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**EFFECT OF EXOGENOUS TRANSFORMING GROWTH
FACTOR BETA ONE ON VASCULAR SMOOTH MUSCLE
CELLS IN DEEP MICROCHANNELS**

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Table of Abbreviations

2D	two-dimensional
3D	three-dimensional
bFGF	basic fibroblast growth factor
LBL	layer-by-layer
PBS	phosphate buffered saline
PCLLGA	poly(ϵ -caprolactone-co-lactide-co-glycolide)
PDGF	platelet-derived growth factor
PDMS	polydimethylsiloxane
PGA	polyglycolic acid
PLGA	poly(lactic-co-glycolic acid)
PTFE	polytetrafluoroethylene
PUR	polyurethane
SMC	smooth muscle cell
TGF	transforming growth factor
UV	ultra-violet

SUMMARY

The creation of a functioning vascular tunica media, with circumferentially positioned contractile vascular smooth muscle cells (vSMCs) and extracellular matrix, suitable mechanical characteristics and vasoactivity has yet to be established for tissue engineering of small calibre blood vessels. Synthetic vSMC is preferred for cell proliferation needed for vessel remodeling but the contractile vSMC is essential for sufficient vasoactivity and prevention of neointima formation. The vessels' tunica media is composed chiefly of vSMCs that when possessing contractile phenotype will endow the vessel with contractility-vasoactivity. The transformation of SMC phenotype from synthetic to contractile at the correct cell development stage has been hypothesized to be crucial for producing a functional vascular tunica media.

Vascular smooth muscle cells have been shown to go through cytoskeletal restructuring and line-up in the direction of micropatterns, triggering a phenotype and genotype switch from synthetic to contractile at confluence (1,2). Transforming growth factor beta one (TGF- β 1) has also been shown to uphold differentiation of vascular smooth muscle cells towards a contractile phenotype (2-4). However the combined effects of micropatterning and TGF- β 1 on VSMC culture have not been studied.

This study aims to examine the combined effect of a microchanneled scaffold and growth factor transforming growth factor beta one (TGF- β 1) on the contractile phenotype of vSMCs. We also investigated whether the temporal addition of TGF- β 1 before cell confluence or after cell confluence made a difference to the vSMC culture in micropatterned scaffold.

Culture of vSMCs in a microchanneled PUR scaffold in combination with the use of TGF- β 1 was found to align vSMCs parallel to microchannels and increase vSMC contractile protein expression (α -actin, calponin, MHC) thereby upregulating the contractile phenotype of vSMCs. This effect of contractile phenotype upregulation was maximal in terms of a statistically significant increase in MHC expression when the TGF- β 1 was added to the micropattern-vSMC culture before cell confluence was achieved.

We believe this novel synergistic effect of culturing vSMCs in a microchanneled scaffold in combination with TGF- β 1 addition before confluence to achieve maximum vSMC contractile phenotype might soon be fully exploited to create a functional vascular tunica media for the purpose of tissue engineering a viable small-diameter blood vessel replacement. We envision for the future using other vascular growth factors in temporal combination with microchanneled scaffolds to further fine-tune and maximize the phenotypic contractile properties of the vSMC culture.

Chapter 1 Introduction

1.1 Background

Cardiovascular disease is a foremost cause of morbidity and death in many developed nations today. Heart disease is the leading killer in North America responsible for 28% of deaths in recent years (2). In Europe, cardiovascular disease such as heart attack and strokes is the most common cause of mortality responsible for 41% of all deaths (2). Depending on the location and function of the small-diameter (< 5mm) blood vessels, their blockage leads to heart attack and lower limb peripheral vascular disease. Current lifesaving surgical treatment involves bypass of blocked small-diameter arteries in the limbs or diseased coronary arteries. Plastic microsurgical reconstructive surgery often requires small-diameter interposition vessels for flap vascularization. About 40% of patients do not possess proper donor vessels for bypass as a result of previous trauma, varicosity or previous surgery. While synthetic substitutes work well for blood vessels larger than 5 mm in diameter, they have poor long-term patency rates and are not suitable for the replacement of small-diameter vessels (1).

It is thought that a biological, rather than synthetic, substitute may be an ideal replacement for blocked or diseased small-diameter blood vessels. Tissue engineering may eventually offer an off-the-shelf readily available small-diameter blood vessel substitute. Tissue engineering involves seeding cells on a scaffold and orchestrating an environment using chemical and/or mechanical factors so as to advance cell growth, propagation and differentiation into the desired tissue phenotype (2).

The blood vessel has three core layers (i.e., the intima, media, adventitia) and the smooth muscle medial layer is the key layer imparting elasticity and vasoactivity to the vessel. It is therefore highly desirable to engineer a vessel with a smooth muscle tissue layer that mimics the native media and inroads have been made to recapitulate the smooth muscle architecture in small-diameter blood vessel engineering (1). One of the exciting patented models in small-diameter blood vessel engineering currently pursued in our laboratory involves using biocompatible micropatterned tubular scaffolds to circumferentially align and control the phenotype of vascular smooth muscle cells (vSMC) like in native vessels (Fig. 1.1) (1,5-8).

Figure 1.1 (A) Schematic showing PCL/LGA micropatterned film synthesis. (B) and (C) Concept of micropatterned films. Actual fabricated micropatterned PCL/LGA tube with inner diameter of 3mm and outer diameter of 5mm. [From Shen et al. (6), copyright 2006 by *Tissue Engineering*]

It has been known that topographical cues can guide cell orientation and growth. Micropatterns and microgrooves created on the scaffold surface can orient a variety of cells including fibroblasts, neurons, osteoblast-like cells, skeletal muscle, cardiomyocytes, smooth muscle and even stem cells (9,10). However, the consequences of topological cues on cellular phenotype has been less known and recently, Shen and Chan-Park et al (8) showed that 3-D microlined scaffolds like in Figure 1.1 can trigger the phenotype change of vSMCs toward more contractile phenotype when cells reach confluence.

Vascular smooth muscle cell behaviour is also affected by diverse locally produced growth factors, of which transforming growth factor-beta ($\text{TGF-}\beta$) is one of the most significant. $\text{TGF-}\beta_1$ usually retards proliferation but upregulates migration of vSMCs and raises both contractile protein expression and extracellular matrix (ECM) production (2).

Researchers have examined the role of interactions between $\text{TGF-}\beta_1$ and other growth factors (epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), or platelet-derived growth factor (PDGF) isoforms PDGF-AB and PDGF-BB) on the proliferation of vSMCs (11). Others have attempted using polymer scaffolds that can deliver angiogenic factors ($\text{TGF-}\beta$, VEGF, PDGF-BB) for engineering blood vessels (2). However there has been no special work done regarding the combined effect of a micropatterned polymer scaffold and growth factors on vascular smooth muscle culture and it is timely to address this research deficit for the purpose of small diameter vessel tissue engineering building on the work previously done in our laboratory.

1.2 Objective and Scope

The overall object of this research is to evaluate the effect of TGF- β 1 on vSMCs cultured in vitro inside deep microchannels.

Specifically, vSMCs were cultured on fabricated polyurethane scaffolds with or without deep microchannel patterns. The temporal effect of TGF- β 1 exposure before or after cell confluence on vSMC tissue cultures was studied in terms of cellular morphology and vSMC contractile protein expression using Western blot.

1.3 Outline

This report is split into 4 chapters. The first Chapter covers the background and objectives of this Masters project. The literature review is presented in Chapter two. Chapter three describes the synthesis of PUR films and its cell culture results involving the use of TGF- β 1. In Chapter four, conclusions are given, salient observations are highlighted, and recommendations for future work are suggested.

Chapter 2 Literature review

2.1 The ideal cardiovascular conduit

The properties of an ideal small-diameter blood vessel substitute are listed below in Table 2.1. To date, no conduit – natural, synthetic, tissue-engineered or otherwise – possesses all of these qualities and attributes (12,13).

Properties of the ideal blood vessel substitute
Strong, long-term tensile strength (burst strength > 1700 mmHg),
Compliant (4-8%/ mmHg x 10 ⁻²), elastic
Kink resistant
Good suture retention
Vasoactive
Remodel well – ease of regrowth of endothelium
Non-toxic
Infection resistant
Non-immunogenic
Low thrombogenicity
Biocompatible, biostable
Readily available in variety of lengths
Easy to handle
Low manufacturing cost

Table 2.1 Properties of an ideal small-diameter blood vessel substitute

Good patency rates are achievable using current commercially available prosthetic materials such as polytetrafluoroethane (PTFE) or Dacron for big caliber vessels, but small diameter prosthetic conduits (less than 5 mm) have unacceptably poor patency rates. This is consequent on low-flow conditions within a narrow tiny conduit and compliance mismatch between prosthesis and native artery, compounded by the predisposition to intimal hyperplasia and inherent thrombogenicity of prosthetic materials.

Again the focus in our vascular engineering laboratory is on tissue engineering a functional media layer of the blood vessel. With a functional stable media layer in a small-diameter blood vessel, we can derive the properties of strength, compliance, kink-resistance, and good suture-resistance - all necessary properties for surgical anastomosis to native vessel. A functional media layer is vasoactive and supports the growth and remodeling of an endothelial layer, and the abundance of phenotypically contractile vSMCs (as opposed to synthetic vSMCs - explained later in the thesis) within that functional media layer reduces the predisposition to intimal hyperplasia and stenosis.

2.2 Current approaches to small diameter blood vessel engineering

Tissue engineering to develop biological blood vessel substitutes has achieved some heartening results. L'Heureux *et al.* summed up the work of foremost tissue engineers engaged in vascular graft tissue engineering, highlighting that results of

implantation in humans of both synthetic grafts and protein-based grafts have not been successful (14).

The chief paradigms to date for the creation of tissue engineered small diameter blood vessels may be classified under the following three categories: (1) cells cultured in collagen, fibrin, decellularized vessels, or other hydrogels; (2) cells cultured in sheets without any scaffold or matrix; (3) cells cultured in porous scaffold supports (Figs. 2.1 and 2.2, Table 2.2).

Figure 2.1 Schematic representation of (a) collagen-based, (b) cell sheet-based, and (c) scaffold-based vascular grafts. [From Chan-Park et al. (2), copyright 2009 by *Journal of Biomedical Materials Research Part A*]

Figure 2.2 Free collagen gel compaction over a nonadhesive central rod to induce circumferential alignment of SMCs and fibrils; SMC-secreted enzymes degrade the fibrils. [From Chan-Park et al. (2), copyright 2009 by *Journal of Biomedical Materials Research Part A*]

However these approaches have significant shortcomings including and not limited to predisposition to thrombosis, unsuitable mechanical properties and vasoactivity, and exceedingly lengthy culture time. Poh *et al.* reported that PGA scaffold-based cellular grafts have not attained suitable mechanical integrity in human research (burst strength less than 300 mmHg) though remarkable mechanical characteristics have been verified in animal setups. Synthetic grafts also carry the risk of chronic inflammation and microbial infection (15). Protein-based grafts have inadequate tensile strength and comparatively fast degradation.

Using autologous live cells within or without a well-defined three-dimensional matrix in vascular engineering might allow the construction of a small diameter blood

vessel replacement with structural and practical properties akin to human vessels - an ultimate ideal we are trying to achieve at our vascular engineering lab here at SCBE NTU using a micropatterned scaffold-based cell culture approach as highlighted earlier. By focusing on replicating a life-like functional media we believe we can achieve superior vasoactivity and compliance compared to other scaffold-based grafts.

Table 2.2 Summary of approaches to tissue engineering of small-diameter blood vessels [Adapted from Chan-Park et al. (2), copyright 2009 by *Journal of Biomedical Materials Research Part A*]

Approach	Detail	Advantage	Disadvantage
Collagen-based	SMCs/ECs in collagen	Remodeling and vasoactivity Well differentiated SMC and functional endothelium	Poor strength and elasticity
	Circumferential collagen fibrils	Circumferential media-like stiffness	Effect lost with increasing culture time
	SMC-induced compaction over a nonadhesive rod	Circumferential media-like stiffness	Long culture time
	Collagen-based vessel modified by cyclic strain	Increased ultimate stress and modulus	Low burst strength
	SMCs in fibrin gel	Higher elastin synthesis than in collagen	In vivo sheep elastin production low
	Decellularized animal vessel	Good mechanical and biological properties	Possible animal to human disease transmission
	Decellularized human vessel	Good mechanical and biological properties	Poor off the shelf availability
	Decellularized small intestine submucosa	Better mechanical properties SMCs and ECs infiltration Vasoactivity	Immunogenicity Thrombogenicity
Cell-sheet-based	Autologous cell based tissue engineered vascular graft	Autologous Well organized adventitia, ECM secretion by SMCs, burst strength > 2000 mmHg	Extensively long culture time Low compliance
Scaffold-based	PGA mesh scaffold	Faster culture time compared to cell-sheet-based Similar histology to native artery High burst strength	Poor vasoactivity Low compliance

2.3 Phenotypic plasticity of the vascular smooth muscle cell

This project has its premise that the contractile differentiated aligned phenotype of vSMCs highly desirable in a practical tissue-engineered small-calibre blood vessel can possibly be maximized through a synergy of effects mediated by TGF- β 1 and a three-dimensional (3-D) microlined scaffold. The synthetic undifferentiated vSMC phenotype is unfavourable for functional blood vessels because it is associated with (1) greater extracellular matrix (ECM) production that predisposes to intimal hyperplasia and vessel narrowing, and (2) lack of contractility (Fig. 2.3) (2). However, the synthetic phenotype is required for in vitro cell culture for cell expansion. So the trigger of phenotype from synthetic to contractile phenotype just before implantation of the confluent tissue into animals/human beings is necessary.

Figure 2.3 Schematic showing the attributes of contractile and synthetic SMCs. Observe the synthetic fibroblast-like morphology of the synthetic SMC phenotype as opposed to the spindle-shape (mechanically advantageous for contraction) morphology of the contractile SMC phenotype. [From Chan-Park et al. (2), copyright 2009 by *Journal of Biomedical Materials Research Part A*]

An interplay of myriad factors mediates the plastic transformation switch of vSMC phenotype from contractile to synthetic and vice versa (Fig. 2.4) (3). It is imperative for tissue engineers to know the standard recognized markers of vascular smooth muscle differentiation and contractility.

Figure 2.4 Cartoon showing the various factors affecting phenotypic switch between the synthetic and contractile states. [From Owens et al. (9), copyright 2004 by *Physiology Reviews*]

2.4 vSMC phenotype marker proteins

The smooth muscle cell (SMC) marker proteins usually used to delineate SMC phenotypes have in recent times been comprehensively appraised (3,4). The markers most pertinent to this project are smooth muscle alpha actin (SM α -actin), smooth muscle-myosin heavy chain (SM-MHC), smoothelin, and smooth muscle calponin (SM-calponin), because these markers are among the most upregulated in the differentiated contractile SMC phenotype (Fig. 2.5) (4). SM α -actin and SM-MHC are structural components of the cellular contractile apparatus. Calponin is a calcium regulatory protein associated

with actin or found in the cytoskeleton, functioning as a regulator of contraction. Smoothelin is also associated with actin, and acts to regulate contraction.

The expression quantity of these contractile marker proteins steadily diminish when SMCs are cultured *in vitro*, though the degree to which their expression is reduced varies amongst the markers. Markers whose expression is increased in the synthetic phenotype are rare. Rather, diminishment of proteins allied with the contractile phenotype is in general accepted as indicative of the synthetic phenotype (Fig. 2.5). SMCs with dissimilar phenotypes exhibit different quantities of these marker proteins instead of totally distinctive marker proteins. For that reason, data on the expression of as a minimum two proteins that are associated with a certain phenotype is requisite to discriminate a contractile from a synthetic SMC, if possible corroborated with information regarding morphology, propagation and migration profiles. As seen in Fig. 2.3, the maximally contractile SMC is spindle-like optimized for contraction with a prominent central nucleus and is stably aligned with other cells, non-proliferative and non-migratory. The synthetic SMC phenotype is fibroblast-like and exhibits greater cell migration and proliferation, ECM secretion and loss of contractility.

Figure 2.5 Schematic showing the relative proportions of SMC protein markers expressed at various stages of differentiation between synthetic and contractile phenotypes. Markers to the left of the indicator bars are expressed predominantly by the synthetic phenotype. Markers to the right are expressed predominantly by the contractile phenotype. [From Rensen et al. (10), copyright 2007 by the Netherlands Heart Journal]

Currently, SM-MHC and smoothelin are the two marker proteins that offer the best characterization of a mature contractile SMC phenotype (3,4). SM-MHC expression has never been identified *in vivo* in non-SMCs, and is the sole marker protein that is also SMC specific during embryogenesis, so that it is the most discerning marker for the SMC acknowledged to date. Even so Owens et al. advises that it would be unsurprising if SM-MHC is discovered in non-SMC and recommends that identification of a contractile differentiated vSMC use SM-MHC in addition to other markers such as SM α -actin, smoothelin and calponin (3). Smoothelin is complementary to SM-MHC as a contractile SMC marker because its expression is more homogeneously and more quickly downsized in cultured vascular SMCs transforming into a synthetic phenotype (4). Both SM-MHC and smoothelin have been found to be missing in myofibroblasts in arterial injury research (4). These marker proteins as well as calponin and SM α -actin should ideally be

carefully searched for by immunoblotting in this thesis project. While calponin and SM α -actin are excellent SMC differentiation marker genes as seen in Fig. 2.5, they may still be expressed in an array of situations in other cell types such as cardiomyocytes and myofibroblasts. Again, the key to successful convincing delineation of a contractile vSMC phenotype is truly a combination of as high and multidimensional a representation of vSMC differentiation markers as possible, and concrete evidence of contractile morphological and culture attributes.

2.5 Effect of micropatterns on vSMC development

The success of the micropatterned biomaterial in inducing alignment and promoting a contractile smooth muscle phenotype has been measured by a variety of endpoints in different studies including cell morphology (8,16-20), actin formation and alignment (1,16-20), nuclear alignment (17,19), smooth muscle markers (ribonucleic acid polymerase chain reaction [RNA PCR] and immunoblotting) (8,21), DNA microarray (22,23). The relevant literature for the effect of micropattern on SMCs is presented in a chronological and/or logical order as follows.

Thakar et al carried out some pioneering investigations into the effect of micropatterns on SMC development (16). They grew bovine SMCs on 2D micropatterned collagen strips and on microgrooved PLGA. SMCs grown on the micropatterned collagen substrate became oriented along the direction of the strips but had lower cell shape index, lesser proliferation rate and fewer actin stress fiber formation compared to those on

unpatterned substrate. SMCs cultured on microgrooved PLGA also showed lesser proliferation rate and fewer stress fibers than those on the control. They concluded that micropattern topography can control SMC morphology and decreases SMC proliferation but is insufficient to promote a contractile phenotype.

Glawe et al used polydimethylsiloxane (PDMS) microchanneled scaffolds to grow SMCs and found that narrow microchannels facilitated a large extent of SMC alignment in culture (17). The degree of cell alignment is greater with narrower channels but this is attenuated as the volume effectively available for cell culture decreases. Sarkar et al also used micropatterned PDMS and reported control over vSMC aspect ratio, alignment, and oriented restructuring of the ECM (18).

A micropatterned scaffold entirely fabricated from biological material came about in the form of a microgrooved fibrillar collagen membrane (19). This was used to separately culture human fibroblasts and SMCs inducing the cells to elongate and align.

Earlier micropatterning techniques have largely placed emphasis on SMC and actin alignment and none highlighted the sought-after phenotype shift from synthetic to contractile at the suitable developmental stage crucial for a vasoactive vessel. 3D micropatterned scaffolds can possibly recreate the 3D tissue with the needed morphology instead of just a monolayer of cells (2). Our group used ultra violet (UV)-embossed polyurethane diacrylate (PUR) microchannels to grow and orientate smooth muscle cell line and skeletal muscle cell line (20). This led to the synthesis of biodegradable UV-

embossed poly (ϵ -caprolactone-co-lactide-co-glycolide) (PCLLGA) microchanneled scaffold (Fig. 1.1) that allowed dense aligned SMC culture at confluence with enhanced smooth muscle α -actin expression accompanied by a corresponding shift towards a more ideal contractile phenotype (7,8). Feng et al progressed to use the same microchanneled PCLLGA scaffold to create a 3D culture of SMC layer-by-layer (LBL) (1). A multilayered 3D SMC construct is produced within a relatively short period by first growing a confluence-aligned SMC cell-layer on the scaffold, then seeding and overlaying another layer, and so on.

Isenberg et al. grew vSMCs on micropatterned thermoresponsive PIPAAm, creating detachable cell sheets whose constituent cells are aligned along the same axis (24). Different types of micropattern-organized cell sheets may then theoretically be layered to create any desired 3D tissue composite – even in the form of tubular structures - that best mimics the native tissue architecture.

The next step is to investigate the combined effects of biochemical factors, biomechanical factors, and micropatterning on vSMCs or its progenitors.

Other vascular engineering attempts have looked at the concomitant effects of cardiomimetic strain on cells, using 3D polymerized gel patterns in the process to guide cell orientation in a fixed vector with respect to the strain vector. Smooth muscle cells oriented by micropatterns in the vector of uniaxial stretch induced phosphorylation of extracellular signal-regulated kinase (ERK)1/2 at physiologic and high strain rate while

low strain rate induced dephosphorylation (21). These outcomes signify that strain rate and cell orientation are important workings of mechanotransduction.

Mesenchymal stem cells (MSCs) have been shown to be able to self-proliferate and differentiate into several cellular lines including smooth muscle cells (2). These stem cells are thus viewed favourably for engineering off-the-shelf non-immunogenic small-diameter blood vessels. The factors driving vascular differentiation of MSCs are therefore decidedly a subject of great interest. Park *et al* found that cyclic uniaxial stress transiently augmented SM α -actin and SM-22 α in MSCs, whose levels normalized after the cells aligned perpendicular to strain direction (22). They postulated that the mechanotransduced upregulation of smooth muscle markers might be sustained by keeping the MSCs oriented in the strain vector in vitro using microgrooves. This was indeed evidenced in a study comparing MSC culture aligned parallel to the strain axis and that aligned perpendicular to the strain axis (23). For cells aligned parallel to strain direction, DNA microarray analysis showed an increase in smooth muscle marker calponin 1, decreased cartilage matrix makers, and cell signaling alterations.

What has not been looked into up to this point then is the combined effect of growth factors and micropatterns on vSMC culture, making a project of this nature a very worthwhile pursuit.

2.6 Effect of growth factors on vSMC development

Soluble chemical factors can initiate signal transduction to adjust cell growth, survival, movement-migration, and differentiation. In the embryonic phase, signaling molecules like cytokines and growth factors intervene in embryonic ordering and growth of the cardiovascular tissues (25). Among vital chemical factors affecting phenotypic regulation and differentiation of vSMCs, the actions of transforming growth factor beta (TGF- β), platelet derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) carry the most impact.

2.6.1 Effect of TGF- β on vSMC

TGF- β isoforms (TGF- β 1, TGF- β 2, TGF- β 3) appear to be fundamental for the development of the contractile SMC phenotype. They evidently propagate the contractile phenotype in mature SMCs. In particular TGF- β 1 signalling has lately been revealed to be requisite for SMC specific gene expression in embryoid bodies. TGF- β 1 upregulates α -SMA, SM-MHC, SM-calponin levels, and SM22 α in cultured SMCs, and TGF- β 2 raises SM α -actin and desmin levels in cultured pig SMCs (4,26,27). On the other hand, TGF- β 2 does not influence expression of more sophisticated contractile phenotype indicators such as SM-MHC and smoothelin, making it a less potent isoform than TGF- β 1 (28). Less is known of TGF- β 3 but it seems to have a greater role in a pathologic state of atherosclerosis. TGF- β 1 generally inhibits proliferation but promotes relocation of vascular SMCs and elevates ECM synthesis (2). The **TGF- β 1** isoform is therefore singled

out as a very important useful growth factor for the project because we are aiming to maximally increase vSMC contractile phenotype expression and differentiation.

2.6.2 Effect of PDGF and bFGF on vSMC

Platelet derived growth factor (PDGF) is a powerful chemoattractant secreted by activated platelets and lesion macrophages that brings about rapid downregulation of SM-selective markers in cultured SMC and induces SMC propagation and migration in arterial injury setups (3). *In vitro* studies show that platelet derived growth factor BB (PDGF-BB) in particular induces SMC relocation, growth, and ECM synthesis, indicating a downshift to a synthetic character (2). PDGF-BB is comparatively more chemotactic than mitogenic. However there is yet no authoritative proof that PDGF-BB is a powerful downregulator of SMC differentiation *in vivo*, though PDGF β -receptor knockout mice showed decreased arteriolar vSMC investment (29).

Basic fibroblast growth factor is known to be a strong angiogenic cytokine that induces SMC proliferation but has modest or zero effect on SMC differentiation marker expression (3,30).

2.6.3 Combined effects of growth factors on vSMC

Few studies examine the combined effects of TGF- β 1, bFGF, and PDGF-BB on regulating the SMC contractile phenotypic switch and differentiation. On the basis of comprehensive evidence supporting the individual roles of TGF- β 1, bFGF, and PDGF-BB in regulating vSMC development, others have concocted “enhanced” culture media containing a mix of PDGF-BB, bFGF, vitamin C to grow engineered small diameter arteries comprising bovine vSMCs and endothelial cells over 8 weeks in a bioreactor (31). The same group proceeded to use the same setup to engineer vessels with walls constructed with human MSC-derived SMCs, albeit this time using a “optimized” protocol of 4 weeks proliferation media (containing only PDGF-BB) and 4 weeks differentiation media (containing only TGF- β 1) (32). Basic fibroblast growth factor was excluded from the proliferation media because it was found to potently inhibit MSC differentiation into SMC lineage. They found that with the two-step process the bioreactor engineered vessels were significantly similar to native vessels both histologically and molecularly speaking. The group concluded that human MSCs can serve as a new source of vSMCs for vascular engineering.

The multidimensional influence in relation to cell growth, survival, movement-migration, and differentiation of growth factors upon vSMCs makes for an interesting number of possible projects one could pursue in relation to vSMC culture in microchannels. The combined effect of TGF- β 1 and micropatterning on vSMC

development and differentiation seemed a very attractive proposal for a Master's project at this point.

2.6.4 Combined effect of growth factor and scaffold for vascular engineering

The concept of culturing cells in a compatible scaffold and fortifying the culture with the most suitable growth factors and chemical factors is intriguing and has been explored by a few groups. Some groups have added chemical factors exogenously while others have attempted infusing or impregnating the scaffold with chemical factors that will be released by the scaffold during the culture period.

Mann et al (33) found that TGF- β 1 can be used in tissue engineering scaffolds like glass and PEG hydrogels to dramatically increase vSMC cell culture matrix production. In addition they found that covalently tethering TGF- β 1 to PEG significantly increases vSMC matrix production compared to the effect brought about by the same amount of TGF- β 1 dissolved in culture media. This also raised the possibility of temporal and quantitative control of vascular growth factor release by the scaffold for vascular engineering.

Sun Q et al (34) found that PLG scaffolds incorporating VEGF resulted in continual VEGF release, enhanced tissue perfusion, superior capillary formation-concentration, and more established vessel networks compared to controls. Another

group had results indicating that adding together FGF2 and VEGF to an acellular collagen scaffold promotes early established vascularisation (35).

Kurane et al worked on *in vivo* vascular engineering and found that by implanting tubular elastin scaffolds with agarose gel containing bFGF in stem/progenitor cell rich adipose tissue, the construct attracted many smooth muscle α -actin positive cells in a homing phenomenon forming a “media” layer surrounded by a collagenous “adventitia” layer (36).

At our vascular engineering lab we envisage that a micropatterned scaffold could be made into microtubular structures functioning as small-diameter blood vessel substitutes that support the growth of a strong native-like differentiated vSMC media layer. Investigating the effect of adding such an established vSMC growth factor as TGF- β 1 to possibly further influence and even enhance the vSMC differentiation within a micropatterned scaffold environs seems a very attractive exciting pursuit now.

2.7 Summary

Small diameter vascular grafts are urgently needed in patients needing coronary artery bypass or peripheral vascular surgery due to cardiovascular disease. Autologous vessels are ideal but approximately 40% of patients who require vascular bypass surgery do not have healthy vessels for grafting. Small diameter synthetic substitutes fail *in vivo*

due to thrombogenicity, compliance mismatch, and neointimal formation causing intimal thickening. Vascular tissue engineering attempts to re-create biological small diameter vascular grafts using live cells within or without a scaffold. A truly competent small calibre tissue engineered vascular graft must meet select physiological and clinical prerequisites including nonthrombogenicity, viscoelasticity, vasoactivity, and sufficient appropriate burst pressure and suture retention strength. It must also be manufactured economically in short response time meeting specific dimensions. However, existing paradigms for forming small diameter tissue engineered vascular grafts are not good enough and fall short in achieving at least one of these requirements.

Native vessel comprises three concentric layers: tunica intima, tunica media and tunica adventitia. Tunica media, one of the key components of the blood vessel wall, is responsible for the mechanical strength, compliance and vasoactivity of the blood vessel. A perfect vascular media is made up of circumferentially aligned contractile vSMCs and their synthesized ECM, such as collagen and elastin fibers. However, current tissue engineering approaches cannot reproduce a native-like media layer because SMCs possess phenotypic plasticity and it is difficult to control much less fine-tune the phenotype of SMCs. SMCs can be contractile or synthetic in phenotype and can switch to either reversibly with the suitable environmental stimuli. The synthetic vSMC character is wanted for cell proliferation considered necessary for vessel remodeling while circumferentially aligned contractile vSMCs are needed for medial strength, contractility and retardation of intimal hyperplasia. The SMC phenotype switch at a suitable developmental juncture is believed to give rise to physiological working vascular media.

We aim to recreate a functional native-like media with contractile phenotype vSMCs for the purpose of small-diameter vessel engineering. In order to characterize the vSMC phenotype in the course of research we need to be well versed with the differentiation marker genes linked with the contractile differentiated SMC phenotype. The current recommendation is that identification of the contractile differentiated vSMC use SM MHC complemented by other markers such as SM α -actin, calponin and smoothelin – this characterization should be abetted by phenotypo-morphological and culture evidence.

Mechanical forces, biochemical factors, and micropatterned physical cues have been harnessed to control SMC phenotype, orientation, and ECM synthesis. Micropatterns in the form of microchannels have been shown capable of aligning vSMCs parallel to each other along the channel-axis and concomitantly inducing a spindle-shaped vSMC morphology even increasing SM α -actin expression thereby increasing the contractile phenotype expression. There is great potential in using a LBL cell culture procedure on micropatterned scaffolds to quickly develop a multi-layered 3D functional vSMC media and in using biocompatible microchannelled tubular scaffolds as small-diameter vessel substitutes.

TGF- β , PDGF, and bFGF are among the most influential vital chemical factors affecting the phenotypic regulation and differentiation of vSMCs. TGF- β 1 in particular increases differentiation marker gene expression in SMCs: α -SMA, SM-MHC, SM-

calponin levels, and SM22; it retards proliferation but stimulates migration of vSMCs and augments ECM synthesis. No study to date has looked into the effect of combining TGF- β 1 and micropatterning on vSMC development and differentiation and we are looking at a potential system-instrument for fine-tuning and even maximizing the differentiated contractile phenotype of the vSMC.

Some groups have looked into vascular culture on suitable scaffolds with the addition of exogenous or tethered TGF- β 1 or other growth factor. Exciting vascular engineering concepts have arisen: temporal and/or quantitative controlled release of vascular growth factor by the scaffold structure and even in vivo homing of vascular cells to the scaffold as a result of tethered growth factors. Again none have investigated the effect of combining TGF- β 1 and micropatterned scaffold for vSMC culture and thereby controlling vSMC phenotype development and differentiation.

Chapter 3 Microchannelled PUR and TGF- β 1 to control contractile phenotype of vascular SMCs

3.1 Introduction

The overall object of this study was to assess the effect of TGF- β 1 on vSMCs cultured in vitro inside deep microchannels.

Specifically, vSMCs was cultured on fabricated polyurethane scaffolds with or without deep microchannel patterns.

The effect of transforming growth factor beta one (TGF- β 1) exposure before or after cell confluence on the tissue cultures was studied in terms of cellular morphology and vSMC contractile protein expression (smooth muscle α -actin, myosin heavy chain, calponin) using Western blot.

This chapter reports on details of scaffold fabrication and outcomes of vSMC culture within the scaffold (flat or patterned) with or without TGF- β 1. We hypothesized a synergism exists between TGF- β 1 and microchannels in promoting a contractile vSMC cell phenotype. We also hypothesized that exposing vSMC-microchannel culture to TGF- β 1 at certain time points of cell growth (i.e., before or after cell confluence) would maximize the contractile phenotype.

3.2 Experimental details

All chemicals were bought from Sigma-Aldrich and utilized as received unless otherwise specified. A polyurethane-based (PUR) formulation was selected because polyurethane is known to be cytocompatible, relatively easy to synthesize from its constituent monomers, and also cost-effective. Its products can also be fine-tuned to the desired rigidity and thickness for the purpose of cell culture.

3.2.1 Materials

The PUR used in this study was prepared by meticulously mixing 68% urethane diacrylate (EB270 supplied by UCB chemicals), 20% dipropylene glycol diacrylate (SR508 supplied by Sartomer Chemicals), 9.8% trimethylolpropane triacrylate (Aldrich chemicals), 2% silicone acrylate (EB350, UCB chemicals), and 0.2% 2,2-dimethoxy-2-phenylacetophenone (Irgacure 651, CIBA chemicals).

3.2.2 Fabrication of PUR films

The microchannelled PUR scaffold was produced by UV embossing, and have deep and wide microchannels (60 μm deep, 300 μm wide) separated by narrow microwalls (25 μm wide). The replication involves making first a Si master mold, replicating an intermediate polydimethylsiloxane (PDMS) intermediate mold and finally a PUR micropatterned scaffold from the PDMS mold.

A silicon master mold was made using SU-8 photoresist and deep reactive ion etching (DRIE) on a 500- μ m thick silicon wafer. A PDMS daughter mold replicated from the master mold was used as an UV embossing mold. SYLGARD 184 silicone rubber (Dow Corning Corporation) was the PDMS used and it is UV transparent.

3.2.3 Fabrication of PUR microstructured films

For the PUR patterned film the PDMS mold was placed on a piece of clean glass, and then the UV resin was dispensed onto the surface of the PDMS patterned rubber mold. For flat PUR film a polyester film (Mylar 300EC, 75- μ m thick, supplied by Higashiyama Film Singapore Pte Ltd) was overlaid on a piece of glass and UV resin was dispensed onto the surface of the Mylar film. The steps for fabrication for both patterned and flat PUR films became identical from this point (Fig. 3.1).

After the resin had spread all over the mold surface, the mold/resin assembly was placed under vacuum at 0.15 torr (0.2 mbar) for 10–15 min to remove all air bubbles and ensure total filling of the mold channels.

A Mylar film was carefully overlaid onto the degassed resin on the mold to avoid the creation of any air bubbles. The resulting “sandwich” was masking-taped on three edges and passed through (open edge of “sandwich” facing operator) a laminator machine to produce a thin resin film of predictable thickness. The resin was then polymerized under UV illumination at 16 mW/cm² for 15 min (from mold side for the patterned film) using a 350-W mercury lamp at 365nm of a mask aligner system (SUSS MicroTech, Bremen, Germany).

The cured PUR was carefully removed from the rubber mold along the long dimension of the channels. The polyester film was then peeled off from the cured resin. These steps yielded micropatterned or flat films composed exclusively of the crosslinked polymer. The crosslinked cured films were distinguished as being optically clear and have a minimum UV light transmission of at least 80 percent measured by a UVA light transmission meter (UV Powermeter OAI 306, Milipilas, CA, USA).

Monomer extraction was done by submerging crosslinked PUR films in methanol (supplied by Baker Analyzed®, A.C.S Reagent) at the w/v ratio of 1:50 (1 g crosslinked polymer in 50 mL methanol) at ambient temperature in a shaker at a speed of 50 rpm for 4 days, to allow unreacted monomers and photoinitiators to leach out.

3.2.4 SEM and optical imaging of photocured PUR films

To study the ultrastructure of the photocured PUR films, optical images were taken using a Zeiss inverted microscope (10x and 20x magnifications). The cross-sectional profile of the microchanneled scaffold was determined using scanning electron microscopy (SEM). SEM was done using the JEOL JSM-5600LV. Before SEM measurement, all samples were coated with a thin layer of gold using the Palaron SC7640 Sputter Coater. The gold coating time was 200s.

3.2.5 Investigating the in-vitro biocompatibility of vSMC (rat aorta) on photocured PUR films

The vSMCs used were from rat aorta (ATCC, CRL-1444). vSMCs were maintained in 75 cm² tissue culture polystyrene flasks in complete growth medium (Dulbecco's Modified Eagle's Medium with 4mM L-glutamine supplemented with 10% fetal bovine serum, 50IU/ml of penicillin and 50 μ l/ml of streptomycin).

Cells were passaged by trypsinization with 0.25% (w/v) Trypsin-0.03% (w/v) EDTA solution before reaching confluence, usually every 3rd day. The PUR micropatterned and flat films were cut into 1.5cm diameter discs and sterilized in 70% ethanol/30% ddH₂O solution for 24h. Before cell seeding, the films were moved to a 24-well culture plate and equilibrated with PBS for 30mins. Then vSMCs together with culture medium were added to the plate wells (1.0×10^5 cells/cm²) and incubated for 7 days.

3.2.6 Investigating the effect of TGF- β 1 in vSMC (human aorta) culture on photocured PUR films

The vSMCs used were obtained from human aortas (human aortic vascular smooth muscle cells from Lonza Singapore Pte Ltd). Cells were routinely maintained in 75 cm² tissue culture polystyrene flasks in complete growth medium (DMEM with

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4mM L-glutamine supplemented with 10% fetal bovine serum, 50 IU/mL of penicillin, and 50 mL/mL of streptomycin).

Cells were passaged by trypsinization with 0.25% (w/v) Trypsin-0.03% (w/v) EDTA solution before reaching confluence, usually every 6 days. Micropatterned and flat PUR films were cut into 6-well culture plate disks and oxygen plasma-treated with a March PX-500 (March Plasma Systems Incorporated, CA, USA) for 2 mins at 100W in 10% oxygen (90% nitrogen), following which the disks were moved to a 6-well culture plate and immediately coated with a collagen I rat tail (Sigma-Aldrich) solution (0.8 mg/ml in 0.1% acetic acid) to facilitate later cell adhesion during culture. After overnight collagen coating, the collagen solution was aspirated and the disks were sterilized overnight with 70% alcohol.

Before cell seeding, the films were rinse-equilibrated with PBS thrice. Then vSMCs together with culture medium were added to the plate wells (1.0×10^5 cells/cm²). Medium was refreshed every 2 days.

Our plan for the cell culture experiment proper would be to have 6 experiment subgroup- cultures of which 2 would act as the control. A pair of flat and micropatterned cultures would be grown without exogenous growth factors; another pair of flat and micropatterned cultures would be grown with the addition of 10ng/ml TGF- β ₁ daily in the 3 days leading to a predetermined day of complete confluence; yet a third pair of flat and micropatterned cultures would be grown with the addition of 10ng/ml TGF- β ₁ daily in the 3 days after a predetermined day of complete confluence. All experiments were done in triplicate.

3.2.7 Optical imaging of cell culture experiments

To quantify cell morphologic changes, phase contrast images were taken using a Zeiss inverted microscope (10x and 20x magnifications).

3.2.8 Immunoblotting for marker proteins of contractility across all cell culture experiment groups

Immunoblotting will be carried out as follows: vascular SMCs grown on micropatterned films and flat films will be washed twice with ice-cold PBS, followed by incubation with 100 μ l ice-cold cell lysis buffer (0.5% Nonidet P-40, 20mM Tris-HCl, pH 7.4, 0.5% sodium deoxycholate, 0.1% SDS, 150mM NaCl, 1mM EGTA, 1mM EDTA, 10mM NaF, 1% Triton X-100, 5 mg/ml leupeptin, 5 mg/ml pepstatin A, 5 mg/ml aprotinin, 5 mM phenylmethylsulfonyl fluoride, 1mMNa₃VO₄, 2mM NaF, 2mM Na₄P₂O₇, and 10mM β -glycerophosphate) for 15 mins on ice. The sample solution of lysed cells is aspirated leaving behind the empty films and cell debris from the aspirated sample solution will be eliminated by a 20-min centrifugation at 20,000g. Proteins will be fractionated by SDS-PAGE on a 10% acrylamide gel under reducing conditions and blotted onto a nitrocellulose membrane (Amersham Biosciences, UK). After blocking with 5% nonfat milk (soluble in TBST) for 1 h, the membrane is incubated with the following antibodies: anti-smooth muscle α -actin (1:500, Sigma); anti-myosin heavy chain 1(SM1, 1:200, Abcam); anti-calponin (1:500, Sigma); anti-GAPDH (1:10000, Abcam), overnight. The membranes will then be washed with TBST and incubated with alkaline phosphatase-linked secondary

antibodies (1/800, Bio-Rad). After being washed with TBST, immunoreactive proteins will be visualized using NBT/BCIP as a substrate. The blots will be scanned with a GS-800 calibrated densitometer (Bio-Rad). The relative levels of detected proteins compared to GAPDH will be assessed by measuring the integrated intensity of all the pixels in each band excluding the local background. Results will be from at least 3 independent experiments. The data shall be analyzed-compared on Microsoft Office Excel 2007 using a paired Student t-test (2-tailed) and a value of < 0.05 is considered statistically significant. The error bars were based on relative intensities.

3.3 Results

3.3.1 SEM dimensional fidelity of microstructured PUR films

Large area patterned PUR films with microchannels (25/300) were successfully prepared over large areas of a few cm^2 . In this study, PUR films were patterned with microchannels (60 μm deep and 300 μm wide) separated by discontinuous microwalls (wall segments 25 μm wide and 160 μm long separated by 40 μm long gaps) and used these scaffolds for vSMC culture. We believe that the gaps between the walls allow for cell-cell communication and circulation of endogenous paracrine chemical factors. Representative SEM images are shown in Fig. 3.1.

(a)

(b)

Figure 3.1 (a) Schematic showing PUR film synthesis. (b) Different views of UV embossed microchannelled PUR film (25/300).

3.3.2 Rat aorta vSMC response to the PUR films (flat and micropatterned) only

Figs. 3.2 and 3.3 show the cultures of rat aorta vSMCs on flat and micropatterned collagen-coated PUR films respectively. It appears that both PUR films had good biocompatibility with rat aorta vSMCs. The vSMCs seeded at a density of 1.0×10^5 cells/cm² adhered to and spreaded extensively on the substrates; proliferation was rapid and 100% confluence was attained within 7 days (Figs. 3.2 & 3.3). The rat vSMCs on flat films had random cellular alignment and adopted a fibroblast shaped morphology. On the other hand, we observed that rat vSMCs on micropatterned films aligned parallel to the microchannels at confluence adopting a spindle shaped morphology.

Figure 3.2 Optical microscopic pictures (10x) of rat aorta vSMCs grown on PUR flat films over 7 days. From above left clockwise: picture at 24 hours, at day 5, at day 7.

Figure 3.3 Optical microscopic pictures (10x) of rat aorta vSMCs grown on patterned PUR films over 7 days. From above left clockwise: picture at 24 hours, at day 5, at day 7.

3.3.3 Human aorta vSMC response to flat and patterned PUR scaffold only

Fig. 3.4 shows the cultures of human aorta vSMCs on collagen-coated plasma treated flat and micropatterned PUR films. It appears that the PUR films exhibited excellent biocompatibility with human aorta vSMCs. The vSMCs seeded at a density of 1.0×10^5 cells/cm² quickly adhered to and spreaded extensively on the substrates,

proliferating rapidly to reach confluence within 5 days, maintaining well through to day 7 of culture (Fig. 3.4). We determined in these preliminary cultures that human aorta vSMC cell confluence is achieved at day 5 on our PUR flat scaffold and used day 5 as our confluence reference point for subsequent experiments.

Vascular smooth muscle cells grown in the deep microchannels were more spindle-shaped and aligned well, parallel to the microchannels as desired without clumping or stacking (Fig. 3.4). Cells grown on flat surfaces appeared more spread out and were fibroblastic in nature-morphology; also there was a lack of order and alignment for vSMCs grown in flat PUR.

Figure 3.4 Microscopic pictures (20x) of human aorta vSMCs grown on PUR films after (I) 3 days, (II) 5 days, (III) 7 days, on (a) flat and (b) patterned surfaces.

3.3.4 Effect of PUR micropatterned scaffold and TGF- β 1 on human aorta vSMC: cell culture and microscopy

Fig. 3.5 shows the cultures of human aorta vSMCs on the collagen-coated PUR films, with the addition of TGF- β 1 before confluence (days 3 to 5).

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Figure 3.5 Microscopic pictures (20x) of human aorta vSMCs grown on PUR films after (I) 3 days, (II) 5 days, (III) 7 days on (a) flat and (b) patterned surfaces with the addition of TGF- β 1 to culture daily from days 3 to 5 before confluence.

The vSMCs seeded at a density of 1.0×10^5 cells/cm² quickly adhered and spread extensively on the substrate, and proliferated rapidly reaching confluence within 5 days; TGF- β 1 was added from days 3 to 5 before confluence and the culture maintained well through to day 7 of culture (Fig. 3.5). In this set of vSMC cultures for both flat and micropatterned film, there was no obvious morphological difference compared to vSMCs cultured on scaffold alone without added TGF- β 1 (Fig. 3.4).

Fig. 3.6 Microscopic pictures (20x) of human aorta vSMCs grown on PUR films after (I) 3 days, (II) 5 days, (III) 7 days on (a) flat and (b) patterned surfaces with the addition of TGF- β 1 to culture daily from days 5 to 7 upon confluence.

Fig. 3.6 shows the cultures of human aorta vSMCs on the fabricated collagen-coated PUR films, with the addition of TGF- β 1 after confluence. The vSMCs seeded

at a density of 1.0×10^5 cells/cm² quickly adhered and spread extensively on the substrate, and proliferated rapidly reaching confluence within 5 days; TGF- β 1 was added from days 5 to 7 after confluence maintaining well through to day 7 of culture. In this set of vSMC cultures for both flat and micropatterned film, there was no obvious morphological difference compared to corresponding vSMCs cultured on scaffold alone without added TGF- β 1 (Fig. 3.4). Also there was no obvious morphological difference compared to corresponding vSMCs cultured on scaffold with TGF- β 1 added pre-confluence (Fig. 3.5).

3.3.5 Effect of PUR micropatterned scaffold and TGF- β 1 on vSMC: western blotting for contractile protein markers

Fig. 3.7 (a) shows the α -actin western blots of human aorta vSMCs cultured on fabricated collagen-coated PUR flat and patterned films with or without the addition of TGF- β 1.

Figure 3.7 (a) Western blot done in triplicate for α -actin of human aorta VSMCs grown on PUR flat or patterned films over 7 days with or without the addition of TGF- β 1 to culture. Column (1) shows the blot for cells grown on patterned PUR, column (2): patterned PUR with TGF- β 1 added before confluence, column (3): patterned PUR with TGF- β 1 added after confluence, column (4) shows the blot for cells grown on flat PUR, column (5): flat PUR with TGF- β 1 added before confluence, column (6): flat PUR with TGF- β 1 added after confluence. (b) Graph of the relative amount of α -actin expression (Y-axis) versus the various scaffold and growth factor combinations. X-axis column (1) represents cells grown on patterned PUR, column (2): patterned PUR with TGF- β 1 added before confluence, column (3): patterned PUR with TGF- β 1 added after confluence, column (4) shows the blot for cells grown on flat PUR, column (5): flat PUR with TGF- β 1 added before confluence, column (6): flat PUR with TGF- β 1 added after confluence.

Fig. 3.7 (b) shows the relative expression of α -actin by the different experiment groups. The greater expression of α -actin by micropatterned substrate cultures compared to flat substrate cultures is apparent. Adding TGF- β 1 to micropatterned culture before or after confluence increases α -actin expression obviously and by about

the same degree; adding TGF- β 1 to flat culture before or after confluence increases α -actin expression only very minimally.

Fig. 3.8 (a) shows the **calponin** western blots of human aorta vSMCs cultured on fabricated collagen-coated PUR flat and patterned films with or without the addition of TGF- β 1.

Figure 3.8 (a) Western blot done in triplicate for calponin of human aorta VSMCs grown on PUR flat or patterned films over 7 days with or without the addition of TGF- β 1 to culture. Column (1) shows the blot for cells grown on patterned PUR, column (2): patterned PUR with TGF- β 1 added before confluence, column (3): patterned PUR with TGF- β 1 added after confluence, column (4) shows the blot for cells grown on flat PUR, column (5): flat PUR with TGF- β 1 added before confluence, column (6): flat PUR with TGF- β 1 added after confluence. (b) Graph of the relative amount of calponin expression (Y-axis) versus the various scaffold and growth factor combinations. X-axis column (1) shows the blot for cells grown on patterned PUR, column (2): patterned PUR with TGF- β 1 added before confluence, column

(3): patterned PUR with TGF- β 1 added after confluence, column (4) shows the blot for cells grown on flat PUR, column (5): flat PUR with TGF- β 1 added before confluence, column (6): flat PUR with TGF- β 1 added after confluence.

Fig. 3.8 (b) shows the relative expression of **calponin** by the different experiment groups. Again, the greater expression of calponin by micropatterned substrate cultures compared to flat substrate cultures is apparent. Adding TGF- β 1 to micropatterned culture increases calponin expression slightly and doing it pre-confluence gives the most difference; adding TGF- β 1 to flat culture before or after confluence increases calponin expression only very minimally.

Fig. 3.9 (a) shows the **MHC** western blots of human aorta vSMCs cultured on fabricated collagen-coated PUR flat and patterned films with or without the addition of TGF- β 1.

Figure 3.9 (a) Western blot done in triplicate for MHC of human aorta VSMCs grown on PUR flat or patterned films over 7 days with or without the addition of TGF- β 1 to culture. Column (1) shows the blot for cells grown on patterned PUR, column (2): patterned PUR with TGF- β 1 added before confluence, column (3): patterned PUR with TGF- β 1 added after confluence, column (4) shows the blot for cells grown on flat PUR, column (5): flat PUR with TGF- β 1 added before confluence, column (6): flat PUR with TGF- β 1 added after confluence. (b) Graph of the relative amount of MHC expression (Y-axis) versus the various scaffold and growth factor combinations. X-axis column (1) represents cells grown on patterned PUR, column (2): patterned PUR with TGF- β 1 added before confluence, column (3): patterned PUR with TGF- β 1 added after confluence, column (4) shows the blot for cells grown on flat PUR, column (5): flat PUR with TGF- β 1 added before confluence, column (6): flat PUR with TGF- β 1 added after confluence.

Fig. 3.9 (b) shows the relative expression of **MHC** by the different experiment groups. The greater expression of MHC by micropatterned substrate cultures compared to flat substrate cultures is apparent, except for the instances where TGF- β 1 was added; MHC expression in “micropatterned” culture was only slightly greater than that in “flat” culture.

Table 3.1 shows the immunoblot data analysed using a paired Student t-test (2-tailed). A p value less than 0.05 is considered statistically significant. A p value of less than 0.001 is considered very significant and this occurred repeatedly in the analysis.

Table 3.1 Paired student t-test data analysis of the immunoblotting data for α -actin, calponin, MHC – all comparing the six experiment groupings of interest. “Pattern” refers to the use of the micropatterned PUR scaffold. “Flat” refers to the use of the flat PUR scaffold. “Early GF” refers to the addition of TGF- β 1 before cell culture confluence, and “Late GF” refers to the addition of TGF- β 1 after cell culture confluence.

	α -actin	Calponin	MHC
Pattern vs Flat	<0.001	<0.001	<0.001
Pattern vs Pattern and early GF	<0.001	0.086	<0.001
Pattern vs Pattern and late GF	<0.001	0.19	<0.001
Pattern and early GF vs Flat and early GF	<0.001	<0.001	<0.001
Pattern and late GF vs Flat and late GF	<0.001	<0.001	<0.001
Pattern and early GF vs Flat	<0.001	<0.001	<0.001
Pattern and late GF vs Flat	<0.001	<0.001	<0.001
Pattern and early GF vs Pattern and late GF	0.87	0.28	<0.05
Flat and early GF vs Flat and late GF	0.50	0.86	0.59
Pattern vs Flat and early GF	<0.001	<0.001	<0.05
Pattern vs Flat and late GF	<0.001	<0.001	<0.05
Flat vs Flat and early GF	0.11	0.17	<0.001
Flat vs Flat and late GF	0.19	0.17	<0.001

3.4 Discussion

The cell adherence and spreading appeared to be superior to those of an earlier published experiment using A7r5 SMCs on the same formulation of PUR but without collagen coating (20). We used primary SMCs here instead of cell lines bearing in mind that primary SMCs are known to be more delicate and difficult to grow. We used rat aorta vSMCs as a cheaper alternative to human aorta vSMCs initially to gauge biocompatibility and experiment feasibility. We believe the excellent cell growth can be attributed to collagen coating to increase cell adhesion and to the prior use of oxygen plasma surface modification before collagen coating. If collagen

coating is not properly carried out the substrate will not be uniformly coated with collagen and in addition the collagen will be less adherent and may come off the substrate more easily and faster compromising the tissue culture quality and stability. The use of plasma greatly improved collagen binding and immobilization to an otherwise relatively hydrophobic PUR surface providing a uniform collagen-coated substrate for the vSMCs (37). We had considered several candidate polymers namely PUR, polydimethylsiloxane (PDMS) and of course our progeny poly(ϵ -caprolactone-co-lactide-co-glycolic acid) (PCLLGA) for use as a scaffold material but finally agreed on PUR because it is cheap, easily synthesized and UV-micropatterned, and has proven biocompatibility both in vitro and in vivo (20,37). Also using PUR we could easily synthesize the micropatterned scaffold with good dimensional fidelity (dimensions specified in Section 3.3.1); dimensions of 25/300 were used over narrower microchannels (e.g., 25/160) because previous studies show that narrow microchannels limit cell proliferation, cause uneven cell alignment and encouraged cell stacking (1,8).

In this experiment, the change in SMC phenotype from a synthetic to a more contractile phenotype was demonstrated on optical microscopy when SMCs achieved confluence in the PUR microchannels. Similarly a genotypic shift of the vSMCs towards a more contractile character was seen in microchannelled scaffold across the experimental groups based on the immunoblotting results, as we shall explain below.

It appeared that compared with vSMC culture on micropatterned substrate alone, vSMC culture on micropatterned scaffold combined with the addition of TGF-

β 1 (whether early or late) gave synergistic maximum contractile SMC phenotype in terms of SM α -actin and SM-MHC expression ($p < 0.001$) (Fig. 3.10).

(a)

(b)

Figure 3.10 Quantitative analysis of α -actin, calponin, MHC expression by (a) vSMCs cultured on micropatterned PUR versus those cultured on micropatterned film with TGF- β 1 added from day 3 to day 5 before confluence, and (b) vSMCs cultured on micropatterned PUR versus those cultured on micropatterned film with TGF- β 1 added from day 5 to day 7 after confluence. Bars with $\pm$ STDEV represent results from at least 3 experiments. α -actin, calponin and MHC expression were significantly increased (*) on micropatterned films with TGF- β 1 added after 7 day culture compared to micropatterned films.

Vascular SMC culture on micropatterned scaffold alone upregulated contractile markers to a significantly greater degree compared to culture on flat scaffold alone without growth factors ($p < 0.001$ for all 3 marker proteins α -actin, calponin, MHC), or compared to flat scaffold with growth factors ($p < 0.001$ for α -actin and calponin) (Fig.s 3.10 and 3.11). The dense parallel alignment of the human aortic vSMCs along the microchannels and concomitant development of their increasingly spindle-shaped morphology was obvious. At our vascular engineering lab, we have previously shown how the micropatterned PCLLGA scaffold induces upregulation of the rat aortic vSMC contractile phenotype giving spindle-shaped morphology and significantly greater SM α -actin expression. From these results we may infer that the micropatterned scaffold acts as a powerful topographical cue for enhancement of the human vSMC contractile phenotype and genotype. It appeared from our results that the micropatterned topographical cues on its own are more influential than TGF- β 1 in upregulating the vSMC differentiated phenotype.

Figure 3.11 Quantitative analysis of α -actin, calponin, MHC expression by vSMCs cultured on flat PUR versus those cultured on micropatterned film. Bars with $\pm$ STDEV represent results from at least 3 experiments. α -actin, calponin, MHC expression were significantly increased (*) on micropatterned films after 7 day culture compared to flat films.

(a)

(b)

Figure 3.12 Quantitative analysis of α -actin, calponin, MHC expression by (a) vSMCs cultured on flat PUR versus those cultured on micropatterned film with TGF- β 1 added from day 3 to day 5 before confluence, and (b) vSMCs cultured on flat PUR versus those cultured on micropatterned film with TGF- β 1 added from day 5 to day 7 after confluence. Bars with $\pm$ STDEV represent results from at least 3 experiments. α -actin and calponin expression were significantly increased (*) on micropatterned films after 7 day culture compared to flat films.

TGF- β 1 added before or after confluence to vSMC culture on flat scaffold significantly upregulated the vSMC contractile phenotype in terms of MHC expression ($p < 0.001$). The use of micropatterned PUR and TGF- β 1 for vSMC culture gave significantly greater upregulation of the contractile vSMC phenotype compared to the combination of flat PUR and TGF- β 1 ($p < 0.001$ for all 3 markers); this did not matter whether the TGF- β 1 was added before or after confluence (Fig. 3.13) and again can probably be attributed to the inference that micropattern effects on its own are more influential than TGF- β 1 effects. We also note that for the results presented in Fig. 3.13, there was a greater absolute intragroup difference (between micropattern-TGF- β 1 and flat-TGF- β 1) for expression of α -actin and calponin compared to MHC. This disparity in quantity expressed is commensurate with the known proportional increase in quantity of α -actin, calponin and MHC expressed by the vSMC as it differentiate-graduates in phenotype from synthetic character to contractile character (illustrated in Fig. 2.5); that is, for the degree of vSMC differentiation effected by micropattern-TGF- β 1 conditions in Fig. 3.13, α -actin and calponin expression is higher than that of MHC. (10).

(a)

(b)

Figure 3.13 Quantitative analysis of α -actin, calponin, MHC expression by (a) vSMCs cultured on flat PUR with TGF- β 1 addition before confluence versus those cultured on micropatterned film with TGF- β 1 addition before confluence, and (b) vSMCs cultured on flat PUR with TGF- β 1 addition after confluence versus those cultured on micropatterned film with TGF- β 1 addition after confluence. Bars with $\pm$ STDEV represent results from at least 3 experiments. α -actin, calponin and MHC expression were significantly increased (*) on micropatterned films after 7 day culture compared to flat films.

There was a trend toward upregulation of contractile vSMC phenotype in terms of increased MHC contractile marker protein expression (statistically significant $p=0.004$) and increased calponin expression (not statistically significant) when TGF- β 1 was added before cell confluence to vSMC culture on micropatterned substrate as compared to TGF- β 1 addition after confluence (Fig. 3.14). This temporal effect of TGF- β 1 was lost for vSMC culture on flat substrate ($p>0.05$ for all 3 markers) (Fig. 3.14).

(a)

(b)

Figure 3.14 Quantitative analysis of α -actin, calponin, MHC expression by (a) vSMCs cultured on micropatterned PUR with TGF- β 1 addition before confluence versus those cultured on micropatterned PUR with TGF- β 1 addition after confluence, and (b) vSMCs cultured on flat PUR with TGF- β 1 addition before confluence versus those cultured on flat PUR with TGF- β 1 addition after confluence. Bars with $\pm$ STDEV represent results from at least 3 experiments. There was a statistically significant increase in MHC expression (*) on micropatterned films with TGF- β 1 addition before confluence, after 7 day culture.

We note that unlike for MHC, the upregulation of SM α -actin and calponin was not statistically significant when TGF- β 1 was added before cell confluence rather than after. However with addition of TGF- β 1 pre-confluence there was trending toward both increased MHC and calponin expression in the micropattern culture that was not at all evident in the flat culture (Fig. 3.14). It may be that vSMC molecular signaling regulatory receptor-mechanisms are so designed that upstream addition of TGF- β 1 pre-confluence to micropattern vSMC culture triggered a downstream increase in contractility marker expression that first began with MHC expression, next

followed by calponin expression (3). This temporally activated mechanisms probably do not affect SM α -actin expression. Although not further investigated, we believe that with a longer culture period (perhaps 12 days) the increase in MHC and calponin expression brought about by pre-confluent TGF- β 1 addition to micropatterned culture would become more significant.

As discussed in previous sections, there is conclusive proof that SM MHC differentiation marker gene expression does not happen in a non-SMC *in vivo* and again SM MHC is therefore the most discerning marker for the SMC. SM α -actin is expressed in a wide diversity of non-SMC cell types under certain circumstances including skeletal and cardiac muscle, myofibroblasts, endothelial cells, tumour cells. Similarly calponin is expressed in cardiomyocytes and a range of tumour cells. That statistically significant enhanced marker gene expression was limited to MHC alone when TGF- β 1 was added pre-confluence demonstrated how specific this differentiation-enhancing intervention was for the vSMC in a micropatterned substrate environment.

Chapter 4 Conclusion and recommendations

4.1 Synthesis of PUR

PUR was successfully synthesized. The resultant resin was amorphous and appeared as a viscous liquid at room temperature.

4.2 Biocompatibility of UV crosslinked PUR films

Liquid-to-solid polymerization was carried out by UV irradiation. The photocrosslinked polymers were amorphous and had some transparency. Films formed from these new polymers without surface modification had good biocompatibility with rat aortic vSMCs. Films formed from these new polymers when subjected to oxygen plasma surface modification and collagen coating had excellent biocompatibility with human aortic vSMCs.

4.3 Fabrication of microchannelled PUR films

Microchannelled polymeric films with wide microchannels (300 μm wide) separated by high aspect ratio (8.7) and high resolution (25 μm) microwalls were successfully fabricated using the liquid PUR by UV embossing. The replication fidelity of the liquid PUR was good. The liquid oligomer can wet the PDMS mold to

fill the microchannels. The resulting microchannelled film was made totally from cytobiocompatible material. This technique of micropatterning by UV embossing is quick and useful.

4.4 Effect of microchannels on human aortic vSMC response

Culture of human aortic vSMCs on microchannelled PUR significantly increased the vSMC contractile phenotype and genotype expression compared to culture on flat PUR. This was evidenced by the elongated spindle-like densely aligned appearance of the vSMCs in microchannels at confluence. This was also evidenced by the significant enhancement of all contractile protein marker expression (α -actin, calponin, MHC) in vSMCs grown in micropatterned environment compared to those grown on flat PUR. Further, micropatterned scaffold alone was more powerful than TGF- β 1 in upregulating the vSMC differentiated phenotype, evidenced by significant enhancement of all contractile protein marker expression in vSMCs grown in micropatterned environment compared to those grown on flat PUR with TGF- β 1 addition.

4.5 Combined effect of microchannels and TGF- β 1 on SMC response

Culture of human aortic vSMCs on microchannelled PUR combined with the addition of TGF- β 1 significantly increased the vSMC contractile phenotype

expression compared to (1) culture on microchannelled PUR alone and to (2) culture on flat PUR combined with TGF- β 1 addition. This was evidenced by (1) significant upregulation of α -actin and MHC expression in vSMCs grown within a micropatterned and TGF- β 1 growth factor enhanced environment compared to those grown on micropatterned PUR only and (2) significant upregulation of all contractility marker proteins (α -actin, calponin, MHC) in vSMCs grown on micropatterned PUR with TGF- β 1 addition compared to those grown on flat PUR with TGF- β 1 addition. There was statistically significant upregulation of MHC contractile marker protein expression when TGF- β 1 was added before cell confluence to vSMC culture on micropatterned substrate as compared to TGF- β 1 addition after confluence.

4.6 Recommendations for future works

We believe this novel synergistic effect of culturing vSMCs in a microchanneled scaffold in combination with TGF- β 1 addition before confluence to achieve maximum vSMC contractile phenotype should be fully exploited in future work at our laboratory to create a functional vascular tunica media for the purpose of tissue engineering a viable small-diameter blood vessel replacement.

Future work should seek to evaluate as well the effects of other chemical factors such as bFGF and PDGF-BB acting alone or in concert on vSMC culture in our PCLLGA biodegradable microchanneled scaffolds. While PDGF-BB and bFGF are more pro-proliferation than pro-differentiation, it would be informative to see the effect of these on microchanneled vSMC culture so that we may consider designing a

proliferative micropattern vSMC culture phase using pro-proliferation growth factors and a differentiation phase using TGF- β 1 towards synthesizing a functional vessel in a bioreactor – akin to earlier vascular engineering work (31,32). Mann et al (33) have shown that TGF- β 1 covalently tethered to PEG hydrogels dramatically increases vSMC cell culture matrix production compared to TGF- β 1 culture media, so in future we may tether growth factors to micropatterned scaffold to evaluate the effect on vSMC differentiation (33). We can also encapsulate a carrier substance like hydrogel with controlled release growth factors and use it to coat the micropatterned scaffold and investigate culture outcome and vSMC differentiation (1).

We may also grow MSCs in microchannels and determine the effects on its growth and differentiation into SMCs with the addition of the various growth factors. The studies may be refined in several permutations with the addition of more culture subgroups and a temporal controlled release component similar to what we had earlier planned, for instance, using a proliferation-inducing growth factor for some time until complete confluence followed by use of a differentiation-inducing growth factor for another week. For logistical reasons smoothelin as a contractility gene marker was not utilized in the research but it is fairly unique to SMCs. Future work should include smoothelin in the immunoblotting analysis for a more complete characterization.

While various endpoints were utilized to analyze the effects of micropatterned topographical cues on cell culture, it is timely to capitalize on the use of proteomics for the purpose. Proteomics, by displaying the entire protein complement to the genome stimulated by topographical and other environmental cues, generates a wealth of data that can be systemically functionalized after processing (38,39). Systemically

functionalizing the resulting proteome means analyzing the type and quantity of proteins produced in response to a stimulus (e.g., response of micropattern vSMC culture to TGF- β 1 addition) and then classifying the proteins according to function like cytoskeletal proteins, matrix synthesis proteins, membrane proteins, metabolic enzymes, etc (40). Inferences such as the effect of a stimulus on cellular differentiation can then be made based on the data. The capability of mass spectrometry in proteomic analysis to recognize and exactly enumerate immense numbers of proteins from composite samples can be anticipated to influence hugely on biology and medicine. Indeed we are in the process of studying the effect per se of microchannels on both vSMC culture and MSC culture using a combination of immunoblotting and iTRAQ coupled LC-MS/MS. We would do well to extend the use of proteomics to profile profound protein changes in particular looking for significant upregulation of the numerous differentiation or related marker genes that occur in culturing vSMCs and MSCs exposed to a milieu of micropatterns and/or growth factors. We also also recommend in future to carry out genotyping characterization (real time PCR using primers specific to cDNA for markers of vSMC differentiation) and immunocytochemistry staining (characterize cell orientation within microchannels by imaging F-actin and α -actin following immunostaining) as additional methods to complement and augment our results (1,8). These are exciting times indeed for vascular tissue engineering research.

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