

Effect of substrate topography on human vascular smooth muscle cell proliferation and phenotype change

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Cardiovascular diseases (CVDs) remain the leading cause of mortality worldwide, with vascular occlusion being a primary contributor. Bypass grafting is a common surgical intervention to restore blood flow, traditionally using autologous grafts such as saphenous veins and internal thoracic arteries. However, the limited availability and invasive harvesting process of autologous grafts have prompted the development of synthetic small-diameter vascular grafts (sSDVGs) as alternatives. Despite advancements, the clinical efficacy of sSDVGs remains unsatisfactory due to high rates of thrombotic occlusion, intimal hyperplasia (IH), and restenosis, primarily caused by dysregulated vascular smooth muscle cell (VSMC) behavior.

VSMCs play a critical role in the progression of IH through their proliferation, migration, and phenotypic plasticity following vascular injury. While extensive studies have explored the influence of substrate topography on endothelial cell (EC) response, the effects on VSMCs remain underexplored. This study investigates the hypothesis that substrate topographies with varying geometries, isotropy, and sizes can differentially regulate VSMC behavior, potentially mitigating IH and improving the functionality of sSDVGs.

To test this hypothesis, a 16-pattern multiarchitecture (MARC) chip was employed to screen various surface patterns for their ability to modulate VSMC phenotype. Five promising patterns were selected and individually fabricated on polydimethylsiloxane (PDMS) substrates for further evaluation. The influence of these topographies on VSMC behavior was assessed under normal and platelet-derived growth factor (PDGF)-stimulated conditions by analyzing protein markers associated with VSMC phenotypic states, including α -smooth muscle actin (α -SMA), phosphorylated myosin light chain kinase (pMLCK), F-actin, desmin, vimentin, phosphorylated focal adhesion kinase (pFAK), and yes associated protein (YAP). Among the tested patterns, the 2 μ m grating emerged as the most effective in inducing a contractile VSMC phenotype. VSMCs cultured on this pattern exhibited reduced proliferation, an elongated spindle-like morphology, and increased expression of muscle-specific proteins, irrespective of PDGF presence. Conversely, VSMCs on the 1.8 μ m convex microlens and unpatterned substrates showed higher proliferation rates and a diminished contractile phenotype. Remarkably, the beneficial effects of the 2 μ m grating pattern were retained when incorporated into a fucoidan-modified polyvinyl alcohol (PVA) hydrogel, a biomaterial known to support EC adhesion and exhibit low thrombogenicity. The 2 μ m grating suppressed PDGF-induced proliferation while

promoting a contractile phenotype and enhancing directional motility. Mechanistic studies revealed elevated pMLCK expression, increased cytoplasmic localization of YAP, and enhanced focal adhesion maturation on 2 μ m gratings, supporting contractility and reducing proliferation. In contrast, unpatterned and 1.8 μ m convex lens substrates induced nuclear YAP localization and reduced pMLCK expression, favoring a proliferative phenotype.

This study introduces a promising strategy for regulating VSMC behavior through substrate topography, leveraging biophysical cues to promote a contractile phenotype while suppressing proliferation. By incorporating these insights into the design of biomimetic graft surfaces, this approach holds significant potential to address the limitations of sSDVGs, reduce complications such as IH, and improve long-term graft patency. Furthermore, the integration of topographical and biochemical modifications into PVA-based hydrogels represents an innovative avenue for the development of next-generation vascular grafts that combine mechanical strength with enhanced biological functionality. This work paves the way for advancing sSDVGs toward better clinical outcomes, reduced graft failure, and improved patient prognosis.

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Dedication

I dedicate this thesis to
My father, David Chacko,
My mother, Somi David,
And my loving husband, Libin Koshy,
I could not have done this without them.

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Chapter 1

Introduction and Literature Review

1.1 Introduction

Despite significant research efforts and the widespread prevalence of cardiovascular diseases, the development of effective treatment options for small arterial grafts remains a major clinical challenge. Synthetic small-diameter vascular grafts (sSDVGs), defined as those with a diameter of less than 6 mm, have consistently failed to demonstrate long-term clinical efficacy. The primary obstacles to their success are the high rates of thrombosis and the formation of anastomotic intimal hyperplasia (IH) at graft sites[1]. In contrast, larger-diameter synthetic vascular grafts (greater than 6 mm) have achieved notable clinical success, primarily due to reduced susceptibility to occlusion. However, when the diameter falls below this threshold, synthetic grafts are prone to rapid occlusion, significantly limiting their utility. A key contributor to the failure of sSDVGs and stents is the pathological behavior of vascular smooth muscle cells (VSMCs). The phenotypic switch of VSMCs from a contractile to a synthetic phenotype, coupled with their excessive proliferation and migration, plays a pivotal role in the development of IH and restenosis[2]. This chapter provides an overview of the structural composition of blood conduits and the vascular diseases that necessitate graft-based surgical interventions. The clinical applications and limitations of currently available vascular grafts are examined, with a particular focus on the challenges impeding their long-term patency and efficacy. Emphasis is placed on the progress and persistent hurdles associated with sSDVGs, including their limited success in achieving sustained clinical outcomes. The chapter also explores the role of platelet-derived growth factor (PDGF) in modulating the phenotypic switching of VSMCs, a critical process contributing to graft failure. Additionally, the potential of substrate topography in regulating VSMC behavior is introduced and analyzed. Finally, previous research on polyvinyl alcohol (PVA) as a candidate material for vascular grafts is reviewed, highlighting its properties and potential in addressing current limitations in sSDVG applications.

1.2 Vascular Diseases Necessitating Graft-Based Surgical Interventions

The vascular system facilitates the transport of oxygen and nutrients to tissues and organs while removing metabolic waste products through a network of arteries, veins, and capillaries. Native blood vessels are structurally composed of three distinct layers: the tunica intima, tunica media, and tunica adventitia (or externa) (**Figure 1**). The innermost layer, the tunica intima, is formed by a monolayer of

endothelial cells (ECs) supported by an endothelial-specific basement membrane[3]. This layer directly interfaces with circulating blood and plays a critical role in regulating hemostasis by responding to both local and systemic signals. The tunica media, the middle layer, is primarily composed of VSMCs and elastin organized into circumferential lamellae (fenestrated sheets) embedded within a matrix rich in collagen and proteoglycans. This structure provides mechanical strength and elasticity, enabling vessel compliance under varying hemodynamic conditions. The outermost layer, the tunica adventitia (or externa), consists predominantly of fibroblasts oriented within a collagen-rich extracellular matrix, along with elastin fibers arranged longitudinally. This layer also contains sparse, quiescent inflammatory cells, contributing to structural support and immune surveillance. The cardiovascular system, comprising the heart and an extensive network of blood vessels, serves as the primary mechanism for circulating blood throughout the body. The heart, functioning as a muscular pump, propels blood through rhythmic contractions that generate the pressure required to sustain blood flow. Blood is directed through a hierarchical vascular network, including arteries, veins, and capillaries, which vary in size and function. Any disruption in the intricate processes of blood flow, oxygen exchange, or nutrient transport can impair the system's efficiency, potentially leading to cardiovascular disease (CVD).

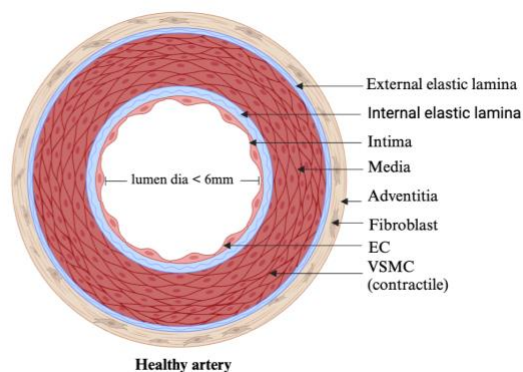


Figure 1. The structure of a healthy artery. [Created with BioRender.com]

1.2.1 Cardiovascular diseases

Cardiovascular disease (CVD) represents a significant global health challenge, ranking as the third leading cause of mortality and contributing substantially to rising healthcare expenditures worldwide. According to the World Health Organization (WHO), the global burden of CVD has increased

markedly, with annual deaths rising from 12.1 million in 1990 to 17.9 million in 2019, accounting for 32% of all deaths globally[4]. Alarming, this trend is projected to persist, with annual CVD-related mortality expected to reach 23.3 million by 2030[5]. CVD encompasses a broad spectrum of congenital and acquired conditions affecting the heart and vasculature. These include ischemic heart disease, stroke, heart failure, congenital heart defects, and peripheral vascular disease. The pathogenesis of CVD is multifactorial, involving a combination of non-modifiable risk factors, such as age, sex, and genetics, alongside modifiable risks, including hypertension, dyslipidemia, diabetes, smoking, obesity, sedentary lifestyle, and poor dietary habits[6]. Despite the diversity of these conditions, many share common underlying mechanisms and clinical manifestations. Atherosclerosis, characterized by the buildup of lipid-rich plaques within arterial walls, is the primary driver of numerous CVDs. The complex interplay of inflammation, oxidative stress, endothelial dysfunction, and thrombogenesis contributes to the pathophysiology of atherosclerosis and its complications[7]. The progression of atherosclerosis can lead to vascular occlusion, reduced tissue perfusion, and ischemic injury, culminating in life-threatening events such as myocardial infarction and ischemic stroke (**Figure 2**).

CVDs are a group of interconnected conditions, and the presence of one CVD often predisposes individuals to develop additional or multiple CVDs. For example, peripheral artery disease (PAD), which is characterized by the narrowing or blockage of arteries in the extremities, not only restricts blood flow to the limbs but also increases the risk of developing coronary artery disease (CAD). CAD, in turn, can lead to life-threatening complications such as myocardial infarction (heart attack) due to reduced oxygen and nutrient delivery to the heart muscle. Heart failure is another complex condition that can arise from multiple vascular and cardiac issues. It may be caused by the malfunctioning of the heart muscle or valves, often as a result of tissue damage sustained during a heart attack or chronic conditions like hypertension. Additionally, inadequate blood supply due to vascular occlusion exacerbates the damage, impairing the heart's ability to pump blood efficiently. Ischemic stroke, another serious consequence of CVD, occurs when a blood clot obstructs a blood vessel supplying the brain. This blockage prevents oxygen and nutrients from reaching the brain tissue, leading to cell death and potentially severe neurological deficits[8]. Such thrombotic events often originate in other parts of the circulatory system, highlighting the interconnected nature of vascular and systemic health. CVD-related complications can be particularly dangerous due to their systemic impact[9]. For instance, atherosclerosis, which underlies many CVDs, can affect multiple vascular beds simultaneously, leading to concurrent conditions such as PAD, CAD, and cerebrovascular disease. This systemic involvement

creates a vicious cycle where the presence of one condition exacerbates the progression of others, compounding the patient's overall cardiovascular risk.

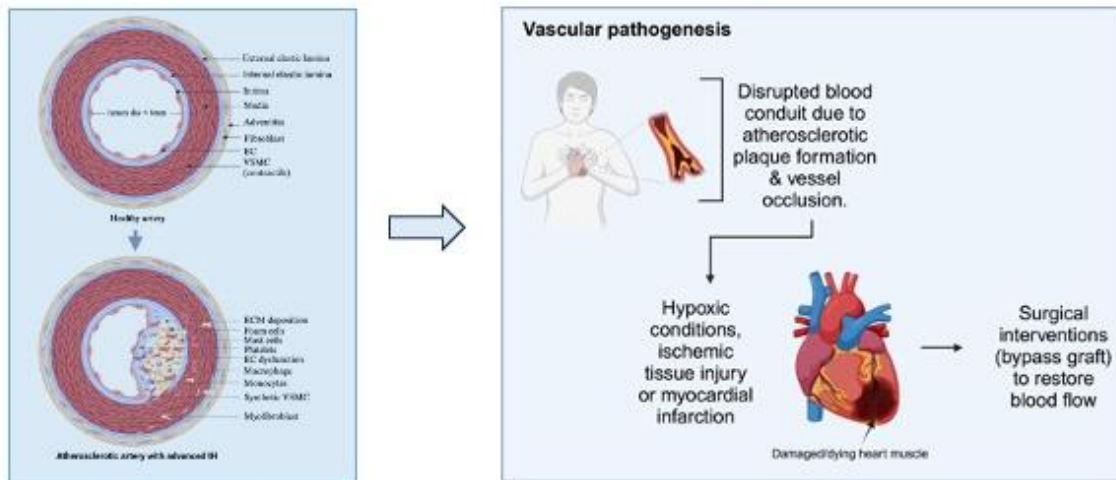


Figure 2. Illustration of atherosclerotic plaques disrupting blood flow and oxygen delivery, necessitating bypass graft surgeries. [Created with BioRender.com]

Given the focus of this thesis on vascular grafts, the discussion will now be limited to vascular complications specifically. Surgical interventions employing vascular grafts are essential to restore blood flow and ensure proper vascular function. Two major applications of vascular grafts are bypass grafts and arteriovenous (AV) grafts[10] (**Figure 3**). Bypass grafts are predominantly used in treating CVDs like coronary artery disease (CAD) and peripheral artery disease (PAD), where they serve to bypass blocked or narrowed arteries, improving circulation to the heart or extremities. On the other hand, AV grafts are commonly utilized in chronic kidney disease patients as access points for hemodialysis, providing a stable and durable connection between an artery and a vein[11]. These applications underscore the indispensable role of vascular grafts in managing both life-threatening and chronic vascular conditions, highlighting their significance in modern surgical and therapeutic practices.

1.2.1.1 Coronary artery disease:

The coronary arteries are vital blood vessels responsible for delivering oxygen-rich blood to the heart muscles, ensuring their proper function. There are two primary coronary arteries: the left main coronary artery, which branches into the left anterior descending and circumflex arteries, and the right coronary artery. Together, they form an intricate network that supplies the myocardium (heart muscle) with the nutrients and oxygen required for continuous pumping action. Blockage in the coronary arteries disrupts this blood supply, placing immense strain on the heart. Such blockages can lead to varying degrees of cardiovascular complications, ranging from angina to severe heart attacks or even heart failure[12]. The nature and extent of the blockage often determine the clinical outcome. When the blockage is partial or minor, the patient may experience angina, characterized by chest pain or discomfort due to reduced oxygen supply to the heart muscles. However, if the blockage is significant—such as when a major artery is narrowed or completely obstructed—it can lead to myocardial infarction (heart attack). To treat severe cases of coronary artery blockage, coronary artery bypass grafting (CABG) is often performed[13]. By restoring adequate blood flow to the heart, bypass surgery alleviates symptoms, reduces the risk of further cardiac events, and improves overall heart function.

1.2.1.2 Peripheral artery disease

Peripheral artery disease (also called peripheral arterial occlusive disease) is a CVD condition in which narrowed arteries reduce blood flow to the lower extremities. PAD is the predominant manifestation of atherosclerosis and results in macro and micro-vascular dysfunctions. Macrovascular disease occurs when the artery supplying to the lower extremity, such as the superficial femoral artery or popliteal artery gets occluded due to atherosclerotic plaque formation[14]. PAD affects mainly the infra-popliteal arteries and induces more damage in small arterial vessels than in large vessels[15]. Microvascular complications are the hallmark of PAD associated with diabetes and are related to foot ulcerations, which is a strong risk factor for amputation. Small vessel occlusion is the usual cause of blue toe syndrome (presented with discoloration and painful cyanotic toe), and cholesterol microemboli from the aorta were suspected to be the cause. It is usually due to embolic occlusion of digital arteries with atherothrombotic material from proximal arterial sources[16]. Abnormalities in the microvasculature represent a presumed candidate mechanism for the development of critical limb events in individuals with PAD. Adopting a healthy lifestyle is strongly recommended as a preventive strategy for PAD. For individuals diagnosed with PAD, initial treatment typically involves a combination of antiplatelet

medications and lifestyle changes. However, in advanced cases where PAD becomes severe and non-responsive to pharmacological treatments or lifestyle interventions, surgical intervention may be required. Bypass surgery is often employed in such cases to restore adequate blood flow to the affected limbs. The use of SDVGs is particularly critical in PAD due to the smaller diameters of peripheral arteries, which necessitate precise and durable graft materials. These grafts must maintain patency under challenging hemodynamic conditions while minimizing the risk of complications such as thrombosis or graft failure.

1.2.2 Chronic kidney diseases

Chronic kidney disease (CKD) is a leading cause of morbidity and mortality globally, affecting over 10% of the world's population, including approximately 4 million Canadians[17]. For patients who progress to end-stage renal disease (ESRD), renal replacement therapies, such as kidney transplantation and hemodialysis (HD), are critical for sustaining life. However, the widespread shortage of compatible organ donors and tissue matches has significantly limited the number of kidney transplants performed[18]. Consequently, the majority of ESRD patients rely on HD for long-term survival. Hemodialysis depends on the establishment of reliable vascular access, which facilitates repeated blood extraction and filtration. The gold standard for HD access is the creation of an arteriovenous fistula (AVF), typically in the non-dominant lower arm. This surgical connection between an artery and a vein provides high blood flow rates, is essential for effective dialysis, and is favored for its durability and lower complication rates compared to other access methods. Despite these advantages, the management of vascular access remains a critical challenge in ensuring the efficacy of HD treatment. However, prolonged use can lead to complications such as stenosis or thrombosis, rendering the AVF insufficient. In such cases, a synthetic vascular graft is often employed to create an arteriovenous shunt, ensuring continued vascular access for repeated cannulation and effective dialysis[19]. This approach highlights the critical role of vascular grafts in maintaining dialysis access for ESRD patients, particularly when conventional options become compromised.

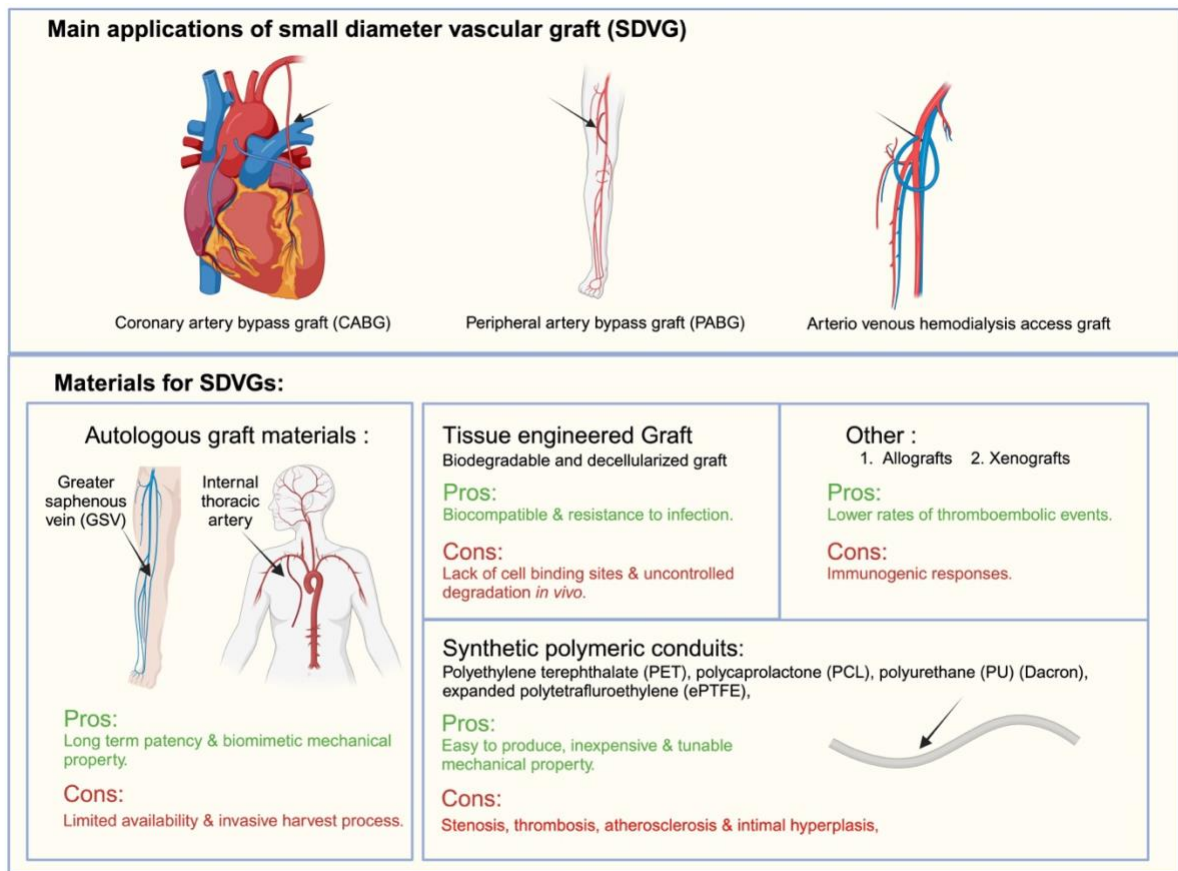


Figure 3. The clinical applications of small-diameter vascular grafts (SDVGs), including coronary artery bypass, peripheral vascular bypass surgeries, and arteriovenous grafts for hemodialysis, along with the current materials used as SDVGs. [Created with BioRender.com]

1.3 Small-diameter vascular grafts: opportunities and hurdles in clinical applications

Surgical interventions are a cornerstone in the treatment of CVD, especially when medications or less invasive procedures are insufficient. Among these interventions, vascular grafts play a crucial role. Conventional vascular grafts for bypass surgery, such as autologous internal thoracic artery and saphenous vein, are the gold standards and have relatively sufficient performance as a large-diameter vascular graft[20]. However, limited availability, surgical cost, significant morbidity and invasive harvesting processes limit their application as SDVGs (**Figure 3**). The greater saphenous vein (GSV) is the most widely used traditional graft because of its fully formed endothelial lining and natural

composition. Nevertheless, venous grafts have a limited patency rate due to the development of neointimal hyperplasia in the peri-anastomotic region[21]. Moreover, vein grafts are unsuitable for one-third of the patients and have poor performance with CABG and femoropopliteal (fem-pop) bypass grafts showing failure rates of around 50% at 10 years[10]. Synthetic materials have attracted broad attention for vascular graft fabrication due to their ease of use, inexpensive to produce and flexibility in tuning the mechanical properties. Synthetic vascular grafts like expanded polytetrafluoroethylene grafts (ePTFE), polyethylene terephthalate (PET) (commercially known as Dacron), polycaprolactone (PCL) and polyurethane (PU) can be used as a replacement for autologous blood vessels. However, established commercial grafts like ePTFE or Dacron are used due to their cost effectiveness, mechanical strength, material choices and surgical preferences rather than with a perspective toward biological functions like the formation of functional endothelium and reduced smooth muscle cells (SMC) proliferation. The ideal graft should be non-thrombogenic, bio-inert, and hemocompatible and must maintain a functional endothelium[22]. The formation of the endothelial cell (EC) monolayer, which is termed endothelialization, is essential for the long-term patency and success of the vascular graft[23]. ECs play an important role in antithrombogenicity using different anticoagulants and antiplatelet mechanisms. Therefore, the lack of ECs in a graft causes thrombosis, leading to stenosis and occlusion of the vascular graft. The proliferation of VSMCs causes IH which is a significant factor in long-term patency. Synthetic biomaterial used as large-calibre synthetic vascular grafts has attracted broad attention in vascular graft engineering, which has shown sufficient long-term *in vivo* performance for more than five years. They are well-established clinically due to their ease and flexibility of tailoring adequate mechanical properties for the benefit of surgery suturing and prevention of blood vessels bursting. However, these polymers fail when applied to small-diameter (less than 6mm in diameter) vessels due to the lack of functional endothelium, increased thrombogenicity, graft restenosis and IH at the anastomotic site, which contribute to low patency rates(**Figure 4**)[24]. The compliant mismatch between the synthetic graft and the native blood vessel at the anastomotic site is one of the major causes of IH[2]. All these factors contribute to the long-term failure of synthetic small-calibre vascular grafts.

End-to-side bypass graft:

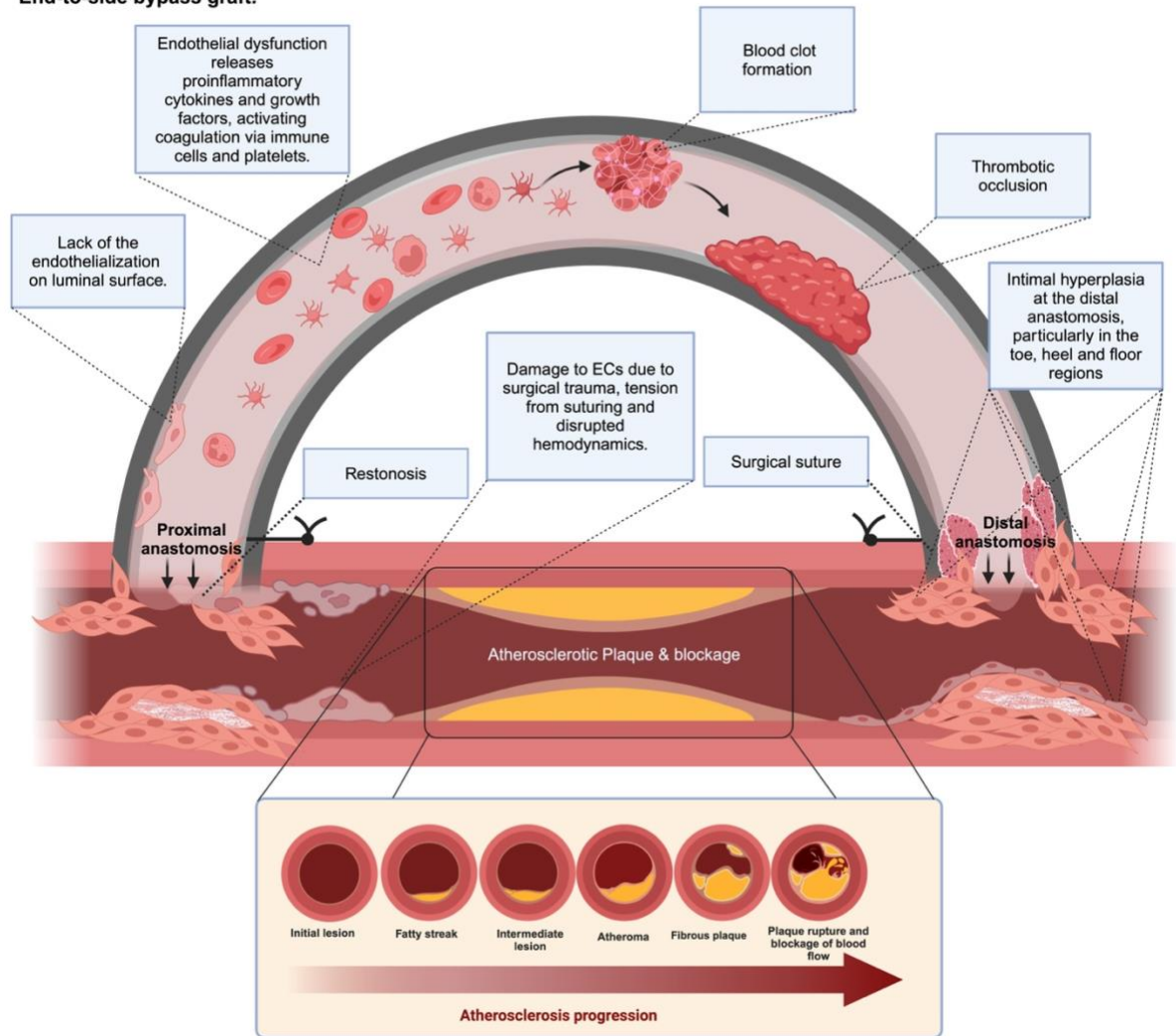


Figure 4. Schematic representation of the primary challenges limiting the clinical application of synthetic small-diameter vascular grafts, including inadequate endothelialization, thrombosis, atherosclerosis, intimal hyperplasia, and restenosis. [Created with BioRender.com] (Inspired from A.A. Conner, D. David, E.K.F. Yim, The Effects of Biomimetic Surface Topography on Vascular Cells: Implications for Vascular Conduits, *Adv Healthc Mater* 13(27) (2024) e2400335” used under Creative Commons CC-BY-NC-ND-4.0 INTERNATIONAL DEED, open access)[25]

1.3.1 Lack of endothelialization:

Endothelialization is a critical factor influencing the long-term success of sSDVGs, primarily because it mitigates thrombosis and IH, which are leading causes of graft failure. Achieving effective endothelialization involves understanding and replicating the native functions of endothelial cells (ECs). ECs play a key role in producing and maintaining an extracellular matrix (ECM) rich in collagen type IV and laminin, which promotes an atheroprotective endothelium[26]. This matrix supports EC adhesion, reduces thrombogenicity, and ensures smooth blood flow. However, synthetic SDVGs often lack this endothelial lining, resulting in poor biocompatibility and thrombogenicity. The main composition of the endothelial ECM is collagen type IV and laminin, which supports an atheroprotective endothelium that is essential for EC adhesion and the maintenance of a smooth, antithrombotic endothelium; whereas matrix protein fibronectin may stimulate an atheroprone endothelium through inflammation or wound repair signaling causing IH *in vivo*[27]. The endothelial ECM has various other functions beyond structural support like vascular remodeling, angiogenesis, blood vessel maturation and vasculogenesis. To attain improved EC monolayer formation of the vascular conduits, it is necessary to incorporate micro and nano features of the regenerative vascular ECM into the scaffold design. Various researchers have attempted to endothelialize the luminal surfaces of the synthetic vascular graft to mimic the blood vasculature[25]. In 2020, Yao *et al.* suggested that the fucoidan with anticoagulant and antithrombotic properties can potentially enhance endothelial cell adhesion and proliferation when the synthetic graft is surface modified with this sulfated polysaccharide[28]. She used fucoidan-modified polyvinyl alcohol (PVA) graft for *in vitro* and *ex vivo* studies which showed low platelet adhesion and activation and decreased thrombin generation. The *in vivo* evaluation of fucoidan functionalized PVA grafts in the right carotid artery rabbit model exhibited monolayer endothelial cell layer formation, increased patency rate (80% patency rate compared to the ePTFE synthetic graft), yielded a lower stenosis percentage and reduced IH formation.

1.3.2 Thrombosis

Vascular graft failures are primarily caused by arterial occlusion, which is driven by thrombosis, atherosclerosis, and IH. Thrombosis occurs when a blood clot, or thrombus, forms within the vessel or graft lumen, leading to partial or complete obstruction of blood flow. This process, known as thrombogenesis, is triggered by an injury response that activates hemostasis, including platelet aggregation and the coagulation cascade, resulting in the formation of a stable fibrin-embedded

clot[29]. In sSDVGs, thrombosis is exacerbated by poor biocompatibility and the absence of an endothelial lining that would normally provide antithrombogenic factors, such as nitric oxide (NO) and prostacyclin[30]. High shear stress, disrupted hemodynamics, and immune responses further promote clot formation, contributing to graft occlusion[2]. Without adequate endothelialization, anticoagulatory signaling is diminished, underscoring the importance of designing grafts that promote EC functionality to mitigate thrombosis.

1.3.3 Atherosclerosis

Atherosclerosis, another major contributor, involves the accumulation of lipid-rich plaques and inflammatory cells within or near the graft. Hemodynamic disturbances, such as low shear stress and turbulence in synthetic grafts, facilitate the deposition of cholesterol onto the vessel wall, forming plaques. These hemodynamic alterations accelerate the formation of atherosclerotic plaques, narrowing the graft lumen and obstructing blood flow[31]. Over time, the progressive plaque build-up not only reduces the graft's functional capacity but also increases the likelihood of complete occlusion or rupture, further compromising graft performance. Atherosclerosis is further complicated by immune responses and inflammation, which can also exacerbate thrombosis. Conversely, thrombosis can amplify the inflammatory responses associated with atherosclerosis, creating a feedback loop that accelerates the blockage of the vascular lumen[32]. Together, these interconnected processes can occur anywhere in the body and are associated with a wide range of cardiovascular complications, including pain, swelling, and tissue ischemia.

1.3.4 Intimal hyperplasia

IH plays a primary role in vascular occlusion and restenosis. Arterial IH is the process by which the cell population increases within the innermost layer of the arterial wall, as a multi-cellular layer instead of a single-cell-layer endothelial lining. The underlying cause of intima thickening of the artery wall is the migration and over-proliferation of VSMCs aggravated by growth factors, integrin receptors and components of the extracellular matrix[33]. The VSMC phenotypic switch from quiescent contractile to proliferative synthetic phenotype after vascular graft implantation has been deemed to be the predominant cause of IH and restenosis[34]. The contractile phenotype of VSMCs regulates vascular contraction, blood vessel diameter and blood flow while the synthetic phenotype controls vascular reconstruction during an injury. Synthetic phenotype is more migratory and proliferative in nature, which is one of the main contributors of IH in an anastomotic site. The differentiated VSMCs

(contractile) are distinguished by low proliferation, ECM production capabilities and migration, and elongated morphology. Moreover, these cells express relatively higher levels of contractile proteins such as α -smooth muscle actin (α -SMA), smooth muscle myosin II (SM-myosin II), smoothelin, calponin, myosin heavy chain[35]. Alternatively, dedifferentiated SMCs (synthetic) possess a higher proliferation rate and increased ECM production[36]. They are defined as cells with spread or fibroblast-like morphology and have no vaso activity (loss of ability to contract). Controlling the phenotype switching from contractile to synthetic and inhibiting the VSMC proliferation is seen as an effective way to reduce IH. IH mainly occurs at graft and native vessel anastomotic sites. Due to compliance mismatch between the graft and blood vessel, hemodynamic factors such as blood flow disturbance, high shear stress and stretch, and injury during surgical intervention. A review by Jeong et al. has thoroughly explained the biomechanical factors that are responsible for the formation and development of IH and their relationship with compliance mismatch[2]. Thus, reducing IH plays a significant role in the long-term patency of SDVGs.

1.4 VSMC phenotypic transformation driven by platelet-derived growth factor

Platelet-derived growth factor (PDGF) is a critical regulator of vascular remodeling, particularly in the context of vascular injury and the development of atherosclerosis[37]. The process begins with vascular injury, which disrupts EC integrity, leading to endothelial dysfunction. This dysfunction creates a pro-inflammatory and pro-thrombotic environment characterized by the increased expression of adhesion molecules, cytokines, and growth factors. Among these, PDGF-BB, the most biologically active isoform of PDGF, plays a pivotal role in orchestrating VSMC responses. In response to PDGF-BB stimulation, VSMCs undergo a phenotype transformation from a quiescent, contractile state to a highly proliferative and migratory synthetic phenotype. This transformation is essential for the repair of vascular injury but also underlies pathological processes such as IH and atherogenesis. The contractile VSMCs, marked by the expression of smooth muscle-specific proteins such as α -smooth muscle actin (α -SMA) and smooth muscle myosin heavy chain (SM-MHC), lose their differentiated markers and gain synthetic properties. These synthetic VSMCs exhibit enhanced proliferation, migration, and secretion of ECM components, such as collagen and elastin, contributing to vascular remodeling and intima thickening.

The phenotypic switch is primarily mediated by the activation of the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway[38]. PDGF-BB binding to its

receptor, PDGFR- β , activates PI3K, which catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 recruits and activates Akt, a serine/threonine kinase that mediates downstream signaling, including mTOR activation. The mTOR pathway integrates signals from Akt to regulate protein synthesis, cell growth, and survival. This signaling cascade promotes VSMC proliferation, migration, and ECM production (**Figure 5A**), driving IH[39]. IH is a precursor to atherosclerotic plaque formation. Over time, the thickened intima narrows the vascular lumen, impairing blood flow and contributing to the clinical manifestations of atherosclerosis, such as ischemia and thrombosis. The interplay between EC dysfunction, PDGF-BB signaling, and VSMC phenotype modulation underscores the complex mechanisms driving vascular remodeling. By shifting VSMCs to a synthetic phenotype, PDGF not only exacerbates the development of atherosclerotic lesions but also promotes restenosis in response to vascular interventions.

A similar scenario occurs during the implantation of synthetic vascular grafts, which induces vascular injury and EC dysfunction (**Figure 5B**). In response to PDGF, VSMCs switch from a contractile to a synthetic phenotype, characterized by increased proliferation and a spread, rounded morphology. Markers of these phenotypic states provide insights into VSMC behavior: desmin, a marker for the contractile phenotype, decreases, while vimentin, indicative of the synthetic phenotype, increases. Alpha-smooth muscle actin (α -SMA), a specific contractile protein and a marker of the contractile, non-proliferative phenotype in adult VSMCs, also decreases. Phosphorylated myosin light chain kinase (pMLCK), a marker of actin-myosin cytoskeletal contractility, is downregulated. Yes-associated protein (YAP), a nuclear mechanosensitive transcriptional coactivator, localizes to the nucleus during proliferative signals and to the cytoplasm during the non-proliferative, contractile state. Focal adhesions (FAs) play a key role in mechanotransduction. Elongated, matured, and aligned FAs promote the contractile phenotype by inducing cytoskeletal tension, while small, immature focal complexes are associated with the synthetic phenotype.

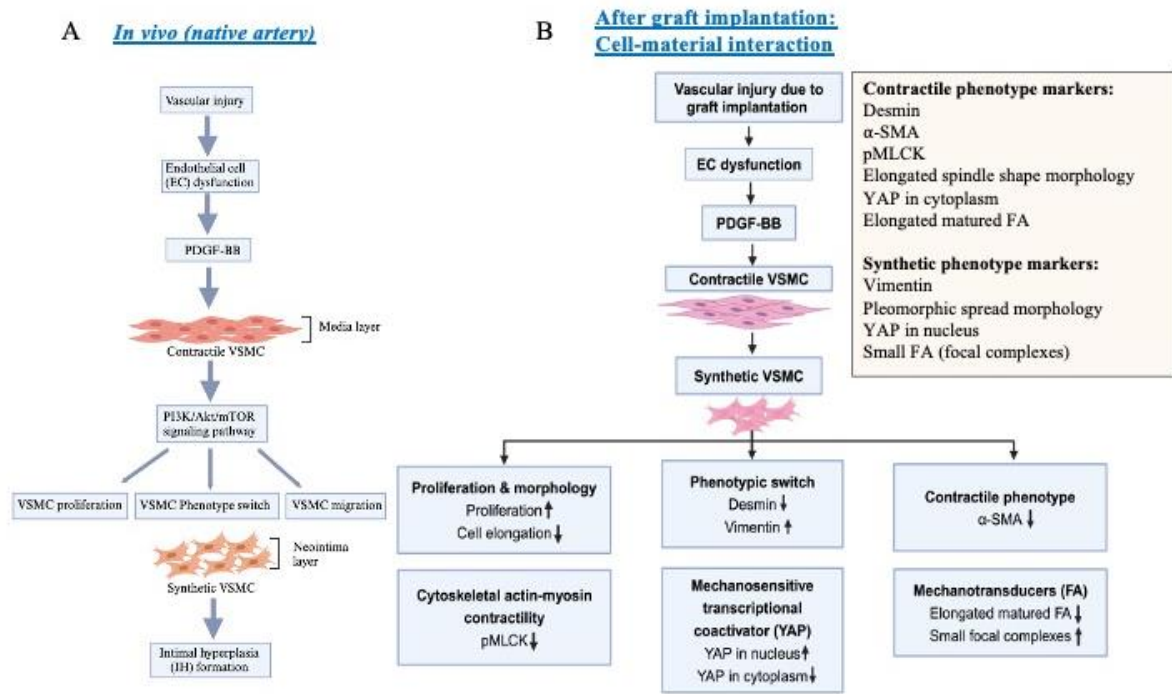


Figure 5. Schematic representation of PDGF-BB mediated VSMC phenotypic switch leading to intima thickening in (A) native artery and (B) Synthetic graft after implantation. [Created with BioRender.com]

1.5 Functional implication of vascular ECM composition and topography

ECM is a dynamic, three-dimensional meshwork of macromolecules that provides structural support and biochemical cues critical for cellular function. It comprises a diverse array of components, including structural proteins (e.g., collagen and elastin), adhesive proteins (e.g., fibronectin and vitronectin), laminins, glycoproteins, glycosaminoglycans, proteoglycans, and polysaccharides[40]. These components create a complex and versatile architecture, which varies in composition and organization depending on the tissue and physiological context.

In blood vessels, the ECM assumes a particularly critical role, constituting roughly half of the vessel wall and adapting to the mechanical demands of hemodynamic forces. The vascular ECM is arranged into micro- and nanoscale structures that influence cell behavior through chemical, mechanical, and topographical cues. These cues regulate ECs and VSMCs, which are essential for vessel wall homeostasis. The natural vascular basement membrane, for instance, comprises fibrillar

proteins, which provide structural integrity, regulate hemocompatibility, and promote endothelialization while suppressing blood leakage[41]. The ECM within blood vessels can be categorized according to the three primary layers of the vascular wall: the tunica intima, tunica media, and tunica adventitia (**Figure 6**). The tunica intima lines the lumen and is composed of a monolayer of ECs atop a subendothelial basement membrane (BM). The BM serves as a scaffold for ECs, facilitating their alignment parallel to blood flow and mediating hemostatic responses. ECs respond dynamically to vascular injury by migrating to reestablished monolayer continuity. The tunica media, located beneath the intima, is composed of helically arranged VSMCs embedded within an ECM rich in elastin, collagen, and proteoglycans[42]. This layer features circumferentially aligned VSMCs, fenestrated elastin lamellae, and interlamellar elastic fibers, creating a resilient and mechanically adaptable structure. VSMCs interact extensively with the ECM, remodeling it via actin-rich lamellipodia, surface proteases, and matrix metalloproteinases (MMPs) to facilitate migration and repair[25]. In contrast, the tunica adventitia forms the outermost layer and consists primarily of fibroblasts embedded in longitudinally aligned collagen and elastin fibers, bordered by the internal and external elastic laminae. These hierarchical structures present challenges in replicating the native ECM in synthetic grafts and scaffolds.

The topographical features of the ECM, such as nanofibers, nanopores, and nanocrystals, play a crucial role in influencing cellular behavior, including adhesion, migration, alignment, proliferation, and differentiation[43]. For example, the vascular ECM's nanoscale intimal architecture not only provides structural stability but also enhances hemocompatibility and endothelialization. Mimicking these micro- and nanoscale topographies in biomaterials has emerged as a central aim in tissue engineering, offering strategies to stimulate EC attachment and guide VSMC behavior toward a contractile phenotype. The interaction between cells and the ECM is highly dynamic, with cells capable of remodeling the ECM in response to physiological and pathological stimuli, such as injury or aging.

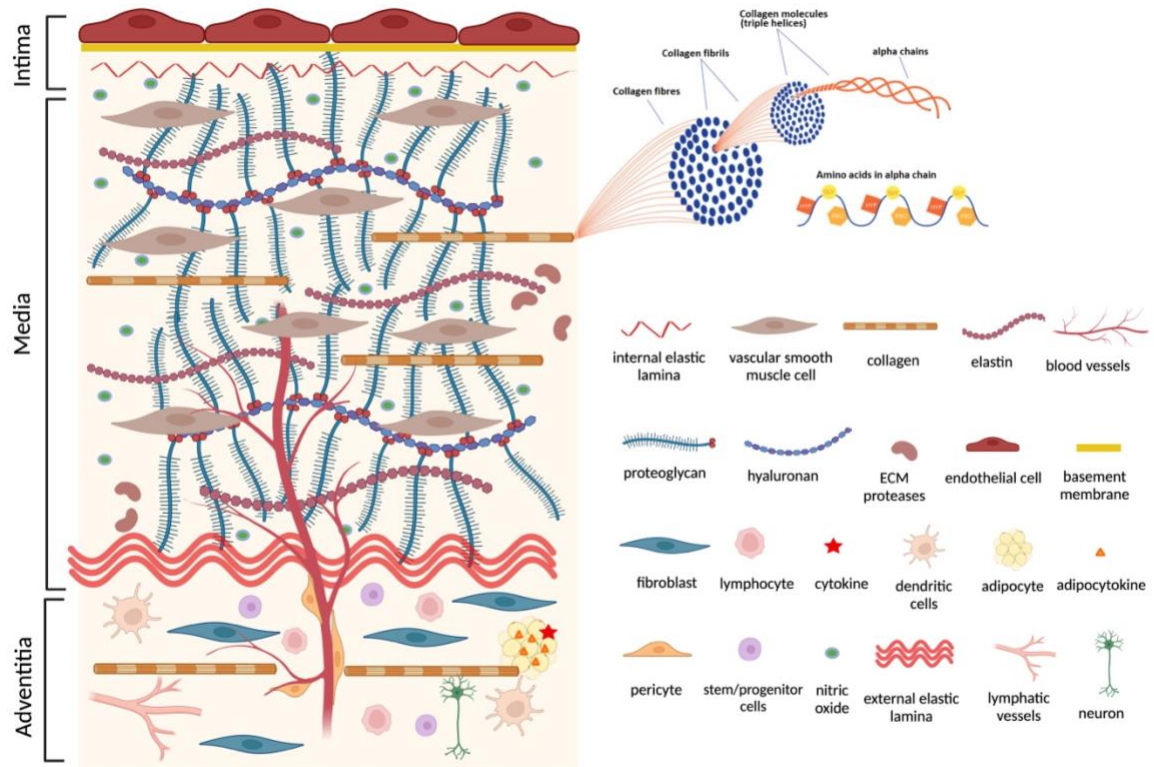


Figure 6. The composition of blood conduit and the vascular ECM. [Created with BioRender.com]

1.6 Topographical cues for modulating vascular cell behaviour

Cells exhibit diverse behaviour on a wide variety of topographies from micro to nano levels. The nanoscale is defined as a length scale of 1–100 nm in the physical domain, while the micro length scale characterizes the feature size larger than 10 μ m. Microtopography (within the length scale of a mammalian cell) mainly affects whole-cell morphology and alignment, while nanotopography is involved particularly with subcellular sensing mechanisms[44]. However, both size scales play important roles in defining the structure and function of biological systems. The introduction of micro and nanopatterning technology created breakthroughs in the field of biotechnology and bioengineering, especially in the field of tissue engineering. Various patterning techniques like photolithography, electron beam lithography, soft lithography, self-assembly, polymer demixing and nanoimprint lithography have been utilized by researchers for the fabrication of various micro-nano isotropic and anisotropic topographies[45]. Understanding each patterning technique's pros and cons will help select a suitable platform for patterning various polymeric-based biomaterials for studying cell behaviours.

Different types of micro and nanostructures can be fabricated using these techniques like gratings, pillars, grooves, pits, wells, lenses and hierarchical patterns with the combination of micro and nano features. Grating and groove topographies are anisotropic topography which has direction vectors. These anisotropic topographies are used for studying cell migration, alignment and elongation. Whereas Isotropic topography such as pillars and wells topography does not have a direction vector and is used to study cell proliferation, adhesion and differentiation.

Many studies have provided compelling evidence that substrate material surface features, or topographies, play a crucial role in influencing cellular responses[46, 47]. These topographies have been shown to exert a significant impact on various aspects of cell behaviour. The topographical patterning of the substrate plays a crucial role in cell adhesion, alignment, migration, phenotype -switch and proliferation. The introduction of surface topographies either in the micrometer or in the nanometer range helps in controlling the cell orientation and adhesion to the implant surfaces. Such cell alignment along the surface patterned structures and directed migration which is termed contact guidance are powerful indicators of the cell's ability to sense its environmental topography[48]. Studies employing both isotropic and anisotropic patterns have elucidated critical aspects of EC behavior[49]. However, the exploration of how bio-interface topography influences VSMCs remains comparatively underdeveloped, highlighting a crucial gap. As this thesis centers on the impact of topographical features on VSMC behavior, the discussion will now specifically focus on VSMC responses to isotropic and anisotropic topographies.

1.6.1 Effect of topography on VSMC behaviour

VSMCs play a crucial role in maintaining vascular tone and regulating blood flow through their ability to actively contract and relax. In response to various stimuli, VSMCs can transition reversibly between contractile and synthetic phenotypes. This phenotypic modulation is characterized by changes in proliferation, migration, ECM protein degradation, and the secretion of pro-inflammatory cytokines and growth factors, particularly in pathological vascular conditions. Topographical features of biomaterial substrates, including micro- and nanopatterning, have been widely investigated to evaluate VSMC responses such as phenotype transformation, cell spreading, alignment, proliferation, and migration. These studies have demonstrated that topographical factors—including shape (e.g., grooves, pillars, wells), dimensions (feature size, width, spacing, and height), and arrangement—profoundly influence VSMC behavior. **Table 1** summarizes key findings on the effects of topography on VSMC

behavior over the last 10 years, highlighting how isotropic and anisotropic patterns have advanced the understanding of VSMC responses and guided the design of synthetic vascular grafts.

Table 1. Summary of works that have covered the responses of vascular smooth muscle cells on substrate topography over the last 10 years. [Adapted from “A.A. Conner, D. David, E.K.F. Yim, The Effects of Biomimetic Surface Topography on Vascular Cells: Implications for Vascular Conduits, Adv Healthc Mater 13(27) (2024) e2400335” used under Creative Commons CC-BY-NC-ND-4.0 INTERNATIONAL DEED, open access][25]

Topography	Dimensions	Material	Cell Type	Major Findings	Study
Microgrooves ‡	Ra: 130nm ± 85nm Rz: 580nm ± 220 nm	Type I Collagen	PASMC	VSMCs cultured on microgrooves exhibit increased nuclear elongation/length, reduced nuclear volume, and decreased nuclear area. Microgrooves result in increased α -SMA, calponin, and SM22- α . The number of cells in the S phase of the cell cycle is significantly decreased on the topography. VSMCs on microgrooves show significantly decreased YAP/TAZ nuclear localization. Nuclear deformation under stretch is reduced on microgrooves.	Nagayama 2021[50]
	Wavelength: 0.5-10 μ m Amplitudes: 0.5-3.5 μ m	PDMS	VSMC (unknown origin)	VSMCs cultured on microgrooves with a wavelength of 3 μ m show increased cell number, density, and proliferation compared to the flat substrate, whereas wavelengths of 0.5 μ m and 10 μ m reduce proliferation. 3 μ m microgrooves increase α -SMA, CNN1, MHC mRNA and protein expression significantly compared to the other patterns. Microgrooves with a wavelength of 0.5 μ m promote VSMC apoptosis via p53 and cleaved capsase-3.	Han et al. 2020[51]
	Width: 5.4 ± 2.1 μ m	Type I Collagen	A7r5 REASMC	A7r5 cells cultured on Type I collagen films with microgroove topography show enhanced alignment elongation in the direction of the groove. Stress fibers are concentrated at the lateral membrane on microgrooves. Nuclear area on microgrooves decreased by 70% whereas the cell height increased by 30%. Cells cultured on microgrooves present homogeneous mechanical properties, alongside a 50% reduction in the elastic modulus of their nuclei. Significant increase in α -SMA and CNN1 gene expression. The number of A7r5 cells in the S-phase of the cell cycle significantly decreased on microgrooves.	Nagayama et al., 2019[52]
	Height: 200nm	Silk fibroin	PASMC	VSMC proliferation is decreased on the microgrooves compared to the unpatterned films. The number of VSMCs	Gupta et al., 2018[53]

	Spacing: 3 μm			in the G0 stage of the cell cycle is significantly higher on the patterned films. Increased SM-MHC expression on patterned films.	
	Width: 3 μm Height: 5 μm	PDMS	RASMC	Primary VSMCs at high passage numbers dedifferentiate and possess an epithelial-like synthetic phenotype yet regain contractile functions and morphology when cultured on microgrooves. Grooves restrict growth in the perpendicular direction and promote an elongated phenotype. Contractile, mesenchymal genes are upregulated on microgrooves. PDGFR β , PCNA, and osteopontin decreased dramatically on the microgrooves whereas α -SMA, myocardin, and N-cadherin increased. Proliferation on microgrooves decreased relative to unpatterned substrate in the presence of serum.	Chang et al., 2014[54]
	Fiber diameter: 0.5-3 μm	PTFE	HASMC	VSMC possess an elongated, spindle-shaped morphology on the electrospun PTFE after 5 days of culture. Cell area greater on electrospun PTFE compared to flat and expanded control, lower than TCPS. Electrospun PTFE resulted in greater α -SMA relative to flat and expanded PTFE, yet considerably lower expression compared to TCPS.	Lamicchane et al., 2016[55]
	Fiber diameter: 1.6 μm	PLLA	HUASMC	Utilized microfibers that possess nanopores of varying aspect ratios/geometries. Increasing nanopore dimension enforces more rapid evolution from the initial rounded shape to an aligned and elongated, spindle-shaped morphology. Greater elastin and collagen production found on larger nanopores. Osteopontin is downregulated on topography.	Zhou et al., 2015[56]
	Fiber diameter: 10 μm	PCL	VSMC (unknown origin)	After 7 days of culture, VSMCs adopt an elongated morphology on the aligned fibers. After 3 days, α -SMA is present on both randomly oriented and aligned fibers. After 7 days, α -SMA is only detectable on the aligned fibers.	Agrawal et al. 2015[57]
Micropillars [†]	Diameter: 3 μm Length: 9 μm Center-to-center spacing: 9 μm	PDMS	PASMC	Micropillars strongly deform nuclei, which insert themselves entirely in the space between pillars, thus “mechanically trapping” the VSMCs. Micropillars significantly reduce VSMC migration and proliferation. The number of cells in the S stage of the cell cycle is significantly decreased. Both contractile and synthetic markers are downregulated on micropillars.	Nagayama et al., 2015[58]

Nanogratings [‡]	Ridge width: 800nm Groove width: 800nm Ridge height: 400nm	PUA	MASMC	Knockout of syndecan-1 in VSMCs negatively affects cellular alignment with the nanogrooves. Loss of syndecan-1 enhances YAP/TAZ and phosphorylated Smad2/3 nuclear localization. Syndecan-1 plays a likely role in mechanosensing of nanotopography.	Le et al., 2018[59]
Nanogratings [‡]	Ridge width: 800nm Groove width: 800 nm Ridge height: 600 nm	PUA	HUASMC	VSMCs are directionally polarized on nanogratings. Cell shape on nanotopography is dependent on the substrate stiffness. α -SMA, CNN1, and desmin upregulated on topography. Nanogratings upregulate components of the Rho-ROCK-LIMK2-cofilin pathway (RhoA, ROCK1, ROCK2, LIMK2, cofilin-2) as well as caveolin-1 and profilin-2. MCP-1 downregulated on nanogratings.	Chaterji et al., 2014[60]
Nanofibers [‡]	Fiber diameter: 280nm <i>(random and aligned fibers)</i>	Fibrinogen	HUASMC	VSMCs cultured on aligned fibers exhibit an elongated spindle-like morphology compared to those on randomly oriented fibers. Increased α -SMA and CNN1 expression on aligned fibers; after 7 days of culture CNN1 not detectable on the randomly oriented fibers. In the presence of TGF- β 1, elastin synthesis was maintained for 14 days of culture on aligned fibers but absent from random fibers. Dense networks of tropoelastin positive fibrillar structures formed on randomly aligned fibers.	Woods et al., 2019[61]
	Aligned fiber diameter: 757 $\pm$ 313 nm Random fiber diameter: 687 $\pm$ 156 nm	PLGA	HASMC	VSMCs aligned on the nanofibers. Cellular alignment is significantly improved in the presence of PDGF. The cell density of VSMCs is also significantly improved by the presence of growth factors.	Zhou et al., 2013
Nanogratings [‡] Nano-wells [†]	Height: 70 nm Width: 70 nm Spacing: 70 nm	TiO ₂	HCASMC	VSMCs show higher proliferation on nano-wells relative to unpatterned surfaces and nanogratings. No significant difference in cell aspect ratio across different topographies.	Muhammad et al., 2014[62]
Nanotubes [†]	Diameter: 30-90 nm	TiO ₂	HCASMC	After 5 days of culture on nanotubes, there are 40% less VSMCs compared to the unpatterned surface. Cell size decreases by ~40% on nanotubes. Nanotubes decrease VSMC proliferation and MCP-1 expression in the presence of TNF α as well. Five-fold decrease in FA formation on nanotubes compared to flat substrates.	Cao & Desai 2020[63]

	Diameter: 30 nm	TiO ₂	HUASMC	HUASMCs deposit ECM on 30nm TiO ₂ , including fibronectin, laminin, and Type IV collagen.	Shen et al., 2015[64]
	Diameter: 110 nm	TiO ₂ (with PDA)	HUASMC	Reduced cell spreading and adhesion on nanotubes compared to unpatterned substrate. After 5 days of culture, VSMC coverage on PDA-coated nanotubes is 60.3%, compared to uncoated nanotubes (72.3%) and unpatterned samples (91.3%).	Zhong et al., 2014[65]

[†] Indicates isotropic topographies; [‡] Indicates anisotropic topographies; Material abbreviations: PLLA = poly (L-lactic acid); PDMS = poly (dimethyl siloxane); TiO₂ = titanium; PCLLGA = poly(ϵ -caprolactone-r-L-lactide-r-glycolide); PDA = polydopamine; PUA = poly (urethane acryl); Cell type abbreviations: HUASMCs = human umbilical artery smooth muscle cells; PASCs = porcine aortic smooth muscle cells; HASMCs = human aortic smooth muscle cells; HCASMCs = human coronary artery smooth muscle cells; RASMCs = rat aortic smooth muscle cells; A7r5 REASMCs = A7r5 rat embryonic aortic smooth muscle cells; MASMCs = murine aortic smooth muscle cells.

1.6.1.1 The impact of isotropic topography on VSMC behavior

Isotropic topographies, characterized by the absence of a directional vector, include features such as pillars, wells, curved surfaces (e.g., concave, and convex microlenses), nanotubes, and nanopores. These patterns are widely employed to study cellular behaviors, including proliferation, adhesion, spreading, and differentiation. Their unique geometries offer insights into how VSMCs respond to non-directional substrate cues, particularly in the context of vascular applications. Micropillars and nanopillars are frequently used to investigate the mechanical forces exerted by cells, such as traction forces and substrate-sensing mechanisms mediated through filopodial probing. Studies have shown that pillar topographies can modulate VSMC behavior, resulting in reduced proliferation, a spread-like morphology, decreased α -SMA expression, and limited migration. For instance, Nagayama *et al.* reported that VSMCs cultured on micropillars exhibited "mechanical trapping," wherein nuclear deformation occurred due to compression between pillars[58]. This deformation, coupled with heightened nuclear stiffness and increased lamin A/C expression, suggested that the inability of the nucleus to adapt to the micropillar topography induced mechanical stress, which negatively affected proliferation and migration. Curved topographies, including microlenses, are used to study VSMC responses such as motility, adhesion, morphology, and differentiation. In native vasculature, VSMCs

wrap circumferentially or helically around the tunica media, emphasizing the relevance of curvature in shaping VSMC behavior. The phenomenon of curvotaxis—cellular guidance in response to substrate curvature—has emerged as distinct from contact guidance[66]. Unlike subcellular-scale features in contact guidance, curvotaxis involves larger curvature radii. Studies have shown that VSMCs exhibit weak alignment in response to curvature and reduced motility on larger curvature features, highlighting curvature's subtle influence on cell behavior[67]. Nanotubes and nanopores further demonstrate the modulatory effects of isotropic topographies on VSMCs. These features are associated with reduced proliferation, a spread-like morphology, and decreased cytokine secretion. For example, studies have documented lower VSMC cell numbers and diminished motility on nanotube surfaces. Interestingly, exceptions exist; for instance, Muhammad *et al.* observed increased proliferation on TiO₂ nanowells compared to both nanogratings and unpatterned surfaces[62]. Despite these variations, isotropic topographies consistently fail to support biomimetic VSMC alignment and elongation, critical for mimicking in vivo vascular structure and functionality.

1.6.1.2 The role of anisotropic topographies in modulating VSMC behavior.

Anisotropic topographies, characterized by directional vectors, include patterns such as grooves, ridges, aligned fibers, wrinkles, and gratings. These patterns have shown significant potential in controlling VSMC behavior, particularly in terms of migration, elongation, alignment, adhesion, and differentiation. Unlike isotropic topographies, anisotropic surfaces offer directional cues that guide VSMCs through a mechanism known as contact guidance, where focal adhesions and filopodia sense and respond to geometric anisotropy[68]. This process involves actin filament confinement along ridges, aligning the long axis of focal adhesions with the surface anisotropy, thereby eliciting specific functional responses. Studies using microgrooves and ridges have consistently demonstrated increased cell elongation, alignment, and upregulation of smooth muscle cell-specific contractile proteins. On the other hand, microgrooves and ridges can suppress VSMC proliferation and DNA synthesis, indicating their potential for controlling IH and maintaining the contractile phenotype in vascular grafts. Maintaining the contractile phenotype of VSMCs, akin to their state in healthy native blood vessels, is crucial for the long-term success of sSDVGs. Studies have shown that anisotropic topographies can regulate VSMC morphology and enhance contractile protein expression[69]. For instance, primary VSMCs cultured on oriented microchannel arrays displayed upregulation of contractile markers, while microgrooves and microfibers reduced the expression of osteopontin, a pro-inflammatory protein implicated in VSMC proliferation[25].

Anisotropic topographies also influence matrix remodeling, a critical function of VSMCs in maintaining vascular integrity. Increased synthesis of elastin and collagen has been reported on microfibers and nanofibers, while reduced expression of matrix metalloproteinases (MMP-2 and MMP-9) was observed on microgrooves[25]. Furthermore, functional assays, such as collagen gel contractility tests, have demonstrated enhanced VSMC contractility on micro-grooved substrates compared to flat surfaces. Continued investigation into the mechanistic underpinnings of VSMC responses to anisotropic surfaces will further refine their application in developing next-generation vascular grafts.

1.7 Previous research in poly (vinyl alcohol) as a vascular graft

Synthetic poly (vinyl alcohol) PVA hydrogel showed great promise as SDVGs due to its hemocompatibility, bio-inertness, limited cytotoxicity, non-immunogenicity, low thrombogenicity, and have tunable mechanical properties[70]. In 2008, Chaouat *et al.* developed a simple process of chemically crosslinking PVA using food-grade crosslinker sodium trimetaphosphate[71]. The preparation of PVA crosslinking doesn't require an organic solvent or melting process to obtain a film with tunable mechanical properties. The fabricated PVA SDVG was mechanically stable, had sufficient burst pressure and compliance, have young's modulus and suture retention comparable to native blood vessels. The implantation of a 2mm diameter tubular graft to replace a segment of an infrarenal aorta in rats proved the compliance, resistance to mechanical stress and biocompatibility of the PVA graft to circulatory blood flow. In 2016, Cutiongco *et al.*, designed the planar and tubular patterning of micro- and nanotopographies on the luminal surface of a PVA hydrogel graft by using the casting method to study the EC responses[72]. It was shown that 2 μ m gratings resulted in long-term graft patency and endothelialization, while unpatterned grafts were occluded 20 days post-implantation in a rat aorta model. Cutiongco *et al.*, in a different study put forward that the 2 μ m grating topographies were promising for maintaining normal endothelial functions such as adhesiveness, angiogenic capacity and cell-cell junction formation, EC elongation and migration[73]. Recently, our lab member, Yao *et al.*, showed that the fucoidan with anticoagulant and antithrombotic properties can potentially enhance endothelial cell adhesion and proliferation when the synthetic graft is surface modified with this sulphated polysaccharide[28]. The author used fucoidan-modified PVA SDVG for *in vitro* and *ex vivo* studies which showed low platelet adhesion and activation and decreased thrombin generation (**Figure 7**). The *in vivo* evaluation of fucoidan functionalized PVA grafts in the right carotid artery rabbit model exhibited monolayer endothelial cell layer formation, increased patency rate (80% patency rate

compared to the ePTFE synthetic graft), yielded a lower stenosis percentage and reduced IH formation[28].

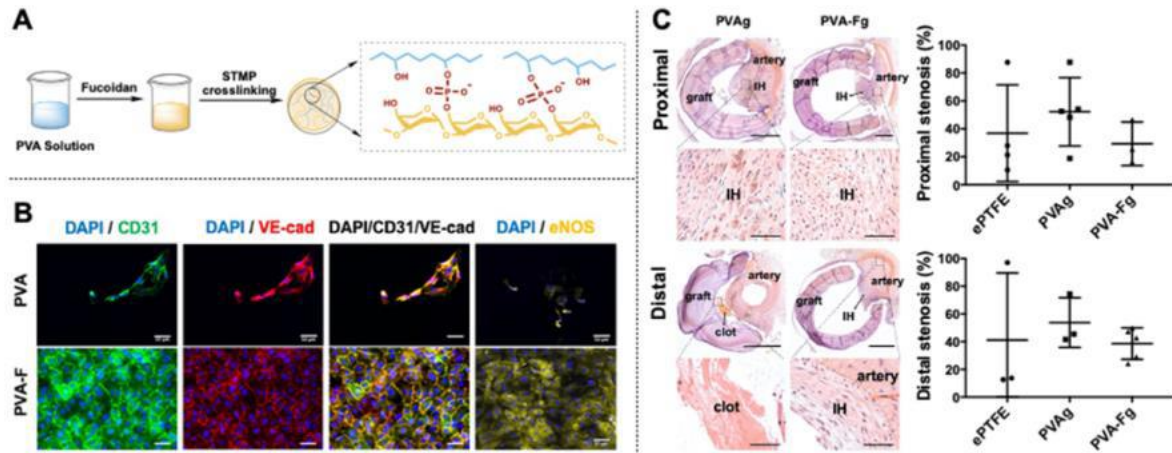


Figure 7. The use of fucoidan as functional molecules for polyvinyl alcohol (PVA) small diameter vascular grafts. (A) Modification of PVA by fucoidan. (B) Fucoidan (PVA-F) significantly improved endothelial cell adhesion on PVA. (C) Fucoidan-modified PVA vascular grafts (PVA-Fg) yielded a lower re-stenosis percentage. Reprinted with permission from Y. Yao, A.M. Zaw, D.E.J. Anderson, M.T. Hinds, E.K.F. Yim, Fucoidan functionalization on poly(vinyl alcohol) hydrogels for improved endothelialization and hemocompatibility, *Biomaterials* 249 (2020) 120011. Copyright © 2020, Elsevier.[28]

Chapter 2

Aims and Hypothesis

2.1 Gaps in Knowledge and Hypothesis

Extensive research has demonstrated that the surface topographies of substrate materials, play a critical role in regulating cellular responses. These topographical cues significantly influence various cellular behaviors, including morphology, alignment, differentiation, adhesion, migration, and proliferation. Substantial progress has been made in understanding how ECs respond to these topographical signals, which has facilitated the design of advanced synthetic vascular grafts. Studies exploring EC responses have utilized both isotropic and anisotropic surface patterns to elucidate the mechanisms by which topographies modulate cell behavior. In contrast, the impact of substrate topography on VSMCs has not been as extensively investigated. The pathological behavior of VSMCs, characterized by a phenotypic switch from a contractile to a synthetic state, along with excessive proliferation and migration, is a major contributor to the development of IH and restenosis. These processes are key factors in the failure of sSDVGs and stents. The overarching aim of this thesis is to explore the potential of substrate topography in modulating VSMC behavior to mitigate IH. Specifically, this study hypothesizes that variations in the geometry, isotropy, and size of substrate topographies can influence VSMC behavior, particularly by regulating proliferation and the transition between the contractile and synthetic phenotypes.

2.2 Research objectives

To address the gaps and limitations, I have divided my research project into two aims.

Aim 1: *In vitro* characterization and optimization of the topographical and chemical modification of PVA film for modulating VSMC proliferation and phenotype change

My aim 1 is further subdivided into two sub-aims.

Aim 1.1: To screen and identify the substrate topography features that modulate VSMC responses by utilizing multi-architectural (MARC) chip and polydimethylsiloxane (PDMS) single patterns.

Aim 1.2: *In vitro* characterization of the topographical cues of the fucoidan-modified polyvinyl alcohol (PVA) to regulate VSMC proliferation and phenotype change.

Different patterned substrates will be utilized to study the influence of surface modification on VSMC behaviour. MARC chamber, PDMS and PVA pattern substrate will be fabricated. The proliferation of primary VSMCs will be studied *in vitro*. Biomarkers such as desmin-vimentin, α smooth muscle actin (α -SMA), and filamentous-actin (F-actin) will be utilized to investigate the phenotype switching, contractility, and cell elongation of VSMCs in the different patterned substrates with and without the presence of human recombinant platelet-derived growth factor (PDGF-BB). The PVA-patterned with the short-listed topography pattern will be chemically modified with fucoidan and will be used to study the VSMC response to modified PVA, which will be the materials for vascular graft fabrication. The wound healing assay/migration of VSMCs will also be studied on modified PVA.

Aim 2: To investigate the mechanistic role of substrate topography in modulating VSMC behavior, such as enhancing contractility, controlling proliferation, and modulating phenotypic switching *in vitro*.

Single pattern PDMS and PVA pattern substrate will be utilized to study the mechanistic role of surface topographies in modulating VSMC responses. Biomarkers such as phosphorylated myosin light chain kinase (pMLCK) and yes associated protein (YAP) will be utilized to study actomyosin contractility and VSMC proliferative phenotype. Phosphorylated focal adhesion kinase (pFAK) will be used to explore the involvement of topography-induced focal adhesion signaling in VSMC migration and cytoskeletal contractility induced by the mechanotransductive signaling pathway.

Chapter 3

Topographical patterns for reduced VSMC proliferation and enhanced contractile phenotype.

3.1 Introduction

The long-term failure of sSDVGs is primarily attributed to the phenotypic switching, proliferation, and migration of VSMCs[2]. VSMCs are a predominant cell type within blood vessel walls, playing a critical role in maintaining structural integrity, mechanical stability, and resistance to hemodynamic stress. These cells exhibit remarkable plasticity, allowing them to alter their phenotype in response to environmental stimuli[74]. Under normal physiological conditions, VSMCs adopt a differentiated, contractile phenotype, remaining quiescent and contributing to vessel stability. However, in pathological states or following vascular injury, VSMCs undergo a phenotypic switch to a synthetic state, characterized by heightened proliferation, enhanced migratory capacity, and increased secretion of extracellular matrix components[75]. This transition has detrimental consequences, as synthetic VSMCs migrate towards the intimal layer, leading to intimal thickening, reduced vascular lumen diameter, impaired blood flow, and recurrent vascular occlusions. These changes not only compromise vessel patency but also undermine the structural and functional integrity of implanted vascular grafts[76]. Understanding the factors that regulate VSMC behavior is thus critical for improving the design and performance of sSDVGs.

VSMC phenotype and behavior are intricately regulated by a dynamic interplay of both biochemical and biophysical cues derived from the extracellular environment. The biochemical factors include a wide range of signaling molecules such as cytokines, growth factors, and ECM components that influence cellular processes like proliferation, differentiation, migration, and phenotype switching[77]. Among these signaling molecules, PDGF plays a pivotal role in driving the transition of VSMCs from a quiescent, contractile phenotype to an activated, synthetic phenotype. This transition is particularly critical during vascular injury and inflammation, where PDGF is released by platelets and other inflammatory cells to initiate the repair process. PDGF stimulates VSMCs to proliferate, migrate, and synthesize extracellular matrix proteins, which contribute to the formation of neointima and the thickening of the vascular wall, ultimately leading to pathological conditions such as intimal hyperplasia and restenosis[78].

In addition to biochemical signals, the biophysical properties of the extracellular environment, particularly the structural and mechanical characteristics of the ECM, also play a significant role in regulating VSMC behavior. The ECM is a dynamic, three-dimensional scaffold that provides both mechanical support and biochemical signals to cells. The structural features of the ECM, such as its topography, also play a vital role in guiding VSMC behavior. Topographical cues, such as surface roughness, micropatterns, and nanostructures, can influence cell alignment, migration, and differentiation. For instance, grooves and ridges on the ECM surface can direct VSMC alignment and orientation, while nanoscale features have been shown to modulate cellular mechanosensation and promote specific phenotypic outcomes. These biophysical signals, working in conjunction with biochemical factors like PDGF, create a complex regulatory network that governs VSMC behavior in both physiological and pathological contexts[79]. Substrate surface topographies have emerged as potent regulators of cellular responses, including morphology, alignment, proliferation, migration, and phenotype. In the context of VSMCs, topographical features have shown promise in modulating both proliferation and phenotypic changes[54, 80]. While substantial progress has been made in understanding how topographical cues regulate vascular ECs and designing EC-compatible synthetic vascular grafts, the study of VSMC responses to bio interface topographies has been comparatively limited[81]. To address this gap, this study systematically investigates the influence of topographical patterns on VSMC behavior using the MultiARChitecture (MARC) chip system, a platform featuring diverse micro- and nanoscale patterns within a single chip.

In this chapter, the use of different topographies to modulate VSMC proliferation and phenotype was explored. Herein, we used a PDMS MARC chip consisting of 16 different topographies with their technical replicates within one chip. VSMC proliferation was evaluated using the EdU assay, while phenotypic changes were assessed by analyzing protein markers, including α -SMA, desmin, vimentin, and F-actin. Following an initial screening, five promising topographical patterns were selected for further investigation and fabricated into single-pattern PDMS substrates to examine VSMC responses under both normal and PDGF-stimulated conditions. Building on previous findings demonstrating the potential of PVA hydrogels crosslinked with sodium trimetaphosphate (STMP) as vascular graft materials, two topographical patterns were replicated on PVA hydrogels. These substrates were evaluated for their ability to regulate VSMC proliferation and phenotypic changes. Furthermore, substrate topography directs VSMC migration by modulating cell alignment, elongation,

and migratory pathways. These findings underscore the ability of biophysical cues to regulate fundamental VSMC processes critical to vascular repair and disease progression.

3.2 Materials and Methods

3.2.1 Preparation of MARC arrays and single patterns

16-pattern MARC arrays were fabricated on PDMS (Sylgard™184, Elastomer Kit, Dow Corning) *via* soft lithography. The fabrication process for the MARC master mold is detailed in works by Moe *et al* [82] and Ankam *et al.* [83]. In brief, an elastomer-to-curing agent ratio of 5:1 was prepared, degassed in a vacuum chamber, and then poured onto a silanized MARC chip master(silicone) mold. The samples were again degassed for at least 30mins to remove any air bubbles, cured inside the 60 °C oven for 3-4 hours, and demolded at room temperature. The obtained mirror MARC chip was surface-treated with perfluoro-decyl trichlorosilane (FTDS) and Triton X (0.01% solution, Sigma-Aldrich) to be used as the mold for the fabrication of MARC chamber with elastomer to curing ratio (10:1.2). Degassing and curing process of MARC chamber is same as that of the MARC chip (**Figure 8**). The MARC chamber mirrors the topographies of the MARC chip master, fitting securely into 6 well-plates or 35mm dishes, minimizing reagent volume for economical use of media and growth factors, and facilitating uniform cell seeding on the patterned area. A list of patterns used in the MARC array study, a description of the pattern features, and the shortened name used to refer to the pattern can be found in **Table 2**. Topographical features were characterized by a reflected laser confocal microscope (Olympus LEXT OLS5000). Similarly, single-pattern PDMS samples were also fabricated using the soft lithography method mentioned above. Once cured, the MARC chamber and PDMS single pattern samples were kept at room temperature until use. The sixth replicas of each sample were used for the cell studies to nullify the effect of silane and the surfactant on cells. PDMS single pattern samples were cut into 1 cm² round film and placed in a 24-well tissue culture plate, and the MARC chamber was placed in a 6-well plate. Prior to cell seeding, the samples were washed in 100% ethanol, air dried and then air plasma-treated for 1 minute. The plasma-treated samples were sterilized in 70% ethanol and ultraviolet irradiation for 30 minutes. MARC chambers and selected single pattern PDMS were coated with bovine collagen type I (collagen I, 2.5µg/cm², Gibco) in acetic acid solution (0.02M solution) for 1 h at 37 °C. Excess collagen was washed off with 1X phosphate-buffered saline (PBS).

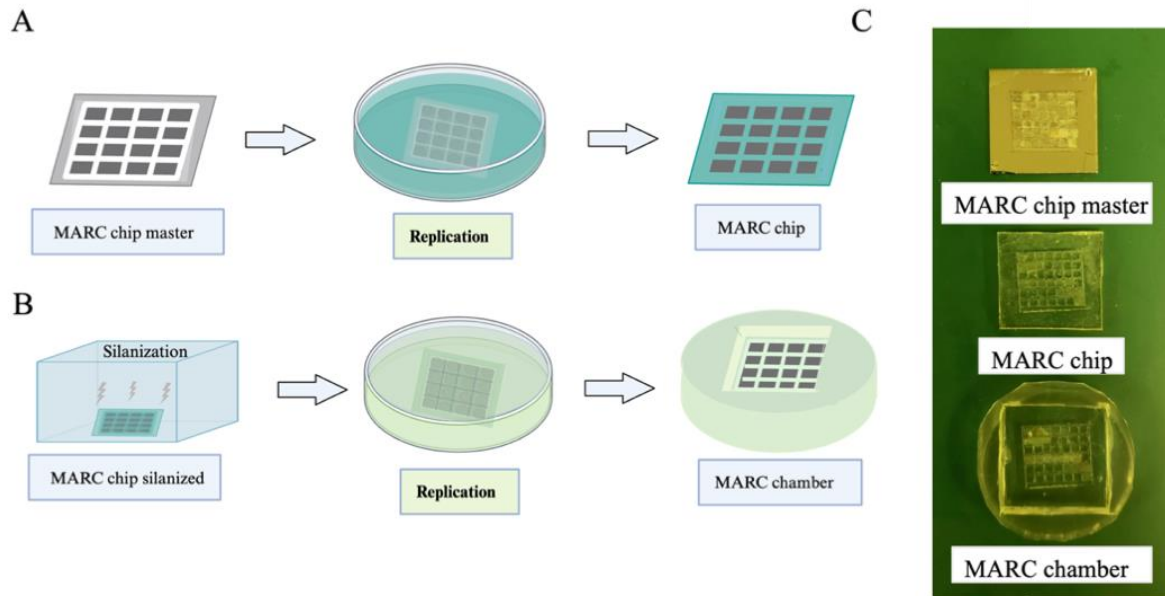


Figure 8. Fabrication of 16 pattern Multiarchitecture (MARC) chip and MARC chamber using soft lithography method. (A) Schematic diagram of replication process of MARC chip (silicone) master into MARC chip. (B) Schematic representation of the fabrication of a MARC chamber from a MARC chip. (C) Photographic images of a MARC chip master, MARC chip and a MARC chamber. (Fabrication method adapted from M. Kukumberg, Y. Yao, S.H. Goh, D.J. Neo, J.Y. Yao, E.K. Yim, Evaluation of the topographical influence on the cellular behavior of human umbilical vein endothelial cells, *Adv Biosyst* 2(6) (2018))[49]. [Created with BioRender.com].

Table 2. Pattern descriptions, pattern shorthand, and numerical key

No	Pattern	Description
	UP	Unpatterned control
1	250nm G	250nm width, 250nm space, 250nm height gratings
2	250x250x110nm G	250nm width, 250nm space, 110nm height gratings
3	460x70x40nm G	460nm width, 70nm space, 40nm height gratings
4	2 μ m G	2 μ m width, 2 μ m space, 2 μ m height gratings

5	1x2 μ m G	1 μ m width, 2 μ m space, 120nm height gratings
6	2x1 μ m G	2 μ m width, 1 μ m space, 80nm height gratings
7	250nmG L 2 μ mG	250nm gratings perpendicular on 2 μ m gratings (Hierarchical grating)
8	250nmG // 2 μ mG	250nm gratings parallel on 2 μ m gratings (Hierarchical grating)
9	250nmG w 2 μ mG	250nm pillars on 2 μ m gratings
10	1 μ m CCV lens	1 μ m diameter and pitch, 0.3 μ m sag concave micro lens
11	1 μ m CVX lens	1 μ m diameter and pitch, 0.3 μ m sag convex micro lens
12	1.8 μ m CCV lens	1.8 μ m diameter, 2 μ m pitch, 0.7 μ m sag concave micro lens
13	1.8 μ m CVX lens	1.8 μ m diameter, 2 μ m pitch, 0.7 μ m sag convex micro lens
14	2 μ m P	2 μ m pillars, 12 μ m pitch, 2 μ m height
15	1 μ m H	1 μ m holes, 6.5 μ m pitch, 1 μ m depth
16	250nm P	25nm pillars, 400nm pitch, 250nm height

3.2.2 The selection of the most impactful patterned surfaces

A systematic ranking of various topographical patterns was performed by assessing VSMC proliferation and contractile to synthetic phenotype switching by MARC chip screening (**Tables 3 and 4**). The quantified values from EdU positive cell % and desmin/vimentin (D/V) ratio were ranked according to their adjusted p-value across the topographies. The topographical patterns that showed higher significance and an adjusted p-value below 0.04 were ranked top. Five top-ranked patterns and the unpatterned control were selected for the single pattern PDMS study.

Table 3. Selection criteria for the most influential topographical pattern from MARC chip screening

MARC Chip Patterns	EdU Positive Cell %			D/V Ratio			Selected Single Patterns
No.	Significance	Adjusted p-value	Ranking	Significance	Adjusted p- value	Ranking	Patterns <0.04 adjusted p-value
1	ns	0.9253	16	ns	0.9362	10	
2	ns	0.1737	9	ns	0.9954	14	
3	ns	0.0948	8	ns	0.7432	7	
4	ns	0.9998	17	*	0.0248	1	P4
5	*	0.0417	6	ns	0.9470	11	
6	*	0.0402	5	ns	0.8286	9	
7	ns	0.4868	12	ns	0.6160	4	
8	ns	0.5763	13	ns	0.9771	13	
9	ns	0.6307	14	ns	0.7423	6	
10	***	0.0009	2	ns	0.9998	16	P10
11	***	0.0010	3	ns	>0.9999	17	P11
12	***	0.0007	1	ns	0.8071	8	P12
13	ns	0.3048	11	*	0.0351	2	P13
14	ns	0.9017	15	ns	0.7423	5	
15	ns	0.1878	10	ns	0.5787	3	

16	*	0.0425	7	ns	0.9509	12	
UP	**	0.0056	4	ns	0.9989	15	UP

Table 4. List of patterns, isotropy, and size of topographical patterns on the MARC chip. Selected patterns for single pattern study are indicated.

Description	Size	Topography on MARC chip	Topography Number	Selected Topography
Anisotropic	Nano	250 nm lines, 250 nm space, 110 nm height	2	
		250 nm lines, 250 nm space, 250 nm height	1	
		70 nm lines, 460 nm space, 40 nm height	3	
	Micro	1 μm lines, 2 μm space, 80 nm height	6	
		2 μm lines, 1 μm space, 120 nm height	5	
		2 μm lines, 2 μm space, 2 μm height	4	2 μm grating
		2 μm lines with perpendicular 250 nm lines	7	

		2 μm line with parallel 250 nm lines	8	
		2 μm lines with 250 nm pillars	9	
Isotropic	Nano	250 nm pillars, 400 nm pitch, 250 nm height	16	
	Micro	1 μm diameter, 1 μm pitch, 0.3 μm sag concave microlens	10	1 μm concave lens
		1 μm diameter, 1 μm pitch, 0.3 μm sag convex microlens	11	1 μm convex lens
		1 μm holes, 6.5 μm pitch, 1 μm depth	15	
		1.8 μm diameter, 2 μm pitch, 0.7 μm sag concave microlens	12	1.8 μm concave lens
		1.8 μm diameter, 2 μm pitch, 0.7 μm sag convex microlens	13	1.8 μm convex lens
		2 μm pillars, 12 μm pitch, 2 μm height	14	

3.2.3 Preparation of PVA hydrogels

3.2.3.1 Fabrication of PVA hydrogels

PVA hydrogels were prepared according to previously established methods[28, 73]. In a concise overview, a 10% PVA solution was created by dissolving PVA powder (average MW 85,000–124,000, 87%–89% hydrolyzed, Sigma-Aldrich) in deionized (DI) water. To crosslink PVA, a 15% (w/v) STMP solution was mixed with the autoclaved 10% PVA solution, and 30% (w/v) sodium hydroxide (NaOH) was added dropwise to activate the functional groups of PVA and STMP. The PVA crosslinking solution was centrifuged at 2500 rpm for 10 minutes to remove any air bubbles within the solution. The resulting crosslinking solution was cast on Petri dishes to form unpatterned PVA films (**Figure 9A**)

For patterned films, PVA crosslinking solution was cast on plasma treated PDMS molds, followed by centrifugation (**Figure 9B**)[72]. Topographical features were characterized by Olympus LEXT OLS5000 laser confocal microscope. Films were left in a controlled temperature and humidity cabinet until fully crosslinked. For rehydration and demolding, 10X PBS, 1X PBS, and DI water were utilized.

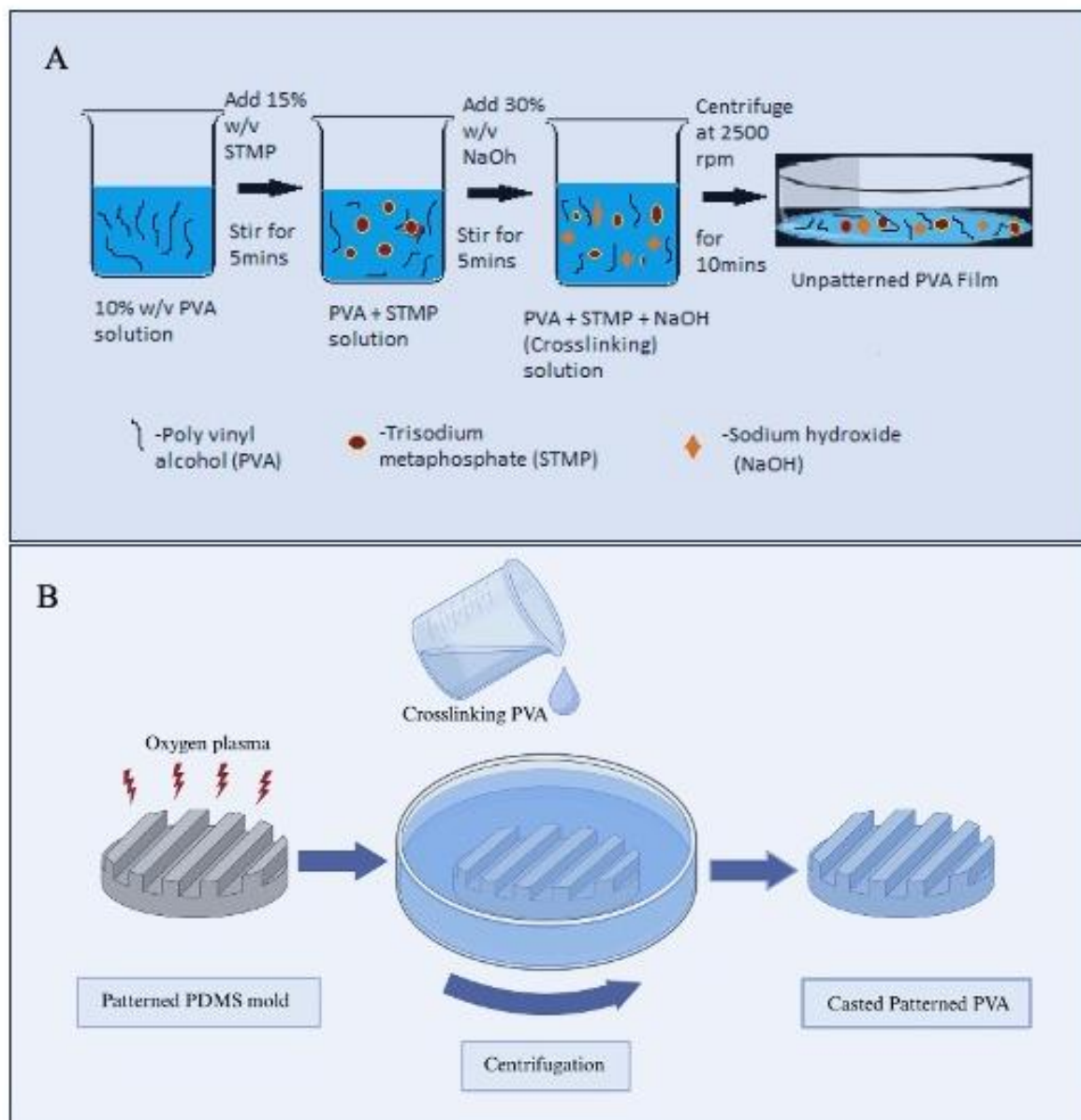


Figure 9. Schematic diagram of fabrication of (A) unpatterned and (B) patterned polyvinyl alcohol (PVA) film using casting method. (Fabrication method adapted from M.F.A. Cutiongco, S.H. Goh, R. Aid-Launais, C. Le Visage, H.Y. Low, E.K.F. Yim, Planar and tubular patterning of micro and nano-topographies on poly(vinyl alcohol) hydrogel for improved endothelial cell responses, *Biomaterials* 84 (2016) 184-195)[72]. [Created with BioRender.com]

3.2.3.2 Modification of PVA hydrogels

PVA hydrogels were modified with fucoidan to promote cell adhesion, as previously published[28]. Briefly, PVA hydrogels were dried overnight at 60 °C to remove moisture, and subsequently activated in a 100 mg/ml carbonyl diimidazole (CDI) solution in dimethyl sulfoxide (DMSO) for 1 hour at room temperature with constant shaking at 100 rpm. The CDI-activated PVA hydrogels were then subjected to three washes in 1X PBS to remove excess CDI, followed by incubation in a 10 mg/ml solution of native fucoidan in PBS at 37 °C overnight. Following the overnight incubation, the samples were washed three times with 1X PBS.

3.2.3.3 Sterilization of PVA hydrogel

PVA films were UV sterilized for 30 min. Concurrently, a fresh antibiotic-antimycotic sterilization solution was prepared by combining 5% w/v Penicillin-Streptomycin (Gibco, Penicillin-Streptomycin 10,000 U/mL solution) with 1% Amphotericin B (Corning, Amphotericin B 250µg/mL solution) in sterile 1× PBS. Following UV irradiation, the films were fully immersed in the sterilization solution and incubated overnight at 37°C. The next day, the sterilization solution was removed, and silastic tube rings (1 cm in height) were placed on the films to secure them. The films were then rinsed with sterile 1× PBS. Subsequently, 500 µL of the antibiotic sterilization solution was added to each sample, which was incubated at 37°C for 1 hour. The PVA films were thoroughly washed (10 times) with sterile 1× PBS to eliminate any residual antimicrobials. Finally, the films were stored in sterile 1× PBS at 4°C until further use.

3.2.4 *In vitro* cell culture and seeding

Human umbilical artery smooth muscle cells (HUASMCs) (Lonza) were maintained in a maintenance medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture-F-12 (DMEM/F-12) with L-glutamine and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (CORNING), fetal bovine serum (FBS) (Avantor), Glutamax TM-I (100x) (Gibco), Penicillin-streptomycin (Pen-Strep) (10,000µg/ml), 40% Glucose, epithelial growth factor and fibroblast growth factor). The cells were cultured until 90% confluency before sub-culture. Confluent cells were washed once with 1X PBS solution and detached by trypsinization. The cells (p 5-9) were seeded at 20,000 cells/cm² on MARC chambers and cultured for three days at 37°C and 5% CO₂ in maintenance media. The cells (20,000 cells/cm²) seeded on the single PDMS and PVA substrates were incubated in maintenance culture

media for 24 hours before replacing with media containing 5 μ g/ml recombinant human PDGF-BB (PDGF) and cultured for another 2 days.

3.2.5 Proliferation assay of HUASMCs on MARC array, single pattern PDMS substrates and PVA hydrogel

The proliferation EdU assay was performed using an established commercially available protocol. 10 μ M EdU solution was prepared in maintenance media. At day 3 of cell culture, the culturing media was replaced with EdU-containing fresh media and incubated for 4 hours before cell fixation with 4% paraformaldehyde (PFA). The cells were blocked with 10% goat serum and 0.03% Triton X-100 (Sigma-Aldrich) in 1X PBS. The samples were then stained with a cocktail solution made of 2029.2 μ L of 1x PBS, 325.8 μ L of 0.93M Tris-buffer, 30 μ L of 100 mM CuSO₄, 15 μ L of 2 mM Alexa Fluor 488 azide (Thermo Fisher Scientific), and 600 μ L of 0.5M ascorbic acid per 3mL of staining solution. The samples stained with the cocktail solution were protected from the light and incubated at room temperature for 30 minutes. The samples were rinsed with 1XPBS three times and stained with DAPI (Sigma Aldrich; dilution from stock 1:2000).

Fluorescence images were taken using a fluorescent microscope (Axio Observer Z1, Zeiss GmbH) and analyzed using ImageJ software. EdU assay is a DNA proliferation assay, where EdU binds with the replicating DNA. The percentage of proliferative cells was obtained by the ratio between the number of EdU-positive cells and the total cell number obtained by quantifying the DAPI count. Three biological replicates (n = 3) were utilized, with images captured from random locations, and over 100 cells were quantified per sample.

3.2.6 Immunofluorescence and image analysis

On day 3 of cell culture, cells cultured on the samples (n = 3) were washed and fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and blocked with 1%(w/v) bovine serum albumin and 10% goat serum. Following blocking, the cells were immunostained with desmin antibody (rabbit monoclonal at 1:50, Life Technologies), vimentin antibody (mouse monoclonal at 1:200, Life Technologies), or α -SMA antibody (mouse monoclonal at 1:200, Life Technologies). Samples were then washed and stained with goat anti-mouse Alexa Fluor 488 (1:750) (Thermo Fisher Scientific) and goat anti-rabbit Alexa Fluor 546 (1:750) (Thermo Fisher Scientific). Samples were counterstained with DAPI solution (1:2000) (Sigma Aldrich) and Alexa Fluor Phalloidin 546 (1:200) (Invitrogen) for 30 min at room temperature and then mounted on glass coverslips for fluorescence microscope analysis.

Fluorescence microscope images were analyzed using the ImageJ software. The average fluorescence intensity of α -SMA signals (intensity normalized to the cell number) was calculated by using ImageJ. For desmin-vimentin analysis, an average between desmin and vimentin-positive cells (D/V ratio) was taken. Three biological replicates ($n = 3$) were employed, with images obtained from random locations, and more than 100 cells were quantified per sample.

3.2.7 Wound healing assay on PVA patterned and unpatterned substrate

HUASMCs (P6-8) were cultured on unpatterned, 1.8 μ m convex lens and 2 μ m grating patterned PVA films ($n = 3$) for 14 hours to allow cell monolayer formation. A wound was created by making a scratch on the cell monolayer with a sterile autoclaved wedge-shaped PDMS strip. The wound on the 2 μ m grating pattern was perpendicular to the grating axis, to allow the cell migration direction to mimic *trans*-anastomotic migration in a vascular graft. Following the creation of the scratch, detached cell debris was removed using sterile 1X PBS and fresh media was added. The migration of the cells was monitored by imaging the wounds at predetermined time intervals (0 h, 2 h, 4 h, 6 h, 12 h, 24 h, and 34 h). The area of the wound at various time intervals was quantified using ImageJ. The percentage of wound closure was determined by dividing the wound area at different time points by the initial wound area at $t = 0$.

3.2.8 Statistical Analysis

All statistical analysis was performed using GraphPad Prism 9. Three biological replicas ($n = 3$) were used across the experiments and were indicated in the methods and/or the figure captions. Statistical analysis of different experimental groups was done using one-way and two-way ANOVA with Tukey's multiple comparisons test. Data values were represented as mean $\pm$ standard deviation. Differences with $p \leq 0.05$ were taken as statistically significant. Statistically significant data was indicated in the figures using *, **, *** and **** for $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$ unless otherwise specified.

3.3 Results

3.3.1 Screening of VSMC response using MARC array

16 topographical patterns were successfully fabricated in the MARC chamber and the fidelity of the MARC arrays was verified by laser scanning confocal microscopy (**Figure 10A**). To evaluate the proliferation of HUASMCs on unpatterned and different patterned samples, an EdU assay was

conducted. Cell proliferation percentage was obtained by dividing the EdU-positive nuclei by the DAPI nuclei count. Cells on pattern 4 - 2 μ m grating with a 1:1 aspect ratio had a reduced proliferation percentage compared to lens patterns (**Figure 11A**). Among the pattern groups, HUASMCs cultured on 1 μ m concave, 1 μ m convex and 1.8 μ m concave lens patterns exhibited the highest proliferation ($40 \pm 8\%$, $p \leq 0.001$), slightly higher than the level of unpatterned substrates ($39.4 \pm 0.6\%$, $p \leq 0.01$) and significantly higher than those on pattern 4 - 2 μ m grating pattern ($22 \pm 2\%$) (**Figure 11C**). In addition, the pattern 5 - 1 μ m width, 2 μ m space and 120nm height grating (34 ± 3 , $p \leq 0.05$); pattern 6 - 2 μ m width, 1 μ m space and 80nm height grating (35 ± 3 , $p \leq 0.05$) and pattern 16 - 250nm pillar, 400nm pitch and 250nm height pillars (34 ± 4 , $p \leq 0.05$) showed significantly higher proliferation compared to pattern 4 - 2 μ m grating with 1:1 aspect ratio. The hierarchical patterns, such as pattern 7 ($31 \pm 6\%$), pattern 8 ($30 \pm 3\%$), pattern 9 ($30 \pm 2\%$); pattern 13 ($32 \pm 1.3\%$) and pattern 15 ($34 \pm 2.3\%$), showed slightly lower proliferation compared to the unpatterned substrate ($39.4 \pm 0.6\%$) (**Figure 11C**), although the differences were not statistically significant.

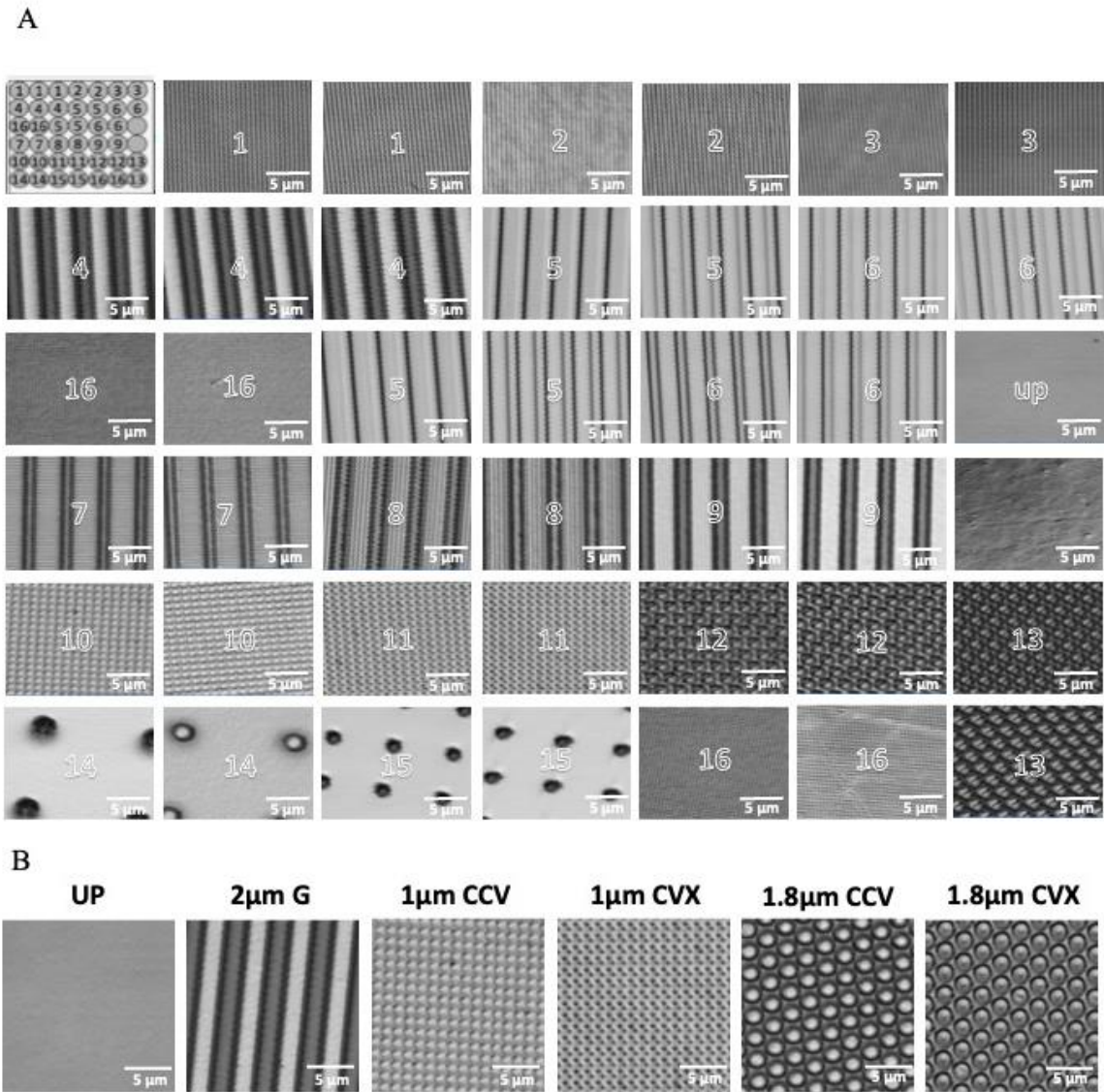
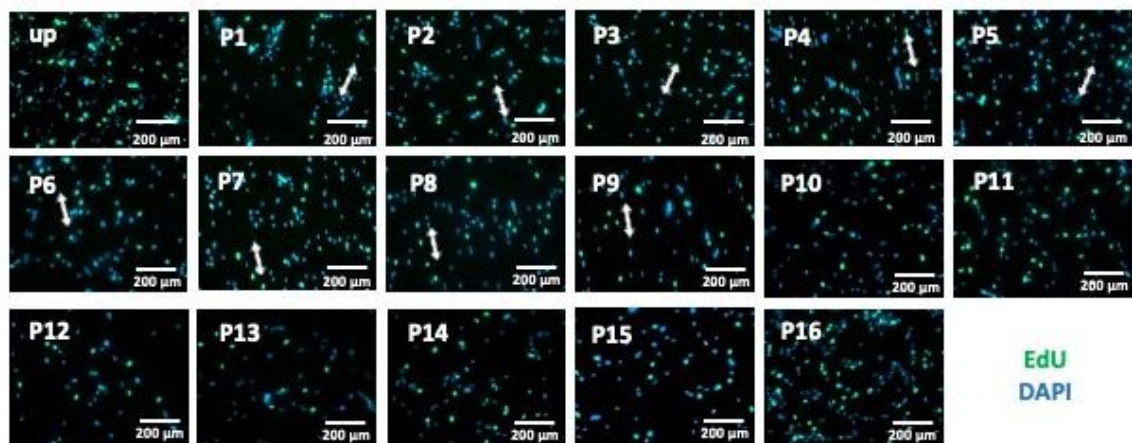


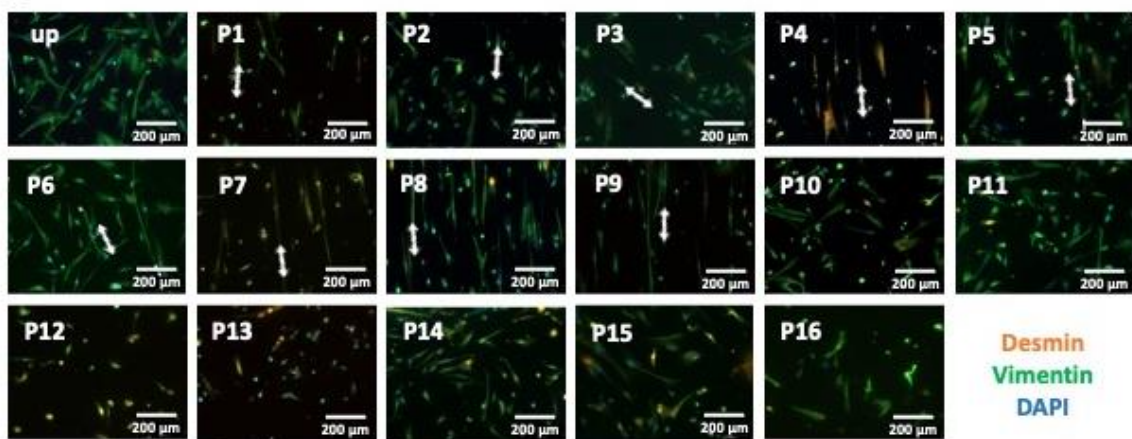
Figure 10. The reflected laser confocal images of (A) 16-pattern MARC array. (B) single-pattern polydimethylsiloxane (PDMS) substrates verified the fidelity of topographical patterns.

To evaluate the VSMC phenotype, the cells were stained with desmin and vimentin (**Figure 11B**). Cells on pattern 4 - 2 μm grating ($0.98 \pm 0.22\%$, $p \leq 0.05$) and pattern 13 - 1.8 μm convex lens ($1 \pm 0.25\%$, $p \leq 0.05$) patterns showed a significantly higher D/V ratio compared to those on unpatterned surfaces ($0.33 \pm 0.12\%$), representing more contractile VSMCs (**Figure 11D**). The screening with MARC chips revealed that certain topographical patterns consistently modulated VSMC behaviour, with either favourable or unfavourable outcomes.

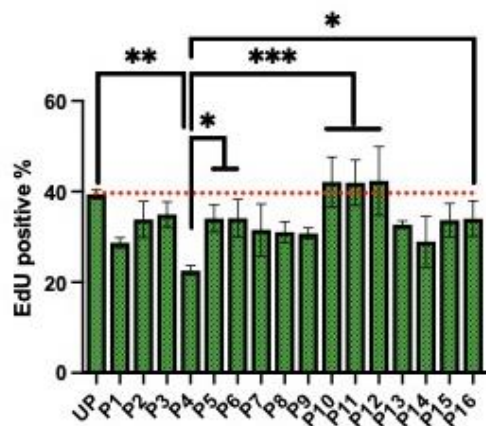
A



B



C



D

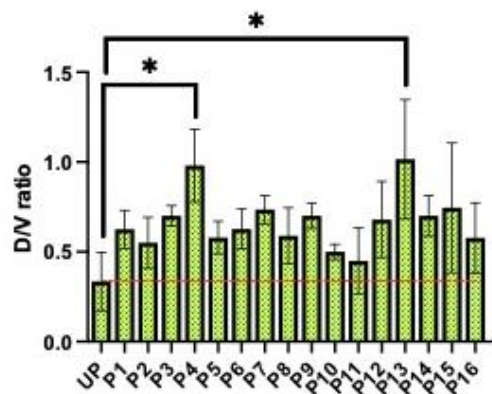


Figure 11. Cell proliferation and phenotypic analysis of human umbilical artery smooth muscle cells (HUASMCs) on Multiarchitecture (MARC) chamber. (A) Representative images of EdU assay. (B) Representative immunofluorescence (IF) image of desmin, vimentin, and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) staining. (C) Percentage of EdU-positive cells. (D) The ratio of desmin-positive cells/vimentin-positive cells. Arrows indicate the direction of gratings. Scale bar = 200µm. Statistical analysis was performed (n = 3 for all surfaces) using a one-way ANOVA test with Tukey's multiple comparisons test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

From a pool of patterns tested in MARC chamber experiments, five patterns showed the most significant impact, with the three most influential being the pattern 10 - 1µm concave lens (1µm diameter and pitch, 0.3µm sag concave microlens), pattern 11 - 1µm convex lens (1µm diameter and pitch, 0.3µm sag convex microlens) and pattern 12 - 1.8µm concave lens (1.8µm diameter, 2µm pitch, 0.7µm sag concave microlens) showing the highest proliferation. On the flip side, the pattern 4 - 2µm grating (2µm width, 2µm space, 2µm height) gratings exhibited the lowest proliferation. Pattern 13 - 1.8µm convex lens (1.8µm diameter, 2µm pitch, 0.7µm sag convex microlens) and pattern 4 - 2µm grating displayed a higher D/V ratio, while unpatterned surfaces exhibited the lowest D/V ratio. Thus, the comparison of proliferation and phenotype transformation screening using the MARC arrays led to the selection of a set of 5 patterns- 2µm grating, 1µm concave lens, 1µm convex lens, 1.8µm concave lens, 1.8µm convex lens, and the unpatterned for further verification of individual topographical patterns using single PDMS patterns.

3.3.2 Single pattern PDMS substrate to assess VSMC behaviour under normal and PDGF-presenting conditions

During vascular injury, inflammatory responses activate the PDGF signaling pathway, promoting the proliferation and migration of VSMCs at the injury site[84]. To mimic the proinflammatory environment, PDGF was used to assess whether topographical modifications would influence the impact of the PDGF on phenotype switching and contractility. To delve deeper into understanding the responses of HUASMCs to substrate modification in the presence and absence of PDGF, the selected five patterns and the unpatterned control were individually fabricated on PDMS substrates. The fidelity of the PDMS single pattern substrates was verified by reflected laser scanning confocal microscopy (**Figure 10B**).

The fluorescently stained F-actin serves as a marker for stress fibers (**Figure 12A**). The elongation of cells was assessed by measuring the length of the long axis divided by the short axis, which is the aspect ratio of the cells (**Figure 12C**). Cells exhibited predominant elongation along the axis of the 2 μ m grating ($34 \pm 3\%$, $p \leq 0.0001$), aligning mostly parallel to one another compared to the unpatterned ($6.1 \pm 0.7\%$). With the presence of PDGF, cells on the 2 μ m grating substrate ($27 \pm 2.5\%$, $p \leq 0.0001$) exhibited a five-fold increase in elongation compared to those on an unpatterned substrate ($5.5 \pm 2.5\%$). In contrast, HUASMCs cultured on other patterned and unpatterned surfaces showed neither elongation nor orientation in the presence and absence of PDGF. The EdU results showed that the cells on the 2 μ m grating had a significant reduction in proliferation ($23.1 \pm 1.4\%$) when compared to those on unpatterned ones ($42 \pm 7\%$, $p \leq 0.001$) and 1.8 μ m convex microlens ($37.2 \pm 3.5\%$, $p \leq 0.05$) in the presence of PDGF (**Figure 12B**), suggesting that the 2 μ m grating can reduce the proliferation of HUASMCs induced by PDGF. We observed the lowest proliferation of cells cultured on 2 μ m grating ($15 \pm 4\%$) without PDGF compared to the other substrates irrespective of the presence or absence of PDGF. PDGF significantly enhanced cell proliferation in both spread-out (lens pattern and unpatterned) and elongated HUASMCs (2 μ m grating) compared to cells on 2 μ m grating without PDGF, suggesting that elongated VSMCs still responded to PDGF, however, proliferation was lower compared to other patterns in the PDGF group (**Figure 12D**).

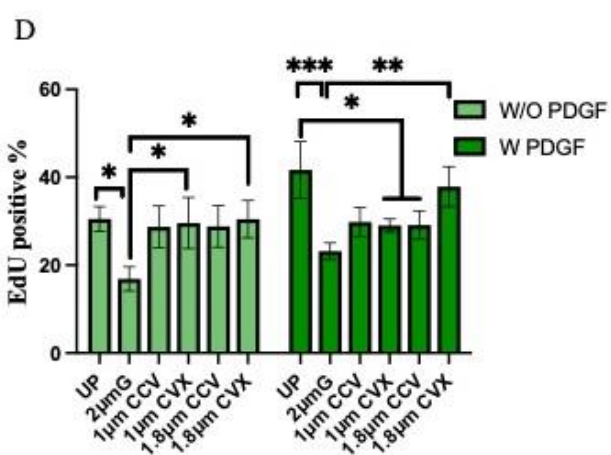
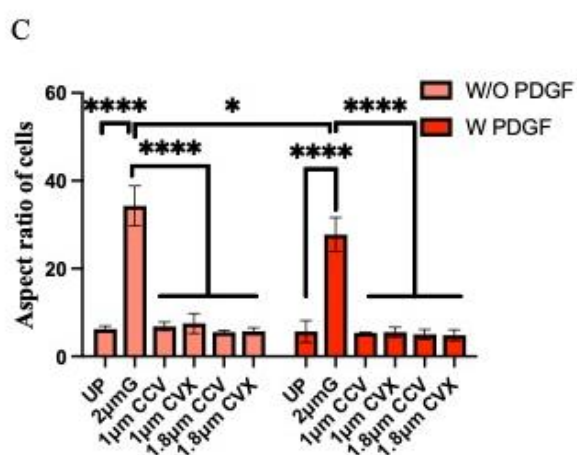
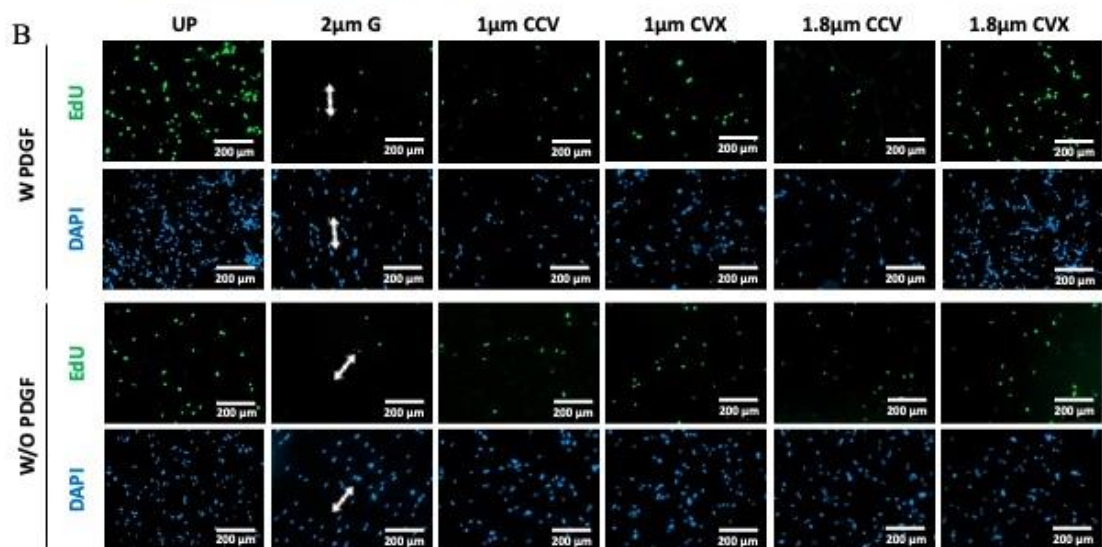
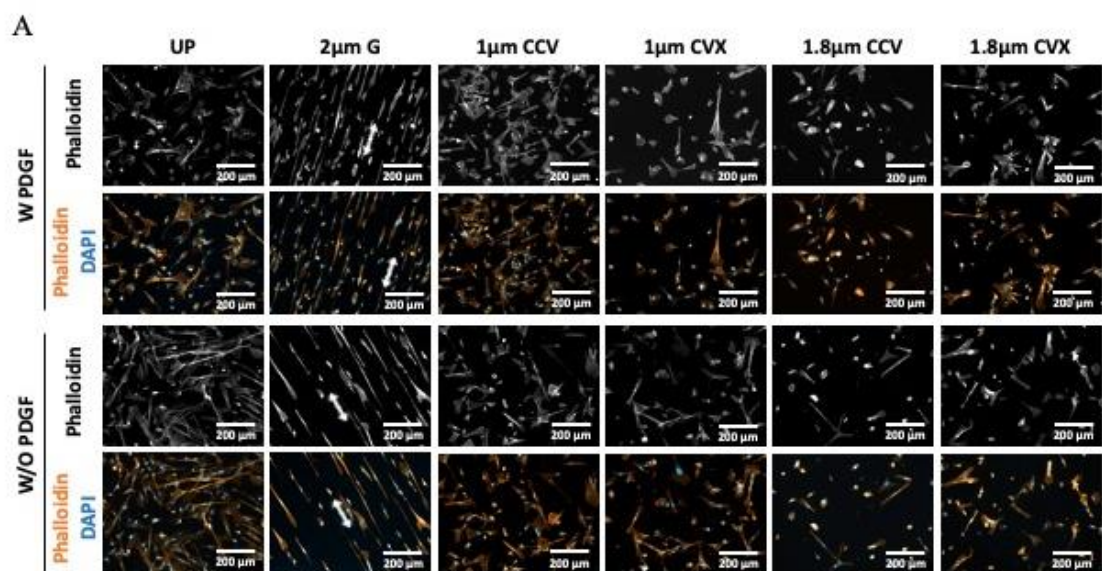


Figure 12. Morphology and proliferation of HUASMCs on polydimethylsiloxane (PDMS) with shortlisted single patterns with or without platelet-derived growth factor (PDGF). (A) Representative images of F-actin staining. (B) Representative images of EdU assay. (C) Cell elongation. (D) Percentage of EdU-positive cells. Arrows indicate the direction of gratings. Scale bar = 200 μ m. Statistical analysis (n = 3 for all surfaces) was performed using a two-way ANOVA test with Tukey's multiple comparisons test. *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001, and **** p $\leq$ 0.0001.

Desmin and vimentin staining were utilized to study the phenotype transformation of HUASMCs (**Figure 13A**). HUASMCs on 2 μ m grating pattern ($1.72 \pm 0.5\%$, p $\leq$ 0.05) showed a significant increase in D/V ratio, implying a change in contractile phenotype when compared to unpatterned substrate ($0.93 \pm 0.18\%$), 1.8 μ m convex lens ($0.95 \pm 0.11\%$) and 1 μ m concave lens ($0.96 \pm 0.35\%$). In the PDGF presenting condition, cells on 2 μ m grating showed a higher D/V ratio ($1.6 \pm 0.11\%$, p $\leq$ 0.01) compared to unpatterned ($0.82 \pm 0.08\%$), 1.8 μ m convex lens ($0.96 \pm 0.14\%$) and 1 μ m concave lens ($0.99 \pm 0.12\%$) (**Figure 13C**). The unpatterned substrate, 1.8 μ m convex lens, and 1 μ m concave lens consistently displayed a lower trend indicative of a synthetic phenotype, characterized by a reduction in the D/V ratio, irrespective of the presence or absence of PDGF.

HUASMCs were stained with α -SMA (**Figure 13B**), a distinctive marker of contractile and non-proliferative phenotype within adult SMCs[85]. **Figure 13D** represents the quantitative analysis of fluorescence intensity divided by the cell population. Cells on the 2 μ m gratings exhibited a significantly higher α -SMA expression (14.40 ± 1.60 a.u., p $\leq$ 0.05) compared to the unpatterned sample with (6.8 ± 3.4 a.u.) (**Figure 13D**).

Following the findings from the single pattern study, 2 μ m gratings and 1.8 μ m convex lens induced the most significant difference in proliferation and phenotypic switch compared to other patterns. As a result, 2 μ m gratings, 1.8 μ m convex lens and the unpatterned control were chosen for fabrication onto PVA hydrogels to further investigate the responses of HUASMCs.

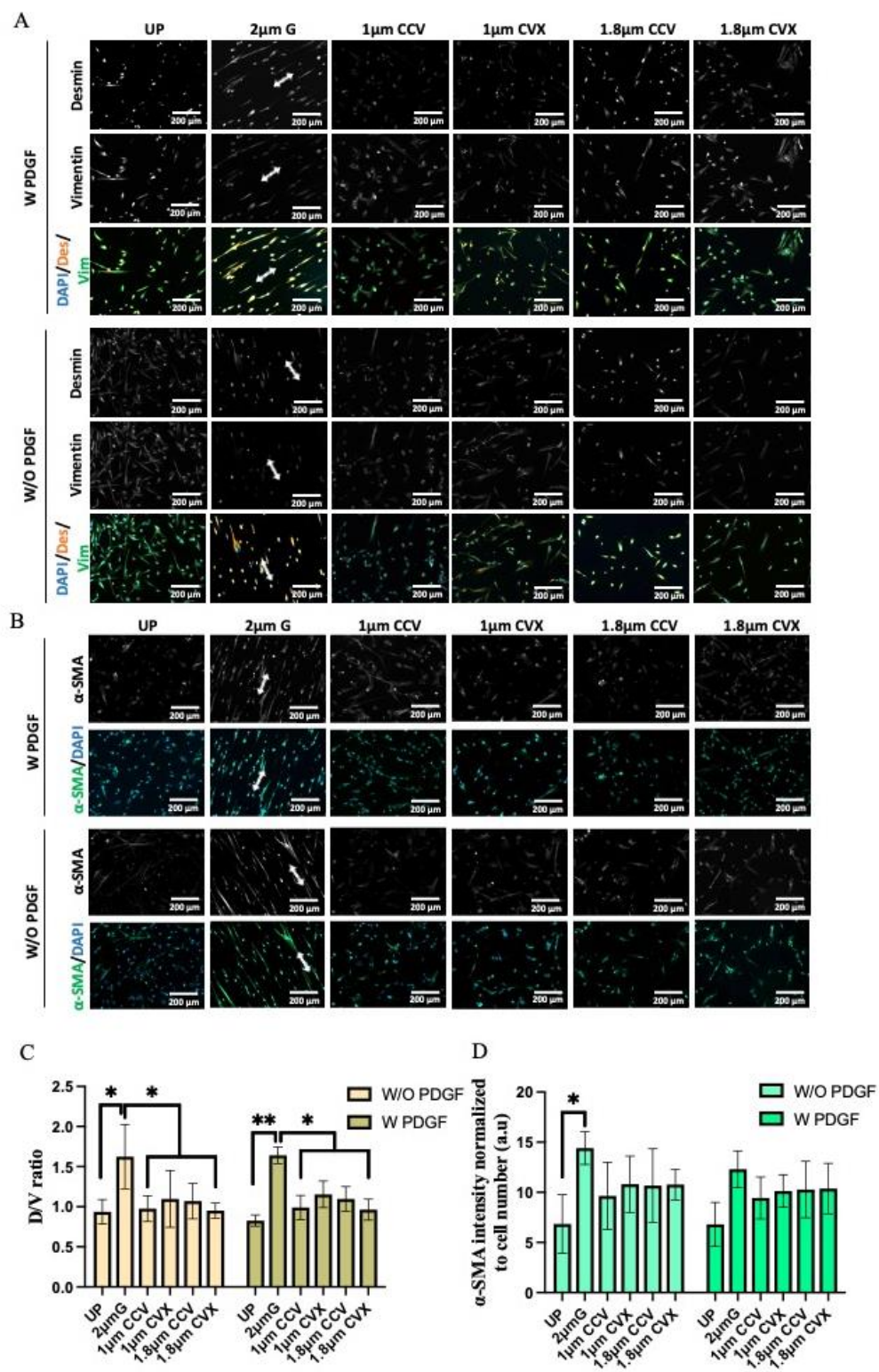


Figure 13. Immunofluorescence staining of desmin-vimentin and α -smooth muscle actin (α -SMA) expression by HUASMCs on PDMS with shortlisted single patterns. (A) Representative images of desmin and vimentin staining. (B) Representative images of α -SMA staining. (C) The ratio of desmin-positive cells/vimentin-positive cells. (D) The average intensity of α -SMA normalized to cell number. Arrows indicate the direction of gratings. Scale bar = 200 μ m. Statistical analysis ($n = 3$ for all surfaces) was performed using a two-way ANOVA test with Tukey's multiple comparisons test. * $p \leq 0.05$, ** $p \leq 0.01$.

3.3.3 Assessment of VSMC behaviour using unpatterned and patterned PVA hydrogel

To further explore how topographical pattern affects the HUASMC behaviours on synthetic vascular graft material PVA hydrogel, cell proliferation, morphology and phenotype change were assessed with and without the presence of PDGF. The fidelity of the patterned PVA hydrogels was verified by laser scanning confocal microscopy (**Figure 14**). F-actin staining images and quantitative analysis show a higher aspect ratio (more than a three-fold increase) for the cells on the 2 μ m grating compared to unpatterned, irrespective of the presence of PDGF (**Figure 15A and 15B**). The proliferation assay (**Figure 15C**) showed that the 2 μ m grating showed low proliferation ($16.6 \pm 2.1\%$) even with the presence of PDGF; conversely, the unpatterned with PDGF showed a three-fold higher proliferation rate ($46.1 \pm 10.4\%$, $p \leq 0.001$) (**Figure 15D**).

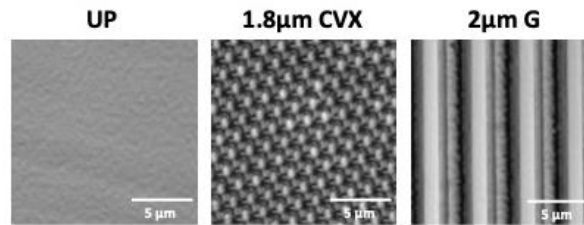


Figure 14. The reflected laser confocal images of unpatterned and patterned PVA hydrogel were captured with the LEXT Olympus 5000 microscope.

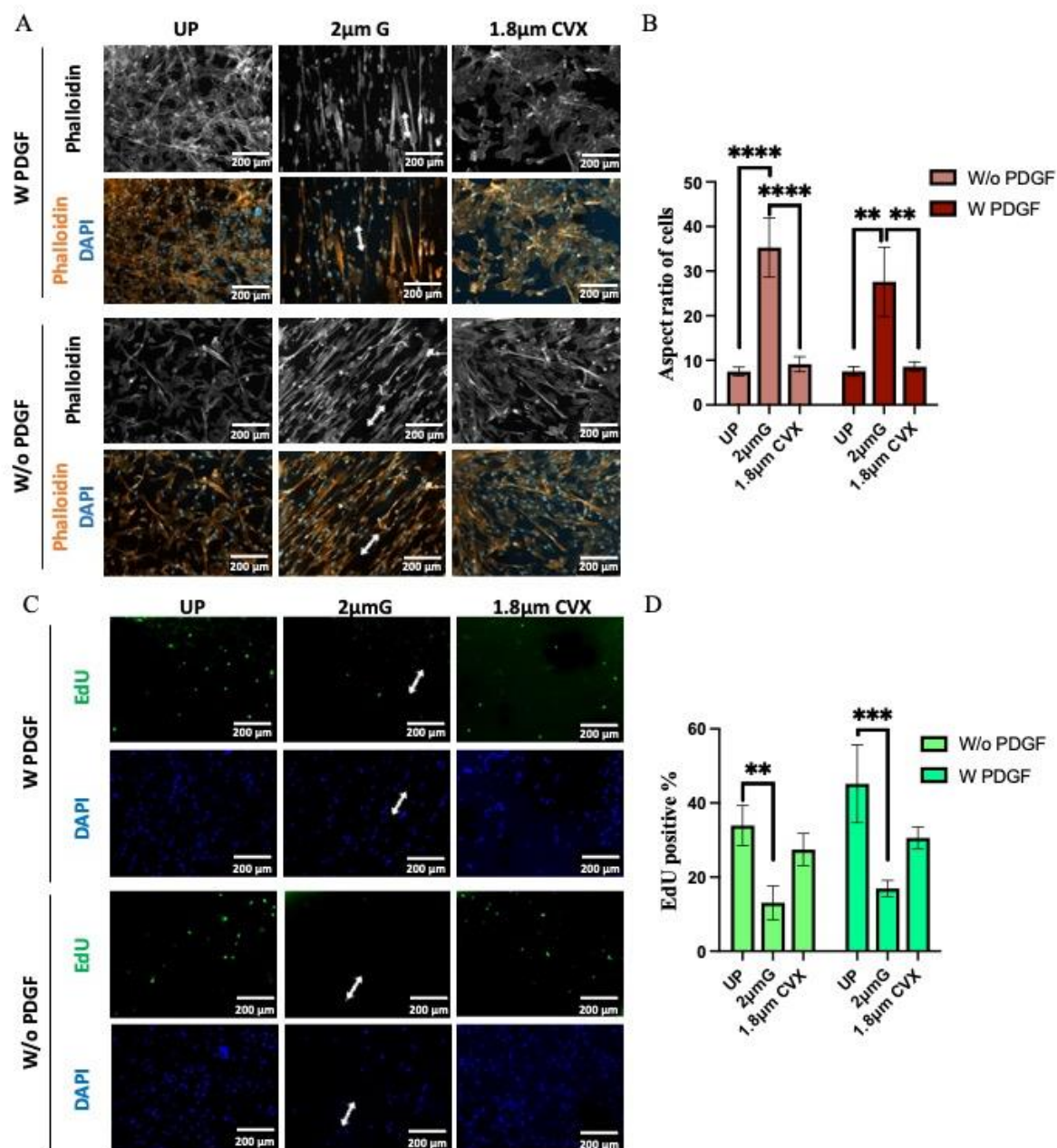


Figure 15. Morphology and proliferation of HUASMCs were assessed on unpatterned polyvinyl alcohol (PVA) and patterned PVA hydrogels with 2 μ m gratings and 1.8 μ m convex lenses. (A) Representative images of F-actin staining. (B) Cell elongation. (C) Representative images of EdU assay. (D) Percentage of EdU-positive cells. Arrows indicate the direction of gratings. Scale bar =

200 μ m. Statistical analysis (n = 3 for all surfaces) was performed using a two-way ANOVA test with Tukey's multiple comparisons test. *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001, and ****p $\leq$ 0.0001.

Desmin-vimentin staining showed a high D/V ratio of cells on the 2 μ m grating topography ($1.2 \pm 0.11\%$, p $\leq$ 0.05) when compared to unpatterned ones ($0.85 \pm 0.05\%$) in the absence of PDGF (**Figure 16A**). However, even with the presence of the PDGF growth factor, the grating structure showed an increase in the D/V ratio ($1.07 \pm 0.18\%$, p $\leq$ 0.01) compared to the unpatterned substrate ($0.5 \pm 0.11\%$) (**Figure 16B**). This shows that the cells on 2 μ m grating can maintain their differentiated state despite the presence of growth factors.

Similar to the single pattern PDMS study, 2 μ m grating without PDGF shows a significantly higher α -SMA expression (22 ± 8.8 a.u., p $\leq$ 0.05) with a two-fold increase compared to unpatterned (9.8 ± 2.7 a.u.) and 1.8 μ m convex lens pattern (10.8 ± 0.4 a.u.) without PDGF representing the contractile feature of VSMCs (**Figures 16C and 16D**). All these quantitative analyses underpin the fact that topographical modification can modulate the VSMC fate.

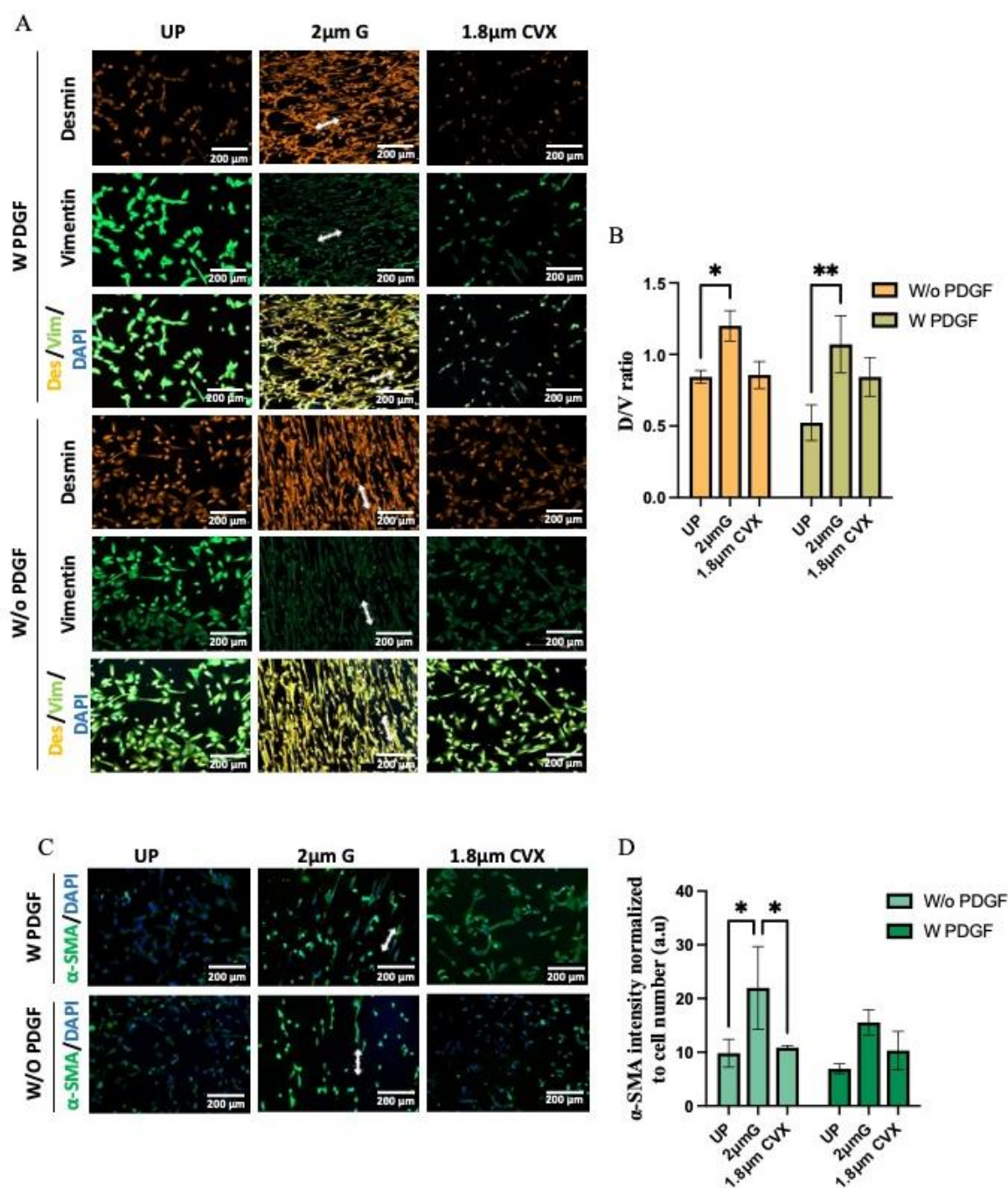


Figure 16. Immunofluorescence staining of desmin-vimentin and α -SMA expression by HUASMCs on PVA hydrogels with shortlisted single patterns. (A) Representative images of desmin and vimentin staining. (B) The ratio of desmin-positive cells/vimentin-positive cells. (C) Representative images of

α -SMA. (D) The average intensity of α -SMA normalized to cell number. Arrows indicate the direction of gratings. Scale bar = 200 μ m. Statistical analysis (n = 3 for all surfaces) was performed using a two-way ANOVA test with Tukey's multiple comparisons test. *p $\leq$ 0.05, **p $\leq$ 0.01.

3.3.4 Comparative analysis of VSMC migration on unpatterned and patterned PVA substrate

HUASMC migration on both unpatterned and patterned PVA samples, incorporating 2 μ m grating and 1.8 μ m convex lens designs, was studied through a wound healing assay. As shown in **Figures 17A and 17B**, after 8 hours, HUASMCs on 2 μ m gratings completely covered the wound area in contrast to those on unpatterned and 1.8 μ m convex pattern, indicating that the cells on 2 μ m gratings migrated faster to cover the wounds. Notably, cells on the 2 μ m gratings showed significantly higher migration, achieving 100% wound closure after 8 hours, in contrast to the 1.8 μ m convex lens (wound closure after 12 hours) and unpatterned substrate (wound closure after 34 hours).

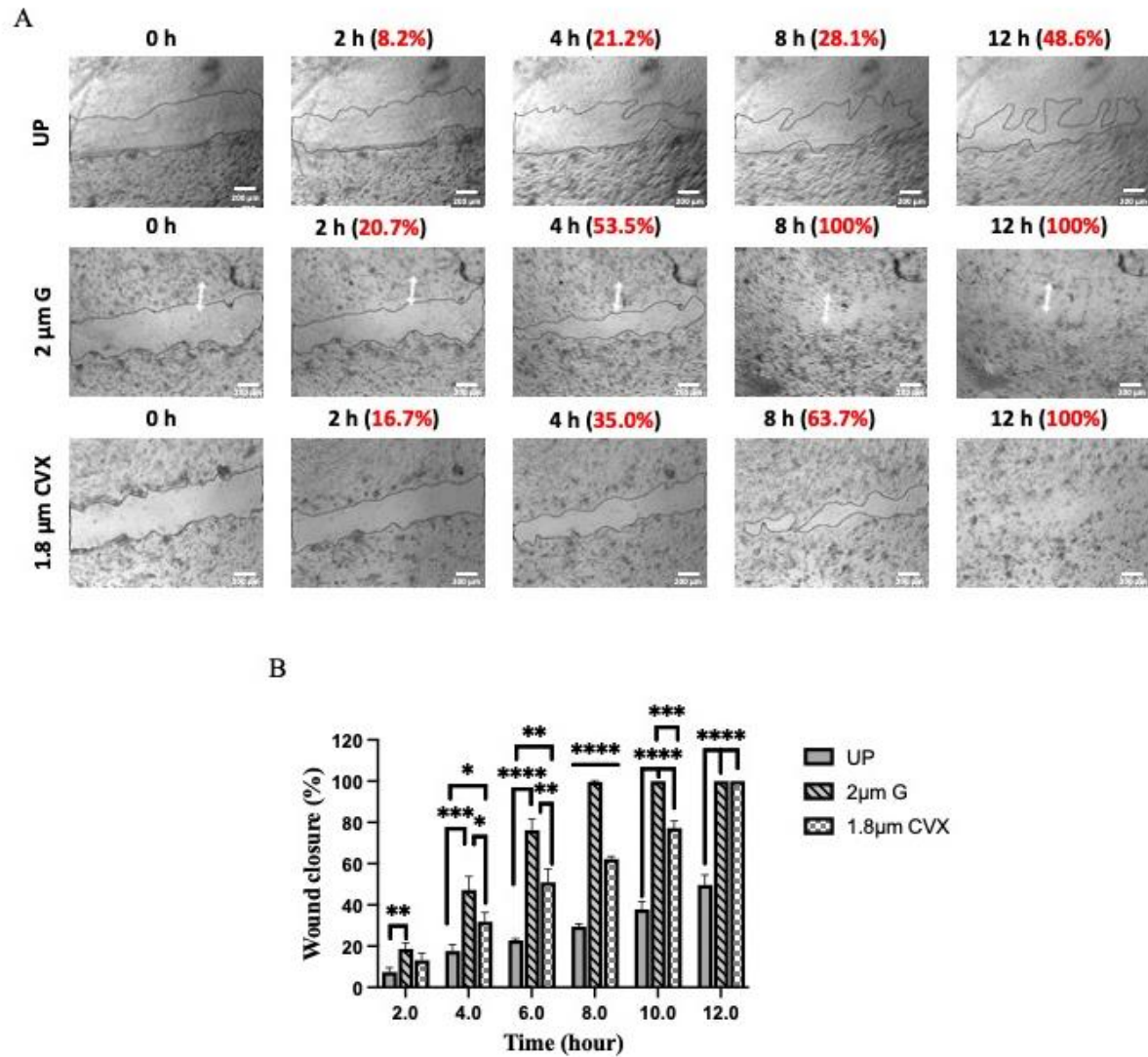


Figure 17. HUASMC migration on unpatterned PVA, and PVA hydrogels with 2 μm gratings and 1.8 μm convex lens using a wound healing assay. (A) Representative images of HUASMCs on unpatterned, 1.8 μm convex lens pattern and 2 μm gratings pattern at 0 h, 2 h, 4 h, 8 h, and 12 h time points. (B) Quantification of wound healing percentage at different time points. Arrows indicate the direction of gratings. Scale bar = 200 μm . Statistical analysis ($n = 3$ for all surfaces) was performed using a one-way ANOVA test with Tukey's multiple comparisons test. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.

3.4 Discussion

The transition from contractile to synthetic phenotype in VSMCs plays a key role in proliferative vascular pathologies, including IH formation and post-angioplastic restenosis. In the processes of atherogenesis and arterial restenosis, VSMCs undergo a transition from contractile phenotype to synthetic one. This transition facilitates their migration to the intima, enhances their proliferative capacity, and stimulates the synthesis of extracellular matrix proteins[34]. The VSMC phenotypic switch from quiescent contractile to proliferative synthetic phenotype after vascular graft implantation has been deemed to be the predominant cause of IH in an anastomotic site and restenosis in sSDVGs [2, 86]. Controlling the phenotype switching from contractile to synthetic and inhibiting the VSMC proliferation is seen as an effective way to reduce IH. Understanding and studying this phenotypic plasticity of VSMCs is crucial for unravelling the complexities of vascular diseases and developing targeted interventions to modulate vascular structure and function. Our study aims to offer significant perspectives into the modulation of VSMC responses by the extracellular environment, particularly substrate topography. This research has the potential to offer significant implications for the long-term performance of sSDVG grafts.

The role of bio-interface topography in mediating cellular responses has been extensively investigated and different investigators have utilized topographical patterning of different biomaterial substrates for evaluating VSMC response toward phenotype transformation, cell spreading, alignment, proliferation, and migration[58]. However, many focused only on limited numbers and types of topography. In our previous work, we engineered a high-throughput topography screening device known as the MARC chip[49]. In this study, we focused on assessing the effects of 16 distinct topographical patterns on VSMCs using the MARC chip. This systematic examination of isotropic and anisotropic patterns, as well as hierarchical topographies, enhances our understanding of how different surface structures impact VSMC behaviour.

The fidelity of the MARC chamber and single patterned PDMS topographies was maintained after replication to PDMS (**Figure 10A**). The screening using the MARC array suggested that the proliferation and phenotype of VSMCs were affected by surface topographies. Specifically, 2 μ m grating with a 1:1 aspect ratio had a lower proliferation rate. In contrast, the 1 μ m concave and convex lens patterns, along with the 1.8 μ m concave lens pattern and the unpatterned surface, exhibited

significantly higher proliferation rates. Additionally, the 2 μ m grating and 1.8 μ m patterns showed an increased ratio of contractile to synthetic phenotype. Following the initial screening results, the aforementioned five patterns and the unpatterned control exhibited the most pronounced impact on HUASMC proliferation and phenotype transition (**Figure 11**) were selected using the ranking method. We organized the quantified data obtained from EdU-positive cell percentages and the D/V ratio by ranking them based on their adjusted p-values across different topographies. Specifically, topographical patterns exhibiting greater significance, with an adjusted p-value below 0.04, were given the top ranking (**Table 3**). Subsequently, we chose five of these top-ranked patterns, along with an unpatterned control, to be included in a single patterned study of cell behaviour of VSMCs on patterned or unpatterned PDMS under normal and PDGF-presenting conditions.

Vascular injuries trigger inflammatory responses, activating pro-inflammatory cytokines, such as PDGF expression and signaling, which set off a series of events leading to cell proliferation and migration [87]. Moreover, accumulating evidence suggests a significant role of PDGF in the pathogenesis of arteriosclerosis, where it tends to enhance VSMC migration, proliferation, and matrix production, contributing to intima thickening[88]. Considering these considerations, PDGF was employed in the single pattern study to examine whether changes in surface features could influence the cellular response to PDGF, offering potential insights into the intricate interplay between topographical cues and growth factor signaling within the context of VSMC behaviour. In the assessment of HUASMC proliferation on various single patterns, an EdU assay was employed. It was observed that cells cultured on the substrate with 2 μ m gratings exhibited a significantly diminished rate of proliferation (**Figure 12B**) induced by PDGF compared to cells on other patterned substrates. Desmin, being a muscle-specific protein and a key subunit of the intermediate filament in cardiac, skeletal and smooth muscle cells, plays a crucial role in maintaining the structural and mechanical integrity of the contractile apparatus in muscle tissues [89]. Consequently, desmin serves as a marker for the contractile phenotype of VSMCs [76]. On the other hand, vimentin is considered a marker for the synthetic phenotype[90]. The D/V ratio is a quantitative measure used to assess the relative prevalence of contractile and synthetic phenotypes within a cell population. An increase in the D/V ratio is indicative of a shift towards a more contractile phenotype, while a decrease suggests a transition towards a synthetic phenotype. In the context of the experiment, the increase in the D/V ratio was specifically observed when VSMCs were cultured on 2 μ m grating, both in the presence and absence of PDGF (**Figure 13A**). This observation implies that the topographical cue provided by the 2 μ m grating

promotes and maintains a contractile phenotype, even with the presence of a growth factor like PDGF that is known to induce a proliferative and migratory phenotype.

Generally, VSMCs in native blood vessels possess an elongated morphology with a well-organized structure [54]. To assess the effect of surface patterns on cell morphology, F-actin staining was done. The morphology of VSMCs is a primary determinant of their contractile or synthetic state. Contractile VSMCs exhibit an elongated, spindle-shaped morphology [69], while synthetic VSMCs demonstrate increased spreading [91] and reduced elongation. Notably, in the case of VSMCs cultured on a surface with 2 μ m gratings, cells displayed an adapted elongated and spindle-shaped morphology, and the stress fibers were predominantly stretched along the long axis of the cells. Conversely, cells on lens patterns and unpatterned surfaces exhibited a pleomorphic morphology, characterized by ellipsoid or rhomboid shapes (**Figure 12A**). The results showed that the cells on 2 μ m grating were elongated in the direction of underlying topography and showed the highest cell elongation both with and without the presence of PDGF (**Figure 12C**) when compared to other patterns, which agrees with the literature [92]. The elongation of cells along the direction of the grating suggest that the topographical features play a significant role in shaping cellular morphology.

Within healthy arteries, VSMCs exhibit high expression of α -SMA along with various other structural and actin-binding proteins such as tropomyosin and troponin. These components delineate the distinctive contractile phenotype characterizing the behaviour and functionality of VSMCs[93]. This α -SMA marker is indicative of the specialized state where the VSMCs exhibit a heightened contractile capacity while remaining in a state of reduced proliferation. Moreover, studies have indicated that α -SMA plays a role in downregulating SMC migration and proliferation[85]. Additionally, the absence of α -SMA has been linked to elevated SMC hyperplasia *in vivo*[94, 95]. In the absence of PDGF, cells on the 2 μ m grating exhibited an increase in α -SMA expression compared to the unpatterned sample (**Figure 13D**). On the contrary, in the presence of PDGF, the difference in the α -SMA expression level between the 2 μ m grating and unpatterned samples was not as significant. The decreased response to 2 μ m grating may be due to the effect of PDGF, which decreases α -SMA expression while affecting VSMC proliferation[87].

Collectively, the single-pattern PDMS investigation reaffirmed that 2 μ m gratings with a 1:1 aspect ratio triggered notable cell elongation, higher contractile phenotype, elevated expression of α -SMA and reduced proliferation. Conversely, the 1.8 μ m convex lens and unpatterned control

demonstrated significant proliferation and synthetic activities compared to the other patterns. Consequently, we opted to include the 2 μ m gratings, 1.8 μ m convex lens, and unpatterned control in our subsequent PVA hydrogel study. Interestingly, while the influence of curvature-shaped geometry on cellular processes and cell mechanics on curved substrates has been studied before in other cell types [96, 97], the impact of microlens topography on VSMC responses remains less explored. This selection aimed to explore how these specific patterns influence cellular responses within the synthetic graft material context.

PVA hydrogels, crosslinked by STMP have emerged as a promising material for SDVGs [72, 98-100]. Prior investigations in this field primarily centered around the behaviour of ECs when interacting with these hydrogels[28]. To delve deeper into the specific interactions with VSMCs, modifications were made to the PVA hydrogels to study VSMC activity. Notably, 2 μ m gratings and 1.8 μ m convex lenses were fabricated onto the hydrogel surface. These specific topographical features were chosen based on their recognized significance in influencing and regulating VSMC activities from the single pattern study. The intention was to explore how these surface patterns could impact the behaviour and responses of HUASMCs, offering insights into potential applications for vascular grafts with enhanced performance and biocompatibility. PVA hydrogel is not conducive to cell adhesion. In a previous study, it was demonstrated that the incorporation of fucoidan could modify PVA without altering the surface topography[101]. Given this, all PVA hydrogels used in this study were pre-modified with fucoidan before seeding with HUASMCs. Cells on PVA hydrogel without patterns or with 2 μ m gratings or 1.8 μ m convex lenses showed similar behaviour to those on single patterned PDMS, even in the presence of PDGF (**Figures 15 and 16**). The structural parameters of biomaterials like ridge depth play a significant role in eliciting cellular response to anisotropy by determining the extent to which cells respond to specific lateral dimensions. Increased cell alignment, elongation and SMC protein expression were evident in studies using microgrooves/ ridges to evaluate VSMC's responses[69]. Culturing SMCs on oriented micro grooves and ridges reduces cell proliferation [54], which aligns with our results that cells on 2 μ m grating exhibit cell elongation (**Figure 15A**) along the grating axis with high contractile protein expression (**Figure 16 A**) and lower proliferation (**Figure 15D**). Conversely, the 1.8 μ m convex lens and unpatterned substrate exhibited higher proliferation, lower cell elongation, and significantly lower expression of α -SMA (**Figures 15 and 16**). Maintaining appropriate VSMC phenotype (contractile) as in the native blood vessel is crucial for the long-term success of the sSDVGs, and it is evident from the studies that geometric anisotropies (2 μ m grating)

play a significant role in regulating cell morphology and VSMC contractile protein expression. This substantiates the notion that PVA when topographically and biochemically modified, can be effectively employed in the construction of sSDVGs to mitigate the formation of intimal hyperplasia.

Despite extensive research on EC wound healing [101] and fibroblast migration [68], in the context of topography, the migration patterns of VSMCs on topographical patterns have not been as thoroughly studied. While prior research, like that conducted by Yim *et al.* [80] and Chang *et al.* [54], has utilized wound healing assays to study the motility behaviour of SMCs using nano gratings and micropatterned grooves; a migration behavioral study using convex microlens pattern is the least explored. To bridge the gap in the literature, our investigation delved into the migration behaviour of VSMCs on surfaces with a 2 μ m grating and a 1.8 μ m convex lens, compared with the unpatterned (UP) control. Gratings are acknowledged for their capacity to impact cellular behaviour, especially in eliciting cell migration [102]. Our study observed that VSMCs cultured on 2 μ m gratings exhibited a significantly faster wound closure compared to cells on unpatterned and 1.8 μ m convex lens (**Figure 17A**). The faster wound closure observed in VSMCs on 2 μ m gratings (**Figure 17B**) suggests that these cells demonstrated accelerated and directional migration. Furthermore, our previous studies have shown that microtubule-organizing centers (MTOCs) become polarized when exposed to nanograting patterns, facilitating directed migration during wound healing [80]. VSMCs can recognize the orientation of gratings, grooves and ridges on the topographically modified biomaterial surfaces and can adhere, self-align, and elongate on these substrates through a contact guidance mechanism. A few studies have shown that VSMCs align weakly in response to curved topography [67], which aligns with our results, which suggest that cells on lens pattern exhibit slower wound closure compared to a 2 μ m grating pattern. The maintenance of a contractile phenotype by VSMCs on the grating pattern may have broader implications for tissue homeostasis. Our findings suggest that 2 μ m gratings can attenuate the proliferative effect of PDGF on VSMCs while retaining their contractile phenotype. Additionally, Chang *et al.* observed that elongated cells on micro-grooved substrates not only displayed improved directional motility compared to those on flat substrates but also exhibited heightened expression of mesenchymal markers [54]. Successful wound healing and tissue regeneration often necessitate coordinated cell migration and the maintenance of specific cell phenotypes. If VSMCs on the grating pattern can achieve this balance, it suggests that such patterns could be utilized in tissue engineering or regenerative medicine approaches to facilitate optimal healing processes.

Chapter 4

Mechanistic role of substrate topography on VSMC behavior *in vitro*

4.1 Introduction

VSMCs play a critical role in maintaining vascular homeostasis and structural integrity, primarily through their contractile function[103]. In their native contractile phenotype, VSMCs exhibit low proliferative activity, high expression of contractile proteins (e.g., α -smooth muscle actin, myosin heavy chain, calponin), and a quiescent state essential for maintaining blood vessel tone[104, 105]. However, under pathological conditions such as vascular injury, atherosclerosis, or restenosis, VSMCs undergo a phenotypic switch from the contractile to the synthetic state. This phenotypic plasticity is marked by increased proliferation, migration, and secretion of ECM components, contributing to abnormal vascular remodeling[104]. The ability of VSMCs to oscillate between these phenotypes is a central feature in vascular pathophysiology, necessitating a deeper understanding of the mechanisms regulating this transition.

While biochemical factors, including growth factors, cytokines, and oxidative stress, are recognized as major regulators of VSMC behavior, the impact of biomechanical and biophysical cues in the cellular microenvironment has gained significant attention in recent years. Among these cues, substrate topography—a parameter describing the physical micro- and nanoscale features of the surface that VSMCs interact with—has emerged as a crucial factor influencing cellular behavior. Substrate topography mimics the structural characteristics of the native ECM, providing cells with mechanical stimuli that are transduced into intracellular signals through mechanotransduction[106]. This process orchestrates critical cellular processes, including adhesion, proliferation, migration, contractility, and the phenotypic modulation of VSMCs.

Mechanotransduction mechanisms are primarily mediated by integrin-based focal adhesion complexes and the cytoskeleton, which serve as conduits for transmitting external mechanical signals into intracellular biochemical responses[107]. These responses activate downstream signaling pathways, including RhoA/ROCK and YAP/TAZ, which are implicated in the regulation of VSMC proliferation, migration, contractile protein expression, and phenotype switching[25]. The influence of substrate topography on these signaling cascades provides an avenue for tailoring VSMC behavior,

highlighting its relevance in both understanding vascular pathophysiology and designing biomaterials for therapeutic applications.

This chapter investigates the mechanistic role of substrate topography in modulating key behaviors of VSMCs, including proliferation, contractility, phenotype switching, and migration. By employing engineered substrates with defined micro- and nanoscale topographical features, we elucidate how distinct patterns influence VSMC responses at the cellular and molecular levels. Our results demonstrate that specific topographical cues can suppress VSMC proliferation, a hallmark of pathological vascular remodeling, while simultaneously enhancing the expression of contractile proteins, thus promoting a quiescent, contractile phenotype.

The implications of this work extend beyond basic mechanistic insights, offering a foundation for translational applications in vascular tissue engineering and regenerative medicine. The ability to design biomimetic surfaces with specific topographical features provides an opportunity to develop advanced vascular grafts and scaffolds that can guide VSMC behavior. By promoting desirable cellular functions—such as reduced proliferation, enhanced contractility, and controlled migration—substrate topography can be leveraged to inhibit pathological vascular remodeling and facilitate vascular repair.

4.2 Materials and Methods

4.2.1 Preparation of PDMS single patterns

Fabrication and sterilization of single PDMS patterns were done by the protocol stated in section 3.2.1.

4.2.2 Preparation of PVA film

Fabrication, modification, and sterilization of patterned and unpatterned PVA film were done by the protocol stated in section 3.2.3.

4.2.3 Cell Culture of HUASMCs on PDMS and PVA substrate

Cell seeding and culturing on the sterile PDMS single patterns, PVA patterned and unpatterned substrate with and without PDGF-BB was done by the protocol in section 3.2.4. After 3 days of incubation, the samples were fixed and stained for immunofluorescence analysis. All experiments were conducted using three biological replicates ($n = 3$), as specified in the figure captions. Unless otherwise stated, more than 100 cells were quantified for each sample.

4.2.4 Immunofluorescence and image analysis

On day 3 of cell culture, cells cultured on the samples (n=3) were washed and fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and blocked with 1%(w/v) bovine serum albumin and 10% goat serum. Following blocking, the cells were immunostained with pMLCK antibody (rabbit polyclonal at 1:50, Invitrogen), YAP1 antibody (mouse polyclonal at 1:400, Proteintech), pFAK (rabbit anti-phosphorylated Y397 FAK at 1:200, Abcam). Samples were then washed and stained with goat anti-mouse Alexa Fluor 488 (1:750) (Thermo Fisher Scientific) and goat anti-rabbit Alexa Fluor 546 (1:750) (Thermo Fisher Scientific). Samples were counterstained with DAPI solution (1:2000) (Sigma Aldrich) and Alexa Fluor Phalloidin 546 (1:200) (Invitrogen) for 30 min at room temperature and then mounted on glass coverslips for fluorescence microscope analysis.

Fluorescence microscope images were analyzed using the ImageJ software. The average fluorescence intensity of pMLCK (intensity normalized to the cell number) was calculated by using ImageJ. YAP nuclear localization (N/C ratio) was quantified by the ratio of the cell population that expressed YAP in the nucleus to the cell population that showed YAP expression in the cytoplasm. Images were randomly acquired from three biological replicates (n = 3), with more than 100 cells analyzed for each sample.

To analyze the focal adhesions (FA), images of pFAK staining were enhanced using contrast limited adaptive histogram equalization (CLAHE) algorithm in MATLAB, as previously described [62, 108]. Briefly, image processing was performed using the software ImageJ to analyze focal adhesions. Initially, each image's color depth was converted to an 8-bit format, followed by background correction adjustments. To enhance the identification of focal adhesions, the CLAHE algorithm was applied to improve local contrast. The images were then converted to binary mode, where focal adhesions appeared as black patches. To eliminate speckle noise, an open operation was executed. Subsequently, using the 'analyze particles' tool in ImageJ, the average FA size was determined, with the particle size set to greater than 0.8 μm^2 to identify relevant structures. Three biological replicas (n = 3) were utilized, and 30 cells were quantified for each sample.

4.2.5 Statistical Analysis

All statistical analysis was performed using GraphPad Prism 9. Three biological replicas (n = 3) were used across the experiments and were indicated in the methods and/or the figure captions. Statistical analysis of different experimental groups was done using one-way and two-way ANOVA with Tukey's

multiple comparisons test. Data values were represented as mean $\pm$ standard deviation. Differences with $p \leq 0.05$ were taken as statistically significant. Statistically significant data was indicated in the figures using *, ** and *** for $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ unless otherwise specified.

4.3 Results

4.3.1 Mechanistic role of topography in regulating VSMC responses: pMLCK and YAP study

We next investigated the impact of topographical cues on HUASMCs contractile phenotype by analyzing pMLCK (**Figures 18A & 18C**) and YAP expression (**Figures 18B and 18D**). In the fluorescence intensity analysis of pMLCK expression, which was normalized to cell number (**Figure 18A**), cells on the 2 μ m grating without PDGF showed higher contractility with significantly higher expression of pMLCK (32.4 ± 1.1 a.u., $p \leq 0.05$) when compared to unpatterned ones (17.3 ± 2.9 a.u.). However, even with the presence of the PDGF growth factor, the grating structure showed an increase in the pMLCK expression (28.2 ± 1.8 a.u., $p \leq 0.05$) compared to the unpatterned substrate (12.9 ± 3.3 a.u.) (**Figure 18C**). This shows that the cells on 2 μ m grating can maintain their cytoskeletal contractility despite the presence of growth factors.

YAP has been recognized as a mechanosensitive transcriptional coactivator. YAP responds to substrate stiffness, cell density, and shear stress by shuttling between the cytoplasm and nucleus [109]. The localization of YAP in the cell provides insights into the phenotypic characteristics, with nuclear expression indicating a synthetic phenotype and cytoplasmic expression indicating a contractile phenotype. Certain studies determined YAP localization using the ratio of nuclear intensity to cytoplasmic intensity [110, 111]. However, we employed an alternative method, inspired by a few researcher's approaches [112-114], wherein we assessed the YAP N/C ratio by dividing the cell population expressing YAP in the nucleus by the cell population expressing YAP in the cytoplasm (**Figure 18D**). The findings demonstrated that the HUASMCs on a 1.8 μ m convex lens exhibited notably higher nuclear localization, as indicated by an elevated N/C ratio ($0.5 \pm 0.17\%$, $p \leq 0.001$), compared to those on 2 μ m grating ($0.073 \pm 0.05\%$) in the absence of PDGF. This suggests a heightened involvement in synthetic activities. Conversely, the cells on the 2 μ m grating substrate displayed minimal nuclear localization with a notably low N/C ratio ($0.063 \pm 0.035\%$) even with the presence of PDGF compared to those on the 1.8 μ m convex lens ($0.6 \pm 0.05\%$, $p \leq 0.0001$). This observation

indicates the localization of YAP in the cytoplasm, indicating a cellular state characterized by an emphasis on contractile activities rather than synthetic processes (**Figure 18D**).

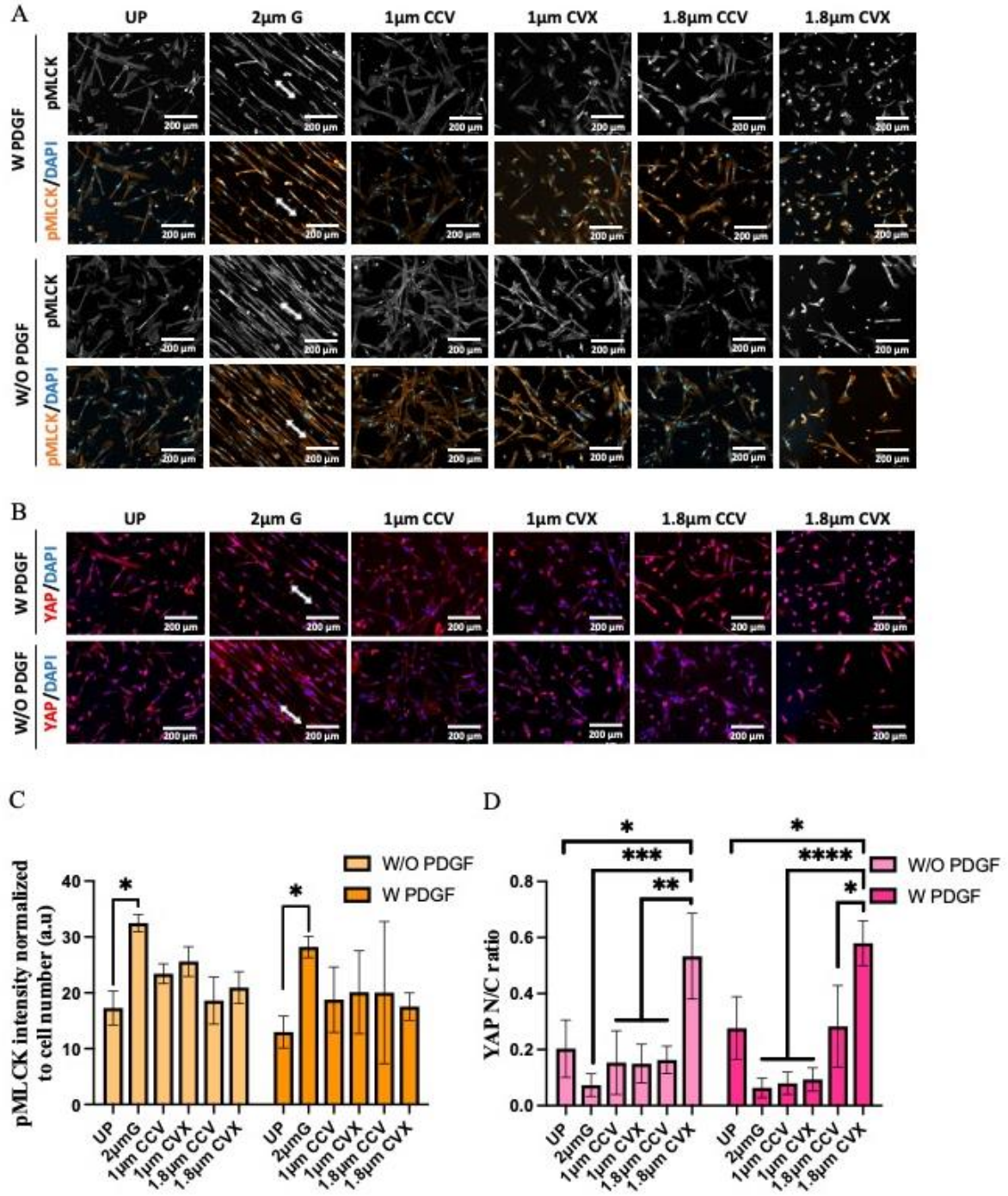


Figure 18. Immunofluorescence staining of phosphorylated myosin light chain kinase (pMLCK) and yes associated protein (YAP) expression by HUASMCs on PDMS with shortlisted single patterns. Representative images of (A) pMLCK staining and (B) YAP staining. (C) Average intensity of pMLCK normalized to cell number. (D) The ratio of cells expressing YAP in the nucleus to cells expressing YAP in the cytoplasm (N/C ratio). Arrows indicate the direction of gratings. Scale bar = 200 μ m. Statistical analysis (n = 3 for all surfaces) was performed using a two-way ANOVA test with Tukey's multiple comparisons test. *p $\leq$ 0.05, **p $\leq$ 0.01, *** p $\leq$ 0.001, **** p $\leq$ 0.0001.

Consistent with findings from the single-pattern PDMS study, HUASMCs cultured on the 2 μ m grating exhibited a significantly elevated expression of pMLCK, demonstrating a markedly elevated expression of pMLCK with a two-fold increase (11.8 ± 2.2 a.u., p $\leq$ 0.05), indicating increased cytoskeletal contractility compared to the unpatterned groups (6.7 ± 1.1 a.u.) (**Figures 19A and 19B**). Moreover, even with the presence of the PDGF growth factor, the grating structure showed an increase in the pMLCK expression (10.2 ± 1.92 a.u., p $\leq$ 0.05) compared to the unpatterned substrate (5.1 ± 1.3 a.u.) (**Figure 19B**). This affirms that the cells on 2 μ m grating can maintain their actin-myosin contractility even in the presence of growth factors. YAP's presence in the cell nucleus is implicated as a key factor influencing the transition of VSMCs to a state characterized by enhanced proliferation. With the presence of PDGF, both unpatterned ($0.109 \pm 0.021\%$, p $\leq$ 0.05) and 1.8 μ m convex lenses ($0.145 \pm 0.015\%$, p $\leq$ 0.001) showed an enhanced amount of nuclear localization, suggesting that PDGF triggered the proliferative phenotype (**Figures 19C and 19D**). YAP expression of cells on the 2 μ m grating was mainly localized in the cytoplasm. The N/C ratio of cells on 2 μ m grating ($0.05 \pm 0.01\%$) was the lowest among all patterns, suggesting that HUASMCs on the grating structure have a contractile phenotype even with the presence of a growth factor.

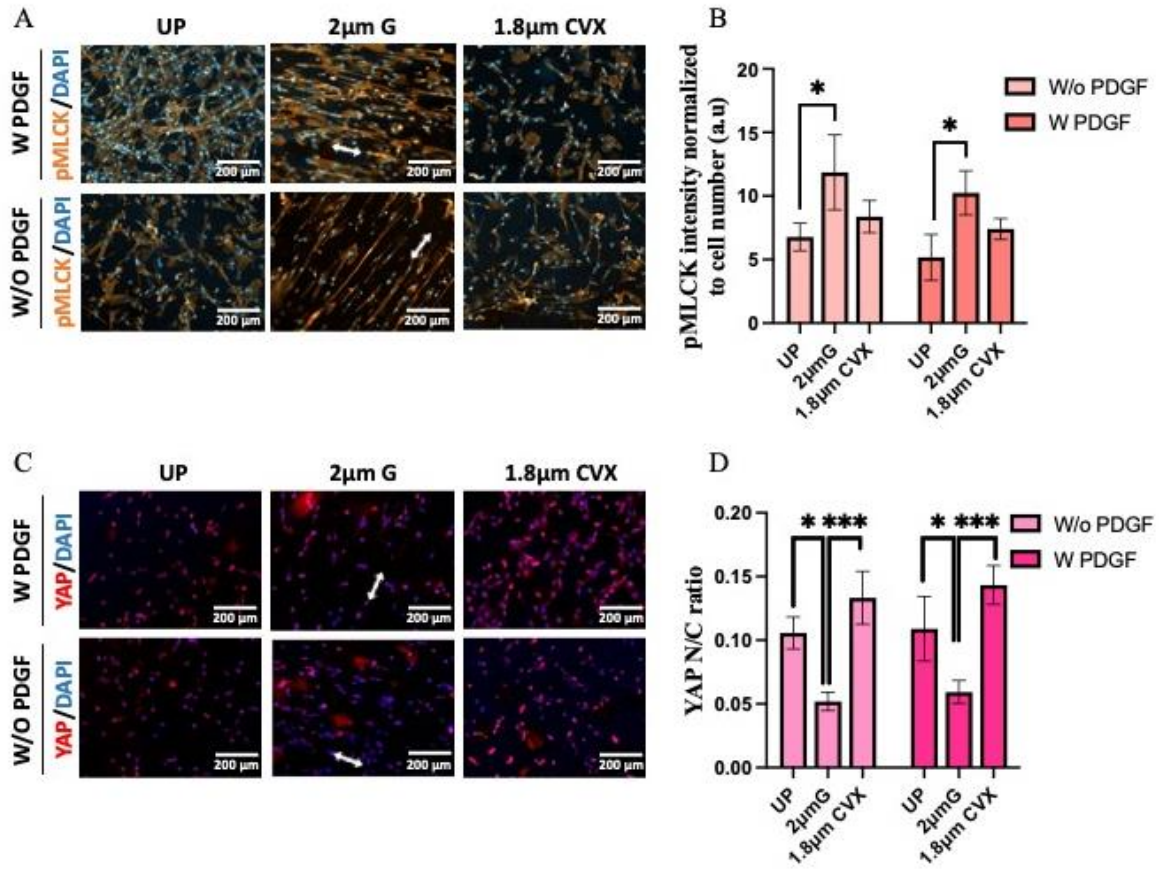
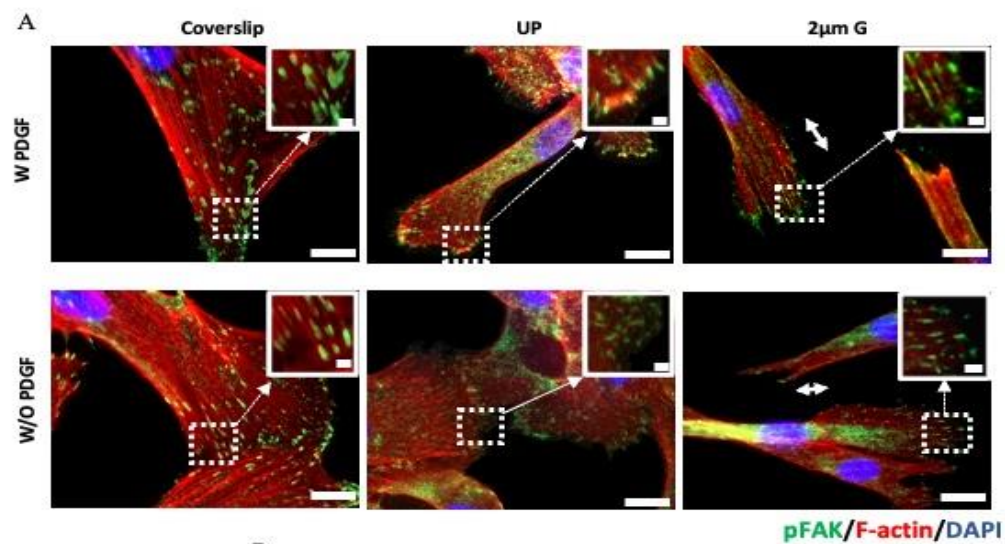


Figure 19. Immunofluorescence staining of pMLCK and YAP expression by HUASMCs on PVA hydrogels with shortlisted single patterns. Representative images of (A) pMLCK staining and (C) YAP staining. Average intensity of (B) pMLCK normalized to cell number. (D) The ratio of cells expressing YAP in the nucleus to cells expressing YAP in the cytoplasm (N/C ratio). Arrows indicate the direction of gratings. Scale bar = 200μm. Statistical analysis (n = 3 for all surfaces) was performed using a two-way ANOVA test with Tukey's multiple comparisons test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

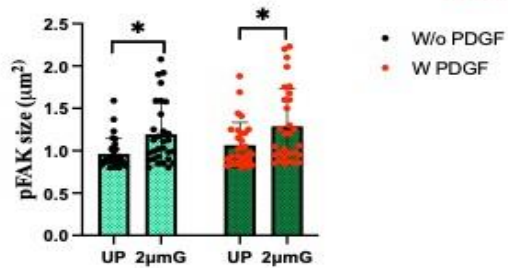
4.3.2 Topography-induced focal adhesion dynamics in VSMCs

To explore the involvement of topography-induced focal adhesion signaling in VSMC migration and cytoskeletal contractility induced by mechanotransductive signaling pathway, HUASMCs on unpatterned and 2μm grating were stained with pFAK to evaluate FA formation. HUASMCs cultured on 2μm gratings exhibited significantly larger and mature FAs (FAs larger than 0.8 μm²) localized predominantly at the leading and trailing edges of the cells compared to cells on unpatterned substrates.

The average FA size on the 2 μ m gratings was significantly larger ($1.19 \pm 0.3 \mu\text{m}^2$, $p \leq 0.05$) and more elongated compared to FAs on unpatterned substrates ($0.961 \pm 0.16 \mu\text{m}^2$) without the presence of PDGF (**Figure 20A, B and C**). In contrast, HUASMCs on unpatterned surfaces predominantly formed smaller FAs (less than $0.8 \mu\text{m}^2$), referred to as focal complexes. The FA size on the 2 μ m gratings further increased significantly ($1.28 \pm 0.48 \mu\text{m}^2$, $p \leq 0.05$) in the presence of PDGF compared to unpatterned surfaces ($1.06 \pm 0.19 \mu\text{m}^2$) (**Figure 20B**). These results suggest that the physical constraints imposed by the 2 μ m grating geometry drive the directional maturation and alignment of FAs.



B



C

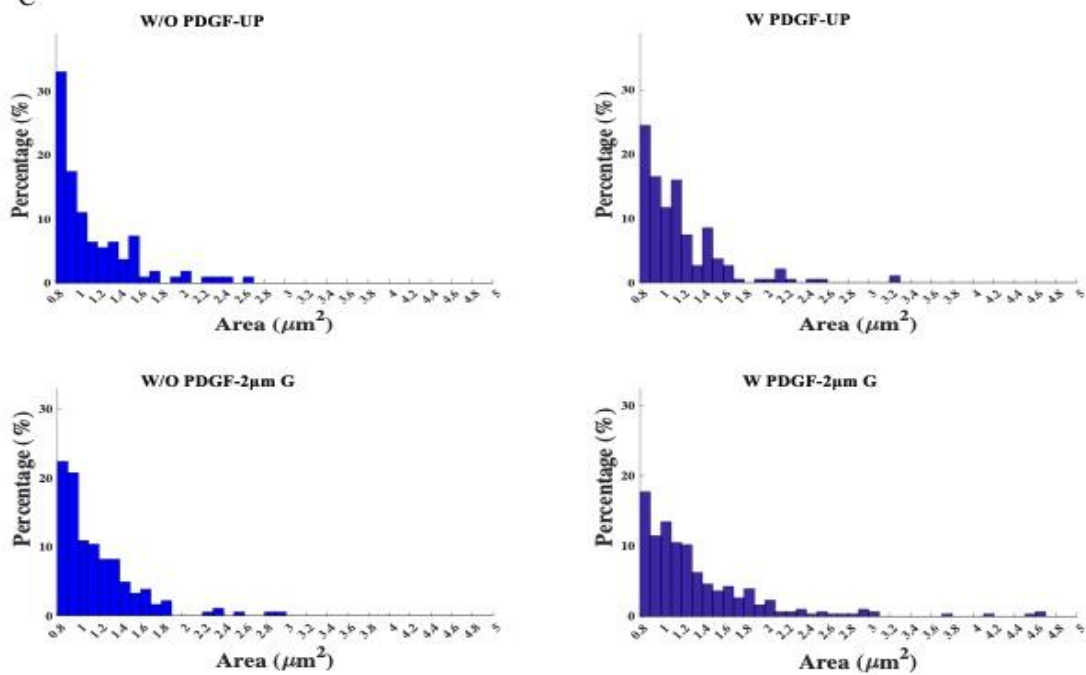


Figure 20. Focal adhesion formation on coverslip, UP and 2 μ mG PVA. (A) Representative immunofluorescence images of focal adhesion formation by HUASMCs on coverslip, UP and 2 μ m grating PVA. Scale bar = 20 μ m. Solid arrow indicates the grating direction. Blue denotes nuclei, green denotes phosphorylated focal adhesion kinase (pFAK), and red denotes F-actin. Inset shows a magnified view. Scale bar = 5 μ m. (B) Focal adhesion (FA) size. 30 cells from 3 samples were quantified. (C) Histogram showing the frequency distribution FAs vs area on UP and 2 μ mG PVA. Statistical analysis was performed using a two-way ANOVA test with Tukey's multiple comparisons test. * $p \leq 0.05$, ** $p \leq 0.01$.

4.4 Discussion

The level of expression of pMLCK is particularly significant in VSMCs exhibiting a contractile phenotype. It is found in the literature that pMLCK can influence the contractility of VSMCs [115]. It serves as a signaling molecule that influences the activation of myosin, a protein involved in smooth muscle contraction [116]. In smooth muscle cells, phosphorylation of the myosin regulatory light chain is an obligatory step involved in the initiation of contraction [117]. Interestingly, when VSMCs were subjected to a topographical cue, specifically in the form of 2 μ m gratings, the cells exhibited the highest level of pMLCK expression (**Figure 18A and 18C**). This observation suggests that the specific surface pattern of 2 μ m gratings has a pronounced effect on the signaling pathways involved in the regulation of pMLCK compared to the isotropic lens patterns and unpatterned control. This finding is particularly noteworthy as it implies that topographical cues, such as the dimensions and arrangement of surface features, can influence the expression of pMLCK, ultimately impacting the contractility and functional characteristics of VSMCs. The pronounced increase in pMLCK expression observed in cells subjected to these specific surface patterns suggests that the physical architecture of the substrate significantly influences the signaling pathways that regulate contractility in VSMCs. In addition to a phenotypic marker of VSMC contractile phenotype, pMLCK is also involved in the actomyosin cytoskeletal contractility. The quantification of pMLCK has also been employed to evaluate actomyosin contractility and the RhoA-ROCK (Rho-associated protein kinase)-MLC pathway[118]. The role of pMLC as a marker of actomyosin contractility has been extensively studied in diverse contexts. The study by Padilla-Rodriguez et al. explored the role of phosphorylated myosin light chain (pMLC) as a marker of actomyosin contractility in estrogen receptor-positive (ER+) breast cancer cells[119]. The study highlights that elevated pMLC levels, regulated via the RhoA-ROCK pathway, enhance cytoskeletal contractility, influencing cell motility and invasive behavior. Similarly, Hernandez et al.

investigated actomyosin contractility in nucleus pulposus (NP) cells under pro-inflammatory tumor necrosis factor- α (TNF α) exposure, a factor in intervertebral disc (IVD) degeneration[120]. TNF α reduced pMLC levels and contractility but activating RhoA via CN03 restored cell morphology and contractility. Additionally, increased actomyosin contractility inhibited NF- κ B nuclear translocation, reducing catabolic gene expression and protecting ECM integrity. These studies underscore pMLCK's critical role in cytoskeletal dynamics and cellular responses to mechanical and inflammatory cues. Thus, our study showing significantly higher pMLCK expression (two-fold increase) on 2 μ m grating compared to unpatterned, also implied the 2 μ m gratings elicited actomyosin contractility and potential mechanotransduction in the VSMC. The implications of these results extend beyond mere expression levels of pMLCK. Given that pMLCK is a critical component in the actomyosin cytoskeletal contractility, the enhanced expression in response to 2 μ m gratings likely translates to increased contractile activity in VSMCs.

In addition to responding to biochemical signals such as growth factors and cytokines from their environment, cells also engage in mechanotransduction, a process through which they sense and interact with the extracellular environment[121]. Cells adhere to the extracellular matrix (ECM), and the mechanical interactions between cells and the ECM can be transduced via cytoskeletal forces to the nucleus, thereby regulating gene transcription and influencing various cellular behaviors[122]. Notably, the Hippo-associated proteins, yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) have been identified as critical nuclear relays that transmit mechanical signals derived from extracellular cues[123, 124]. The study by Mason *et al.* elucidates the critical role of the transcriptional regulators YAP and TAZ in mechanotransduction, particularly in endothelial cell motility and vascular morphogenesis[125]. YAP and TAZ are activated by mechanical cues, leading to transcriptional suppression of myosin II activity, which is essential for maintaining dynamic cytoskeletal equilibria. This feedback mechanism prevents excessive maturation of focal adhesions (FAs), thereby facilitating persistent cell migration. The study identifies a characteristic time scale of approximately 4 hours for the initial closure of this mechanotransductive feedback loop, resulting in full motile equilibrium by 8 hours. Through a combination of *in vitro* and *in vivo* experiments, the authors demonstrate that YAP/TAZ-mediated transcriptional feedback is crucial for translating mechanical changes into cellular responses, influencing cytoskeletal organization and the dynamics of focal adhesion formation[125]. YAP is a key cellular regulator that plays a pivotal role in modulating cell behaviour, particularly in the context of proliferation and differentiation[126]. In the absence of

proliferative signals, YAP is typically found scattered throughout the cytoplasm. However, when cells transit into a proliferative phenotype, YAP undergoes nuclear localization. This nuclear translocation is often associated with increased cell proliferation and altered gene expression[109]. In the nucleus, YAP mainly binds to TEAD (TEA domain transcription factor) and influences the expression of genes that regulate cell proliferation, differentiation, and apoptosis. When the inhibitory kinases of the Hippo pathway are active, YAP/TAZ is phosphorylated and sequestered in the cytoplasm[109]. Consequently, the nuclear localization of YAP is an indicator of the proliferative phenotype in VSMCs. In this study, we quantify the contractility of VSMCs through the assessment of the N/C ratio, where "N" represents the number of cells expressing YAP in the nucleus and "C" represents the number of cells expressing YAP in the cytoplasm. The N/C ratio serves as an indicator of the cellular phenotype, where a higher N/C ratio suggests a prevalence of YAP expression in the nucleus, signifying an elevated proliferative VSMC phenotype.

The Hippo-YAP signaling pathway, particularly through the action of YAP, plays a crucial role in regulating the proliferation and phenotypic switch of VSMCs. A study by Xie *et al.* has shown that YAP expression is rapidly upregulated in response to PDGF-BB stimulation, a response also observed in injured vascular tissue, highlighting its role in the vascular injury response[127]. This upregulation facilitates the shift of VSMCs from a contractile phenotype to a synthetic state, characterized by enhanced proliferation, migration, and matrix synthesis. Mechanistically, YAP interacts with myocardin, impairing its ability to bind serum response factor (SRF) at VSMC-specific gene promoters, which leads to the downregulation of contractile markers such as smooth muscle actin (SMA), smooth muscle myosin heavy chain (SMMHC), and myocardin (MyoCD). Conversely, the knockdown of YAP disrupts VSMC proliferation and promotes the expression of contractile phenotype-specific genes by stabilizing the myocardin-SRF-CArG complex. This suggests that YAP acts as a central molecular switch that mediates the phenotypic modulation of VSMCs in response to environmental cues and vascular injury[127]. The localization of YAP is strongly influenced by physical cues provided by the cellular microenvironment, such as substrate topography. Notably, VSMCs cultured on 2 μ m grating had the lowest nuclear localization of YAP (**Figures 18B and 18D**), suggesting a tendency to maintain the contractile phenotype of VSMCs. This reduced nuclear localization suggests that the physical constraints of the 2 μ m grating suppress YAP-mediated transcriptional activity, favoring the preservation of contractile function and reduced proliferative potential. In contrast, cells cultured on a 1.8 μ m convex lens pattern displayed the highest nuclear YAP localization, consistent with

dedifferentiated, synthetic activities. This observation indicates that the curvature and geometry of the lens pattern provide physical cues that promote YAP nuclear translocation and the corresponding proliferative phenotype. These findings highlight the critical role of substrate geometry in modulating YAP activity and influencing VSMC behavior.

Culturing SMCs on oriented micro grooves and ridges reduces cell proliferation[54], which aligns with our results that cells on 2 μ m grating exhibit high contractile protein expression (**Figure 19A**) and lower proliferative phenotype (**Figure 19C**). Conversely, the 1.8 μ m convex lens and unpatterned substrate exhibited higher proliferative phenotype by nuclear localization of YAP and significantly lower expression of pMLCK (**Figure 19B and 19D**). Maintaining appropriate VSMC phenotype (contractile) as in the native blood vessel is crucial for the long-term success of the sSDVGs, and it is evident from the studies that geometric anisotropies (2 μ m grating) play a significant role in regulating cell morphology and VSMC contractile protein expression. This substantiates the notion that PVA when topographically and biochemically modified, can be effectively employed in the construction of sSDVGs to mitigate the formation of IH.

FAs play a critical role in mechanotransduction, particularly when cells are subjected to physical constraints such as 2 μ m grating patterns. Our previous studies have demonstrated that the cells on grating substrates exert higher and more polarized traction stresses than those on flat substrates, which correlates with increased alignment of FAs along the direction of the gratings[128]. This anisotropy in traction stress likely provides the mechanical forces necessary to drive cell elongation parallel to the grating direction. In addition, it was observed that the ratio of the pMLCK intensities and total FA areas is significantly higher in cells on gratings than on flat substrates, suggesting that cellular contractility per FA is enhanced on grating substrates compared to flat surfaces[128]. Moreover, FA size and distribution are critical factors in regulating cell migration[129]. For instance, Muhammad *et al.* demonstrated that patterned TiO₂ topography significantly influences the formation and maturation of FAs[62]. The polar organization of FAs has been shown to enhance the speed of cell migration [130], while stress fiber alignment directly correlates with anisotropic traction forces, promoting cell polarization and a more organized, contractile cytoskeletal architecture[53, 54, 81]. Similarly, in this study, physical constraints imparted by 2 μ m grating patterns induce significant changes in FA dynamics and cytoskeletal organization compared to unpatterned substrates. FAs formed by VSMCs on 2 μ m gratings exhibit distinct elongation and alignment along the grating axis (**Figure 20A**). The spatial constraints imposed by the grating pattern restrict the isotropic growth of FAs, which results in the

organization of actin stress fibers along the grating direction. Furthermore, on 2 μ m gratings, the enhanced FA maturation, elongation, and alignment induce a contractile phenotype, as the cells experience increased cytoskeletal tension and maintain robust stress fiber integrity. Conversely, the absence of defined directional cues on unpatterned substrates results in less coordinated FA maturation and stress fiber orientation, leading to a more synthetic-like phenotype, characterized by increased proliferation and reduced contractility. Moreover, the predominance of smaller FAs (focal complexes) we have observed on unpatterned substrates (**Figure 20B**) reflects a less mature adhesive state, which is associated with reduced mechanotransduction and weaker cytoskeletal tension. In this study, we propose that the differences in FA formation driven by the topographical constraints of 2 μ m grating patterns are the primary contributors to the observed contractile phenotype and reduced proliferation of vascular smooth muscle cells (VSMCs), as these patterns induce significant changes in FA dynamics and cytoskeletal organization, leading to enhanced FA maturation, elongation, and alignment that promote increased cytoskeletal tension and robust stress fiber integrity compared to unpatterned substrates.

Topography-induced cell alignment positions FAs as a key mechanosensing component, enabling cells to respond to physical cues from their microenvironment[25]. This alignment suggests that FA formation and cytoskeletal rearrangement are fundamental to the behaviors observed on patterned surfaces. The ability of cells to transduce topographical information into morphological changes and lineage differentiation relies on a sensing mechanism that involves cytoskeletal contractility against the ECM[108]. These cytoskeleton-generated signals play a pivotal role in regulating FA formation and maturation. The transition of cell-matrix adhesions from initial punctate focal complexes into mature, elongated focal contacts requires the activity of Rho GTPases [131]. Specifically, myosin II-driven contractility, mediated by Rho-associated kinase (ROCK), is essential for focal contact formation. On micropatterned substrates, the elongation and orientation of FAs are mechanotransductive events that regulate cytoskeletal tension through integrin-mediated signaling pathways, such as the RhoA/ROCK pathway[132, 133]. These pathways enhance MLC phosphorylation, promoting actomyosin contractility, which is essential for maintaining the contractile phenotype of VSMCs[60]. Similarly, the specific geometry of 2 μ m gratings provides physical cues that influence FA dynamics, promoting both the directional maturation and orientation of FAs along the grating axis (**Figure 20A**). The directional maturation of FAs on the grating is facilitated by enhanced integrin clustering and activation, which promote stronger and more stable FA complexes[101, 134].

These elongated FAs serve as nucleation sites for stress fibers, which also align along the grating axis, creating a highly anisotropic cytoskeletal structure. Therefore, the elongation and directional alignment of matured FAs, coupled with enhanced cytoskeletal contractility, support the generation of anisotropic traction forces, which contribute to cell polarization and directed migration.

Chapter 5

Conclusion and Future Recommendations

5.1 Conclusions

This thesis presents a comprehensive investigation into the role of substrate topography as a modulator of VSMC behavior, particularly focusing on the antithrombotic phenotype. This study identifies specific topographical patterns, notably a 2 μ m grating, that significantly influence VSMC proliferation and facilitate the transition from a synthetic to a contractile state. This work also demonstrates that various isotropic and anisotropic topographical cues can modulate VSMC phenotype, proliferation, morphology, and migration. Among these, the 2 μ m grating pattern stood out for its ability to reduce proliferation, increase the expression of contractile proteins, and induce an elongated cellular morphology. Importantly, these effects were observed even in the presence of PDGF, a potent stimulator of VSMC synthetic phenotype. The study further confirmed that 2 μ m gratings could attenuate PDGF-induced VSMC proliferation and phenotype switching, underscoring their robust regulatory perspective and their potential for future applications in both *in vitro* and *in vivo* contexts.

The mechanistic exploration reveals that substrate topography impacts VSMC behavior through key molecular pathways, including pMLCK and YAP signaling. The elevated expression of pMLCK on 2 μ m gratings indicates that specific surface features can effectively modulate the signaling mechanisms that govern VSMC contractility, thereby influencing their functional characteristics. Furthermore, the study elucidates the role of mechanotransductive feedback mechanisms, highlighting the Hippo-YAP signaling pathway as a critical regulator of gene transcription and cellular behavior in response to substrate properties. The findings emphasize the importance of focal adhesion dynamics and cytoskeletal organization in the context of VSMC contractility. Enhanced focal adhesion formation and alignment on 2 μ m gratings not only influence cellular morphology but also augment mechanotransduction, promoting a more contractile state in VSMCs. This intricate interplay between topographical cues and cellular signaling mechanisms provides valuable insights into the design of biomaterials for vascular applications, particularly in the development of synthetic vascular grafts.

Overall, this research highlights the efficacy of 2 μ m gratings in modulating VSMC bioactivity and proposes their broader application in the development of synthetic vascular grafts. The findings not only advance the understanding of topography-mediated cellular regulation but also offer promising

strategies for enhancing the performance and longevity of vascular grafts, with significant implications for the field of vascular surgery and patient care.

5.2 Future directions

Despite the demonstrated alterations in properties of topographically modified biomaterials, the cellular responses to such topographies remain inadequately understood. The mechanisms by which scaffold topographic patterns influence cellular characteristics are intricate, and the processes through which cells discern the limitations of their environments—specifically, the boundaries of the patterns on which they proliferate—are still largely unidentified[135]. The mere observation of a cell adopting a 'native' morphology on synthetic or natural graft materials *in vitro* does not guarantee its biological functionality *in vivo*. Therefore, it is imperative to conduct comprehensive studies aimed at characterizing the native architecture of blood vessels, which encompasses the essential features of vascular ECMs relevant for investigating cell adhesion, proliferation, differentiation, tissue development, and repair.

Subsequent investigations should prioritize the interactions between cells and substrates, meticulously examining the biological functions of scaffold biophysical cues to elucidate fundamental mechanisms[136]. A diverse array of fabricated patterned substrates has been utilized in cell culture models, yielding extensive biological insights into cellular characteristics, and facilitating the exploration of individual biophysical cues on cellular responses. However, it is crucial to recognize that signals in native tissues are rarely isolated. This necessitates studies that incorporate multiple biochemical and biophysical variables, enhancing the informativeness of research once cellular responses to individual cues have been thoroughly established.

To address the remaining questions and limitations, the following studies are proposed to further expand this thesis:

To study the synergistic effect of topographical cues with biochemical signals or bioactive molecules on VSMC responses: Investigate a broader range of topographical patterns by exploring various grating widths, depths, and orientations. Additionally, assess the combined effects of topographical cues with biochemical signals or bioactive molecules, such as DNA, mRNA, siRNA, microRNA etc, on VSMC contractility and proliferation.

To utilize advanced imaging techniques and RNA sequencing methods to study the mechanistic role and molecular pathway: Utilize advanced imaging methodologies, including live-cell imaging and super-resolution microscopy, to gain insights into the real-time dynamics of focal adhesion formation,

cytoskeletal reorganization, and YAP localization in response to topographical cues. This would facilitate a more detailed understanding of the mechanotransductive processes involved. Further evaluating the molecular mechanisms underlying the effects of substrate topography on VSMC behavior, employ advanced techniques such as RNA sequencing and proteomics to identify additional signaling pathways and gene expression changes associated with VSMC responses to different topographical cues.

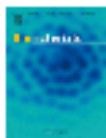
To study patient derived VSMC response to substrate topography: Investigate VSMC responses derived from patients with diverse vascular pathologies. This research will help determine how underlying conditions may influence VSMC responsiveness to topographical cues and inform personalized approaches to graft design.

To conduct long-term studies on grating effects: Conduct long-term studies to evaluate the sustained effects of 2 μ m gratings on VSMC behavior over extended culture periods. This will provide insights into the durability of the contractile phenotype and the potential for long-term performance of vascular grafts.

To evaluate the molecular pathways affected by substrate topography, with a focus on the different signaling pathways and their downstream effectors: Employ pharmacological inhibitors or genetic manipulation to clarify the specific roles of different signaling pathways in VSMC behavior.

To incorporate physiologically relevant conditions into experimental designs, such as introducing pulsatile flow and cyclic stretching, to better mimic the mechanical forces experienced by VSMCs in vivo: Conduct longitudinal studies to examine the long-term effects of topographical cues on VSMC behavior in dynamic environments, including the presence of flow or mechanical strain. Such investigations would provide valuable insights into the physiological relevance of these findings and elucidate how VSMCs adapt to fluctuating mechanical conditions *in vivo*, particularly in the context of vascular injury and repair.

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Fucoidan functionalization on poly(vinyl alcohol) hydrogels for improved endothelialization and hemocompatibility

Author: Yuan Yao, Xiang Moe, Zhen Deirdre E.J., Anderson, Monica T., Hink, Evelyn K.F., Yin

Publication: Biomaterials

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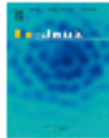
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Planar and tubular patterning of micro and nano-topographies on poly(vinyl alcohol) hydrogel for improved endothelial cell responses

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