Cell mechanics study on single cell and collective behavior

Han, Jingjing

2017

Han, J. (2017). Cell mechanics study on single cell and collective behavior. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/70071>

<https://doi.org/10.32657/10356/70071>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**CELL MECHANICS STUDY ON SINGLE CELL AND
COLLECTIVE BEHAVIOR**

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

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**CELL MECHANICS STUDY ON SINGLE CELL AND
COLLECTIVE BEHAVIOR**

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**A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy**

2017

Abstract

The investigation of cell-cell and cell-substrate interactions from the perspective of physics is fundamental to the study of cell mechanics. Based on the fact that cells are exposed to various biochemical as well as mechanical cues from their environment, we have conducted research on the influence of these cues on their biomechanical behaviors. Specifically, we focus on single cell contraction and cells' collective motion in the space of 2-dimensional, and single cell morphology study in the space of 3-dimensional.

Fibronectin and collagen type I are extracellular matrix proteins that play important role in the modulation of cell mechanics and other physiological processes such as angiogenic sprouting. In the first project, we have conducted comparative analyses of cell contraction on fibronectin and collagen. Our study has found that during the early stage of cell spreading, human umbilical vein endothelial cells (HUVECs) exhibit stronger and more directed contraction on fibronectin than collagen type I coated microposts. In Particular, greater cell traction force, larger spreading area, and higher directionality are all observed in microposts coated with fibronectin.

In addition to being regulated by biochemical signals transmitted through various molecules, cellular functions also depend on the mechanical properties of the substrate like its nanotopography. Meanwhile, the coordination among cells is important in maintaining the integrity of group behavior during collective cell migration. The key question we investigate in the second project is on the influence of the substrate property of roughness on collective cell migration. Through our experimental investigation and analysis, we have uncovered that cells tend to display a slower and less directional collective migration on nanoroughened substrate.

Moreover, the expression of adhesion proteins is also observed to decrease on a rough substrate.

Currently, there are various in-vitro studies of cell morphology in 2D, but this does not represent the more physiological profile of in-vivo cells. Hence, in the third project, we have initiated a study on cell attachments in 3D nanoroughened scaffold to mimic the in-vivo cellular environment. Our objective is to investigate how matrix nanotopography impact cell function in 3D relative to 2D. We found that cells exhibit distinct adhesions with different number of focal adhesions between a smooth scaffold and a rough scaffold with nano-spikes. More importantly, our analysis on cell surface curvatures indicates significant correlation between the distribution of cortical actin and the local curvature of cell surface.

In summary, our studies have provided insights into the physics of cellular mechanics through the complex interactions of a single cell or a collection of cells with the biochemical and mechanical cues of the environment. I believe this thesis had brought us closer to a deeper understanding on the mechanical behavior of cells with potential application in the design of biomedical devices for the purpose of cellular manipulation.

Acknowledgements

First and foremost, I want to give my sincere appreciation and thanks to my supervisor, Prof. Chew Lock Yue for his continuous support, guidance, and invaluable advice even during my hardship. He has been a tremendous mentor to teach me consciously and un-consciously, how good physics research is done and how to think critically like a scientist. I appreciate all his enormous contributions of time, patience, knowledge, encouragement and funding to make my research inspiring, and productive. His brilliant comments and suggestions are of great insight for me. I am also grateful for the excellent role model he has provided as a successful professor and physicist.

I would also like to thank my group members for all the happy time together as great friends and all the precious advice to my research as amazing colleagues. Especially during our weekly meeting, all the members are actively involved in discussions for everyone's latest research progress and generously propose great suggestions to others' work.

The collaborators also contributed immensely to my work for the fruitful discussion and continuous support throughout our research. In regards of cell contraction study, I want to express my thanks to Prof. Tan Suet Mien, who is also my thesis committee member, and Miss Tan Hui Foon. They devoted so much intelligence and diligence to our joint work and they teach me a lot about experimental biology. I also want to thank Prof. Kang Yue Jun and Dr. Nishanth Menon for their contributions in our study on cell collective migration, especially for the illumination on substrate design and migration mechanism analysis.

In my third year of PhD study, I had a fantastic academic attachment experience in Dr. Lin Kenghui's lab in Academia Sinica, Taiwan. I would like to appreciate Dr. Lin's hospitable host and the great suggestions on my research. I would not have fulfilled the collaborating project on 3D cell morphology without her contributions to 3D scaffold design and data analysis. Her great performance as a female scientist as well as a mommy for three young kids also inspire me a lot on the balance of work and family. I also want to give thanks to all

the members in Dr. Lin's lab for their kind help during my early days in Taiwan and all the inspirational discussions on the experiments.

For this dissertation I would like to thank my thesis committee members, Prof. Phan Anh Tuan and Prof. Tan Suet Mien for their time and insight suggestions on my study.

I gratefully acknowledge Nanyang Technological University for the research opportunities and financial support throughout my study.

Finally I want to give special thanks to my dearest family. I am so grateful to my parents for all the sacrifices that they have made on my behalf. Their unconditional love and support for me is what sustained me thus far. I would also like to thank my grandma, who has been in heaven now, for taking care of me since I was an infant.

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Chapter 1

1 Introduction and motivation

1.1 Study scope of cell mechanics

Cell mechanics is a sub-field of biophysics focusing on the mechanical properties and mechanical behaviors of living cells and how it correlates to cellular biochemical functioning. As a newly-emerging cross-disciplinary field, it involves the study of physics, biology, materials, and computer science. Unlike traditional biological research which mainly focused on descriptive and qualitative study of cell growth, modern new techniques in bioengineering and computational science are applied to pave the way for a more quantitative investigation on cell mechanical behaviors. Living cells live in a complex environment in which cells are exposed to the neighboring cells as well as the extra cellular matrix (ECM). Therefore, cellular behavior results from a complex combination of influence from other cells and the property of ECM. Many cell types are highly responsive to physical as well as biochemical stimuli from the surroundings [1], after which mechanosensitive and chemical signaling pathways are activated to help adjust the cell behavior [2], ranging from changes in cell morphology to changes in cell phenotype [3]. As is well known that cell mechanics is closely related to the pathophysiology of disease, the change of cell mechanical behavior could be used as possible detection of associated disease. For example, in the vascular system, reduced cell deformability and enhanced adhesiveness have great influence on the microcirculatory of blood flow and could even result in cerebral and organ failure [4]. Therefore, the understanding of cellular mechanical behavior could contribute to the study of disease mechanism from the cell mechanics perspective. Also, cells are found to be capable of adjusting their behavior,

like morphology, adhesion, and even differentiation to the mechanical cues from the external environment. For example, it was reported that epithelial cells were able to modulate the shape of intercellular junctional contact based on the physical cellular properties and disordered proliferation [5]. Also, stem cells were found to adjust their differentiation based on the substrate stiffness which is sensed by the active pulling from cells on the environment with forces generated by actomyosin motor proteins. Thus, osteogenesis was more likely to happen on rigid substrate while adipogenesis was more likely to happen on soft substrate [6]. Moreover, differentiated cells are reported to be living in a stable environment of isometric tension, which is usually exerted by a cell with an equilibrium force to the local tension from the environment [7].

1.2 Cell traction force of single cell

Cell traction force is the most important and fundamental concept of cell mechanics study. Adherent cells need to get attached to a substrate in order to grow and exert contractile force on it. As the endogenous forces produced by cells, cell traction force could be transferred to substrate through integrin-mediated cell focal adhesion sites [8] (Figure 1. 1). The leading edge of the cell usually develops a lamellipodia, where strong traction forces pull the cell forward. The small net imbalance in the traction forces across the length of the cell, between the front and rear, drives the cell forward [9] [10].

Mechanotransduction through integrins

Figure 1. 1 Schematics of cell traction forces transmitted through ECM (A) or directly to cell surface (B). Cell traction force was transmitted to the focal adhesions anchored at integrins via substrate adhesions or cytoskeletons. Internal tension and force transmitted through cell-cell contacts arrive at the focal adhesions via cytoskeletal filaments. Force concentrated within focal adhesions could stimulate the clustering of α and β integrin receptors and thus induce recruitment of focal adhesion molecules which were directly connected to microfilaments and indirectly to microtubules and intermediate filaments (reproduced from Figure 1 in [11]).

1.2.1 The role of cell traction force in cell function regulation

It was broadly studied that cell traction forces play critical roles in regulating biological and physiological processes, including inflammation, wound healing,

angiogenesis, remodelling of the surrounding tissues, and metastasis [12, 13]. For example, at the early stage of wound healing, traction forces which points away from the wound were generated to support cell crawling, which was for driving wound closure [14]. Also, in three-dimensional tissue engineering, the spatial distribution of mechanical stress across the epithelium was reported to be defined by tissue geometry [15]. Evidence strongly suggested that the generation of these cellular forces primarily come from the interactions between myosin II motor proteins and different types of cytoskeleton networks [16-18] and cytoskeleton also plays important role in transmitting forces to the surrounding matrix [19]. It was reported that in either 2D or 3D environment the disruption of actin, myosin and microtubules would disturb the generation of cell traction force [19]. On the other hand, increased actomyosin contractility within cells accompanying an established actin cytoskeleton network could enhance cell traction force, while it would inhibit the temporal force redistribution [20]. In the meantime force generation is also coordinated with other cellular events, like cell adhesion and deadhesion [21]. For example, during the typical cell movement process cell extends a protrusion in motion direction by actin polymerization, and as adhesion happens in the leading edge, deadhesion occurs at cell body and rear to help generate contractile forces for pulling the cell body forward [10].

1.2.2 Popular methods for cell traction force measurement

The characterization of mechanical interactions is the key for us to have a deeper and quantitative understanding of cell-cell communication and cell-substrate adhesion. In recent years, several methods have been proposed to measure cell traction forces with different transparent, non-toxic, and flexible substrata [21], which facilitates the use of optical microscopy and biocompatibility with cell culture. The popular methods

include cell-embedded collagen gel, cell traction force microscopy, thin silicone membrane, and force sensor arrays [12]. For example, the original idea was based on the fact that the compressive force exerted by adherent cells could result in wrinkles on surface (Figure 1. 2). The pattern of wrinkles could be converted to cell traction force map [21]. Due to the difficulty of modelling involved in the conversion of wrinkle pattern and its shortcoming in measuring complex force pattern rather than compressing force alone, microposts made of PDMS (Polydimethylsiloxane) stands out for its advantage of easy and accurate quantification of cell traction force.

Figure 1. 2 The cell contraction of moving fish keratocyte induced wrinkle on a silicone substrate. Arrow suggests the direction of motility (reproduced from Figure 1 in [21]).

1.2.3 Advantage of the application of PDMS microposts in cell mechanics study

Siloxane-based polymers like PDMS are popular materials in fabricating stamps and molds in soft lithography industry [22]. The Young's modulus of PDMS is around 1 MPa, making it a moderately stiff elastomer [23]. Besides, PDMS is optically clear, transparent at 300 nm, inert and non-toxic, and it becomes hydrophilic from a very hydrophobic state through simple plasma treatment. Hence, it is a suitable material for biological applications like microfluidics and micropattern.

Micropost array is composed of uniformly distributed grids of deformable silicone pillars, and different geometries could be applied for various applications. Nowadays, the most popular fabrication method is the application of high-resolution photolithography and deep reactive ion-etching techniques to achieve the fabrication of silicon micropost masters in a large batch [24]. Compared to modulating the cross-linker ratio of synthetic ECM analogs like polyacrylamide or polyethylene, it is more precise to control substrate rigidity by altering the ratio of pillar radius to height as well as spacing distance. This avoids the problem of changing the properties of the material, which includes backbone flexibility and the porosity of hydrogels. As a result, the use of microposts broaden the study on the influence of substrate mechanical properties on cell growth from sensing stiffness between adhesion sites at microscale to sensing local alterations in receptor-ligand binding at nanoscale [24]. Cell contraction, spreading, and focal adhesions can all be examined on microposts after cells are cultured on it.

1.2.4 Two methods of cell traction force quantification based on micropost deflections

Based on Euler-Bernoulli beam theory, an approximation of the magnitude of the nominal spring constant of the beam K can be quantified as long as its Young's modulus (E), diameter (d), and length (L) is known:

$$K = \frac{3\pi E d^4}{64 L^3} \quad (1.1)$$

Finite element analysis is then applied to characterize the spring constant of the post [24]. Due to cell contraction, the posts occupied by the cells become deflected (Figure 1. 3). Using beam bending theory, the cell traction force can be accurately resolved from the deflection of the posts through image analysis techniques by tracking the centroid of each post.

Figure 1. 3 Scanning electron microscopy (SEM) image of a 3T3 fibroblast cell spreading on the microposts shows posts' deflection (reproduced from Figure 2.1 in [25]).

There are two methods to determine post deflections. The first method is known as the Top-Idea method. It involves labelling the top surfaces of the posts occupied by the cells and compares them against the theoretical unbended post positions before cell occupation. The difference between them gives the deflection (Figure 1. 4A). The second method is the Top-Base method. In this method, both the top and bottom of the microposts are captured and the locations of the bottom of the posts are used to represent the locations of the undeflected posts [26] (Figure 1. 4B). As for imaging of the microposts, common phase contrast microscopy works well enough for the Top-Ideal method and no extra staining of posts is required, while the posts need to be labelled with molecular probes for visualisation in the Top-Base method. Moreover, Top-Base method is more sensitive to vibrations from the external environment compared to Top-Idea method. Therefore, we chose Top-Idea method to calculate post deflections in our study.

Figure 1. 4 (A) For T-I Method, unoccupied posts (gray) are used to determine the original positions of the occupied grid with linear fitting method. Deflections of occupied posts (white) are equal to the differences between the locations of actual centroids (center dot) and locations of the theoretical undeflected centroids

(intersections of dashed lines). (B) For T-B Method, white arrows indicate the deflections of posts. Both the top layer of microposts (light gray) and the base surface (dark grey) are captured. Deflections equal to the difference of centroid position of posts (reproduced from Figure 2.C and Figure 4.A in [26] respectively).

1.2.5 The role of focal adhesion as mechano-sensor

Several different types of adhesions are formed by cells following engagement with the extracellular matrix (ECM). These cell-matrix adhesions can be classified on the basis of various factors, such as protein composition, lifespan and proteolytic properties. Based on the lifespan and size, the adhesion structures are classified into focal contacts (FCs), focal adhesions (FAs), and fibrillary adhesions (FBs). Focal contacts (FCs) are small and the shortest-lived adhesion structures. They are observed to assemble and disassemble within the order of seconds or minutes. FAs are larger and more stable than FCs, with lifespan on the order of tens of minutes [27]. Fibrillar adhesions are even larger and more stable than focal adhesions, which are found to be highly concentrated in tensin and $\alpha 5\beta 1$ integrin [28].

Focal adhesions (Figure 1. 5) are sites where integrin and proteoglycan mediated adhesion links to the actin cytoskeleton. The components of focal adhesions are diverse and include scaffolding molecules, GTPases, and enzymes such as kinases, phosphatases, proteases, and lipases [29].

Figure 1. 5 Fluorescent image of a fibroblast like cell cultured *in-vitro* with vinculin (green) containing focal adhesions, actin stress fibers (red). (reproduced from figure in <https://www.pediatrics.wisc.edu/research/research-groups/huttenlocher>).

Besides playing the role of physical linkages between substrate and cells, focal adhesions are also important as signaling hub to concentrate and direct numerous signaling proteins, like $\alpha v \beta 3$ and $\alpha 5 \beta 1$ integrins, vinculin, paxillin, talin, zyxin, tensin, protein tyrosine kinases, and phosphatases [12]. Focal adhesions are also sensitive to the change of cellular environment, for example, alterations in ECM composition might change the relevant signaling complex composition [29]. Besides, it is known that the recruitment of focal adhesion kinase (FAK), vinculin, together with paxillin to focal adhesions, could happen before tyrosine phosphorylation [29].

In particular, mechanosensing is critical to the regulation of cell adhesion and adhesion-associated actin cytoskeleton organization. Cells can transduce mechanical signals into a biochemical response. This process is known as mechano-transduction.

Mechanical forces applied to cells, which transmit signals ~40-fold faster than diffusion of some chemical signals, can cause conformational changes in heterodimeric integrin proteins in cell-matrix adhesion sites. This can ultimately alter signaling pathways and gene expression [30]. In addition to integrin binding, mechanical tension also serves an indispensable role in inducing the relocation of ribosomes and mRNA to focal adhesions [7]. Moreover, cells could adjust focal adhesions in response to other exterior physical forces [29]. It was found that total traction force and total focal adhesion intensity decreased almost simultaneously at an extremely small time scale of faster than a few seconds [31]. Past studies also have shown that focal adhesions increase with cell traction force [24]. More specifically, micromolded elastomeric micropost arrays have been used to map traction forces to individual FAs and to spatially characterize subcellular-level distributions of focal adhesion area and cell traction force. It was found that higher stress was exerted on peripheral focal adhesion, which was correlated with the enhanced traction force at cell periphery. In addition, with the precise micropatterning of hydrogels, it was uncovered that cell traction force was primarily determined by measuring the distance from cell center to the periphery. In consequence, distance could control traction force by regulating focal adhesion size [32].

1.2.6 The influence of ECM proteins on cell function

Cells in tissues are mechanically coupled both to the ECM and neighbouring cells. As a result, the biochemical signals from ECM proteins is an important factor that influences cell behavior. Ligand-induced clustering of integrins plays an important role in mechanotransduction because they provide physical linkages between cytoskeleton and ECM. They serve as nucleating factors for the maturation of nascent adhesive contacts into focal adhesions [33]. Signaling specificities amongst integrins

have been reported [34], which leads to a difference in affinity to various ECM proteins and cells. Thus different kinds of ECM proteins affect cell growth in different ways. Temporal differences in the synthesis and deposition of ECM and matricellular proteins is known to occur in wound healing [35]. As two important ECM molecules, fibronectin and collagen are critical for angiogenesis to take place during the healing process. Past studies have shown that there are differences of in the way fibronectin and collagen influences cell behaviour. For example, HUVEC cell proliferation and migration rates are reported to be higher on collagen coated gels compared to fibronectin coated gels [36]. Also, M2 melanoma cells are found to respond differently to stiffness gradient on gels coated with either collagen or fibronectin [37].

1.2.7 Motivation of studying the influence of ECM proteins on cell mechanical behaviour

From the integrin perspective, it was reported that integrins $\alpha 5 \beta 1$ and $\alpha v \beta 3$ bind fibronectin whereas integrins $\alpha 1 \beta 1$ and $\alpha 2 \beta 1$ bind collagen [37-39]. Most importantly, fibronectin and collagen are commonly used to mimic ECM in many in-vitro cell mechanics studies. However, the difference in their separate role in cell traction force study is still unknown. Hence, a comprehensive comparison of fibronectin and collagen as ECM proteins in cell contraction is extremely important. This shall include their influence on cell contraction force, cell spreading area, and focal adhesion formation. Moreover, our comparative investigation is expected to be helpful to researchers in choosing the appropriate ECM proteins during in-vitro studies.

1.3 Cell collective migration in 2-dimensional environment

1.3.1 Collective behaviours in biological systems

A fundamental question in biology is, “how do cells collaborate to build and change the structure of epithelial tissues, while maintaining characteristically epithelial features such as cell-cell contact and barrier function [40, 41]?” In addition to studies on single cell mechanics, it is also important to investigate cell collective behaviour as in vivo cells are always coupled to neighbouring cells (Figure 1. 6), and maintaining the cell-cell integrity is important for collective behaviour. Besides, investigating the collective behaviour of cancer cells would be important for the in-vitro modelling of cancer metastasis and invasion. Collective behaviour has long been observed among many biological systems in nature, especially in schools of fish, bird flocks, and social insect colonies [42]. Collective behavior in a biological system is an intriguing phenomenon from the biological and physical point of view. This explains its increasing interest within the physics community in recent years [43]. Complex collective behaviour is not simply summing the individual behaviours of all members. Thus, a deeper understanding on the group behaviour of large interacting systems and the principle behind cooperative behaviour among diverse individuals is crucial in recent interdisciplinary studies on the life sciences.

Figure 1. 6 Phase contrast image of a confluent human hepatocellular carcinoma cell 2D layer after culturing for 24 hours in vitro. The cells are coupled to each other to form an intact cell sheet.

1.3.2 Cell migration mode – single cell migration and collective migration

Cell migration is one kind of remarkable collective behaviour observed in physiological processes like morphogenesis [44] and wound healing [45]. Cell migration is a highly integrated multistep process involving highly polarized cells, and it is critical for many biological processes. For example, it plays an important role in tissue repair and regeneration, and it could orchestrate embryonic morphogenesis. Moreover, a lot of diseases are also closely associated with cell migration, like mental retardation, disease progression in cancer, arthritis, and atherosclerosis [46]. For example, neurofibroma, a kind of benign tumour caused by abnormal cell migration during embryonic development, affects the central nervous system and results in mild

retardation and learning disabilities [47]. During the invasion of cancer cells into the surrounding stroma, the loss of cell-cell adhesion capacity allows malignant tumour cells to migrate away from the primary tumour mass which causes a change to the cell-matrix interaction [48]. The regulations or interventions of abnormal cell migration are promising treatment strategies of cell migration related diseases. For example, podosomes and invadopodia are two specialized cellular structures present in cells with physiological or pathological invasive behaviours, and the inhibition of these two structures might be applicable in the treatment of vascular diseases and cancers [49]. There are two modes of cell migration (Figure 1. 7).

Figure 1. 7 Different modes and various stages of transitions for cell migration: The mechanisms and steps of the transition from single cell to collective migration (reproduced from Figure 1 in [50]).

Single cell migration is the most widely investigated mechanism of cell migration in vitro. This mode of migration allows cells to locate themselves in tissues. It is found during morphogenesis and tumour generation, or a result of transitory travel of the immune cells [51]. Cell collective migration is another mode of migration which preserves the cell-cell junction integrity. Collective cell movement occurs when two or more cells that retain their cell-cell junctions move together across a 2D layer of extracellular matrix (ECM) or through a 3D interstitial tissue scaffold [52]. Cells migrate collectively via simultaneous impact on each other through cell-cell adhesion [53]. Cell-cell adhesion via cadherins and other adhesive proteins, together with cell-cell signaling, through gap junctions, are the most critical characteristics of cell collective behaviour [54]. Cell collective migration exhibits a high degree of complexity that encompasses cell-substrate interplay as well as cell-cell interaction compared to single cell migration. In this process complex movement patterns emerge over much greater distances than the individual component scale [55]. Moreover, collective cell dynamics give rise to complex changes in multicellular tissue structures, including epithelial regeneration, the sprouting of vessels and ducts in angiogenesis and branching morphogenesis, and the deregulated invasion of cell masses during cancer progression and consecutive tissue destruction [52].

1.3.3 The mechanism of tumour cell migration and invasion

Most deaths from cancer are caused by secondary tumours arising via invasion and metastasis [56] instead of the primary tumour. The ability to migrate is critical for tumour cells to change locations inside tissues. Cancer cells could even adjust their migration mechanisms according to different situations, which increases the difficulty of targeting adhesion receptors or proteases for cancer therapeutics [54]. Cancer cells possess a wide range of invasion and migration mechanisms (Figure 1. 8). Broadly, the mechanism of tumour cell dispersion and invasion includes the movement of single cells since individual tumour cells are highly motile in vitro and in vivo [57, 58], moreover cells could gather together and migrate in the form of a functional unit through cell-cell adhesion. While both individual cell and cell groups are present at the same instance in tumours [54], it is being recognized that collective migration is the dominant mode of invasions during cancer metastasis [42]. Hence it is crucial to investigate the collective behaviour of cancer cells in order to gain a deeper understanding on the intrinsic cancer mechanism. Besides the importance in tumour metastasis and invasion, collective cell migration is also crucial for lots of other biological and physiological activities, like morphogenesis and wound healing [51, 59-61].

Figure 1. 8 Heterogeneity of tumour invasion modes. Different molecular programmes (triangles) determine the strategies of tumour cell migration. From single cell move to cell group movements, matrix-degrading proteases and integrins provide increased regulation of cell-matrix interactions. There are two specific features of cell collective behaviours, one is the cell-cell adhesion via adhesion molecules like cadherin, the other is the cell-cell communication through gap junctions (reproduced from Figure 1 in [54]).

1.3.4 The contribution of substrate roughness to cell function

The regulation of cell collective migration relies mostly on the interplay between cell and the extracellular matrix (ECM) through direct binding between integrin and ECM molecules [62]. Hence, besides the bio-chemical signalling from ECM and other cells,

the physical cues of ECM also contribute to cell collective migration [63, 64]. Past studies have shown the influence of various substrate mechanical properties on many aspects of cell growth and because of which, cells are able to modulate their behaviour accordingly. For example, it is reported that mesenchymal stem cells (hMSCs) cultured on substrates of different stiffness exhibit biphasic changes in vimentin detergent solubility [65]. Furthermore, cells cultured on flexible substrates are found to possess focal adhesions that are highly dynamical and exhibit irregular shape. On the other hand, for cells grown on firm substrates, their focal adhesions are found to show a normal and highly stable morphology [66]. As for the mechanical properties of cell itself, its cortical stiffness was observed in proportion to both its spreading area and matrix stiffness [67]. Roughness is another important mechanical properties for cell function *in vivo*. During migration and invasion, tumour cells encounter matrices of diverse topography, and roughness is a typical parameter used to characterize topography in materials study [68]. A lot of work have revealed the role of substrate roughness as a regulator on fundamental cell function, like cell spreading, cell adhesion, cell division, and single cell migration [69-72]. For instance, enhanced cell adhesion was found on polymer surface of higher roughness [69], and the proliferation rate of osteoblast dropped significantly on polymer material whose roughness is around 1.1 nm [70].

1.3.5 Motivation for studying the influence of substrate nanotopography on tumour cell collective migration in 2-dimensional environment

Interestingly, scientists from USA proposed a simple yet effective method of capturing circulating cancer cells with glass surface of nanotopography (Figure 1. 9). Unlike other capturing methods using antibodies to trap cell, this study focused on the affinity preference of tumour cells to nanoroughened substrates. Hence, it did not rely

on the cell size and membrane proteins expression [73]. This gives us some inspiration and hint about utilizing substrate nanotopography to regulate tumour cell collective migration and its further influence on relevant gene expression. Therefore, a comprehensive research on the collective mode of tumour cell migration on substrates of different roughness is essential to attain a better understanding on tumour cell metastasis mechanism.

Figure 1. 9 Fluorescence microscopy of MCF-7 cells got captured after seeding at the density of 10^5 mL^{-1} for 1 hour on glass substrates (bottom panel) of different RMS nanoroughness indicated (SEM images of top panel) (reproduced from Figure 1 in [73]).

1.4 Cell morphology in 3-dimensional environment

We have obtained some understanding on the important role played by matrix nanotopography on cell function regulations by providing spatial as well as mechanical cues. While most studies on nanotopography were carried out on two-dimensional cultural substrates, only a handful of study the nanotopographic effect on

cells in 3-dimension. Nevertheless, the 3D matrix structures used in these studies at microscale are quite heterogeneous. In fact, the effect of the nanotopography on cells is not necessarily at the single cell level.

1.4.1 The importance of dimensionality in cell growth – 2-dimension vs 3-dimension

Early studies comparing cell behaviours in 2-dimensional (2D) and 3-dimensional (3D) matrix have exhibited the distinction in migration, morphology, adhesions, and actin cytoskeleton distribution [74-76]. These findings have indicated the significance of matrix dimensionality, that is, 3D vs 2D. Thus it has become extremely essential for us to further our study into the interactions between single cell and nanotopography in 3D environment from a physiological view point.

Morphology is the most prominent difference between cell culture on 2D flat plate and 3D space (Figure 1. 10). Cells exhibit elongated and unnatural cell shape in 2D cell cultures. In 3D cell cultures, cell shape is more natural and closer to cell shape in vivo. It is highlighted in past studies that cell morphology is closely correlated with cell function [77, 78], indicating the possibility of manipulating cell behaviour through regulating cell morphology.

Figure 1. 10 SEM images of the morphologies of Primary rat hepatocytes cultured on 2D collagen I (a), 3D collagen I (b), and 3D matrigel (c) respectively. (Figures are reproduced from <http://cellculturedish.com/2015/03/materials-and-assay-systems-used-for-3d-cell-culture/>).

1.4.2 Motivation of studying the interactions between cell morphology and matrix nanotopography in 3-dimensional environment

The shape of cell is greatly influenced by the physical environment where it is grown. Unlike micro scale feature causing whole-cell contact guidance, nanoscale feature is of similar size to individual receptor. Hence, it affects integrin distribution and trigger receptor-driven pathways. This alters biochemical signaling [79], which in turn influences cell function, including cell morphology. Cortical actin and focal adhesions are important to maintain cell morphology and resist deformation [80, 81]. Although in 2D it has been reported the influence of cell shape on actin distribution [82], it still remains unclear about the correlation between cortical actin and cell geometry in 3D environment. Hence a novel study on the complex interactions between matrix geometry and cell morphology, as well as their regulation of cortical actin and focal adhesion is important for studying cell mechanics in a way that is closer to natural growth.

Chapter 2

2 Measuring traction forces of human umbilical vein endothelial cells on fibronectin and on collagen type I-coated microposts

2.1 Introduction

The endothelium is a monolayer of endothelial cells that forms the inner lining of blood and lymphatic vessels. It plays an important role as a semi-permeable barrier between the circulation and surrounding tissues to regulate the transfer of molecules of various sizes. In addition, endothelium is critical for glucose and amino acid transport, infection and inflammation, haemostasis and coagulation, angiogenesis and blood flow [83]. Impairment of endothelium function is also associated with diseases such as type 2 diabetes, pre-eclampsia, and obesity [84]. Endothelial functional integrity is closely correlated with endothelial cell-cell and cell-extracellular matrix (ECM) adhesions mediated by cellular surface adhesion molecules [85, 86]. As a result, cellular response is highly dependent on the interactions between cells and ECM molecules. Cell traction force on ECM is crucial to many biological and physiological processes, including embryogenesis, wound healing, inflammation, and angiogenesis[12, 87]. Fibronectin and collagen type I (hereinafter referred to as collagen) are two abundant ECM proteins. Temporal difference in the synthesis and deposition of fibronectin and collagen modulates the angiogenic response during wound healing [35]. Human umbilical vein endothelial cells (HUVECs) are commonly used for investigations on biological processes in endothelial cells under physiological or pathological conditions, and these include the transportation of

macromolecules across endothelial barriers, inflammatory responses, platelet activation and vascular fibrinolysis [88]. It has been shown that the proliferation and migration rates were different between HUVECs on fibronectin and collagen [36]. A recent study showed that endothelial progenitor cells formed colonies much earlier on fibronectin substrate than on collagen [89]. Using M2 melanoma cells, Byfield et al., reported that cells responded differently to stiffness gradient on substrates coated with either fibronectin or collagen [37]. Thus differences in mechano-sensing as a result of different ECM substrates can lead to distinct cellular responses.

HUVECs express fibronectin-binding integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ and collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ [37, 90, 91]. Integrins are heterodimeric adhesion proteins mediating the cell attachment to ECM [92]. In humans, 24 different integrin heterodimers are formed with noncovalent association between 18 α and 8 β sub-units, and signaling specificities amongst integrins are known to induce different cellular responses [34].

Given that HUVECs respond differently to fibronectin and collagen substrates, and that traction force plays an important role in cellular responses, we therefore performed comparative analyses of traction forces and directional contractions of HUVECs on fibronectin- and collagen-coated microposts.

2.2 Materials and Methods

2.2.1 Cell culture

HUVECs were bought from commercial source (Lonza, Basel, Switzerland) and cultured in endothelial cell growth medium containing supplements (Promocell, Germany) at 37°C in a humidified incubator with 5% CO₂.

2.2.2 ECM coating of microposts

The micro-posts were kind gifts from Prof. Jianping Fu (University of Michigan, U.S.A.). Microposts fabrication method has been reported [24]. The parameters of the microposts are: height (8.3 μm), spacing between posts (6 μm), spring constant (7.22 nN/ μm) and effective modulus (5.65 kPa). Microposts were coated with 20 $\mu\text{g/ml}$ human plasma fibronectin (Sigma Aldrich, St Louis) in PBS for 2 h at 37° C. Microposts were coated with 20 $\mu\text{g/ml}$ rat tail collagen type I (BD Bioscience) in 0.02 N acetic acid for 2 h at 37 °C. All microposts were rinsed in PBS before used. Besides, the microposts embedded on a cover-glass culture dish were cleaned by ultrasound sonicator (Elma, Schmidbauer GmbH, Germany) with water bath.

2.2.3 Live cell imaging

HUVECs (4×10^5 cells) were settled onto ECM-coated micro-posts that were embedded on a cover-glass culture dish. Cells were settled for 10 min under culture conditions. The culture dish was then placed in an adaptor (with 5% CO₂ supply) on the stage of an inverted fluorescence microscope (Olympus IX70, 40X/0.60 Ph2 objective, equipped with CCD camera) which was housed in a custom-built plastic box connected to a temperature controlled 37 ° C incubator. Individual cell was chosen in a random manner within 3 min after placing the micro-post dish on the microscope stage. Only cell with no contact with other cells was chosen to avoid the interference from cell-cell contact, especially it might influence cell spreading and contraction. Also, to achieve proper focusing and to capture continuous images in 2 hour session, only 1 isolated cell was selected for each run of experiment. We captured phase contrast images every 1 min for 2 h and processed with the MetaMorph software.

2.2.4 Quantification of cell traction force

Acquired images were exported as 16-bit TIFF images and the centroid of each pillar was identified with a custom 2D particle tracking program (31) (Mathworks, Novi, MI). The Top-ideal Method(T-I) (32) was used to calculate the pillar deflection. In brief, all the pillars are supposed to be spaced uniformly, then based on the positions of the undeflected pillars at the first frame, we estimate the original positions of the occupied pillars using two-dimensional linear interpolation to get the theoretical unoccupied frame (TUF). The deflection is determined by the difference between the pillar centroid in the TUF and that in the actually captured frame. The drift of microscope setup during experiment is calculated as the average vectorial displacement of the unoccupied pillars in each frame compared to the corresponding initial positions in TUF. After drift elimination, there is still small deflection for the unoccupied pillars, then based on our trial tests, 0.06 to 0.10 μm is chosen as a threshold that only the pillars whose deflection magnitudes are above the threshold are considered to be occupied by cell contraction. The traction force is obtained by multiplying deflection with nominal spring constant of pillar. The total absolute force exerted by each cell and the number of pillars under each cell were calculated and plotted against time. The number of pillars under each cell, which corresponds to the spread area of the cell, was determined by inspecting each cell-image captured over the 2 h duration. Values were plotted against time. The average force exerted on each pillar per cell was also calculated and plotted against time. We performed unpaired Student's t test to calculate the statistical significance of the difference between pillars that experienced contraction force in the range of 0–2 nN amongst different groups, namely FN (fibronectin) vs Uncoated, COL (collagen) vs Uncoated, and FN vs COL.

Pillars in the force range of 0–2 nN were chosen because they formed the major population in the normalized distribution of force in all three groups of cells.

2.2.5 Immunofluorescence staining and confocal microscopy

HUVECs (4x10⁵ cells/dish) are seeded onto coated microposts and incubated (as described earlier) for 15mins, 30mins, and 50mins respectively. Cells are fixed with 3.7% paraformaldehyde(Sigma Aldrich) in 1xPBS for 10mins at room-temperature, then permeabilized with 0.3%(v/v)Triton X-100 (Sigma Aldrich) in cytoskeleton stabilization buffer containing 100mM NaCl, 300mM sucrose,3mM MgCl₂,1mM EGTA, and 10mM PIPES for 1min at room temperature. After washed with 1x PBS, the micropost dish is coated with 1% Albumin from Bovine Serum(BSA; Sigma Aldrich) in 1x PBS at room temperature for 30mins and then rinsed with 1x PBS again. 5µg/ml Anti-Paxillin, 5H11 PC 12-305(Merck) in 1% BSA is added to the dish, then the dish is incubated at 4°C overnight. After washing the dish with 1x PBS, it is incubated in dark with 1:600 Alexa Fluor ® 488 Conjugated goat antimouse IgG secondary antibody(Invitrogen),1:100 DAPI(Sigma Aldrich) and 1:100 Alexa Fluor® 594 Phalloidin (Life Technologies) in 1x PBS for 1hr at room temperature. The dish is then washed with 1x PBS several times and 2ml of 1x PBS is added to the dish. The dish is examined under Zeiss LSM 510 confocal laser scanning microscope with 63x oil immersion objective lens.

2.2.6 Focal adhesion size and cell size quantification

To quantify the focal adhesion size, we used ImageJ (National Institutes of Health) to produce a black-and-white image from fluorescence microscopy of paxillin, then after subtracting the noise from background, we chose threshold of the grayscale image to get a binary image. The number of pixels with value above the threshold was for the size of focal adhesions. The brief process was depicted in Figure 2. 1. Moreover, to

quantify the cell spread area, fluorescent images were converted to black-and-white images using ImageJ, and Canny edge detection method was applied to render the binary images (Mathworks, Novi, MI). Image dilation, erosion, and fill operations were applied to fill in the gaps. The number of pixels with value ‘1’ was summed to determine the cell spreading area.

Figure 2. 1 An example of how to calculate focal adhesion area with imageJ. From left to right: (a) fluorescent image of paxillin, (b) black-and-white image produced from fluorescent image, (c) image after subtracting noise from background, (d) binary image after thresholding of (c).

2.2.7 Statistical analysis

Since 0-2 nN is the dominant range of the normalized distribution of force on single pillar for cells in all the three groups, we performed Student’s *t* test to determine the statistical significance of the difference between pillars whose corresponding contraction force is in the range of 0-2 nN amongst different groups, namely Uncoated vs COL, Uncoated vs FN, and COL vs FN.

2.3 Results and discussion

2.3.1 Differences in cell shape of HUVECs on fibronectin compared with collagen

HUVECs were allowed to adhere and spread under culture conditions on glass-coverslip bottom culture dish that was coated with either fibronectin or collagen type I.

At 60 min, the shape of HUVECs on fibronectin was different from those on collagen (Figure 2. 1 a). Furthermore, cells on fibronectin generally have larger cell spread area compared to those on collagen. Cells were also subjected to immunofluorescence staining followed by confocal microscopy analyses to visualize actin filaments and the focal adhesion protein paxillin (Figure 2. 1 b). Cells on fibronectin or collagen showed punctate mature focal adhesions as reported by paxillin staining and filamentous actin were clearly visible.

Figure 2. 1 Microscopy images of HUVECs on fibronectin or collagen-coated glass coverslips. (a) Phase contrast images of cells on ECM-coated glass coverslips at 60 min. Scale bar 50 µm. The brightness and contrast of the whole images are adjusted

using the software photoshop for better clarity. (b) Cells were fixed, permeabilized and immunofluorescence staining performed followed by visualization under confocal microscopy. Focal adhesion protein paxillin (green), actin (red) and nucleus (blue). Scale bar 20 μm .

2.3.2 Total traction force of HUVECs is greater on fibronectin than on collagen but the average force per pillar is in the same range

Integrins $\alpha 5\beta 1$ and $\alpha V\beta 3$ bind fibronectin and integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ bind collagen [92]. These integrins are expressed in HUVECs [37-39]. Force sensing is an important mechanism by which cells establish stable adhesion that is necessary for effective spreading. We therefore examined the traction forces exerted by cells on these ECM proteins mediated by different integrins. The applications of micro-post platform in single-cell or cell monolayer traction force measurements have been reported [93, 94].

HUVECs were settled onto non-coated, or fibronectin- or collagen-coated micro-posts and the deflection of pillars monitored for 2 h by live-cell imaging. The total absolute force exerted by each cell and the number of pillars under each cell were calculated and plotted against time (Figure 2. 2). Total absolute force is an important quantitative indication of cell contractility, which is caused by the interactions between cell surface receptors and molecules of the extracellular matrix [95]. In all three experimental groups, the total traction force per cell increased with time and reached saturation plateau after almost 40 min (Figure 2. 2 a, c, e). The total absolute force per cell on fibronectin coated microposts was higher than on collagen type I during the early stage of cell spreading (0 - 20 min) and the later stage of saturation plateau (fibronectin : ~ 200 nN versus collagen: ~ 150 nN). The total absolute force per cell on uncoated pillars was the lowest amongst the three groups. The number of pillars under each cell, which is used to represent the cell spreading area, increased with time albeit

at different levels for all three groups of cells over the 2 h duration (Figure 2. 2 b, d and f). The steady value of number of pillars is around 65, 90, 65 for uncoated, FN, and COL group respectively.

Figure 2. 2 Force measurements of HUVECs on uncoated (a-b), fibronectin (c-d) or collagen type I (e-f)-coated micro-posts. (a, c, e) Plots of total absolute force (nN)

against time (min) (duration = 2 h) of cells on micro-posts. (b, d, f) Plots of number of pillars occupied per cell against time (min), (a, b, c, d, e, f) N=7 for each plot. Data are presented as mean (dark blue line) $\pm$ SD (pale blue region).

To have a quantitative understanding on the average force density, the average force exerted on each pillar is also an important indicator for cell contraction. Thus, from the plot of the average force against time (Figure 2. 3 a, c and e), we can see that for cells on either the fibronectin or collagen type I the average force exerted on each pillar increase with time, while there was a clear difference on the time taken to reach the saturation plateau. Cells on fibronectin and on collagen type I reached saturation plateau around 10 min and 25 min, respectively. Previous study on cell contractile response to microposts array suggests that with a larger number of pillars under one cell, a lower average force is exerted per pillar [96]. While in our study, there was no significant difference between the average forces exerted per pillar by cells on fibronectin and collagen after reaching the saturation plateau, despite that we observed more fibronectin-coated pillars compared with collagen-coated pillars under each cell with time. Cells on uncoated pillars showed the lowest average force per pillar, which indicates the deficiency of local cell contraction on uncoated pillars. And we speculate it might be due to the lack of ECM proteins that caused the decrease of binding affinity, which further resulted in the contraction deficiency. To check the distribution of force on individual pillar, we plotted the normalized force histogram for all the pillars in all time frames of all cells (Figure 2. 3 b, d and f), which is over 45000 data points. Similar to the result of average force on each pillar, there is no significant difference of force distribution between two groups in five force ranges, 0 to 2 nN, 2 to 4 nN, 4 to 6 nN, 6 to 7 nN, and above 7 nN. Almost 60% of the pillars

are exerted forces smaller than 2 nN in two groups. Therefore, combining results of average force and normalized force range distribution, the distinctive total absolute force of cells on two substrates primarily results from the difference in cell spreading area rather than the variance of force distribution within the cell body.

Figure 2. 3 Force measurements of HUVECs on uncoated (a-b), fibronectin (c-d) or collagen type I (e-f)-coated micro-posts. (a, c, e) Plots of average force exerted on each pillar against time (min). (b, d, f) Normalized distribution of forces exerted on all

the pillars for 2 h, (a, b, c, d, e, f), N=7 for each plot. Data are presented as mean (dark blue line) $\pm$ SD (pale blue region).

Throughout each trial of our experiment, we select healthy living cells that remain spread on the pillar and exhibit contraction even at the end of 2 h (Figure 2. 4). We are aware that the cells are still living and not undergoing cell deaths. Because the latter cells usually exhibit a circular and detached shape, which indicates that they have lost their ability to contract and spread readily. In the subsequent sections, we shall focus only on cells that spread on fibronectin and collagen type I-coated micro-posts. Cells on uncoated pillars are not included because they show low contraction force compared to those on the ECM-coated pillars. The apparently lower average force per pillar for cells in the non-coated group also indicates a weaker local contraction and spreading. In fact, such a decrease in the affinity of cells to the substrates results from a lack of ECM proteins.

Figure 2. 4 Representative still images of cells on uncoated, fibronectin (FN)-coated or collagen type I (COL)-coated micro-posts. Representative still images of cells at the end of 2 h are shown. The cell outline was traced using the Image J software. All images are of the same magnification. Reference scale bar (10 μm) is shown in the left panel (uncoated group).

2.3.3 HUVECs on fibronectin- but not collagen-coated microposts showed directional contraction

Cell contraction is not only dictated by the magnitude of force, but also the directionality. The magnitude and the direction of force transmission are important parameters regulating cell spreading and migration. Force vectors directed towards the cell center was evident for cells on fibronectin-coated microposts (Figure 2. 5a). By contrast, cells on collagen-coated microposts exhibited a somewhat disordered distribution of force vectors (Figure 2. 5a and b). We then determined the directionality of contraction (gripping and pulling) of HUVECs on fibronectin and on collagen. The directionality of contraction is defined as the magnitude of the sum of force vectors divided by the sum of their magnitudes [97]. A value of 1.0 suggests parallel forces whereas a value of 0 suggests balanced forces. During the entire duration (2 h) of the experiments, directional contraction was more pronounced in cells on fibronectin than on collagen (Figure 2. 5b and e).

For cells on the fibronectin coated microposts, the neighboring pillars are found to orient in a relatively parallel direction. This is different from the pillars of cells on collagen coated substrate, which are deflected in a much more chaotic way (Figure 2. 5a). Similar diversity is reported between cells cultured on micron and submicrometer pillars [98]. To quantify such correlation between neighboring pillars, all the neighboring pillars are divided into pairs. The force correlation of a pair is defined as the cosine of the included angle of the two force vectors exerted on each pillar. A correlation value of 1.0 is attained when the angle is 0 (i.e. when the two neighboring force vectors are parallel). Cells on fibronectin generally showed higher correlation values compared with cells on collagen throughout the duration of the experiments (Figure 2. 5c, f). The higher correlation, along with the greater directionality for cells

on fibronectin coated microposts may be caused by larger cell spreading area since actin filaments tend to be aligned more parallel and tightly in cell of greater size, and the greater force and larger focal adhesion will also strengthen the parallel alignment of actin, which together lead to the greater contraction directionality of cell as a whole as well as the larger correlation between neighboring pillars.

Figure 2. 5 Measurements of directional contractions of HUVECs on micro-posts coated with either fibronectin (a, b, c) or collagen type I (d, e, f). (a, d) Representative

force maps of cells on micro-posts at ~ 60 min. The yellow arrow indicates the force vector on each pillar. Scale bars: force magnitude 5 nN (yellow) and dimension 10 μm (white). (b, e) Plots of directional contractions against time (min). (c, f) Plots of the average correlation of force on all neighboring pillar pairs against time (min). (b, c, e, f), N=7 for each plot. Data shown are mean (dark blue line) $\pm$ SD (pale blue region).

2.3.4 Focal adhesion size increased as cell area

Next, to examine and compare the extent of focal adhesion formation, cells on ECM-coated micro-posts at different time points (2, 17 and 37 min) were fixed followed by immunofluorescence staining protocol of focal adhesion protein paxillin [99] (Figure 2. 6 a, c). We choose paxillin as marker protein for focal adhesion (FA), since paxillin is one of the first components appearing visible at cell-matrix adhesion sites [99], which matches our study on cell early spreading. Horse-shoe shape focal adhesion formation around each pillar was observed. Similar structures of focal adhesions on ECM micro-patterns have been reported by others [100-102]. It should be noted that the force exerted on the post is inward-directed to the cell center (Figure 2. 6a, d), which is the direction of the open end of the horse-shoe shape focal adhesion. Also, both experiments and simulations have shown a close correlation between subcellular distribution of focal adhesions and local traction forces [102, 103]. Overall, the increase of sum of the focal adhesion areas of cells on fibronectin and on collagen correlates with an increase in cell spread area (Figure 2. 6b, d). Centroids of the cluster of points at each time step were used to show the overall increase of focal adhesion areas and cell area against time for cells on fibronectin and collagen (Figure 2. 8).

The connection between the actin cytoskeleton and the focal adhesion sites coupled with myosin activity are necessary for force transmission in cells [87]. Different integrins are involved in binding fibronectin and collagen type I [92]. It is possible that different integrin signaling pathways are activated in HUVECs on fibronectin and on collagen type I, leading to differences in cell spreading area and directional contraction. Notably, endothelial cells interacting with fibronectin under shear stress have been shown to exhibit faster activation of JNK signaling and alignment of cells along the direction of flow compared with cells interacting with collagen [104]. In order to validate the molecular mechanisms that cause the difference in cell contraction on fibronectin and collagen, we can perform a gene-knockout for the fibronectin-binding integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$, and the collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ [37, 90, 91] to observe the changes in traction force, spreading area, and directionality compared to the cells before the gene-knockout. The distinct biomechanical behaviors of HUVECs on fibronectin and collagen type I could be important in angiogenic response during wound healing when there is a temporal difference in the synthesis and deposition of these ECM proteins at the sites of injuries [35].

Figure 2. 6 Measurements of focal adhesion area of HUVECs on micro-posts coated with either fibronectin (a, b) or collagen type I (c, d). (a, c) Representative

immunofluorescence microscopy images of fixed cells on micro-posts stained for focal adhesion protein paxillin at the 17th min. (b, d) Plots of total focal adhesion area (μm^2) per cell against cell spread area (μm^2) at indicated time points. Each blue dot represents one cell.

Figure 2. 8 Plots of time against cell spread area and total focal adhesion area of HUVECs on micro-posts coated with either fibronectin (a) or collagen type I (b).

2.4 Conclusion

In contrast to previous reports on collagen or fibronectin alone as ECM proteins, our investigations have made a comparative investigation into the influence of these two proteins on cell biomechanical behaviours. Our study has uncovered that during the

early stage of cell spreading, HUVECs exhibit a stronger cell traction force and a larger spreading area on fibronectin coated microposts, while the average force on each pillar is comparable between the two groups. The correlation between the spreading area of the cell and the size of focal adhesion indicates the interplay between adhesion molecules and binding integrins in cell mechano-transduction via the formation of focal adhesion. Our findings have not only provided the basis for comparative cell mechanics research, they may also help to reveal the mechanism of endothelium dysfunction, which is usually accompanied by fluctuations of fibronectin and collagen concentrations.

Chapter 3

3 Substrate nano-roughness regulates tumour cell collective migration via mediating the expression of adhesion proteins

3.1 Introduction

Collective cell migration is a phenomenon where complex movement patterns emerge over a much larger distance than the scale of individual cells [55]. It is crucial to many pathological and physiological processes, including wound healing, morphogenesis, invasion, and metastasis [51, 59-61]. The ability of migration is crucial for tumour cells to change locations inside tissues. Most deaths in cancer arise from secondary tumours via invasion and metastasis [56] rather than from primary tumours. Even though both single cells and groups are present simultaneously in a broad range of tumours [54], the mechanisms of tumour cell movement can be divided into single cell dispersion and group movement in the form of clusters or sheets. In fact, collective cell migration has been shown to be the dominant mode of invasions [42].

As cells in vivo live in a complex environment where they couple to other cells or the matrices around them, cell behaviors are also greatly influenced by the mechanical and physical signal from the environment apart from biochemical signals [63, 105]. During tumour metastasis and invasion into its target tissues, the tumour cells encounter environments of different geometrical topography, which are in a range of different roughness. A lot of work has been done to indicate the important role of substrate roughness on fundamental cellular functions, like cell spreading, cell

attachment, cell division, and single cell migration [62, 69-71]. For single cell migration, researchers had found that the speed of migration of the human embryonic stem cell increases with the RMS roughness of the substrates. Meanwhile, punctuate adhesion structures of smaller size and of a larger density were found to distribute randomly over the entire cell when cultured on nanoroughened surfaces. These punctuate adhesions imply fast FA turnover and weak actomyosin contraction, which could lead to disorganized actin filamentation and rapid cell migration [106]. However, in another study, the migration of epithelial cells was inhibited on the sandblasted-acid-etched topography [107]. These contrasting results suggest that the influence of nanoroughness on single cell migration is specific to cell-type.

There are several techniques for the fabrication of substrate roughness, which includes plasma treatment, polymer demixing technique, and chemical etching [108]. Chemical etching stands out due to its low cost, greater accessibility, and high effectiveness compared to other techniques which require expensive specialized facility.

As no in-depth research has been done on the role of substrate roughness in tumor cell collective migration and how substrate roughness regulates cell-cell and cell-substrate adhesion proteins expression, we have explored into this question by developing a simple while effective method to fabricate and control roughness gradient on a single surface with chemical etching technique. More importantly, we have conducted a novel quantitative investigation into how the substrate roughness influences tumor cell collective migration from the perspective of physics. In this study, we generated regulated roughness contrast on one piece of polystyrene surface with chemical etching. We then transferred the roughness onto the polydimethylsiloxane (PDMS) via pouring the pre-mixture of elastomer and curing agent over the polystyrene surface and thermally cured it. Next we cultured cells and performed cell migration

study on the nanoroughened PDMS substrate. The method allows for the fabrication of different degree of roughness on the same PDMS surface. Such roughness contrast facilitates a comparative investigation of cell collective migration on different roughness simultaneously. Hepatocyte derived carcinoma cells (HuH7) were utilized for experiment and the expressions of different adhesion proteins were analyzed to study the influence of nanoroughness on cell collective migration. The results of cell mechanical behaviors together with bio-chemical functioning indicate that substrate nanoroughness plays an important role in collective migration and give us hints on developing efficient strategy to regulate tumor cell behaviors.

3.2 Materials and methods

3.2.1 Substrate preparation

Chemical etching is a widely used method for creating roughness because of its low cost and high effectiveness. Different substrates like glass and silicon have been etched using different chemicals. In this study we have etched polystyrene based tissue culture petri dishes (Iwaki, Japan) with the help of acetone (HPLC grade, Fischer Scientific, Singapore), after which the polystyrene was resolved and as a result an increase in roughness of the substrate was created. The roughness degree was regulated with different concentrations (100%, 75%, 50%, 25%, 0) of acetone diluted with ethanol (ACS grade, Merck, Germany).

PDMS (polydimethylsiloxane) (Dow Corning, USA) is a silicone polymer with a range of advantages like bio compatibility and optical transparency. It was prepared by pre-mixing the elastomer and curing agent with a weight ratio of 10:1 and curing them at a temperature of 70°C for one hour. In our study to observe the collective migration of tumour cells on rough and smooth substrate simultaneously, we have managed to fabricate substrate with controlled roughness contrast (partially smooth

and partially nanoroughened) on the same piece of PDMS. This was done by covering half part of petri dish with solidified PDMS and exposing the other part to acetone for 3 minutes following which the dish is washed with ethanol once, air dried and next rinsed with distilled water twice and air dried again (Figure 3. 1). Thus we could create a roughness contrast with one half being rough (100% acetone) and the other half remaining smooth (no acetone).

The reaction between acetone and the petri dish normally leaves the surface to be rough and blurry which will cause problem during optical microscopy for the cell imaging. To overcome this, the partially nanoroughened polystyrene substrate was adopted as a mold and thus the roughness was transferred onto PDMS by prototyping. Pre-mixture of PDMS elastomer and curing agent were casted onto the partially nanoroughened substrate in the polystyrene dish. After degassing and curing at 70°C oven for 1 h, the solidified PDMS layer was peeled off from the dish to maintain the contrast roughness from the polystyrene substrate, while retaining its transparency for imaging. An 8 mm punch was used to punch a piece of circular PDMS on the boundary to obtain PDMS surface that is half smooth and half rough.

Figure 3. 1 Schematic of fabricating controlled roughness contrast on the same piece of PDMS layer. Briefly, this was achieved by covering half part of petri dish with solidified PDMS as a stencil and exposing the other part to acetone for etching. Fixing reaction time of 3 min, we added different concentrations of acetone to control different degrees of roughness. In this way the partial rough and partial smooth surface was generated on the polystyrene dish. The obtained dish with roughness contrast was utilized as the stencil for prototyping. The PDMS pre-mixture with elastomer and curing agent was cast over the bottom of polystyrene dish. Once the PDMS get cured, the nanotopography of the dish bottom with roughness contrast was transferred onto the PDMS, which was then peeled off from the dish and used for cell culture in later experiment.

3.2.2 PDMS treatment

PDMS is not well suited for cell culture in its original state due to its hydrophobicity. Different surface treatment methods are available to improve cell growth on PDMS. In our study, after oxygen plasma treatment we used 20 µg/ml of fibronectin (Life technologies, Singapore) in 1X PBS to coat the PDMS substrate. The substrate was immersed completely in fibronectin solution for 1 hour at 37°C following which they were washed twice with 1X PBS and cell seeding was performed.

Cell culture

Human hepatocarcinoma cells (Huh 7) were cultured in a T-25 flask in Duplecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. The cells were trypsinized with trypsin-EDTA when around 80% confluent, and approximately 30,000 cells were seeded onto the 8mm-diameter PDMS surface. To evaluate the influence of substrate roughness on collective cell migration, we seeded Huh7 cells onto area close to the midline that separates the rough and smooth side. After incubating at 37°C with 5% CO₂ in a humidified incubator for 24 hours, a continuous cell monolayer with no boundary restriction was developed covering both the rough and smooth surface. All the medium and chemicals for cell culture are purchased from Life Technologies, Singapore.

3.2.3 AFM measurements

Silicon cantilevers (OMCL-160TN-C3, Olympus, Japan) with spring constant around 20 N/m were mounted on a Nanowizard III AFM (JPK Instruments AG, Berlin, Germany), which was setup on an inverted Olympus IX71 microscope (Olympus, Japan) with a monochrome charge-coupled device (CCD) camera (DFK 31AF03, The

Imaging Source, Germany). Tapping mode was conducted to obtain the height map in a $15 \times 15 \mu\text{m}$ area; CCD together with phase contrast microscopy was combined to visualize the cantilever tip and substrate. Root mean squared roughness (RMS) was calculated by the standard deviation of the heights for the scanning area.

3.2.4 Live cell microscopy

Before moving to microscopy stage, the cell culture medium was replaced with CO₂ independent medium (Invitrogen, USA) supplemented with 10% FBS and 1% penicillin streptomycin. The cells were imaged with a 10X objective mounted on an inverted Olympus IX71 microscope (Olympus, Japan). A stage-top thermostatic incubator was used to enclose the microscope to maintain 37°C. The frame for imaging was chosen such that both the rough and the smooth sides are visible in the same frame and hence collective migration on rough and smooth sides were observed simultaneously. Imaging was performed continuously for a period of 10 hours with a time lapse of 10 minutes between consecutive images.

3.2.5 Image and data analysis

After de-drift operations for all the captured images, only the images from 200th minute onwards were used in real analysis to further exclude the effect of the relatively large drift that happens during the initial imaging period. Drifts in image sequence were removed using Image stabilizer plugin for ImageJ (National Institutes of Health, USA). Particle image velocimetry (PIV) was applied to quantify the velocity field of the tumor cell sheet. PIV is powerful for computing local displacement using imaging cross-correlation algorithm. Originating from fluid research for flow dynamics, PIV or PIV-like techniques have now been successfully applied in cell motion study [109-111]. It helps to quantify the coordinated motion of cell sheet as a whole as well as to characterize the details caused by the movement of

organelles and membranes [111]. In our experiment, the window size for PIV analysis is 32 pixels $\times$ 32 pixels with step size of 16 pixels. PIV was performed using PIVlab package implemented in MATLAB (Mathworks, USA) [112]. All the remaining data analysis was assisted with custom-built MATLAB programs.

3.2.6 Quantitative Reverse transcriptase PCR

qRT PCR was conducted to quantify the gene expression level. In this study GAPDH was chosen as the housekeeping gene and Ki67 was chosen as the gene targeting cell proliferation. Other five target genes involving in cell-cell adhesion and cell-substrate adhesion were picked, including three kinds of cell-cell junction proteins, E-cadherin, ZO-1, and beta-catenin, and one kind of focal adhesion protein, paxillin. Huh7 cells were passaged and separated into three groups. The first group was indicated as D0 (day 0) because RNA extraction was done immediately after passaging from culture flask; the second group was seeded onto the rough PDMS surface; and the last group was seeded onto the smooth surface. Both rough and smooth substrate group contained 3 samples each. Around 30000 cells were seeded onto each of the samples and they were incubated for 30 hours in a humidified incubator maintained at 37°C and 5% CO₂. Total cellular RNA was first extracted from all the samples using RNeasyR Mini Kit 250 (Qiagen, Singapore), followed by its density quantification with NanoDrop lite spectrophotometer (Thermo Scientific, USA). 100 ng RNA of each sample was reversely transcribed into cDNA with iScriptTM Reverse Transcriptase Supermix (Biorad, Singapore). Real-time PCR was performed on 96-well optical reaction plates (Bio-Rad, USA). The mixing solutions in each well had a volume of 20 μ l composed of 10 μ l of Power SYBR Green PCR Master Mix (Invitrogen), 0.24 μ l of forward primer, 0.24 μ l of reverse primer, 10 ng of cDNA product, and the remaining volume of dnase-free water. The primer sequences for

PCR were as follows: human E-cadherin forward primer, 5'-GAAGGTGACAGAGCCTCTGGAT-3'; human E-cadherin reverse primer, 5'-GATCGGTTACCGTGATCAAAATC-3'; human ZO-1 forward primer, 5'-TGCCATTACACGGTCCTCTG-3' [113]; human ZO-1 reverse primer, 5'-GGTTCTGCCTCATCATTTTCCTC-3'; human Paxillin forward primer, 5'-AAACCCGACTGAACTGGAACCCT-3' [114]; human β -catenin forward primer, 5'-GTGCTATCTGTCTGCTCTAGTA-3'; human β -catenin reverse primer, 5'-CTTCCTGTTTAGTTGCAGCATC-3' [115]. human ki-67 (5'-ACTTGCCCTCCTAATACGCC-3'), human GAPDH (5'-GCCAAATCCGTTCACTCCGACCTT-3'). The amplification of housekeeping gene GAPDH was conducted as a control for sample loading and normalization. All the primers were purchased from IDT, Singapore and the data were analyzed with Step One software version 2.3. Unpaired Student's t-test was performed to estimate the significance of gene expressions on two substrates.

3.3 Results and discussion

3.3.1 Roughness of different orders was created by varying acetone concentration

For chemical etching, chemical concentration and application time are two most important factors influencing the result [116]. In our study, we controlled the etching time, and used different concentrations of acetone in ethanol to etch the petri dishes to get roughness of different orders on PDMS substrate, ranging from 1 nm to 10^3 nm (Figure 3. 2). The RMS reached the maximum value around 75% concentration (Figure 3. 2b), rather than 100% as we expected before.

We used the substrate partially etched by 100% acetone for cell culture, which had RMS roughness of about 60 nm on the roughened side and about 2 nm on the smooth

side. Why we chose 60 nm is because nanoscale feature is comparable to the interspacing of integrins (45 nm) and the integrin-mediated adhesion is important for cell growth and spreading. While the microscale topographical feature is nearly comparable to the size of individual cell that usually result in whole-cell contact alignment [79]. So in our study of cell collective migration it is more appropriate to choose nanoroughness to study since the adhesion-based activities are more sensitive to it compared to microroughness.

Figure 3. 2 (a) Representative AFM figures of rough surface etched by acetone of different ratios with ethanol: (I) 0%, (II) 25%, (III) 50%, (IV) 75%, (V) 100%. The

scan size is 15 μ m*15 μ m, comparable to cell size in our study. (b) The RMS roughness under different concentrations of acetone. The RMS did not reach the maximum at the 100% concentration, but around concentration of 75%. Data shown as means $\pm$ SD.

3.3.2 Cells migration was slower and less directional persistent on nanoroughened substrate compared to the smooth one

After cells reached confluence to form cell sheet, 10 h long imaging was taken to capture the collective migration of cells. We applied particle image velocimetry (PIV) to quantify the velocity field of tumor cell sheet. PIV is very powerful in quantifying local displacement with imaging cross-correlation techniques. After computing the local velocity field, we found clear difference in large-scale cell behaviour patterns between smooth surface and surface with nanoroughness. Interestingly, the average velocity of the cell migration was lower on the surface with nanoroughness compared to those on smooth surface (Figure 3. 3a). After calculating the mean of the magnitudes of all velocity vectors in each frame, we found that the speed of the collective cell migration is always lower on the rough side of the substrate throughout the whole experiment (Figure 3. 3b), indicating the impact of substrate roughness on the collective migration of tumor cells. This finding was consistent with the study that the migration of epithelial cells was inhibited on rough PLGA membranes [107]. However, another study found that the migration area of vascular cells and corneal cells could be enhanced by roughened PMMA substrate which was sandblasted by aluminium grain of size 50 to 150 μ m [117]. This discrepancy indicates the influence of substrate roughness on cell movement is cell-lineage specific.

Figure 3. 3 PIV analysis of migrating huh7 monolayer and individual cell tracking. (a) A representative snapshot of velocity field distribution. The black midline roughly separated the substrate into nanoroughened (left, RMS=60 nm) and smooth (right, RMS=2 nm) part. The overall velocity of cells on the smooth substrate was larger than on rough substrate. (b) The velocity of cells on rough and smooth substrate was monitored continuously for 10 h, after culturing for 24 hours. (c) The migration trajectory of chosen individual huh7 cells for 10 h on rough and smooth substrate respectively (n=19 for nanoroughened part, n=17 for smooth part). Upper-right inset: the migration persistence of cells based on the corresponding movement path. (d) Mean squared displacement (MSD) of cells on rough and smooth substrate with different time intervals. Data is shown as mean $\pm$ SD.

To further investigate the difference of tumor cell collective migration on nanoroughened and smooth surface, we manually tracked the movement path of individual cells within the cell sheet with ImageJ (Figure 3. 3c). To compare the spatial extent of cell migration on two regions of the surface with different nanoroughness, mean squared displacement (MSD) was calculated under different time interval. MSD is defined as:

$$\langle \Delta \mathbf{r}(\tau) \rangle = \langle [\mathbf{r}(t + \tau) - \mathbf{r}(t)]^2 \rangle \quad (3.1)$$

where $\langle \dots \rangle$ designates the mean value over t with all cells tracked. $\mathbf{r}(t)$ is the location of a cell at time t , and $\mathbf{r}(t + \tau) - \mathbf{r}(t)$ is the displaced distance of a cell over the lag time τ . Almost for all the different time intervals, the MSD of cell movement on smooth region of the substrate was larger than that of cell migration on rough region (Figure 3. 3d), which was consistent with the data of dynamic velocity obtained from PIV analysis. Moreover, fixing the starting position as the origin of coordinate, we could see from the trajectory of cells on rough and smooth parts that the cells on the smooth part diffused further and more persistent (Figure 3. 3c). Cell migration is dictated not only by locomotion rate, but also how persistent a cell maintains the migration direction [118, 119]. The persistence of migration is defined as the ratio of the start to end point distance over the length of traversed path [120]. In our study, for a period of 10 h, the directional persistence for cell migration on rough substrate was smaller compared to smooth substrate (Figure 3. 3c inset), indicating the increased difficulty of tumor cells migrating further on rough substrate. Directional migration is an important component of cell motility when cells navigate in a complex environments. In our study, the result of cell migration directionality together with the

lower velocity and smaller MSD for the cells on the nanoroughened substrate indicated that the nanotopography might modulate the collective behavior of cell sheet accordingly, result in slower as well as less directionally persistent mode of tumor cell collective migration on a nanoroughened substrate.

3.3.3 The correlation of cooperative motions for individual cells decreased as the distance between cells increased

Cooperative migration is the most crucial characteristic of collective cell migration as there is no free space within cell monolayer. To estimate the distance over which the motility are correlated, we calculated the mean spatial autocorrelation function of velocity fluctuation vectors over all time points, which is a quantitative indication of intercellular coordination:

$$C(\mathbf{R}) = \left\langle \frac{\sum_t \sum_i d\mathbf{v}(\mathbf{r}_{i,t}) \cdot d\mathbf{v}(\mathbf{r}_{i,t} + \mathbf{R})}{\sqrt{\left(\sum_t \sum_i d\mathbf{v}^2(\mathbf{r}_{i,t}) \right) \left(\sum_t \sum_i d\mathbf{v}^2(\mathbf{r}_{i,t} + \mathbf{R}) \right)}} \right\rangle_{\phi}. \quad (3.2)$$

Note that $d\mathbf{v}$ is the velocity fluctuation, obtained by subtracting the average velocity from the velocity field, $d\mathbf{v}(\mathbf{r}_{i,t})$ indicates the velocity fluctuation of position $\mathbf{r}_i$ at time point t , and $\mathbf{R}$ is the distance vector between two positions. $\langle \cdots \rangle$ refers to the mean over all directions of $\mathbf{R}$. The spatial correlation for velocity fluctuation decays over short distances. Interestingly, even though the collective migration on rough and smooth regions of surface was evidently different (Figure 3. 3) for migration velocity and migration directionality, the correlation function for these two cases were comparable to each other (Figure 3. 4). The correlation function exhibits a rapid decay over about 100 μm for both groups. A closer examination indicated that the correlation decays faster on the rough surface, which may reveal the fact that the cell-

cell interactions on the rough substrate are reduced because of their limited motility. Until now, several factors have been raised with regards to their influence on correlated motion. These factors are cell density [110], substrate viscosity [121], and substrate rigidity [120]. In this study, there is negligible cell density difference at the rough and smooth parts of the substrate. In addition, other mechanical properties except for roughness are the same for both the rough and smooth surfaces, thus ruling out the possibility of known effects on their correlated motion. Our results thus indicate the limited influence of substrate roughness on the modulation of intracellular coordination compared to the other reported substrate mechanical properties like substrate stiffness and viscosity.

Figure 3. 4 The coordination of tumour cell collective migration on nanoroughened and smooth substrates. It is quantified by the average correlation of cell velocity fluctuation field for all the time points, as a function of cell-cell distance.

3.3.4 The expressions of cell-cell and cell-substrate adhesion proteins were downregulated on nanoroughened substrate compared to the smooth one

It is important to find out the biochemical signaling behind the diverse physical behaviors of tumor cells as a group. In particular, focal adhesion proteins and intercellular junction proteins serve to mediate the interactions between cell to substrate, and cell to cell, thus influencing cell behavior in many respects. Adhesion proteins can be broadly divided into cell-cell adhesion proteins which regulate cell-cell communication, and cell-substrate adhesion proteins which regulate cell-substrate interactions. Unlike single cell movement, collective cell migration requires not only cell-substrate interaction, but also continuous cell-cell communication to maintain cell sheet integrity. The expression of cell-cell adhesion proteins would control monolayer integrity, and might further regulate the collective migration. Cell-cell adhesion and cell-substrate adhesion are both required in collective migration [122]. In consequence, we performed RT-PCR to measure the gene expression of several kinds of representative adhesion proteins for cells growing on nanoroughened and smooth substrates respectively. First, we quantify the expression of Ki-67 to make sure that the cells are proliferating. It was observed that the Ki-67 expression level has increased 2 folds after culturing on rough/smooth substrate for 30 hours (Figure 3. 5a), indicating that they were growing well on both smooth and rough PDMS surfaces. Interestingly, the gene expressions of cell-ECM adhesion protein, paxillin, and cell-cell adhesion proteins ZO-1 and β -catenin were all found to be significant less on rough surface than those on smooth surface (Figure 3. 5b) , though the expression of E-cadherin, one kind of cell-cell adhesion protein on two kinds of substrates was not significantly different in statistic. Together with the findings of collective cell migration on smooth and nanoroughened substrates, it implies that rough surface

could downregulate the gene expression of cell-cell and cell-substrate adhesion proteins and in this way weaken cell migration as a group.

Figure 3. 5 RT-PCR analysis of RNA extracted from huh7 cells growing on smooth and nanoroughened substrates for 30 h. (a) Fold increase in mRNA level of Ki-67 (indicated the proliferation during 30 h) on two kinds of surfaces. (b) The mRNA levels of paxillin, ZO-1, β -catenin, and E-cadherin are normalized to GAPDH mRNA levels. The data is shown as mean $\pm$ SD. Unpaired student t-test was performed to calculate the significance of difference (** $p < 0.01$).

Some earlier investigations have suggested that the upregulated expression of adhesion molecules could enhance tumor cell migration. For instance, the cytoplasmic localization of ZO-1 was found to induce a dramatic epithelial to mesenchymal transition of Madin-darby canine kidney cells [123]. Besides, the enhanced expression of β -catenin was reported to facilitate the motility of different kinds of carcinoma cells, like cervix carcinoma cells, colorectal carcinoma cells, and oral squamous carcinoma cells [124-126]. Also, the role of Wnt pathway in maintaining carcinoma cell migration and stemness was reported [126], showing the correlation between the

expression of β -catenin and tumor metastasis. Resulting from increased expression of β -catenin, the upregulation of paxillin further enhanced phosphorylation and in turn led to directional cell migration [127]. The earlier results were consistent with our findings, which evidently suggested the impact of surface nanoroughness on metastasis potential and gene expression of HuH-7 cells.

3.4 Conclusion

In conclusion, we have initiated a novel investigation on the influence of substrate nanoroughness on the collective migration of tumour cells and the expression of relevant adhesion proteins. The effective and low-cost method of creating nanoroughness can be used to fabricate a great batch of surfaces with a broad range of nanoroughness. Moreover, this simple way of introducing solidified PDMS as mask may enable a fast fabrication of roughness gradient in a bulk substrate. The interactions between cell and substrate is a complex process in which both the physical behaviours of cell and molecular signalling are involved. In our study, the cells exhibit slower and less directional collective migration on nanoroughened substrate. Also, the cell-cell and cell-substrate adhesion molecules are expressed less on rough substrate, suggesting the regulation of substrate nanotopography on cell biochemical functioning as well as physical response. Furthermore, our findings highlight the potential of manipulating tumour cell behaviours via regulating the mechanical properties of substrate in cancer study.

Chapter 4

4 Study on the Regulation of Cell Attachments by Matrix Nanotopography in 3D Environment

4.1 Introduction

Designing biomaterials, or scaffolds, is a long-standing challenge in the field of regenerative medicine and tissue engineering. Recently more and more attention has been paid to understand the impact of the mechanical cues of the scaffolds on cellular behaviors such as differentiation, proliferation, and so on. The mechanical cues include architectural structure of the scaffolds, and the matrix nanotopography provides spatial as well as mechanical cues for cells to sense and respond [72, 128, 129]. The effect on cellular behaviors ranges from microscale to nanoscale. The microscale feature would cause whole-cell contact guidance and thus control its shape, while the nanoscale feature is of similar size to individual receptor which affects integrin distribution and trigger receptor-driven pathways and alter biochemical signaling [79]. The mechanism of how nanotopography affects cellular behaviors is yet to be elucidated. It has been proposed that nanoscale topography is possible to modulate the tensions which direct the formation of cytoskeleton and the organization of membrane receptor within the cell, which in turn could regulate the intracellular mechanical and biochemical signaling [72, 130]. Most studies on nanotopography were carried out on two-dimensional cultural surfaces. Only a handful of work studies the nanotopographic effect on cells in 3D. Nevertheless, the 3D matrix structures used in these studies at the microscale are quite heterogeneous. The effects of the nanotopography on cells are not at the single cell level.

In this work, we will investigate the impact of nanotography on cells at the single cell level by creating 3D microenvironment which is uniform at micro-scale but with different surface roughness at nano-scale. The 3D microenvironment is a 100 μm spherical pore on which we generate two kinds of pore surfaces. One is smooth and the other is rough and full of nano-spikes. We compared cell attachments on smooth and rough surface. Inspired by the observation that strong stress fibers frequently appear in the tube-shaped structure of cell surface and a recent work of Elliott et. al. [131] where they had computed the surface curvature of endothelial cells in 3D collagen matrix to quantify cell surface, we also adopted such a method to quantify cell surface curvature in our 3D pore and further analyze the relation between surface curvature and cortical actin distribution. We found that cells tend to have more attachments to the smooth scaffold with more focal adhesions, while the cells on the rough surface exhibit less anchoring sites with reduced number of focal adhesions. By applying differential geometry and computer vision techniques, it is found that when cortical actin intensity increases, the corresponding curvature range becomes smaller with lower values. High intensity of actin mostly concentrates in region where the Gaussian curvature is close to 0, and stress fibers tend to form with tube-like structure. Moreover, the major range of the distribution for both actin and the curvature are different for cells cultured in the two kinds of scaffold, which might be affected by morphology and FAs distribution. Meanwhile, different parts of the cell surface also exhibit specific range of surface curvature and actin intensity. As a whole, our study indicates that in 3D environment, matrix nanotopography influences the adjustment of cellular attachments to matrix via the formation of focal adhesions. Moreover, cortical actin, which is important for supporting cell attachment, is greatly influenced by the local curvature of cell surface.

4.2 Materials and methods

4.2.1 Fabricating 3D microenvironment

The 3D microenvironment, which are semi-open spherical pores in polydimethylsiloxane (PDMS) matrix, is template by polystyrene microspheres (Polyscience, USA) of diameter 100 μm to be removed later. (Figure 4. 1) To create nano-roughened surface, the polystyrene particles were etched by 70% acetone diluted with ethanol for 3 min, followed by rinsing with ethanol for 5 times [132]. We deposited 0.1 ml polystyrene microspheres suspended in ethanol on a glass slide salinized with 1H,1H,2H,2H-perfluorodecyltrichlorosilane (Alfa Aesar, USA) and bake the solution in the oven for 1 minute at 70 °C to evaporate ethanol and leave beads spread on slide. We next cast mixed PDMS at 10:1 ratio of pre-polymer and curing agent (Sylgard 184, Dow Corning, USA) onto glass slide to form a thin layer. The cast PDMS layer was cured at 80 °C for 2 h and then the film was gently peeled off. The PDMS film was treated in dimethyl sulfoxide (DMSO, J.T.Baker, USA) at 140 °C to dissolve polystyrene microspheres [133] to create hollow spherical pores in PDMS film, which we refer as PDMS scaffolds. The PDMS scaffold was removed from DMSO and heated at 80 degree for 12 hours to remove residual DMSO from PDMS scaffolds. Before seeding the cells, we first treated the PDMS scaffold with oxygen plasma and next incubated PDMS scaffolds in 50 $\mu\text{g/ml}$ fibronectin (Sigma, USA) with 5X penicillin for 2 hours at 37 degree and finally rinsed the scaffolds with phosphate buffered saline (PBS) twice. To visualize the spherical pore, we conjugate cy5 (GE Healthcare) on to fibronectin. For the following discussion, we refer the scaffold made from etched beads as rough scaffold, while the scaffold from unetched beads smooth scaffold.

Figure 4. 1 Schematics of fabrication of PDMS spherical scaffold with topography cast from topography of polystyrene beads. Briefly, polystyrene beads in ethanol was dropped onto salinized glass slide, and after ethanol evaporates, PDMS was poured on glass slide. Then after heated at 80 degree for 2 hours, solidified PDMS layer was peeled off from glass slide. PDMS was put in DMSO at 140 degree to dissolve polystyrene beads to get PDMS scaffold and in this way the topography of microsphere was transferred to the scaffold.

4.2.2 Cell culture and immunostaining

REF cells stably expressing YFP-Paxillin were cultured in cell culture dish in Dublecco's Modified Eagle Medium (DMEM) (Life Technologies, USA), which was supplemented with 10% fetal bovine serum (FBS) (HyClone, USA) and 1% penicillin streptomycin (Life Technologies, USA). The cells were trypsinized after reaching 80% confluence and a small number of cells were seeded into PDMS spherical scaffold to observe single cell behavior.

Cells were cultured for 5 hours for full attachment and then fixed with 4% paraformaldehyde (Sigma, USA) for 10 min, and next permeabilized using 0.5% Triton X-100 for 10 min. Cells were stained with phalloidin-iFluor555 (1:1000 dilution) (AAT Bioquest) for 20 minutes to visualize F-actin. The samples was embedded in mounting medium made of 60% glycerol, 40% PBS, and 2.3% 1,4-Diazabicyclo[2.2.2]octane for matching refractive index and reducing photobleaching.

4.2.3 Surface characterization by AFM

The topography of etched and unetched polystyrene microsphere was measured with an atomic force microscope (AFM) (Nanowizard II, JPK, German). 6 μm x 6 μm area of bead top surface was scanned using a silicon cantilever (OMCL-AC240TN-C3, Olympus) with spring constant 2 N/m in tapping mode. To characterize topography of the microsphere, firstly we need to remove the surface curvature and we followed the approach from Karl Shellenberger's work on colloidal beads [134]. Briefly, each individual scan trace over the polystyrene surface was fit to a circle and then the height due to the arc was subtracted from the original scan. Next we chose 10 nm as the threshold for local peaks and calculated the number of peaks per 6 μm x 6 μm scan area [135] based on surface topography map after such flattening operations. The significance of the difference for the number of local peaks in two groups of substrate was estimated with an unpaired student t-test.

4.2.4 Imaging and analysis

The image stacks were taken with a 63X/1.3NA glycerol objective on a Leica DMI 6000 microscope equipped with a CSU22 spinning disk confocal unit.

To calculate the individual focal adhesion length, the 2D images were first deconvolved with Huygens 4.5 (Scientific Volume Imaging), and then processed with Imaris 8.1.2. We identify focal adhesion by the built-in surface function with appropriate threshold and then used the bounding box function of Imaris to calculate the length of focal adhesion as the maximum length of the bounding box. Cell surface was segmented from intensity signals combining from paxillin and F-actin. The combined signal was smoothed by Gaussian filter of kernel size 0.102 μm and then segmented by manual contour surface function embedded in Imaris. The cell surface was denoted as triangulated-mesh and exported to MATLAB for later analysis. The

local surface curvature of cell surface was calculated based on algorithm from [136]. Throughout the study we were using locally averaged curvatures, which was the mean value of all curvatures within a sampling sphere of radius 1 μm [131]. The same sampling scale was also applied to actin cortex intensity calculation such that the intensity value was calculated as the average value of all vertices falling within sampling sphere. Actin intensity was normalized using the method proposed in [131].

In order to further study the roles play by different parts of the cell surface in regulating cell morphology, we split the cell surface into three parts to investigate them separately. First, we rotate the 3D cell object along the x and y axis at various angles to obtain the maximum 2D projection onto the xy plane, and then obtain the corresponding cell boundary in 2D. Next, we took solely the x and y coordinates of all the vertices so that all the vertices at a distance of 1~1.5 μm from the 2D boundary in the xy plane, together with vertices composed of the 2D cell boundary, to define the cell boundary in 3D. After removing the defined boundary from the cell surface, there remain two major vertices clusters. The clusters are split into the basal part which gets attached to the scaffold, and the apical part which is away from the scaffold.

4.3 Results and discussion

4.3.1 Numerous nano spikes were created on polystyrene beads after etching with acetone

Even though it is difficult to measure the topography of the surface inside the pore directly, we measured the nano-topography of the template particles instead (Figure 4. 2a) since PDMS has been used for nano-imprint (reference) and thus we believe that the nano-features created on template particles are preserved in the PDMS scaffolds. After flattening operations for AFM measurement to exclude the effect of surface curvature (Figure 4. 2b), local spikes higher than 10 nm are defined as peaks, which is

a commonly defined spacing parameter [135]. The AFM results showed that there were around 60 nanospikes on $6 \times 6 \mu\text{m}^2$ scanning area on etched microsphere, while there were only around 15 nanospikes on that of unetched microsphere (Figure 4. 2c).

Figure 4. 2 (a) AFM characterization of polystyrene beads topography obtained from beads before (left) and after (right) etching by acetone. (b) The topography of unetched beads (left) and etched beads (right) after removing the effect of surface curvature. (c) Number of peaks per scan area for two groups of beads. $n=14$, 11 measurements respectively. Data is shown as mean $\pm$ SD.

4.3.2 Cells tend to attach to scaffold inner surface with a larger number of focal adhesions when cultured in unetched scaffold compared to growing in the etched scaffold

Cells exhibited distinctive attachments on smooth and etched scaffolds. In the smooth scaffold, cells tend to have more attachments to scaffold inner surface than cells in the etched scaffold (Figure 4. 3a). Interestingly, we found there are more focal adhesions of cells on smooth scaffolds than cells on rough scaffolds (Figure 4. 3b), which might arise from greater attached area with scaffold. In 2D, cell area and total focal adhesion

area was reported to be positively correlated [100], and less focal adhesions were found for cells on micro-rough surface compared to that on smooth surface [137], which are all consistent with our study. Moreover, cell adhesion was reported to be higher on rough substrate in 2D study [69, 71]. Thus if we assume the adhesion is also stronger for cells cultured in etched scaffold, then such stronger adhesion makes it possible that less adhesion sites might be sufficient for cellular adhesion in etched 3D scaffold.

Figure 4. 3 (a) Representative figure of cells expressing YFP-paxillin (green) in unetched (top) and etched (bottom) scaffold. Blue: scaffold, red: actin. (b) The

number of focal adhesion streaks per cell. $n=64$, 60 cells respectively. Used unpaired student t-test, $p<0.001$. (c) Normalized individual FA length distribution for cells in unetched (top) and etched (bottom) scaffold group.

However, there is not much difference between the focal adhesion size for cells on both surfaces. The focal adhesion size ranges from 0.5 μm to 3 μm (Figure 4. 3c) and are generally smaller than focal adhesions on 2D substrates, which indicated that the nanotopography of the scaffold is not a dominant factor in regulating the size of focal adhesions in 3D environment.

4.3.3 The local mean and Gaussian curvatures of cell surface are mainly concentrated in a narrower range for cells grown in etched scaffold than in unetched scaffold

As we observed stress fibers more likely to appear in tube-shaped structure of cell surface, we speculate this might be related with surface local geometry. To quantify cell surface further, we computed the local mean and Gaussian curvature (Figure 4. 4b, c) for cell surface segmented from Imaris (Figure 4. 5a, b). Two principal curvatures determine the local shape of a point on a surface. One characterizes the rate maximum bending of the surface and the tangent direction in which it occurs, while the other characterizes the minimum bending. Let k_1 and k_2 be the principal curvature of a surface patch. The corresponding Gaussian curvature is $G = k_1 k_2$, and its mean curvature is $H = \frac{1}{2}(k_1 + k_2)$. In our study, triangulated-mesh surface was created with Imaris. To estimate the curvature tensor of a triangular mesh, a simple normal based estimation algorithm was proposed in [136]. Because the curvature tensor of a smooth surface is completely defined by its first order partials and the first order

partials of its normals, for each triangle patch, we use linear interpolation of three vertices to get the surface and its first order, and the corresponding normals of vertices are used to calculate the normal and its first order partials [136]. For consistency in the whole study, curvature and actin intensity are both determined after spherical sampling with a radius of 1 μm .

Figure 4. 4 (a) Representative cortical actin intensity map of a cell grown in etched scaffold. (b) Mean curvature map. (c) Gaussian curvature map.

For cells in both groups, the plot of mean curvature against Gaussian curvature shows that most of the curvature value falls around the region where Gaussian curvature is close to 0 while mean curvature falls within the range of -0.3 to 0.3. Note that the least number of data points are found to fall within the second quadrant (Figure 4. 5c, d), like the invagination-shaped structure in [131]. The pattern indicates that the local curvature of cell surface is not some random value, but is limited to a certain range where the extremely large curvature is unlikely. Similar findings were also reported for primary aortic endothelial cells cultured in 3D collagen matrix [131].

Figure 4. 5 (a, b) Representative surface segmentation for cells in unetched (a) and etched (b) assisted by Imaris. (c, d) Gaussian curvature VS mean curvature for cells in unetched (c) and etched (d) scaffold. The color indicates log10 of surface points counted. $n=12$, 12 cells respectively. (e, f) Mean curvature normalized distribution for cells in unetched (e) and etched (f) scaffold. Only major range $[-1, 1]$ was shown.

n=12, 12 cells respectively. (g, h) Gaussian curvature normalized distribution for cells in unetched (g) and etched (h) scaffold. Only major range [-0.5, 0.5] was shown. n=12, 12 cells respectively.

Importantly, for cells in etched group, the peaks of both histograms for mean and Gaussian curvature are higher and narrower than those in unetched group (Figure 4. 5e, f, g, h), which might result from fewer attachment sites in etched scaffold. Full Width Half Maximum (FWHM) was used to quantify the difference of curvature distribution. It was shown that the FWHM of Gaussian curvature distribution plot is 0.0122 for etched group, much smaller than 0.0217 for unetched group. Similarly, the FWHM of the mean curvature distribution plot for etched group is 0.2092, still smaller than 0.2536 for unetched group. The Gaussian distribution being relatively more concentrated around 0 suggests that there are more tube-shaped structures formed on the cell surface for cells cultured on etched scaffold, which might arise from fewer anchoring sites.

4.3.4 “Negative” correlation was found between the distribution of cortical actin intensity and absolute value of the mean/Gaussian curvature

We found actin stress fibers to mostly appear in area where cell body are elongated (Figure 4. 6), while they are seldom observed at highly curved cell surface area. This suggests that actin distribution might be linked to local cell surface curvature.

Figure 4. 6 Observed prominent stress fibers for cells in etched scaffold.

Then we investigated the correlation between cortical actin intensity and the absolute value of local mean curvature (or Gaussian curvature) (Figure 4. 7a, b, c, d). The plots of normalized actin intensity against mean curvature showed that for either cell in unetched or etched scaffold, when cortical actin intensity increases, the corresponding curvature range becomes smaller with lower absolute values. High actin intensity was found to concentrate mostly in regions where Gaussian curvature is close to zero, and here highly-aligned stress fibers are observed to form at tube-like structures. Such “negative” correlation is consistent with experimental findings that stress fibers mostly appear at tube-shaped area on cell surface and also frequently appear in 2D studies. Moreover, the major actin intensity distribution for cells in etched scaffold are found to concentrate in a smaller range of lower value compared to the more scattered distribution observed for cells in the smooth scaffold. The decrease of focal adhesions

for cells in etched scaffold may cause a decrease in ways for actin to be distributed or aligned, which results in a smaller actin intensity range.

Figure 4. 7 (a, c) Cortical actin intensity VS mean curvature for cells in unetched (a) and etched (c) scaffold. The color indicates $\log_{10}$ of data points counted. $n=12$, 12 cells respectively. (b,d) Cortical actin intensity VS Gaussian curvature for cells in unetched (b) and etched (d) scaffold. The color indicates $\log_{10}$ of data points counted. $n=12$, 12 cells respectively.

Most importantly, FAs and actin filaments are closely correlated with each other. FAs contain a lot of actin-binding proteins and the regulation of actin dynamics is also associated with FAs [138], and actin in turn could influence the stability of FAs [139, 140]. In our study, we found that most focal adhesions appear at two ends of the actin

filaments, similar to most findings in 2D environment. It was observed in 2D that a decreased FA area favored of higher F-actin alignment [141], which was consistent with our study that highly aligned stress fibers were more likely to occur for cells in etched scaffold with less focal adhesions compared to cells in smooth scaffold with more focal adhesions.

4.3.5 Different parts of cell surface play independent while interconnected role in regulating surface curvature as well as cortical actin distribution

After obtaining knowledge on the correlation between cortical actin intensity and cell surface curvature for the whole cell, it remains unclear whether different parts of the cell surface play different roles in actin intensity and surface curvature regulation. To understand this, we first obtain the maximum 2D projection of 3D cell morphology, and define the projection boundary together with all the neighboring points from the boundary in 3D (Materials and Methods). Next the cell surface except the cell boundary is divided into the apical and basal parts depending on whether they get attached to the matrix (Figure 4. 8).

Figure 4. 8 Representative figure of cell surface split into boundary (green), basal (blue), and apical (red) part for unetched group (panel a) and etched group (panel b).

After segmenting one complete cell surface into three parts: boundary, apical, and basal, we then studied the correlation between actin intensity and curvature for each part in unetched (Figure 4. 9) and etched (Figure 4. 10) group.

Figure 4. 9 Cortical actin intensity VS mean curvature for cell whole surface (a), boundary (b), apical (c), and basal (d) part of one representative cell from unetched group. Cortical actin intensity VS Gaussian curvature for total cell surface (e), boundary (f), apical (g), and basal (h) part for one representative cell.

Figure 4. 10 Cortical actin intensity VS mean curvature for cell whole surface (a), boundary (b), apical (c), and basal (d) part of one representative cell from etched group. Cortical actin intensity VS Gaussian curvature for total cell surface (e), boundary (f), apical (g), and basal (h) part for one representative cell.

Interestingly, the scattering of actin with respect to mean curvature (Figure 4. 9a, b, c, d and Figure 4. 10a, b, c, d) and Gaussian curvature (Figure 4. 9e, f, g, h and Figure 4. 10a, b, c, d) exhibit a similar pattern for the whole cell and the cell boundary. This

indicates that cell boundary is critical to determine the cell shape. The high curvature is likely to happen in boundary part (Figure 4. 10 a), while it is also possible for the tiny structures, like some local bumps, on basal or apical surface to possess high curvature, as the arrow indicated (Figure 4. 10 a, c). Highest actin intensity is frequently found to appear at the region where cell adheres to the scaffold. For cells in unetched scaffold, it is more likely to happen in basal part, due to the great attachments. While, if most of the adhesions come from the attach of boundary part to scaffold, which is more likely to happen in cells of etched group, the highest actin may also arise from the boundary region (Figure 4. 10 b). Another impact factor is the formation of stress fibers, which has been observed to have a preference towards cell boundary (Fig. 4.3 a, b) and is of great intensity. Through our investigation, in terms of different subunits, we have observed that each part of the cell surface plays independent while interconnected role in regulating surface curvature as well as cortical actin distribution.

4.4 Conclusion

In summary, based on a highly effective method of fabricating 3D spherical hollow scaffold with controlled topography, we have conducted a study on the influence of matrix topography on cell adhesions in 3D. The cells are observed to exhibit dissimilar attachments and different number of focal adhesions in two kinds of scaffold: unetched scaffold and etched scaffold with nano spikes. Our results show the presence of correlation between cortical actin, the important component for supporting cell attachment, and local cell geometry, which implies the influence of 3D matrix topography on cell attachments through the regulation of actin and focal adhesion distribution. It highlights the potential of applying 3D matrix topography to manipulate cell behavior for the design of biomedical devices.

Chapter 5

5 Conclusion and future work

5.1 Summary of my work and contribution to the field of cell mechanics

Cells in tissues are mechanically coupled both to the ECM and neighbouring cells, thus a quantitative investigation of the cell-cell and cell-substrate interactions becomes crucial for the cell mechanics study. Cell mechanics is a cross-disciplinary field, and the application of new emerging computer techniques and advanced microscopy contribute greatly to the quantification of cell mechanical behaviour study. In particular, my research has spanned the range of cell mechanics from single cell level to the collective level of a group of cells, and from two-dimension to three-dimension.

My thesis begins in the extremely important and fundamental concept of cell mechanics – cell traction force. As adherent cells need to attach to a substrate in order to survive, it exerts contraction forces which are transferred to the substrate by means of integrin-mediated focal adhesions. Traction force exerted by a cell to ECM is necessary for cell spreading and migration [87]. In our study, we have used microposts to facilitate an accurate quantification of cell traction force. During cell contraction, ligand-induced clustering of integrins are critical in mechanotransduction. Thus, biochemical signals from ECM proteins is an important factor that influences cell traction force.

In the first part of my thesis, I have detailed our measurement and comparison of the traction forces of HUVEC on either fibronectin or collagen type I, which are two abundant and essential components of the ECM. Although HUVEC is found to adhere to both type of proteins on glass surface and the microposts by expressing collagen-

binding integrins $\alpha1\beta1$ and $\alpha2\beta1$ or fibronectin-binding integrins $\alpha5\beta1$ and $\alpha v\beta3$ [37-39], we observe that the cell spread area is larger on fibronectin than on collagen. We found that this difference is not attributed to a lack of ligand-specific integrins. Moreover, the average force exerted per pillar is not significantly different between the two groups of cells, suggesting that any difference in force transmission signaling between them is unlikely a result of a difference in force magnitudes. The profiles of the force range distributions between the two groups are also similar. Besides the magnitudes of cell traction force, we have also investigated cell contraction by quantifying its directionality. A higher level of directional contraction of cells is found on fibronectin than on collagen after sustenance for 2h. The angle of neighboring force vectors is also smaller, depicting close-to parallel orientation in cells on fibronectin than on collagen. The connection between the actin cytoskeleton and the focal adhesion sites coupled with myosin activity is necessary for force transmission in cells. In our study, we found that total focal adhesion area increased with cell spread area in both group of cells, but cells on fibronectin exhibit larger value for both parameters, which are evident at a later time point of 37 min. Different integrins are involved during binding with fibronectin or collagen type I. Hence, it is possible that different signaling pathways have been triggered that results in differences in cell spread area and directional contraction of the two groups. We have for the first time comprehensively demonstrated the distinct cell biomechanical behaviours on fibronectin and collagen. This comparative investigation is expected to not only provide some hints for other researchers when choosing ECM proteins in in-vitro studies, but also help the investigation on the mechanisms of physiological behaviours that are correlated with ECM molecules concentration and composition. For example, during wound healing there is a temporal difference in the synthesis and

deposition of ECM proteins, including fibronectin and collagen at the site of injury [35]. We speculate that distinct biomechanical behaviors of endothelial cells would arise on these ECM proteins during the modulation of the angiogenic response.

After investigating single cell mechanical behaviour, I proceed to study the behaviour of cells in groups rather than in an isolated state, and how individual cells would influence each other as they couple with neighbouring cells. For tumour cells, the ability to migrate collectively is necessary during tumour invasion. And glass surface with nanotopography was reported to capture circulate cancer cells [73]. Collective cell behaviour is a fundamental question in biology that motivates my research. For this, I find that substrate nanotopography, which is characterized by roughness, is an important factor that influences cell growth and modulation of cell behaviours that yet to be properly understood. Thus, in my second project, I have conducted a comprehensive investigation on the influence of substrate nanoroughness on cancer cell collective migration.

An effective while low-cost fabrication method of nanoroughened surface was proposed to create a broad range of nanoroughness from 10 to 10^3 nm. Moreover, controlled roughness gradient in a bulk substrate was achieved by introducing an efficient way of utilizing solidified PDMS layer as mask. After culturing cells on PDMS substrate containing half smooth and half-roughened surface, we observed huh7 cell monolayer to exhibit a slower collective migration on roughened substrate compared to smooth substrate. Moreover, the directional persistence of migration on nanoroughened substrate is smaller than that on smooth surface, which indicates the increased difficulty of tumour cells migrating on rough substrate. These findings indicate the potential of repressing tumour cell migration by controlling the magnitude and directionality of substrate roughness. Spatial autocorrelation function

of the velocity fluctuation fields was applied to quantify the cooperative motion of individual cells and their neighbours, and it was shown that for both groups the correlation decays quickly as the distance between the two cells increases, typically over the first 100 μm . However, a closer examination indicates that the correlation decays faster on rough surface, which suggest that the interactions between cells on the rough substrate has been reduced by their limited motility. To study the relevant biochemical signalling of cells under different roughness, we measured the gene expression of adhesion molecules on the rough and smooth surface with RT-PCR. It was shown that the expressions of both cell-cell adhesion and cell-substrate adhesion molecules were downregulated on nanoroughened substrates. Together with the collective migration results, our study suggest the regulation of surface nanotopography on cell mechanical behaviours as well as biochemical signalling. Our results highlight the potential of manipulating tumour cell behaviour through artificially modifying substrate mechanical properties in biochemical device design.

My first two projects focus on the study of the interactions between cells and its environment in 2-dimension (2D). It is however known for more than thirty years that cells which grow on 3-dimensional (3D) scaffolds are more representative of the *in-vivo* tissues and organs than the cells cultured on 2D surface. Past 2D studies have shown that substrate nanotopography is an important mechanical cues for cell function. Therefore in my third project, I investigate into the interplay between single cell and nanotopography in a 3D environment.

The design of biomaterials, or scaffolds, is known to be a long-standing challenge in the field of regenerative medicine and tissue engineering. In this project, we proposed a simple while effective method to fabricate hollow spherical PDMS scaffold whose topography could be determined by different degree of etching of mold during

fabrication, and in our case we use polystyrene microsphere as the mold. Based on such a platform, we cultured cells in smooth scaffold (the unetched scaffold) and etched scaffold to study the influence of matrix topography on cell morphology in 3D environment. Cells tend to have more attachments to the smooth scaffold with more focal adhesions, while the cells on the etched surface exhibit less anchoring sites with reduced number of focal adhesions. Furthermore, we observe that actin stress fibres appear mostly in area where the cell body is elongated, instead of the highly curved cell surface area. We quantify the local curvature of cell surface and find that when cortical actin intensity increases, the corresponding curvature range becomes smaller with lower values. Moreover, for cells in the etched group, the peak of both histograms for the mean and Gaussian curvature is higher and narrower than those in the unetched group. Together with the findings on focal adhesions, our study has contributed to a quantitative understanding on the influence of 3D matrix topography on cell attachments through the regulation of actin and focal adhesion distribution. It also highlights the potential of applying 3D matrix topography to regulate cell behavior in biomedical device design.

All the three research projects in this thesis focus on the cellular mechanical interactions between cells and environment. Computer techniques have been applied in our quantitative analysis of cellular functioning. Together with experimental analysis, our research has contributed to a better understanding on the mechanism of cellular response to different kinds of biochemical or physical cues. We believe our results would benefit biomedical device design for the purpose of cell behaviour manipulation.

5.2 Future work

In the third project we have found that matrix nanotopography is crucial for cell morphology regulation at the single cell level in 3D environment. We found that cell exhibits a more distinct and physiological morphology in 3D compared to the flat shape commonly seen in 2D. For a more comprehensive understanding of cell mechanical behaviours in 3D and under in-vivo conditions, it becomes necessary for us to advance our knowledge on the collective behaviour of cells in 3D since cells do not live isolated from other cells *in vivo*.

The design of biocompatible scaffold is critical for 3D cell mechanics study and a lot of attention has been paid to the impact of the mechanical properties of the matrix on cell function. It is better to choose materials whose mechanical properties can be regulated and apply different structured design to mimic different *in-vivo* conditions. As an important component of the study of cell collective behaviour, understanding the coordination between neighbouring cells in 3D environment is critical to resolve the question of structural integrity, which is closely correlated with many diseases.

The study of cell collective behaviours in 3D, especially the interplay between cells and matrix mechanical properties, is expected to contribute to regenerative medicine and tissue engineering. In particular, the targeted manipulation of tumour cell behaviour for cancer research, and adjunctive therapy for impaired tissue integrity.

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Publication

Han, J., Menon, N. V., Kang, Y., & Tee, S. Y. (2015). An in vitro study on the collective tumor cell migration on nanoroughened poly (dimethylsiloxane) surfaces. *Journal of Materials Chemistry B*, 3(8), 1565-1572.

Han, J., Tan, H. F., Feng, C., Wee, W. K., Tee, S. Y., & Tan, S. M. (2016). Data on cell spread area and directional contraction in human umbilical vein endothelial cells on fibronectin and on collagen type I-coated micro-posts. *Data in brief*, 6, 803-810.