC TGA CTT CAA CA, and reverse primer, GTT GCT GTA GCC AAA TTC GTT GT, were used[3]. Cycling parameters were optimized as follows: denaturation 95°C (10 s), gradient annealing 50°C/65°C (10 s), extension 72°C (30 s), and running for 39 cycles.Notch3, SM-α-actin and calponin gene expressions in HCASMCs were normalized to GAPDH with at least three repeats per experimental group and expressed as relative ratios using the Gene Expression Macro analysis software.

3.2.14 Protein Extraction and Western Blot

Expression levels of Jagged1, Notch3, SM-α-actin and calponin in 3D cultures were evaluated using Western blotting. Scaffolds from different culture conditions were incubated in 0.25% Trypsin/EDTA for 5 minutes at 37^{o}C. The trypsin solution was pipetted repeatedly into the scaffolds to recover cells after which the cells were centrifuged and collected. Cells from co-culture were separated using anti-PECAM conjugated Dynabeads (as described above) while cells from monocultures were immediately immersed in 100 μL of SDS sample buffer containing 5% (v/v) β-mercaptoethanol. Lysates were micro-centrifuged and the protein concentrations were determined using 660 nm Protein Assay. 20 μg/well of protein was loaded and separated by 10% SDS-polyacrylamide gel electrophoresis for 50 minutes then subsequently transferred overnight at 4°C onto a nitrocellulose membrane while submerged in a Tris-glycine buffer. Ponceau S stain was used to verify proper transfer. Membranes were then blocked with 5% non-fat dry milk in 1 x PBS for one hour and incubated in primary antibodies (diluted in 5% non-fat dry milk in 1 x PBS): anti-SM α-actin (1:1000 dilution), anti-calponin (1:1000 dilution), anti-Jagged1 (1:200 dilution), anti-Notch3 (1:200 dilution) and anti-GAPDH (1:2000 dilution) for 2 hours. After incubation with HRP-conjugated secondary antibodies for 45 minutes, membranes were incubated for 5 minutes in SuperSignal®West Pico Chemiluminescent substrate. Bio-Rad's ChemiDoc™ XRS+ System (Mississauga, ON, Canada) was used to image the membranes and blots were quantified using Image Lab™ software.

3.2.15 Statistical Analysis

Quantified data for RNA and protein expression levels were plotted and analyzed using GraphPad Prism 5. Values were normalized against GAPDH and graphs were constructed using data from at least three independent experiments. Quantified data are presented as mean ± standard deviation and were statistically analyzed by one-way ANOVA followed by Tukey's post hoc test to compare differences between two groups. Values of $p < 0.05$ were considered to be statistically significant.

3.3 References

1. Grenier, S.; Sandig, M.; Mequanint, K., Polyurethane biomaterials for fabricating 3D porous scaffolds and supporting vascular cells. *J Biomed Mater Res A* **2007,** 82A, (4), 802-809.
2. Grenier, S.; Sandig, M.; Mequanint, K., Smooth muscle alpha-actin and calponin expression and extracellular matrix production of human coronary artery smooth muscle cells in 3D scaffolds. *Tissue Eng Pt A* **2009,** 15, (10), 3001-11.
3. Liu, H.; Kennard, S.; Lilly, B., NOTCH3 expression is induced in mural cells through an autoregulatory loop that requires endothelial-expressed JAGGED1. *Circ Res* **2009,** 104, (4), 466-75.

Chapter 4 – Results and Discussion

4.1 Gene Expression Levels in HCASMCs and HCAECs from 2D Co-culture

One of the main objectives of this study was to examine possible phenotype changes that HCASMCs may undergo in the presence of HCAECs. Before introducing HCAECs into co-culture studies with HCASMCs, we conducted a series of experiments to determine if an EC secreted molecule, Jagged1, exogenously added to HCASMC cultures could affect the regulation of gene expression leading to phenotype modulation. To this end, a series of experiments that analyzed gene expression in HCASMCs cultured in the presence of soluble Jagged1, immobilized Jagged 1 and EC-surface expressed Jagged1 were compared.

Although it has been detected in arterial SMCs, Jagged1 is a transmembrane protein that is predominantly expressed by VECs[1-4] while the Notch3 receptor is a membrane-bound protein that is expressed in VSMCs. Binding of the Notch receptor with its ligand (Jagged1) results in activation of the Notch signaling pathway and ultimately prompts differentiation in VSMCs[5]. A host of *in vivo* and *in vitro* studies have demonstrated a vital function of Jagged1 in regulating SMC differentiation[2,5-9]. These studies formed the basis for our examination of gene expression levels of Notch3 and various differentiation markers in HCASMCs and HCAECs.

Several reports have demonstrated the ability of soluble Jagged1 to induce differentiation of keratinocytes[10-11], mesenchymal stem cells into cadriomyocytes[12] and

cochlea progenitor cells into primary sensory cells[13]. Other studies have shown contrasting findings in which Notch ligands in immobilized form is a requirement for inducing differentiation[14-17]. Alternatively, considering that the Jagged1 ligand is an EC transmembrane protein, signaling is generally assumed to be mediated by cell-cell contact[18]; hence VSMC phenotype modulation may be contact-dependant[19-20]. If soluble Jagged1 was able to induce contractility in HCASMCs then it could be delivered to cells via culture media during vascular tissue engineering. If, on the other hand, Jagged1-immobilized beads produced a significant enhancement of Notch3 and SMC contractile gene expression, Jagged1-functionalized scaffolds could be designed. Given the complexity of 3D culture systems a 2D model was chosen to simplify and ensure delivery of these molecules to cultured cells.

4.1.1 Effects of Soluble and Immobilized Jagged1 on HCASMC Gene Expression

In the first of these experiments, HCASMCs were cultured on a 2D substrate of tissue culture–treated polystyrene for 48 hours in media containing 5 μg/ml soluble Jagged1. This was followed by RNA extraction for RT-PCR as described in Chapter 3. The role of soluble Jagged1 in HCASMC phenotype modulation was assessed through examination of Notch3, SM-α-actin and calponin gene expression levels. SM-α-actin and calponin are early-to-mid stage SMC differentiation markers that are known to be upregulated in the contractile phenotype[21-22]. SM-α-actin was also chosen because it is a direct target of Notch activity[23-24]. Results from Figure 4.1A-C indicate that soluble Jagged1 was unable to produce any significant changes in terms of HCASMC gene

expression compared with the controls in which HCASMCs were cultured in pure SmGM as well as a second condition consisting of HCASMCs cultured in IgG-supplemented SmGM ($p>0.05$). The IgG control accounts for any non-specific effects of the Fc-fragment found on the Jagged1 protein. These results suggest that Jagged1 protein in and of itself is not sufficient in promoting Notch signaling activity.

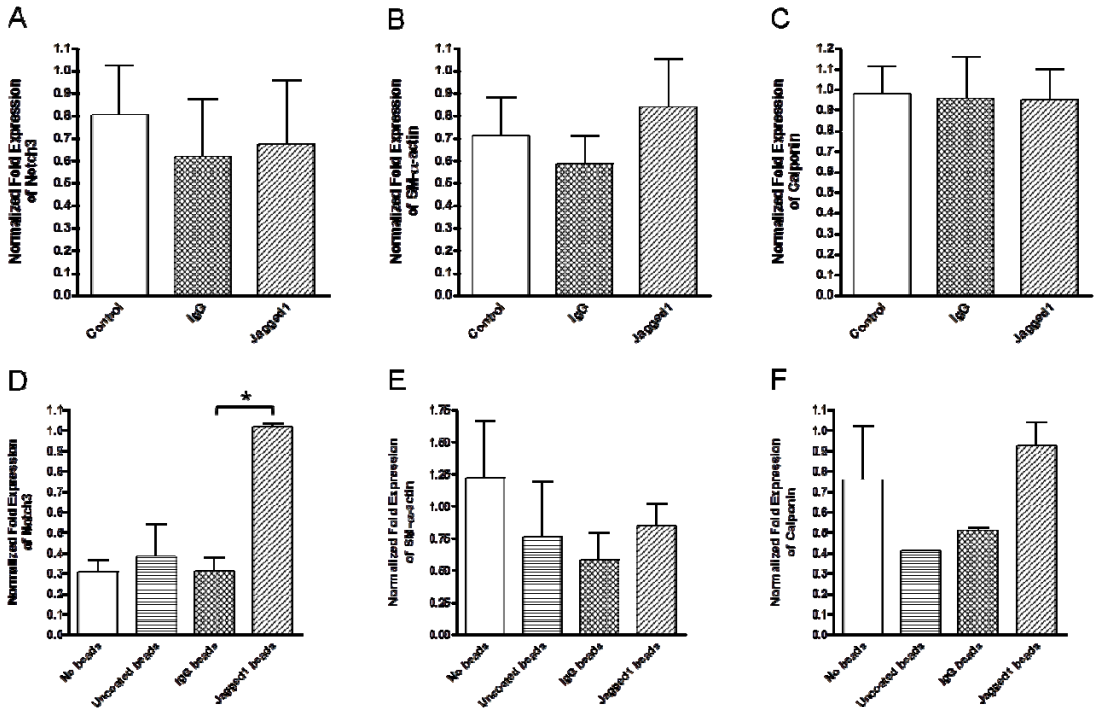

Figure 4.1: Effect of Soluble Jagged1 and Immobilized Jagged1 on Notch3 and HCASMC Gene Expression. HCASMCs were culture for 48 hours with soluble Jagged1 (A-C) or Jagged1-immobilized protein G beads. Gene expression levels of Notch3 (A, D), SM-α-actin (B, E) and Calponin (C, F) are shown. The asterisks indicate statistically significant difference ($p < 0.05$). In Figs A-C, control refers to HCASMCs cultures in pure SmGM.

Since soluble Jagged1 did not affect HCASMC gene expression, the role of Jagged1 immobilized to the surface of protein G beads could influence Notch and SMC contractile gene expression. The reasoning behind this was that the bead surface could potentially mimic a signaling cell surface by presenting the Jagged1 ligand to adjacent

HCASMCs. Furthermore, it would provide insight into biomaterial-mediated Notch3 signaling. Results from Figure 4.1D-F show that although Jagged-immobilized beads were able to significantly upregulate Notch3 gene expression in HCASMCs ($p<0.05$), a corresponding increase in contractile gene expression was not detected ($p>0.05$) which is presumably due to an already high basal level of contractile gene expression. This suggests that other biochemical or structural cues – that are not offered by the Jagged1-immobilized beads – may be necessary to activate the signaling cascade that results in HCASMC differentiation. Alternatively, the upregulation of the Notch3 gene may not translate into Notch3 protein expression which may be elementary in inducing the contractile phenotype in HCASMCs. Nonetheless, these results indicate the need for a model that recapitulates the interactions between Jagged1 and Notch3 *in vivo*. Complementary experiments that support these results are shown in our previous publication[25]. The general finding was that Jagged 1 in soluble or immobilized form is not capable of producing an increase in HCASMC contractile protein expression.

4.1.2 Effect of HCAEC Jagged1 Knockdown on HCASMC Gene Expression

The next step to investigate Notch-induced gene expression in 2D cultures was to examine the effect of EC-bound Jagged1 on Notch activity. In order to do this, a direct co-culture model was chosen to allow for heterotypic cell-cell contact. This type of interaction has been shown to be a requirement for Notch3 activation and subsequent SMC contractile marker expression[2]. Jagged1 was knocked down in HCAECs using

siRNA and was followed by 48hours of co-culture of HCAECs with HCASMCs. Cells were separated using PECAM-coated magnetic beads prior to RNA extraction as described in Chapter 3. Results are shown in Figure 4.2 below.

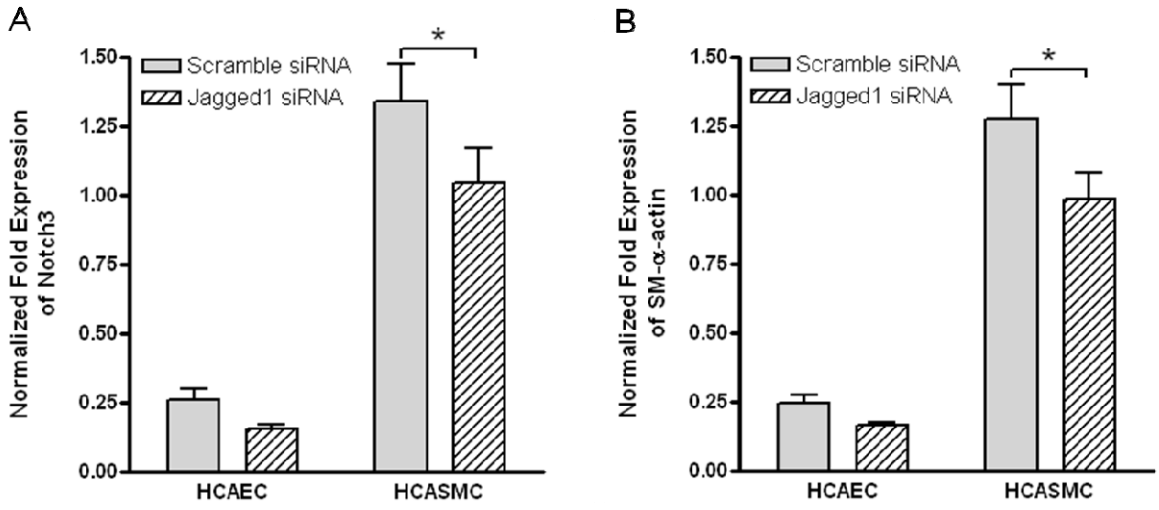

Figure 4.2: Effect of siRNA knockdown of Jagged1 on gene expression levels in co-culture. Expression levels of Notch3 (A) and SM α-actin (B) in HCASMCs and HCAECs co-cultured for 48 hours and subsequently separated using PECAM-conjugated magnetic beads. Asterisks indicate statistically significant data ($p<0.05$).

As is shown in Figure 4.2, siRNA knock down of Jagged1 in HCAECs significantly downregulated the expression of Notch3 in HCASMCs in co-culture ($p<0.05$). This was further translated into a significantly lower expression of SM-α-actin in HCASMCs suggesting a direct link between HCAEC-bound Jagged1 and HCASMC differentiation ($p<0.05$). Unlike immobilized Jagged1, which was able to upregulate the expression of Notch3 in HCASMCs but failed to upregulate contractile marker expression, HCAEC-bound Jagged1 appears to have a direct effect on the expression of Notch3 and SM-α-actin in HCASMCs. Complimentary experiments were performed to examine the effects of HCAEC-bound Jagged1 on HCASMC protein expression. The results, shown in our

recent publication[25], were in agreement with the gene expression data; that is, direct heterocellular cell-cell contact is necessary for HCASMC differentiation via Notch3 signaling. This in turn validates the need for a direct co-culture system in 3D for studying the controlled modulation of HCASMC phenotype. Based on these exploratory experiments, a 3D culture approach for SMCs phenotype modulation is now needed.

4.2 Scaffold Characterization

Scaffolds were fabricated using a SCPL method by the application of pressure to force polymer solution through a tightly packed porogen bed. SCPL was used because it allows for easy control of pore size and porosity[26]. The polymer and porogen of choice were PCU and NH_4CL, respectively. Polyurethanes are among the various natural and synthetic polymers that are commonly used for tissue engineering applications. With regard to vascular tissue engineering, degradable polymers are generally favoured as scaffolding materials; however, traditional polyesters such as PLLA, PGA and their copolymers, although promising, tend to form acidic degradation products *in vivo* that can impact VSMC phenotype[27] and cause inflammation[28]. Protein hydrogels composed of natural polymers such as collagen type I[29], fibrin[30-31] and elastin[32] have displayed a high degree of biocompatibility but generally lack the necessary mechanical properties that are required of a blood vessel substitute[33] even after weeks of *in vitro* maturation[34]. Furthermore, biodegradable scaffolds may prematurely degrade *in vivo* before sufficient graft remodeling has taken place, leading to weak neotissue[35-36]. In this respect, biostable scaffolds are advantageous since they have the capacity to carry mechanical loads

enabling the graft to function immediately upon implantation – a requirement for engineered vascular tissues. Biostable polyurethane elastomers have been extensively used for biomedical and vascular applications due to their acceptable biocompatibility and excellent mechanical characteristics[37-41]. PCU was utilized in this study based on (1) its proven elasticity[42-44], (2) the fact that there are no degradation products that can adversely modulate VSMC behavior and interfere with evaluation of VSMC phenotype[27], (3) the ability to recover cells for biochemical analysis without contamination by scaffold fragments and (4) its capacity for supporting the growth of vascular cells[38,45-46]. Before commencing cell culture studies it was important to first verify the structure of the scaffolds in use since scaffold geometry and pore structure may significantly impact cell infiltration and communication[46]. The structure of the fabricated PCU scaffolds, show in Figure 4.3, was highly porous, uniform and interconnected.

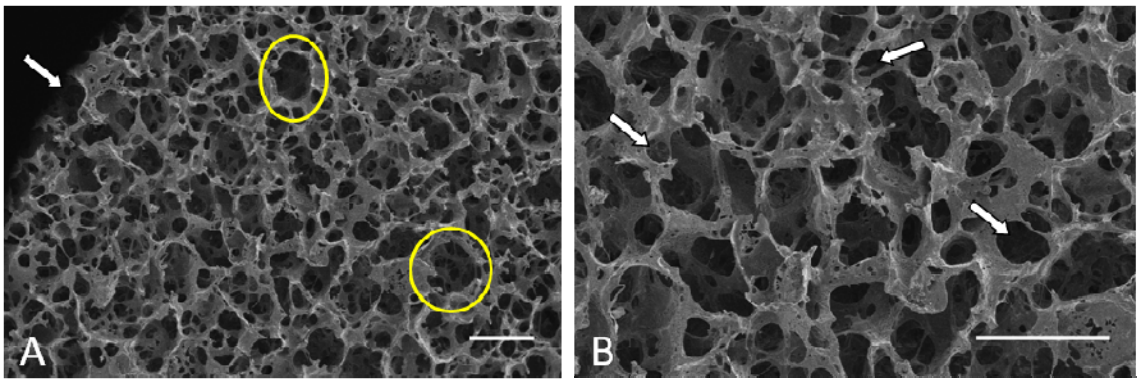

Figure 4.3: Scanning electron micrographs of porous PCU scaffolds. Scanning electron micrographs of scaffolds fabricated from PCU with porogens ranging from 180-212µm in diameter; A) Arrow indicates scaffold edge, pores are highlighted in yellow; B) Arrows indicate interconnecting pores. Scale bar, 250µm.

Since the pores are defined by the x, y and z planes, it was difficult to take an accurate measurement of pore size based solely on qualitative analysis. However, because PCU scaffolds fabricated from the SCPL method were extensively studied by Mequanint and co-workers[46-47] it was not necessary to analyze the pores in depth. The pores outlined in Figure 4.3A gives values ranging from 142μm to 254μm for pore diameter. There are several possible reasons for the variance in pore size. Firstly, the isotropy of the pore dimensions can be attributed to the irregular shape of the ground NH_4Cl particles[46]. Particles having a narrow diameter in one plane but a large diameter in another plane may easily become trapped in the sieve or pass right through depending on the particle's orientation during sieving. As a result, some pore sizes fall outside the expected range of 180-212μm. Secondly, evaporation of the DMF solvent likely results in volume reduction of the scaffold struts; consequently, the pore size increases. The reasoning behind the choice of porogen size was two-fold. First, it has been identified that the optimal pore size range for vascular tissue engineering applications is 150-300μm[48]. Second, a large surface area to volume ratio is necessary for the recruitment and maintenance of a dense cell population[38,49] and a reduction in pore size results in a higher surface area to volume ratio[45]. Thus, particles were chosen from the lower end of the range to meet both of the above criteria. Although porosity calculations were not performed in this study, previous work in our laboratory has shown that PCU scaffolds fabricated using NH_4Cl and SCPL result in structures with 84% porosity – which falls into the optimal range of 80-90%[38]. In comparison to those scaffolds, those used in this study have thinner struts and so the porosity value may actually be higher. Scaffold architecture has been identified as an important factor in facilitating cell-cell and cell-

matrix communication and in providing the necessary mechanical strength. High porosity and large interconnected pores promote tissue ingrowth, vascularization, interstitial fluid flow and diffusion of nutrients and waste[50-52]. Furthermore, cell migration is discouraged if there are no interconnecting pores[53] and a lack of structural uniformity produces inferior mechanical properties[54]. Since the porogen particles were ground and sieved into a narrow size range, a relatively uniform pore size could be achieved. Microporosity is another critical property to be considered. A delicate balance must be struck to limit the microporosity to a reasonable value in order to retain the mechanical integrity of the scaffold. Micropores are essential in allowing capillary ingrowth during *in vivo* remodeling[54]. On the other hand, an overabundance of micropores – specifically those that do not link the macrovoids – can detract from the mechanical strength of the scaffold. This is crucial in the case of vascular grafts since compliance and structural integrity are imperative[48]. SEM images (Figure 4.3) of scaffolds fabricated for this study show the presence of micropores on the order of 16 μm or less. These pores should significantly improve the formation of a 3D tissue by facilitating cell-cell communication, mass transfer and capillary infiltration[55]. Studies previously conducted by our group demonstrated that PCU scaffolds fabricated using NH_4Cl and employing the SCPL method have mechanical properties comparable to that of native blood vessels[47]. Therefore, the degree of microporosity seen here may not adversely affect the strength of the scaffold. The relative rate at which solvent is exchanged for nonsolvent during scaffold fabrication determines the extent of micropore formation[48]; so if required, microporosity can be controlled through careful selection of solvent/ nonsolvent pairs. As demonstrated by these findings, scaffolds constructed for the current study exhibit the

qualities that are essential for vascular tissue engineering applications: they are structurally uniform, highly porous and highly interconnected.

4.3 Optimizing Conditions for Co-Culture

4.3.1 Effect of Varying FN Concentration on HCASMC Attachment and Proliferation

Cell adhesion plays a crucial role in guiding cell fate[56]. In order to promote HCASMC adhesion, PCU scaffolds were made bioactive through surface modification with FN. In native blood vessels FN is a glycoprotein of the ECM that serves many functions. In conjunction with the other components of the ECM, it provides mechanical support and anchorage for the cells, participates in mechanotransduction and guides the biological functions of the tissue under both physiological and pathological conditions[47,57-58]. FN can also bind many ECM molecules into a continuous network and binds cells through interactions between the α5β1 and αvβ3 integrins (transmembrane receptors) and the RGDS (Arg-Gly-Asp-Ser) sequence on the FN molecule[59]. A host of *in vitro* culture studies have shown a correlation between FN and SMC phenotype[60-63]. Indeed the presence of a FN substrate shifts SMCs into a synthetic phenotype by enhancing cell attachment and spreading combined with the formation of an extensive rough endoplasmic reticulum and large Golgi complex and the loss of myofilaments[62]. FN has also shown to have superior results as an adhesion substrate when compared with others such as vitronectin[61]. There is also evidence that FN promotes this behavior in SMCs on 3D substrates. Grenier et al.[47] observed increased infiltration, proliferation and

collagen deposition by HCASMCs on PCU scaffolds compared with uncoated scaffolds. This was the primary justification for functionalizing PCU scaffolds prior to cell culture. The secondary purpose for the addition of FN was to partially recreate the ECM of the arterial media- since the short culture duration is likely insufficient for the formation and deposition of an extensive network of ECM molecules. As the 3D geometry of the scaffold makes it difficult to assess cellular morphology and adhesion characteristics, a 2D substrate was instead used to determine the optimal FN density for enhancing HCASMC attachment. The range of FN concentrations was chosen based on values reported in literature[64-65].

HCASMCs were cultured for 2, 4 or 7 days on FN concentrations of 2, 5 or 10 $\mu g/cm^2$. Cells were more sparsely distributed on a substrate of 2 μg FN/cm^2 (Figure 4.4 A-C) as compared with those cultured on substrates of 5 and 10 μg FN/cm^2 (Figure 4.4 D-I) as evidenced by the black areas (bare coverslip). This is likely due to incomplete coverage of the glass coverslip by FN molecules resulting in cells adhering specifically at sites where the adhesion sequence is detected by integrins. All subsequent experiments for HCASMC monocultures and co-cultures employed FN as a basal adhesion protein at a concentration of 5 $\mu g/cm^2$ or 5 μg/scaffold.

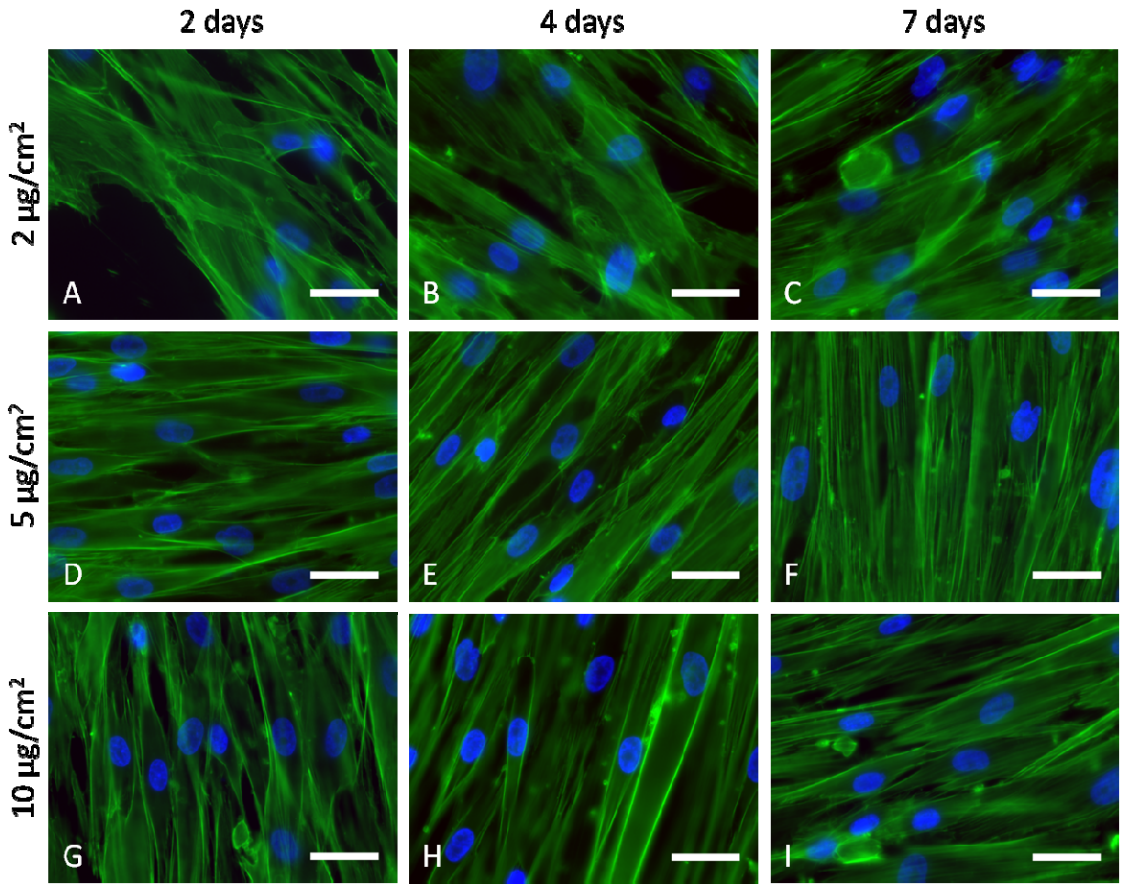

Figure 4.4: HCASMCs grown on varying amounts of FN. Representative Fluorescence microscopy images of HCASMCs cultured on FN coated glass coverslips. HCASMCs were cultured over 2 (A, D, G), 4 (B, E, H), and 7 (C, F, I) days on 2 (A-C), 5 (D-F) or 10 (G-I) µg/cm2 FN coated coverslips. F-actin is stained with Alexa Fluor®488 Phalloidin (green) and nuclei are stained with Hoechst 33342 (blue). Scale bar, 10µm.

It has been acknowledged that scaffold surface area is not 1 cm^2 and that 5 $\mu g/cm^2$ is not analogous to 5 µg/scaffold, nevertheless, it has been proven effective by others[21]. Since porosity measurements were not calculated, the exact surface area that is available for FN adsorption is unknown; as a preliminary experiment it was not necessary to investigate this topic further.

4.3.2 Effect of Growth Media Composition on HCASMC and HCAEC Viability

In addition to determining the optimal FN concentration it was also necessary to establish the optimal media compositions for maintenance of cells in co-culture. ECs and SMCs have different nutrient requirements, hence the need for specific nutrient composition for growth during cell culture. During co-culture of these cells, it was imperative that the media used in maintaining co-cultures not compromise the viability or change the behavior of either cell type. Therefore, monocultures were conducted on 2D substrates of FN-coated glass coverslips for identifying media composition.

As shown in Figure 4.5, HCASMCs exhibited a spindle-like morphology and were able to maintain a dense population irrespective of media composition. On the other hand, HCAECs appear to be more stringent in their requirements. HCAECs from panels D through F in Figure 4.5 displayed a cobblestone morphology which is indicative of a healthy phenotype[65]. However, in panel F, which shows HCAECs cultured in SMC-specific media, HCAECs failed to achieve a high enough density to form cell-cell junctions. Junction formation is critical in achieving two distinct cell layers in co-culture.

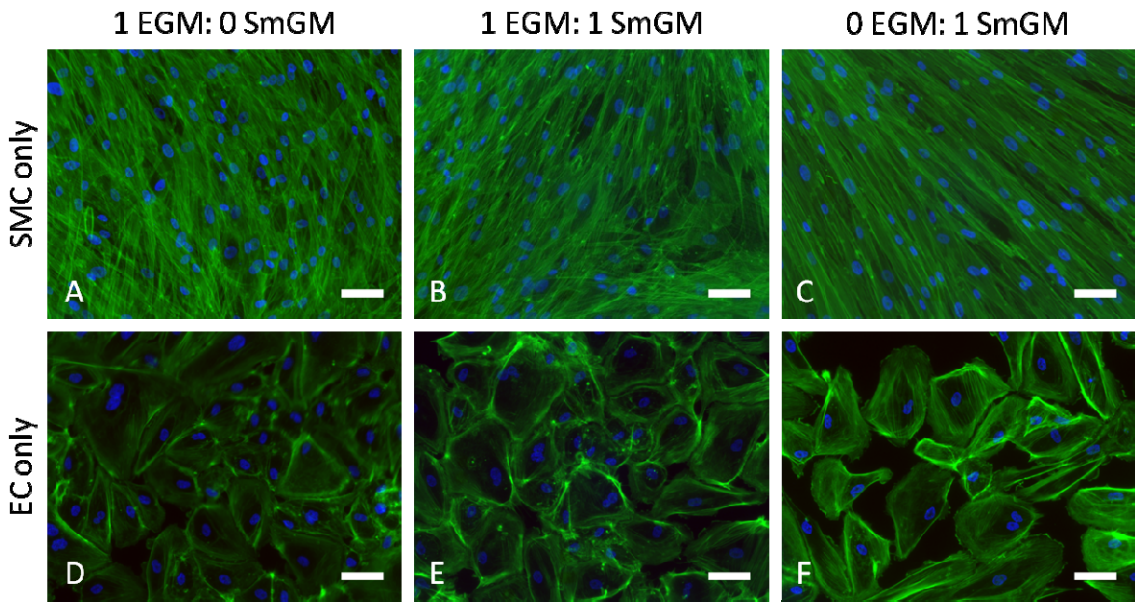

Figure 4.5: HCASMC and HCAEC monocultures in varying growth media compositions. HCASMCs (A-C) and HCAECs (D-F) were cultured alone for 4 and 2 days respectively, in EGM (A, D), 1 part EGM and 1 part SmGM (B, E) or SmGM (C, F). F-actin is stained with Alexa Fluor®488 Phalloidin (green) and nuclei are stained with Hoechst 33342 (blue). Scale bar, 20µm.

HCASMCs may tend to shift to a synthetic phenotype, migrate through the voids and disrupt the HCAEC layer thereby mimicking the injury response commonly seen during atherosclerosis. In an engineered vascular substitute this type of cell behavior would render the construct dysfunctional and upon implantation would result in intimal hyperplasia, stenosis and graft failure[66]. The underlying cause for decreased HCAEC growth in SmGM is likely explained by the absence of essential media components that are found in EC-specific media (EGM). Examination of the constituents of both SmGM and EGM reveals that vascular endothelial growth factor (VEGF), recombinant insulin-like growth factor-1 (IGF-1), ascorbic acid and heparin are absent in SmGM. VEGF is known to have potent mitogenic and angiogenic effects on ECs, a process which is strongly mediated by heparin[67-68]. *In vitro* studies of human umbilical vein ECs have

suggested that IGF-1 molecules present in the blood *in vivo* migrate through the endothelium and act on the subendothelial ECM to have a mitogenic and growth promoting action on ECs. Furthermore, these studies suggest that IGF-1 may be involved in the survival and stability of ECs[69-70]. Finally, the role of ascorbic acid is in the regulation of nitric oxide (NO) bioactivity. *In vivo*, NO is produced by ECs and maintains them in a healthy state. It is also critical for proper control of vascular tone, arterial pressure, platelet adhesion and SMC proliferation. In fact, the manifestation of vascular disease may in part be attributed to impaired NO production[71]. Clearly these components are vital in sustaining ECs in a healthy proliferative state. Therefore, their absence in SmGM is the likely cause for reduced HCAECs density in cultures sustained in this media. These components are also required in the co-culture media to ensure that HCAECs spread to form a continuous monolayer over the HCASMCs to create a structure that mirrors the arterial wall. Thus, it was decided that all subsequent co-cultures would be sustained in growth media composed of equal parts SmGM and EGM since under this condition both HCASMCs and HCAECs were able to proliferate and maintain healthy morphologies.

4.3.3 Long-term HCASMC Monoculture on PCU Scaffolds

The next stage of the study involved examining the interactions between HCASMCs and PCU scaffolds. *In vitro* tissue maturation is generally a lengthy process that spans several weeks. In order for the co-culture model being developed to be applicable in the fabrication of engineered vascular substitutes, PCU scaffolds must be

able to support the growth of vascular cells over long periods of time. Specifically they must enable VSMCs to maintain a healthy state that encourages cell migration, expansion and ECM deposition. This process should culminate in the formation of a vascular media that closely resembles the tunica media of the coronary artery. VECs can then be incorporated to form the tunica intima and regulate the activities of the underlying VSMC layers. Cell retention on PCU scaffolds is also a good indicator of how these cells would behave in an *in vivo* environment; detachment of cells upon implantation may severely compromise the function of a tissue engineered vascular substitute. In view of this, long-term HCASMC monocultures were investigated.

Figure 4.6 shows the degree to which HCASMCs infiltrated PCU scaffolds over an 8 and 16 day period. Z-sections were taken at 20 μm increments through the depth of the scaffold and selected slices are shown at 0 μm, which corresponds to the seeding surface, 40 μm and 140 μm.

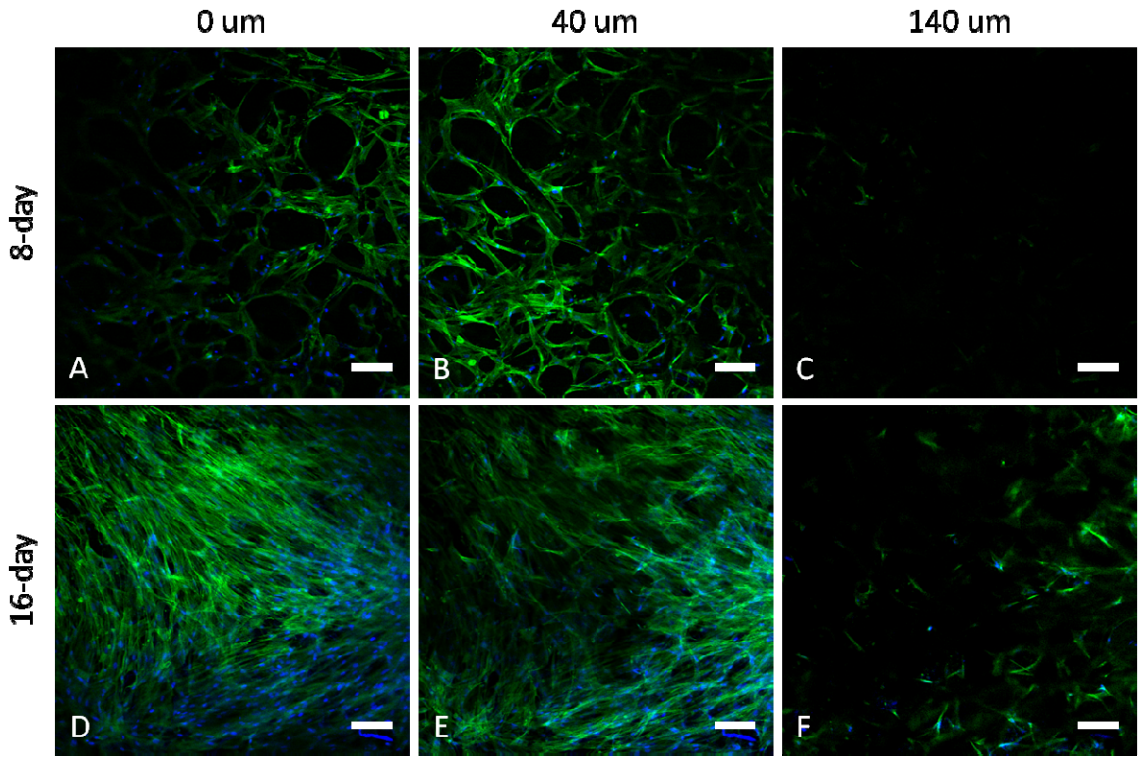

Figure 4.6: Serial optical sections of long-term HCASMC cultures on PCU scaffolds. Images of HCASMCs cultured on PCU scaffolds for 8 (A-C) and 16 (D-F) days at depths of 0 µm (A, D), 40 µm (B, E) and 140 µm (C, F) from the seeding surface. F-actin is stained with Alexa Fluor®488 Phalloidin (green) and nuclei are stained with Hoechst 33342 (blue). Scale bar, 100 µm.

As shown above, HCASMCs adhered to the scaffold struts initially and over time extended cytoplasmic projections across the entire pore diameter. After 16 days of culture, the cell population dramatically increased and pores were no longer visible. Interestingly, a majority of the cell migration and proliferation took place within 40 µm of the seeding surface of the scaffold. It is possible that the adsorbed FN molecules were focused within this section of the scaffold resulting in increased initial HCASMC attachment at these locations. Additionally, because static culture conditions were utilized, the nutrition and oxygen content of the medium likely becomes depleted with

increasing depth through the scaffold, thereby affecting cell growth at greater depths. Although cell infiltration in the vertical direction was slow, a significant difference was observed between day 8 and day 16 of culture; cell density at 140 μm was much greater after 16 days as compared with 8 days of culture. Maximal cell penetration at 16 days of culture was close to 200 μm (Figure Appendix A1); however, only a few cells could be seen. A study by Grenier et al.[45] showed that HCASMCs cultured on PCU scaffolds achieved a maximum depth of 300 μm after 14 days of culture. Similar to the current results, the majority of the cell population resided close to the seeding surface of the scaffold and only one or two cells could be seen at a depth of 300 μm. However, their presence could not with certainty be attributed to active cell migration[45]. For instance, during cell seeding, those cells that show delayed attachment may fall through the pores to a greater depth within the scaffold before they extend adhesion processes. In the current study, images were taken at relatively small intervals so the path of cell migration could be mapped. This confirmed that the presence of cells at 200 μm was a direct result of active cell migration from the focal planes above. It is possible that some cells were present at depths greater than 200 μm, but due to limitations placed by the scaffold thickness or the mounting procedure used, in-focus images could not be taken beyond this point.

A previous work by our research group[46] showed that PCU substrates have the capacity to support HCASMCs for a period of at least 14 days. In this cited study, interactions between HCASMCs and PCU films were examined to eliminate any effects of 3D geometry that PCU scaffolds may impart. Results indicated that HCASMCs do not attach as readily to bare PCU as compared with Matrigel-adsorbed PCU. Furthermore,

after 7 days of culture bare PCU films remained sparse[46]. In a subsequent study[45], interactions between HCASMCs and PCU scaffolds were investigated. In agreement with the prior results, cells did not attach to bare PCU scaffolds as readily as they did to Matrigel- coated PCU scaffolds[45]. Contrary to these results, in the present study, HCASMCs showed no aversion to bare PCU scaffolds. In fact, examination of Confocal images showed no noticeable differences in cell density between bare PCU scaffolds and FN-coated scaffolds after 48 hours of culture (Figure Appendix A2). However, FN was still employed based on 2D culture results. Moreover, Lin et al.[21] noted that HCASMCs readily adhere to bare PCU scaffolds but do not achieve as dense population as they do on FN-coated PCU scaffolds after 7 days. Therefore, although noticeable differences could not be observed over the short culture duration used in the current work, the FN coating would likely have a long-term effect. Prior to HCASMC seeding, FN-adsorbed scaffolds were kept hydrated in HBSS to increase their hydrophilicity – which is known to improve VSMC adhesion, spreading and proliferation[72].

Overall, these results reiterate the value of using PCU scaffolds for vascular tissue engineering. Their ability to support long term growth of HCASMCs is elemental in the creation of vascular grafts and is a prerequisite for the successive seeding of HCAECs. This particular experiment was conducted under static conditions using a relatively low cell density. The seeding of a high HCASMC density and transition to dynamic culture conditions in a bioreactor would likely enhance and expedite their growth and synthesis of ECM molecules by improving oxygen, nutrient and waste exchange and by providing biomechanical cues. In fact experiments involving dynamic culture of HCASCMs on PCU scaffolds have already been executed with positive results[73].

4.3.4 Effect of Varying HCASMC and HCAEC Seeding Density on Co-culture Stability

HCASMCs were seeded at varying densities on PCU scaffolds in order to investigate its effect on scaffold coverage and ultimately HCAEC attachment. High cell densities were chosen since the culture duration was short and so would not provide sufficient time to allow cells to migrate or proliferate extensively. Moreover, low seeding densities (less than $1x10^5$ SMCs/scaffold) reported in other studies[47,74] were not effective in forming a confluent cell layer over the scaffold surface after 4 days of culture so higher cell densities were chosen for the present work. HCASMCs cultured alone at three different densities for 72 hours on PCU scaffolds are shown in Figure 4.7.

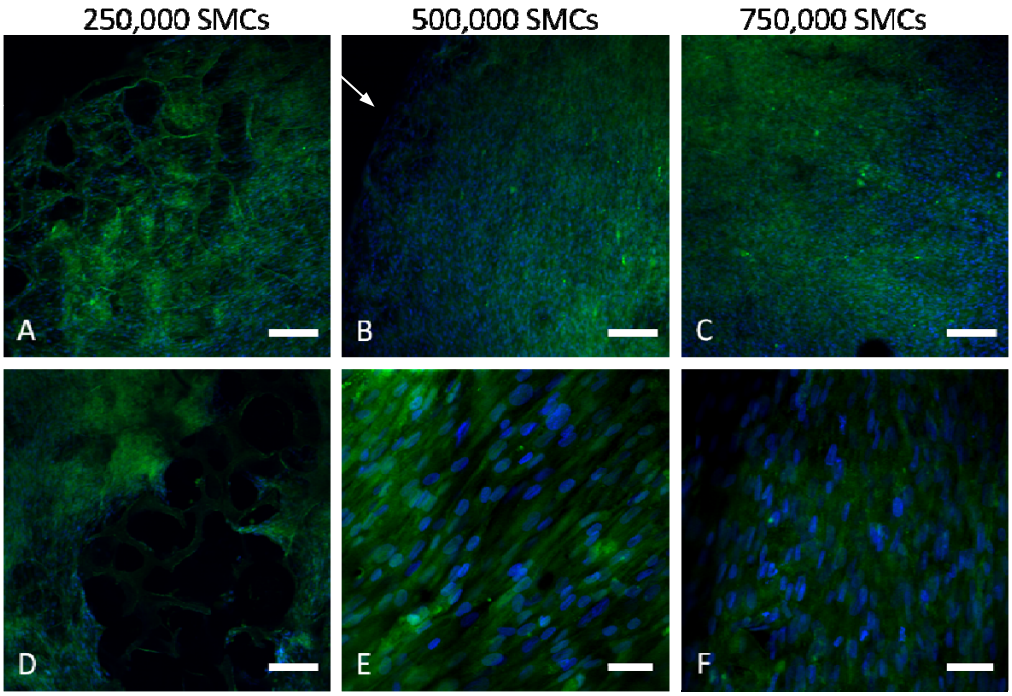

Figure 4.7: HCASMCs cultured at varying initial densities for 72 hours on PCU scaffolds. HCASMCs seeded at initial densities of 250,000/scaffold (A,D), 500,000/scaffold (B,E) and 750,000/scaffold (C,F) shown at 10x (A-D) or 40x (E,F) magnification. Cells are stained with a the cytoskeletal marker CTG (green) and Hoechst 33342 (blue) for the nuclei. Arrows indicate scaffold edge. Scale bars, 200µm (A-D) and 20µm (E, F).

A seeding density of 250,000 cells/scaffold produced scaffolds that were sparse in some areas and moderately confluent in others. At 500,000 cells/scaffold the majority of the scaffold surface was covered by a confluent layer of cells. Some areas such as the scaffold periphery (indicated by arrows in Figure 4.7) had pores that were still visible. Upon further magnification (Figure 4.7E) it can be seen that confluent areas consisted of a single monolayer of cells. In contrast, scaffolds cultured with 750,000 cells had densely packed cells that covered the entire scaffold surface. A corresponding high magnification image (Figure 4.7F) shows that cells were tightly packed together and appeared to have multiple layers making them indistinguishable from one another. The presence of multiple HCASMC layers is also confirmed in Figure 4.8.

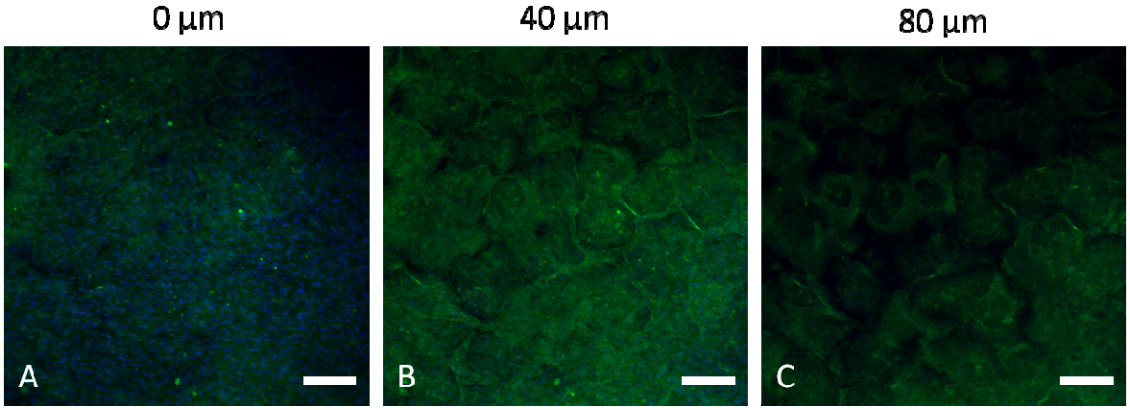

Figure 4.8: Serial optical sections of HCASMC cultured on PCU scaffolds. HCASMCs seeded at an initial density of 750,000/scaffold and cultured for 72 hours on PCU scaffolds. Selected images from a single image stack are shown at depths of 0 µm (A), 40 µm (B) and 80 µm (C) from the seeding surface. Cells are live-stained with a the cytoskeletal marker CTG (green) and Hoechst 33342 (blue) for the nuclei Scale bar, 200 µm.

Panels A through C from Figure 4.8 show cell penetration through the scaffold depth. The high seeding density (750,000 SMCs/scaffold) facilitated the formation of densely packed layers of cells within (at least) the top 80 µm of the scaffold. In confluent

areas cells were aligned in parallel to each other and oriented in coordination with neighboring cells regardless of seeding density; that is, cells throughout the entire surface seem to be aligned in the same general direction. Interestingly, at low cell densities (such as those shown in Figure 4.6) cell orientation was guided by the scaffold struts, whereas at high cell densities, cell alignment was self-guided and coordinated with neighbouring cells. This created a uniform yet crowded environment rather than clusters of cells that were randomly aligned. This rearrangement could be an effort to conserve space as cell density increases and to improve intercellular communication. Unfortunately, the constituent cells from different cell layers were aligned in different directions (Figure Appendix A3) – an issue that would likely be resolved under dynamic culture with mechanical stimulation[75-77]. With respect to morphology, there appeared to be some heterogeneity within the scaffold. At seeding densities of 250,000 SMCs/scaffold, sparse areas of the scaffold contained cells that had a fibroblast-like/ polygonal appearance neighboring cells that had a spindle-shaped appearance. Furthermore, at high seeding densities, cells along the seeding surface had a homogenous spindle-shaped morphology while cells in the underlying layers had a fibroblast-like/ polygonal morphology (Figure Appendix A3). The spindle-shaped and fibroblast-like morphologies are distinguishing characteristics of SMCs in the contractile and synthetic phenotypes respectively[78]. Whereas cells in the contractile phenotype show a very low proliferative index, cells in the synthetic phenotype are highly proliferative, migratory and lay down an abundance of ECM proteins[79-80]. It is possible that the morphological differentiation is induced by the scaffold geometry; however, it is unclear why HCASMCs in the upper levels of the

scaffold undergo this differentiation while cells deep into the scaffold remain in the synthetic phenotype and continue to populate the scaffold.

Confocal images of co-cultured HCASMCs and HCAECs are shown in Figure 4.9. Images are shown deconstructed into their component channels alongside their composite images. Cadherin molecules from cell-cell junctions between individual HCAECs are shown in red, HCASMCs are stained green and nuclei are stained blue. Mild autofluorescence of PCU lends to its visibility in Figure 4.9D and 4.9G.

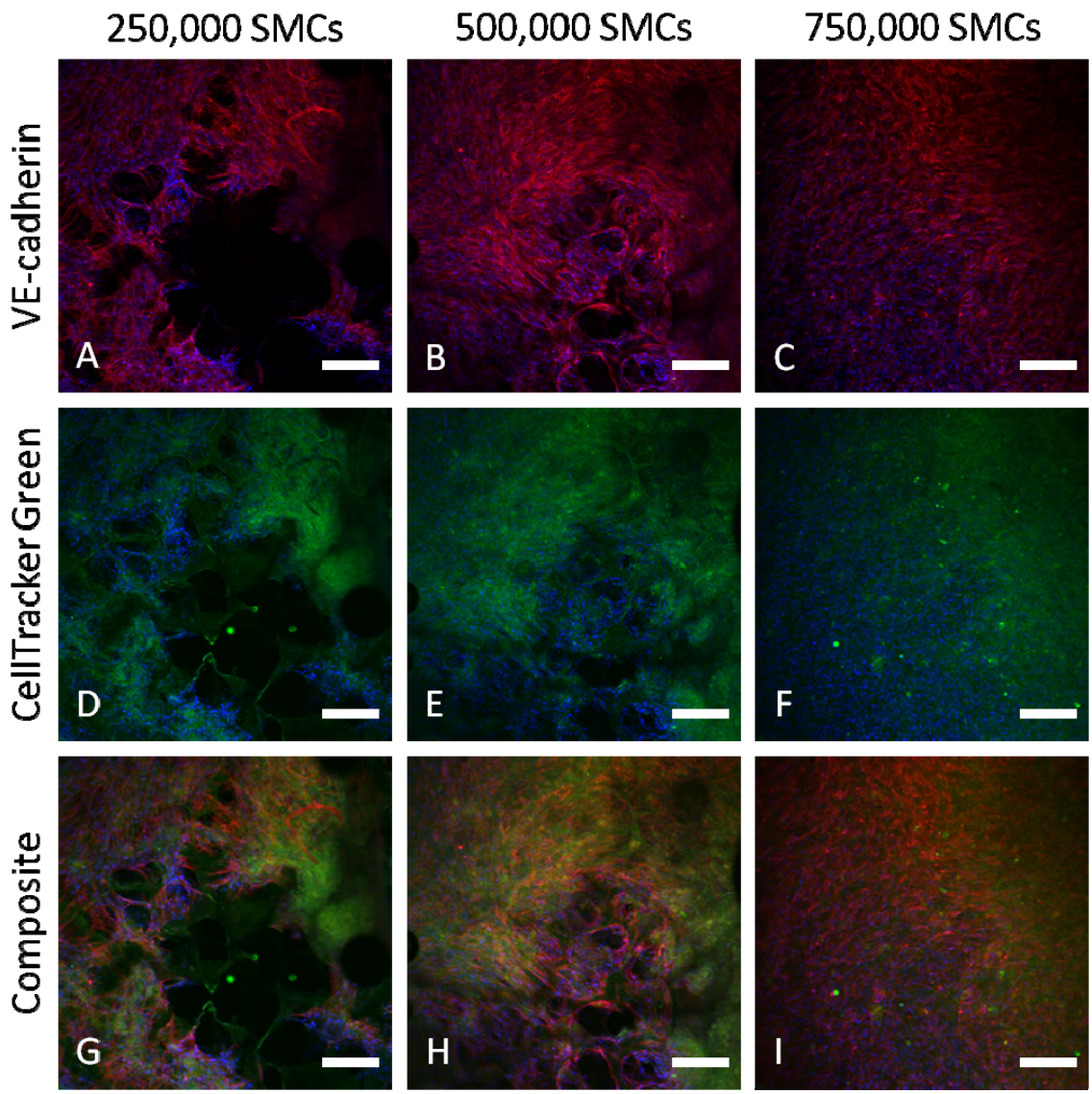

Figure 4.9: HCAECs cultured with varying densities of HCASMCs for 48 hours on PCU scaffolds. HCASMCs seeded at initial densities of 250,000/scaffold (A,D,G), 500,000/scaffold (B,E,H) and 750,000/scaffold (C,F,I). HCAEC-cell junctions are stained with VE-cadherin (red) and HCASMCs are live-stained with the cytoskeletal marker CTG (green).All nuclei are labeled with Hoechst 33342 (blue). All images are at 10x magnification. Scale bar, 200µm.

Intriguingly, HCAECs preferentially adhered to the HCASMCs over the scaffold struts. This is evidenced by the fact that areas where the scaffold appears bare in the red channel (appears black in images) also appear bare in the green channel (appears black or

faint green in images). Bare areas on the scaffold should have molecules of FN adsorbed onto their surface and so should promote HCAEC attachment. However, it is possible that the ECM molecules produced specifically by the HCASMCs provided a better substrate for HCAEC attachment. The HCAECs also seemed to organize themselves into a pattern that very closely follows the HCASMC alignment. This is likely due to the pattern in which the HCASMC-produced matrix molecules were deposited. Not surprisingly then, scaffolds seeded with 750,000 SMCs resulted in full coverage of the scaffold surface by HCAECs. Moreover, the HCAECs formed a confluent layer and no clusters of cells were observed. This observation is in agreement with results obtained by other investigators[81-82]. Wallace et al.[81] reported alignment and arrangement of ECs in a similar pattern as the underlying SMCs over which they were cultured. They found that whereas FN adsorbed onto culture dishes was uniform and diffuse, FN synthesized by SMCs were organized into fibrils along the cell surface. Therefore, they conjectured that the pattern of ECM deposition was responsible for the alignment of ECs with the underlying SMCs[81]. This was later confirmed in a subsequent study[82] where they showed that the primary protein that controls EC attachment in co-culture is fibrillar FN and is mediated by it receptor (the α5β1-integrin complex). It is worth mentioning that co-cultures on subconfluent HCASMCs (Figure 4.9A, D and G) displayed heterogeneous attachment of HCAECs. At higher magnifications these cultures had some areas in which HCAECs were well spread out and aligned with the underlying elongated HCASMCs and other areas in which HCAECs formed clusters over underlying fibroblast-like HCASMCs. Moreover, HCAECs in the latter region: were seen adhering to the scaffold struts, were intermixed with the HCASMC population and had ill-defined junctions

(Figure Appendix A4). Although the phenotype of the HCASMCs had not been confirmed at this point it seems as though the HCAECs preferred to adhere to morphologically differentiated HCASMCs with an elongated, spindle-like morphology. The pattern of FN deposition by the differentiated HCASMCs versus the synthetic HCASMCs is likely responsible for this type of attachment pattern. Chaterji et al.[83] demostrated that differentiated SMCs produce fibrillar FN while proliferating SMCs secrete diffuse FN and that this resulted in uniform attachment of ECs to differentiated SMCs and clustering of ECs on proliferating SMCs. Other reports in literature suggest that uneven topography results in uneven cell spreading and reduced focal adhesions as compared with flat surfaces[84-85]. Together these two factors could account for the preferred attachment of HCAECs to the relatively horizontal topography (of elongated HCASMC) rather than the scaffold struts. It has also been suggested that the deposition of ECM proteins in fibrillar form may be a prerequisite for conserving a quiescent state in ECs over a long period of time[86]. Long-term co-culture stability was not investigated in the present study but the observed arrangement of ECs is akin to that observed in stable long term co-cultures by other investigators. This suggests that the present 3D co-culture model could potentially be sustained for extended periods of time.

To verify the existence of an EC monolayer, high magnification optical sections were taken in the z direction. Images were taken from several different scaffolds to ensure reproducibility and are shown in Figure 4.10. Images were taken at the seeding surface (0 μm) and at increasing depths through the scaffold (10 and 20 μm). Since the HCAECs appear in focus near the seeding surface while the HCASMCs appear in focus

further into the scaffold, it is clear that the HCAECs were residing above the HCASMCs. In addition, the HCAECs were organized into a confluent monolayer.

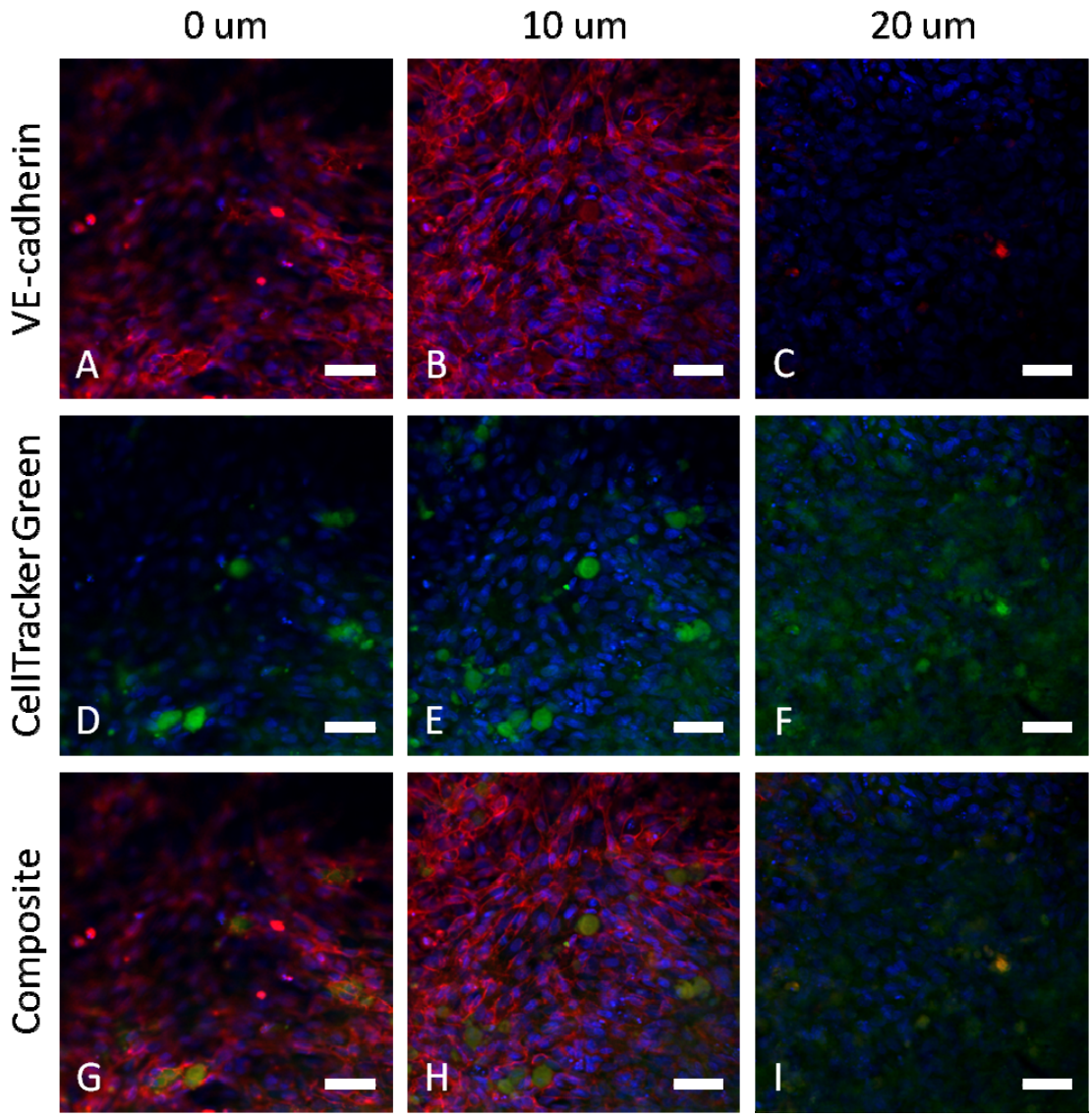

Figure 4.10: Serial optical sections of 750,000 HCASMCs and 200,000 HCAECs co-cultured for 48 hours. Images taken at depths of 10 µm increments starting at the seeding surface (A,D,G) and at increasing depths of 10 µm (B,E,H) and 20 µm (C,F,I). VE-cadherin (red) staining shows HCAEC-cell junctions while HCASMCs are stained with a cytoskeletal marker CTG (green). All nuclei are labeled with Hoechst 33342 (blue). Scale bar, 20µm.

Examination of the bottom right corner of Figure 4.10A shows HCAECs that are in focus. Upon inspection of the same area in Figures 4.10B&C it can be concluded that there were no HCAECs inhabiting that area at 10 or 20 μm depths. Similar observations can be made for all other areas of the images shown in Figure 4.10 thus proving that HCAECs were organized into a single monolayer. Junctions between individual HCAECs were well-defined as demonstrated by the intense VE-cadherin staining. VE-cadherin is a major component of EC-cell junctions and is the primary determinant of contact integrity. Its presence is critical in controlling vascular permeability to cells and other substances. Unlike other EC junction molecules (such as PECAM) cadherins are linked to the actin cytoskeleton through catenins thereby strengthening intercellular contacts. VE-cadherin also mediates cell proliferation through contact inhibition via various signaling processes[87-88]. This suggests that the formation of a confluent monolayer contributes to quiescence in ECs. The presence of intense VE-cadherin staining in the current 3D co-cultures is a positive sign suggesting that the HCAECs are in a healthy quiescent state with strong junctions that inhibit overgrowth of HCASMCs into the HCAEC layer and that the structure as a whole is representative of a native artery. This property is important for tissue engineered vascular substitutes since wide junctional gaps may result in dysfunction causing the tissue to behave as a pathological vessel[66]. The alignment of the underlying HCASMCs in Figure 4.10 is difficult to make out partly due to the high cell density and partly due to the varying directions in which different layers of HCASMCs are aligned. It seems as though some of the HCASMCs were organized perpendicular to the direction of the HCAECs as seen *in vivo*.

Realignment of HCAECs in co-culture has been observed by other investigators[81] as a result of reorganization of the ECM between the two cell layers.

A study by Lavender et al.[65] examining the effects of culturing ECs on proliferating SMCs showed that ECs adhered with lower efficiency and attached as clusters over the underlying SMCs, failing to form a confluent monolayer. In addition, the EC population continuously declined and after 6 days in co-culture, only few cells were left. In comparison ECs seeded onto a confluent, quiescent layer of SMCs attached uniformly and formed a confluent layer that could be sustained for at least 10 days[65]. Chaterji et al.[83] reported similar observations. It should be noted that in both investigations serum withdrawal was used to induce a quiescent, contractile phenotype in SMCs. Although the present study did not induce contractility in the HCASMCs prior to HCAEC seeding, varying HCASMC densities were used to examine their effects on EC attachment. In co-cultures employing sub-confluent HCASMC densities it was observed that although HCAECs preferentially adhered to the underlying HCASMCs, the attachment was non-uniform. Over longer culture durations this could result in HCAEC detachment as well as heterogeneous HCASMC phenotypes as reported in literature[65]. It is also likely that there would be overgrowth of one cell layer into the other resulting in the loss of a distinct layered structure. Therefore, in order to avoid this undesirable outcome, a seeding density of 750,000 – which resulted in multiple dense and confluent layers of HCASMCs - was chosen for subsequent experiments.

Similar experiments were carried out to determine the effect of varying HCAEC seeding density on co-culture stability. A confluent endothelium has important implications for tissue engineered vascular substitutes. *In vivo*, the endothelial monolayer

regulates the transport of molecules into the vascular bed, prevents thrombosis, and regulates the growth and function of the underlying SMCs[89-91]. Loss of an intact endothelium can result in intimal hyperplasia – characterized by migration and proliferation of SMCs[92]. Similar pathologies have been observed in vascular grafts lacking an intact endothelium. This exemplifies the importance of an intact confluent endothelium in the maintenance of a differentiated SMC phenotype. Studies conducted by Chaterji et al.[83] revealed that culturing of SMCs with a subconfluent layer of ECs induced a hyperplastic state and loss of differentiation markers after 48 hours. On the other hand, a confluent layer of ECs permitted co-culture stability for 40 days[83]. In the present work, HCAECs were seeded at densities of 200,000 cells/scaffold or 400,000 cells/scaffold. Since cultures utilizing 200,000 ECs/scaffold were successful in achieving a confluent monolayer over the entire scaffold surface, the data for the experiment involving 400,000 cells/scaffold is not shown. With the exception of one notable difference (described below), the co-culture structure appeared identical irrespective of HCAEC seeding density and so a seeding density of 200,000 ECs/scaffold was used in subsequent experiments. Figure 4.11 shows images taken from co-cultures employing 400,000 ECs/scaffold. Interestingly, there appeared to be tubular structures at the top surface of the scaffold. Though difficult to conclusively assert, some areas of these structures are composed of both HCASMCs and HCAECs and somewhat resemble an inverted artery wall with a very narrow lumen (indicated by arrows in Figure 4.11). A possible explanation for the rearrangement of cells into these structures is the overcrowding of cells resulting from an excess of HCAECs during seeding. By virtue of

the nature of ECs, and as a result of contact inhibition, the excess cells likely preferred arrangement into tubular structures rather than a second monolayer.

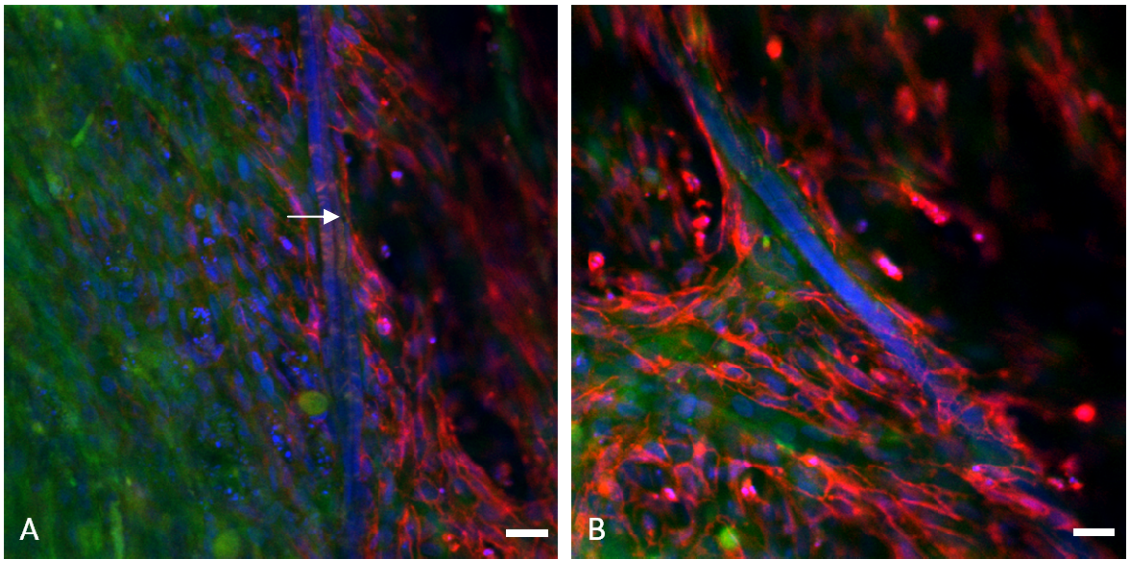

Figure 4.11: Formation of tubular structures in HCASMCs co-cultured with excess HCAECs. Images taken at the seeding surface (0 µm) of scaffolds used for culturing 750,000 HCASMCs and 400,000 HCAECs. HCASMCs are stained with CTG (green) while HCAEC-cell junctions are stained with VE-cadherin (red). All nuclei are labeled with Hoechst 33342 (blue). Arrow indicates the hollow lumen of the tubular structures. Scale bar, 20 µm.

These cells appear to be arranged end-to-end as evidenced by the gaps between individual nuclei; this organization is typically seen in *in vitro* angiogenesis models[93-95]. Another structural feature that was observed in these cultures (and other monocultures and co-cultures) was the hill-and-valley growth pattern of HCASMCs. Because HCAECs (likely) attached to the *in situ* fibrillar FN molecules, they adhered to the underlying HCASMCs regardless of their topography and so the confluence of the HCAEC monolayer was unaffected. Hill-and-valley formation is characteristic of SMCs that have been monocultured and has been noted by several investigators[96-98]. In the cited studies, the hill-and-valley growth pattern was inhibited in co-culture by ECs. However, these

studies utilized a bi-layered co-culture configuration in which ECs and SMCs were cultured on opposite sides of a PET membrane and were cultured together for durations exceeding 48 hours. On the other hand, Powel et al.[96] showed that whereas hill-and-valley formation was arrested in co-cultures using a bi-layer format, the same was not observed in co-cultures using a monolayer format (ECs grown directly over SMCs). It is possible that extended co-culture durations in the present work would result in the rearrangement of cells into a more planar structure but the use of dynamic culturing would likely correct this problem prior to the initiation of co-culture[99].

The formation of multiple confluent SMC layers and a monolayer of ECs are critical in the replication of both the normal arterial structure and physiological functions. *In vivo*, ECs line the innermost surface of the artery wall, creating a barrier between the blood and the subendothelial tissue. This lining consists of a confluent cell monolayer that if disrupted can lead to a pathogenic response such as that seen in atherosclerosis. Therefore, in the current study, it was important to achieve confluent distinct layers of HCAECs and HCASMCs.

4.3.5 Effect of Varying Matrigel Concentration on HCAEC Attachment

Matrigel was incorporated into co-culture constructs in an attempt to mimic the basement membrane found in native blood vessels. *In vivo*, the ECs that line the lumen of the vessel are bound to a substrate of ECM molecules (collagen IV, heparin sulfate proteoglycans and the glycoproteins LN and nidogen/entactin) that comprise the basement membrane[95]. These proteins play a role in maintaining the stability of the

vessel, in regulating EC behavior and in sustaining the ECs in a well-differentiated state[94]. *In vitro* studies have tested the effects of these proteins (alone or in combination) on EC attachment and differentiation. For instance, culturing of rat microvascular ECs on substrates of FN, collagen III or collagen I enhances proliferation while culturing on substrates of LN or collagen IV (basement membrane components) promote attachment and differentiation[94]. Matrigel has been extensively used to promote morphological differentiation of ECs[93-94]. Tubule formation in these and other studies[95] has been attributed to the actions of LN. LN is the primary component of Matrigel followed by collagen IV, heparin sulfate proteoglycans, entactin and nidogen. Therefore, it was hypothesized that a low concentration of Matrigel would enhance HCAEC attachment and would maintain a (quiescent) differentiated state through activities mediated by LN. As per the manufacturer's instructions, a highly diluted solution of Matrigel was applied to bare PCU scaffolds and also to co-cultures in order to avoid HCAEC capillary formation while still promoting cell attachment. The formation of capillaries in this co-culture model would be disadvantageous in that it would firstly create a heterogeneous structure and may secondly result in heterogeneous signaling and cell behaviors- thereby hampering our ability to study the interactions between HCAECs and HCASMCs in direct contact in a controlled manner. Two different dilutions were investigated to determine the extent to which Matrigel promoted HCAEC attachment. HCAEC distribution on Matrigel-coated scaffolds and Matrigel-incorporated co-cultures are shown in the Figures 4.12 and 4.13 respectively.

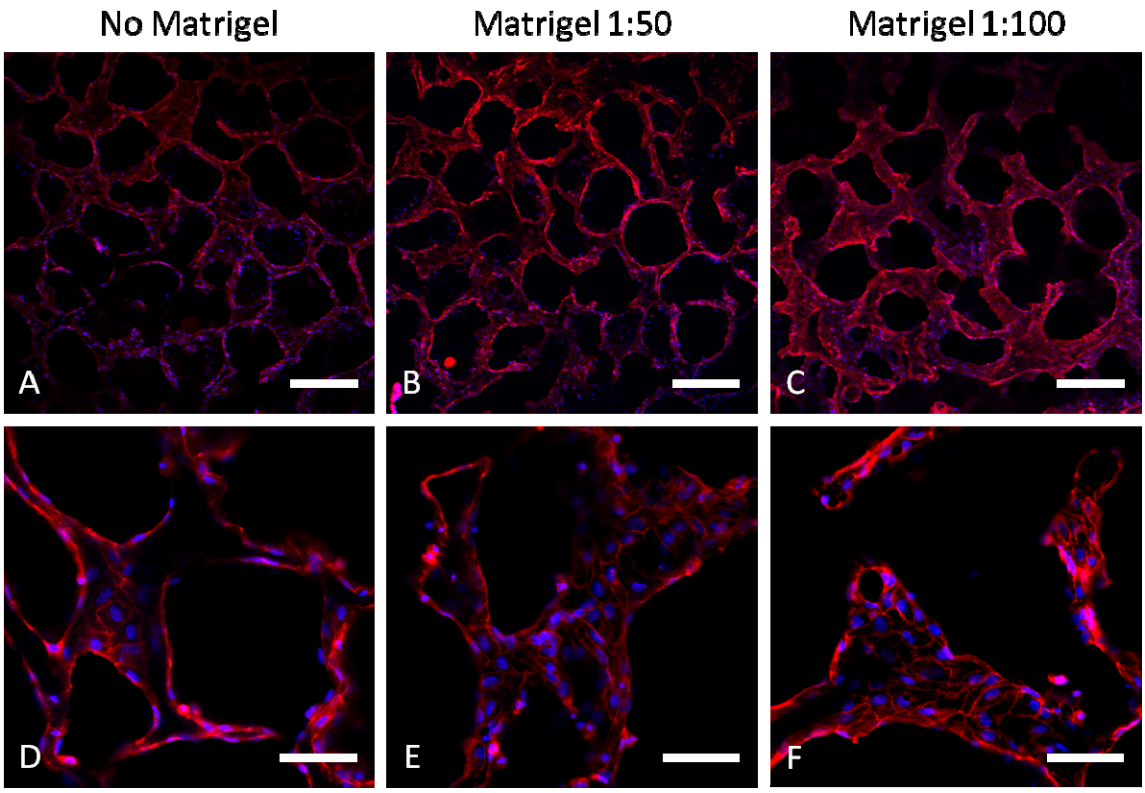

Figure 4.12: Effect of Matrigel on HCAEC attachment to PCU. ECs cultured for 48 hours on PCU scaffolds containing no Matrigel (A,D), Matrigel at 1:50 dilution (B,E) and Matrigel at 1:100 dilution (C,F) at 10x (A-C) and 40x (D-F) magnifications. Cell-cell junctions are stained with VE-cadherin (red) and nuclei are labeled with Hoechst 33342 (blue). Scale bars, 200µm (A-C), and 20µm (D-F).

Images of monocultures show that HCAECs readily adhered to the scaffold in all instances and no areas lacking cells could be seen. On Matrigel-coated scaffolds, cells appeared as though they were more densely packed together so the addition of Matrigel may have mildly promoted cell attachment. In addition, there were a greater number of cells exhibiting intensely stained VE-cadherin based junctions on scaffolds coated with Matrigel. This is not surprising since LN promotes a differentiated phenotype in which ECs form well-defined junctional complexes (a characteristic of native vascular ECs).

When comparing the images from monocultures (Figure 4.12) to images from co-cultures (Figures 4.10 and 4.13) a difference in morphology, alignment and cadherin staining can be discerned. HCAECs cultured alone on scaffolds had a cobblestone shape where as HCAECs cultured over SMCs had an elongated shape. This finding is consistent with literature reports which have shown a shift in EC morphology from cobblestone in monoculture to elongated in co-cultures with differentiated SMCs[65, 81, 83, 100]. Whereas HCAECs showed no particular pattern of alignment in monocultures, co-cultured cells were relatively aligned in the direction of the underlying HCASMCs. As discussed earlier, this could be attributed to the organization of the fibrillar FN that is produced by HCASMCs. Lastly, HCAECs in co-culture stained more intensely for VE-cadherin than HCAECs cultured alone. This observation is also consistent with reported findings where an increase in tight junction formation in co-cultures of ECs and SMCs were observed compared with monocultured ECs[101]. Although tight junctions are not the same as cadherins junctions, they are a measure of contact integrity.

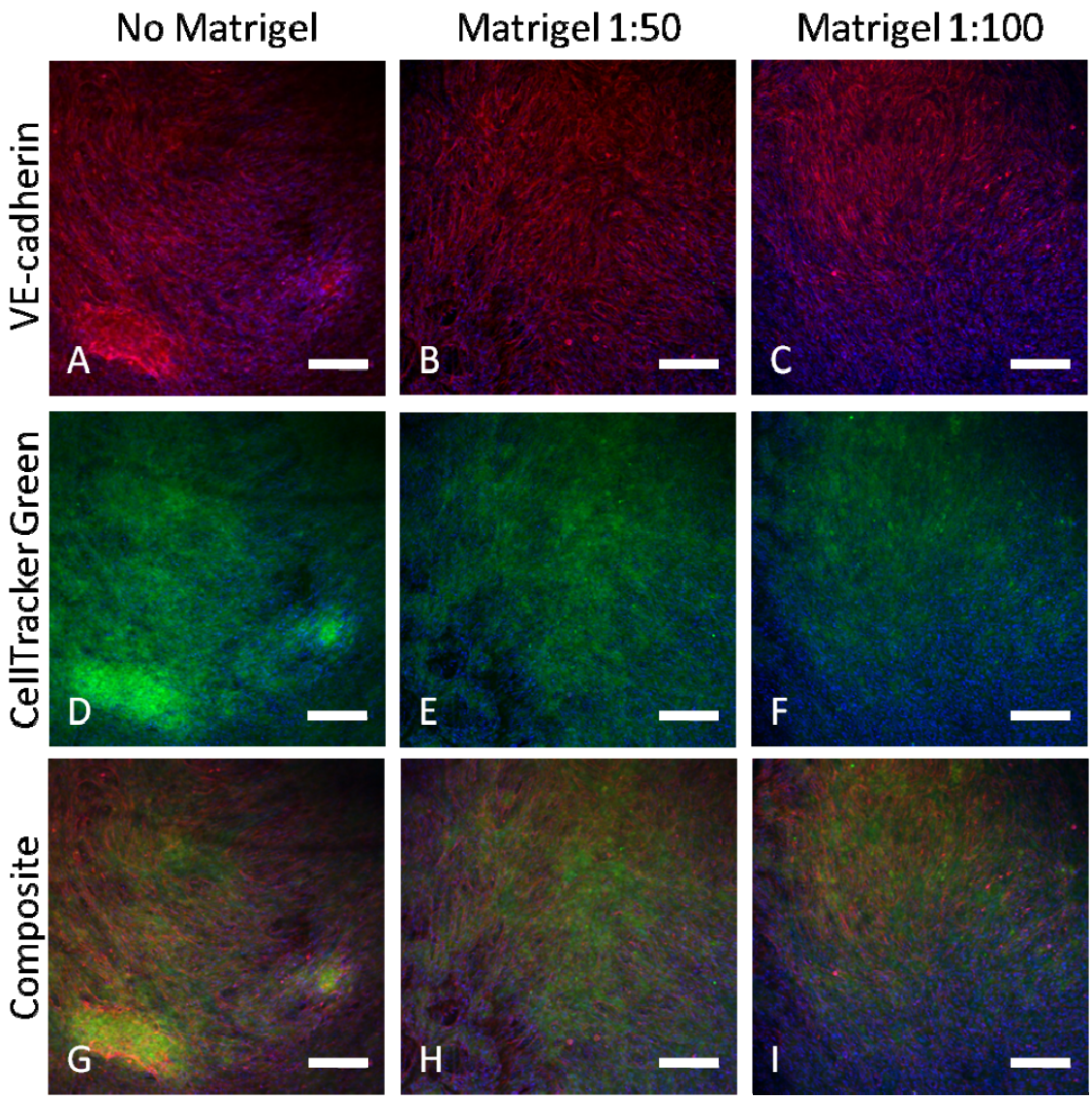

Figure 4.13: Effect of Matrigel on HCAEC attachment in co-culture. HCAECs cultured for 48 hours on HCASMC-scaffold constructs containing no Matrigel (A,D), Matrigel at 1:50 dilution (B,E) and Matrigel at 1:100 dilution (C,F) at 10x (A-C) and 40x (D-F) magnifications. HCAEC-cell junctions are stained with VE-cadherin (red) while HCASMCs are stained with the cytoskeletal marker CTG (green). All nuclei are labeled with Hoechst 33342 (blue). Scale bars, 200μm.

Images of co-culture constructs employing a medial layer of Matrigel are shown in Figure 4.13. Since the effects of Matrigel were not drastic in HCAEC monocultures it was not expected that Matrigel addition in co-culture would have any profound effects.

Consistent with this, no differences could be seen among the three conditions with respect to HCAEC attachment and density. This may not be surprising since it has already been discussed that ECs prefer attachment to extracellular proteins from an in situ source (SMCs) rather than an exogenous source. Moreover, if the HCASMCs have already deposited enough fibrillar FN by the onset of co-culture to achieve full coverage of HCAECs then Matrigel's presence should not have any added benefit (with respect to initial attachment). Matrigel (at a 1:100 dilution) was still incorporated in the experiments to follow since firstly, it did not produce any adverse effects, secondly, it may have had some benefit (such as creating a more extensive medial ECM) that could not be observed through gross examination, and finally, Matrigel may be required to maintain monolayer integrity over longer culture durations[46]. The latter point has been shown to be true in monocultures of ECs on PCU films so it may or may not be applicable to this scenario.

4.3.6 Conditions Resulting in Successful Co-culture

The conclusions of all above experiments are summarized in Table 1 below.

Table 1: Summary of conditions required for successful co-culture

Factor	Preferred condition
Fibronectin concentration	5 μg/scaffold
Co-culture growth media composition	1 part EGM and 1 part SmGM
HCASMC seeding density	750,000 cells/scaffold
HCAEC seeding density	200,000 cells/scaffold
Matrigel dilution	1:100 dilution in growth media

These are the culture conditions that resulted in the formation of a healthy and stable 3D co-culture construct. As mentioned previously, it was important to ascertain these optimal conditions in order to study the interactions between HCAECs and HCASMCs with relevance to the interactions in native states.

4.4 Protein Expression Levels in HCASMCs and HCAECs from 3D Co-culture

One of the main objectives of this study was to examine any phenotype changes that HCASMCs may undergo in the presence of HCAECs. Up until this point, results were largely qualitative observations made based on confocal images. HCASMCs cultured alone on PCU scaffolds had mixed morphologies with cells in the upper layers having an elongated, spindle-like shape characteristic of the contractile phenotype while cells deeper in the scaffold displayed a fibroblast-like morphology characteristic of the synthetic phenotype (Figure Appendix A3). The initiation of co-culture did not produce any noticeable changes in HCASMC morphology. Based on reports from literature, we had hypothesized a phenotypic shift from synthetic in monocultures to contractile in co-cultures[77,97,102]. However, HCASMCs on the seeding surface of the scaffold already displayed a spindle-like morphology and so if the presence of ECs did activate a contractile phenotype shift, morphological changes would not be observed. However, the contractile morphology of the HCASMCs, may not necessarily correlate with the contractile phenotype, that is, the elongated HCASMCs may have been in a non-proliferative state without a simultaneous expression of contractile marker proteins. For

instance, Grenier et al.[47] noticed most HCASMCs cultured on PCU scaffolds to be elongated but cells expressing contractile marker proteins were found next to cells devoid of any contractile marker protein expression. Similarly Lin et al.[21] observed a heterogeneous expression of contractile protein markers in elongated HCASMCs cultured on PCU scaffolds. Therefore, qualitative observations alone cannot be used to definitively ascertain the phenotype of the SMCs. For this reason it was necessary to examine the relative contractile protein expression levels to deduce if there was a differential regulation between HCASMCs in monoculture and HCASMCs in co-culture.

4.4.1 Effect of Co-culture on Protein Expression Levels

At the start of this study we hypothesized that HCASMC phenotype would be modulated from synthetic in monoculture to contractile in co-culture with HCAECs. This speculation was based on reports generated by other investigators[77,97,102]. For example, in a 2D direct co-culture system, Chaterji et al.[83] described a dramatic increase in the expression of SM-α-actin by SMCs in co-culture compared to expression levels in monoculture and a less noticeable increase in calponin. Wallace et al.[81] noticed a distinct upregulation of calponin in co-cultured SMC in a similar 2D direct co-culture system. However, there have not been any studies (that we are aware of) that have focused exclusively on the interactions between SMCs and ECs in a 3D environment in the absence of other stimuli (ie. mechanical or biochemical). Furthermore, the mechanisms regulating their interactions are not well understood. Therefore, in order to create a functional vascular substitute that closely mimics the native artery it is important to

understand the interactions between the individual cellular components of the substitute so that the tissue engineered vascular substitute can be optimized prior to implantation.

In preliminary screening experiments, the cell-scaffold constructs were directly lysed to extract proteins for Western blot analysis. However, this proved to be insufficient because protein lysates from both cell types were mixed in the co-culture condition, thus limiting our ability to determine which cell type was contributing to the expression levels. This led to the use of PECAM –conjugated magnetic beads for the separation of HCAECs from HCASMCs. PECAM is a cell surface molecule that is expressed by ECs, platelets and leukocytes (among others) and is not expressed by SMCs[87]. Cells were first detached using trypsin which has been shown to be effective in removing cells from PCU scaffolds[21,47]. The cell mixture was then incubated with PECAM- conjugated magnetic beads to facilitate binding of HCAECs to the beads. The beads were then separated from the remaining suspension (containing HCASMCs). Separation efficiency was tested to ensure cell extracts were not contaminated to any significant extent that it would limit Western blot analysis. Following SDS-PAGE, membranes were probed for the following proteins: SM-α-actin, calponin, Notch3, Jagged1 and GAPDH. SM-α-actin and calponin are SMC differentiation markers that are known to be upregulated in the contractile phenotype[21-22] while Notch3 and Jagged1 are involved in a mechanism pathway that has been implicated in the regulation of SMC phenotype[2-3,5-6,8-9,103]. GAPDH is a housekeeping gene that is commonly used as an internal control. It is expressed at a relatively constant level regardless of experimental conditions and as such it provides a good method of normalizing quantitative gene/ protein expression data[104]. Normalized protein expression levels are shown with their

corresponding blots in Figure 4.14. Four culture conditions were compared: SMCs cultured alone (SM), SMCs co-cultured and subsequently separated (SM cc), ECs co-cultured and subsequently separated (EC cc) and ECs cultured alone (EC).

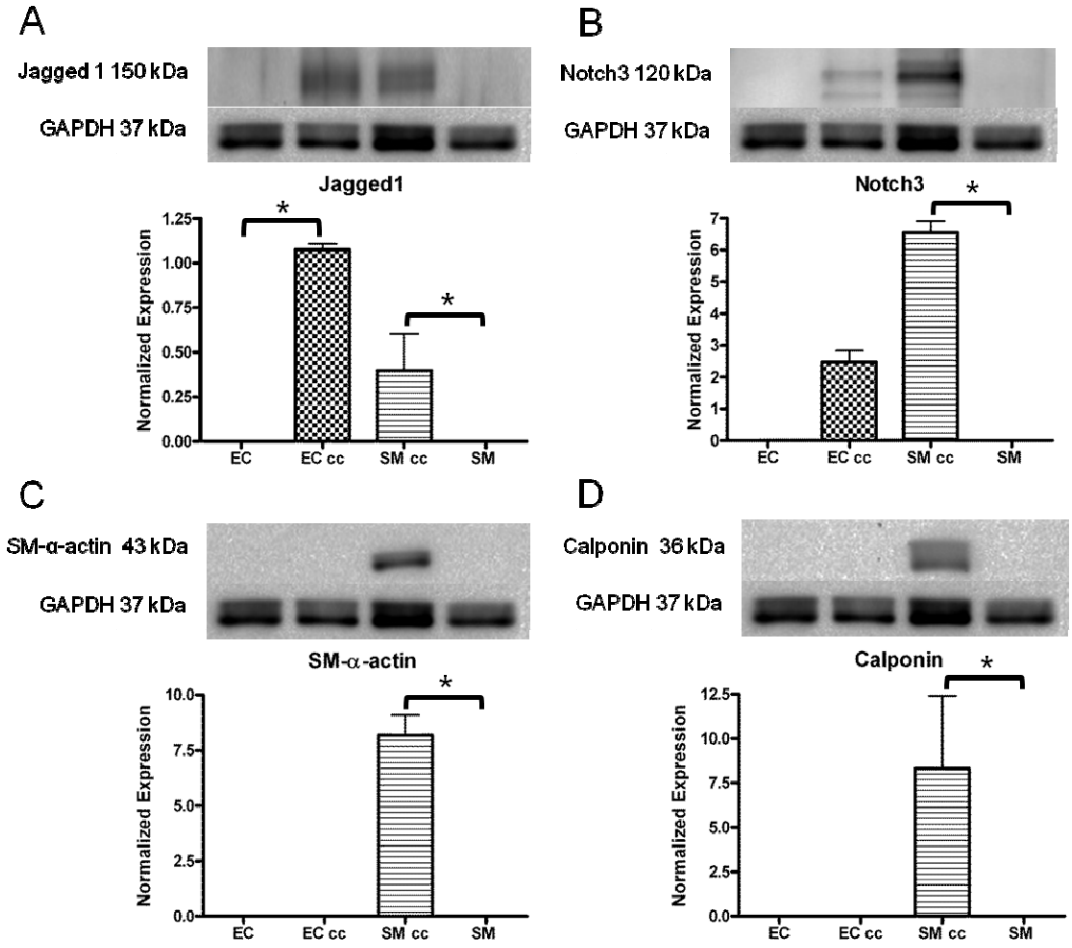

Figure 4.14: HCASMC and HCAEC protein expressions levels after 48 hours of co-culture on PCU scaffolds. Expression levels of Jagged1 (A), Notch 3 (B), SM α-actin (C) and Calponin (D) in HCASMCs and HCAECs cultured alone or together. Asterisks indicate statistically significant data ($p<0.05$). Nomencalture: EC (HCAECs from monoculture), EC cc (HCAECs in co-culture with HCASMCs and separated), SMC cc (HCASMCs in co-culture HCAECs and separated) and SM (HCASMCs from monoculture).

When comparing HCASMCs cultured alone to HCASMCs from co-culture it is clear that co-culture induced a significant upregulation in contractile proteins ($p<0.05$).

Surprisingly, even basal levels of contractile proteins could not be detected in HCASMCs cultured alone. SM-α-actin and calponin are known to be present in the early to mid stages of VSMC differentiation[25,105-106]. This suggests that even after 4 days of HCASMC monoculture cells either did not begin a transition to the contractile phenotype or that very small populations of cells were expressing contractile markers in monoculture but these proteins were not at a high enough concentration to allow detection. Previous work involving the culture of HCASMCs on PCU scaffolds described some expression of contractile protein markers[21,47]. However, these studies involved longer culture durations or much larger samples from which proteins were collected. The absence of SM-α-actin and calponin from HCAECs in co-culture indicates that the separation protocol used was highly efficient.

As part of the hypothesis that was earlier stated, we also speculated that the any observed modulations in HCASMC phenotype may in part be due to the actions of Notch3 and Jagged1. The Notch family of membrane-bound receptors has been shown to play a critical role in vascular development and remodeling. These receptors are part of a short-range signaling pathway that involves membrane-bound receptors and ligands that are expressed on adjacent cells[5]. Four Notch receptors (Notch1 to 4) and their ligands (Delta-like 1 and 2 and Jagged1 and 2) are expressed in ECs and SMCs in differing combinations[2]. Notch 3 is the predominant receptor that is expressed in VSMCs while Jagged1 is prominently expressed by vascular ECs and has also been detected in arterial SMCs[1-4]. Their crucial roles are exemplified in diseases such as Alagille syndrome and CADASIL which are consequences of mutations in Jagged1 and Notch3[3]. Binding of the Notch receptor with its ligand (Jagged1) results in proteolytic cleavage of the receptor's

intracellular domain which then translocates to the nucleus. This signal transduction pathway involves several effectors and ultimately prompts differentiation in VSMCs[5]. A host of *in vivo* and *in vitro* studies have demonstrated a vital function of Jagged1 in regulating SMC differentiation[2,5-9]. These studies formed the basis for our examination of expression levels of Notch3 and Jagged1 in HCAEC and HCASMCs.

As shown in Figure 4.14, expression of both Notch3 and Jagged1 were upregulated in co-culture, indicated by the blots and the corresponding bar graph. As expected, Jagged1 was predominantly expressed by HCAECs and to a lesser but detectable degree by HCASMCs. Unexpectedly, there was also no basal expression of Notch 3 or Jagged1 in monocultures. In the case of Jagged1, the differential expression was not as dramatic as in the other proteins. This is evidenced by the intensity of the bands on the Western blots as well as the scales used in the graphs. The absence of Jagged1 and Notch3 in monocultures was not in agreement with findings reported in literature. For instance, in a study by Ying et al.[25] Jagged1 and in particular Notch 3 were noticeably expressed in HCAECs and HCASMCs respectively. Similarly Liu et al.[2] noticed a robust basal expression of Notch3 and Jagged1 in HCASMCs. In both cases, the expression of Notch3 in monocultured SMCs was accompanied by detectable amounts of SM-α-actin and calponin. In spite of these differences, the general trend in the current study was similar to that seen in the aforementioned work; co-culture augmented the expression of all four target proteins: Jagged1, Notch3, SM-α-actin and calponin.

4.4.2 Effect of siRNA Knockdown of Jagged1 on Protein Expression Levels

At this point, no correlations between the target proteins could be inferred from the data. Therefore, the next logical step was to abrogate a component of the Notch signaling pathway and observe the downstream effects on differentiation markers. To this end, we knocked down the expression of Jagged1 in HCAECs using siRNA. It has previously been shown that EC derived Jagged1 and a model that enables heterotypic cell-cell contact are both requisites for enhancing Notch3 expression and subsequent contractile protein expressions[25]. One day prior to the initiation of co-culture, HACECs were transfected with either Jagged1 siRNA or control siRNA. 20 hours later, HCAECs were seeded over HCASMCs and cultured for 48 hours as per the co-culture protocol described in Chapter 3. Transfection efficiency was tested to ensure sufficient knockdown of Jagged1 in HCAECs.

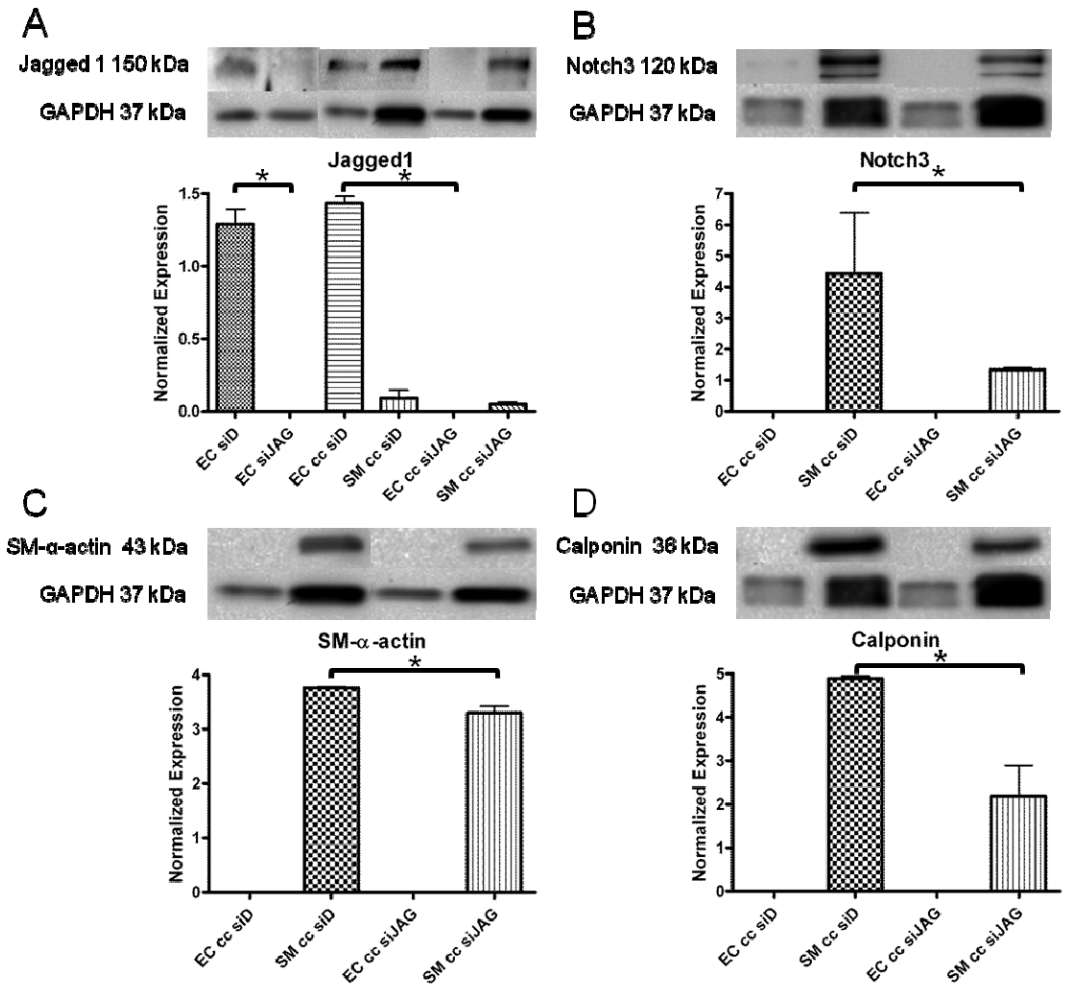

Figure 4.15: Effect of siRNA knockdown of Jagged1 on protein expression levels in co-culture. Expression levels of Jagged1 (A), Notch3 (B), SM α-actin (C) and calponin (D) in HCASMCs and HCAECs cultured alone or together. Asterisks indicate statistically significant data ($p<0.05$). Nomenclature: EC siD (control siRNA-transfected HCAECs in monoculture), EC siJAG (Jagged1 siRNA-transfected HCAECs in monoculture), EC cc siD (control siRNA-transfected HCAECs in co-culture with SMCs and separated), SM cc siD (HCASMCs co-cultured with EC siD and separated), EC cc siJAG (Jagged1 siRNA-transfected HCAECs in co-culture with SMCs and separated) and SM cc siJAG (HCASMCs co-cultured with EC siJAG and separated)

As shown in the Figure 4.15 Jagged1 in siRNA transfected HCAECs was not detected in neither monoculture nor co-culture indicating successful knockdown of Jagged1. As expected, the result of culturing HCASMCs with Jagged1 siRNA transfected- HCAECs was a significant (approximately three-fold) downregulation of

Notch3 in HCASMCs. Interestingly, although the reduction in SM-α-actin expression was significant it was not as marked. This was surprising since Notch has been shown to directly target SM-α-actin expression[23].In comparison the reduction in calponin expression was more than two-fold. Nonetheless, it is possible that because Notch3 expression was not entirely diminished in co-culture with Jagged1 siRNA-transfected HCAECs, a low level of Notch activation was sufficient in propagating its own expression and that of α-actin. Using a series of Notch pathway inhibitors and activators, Liu et al. showed that Notch3 receptor activation is essential in promoting further Notch3 expression. They suggested that initial Notch3 activation by Jagged1-presenting ECs initiates an autoregulatory positive feedback loop in SMCs whereby the SMCs increase expression of Notch3 and Jagged1, thus maintaining themselves in a differentiated state[2]. Hence, the sustained expression of Notch3 and SM-α-actin in the present experiment may be a result of initial Notch3 activation by a few Jagged1-expressing HCAECs and subsequent amplification of this signal by homotypic cell-cell interactions between HCASMCs that are expressing Notch3 and neighboring HCASMCs that are expressing Jagged1. It may be of interest to examine if the expression of these proteins is time-dependant. Perhaps the expression of both Notch3 and SM-α-actin are considerably abrogated within the first few hours of co-culture but are recovered overtime. Furthermore, because SM-α-actin expression precedes that of calponin during the process of SMC differentiation, calponin expression may be recovered more slowly. Alternatively, it is possible that other regulatory mechanisms were in place that counteracted the downregulation of SM-α-actin. For instance, the protein kinase A and phosphoinositide 3-kinase/Akt pathways have also been implicated in EC-induced

VSMC differentiation. Fetalvero et al.[107] reported an increase in SM-α-actin, calponin, MHY and h-caldesmon expression in VSMCs cultured with iloprost. They showed that iloprost activated the VSMC-expressed prostacylin receptor and in turn activated the protein kinase A pathway. Iloprost is an analog of prostacyclin which is an EC-derived molecule that is important in the maintenance of a differentiated VSMC phenotype *in vivo*[107]. In a bilayered EC-SMC co-culture model, Brown et al.[108] demonstrated that bovine aortic ECs augmented the expression of differentiation markers in bovine aortic SMCs through activation of the phosphoinositide 3-kinase/Akt signaling pathway in SMCs. Therefore, these signaling pathways are likely activated simultaneously by EC-expressed factors so disruption of one pathway may not result in complete suppression of VSMC differentiation markers. This is especially true if the signal strength of one of these pathways is far greater than the signal strength of another's. A comparative study to determine the extent to which each of these pathways plays a role in HCASMC differentiation may be helpful in revealing the optimal method through which the phenotype of these can be modulated. Although it has been shown, in the present study and in others, that direct co-culture of VECs and VSMCs is a requirement for Notch signaling, the same may not be true for Akt activation[108] or prostacylin receptor activation[107]. Incorporation of multiple factors into PCU scaffolds and into the co-culture model described in this study could conceptually have a synergistic effect on HCASMCs differentiation.

4.4 References

1. Ghosh, S.; Paez-Cortez, J. R.; Boppidi, K.; Vasconcelos, M.; Roy, M.; Cardoso, W.; Ai, X.; Fine, A., Activation dynamics and signaling properties of Notch3 receptor in the developing pulmonary artery. *J Biol Chem* **2011,** 286, (25), 22678-87.

2. Liu, H.; Kennard, S.; Lilly, B., NOTCH3 expression is induced in mural cells through an autoregulatory loop that requires endothelial-expressed JAGGED1. *Circ Res* **2009,** 104, (4), 466-75.

3. Iso, T.; Hamamori, Y.; Kedes, L., Notch signaling in vascular development. *Arterioscl Throm Vas* **2003,** 23, (4), 543-53.

4. Villa, N.; Walker, L.; Lindsell, C. E.; Gasson, J.; Iruela-Arispe, M. L.; Weinmaster, G., Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mech Develop* **2001,** 108, (1-2), 161-4.

5. High, F. A.; Lu, M. M.; Pear, W. S.; Loomes, K. M.; Kaestner, K. H.; Epstein, J. A., Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. *P Natl Acad Sci USA* **2008,** 105, (6), 1955-9.

6. Caolo, V.; Schulten, H. M.; Zhuang, Z. W.; Murakami, M.; Wagenaar, A.; Verbruggen, S.; Molin, D. G.; Post, M. J., Soluble Jagged-1 inhibits neointima formation by attenuating Notch-Herp2 signaling. *Arterioscl Throm Vas* **2011,** 31, (5), 1059-65.

7. Doi, H.; Iso, T.; Shiba, Y.; Sato, H.; Yamazaki, M.; Oyama, Y.; Akiyama, H.; Tanaka, T.; Tomita, T.; Arai, M.; Takahashi, M.; Ikeda, U.; Kurabayashi, M., Notch signaling regulates the differentiation of bone marrow-derived cells into smooth muscle-like cells during arterial lesion formation. *Biochem Bioph Res Co* **2009,** 381, (4), 654-9.

8. Tang, Y.; Urs, S.; Boucher, J.; Bernaiche, T.; Venkatesh, D.; Spicer, D. B.; Vary, C. P.; Liaw, L., Notch and transforming growth factor-beta (TGFbeta) signaling pathways cooperatively regulate vascular smooth muscle cell differentiation. *J Biol Chem* **2010,** 285, (23), 17556-63.

9. Wu, X.; Zou, Y.; Zhou, Q.; Huang, L.; Gong, H.; Sun, A.; Tateno, K.; Katsube, K.; Radtke, F.; Ge, J.; Minamino, T.; Komuro, I., Role of Jagged1 in arterial lesions after vascular injury. *Arterioscl Throm Vas* **2011,** 31, (9), 2000-6.

10. Aho, S., Soluble form of Jagged1: Unique product of epithelial keratinocytes and a regulator of keratinocyte differentiation. *J Cell Biochem* **2004,** 92, (6), 1271-1281.

11. Nickoloff, B. J.; Qin, J. Z.; Chaturvedi, V.; Denning, M. F.; Bonish, B.; Miele, L., Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. *Cell Death Differ* **2002,** 9, (8), 842-55.

12. Li, H.; Yu, B.; Zhang, Y.; Pan, Z.; Xu, W., Jagged1 protein enhances the differentiation of mesenchymal stem cells into cardiomyocytes. *Biochem Bioph Res Co* **2006,** 341, (2), 320-5.

13. Savary, E.; Sabourin, J. C.; Santo, J.; Hugnot, J. P.; Chabbert, C.; Van De Water, T.; Uziel, A.; Zine, A., Cochlear stem/progenitor cells from a postnatal cochlea respond to Jagged1 and demonstrate that notch signaling promotes sphere formation and sensory potential. *Mech Develop* **2008,** 125, (8), 674-86.

14. Beckstead, B. L.; Santosa, D. M.; Giachelli, C. M., Mimicking cell-cell interactions at the biomaterial-cell interface for control of stem cell differentiation. *J Biomed Mater Res A* **2006,** 79A, (1), 94-103.

15. Beckstead, B. L.; Tung, J. C.; Liang, K. J.; Tavakkol, Z.; Usui, M. L.; Olerud, J. E.; Giachelli, C. M., Methods to promote Notch signaling at the biomaterial interface and evaluation in a rafted organ culture model. *J Biomed Mater Res A* **2009,** 91A, (2), 436-446.

16. Goncalves, R. M.; Martins, M. C. L.; Almeida-Porada, G.; Barbosa, M. A., Induction of notch signaling by immobilization of jagged-1 on self-assembled monolayers. *Biomaterials* **2009,** 30, (36), 6879-6887.

17. Varnum-Finney, B.; Wu, L.; Yu, M.; Brashem-Stein, C.; Staats, S.; Flowers, D.; Griffin, J. D.; Bernstein, I. D., Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. *J Cell Sci* **2000,** 113 Pt 23, 4313-8.

18. Kume, T., Novel insights into the differential functions of Notch ligands in vascular formation. *J Angiogenes Res* **2009,** 1, 8.

19. Ray, L. B., Endocytotic notch signal. In *Sci. Signal*, 2010; Vol. 3, p ec235.

20. Regan, J. N.; Majesky, M. W., Building a vessel wall with notch signaling. *Circ Res* **2009,** 104, (4), 419-21.

21. Lin, S.; Sandig, M.; Mequanint, K., Three-dimensional topography of synthetic scaffolds induces elastin synthesis by human coronary artery smooth muscle cells. *Tissue Eng Pt A* **2011,** 17, (11-12), 1561-71.

22. Manabe, I.; Nagai, R., Regulation of smooth muscle phenotype. *Curr Atheroscler Rep* **2003,** 5, (3), 214-22.

23. Noseda, M.; Fu, Y.; Niessen, K.; Wong, F.; Chang, L.; McLean, G.; Karsan, A., Smooth Muscle alpha-actin is a direct target of Notch/CSL. *Circ Res* **2006,** 98, (12), 1468-70.

24. Tang, Y.; Urs, S.; Liaw, L., Hairy-related transcription factors inhibit Notch-induced smooth muscle alpha-actin expression by interfering with Notch intracellular domain/CBF-1 complex interaction with the CBF-1-binding site. *Circ Res* **2008,** 102, (6), 661-8.

25. Xia, Y.; Bhattacharyya, A.; Roszell, E. E.; Sandig, M.; Mequanint, K., The role of endothelial cell-bound Jagged1 in Notch3-induced human coronary artery smooth muscle cell differentiation. *Biomaterials* **2012,** 33, (8), 2462-72.

26. Reignier, J.; Huneault, M. A., Preparation of interconnected poly(epsilon-caprolactone) porous scaffolds by a combination of polymer and salt particulate leaching. *Polymer* **2006,** 47, (13), 4703-4717.

27. Higgins, S. P.; Solan, A. K.; Niklason, L. E., Effects of polyglycolic acid on porcine smooth muscle cell growth and differentiation. *J Biomed Mater Res A* **2003,** 67, (1), 295-302.

28. Mendelson, K.; Schoen, F. J., Heart valve tissue engineering: concepts, approaches, progress, and challenges. *Ann Biomed Eng* **2006,** 34, (12), 1799-819.

29. Weinberg, C. B.; Bell, E., A blood vessel model constructed from collagen and cultured vascular cells. *Science* **1986,** 231, (4736), 397-400.

30. Grassl, E. D.; Oegema, T. R.; Tranquillo, R. T., Fibrin as an alternative biopolymer to type-I collagen for the fabrication of a media equivalent. *J Biomed Mater Res* **2002,** 60, (4), 607-612.

31. Swartz, D. D.; Russell, J. A.; Andreadis, S. T., Engineering of fibrin-based functional and implantable small-diameter blood vessels. *Am J Physiol-Heart C* **2005,** 288, (3), H1451-H1460.

32. Leach, J. B.; Wolinsky, J. B.; Stone, P. J.; Wong, J. Y., Crosslinked alpha-elastin biomaterials: towards a processable elastin mimetic scaffold. *Acta Biomater* **2005,** 1, (2), 155-164.

33. Stegemann, J. P.; Kaszuba, S. N.; Rowe, S. L., Review: Advances in vascular tissue engineering using protein-based Biomaterials. *Tissue Eng* **2007,** 13, (11), 2601-2613.

34. Patel, A.; Fine, B.; Sandig, M.; Mequanint, K., Elastin biosynthesis: The missing link in tissue-engineered blood vessels. *Cardiovasc Res* **2006,** 71, (1), 40-49.

35. Poh, M.; Boyer, M.; Solan, A.; Dahl, S. L. M.; Pedrotty, D.; Banik, S. S. R.; McKee, J. A.; Klinger, R. Y.; Counter, C. M.; Niklason, L. E., Blood vessels engineered from human cells. *Lancet* **2005,** 365, (9477), 2122-2124.

36. Yang, S. F.; Leong, K. F.; Du, Z. H.; Chua, C. K., The design of scaffolds for use in tissue engineering. Part 1. Traditional factors. *Tissue Eng* **2001,** 7, (6), 679-689.

37. Gogolewski, S., In vitro and in vivo molecular stability of medical polyurethanes: A review. *Trends Polym Sci* **1991,** 1, 47-61.

38. Ratcliffe, A., Tissue engineering of vascular grafts. *Matrix Biol* **2000,** 19, (4), 353-357.

39. Stokes, K.; McVenes, R.; Anderson, J. M., Polyurethane elastomer biostability. *J Biomater Appl* **1995,** 9, (4), 321-54.

40. Szycher, M.; Reed, A. M., Biostable polyurethane elastomers. *Med Device Technol* **1992,** 3, (10), 42-51.

41. Zhao, Q.; Agger, M. P.; Fitzpatrick, M.; Anderson, J. M.; Hiltner, A.; Stokes, K.; Urbanski, P., Cellular interactions with biomaterials: in vivo cracking of pre-stressed Pellethane 2363-80A. *J Biomed Mater Res* **1990,** 24, (5), 621-37.

42. Alperin, C.; Zandstra, P. W.; Woodhouse, K. A., Polyurethane films seeded with embryonic stem cell-derived cardiomyocytes for use in cardiac tissue engineering applications. *Biomaterials* **2005,** 26, (35), 7377-86.

43. Danielsson, C.; Ruault, S.; Simonet, M.; Neuenschwander, P.; Frey, P., Polyesterurethane foam scaffold for smooth muscle cell tissue engineering. *Biomaterials* **2006,** 27, (8), 1410-5.

44. Ramrattan, N. N.; Heijkants, R. G.; van Tienen, T. G.; Schouten, A. J.; Veth, R. P.; Buma, P., Assessment of tissue ingrowth rates in polyurethane scaffolds for tissue engineering. *Tissue Eng* **2005,** 11, (7-8), 1212-23.

45. Grenier, S.; Sandig, M.; Holdsworth, D. W.; Mequanint, K., Interactions of coronary artery smooth muscle cells with 3D porous polyurethane scaffolds. *J Biomed Mater Res A* **2009,** 89A, (2), 293-303.

46. Grenier, S.; Sandig, M.; Mequanint, K., Polyurethane biomaterials for fabricating 3D porous scaffolds and supporting vascular cells. *J Biomed Mater Res A* **2007,** 82A, (4), 802-809.

47. Grenier, S.; Sandig, M.; Mequanint, K., Smooth muscle alpha-actin and calponin expression and extracellular matrix production of human coronary artery smooth muscle cells in 3D scaffolds. *Tissue Eng Pt A* **2009,** 15, (10), 3001-11.

48. Bezuidenhout, D.; Davies, N.; Zilla, P., Effect of well defined dodecahedral porosity on inflammation and angiogenesis. *ASAIO J* **2002,** 48, (5), 465-71.

49. Yarlagadda, P. K.; Chandrasekharan, M.; Shyan, J. Y., Recent advances and current developments in tissue scaffolding. *Bio-med Mater Eng* **2005,** 15, (3), 159-77.

50. Contreras, M. A.; Quist, W. C.; Logerfo, F. W., Effect of porosity on small-diameter vascular graft healing. *Microsurg* **2000,** 20, (1), 15-21.

51. Mikos, A. G.; Bao, Y.; Cima, L. G.; Ingber, D. E.; Vacanti, J. P.; Langer, R., Preparation of poly(glycolic acid) bonded fiber structures for cell attachment and transplantation. *J Biomed Mater Res* **1993,** 27, (2), 183-9.

52. Wang, Z.; Liu, S.; Guidoin, R.; Kodama, M., Polyurethane vascular grafts with thorough porosity: does an internal or an external membrane wrapping improve their in vivo blood compatibility and biofunctionality? *Artif Cell Blood Sub* **2004,** 32, (3), 463-84.

53. Mooney, D. J.; Baldwin, D. F.; Suh, N. P.; Vacanti, J. P.; Langer, R., Novel approach to fabricate porous sponges of poly(D,L-lactic-co-glycolic acid) without the use of organic solvents. *Biomaterials* **1996,** 17, (14), 1417-22.

54. Carletti, E.; Motta, A.; Migliaresi, C., Scaffolds for tissue engineering and 3D cell culture. *Methods Mol Biol* **2011,** 695, 17-39.

55. Gao, J.; Crapo, P. M.; Wang, Y., Macroporous elastomeric scaffolds with extensive micropores for soft tissue engineering. *Tissue Eng* **2006,** 12, (4), 917-25.

56. Bouten, C. V.; Dankers, P. Y.; Driessen-Mol, A.; Pedron, S.; Brizard, A. M.; Baaijens, F. P., Substrates for cardiovascular tissue engineering. *Adv Drug Deliver Rev* **2011,** 63, (4-5), 221-41.

57. Friedland, J. C.; Lee, M. H.; Boettiger, D., Mechanically activated integrin switch controls alpha5beta1 function. *Science* **2009,** 323, (5914), 642-4.

58. Vogel, V.; Sheetz, M. P., Cell fate regulation by coupling mechanical cycles to biochemical signaling pathways. *Curr Opin Cell Biol* **2009,** 21, (1), 38-46.

59. Glukhova, M. A., Koteliansky, V.E., Integrins, cytoskeletal and extracellular matrix proteins in developing smooth muscle cells of human aorta. In *The Vascular Smooth Muscle Cell. Molecular and Biological Response to the Extracellular Matrix.*, Schwartz, S. M., Mecham R.P., Ed. Academic Press: San Diego, 1995; pp 37-79.

60. Bottger, B. A.; Hedin, U.; Johansson, S.; Thyberg, J., Integrin-type fibronectin receptors of rat arterial smooth muscle cells: isolation, partial characterization and role in cytoskeletal organization and control of differentiated properties. *Differentiation* **1989,** 41, (2), 158-67.

61. Hedin, U.; Bottger, B. A.; Luthman, J.; Johansson, S.; Thyberg, J., A substrate of the cell-attachment sequence of fibronectin (Arg-Gly-Asp-Ser) is sufficient to promote transition of arterial smooth muscle cells from a contractile to a synthetic phenotype. *Dev Biol* **1989,** 133, (2), 489-501.

62. Hedin, U.; Thyberg, J., Plasma fibronectin promotes modulation of arterial smooth-muscle cells from contractile to synthetic phenotype. *Differentiation* **1987,** 33, (3), 239-46.

63. Yamamoto, M.; Yamamoto, K., [Regulation of differentiated properties of arterial smooth muscle cells]. *Nihon Rinsho* **1993,** 51, (8), 1980-6.

64. Hedin, U.; Bottger, B. A.; Forsberg, E.; Johansson, S.; Thyberg, J., Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. *J Cell Biol* **1988,** 107, (1), 307-19.

65. Lavender, M. D.; Pang, Z.; Wallace, C. S.; Niklason, L. E.; Truskey, G. A., A system for the direct co-culture of endothelium on smooth muscle cells. *Biomaterials* **2005,** 26, (22), 4642-53.

66. Grainger, S. J.; Putnam, A. J., Assessing the permeability of engineered capillary networks in a 3D culture. *PLoS One* **2011,** 6, (7), e22086.

67. Ashikari-Hada, S.; Habuchi, H.; Kariya, Y.; Kimata, K., Heparin regulates vascular endothelial growth factor165-dependent mitogenic activity, tube formation, and its receptor phosphorylation of human endothelial cells. Comparison of the effects of heparin and modified heparins. *J Biol Chem* **2005,** 280, (36), 31508-15.

68. Nauck, M.; Karakiulakis, G.; Perruchoud, A. P.; Papakonstantinou, E.; Roth, M., Corticosteroids inhibit the expression of the vascular endothelial growth factor

gene in human vascular smooth muscle cells. *Eur J Pharmacol* **1998,** 341, (2-3), 309-15.

69. Bastian, S. E.; Walton, P. E.; Belford, D. A., Paracellular transport of insulin-like growth factor-I (IGF-I) across human umbilical vein endothelial cell monolayers. *J Cell Physiol* **1997,** 170, (3), 290-8.

70. Grulich-Henn, J.; Ritter, J.; Mesewinkel, S.; Heinrich, U.; Bettendorf, M.; Preissner, K. T., Transport of insulin-like growth factor-I across endothelial cell monolayers and its binding to the subendothelial matrix. *Exp Clin Endocr Diab* **2002,** 110, (2), 67-73.

71. Huang, A.; Vita, J. A.; Venema, R. C.; Keaney, J. F., Jr., Ascorbic acid enhances endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin. *J Biol Chem* **2000,** 275, (23), 17399-406.

72. Serrano, M. C.; Portoles, M. T.; Vallet-Regi, M.; Izquierdo, I.; Galletti, L.; Comas, J. V.; Pagani, R., Vascular endothelial and smooth muscle cell culture on NaOH-treated poly(epsilon-caprolactone) films: a preliminary study for vascular graft development. *Macromol Biosci* **2005,** 5, (5), 415-23.

73. Seifu, D. G., Mequanint, K., Fabrication of vascular tissue engineering scaffolds with enhanced oxygen diffusivity pore interconnectivity. *Biomed Mater* **2012,** (under review).

74. Dubey, G.; Mequanint, K., Conjugation of fibronectin onto three-dimensional porous scaffolds for vascular tissue engineering applications. *Acta Biomater* **2011,** 7, (3), 1114-25.

75. Williams, C.; Wick, T. M., Perfusion bioreactor for small diameter tissue-engineered arteries. *Tissue Eng* **2004,** 10, (5-6), 930-41.

76. Kanda, K.; Matsuda, T., In vitro reconstruction of hybrid arterial media with molecular and cellular orientations. *Cell Transplant* **1994,** 3, (6), 537-45.

77. Williams, C.; Wick, T. M., Endothelial cell-smooth muscle cell co-culture in a perfusion bioreactor system. *Ann Biomed Eng* **2005,** 33, (7), 920-8.

78. Mitchell, S. L.; Niklason, L. E., Requirements for growing tissue-engineered vascular grafts. *Cardiovasc Pathol* **2003,** 12, (2), 59-64.

79. Owens, G. K., Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* **1995,** 75, (3), 487-517.

80. Owens, G. K.; Kumar, M. S.; Wamhoff, B. R., Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* **2004,** 84, (3), 767-801.

81. Wallace, C. S.; Champion, J. C.; Truskey, G. A., Adhesion and function of human endothelial cells co-cultured on smooth muscle cells. *Ann Biomed Eng* **2007,** 35, (3), 375-86.

82. Wallace, C. S.; Strike, S. A.; Truskey, G. A., Smooth muscle cell rigidity and extracellular matrix organization influence endothelial cell spreading and adhesion formation in coculture. *Am J Physiol-Heart C* **2007,** 293, (3), H1978-86.

83. Chaterji, S.; Park, K.; Panitch, A., Scaffold-free in vitro arterial mimetics: the importance of smooth muscle-endothelium contact. *Tissue Eng Pt A* **2010,** 16, (6), 1901-12.

84. Dalby, M. J.; Childs, S.; Riehle, M. O.; Johnstone, H. J.; Affrossman, S.; Curtis, A. S., Fibroblast reaction to island topography: changes in cytoskeleton and morphology with time. *Biomaterials* **2003,** 24, (6), 927-35.

85. Dalby, M. J.; Riehle, M. O.; Johnstone, H. J.; Affrossman, S.; Curtis, A. S., Polymer-demixed nanotopography: control of fibroblast spreading and proliferation. *Tissue Eng* **2002,** 8, (6), 1099-108.

86. Ziegler, T.; Alexander, R. W.; Nerem, R. M., An endothelial cell-smooth muscle cell co-culture model for use in the investigation of flow effects on vascular biology. *Ann Biomed Eng* **1995,** 23, (3), 216-25.

87. Hordijk, P. L.; Anthony, E.; Mul, F. P.; Rientsma, R.; Oomen, L. C.; Roos, D., Vascular-endothelial-cadherin modulates endothelial monolayer permeability. *J Cell Sci* **1999,** 112 (Pt 12), 1915-23.

88. Vestweber, D., VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arterioscl Throm Vas* **2008,** 28, (2), 223-32.

89. Chien, S., Mechanotransduction and endothelial cell homeostasis: the wisdom of the cell. *Am J Physiol-Heart C* **2007,** 292, (3), H1209-24.

90. Redmond, E. M.; Cullen, J. P.; Cahill, P. A.; Sitzmann, J. V.; Stefansson, S.; Lawrence, D. A.; Okada, S. S., Endothelial cells inhibit flow-induced smooth muscle cell migration: role of plasminogen activator inhibitor-1. *Circulation* **2001,** 103, (4), 597-603.

91. Sprague, E. A., Endothelial and Smooth Muscle Cell Injury in Contrived Models and Natural Disease. In *Comprehensive Toxicology*, Bishop, S. P., Kerns, W.D., Ed. Pergamon: Oxford, 1997; pp 213-240.

92. Lee, Y. U.; Luo, J.; Sprague, E.; Han, H. C., Comparison of artery organ culture and co-culture models for studying endothelial cell migration and its effect on smooth muscle cell proliferation and migration. *Ann Biomed Eng* **2010,** 38, (3), 801-12.

93. Kiec-Wilk, B.; Polus, A.; Razny, U.; Cialowicz, U.; Dembinska-Kiec, A., Modulation of endothelial cell proliferation and capillary network formation by the ox-LDL component: 1-palmitoyl-2-archidonoyl-sn-glycero-3-phosphocholine (ox-PAPC). *Genes Nutr* **2011,** 6, (4), 347-51.

94. Grant, D. S.; Kleinman, H. K.; Martin, G. R., The role of basement membranes in vascular development. *Ann NY Acad Sci* **1990,** 588, 61-72.

95. Kubota, Y.; Kleinman, H. K.; Martin, G. R.; Lawley, T. J., Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol* **1988,** 107, (4), 1589-98.

96. Powell, R. J.; Bhargava, J.; Basson, M. D.; Sumpio, B. E., Coculture conditions alter endothelial modulation of TGF-beta 1 activation and smooth muscle growth morphology. *Am J Physiol* **1998,** 274, (2 Pt 2), H642-9.

97. Powell, R. J.; Cronenwett, J. L.; Fillinger, M. F.; Wagner, R. J.; Sampson, L. N., Endothelial cell modulation of smooth muscle cell morphology and organizational growth pattern. *Ann Vasc Surg* **1996,** 10, (1), 4-10.

98. Nackman, G. B.; Bech, F. R.; Fillinger, M. F.; Wagner, R. J.; Cronenwett, J. L., Endothelial cells modulate smooth muscle cell morphology by inhibition of transforming growth factor-beta 1 activation. *Surgery* **1996,** 120, (2), 418-25; discussion 425-6.

99. Lyons, E., Pandit, A., Design of bioreactors for cardiovascular applications. In *Topics in Tissue Engineering*, Ashammakhi, N., Reis, R.I., Ed. 2006; Vol. 2.

100. van Buul-Wortelboer, M. F.; Brinkman, H. J.; Dingemans, K. P.; de Groot, P. G.; van Aken, W. G.; van Mourik, J. A., Reconstitution of the vascular wall in vitro. A novel model to study interactions between endothelial and smooth muscle cells. *Exp Cell Res* **1986,** 162, (1), 151-8.

101. Korff, T.; Kimmina, S.; Martiny-Baron, G.; Augustin, H. G., Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J* **2001,** 15, (2), 447-57.

102. Powell, R. J.; Hydowski, J.; Frank, O.; Bhargava, J.; Sumpio, B. E., Endothelial cell effect on smooth muscle cell collagen synthesis. *J Surg Res* **1997,** 69, (1), 113-8.

103. Shawber, C. J.; Kitajewski, J., Notch function in the vasculature: insights from zebrafish, mouse and man. *Bioessays* **2004,** 26, (3), 225-34.

104. Barber, R. D.; Harmer, D. W.; Coleman, R. A.; Clark, B. J., GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol Genomics* **2005,** 21, (3), 389-95.

105. Frid, M. G.; Shekhonin, B. V.; Koteliansky, V. E.; Glukhova, M. A., Phenotypic changes of human smooth muscle cells during development: late expression of heavy caldesmon and calponin. *Dev Biol* **1992,** 153, (2), 185-93.

106. Abberton, K. M.; Healy, D. L.; Rogers, P. A., Smooth muscle alpha actin and myosin heavy chain expression in the vascular smooth muscle cells surrounding human endometrial arterioles. *Hum Reprod* **1999,** 14, (12), 3095-100.

107. Fetalvero, K. M.; Shyu, M.; Nomikos, A. P.; Chiu, Y. F.; Wagner, R. J.; Powell, R. J.; Hwa, J.; Martin, K. A., The prostacyclin receptor induces human vascular smooth muscle cell differentiation via the protein kinase A pathway. *Am J Physiol-Heart C* **2006,** 290, (4), H1337-46.

108. Brown, D. J.; Rzucidlo, E. M.; Merenick, B. L.; Wagner, R. J.; Martin, K. A.; Powell, R. J., Endothelial cell activation of the smooth muscle cell phosphoinositide 3-kinase/Akt pathway promotes differentiation. *J Vasc Surg* **2005,** 41, (3), 509-16.

Chapter 5 – Conclusions and Future Directions

5.1 Conclusions

The present study involved the examination HCASMCs phenotype modulation in the presence of HCAECs. Initial exploratory experiments that involved the assessment of gene expression in HCAECs and HCASMCs in 2D cultures indicated that the contractile phenotype can be induced via the Notch3 signaling pathway only when HCASMCs are directly cultured with HCAECs. Soluble Jagged1 protein and Jagged1-immobilized Dynabeads failed to upregulate SM-α-actin and calponin gene expression levels; however, siRNA knockdown of Jagged1 in HCAECs demonstrated a direct link between HCAEC-expressed Jagged1 and contractile gene expression in HCASMCs. This led to the investigation of direct co-culture of HCAECs and HCASMCs on 3D PCU scaffolds. In order to create a cell-scaffold construct that mimics the *in vivo* artery structure a series of experiments were carried out to optimize the culture conditions. It was determined that a FN concentration of 5 $\mu g/cm^2$ resulted in increased HCASMCs attachment and that a growth media composition of 1 part SmGM and 1 part EGM enabled the maintenance of high HCASMC and HCAEC densities. Long-term HCASMC cultures on PCU scaffolds showed that cells were able to proliferate and maintain a healthy morphology which illustrated the value of using PCU scaffolds as a support structure for engineered vascular substitutes. Short-term cultures were then carried out using high HCASMC densities. A seeding density of 750,000 HCASMCs/ scaffold resulted in the formation of a dense, confluent HCASMCs layer which facilitated the formation of a confluent layer of

HCAECs. Matrigel was then incorporated into the co-culture model to mimic the basement membrane matrix that is found underlying the endothelium of native blood vessels. The addition of Matrigel did not produce a significant difference, with respect to HCAEC attachment, however, a dilution of 1:100 (Matrigel: growth media) was still employed in subsequent cultures. To conclusively affirm the phenotype of HCASMCs in co-culture with HCAECs, Western blot was performed and protein expression levels were analyzed. It was demonstrated that co-culture of HCASMCs with HCAECs upregulated the expression of the contractile proteins SM-α-actin and calponin as well as Notch3 and Jagged1. Gene knockdown of Jagged1 in HCAECs using siRNA established a direct link between the Notch3 signaling pathway and HCASMC phenotype. A reduction in Jagged1 expression by HCAECs in co-culture resulted in decreased expression of SM-α-actin and calponin by HCASMCs in co-culture.

These results demonstrate that co-cultures of HCAECs and HCASMCs can be successfully sustained on PCU scaffolds and that HCAECs modulate HCASMC phenotype in 3D cultures via the Notch3 signaling pathway. These findings are highly relevant in the context of vascular tissue engineering. The success of a tissue engineered vascular substitute is highly dependent on the phenotype of the VSMCs that reside within it. Based on these findings the Notch3 signaling pathway can potentially be exploited for regulating the phenotype of VSMCs during *in vitro* tissue maturation. This could be achieved through augmenting the expression of one or more of the components involved in the Notch3 signaling pathway.

5.2 Future Directions

Follow-up studies should be performed to determine the long term effect of HCAEC on HCASMC phenotype. It would be undesirable for HCASMCs to revert back to a synthetic phenotype so it should be verified that HCAECs are capable of maintaining HCASCMs in a contractile phenotype for periods greater than 48 hours. Furthermore, it would be beneficial to perform a comparative study to examine the relative effects of different phenotype regulatory molecules and mechanisms. These include illoprost and TGF-β1 which have both been shown to induce the contractile phenotype in VSMCs[1-3].

5.3 References

1. Fetalvero, K. M.; Shyu, M.; Nomikos, A. P.; Chiu, Y. F.; Wagner, R. J.; Powell, R. J.; Hwa, J.; Martin, K. A., The prostacyclin receptor induces human vascular smooth muscle cell differentiation via the protein kinase A pathway. *Am J Physiol-Heart C* **2006,** 290, (4), H1337-46.
2. Lin, S.; Sandig, M.; Mequanint, K., Three-dimensional topography of synthetic scaffolds induces elastin synthesis by human coronary artery smooth muscle cells. *Tissue Eng Pt A* **2011,** 17, (11-12), 1561-71.
3. Tang, Y.; Urs, S.; Boucher, J.; Bernaiche, T.; Venkatesh, D.; Spicer, D. B.; Vary, C. P.; Liaw, L., Notch and transforming growth factor-beta (TGFbeta) signaling pathways cooperatively regulate vascular smooth muscle cell differentiation. *J Biol Chem* **2010,** 285, (23), 17556-63.

Appendix – Supplementary Data

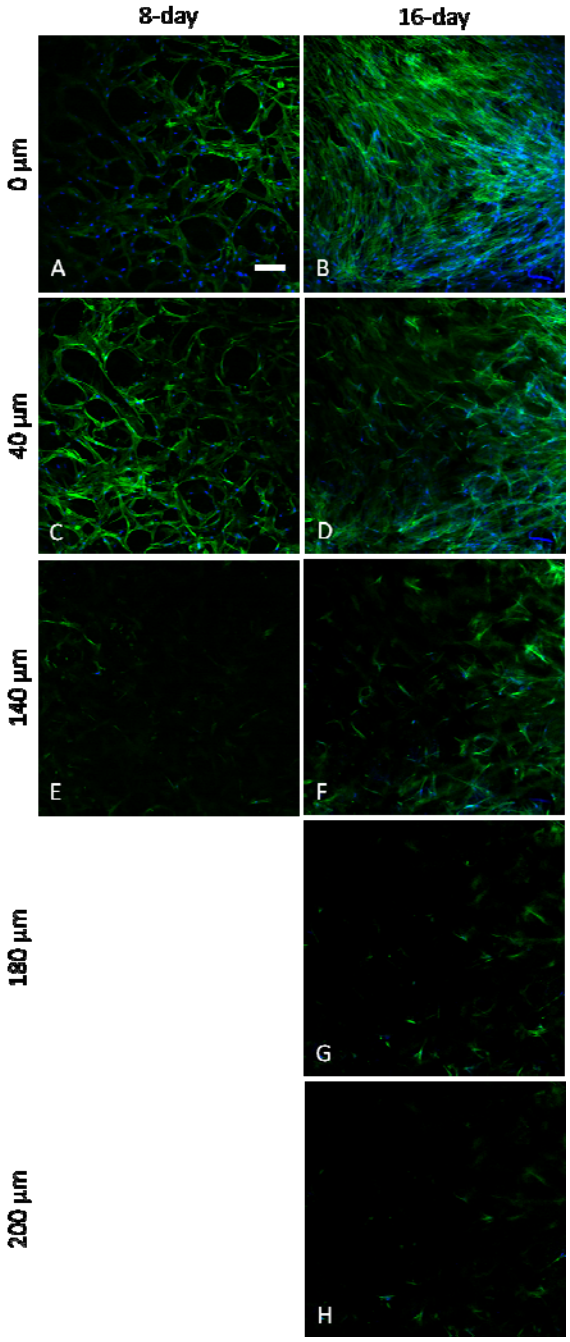

Figure A1: Cell infiltration in long-term cultures of HCASMCs on PCU scaffolds. Images of HCASMCs cultured on PCU scaffolds for 8 (A, C, E) and 16 (B, D, F, G, H) days at depths of 0 µm (A, B), 40 µm (C, D), 140 µm (E, F), 180 µm (G) and 200 µm (H) from the seeding surface. F-actin is stained with Alexa Fluor® 488 Phalloidin (green) and nuclei are stained with Hoechst 33342 (blue). Scale bar, 100 µm.

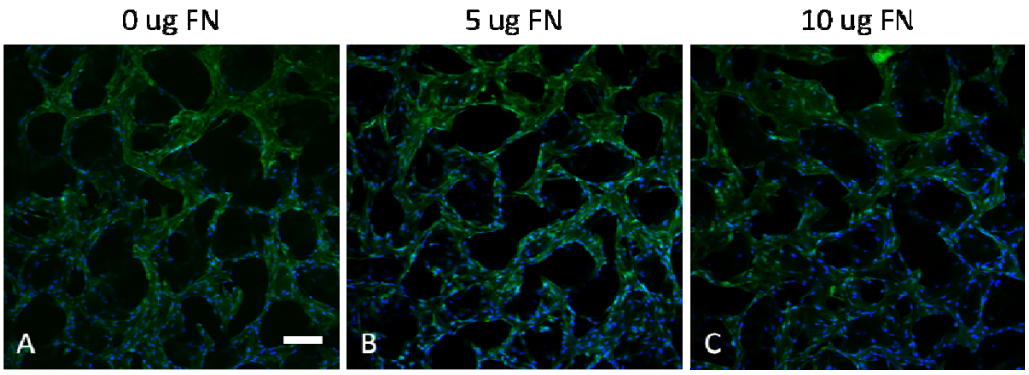

Figure A2: HCASMCs cultured for 72 hours on PCU scaffolds coated with varying amounts of FN. HCASMCs cultured on PCU scaffolds coated with 0 (A), 5 (B) and 10 (C) µg of FN/scaffold. Cells are live-stained with a the cytoskeletal marker CTG (green) and Hoechst 33342 (blue) for the nuclei Scale bar, 200 µm

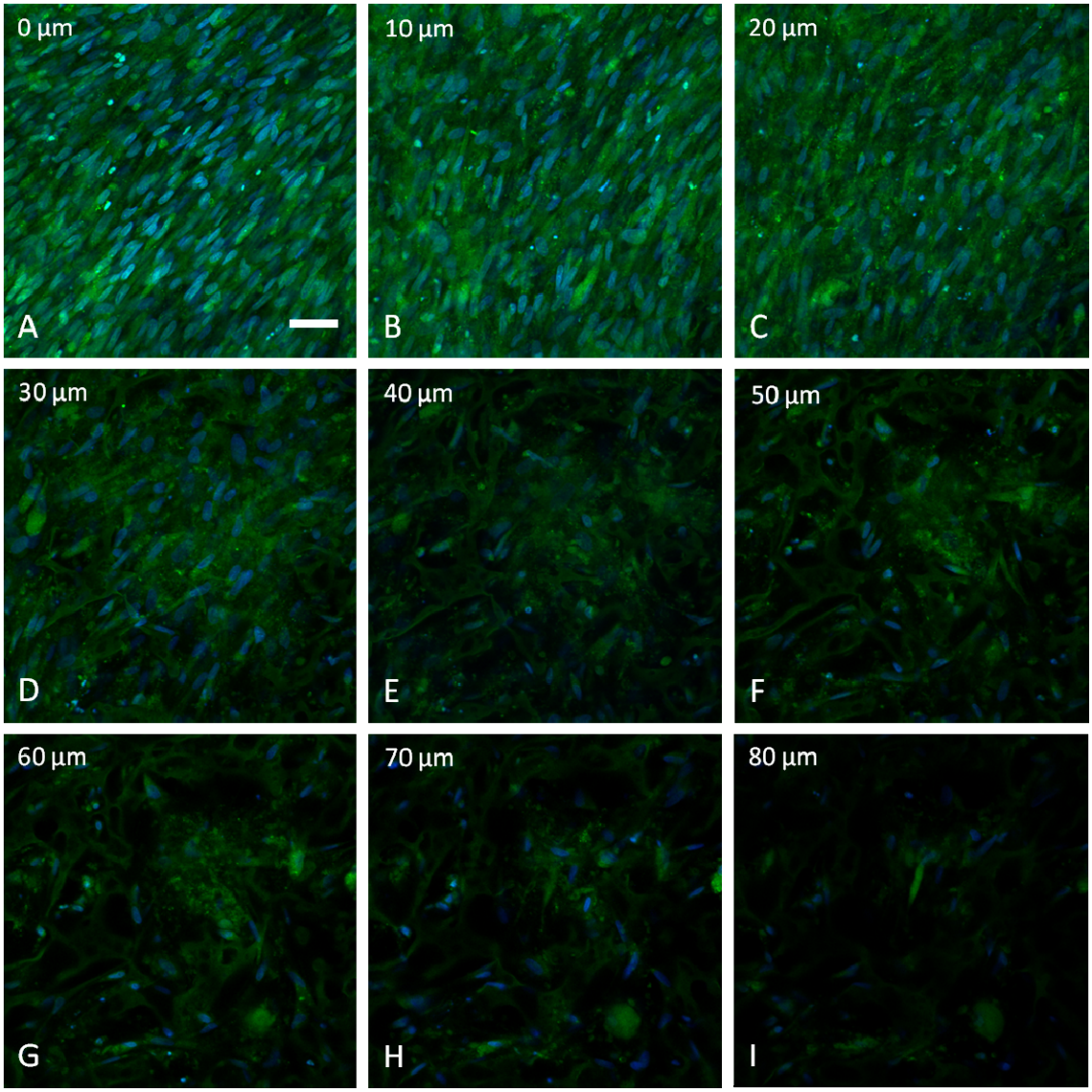

Figure A3: Serial optical sections of HCASMCs cultured for 72 hours on PCU scaffolds at a density of 750,000 cells/scaffold. Selected images from a single image stack are shown at depths of 0 µm (A), 10 µm (B), 20 µm (C), 30 µm (D), 40 µm (E), 50 µm (F), 60 µm (G), 70 µm (H), 80 µm (I)from the seeding surface. Cells are live-stained with a the cytoskeletal marker CTG (green) and Hoechst 33342 (blue) for the nuclei Scale bar, 20 µm.

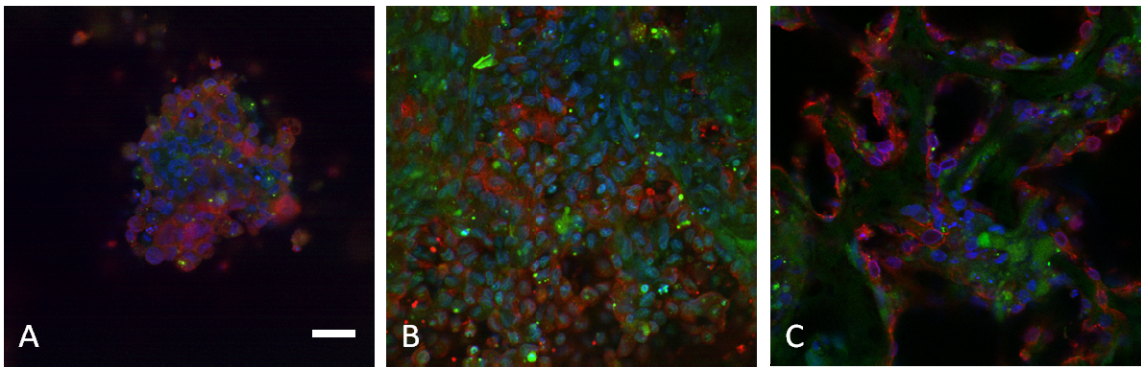

Figure A4: 48-hour co-cultures of HCAECs with sub-confluent HCASMCs on PCU scaffolds. HCAECs can be seen forming clusters (A), intermixing with the HCASMC layer (B) and adhering to the scaffold struts (C). PCU scaffold is shown as mild green fluorescence in panel C. HCASMCs are stained with CTG (green) while HCAEC-cell junctions are stained with VE-cadherin (red). All nuclei are labeled with Hoechst 33342 (blue). Scale bar, 20 μm.

Curriculum Vitae

Aparna Bhattacharyya

EDUCATION

2009-2012 **M.E.Sc. Biomedical Engineering**
The University of Western Ontario, London, ON, Canada

2005-2009 **B.E.Sc. Chemical and Biochemical Engineering**
The University of Western Ontario, London, ON, Canada

RELATED WORK EXPERIENCE

2009-2011 **Graduate Teaching Assistant**
Department of Chemical and Biochemical Engineering
The University of Western Ontario, London, Ontario, Canada

PUBLICATIONS IN REFEREED JOURNALS

Xia, K; **Bhattacharyya, A**; Roszell, E; Sandig, M; Mequanint, K., The role of endothelial cell-bound Jagged1 in Notch3-induced human coronary artery smooth muscle cell differentiation. *Biomaterials* **2012,** 33, (8), 2462-72.

CONFERENCE PRESENTATIONS

Bhattacharyya, A., Mequanint, K. Co-culture of Smooth Muscle Cells and Endothelial Cells on Porous 3D Polyurethane Scaffolds for Vascular Tissue Engineering.
61st Chemical Engineering Conference, London ON Canada, October 2011 (oral)

HONOURS AND AWARDS

2009-2011 **Western Graduate Research Scholarship**

2008 **1st Place, Capstone Design Day**
Manufacture of Biobutanol from Starch
Sarnia, ON, Canada