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**REGULATION OF SUBCELLULAR CONTRACTILE
ACTIN NETWORK BY EXTRACELLULAR
GEOMETRY**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2017

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

2017

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

25 May 2017

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Date

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Abstract

Cells are active materials which can undergo division, shape changes, and migration. The ability to generate force, which is regulated by the actin cytoskeleton in the spatial and temporal dimensions, is essential for these dynamic behaviors. The distribution and localization of force are controlled by actin organization in cells and determined by the biochemical and biophysical properties of ECM. Actomyosin, which is composed of actin filaments, motor proteins, and crosslinkers, constitutes the core machinery for contraction generation. Focal adhesions serve as the mechanical linkage between ECM and the actin cytoskeleton which are responsible for transmitting mechanical forces and regulatory signals. Using high-resolution light microscopy, researchers can determine the location of proteins on actin networks with high spatial and temporal precision. To test how actin binding proteins collaborate to form functional actin structures, a reconstituted system with purified proteins are often used. However, the results obtained from this over-simplified system cannot reflect the real situation inside living cells, for example, the function of focal adhesion and ECM are neglected in this experiment set up. Until now, the formation of actomyosin structure in living cells is still not clearly understood.

In this thesis, the primary objective is to study the actin structure in living cells during their migration across discontinuous ECM, by using a pattern which can constrain cells movements directionally. The behaviors of different types of cells when migrating across these discontinuous gaps were then studied. Interestingly, a unique multinodal actin network was discovered for the first time by merely varying the continuous properties of ECM in HUVEC, which provides us a living cell model to study the formation of the actomyosin structure. Using traction force microscopy, it is demonstrated that this subcellular actin structure is contractile. Lastly, the function of a motor protein - myosin II and crosslinker-filamin A in this actin structure was elucidated in detail. The work in this thesis suggests that activities of myosin II and filamin A are controlled by the ECM distribution, which determines the actin architecture.

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Abbreviations

ECM	Extracellular Matrix
PAA	Polyacrylamide
SEM	Scanning Electron Microscopy
TFM	Traction Force Microscope
CTF	Cell Traction Force
HUVEC	Human Umbilical Vein Endothelial Cells
PDMS	Polydimethylsiloxane
STED	Stimulated emission depletion
ROCK	Rho-associated coiled-coil kinase
FA	Focal Adhesion
DIC	Differential Interference Contrast
PFA	Paraformaldehyde
BSA	bovine serum albumin
MRLC	Myosin Regulatory Light Chain
siRNA	Small Interfering RNA

Chapter 1

Introduction

The ability of cells to divide into two daughter cells, change or maintain their shape depends on cell mechanics. The studies of mechanical interactions between cells and extracellular environment have become an intense area. The cytoskeletons and focal adhesions organized into hierarchical structures are the basic elements for dynamic behaviors. How cytoskeletal networks generate and respond to external chemical and mechanical signals remains unclear. The distribution of ECM is heterogeneous, and the degradation and remodeling occur in development and disease. In this chapter, first, the importance of discontinuous ECM in the physiological and pathological state is discussed, previous works on studying the interactions between cells and discontinuous ECM are summarized, and it is agreed that the challenge is to understand how actin network are generated when cells migrate across non-adhesive ECM. In this thesis, the multi-nodal actin network localized on gaps was observed and studied in detail. It is demonstrated that the formation of this unique actin network is due to the activation of myosin II and filamin A by the discontinuous ECM. Finally, a concise introduction to the contents of each chapter is presented. Also, the novel findings have been listed at the end.

1.1 Hypothesis

The extracellular matrix (ECM), which provides mammalian cells with both chemical and physical information, is a mesh composed of various types of protein fibers such as elastin and fibrillar collagens with the length from 10 to hundreds of nanometers. Adhesive proteins, such as laminin and fibronectin are coated on the mesh and provide specific attachment sites such as RGD (Arg-Gly-Asp) for cell adhesion. The ECM of different organs differ in the chemical composition and physical organization. Therefore they can supply specific signals for tissue morphology and physiology (Figure 1.1). For example, the ECM in hearts is an 3D network composed of an interweaving pattern of fibrillary collagen and elastin bundles that form an elastic network with proteoglycans and adhesive molecules. In this defined network, the cardiomyocytes are forced to couple mechanically to each other, synchronized with each other and generate contraction force for heart beating^[1]. But in the bone, ECM is a nanocomposite material composed of inorganic nanocrystalline hydroxyapatite and an organic collagen-rich matrix.

Figure 1.1 The ECM in different types of tissues. Illustrations of cell-matrix interactions at different organs, including heart, liver, and bone^[2]. Scanning electron micrographs of corresponding biomimetic engineered scaffolds were shown on the right side.

Alterations to the ECM occur during the formation of many diseases. For example, in central nervous system (CNS), there are three major compartments of ECM (Figure 1.2). The basement membrane is a sheet-like layer used to connect endothelial cells with astrocytic endfeet and maintain the integrity of the blood-brain barrier (BBB). This continuous structure is comprised of basal lamina, collagen, fibronectin, perlecan, and dystroglycan. But in cerebral ischaemia, this continuous ECM breaks down to discontinuous because of the increased concentration of matrix metalloproteinase 9 (MMP9), which is a class of enzymes regulating the degradation of the ECM. This discontinuous ECM can cause in the leakiness of the BBB. In summary, the composition of ECM differs in temporal and spatial scales. During diverse physiological and pathological processes, cells encounter various external signals, including varied biochemical composition, topography or stiffness. Among this broad range of extracellular environments, the discontinuous ECM is an important factor in causing disease and which is not sufficiently studied. Therefore, it is important for studying cell reorganization during the interaction with discontinuous ECM.

Figure 1.2 The three principal compartments of ECM localized on CNS^[3]

Figure 1.3 Studies of actin self-organization responding to ECM. (a) Basal confocal section of epithelial bridges immunostained of E-cadherin and actin (white arrow shows the cell-cell junction)^[4]. (b) The stain of myosin, cortactin, and actin when collective cells placed on well-defined geometry after 15 mins^[5]. (c) Actin bundles accumulated along non-adhesive edges and thin actin bundles formed along adhesive edges^[6]. (d) Immunostaining of vinculin, F-actin, and cortactin were averaged over 16–97 cells (Lower)^[7]. (e) Cellular bridges are generated when cells migrate on patterned fibronectin lines. The actin network is composed of ‘bow-tie’-shaped cytoskeleton and concave edges^[8]. (f) STORM images of actin structures before and after LatA treatment. Blue arrowheads indicate actin nodes^[9].

To systematically investigate the cell deformation regulated by geometrical constraints,

researchers developed a microcontact printing technology to fabricate micropatterned substrates. At the tissue level, previous works have demonstrated that multi-cellular bridge can be formed to maintain tissue integrity by human keratinocytes when migrating along non-adhesive strips. But when the epithelial cell line (MDCK) was placed on the same substrate, no epithelial bridges were observed (Figure 1.3a)^[4]. This is because the expression of cell-cell junction proteins depends on cell types. These multicellular suspended structures require large-scale tension driven by antiparallel actin stress fibers connected to intercellular adhesion and focal adhesion proteins. The closure mechanism of wounds and holes within tissues is another interesting research area in collective cell behavior. Lamellipodium crawling and actomyosin-mediated purse-string contraction are the two mechanisms to influence the closure behavior of epithelia^[5]. The thick actomyosin cables localized at the cell edge is responsible for generating tangential forces (Figure 1.3b). Moreover, in the studies of single cell sensing the mechanical heterogeneity of external environment, increasing actin cable tension was observed over unattached edges (Figure 1.3c,d)^{[6],[7]}. Also, cellular bridges were obtained in single cell embedded on heterogeneous ECM with gaps (Figure 1.3e)^[8]. Cells remodel their cytoskeleton to arc shape structure connected with focal adhesion. With the treatment of Latrunculin-A (Lat A) which is an inhibitor of actin polymerization, actin nodes appear on the non-adhesive area (Figure 1.3f)^[9]. In summary, to accomplish different physiological functions on different ECM, a cell constructs several distinct cytoskeleton structures as described in Chapter 2 in detail. The challenge is to clearly understand how cells spatially and temporally build these architectures with different components, including integrins, signaling molecules, actin, myosin family, crosslinkers (Figure 1.4).

Previous work on discontinuous ECM always uses micropatterned strips as the simplified model. The disadvantage of this model is that cells prefer to migrate along the strips which make the ECM continuous at one dimension. In this thesis, to constrain cells to migrate across the non-adhesive area and study the remodeling of actin structure, a new pattern was designed at the subcellular level.

During the study of single cell migrating across gaps, a unique actin structure connected by actin nodes was observed on the non-adhesive area. I studied this actin network in detail and identified that this is a highly dynamic contractile network. Although a great

deal of work focused on the understanding the organization of actin network, how the force generation unit – actomyosin structure is generated in the living cell remains an open issue. Studies on actomyosin in reconstitute systems have demonstrated that contractility depends on the connectivity of actin network, which regulated by the concentration of motor proteins - myosin II and crosslinking proteins, such as formin or filamin A^[10]. Also, it is demonstrated that filamin A is a key element in stress fibers^[11]. Thus, I proposed that this unique contractile structure formed on the non-adhesive area is the result of interaction between actin filaments, myosin II and filamin A.

Figure 1.4 The scheme of the interplay between actin structure of focal adhesion linked ECM^[12].

1.2 Objectives and Scope

To study the strategy that different cell line uses to migrate across the non-adhesive area, a discontinuous pattern was designed. The cells were placed on fibronectin blocked

fabricated by microcontact printing on polyacrylamide (PA) gel. Interestingly, an actin network connected by several actin nodes was observed. This is reminiscent of the actin cluster observed in living systems which have an important function, such as the foci coalesce in the early development of the embryo. Using transfection technology and advanced light microscope, I recorded the dynamic formation process of this multinodal actin structure, including actin condensation, actin nodes merging and actin turnover. The result of focal adhesion immunostaining indicates that the actin network is contractile.

The actomyosin-mediated contractility is essential for various physiological processes. However, it is only well studied in simplified reconstituted system. This work provides a model for studying the formation of actomyosin structure in the living cell. Using traction force microscope, I quantify the forces generated on the continuous pattern and discontinuous one with the different size of the gap. The result by the quantification data of focal adhesions demonstrates this multinodal actin network is contractile.

To investigate the mechanism of contractile multinodal actin network formation, I focused on elucidating the function of myosin II and filamin A. The results show that the localization and distribution of these two kinds of proteins are determined by the discontinuous property of ECM.

This finding of actin architecture reminds us the complexity of cytoskeleton and the potential of extracellular signals in regulating cytoskeleton architecture, also highlights that filamin A plays a key role in subcellular force generation.

1.3 Dissertation Overview

The thesis addresses the findings in how mechanical behavior at cellular scales is regulated by the chemical and physical properties of ECM. It contains seven chapters and here are the details of each chapter.

Chapter 1 provides a rationale for the research. The importance of studying the interaction between cells and the extracellular environment is elucidated. And the hypothesis of how cells sense and respond to the discontinuous ECM is also described in detail.

Chapter 2 reviews the basic knowledge about cytoskeleton and summarized recent

progress on the dynamic reorganization of the cytoskeleton, focal adhesion in living cells in responding to different physical properties of ECM, including confinement, stiffness, gaps. In particular, I summarized the studies of actomyosin structure, which is the core machinery of contractile stresses generation.

Chapter 3 addresses the experimental methodology mentioned in this thesis including hydrogel fabrication, photolithography, microcontact printing, super-resolution microscope, RNA interference and so on.

Chapter 4 elaborates actin reorganization in different types of cells after placed on discontinuous ECM. Interestingly, a multinodal actin network was observed in endothelial cells. Then I studied this special actin structure in details. As a result, the orientation of actin filaments is controlled by the gap size on the pattern. Also, the dynamic organization process of this actin structure was studied using transfection method.

Chapter 5 elaborates focal adhesion distribution and corresponding subcellular force generation on discontinuous ECM.

Chapter 6 elaborates the localization of myosin II and filamin A on contractile actin network using super-resolution microscope. With the treatment of siRNA, the forces generated on the discontinuous pattern was decreased indicating that myosin II and filamin A cooperatively enable cells to generate subcellular contractile actin network.

Chapter 7 discusses the major findings of this thesis. Moreover, some unanswered questions and future perspective were proposed in this chapter.

1.4 Findings and Outcomes

This research led to several novel outcomes by:

1. Establishing a platform to constrain single cell to migrate across the non-adhesive area. This design mimics the physiological process in vivo when single cell transfer between discontinuous surfaces.
2. Finding out a new multinodal contractile actin network linked with focal adhesion, which is generated only by varying ECM distribution. This result provides new insight on intracellular contraction generation.

3. Elucidating the function of myosin II and filamin A in regulating the formation of subcellular anisotropic contraction.

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Chapter 2

Literature Review

A living cell can sense and respond to both biochemical and physical signals in its extracellular environment through the regulation of crosstalk between the forces generated by actomyosin contraction and the assembly and disassembly of focal adhesions. Different contractile stresses generated by the actomyosin structure is essential for diverse physiological processes such as cell division, migration, muscle contraction and tissue morphogenesis. To study the mechanism of the actin network organization, an active gel system composed of actin filaments, motor proteins and crosslinkers was developed and studied. However, the process through which this contractile network is generated and controlled in living cells remains unclear. This literature review outlines the progress of cytoskeleton mechanics and focal adhesions formation in response to ECM environments. This knowledge is crucial for establishing the predictive model of physiological processes.

2.1 Cytoskeleton

The cytoskeleton consists of three major types of proteins: actin filaments, microtubule^[1] and intermediate filaments that differ in structure and composition. It is well demonstrated that the biological function of cytoskeleton depends on its physical structure, such as cilia and mitotic spindle. Cells have the ability to migrate, cytokinesis or change their shape responding to extracellular environment mainly relies on the dynamic assembly of actin protein. Here, I summarized previous works reporting the different actin organization and mechanical function.

2.1.1 Actin organization and mechanics

The actin monomer, a globular protein of 375 amino acids (45 kDa), is a basic unit which polymerizes into filaments. Single actin filament is a polar polymer with the barbed end and pointed end. The rate of elongation at the barbed end is $11.6 \mu\text{M}^{-1}\cdot\text{s}^{-1}$ ^[2], faster than the pointed ends. Studies reveal that single actin filaments behave like rigid rods when the length is shorter than the scale of a cell ($\sim 10 \mu\text{m}$), whereas can bend when the length is longer than $10 \mu\text{m}$, called persistence length^[3]. Because the actin assembly rate at barbed ends can be as fast as 3000 subunits/s, and cellular actin monomer concentration can be as high as $300 \mu\text{M}$ so that actin filaments can reach persistence length within 2 s ^[4]. At cell scale, actin filaments are virtually straight, however, can be bent by motor proteins or mechanical constraints imposed by geometry. This behavior is called actin buckling.

Distinct actin organization observed in migrating cells are generated by single actin filaments because of their association with actin-binding proteins (ABP), include branched actin networks such as lamellipodia, crosslinked actin networks such as cell cortex, parallel bundles such as filopodia and antiparallel bundles such as stress fibers (Figure 2.1).

Until recently, lamellipodia was definitively visualized as branched actin network by electron tomography^[5]. Lamellipodia is composed of Arp2/3, a complex made of seven proteins, nucleated actin filaments as branches of existing actin filaments. The activation

of Arp2/3 is controlled by SCAR–WAVE regulatory complex^[6]. The mechanical property, such as stiffness, of this branched actin structure, is regulated by the mesh size which is controlled by the degree of branching and the concentration of actin monomer. Steering cell migration is the most important function of lamellipodium^[7]. Cells can control the direction of their migration, by responding to substrate rigidity (durotaxis), ECM density (haptotaxis), gradients of chemokines (chemotaxis) or electric fields (electrotaxis)^[8]. Cells also control their directional persistence during random migration by regulating lamellipodium persistence which determined by the balance between actin elongation and branching^{[7], [9]}.

Figure 2.1 Distinct actin organization and mechanical properties^[10].

Filopodia are fingerlike parallel bundles composed of Ena/VASP proteins and formin at the front of migrating cell. The elongation of filopodia is due to the binding of formin homology 2 domain on the barbed ends^[11]. However, the initiation of filopodia formation is still a mystery. There is a debate on whether the initiation is activated by Arp2/3 complex or formin nucleation. Filopodia can facilitate stiffness sensing and generate retraction force during cell migration. The mechanics rely on a retrograde flow controlled by actin assembly and disassembly, not the motor protein such as myosin II^[12]. Recently study reveals another mechanism for force generation by filopodia, that is helical coiling and rotational motion. Also, the myosin II motors didn't play a role in this observation^[13].

Unlike filopodia, cell cortex is a contractile network due to the presence of myosin. The initiation of actin cortex formation is based on two actin nucleators, Arp 2/3 complex and formin Diaph1. The actin cortex attached to the cell membrane through various proteins, among which ERM family is one of the most important players, such as ezrin, moesin^[14]. This interplay between cell cortex and plasma membrane plays an important role in cellular morphogenetic processes. Cellular inward pressure can be generated by cortical contractility resulting in cell shrink, for example, the round shape of cells before mitosis. The bleb, which is a special structure, also is a result of cortical contractility.

Another actomyosin structure in the cell is contractile fiber. There are two main types of contractile fibers: myosin-II-enriched ventral stress fibers anchored directly to focal adhesion (FA) sites, run approximately parallel to the direction of cell migration, and transverse arcs which not linking to FAs and connected with non-contractile dorsal stress fibers, run parallel to the leading edge. A more recent work discovered a radially symmetrical system of actin bundles which were composed of non-contractile radial fibers and contractile transverse fibers when living cells were confined to a circular island^[15]. In this highly dynamic system, the self-organization of actin results in five distinct patterns. Until now, the formation of dynamic contractile actin network in vivo is still not clear.

2.1.2 Motor proteins

Different deformation of contractile stresses exerted by actomyosin structures is essential for regulating diverse cell behaviors (Figure 2.1). For example, isotropic contraction is

generated in platelets to keep it in round shape and reduce the overall size. Anisotropic stresses are generated in fibroblasts to get a front-back polarity, localized at the cell equator to drive cell division and localized at the tail to break cell symmetry and facilitate cell migration (Figure 2.2). This actomyosin structure is composed of F-actin and myosin II. Myosin II moves towards the barbed ends of actin filaments. In muscle tissue, the actomyosin which organized into sarcomeres by myosin II is well studied. Also, in non-muscle eukaryotic cells, similar functions generated by non-muscle myosin II (NM II) have been observed and studied.

Figure 2.2 Types of contraction generated in cells^[16].

At the molecular level, forces are generated by converting chemical energy into mechanical work. NM II is a hexamer comprised of three pairs of peptides: two isoform-specific heavy chains of 230 kDa, two 17 kDa essential light chains (ECLs) and two 20 kDa regulatory light chains (RLCs) (Figure. 2.3). NM II assembles into bipolar thick filaments through interaction between coiled-coil rod domains on heavy chains. Single-molecule techniques, such as optical tweezers, sing-molecule fluorescence microscopy,

and atomic force microscopy, are often used to study motor proteins^[17]. In mammalian cells, there are three types of NM II heavy chain (NMHC) proteins, which are named NMHC IIA, NMHC IIB, NMHC IIC, encoded by three different genes (*MYH9*, *MYH10*, *MYH14*, respectively). Unlike cardiac and skeletal muscle myosins which are regulated by a set of proteins binding to actin filaments, the regulation of smooth muscle myosins^[18], NM IIA, NM IIB and NM IIC are controlled by the reversible phosphorylation of RLCs and the heavy chains through increasing the Mg^{2+} -ATPase activity on myosin heads^[19]. Previous studies demonstrate the two major isoforms: NM IIA and NM IIB play a different role in cell migration. NM IIA localized mainly at the front of the cell and promote the stress fiber formation and adhesion maturation behind the lamellipodium. Whereas, NM IIB determines the rear contraction of migrating cells by increasing actomyosin bundles^[20].

Figure 2.3 Subunit of non-muscle myosin II^[21].

The localization of contractile force as described in Figure 2.2 is spatiotemporally controlled by the phosphorylation of myosin II through factors that influencing the upstream of signaling pathways, including RhoA, myosin light chain kinase and Rho-kinase^[22].

From the materials perspective, the self-organization of contractile actin networks differs

from the self-assembly of common soft materials which is out of thermodynamic equilibrium driven by external stress in the fact that it is a non-equilibrium system with internally driven matter^[23]. The internal driving force produced by the force generators-motor proteins drive the actin networks into non-equilibrium networks^[24]. However, the behavior of dynamic and heterogeneous actin network rely on not only the motor protein but also other complementary components, in particular, crosslinking proteins in cells.

2.1.3 Crosslinkers

It has turned out that the complexity of cytoskeleton is regulated by crosslinkers as each one has an own characteristic effect on mechanical properties of the network. The crosslinking proteins that play a role in contractile force generation have been listed in Table 2.1^[25-37]. These proteins typically share an F-actin binding domain (ABD). Studies have demonstrated that in the absence of F-actin crosslinkers, motor proteins weaken F-actin network by active filament sliding^[38]. Whereas, cross-linked F-actin network exhibit nonlinear stiffening with strain. This phenomenon is particularly pronounced when F-actin is cross-linked by filamin A.

Table 2.1 Summary of crosslinking proteins on different kinds of actin structure and in related to force generation

Crosslinkers	Localization in cell	Effect on actomyosin architecture	References
Fascin	Filopodia, nucleus	Unipolar bundling	23-25
α-actinin	Stress fibers, contractile ring, and cortex	Apolar bundles and isotropic networks	26, 27
Anilin	Cleavage furrow	Apolar bundles	28, 29
Cortexillin	Cleavage furrow	Apolar bundles	30
Filamin	Stress fibers and cell cortex	Apolar bundles and isotropic networks	31-35

The filamin family is composed of three proteins (filamin A, B, and C) which are encoded by different genes. Filamin A, also called human actin-binding protein 280 (ABP 280), is crucial for cell movement and fetal development. Filamin A is responsible for orthogonal actin cross-linking (Figure 2.4). This orthogonal network protects the cell

from different shear stresses. Filamin A also binds cell cortex to the plasma membrane and connects actin filaments into 3D networks through interaction with signaling molecules and transmembrane proteins, such as integrin. It is reported that when increasing the binding between filamin to β -integrin, cell migration was inhibited^[39]. Recently, Liu et al., for the first time, provided structural evidence of filamin-mediated integrin inactivation, suggesting a new mechanism for regulation of cell adhesion^[40]. Moreover, the regulation of contractile actin network by crosslinkers has been well studied in the simplified model.

Figure 2.4 Structure of human filamin A protein. Filamin-A is a V-shaped homodimer containing 2647 amino acids. There are three major domains in filamin a: Rod regions, C-Terminal Domain (FCTD) and F-Actin-Binding Domain (ABD)^[41].

To reveal the process F-actin network remodeling in vitro, recent studies use the simple reconstituted active gel system which consists of only three components: purified actin, motor proteins and crosslinking proteins. By combining fluorescence microscope, image analysis of actin structure tracking and simulation, researchers studied the dynamic process of actin network organization. Their works provided insights of the distribution of cluster size, network connectivity by changing the concentration ratio of motor proteins and static crosslinker^[26, 27, 42-45] (Figure 2.5). Using the minimal model composed of actin filaments, myosin II and fascin, the coarsening process of actin clusters has been identified. At high myosin II concentrations, a broad cluster-size distribution was observed in a dynamic steady state. The diameter of actin cluster was limited by

decreasing connectivity depending on fascin and the subsequently decreased activity of myosin II, as motor proteins need tracks to apply forces on (Figure 2.5a). Other crosslinkers, such as filamin, result in the much more complicated network. Two distinct regimes have been distinguished when varying the concentration of actin and filamin. In the first regime, actin bundles were induced by filamin which enhanced the network stiffness. Purely bundled actin network emerged in the other regime with high filamin concentration, and bundle clusters were observed with high actin concentration (Figure 2.5e).

Figure 2.5 Studies on in vitro reconstituted actomyosin structure. (a) Fusion and rupture events were shown in a dynamic steady state of actin network^[27]. (b) Experiment behavior consistent with the simulated result^[26]. (c) Time-series of myosin II-induced actin network contraction on a micropatterned line. Actin density was quantified with a scan line along the pattern at different time points. After 55 min, actin network was disassembled^[24]. (d) Actin network contraction with molecular motors during 45 mins after stimulation. Corresponding result of static spring network model (right)^[46]. (e) The structure of actin network is determined by the actin concentration and the molecular ratio of filamin^[47].

These findings are reminiscent of myosin-containing actin nodes observed in a living system, for example, the foci coalesce in early development embryo^[48, 49] and actomyosin contractile ring during cytokinesis^[50]. However, this bottom-up model is oversimplified in compared with a living system which including other functional proteins, such as focal adhesions^[51]. Therefore, works are needed to elucidate the actomyosin cluster formation in living cells with the regulation of ECM^[52].

2.2 Focal adhesion

Integrin-mediated adhesions are dynamic complexes that link the intracellular actin cytoskeleton to the extracellular environment. The hierarchal structure of the linkage is composed of ~180 protein-protein interaction termed adhesome. Mammalian cells co-express several integrins which are transmembrane heterodimeric receptors binding to the specific amino acid groups, such as $\alpha_5\beta_1$ and all α_v -class integrins specifically binding to the Arg-Gly-Asp motif (RGD)^[53]. This attachment site RGD was discovered in fibronectin in 1984^[54] and subsequently extended to other ECM proteins. (Figure 2.6). The adhesion of cells to the ECM is crucial for the forces generated by actin network to be translated into motion. The integrin-mediated adhesion complexes can be strengthened when forces are applied to them. This allows cells to modulate the adhesion level depending on the forces generated by cells specifically through the binding of talin and kindlin to the intracellular tail of integrin β -subunits.

Figure 2.6 Mechanosensitivity of focal adhesion molecular clutch^[55].

The dynamic process of adhesion formation, maturation and disassembly are driven by actin network self-organization. During cells migrating on the fibronectin-coated substrate, nascent adhesions ($< 2 \mu\text{m}^2$) are formed at the leading edge. After a short time (~ 60 s), these small adhesions can turn over or mature to larger focal adhesion complexes. As migration continues, actin bundles or stress fibers are organized and linked to larger mature focal adhesion. This focal adhesion maturation performance is the result of contractile forces generated by the activity of myosin II on actin, suggesting that the forces can be transmitted down to the adhesions several micrometers away from actin filaments. Integrin linker proteins, such as talin and filamin, plays a key role in force sensing through domain unfolding responds to stretching. After stretching, the more binding domain could be exposed to other molecules, such as vinculin^[56].

The distribution of different types of adhesions is dependent on the chemical composition and physical properties of ECM and cell type. For example, cells placed on softer substrates produce smaller and more dynamic adhesions, whereas cells spreading on stiffer substrates contain larger and more stable adhesions. Therefore, the size and distribution of adhesions reflect the contractile nature of cells. Both adhesion formation and disassembly are regulated by the actin polymerization and contractile actomyosin network. There are some remaining questions in this model, such as how cells sense the physical properties of the ECM to control actomyosin structure and subsequently regulate focal adhesion assembly.

2.3 Physical influences of extracellular matrix on cell mechanics

Living cell migration responses to the extracellular environment go far beyond biochemical properties (such as different membranes of other cells or protein types of ECM) and encompass various physical cues including confinement, stiffness, or whether the ECM is continuous or discontinuous. The core molecular machinery, actomyosin structure, drives cell movement can differ and adapt to external signals and generate different migration strategies. Another important component for mechanosensing is focal adhesion (Figure 2.7). The challenge is to understand how the physical properties of extracellular environment modulate the actomyosin structure and focal adhesion to produce different cell behaviors.

Figure 2.7 The structures of two essential components at different length scales, focal adhesion and stress fiber, involved in ECM mechanosensing^[57].

When cells are migrating *in vivo*, they are confined either to other cells or ECM. Using microfabrication techniques, the influences of confinement can be studied independently of changes in ECM composition and stiffness. Researchers confirm that the strategy to push plasma membrane forward are altered by ECM confinement. On a flat surface, branched actin network, lamellipodia, dominates the cell protrusion. Whereas, when cells migrate in confined tunnels, different actin structures were observed, such as 3 dimensional actin-rich slab comprises a free network at cell front and an adherent network localizes perpendicularly to microchannel (Figure 2.7a)^[58], large stable bleb at the front of Hela cell during amoeboid migration induced by confinement in the absence of focal adhesion. The confinement ECM provides a model to study the transitions between

mesenchymal migration and amoeboid migration (Figure 2.7b)^[59].

Figure 2.7 Actin structure regulated by ECM confinement. (a) Left: Confined space comprises two parts: PLL-PEG and PLL-PEG-RGD to control cell adhesion area, a PDMS micro-spacer treated with PEG. Right: Live staining of vinculin and actin in Hela cells placed on two different substrates: a 2D flat surface coated with fibronectin, and confined space with the height under 3 μm and non-adhesive area^[59]. Scale bar: 10 μm . (b) Left: SEM image of SU8 photoresist chamber. The top PDMS lid is functionalized with PEG, and the whole chamber is coated with κ -casein to prevent actin interactions. Right: Histograms of actin orientation when 7 mg/mL actin confined in designed chambers with different aspect ratios^[60]. (c) Confocal images of actin network reorganization without confinement (upper) and with confinement in spherical droplets (lower)^[61]. Scale bar: 20 μm .

Recent in vitro experiment composed of purified proteins in cell-sized confinement revealed that the boundary of cells determined actin structure orientation. In a cell-sized rectangular microchamber, actin filaments are forced to assemble into bundle-like structures oriented along the long axis in order to minimize the bending energy of semiflexible filament^[60]. In a spherically confined space, actin assembled independently into a ring-shaped structure, which enhanced the understanding of contractile ring

formation during cytokinesis (Figure 2.7c)^[61].

Figure 2.8 Actin structure and focal adhesion regulated by ECM rigidity. (a) Immunostained of myosin IIa in hMSCs after seeding on substrates with different Young's moduli^[62]. (b) Immunostained of actin and phosphorylated focal adhesion kinase in cells plated on 5 kPa and 29 kPa gels^[63]. (c) Illustration of force-mediated integrin regulation on stiff vs. soft ECM^[64].

Rigidity is another physical factor. The elastic modulus of different normal tissues can vary by orders of magnitude, for example, the elastic modulus of brain tissue is around 100 Pa and muscle of around 10^3 Pa. The stiffness of ECM can be remarkably affected when disease occurs in a patient. For example, the state of cardiac fibrosis is determined by the changes of matrix metalloproteinase (MMPs) and their inhibitors. MMPs are capable of degrading ECM proteins, thus altering the composition of ECM. Many cells can sense the rigidity of ECM, alter their internal signaling pathway, and further regulate the polarization of stress fibers which is a process known as mechanotransduction (Figure 2.8). The theoretical model has already been carried out to predicts the alignment of stress fibers depends non-monotonically on ECM rigidity^[62]. Cells sense the matrix rigidity through the focal adhesion molecular clutch (Figure 2.6). On the soft substrate, several

types of integrins have been identified to transmit forces generated by the movement of the actin cytoskeleton to surrounding ECM and induced ECM deformation^[64]. On the stiff substrate, the force transduction is mediated by talin unfolding. There is a force threshold for talin unfolding, and above this threshold, vinculin can bind to talin, which results in focal adhesion maturation^[63]. An open question of rigidity sensing is that how the force transmitted by molecular clutch affects the biochemical signaling pathway, therefore regulate actin structure.

The importance and influence of discontinuous ECM have been summarized in Chapter 1. Basically, the strategy that cells use to maintain cytoskeleton homeostasis when migrating across gaps is still an open question.

2.4 Ph.D. in Context of Literature

Our work focuses on studying actin architecture regulated by the physical factor-discontinuous ECM. Interestingly, I observed a highly dynamic actin network which linked by actin nodes and generates anisotropic subcellular forces. Previously, a wealth of studies has explored the mechanisms of cytoskeleton contractility using actin filaments, motor proteins, and actin cross-linkers in reconstitute system. However, these findings might not directly reflect biophysical properties inside living cells. The existing data indicates that actin self-organization in cells cannot only be controlled by the molecular composition but also determined by extracellular environment. Until now, how the mechanics of actin contractile network assemblies at subcellular and cellular scales in responding to extracellular environment is still not well understood. Our work provides a living system for studying actin contractile network remodeling activated by discontinuous ECM.

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Chapter 3

Experimental Methodology

The principles underlying the techniques employed and the data analysis methods are discussed in this chapter. Substrate fabrication methods are also introduced in this chapter, including hydrogel fabrication, photolithography, microcontact printing. To obtain high-resolution images, advanced light microscopy such as confocal laser scanning microscopy, spinning disk microscopy and STED (Stimulated Emission Depletion) were employed. Biological techniques, including cell culture, immunostaining, western-blot, transfection and RNA interference, were also used. The most important technique that was used in the measurement of cell mechanics is traction force microscopy. The analysis of data was then conducted using ImageJ, Matlab, and Origin.

3.1 Rationale for selection

Polyacrylamide (PAA) has been used in molecular biology for a long time, notably for the application in western blots, because of the mesh size inside the gel can be modulated by changing the ratio of monomers and crosslinkers. The mesh size also determines the stiffness of PAA gel and this property has been used for fabrication cell culture substrate with a wide range of flexibility^[1]. In addition, the acrylamide groups in the gel can provide covalent bonding with specific proteins and the constitutive antifouling property of PAA gel help prevent nonspecific protein. These properties make PAA gel is an ideal cell culture substrate with tunable stiffness and protein composition. In our study, I used 10 kPa PAA gel to mimic the physiologic stiffness. This is the gel with elasticity that approximates that of relaxed muscle bundles ($E \sim 10$ kPa)^[2] and heart tissue^[3]. Because endothelial cells were investigated in the thesis and in human body, endothelial cells which form blood vessels is surrounding by muscles and heart tissue. Therefore, 10 kPa PAA gel was selected.

With the development of micro-engineered functional biomaterials, researchers are able to characterize cell culture environment and study the interactions between cells and extracellular environment with different chemical and physical properties. To generate cell culture substrate with various microscale topographies, microcontact printing is the most popular methods. This method is the combination of lithography and stamp printing, which makes the substrate fabrication can be easily done in the traditional laboratory without the constant use of a clean room. Using this microcontact printing method, I optimized the pattern size and produced a discontinuous pattern for constraining single cell moving across the non-adhesive area.

To measure the mechanical properties of cells, traction force microscopy (TFM) has been developed. TFM calculate the traction force using the observation of ECM displacement. Submicron-sized fluorescent beads embedded in transparent hydrogel substrate is used as fiduciary markers. The displacement of fluorescent beads was recorded by high-resolution light microscope. Microcontact printing on hydrogel with fluorescent beads makes it possible for investigating cell traction force in response to ECM geometry. Therefore, I use this TFM as an important tool to study forces distribution in single cell

regulated by discontinuous ECM.

To study the actin architecture and focal adhesion distribution, immunostaining was used to visualize the structure. To understand the dynamics of actin network, cells were transfected with the plasmid. Moreover, siRNA was used to inhibit selected proteins. In the following part, detailed introduction to the methods used in this thesis was discussed.

3.2 Hydrogels for cell mechanics study

Figure 3.1 Cell morphology in response to the substrate with different stiffness and adhesive protein. Human mesenchymal stem cells (MSCs) spread on a substrate with higher stiffness. And the spreading area of MSCs on a substrate coated with laminin is smaller than coated with other proteins. Scale bar, 50 μm .^[4]

Extracellular environment plays an important role in regulating cell behavior. Previously, most of the experiments were performed on flat and stiff materials, such as glass and polystyrene. However, these materials can not reflect the real physiological environment in the human body. Thus, various biomaterials have been developed to control the chemical and mechanical cues which more accurately represent features of our tissue, including hydroxyapatite ceramics, elastomeric films, and hydrogel. The hydrogel is a water-swollen material composed of a network of hydrophilic polymers chains. Due to their mechanical properties which are similar to soft tissues, hydrogel has been used as a promising material for cell culture in vitro. There are many kinds of hydrogels can be used, including the natural materials such as collagen and alginate, synthetic materials, such as PAA gel and hybrid materials, such as polypeptides. PAA gel is the most popular

hydrogel for study cell mechanics with tunable stiffness and coupling of different kinds of ECM protein (Figure 3.1).

3.3 Micro-topographically modified surfaces

George M. Whitesides and Amit Kumar at Harvard University first introduced the soft lithography as an important method to the biological application (Figure 3.2).

Figure 3.2 Schematic illustration of softlithography^[5].

Even though the mold composed of photoresist generated by photolithography are used directly as a substrate with various physical properties (Figure 3.3a)^[6], it is the transfer methods which enable patterns from mold to secondary substrates increase the versatility of obtained structure. To transfer designed micro/nanoscale topographies, a stamp is often used. The stamp is generated by pouring organic polymers on mold pre-treated with trichloro (1H,1H,2H,2H-perfluorooctyl) (FOTS) to process lower surface energy, and then cured by thermal or UV treatment. Polydimethylsiloxane (PDMS) (Sylgard 184) is the most often used material for fabricating stamp, due to its unique properties including

optical transparency, non-toxicity, and elasticity. The limitation of replica mold from photoresist using 184 PDMS is 500 nm because the structures on stamp tend to deform because of the high compressibility of PDMS which is around 2 N/mm².

Figure 3.3 Replica mold used as a stamp for transferring topography. (a) SEM image of epithelial cell plated on a nanostructured wafer coated with silicon oxide^[6]. (b) Left: a schematic illustration of the fabrication process for nanopatterned PEG scaffold. Right: photograph of a round cover glass with nanostructures and SEM image of cross-sectional PEG hydrogel structure^[7]. (c) Schematic of the procedures for fabricating replica mold using conventional Sylgard 184 and composite stamp consists of h-PDMS and Sylgard 184^[8]. (d) Upper: SEM images of photoresist patterns produced on Si wafers through different stamp illustrated in (c). Lower: the Cross-sectional image of the photoresist line^[9].

To fabricate stamp with higher structure density, harder materials were developed, such as polyurethane acrylate (PUA) or h-PDMS. Using capillary force lithography, the PUA

pattern with nanoscale arrays can be obtained after exposed to UV light (Figure 3.3b)^[7]. Schmid et al. improved the elastic modulus of PDMS to $\sim 9 \text{ N/mm}^2$, so called h-PDMS by employing end-linking reactions and achieved the replica mold at 100 nm scale^[10]. Teri W. Odom et al. first combined the h-PDMS with conventional PDMS and generated composite stamps (Figure 3.3c)^[8], therefore further improved the free-standing features of a stamp down to 50 nm^[9]. The lines made of photoresist produced through the h-PDMS mask was 50 nm and stable in width (Figure 3.3d).

Figure 3.4 Microscale patterning for biomaterial engineering using PIPAAm. (a) A schematic illustration of patterning on topography^[11]. (b) Top-down and cross section view of the actin structure in cardiomyocytes on printed pattern using techniques shown in (a). Actin is stained with phalloidin (red), the nucleus is stained (blue) and 20 μm wide fibronectin line is stained (green). (c) Images of C2C12 cells plated on fibronectin patterned free-standing alginate microfibers after cultured for day 0, day 3 and day 7^[12].

Moreover, other methods have been introduced to generate ECM with more complex geometry. For example, poly(N-isopropylacrylamide) (PIPAAm), a thermally sensitive polymer, has been used to generate 3D and free standing ECM (Figure 3.4). As the critical solution temperature for PIPAAm is at $\sim 35^{\circ}\text{C}$ when the temperature is higher than this, PIPAAm swells and when the solution is at room temperature.

Soft lithography and derivative techniques are the most powerful and frequently used methods to fabricate micro/nanoscale ECM patterns. Here I use this technology to generate unique topography for studying the interaction between cell mechanics and ECM geometry.

3.4 Traction force microscopy

Cell traction forces (CTFs) are essential in various physiological and pathological process, such as wound healing, cancer metastasis. To grow and survive, most cells need to attach and spread on ECM. Once attached to substrates, the cells can exert tractions on their surrounding environments generated by actin polymerization and actomyosin interactions through focal adhesions with ECM. Therefore, the CTF is regulated by the molecules which are responsible for regulating actin organization and focal adhesion formation, such as ROCK and actin-binding proteins. To measure CTF, traction force microscope (TFM) was developed fast in the past decades.

Basically, current TFM for measuring CTF on 2D surfaces includes the following three major steps. First, fabricate a PAA gel surface embedded with nano-/micro-sized fluorescence beads. Next, the displacement field is obtained by comparing the movement of fluorescence beads before or after the detachment of spreading cells. Finally, different methods are developed to compute CTF from the measured displacement field. Dembo and wang were first one to show that with the knowledge of displacement field, cell traction can be mapped^[13].

In the beginning, there are two main methods were introduced by Burler et al.^[14] in 2002 and Marganski et al.^[15] in 2003. The limitation of these two methods is that they assumed the thin PAA gel substrate is an infinite half-space. With this assumption, they used Boussinesq analytical solution^[16] to get a surface displacement of an infinite half-space

due to a point surface load. This assumption is only valid when in compared with the thickness of gel, the lateral dimension of the cell and lateral distance that are measured are both small. However, the PAA gel substrate is of finite thickness and the lateral dimension of cells placed on the substrate often close to the PAA gel thickness. Therefore, the Boussinesq analytical solution is bound to generate errors. In 2006, Yang et al.^[17] adopted 3-D finite element method (3D FEM) instead of the Boussinesq solution to evaluate finite substrate. They also evaluated the errors using 3D FEM. The experiments used a PAA gel with an area of $1000\ \mu\text{m} \times 1000\ \mu\text{m}$ and a different thickness. And the results show that the thickness of the substrate can influence the displacement field generation in how fast and magnitudes. When the thickness is $1000\ \mu\text{m}$, the results obtained from Boussinesq solution and 3D FEM are close. Whereas, when the substrates become much thinner, the displacement decay faster and magnitude become smaller, the results obtained by the Boussinesq solution is larger than by 3D FEM because of errors. In terms of computing procedure, 3D FEM is equivalent to the constraint methods used in Butler et al.'s approach (Figure 3.6), and with two advantages, first one as described before is the consideration of substrate thickness, the second one is no need for iteration. Thus, the 3D FEM is very efficient.

3D forces measurement is a challenge for researchers in the last decades. In vivo, the ECM environment provides for cells are three dimension and studies have been shown different migration strategies used by cells to migration on 2D or in 3D environments^[18]. Therefore, 3D forces measurement can provide more information about the physiological processes in human body.

Figure 3.5 The application of traction force microscopy (TFM) in 2D and 3D^[19].

Figure 3.6 Fourier transform traction cytometry (FTTC) applied in traction force microscope. (a) Phase-contrast image of human airway smooth muscle cell placed on collagen coated PAA gel. (b) Fluorescence image of $0.2\ \mu\text{m}$ beads embedded in PAA gel. To ensure the forces produced at the cell boundary and avoid the defined discretized lattice, the area selected here is larger than the cells presented in (a). (c) The result of displacement field using the fluorescence image of beads before and after the removal of the cell. (d) The traction field calculated by unconstrained FTTC (e) The traction field calculated by constrained FTTC^[14].

TFM is the most reliable and informative method to measure the forces produced by the

entire cell. However, it cannot give the information about piconewton-scale force generated by specific focal adhesion on cells. Recently, with the development of Förster resonance energy transfer (FRET)-based tension sensors, pN-scale forces measurement become possible. This tension sensor module is made up of two fluorophores linked by a spring-like peptide and when mechanical forces applied on this peptide, it can stretch or unfold. Increasing distance between these two fluorophores leads to lower energy transfer rates, therefore mechanical forces can be measured. The most important procedure in this method is to calibrate the tension sensor module (Figure 3.7). Insufficient calibration leads to data misinterpretation. Currently, single-molecule force spectroscopy is the only way to calibrate a tension sensor module.

Figure 3.7 Calibration of a tension sensor module using Single-Molecule Force Spectroscopy^[20].

Techniques to measure CTF are still developing for higher sensitivity and less limitation. In this work, I am focusing on the total cell traction forces. Therefore, TFM has been chosen and used as an important tool in this thesis.

3.5 Transfection

Visualization actin cytoskeleton is essential for mechanobiology research. Actin dynamics is imaged by actin-GFP fusion proteins. However, previous work shows the

actin fusions are functionally impaired. Recently, fusions of GFP or RFP to actin-binding domains have been proposed and used (Figure 3.8). And Abp140-GFP has been reported as the only stable probe to label actin in a yeast cell. Reidl et al. introduced Lifeact, a small polypeptide, to visualize the actin filaments. This Lifeact is a 17-amino-acid peptide, as the first 17 amino acids of Abp 140 and can be used to stain F-actin in various mammalian cell lines without interfering the actin dynamics. In this study, I used the fusion protein provided by ibidi (Figure 3.9) and got a stable F-actin expression.

Figure 3.8 Identification of Lifeact. (a) Total internal reflection fluorescence (TIRF) microscopy image of Abp-140 in a yeast cell. (b) The actin-binding sequence in fungi. (c) F-actin stained with F-Lifeact (green) and phalloidin-Cy3 (red) in mouse skeletal muscle (merged in yellow)^[21].

Figure 3.9 The vector encoding LifeAct®–TagRFP fusion protein. Provided by ibidi.

3.6 RNA interference vs. gene knockdown

RNA interference is the process of post –transcriptional gene silencing in mammalian cells. Small interfering RNAs (siRNAs) which is generated by ribonuclease III cleavage from longer double-stranded (dsRNAs) has been demonstrated can specifically suppress the express genes in various cell lines^[22]. siRNA composed of two RNA strands forms a duplex 19 or 25 base pair (bp) in length with 3’ dinucleotide overhangs (Figure 3.10). One

strand is for sensing, and the other strand is called antisense strand which has a perfect reverse complement of the target mRNA. Transfection reagents, such as cationic lipid or polymer-based reagents, are needed to deliver the siRNA into cells. Another delivery method can also be used including electroporation or chemical modification. With improved efficiency of gene knockdown and mitigation of unintended target^[23, 24], siRNAs are widely used to study the contributions of genes to the cell functions. In this thesis, I used this gene silencing method to study the roles of myosin II and filamin A in regulating cell mechanics.

Figure 3.10 Structure of siRNA.

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Chapter 4

Multi-nodal Transverse Actin Network Regulated by ECM Geometry

The actin cytoskeleton, an active material, is responsible for various cellular functions, such as maintain cell shape, exert intracellular forces. The dynamic actin architecture is precisely controlled by the physical properties of ECM in spatial and temporal. By developing a platform for constraining single cell migrating across gaps, the actin assembly process on the non-adhesive area has been investigated in a different cell line, including an epithelial cell, endothelial cell, cardiomyocyte and cancer cells. Among all these cell lines, only HDFs and HUVECs keep migration when plated on discontinuous ECM. Interestingly, a unique actin architecture was observed in HUVEC, which is a multi-nodal actin network run transversely to migration direction, and the orientation of actin filaments inside this network is influenced by gap size. Also, the dynamic formation process of this network was analyzed, showing that the large actin node is merged by small nodes.

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4.1 Introduction

Cells are active materials, capable of migration, divide, and communicate with each other. The dynamic cell behavior depends on the spatiotemporal regulation of actin structures. Despite the importance of the actin network, our understanding of it remains incomplete. Studies have demonstrated that actin structure is highly influenced by the topography of ECM environment, such as the intensity of stress fibers is reinforced at non-adhesive edges^[1].

Figure 4.1 Overall SEM image of the network of corneal fibroblasts in rat^[2]. The cells were flattened, polygonal and contacted with each other. The bar indicates 10 μm .

The geometry of ECM in vivo is a fibrous network. This extracellular fibrous network is essential to keeping the tissue integrity. However, architectures of ECM are not continuous for cells to grow on, they are discontinuous and separated by micron-sized gaps, such as in the cornea (Figure 4.1)^[2] or the intestinal mucosa^[3]. Therefore, it is a challenge for individual cell, or collective cells migrate on the matrix overcome the gaps between the adhesive area. Existing findings have studied the actomyosin network when cell migration along discontinuous ECM architectures^[4-6], but little attention has been paid to the process of cell migration across the gaps on heterogeneous ECM network.

During the process of cells relocation in the human body, cells need to migrate across various geometry, in particular, when vessel is sprouting in angiogenesis, the tip cell needs to migrate on different tissues with manifold ECM structures^[7]. Thus, it is important for establishing a platform to study cells migrate across the non-adhesive area.

Microcontact printing is a useful tool to fabricate a patterned substrate with different ECM composition. When plating on a patterned substrate, cells can deform their shapes and reorganize their actin network in response to different ECM geometry. Here I developed a microscale discontinuous pattern to constrain the direction of cell migration and further force cell to span over non-adhesive gaps. I found that there was a gap size threshold for cells migrate across and this threshold varied between cell types. Furthermore, I identified the size of the non-adhesive area determined the orientation and organization of subcellular actin network. Interestingly, several separated multinodal actin networks were observed in single endothelial cell plated on discontinuous ECM.

4.2 Experimental Methods

4.2.1 Cell culture

Human umbilical vein endothelial (HUVEC) were purchased from Lonza and cultured in EGM-2 medium (Lonza). HUVEC were used between passages 2 and 9. Human keratinocytes (HaCaT) were purchased from Lonza and cultured in DMEM medium (Lonza). Rat smooth muscle cell (A7r5), human dermal fibroblast (HDF), human squamous cell carcinoma cell (TR146) were purchased from Sigma-Aldrich and cultured in DMEM medium. Human liver cancer cell (Hep G2) and mouse fibroblast cell (MEF) were purchased from American Type Culture Collection (ATCC) and cultured in DMEM.

The neonatal ventricular myocyte was isolated as previously published.^[8] For isolation, ventricles were dissected from 2 days old Sprague Dawley rat (InVivos). Then the tissue was manually minced and digested in 4 mL of 0.1% trypsin (Lonza) at 4 °C for 12 h. After cell solution was centrifuged for 5 min, trypsin was removed and replaced with 0.2% type II collagenase (Sigma-Aldrich) for the next three times enzymatic digestion at 37 °C with gentle rocker agitation. Next, the suspended cell solution was then centrifuged for 5 mins and resuspended in medium (Lonza) with 200 μ M BrdU solution to prevent the

overgrowth of fibroblasts. Before seeding on the substrate, cells were filtered through a 40 μm cell strainer to get isolated individual cell and pre-plates at 37 $^{\circ}\text{C}$ for 45 min to remove fibroblast.

For each cell seeding, cells in resuspended solution were counted and pipetted on the substrate with the concentration of 2000 cells/ml. Then the sample was incubated for 12 h to allow cells to spread on the fabricated substrate.

4.2.2 Photolithography and fabrication of PDMS stamp

Surfaces with designed topographies were obtained by using photolithography according to published methods^[9]. The photoresist layer with a thickness of 5 μm was obtained by spin-coating SU-8 2005 photoresist (Microchem) onto prebaked silicon wafer at 3000 rpm for 45 s. After soft bake at 95 $^{\circ}\text{C}$ for 5 min, the negative photoresist layer was exposed to UV light through chromium photomask (CikaTech), washed using developer (Microchem) and post-baked for 5 min. Finally, photoresist master mold was produced for fabricating polydimethylsiloxane (PDMS, Sylgard 184, Dow-Corning) stamp with designed microtopography. The silicon wafer was passivated with trichloro (1H,1H,2H,2H-perfluorooctyl) silane in vapour-phase for 30 mins to prevent PDMS adhesion. Then PDMS (base resin: curing agent = 10:1) was cast on the wafer, cured for 1h at 90 $^{\circ}\text{C}$ and then peeled off.

4.2.3 Micro-contact printing on polyacrylamide hydrogel

Figure 4.2 Fabrication process of polyacrylamide hydrogel.

PA gel was prepared as a substrate for microcontact printing. The bottom 13 mm circle coverslip was exposed to trichloro (1H,1H,2H,2H-perfluorooctyl) silane in vapour-phase to prevent PA gel adhesion. For fibronectin conjugated to PAA gel, the crosslinker N-succinimidyl ester of acrylamino hexanoic acid (N6) dissolved in ethanol at a concentration of 20 μ M is added to the acrylamide and bis-acrylamide solution. After adding ammonium persulfate (10% wt/vol solution, 1:125 volume), TEMED (1:1,250 volume; all from Bio-Rad Laboratories) into the solution, a 3 μ L drop of this solution was put on the bottom coverslip. Top coverslip treated with the bind-silane solution was placed over the drop and left polymerizing. After 10 min, the top coverslip was removed, and the PAA gel was made on the top coverslip.

The PDMS stamp was inked with fluorescence labeled fibronectin for 1 h. The coating fibronectin was a mixture with fibronectin diluted to a concentration of 50 μ g/mL in distilled water and fibronectin conjugated to Alexa Fluor 405, 488 or 561 fluorescent dye in PBS (ratio=5:1). After fibronectin deposition, the stamp was dried under nitrogen and placed in conformal contact with PAA gel for 5 min. Gentle pressure was applied by tweezers for seconds. After removal of PDMS stamp, fibronectin pattern was transferred onto PAA gel. The substrate was washed twice with PBS before cell culture.

4.2.4 Immunostaining

Cells were deposited on patterned PA gel at a concentration of 1000 cells/ mL. Cells were incubated for 24 h, removed from the incubator, washed with PBS for twice and then fixed for 15 min with a solution of 4% paraformaldehyde (PFA) at room temperature. Next, cell membranes were permeabilized 0.1% Triton X-100 in PBS were used for 5 min. After that, samples were washed with PBS and blocked with 1% bovine serum albumin (BSA) and incubated with Alexa Fluor® 568 Phalloidin (ThermoFisher Scientific, cat. no. A12380, dilution 1:150) to stain actin. The nucleus is stained by NucBlue Live ReadyProbes Reagent Thermo Fisher).

Figure 4.3 Actin organization in responding to different ECM geometry. Fibronectin is conjugated to Alexa Fluor 488 fluorescent dye (green), actin structure is stained with phalloidin (red), and the nucleus is stained with NucBlue Live ReadyProbes Reagent (blue). Scale bar is 10 μm . Left: The length of triangle sides is 50 μm .

4.2.5 Transfection

HUVECs seeding on the substrate at a concentration of about 10000 cells/mL in the antibiotic-free medium one day before transfection. p^{CMV} LifeAct-TagRFP (ibidi) was used to transfected cells and delivered by Torpedo^{DNA} (ibidi). After transfection, cells were incubated with the antibiotic-free medium at 37 °C in a CO₂ incubator for more than 18 h prior to imaging actin dynamics. Transfected cells were imaged after replaced with complete culture medium.

4.2.6 Fluorescence imaging

Samples were imaged with a laser scanning confocal microscope (Leica SP8) with 40 $\times$ or 100 $\times$ oil immersion objective (NA=1.40). Live cell imaging was performed on a confocal microscope or Andor CSU-W1 spinning disk confocal microscope (SingHealth) equipped with 37 °C, 5% CO₂ incubation chamber. Live cells were washed with PBS before imaging to remove unattached cells. Images were obtained using Nikon NIS-Elements AR software on spinning disk confocal microscope.

4.2.7 Image analysis

Images were performed with ImageJ (National Institutes of Health, NIH) or Imaris (Bitplane). Actin orientation profiles were generated by Orientation J (plugin for ImageJ).

4.3 Principle outcomes

4.3.1 HUVEC moving across discontinuous pattern

Since our aim was to study how single cell migrate across the non-adhesive area in extracellular matrix, guided moving was controlled using fibronectin-patterned substrates comprised of 8- μm -wide, 20- μm -long adhesive islands and 4-10- μm -wide, 20- μm -long non-adhesive gaps which are created on 10 kPa polyacrylamide (PAA) hydrogels (Figure 4.3a). The fibronectin coated on the adhesive area is labeled with Alexa Fluor so that the pattern can be imaged by fluorescence microscope.

When human umbilical vein endothelial cells (HUVECs) were plated on these surface, they spread and spanned over non-adhesive gaps. After 30 mins -1 h spreading, HUVECs reached their fully spread area and started to move across the discontinuous substrates (Figure 4.3b).

Figure 4.4 Experiment setup. (a) Schematic illustration for fabricating micropatterned hydrogel substrate. (b) Single HUVEC cell migrating across a discontinuous pattern in 1 h. The gap size is 6 μm . The cell is migrating upward. The time interval is 5 min. Fibronectin is conjugated with Alexa Fluor 561, shown as red in the image.

There was a big difference of nucleus position when cells migrate on the different pattern (Fig. 14). When the crawling process happened on less confined ECM, such as on the line or non-adhesive with 4 μm gap size, the nucleus position was near the cell rear. Subsequently, the cell front protrusion and rear retraction happened at the same time. However, when cells migrated on discontinuous ECM, such as the non-adhesive gap size was 6-10 μm , the nucleus position was close to the cell centroid, and the migration behavior was a step-by-step process: first the cell protrusion span over a non-adhesive gap, then cell body moves forward. Finally the cell rear retracted and released the attachment to adhesive blocks (Figure 4.5). Nucleus position which is an indicator of cell polarity in moving cells is regulated by an unclear actin-dependent mechanism^[10]. Previous works demonstrate the perinuclear actin network can be modulated by cell geometry, from a mesh-like actin network to apical stress fibers^[11]. Thus, the different position of the nucleus in cells moving across gaps suggesting the actin architecture supporting these cells can be influenced by discontinuous ECM.

Figure 4.5 Nucleus position in single cell is moving across the different pattern. The cell center is defined as "0". Negative values are localized near cell rear; positive values are near cell protrusion. (n=20 cells)

4.3.2 Actin architecture in response to discontinuous ECM in HUVECs

To study the actin architecture in moving HUVECs across these micron-sized gaps, cells were fixed, stained with fluorescent phalloidin and observed by laser scanning confocal microscopy. On confined continuous fibronectin strips, the antiparallel stress fibers were formed along the direction of cell migration at the basal plane, which is consistent with previous studies^[12]. In contrast, when gap size is 4 μm , transverse actin filaments which span both adhesive area and gaps were observed in some moving HUVECs on confined discontinuous substrate. This result suggests a reorganization of the actin cytoskeleton are triggered by the gap between two adhesive fibronectin block. Interestingly, with the increasing of non-adhesive gap size (6-10 μm), a transverse actin network with actin nodes appeared especially on the non-adhesive area (Figure 4.6). At the same time, the stress fibers run parallel to the direction of cell moving decreased. There were few actin filaments localized on adhesive blocks linked these actin nodal structure together, which made this unique actin nodal structure separated from each other. Therefore, this actin structure can be regarded as an independently unit formed on non-adhesive areas, like a subcellular bridge linked two adhesive blocks.

Figure 4.6 Confocal images of distinct actin organization in moving HUVEC on a different pattern. (Merge: Alexa 568 phalloidin labeled F-actin, red; Alexa 405 labeled fibronectin coated pattern, blue) Left: continuous line, width = 20 μm ; the spacing between surfaces of chosen patterns from left to right, respectively, 4 μm , 6 μm , 8 μm , 10 μm .

Actin runs transversely with the direction of cell movement has been reported before. It

is called transverse arcs which are localized behind the lamellipodium and not linked with focal adhesion, but linked with dorsal stress fibers. The formation of this transverse arcs is regulated by Arp2/3 protein complex and myosin^[13]. To elucidate the position of the transverse actin nodal structure found in our system, a 3D scanning image was obtained using a confocal microscope. As shown in Figure 4.7, the actin node is localized at the basal plane of HUVECs indicating this is a ventral actin network. And the linkage between this transverse actin nodal structure and dorsal actin network was not observed.

Figure 4.7 The multinodal actin structure localized at the basal surface of moving cell. Lower: the Cross-sectional image of 3D actin structure. Actin is stained with phalloidin (red), fibronectin pattern is conjugated with Alexa 488 (green), and the nucleus is stained (blue).

4.3.3 Actin architecture in response to discontinuous ECM depends on cell types

Finger-like filopodia structure formation was observed at the tip of HUVECs, known as the first step when cells are moving across gaps. Filopodia is responsible for exploring ECM environment using its distinct actin architecture which is a thin (100 nm to 300 nm), parallel actin structure crosslinked by fascin^[14]. The mechanical performance and dynamics of filopodia rely on actin assembly and crosslinkers varying in different cell lines. Thus, the maximum length, growth and retraction speed of actin filaments during filopodia formation depends on cell types^[15, 16]. To probe the versatility of actin architectures in different cell lines in response to discontinuous ECM, I placed 7 different cell lines both on the continuous and discontinuous substrate.

Figure 4.8 Actin remodeling in different cell lines in responses to the continuous and discontinuous pattern. Upper: 5 different types of cells were placed on continuous fibronectin coated line or 10- μm gap discontinuous pattern. All these 5 cell lines only can span over two non-adhesive gaps. Scale bar, 10 μm . Lower: nHDF were plated on the pattern with different gap size. Scale bar, 10 μm .

To study the actin structure in different cell lines and find out whether the transverse actin structure formation depends on cell types or not, cells were plated on a patterned substrate, cultured for 12h to spread sufficiently, and then fixed using 4% PFA. To reveal the actin structure, I stained actin structure with phalloidin as shown in Figure 4.8. In smooth muscle cell A7r5 and mouse embryonic fibroblasts, the cells normally spread on the continuous pattern, whereas cell spreading area decreased dramatically when cells plated on the discontinuous pattern (gap size = 10 μm). In HaCaT epithelial cell, the cell

spreading area is small on the continuous pattern, and also cells cannot continuously span over the non-adhesive area. In cancer cell lines, HepG2 and TR146, small actin nodes were observed when cells spread on the continuous pattern and more concentrated when spreading across two 10 μm gaps. However, in normal human dermal fibroblasts (nHDF), cells showed the ability to spread and to move across gaps with different sizes. Similarly, transverse actin structure was observed in nHDF on the discontinuous pattern (gap size = 8 μm), but without actin nodes. This finding verified previous results in HUVECs and indicated the actin filaments orientation could be regulated by discontinuous ECM. In summary, the actin architecture in response to discontinuous ECM highly depends on cell types.

Figure 4.9 Schematic illustration of myofibrillogenesis in pre myofibril model^[17].

I also studied the actin organization in cardiac myocytes. The contraction force produced by cardiac myocytes is essential for a living system. The basic machinery for contraction force generation is called myofibril. Understanding the initiation, assembly and maintenance of myofibril structure is very important but still a challenge for scientists. The contractile unit in myofibril is called sarcomere. The disruption of sarcomere in vivo can lead to disease^[18]. Therefore, the understanding of sarcomere stability in response to discontinuous ECM is meaningful. Cells were cultured for two days for the maturation of myofibril. Using the same methods as previous, the multinodal actin structure was acquired instead of sarcomere in beating cardiac myocyte on the discontinuous pattern (gap size = 4 μm and 6 μm), and observed no beating cells attached on the pattern with larger gaps. The results indicates that myofibril is highly regulated by non-adhesive gaps

in ECM

Figure 4.10 Actin architecture in cardiac myocyte in responses to the continuous and discontinuous pattern.

4.3.4 Distribution and organization of actin architecture is regulated by ECM geometry in HUVEC

Focusing on the transverse actin nodal structure formed in HUVECs, it is found that this unique actin organization comprising actin filaments connected by actin nodes and anchored to the corner of adhesive fibronectin block was more concentrated on the non-adhesive area when gap size was larger (Figure 4.13).

Figure 4.11 Colormap of actin filaments orientation. The images are analyzed using Orientation J.

Figure 4.12 Actin orientation in response to different ECM geometry. (a) Color map showing the corresponding orientation plots for actin filaments staining on the non-adhesive area. The microdomains of actin network architecture can be identified by the oriented features. Upper: spacing = 0 μm; Lower: spacing = 10 μm. Scale bar, 10 μm. (b) The orientation analysis of actin network formed on a different pattern. Only the center part of the cell is analyzed as the cell cortex at the periphery are always at the same angle. Each curve represents orientation analysis of the area observed in an individual cell. (n= 5) Red line: spacing = 0 μm; Blue line: spacing = 10 μm.

Figure 4.13 Box plot with no pattern: the non-adhesive area percentage in single cell spreading across fibronectin coated pattern. Box plot with the pattern: the percentage of actin

intensity on non-adhesive area against the actin intensity in the whole cell.

The orientation of actin filaments inside this structure on the non-adhesive area (gap size is 8 μm and 10 μm) was further quantified by using the method published before^[18]. Images of each actin structure were cropped to remove the impact of the actin cortex at the periphery. The angular distribution of actin filaments is presented as colored orientation plots in Figure 4.11 and quantified in Figure 4.12b. These results clearly indicate the orientation of actin microdomains can be tuned by the gap size, but remain transverse to the longitudinal axis of cells. In summary, a multinodal actin network in cells was observed and determined only by the ECM geometrical confinement. The dynamic formation process of this network is analyzed in the following section.

4.3.5 Dynamics of multinodal actin network assembly

To dissect the forming process of contractile transverse actin structure, cells were transfected using LifeAct Plasmids. After 24 hours, cells were visualized using confocal microscope and spinning disk microscope. Actin nodes were observed on the non-adhesive area which is consistent with the previous result. The actin nodes were tracked with an interval of 2 mins and 1 mins using confocal microscopy. The typical example shows actin network self-organization at the front of the cell when moving across 8 μm gap is depicted in Figure 4.14. First, cell protrusion was formed and then spanned over 8 μm gap within 4 mins. Next, small actin filaments were concentrated on the non-adhesive area and assembly. Actin nodes in the center of the non-adhesive area appeared within 20 mins. The second examples illustrated the process of three small actin nodes merging into one within 30 mins in Figure 4.14. The mean area of actin nodes was analyzed during the dynamic process of actin nodes formation. The decrease of mean actin nodes area suggesting actin turnover behavior also performed within the confinement of non-adhesive area (Figure 4.15). To further investigate the actin nodes formation in short time scale, spinning disk microscopy was used and the time interval was set to 200 ms. The result shows the moving of actin nodes was not obvious (Figure 4.16).

Figure 4.14 The dynamic process of transverse actin network formation at the front of cells. Scale bar, 10 μm .

Figure 4.15 Actin nodes condensation on the non-adhesive area. (a) Time-lapse montage of actin organization in HUVEC moving across discontinuous ECM. Spacing = 10 μm . Zoom: Movement of actin nodes (white arrows) in cells moving across the non-adhesive area. (b) The mean area of actin nodes shown in (a) over time.

Figure 4.16 Time-lapse montage of actin nodes assembly on non-adhesive gaps. Image acquisition was performed on an Andor CSU-W1 spinning disk confocal microscope.

4.4 Conclusions

A substrate to constrain cell to move across gaps was developed with different sizes and studied the actin assembly regulated by discontinuous ECM. By staining actin structure inside different types of cells, it is recognized that the actin organization highly depending on cell types. However, both in nHDF and HUVEC the transverse actin structure was observed, suggesting the actin orientation is influenced by non-adhesive gaps in ECM.

Interestingly, the transverse actin network found in HUVEC containing large actin nodes. To our knowledge, this is the first time to observe actin nodes formation without chemical stimuli. Previously, to establish an in vivo model with multiple actin nodes, different drugs treatments need to be used to interfere the cellular actomyosin network, such as cytochalasin D, an actin polymerization inhibitor, and latrunculin A, an actin monomer sequester^[5, 6, 19]. Using this chemical approach, researchers investigate the formation of multinodal actin filaments. However, few studies report the actin node structure in untreated cells^[20]. Our finding highlights the potential that multinodal actin structure can also be triggered only by ECM heterogeneous geometry.

To better understand how small actin filaments assemble into large actin network linked by actin nodes, HUVECs were transfected to study the dynamic formation process. The recording and analysis of dynamic data revealed both actin condensation and turnover play important roles in multi-nodal actin network formation. This phenomenon is reminiscent of the actin clusters studied in a reconstituted system with actin filaments, motor proteins, and cross-linking proteins, indicating the possibility for subcellular force generation by this unique dynamic multi-nodal actin network.

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Chapter 5

Subcellular contractility generated by multi-nodal actin network

Cell mechanics can be modified by surrounding environment. However, the mechanism of subcellular force generation and localization in living cell when migrating on a substrate with different geometry is still unclear. With the design of discontinuous pattern that constrains cells to migrate across gaps, here I investigate how the non-adhesive area of ECM affects the size and distribution of focal adhesion and the orientation of cell traction force. Our data indicate that mature focal adhesions provide the linkage between multinodal actin network localized on the non-adhesive area and the adhesive area coated with fibronectin. The size of focal adhesion is determined by the gap size. Within single migrating cell across gaps, separate anisotropic forces are generated and localized between two adhesive islands. Also, the single cell traction force increases with the increasing of gap size. Therefore, the geometry of ECM can significantly influence cell mechanical properties.

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5.1 Introduction

Contractile stresses are essential for cell shape changing, cell movements and other tissue scale physiological functions, including muscle contractility and heart beating. Different types of contractile forces have been reported before, for example, in platelets, isotropic stresses are responsible for reducing their volume and producing compaction; during cytokinesis process, contractile rings are formed on cell equator to generate contractile stresses; in migrating cells, such as epithelial cells and fibroblasts, anisotropic contraction are generated to perform tissue morphogenesis.

Forces generation are regulated by diverse, dynamic protein-based structures. Compared with the force generation in the striated muscle which is well studied in the last decades, non-muscle cells show high variability in the magnitude of contractile force generation and shape deformation. For example, during cell migration, the lamella formation needs steady state contractile actin flows to facilitate focal adhesion assembly at the front of the cell, whereas at the rear, much higher contractile stresses are produced to perform tail retraction. To support the various force generation, contractile actin network in non-muscle cells are highly dynamic. At the molecular scale, forces are generated by actomyosin structure composed of motor proteins, such as myosin II and actin filaments. In muscle cells, the force generation unit is called sarcomere, which myosin II localized at the center of polarized actin filaments with barbed ends at the edges and pointed ends at the center of the sarcomere (Figure 5.1a). Thus the forces generated in muscle cell contract in one direction with sarcomere. However, the organization of actomyosin inside non-muscle cells is less ordered. For example, actin network in cell cortex and apical surfaces are proved to exhibit multi-polarities. The activation of non-muscle myosin II is regulated by Rho-associated coiled-coil kinase (ROCK). Jonathan S. Coravos et al.^[1] recently has found out a-actin structure with mixed-polarity, called a radial actin network in epithelial cells, which is responsible for two-dimensional apical contraction and regulated by ROCK kinase activity (Figure 5.1 b and 5.2). The radial actin network is a sarcomere-like structure with barbed ends at the cell-cell junction edge and pointed ends in the center of actin cluster with myosin. This work extends the boundary of actomyosin actin structures studies. Similar radial actin clusters have been observed and studied in a

reconstituted system, in contrast with barbed ends at the center of actin cluster with myosin (Figure 5.1c)^[2]. This emphasizes the importance of the linkage between actin filaments and cell membranes for actin network organization.

Figure 5.1 Different organization of contractile actin structures. (a) Sarcomere structure localized in muscle cells. Green: actin filaments, red: myosin bipolar filaments, blue: Z line. (b) A radial actin network observed in the apical surface of epithelial cells. (c) In vitro system of actin polar cluster formation composed of actin filaments (green) and myosin (red).^[3]

Figure 5.2 The localization of ROCK is in the middle of epithelial cells. Right: Illustration of radial actin network linked at cell-cell junctions cooperatively contract. The minus ends refer to pointed ends and plus ends mean barbed ends.^[1]

The radial actin network was also observed in our system as multinodal actin network triggered by non-adhesive area at the basal plane of the single cell. Unlike the radial actin network in the apical surface of epithelial tissues with the cell-cell junction, the multinodal actin network is linked with ECM through focal adhesions. Our question here is whether multinodal actin network can generate contractile force and how it would be influenced by ECM geometry.

Focal adhesions play a crucial role in transforming the mechanical properties of ECM to actin filaments and after the remodeling of actin network, transmitting the forces generated by various actin structure to ECM in turn. The size of focal adhesion is significantly modulated by ECM adhesive area (Figure 5.3). Boris Hinz's group found when stress fibers exert high traction force, then supermature focal adhesion (8-30 μm long) were generated. This indicates that focal adhesion plays an important role in contractile force generate and the size of focal adhesion would reflect the magnitude of forces exerted on the area.

Figure 5.3 The size of focal adhesion linked to stress fibers is regulated by ECM adhesion area. Blue: Fibronectin patterned on glass with 1.25- μm width, 20- μm length and the spacing between two adhesive islands is 6 μm . The non-adhesive area was passivated with PLL-g-PEG. Green: F-actin, red: vinculin, white, α -smooth muscle actin. Scale bar, 20 μm .^[4]

The ability of focal adhesion sensing and response to chemical and physical cues is completed by integrins. Integrins are transmembrane proteins with an extracellular region binding to ECM proteins and a cytoplasmic tail binding to cytoskeletal. The activation of integrins result in the recruitment of other actin -binding proteins and also can lead to changes in the signaling pathway. Finally, the chemical and physical signals can be transmitted into cells. Talin is one of the most important mechanical sensing integrin protein. Talin is essential for integrin activation by binding the head domain directly to the cytoplasmic tail of β -integrin^[5, 6]. There are multiple sites on the rod domain for vinculin binding and at least two sites for actin binding. The helix bundles in talin rods is a compact structure, and when forces are applied to this domain, the helix bundles unfold and make the vinculin binding sites (VBS) exposed (Figure 5.4a). Single molecule experiments confirm that stretching of talin rod activates the VBS site^[7]. The dynamic of

focal adhesion recruitment highly depends on the forces generated actin cytoskeleton. Previously, I have introduced that the contractile forces in cells are particularly generated by the actomyosin structure and with a different organization. In vitro, reconstituted studies have proved that the binding between talin and vinculin controlled by actomyosin structure is a reversible manner (Figure 5.4b). The activation of vinculin binding induces the reinforcement of actin anchoring^[8].

Figure 5.4 Model of talin structure and mechanosensitive process. (a) The domain organization of talin 1. The talin head is composed of F0-F3 domains and linked with flexible talin rod by a ~80 residues linker. The talin rod is composed of 13 helix bundles (R1-R13)^[9]. (b) Vinculin binding is a reversible process. When actomyosin filaments stretch talin, the vinculin is recruited and binds on the talin rod. And the detachment of actomyosin filaments leads to vinculin releasing and talin refolding.

By quantification of the focal adhesion sizes in cells on discontinuous ECM, it is shown that the focal adhesion maturation is triggered by non-adhesive area. To study the mechanical regulation of actin network with geometrically regulated substrates, I use traction force microscope and reveal the contractile property of multinodal actin structure.

5.2 Experimental Methods

5.2.1 Immunostaining

Cells were deposited on patterned PA gel at a concentration of 1000 cells/ mL. Cells were incubated for 24 h, removed from the incubator, washed with PBS for twice and then fixed for 15 min with a solution of 4% paraformaldehyde (PFA) at room temperature. Next, cell membranes were permeabilized 0.1% Triton X-100 in PBS were used for 5 min. After that, samples were washed with PBS and blocked with 1% bovine serum albumin (BSA) and then incubated with appropriate primary antibodies at different concentration: a monoclonal Anti-Vinculin-FITC antibody produced in mouse (Sigma, cat. no. F7053, dilution 1:100). Actin was stained with Alexa Fluor® 568 Phalloidin (ThermoFisher Scientific, cat. no. A12380, dilution 1:150).

5.2.2 Traction force microscope

Traction force measurements were conducted on PAA gel embedded fluorescent beads (diameter is 0.1 μm). Briefly, fluorescent beads were added into the mixture of crosslinker N-succinimidyl ester of acrylamino hexanoic acid (N6), acrylamide and bis-acrylamide solution, ammonium persulfate (APS and TEMED. And a droplet of 3 μL of the mixture was added on the coverslip and covered by another coverslip treated with trichloro (1H,1H,2H,2H-perfluorooctyl) silane for 30 mins. After polymerization about 5 mins, the coverslip was removed, and PAA gel was formed. Cells were a culture on the micropatterned PAA gel substrate for 12 h, and images acquisition was performed on the laser scanning confocal microscope.

DIC image cells and fluorescent images of embedded fluorescent beads were obtained with 40 $\times$ objectives (NA=1.4). At the end of measurements, the adhering cells were treated with trypsin and the image of fluorescent beads position was acquired as a reference. The displacement field of fluorescent beads was acquired by comparing the position of the bead before and after removal of the adhering cells. With assuming that beads displacements were caused by forces generated by cells through contact area with gel, the map of gel deformation was then translated into a map of cell forces based on Green's function as described previously^[10]. Finally, overlay of the cell traction stress

and cell DIC image was obtained with the colormap.

5.3 Principle Outcomes

5.3.1 Focal adhesion localization and distribution is determined by discontinuous ECM

The actin network in moving cells is coupled to ECM via the spatiotemporal regulation of FA assembly^[11]. To test the FA distribution in responding to different ECM geometry, HUVECs were fixed and immunostained of vinculin, which is known as a mediator of FA maturation^[12]. After immunostained with phalloidin and vinculin antibody, I used a confocal microscope to take images of pattern, actin, and focal adhesion. As shown in Figure 5.5, vinculin is the linkage between actin network and adhesive pattern.

Figure 5.5 Immunostaining images of single HUVEC cell placed on the pattern (blue) for FITC-vinculin (green) and F-actin (red). Scale bar, 10 μm . Lower: the corresponding zoomed images of focal adhesions in the cells.

Most of the FAs in cells moving on continuous strips were small nascent adhesions ($< 2 \mu\text{m}^2$) (Figure 5.8) disperse localized on the fibronectin pattern (Figure 5.5, upper). On the discontinuous substrate, FAs were mostly localized along the edge of the adhesive blocks, corresponding to the ends of stress fibers network formed on the non-adhesive area

(Figure 5.5, lower). Quantification data reveals that the FA size distribution is determined by ECM geometry and large FAs ($> 2 \mu\text{m}^2$) dominated on the discontinuous pattern when gap size is 8 or 10 μm (Figure 5.8). This result shows that multinodal actin network anchored on the adhesive block using mature FAs. Studies have demonstrated that the transition of nascent adhesions to mature FAs is stimulated by mechanical tension (Figure 5.7)^[13], therefore, the enrichment in large FA around adhesive block on discontinuous ECM is an indication that the multinodal actin network can exert more tension than antiparallel stress fibers in moving cells on continuous ECM.

Figure 5.6 Immunostaining images of a single nHDF cell placed on the pattern (blue) for FITC-vinculin (green) and F-actin (red). Scale bar, 10 μm . The White box indicates focal adhesion located at the edge of adhesive blocks.

Figure 5.7 The schematic illustration of changes in morphology and composition during adhesion maturation. ^[11]

Figure 5.8 Boxplots indicates the distribution of focal adhesion classes in an individual moving cell triggered by a different pattern.

In Chapter 4, I investigated actin architecture in different cell lines in response to ECM geometry, and find that not only actin network in HUVEC cell is modulated by the discontinuous substrate, the orientation of actin filaments in nHDF is also influenced by non-adhesive gaps. With focal adhesion staining of nHDF (Figure 5.6), the localization of vinculin also was regulated by non-adhesive gaps and around the adhesive block (gap size = 4 μm). This result confirms that the transverse actin structure reoriented by non-adhesive area is linked with focal adhesion.

5.3.2 Subcellular force generated by multinodal actin network

Figure 5.9 Quantification of total traction force per cell in moving cell after seeding 12 hours on a different pattern.

To test whether the presence of multinodal actin network would induce an increase in cell traction force (CTF), monolayer traction force microscopy was used as described previously^[10]. I quantified the total CTF generated by single cells placed on different substrates. There is a significant difference in the level of contractility between different ECM patterns. The total contractile energy increased with the gap size between adhesive blocks (Figure 5.9). The average total CTF in cells moving across the gap (10 μm) is

approximately twice as the cell moving on continuous strips. As the ability to spread is different for each cell, I analyzed the CTF of an individual cell with the different spreading area (Figure 5.11). The result shows that with the same spreading area, the cell still exerts much higher CTF on the discontinuous substrate. The finding highlights the potential of multinodal actin network in contractile force generation.

To confirm the presence of subcellular anisotropic force in moving a cell, I analyzed the spatiotemporal distribution of traction stress when single cell is moving persistently across the discontinuous pattern. During the moving process of single HUVEC on the discontinuous pattern (gap size= 10 μm) (Figure 5.13b), the subcellular forces were consistently formed according to the pattern. The quantification result indicates the total traction force generated in moving process is highly correlated with the number of gaps cell span over, thus depends on the number of multinodal actin structure (Figure 5.13c). And individual contractile network produces approximately 200-400 nN traction force (Figure 5.12).

Figure 5.10 Localization and quantification of cell traction forces in response to ECM geometry. Representative stress color map showing the different localization and distribution of traction stress when HUVEC is moving on a different pattern. Upper: continuous pattern; lower: discontinuous pattern, spacing = 10 μm . Color bars at the left side indicate stress values from 0

to 1 kPa. Cells are moving upward.

Figure 5.11 Quantification of individual cell traction force when moving on designed pattern with the different spreading area.

Figure 5.12 The dynamic of force generation located at the rear of moving cell across 10 μm gaps.

Figure 5.13 The dynamic distribution of cell traction force in sing cell moving on a different pattern. (a)(b) Spatiotemporal kymograph of traction stress colormap. The color bar is same as shown in Figure 5.9. (a) The cell is moving upward as the arrow shown. (b) The spacing between non-adhesive area is 10 μm . (c) Total traction force for the representative moving cell showing in (a) and (b).

5.4 Conclusions

By a combination of immunostaining of focal adhesion and traction force microscope, it is demonstrated that the multimodal actin network observed previously is a contractile actin structure. The colormap of force generation result clearly show the localization of forces, that is at the corner of adhesive blocks and oriented toward the center of the non-adhesive area. There are questions rising from this subcellular force generation, such as what molecular composition comprised this contractile multinodal actin network.

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Chapter 6

Myosin II and filamin A regulate the formation of contractile multi-nodal actin network

Actomyosin structure is essential for cell mechanics. Using reconstituted system composed of pure proteins including actin, motor proteins, and crosslinkers, it is demonstrated that this active polymer network is highly regulated by external stress. However, in living cells, the function of the motor protein and crosslinker in the formation process of actin contractile structure is still unclear. Here, it is demonstrated the multinodal actin structure observed previously is contracted by myosin II and cross-linked by filamin A. In particular, the activity of filamin A, which determines the connectivity of actin network, depends on the chemical composition of ECM. With the inhibition of myosin IIA and filamin A, it is demonstrated that they play an important role in multinodal actin network formation and anisotropic subcellular force generation.

6.1 Introduction

The physiological functions of the cell are controlled by the architecture of actin cytoskeleton. Various contractile actin structures have been found in living cells, including stress fibers, cytokinetic rings, and cortex actin. The structure of actin cytoskeleton determines its mechanical property. For example, stress fibers are contractile bundles comprised of antiparallel filaments anchored on the substrate with two end focal adhesions. And the cellular chirality observed when cells plated on circle pattern is controlled a radial actin structure composed of a radial fibers, and transverse fibers have been found recently^[1]. These highly organized actin structures are built by the assembly and disassembly of unorganized actin filaments and accessory proteins in spatial and temporal.

Figure 6.1 Dynamic reorganization of F-actin and myosin II during the early stage of cytokinesis. Fixed embryos stained with phalloidin and p19-MRLC. Scale bar, 10 μm ^[2].

Recent studies have focused on a specialized cytoskeleton structure - actin nodes, which is a basic unit supporting the contractile network. Actin nodes have been observed in many physiological processes. For example, in the early stage of cytokinesis, the actin nodes can merge into a contractile ring at the equator of cells (Figure 6.1). The 3D molecular composition of actin nodes in live fission yeast has been determined using super-resolution microscopy. However, the actin nodes formation in a mammalian cell is still unclear^[3]. Another example is the actin cluster observed at the apical surface of epithelial cells and is responsible for apical constriction during tissue morphogenesis. Using the simplified model system in vitro which composed of the basic core players, including actin filaments, motor proteins, and crosslinkers, researchers have clarified their roles in regulating actin nodes formation. Molecular motors, in particular, myosin II, can exert internal stresses in the actin network and induce the dynamic actin reorganization. Modulating the activity of motor proteins can dramatically change the mechanical properties of active actin network. Although the motor protein itself can bring actin filaments into dense foci structure and induce contraction^[4], to generate macroscopic contractile forces, network connectivity is required.

Figure 6.2 The function of filamin A binding partners in regulating cell polarity. ROCK phosphorylates FilGAP, then inactivate Rac and finally promotes the disassembly of actin filaments^[5].

Until recently, researchers shifted attentions to the importance role of crosslinkers which provides connectivity in the actin network. In a weakly connected actin network, motor proteins drive actin filaments sliding into dispersive actin clusters. Also buckling can happen easily under the force generated by motors^[4]. Whereas, in a well-connected actin network, contractile stresses exerted by motors on crosslinked network can lead to nonlinear stiffening or cause macroscopic contraction^[6]. Various crosslinkers have been proved to play important roles in enhancing actin network connectivity, including fascin, α -actinin, anilin and filamin. They are located at different actin structures in cells as shown in Table 2.1, and filamin A has been shown as a widely presented protein on contractile stress fibers. Filamin A, a V-shape F-actin crosslinker, is essential for the formation of the orthogonal F-actin network^[7, 8]. FilGAP has been identified as an important filamin A binding partners. In cells, FilGAP phosphorylation by activated ROCK can regulate the actin organization (Figure 6.2). The FilGAP binding site - domain repeat 23 on filamin A can be deformed by external stresses. When mechanical stresses applied on the filamin A, the FilGAP can be released, and integrin binding sites are exposed (Figure 6.3). Thus, filamin A is a mechanosensitive crosslinker on actin network, which plays an important role in regulating actin organization.

The multinodal actin network observed before on discontinuous ECM is similar to the actin nodes structure found in the reconstituted system. This actin network can generate large subcellular traction forces as proven in Chapter 5. As introduced previously, high connectivity is essential for generating macroscopic contractile force. Therefore, it was proposed that crosslinkers could be activated discontinuous ECM and lead to high connectivity of actin network, therefore generation large cellular traction force. To confirm the hypothesis and better understand the mechanism of contractile actin network organization in response to discontinuous ECM, here I studied the function of a motor protein - myosin II and crosslinkers - filamin A in detail. By studying the localization of these two kinds of proteins, it is demonstrated that both myosin II and filamin A can be activated by the non-adhesive gap. Furthermore, with the inhibition of filamin A, total cell traction forces decreased dramatically, indicating the important role of filamin A in subcellular force generation.

Figure 6.3 Mechanotransduction through filamin A deformation in response to mechanical stresses. (a) Orthogonal actin network crosslinked by filamin A. (b) Deformation of filamin A when actin network is strained. (c) The molecular composition of filamin A. FilGAP binds on repeats 23 domain on the filamin A and the domain of β_7 integrin is unexposed. (d) When filamin a is deformed by external stresses, the repeat 21 domain is exposed for β_7 integrin binding, at the same time the two ends of repeat 23 are separated, therefore preventing FilGAP binding^[9].

6.2 Experimental Methods

6.2.1 Drug treatment

For myosin II contractility inhibition studies, the following pharmacological agents were used: 10 μ M and 20 μ M blebbistatin, 10 μ M ROCK inhibitor Y-27632. Cells were placed on the substrate for 24 h; then drugs were added for 1 hours before washed out using PBS.

6.2.2 Immunostaining

Cells were deposited on patterned PA gel at a concentration of 1000 cells/ mL. Cells were incubated for 24 h, removed from the incubator, washed with PBS for twice and then fixed for 15 min with a solution of 4% paraformaldehyde (PFA) at room temperature. Next, cell membranes were permeabilized 0.1% Triton X-100 in PBS were used for 5 min. After that, samples were washed with PBS and blocked with 10% goat serum and then incubated with appropriate primary antibodies at different concentration: anti-FLNA (Sigma, cat. no. HPA002925, dilution 1: 100), anti-Myosin IIA non-muscle antibody produced in rabbit (Sigma, cat. no. M8064, dilution 1:50), anti-Myosin IIB non-muscle antibody produced in rabbit (Sigma, cat. no. M7939, dilution 1:200) at room temperature for 1 hour. Cells were subsequently washed with PBS for three times and incubated for 1 hour at room temperature with secondary antibody conjugated to Alexa Fluor 488 or 568 (ThermoFisher Scientific, dilution 1:500). Actin was stained with Alexa Fluor® 568 Phalloidin (ThermoFisher Scientific, cat. no. A12380, dilution 1:150).

6.2.3 RNA interference

To achieve particular gene knockdown in HUVEC, siRNA was delivered using transfection reagents (Dharmacon, Cat. no. T-2001-03). Cells were transfected with a final concentration of 25 nM siRNA using ON-TARGETplus Human FLNA (2316) siRNA-SMART pool (Dharmacon, Cat. no. L-012579-02-0005), or ON-TARGETplus Human MYH9 (4627) siRNA-SMART pool (Dharmacon, Cat. no. L-007668-00-0005), or non-targeting siRNA as control. Transfection medium (antibiotic-free) was added and replaced with the complete medium after 24 h to reduce cytotoxicity. Transfected cells were incubated for 72 h-96 h before analyzed using western blot, immunofluorescent staining or traction force microscope.

6.2.4 Fluorescent imaging

Fixed cells were imaged by laser scanning microscope and super-resolution microscope (Leica TCS SP8 STED) with a $\times 100$ oil immersion objective (NA=1.40). Deconvolution of STED images was performed by Huygens software.

6.2.5 Western blot

Transfected cells were lysed on day 5 in NP40 buffer (20 mM Tri-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride and recommended concentration of cOmplete EDTA-free protease inhibitor cocktail)^[10]. Proteins were separated by 4-20% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Bio-Rad) before incubation at 4 °C overnight with primary antibodies: anti-FLNA (Sigma, cat. no. HPA002925, dilution 1: 200), anti-Myosin IIA non-muscle antibody produced in rabbit (Sigma, cat. no. M8064, dilution 1:2000) mouse monoclonal anti-Gapdh (1:10000, Ambion). Next, the membranes were visualized after proteins were incubated with corresponding secondary antibodies conjugated with horseradish peroxidase (GE Healthcare) and Immobilon Western ECL reagents (Millipore).

6.3 Principle outcomes

6.3.1 The localization of myosin II and filamin A

Recent studies using the simple reconstituted active gel system which consists of only three components: purified actin, motor proteins, and crosslinking proteins reveal the process of actin network remodeling *in vitro*. Their works provided insights of the distribution of cluster size, network connectivity by changing the concentration ratio of motor proteins and static crosslinker^[4, 6, 8, 11-13]. To understand the function of the motor protein and static crosslinker protein in this contractile actin cluster, I examined the localization of myosin II and filamin A using immunostaining methods.

As shown in Figure 6.4a, myosin IIA localized on the multinodal actin network. This indicates the actin nodes are myosin foci. The analysis of myosin IIA localization reveals that when cells are moving across the continuous pattern, myosin IIA presented in the whole cell body, whereas on the discontinuous pattern, myosin IIA concentrated on the nonadhesive area, the cell front, and cell rear. (Figure 6.4c) The colocalization of myosin IIA and myosin IIB is shown in Figure 6.6 revealed that myosin IIB also has an effect on actin reorganization. Both myosin IIA and myosin IIB can be activated on non-adhesive gaps. After enlarged the front part of one moving cell on the non-adhesive area (gap size

= 10 μm), the actin filaments were observed parallel to the direction of moving, and myosin IIA mini filaments were transverse (Figure 6.5a). However, when transverse actin filaments formed, the myosin IIA thick filaments were run perpendicularly to the axis of actin filaments (Figure 6.4d). The distance between two myosin IIA filaments is around 1 μm . The localization of myosin II on the multinodal actin network in HUVEC is reminiscent of the Z-line in muscle sarcomere, where myosin bipolar thick filaments localized in the middle and pull antiparallel actin filaments together to exert a contractile force (Figure 6.4d). These observations suggest that on non-adhesive gaps, motor protein myosin II drive actin filaments into actin nodes through a multistage process.

Figure 6.4 Localization of myosin IIA and filamin A. (a) Immunostaining images visualized by confocal microscope show myosin IIA localizes with actin network in HUVEC cell placed on the discontinuous pattern. Spacing = 10 μm .) (b) Immunostaining of actin (red), filamin A (green) and fibronectin-coated pattern (blue). Spacing = 10 μm . (c) (d) Fluorescence intensity profile of the indicated staining along the depicted dash line. Scale bar is 10 μm .

The immunostaining images of filamin A (Figure 6.4b) elucidate that lack of focal adhesion has an effect on the distribution of actin crosslinker protein. Using super-resolution microscope, it is clearly shown that the concentration of filamin A increased

on the non-adhesive area.

Figure 6.5 Super-resolution image of myosin IIa And filamin A localized on the non-adhesive area. (a)STED images of enlarged actin structure and myosin IIA in HUVEC cell moving across 10 μm gap. Upper: front part of moving cell; lower: the middle part of moving cell. Red: actin filaments; green: myosin IIA; blue: fibronectin. Spacing = 10 μm. (b) STED images of enlarged actin structure and filamin A in the front part of moving cell across 8 μm gaps. Red: actin filaments; green: filamin A; blue: fibronectin. Scale bar, 10 μm.

Figure 6.6 Colocalization of myosin IIA and myosin IIB in moving cells across the non-adhesive area on a different pattern. Scale bar, 10 μm .

6.3.2 Myosin II and filamin A are required for multinodal actin network formation

To understand the function of myosin II and filamin A in contractile actin structure, inhibitors were used, including myosin II inhibitor blebbistatin, ROCK-specific inhibitor-Y27632 and short interfering RNA (MYH-9 siRNA and FLNA siRNA) to silence the protein expression. All drugs were added after cells placed on the pattern for 12 hours.

Figure 6.7 Confocal images of actin organization when HUVEC placed on designed pattern treated with myosin II inhibitor blebbistatin (10 μM and 20 μM), or 10 μM Rho-kinase inhibitor Y-27632. (Merge: Alexa 568 phalloidin labeled F-actin, red; Alexa 405 labeled fibronectin coated pattern, blue) Scale bar, 10 μm .

Figure 6.8 Confocal images of actin organization after treated with MYH9 and FLNA siRNA for 4 days. Scale bar, 10 μm . (Merge: Alexa 568 phalloidin labeled F-actin, red; Alexa 405 labeled fibronectin coated pattern, blue)

In the drug treatments experiments using blebbistatin and Y-27632, drugs were added in for 1 h and then fixed cells with PFA. In the siRNA experiments, siRNAs were added in for 72 h and then fixed cells.

After the inhibition of myosin II with 10 μM blebbistatin, multinodal actin structure was disappeared on 10- μm -gap pattern, still presented on the smaller sized pattern. However, after increasing the concentration to 20 μM , the actin structure was inhibited on all kinds of the pattern (Figure 6.7). This dose-dependent manner indicates the different concentration of myosin II in cells can lead to different actin structure. With the treatment of Y-27632, which is Rho-associated protein kinase (ROCK) inhibitor, the multinodal actin structure is also completely inhibited. ROCK is a major downstream effector of the small GTPase RhoA. They induce the formation of stress fibers and focal adhesions by

phosphorylating MLC (myosin light chain). Therefore, this result also indicate that myosin II plays an important role in the formation of multibodal actin network. Also, with the depletion of myosin IIA and filamin A using MYH9 siRNA and FLNA siRNA (Figure 6.11), the multinodal actin structure completely disappear (Figure 6.8), demonstrate that filamin A is essential for multinodal actin structure formation on non-adhesive gaps.

6.3.3 Myosin II and filamin A are required for subcellular force generation

To further investigate the roles of two main components myosin II and filamin A in regulating mechanical properties, the TFM was used again. During the 5-day siRNA transfection experiments, it was found that with the increased cell duration, the total cell traction forces were also increasing. By comparing the traction forces generated by cells on day one and day 5, it shows that cell traction forces significantly increased on all patterns. Therefore, the day 5 traction force produced by untreated cells was used as the control experiments (Figure 6.9). The different spreading area for the individual cell was also considered (Figure 6.10).

Figure 6.9 A comparison of traction force generation in HUVECs after placing on different patterns for one day and five days.

Figure 6.10 Quantification of individual cell traction force on day 5 when moving on designed pattern with the different spreading area.

Figure 6.11 Western blot result and force distribution after cells treated with siRNA.

Figure 6.12 Quantification of cell traction force in moving cells transfected with siRNA reagents after 72 hours.

The quantification of CTF after 72h transfection indicates that CTF was decreased after inhibition of motor protein and crosslinkers on all patterns. Interestingly, CTF was slightly decreased after depletion of myosin II and filamin A on patterns when gap size is smaller 6 μm , however, significantly decreased when gap size is 8 μm and 10 μm (Fig. 2b). It was shown before; the contractile multinodal actin network was mainly formed on 8- μm gap and 10- μm gap pattern, therefore the decrease of CTF was the result of inhibition of the actin network. These findings confirmed the conclusion that myosin II and filamin A are both indispensable to contractile multinodal actin network formation. By a combination of actin transfection and TFM, the subcellular forces generated by different actin structure can be clearly visualized. For example, as shown in Figure 6.13, the inhibition of myosin IIA lead to successfully inhibition of subcellular force generation and multinodal actin formation.

Figure 6.13 The dynamic distribution of cell traction force and actin structure in single cell moving on the different pattern after treated with MYH9 siRNA.

6.4 Conclusions

In this study, I demonstrated that myosin II and filamin A are both essential for actin network organization on the non-adhesive area. With the inhibition of these two components by drug treatment and siRNA transfection, the multinodal actin network was disassembly, and total cell traction force was decreased significantly when cells were plated on the discontinuous pattern.

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Chapter 7

Discussion and Future Work

In this chapter, the summary of main findings in this thesis is discussed. Basically, the mechanical regulation of actin network by discontinuous ECM was studied. Interestingly, multinodal transverse actin structure was triggered only by changing the gap size between adhesive blocks. Unlike the transverse arcs found previously, this actin network was linked with focal adhesion and generated large contractile traction forces. Furthermore, the molecular composition of this subcellular contractile actin network was revealed. The most important two components were myosin II and filamin A. With siRNA transfection techniques, the important role of filamin A was proved. Filamin A is essential for multimodal actin network formation and subcellular force generation. Therefore, it is demonstrated that filamin A could be activated by the non-adhesive area. Future works are also introduced in this chapter, including actin organization in collective cells in response to ECM geometry, the actin organization in response to 3D ECM.

7.1 General Discussion

In this thesis, I focused on studying actin network organization and mechanical properties in response to discontinuous ECM. Previous studies on discontinuous pattern focused on the formation and maintenance of cellular bridges which composed of a rich actin meshwork above non-adhesive gaps^[1]. Also, earlier studies showed that the actin structure of bridges could produce a force on the substrate and elucidated the dynamics of actin structure formation which relies on the function of myosin. However, in these studies, cells migrated along the adhesive fibronectin strip which made the ECM continuous at one dimension. This study helps complete the picture by optimizing the pattern to constrain the cell to migrate directly across the gaps. In the human body, the ECM composition and topography is heterogeneous. Therefore, the ability for cells to span over non-adhesive gaps is crucial for the various physiological process.

The cell lines experiments revealed the reorganization of actin architecture on discontinuous ECM depending on cell types. Most of the selected cell lines cannot spread on the discontinuous pattern with 10 μm gap size, including cancer cells, smooth muscle cells, MEFs and HaCaT. But actin nodes appeared in these cell lines, indicating the potential that actin clusters can be triggered by non-adhesive area. Previously, to establish an in vivo model with multiple actin nodes, different drugs treatments are used to interfere the cellular actomyosin network, such as cytochalasin D, an actin polymerization inhibitor, and latrunculin A, an actin monomer sequester. However, few studies reported the actin node structure in untreated cells. These findings demonstrated that actin clusters could be obtained by simply modification of ECM topography.

Furthermore, after placing HUVECs and nHDFs on the discontinuous pattern, transverse actin networks were observed and localized specifically on the non-adhesion area. Unlike the transverse arcs previously reported, this transverse actin network was linked with focal adhesion and attached on fibronectin coated area. Interestingly, actin network in HUVECs composed of large actin nodes anchored on mature focal adhesion. With the quantification analysis of actin filaments orientation and focal adhesion sizes, it is demonstrated that the actin orientation and focal adhesion distribution were modulated by the gap size between two adhesive blocks. Then the cells were transfected for studying

the formation process of this actin structure on the discontinuous pattern. When a cell is moving across a 8 μm gaps, the multinodal actin network was formed within 30 mins, and after formation, the actin nodes keep in a highly dynamic manner. During the process, reorganization of actin architecture was observed, including actin nodes appear, actin nodes merge and actin turnover. These findings indicate the multistage formation process of the actin structure.

This work also provides insights into subcellular mechanics. To investigate whether this multinodal actin network is responsible for force generation, traction force microscopy was used. It is found that the localization and magnitude of cell traction forces were highly dependent on the gap size on the discontinuous pattern. In contrast, Kalpana Mandal et al. has reported the cell traction force is independent of the ECM geometry^[2]. This is because the adhesive areas are continuous and confined in their study. Therefore, it is concluded that the subcellular traction forces can be triggered and modulated by discontinuous ECM.

This study uncovered a new structure of actin stress fiber, which connected by actin nodes and can generate radically contractile traction force. This finding is reminiscent of myosin-containing actin nodes observed in a living system, such as the actin clusters at the early stage of cytokinesis. The formation of actin nodes has been well studied in a reconstituted system using pure proteins. These studies confirmed that network connectivity is required to allow motor proteins to generate contractile forces. To better understand the mechanism of contractile multinodal actin network formation, the activity of motor protein-myosin II and crosslinker-filamin A were studied in cells moving across the discontinuous pattern. With the inhibition of these two components, actin network on non-adhesive area disappeared, and the forces generated on substrates were decreased. This result obtained in cells is consistent with the findings in the reconstituted model, which highlights the importance of actin network connectivity produced by crosslinkers, particularly filamin A in contractile force generation. In the absence of filamin A, myosin II drive actin filaments sliding may induce actin buckling. Also, the filamin A plays the role here as a mechanotransduction protein, which can sense the applied tension and regulate actin assembly and disassembly. Finally, with the all these findings I proposed a mechanism to explain the actin organization when cells are moving across discontinuous

ECM (Figure 7.1).

Figure 7.1 Proposed mechanism for multinodal actin network organization on the discontinuous pattern. Stage 1: Myosin II and filamin A are activated by non-adhesive area, actin filaments aggregates, finally a well-connected actin network formed which supporting HUVEC span over gaps. At the same time, nascent focal adhesion formed around adhesive blocks. Stage 2: The motor protein - myosin II drives actin filaments moving together, which induces the actin filaments buckling, stiffen and myosin foci formation. Small actin nodes exert stresses on focal adhesion which lead to the exposure of vinculin binding site and vinculin recruitment. Stage 3: Small actin nodes merged into large actin nodes driven by myosin II, as the self-reinforcing loop exists, and generate large subcellular contractile force (as shown with the yellow arrows). The stresses exerted on crosslinker-filamin A could help release the FilGAP binding on repeat 23 domain on filamin A, therefore increase the assembly of actin filaments. Stage 4: Actin nodes turnover and disassembly.

In summary, I studied the actin structure organization across the non-adhesive area, found a new multinodal actin network structure which is responsible for subcellular force generation and demonstrated that motor protein-myosin II and crosslinker-filamin A are two key mechanotransduction proteins in this across the process. However, to complete the picture of mechanosensing proteins is still a big challenge for biophysical scientists.

There are some drawbacks in this developed platform and could be improved in the future. First of all, in this work, we only use PA gel as the platform to study cell behavior on discontinuous ECM, however, there are other hydrogel which has been proposed as good platform to study cell behaviors, such as as poly (2-hydroxyethyl methacrylate) (PHEMA), poly(ethylene glycol) (PEG) and polyvinyl alcohol (PVA) hydrogel. Also, we only focused on cell behavior on 2D substrate, however, in vivo the ECM is a 3D environment. Finally, to increase the material complexity and mimic the biological

processes such as tissue development, dynamic hydrogel could be used^[3].

7.2 Reconnaissance work not included in main chapters: Actin organization in response to different ECM environment

7.2.1 Actin organization in response to discontinuous ECM with different stiffness

As discussed in Chapter 2, the stiffness of ECM can remarkably regulate the actin structure (Figure 2.8). During the process of mechanotransduction, cells can sense the rigidity of ECM and alter internal signalling pathway, resulting in different actin structure. In the future work, the multinodal actin network regulated by ECM with different stiffness should be studied. Here shows the preliminary data in Figure 7.2. When a single cell plating on the soft substrate (2 kPa), the cell cannot spread on the discontinuous pattern with large gap size, and there is only actin nodes observed, no multinodal actin network are observed. However, on stiff substrate, the cell is well spreading and multinodal actin network is obtained. The specific signalling pathway which ECM regulated in this process is needed to be investigated in the future work.

Figure 7.2 Immunostaining images of a single HUVEC cell placed on the pattern (blue) for F-actin (red). The width of pattern: 20 μm.

7.2.2 Actin organization in single cell exploring continuous ECM grooves

In order to mimic the cell behaviors *in vivo*, 3D ECM has been developed recent years. Previous works show that cell can deform and migrate across discontinuous gaps on the 2D surface by actin remodeling. Next, I focus on that what the strategy cell uses in migrating in 3D ECM with different topographies. The model designed to mimic the 3D ECM is shown Figure 7.2a using the methods introduced before^[4]. With this method, the height of grooves and the gaps between the grooves can be modulated, therefore, further study the mechanism of actin network organization in a 3D environment. In particular, how filopodia sensing the spatial angle will be a new point. The role of associated proteins in filopodia during this process, such as Ena/vasp and Arp2/3 complex can also be explored.

Figure 7.3 HUVEC spreading on the pattern with topography. (a) 3D confocal image of a pattern coated with fibronectin conjugated with Alexa Fluor 488. Width: 15 μm , gap size: 15 μm , groove height: 15 μm . (b) HUVECs placed on the pattern shown in (a) for 12 hours. (c) Basal

and apical image of actin structure.

7.2.3 Collective cell migration on grooved ECM

Collective cell migration has an important role in wound healing, cancer spreading, tissue renewal and morphogenesis in vivo. Also in 3D bioprinting or tissue engineering, after fabricating scaffold, cells are plated, following the most important issue is whether cells can spread well on this designed scaffold. So it is essential to study collective cell migration on ECM in responding to different physical properties. As shown in Figure 7.3, MDCK cells were plated on the grooved PDMS coated with fibronectin. At the leading edge, cells migrated with the larger spreading area than the cells in the middle part of collective cells. Compared with the isolated small cell clusters, this spreading area is also much larger indicating that this is not because of cell density. To reveal what is the regulator here when collective cells migrating across grooves at the leading edge will provide useful information for scaffold fabrication.

Figure 7.4 The actin network in different part of collective MDCK cells migrating on the groove (width = 5 μm). Left: the leading edge of collective cells. Upper: DIC image of cells merged with fluorescence image of actin structure.

7.3 Future work: actin organization regulated by other geometrical cues

An intriguing question of biology is how biological patterns arise without preexisting positional information^[5]. This question was inspired by Turing in 1953, and he suggested pattern is generated by the reaction-diffusion process^[6]. However, until now, the biomolecular and mechanical understanding of this process is still elusive. In the human body, at the cellular level, actin cytoskeleton has the ability to generate various structures. Actin organization highly depends on the interactions with ECM as discussed before. Recently, more findings of new actin structures were reported *in vivo*^[7]. For example, a concentric actin ring pattern has been found in many species, and all are cellular cylinders (Figure 7.4). These findings highlight the importance of curvatures as a geometrical cue for mammalian cells. In summary, comprehensive studies are still needed to fully understand actin self-organization.

Figure 7.5 The periodic actin rings formed in the tracheal tubular system.^[8]

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