Characterization of the real-time mechanobiology of engineered tissue equivalents

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NANYANG
TECHNOLOGICAL
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**CHARACTERIZATION OF THE REAL-TIME
MECHANOBIOLOGY OF ENGINEERED TISSUE
EQUIVALENTS**

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SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING
2016

**CHARACTERIZATION OF THE REAL-TIME
MECHANOBIOLOGY OF ENGINEERED TISSUE
EQUIVALENTS**

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ABSTRACT

Engineering functional blood vessels play an important role in both tissue regeneration and wound healing. However, engineered blood vessel equivalent remains to be far from clinical realization due to the difficulty in reconstituting the complex three-layer capillary histology. Among all major challenges, the elucidation on the intricate mechanisms involved in tissue morphogenesis of functional blood vessels engineering *in vivo* significantly hinders the development of engineered blood vessel equivalent *in vitro*. Thus the search for a highly biocompatible material for vascular tissue culture is inevitable in the design of artificial blood vessel. During the past several decades, novel biomaterial scaffold for cell attachment and culture have been developed for applications in tissue engineering, biosensing and regeneration medicine.

In order to engineer functional blood vessels, it is essential to recapitulate the characteristics of the tunica media consisted of vascular smooth muscle cells (SMCs) which is known to be critical for triggering vasoconstriction and vasodilatation in circulatory system. To simulate the physiological functions of blood vessel, the establishment of hyperplasticity of SMCs *in vitro* has been focused in several previous studies to produce viable synthetic SMCs in the initial stage and develop into quiescent contractile SMCs with highly aligned orientation by the use of microchannel arrays. However, the mechanism of such hyperplastic transformation occurring inside a microchannel to produce highly regulated orientation and phenotype of SMCs sheet remained to be elucidated.

Complex mechanotransduction between cells and the surrounding microenvironment such as the neighboring cells, the extracellular matrix (ECM) and biomaterial scaffold, plays an important role in the regulation of various

physiological processes. Among these interactions, cell traction force (CTF) is a crucial representative parameter in cellular functions. The conventional traction force assay for single cell measurement was extended herein for applications in three dimensional cell aggregates. Generally, the cooperative multiple cell-cell and cell-microwall interactions were accessed quantitatively by the newly developed assay with the aid of finite element modeling. Therefore, the main objective of my thesis is to develop different types of micropatterned cell culture platforms for carrying new cell-microenvironment mechanotransduction study *in-vitro*. At the same time, conventional cell traction force microscopy (CTFM) has been further developed for executing biophysical study of collective SMCs grown in the micropatterned scaffolds as mentioned above.

In tissue regeneration, the geometrical and mechanical properties of the microenvironment surrounding cells play an important role in cell physiology including migration, proliferation, differentiation and apoptosis. To mimic physiological conditions *in-vivo*, micropatterning of cells by applying soft lithography provides new possibilities for conducting experimental studies on cell mechanotransduction. In my thesis, I have designed and developed several types of biomaterial scaffolds with various unique features such as parallel discontinuous microwalls, concentric microwalls and bifurcate microgrooves by applying microfabrication technology and soft lithography. First of all, such microfabricated arrays were coated by polyacrylamide gel with embedded fluorescence microbeads in order to probe the mechanotransduction of multi-cell system such as smooth muscle cell layer. Secondly, atomic force microscopy in combination with regression analysis was applied to measure the elastic modulus of polyacrylamide gel coating on the microfabricated arrays. In the first part of my thesis, a novel

microfabricated array of discontinuous microwall was developed for stimulating the formation of highly aligned layer of SMCs. At the same time, the cell shape, density and alignment orientation of SMCs during cell alignment and cell layer formation were probed with CTFM. Secondly, the collective mechanotransduction of vascular tissue during circumferential alignment was studied with the use concentric micro-walls and CTFM. Finally, the morphological switch and orientated adaptation process of SMCs within the bifurcate microgrooves were monitored to reveal the influences of different bifurcation angles on the mechanotransduction of SMC layer.

In the first part of my thesis, our results from discontinuous microwalls showed that the traction forces of highly aligned cells lying in the middle region between the two opposing microwalls were significantly lower than those lying adjacent to the microwalls. Moreover, the spatial distributions of von Mises stress during the cell alignment process were dependent on the collective cell layer orientation. In the second part of my work, it was found that the mechanotransduction of SMC layer grown between the circular microwalls was significantly altered compared to the SMC layer grown on a flat or planar substrate. The results indicated the presence of an optimal region for formation of circumferentially aligned SMC layer verified by both quantitative analysis and biomechanical study. Besides, the distributions of traction forces and von Mises stresses during cell alignment were affected by the cellular mechanotransduction from the extracellular physical constraints and cell-cell interactions. In the last part of my thesis, the relatively low stress zone occurred around 90° bifurcation provided a novel explanation for the early localization and development of atherosclerosis.

In order to verify our previous postulation on the change of cytoskeleton structure of SMCs due to the formation of collective cell sheet and establish the biological differences between microwall-constrained SMCs and control SMCs, the 2D nano-LC-MS/MS and real time quantitative polymerase chain reaction (RT-qPCR) analyses were carried in addition to our biophysical studies. The online 2D LC-MS/MS analysis verified the modulation of focal adhesion formation under the influence of parallel microwalls through the regulation in the expression of three key cytoskeletal proteins. The real time quantitative polymerase chain reaction (RT-qPCR) analysis of actin further revealed the relation between the monolayer-generated traction forces and the direction of circumferential aligned actin filaments. Also, the immunofluorescence staining of SMC sheet demonstrated that the collective mechanotransduction induced by 3D topographic cues was correlated with the variation of actin and vinculin expression.

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LIST OF ABBREVIATIONS

2D	Two Dimensional
3D	Three Dimensional
AFM	Atomic Force Microscopy
Ar	Argon
BIS	Bis-acrylamide
BSA	Bovine Serum Albumin
CTF	Cell Traction Force
CTFM	Cell Traction Force Microscopy
CPCG	Cell-populated Collagen Gel
DRIE	Deep Reactive Ion Etching
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
FA	Focal Adhesion
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FEM	Finite Element Method
FITC	Fluorescein Isothiocyanate
GFP	Green fluorescent protein
HA	Hydroxyapatite
HaSMC	Human aortic smooth muscle cells
HEPES	4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid
MEMS	Microelectromechanical Systems
PAG	Polyacrylamide Gel
PBS	Phosphate Buffered Saline

PCL	Poly-caprolactone
PDMS	Poly(dimethyl siloxane)
PEG	Poly(ethylene glycol)
PEG-DA	Poly(ethylene glycol)-diacrylate
PGA	Polyglycolic Acid
PIV	Particle Image Velocimetry
PLGA	Poly(D, L-lactide-co-glycolide)
RPBOD	Rapid Prototyping Robotic Dispensing
SAP	Self-assembling Oligopeptide
SEM	Scanning Electron Microscopy
SFF	Solid Freeform
SMC	Smooth Muscle Cell
SmGM	Smooth Muscle Growth Medium
Sulfo-SANPAH	Sulfosuccinimidyl-6(4'-azido-2'-nitrophenylamino) Hexanoate
TEMED	N,N,N',N'-Tetramethylethylenediamine
UV	Ultra-violet

Chapter 1 Introduction

1.1 Background

Cell morphogenesis, which is a fundamental process in the developmental biology, plays an important role in the pattern formation of higher organisms. The cellular signaling during the cell morphogenesis has emerged as a crucial part for tissue engineering. Over the past decades, various advances in the developmental biology have been achieved from the tools in chemical and molecular sciences, including the morphogenetic signaling and ECM molecular interactions [1]. However, the study of the mechanical cues underlying between cells and their surroundings is lacked. On the other hand, a variety of synthetic biomaterials are developed in the form of 3D scaffold for tissue regeneration. The contact guidance conducted by the biomaterials may provide mechanical stimulus and facilitate the cellular activities such as cell adhesion, cell proliferation and cell migration.

Vascular smooth muscle locates at the middle layer of the blood vessel known as tunica media and forms the wall of blood vessels. Because of the phenotype hyperplasticity, the principle functions of SMC in mature mammals are proliferation and contraction. It is well known that SMCs with the expression of contractile phenotype are quiescent, while SMCs with the expression of synthetic phenotype tend to differentiate and proliferate. In order to engineer functional blood vessels, controlling the change from synthetic to contractile phenotype of SMCs becomes a key challenge and the confluence-triggered switch of SMC phenotypes within 3D microchannels has been recently reported [2-4]. The studies showed that the elongation of vascular SMCs not only ensure the vasoactivity in cardiovascular tissue engineering, but also express larger amount of contractile proteins. To

investigate the collective biomechanical behaviors of SMCs in tissue engineering systems, we have further modified the 3D scaffold and developed different micropatterned scaffolds, which are more convenient for the biophysical and biomechanical study.

Among several mechanical interactions between cells and their surroundings, cell traction force (CTF) plays a critical role in cellular functions of anchorage-dependent cells. CTF is known to be generated by the cytoskeleton, regulated by the intracellular proteins and transmitted to the ECM via focal adhesion. While previous study has been focused on the measurement of the cell traction force of a single cell, the understanding in the collective mechanotransduction of multiple cells is currently lacked. In the thesis, we developed an optimum experimental platform for the biophysical and mechanical study of collective cellular mechanotransduction. At the same time, we integrated the novel platform as mentioned above with cell traction force microscopy (CTFM) to investigate the traction forces of confluent SMC layer exerted on the soft substrate. In addition, we monitored the dynamic alignment and morphological transformation of SMC by real-time cell imaging and quantitative analysis. Furthermore, because of the contact guidance provided by the 3D microwalls, the influences of mechanical signals during the process of cellular assembly were systemically elucidated.

1.2 Objective and Scope

The overall objective of my PhD project is to develop different micropatterned scaffolds to characterize the biophysical study of collective SMCs from parallel alignment, to circumferential alignment and branched distribution. The developed models in this thesis are expected not only to facilitate the collective

mechanotransduction underlying *in-vivo-like* micropatterned SMC sheets, but also to provide a fundamental bio-mechanistic understanding for the development of novel tissue engineered platforms.

1). To develop 3D experimental platform that regulates the orientation and phenotypes of SMCs and enables real-time collective cell traction force assessment.

In healthy blood vessel, SMCs are in the contractile state and are well aligned long the vessel wall. When SMC are cultured *in vitro*, SMCs must attach to the substrate and spread randomly in the synthetic phenotype in order to survive and grow. It is well known that both microchannels and discontinuous microwalls can facilitate the switch from synthetic to contractile phenotype of SMCs [4]. However, the morphological shift rate and cell alignment percentage are varied according to different scaffolds. As primary SMCs are larger and harder to be cultured than cell line-derived SMCs, microwalls will be used to replace microchannels for parallel-aligned cell sheets for a better cell-cell communication, which can simulate the confluence and facilitate the phenotype switch. For circumferential alignment and bifurcating structures, in-depth microwalls will be applied.

2). To quantify the morphological changes of SMCs and alignment of cell sheet over the period of functional SMCs sheet formation.

After recording the process of functional SMCs sheet formation, we want to quantify the cell density and morphological changes by combining the phase contrast images with MetaMorph software. Besides, the degree of collective SMCs orientation will be determined by calculating the alignment percentage (parallel and circumferential alignments) and orientation-specific adaptations (bifurcations).

3). To develop a novel CTFM assay for the collective cell traction force measurement under the contact guidance of 3D scaffolds.

As several advances in studying single cell mechanotransduction have been achieved with conventional CTFM assay, the research efforts will focus on the novel experimental and theoretical methods of collective CTFM. In addition to the geometry of 3D matrix, the mechanical property of the matrix including elasticity is another key factor that influences the cell traction force assessment. In our novel CTFM assay, the 3D platforms will be coated with a thin layer of polyacrylamide-based hydrogels [5], which is an ideal experimental substrate for probing cell traction forces through the displacement of embedded fluorescent beads. The linearly elastic feature of PAG ensures a complete recovery after removal of the stress and the substrate is soft enough to distinguish beads displacement. To determine the stiffness of the substrate, the contact mode of atomic force microscopy (AFM) will be chosen to measure the Young's modulus. As the PAG is chemically inert, we need to activate the surface and then conjugate ECM proteins before cell seeding.

4). To investigate the mechanical influences of different micropatterned scaffolds to the collective cell traction forces and stresses of cell sheets.

With scaffolds depth of ~70 μm and width of 300 μm , we aim to characterize the mechanotransduction of SMCs grown within the micropatterned scaffolds together with SMCs grown on 2D platform, which will be used as negative control, during the entire process of collective cell alignment. Collective CTF will be studied on the aligned cell sheet grown within the different micropatterned scaffolds, which have been proved to be useful in inducing the confluence-induced phenotype switch of SMCs. We tend to elucidate the influence of 3D constraints on the spatial distributions and magnitudes of CTFs and stresses.

5). *To provide a new possible explanation for the early localization and development of atherosclerosis according to the biomechanistic study of SMC layers.*

Atherosclerotic plaques do not develop randomly within the arterial tree, but preferentially at arterial curvatures, branches and bifurcations where the blood flow is disturbed [6]. Together with the micropatterned techniques, collective CTFM assay will be applied to reveal possible connections between different bifurcating SMC layers and atherosclerosis lesions.

6). *To verify the findings on the change of cytoskeletal structures of SMC layers due to different micropatterned constraints via biological methods.*

At the cellular and molecular levels, immunofluorescence staining, online 2D LC-MS/MS analysis and RT-qPCR assay will be further used to provide further support for the monolayer-generated traction forces and demonstrate different gene expression levels.

1.3 Outline

This thesis is divided into six parts. In Chapter 1, the background of biophysical study is provided and the objectives and outline of this thesis are introduced. Chapter 2 provides a detailed description of cell mechanotransduction in 3D systems. In Chapter 3, the optimal experimental platform for mechanotransduction study of cell sheet is fabricated and characterized. And the collective CTFM assay is newly developed and applied for the study of biophysical effects of discontinuous microwalls on the mechanotransduction of SMC layer undergoing parallel alignment. Chapter 4 further extends the application of collective CTFM assay to the biomechanistic of SMC layer during circumferential

alignment in circular micropatterns. In addition, the immunofluorescence staining and RT-qPCR analysis are used together to verify the cytoskeletal changes. Chapter 5 focuses on the role of bifurcation angles on collective SMC biomechanics and atherosclerosis development. The expression levels of specific genes are also measured to provide biological support for atherosclerotic localization. In the last chapter, overall conclusions are made and possible future work is discussed.

Chapter 2 Literature Review

2.1 Micropatterned Substrate

In cell and tissue regeneration, the geometrical and mechanical properties of microenvironment surrounding cells or tissues play an important role in the resulted cell physiology including migration, proliferation, differentiation and apoptosis, etc. [7, 8]. As such, researchers in tissue engineering have traditionally focused their efforts in the development of various types of biomaterial scaffold with specific porosity and engineered compliance for various applications in specific cell culture and tissue regenerations. On the other hand, conventional biomaterial scaffold doesn't provide a highly engineered microenvironment with precise control in positions and shape of various types of cell for reestablishing the intricate organizations in functional organ subunit. Alternatively, micropatterning of cells on various planar substrates has been exploited with microfabrication techniques such as soft lithography over the past two decades. In general, several popular techniques including microcontact printing [9], microfluidic patterning [10], photolithography [11, 12], plasma polymerization [13] for patterning cells on two-dimensional surfaces (with features down to the dimension of 1 μm) include have been under extensive development [14]. Precise fabrication of three-dimensional (3D) microstructures on biomaterial scaffolds is critical not only for controlling cell placement but could also serves as the prerequisite for engineered tissue constructs for *in vitro* applications [15]. For the effective development of tissue engineering scaffolds [16], several technical requirements in selected materials including mechanical properties, biocompatibility and easy manipulation need to be fulfilled. In addition, the advancement in 3D fabrication techniques emerges as a major mean

for imposing spatial control of specific cell types in engineered tissue equivalents. Therefore a thorough discussion on recent development in 3D cell patterning techniques which would be increasingly crucial for the realization of tissue engineering products would be worthwhile to be carried herein.

2.1.1 Polymeric microtextured scaffold

The most commonly used synthetic polymers for 3D fabrication include polyglycolic acid (PGA) [17], poly-caprolactone (PCL) [18], polydimethylsiloxane (PDMS) [19] and poly(ethylene glycol) (PEG)-based hydrogels [20]. For example, polyglycolic acid (PGA) and its copolymers are widely used in as scaffolds for tissue engineering applications because they are biodegradable and non-toxicity [21]. However, the native surface of PGA is not suitable for direct cell attachment and regeneration. Thus the modification of PGA with biological active ligands are essential for cell culture applications, e.g., the addition of β -tricalcium phosphate (β -TCP) in porous 3D PGA scaffolds has been applied for repairing bony defects [22]. Moreover, copolymers built from PGA such as poly(lactic-co-glycolic acid) (PLG) matrices has been used as a scaffold with the controlled release capability of vascular endothelial growth factor *in vivo*. The PLG system as mentioned above facilitated the blood vessel development and enhanced local vascularization [23]. Besides, the microporous poly(D, L-lactide-co-glycolide) (PLGA) membrane has been applied for the 3D stacked culture of hepatic tissues [24].

In spite of the development microporous polymeric scaffolds, there has been a lack of precisely engineered 3D experimental systems for simultaneous culture and monitoring of cells or tissues. Recently, Chan-Park et al. has successfully developed high-aspect ratio microchannel by UV embossing on a liquid of UV polymerizable biodegradable macromer composed of poly(ϵ -caprolactone-r-L-

lactide-r-glycolide) diacrylate [2]. In detail, the master Si mold is prepared from a lithography system, which uniquely combines deep reactive ion etching (DRIE) with passivation treatments. After obtaining the daughter PDMS mold, UV resin liquid formulation and a polyester film are successively dispensed onto the PDMS mold. Lastly, the resin is polymerized under UV illumination and separated from the PDMS mold and polyester film [25, 26]. The depth of the micropatterned biodegradable scaffold synthesized by this method can reach up to 70 μm . Furthermore, the layer-by-layer (LBL) process has been successfully applied to build multilayers of confluent SMCs on the micropatterned biodegradable scaffold as mentioned above and to trigger the expression of contractile phenotype of SMCs [3]. In addition to the highly engineered spatial order in cells, effective diffusion of dissolved gas and essential nutrient to cells seeded on the micropatterned biodegradable scaffold are critical for retaining cell viability and functionality.

Sarkar et al. [18] has developed the porous micropatterned PCL by combining soft lithography, melt molding and polylactic-co-glycolic acid (PLGA) micro/nanoparticles leaching. The group has further demonstrated that diffusion of culture media into the PLGA-leached PCL scaffolds as mentioned above was enhanced by six times in comparison with that through the non-porous PCL scaffolds. Recently, biodegradable microstructured polycaprolactone construct has been chemically functionalized with poly(ethylene glycol)-diacrylate (PEG-DA) gel as an adhesive sandwich layer for fabricating 3-D composite structure of vascular smooth cell-seeded layered with precisely control cell orientations [27]. Until now, most emerging techniques as mentioned above were aimed at the precise fabrication of 3D micropatterns on polymeric scaffold for tailored cell culture instead of

providing an experimental platform for probing cellular behavior under the prescribed patterns.

Recently, Deutsch et al. [19] has developed new fabrication process of microgrooved topographical cues on PDMS scaffold by combining photolithography and soft lithography. Briefly, the silicon wafer is spun coated with a photo resist layer and exposed under UV light through a contact mask. The micropatterned PDMS scaffolds are created by casting and curing on the wafer surface. The fibronectin-coated PDMS scaffolds fabricated by this method has been shown to direct the spatial organization of cells and extracellular matrix [28]. In another study, PEG-based hydrogel microstructures are fabricated on silicon or glass substrates by using photolithography-based patterning [20, 29]. In detail, glass substrate was first functionalized with 3-(trichlorosilyl)propyl methacrylate, then spin-coated with poly(ethylene glycol) (PEG) derivative and a photoinitiator and finally exposed to UV light through a photomask for PEG crosslinking. Moreover, PEG-based hydrogel are successfully developed into cylindrical or cubic shape for the encapsulation of cells, drug-drug interaction detection and tumor sphere formation study [30-32]. Hahn et al. [33] develop confocal-based laser scanning lithography for 3D surface patterning (feature under $\sim 5\ \mu\text{m}$) of PEG-based hydrogel substrates. The micropatterned PEG-based hydrogels embedded within independently PDMS housing are applied in microfluidic device to study the cellular viability and metabolic density, which contributes to emerging applications for *in vitro* diagnostics and regenerative medicine [34]. At the same time, the PEG-based hydrogels can be further developed into porous 3D structure to control spatial organization, enhance cell-binding affinity for engineered tissue equivalent and angiogenesis characterizations [35, 36].

2.1.2 Natural polymers

Naturally derived polymer such as proteins and polysaccharides are ideal materials for the fabrication of biomimetic 3D scaffolds with distinct properties compared those of synthetic polymers. Collagen-based scaffold is one of the most important natural polymers serving as adhesive ligand and is widely found in most ECM surrounding tissues *in vivo* [37]. Recently, phase change ink-jet printing and indirect 3D printing technique have been applied to fabricate the collagen scaffolds with micropatterns such as internal channels and capillary networks [38, 39]. Indirect 3D printing of collagen involves three steps. Firstly, a negative mold is created by solid freeform (SFF) fabrication technique in combination with computer-aided design [40, 41]. Secondly, collagen solution is casted into the mold and frozen. At last, the predefined scaffold is achieved by and dissolution of the mold with ethanol followed by the critical point drying with liquid carbon dioxide. Another preferred method for fabricating collagen-based scaffold with uniform pore structure is more homogeneous freezing with controlled freezing rate [42]. The cell attachment and viability on the freeze-dried 3D scaffold as mentioned above are shown to be influenced by specific surface area and the pore size [43].

Beside protein, chitosan that is formed from the deacetylation of chitin is another important naturally derived polysaccharide for fabricating biomaterial scaffold in various applications of tissue engineering such as hepatocyte culture [44, 45]. Recently, a rapid prototyping robotic dispensing (RPBOD) system has been developed to fabricate 3D scaffold of chitosan and chitosan-hydroxyapatite (HA) with reproducible macropore architecture by injecting chitosan and chitosan–HA solution (in acetic acid) into sodium hydroxide–ethanol solution (in ratio of 7:3) [46]. Moreover, a hydrogel formed from the self-assembly of oligopeptides has

been molecularly engineered with ordered filaments which form microporous structure such as that found in native ECM for 3D cell culture system [47, 48]. Recently, one self-assembling oligopeptide (SAP) designed with specific cell adhesion, cell differentiation and bone marrow homing motifs has been successfully developed into nanofiber scaffolds for the functional maintenance of mouse adult neural stem cells in 3D environment. SAP gels contain high concentration of peptides by using standard solid phase synthesis method and cells are embedded inside the matrices. To date, the SAP gels have been further extended into tunable properties such as nano-scale architecture and biological functionality with for various applications in tissue engineering such as vascular graft [49, 50]. In spite of the recent advances in the natural material fabrication, the quantitative correlation between scaffold structure and cellular responses for the design of an appropriate 3D scaffold for tissue regeneration remains to be elucidated. In general, a 3D biomaterial scaffold should mimic the physical microenvironment and biochemical cues of native ECM as much as possible. Therefore a better understanding of cell-scaffold interaction and cell behavior in the 3D environment will facilitate the design of highly tailored biomaterial scaffold for tissue regeneration.

2.2 Cell - Microenvironment Biomechanics

It is known that the elasticity of substrate directly imposes control on the contact mechanics of cells while microfabrication technologies such as micropatterning provides the geometric control of cell morphology on the opposing biomaterial scaffold. Regardless of the scaffold geometry, the elasticity of the scaffold material ranges from soft to rigid. Simultaneously, cells including fibroblasts, SMCs and others respond differently to the substrate with various range

of elasticity through intricate mechanotransduction and cytoskeleton organization [51]. In a recent study, it has been shown that the cell spreading and motility is reduced and increased, respectively, on flexible polyacrylamide gel substrate [48]. Moreover, cells have demonstrated preferential migration from soft region to stiff region on a polyacrylamide gel substrate but not from stiff to soft region on the same substrate [52, 53].

The communication between cells and apposing ECM or biomaterial scaffold is driven by the intricate mechanotransduction through the active reorganization of internal cytoskeletal elements in the cytoplasm [54]. In general, cell-ECM interaction is strengthened by the formation of focal adhesions (FAs), which are dynamic protein complexes and are formed from specific transmembrane proteins, known as integrins. At the same time, the increase of ECM stiffness has been shown to enhance cell motility as cells exert higher contractile forces towards stiffer substrate during cell adhesion and migration. The geometry of the sub-region functionalized with adhesive islands can be precisely engineered with the use of microcontact printing techniques, which are instrumental for studying the influence of physical constraints to the regulation of cellular functions on model substrate. Several studies have shown that cell adhesion is not only determined by the integrin binding with ligand, but also related to the biochemical signaling pathway and structural deformation of intracellular organelles of adherent cells. It has been revealed that both the formation of focal adhesion and the emergence of actin stress fibers of adherent cells are dependent on the ECM stiffness. Moreover, the optimal value of substrate stiffness for supporting maximal migration is correlated with the concentration of ECM ligands covalently attached to the substrate [55].

New advances in the development of model systems to study the mechanotransduction of cells at the cell-substrate interfaces have been recently established. In the first report, the elastic distortion and wrinkling of silicone substrate induced by the adherent cells through cytoskeleton remodeling during cell locomotion can be easily observed via optical microscope [56] and can be further quantified through the variation of substrate elasticity [57, 58]. Thereafter, Balaban et al. has combined the transparent micropatterned elastomer and focal adhesion characterization through the expression of green-fluorescent protein (GFP) labeled vinculin in adherent cells for elucidating the relationship between the assembly dynamics of focal adhesions and the alteration of cell traction forces [55]. The method as mentioned above hinges on the use of a non-wrinkling substrate with micropatterns composed of dots or lines for the determination of cell-induced surface deformation.

Recently, Tan et al developed a microfabricated array of elastomeric microposts, each deformable structure independently measures the mechanical interactions at a specific area between adherent cells and apposing substrates [59]. With the use of micropost as the mechanical probe, the spatial deformation map caused by the cell traction forces across the entire substrate can be quantitatively measured [60]. By changing the height of micropost, a series of micro-post arrays reported with a range of substrate rigidity further demonstrates the influence of microscale geometry and elasticity on cell morphology, focal adhesion organization and cell contractility [61]. By combining micropost array technology with super-resolution cell imaging, the precision map of cellular traction force with a detection precision of 500 pN and the nanoscale architecture of individual force-bearing focal adhesions have been simultaneously measured [62]. Moreover, the micropost array

has been developed to measure the real-time stiffness of entire cells through the integration with a cell stretching device, which enables the quantitative study on the force-sensing mechanisms of live cells and provide better understanding of real-time mechanotransduction [63, 64]. For all the silicone-based substrata used in micropost technology, the surface coating of extracellular matrix (ECM) proteins is essential to create a more physiological environment for cell adhesion. In addition to the PDMS based microarrays, polymeric hydrogels are also used in micropost array fabrication [65]. First of all, the hydrogel monomers such as hydroxyethyl methacrylate were mixed with photoinitiator and followed by replica molding and UV exposure to transform into partially polymerized precursor solutions. Secondly, photo-crosslinking of the partially polymerized precursor solutions with ethylene glycol dimethacrylate leads to the formation of stable hydrogel micropost array. Moreover, the stability and surface clustering of a range of high-aspect-ratio hydrogel microarrays under capillary forces have been studied by controlling the geometry and elastic moduli of these microposts [66].

Another widely reported substratum for studying cell-substrate mechanics is polyacrylamide-based hydrogel, a microporous material that has been proven to be an ideal experimental system for various cell types due to its tunable chemical and mechanical properties [5, 67]. Firstly, polyacrylamide-based hydrogel can be engineered with precise mechanical properties, e.g., it can be easily tuned from extremely soft to stiff material with distinct elastic modulus by adjusting the concentration ratio of acrylamide and bis-acrylamide during the course of polymerization. Secondly, the polyacrylamide-based hydrogel can be loaded with fluorescently labeled microbeads, which serve as position markers throughout the entire 3D hydrogel matrix. More importantly, polyacrylamide-based hydrogel can

be linearly deformed in response to the applied forces and can be fully recovered to its original state upon the removal of force. Thirdly, the low thickness and transparent nature of polyacrylamide-based hydrogel ensures the detection of small displacements of the fluorescent beads as mentioned above. Furthermore, the gels are naturally non-adhesive for mammalian cells unless specific ECM molecules are (type I collagen) covalently linked onto the surface. The embedded fluorescent microbeads actually serve as position markers for the detection of local deformation across the entire region of interest of the hydrogel under microscopy monitoring [68].

In addition to the planar flexible substrata, the microelectromechanical systems (MEMS) were applied on polysilicon device for the installation of force transducers in cell mechanics study [69]. By measuring the deflections of micrometer-sized pads connected to the cantilever, the cell contraction force was measured [70]. A substrate composed of close-packed standing cantilevers has been designed with different cantilever geometry and spatial arrangement for probing the local mechanical forces at cell adhesion contact [71]. When it comes to the geometry control of adherent cells, both planar substrate and ECM can be modified by using 3D microfabrication techniques as mentioned above. In addition to micropatterned substrate, the spatial organization of focal adhesions for cells in 3D microenvironment remains to be elucidated. It is known that cell spreading and migration within 3D microenvironment hugely depends on the topological properties [72] and mechanical stiffness [73-75] of surrounding ECM. The stiffness of 3D collagen matrix has been engineered under same collagen density to study its sole influence of matrix stiffness on cell behavior; the study showed that endothelial cell spreading is enhanced by the increase of matrix stiffness [76]. When cells are

partially or completely embedded in a 3D matrix, the cellular responses are quite different from those observed in 2D culture dish, e.g., FAs become smaller, diffusively distributed and hard to be detected in the cell cytoplasm [77, 78]. Moreover, cell adhesive traction forces in 3D matrix are influenced by the sparse distribution of focal adhesion molecules as cells tend to pull instead of push their surrounding matrix and the matrix traction always concentrate at the end of membrane protrusions at the pulling tail of the cells [79].

2.3 Mechanotransduction and Cell Traction Force

Mechanotransduction that generally occurs at the cell-ECM interface and cell-cell contact, is the transmission of mechanical forces to biochemical cues and vice versa for the regulation of cell behavior. Mechanical stresses are present in the microenvironment surrounding cells, whether they are generated from the external applied forces or internal traction forces produced by the cytoskeleton, have been intensely studied because of their important role in maintaining homeostasis in tissues *in vivo*. Although the involvement of cell traction force on cellular signaling and function has been recently revealed, the mechanism of mechanotransduction in 3D systems still remains unclear. In physiological environment, both cells and subcellular organelles can sense mechanical stresses from various sources such as shear stress of flowing blood, mechanical stress from the surrounding ECM, contractile forces from adjacent cells [80]. There are significant differences between external and cell-generated forces, which can be characterize from the differences in magnitudes, directions and distribution. However, certain indications on the existence of tight coupling between external applied and cell-generated forces have been revealed [81, 82]. Recent study has shown that the intracellular organelles

which perform mechanotransduction directs cellular responses towards the mechanical stress induced from blood flow [83]. Inside blood vessel, the blood pressure and shear stress induced by flowing blood act as the external forces towards endothelial cells (Figure 2.1(a)), while the mechanotransduction takes place at both cell-cell junctions and cell-matrix contacts (Figure 2.1(b)). Here, we discuss the mechanotransduction process via the pathways of contact formation.

Figure 2.1. (a) Schematic illustration of endothelial cells that form the interior surface of blood vessels. (b) The cell-cell junctions and cell-substrate tractions

exerted by endothelial cells. (c) Positive feedback in cell-ECM mechanotransduction.

2.3.1 Cell-Matrix Contacts

An adherent cell binds to ECM through the FAs, where several structural proteins (e.g. vinculin, talin, paxillin and α -actinin) [84] stabilize the cell adhesion by anchoring FAs to the actin cytoskeleton via signaling proteins (e.g. FAK, Ras, Src) [85]. The biochemical processes in the generation of contractile force after cell attachment to and spreading on the substrate involves actomyosin interactions and actin polymerization [86, 87]. The traction forces are then transmitted to ECM via focal adhesions (FA), which are crucial for connecting the cytoskeleton of adherent cells to the ECM [88]. One of the various components in FA, a transmembrane receptor known as integrins, acts as a cross bridge between the cytoskeleton and ECM. These cell-generated forces as mentioned above are called ‘cell traction forces’. A variety of molecular biology studies have revealed that CTF involves in many cellular functions like regulating cell-signaling pathway. For SMC-generated CTF, it is mainly regulated by the Rho-kinase/ROCK, which affects the mitogen-induced DNA synthesis [89] and the assembly of focal adhesions [90]. As such, the presence of CTF plays a key role in modulating cell differentiation, migration, apoptosis and maintaining the cell homeostasis [91]. However, with the discovery of mechanochemical transduction between the CTF and cellular response, a new question about the involved mechanism needs to be addressed.

As cells exist in 3D microenvironment *in vivo*, three-dimensional ECM has been artificially developed and applied for the mechanotransduction study. The materials and techniques for the 3D micropatterning fabrications are already discussed above. Three dimensional collagen matrices have been widely used to

study cell adhesions and motility. For the signaling transduction, focal adhesion kinase (FAK) phosphorylation level decreases compared to 2D culture, while focal adhesion in 3D collagen gel depends on $\alpha_5\beta_1$ instead of $\alpha_v\beta_3$ integrins. For the 3D culture of SMCs *in vitro*, the down-regulated expressions of p21, TGF β_1 and FAK phosphorylation are revealed by DNA microarray, which indicate the inhibit of cell proliferation and FA formation [92, 93]. Similarly, increased matrix stiffness facilitates the formation of activated 3D-matrix adhesions and enhances the outside-in/inside-out FAK-Rho signaling loop [94]. During the cell migration in 3D scaffold, primary dermal fibroblasts can switch the mode between lamellipodia and lobopodia according to the ECM elasticity [95]. These results suggest that the architectures of ECM mediate the focal adhesions and subsequently influence the mechanotransduction process in both 2D and 3D platforms.

While the mechanical behavior of single cells has been studied for the past two decades, the collective mechanisms of cell mechanotransduction still remain unclear. Cell traction forces, which are generated by the cytoskeleton reorganization, exert on the substrate via the formation of focal adhesions and are influenced by the ECM or neighboring cells. *In vivo*, individual cell migrates by applying traction forces on its surrounding ECM through cell's leading and trailing edges, while the mechanism migration of a group of neighboring cells, e.g., cell sheet, that influences many biological processes containing morphogenesis, wound healing and tissue repair, is poorly understood. A recent study has reported the traction force map exerted by an advancing epithelial cell sheet [27]. The distribution and magnitude of traction forces are coupled to the colony size and generally localize at the periphery of the cell colony [96]. The study has demonstrated the tractions force in the leading edge of cell sheet during migration is

predominately conducted by many backward cells instead of the leading cells [97]. Compared to individual cells, the maximal-traction forces and stresses at the edge of the monolayer are higher [98]. The cell-matrix tractions, together with cell-cell interactions, drive the collective cell migration.

It is believed that physical interactions as well as chemical cues are essential in the mechanotransduction process. According to our knowledge, the mechanical properties in different tissues and organs vary a lot *in vivo*. As these changes are related to the cellular cytoskeleton and ECM composition, extensive studies have been focused on fabricating substrate stiffness and micropatterning substrate structure for a deep understanding of mechanochemical transduction [51]. The features of individual cells and matrices play an important role in the biomechanics study, while the organization of groups of cells and their surrounding microenvironments provide significant cues for tissue mechanotransduction. Cells apply traction forces on the microenvironment, and at the same time make cellular responses to the mechanosensing from the surroundings (Figure 2.1(c)). Through CTF measurement under various conditions, the cell-signaling pathway in many physiological and pathological events can be reflected and tracked. Besides, as the fundamental forces in the mechanotransduction process, CTF measurement makes the biomimetic study of morphogenesis *in vitro* possible. It is believed that the deep understanding of the complex driving force underlying the cell-ECM interactions is a key factor in the success of tissue regeneration.

2.3.2 Cell-Cell Contacts

Another important mechanosensing type is via cell-cell contacts, which contain adherens junctions, gap junctions and tight junctions. In addition to promote cell-cell adhesions, these junctions act as the bridge to transmit intracellular signals

directly to the actin cytoskeleton [99-101]. Mechanical stresses produced from the surrounding physiologically environment, such as substrate, ECM, neighboring cells and so on, could influence the collective cell proliferation and migration, which eventually contribute to the vasoactivity of blood vessel. A simple form of inhibition to cell proliferation and movement is known as “contact inhibition”. In cultured epithelial cells, the freely growing, nonconfluent cells can switch to fully differentiated, densely packed monolayers because of the cell-cell contact [102, 103]. The phenomenon of contact inhibition also exists in many different kinds of cells [104, 105]. Quantitative characterization of contact inhibition dynamics on collective cell behavior shows that cell-cell contact is a necessary but not sufficient condition for the growth inhibition [106]. It has been suggested that mechanical compression may provide an inhibitory signal for cell division [107].

In addition to the inhibition of cell proliferation, cell-cell contact may conduct stresses between cells, which guide the orientation of cell migration along the minimal intercellular shear stress [108]. As epithelial cells undergo stable cell-cell adhesions and mesenchymal cells rely on transient and dynamic cell-cell contacts, the strategies of collective migration are different based on cooperative cell behaviors [109]. Recent study has shown the chase-run phenomenon between placode cells and neural crest cells involved in a mechanism of chemotaxis and N-cadherin signaling transduction, which provide the explanation of coordinated migration of different cell populations [110]. With the assumptions about the existence of mechanical coupling between neighboring cells, cell-cell interactions are sufficient to guide cell sheet direction of movement, without the physical directional cue [111-113].

To understand the principles and mechanisms involved in the processes as mentioned above remains a tremendous challenge, because the mechanical stresses are difficult to be experimentally measured. Traction force, as one of the most significant mechanical properties, is recently used as an ideal model for analyzing the complex mechanotransduction of multiple cells [114-116].

2.4 Cell Traction Force Measurement

Mechanotransduction, which is an emerging research area, requires extensive knowledge from many other subjects. A variety of methods have been developed to measure CTFs of both individual cells and collective cells during the past few years [117], including cell-populated collagen gel (CPCG) [118], thin silicone membrane[56], force sensor array and improved micropost force sensor [59]. Currently, the most reliable and comprehensive method recently used in the measurement of CTF is CTFM assay, which was developed by Dembo and Wang in 1999 [119].

In CTFM assay, PDMS, a suitable stretchable matrix to facilitate the study of overlying cell populations, was chosen as the platform. Chemically, the properties of PDMS can be adjusted by the polymerized ratios. Biologically, the transparent biocompatible surface can be used to develop more physiologically substrates for appropriate cell culture. Mechanically, the flexibility of substrate material and the ability to create features on the micron and submicron size scale can be easily micropatterned. However, PDMS is inherently hydrophobic, which requires further treatment for the conjunction of microbeads-embedded hydrogen gel.

In CTFM, the fluorescent microbeads embedded in the substrate serve as markers to record and track the deformation caused by CTFs. By taking a pair of

‘force loaded’ and ‘null force’ images of the same image frame, the deformation of the elastic substrate is determined and used for the CTF computation. By dividing images into overlapped windows with a constant distance, a pair of small windows respectively from ‘force loaded’ and ‘null force’ images are obtained and applied to calculate the displacements. There are another two methods of CTFM assay that are developed by Butler et al. [86] and Yang et al. [120]. Both methods as mentioned above rely on the setting window and conduct the correlation computation by using Fourier transform. But the bead displacement calculation used in Dembo and Wang method is not fixed to the pair windows as mentioned above. The correlation-based matching method makes it more flexible than Butler et al. method. While the former approaches by using pixel windows ignore the local rotation of displacements, Yang et al. method chooses a suitable microbead to set it as the threshold for beads identification before beads matching. All the derived CTFM methods suffer from a same problem – the accuracy of the displacement calculated from the microbeads. In addition, all the measurement methods mentioned above are limited to the 2D substrate surface. CTFM method can achieve a broader application if the topology can be extended to 3D matrix.

2.4.1 3D cell traction force

Recently, more efforts have been devoted to developing novel experimental and numerical methods for determining 3D CTFs, which have similar elastic moduli and physiological features to *in vivo* situations. By using laser scanning confocal microscopy and digital volume (3D) correlation, 3D traction force fields can be computed by 3D CTFM techniques [72, 121-123]. In this method, 3D traction forces of single cell are mapped over the 2D surface between cell and polyacrylamide gel and the dynamic traction forces during cell migration and

locomotion can be calculated. Similarly, Hur et al. [124] and Delanoë Ayari et al. [125] develop 3D CTFM techniques to measure the traction forces in three dimensional. The differences among them are the maximum limit of beads distance and computation algorithms [126]. What's more, compared to the traditional linear deformation framework, new large deformation methodology together with high-resolution digital volume correction technique are developed recently [127].

For the full 3D method that cells are surrounded by ECM in all directions, collagen rather than synthetic gel are used. The limitations in 3D scaffold are the control of mechanical properties of the natively derived gel and the difficulty of fabrication process. Legant et al. [128] encapsulate GFP (EGFP)-expressing cells in the well-defined 3D hydrogel matrices, track the displacements of embedded fluorescent beads and calculate the tractions exerted by the inside living cells. They apply linear elastic theory and the finite element method developed from confocal imaging of hydrogels. The stiffer the hydrogels are, the stronger tractions cells exert. Strong inward forces have been revealed to locate predominantly near the long extensions, while the shear tractions are the main tractions produced by the cells encapsulated in 3D hydrogel. By combining with 3D collagen network, Gjorevski et al. [129] and Koch et al. [130] also obtain the 3D traction mapping that exists throughout the surrounding matrix. One challenge in this method is the difficulty to interpret the traction field from the cell types that can degrade surrounding collagen and subsequently change its mechanical properties, like fibroblasts, some tumor cells [77].

2.4.2 Collective cell traction force

Based on the CTFM techniques, additional studies focused on confluent cell monolayers has been achieved to improve our knowledge about cell-cell and

intracellular forces, including aligned SMC layer with contractile morphology [131], endothelial cell sheet under both static and shear flow conditions [132]. Recent studies have shown that the spatial distribution of traction forces exerted by cohesive cell colonies is significantly localized at the periphery of the colony [96]. In other words, collective cell traction forces are strongly influenced by the shape of the adhesive island. For better understanding of collective cell activities, novel CTFM has been developed to show the dynamic traction domains and the compressions among cell clusters [133]. To quantify the cell-generated mechanical stress *in situ* within living tissues, a newly technique with 3D microdroplet has been developed for the study of both individual living cells and groups of cells [134]. After obtaining the shape deformations of the droplets microinjected among cells in the tissue, the anisotropic stresses generated by epithelial cell colonies can be quantified via fluorescent imaging and computerized analysis.

Although the 3D CTFM attracts much attention, the techniques are still immature and not widely used. Several challenges, including the fabrication and understanding of the 3D matrix, the techniques of high resolution 3D imaging, and the necessity for complex computation algorithms, need more efforts to conquer them. In addition, these methods mentioned above to measure the collective cell traction field are limited by both the disadvantages of CTFM and the multiple-cell behaviors. Considering that multiple-cell sheet instead of individual cells evolve in most physiological processes, there is an essential need to develop the measurement methods for 3D traction forces that resulted from collective cells.

Chapter 3 Collective Cell Traction Force Analysis on Aligned Smooth Muscle Cell Sheet between Three Dimensional Microwalls

3.1 Introduction

In tissue engineering, the mechanical properties of the external microenvironment surrounding cells play an important role in cell physiology, including migration, morphogenesis, proliferation, differentiation and apoptosis [7, 8, 52, 91, 135-137]. For example, the formations of focal adhesion and stress fibers in adherent cells are dependent on the stiffness of extracellular matrix (ECM) [138-141]. Also, the optimal ECM stiffness for mediating strong cell adhesion is affected by the concentration of immobilized adhesive ligands on ECM surface [55]. Generally, the elasticity of substrate dictates the responses of cells including fibroblasts, smooth muscle cells (SMCs), etc., through the cytoskeleton organization [51]. Mechanical stresses generated from the external microenvironment or cell cytoskeleton (e.g., cell traction force) in cell signaling play important roles in regulating many biological functions [139]. To date, the effect of cell traction force on cell migration, proliferation and metastatic potential has been demonstrated in two-dimensional (2D) model systems [2]. Moreover, recent studies have demonstrated significant coupling between external and cell-generated forces at the single cell level [81, 82]. In our recent study, cell traction forces (CTF) which are generated by the actomyosin interactions and actin polymerization has been shown to transmit to ECM via focal adhesions on 2D hydrogel [86, 88]. Such CTF investigations in 2D system have so far paved the way for carrying quantitative analysis of cell morphogenesis *in vitro*.

Several *in vivo* studies have demonstrated that single cell migrates by exerting CTF on its surrounding ECM through cell's leading and trailing edges. On the other hand, the mechanotransduction for a group of cells, e.g., cell sheet, which is actually involved in major biological processes such as morphogenesis, wound healing and tissue repair has not been thoroughly understood [142-144]. A recent study has shown that the distribution and magnitude of CTF concentrate on the periphery of the cell colony on planar substrates [96, 98]. Another study of advancing epithelial cell sheet has demonstrated that CTF in its leading edge during migration is predominately driven by cells at rear end instead of those at leading edge [97]. Collective mechanotransduction of cell sheet has been known to trigger global phenotypic transformation. In cultured epithelial cells, non-confluent cells can switch from individual cells to fully differentiated, densely packed monolayers as a result of the formation of cell-cell contact [102, 103]. The phenomenon of contact inhibition as mentioned above has been observed in other types of cells as well [105]. By tracking individual cell contour within MDCK cell sheet, cell-cell contact may not be the only condition required for growth inhibition. However, such study has not provided any information on the dynamic behaviour of CTF during the contact inhibition of cell sheet. Mechanical compression between opposing cells may provide an inhibitory signal against cell division [107]. In addition, cell-cell contact actually conducts stresses between adjacent cells, which eventually guides the orientation of cell along the minimal intercellular shear stress [108]. Cadherins, major transmembrane proteins at the cell-cell adherent junctions act as the intercellular bridge between the cytoskeleton of two adjacent cells [142, 145]. For instance, E-cadherin, which is essential for the collective directional migration [146] is connected with integrin-based focal adhesions [147] and

conducts the loading forces exerted by actomyosin cytoskeleton at the cell-cell adherent junctions in epithelial cell sheet [148]. Interestingly, collective cells grown under 2D geometrical constraints can form different modes of collective migration under the cell-cell interactions [149]. The elucidation on the mechanisms of 3D collective mechanotransduction remains a tremendous challenge because the mechanical stresses of cell sheet are difficult to be quantified with existing physical models.

Recent advances in the development of 2D model systems for studying cell-substrate mechanics of single cells have been reported by several groups [59, 97, 98, 119, 150]. Among various biomaterials, polyacrylamide-based hydrogel (PAG) which can be engineered with variable chemical and mechanical properties has been proven to be an ideal experimental system for such study [5]. It has been first shown that the stiffness of PAG regulates cell motility through the alteration of contractile forces exerted by cells to surrounding gel medium [51, 151]. At the same time, the geometric cue imposed on adherent cells from external microenvironment has a significant impact on cellular functions [21]. Thus micropatterning of cells on the flat 2D PAG surface has been exploited over the past two decades for controlling the geometry and position of mammalian cells. For instance, 2D micropatterned substrate has been used to investigate the cell shape index (CSI) and proliferation rate of single smooth muscle cells (SMCs) [152]. More recently, 3D architecture of microchannel has been used to control SMC morphology and phenotypes [2]. SMC-generated CTF is regulated by the Rho-kinase/ROCK, which affects the mitogen-induced DNA synthesis [89] and the assembly of focal adhesions [90]. It is believed that mechanical homeostasis as well as chemical cues is essential in the mechanotransduction of tissues [91]. In order to elucidate the

mechanochemical transduction of the actual cardiovascular system, the measurement of CTF of SMC sheet or layer will be necessary. The experimental and numerical data, particularly in terms of fabricating artificial arteries or aortas, are key to well control of the *in vivo* cell growth and tissue formation. Even in the surgical phase, the understanding of CTF of SMC sheet would add another guarantee for safe transplant [153].

Various experimental methods including cell-populated collagen gel (CPCG) [118], thin silicone membrane [56] and force sensor array have been developed to probe selected parameters involved in mechanotransduction of both individual cells and cell layer during the past few decades. More recently, the arguably most acceptable method for quantifying mechanotransduction at the cell-substrate interface is CTF microscopy (CTFM) developed by Dembo and Wang [119]. There are another two other types of CTFM which are modified from the original versions as reported by Butler *et al.* [86] and Yang *et al.*. All CTFM methods as mentioned above rely on the correlation computation of traction force with the use of Fourier transform. As a result, all conventional CTFM methods suffer from the same limitation in achieving high accuracy in the calculation of microbead displacement before/after cell detachment. Generally, 3D microenvironment is the predominant *in vivo* culture systems for most vascular cells and tissues. For instance, both cells and cellular organelles sense 3D mechanical forces such as shear stress of hydrodynamic blood flow, mechanical loading from surrounding ECM and contractile forces exerted by adjacent cells [80]. Among various cells and tissues, SMC functions and fate are highly subjected to the mechanotransduction between cells and surrounding 3D microenvironment. Mechanical stresses produced from the surrounding physiologically microenvironment, such as ECM, neighbouring

cells and so on, can influence the collective SMCs proliferation and morphogenesis, which eventually contribute to the vasoactivity of blood vessel. Until now, a mechanistic understanding on the mechanotransduction of SMC layer in 3D experimental systems which mimics the tunica media of blood vessels still remains to be elucidated. This shortage ushers the cooperation of numerical simulation to analyze the mechanical deformation and stresses on substrate induced by cultured cells.

CTFM is the most reliable approach for studying cell layer-ECM mechanotransduction if a 3D model system incorporated with the PAG-microbead layer would be developed. In this study, we developed new traction force microscopy assay modified from the conventional CTFM to probe the collective CTF of SMC layer between two adjacent microwalls [5]. We first capitalized on our soft lithography approach in fabricating 3D microstructures with microwall features in PDMS as the precursor of the 3D microenvironment for probing mechanotransduction of collective cell sheet [4]. Afterwards, a thin layer of PAG which contained well dispersed florescent microbeads was coated on the PDMS microwalls. To measure the elastic modulus of the PAG layer, atomic force microscopy in combination with regression analysis was applied. Then the dynamic process of cell alignment was probed by real-time cell imaging and quantitative analysis during the formation of functional SMC sheet. The cooperative mechanical forces of SMC layer were accessed by our recently developed finite element analysis [154]. We demonstrated that the mechanotransduction of collective cells are affected by the topographic cues provided by the microwalls. At the same time, both the cell-cell contact and cell-microwall contact influenced the cell proliferation, cytoskeleton remodeling, focal adhesion, cell alignment and protein

expression. Overall, we systemically elucidated the influences of contact guidance provided by the microwalls and cell-cell adherent interaction on the CTF distribution and mechanical stresses during the process of cell sheet assembly.

3.2 Materials and Methods

3.2.1 Cell Culture

Human aortic smooth muscle cells (HaSMC obtained from Lonza, Switzerland) were used herein. Cells were cultured in growth medium (SmGM basal medium supplemented with SmGM-2 SingleQuot Kit Suppl. & Growth Factors obtained from Lonza). Cells were kept in CO₂ incubator at 37°C under 5% CO₂ atmosphere. The culture medium was changed every two days. Before cell passage and seeding, cells were washed with HEPES buffered saline solution (ReagentPack™ subculture reagents obtained from Lonza) and detached from the flask by adding trypsin/EDTA solution (ReagentPack™ subculture reagents obtained from Lonza). The cell suspension was then transferred to a 15 ml Falcon tube and was centrifuged at 1000 rpm for 5 minutes. After the removal of supernatant, the remaining cells at the tube bottom were resuspended by adding culture medium and transferred them back to the cell culture flasks.

3.2.2 Fabrication of Microstructured PDMS Stamps

Master silicon mold was first prepared by a deep reactive ion etching (DRIE) system (SURFACE systems + technology GmbH & Co.). Photoresist AZ5214 was coated on the silicon wafer mold by spin coater (Delta 80BM) at a rotation speed of 4000 rpm for 30 seconds. Then prebaking of the photoresist coated silicon mold

was carried at 110 °C hotplate for 105 s. After exposure and developing, the pattern was transferred from the silicon mask to the photoresist layer. The 3D pattern of microwalls was etched into the silicon wafer by deep reactive-ion etching. After dry etching, the silicon wafer surface was passivated with a Teflon-like polymer for cleaning and demolding. We fabricated the microwalls with height of 30 µm, width of 60-70 µm and length of 160 µm. The distances between the two opposing walls were 300 µm. In addition, the two adjacent microwalls along the same long axis were separated by a distance of 40 µm, which were found to increase the degree of confluence of primary SMCs by providing more free space among cells.

The silicone base and curing agent (Sylgard 184 kit) were mixed at a weight ratio of 10:1 and then casted over the silicon wafer with the inscribed microwalls. Then the silicon mold with silicone curing reagents was put under vacuum for ten minutes, repeated for three times and then stored at 80 °C overnight. In the following day, the sample was heated at 120 °C for two hours. When the silicon mold was cooled down to room temperature, the polymerized PDMS was peeled off and cleaned in hexane bath overnight. Lastly, PDMS was washed in ethanol overnight and dried in oven. The depth of the PDMS microstructure was validated by using surface profiler (Alpha-Step IQ, KLA-Tencor Corporation). The micropatterned PDMS was cut into several circles of 15-mm in diameter and put onto the 35-mm-diameter Petri dishes with the glass bottom. The sample was treated by Argon plasma to generate radicals on the PDMS surface before coating with a layer of polyacrylamide gel [155].

3.2.3 Preparation and Functionalization of Polyacrylamide Gels

Acrylamide (40% w/v, Sigma) and N, N'-methylene-bis-acrylamide (BIS, 2% w/v, Sigma) were mixed at the ratio of 10% v/v to 1% v/v in a solution of distilled

water and ethanol. Solutions were degassed for 30 minutes using argon gas to optimize the polymerization. Then 0.2µm fluorescent latex beads (Invitrogen Inc.) were ultrasonicated at 1/1000 volume concentration and were added to the solution. To obtain a thin layer of PAG, the solutions were cast over the PDMS surface and put under the UV radiation at a distance of 15 cm for 15 minutes. To check the beads density, fluorescence microscopy was used to observe the beads distribution.

As PAG is highly hydrophilic and chemically inert, a heterobifunctional cross-linker known as sulfosuccinimidyl-6(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH, Sigma Pte Ltd) was attached to PAG surface before the binding of adhesive ligand. Approximately 250 µl of the sulfo-SANPAH solution (dissolved into 1mg/ml) was added per sample which was then exposed to 365nm UV-light for at least 15 minutes until the solution turns from orange to dark red color. The phenylazide group of sulfo-SANPAH was photoactivated and covalently linked to the PAG functional groups within the gel, while the succinimidyl ester group at the other end was then reacted to the primary amines of cell adhesion protein such as collagen. The gels were rinsed with PBS and stored in the dark place. Afterwards, 0.2 mg/ml collagen type I (BD Bioscience Inc.) which was diluted by 10 times in PBS from 2 mg/ml stock solution was added onto the PAG surface and kept in the shaker at room temperature for 4 hours under dark. After the collagen binding, the samples were rinsed with PBS for 3 times under the sterile conditions. 1.5 ml of culture medium without FBS was added to the sample dishes which were put into incubator at 37 °C for at least 30 minutes. Before cell seeding, a final sterilization of the PAG coated PDMS microwalls with UV illumination for 15 minutes was required. Figure 3.1 shows the schematic illustration about fabrication process of

PDMS micropatterned scaffold and then coating with a thin layer of fluorescent microbeads embedded PAG.

Figure 3.1. Schematic illustration about fabrication process of PDMS micropatterned scaffold coated with a thin layer of fluorescent microbeads embedded PAG.

3.2.4 Cell Seeding and Image Collection

The seeding density of HaSMCs on PAG coated PDMS microwalls was around 10^4 cells/cm² as confirmed by cell counting with hemacytometer. Cell culture medium was refreshed every day. At certain time, transmitted light images of the HaSMCs seeded on the microwalls were taken using 40X objective on a motorized microscope stage (BioPoint 2, Ludl Electronic Products) of Olympus IX71 inverted microscopy. During the experiment, the samples containing HaSMCs were kept at 37 °C with Temperature control 37-2 Digital and Heating Unit (Leica Inc.). The humidity of the stage and 5% carbon dioxide were maintained by the humidifier system (CTI-Controller 3700, Leica). In order to perform CTF calculation of collective cells in the cell layer, both transmitted light and fluorescence images of the cell layer were taken as the ‘force load’ images. The phase contrast image determines the location and locomotion of collective cells at certain time (1 h, 4 days or 7 days) after cell culture in the 3D microwalls. At the same time, the fluorescence taken with a light source 490 nm and emission at 515 nm determines the positions of fluorescent microbeads. After trypsinization, cells were detached from the substrate and ‘null force’ images were taken again in the same image frame with both phase contrast and fluorescence microscope. From the pair of fluorescent images (before and after trypsinization), the displacement of the fluorescent microbeads was determined and used for the CTF computation.

3.2.5 Substrate Displacement and CTF Determination

A particle image velocimetry (PIV) program coded in Matlab was used to determine the displacements of fluorescent microbeads in the PAG layer on top of the PDMS microwall substrate. PIV is a visualization tool for calculating the bead displacement map by maximizing the intensity cross-correlation function between

two pixel points on a pair of fluorescence images before and after trypsinization.

The cross-correlation function is defined as

$$C\left(u_{ij}, v_{ij}, \frac{\partial u_{ij}}{\partial X_i}, \frac{\partial u_{ij}}{\partial Y_i}, \frac{\partial v_{ij}}{\partial X_i}, \frac{\partial v_{ij}}{\partial Y_i}\right) = \frac{\sum_{i=1}^N \sum_{j=1}^N [f_1(X_i, Y_j) - \bar{f}_1][f_2(X_i + \Delta X, Y_j + \Delta Y) - \bar{f}_2]}{\sqrt{\sum_{i=1}^N \sum_{j=1}^N [f_1(X_i, Y_j) - \bar{f}_1]^2} \sqrt{\sum_{i=1}^N \sum_{j=1}^N [f_2(X_i + \Delta X, Y_j + \Delta Y) - \bar{f}_2]^2}} \quad (3.1)$$

The subscripts denote the (i, j) pixel point. The relations between the pixels (X_i, Y_j)

and $(X_i + \Delta X, Y_j + \Delta Y)$ in two images are

$$X_i + \Delta X = X_i + u_{ij} + \frac{\partial u_{ij}}{\partial X_i} \Delta X + \frac{\partial u_{ij}}{\partial Y_i} \Delta Y \quad (3.2)$$

$$Y_j + \Delta Y = Y_j + v_{ij} + \frac{\partial v_{ij}}{\partial X_i} \Delta X + \frac{\partial v_{ij}}{\partial Y_i} \Delta Y \quad (3.3)$$

where u_{ij}, v_{ij} are x and y displacements of the (i, j) pixel point; N is the total pixel number inside the interrogation window; $f_1(X_i, Y_j)$ and $f_2(X_i, Y_j)$ are intensities of image at pixel point (i, j) in each image; $\bar{f}_1, \bar{f}_2$ represent the average values of $f_1(X_i, Y_j)$ and $f_2(X_i, Y_j)$ in each window; $\Delta X, \Delta Y$ are the shift (or overlap) of the interrogation window between two images.

Note that the value of C is between 0 and 1, the higher the value of C, the more closely related between the images at (X_i, Y_j) and $(X_i + \Delta X, Y_j + \Delta Y)$. Therefore, the objective is to numerically search a location in the second image that is most consistent with the one in the first image, i.e. finding the location with maximum C. Since the search for maximum C involves all six variables $\left(u_{ij}, v_{ij}, \frac{\partial u_{ij}}{\partial X_i}, \frac{\partial u_{ij}}{\partial Y_i}, \frac{\partial v_{ij}}{\partial X_i}, \frac{\partial v_{ij}}{\partial Y_i}\right)$, the computation has to be carried numerically. A free program MatPIV distributed under the GNU general public license was adopted for our study [156-159]. The window size used for calculation is 8×8 pixels and 64×64 pixels for different trials. The location of the maximum value in C is then recognized as the mean particle displacements in the window.

3.2.6 Estimation of Mechanical Properties of Collective CTF

The results obtained from the quantitative PIV process on the displacements of microbeads are input as boundary conditions for the subsequent finite element analysis. The finite element method (FEM) was implemented by ANSYS®11 to determine the formation and stress of PAG substrate.

3.2.7 Characterization of Elastic Mechanical Properties of Substrate

Atomic force microscopy (AFM, Asylum research, model MFP-3D) was used to measure the mechanical properties of PAG layer in physiological condition [160, 161]. The contact mode was chosen for the indentation of PAG layer. The AFM probe is a silicon nitride cantilever (Sharpened Microlevers, Crest Technologies) with spring constant of 8.6 mN/m. Calibration was carefully carried out for the unloaded cantilever probe every time before the test in order to reduce the thermally deviation. By controlling the velocity and indentation depth, the displacement of the AFM cantilever tip during the course of indentation in the z-direction and its deflection of cantilever were recorded as the x-axis and y-axis of the force curve, respectively. The parameters shown in the picture (Figure 3.2) are depicted in the graph [154, 162].

Figure 3.2. Schematic illustrations of the AFM indentation.

To estimate the elasticity of PAG substrate, Hooke's law and Hertz's model were applied in the data analysis [162]. The indentation and deflection are assumed in the z -direction (Figure 3.2) and for elastic modulus, the applied loading force F and elastic deflection of the cantilever d is:

$$F = kd = k(z - \delta) \quad (3.4)$$

On the other hand, the relation between the applied loading force F and small deflection δ on the elastic substrate can be modelled by Hertzian model as follows:

$$F = \frac{2}{\pi} \cdot \left[\frac{E \cdot \delta^2}{1 - \nu^2} \right] \cdot \tan \alpha \quad (3.5)$$

where E is the elastic modulus, ν is Poisson's rate of polymer and α is the half-cone angle of the tip shown in the Figure 3.2.

By combining the equations (3.4) and (3.5), we get the following indentation-deflection relation for the elastic modulus calculation.

$$z - z_0 = d - d_0 + \sqrt{\frac{k(d - d_0)}{(2/\pi)[E/(1 - \nu^2)] \tan \alpha}} \quad (3.6)$$

where z_0 is the arbitrary tip position as long as it is above the zero contact point which locates at infinite end where the surface is not deformed. d_0 represents the position of non-deflected beam position. The experiment provides measures on $z - z_0$ and $d - d_0$ to allow us to calculate the Young's modulus E of the indented surface.

3.2.8 Analysis of SMC Morphology and Orientation

To track changes in collective cell morphology, phase contrast images were taken and 850 x 1000 pixels field was chosen to quantify the cell morphology against culture time. The boundaries of SMCs were first outlined by MetaMorph[®]. At least 50 cells from 3 frames were randomly chosen and analyzed. Cells grown on flat PAG coated PDMS substrates without microwalls were also studied as control groups. The deformation of cells can be quantified as following:

$$CSI = \frac{4\pi S}{L^2} \quad (3.7)$$

where S is the spreading area (projection area) and L is the perimeter of the cells. If CSI's value approaches 0, cells are highly elongated while cells are more spread when CSI equals 1.

The degree of orientation of a single cell was defined by calculating the percentage of alignment.

$$\%Alignment = \frac{(B-A)}{(B+A)} \times 100 \quad (3.8)$$

where A is the cell width in the y-axis and B is the cell length in the x-axis. For the micropatterned substrate, the x-axis defines as the microwalls' longitudinal axis.

3.2.9 Immunofluorescent Staining

SMCs grown between microwalls or on flat films were washed twice with prewarmed PBS, fixed in 3.7% paraformaldehyde for 10 minutes, permeabilized by 0.05% Triton X-100 for 5 minutes and then blocked in 1% BSA/PBS solution at room temperature. For vinculin staining, monoclonal anti-vinculin FITC antibody (Sigma) was applied at 1:50 dilution at 4 °C overnight. For cytoskeleton staining, samples were incubated in Alexa Fluor 546 phalloidin (Invitrogen) with 1:40 dilution in 1% BSA/PBS solution at room temperature for 1 hour. After washing twice with PBS, DAPI (Invitrogen) staining was performed for 10 minutes after all other staining. Images were taken with an Olympus IX71 fluorescence microscope.

3.2.10 Protein extraction, digestion, labeling with iTRAQ Reagents and online 2D LC-MS/MS Analysis

The sample was prepared and analyzed according to the previously published work [163, 164]. Cells were harvested and lysed in 100 µL of 8M urea, 4% (w/v)

CHAPS and 0.05% SDS (w/v) on ice for 20 min with regular vortexing. Then samples were centrifuged at 15 000 for 60 min at 4 °C. The supernatant was removed and protein was quantified by using the 2-D Quant Kit (GE Healthcare). After adding 4 times the sample volume of cold acetone at -20 °C for 2h for precipitation, each sample was dissolved in the solution buffer and denatured, and cysteines were blocked as described in the iTRAQ protocol (Applied Biosystems). Each sample was digested with 20 µL of 0.25 µg/µL sequence grade modified trypsin (Promega) solution at 37 °C overnight. SMCs grown between microwalls were labeled with iTRAQ 117 and control SMCs grown on the flat substrate were tagged with iTRAQ 116. The labeled samples were then pooled in a fresh tube before analysis.

The analysis was performed on an Agilent 1200 nanoflow LC system (Agilent Technologies) interfaced with a Q-TOF 6530 mass spectrometer (Agilent Technologies). In the first step of separation, 4 µg of the combined peptide mixture was loaded onto the PolySulfoethyl A strong cation exchange column (0.32 × 50 mm, 5 µm) and was eluted by injecting gradient ammonium formate solution with concentration of 20, 40, 60, 80, 100, 300, 500mM, and 1 M. The eluted peptides were analyzed by chip (Agilent Technologies) that is integrated with large capacity enrichment column and C18 reverse phase column. In each elution, the retained peptides were eluted with gradient mixture of buffer A (ddH₂O/0.1% Formic Acid) and buffer B (Acetonitrile with 0.1% Formic Acid) at a flow rate of 0.3µl/min [164]. MS/MS generated spectra were deconvoluted and analyzed using Spectrum Mill (Agilent Technologist, CA). The peptides were identified against UniProtKB/Swiss-Prot protein database (Geneva, Switzerland) for species of *Homo Sapiens* [164].

3.3 Results and Discussions

3.3.1 PDMS Microwalls for CTF Measurement

The polymerizations of PDMS were conducted by mixing of silicone base and curing agent (Sylgard 184 kit). The depth of microwalls used herein was confirmed as 60 μm [4]. In order to measure the CTF exerted on a deformable substrate, the PDMS microwall substrate were evenly coated with a thin layer of PAG with optimized stiffness. The thickness of PAG was around 10 μm . At the same time, the PAG layer was embedded with 0.2 μm fluorescent microbeads for tracking the displacement of gel micro-domain in the CTFM assay. First, phase contrast image demonstrated the successful formation of rather clear PAG layer without any detectable particulate on the surface of PDMS microwalls (Figure 3.3(a)). The excellent optical quality facilitates the microscopy monitoring of cellular processes and immunofluorescence staining. Secondly, the distribution of fluorescent microbeads dispersed in the PAG layer was uniform as confirmed with fluorescence microscopy (Figure 3.3(b)). Figure 3.3(c) showed the scanning electron microscopy (SEM) images of the discontinuous PDMS microwalls coated with a thin layer of PAG. Compared to the smooth surface of PDMS (Figure 3(d)), the result indicated the uniform distribution of PAG layer which was composed of closely packed meshwork [67]. Moreover, the microstructure of PAG coating is supported by the cross-linked topographic feature on the SEM image under high magnification (Figure 3.3(e)).

Figure 3.3. Micropatterned PDMS coated with a layer of PAG. (a, b) Phase contrast images and fluorescent images of PDMS microwall were taken under 20X and 40X magnifications. The microbeads were uniformly distributed in the PAG layer. (c, d) SEM images of the PDMS-PAG substrate and only PDMS microwalls to show the

PAG coated on the PDMS. (e) SEM image of the PAG microstructure under the optimal PAG concentration used in our CTFM study.

3.3.2 PAG Substrate Stiffness Measured by AFM

The stiffness data of PAG measured by AFM was shown in Figure 3.4 (inset). The deflection of cantilever probe ($d - d_0$) vs indentation ($z - z_0$) was presented. This functional relation between the two parameters can also be calculated using the theoretical Hertzian model from equation (3.6) if Young's modulus is provided. With the given experimental data, the Young's modulus can be estimated by minimizing the errors between experimental data and theoretical calculations [52]. One example calculation is shown in Figure 3.4(a) as an appropriate Young's modulus is chosen. The loading curve by AFM can be fitted with minimum errors between the experimental data and the calculated values. The five set AFM measurements were analyzed with regression method for determining the optimal values of Young's modulus for each set of data as shown in Figure 3.4(b). The results indicated that the average value of Young's modulus was 17.5kPa with standard derivation of 6.52kPa. The result determined herein agreed well with other Young's modulus value of PAG which was probed by AFM indentation or other mechanical tests reported by other groups [55, 165]. Moreover, numerical values of the fitted Young's modulus were required for the subsequent finite element simulation of the CTF induced by SMCs on PAG-PDMS substrate.

Figure 3.4. (a) One deflection - indentation curve for measuring Young's modulus of PDMS-PAG substrate using the AFM contact mode. (b) Errors between the calculated and experimental measures of AFM indentation curve for Young's modulus measurement of PDMS-PAG substrate by using the contact mode. The average value of Young's modulus is 17.5kPa and the standard derivation is 6.52kPa.

3.3.3 The Morphology and Phenotype Control of SMC

To observe the role of 3D geometric constraints on the collective mechanical behaviours of multiple-cell sheet, human aortic SMCs were directly seeded on PAG

layer containing the fluorescent microbeads in all regions between the microwalls. The cell morphology and alignment within the cell layer were monitored for 10 days under physiological conditions. During the course of imaging, the sample was secured on the microscopic stage at 37 °C and supplied with 5% carbon dioxide in the incubator. Figure 3.5 showed a series of phase contrast images of the cell sheet cultured on the PAG coated PDMS microwall substrate during the 10 days period. When the cell density on the microwall substrate was low immediately following cell seeding (Day 0), SMCs were found to express fibroblast-like morphology and to adopt random orientation as shown in Figure 3.5(b). Moreover, there was no significant difference in the cell orientation and morphology between SMC on microwall substrate and flat PDMS substrate (Figure 3.5(d)). The result indicated that the constraints from a pair of adjacent microwalls didn't significantly affect the cell shape and morphology when the cell-cell contacts were absent under low cell density at Day 0. From Day 0 to Day 4, SMCs seeded on the PAG layer between the PDMS microwalls significantly elongated following the increase of cell density. At the same time, SMCs started to align along the direction of the long axis of the microwall (Figure 3.5(b)). The result as mentioned above was supported by the constraint on SMC alignment which had been reported in SMC grown between PDMS microchannel [4]. During the intense period of cell proliferation, cells reached confluence after 7 days of culture. As shown in Figure 3.5(c), SMCs on microwall substrate were mostly found to be switched from fibroblast-like to elongated morphology and aligned well along the microwall's long axis after 7 days of culture. In contrast, various groups of SMCs grown on the flat PDMS substrate randomly aligned in different directions within significantly smaller region with area of around $150 \times 150 \text{ } \mu\text{m}^2$ with the absence of highly collective cell alignment

across larger area found in SMCs on microwall substrate, e.g., 300 x 600 μm^2 (Figure 3.5(f)). In other words, flat PDMS didn't provide any uni-directional guidance on the intended direction of cell sheet as compared the cases of microwalls. Of course, it does not mean that there is no alignment of cells if no microwalls present on the substrate. Instead the alignment is due to the contact from nearby cells. Figure 3.5 indicated that imprinted discontinuous microwalls successfully guided the direction of collective alignment of SMCs via the imposed boundary constraints.

Figure 3.5. Phase contrast images of SMCs grown on PDMS microwalled substrate (a, b, c) and flat PDMS (d, e, f) substrate under 20X and 40X magnifications, both

with PAG coating at various time after cell seeding including 1 hour (a & d), 4 days (b & e) and 7 days (c & f).

In addition to the temporal tracking of the morphology switching of SMC layer, quantitative analysis was needed to elucidate the effect of constraints from microwalls on cells. Figure 3.6(a) shows the SMC density on PDMS microwall and flat PDMS substrates against time of culture. In general, the result indicated that intense cell growth only occurred during the first four days of cell culture. Also, the initial growth rate of cells (slope of the cell density vs. time plot from day 0 to day 4) for SMC on microwalled substrate is 33% lower than that on flat substrate. The trend as mentioned above was likely caused by the combined 3D geometrical and mechanical constraints induced by the parallel microwalls on the highly proliferative SMC during early cell culture. After 5 days of culture, the SMC density ceased to increase further on both types of substrates as cells reached confluence. At the same time, the projected area of individual cell was reduced to a critical value when cell-cell contact inhibition totally dominated over further cell division. In addition, the steady-state cell density of SMCs grown on microwalled substrate after day 5 was 20% lower than that on flat PDMS. The result as mentioned above further exemplified the physical effect of the parallel discontinuous microwall in moderating the extent of cell proliferation. After 7 days of culture, the cells completely shifted from the fibroblast-like to elongated morphology as the CSI value was significantly reduced as shown in figure 3.6(b). The reduction of CSI indicated that the cell body was significantly elongated after 3 days of culture compared to the non-polarized cell shape immediately after cell seeding on the two substrates. Moreover, the reduction of CSI from day 0 to day 2 for SMCs grown on microwalled substrate is quite significant, indicating cells

found their preferred alignment if there are microwalls present. This change of CSI suggested that the microwalls accelerated the phenotype shift of SMC. Also, the percentage of cell alignment on microwalled substrate significantly increased from 16% to 76% after 7 days of culture, while the percentage error reached maximum level around 2 days and continued to decline as shown in Figure 3.6(c). The percentage of cell alignment is not applicable to SMCs grown on flat PDMS herein because of the lack of a reference axis. Figure 3.6(d) shows the contrast images of cells at various locations on day 0 and day 7 where the uni-direction alignment of cells can be clearly observed under the presence of microwalls on day 7. The cells on flat PDMS substrate have different alignment at different locations, which is mostly affected by the neighbouring cells.

Figure 3.6. Cell density, morphology and orientation of SMCs grown on microwalls and flat PDMS substrate. (a) Density of SMCs during 10 days' culture.

(b) CSI of SMCs during 10 days' culture. Error bars represent mean values $\pm$ SD from at least 40 cells in 3 different frames. The cut frame is 850×1000 pixels (1 pixel = $0.334572 \mu\text{m}$). (c) %Alignment and %Alignment error of SMCs grown between microwalls. (d) Selected locations for cells at day 0 and day 7.

To quantify the statistical differences for cells cultured on microwalled channels and flat PAG substrate, a t-test was performed for the differences in cell density and CSI. Table 3.1 showed the p-values of the two parameters for different days of culture and as expected from Figure 3.6(a) and (b). The result indicated that the cell density was significantly higher on flat substrates than that on microwalled channels starting from day 4 and onwards. The result indicated that microwalls are effective in guiding the directional alignment of confluent cell sheet while the flat substrate is more suitable for cell proliferation.

Table 3.1. List of p-value from t-test for the cell density and CSI shown in Figure 3.6. The shadowed data indicated significant difference between cells on patterned and flat PAG substrate.

Day	CSI		Density	
	t_0	p-value	t_0	p-value
0	-0.8531	0.2281	0.7348	0.2695
1	0.8987	0.2175	1.3093	0.1408
2	1.7071	0.0932	-1.5119	0.1139
3	1.0434	0.1867	-3.8125	0.0312
4	1.0892	0.1779	-3.7417	0.0323
5	0.8047	0.2399	-4.8107	0.0086
6	0.7524	0.2532	-6.7361	0.0107
7	0.0839	0.4692	-7.8001	0.0022
8	0.0210	0.4923	-7.1119	0.0029
9	0.0518	0.4810	-2.7386	0.0357
10	-0.0463	0.4830	-5.2842	0.0170

3.3.4 Traction Forces and Stress Distribution of Aligned Multiple-cell Sheet

Figure 3.7. Coordinates and geometry of PAG of $334\ \mu\text{m} \times 284\ \mu\text{m} \times 10\ \mu\text{m}$ for finite element analysis.

The deformation and stress analysis of PAG layer is implemented by the FE software ANSYS[®]12. Starting with the geometry of PAG gel with dimension of $334\ \mu\text{m} \times 284\ \mu\text{m} \times 10\ \mu\text{m}$, the whole domain (PAG substrate) is discretized by isoparametric, 20-node brick element SOLID186 (Figure 3.7). The boundary conditions were *specified displacements* from PIV results, as shown in Figure 3.8(a2 - d2), at corresponding nodes on the surface underneath the cell. The nodes on the bottom of substrate are *fixed* because PAG is constrained on the microchannel. A flat PAG-PDMS substrate in Figure 3.8(d1) was used as a control group for comparisons. Material properties of PAG were listed in Table 3.2 for readers' reference.

Table 3.2. Material property for finite element analysis.

Young's modulus	Poisson's Ratio	Mooney-Rivlin constant C_{10}	Mooney-Rivlin constant C_{01}	Mooney-Rivlin constant compressibility
1.75E+04	0.495	1.75E+04	1.75E+04	0.00669

On day 1 after cell seeding (Figure 3.8(a1)), SMCs attached well to the PAG-PDMS microwalled substrate, while some cells adopted fibroblast-like morphology and others were on the verge of transforming into elongated morphology. The result of CTF map in the selected area (outlined with red dotted line) indicated that the directions and magnitudes of CTFs located adjacent to the microwalls were non-polarized and random, respectively (Figure 3.8(a2)). The Von Mises stress was calculated as follows:

$$\sigma_{\text{VonMises}} = \sqrt{\frac{1}{2}[(\sigma_1 - \sigma_2)^2 + (\sigma_2 - \sigma_3)^2 + (\sigma_3 - \sigma_1)^2]}$$

At this early stage, the intercellular interactions were not strong enough so that the orientations of individual cells as well as the high stress areas were quite random and discrete (Figure 3.8(a3)). Such a stress represents the energy equivalent magnitude of all stress components. High Von Mises stress indicates either a high tensile or compressive stress. Also noted that the microwalls imposed constraints on cells at single-cell level and therefore their effects were not significant.

Figure 3.8. The distribution of CTFs and Von Mises stresses of the collective SMCs against days of cell culture on PAG-PDMS microwall patterned substrate and flat PAG-PDMS substrate. (a1, b1, c1, d1) The phase contrast images were taken under 40X magnification with resolution of 850×1000 pixels. (a2, b2, c2, d2) The displacements of microbeads processed from the pair of fluorescence images before/after trypsinization with the calculation of PIV. The window sizes were chosen to be 64×64 pixels. (a3, b3, c3, d3) Von Mises stress distributions of collective SMCs on PAG-PDMS simulated by FEM analysis.

As the cell culture proceeded to day 4, many cells had already spread out but not overlapped with each other yet (Figure 3.8(b1)). At this stage, most cells started to adopt a highly elongated morphology. Unlike the arbitrary orientations of cells on the day 1 (Figure 3.8(a2)), the CTF vectors caused by the incomplete cell sheet on day 4 (Figure 3.8(b2)) were mostly pointing towards the right hand side of the microwalls. Moreover, a zone of low stress started to evolve at the entire bottom half and partial top half of the selected area of SMC layer between the parallel microwalls (Figure 3.8(b3)). The results indicated that the intercellular interactions

played an essential role in the formation of multiple-cell sheet, while the microwalls confined the direction of the cell alignment and triggered stress reductions across the SMC layer. A major component of intercellular adhesion is the cadherin complex that is tightly connected with the cytoskeleton inside cells. Meanwhile, the organization and transformation of cytoskeleton can influence the CTFs transmission to the ECM through integrin-based focal adhesions. In other words, intercellular interactions among multiple cells could affect the distribution of CTFs. In addition, the microwalls have imposed much more significant boundary conditions on the edges of incomplete cell sheet.

As cell density increased, the intact cell layer became confluence around 7 days, which was proved by the slightly overlapping between the cell membrane of adjacent cells (Figure 3.8(c1)). There was no more free space for additional SMC to adhere on the microwall substrate. The spatial map of CTFs within the controlled region during the SMC alignment from Day 4 to Day 7 (Figure 3.8(b2) and (c2)) was found to be altered. The traction forces became more uniform as cells were well aligned along the microwalls and evolved as a cohesive structure in the selected area of the PAG layer (Figure 3.8(c2)). As shown in Figure 3.8(c2), the magnitudes of CTFs at the center of aligned cell sheet became significantly smaller than those at the periphery of cell sheet, which is due to the boundary effects of cell colony induced by microwalls [96]. At the same time, the low stress zone had completely covered the entire selected area of aligned cell sheet (Figure 3.8(c3)). For cells grown on the flat substrate, CTFs with similar magnitude across the entire controlled region were all pointing towards a random direction of cell elongation (Figure 3.8(d2)). The result basically indicated that cell-microwall interactions significantly changed both the magnitude and direction of local CTFs in the

controlled region. This was also clearly demonstrated by the fact that the directions of the CTFs near the microwalls were aligned along the wall, while the CTFs near the gap between microwalls pointed more randomly. These observations were reinforced by the case of control group in Figure 3.8(d2) – (d3) where the free grown cells on flat substrate are less organized, the CTFs were distributed more randomly and low stress zone was absent. Figure 3.8(a2) – (c2) revealed that as cells from a more complete sheet, so more substantial became the imposed boundary constraints by microwalls. The well-aligned cells on day 7 implied that individual cell relied less on the cytoskeleton remodeling such as actin polymerization since interactions among cells could uphold them as a cohesive unit without highly dense internal cytoskeletal structures and focal adhesion. This idea can be schematically illustrated as shown in Figure 3.9, which provided a sound explanation to the computational results in Figure 3.8(c2). It must be noted that the geometry of the system used herein was rather different from the traditional assay with cells surrounded by an isotropic medium.

Figure 3.9. The schematic drawing of CTFs by the collective SMCs when cell sheet is completely formed.

3.3.5 Immunofluorescent Staining

In order to verify our previous postulation on the change of cytoskeleton structure of SMCs due to the formation of collective cell sheet, immunostained SMCs grown on microwall and flat substrates were imaged with fluorescence microscope. As shown in Figure 3.10 (a1, b1, c1 & a2, b2, c2), actin immunostaining (red) clearly indicated the presence of stressed fibers in cells. The intensity of stained actin for the case of flat PDMS on day 7 was much higher than that of microwall substrate, which supported our claim that significantly less actin was polymerized when the complete cell sheet was formed. The morphology of SMCs switch from fibroblast-like to elongated-like was also consistent with phase contrast images in Figure 3.5. For SMCs cultured between microwalls (Figure 3.10(a1, b1 and c1)), the levels of actin expression were all lower than those grown on flat substrates (Figure 3.10(a2, b2 and c2)).

Figure 3.10. Spatial distributions of F-actin (red: TRITC), vinculin (green: FITC) and nucleus (blue: DAPI) for SMCs cultured on microwall patterned (a1, a3, b1, b3,

c1, c3) and flat (a2, a4, b2, b4, c2, c4) PDMS-PAG substrates on day 1, day 4 and day 7.

In addition to actins, vinculin is another cytoskeletal protein of our interest because vinculin concentrate at the focal adhesion plaques on the surfaces of mammalian cells. On Day 1, focal adhesions composed of vinculin clusters (green spots on Figure 3.10(a3 and a4)) appeared for SMCs between microwalls and on flat substrate. Cells generally prefer to establish cell-wall interactions along the wall. This might be due to the additional physical cue provided by the wall to form focal adhesions. Interestingly, the density of vinculin clusters (evenly distributed throughout the cytoplasm) found in SMCs on the flat substrate was significantly higher than that on microwalled substrate. The presence of microwalls alone moderated the formation of focal adhesion in the absence of cell-cell interaction.

On Day 4, the density of vinculin clusters found in SMCs on microwalled substrate slightly increased due to the confluence-triggered contractile phenotype, particularly at locations close to the microwalls (Figure 3.10(b3)). In contrast, vinculin clusters significantly increased in numbers throughout the cytoplasm of non-aligned SMCs on flat substrate (Figure 3.10(b4)). The trend as mentioned above strongly indicated that the microwalls induced unidirectional constraints to both the cell-microwall and cell-cell interactions. From Day 4 to Day 7, the density of vinculin clusters for SMCs on microwalled substrate was significantly reduced while the stressed fibers were well oriented along the along the microwalls (Figure 3.10(c3)). The density of vinculin clusters reached the highest level for those elongated cells on flat substrate after 7 days of culture (Figure 3.10(c4)). The fluorescence staining of vinculin during 7 days culture implied that the microwalls was instrumental in down regulating the formation of focal adhesion which yields

lower CTF and low stress zone for SMCs located in the center between the two adjacent microwalls (Figure 3.8).

More recently, Wu et al. summarized several systematic approaches on the CTFM analysis [126]. They indicated two different computational methods for characterizing the cellular interactions with collagen matrix i.e. the inverse and the forward computation methods. Both methods are useful in study of cells completely embedded within extracellular matrix. For cells cultured on a substrate is somewhat different from the 3D embedded case but the forward computational method is simplify preferred due to the widely available FEM commercial programs. The forward computational method is also found in other studies, for instance, Koch and Fabry [166] presents a computational approach based on the strain energy (deformation of matrix described by a mechanical strain energy function) in order to derive the stress field for the invasion of carcinoma cells into surrounding matrix. This approach is essentially the same as the finite element analysis except that they depend on the presumed energy functions from experiments. The value of T. M. Koch and Ben Fabry's work was that they captured the deformation and casted them in terms of strain energy, which is more illustrative because energy is a scalar rather than a tensor.

3.3.6 iTRAQ Analysis and Differentially Expressed Proteins

In order to establish the biological difference between microwall-aligned SMCs and control SMCs, the protein expression profile for each type of cell was analyzed by 2D nano-LC-MS/MS procedure. In our study, iTRAQ 117 represented cells grown on microwall substrate, while iTRAQ 116 represented control SMCs grown on flat platform. Each MS/MS spectrum was searched against the Human IPI protein database and more than 200 proteins were detected. In our study, two or

higher confidence unique peptides were identified and only protein quantification data with relative expression of >1.2 or < 0.8 was chosen for further analysis. According to these criteria, a total of 14 unique proteins, 8 significantly up-regulated and 6 significantly down-regulated were identified as shown in Table 3.3.

Table 3.3. List of differentially expressed proteins in SMCs grown between microwalls. No. peptides indicate the detected peptides (with 99% confidence) for the individual protein.

Accession no.	Protein Name	No. peptides	iTRAQ 117/116	Protein Function
Up-regulation proteins				
P52907	F-actin-capping proteins	2	1.28	Cytoskeletal organization
P23246	Splicing factor, proline- and glutamine-rich	2	1.21	DNA and RNA binding protein
P48444	Coatomer subunit delta	2	1.63	Intercellular protein transport
P21796	Voltage-dependent anion-selective channel protein 1	2	1.37	Membrane protein, plays a role in ion transport and apoptotic process
Q13162	Peroxiredoxin-4	3	1.35	Regulates the activation of NF-kappa-B in the cytosol
Q96AG4	Leucine-rich repeat-containing protein 59	2	1.46	Required for nuclear import of FGF1
Q8NBS9	Thioredoxin domain-containing protein 5	2	1.23	Influences thioredoxin activity
P15144	Aminopeptidase N	2	1.25	Required in the final digestion of peptides
Down-regulation proteins				
P67936	Tropomyosin alpha-4 chain	6	0.13	Binds to actin filaments, response to oxidative stress
P48643	T-complex protein 1 subunit epsilon	2	0.26	Involved in the folding of actin and tubulin
Q09666	Neuroblast differentiation-associated protein AHNK	6	0.10	Required for cell differentiation
O00299	Chloride intracellular channel protein 1	4	0.26	Ion transportation
Q02809	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	3	0.24	Form hydroxylysine residues in -Xaa-Lys-Gly-sequences in collagens
P07195	L-lactate dehydrogenase B chain	2	0.21	Involved in glycolysis

Based on the different molecular functions, 14 proteins could be subsequently classified into four catalogues. The group composed of 3 cytoskeletal proteins, 5 proteins involved in DNA synthesis/protein transportation, 4 signalling molecules and 2 metabolic enzymes. Among the group of three cytoskeletal proteins, F-actin-capping protein subunit alpha-1 (CapZA1), Tropomyosin alpha-4 chain (TPM4) and T-complex protein 1 subunit epsilon (TCP-1) were all involved in the actin polymerization and folding. For instance, F-actin-capping protein (CapZ) is an $\alpha\beta$ heterodimer that is widely distributed in cells for limiting the growth of the actin filaments. It also plays a significant role in the regulation of F-actin conformation, protein kinase C signalling, calcium sensitivity as well as focal adhesion reduction [167]. CapZA1 which is the α subunit of CapZ has been found to exhibit abnormal

expression level in the breast-cancer tissues [168]. At the same time, the up-regulation of CapZA1 would decrease the myofilament calcium sensitivity and inhibit the polymerization at the fast growing end of actin filaments. In contrast to the up-regulation of CapZA1, TPM4 and TCP-1 were significantly down-regulated. Tropomyosin alpha-4 (TPM4) is one of the four isoforms of tropomyosins in human. A role of tropomyosin in muscle cells is to stabilize actin filaments and to alter focal adhesion structure [169]. T-complex protein 1 subunit epsilon (TCP-1) is essential in cytoskeletal functions by assisting in the folding of actin and tubulin. Therefore, the reduction in TPM4 and TCP-1 levels may affect the stability of cytoskeleton associated cluster like focal adhesion under the effect of the physical cell-wall interactions. In other words, CapZA1, TPM4 and TCP-1 are all likely to play a key role in altering the motility and contractile function of vascular SMCs. Our findings were strongly by the fact that the focal adhesion composed of vinculin was significantly moderated under the influence of the microwalls.

3.4. Conclusions

We demonstrated the use of 3D discontinuous microwalls for collective CTF measurements by coating a thin layer of with PAG embedded microbeads onto the microwall substrate. Meanwhile, AFM was used to measure the elastic modulus of optimal PAG. To observe the influence of microwall constraints on the multiple-cell sheet, we tracked the process of morphology switches and cell alignment over culture of 7 days. The grown collective cell sheet can be potentially used a patch for repairing damaged artificial arteries. As the sheet is grown between microwalls, the shape and thickness is well controlled and this uniform sheet is ready for further biological or clinical test.

With the newly developed collective CTFM assay, the results indicated the significantly higher CTFs of aligned cells were found near the microwalls compared to those in the middle region between the microwalls. Interestingly, the spatial distributions of mechanical stress during the cell alignment process were correlated with the orientation of collective cell layer. Moreover, we demonstrated the influence of physical constraint induced by microwalls on the formation of low stress zone for SMC layer. Immunostaining results showed the significant reduction in the expression of focal adhesion composed of vinculin for SMCs under the influence of microwalls. The results were further validated by the altered expressions of three major cytoskeleton associated protein by performing differential proteomic analysis between SMC on microwall substrate and control SMCs.

Chapter 4 Biomechanistic Study of Smooth Muscle Cell Sheet during Circumferential Alignment in Circular Micropatterns

4.1 Introduction

One of the major challenges in tissue engineering is to mimic native physiological system for inducing cell morphogenesis and tissue regeneration in 3-dimensional (3D) microenvironment [170-172]. In order to trigger the remodeling of blood vessel equivalent *in vitro*, it is essential to recapitulate the characteristics of vascular smooth muscle cells (SMCs) which are circumferentially aligned inside the tunica media [173, 174]. In general, SMCs tend to adopt spindle-like morphology and express contractile phenotype in a native wall of blood vessel. On the other hand, SMCs cultured on a tissue culture plate or conventional biomaterial scaffold *in vitro* adopt more round-shaped morphology and express proliferative synthetic phenotype [175]. Recently, micropatterning techniques based on soft lithography have emerged as an attractive approach for controlling cell shape and spatial organization of connective cells in the formation of functional small-diameter blood vessel *in vitro* [2, 4, 8]. For instance, previous studies have shown that rectangular microchannels fabricated on polydimethylsiloxane (PDMS) scaffold can regulate the aspect ratio, actin alignment and phenotype switch of adherent SMCs [28]. However, the rectangular microchannels failed to induce the formation of spindle-like morphology of SMCs inside the native blood vessel. To date, the construction of small-diameter vascular graft remains to be a tremendous challenge due to the lack of *in-vitro* culture system for inducing circumferential alignment of SMCs during cell sheet formation [176, 177].

The mechanical cues imposed on cells from the surrounding microenvironment, such as biomaterial scaffold, extracellular matrix (ECM) and neighboring cells are crucial for the regulations of cell morphogenesis, proliferation, differentiation, migration, etc [7, 8, 52, 135, 136, 178, 179]. Recent advances in the fabrication of micro-textured scaffolds have enabled the culture of organized SMC layers *in vitro* as a step towards the realization of functional vascular graft. Along the way, it has been demonstrated that the topographical cues and ECM proteins on micro-textured scaffold directly influences the phenotype and vasoactivity of blood vessel equivalents either using linear constraints [25, 28, 152, 180, 181] or 3-dimensional curved constraints [177, 182]. In addition to the external stimulations, cells usually adopt and respond actively to the surrounding microenvironment through the transmission of stress or tension via the cytoskeletal network between cell nuclei and ECM proteins [86, 88]. Recently, cell traction force (CTF) that is generated through actomyosin interaction and actin polymerization has been recognized as the mechanotransduction marker for quantitative analysis of the morphogenesis and angiogenesis of single cells in a 2-dimensional (2D) system [154]. In contrast to single cells, the mechanotransduction of cell sheet or layer, which actually represents tissue remodeling and regeneration *in vivo*, has not been systematically investigated [144, 183]. Thus collective CTFs exerted by cohesive cell colony have been shown to distribute like the pattern found in a single cell but have higher values at the cell layer boundary [98]. It has been revealed that the formation of densely packed cell monolayers from cultured epithelial cells is mediated by the cell-cell contact inhibition [103, 105]. The achievement of uniform growth in tissues deeply hinges on the integral-feedback mechanism of mechanical forces among neighboring cells [107]. In addition, cell-

cell contact serves as the communication signaling network between neighboring cells to transmit appreciable shear stress and to dictate the direction of cell growth and migration [108]. On the other hand, the dynamics of CTFs during the formation of well-organized cell sheet induced by both physical constraints and cell-cell interactions pertaining to specific tissue morphogenesis has not been systematically elucidated.

In order to probe the mechanotransduction between cell and external microenvironment, several methods including cell-populated collagen gel (CPCG) [118], micromachined cantilevers, thin silicone membranes [56, 58], micropost force sensor arrays [59, 184], and cell traction force microscopy (CTFM) [119] have been developed. In particular, CTFM assay has been applied to measure the traction forces for single cells on polyacrylamide-based hydrogel (PAG) supported on a planar rigid substrate [86, 120]. CTFM is based on the local deformation of PAG substrate, which linearly responds to a wide range of stress imposed by adherent cells through active cytoskeletal remodeling [5]. The stiffness of PAG can be easily manipulated via changing the degree of polymerization for various applications. In addition, the PAG surface can be conjugated with different ECM proteins for the CTF measurement of different cell types [67]. Thus the successful development of PAG for single cell study paves the way for probing collective mechanotransduction of SMC layer confined in a PAG-based scaffold, which mimics the tunica media of blood vessels.

In this work, human aortic smooth muscle cells (HaSMCs) that serve as the main component of tunica media in blood vessels are selected as the *in-vitro* model system. Specifically, we aim to study the collective mechanotransduction of vascular tissue during circumferential alignment under the influence of physical

constraints with different curvatures, which has not been considered in previous work using curved constraints but with a fixed curvature [177, 182]. By applying soft lithography techniques, we fabricated micropatterns comprising a series of concentric microwalls to create culture zones with various curvatures. Moreover, a thin layer of PAG with embedded fluorescent microbeads and immobilized ECM proteins was coated onto the PDMS patterns for the mechanotransduction study of SMC cell culture and alignment. First, the dynamic process of cell population alignment between the concentric microwalls was tracked by real-time optical microscopy. Then cell density, cell shape index (CSI) and the orientation angle of adherent SMCs were measured in order to quantify the SMC morphology and polarization during the circumferential alignment. The cooperative traction forces and mechanical stresses underlying the collective SMCs were then determined by CTFM assay with the aid of finite element modeling [154]. The distributions of CTFs and mechanical stresses together with the immunofluorescence staining of actin filaments during circumferential cell alignment shed new light on the mechanotransduction of SMC layer with the surrounding microenvironments.

4.2 Materials and Methods

4.2.1 Cell Culture

Human aortic smooth muscle cells (HaSMCs, Lonza, Switzerland) were maintained in selected growth medium (SmGM basal medium supplemented with SmGM-2 SingleQuot Kit Suppl. & Growth Factors obtained from Lonza). Cells were cultured in a humidified incubator at 37 °C and 5% CO₂. The culture medium

was changed every two to three days. HaSMCs undergoing passages 6-9 were used for the study herein.

4.2.2 Fabrication of Micropatterned PDMS Scaffolds

First, master silicon mold was prepared by the surface modification of negative photoresist as mentioned previously [185]. Then SU-8 3025 (Microchem, MA, USA) was coated onto a silicon wafer by a spin coater (SCS G3P-8) at 2000 rpm for 30 s. The excess solvent was evaporated by heating for 15 min at 95 °C. After UV irradiation for 15 s and baking for 1 min at 65 °C and 5 min at 95 °C, the master mold was developed with the thickness of around 30 µm.

The silicone base and curing agent (Sylgard 184 kit, Dow Corning) with a weight ratio of 10:1 were then thoroughly mixed and casted over the master silicon wafer. After degassing under vacuum for 1 h, the PDMS were stored at 80 °C overnight. The oven temperature was increased to 120 °C to heat the sample for 2 h in the following day. After cooling down to room temperature, the polymerized PDMS scaffold was peeled off and washed in hexane bath overnight. Moreover, the PDMS scaffold was cleaned in ethanol overnight and then dried in oven. A surface profiler (Alpha-Step IQ, KLA-Tencor Corporation) was used to measure the depth of PDMS microstructure. Lastly, PDMS surface was sterilized and treated by Argon plasma (MARCH PX-500) for 100 s to generate free radicals [155].

4.2.3 Preparation and Functionalization of Polyacrylamide Gels

Figure 4.1 illustrates the schematics for fabrication of PDMS micropatterned scaffold, which is coated with a thin layer of PAG embedded with fluorescent microbeads. First, acrylamide (40% w/v, Sigma) and N-N'-methylene-bis-acrylamide (BIS, 2% w/v, Sigma) with a ratio ranging from 10% v/v to 1% v/v were mixed in a solution containing equal volume of distilled water and ethanol.

Argon gas was used to degas the solutions for 15 min in order to optimize the polymerization kinetics. Then 0.2 μm fluorescent latex beads (Invitrogen) were ultrasonicated and added to the solution at 0.1% volume concentration. After casting the solutions over the PDMS surface, polymerizations were initiated by UV radiation for 15 min.

Figure 4.1. Schematic illustration of fabricating the micropatterned PDMS and cell seeding on PAG-PDMS substrate embedded with a thin layer of fluorescent microbeads.

As PAG is highly hydrophilic and chemically inert, sulfosuccinimidyl-6(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH, Sigma), a heterobifunctional cross-linker, was exposed to 365 nm UV-light for 15 min and then attached to PAG surface before the coupling of adhesive ligand. After the exposure, 2 mg/ml collagen type I (BD Bioscience) was diluted by 100 times in 1 $\times$ PBS and then added onto the PAG surface at room temperature for at least 4 h in

the darkness. Samples coated with collagen type I were then pre-incubated with culture medium without FBS for at least 30 min at 37 °C. All the samples were sterilized with UV illumination for 15 min prior to cell seeding.

4.2.4 Cell Imaging

HaSMCs were seeded onto PAG-coated PDMS scaffold at a density of 10^4 cells/cm². Unbound cells were washed away after 2 h of seeding and cell culture medium was refreshed every day. The cell imaging was performed with an Olympus IX71 inverted microscope with 10X and 40X objectives on a motorized microscope stage (BioPoint 2, Ludl Electronic Products). The samples were located on the microscope stage fitted with Temperature Control 37-2 Digital and Heating Unit (Leica) and maintained at 37 °C. The humidity of the stage and 5% CO₂ were maintained by a humidifier system (CTI-Controller 3700, Leica). In order to perform CTF calculation of collective cells, both transmitted light and fluorescence images of the cell layer were taken as the ‘force load’ images. Before trypsinization, transmitted light microscopy was first applied to visualize the cell shape and location of the HaSMCs seeded on the PDMS scaffold at different time during cell culture. Afterwards, the positions of fluorescent microbeads inside the PAG layer were measured by fluorescence microscopy with excitation at 490 nm and emission at 515 nm. After trypsinization, cells were detached from the substrate and ‘null force’ images were taken again in the exactly same frame. From the pair of fluorescence images (before and after trypsinization), the displacement of the fluorescent microbeads was determined and used for the CTF computation.

4.2.5 Calculation of the Substrate Displacement and Collective CTF

The fluorescent microbeads embedded in PAG on top of the PDMS scaffold serve as markers for tracking the substrate deformation caused by CTFs. A particle

image velocimetry (PIV) program encoded in MATLAB[®] (MathWorks) was used as a visualization tool to calculate the bead displacement map by maximizing the intensity cross-correlation function between two pixel points on a pair of fluorescence images before and after trypsinization. The cross-correlation function is defined as

$$C\left(\mathbf{u}_{ij}, \mathbf{v}_{ij}, \frac{\partial \mathbf{u}_{ij}}{\partial X_i}, \frac{\partial \mathbf{u}_{ij}}{\partial Y_i}, \frac{\partial \mathbf{v}_{ij}}{\partial X_i}, \frac{\partial \mathbf{v}_{ij}}{\partial Y_i}\right) = \frac{\sum_{i=1}^N \sum_{j=1}^N [f_1(X_i, Y_j) - \bar{f}_1] [f_2(X_i + \Delta X, Y_j + \Delta Y) - \bar{f}_2]}{\sqrt{\sum_{i=1}^N \sum_{j=1}^N [f_1(X_i, Y_j) - \bar{f}_1]^2} \sqrt{\sum_{i=1}^N \sum_{j=1}^N [f_2(X_i + \Delta X, Y_j + \Delta Y) - \bar{f}_2]^2}} \quad (4.1)$$

where the subscripts denote the (i, j) pixel point. The relations between the pixels (X_i, Y_j) and $(X_i + \Delta X, Y_j + \Delta Y)$ of two images are

$$X_i + \Delta X = X_i + \frac{\partial u_{ij}}{\partial X_i} \Delta X + \frac{\partial u_{ij}}{\partial Y_i} \Delta Y \quad (4.2)$$

$$Y_j + \Delta Y = Y_j + \frac{\partial v_{ij}}{\partial X_i} \Delta X + \frac{\partial v_{ij}}{\partial Y_i} \Delta Y \quad (4.3)$$

where u_{ij}, v_{ij} are x and y displacements of the (i, j) pixel point; N is the total number of pixels inside the interrogation window; $f_1(X_i, Y_j)$ and $f_2(X_i, Y_j)$ are intensities of image at pixel point (i, j) in each image; $\bar{f}_1, \bar{f}_2$ represent the average values of $f_1(X_i, Y_j)$ and $f_2(X_i, Y_j)$ in each window; $\Delta X, \Delta Y$ are the shift (or overlap) of the interrogation window between two images.

It should be noted that the value of C is between 0 and 1. The higher the value of C, the more closely related between the images at (X_i, Y_j) and $(X_i + \Delta X, Y_j + \Delta Y)$. Therefore the objective is to numerically search for a location in the second image that is most consistent with the one in the first image, i.e. finding the location with maximum C. Since the search for maximum C involves all six

variables $\left(u_{ij}, v_{ij}, \frac{\partial u_{ij}}{\partial x_i}, \frac{\partial u_{ij}}{\partial y_i}, \frac{\partial v_{ij}}{\partial x_i}, \frac{\partial v_{ij}}{\partial y_i}\right)$, the computation has to be carried out numerically. A free program MatPIV distributed under the GNU general public license was adopted for this study. The window size used for calculation is 8×8 pixels to 64×64 pixels for different trials. The location of the maximum value in C was then recognized as the mean particle displacements in the window.

4.2.6 Analysis of Mechanical Properties

The finite element method (FEM) was used with ANSYS®12 in order to determine the stress exerted on PAG substrate by the cell sheet. The results obtained from the PIV calculation on the displacements of microbeads were input as boundary conditions for the subsequent finite element analysis.

4.2.7 Determination of SMC Morphology and Orientation

To quantify cell morphological changes against the time of culture, phase contrast images of different pixel fields (C1: 300 × 1000, C2: 530 × 1000, C3: 770 × 1000, C4: 1000 × 1000) in four concentric annular regions were taken every day. The boundaries of SMCs were first outlined by MetaMorph®. At least 45 cells from 3 different frames were randomly selected and analyzed. Cells grown on flat PAG-coated PDMS substrates without circular patterns were used as negative control (NC) in this study. The cell shape index (CSI) was calculated as follows:

$$\text{CSI} = \frac{4\pi S}{L^2} \quad (4.4)$$

where S is the spreading area (projected area) and L is the perimeter of the cells. If $\text{CSI} \approx 1$, cells spread well with a circular shape, while cells are highly elongated when CSI approaches 0. The degree of alignment for an elongated single cell

against the tangent of inner microwall can be characterized by calculating the orientation angle θ (Figure 4.2).

$$\theta = \cos^{-1} \frac{OA^2 + AB^2 - OB^2}{2 \cdot OA \cdot AB} + \frac{1}{2} \cdot \cos^{-1} \frac{OA^2 + OB^2 - AB^2}{2 \cdot OA \cdot OB} - 90^\circ \quad (4.5)$$

This calculation is based on the centre O of the circles and the two poles A and B of the cell. The smaller the value of θ , the better alignment of the cell along the adjacent microwall.

Figure 4.2. Schematic illustration of how to determine the cell orientation angle θ .

4.2.8 Fluorescence Staining

For fluorescence staining, HaSMCs grown on the micropatterned PDMS scaffold were washed twice with 1×PBS at 37 °C and fixed in 3.7% paraformaldehyde for 10 min at room temperature. Cells were eventually permeabilized by 0.05% Triton X-100 for 5 min and then blocked in 1% BSA/PBS solution to minimize background fluorescence signals. For cytoskeleton labeling, samples were stained with Alexa Fluor[®] 546 phalloidin (Invitrogen) at a dilution of 1:40 in 1% BSA/PBS solution for 1 h. After washing twice with 1×PBS, DAPI (Invitrogen) staining was performed for 10 min. The stained cells were imaged with an Olympus IX71 fluorescence microscope.

4.2.9 Statistical analysis

Student's *t*-tests were used to study the statistical significance between different sets of experimental data. The results were analyzed based on two-tailed test. A *p*-value of 0.05 or less between two sets of data was considered to be statistically significant.

4.2.10 Reverse transcription and real-time quantitative PCR analysis

HaSMCs were harvested by trypsinization and centrifugation. The total RNA of HaSMCs was extracted by using PureLink™ RNA Mini Kit (Life Technologies, Singapore). The concentration of total RNA was measured by NanoDrop ND2000 (Thermo Scientific, Delaware, USA). The RNA samples were then reverse transcribed into cDNA with iScript™ cDNA Synthesis Kit (Biorad Laboratories, Singapore). Real time polymerase chain reaction (PCR) assay was performed to quantify the mRNA expression level of alpha smooth muscle actin (α SMA) in StepOnePlus™ Real Time PCR Systems (Life Technologies, Singapore) with SYBR PCR Master Mix Kit (Life Technologies, Singapore). The primer sequences of selected genes were listed in Table 4.1. The data of relative gene expression level were normalized by mRNA level of human housekeeping gene GAPDH and quantified with the Livak method. The experiments were repeated three times.

Table 4.1. The primers used in real time PCR assay.

Gene Name	Description	Sequence
α SMA	Forward primer	5' GACAGCTACGTGGGTGACGAA 3'
α SMA	Reverse primer	5' TTTTCCATGTCTGT CCCAGTTG 3'
GAPDH	Forward primer	5' ATGGGGAAGGTGAAGGTCG 3'
GAPDH	Reverse primer	5' TAAAAGCAGCCCTGGTGACC 3'

4.3 Results and Discussions

4.3.1 Micropatterned PDMS

In this study, we applied soft lithography to fabricate a series of concentric microwalls on PDMS scaffold which led to the formation of circular microchannels with different outer/inner perimeters for guiding specific circumferential alignments of HaSMCs. Figure 4.3(a) showed the scanning electron microscopy (SEM) image of the PDMS substrate fabricated with circular microchannels between a series of concentric microwalls. The external radii of the four circular microchannels were measured as 450 μm , 780 μm , 1110 μm and 1440 μm , respectively, starting from the centric circle with a radius of 150 μm . Moreover, the width and depth of the concentric circular microwalls were both 30 μm , while the distance between two adjacent microwalls (i.e., width of the resulted microchannel) was around 300 μm (Figure 4.3(b)). HaSMCs were seeded and cultured in those circular micro-channels in order to induce circumferential alignment of individual cells under various geometric confinements. In order to study the mechanotransduction between cells and the model ECM, the micropatterned PDMS substrate were covered with a thin layer of transparent PAG containing 0.2 μm fluorescent microbeads for measuring the substrate deformation in the CTFM assay [67]. Under higher magnification, the SEM image further revealed that the uniformly coated PAG layer exhibited highly crosslinked meshwork on PDMS (Figure 4.3(b)). The phase contrast image indicated that the PAG layer was transparent (Figure 4.3(c)), which facilitated the real time visualization of cell morphology and fluorescent beads. In addition, the dense and homogeneous distribution of fluorescent microbeads (Figure 4.3(d)) validated the successful

formation of PAG layer on the entire PDMS scaffold, which was necessary for accurate analysis of the CTFs.

Figure 4.3. The micropatterned PDMS substrate coated with a layer of PAG embedded with fluorescent microbeads. (a, b) SEM images of the PAG-PDMS substrates. (c, d) Phase contrast image and fluorescent image of the PAG-PDMS substrates taken under 10X magnification.

4.3.2 Cell Morphology in Microchannels

In order to investigate the phenotypic responses of HaSMCs under specific physical constraints, primary HaSMCs were cultured inside all four circular microchannels (C1, C2, C3, C4 from inner to outer) imprinted in the PAG-PDMS substrate for 7 days. Before cell seeding, collagen was linked to the PAG surface for enhancing the biological signaling between cells and supporting substrates. As a negative control, the same HaSMCs were cultured on a planar surface of PAG-PDMS without any imprinted micropattern. The phase contrast images of seeded

cells in the circular microchannels and on planar substrate were taken every day. In general, the culture on Day 0, Day 2 and Day 5 were selected to compare the behavior between the HaSMCs grown in these circular microchannels and those grown on the planar substrate (Figure 4.4(a, b)).

Figure 4.4. Phase contrast images of HaSMCs grown on the concentric micro-patterns and flat PAG-PDMS substrate under 10X (a) and 40X (b) magnifications at Day 0 (4 h after cell seeding), Day 2 and Day 5, respectively. Scale bars in (a): 250 μm ; Scale bars in (b): 100 μm . (c) Density change of SMCs grown in all regions and flat substrate during 7 days culture. (d) Schematic illustration of the micro-patterned scaffold. The rectangular frames (red) were selected for quantitative analysis. (e) CSI of SMCs during 7 days culture. Student's t-test between C3 and other 4 groups at Day 5 was performed. * Significant at $p < 0.05$, ** significant at

$p < 0.005$, *** significant at $p < 0.001$. Error bars in (c) and (e) represent mean $\pm$ SD calculated based on at least 45 cells in 3 different frames.

The cell density on all circular microchannels and planar substrates was relatively low at 4 h after cell seeding (Day 0). The results indicated that both circular microchannels and planar substrates were sparsely covered with adherent SMCs due to the relatively short time of cell culture. At the same time, most SMCs adopted rather round shape on planar substrate without significant cell body elongation (Figure 4.4(b)). However, significant number of SMCs extended their bodies and adopted an elongated shape inside the circular microchannels (e.g., C3 region on Figure 4.4(b)). At Day 2, most SMCs were significantly elongated on both circular microchannels and planar substrate. Interestingly, individual cells on planar substrate adopted rather random orientation, while most cells inside the circular microchannels showed certain degree of alignment between the two adjacent microwalls according to the channel curvature. At Day 5, the development of synthetic phenotype in SMCs became apparent as cells continue to proliferate against the increase of cell density. From Figure 4.4(a), it was shown that all cells grown on circular microchannels and planar substrate reached confluence in about 5 days after seeding. From Figure 4.4(c), the initial cell growth rate could be determined from the slope of cell density vs. time plot (From Day 0 to Day 2). It was shown the initial growth rate of SMCs on PAG-PDMS substrate was dependent on the geometry of imprinted micropatterns according to the following decreasing order: $C1 > C2 > NC > C3 > C4$ (Figure 4.4(c)). Highest cell growth rate was found in C1 region, which might be due to the enhanced cell-cell interactions between neighboring cells constricted by the higher curvature of the smaller concentric circles. Moreover, cells grown in C1 region reached confluence within 3

days, which was much earlier than those grown in other geometries. Cell density in C1 region remained constant from Day 3 to Day 5 followed by a sharp growth phase, which might be due to the formation of multiple layers of SMCs. As a result, the density of adherent cells inside C1 circular microchannel was significantly higher than that on planar substrate after 6 days of cell culture. Meanwhile, the cell density in other circular microchannels including C2, C3 or C4 was lower than that in NC, which suggested the growth inhibition was only triggered by the mechanical constriction under lower curvatures.

With the increase of cell density, SMCs grown either inside the circular microchannels or on the planar substrate became highly elongated. It is worthwhile to elucidate the effect of physical constrain on the transformation of SMC morphology during the 6-days culture. Thus the temporal change in cell morphology under different geometries was quantified by the calculation of cell shape index (CSI). In order to make the comparison logical, the same angle of circle segment was chosen for all four types of circular microchannels herein. The frame size of C1, C2, C3, C4 and planar substrate (i.e., negative control) was $100 \times 335 \mu\text{m}^2$, $177 \times 335 \mu\text{m}^2$, $258 \times 335 \mu\text{m}^2$, $335 \times 335 \mu\text{m}^2$ and $258 \times 335 \mu\text{m}^2$, respectively (the red regions in Figure 4.4(d)). CSI of SMCs was high at around 0.78, which indicated cells adopted circular shape in all four circular microchannels and on NC immediately after cell seeding (Day 0). After reaching confluence in Day 7, the lowest extent of SMC elongation was found in C1 region with a CSI of 0.39. In contrast, SMCs in C3 region with CSI value of 0.25 was more elongated than those in C2 and C4 regions that had greater CSI of 0.32 (Figure 4.4(e)). In addition, SMCs on NC demonstrated significant change in morphology as shown by the reduction of CSI value from 0.77 to 0.22. These results indicated the

microchannel simultaneously moderated cell elongation and enhanced the density of adherent cells.

4.3.3 Cell Orientation between Concentric Microwalls

Similarly, the collective cell orientation was measured in order to determine how the circular microchannel facilitated the circumferential alignment of SMC layer and which size of circular microchannel was optimal for the formation of functional equivalents of SMC tissues. The major difference among the four circular microchannels was the radius (or the curvature). Figure 4.5 shows the optical contrast image and the difference of orientation angle θ for SMCs inside four microchannels after several days of culture. The reduction of radius leads to an increase of curvature, which prevented SMCs from making parallel alignments with respective to each other as supported by the multiple cell layer and randomly orientated cells grown in C1 region (Figure 4.5(a)). Therefore both cell-cell interaction and cell-channel interaction could influence the collective cell alignment. Moreover, SMCs grown in C3 region presented better collective alignment compared to those in C2 and C4 regions. These results suggest that there exists an optimal range of curvature in the circular microchannel that can promote SMC alignments.

Figure 4.5. (a) Collective SMCs grown on the micropatterned PAG-PDMS after 5 days culture. Scale bar: 250 μm . (b) The change of orientation angles of SMCs grown between the circular microwalls in 7 days culture. Student's t-test between C3 and other 3 regions at Day 5 was performed. ** Significant at $p < 0.005$, *** significant at $p < 0.001$. Error bars represent mean $\pm$ SD obtained from at least 30 cells in 3 different frames.

To quantify the circumferential alignment, we introduced the orientation angle θ that was defined as the angle between the cell's longitudinal axis and the microwall tangent direction (Figure 4.2). Smaller θ indicates better cell alignment with regard to the local channel wall (Figure 4.5(b)). At Day 0, there was no significant difference in θ among cells in C1, C2, C3, C4 regions and the NC

(~50 °). θ of SMCs in C1 region was reduced from 52 ° to 28 ° after 1 day of culture and remained at around 30 ° from Day 2 to Day 7. The result indicated that the highest curvature of C1 was least effective in inducing collective SMCs alignment. In contrast, the largest extent in the reduction of θ (from $50 \pm 12^\circ$ to $7 \pm 4^\circ$) for SMCs was found inside the C3 region. For cells grown on planar (NC) substrate, however, their orientations are quite random even after 5 days of culture. Among the four circular microchannels, C3 exhibited the optimal curvature in guiding the circumferential alignment of SMC cell sheet.

4.3.4 Traction Forces and Stress Distribution of Optimal Circularly-aligned Collective Cell Sheet

Using the analytical technique developed in our previous study, the collective cell traction forces during the formation of circumferentially aligned SMC layer (Figure 4.6(a)) were determined by the displacement field of fluorescent microbeads embedded in the thin layer of PAG coated on top of the PDMS micropatterns. Within the selected rectangular frames, PIV method was used to analyze the displacement vector of the fluorescent microbeads captured between the images before and after cell detachment (Figure 4.6(b)). Then the displacement fields were used as the boundary conditions for analyzing the stress distributions using finite-element software ANSYS®12. SMCs grown on the planar substrate after 5 days culture were used as the negative control.

The cell spreading and alignment depend on the interactions between F-actin cytoskeletons and microenvironment through transmembrane receptors [179, 186]. Since the actin filaments are randomly oriented within the complex cytoskeleton network in subcellular scale, the traction forces at the cell-substrate interface may not be perfectly along the circumferential direction even though the

individual cells are aligned in such a way. The off-circumferential (radial direction) components of the stress tensor should also be considered in order to accurately characterize the entire loading on the cells. Therefore, we present all the stress levels in terms of von Mises stress, which provides a comprehensive and accurate measure of the stress on cells due to the randomly oriented traction forces at the cell-substrate interface. The von Mises stress represents the energetically equivalent magnitude of all principle stress components to the uniaxial tension, and is defined as

$$\sigma_{\text{vonMises}} = \sqrt{\frac{1}{2}[(\sigma_1 - \sigma_2)^2 + (\sigma_2 - \sigma_3)^2 + (\sigma_3 - \sigma_1)^2]}$$

where σ_1 , σ_2 , σ_3 represent three principal stresses, respectively.

Figure 4.6. The variation of CTF and von Mises stress distributions during the formation of collective SMCs in C3 region at Day 0, Day 2 and Day 5, compared to the control sample on a flat PAG-PDMS substrate at Day 5. (a) The phase contrast images were taken under 40X magnification. The pixel dimension (height $\times$ width) of the selected frame is 770×1000 (1 pixel $\approx 0.3346 \mu\text{m}$). Scale bars: $100 \mu\text{m}$. (b)

The distributions of microbead displacements in the framed region induced by the SMCs using PIV analysis. The unit window size for comparison is 32×32 pixels.

(c) The distributions of von Mises stresses simulated by FEM analysis.

Figure 4.6 indicated the dynamic change of SMC cell morphology within 5 days during circumferential alignment under the influence of the circular micropatterns. The stress analysis for SMCs grown in C3 region aimed to elucidate the cell mechanotransduction in this region with a particular curvature for enhanced cell alignment. From Day 0 to Day 5 as shown in Figure 4.6(a), the cell density significantly increased and cell morphology changed from round to spindle shape. The individual cells attached and spread within 4 h after cell seeding (Day 0), which led to evolution of the random patterns of CTF and von Mises stress (Figure 4.6(b, c)). At Day 2, the directions of the CTF in the center of C3 region mostly pointed along the longitudinal axis of the circumferentially aligned SMCs, while those adjacent to the microwalls were oriented in a direction normal to the microwall (Figure 4.6(b)). Moreover, higher stresses were found near the boundary of the incomplete cell sheet compared to those in the central area (Figure 4.6(c)), which was probably due to the physical constraints imposed by the adjacent microwalls. Beneath the partially formed cell sheet, the stress level was lower but non-uniform since some cells were still in the process of proliferation. In other words, the transformation and reorganization of cytoskeletons induced by both intercellular interactions and the extracellular physical constraints could have an impact on the distribution of CTFs and stresses at this stage. As the cell culture continued to Day 5, a confluent cell sheet was formed and well aligned along the micro-circular direction (Figure 4.6(a)). Compared to the randomly distributed CTFs on the negative control grown on a flat substrate, the traction forces of the aligned cell

sheet were much more uniform with a consistent pattern (Figure 4.6(b)). Moreover, a uniform region of low stresses evolved in the central region of SMC cell sheet (Figure 4.6(c)). Similar to a prior study on SMCs grown between parallel microchannel walls, the low stress zone almost covered the entire selected zone of aligned cell sheet.

In addition to the dynamic change in C3 region as shown above, we also compared the stress distribution of all annular regions (C1 to C4). As shown in Figure 4.7(a), the cell layer in all four regions reached confluence after 5 days of culture. For the distribution of CTFs, the forces in C1 and C2 regions were mostly horizontally orientated, which were along the radial direction with respect to the circular microwalls (Figure 4.7(b)). On the contrary, the CTF distribution in C3 and C4 regions became more aligned along the circumferential direction between microwalls, where the longitudinal axes of SMCs were parallel to the same orientation. The more uniform CTF distribution in C3 and C4 regions indicated the formation of intact cell sheet with smaller stresses at the center and higher stress near the cell sheet boundary adjacent to the microwalls (Figure 4.7(b, c)). The results herein revealed that the interactions between cells and microwalls were important on the distribution of CTFs. The SMC layer grown between two microwalls with smaller curvatures such as in C3 and C4 prefer to align along the circumferential direction. The distribution of von Mises stresses (Figure 4.7(c)) further showed that C3 represented the most uniform region with low stress at the center of the confluent cell sheet. These results suggested that the curvature in C3 region could provide the optimal scaffold to promote the circumferential alignment of SMCs *in vitro*.

Figure 4.7. The distributions of CTFs (b) and von Mises stresses (c) of the confluent SMC sheet on the circular PAG-PDMS micropatterns at Day 5 (a). Scale bar: 250 μm .

It is noted that the PIV analyses in Figure 4.6 and Figure 4.7 are based on selected frames with limited window size in each microchannel. Ideally, the entire annular channels should be analyzed for the CTF and stress fields. However, the lower magnification necessary to capture the entire channel is not enough to discern the displacement of the microbeads in PAG, which is required for subsequent PIV analysis. Therefore we have to select discrete rectangular windows that can be captured by camera under much higher magnification, which is one of the major limitations of the present methodology. On the other hand, since the cells become aligned circumferentially due to the concentric microwalls, the entire system is axisymmetric. This implies that the variations (or non-uniformity) of CTFs and stress fields along the circumferential direction (within a single circular channel) are

much lower than those in the radial direction (across multiple channels). Similar results as shown in Figure 4.6 and Figure 4.7 have been found on multiple chips of the same design using PIV analysis.

4.3.5 Actin Organization and Expression of HaSMCs on Micropatterned Scaffold

Actin is one of the most important cytoskeletal proteins in maintaining the structural integrity and contractile functions of SMCs within functional vascular tissues [186]. Actin is present in all eukaryotes and its protein sequence is highly conserved. The quantitative change of actin expression can effectively signal the variation of cytoskeletons. In view of the current study, when cells are cultured in the circular microchannels they are mostly restricted to move along the circumferential direction. This implies fewer demands on the assembly of actin in contrast to the case of cells on a flat substrate, under which the constant assembly of actins is necessary by chemokinesis. To verify this hypothesis, F-actin filaments of cells cultured in circular microchannels were immuno-stained and imaged by fluorescence microscopy on Day 0, Day 2 and Day 5. As shown in Figure 4.8, adherent cell density in microchannels was significantly increased from Day 0 to Day 5 of culture. Meanwhile, the organization of actins (red) in Figure 4.8(c) shows the cellular morphology switched from compact to more elongate as compared to those in Figure 4.8(a). After 5 days culture, F-actin filaments were highly aligned along the circumferential direction of concentric microwalls, which demonstrated the similar SMC phenotype as obtained previously using 3D circular tubes [177, 182]. The intensity of stained actin in center region (C1) was much higher than that in peripheral regions (C3 and C4), and more actin filaments were observed in cells adjacent to the microwalls. This observation is consistent with the quantified results

in Figure 4.5, indicating that the concentric walls are able to guide and confine the morphological development of SMCs along the circumferential direction, which effectively reduces the needs for F-actin filaments. The less F-actin, the lower cell traction force would be as the actin filaments are closely related to cell-generated contractile forces (Figure 4.7).

Figure 4.8. Immunofluorescence staining of F-actin (red: TRITC) and nucleus (blue: DAPI) for HaSMCs grown between the concentric microwalls on Day 0 (a), Day 2 (b) and Day 5(c). Scale bar: 250 μm. (d). The relative gene expression of αSMA by HaSMCs cultured on the micropatterned scaffold for 4 h (Day 0), 2 days, and 5 days after cell seeding. HaSMCs grown on flat PAG-PDMS substrate on Day 0, Day 2 and Day 5 are used as calibrator. The expression levels, quantified by RT-qPCR, are normalized to the housekeeping gene GAPDH. * indicates significant difference at $p < 0.05$.

One remaining question is related to whether the micropatterned scaffold could impact the genetic expression for actin during the alignment process. Since alpha smooth muscle actin (α SMA) is one of the six identified actin isoforms in mammalian cells and of great importance in the cellular contractile apparatus, we used real time reverse transcription polymerase chain reaction (RT-qPCR) assay to test the relative mRNA expression of α SMA. The normalized expression level of cells cultured on micropatterned scaffold was then compared to cells grown on flat PAG-PDMS substrate in Figure 4.8(d). On Day 0 and Day 2, the α SMA expression was slightly lower but generally similar to that on a flat substrate, which suggested that most cells were still in active proliferation and had not been affected by the physical constraints due to the channel walls. In contrast, it was observed that down-regulation of α SMA became statistically significant on Day 5. These results were consistent with the findings in previous studies on circumferential alignment of SMCs in 3D hollow tubes [177, 182], where the α SMA expression were also reported to be down-regulated on aligned micropatterns as compared to randomly-oriented patterns or flat substrate. These quantitative analyses evidently support our earlier conjecture that the micropatterned scaffold constrained the alignment of collective cells along a specific direction, which decreases the quantity of actin filaments at cellular level and this reduction is proved by the inhibited expression of actin related gene marker.

4.4 Conclusions

With a concentric micropatterned PAG-PDMS scaffold, we demonstrated the formation of microscale circumferentially aligned smooth muscle cell sheet. In order to study the influence of the surrounding microenvironment, we tracked the

dynamic processes of cell growth, morphological switches and orientation angle changes for 7 days. By quantitative comparison among four regions with different curvatures, we indicated that there existed a special curvature zone to induce the optimal circumferential alignment of collective SMCs. It was found that the distribution of cell traction forces and von Mises stresses were influenced by the physical constraints of circular scaffolds and significantly different from those on a flat substrate. Assisted by PIV analysis, it was further demonstrated that the complete monolayer of SMCs could generate traction forces along the direction of aligned actin filaments. A typical homogeneous zone of lower stress in the center was observed after forming a confluent cell sheet and the higher stresses occurred near the boundary, which indicated the cellular mechanotransduction under the effect of surrounding microenvironment. Lastly, at the cellular and molecular levels, immunofluorescence staining and RT-qPCR assay further confirmed that the SMC sheet demonstrated a significant down-regulation of actin expression after the cells became confluent and circumferentially aligned. As the cell sheet was well organized and grown on a removable platform, the number of cell layers and the diameter of circularly aligned cell sheets could be easily adjusted. The resulted cell layers together with natural or synthetic scaffolds could be further applied to make tubular constructs and develop functional arterial replacement grafts with different diameters. This *in-vitro* study has demonstrated an obvious effect on the mechanotransduction of SMCs induced by variable curvatures at both cellular and biomolecular levels, which may shed new light on designing the future engineered tissues in regenerative medicine for treatment of vascular diseases.

Chapter 5 The Role of Bifurcation Angles on Collective Smooth Muscle Cell Biomechanics and the Implication in Atherosclerosis Development

5.1 Introduction

The middle layer of an artery or vein known as tunica media is mainly made up of vascular smooth muscle cells (SMCs). In the arterial system, vascular SMCs which are specifically differentiated play a critical role in the expression of contractile phenotype for controlling the blood pressure and blood flow distribution [187]. The principal physiological response resulted from the quiescent contractile phenotype of vascular SMCs is blood vessel contraction. Interesting, the switch of SMCs from contractile phenotype to active synthetic state would lead to cell proliferation and migration. Under *in vivo* conditions, mature SMCs retain remarkable phenotypic plasticity that allows rapid adaptation to the change of physiological microenvironment such as the development and progression of atherosclerosis [188].

Atherosclerosis and the involved pathophysiological complications such as heart attacks and strokes, is the leading cause of death and disability for general populations in most developed countries. For instance, the lesions induced by atherosclerosis are triggered by a series of cellular and molecular events leading to the accumulation of lipids, proteins and cellular components within the blood vessel. To date, the mechanisms underlying the multi-stage development of atherosclerosis in human remains to be elucidated [189]. The earliest event of atherosclerosis development takes place in the endothelium which is the innermost layer of arterial wall known to be responsible for the macromolecular transportation

in circulation. Atherosclerotic lesions (atheroma) can develop in response to the dysfunction of endothelium that leads to an increase of vascular permeability. After endothelium injury, the subsequent inflammatory responses promote the phenotype switch of vascular SMCs and further stimulate proliferation and migration of vascular SMCs [190, 191]. Vascular SMCs expressing migratory, synthetic phenotype *in vivo* have been shown to be directly involved in the development of arterial intimal lesions like atherosclerosis [187, 192]. The accumulations of vascular SMCs, lipid, macrophage, T-lymphocytes and foam cells lead to thickening of the injured endothelium as well as the narrowing the blood vessel.

In general, the common risk factors of atherosclerosis including aging, smoking, stress, diabetes, hypertension and high cholesterol level are well defined. Atherosclerotic plaques do not develop randomly within the arterial tree, but preferentially locate at arterial curvatures, branches and bifurcations where the blood flow is disturbed into different streams [6]. This observed phenomenon indicates the presence of highly localized physiological responses at the branching points of arteries leading to early disease development in addition to the common risk factors. The mechanisms in ECs' biological responses to the shear stress of flowing blood and mechanotransduction studies on bifurcation or branch geometries have been previously reported [193], while the mechanotransduction underlying branched SMC layer remains unclear.

Mechanical stresses present in the microenvironment surrounding cells, whether generated from the external applied forces or internal traction forces generated by the cytoskeleton of cells, have been intensively studied because of their important roles in regulating many physiological processes. Under *in vivo* condition, both cells and subcellular sites [80] can sense stresses from many sources

such as shear stress of blood flowing, physical induced stress of the cytoskeleton system and contractile forces exerted on the surrounding extracellular matrix (ECM) in 3D space. There are significant differences between external and cell-generated forces, which can be characterized from the differences in the magnitudes, directions and dynamics of forces. However, some indications of coupling between externally applied and cell-generated forces are revealed in recent studies [81, 82]. The biochemical processes in the generation of contractile force after SMCs attachment and spreading on the substrate involve actomyosin interactions and actin polymerization in the cytoskeletal network of cells [86]. The traction forces are then transmitted from cell to ECM via focal adhesions (FAs), which are crucial for connecting the cytoskeleton of adherent cells to the binding sites of ECM molecules [88]. These cell-generated forces as mentioned above are known as cell traction forces (CTF). The presence of CTF plays a key role in modulating cell differentiation, migration, apoptosis and in maintaining the cell homeostasis [91]. For SMC-generated CTF, it is mainly regulated by the Rho-kinase/ROCK, which directly affects the mitogen-induced DNA synthesis [89] and the assembly of focal adhesions [90].

To measure the CTFs of both individual cell and cohesive cell mass, a variety of methods including cell-populated collagen gel (CPCG) [118], thin silicone membrane [56], micromachined cantilevers and micropost force sensor arrays [59, 184] have been developed during the past two decades. However, the arguably most reliable and comprehensive method for quantifying cell-substrate mechanotransduction is CTF microscopy (CTFM) assay developed by Dembo and Wang [119]. It is believed that physical interactions as well as chemical cues are essential in the mechanotransduction process. As the fundamental forces in the

mechanotransduction process, CTF measurement makes the biomimetic study of morphogenesis *in vitro* possible. Mechanical stresses produced from the surrounding physiologically microenvironment, such as substrate, ECM, neighboring cells and so on, could influence the collective cell proliferation and migration, which eventually contribute to the vasoactivity of blood vessel.

Recently, micropatterning of cells by applying microfabrication techniques and soft lithography have been proved to be an attractive approach for *in vitro* cell shape control and spatial organization of connective cells [2, 4, 8]. Additionally, in some recent studies to investigate the adaptive alignment of smooth muscle cell sheet in straight and circular [131, 194] micropatterns, we have revamped the traditional single-cell CTFM assay so it can be used to analyze a batch of cells, which is very convenient for biophysical and biomechanical studies on the collective cell behavior under cell-substrate interactions. By taking advantage of both microarrays and collective CTFM assay, we aim to use bifurcate micropatterns with different angles for investigating the mechanotransduction of branched human aortic smooth muscle cell (HaSMC) layer in response to these unique physical cues. More specifically, a series of branched microgrooves with different bifurcation angles are fabricated using soft lithography method. These grooves are further coated with a thin layer of polyacrylamide gel (PAG), which contains monodispersed fluorescent microbeads as indicators to measure the cell traction force. The cell shape, density and orientation-specific adaptation of SMCs during branched cell layer formation were measured during 5 days cell culture. And the collective CTFM assay with the aid of finite element modeling [131, 154] was applied to measure the cell sheet traction forces around the bifurcations. Moreover, the immunofluorescence staining and real time quantitative polymerase chain

reaction (RT-qPCR) analysis provide further support for the monolayer-generated traction forces and localizations of atherosclerotic lesions.

5.2 Materials and Methods

5.2.1 Device Fabrication

To fabricate the bifurcate PDMS microgrooves for cell culture (Figure 5.1), a master mold was made by surface patterning of negative photoresist SU-8 3025 (Microchem, MA, USA) [185]. Briefly, the photoresist was cast onto a cleaned silicon wafer with a spin coater (SCS G3P-8) at 1000 rpm for 30 s. The excess solvent in the photoresist was evaporated by heating for 30 min at 95 °C. The selective crosslinking of SU-8 was achieved via UV irradiation through a film photomask for 8 s. After post-baking for 1 min at 65 °C and 5 min at 95 °C, the master mold was created by rinsing and dissolving the uncrosslinked photoresist in the SU-8 developer. The final branched mold structure was 300 µm in width and 70 µm in height.

The silicone prepolymer base and curing agent (Sylgard 184 kit, Dow Corning) were mixed (10:1 w/w) thoroughly and casted over the master mold with bifurcate patterns. The evenly distributed reagents were degassed under vacuum for 30 min. After curing at 80 °C overnight and 120 °C for 2 h in the following day, the PDMS scaffold was polymerized, followed by washing with hexane bath and sterilizing with ethanol overnight. A surface profiler (Alpha-Step IQ, KLA-Tencor Corporation) was used to measure the depth of the bifurcate groove structure.

Figure 5.1. Schematic illustration of bifurcating PDMS scaffold fabrication.

To further modify the PDMS surface for CTFM assay, they were sliced into circular slabs of 15 mm in diameter before being treated by Argon plasma (Harrick Plasma) for 120 s to generate free radicals on the surface [155]. The acrylamide (40% w/v, Sigma) and N-N'-methylene-bis-acrylamide (BIS, 2% w/v, Sigma) were mixed at a ratio of 10% (v/v) to 1% (v/v) in a solution containing equal volumes of distilled water and ethanol. The air was replaced by argon gas to optimize the polymerization kinetics. The mixed solutions containing 0.2 μm fluorescent latex beads (Invitrogen) dispersed at 1/1000 volume concentration were cast over the PDMS surface. The polymerization process was induced under UV irradiation for 15 min. The beads density was characterized with fluorescence microscopy.

As PAG is highly hydrophilic and chemically inert, a heterobifunctional cross-linker, sulfosuccinimidyl-6(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-

SANPAH, Sigma), was coated on the surface after exposure to 365 nm UV-light for 15 min. The photoactivated phenylazide group of sulfo-SANPAH was covalently coupled to PAG surface, while the succinimidyl ester group at the other end was linked to the primary amines of ECM proteins. Then 0.2 mg/ml collagen type I (BD Bioscience) was added onto the sample for at least 4 h at room temperature in the dark. After collagen binding, samples were rinsed with PBS and pre-incubated with culture medium without FBS for 30 min at 37 °C. The final platform was sterilized with UV illumination prior to cell seeding.

5.2.2 Cell Preparation and Imaging

The human aortic smooth muscle cells (HaSMCs, Lonza, Switzerland) were cultured in growth medium (SmGM basal medium supplemented with SmGM-2 SingleQuot Kit Suppl. & Growth Factors, Lonza). Cells were maintained in an incubator at 37 °C with 5% CO₂. For cell passage and seeding, cells were first washed with HEPES buffered saline solution and then detached from the flask using trypsin/EDTA solution (ReagentPack™ subculture reagents, Lonza). The culture medium was changed every two or three days. The cells undergoing passages 6-9 were used for this study.

Cells were seeded onto the bifurcate scaffold at density of 2×10^4 cells/cm². The culture medium was refreshed every day. To determine cell morphology and orientation, the transmitted light images of SMCs grown in the microgrooves were taken on a motorized microscope stage (BioPoint 2, Ludl Electronic) using an Olympus IX71 inverted microscope. For CTF measurement, live cell samples were monitored at 37 °C on the stage controlled by a Temperature Control 37-2 Digital and Heating Unit (Leica). The required humidity and 5% CO₂ were maintained by a humidifier system (CTI-Controller 3700, Leica). After 3 days

culture, both transmitted light and fluorescence images of the cell layer were taken as the ‘force load’ images before trypsinization. The positions of fluorescent microbeads in the PAG layer were recorded under excitation at 490 nm and emission at 515 nm. After cell detachment by adding trypsin, ‘null force’ images were taken again in the same frame using both phase contrast and fluorescence microscopy. Combining the pair of fluorescence images before and after trypsinization, we could determine the displacement of the fluorescent microbeads for CTF computation.

For fluorescence staining, SMCs grown in the bifurcate scaffolds were washed twice with prewarmed PBS. After being fixed in 3.7% paraformaldehyde for 15 min and permeabilized by 0.05% Triton X-100 for 5 min, cell samples were blocked in 1% BSA/PBS solution for 15 min to minimize background signals. The cytoskeleton was labelled with Alexa Fluor[®] 546 phalloidin (Invitrogen) diluted by 40 times in 1% BSA/PBS solution at room temperature for 1 h. After washing with PBS, the cell nuclei were stained with DAPI (Invitrogen) for 10 min. The images of stained cells were taken by an Olympus IX71 fluorescence microscope.

5.2.3 Measurement of Collective CTF

The fluorescent microbeads were embedded into PAG as markers to track the substrate deformation caused by CTFs. A particle image velocimetry (PIV) program encoded in MATLAB[®] (MathWorks) was used to determine the bead displacements. This program worked as a visualization tool for calculating displacement map by maximizing the intensity cross-correlation function between two pixel points from a pair of fluorescence images before and after trypsinization. The cross-correlation function is defined as

$$C\left(u_{ij}, v_{ij}, \frac{\partial u_{ij}}{\partial X_i}, \frac{\partial u_{ij}}{\partial Y_i}, \frac{\partial v_{ij}}{\partial X_i}, \frac{\partial v_{ij}}{\partial Y_i}\right) = \frac{\sum_{i=1}^N \sum_{j=1}^N [f_1(X_i, Y_j) - \bar{f}_1] [f_2(X_i + \Delta X, Y_j + \Delta Y) - \bar{f}_2]}{\sqrt{\sum_{i=1}^N \sum_{j=1}^N [f_1(X_i, Y_j) - \bar{f}_1]^2} \sqrt{\sum_{i=1}^N \sum_{j=1}^N [f_2(X_i + \Delta X, Y_j + \Delta Y) - \bar{f}_2]^2}} \quad (5.1)$$

where the subscripts represent a pixel point (i, j). The relations between the pixels (X_i, Y_j) and $(X_i + \Delta X, Y_j + \Delta Y)$ from two images are described as follows:

$$X_i + \Delta X_i = u_{ij} + \frac{\partial u_{ij}}{\partial X_i} \Delta X + \frac{\partial u_{ij}}{\partial Y_i} \Delta Y \quad (5.2)$$

$$Y_j + \Delta Y_j = v_{ij} + \frac{\partial v_{ij}}{\partial X_i} \Delta X + \frac{\partial v_{ij}}{\partial Y_i} \Delta Y \quad (5.3)$$

where u_{ij}, v_{ij} are x and y displacements of the (i, j) pixel point; N is the total pixel number inside the interrogation window; $f_1(X_i, Y_j)$ and $f_2(X_i, Y_j)$ are the intensities of image at pixel point (i, j) in each image; $\bar{f}_1, \bar{f}_2$ represent the average values of $f_1(X_i, Y_j)$ and $f_2(X_i, Y_j)$ in each window; $\Delta X, \Delta Y$ are the shift (or overlap) of the interrogation window between two images.

The value of cross-correlation function C varies between 0 and 1. The higher the value of C, the more closely related between the images at (X_i, Y_j) and $(X_i + \Delta X, Y_j + \Delta Y)$. Therefore, the objective is to numerically search for a location in the second image that is most consistent with the one in the first image, i.e. finding the location with maximum C. Because the search for maximum C involves all six variables $\left(u_{ij}, v_{ij}, \frac{\partial u_{ij}}{\partial X_i}, \frac{\partial u_{ij}}{\partial Y_i}, \frac{\partial v_{ij}}{\partial X_i}, \frac{\partial v_{ij}}{\partial Y_i}\right)$, the computation has to be done numerically. A free program MatPIV distributed under the GNU general public license was adopted for this study. The window size used for calculation varied from 8×8 pixels

to 64×64 pixels for different trials. The location of the maximum value of C was then recognized as the mean particle displacement in the window.

The displacements of microbeads calculated by quantitative PIV analysis were input as boundary conditions for subsequent finite element analysis using ANSYS[®] 12 to determine the stress distribution at the cell-substrate interface.

5.2.4 Cell Morphology and Orientation

To characterize the cell morphological changes over time, phase contrast images of a 500 × 800 (pixels) field in the branched microgrooves with 30°, 60°, 90° and 120° bifurcation angles were taken every day. The boundaries of SMCs grown near the branch points (within the red circle in Figure 5.2) were first outlined by MetaMorph[®]. At least 45 cells from 3 different frames were randomly selected and analyzed. The cells grown on flat PAG-coated PDMS substrates were used as negative control (NC). The cell shape index (CSI) is defined as

$$CSI = \frac{4\pi S}{L^2} \quad (5.4)$$

where S is the spreading area (projected area) and L is the perimeter of the spreading cells. The cells are close to round shape if CSI equals to 1, while cells are highly elongated when CSI approaches 0. The orientation angle θ of a single cell (Figure 5.2) is defined as

$$\theta = \tan^{-1} \frac{y_B - y_A}{x_B - x_A} \quad (5.5)$$

which is based on the coordinates of two longitudinal ends $A (x_A, y_A)$ and $B (x_B, y_B)$ of the cell. The orientation-specific adaptation angle α can be defined as the difference between the orientation angle θ and half of the bifurcation angle β .

$$\alpha = \theta - \frac{\beta}{2} \quad (5.6)$$

The smaller the adaptation angle α , the better adaptation of the cell to the bifurcation.

Figure 5.2. Schematic illustration of the calculation of cell orientation angle θ .

5.2.5 Gene Analysis

For reverse transcription and real-time quantitative PCR (RT-qPCR) analysis, SMCs were collected after trypsinization. The RNA was extracted using PureLinkTM RNA Mini Kit (Life Technologies). NanoDrop ND2000 (Thermo Scientific, Delaware) was used to measure the concentration of total RNA. The RNA samples were then reverse-transcribed into cDNA with iScriptTM cDNA Synthesis Kit (Biorad Laboratories). RT-PCR was induced to quantify the mRNA expression level of selected genes in StepOnePlusTM Real Time PCR Systems (Life Technologies) with SYBR PCR Master Mix Kit (Life Technologies). The primer sequences of selected genes are listed in Table 5.1. All data of relative gene expression level were quantified with Livak method and normalized according to

the mRNA level of human housekeeping gene GAPDH. Each test was repeated three times.

Table 5.1. The primers used in real time PCR assay.

Gene Name	Description	Sequence
α SMA	Forward primer	5' GACAGCTACGTGGGTGACGAA 3'
	Reverse primer	5' TTTTCCATGTCGTCCCAGTTG 3'
MMP2	Forward primer	5' TACAGGATCATTGGCTACACACC 3'
	Reverse primer	5' GGTCACATCGCTCCAGACT 3'
β -catenin	Forward primer	5' GTGCTATCTGTCTGCTCTAGTA 3'
	Reverse primer	5' CTCCTGTTTAGTTGCAGCATC 3'
GAPDH	Forward primer	5' ATGGGGAAGGTGAAGGTCG 3'
	Reverse primer	5' TAAAAGCAGCCCTGGTGACC 3'

5.2.6 Statistical Analysis

To study the statistical significance, student's *t*-tests were applied between different sets of experimental data. The results were analyzed based on two-tailed test. A *p*-value smaller than 0.05 was considered to be statistically significant.

5.3 Results and Discussions

5.3.1 Scaffold Functionalization

In this study, we applied soft lithography to fabricate different bifurcate microgrooves on PDMS scaffold (Figure 5.1). The width, depth and bifurcation angles of the microgrooves were illustrated in Figure 5.3(a). In order to study the mechanotransduction underlying the SMC sheet near the bifurcations, a thin layer of hydrogen gel embedded with 0.2 μ m fluorescent microbeads was coated onto the micropatterned PDMS substrate to measure the cell-layer-induced deformations via CTFM assay [67]. Fluorescence microscopy (Figure 5.3(b)) indicated that PAG

layer was successfully formed and the microbeads were uniformly distributed on top of the PDMS substrate. The fluorescent microbeads were densely coated to ensure accurate measurement of the collective CTF. After chemical activation and collagen coating, HaSMCs were seeded and cultured in these bifurcate microgrooves to form confluent cell layers. The phase contrast images (Figure 5.3(c)) showed the micropatterned SMCs grown on the platforms after 3 days, indicating the intact cell sheets inside the microgrooves.

Figure 5.3. The experimental process to functionalize the bifurcate microgrooves for cell culture. (a) Designed scaffolds with different bifurcation angles. (b) Fluorescence images of micropatterned PAG-PDMS scaffolds under 10X magnification. The microbeads were densely and uniformly distributed in the PAG layer. (c) Phase contrast images of SMCs grown inside the bifurcate microgrooves under 10X magnification. The images were taken after 3 days' cell culture. Scale bar: 250 μm .

In order to study the influences of the micropatterned scaffolds, primary HaSMCs were cultured inside the PAG-PDMS microgrooves with four different bifurcation angles (30° , 60° , 90° and 120°) and on the flat PAG-PDMS substrate (NC) for 5 days. The phase contrast images of seeded cells were taken every day to

record the morphological changes and orientation-specific adaptation processes. Cells located within the red circle (Figure 5.2) were chosen to study the impacts caused of different bifurcation angles. When cells became confluent after 3 days culture, their density (Figure 5.4(a)) and orientation-specific adaptation (Figure 5.5(b)) were compared with those on day 0 (4 h after cell seeding).

Figure 5.4. The change of cell density and morphology of SMCs grown near bifurcations and on flat PAG-PDMS substrate. (a) Density comparison between day 0 and day 3. (b) Change of CSI during 5 days culture. Error bars represent mean values $\pm$ SD from at least 45 cells in 3 different frames.

The density of SMCs grown under four types of bifurcate microgrooves and on flat substrates almost doubled from day 0 to day 3 (Figure 5.4(a)). And there was no significant difference of the cell density among different scaffolds. The results implied that the microgrooves had no considerable effect on the general cell proliferation. Characterization of cell shape index (CSI) revealed the temporal change in cell morphology under different scaffolds. All scaffolds were initially covered with adherent SMCs that adopted rather round shape with CSI of 0.7~0.8 (Figure 5.4(b)). Significant decrease of CSI was observed from day 0 to day 3 indicating that the SMCs elongated to a spindle-like shape due to cell-cell interactions. After 3 days culture, CSI on all scaffolds remained at about 0.3, which

was similar to the natural cell morphology *in-vivo*. On the other hand, there was no considerable difference of the stabilized CSI under different bifurcation angles or on planar substrates. This result indicated that the microgroove constraints did not affect the morphological changes of the SMCs.

The orientation-specific adaptation of collective cell sheet was analyzed to investigate how SMCs responded to different bifurcation angles. The phase contrast images of cells grown in a 30D bifurcate microgroove on day 0 and day 3 respectively (Figure 5.5(a)) indicated notable morphological switch and orientation-specific adaptation process. All the adaptation angle α decreased significantly (t-test, p-value < 0.05) on day 3 due to cell alignment with either branch of the bifurcate microgroove (Figure 5.5(b)). Moreover, in order to compare the level of orientation changes under four bifurcation angles, the variation of adaptation angles from day 0 to day 3 (Figure 5.5(c)) were calculated based on the data in Figure 5.5(b). The results exhibited significantly lower level of cell orientation change in the 90° bifurcate microgroove compared to other microgrooves with different bifurcation angles, suggesting that SMCs near a 90° bifurcate blood vessel responded more slowly to the geometrical cues induced by the surrounding microenvironment.

Figure 5.5. (a) Phase contrast images of SMCs grown in a 30° bifurcate microgroove on day 0 and day 3, respectively. The images were taken under 10X magnification. Scale bar is 250 μm . (b) Orientation-specific adaptation angle of SMCs on day 0 and day3. (c) Orientation changes between day 0 and day3 under different bifurcation angles. Error bars represent mean values $\pm$ SD from at least 45 cells in 3 different frames. *significant at $p < 0.05$.

5.3.2 Distributions of Collective CTF and Stress near Bifurcations

Using the similar methodology as developed in our previous study [131], the collective cell traction forces of SMC sheet near the branched microgroove (Figure 5.6(a)) were analyzed using the displacement field of the fluorescent microbeads imbedded in the substrate. And the selected areas for analysis were marked as red rectangles (Figure 5.6(a)). The stress distribution on PAG-PDMS substrate was analyzed using finite-element software ANSYS®12. The geometry of PAG gel layer (dimension: 167 $\mu\text{m} \times 268 \mu\text{m} \times 10 \mu\text{m}$) was discretized by isoparametric, 20-node

brick element SOLID186. The microbead displacements computed by PIV method (Figure 5.6(b)) were input as the boundary conditions at corresponding nodes underneath SMC layer. Because the traction forces at the cell-substrate interface were randomly oriented, we analyzed the von Mises stress to represent the energetically equivalent magnitude of all principle stress components with regard to the uniaxial tension. The von Mises stress is defined as

$$\sigma_{\text{vonMises}} = \sqrt{\frac{1}{2}[(\sigma_1 - \sigma_2)^2 + (\sigma_2 - \sigma_3)^2 + (\sigma_3 - \sigma_1)^2]}$$

where σ_1 , σ_2 , σ_3 represent three principal stresses, respectively. High von Mises stress indicates either a high tensile or compressive stress.

Figure 5.6. The distributions of collective CTF and von Mises stresses underlying SMC sheets near different bifurcations. (a) Phase contrast images of SMCs grown in branched microgrooves with different bifurcation angles at day 3. Red ovals represent the edge next to the channel wall. Images were taken under 40X magnification. Scale bar: 100 μm . Pixel dimension (height $\times$ width) of the selected frame: 500 $\times$ 800 (1 pixel $\approx$ 0.3346 μm). (b) The distributions of microbeads

displacements in the selected region induced by the SMCs. Window sizes: 16×16 pixels. (c) The distributions of von Mises stresses simulated by FEM.

Figure 5.6(a) showed the collective SMCs grown near different bifurcations after 3 days culture. The cell morphologies on all four platforms were quite similar, while the cell orientations were adapted to the different bifurcation angles. The characteristic distributions of collective CTFs with smaller force in the center region (Figure 5.6(b)) indicated the formation of intact cell layer [131]. The red oval areas outlined in Figure 5.6(b) were the locations of the different bifurcations. Compared to the traction forces adjacent to the channel walls of 30° , 60° and 120° bifurcations, the traction forces near the 90° bifurcation were considerably lower (Figure 5.6(b)). These results suggested the lower tendency of SMCs to migrate along the 90° bifurcate microgroove. Moreover, all the traction forces located left to the oval areas where were the branched points were relatively small. As SMC layers in-vivo are located at the outer layer of the endothelium, the smaller traction forces near the bifurcating points exerted onto the endothelium, together with the high wall shear stress from blood flow [193, 195], become a new localizing factor for early atherosclerotic development.

The corresponding distributions of von Mises stresses near different bifurcations were shown in Figure 5.6(c). In the 90° bifurcate microgroove, the lower stress zone was found to be located near the channel walls, which was quite different from the situations under other three bifurcation angles (Figure 5.6(c)). This finding further suggested that the mechanotransduction of SMC layers was affected by bifurcation angles. Previous studies usually focus on the biochemical aspects of SMC development in late stages of atherosclerosis, such as how the growth factors and cytokines promote phenotypic changes of SMCs and then

further induce SMCs migration and proliferation [188]. On the other hand, the present study provides further insight, from the mechanotransduction point of view, that SMC layer may be also related to the early localization of atherosclerosis. Moreover, the intact low stress zone around the bifurcating micro-walls further indicated that atherosclerosis lesions tend to occur around 90 °bifurcation.

5.3.3 Immunofluorescent Staining and RT-qPCR Analysis

As one of the most important assembly proteins for microfilaments, actin expressed by SMCs within functional vascular tissues is responsible for maintaining the structural integrity and contractile functions [186]. In order to examine the orientation-specific adaptation of cytoskeleton near bifurcations, the SMCs after 3 days culture were immunostained and imaged with fluorescence microscopy. The organization of F-actin filaments (red, Figure 5.7(a)) clearly indicated the presence of elongated and stressed fibers inside the SMCs. Meanwhile, SMC layers grown near 30 °, 60 ° and 120 ° bifurcations aligned much better along the microgrooves than those grown near the 90 ° bifurcation. This finding was consistent with the results with regard to the distributions of collective CTFs and von Mises stresses (Figure 5.6).

Figure 5.7. Immunofluorescence staining of F-actin (red: TRITC) and nuclei (blue: DAPI) of the HaSMCs grown near different bifurcations. Images were taken under 40X magnification. Scale bar: 100 μm .

In addition, we used real time qPCR assay to quantitatively measure the gene expression levels of different proteins on micropatterned substrates relative to those on the flat PAG-PDMS substrate. Alpha smooth muscle actin (αSMA), as one of the six mammalian isoforms of the cytoskeletal proteins, plays a key role in cellular contractility. Figure 5.8(a) indicated relative αSMA expression level of SMC sheet cultured in different bifurcate microgrooves after 3 days culture. Although down-regulated αSMA was found in dedifferentiated (synthetic) vascular SMCs [196], significant down-regulation of αSMA induced by micro-walls was reported in one of our previous studies [194]. Similarly, in the present study, the general αSMA expression levels of SMCs decreased under the physical constraints

of microgrooves in the present study (Figure 5.8(a)). Furthermore, α SMA expression level in 90 ° bifurcation was significantly higher than those in 30 °, 60 ° and 120 ° bifurcations. This interesting result suggested lower response of SMCs to the physical cues in 90 ° bifurcate microgroove.

In order to quantitatively verify the hypothesis about the correlation between localization of atherosclerotic plaques and vascular bifurcation angles, two typical SMC marker genes related to SMCs were analyzed by RT-qPCR including matrix metalloproteinase-2 (MMP2) and β -catenin. MMP2 is a protease responsible for extracellular matrix degradation during changes in vascular structure. It has been shown that the stimulation of MMP2 is closely related to the proliferation and migration of vascular SMCs [197]. Significant up-regulation of MMP2 *in vivo* has been found at vulnerable sites in many pathological situations such as atherosclerotic plaques [198]. Figure 5.8(b) indicated significantly up-regulated MMP2 expression level of SMCs grown in 90 ° bifurcate microgroove, while the same gene expression were down regulated under other three bifurcation angles. The result revealed that 90 ° vascular bifurcation is more susceptible to develop atherosclerosis lesions compared to other bifurcation angles.

As a protein to regulate cell-cell adhesion, β -catenin interacts with cadherin at one end and binds to α -catenin at the other end to further associate with cytoskeletal proteins. The Wnt/ β -catenin signaling pathway is able to regulate osteoblastogenesis and vascular SMC calcification, which is associated with stable atherosclerotic plaques [199, 200]. In Figure 5.8(c), significant up-regulation of β -catenin expression level was found in SMCs grown cultured in 90 ° bifurcation, while the variation of β -catenin expression under other bifurcation angles was insignificant. This result again implied that 90 ° vascular bifurcation was more

amicable for formation of atherosclerotic plaques. Together with the above results for MMP2, the quantitative analyses of these two important marker genes confirmed that vascular bifurcation angle affected the mechanotransduction of SMC layers associated with early atherosclerotic development, which could be another important pathological factor in addition to the widely investigated biochemical or flow-induced physical factors leading to atherosclerosis.

Figure 5.8. The relative gene expression levels of α SMA (a), MMP2 (b) and β -catenin (c) by HaSMCs grown in different bifurcate microgrooves at day 3

measured by RT-qPCR. HaSMCs grown on flat PAG-PDMS substrate on day 3 were used as calibrator. The expression levels, quantified by RT-qPCR, are normalized to the housekeeping gene GAPDH. *significant difference at $p < 0.05$.

5.4 Conclusions

In this study, the collective cell traction force microscopy assay was applied to study the mechanotransduction of smooth muscle cell sheet cultured in microgrooves with different bifurcation angles. The cell density, shape and orientation-specific adaptation during branched cell sheet formation were analyzed quantitatively. The results revealed the distributions of collective cell traction forces and von Mises stresses in response to different bifurcation angles. It was found that there was a characteristic zone with relatively low stress near the 90° bifurcation. Interestingly, using optical microscopy and immunofluorescence staining, we found that orientation-specific adaptation of the SMCs in the 90° bifurcate microgroove over 3 days culture was significantly less than those under other bifurcation angles. RT-qPCR assays further revealed relatively increased α SMA expression and significantly up-regulated expressions of MMP2 and β -catenin in the SMCs cultured under 90° bifurcate microgroove. From both mechanistic study and gene analysis, the SMC layer exhibited less tendency of responding to 90° bifurcations with distinct proliferative and migratory state, indicating that the mechanotransduction of SMCs was affected by the geometrical cues induced by bifurcation angles. This interesting finding may provide further insight on the localization and progression of atherosclerotic lesions, however, from totally new

viewpoint related to the mechanotransduction induced by the geometrical and physical cues.

Chapter 6 Overall Conclusions and Future Work

6.1 Overall Conclusions

During the past two decades, novel biomaterial scaffold for cell attachment and culture have been developed for applications in tissue engineering, biosensing and regeneration medicine. The tissue engineering of blood vessel remains to be a challenge due to the complex three-layer histology involved. In general, the reconstruction of tunica media from SMCs that mediate vasoconstriction and vasodilation of circulatory system is critical for engineering functional blood vessels. To date, the mechanotransduction during parallel and circumferential alignments of SMC layers in tunica media remains to be elucidated.

In my PhD project, I first started with the improvement of microfabrication methodology and the development of collective CTFM assay to probe the mechanotransduction of SMC layers via the hydrogel deformations. A variety of methods have been developed to measure CTFs of both individual cells and collective cells during the past few years, including cell-populated collagen gel (CPCG) [118], thin silicone membrane [56] and force sensor array. More recently, the arguably most acceptable method for quantifying mechanotransduction at the cell-substrate interface is CTF microscopy (CTFM) developed by Dembo and Wang [119]. Until now, all the derived CTFM methods suffer from a same problem – the accuracy of the displacement calculated from the microbeads. In addition, CTFM cannot measure the real-time stiffness of entire cells, while micropost arrays enables the quantitative study on the force-sensing mechanisms [63, 64]. CTFM method can achieve a broader application if the topology can be extended to 3D matrix. Compared to other methods, CTF microscopy (CTFM) assay is particularly

convenient to quantify the individual or massive cell-substrate mechanotransduction on a highly compliant substrate. CTFM allows for biomimetic study of cell morphogenesis in vitro and characterization of mechanical stresses produced by cellular interaction with surrounding physiological environment, which could affect the SMC proliferation, migration and hence their role in regulating the vasoactivity of blood vessels.

In Chapter 3, microfabricated arrays of discontinuous microwalls coated with fluorescence microbeads were developed to evaluate the cellular mechanical responses. Firstly, the system was exploited for stimulating the formation of highly aligned orientation of SMC in native tunica media. Secondly, atomic force microscopy in combination with regression analysis was applied to measure the elastic modulus of polyacrylamide gel layer coated on the discontinuous microwall arrays. Thirdly, conventional traction force assay for single cell measurement was extended for applications in three dimensional cell aggregates. Then the biophysical effects of discontinuous microwalls on the mechanotransduction of SMC layer undergoing cell alignment were probed. Generally, the cooperative multiple cell-cell and cell-microwall interactions were accessed quantitatively by the newly developed assay with the aid of finite element modeling. The results show that the traction forces of highly aligned cells lying in the middle region between two opposing microwalls were significantly lower than those lying adjacent to the microwalls. Moreover, the spatial distributions of Von Mises stress during the cell alignment process were dependent on the collective cell layer orientation. The immunostaining of SMC sheet further demonstrated that the collective mechanotransduction induced by 3D topographic cues was correlated with the reduction of actin and vinculin expression. Also, the online 2D LC-MS/MS analysis

verified the modulation of focal adhesion formation under the influence of microwalls through the regulation in the expression of three key cytoskeletal proteins.

In Chapter 4, concentric microwalls fabricated on a PDMS substrate were developed for CTFM assay of SMC layer. Firstly, the cell shape, density and alignment orientation of SMCs during circumferential cell sheet formation were measured during 7-day cell culture. Secondly, a collective CTFM assay was carried out to measure the traction forces exerted by the confluent cell layer at various stages of circumferential alignment. It was found that the mechanotransduction of SMC layer grown between the circular microwalls was significantly altered compared to the SMC layer grown on a flat substrate. The results indicated the presence of an optimal region for formation of circumferentially aligned SMC layer verified by both quantitative analysis and biomechanical study. Moreover, the distributions of traction forces and von Mises stresses during cell alignment were affected by the cellular mechanotransduction from the extracellular physical constraints and cell-cell interactions. The immunofluorescence staining and RT-qPCR analyses of actin filaments further revealed the relation between the monolayer-generated traction forces and the direction of aligned actin filaments.

Atherosclerotic lesions are mainly found at sites of low or disturbed blood flow, like arterial branch points and bifurcations. Although migratory and proliferative activities of SMCs together with phenotype switch are widely studied, the mechanotransduction of SMC layer underlying atherosclerotic plaques remains unclear. In Chapter 5, bifurcations with different angles fabricated with PDMS and PAG were developed for CTFM of SMC layer. First, the cell shape, density and orientation-specific adaptation of SMCs during branched cell layer formation were

measured during 5-day cell culture. The orientation change caused by 90-degree bifurcation was significant less than other situations. Second, the collective CTFM assay was applied to measure the cell sheet traction forces around the bifurcation points. The results indicated the mechanotransduction of SMC layers grown between the bifurcating micro-walls. And the distributions of traction forces and von Mises stresses were significantly affected by bifurcation angles. The relatively low stress zone occurred around 90-degree bifurcation provided a novel explanation for the atherosclerosis development. Third, the immunofluorescence staining and real time quantitative polymerase chain reaction (RT-qPCR) analysis provide further support for the monolayer-generated traction forces and localizations of atherosclerotic lesions. The less tendency of cells respond to 90-degree bifurcations was proved by the relatively increased α SMA expression.

Overall, in addition to the biophysical understanding, these grown collective cell sheets can be potentially used as a patch for repairing damaged artificial arteries. And the findings may shed new light on designing the future engineered tissues in regenerative medicine for treatment of vascular diseases. Besides, the interesting finding may provide further insight on the localization and progression of atherosclerotic lesions from another new viewpoint related to the mechanotransduction induced by geometrical cues.

6.2 Future Work

In addition to the traditional CTFM assay, novel methods to measure collective CTFs can be developed based on this thesis. Recently, our detection of the CTF measurement is cooperated with optical fiber sensor to probe the collective CTFs of the cell layer between two adjacent microwalls. Optical fiber sensors have

been intensively studied due to their many intrinsic advantages such as electrically passive operation, long life-time and immunity to electromagnetic interference. One of the optical fiber sensors, Sagnac fiber loop we used based on polarization maintain fiber with relatively high strain and curve sensitivity. The polarization-maintaining fiber has been immersed into PDMS as a mechanotransduction sensor.

The other future projects that can be based on this thesis will be the development of 3D traction force measurement and the determination of cellular mechanobiological behaviors in different cell or tissue systems. The development of novel experimental and numerical methods for determining 3D CTFs, which have similar elastic moduli and physiological features to *in vivo* situations, would be an important tool to study the cellular mechanosensing and mechanotransduction. As the clinical trial of *in vivo* experimental studies on human body is unsafe, expensive and time consuming, *in vitro* culture systems that mimic the native microenvironment would become a promising approach for tissue engineering, drug delivery and toxicology study.

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Appendix: Publication

- 1) Zhang, Y., Kang, Y., Chan, V., *Cell Mechanotransduction in Three Dimensional System*, 2015, *In writing*.
- 2) Zhang, Y., Menon, N.V., Li, C., Chan, V., Kang, Y., *The Role of Bifurcation Angles on Collective Smooth Muscle Cell Biomechanics and Atherosclerosis Development*, 2015, RSC Biomaterials Science, *In press*.
- 3) Menon, N.V., Chuah, Y.J., Phey, S., Zhang, Y., Wu, Y., Chan, V., Kang, Y., *A microfluidic assay to study the combinatorial impact of substrate properties on mesenchymal stem cell migration*, 2015, ACS Applied Materials & Interfaces, 2015.**7**(31):p. 17095-17103.
- 4) Zhang, Y., Li, C., Chan, V., Kang, Y., *Biomechanistic Study of Smooth Muscle Cell Sheet during Circumferential Alignment in Circular Micropatterns*, ACS Biomaterials Science & Engineering, 2015.**1**(7):p. 549-558. **(2015 July cover story)**
- 5) Chuah, Y.J., Zhang, Y., Wu, Y., Menon, N.V., Goh, G.H., Lee, A.C., Chan, V., Zhang, Y., Kang, Y., *Combinatorial effect of substratum properties on mesenchymal stem cell sheet engineering and subsequent multi-lineage differentiation*, Acta Biomaterialia, 2015.**23**:p. 52-62.
- 6) He, T., Zhang, Y., Lai, Alvin. C.-K., Chan, V., *Engineering Bio-Adhesive Functions in Antimicrobial Polymer Multilayer*, Biomedical Materials, 2015.**10**(1):015015.
- 7) Zhang, Y., Ng, S. S., Wang, Y. L., Feng, H. X., Chen, W. N., Chan-Park, M. B., Li, C., Chan, V., *Collective cell traction force analysis on aligned smooth muscle cell sheet between three-dimensional microwalls*. Interface Focus, 2014. **4**(2).
- 8) Zhang, Y., Ng, S. S., He, T., Fang N., Neoh, K. G., Kang, E. T., Chen, W. N., Chan, V., *Effect of adhesive ligand on cell deadhesion kinetics on poly(N-isopropylacrylamide)*. Bio-Medical Materials and Engineering, 2014. **24**(2): p. 1433-1445.