Engineering cellular bridges to collectively regulate cell migration and morphology

Hu, Benhui

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NANYANG
TECHNOLOGICAL
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**ENGINEERING CELLULAR BRIDGES TO
COLLECTIVELY REGULATE CELL
MIGRATION AND MORPHOLOGY**

HU BENHUI

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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Abstract

In living systems, cells rely on cell signaling to explore and respond to stimulus from their surroundings. The process promotes the individual cells to realize proper functioning, and more importantly, this process facilitates the communication among cells and groups of cells. Collectively, cellular communication contributes significantly for regeneration and developmental biology.

Based on the essential role of cellular bridge in cellular communication, we systematically investigated the cellular bridges at varied scales and their functions in regulate cell migration and morphology. Firstly, via regulating the subcellular bridges formed on extracellular matrix (ECM) with discrete adhesions sites and variable rigidity, the multicellular morphology and its related protective function determined by the efficiency of cells in maintaining its continuity and integrity can be switched on or off by modifying their environment. Then, by forming cellular bridge between muscle cell pair and utilizing the high tension within the bridge, we revealed an unanticipated mode of oscillation induced cell migration, providing an effective way to inhibit contact inhibition locomotion (CIL), which may help minify intimal thickening during in-stent restenosis and prevent vein graft failure. Lastly, through increasing the cellular bridge to multicellular scale, we investigated the formation and regulation of multicellular bridges over large non-adhesive regions. The studies on the geometry of ECM and orientation of cell migration provide in-depth understanding of the biophysical mechanism for cellular bridge formation and the corresponding healing efficiency. The elucidated interplay between the geometrical aspects of ECM and the multicellular bridges would allow us to optimize the migratory strategies for wound healing.

As cell migration and morphology are critical for regeneration and developmental biology. Our findings provide unanticipated modes to engineer artificial, bio-compatible scaffolds, which are then potentially applied for developmental and regenerative biology.

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Chapter 1 Introduction

This chapter firstly introduces the engineered biomaterials for investigating the interactions between cells and extracellular matrix (ECM) or adjacent cells. Next, the micropatterning technique was reviewed, especially on how this technique affect cell morphogenesis and functions. Furthermore, utilizing the platform mentioned above to study cellular bridges and their correlation to tissue integrity was introduced. Finally, the research motivation, objectives, and the organization of the thesis are presented.

1.1 Engineered Biomaterials to Investigate Cell-ECM and Cell-Cell Interactions

1.1.1 Introduction of Cell-ECM and Cell-Cell Interactions

In *vivo*, adherent cells pull the ECM or neighboring cells they attach to, by myosin-II based cytoskeleton contractile machinery.^[1-3] Such a type of interaction between cells and the environment surrounding them is critical for cellular sensing of ECM rigidity via integrin-mediated signaling.^[4,5] Recently, cumulative evidences has shown that cell traction force hold a significance on mechanoresponsive cellular behaviors like cell differentiation,^[6-8] morphogenesis^[1] and cancer metastasis.^[9,10] Investigation of the force transmission between cells and their environment is vital for developmental biology and revealing the mechanisms underlying mechanotransduction.^[1] As the stress is not a quantity that can be easily or directly measured, efforts are thus been devoted to record the deformation of the local cell environment. In conjunction with

advanced imaging technologies, these deformations are further converted into mechanical forces.^[11]

1.1.2 Elastomeric Beams as Strain Sensors

In order to visualize the deformation of the substrate underneath adherent cells, people have developed micro/nanoscale beam structures that can deflect or bend while serving as an adhesive surface for cells. Typically, the top surface of the beam array is labeled with fluorescent markers. As shown in Figure 1.1, the cells are cultured on the substrate and pull the beam tops, displacements of which can be precisely measured by a fluorescence microscope and further converted into cell traction forces.^[12] So far, PDMS post array has been successfully applied for studying the cell-ECM force at both individual cellular and multicellular level (Figure 1.1c).^[13] Furthermore, the E-cadherin mediated intercellular force are also been investigated by assuming a mechanical equilibrium between cell-cell and cell-ECM forces (Figure 1.1d).^[14]

The beam-based stress sensors have recently been utilized to investigate cell-ECM interactions with conditions mimicking physiology, via combination with micro-engineered actuating systems. As demonstrated in Figure 1.1e, the cell-ECM forces during morphological remodeling of endothelial cell in response to laminar shear flow were recorded by integration of strain sensors and microfluidics.^[15]

Figure 1.1 Quantification of cell traction force by beam-based stress sensors. (a) Schematic of the beam-based stress sensors. (b) Processed image demonstrating cell traction forces within a single cell (Reprinted with permission from reference [12]). (c) Bright field image and processed force mapping beneath a micropatterned multicellular

cluster (Reprinted with permission from reference [13]). (d) Fluorescent image illustrating the application of stress sensor in intercellular tugging force (Reprinted with permission from reference [14]). (e) Image (left) and schematic (right) of a microfluidic channel with integrated stress sensor (Reprinted with permission from reference [15]). (f) Schematic representation of a microfluidic gradient generator with integrated stress sensor for investigating mechanical crosstalk during chemotaxis (Reprinted with permission from reference [16]).

Briefly, the microbeam array was fabricated by soft lithography, followed by functionalization with ECM proteins, and finally sealed into a microfluidic channel through plasma-treated bonding. Another application adapting this combined platform is to study the cell-ECM mechanical crosstalk during chemotaxis, as the gradient of chemoattractant is easily established and maintained in microfluidic channels.^[16] The results suggest that cell-ECM force is correlated with the orientation of cell migration, as the maximum principle force is always concentrated at the front of cell.

Besides these mechanically actuating systems, the beam-based strain sensors have been combined with magnetic actuation to induce local mechanical perturbations into the ECM surrounding cells.^[17] As demonstrated in Figure 1.2, the magnetic nanowire embedded in the micropost would bend the post and thus impose an external force on the focal adhesion locally, in the presence of an external magnetic field. Immunostaining of cells revealed that the external force could pluck the stress fiber bridging over several posts and spread out the stimulation.

Figure 1.2 Magnetic micropost array. (left) Schematic representation of magnetic actuation. (middle) Schematic of traction force monitoring with the magnetic actuation. (right) Phase contrast image of the magnetic actuation (Reprinted with permission from reference [17]).

1.1.3 Hydrogels Embedded with Fiduciary Micro/nanoparticles

In order to visualize the strain, a most straightforward way is utilizing nanoparticles embedded in the ECM as fiduciary markers to record their displacements under the cellular forces. Traction force microscopy (TFM) is the most typical example applying this principle to detect cell traction forces on a compliant substrate.^[18]

Basically, in TFM, the hydrogel precursor solution is mixed with fluorescent nano-beads before polymerization with subsequent functionalization with adhesive coating for cell adherence. High-resolution fluorescent microscope or confocal microscope is used to record the positions of fluorescent beads near the hydrogel top surface both when cells spread on and are detached from the surface (Figure 1.3). Reconstitution of traction force exerted on the substrate can be calculated by using Green's function from Boussinesq's theory.^[18] Recently, the improvement for the accuracy of TFM has been performed by

using two sets of fluorescent nano-beads with different colors, conjunct with advanced imaging processing and optimized algorithm.^[19,20]

Figure 1.3 Microengineered traction force microscopy(TFM). (top) Schematic of TFM. (middle) Extracted beads displacement field and calculated traction force field (Reprinted with permission from reference [19]). (bottom) Schematic and fluorescent image demonstrating the application of TFM in studying intercellular tugging force (Reprinted with permission from reference [20]).

TFM has been successfully utilized for investigate cell-ECM and cell-cell interactions.^[20] A recent trend for application of TFM is to study the traction forces

within a cluster of cells during collective cell migration.^[21] Such investigations revealed that traction forces driving collective cell migration can be generated many cell rows behind the leading front, which contradict the traditional theory only emphasizing the important role of leader cells.^[22] Therefore, TFM has been proved as a powerful method to characterize the functional roles of mechanical forces involved in mechanoresponsive cellular behaviors.

1.1.4 Force Sensors Adapting Single Molecule Fluorescence Force Spectroscopy

TFM and beam-based strain sensors can neither detect cell traction force down to molecular level nor provide information on force generated on curved surfaces due to their specific fabrication procedure. Over the last few years, efforts have been devoted to develop molecular force sensors that can be combined with fluorescence resonance energy transfer (FRET) or distance dependent fluorescence quenching to probe cellular forces in range of 1-50 pN, enough for driving conformational changes in molecular assemblies.^[23]

Recently, a genetically encoded FRET-based force sensor was developed to study force transmission through vinculin in living cells and its role in focal adhesions.^[24] A pair of FRET donor and acceptor fluorophores was inserted between the head and tail domains of vinculin, serving as an entropic nanospring to measure forces at piconewton range (Figure 1.4a and Figure 1.4b).^[24,25] FRET has been applied as sensors not only for intracellular molecular force but also for extracellular molecular force. For example, a FRET-based fibronectin sensor with broadened effective working distance has been developed by inserting multiple donors and acceptors (Figure 1.4c).^[25] In addition, a

FRET-based extracellular molecular force sensor was developed for mapping forces both spatially and temporally. The sensor contained a linker, which was anchored onto a surface at one terminus and conjugated to a ligand at the other terminus. The surface and the ligand were functionalized separately with quencher molecules and fluorophore. Therefore, when cellular forces was large enough to extend the linker and remove the fluorophore from proximity to the quencher, the fluorescence intensity would enhance and reveal the signal for mapping the mechanical forces (Figure 1.4d).^[26]

Figure 1.4 Nanoengineered fluorescent sensors for monitoring single-molecule force. (a) Schematic of FRET vinculin force sensor (Reprinted with permission from reference [25]). (b) Images showing FRET index in the rear (R) and protrusion (P) of a cell (Reprinted with permission from reference [24]). (c) Schematic of a FRET fibronectin

strain sensor (Reprinted with permission from reference [25]). (d) Schematic description of an extracellular molecular force sensor (Reprinted with permission from reference [26]).

1.2 Micropatterning to Decode Cell Morphogenesis and Functions

1.2.1 Oriented Cell Spreading and Cell Polarity

The cell adhesion machinery is the cellular component that encounters the physiological cell microenvironment, which consists of ECM, extracellular fluids and adjacent cells. Via binding of extracellular ligand, the assembly of cytoskeleton and thus the entire cell architecture can be affected by these localized signaling.^[27] Since many cells within tissues have elongated shapes and this elongation may have influence on the cell architecture, experiments have been performed to test this whether this influence exists.^[28] The results show that cells are geometrically constrained by the micropatterns. The anisotropic cell adhesion will result in cytoskeleton rearrangement to form similar anisotropic organization of actin filaments. People also found that the micropatterning has a cell type specific regulation of contractility in elongated cells. The higher levels of stress fibers can be observed in mesenchymal cells, whereas for other cell lines such as vascular smooth muscle cells and endothelial cells, contractility and cytoskeleton are reduced by cell elongation.^[29] Regarding the effect of cell contractility on cell growth, the regulation of cell growth via cell elongation is thus dependent on cell type.

Cell polarity, reflected by the intracellular organization, is impacted by the oriented assembly of cytoskeleton component such as actin filaments.^[30] Since cytoskeleton networks are highly dynamic and spatially responsive to external cues, the

geometry and architecture of adhesive conditions from ECM can direct cell polarity, as well as other cellular processes.^[31]

Unlike highly spread cells, whose stress fibers perturb positioning of centrosome and also inhibit the growth of the primary cilium, the spatially confined cells form a primary cilium via branched actin meshwork (Figure 1.5a).^[30] By controlling the architecture of actin, the micropatterns exert local constraints on microtubules. The microtubules grow along contractile stress fibers and stop in the regions where membrane protrusions are induced by actin polymerization (Figure 1.5a).^[30] As the Golgi complex is associated to the centrosome,^[31] the micropatterns in crossbow shapes demonstrated that intracellular trafficking from Golgi complex is oriented towards adhesive regions.^[32] Generally, the nucleus is off-centered, directed either towards cell-cell junction or regions that deprived of ECM (Figure 1.5b).^[32] Moreover, cell division is also oriented in according to the geometry of ECM (Figure 1.5c).^[33] In conclusion, the nucleus-centrosome-Golgi axis is oriented from regions that deprived of cell adhesions towards regions where adhesions are sufficient.^[32]

Figure 1.5 Effect of the cell adhesion micropattern on the cell polarity and migration. (a) A cell confined in a small pattern grows a primary cilium on its dorsal surface (top), whereas for a cell grown on a pattern with increased size, the centrosome is close to the ventral surface (Reprinted with permission from reference [30]). (b) On a crossbow-like pattern, microtubules grow along stress fibers and stop once they reach the branched actin meshwork. The orientation of off-centred nucleus is towards the contractile non-adhesive edge (Reprinted with permission from reference [32]). (c) Microenvironment geometry orients cell division (Reprinted with permission from reference [33]). (d) For a cell migrates on wide pattern (top), its centrosome locates in the front of its nucleus, whereas for a cell migrates on narrow pattern (down), its centrosome falls behind. F-actin staining reveals the membrane protrusions (Reprinted with permission from reference [36]).

1.2.2 The Orientation of Cytoskeleton Governs Cell Migration

The cell migration is governed by the orientation of the actin cytoskeleton and cell polarity in response to the geometrical constraints. A teardrop-like pattern could orientate the cell polarity from pointed edges towards curved edges.^[34] Further release of cells from the pattern make cells escape from the curved edge, which indicates that cell migration is dependent on external geometrical constraints.^[34] Actually, not only the geometrical cues but also the mechanical cues impose the orientation on cell migration. For instance, the cell could migrate along the stiffness gradient on a microfabricated substrate, with front toward the stiffest end.^[35]

Additional to these asymmetric patterns mentioned above, strikingly, symmetric environments can also have a dramatic impact on cell migration. As shown in Figure 1.5d, the centrosome position is significantly different between cells plated on wide tracks and narrow tracks of fibronectin. Centrosome is located in front of the nucleus for cells grown on wide tracks, whereas for cells on narrow tracks, the centrosome shifts to the back.^[36] Despite the orientation, the migration velocity can also be affected by the adhesion width, as previous investigation demonstrated that cells plated on a thinly lined substrate migrated faster than those on a homogeneous substrate.^[36]

1.2.3 Coordination of Micropatterning in Multicellular Arrangements

The shape of multicellular groups can also be controlled by micropatterning. Considering the simple case of cell pair, two endothelial cells confined in a micropattern tend to turn around each other via coordinating the orientations of their leading edges.^[37] This kind of coordinated movements of cells is highly relevant for the building and

maintenance of the mechanical equilibrium *in vivo*. In migrating epithelial cell sheet, coordinated movements were analyzed via removing the pattern limitation to allow cells to move forward.^[38] Instead of migrating all at once, the cells form finger-like multicellular outgrowths that arise from the leader cells. The leader cells migrate faster than the others and act as a guide, which could influence not only neighboring cells but also cells many rows behind.^[39]

In vivo, the multicellular coordinations lead to morphogenetic changes at large scale, and dedicated micropatterning methods can also be applied to study this process. For example, endothelial cells could follow the guiding cues on 2D substrates to form hollow tubes, mimicking the blood vessels.^[40] Noteworthy, elongation of endothelial cells on micropatterned lines was proved to promote the expression of immunogenic genes, reducing inflammatory capability of blood vessels. The results demonstrated that modulation of cell shape plays an important role on the recapitulation of cell function.^[41] Moreover, the micropatterning provide co-culture of two cell types and thus allow to identify the parameters in heterotypic interactions.^[42]

In summary, the micropatterning methods have revealed insights into how the geometric cues impact on cellular physiology, both for intracellular organization and multicellular morphogenesis.

1.3 Cellular Bridges and Tissue Integrity

1.3.1 Structure and Function for Cellular Bridges

In multicellular organisms, intercellular communication contributes to the development of complex form and viability in variable environments. Although

biochemical interactions can be effective over distances, the physical continuity remains the most direct ways for achieving cellular communication. Typically, thin cytoplasmic channels transfer signals and components between cells, serving as one type of cellular bridges. Recent investigations discovered two types of epithelial bridges, which are structurally distinct from cellular channels. The first epithelial bridge type can facilitate transport of material between cells and the second type regulate migration of cells between epithelial cell colonies.^[43]

The cytoplasmic connections between animal cells have been identified as tunneling nanotubes (TNTs), which are the longest cytoplasmic connections. TNTs contain actin filaments as the prominent cytoskeletal component, yet most of them are lack of microtubules.^[44] The diameters of TNTs are very small, from tens to hundreds of nanometers, but the lengths are typically much longer, up to tens of micrometers.^[44] Distinct from TNTs, both types of epithelial bridges represent the longest direct tubular connections in vivo reported to date. For function, the type I epithelial bridge is basically similar to other intercellular channels such as TNTs, but the type II epithelial bridge demonstrate a totally new mechanism as it provides a conduit for transport of whole cells.^[43]

Depending on the cellular system, TNTs regulate intercellular transfer of materials including ions, organelles and membrane components.^[43] In contrast, epithelial bridge lengths are larger ranging from micrometers to over a millimeter.^[45] Confocal 3D reconstruction shows that both TNTs and epithelial bridges are cellular structures hover above the underlying substrates (Figure 1.6a).^[45] Actin filament is the only backbone constituting most TNTs, whereas components of epithelial bridges include both actin and

microtubules (Figure 1.6b and 1.6c). Furthermore, epithelial bridges are structurally stable for longer periods of time, up to a couple of days as opposed to the transient integrity of TNTs. The prolonged survival reflect decreased sensitivities of epithelial bridges exposed to external stimulus that readily disrupts TNTs.^[46]

Figure 1.6 Epithelial bridges. (a) Sectional view of confocal image showing an epithelial bridge hovering above the substratum. (b) Microtubules (green) involved in epithelial bridges. (c) F-actin (red) involved in epithelial bridges (Reprinted with permission from reference [45]).

The transfer of cellular components in type I epithelial bridge shares the similar mechanism employed by TNTs. The small membrane components and cytoplasmic molecules appear to be transferred by passive diffusion, while larger components such as organelles seem to be transported in an actin-myosin dependent system.^[46] In contrast, the architecture of type II epithelial bridges varies in complexity to facilitate the migration of single cell and multiple cells. This kind of cell migration may be an

integrated effort of the cytoskeletal networks from both migrating cells and epithelial bridges.^[43]

1.3.2 Formation of Cellular Bridges

Two mechanisms have been reported for the formation of cellular bridges, employed for both TNTs and epithelial bridges. The first involves a filopodia extension that initiates from one cell and adheres to a neighboring cell. The connection of the cellular extension is subsequently remodeled to an intracellular tube and thus forms the bridge. The other mechanism giving rise to cellular bridges between cells initially in contact with one migrates apart while maintaining the connectivity. During the formation and maintenance of cellular bridges, actin polymerization plays an important role via propelling extension of filopodia-based structures and stabilizing the elongation.^[46] The disruption of cellular bridge can also occur by either breaking from one end of the connection or fusing of two ends via progressively shortening the bridge.

Increased levels of inflammatory molecules could result in lower numbers of epithelial bridges, indicating the downregulation of bridge formation by inflammatory molecules.^[47] Moreover, investigations also revealed reactive oxygen species (ROS) could induce actin polymerization, which result in formation of TNT-like connections.^[48] However, blocking ROS with antioxidants did not affect the number of cellular bridges, implying under control conditions, ROS levels do not contribute.^[48] Therefore, the epithelial bridge biogenesis seems to be dependent on biochemical interactions and partially through the regulation of inflammatory pathways.

Despite the live-cell imaging by normal microscopy, several characterization methods have been summarized in order to decoding the formation and structure of cellular bridges. First of all, immunofluorescence staining is the technique using the specificity of antibodies to their antigen for targeting fluorescent dyes to specific bimolecular targets, and thus allows visualization of the distribution of the target molecule within a cell. By this method, the components and structure of cellular bridges could be clearly analyzed. Secondly, the scanning electron microscopy and transmission electron microscopy could be utilized to further decode the structure of cellular bridges at three-dimensional view, as well as the detailed information of the surface texture. Last but not least, atomic force microscopy could be applied to study the mechanical properties of various locations on the cellular bridges by attachment with the end of a cantilever. This method may prove helpful in investigating the tension distribution within the cellular bridges.

1.3.3 Epithelial Bridges Contribute to Regeneration

The repair of wounds contributes to regeneration, which is one of the most complex biological processes during human life.^[49] Despite retaining tissue integrity, wound healing varies depending on the process occurs. For example, wound healing in embryos is distinguished from the process in adults, as the scarring may happen for the latter.^[50] In-depth comprehension of the factors regulating wound healing may pave the way for better alternatives overcoming the limitations to promote satisfactory regeneration. Re-epithelialization is critical for wound healing response, wherein keratinocytes collectively migrate and cover the epidermal barrier.^[51] Hence, in the

context of collective migration, decoding how cells respond to physical constraints of ECM and restore the integrity is particularly important.

Recently, a brilliant work revealed that pluricellular suspended epithelial bridges can be formed on micropatterned ECM composed of adherent stripes and non-adherent gaps.^[52] The formation of epithelial bridges contributes to re-establish continuity of cell sheet and presents a significantly different architecture from other cellular bridges. The multicellular structures in the epithelial bridges require build-up of tension by actomyosin contractility at large scale. As shown in Figure 1.7a, keratinocytes migrated into the adhesive stripes on the substrate and formed the suspended multicellular bridges over the non-adhesive regions in between. However, another epithelial cell line (MDCK) on the same patterned substrate only followed the adhesive stripes, without bridges formation. Some nascent cellular extensions could be found at the junction area between the stripes and reservoir (Figure 1.7b). Confocal sections of these epithelial bridges immunostained for paxillin and phosphor-paxillin demonstrated that the focal adhesions were mostly restricted on the fibronectin stripes (Figure 1.7c). Moreover, scanning electron microscopy (SEM) images again showed the epithelial bridges were not attaching to the underlying surface (Figure 1.7e and 1.7f). The three-dimensional PDMS substrate was raised from the surface and coated by fibronectin only on the raised regions (Figure 1.7d). They further demonstrated that the pulling force from cells migrating on the adhesive stripes drove the formation of epithelial bridges, and the overall integrity of the bridges relied on the adherent junctions.

Figure 1.7 Multicellular suspended epithelial bridges formed by keratinocytes migrating on fibronectin patterns from microcontact-printing. (a) Keratinocytes migrating into the fibronectin strips from the reservoir and forming the multicellular suspended epithelial bridges over the non-adhesive regions. (b) In contrast, MDCK cells do not form the suspended epithelial bridges. Only some ‘nascent’ bridge-like structures can be found at the junction area of the fibronectin strips and the reservoir. (c) Basal sections of epithelial bridges with immunostaining for paxillin (top panel) and phospho-paxillin (bottom panel). The inset in the top panel demonstrates F-actin in the region, which is enclosed by the red square. White dotted lines indicate the concave leading edge of the suspended epithelial bridge. (d) Schematic description of patterned PDMS substrates with raised features

printed with fibronectin (red). (e) SEM image of keratinocytes illustrating the formation of epithelial bridges between the fibronectin-coated raised PDMS ridges. The inset demonstrates an enlarged view of the epithelial-bridge region bounded by the red square. (f) 'End-on' view of the epithelial bridge on the PDMS substrates with raised features. Scale bars, 50 μm (a,b) and 20 μm (c,e,f) (Reprinted with permission from reference [52]).

In contrary to conventional view on wound closure and collective cell migration, the epithelial bridges over large non-adhesive areas provide a means to ensure tissue integrity and maintain complete epithelial barriers regardless of ECM. Furthermore, the epithelial bridges could advance over regions lack of ECM and thus may help in accelerating wound healing. This unanticipated mode of cell migration has potential application in engineering artificial scaffolds for regeneration.

1.4 Research Motivation and Objectives

In living systems, cells rely on cell signaling to explore and respond to stimulus from their environment. The process not only plays a role in the proper functioning of individual cells but also contributes to the communication among cells and groups of cells. Collectively, cellular communication contributes significantly for regeneration and allows development of complicated forms and viability in dynamic environments. Cellular bridges, via physical continuity, remain the most straightforward means for cellular interactions. So far, cellular bridges have been proved to facilitate transport of signal, material and even cells between cells and cell masses. Therefore, cellular bridges play potential roles in health and disease.

However, how to engineer ECM properties to modulate cellular bridges at variable sizes (subcellular, cellular and multicellular) remains less explored. An in-depth investigation on formation and regulation of cellular bridges, especially their potential application in morphogenesis and wound healing are required in my PhD study. Based on the essential role of cellular bridge in cellular communication, we systematically investigated the cellular bridges at varied scales and their functions in regulate cell migration and morphology. The objectives of this PhD work are summarized as below:

- To investigate the accumulating effect of subcellular bridges on multicellular morphology via regulating discrete adhesions. Decoding how multicellular morphology is affected by the geometrical properties of subcellular non-adhesive gaps, as well as ECM rigidity, would help us to predict multicellular behaviors and design strategies for eliminating health-threatening effects.
- To establish cellular bridge between smooth muscle cell pair and study its components. Moreover, to utilize the cellular bridge to guide cell bidirectional migration by its gradual extending or contracting in a controllable way, indicating an effective way to inhibit contact inhibition locomotion (CIL) and may help minimize intimal thickening during in-stent restenosis and prevent vein graft failure.
- Study the formation of multicellular bridges over large non-adhesive regions to reveal the interplay between the geometrical aspects of ECM and the epithelial bridges, which allow us to optimize the migratory strategies for wound healing.

1.5 Organization of the Thesis

This thesis is composed of six chapters. Chapter 1 provides a detailed introduction into the field of mechanobiology and bioengineering, starting with the engineered

biomaterials to investigate cell-ECM and cell-cell interactions. Subsequently, microfabrication technologies have been described, with the application to regulate cellular behaviors such as morphogenesis and migration. Finally, cellular bridges and their related functions have been reviewed, as the in vitro study of cellular bridges relies on these advanced bioengineering methods. In Chapter 2, the subcellular bridges formed on discrete adhesive micropatterns are presented. Furthermore, the collective effect of orthogonal modulation of ECM topography and rigidity on multicellular morphology has been elaborately studied. Chapter 3 and Chapter 4 focus on the investigation of cellular bridges between a pair of smooth muscle cells. Unlike the subcellular bridges discussed in Chapter 2, the bridge between cell pair is at cellular level. We first study the relaxation and contraction of individual smooth muscle cell in Chapter 3 and establish the correlation between cytoskeleton and mechanical forces transmitted to ECM. Then, in Chapter 4, the oscillation from periodic alternations between contraction and relaxation of smooth muscle cells have been utilized to guide cell migration, which is regulated via extension and shortening of the cellular bridge in between. By oscillation, we provide an effective way to inhibit contact inhibition locomotion (CIL) and may help minify intimal thickening during in-stent restenosis. In Chapter 5, the size of cellular bridge is further increased to multicellular level, as the epithelial bridges over large non-adhesive regions are addressed. By regulating the geometrical aspects of ECM, the epithelial bridges demonstrate variable dynamics for their formation, which allow us to optimize the migratory strategies for wound healing. Finally, the thesis closes with conclusions and future perspectives for the whole project as addressed in Chapter 6.

Chapter 2 Orthogonally Engineering Matrix Topography and Rigidity to Regulate Multicellular Morphology

In this chapter, the subcellular bridges formed on discrete adhesive micropatterns are presented. Furthermore, the collective effect of orthogonal modulation of ECM topography and rigidity on multicellular morphology has been elaborately studied. The mechanism of such morphological organization has been explored, including analysis of the patterned substrates using molecular dynamics simulation, and immunostaining. The multicellular morphology is dependent on the competition between cell-matrix adhesion and cell-cell adhesion and the decreased cell-matrix adhesion would cause cytoskeleton reorganization, inhibit lamellipodial crawling, and thus enhance the leakiness of multicellular morphology.

2.1 Introduction

The formation and regulation of multicellular morphology, during which cells can actively respond to their environment and collectively build into the entire functional organization, are vital in synthetic tissue biology.^[53-55] Many important events in physiology are strongly related to multicellular morphology,^[56] such as tissue maintenance,^[57,58] endothelial function^[59,60] and morphogenesis,^[61] as it confers structure complexity and functional output.^[62,63] In addition, multicellular morphology is a critical indicator for life-threatening cancer progression,^[64] such as the morphological change in epithelial-to-mesenchymal transition and dysplasia.^[65-67] This indicates that methodologies to regulate multicellular morphology may possibly be efficient for cancer

therapy.^[68-70] In order to identify the cues that direct collective cellular behavior, numerous efforts have been devoted to recapitulate these cues in vitro growth environment.^[71-77] For example, manipulation of DNA, RNA and protein can provide the foundation for the synthetic morphology towards which cells are genetically engineered, in order to achieve biological constructs on the scale of organisms.^[78-81] Further biochemical cues, such as growth factors and cytokines, can promote multicellular assembly into suitable morphology, thereby forming proper shape for tissue and organs.^[82]

While engineering such biochemical cues is straightforward, technologies have only recently been developed to enable one to investigate the structural and mechanical cues from the cells' physical environments, including the extracellular matrix (ECM) and neighboring cells.^[83-91] Several *in vitro* studies have been reported on utilizing substrates with variable ingredients or porosity to affect multicellular morphology.^[92,93] However, these substrates remained highly artefactual as they cannot impose precise geometrical constraints on cell adhesion, which would serve as the communication centers between environmental cues and intracellular signaling pathways.^[94,95] In addition, most investigations on in vitro cultured cells that utilize recently developed micropatterning are mostly related to the differentiation, proliferation, and migration of individual cells,^[28,30,96-100] rather than collective cell responses.^[101-106] Cells seeded on these patterned substrates are simply confined in the ECM coated region and do not adhere to the non-adhesive regions.^[107] The effect of discrete adhesions on multicellular morphology remains an open question. Furthermore, decoding how multicellular morphology is affected by the geometrical properties of subcellular non-adhesive

gaps,^[108] as well as ECM rigidity, would help us to predict multicellular behaviors and design strategies for eliminating health-threatening effects, especially for tumor cells.^[109]

2.2 Materials and Methods

2.2.1 Fabrication of micropatterned substrates

Microcontact printing based on polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, Midland, MI) was applied to pattern extracellular protein, fibronectin (Sigma, St. Louis), onto PDMS substrates of various stiffness. Briefly, a PDMS stamp (curing-base weight ratio, 1:10) was microfabricated using standard photolithography methods. After curing on the mold at 90 °C for 1 h, the stamp was peeled from the mold and slightly treated by plasma cleaning. Meanwhile, the FITC conjugated fibronectin was coated onto another PDMS stamp with a flat surface via direct coating in solution for 1 h. The flat surface was then dried by Nitrogen gas and placed in contact with the microfabricated PDMS stamp so that fibronectin was transferred to the top surface of the microfabricated stamp. The stamp was then gently pressed against the target PDMS substrate (curing-base weight ratio at 1:40, 1:50, and 1:60, respectively). After around 1 min, the stamp was carefully lifted and the substrate immersed by a solution of 0.2% Pluronic F127 (Sigma, St. Louis) for half hour to prevent cell attachment on the unprinted areas. Lastly, the substrate was twice rinsed with phosphate buffered saline (PBS) and ready for cell seeding.

2.2.2 Cell Culture and Immunostaining

MCF-7 cells (human breast adenocarcinoma cell line) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (Sigma, St. Louis) at 37 °C in cell incubator with 5% CO₂. MCF-7 Cells were cultured on PDMS substrates patterned or fully coated with fibronectin. Prior to staining, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature and made permeable by 0.2% Triton X-100 in PBS for 10 min. Cells were then incubated with anti- α -Tubulin-Alexa 488 (Invitrogen, Carlsbad, CA) for 20 min in order to stain the microtubules. For cell-cell adhesion studies, cells were incubated with FITC conjugated anti-E cadherin (Abcam, Cambridge, UK) for 1 h. 4'-6-Diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA) was used to stain cell nuclei for 5 min.

Cells attached on PDMS substrate were imaged on a Leica TCS SP8 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with stage top incubator (37 °C and continuous pumping in of 5 % CO₂; Tokai Hit, INUB-WELS-F1 series). The images were recorded using 20 $\times$ /0.7 NA dry objective lens and 40 $\times$ /1.30 NA oil-immersion objective lens with scanning resolution at 2048 $\times$ 2048 pixels.

2.2.3 Simulation Method

In our molecular dynamics simulations, the substrate was composed by 9 $\times$ 9 unit cell. The ratio of the O and C atoms for the unit cell is 49/351 and 196/204, respectively. The distance between the substrate atoms is 2 Å. The position of the substrate atoms are

frozen during the simulation. Two different molecular models $C_{200}H_{402}$ and $C_{100}O_{100}H_{201}$ with different rigidity and charge distribution were employed in this study. Bond lengths of molecules were constrained using the LINCS algorithm. Simulations were done using the NVT ensemble. In this ensemble, the number of molecules (N), the normal volume (V), and the temperature (T) are fixed. By applying the Berendsen temperature coupling method, the temperature was kept at 300 K. A constant normal pressure of 1 bar was maintained by utilizing Berendsen bath coupling scheme. The cutoff distance of short-range nonbonded interactions (van der Waals and real-space Coulomb) was set as 12 Å and the Reaction-Field (Particle Mesh Ewald) approach was utilized for computing long-range electrostatic forces. A time step was chosen as 2 fs.

2.3 Results and Discussion

2.3.1 Topologically and Mechanically Co-modulated Multicellular Morphology

Herein, we developed a platform to control the multicellular morphology by orthogonally modulating the mechanical and topological cues of extracellular matrix based on the biocompatibility of polymer materials and patterning flexibility of micro-contact printing (μ CP). As a proof of concept, MCF-7, a human breast adenocarcinoma cell line, was chosen as the model system for the study. Specifically, different types of multicellular morphology strongly depending on the ECM, such as highly confluent monolayer, leaky network with ring structure, and isolated islands, were observed. It was found that cell-matrix anchorage via either modulating non-adhesive gap size or the rigidity of ECM plays an important role for the multicellular morphology, and it was confirmed by the molecular dynamics simulations. Our findings on such coupling effect

of the topological and mechanical cues would provide in-depth understanding of the regulation of multicellular morphology and facilitate materials development to specify the multicellular assembly with local variations in ECM.^[110,111]

To precisely control both micro-topography and elasticity for orthogonally engineering, polydimethylsiloxane (PDMS) was chosen as the substrate material due to its tunable stiffness and high binding capability for extracellular protein.^[112] However, due to the ease of deformation on an extremely soft and viscous substrate, it was challenging to accurately print extracellular proteins on PDMS cured with an ultra-low cross-linking reagent. During a conventional contact printing process, redundant proteins would be present on the inner areas of the stamp that was not part of the printing pattern. The deformation of the substrate would allow the transfer of these redundant proteins from the stamp to unwanted areas, thus yielding an undesired blurred pattern. In order to circumvent this problem, we developed a simple and efficient method, a twice-transfer process, for protein printing. As seen from the schematic illustration in Figure 2.1a, instead of direct immersion of the stamp in the protein, a flat surface was first coated with the protein, against which the stamp was applied to complete the protein transfer.^[113] This ensured that only the top surface of the stamp would be coated with protein; thus despite substrate deformation during the contact printing process, the printed patterns remained consistent with that of the stamp. It can be surmised that additional protein transfer step was effective in the removal of redundant protein to ensure high resolution printed patterns.

Figure 2.1 (a) Micropatterning on soft substrate and subsequent cell culture. Briefly, protein transfer was applied to coat ECM on only the top surface of PDMS stamp, followed by contact printing upon the PDMS substrate with extremely low stiffness. Finally, MCF-7 cells were seeded on the patterned substrate, with the unprinted area blocked by poly (ethylene glycol) (PEG). (b) Matrix of results showing the interplay between substrate rigidity and fibronectin square length/non-adhesive gap width ratio on cell spreading. Decreasing either substrate rigidity or adhesive pattern size would negate cell spreading. Scale bar: 20 μm (same scale bar applied to all images in this Figure).

Through orthogonally varying the PDMS stiffness and size of the extracellular protein micropatterns, we were able to investigate the concomitant effect of both key factors. Since substrate rigidity inflicts a mechanical constraint on cell spreading,^[114] there is a positive correlation existing between substrate stiffness and the projected spreading area. On the other hand, the extracellular protein micropatterns impose a chemical limitation towards cell spreading, so cell-substratum adhesivity is modified by the decoration of the substrate with extracellular protein.^[115-117] As demonstrated in Figure 2.1b, a ‘library’ of substrates was generated for the investigation of cell spreading; and the PDMS substrates of three different Young’s modulus (15, 30, and 70 kPa) were fabricated by varying the curing reagent/substrate weight ratio, upon which four types of fibronectin micropatterns were imprinted.^[118] These micropatterns consist of repeated squares with four different side lengths (7, 10, 12, and 14 μm), as well as a fixed period at 20 μm , thus yielding various non-adhesive gap widths (13, 10, 8 and 6 μm). The results from the combination of these two parameters demonstrate that either low substrate stiffness or large non-adhesive gap inhibits cell spreading. The illustrated red border divided the results matrix into two phases: one is high extent of cell spreading, which would lead to confluent cell monolayer and the other is poor cell spreading, concomitant with cell aggregation and multicellular ring formation. From this, it could be deduced that a broader range of non-adhesive gap widths can be tolerated with higher substrate stiffness in order to achieve confluent cell monolayer, whereas lower substrate stiffness lead to poor cell spreading even with sufficiently small non-adhesive gap. An interesting phenomenon could be observed with the micropatterned PDMS substrate of the lowest rigidity (Young’s modulus 15 kPa), on which the MCF-7 tumor cells displayed

striking morphogenetic changes, forming a large number of hollow ring structures. This was in sharp contrast to the uniform, confluent cell monolayer on a rigid substrate fully coated with protein (Figure 2.2).

Figure 2.2 Bright field image showing that a uniform, confluent MCF-7 cell monolayer could be formed on a PDMS substrate (15 kPa) fully coated with fibronectin. Scale bar: 20 μm .

Similar morphological changes of MCF-7 cells were also observed by replacing PDMS substrate to polyacrylamide (PAA) gel films with Young's modulus of 40 kPa and 10 kPa, respectively. The hollow ring structures were formed by MCF-7 cells on PAA gel films (10 kPa) with printed fibronectin, as demonstrated in Figure 2.3. This indicates a broad range of materials could be utilized to regulate the multicellular morphology.

Figure 2.3 Matrix of results showing the interplay between rigidity of PAA gel substrate and fibronectin square length/non-adhesive gap width ratio on MCF-7 cell spreading. Decreasing either substrate rigidity or adhesive pattern size would negate cell spreading and thus facilitate the formation of multicellular ring. Scale bar: 50 μm (same scale bar applied to all images in this Figure).

To further generalize this ECM-dependent multicellular morphology, we also cultured Madin-Darby Canine Kidney (MDCK) cells on the same orthogonally modulated PDMS substrate and found a very similar tendency for morphological changes. Based on the size and distribution of hollow rings, the leakiness of multicellular morphology would be enhanced by enlarged non-adhesive gap width (reduced pattern size) and lower rigidity of substrate, as shown in Figure 2.4. The hollow rings resembled the epithelial gaps observed in vivo, of which the size and closure were important variables with regards to health and diseases.^[119]

Figure 2.4 Matrix of results showing the interplay between rigidity of PDMS substrate and fibronectin square length/non-adhesive gap width ratio on MDCK cell spreading. Decreasing either substrate rigidity or adhesive pattern size would negate cell spreading and thus facilitate the formation of multicellular ring. Scale bar: 50 μm (same scale bar applied to all images in this Figure).

2.3.2 Theoretical Studies of Multicellular Assembly

The multicellular morphology is actually established and maintained from the cooperation between integrin mediated cell-matrix adhesions and cadherin-based intercellular adhesions. In order to predict the shape of multicellular assembly, previous model studies have compared the morphogenetic cell movements to the behaviors of

immiscible liquids based on their similarity.^[120] But the model is limited to reveal local morphogenetic information since it ignores the shape change of every individual cell,^[121-123] which is an important factor in cell-matrix interaction.^[124] Herein, we improve the model to simplify this morphogenetic process by applying molecular dynamics based simulation to treat cells as extended objects with capability of changing shape. Due to the similarity in structural and mechanical properties for both cytoskeleton and macromolecule, the alkyl-chain macromolecule was chosen to represent every tumor cell. Specifically, since the flexibility of cytoskeleton would be increased for cells cultured on substrate with lower rigidity, we increased the flexibility of macromolecule by replacing alkyl chain with ether chain as a similar effect of rigidity decreasing. To understand the role of non-adhesive gap in the multicellular morphology, the substrate composed of carbon (C) and oxygen (O) atoms was introduced to represent the fibronectin printed substrate. Based on the Lennard-Jones potential calculation, the basic units of both alkyl chain and ether chain exhibit higher affinity to O atoms than to C atoms. Hence, the C atoms represented the non-adhesive gap and O atoms represented fibronectin. For simplification, we chose the cases of smallest (6 μm) and largest (13 μm) non-adhesive gap width for simulation. Since the corresponding side lengths for fibronectin squares are 14 μm and 7 μm , yielding the fibronectin coverage ratios of 196/400 and 49/400, we modulated the ratio of O to C from 196/204 to 49/351 on the substrate in simulation to represent the same geometrical properties (the O atoms coverage ratios are 196/400 and 49/400, consistent with the corresponding fibronectin coverage ratios). In addition, rigidity increasing would enhance cell spreading over a surface, thus we introduced charge distribution to O atoms on the rigid substrate (Figure 2.5a and 2.5b) for enhancing

Figure 2.5 Snapshots of simulation results of molecules adhering on substrate composed of C atoms and O atoms: (a) $\text{CH}_3-(\text{CH}_2)_{198}-\text{CH}_3$ on substrate with the ratio of O to C of 196/204 (the O and $\text{CH}_3-(\text{CH}_2)_{198}-\text{CH}_3$ with charge distribution), (b) $\text{CH}_3-(\text{CH}_2)_{198}-\text{CH}_3$ on substrate with the ratio of O to C of 49/351 (the O and $\text{CH}_3-(\text{CH}_2)_{198}-\text{CH}_3$ with charge distribution), (c) $\text{CH}_3-\text{O}-(\text{CH}_2-\text{O})_{98}-\text{CH}_2-\text{OH}$ on substrate with the ratio of O to C of 196/204 (the O and $\text{CH}_3-\text{O}-(\text{CH}_2-\text{O})_{98}-\text{CH}_2-\text{OH}$ without charge distribution), and (d) $\text{CH}_3-\text{O}-(\text{CH}_2-\text{O})_{98}-\text{CH}_2-\text{OH}$ on substrate with the ratio of O to C of 49/351 (the O and $\text{CH}_3-\text{O}-(\text{CH}_2-\text{O})_{98}-\text{CH}_2-\text{OH}$ without charge distribution).

macromolecule affinity, representing the effect from higher rigidity. The results showed that for either enlarged non-adhesive gap (from Figure 2.5a to Figure 2.5b) or increased flexibility of macromolecule (Figure 2.5a to Figure 2.5c), the morphology of macromolecule assembly became a more porous network, whereas the combined change would lead to morphology of isolated islands (Figure 2.5d). Hence, simulation of macromolecule assembly illustrated the similar trend in the multicellular morphology: either increasing non-adhesive gap width or decreasing substrate rigidity would attenuate the cell-ECM interaction and thus enhance the aggregation.

2.3.3 Mean Sizes and Edge Roughness of Multicellular Rings

Due to the importance of maintaining tissue continuity and function, much effort has been contributed to the investigation of wound healing and gap closure.^[125,126] Proposed mechanisms include purse-string contraction of an assembled actomyosin ring for smaller wounds,^[127] as well as proliferation and cell migration/lamellipodia extension for relatively large wounds. This highlights the importance of size with regards to wound closure in cell tissues. To verify the influence of size on the repair mechanism, the mean sizes of MCF-7 multicellular rings were quantitatively analyzed on the 15 kPa PDMS substrates with different non-adhesive gap widths. Due to the square array, the adhesion sites for cells are discrete along both axes on the substrate; whereas anisotropic patterns such as the line pattern would result in cells aligned along those lines (adhesions are continuous in this direction) and thus deform the ring structures, as shown in Figure 2.6.

Figure 2.6 MCF-7 cells grown on PDMS substrate (15 kPa) with line pattern were aligned along those lines and deformed the ring structures. Scale bar: 20 μm .

Figure 2.7 presented the bright field images of morphogenetic changes with gradually decreasing non-adhesive gap width. Figure 2.8a demonstrated that the statistically averaged ring size decreased exponentially from $72,000 \mu\text{m}^2$ at 13 μm non-adhesive gap (0.65 gap width/periodic length ratio) to $13000 \mu\text{m}^2$ at 6 μm non-adhesive gap (0.3 gap width/periodic length ratio). This is because cell-substrate adhesion is produced via the binding of integrin to fibronectin,^[128] in which integrin acts the receptor mediating cell attachment to extracellular ligands by specific interaction. Thus an decrease in non-adhesive gap width could enlarge the ECM coverage and thus enhance the cell-substrate adhesion energy per unit area, leading to a larger confluent cell area and in turn smaller average ring size.

Figure 2.7 The bright field images of MCF-7 cells morphogenetically changing with gradually increased gap width/periodic length ratio from 0.3 to 0.65 on PDMS substrate (15 kPa). Scale bar: 100 μm .

Figure 2.8 (a) Quantitative study of multicellular ring size distribution versus different non-adhesive gap width/periodic length ratios on the 15 kPa PDMS substrate (Using student t-test, $n=12$, $p<0.05$ in comparison of 0.3 and 0.4 gap width/periodic length ratio; $n=15$, $p<0.01$ in comparison of 0.5 and 0.65 gap width/periodic length ratio). (b) Bright field images of MCF-7 cells on PDMS substrate (15 kPa) with 0.4 gap width/periodic length ratio and full fibronectin coverage. The red curves outline the ring borders and the border roughness was then calculated by comparing contour length of the ring against that of a circle with equal area. Scale bar: 50 μm . (c) The calculated shape factors indicating the roughness of ring border for MCF-7 cells grown on four types of patterned

PDMS substrate plus the fully coated substrate (by student t-test, $n > 10$, $p < 0.001$ for all comparison between all substrates).

Unlike previous studies on the coordinated movement for epithelial monolayers, or the unblocked multicellular outgrowth that arose from leader cell movement,^[119] no finger-like outgrowth seemed to have occurred at the border of the MCF-7 multicellular rings on the patterned soft substrate. Experimental results revealed that for cells well spreading on substrates fully coated with fibronectin, gaps or holes could be closed by cell proliferation and migration (see Figure 2.9). However, for the multicellular rings, no hole closure was observed even with prolonged culture of up to 2 weeks. When cell proliferation occurred at the boundary, the divided cells would rapidly adhere to the multicellular rings, thus returning the border to its original smoothness (Figure 2.10). To provide a quantitative comparison between the smoothness of the multicellular ring borders, the shape factor of the rings were defined as $P_{\text{cell}}/P_{\text{circle}}$, which is the ratio of the ring inner contour length to the contour length of a circle with equal area to the ring, as schematically displayed in Figure 2.8b. The shape factors demonstrated significant differences between rings of smooth borders and those of rough borders. For rings present on all four types of micropatterned 15 kPa PDMS substrates, the shape factors ranged from 1.2 to 1.5. In contrast, for holes present on PDMS substrates of the same rigidity but fully coated with fibronectin, the shape factor increased dramatically in value to above 2.1, indicating that that cellular protrusions are highly extended into the available space. Interestingly, a biphasic effect was observed from the curve illustrated in Figure 2.8c. An increment in non-adhesive gap width would result in the formation of multicellular rings with smoother borders and hence smaller shape factors; however,

excessively larger non-adhesive gap widths would lead to significantly enlarged ring sizes, resulting in larger deviations from the circle shape, and in turn slightly increased shape factor values. This suggests that within a fixed period of micropattern, the circular multicellular rings could only be formed by coating fibronectin with moderate gap width on 15 kPa PDMS substrate; whereas smaller gap width would lead to confluent cell monolayer and larger gap width would result in large, deformed multicellular rings which could easily develop into isolated cell colonies. The summation of these data points to the conclusion that the geometrical property of extracellular protein on the substrate is an important factor controlling the coordinated healing of wounds or holes present in the cell sheets.

Figure 2.9 For MCF-7 cells well spread on the PDMS substrate fully coated with fibronectin (70 kPa), the ring with of inner diameter about 200 μm was closed by cell proliferation and migration within 20 h. Scale bar: 50 μm .

Figure 2.10 Bright field images recording phenomenon in which divided cells would quickly adhere to and flatten the border of holes in multicellular rings. The substrate is fabricated with soft PDMS (15 kPa) with geometrical topography, partially coated with fibronectin (0.3 gap width/periodic length ratio). Scale bar: 75 μm .

2.3.4 Mechanism of Formation of Multicellular Ring and Confluent Monolayer

Herein, we propose that the mechanism of formation of the unclosed rings stems from the transmission of extracellular mechano-stimulation into cytoskeleton reorganization,^[129] leading to collective cellular morphological change. To demonstrate this hypothesis, immunostaining was employed to study the microtubules distribution of both MCF-7 cells with ring formation and confluent cell monolayer. For the former, microtubules accumulation occurred around the nuclei of the MCF-7 tumor cells at the regions of ring structure formation. For the latter, the microtubules were well distributed throughout every cell, as shown in Figure 2.12a. Figure 2.12b charted the quantitative measurements of microtubules amount distributed in the extranucleus region constituting half the area of a nucleus. It could be seen that multicellular ring formation occurred with dramatically enhanced microtubules accumulation, with a distribution value of around 0.8, as compared to the value of 0.4 for the confluent cell monolayer. Since microtubules served as the compression resistant component within cells,^[130] the

increased concentration of microtubules around the nuclei suggested an enhancement in the flexibility of microtubules.^[131] This negates the cells' ability to be as extended as those with uniformly distributed microtubules, and such cytoskeleton reorganization of individual cells manifested as a collective cellular behavior, culminating in the formation of multicellular rings. To further demonstrate that reduced cell-matrix anchorage resulted in enhanced cell-cell adhesion due to their competitive nature, immunostaining of E-cadherin was applied to quantify the average fluorescence intensity for MCF-7 cells both well-distributed on a rigid (70 kPa) substrate with small (6 μm) non-adhesive gap and with multicellular rings formed on a soft (15 kPa) substrate with large (10 μm) non-adhesive gap (Figure 2.11).^[132]

Figure 2.11 Immunostaining of E-cadherin was applied to quantify the average fluorescence intensity for MCF-7 cells forming multicellular rings and confluent monolayer, respectively. Scale bar: 25 μm .

Through three-dimensional scanning of the aforementioned MCF-7 cells, it was discovered that cell aggregation during ring formation was concomitant with the agglomeration of the nuclei (Figure 2.12c). Quantitative measurement in Figure 2.12d demonstrated that the E-cadherin fluorescence intensity at cell–cell contacts in regions of ring formation increased significantly by ~40% relative to that of cells in confluent monolayer, which indicated the enhancement of cell-cell cohesivity. Such increment in adhesiveness between cells constituted the reason for the restrained cell spreading, as well as the subsequent aggregation of cells to form ring structures.

Figure 2.12 (a) Immunostaining of nuclei (blue), microtubules (green) for MCF-7 cells grown on both patterned and fully coated PDMS substrates (15 kPa). Scale bar: 50 μm . (b) Statistical study of tubulin accumulation around nuclei by fluorescence image processing. The results show that, for cells comprising ring formation, tubulin accumulated in extranucleus region at half nucleus area are almost twice that of cells well spread on

fibronectin fully coated substrate (n=20 cells, $p<0.001$ by student t-test). (c) A three-dimensional construction of immunostained MCF-7 cells well distributed on a patterned stiff PDMS substrate (70 kPa, 0.3 gap width/periodic length ratio); as well as construction of multicellular ring formed on patterned soft PDMS substrate (15 kPa, 0.5 gap width/periodic length ratio). Scale bar: 20 μm . (d) Statistical study of the fluorescence intensity for E-cadherin (intercellular adherence protein). The results suggest that, for the cells with ring formation, fluorescence of E-cadherin located at adherent junction are enhanced by ~40% compared to that of cells well spread on substrate (n>60 cells, $p<0.05$ by student t-test).

Figure 2.13 Model explaining cell-matrix anchorage dependent multicellular organization. The cell sheet homogeneously distributed over the substrate surface could

be generated by high cell-ECM anchorage via increasing substrate rigidity and ECM coverage (smaller ratio of gap width/periodic length). On the other hand, cytoskeleton reorganization (tubulin accumulation) and enhanced intercellular adhesion (increased expression of E-cadherin) could be induced by sufficiently low cell-ECM anchorage, which culminated in the formation of multicellular ring structures.

Based on the aforementioned experimental results, a simple working model could be established elucidating the formation of multicellular rings on PDMS substrate with sufficiently low rigidity and large non-adhesive gap width (Figure 2.13). The ring formation originated from the competition between cell-substrate interaction and cell-cell interaction. A decrease in either substrate stiffness or adhesive pattern size would lead to reduced cell-ECM interaction, which enabled cell-cell adhesion to dominate cell-substrate anchorage, resulting in cytoskeleton reorganization and cell spreading inhibition. Cell traction force, indicating cell-substrate adhesion,^[20] was dramatically reduced on the micropatterned substrate (with 10 μm non-adhesive gap) compared to that fully coated with fibronectin (substrate rigidity was kept constant at 15 kPa) (Figure 2.14). Moreover, the entire cell sheet containing ring structures could be easily removed with slight liquid flow, indicating that cell-substrate interaction was indeed significantly decreased (Figure 2.15). The resultant reduction in cell spreading not only promoted the formation of rings, but also inhibited the cellular crawling or protrusion necessary for ring closure. The multicellular rings remain unclosed even when excess cell proliferation was effected by extended culture, since the divided cells would adhere to neighboring cells rather than migrate into the available free space within rings.

Figure 2.14 Calculation of cell traction forces intensity indicated that the cell-ECM adhesion for MCF-7 cells dramatically decreased on patterned substrates compared to substrates fully coated with fibronectin (15 kPa).

To sum up, multicellular morphogenesis was the result of integration among cell-ECM anchorage, cytoskeleton organization and cell-cell cohesivity, with cell-ECM anchorage comprising the main inducing factor. Thus, by enhancing cell-ECM anchorage through the increase of substrate rigidity and ECM coverage, a confluent cell monolayer consistently spreading over the substrate surface could be achieved, while reducing the cell-ECM anchorage would promote the formation of multicellular ring structures.

Figure 2.15 Bright field image of the entire MCF-7 cell sheet with ring structures being easily peeled off the substrate by slight liquid flow. Scale bar: 100 μm .

2.4 Conclusion

In conclusion, a polymer substrate printed with an extracellular protein pattern has been rationally designed to control the collective multicellular formation in MCF-7 cells by orthogonally modulating substrate rigidity and non-adhesive gap width. It was found that morphogenetic ring formation process is the result of a mechanical equilibrium established and maintained by multicellular cooperation with the extracellular matrix. This demonstrates that the extracellular matrix parameters are comparable to biochemical cues from growth factors in profoundly influencing cell behavior.^[133] Weakened cell-matrix anchorage may lead to the smoothening of multicellular ring borders and enlargement of ring size, leading to unclosed rings even with long-term cell proliferation. The formation of rings can increase the permeability of the cell sheet and thus allow better delivery of nutrients or therapeutics,^[134,135] while enhanced cell-matrix anchorage

would promote the formation of confluent monolayer, serving as a protective barrier which can resist pressure in vivo.^[136] Consequently, this protective function determined by the efficiency of tumor cells in maintaining its continuity and integrity can be switched on or off by modifying their environment, thereby suggesting ways in developing implantable materials to specify the assembly of biological structures and contributing to a better understanding of synthetic tissue biology.

Chapter 3 Contraction and Relaxation of Individual Cell

In this chapter, by investigating the relaxation and contraction of individual smooth muscle cell, the correlation between cytoskeleton and mechanical forces transmitted to ECM has been established. The mechanical forces were measured by cell traction force microscope (CTFM). Based on the real-time monitoring of CTF and dynamics of actin filaments, two types of theoretical models have been raised for contraction and relaxation, respectively. These mechanical analyses also facilitate elaborating tension development within cellular bridges in the following chapters.

3.1 Introduction

Adaptability is one kind of capabilities make lives survive under different external stimuli. Vascular smooth muscle (VSM) cells, which are key components for both blood vessel and respiratory system, can contract due to external stimuli that then lead to intracellular calcium increase and activation of Ras homolog gene family, member A (RhoA, known to regulate actin cytoskeleton). And they can also be able to spontaneously relax to its initial state after contraction in an opportune while without any further stimuli. Hence, the spontaneous relaxation dynamics demonstrate the adaptability of VSM cells and the fast and stable spontaneous relaxation relates to high adaptability. Here we induced drug stimuli to VSM cells to elevate the intracellular calcium concentration. Most cells underwent contractions via drug stimuli and their robust spontaneous relaxations make them recovery and keep survived, for many cycles.

3.2 Materials and Methods

3.2.1 Materials

Vascular smooth muscle cell line (A7R5) was purchased from ATCC (Rockville, MD). Dulbecco's Modified Eagle's Medium (DMEM) and collagen (type I, Rat tail) were purchased from GIBCO (Gaithersburg, MD). Calcium ionophore A₂₃₁₈₇ and dimethyl sulfoxide (DMSO) were obtained from Sigma. Phosphate buffered saline (PBS), anti- α -Tubulin-Alexa 488[®], Alexa Fluor[®] 594 phalloidin, and 4'-6-Diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. PDMS base and crosslinker (Sylgard 184) were obtained from Dow Corning Corp. (Midland, MI, USA)

3.2.2 Preparation of Elastic Substrate Embedded with Fluorescent Beads

The elastic substrate for recording cell traction force is composed of chemically modified glass coverslips, PDMS(Sylgard[®] 184 silicone elastomer kit, Dow Corning) and fluorescent beads (FluoSpheres[®] microspheres, 0.1 μ m, carboxylate-modified, 0.1 vol %, Molecular Probes). Briefly, coverslips were rinsed with acetone and isopropanol and then cleaned by oxygen plasma before being placed in a vacuum desiccator containing 5 μ L of 1H,1H,2H,2H-perfluorooctyltrichlorosilane for half an hour. The coverslips were taken out and 100 μ L of diluted yellow-green FluoSpheres[®] (0.1 vol %) aqueous solution was utilized to cast a single layer of fluorescent microspheres on the top of glass coverslips. A thin film of PDMS were generated by spreading 18 μ L of PDMS mixture (uncured, 1:60 w/w of crosslinker to base ratio) on the fluorescent beads coated glass coverslips. One layer of Scotch tapes was used as a spacer prior to putting the other

bare glass coverslip on the top of the spread PDMS film. The PDMS droplet was further flattened by the weight of the upper coverslip. The whole assembly was then placed in 37 °C incubator overnight. Afterwards, before the upper bonded PDMS thin film with fluorescent beads embedded in its top was peeled off. Only substrates with suitably dispersed beads were used. To promote cell attachment on PDMS substrate, substrate after UV sterilization was incubated with collagen solution (50 µg/mL in 0.02 M acetic acid) overnight before further rinsing by PBS (pH 7.4) solution, and then stored in 4 °C fridge before use. The rheological properties of PDMS were measured by Physical Rheometer (Model: MCR 501, Anton Paar, Germany).

3.2.3 Cell Traction Force Calculation Method

Calculation of cell traction force, strain energy (forces multiplied by displacements of tracked beads), and polarity (ratio of force along principle axe to force along vice axe which is vertical to principle one) were based on the elasticity of PDMS substrate and displacements of fluorescent beads. Figure 3.1 shows the image of fluorescent beads, and the displacement of every single bead was measured based on comparison between the null image before seeding cells and the force loaded image after seeding (as shown in Figure 3.2a).

Figure 3.1 Image of fluorescent beads embedded in substrate by confocal microscopy.

Scale bar: 10 μm .

Figure 3.2 (a) Calculation for displacement for tracked fluorescent beads; (b) examples of calculated deformation field and stress field.

The cell traction force mapping was based on the images of VSM cells on PDMS substrate and the corresponding traction force mapping in pseudocolor, which indicated regions of traction force. Figure 3.2 demonstrate an example about how to track fluorescent beads, as well as the deformation field and stress field.

Briefly, the traction force located at the position (x_i, y_i) , at discrete point $\vec{f}_i$, was generated according to the following formulation: $\vec{u}_t(x, y) = \sum_{i=1}^n \vec{G}(x - x_i, y - y_i) \vec{f}_i$, where $\vec{G}$ stated the Greens' tensor and $\vec{u}_t$ stated the experimental displacements of fluorescent beads at position (x_i, y_i) . The overall force of the cell (F) is calculated from an integral of the traction field magnitude over the area, $F = \int \int \sqrt{T_x^2(x, y) + T_y^2(x, y)} dx dy$, where $T(x, y) = [T_x(x, y) + T_y(x, y)]$ is the continuous field of traction vectors, which is defined at spatial position (x, y) .

The current cellular traction force mapping relies on the validity of the following assumptions, which is also the limitation of our CTF method. Forces normal to the surface of the substrate has to be very small since the vertical component of traction force is not measurable based on our monolayer distributed fluorescent nano-beads.

3.3 Results and Discussion

3.3.1 Oscillations between Contraction and Relaxation

Here we present the dynamic processes for contraction and relaxation of VSM cells with optimal control and also the oscillations between these two states. Basically, cells state can be distinguished by its morphology. For cells with higher aspect ratio, the

state is identified as relaxed state, and the one with respectively lower aspect ratio correlates to contracted state.

Figure 3.3 Difference in the morphology of relaxed and contracted VSM cell, respectively.

After seeding VSM cells on our PDMS substrate coated with collagen type I, inducing calcium ionophore A_{23187} will result in increase of intracellular concentration and hence initiate cells contraction which typically could be completed in 20min. Rather than a transient response of cardiac muscle cells, VSM cell demonstrates a slow tension development since it requires a dynamic remodelling of actin cytoskeleton during the contraction.^[137] After that, spontaneous relaxation will occur. Therefore, by repeating A_{23187} stimuli, the oscillations between contraction and relaxation will be triggered, as shown in Figure 3.4.

Figure 3.4 VSM cell oscillation between contraction and relaxation for three cycles (a-b, c-d, respectively), scale bar: 20 μm .

The cell traction force was carefully investigated by the methods described above. Briefly, as Figure 3.5a shows, the cell starts being shortened during contraction followed by the detachment of actin filaments from the focal adhesion sites and further the decrease of traction force, whereas during relaxation the traction force will increase due to the elongated cytoskeleton composed of stiff and parallel bundles of actin filaments. The elastic property of cytoskeleton is similar to a spring, following the rule of more strain, more stress. On the other hand, the contractile force generated during cell contraction can only be detected when the actin filaments keep anchored to the focal adhesion sites so that force can be successfully transmitted to the substrate, which in turn result in traction force increase, as shown in Figure 3.5b (indicated by green circle). The

overall tendency for traction force during contraction is decreasing since increased actomyosin-based contractile force will eventually pull up the rear cell edge followed by disassembly of cell-substratum adhesions^[138]. Normally, the traction force changes during continuous oscillations will demonstrate in an undamped form.

Figure 3.5 Cell traction force (black curve) and strain energy (blue curve) for two individual VSM cell during scillation between contraction and relaxation for three cycles, respectively.

The occasional occurrence of the spike for CTF curve is due to the following reason: during cellular contraction, the force generated from actin-myosin contractile system would actually further deform the underneath elastic substrate and thus result in slightly enhanced CTF. However, this type of slight enhancement is transient since the cell length would be reduced by the contractile force. According to the Hooke's law, less cell length results in less CTF. Therefore, occasionally measured spike could be happen during cellular contraction.

Damped oscillation indicates cell adaptation which will reduce the response of cell to external stimuli and usually demonstrates as a small phase delay to the preferred

direction in the response/cycles figure. However, in our statistical study, the cells behave mostly in normal oscillation. As shown in Figure 3.6, four parallel experiments recorded the robust oscillations of individual cell between contraction and relaxation for four cycles, with each cycle of 40 minutes.

Figure 3.6 Parallel experiments for cell traction force recording of four individual VSM cell during oscillation between contraction and relaxation for four cycles, respectively.

3.3.2 Investigation of Cytoskeleton

In order to clarify the F-actin dynamics during VSM cells oscillations, especially the orientation relationship between cell pair and parallel aligned actin filaments, transfection must be applied to our systematic study. Recently, we have stained actin filaments and microtubulin in fixed cells and also transfected VSMC and Hela by

complex of lipofectamine and plasmid. Both RFP (red fluorescence protein)-actin and RFP-paxillin plasmid are used. Alternatively, one commercial product named as LifeAct can be used to stain filamentous actin (F-actin) structures in living cells and also does not interfere with actin dynamics.

Figure 3.7 Left: Micro-tubulin of smooth muscle, scale bar: 10 μm . Right: Actin filament of smooth muscle cell, scale bar: 20 μm .

To build our own model, we need to combine these classic viscoelastic models with current model designed for cell migration^[139] to make qualitative predictions about the events happened in our own system, especially the force raised from actin polymerization, which directly leads to an increase of the cytoskeletal stiffness and also promotes attachments at the new sites at leading edge, as well as detachments at the tailing edge of cells.

3.3.3 Basic Model for Viscoelastic Elements

Viscoelastic issues can be modeled typically and basically by the Maxwell model or the Kelvin-Voigt model in order to generate the stress and strain interactions, including their temporal dependencies.

Figure 3.8 The different organization of Maxwell model and Kelvin-Voigt model.

The Maxwell model is represented by a Newtonian damper and a Hookean elastic spring connected in series, whereas the Kelvin-Voigt model consists of a Newtonian damper and Hookean elastic spring, with connection in parallel. The Maxwell model predicts that stress decays exponentially with time, and Kelvin-Voigt model is used to explain the creep behavior. We demonstrate these two models here since the cell is actually also viscoelastic and hence could apply one of them into our model.

3.3.4 Stick-slip Model for Cell Contraction

The description of the anchorage of the cell to ECM as a spring is only valid when the force strength is not extremely high. Once it exceeds the threshold $f = k_m/2\pi$ (k_m defines the adherence properties of ECM), the adhesion moves rather than deforms and makes the junction between cell and ECM undergo a stick-slip motion^[140]. Actually the stick-slip motion is caused by calcium-dependent detachment which includes the retraction of cell edges pulled by increased actomyosin-based contractile force^[138] and disassembly of cell-ECM adhesions.^[141] The sudden jump-down of cell traction force

could be explained as reduced dynamic friction and also the decreased cytoskeleton tension.

Figure 3.9 Model for cell contraction.

Here we apply modified Kelvin–Voigt model to describe the viscoelasticity property of one cell, hence the cell traction force can be deducted from the total contractile force generated inside the cell plus the force from elastic part but minus the viscous part, following the equation $F_{tract} = aK * L + F_{contr} - \eta v$, where F_{tract} , F_{contr} , stand for cell traction force and contractile force generated inside the cell, respectively; and K is the spring constant; L stands for cell length; η is viscosity coefficient; v stands for cell contraction rate; a is the cell stiffness factor which also dependent on the cell-ECM interaction.^[73] The cell traction force detected by us is mainly the elastic part according to the cytoskeleton-ECM linkages. As Figure 3.10 shows, the cell traction force is almost proportional to cell length and the jump-up contraction rate leads to a jump-down of the traction force. Both confirm the above equation experimentally.

The Kelvin–Voigt element applied in our model to represent each single cell, which consists of a spring of constant K and a damper of impedance η . In the simulation

part of Figure 3.10, Traction Force could be deduced just by recording the cell length dynamics during contraction under fluorescent microscopy. Based on the following equation containing presumed parameters $F_{tract}=5nN \text{ } \mu\text{m}^{-1} \square L+50nN \text{ } -v \square 5nN \text{ min } \mu\text{m}^{-1}$, the kinetics of CTF yields good agreement with the measured values, as demonstrated in the upper diagrams.

Figure 3.10 Relationship between cell traction force, cell length and cell contraction rate during a typical contraction for VSM cell. Left: experimental data; Right: simulated results.

3.3.5 Spring Extension Model for Cell Relaxation

Due to the elastic properties of cytoskeleton and mechanosensing by focal adhesions, a ‘two spring model’^[142] was developed to describe the cell attachment to the

compliant substrate by integrin-mediated adhesions, where cytoskeleton and ECM stiffness (K_c, K_{ex}) are represented by two linear springs with connection by integrin-ligand bonds. Since cell relaxation will extend the cytoskeleton along the principle axis and generate higher tension like the one from more elongated spring. Furthermore, the spring constant should also increase somewhat during relaxation by reassembly of the stress fibers, which needs to be clarified by dynamic visualizing of actin filaments. Hence, cell traction force increase between each end of the cell during relaxation following Hook's law $F = K \cdot \Delta L$, where $K = (K_c^{-1} + K_{ex}^{-1})^{-1}$. Previous study^[143] also support this kind of tendency for tension change.

Figure 3.11 Model for cell spontaneous relaxation.

This model is useful in analyzing or predicting the relaxation for specific cell lines such as smooth muscle cells, since the shape of an adherent smooth muscle cell is in high

polarity, as cell would elongate significantly during spontaneous relaxation. This geometric transition make the actin-myosin system based cell more like an elastic spring and thereby validate the Hooke's law. However, for adherent cells with a high roundness, e.g. on patterned surface, this model may not be suitable.

3.4 Conclusion

In summary, a novel way to check VSM cells adaptation for specific repeated stimuli via cell traction force recording of its oscillation between contraction and relaxation was achieved. Typically, VSM cell shortens itself during contraction followed by detachment of actin filaments from the focal adhesion sites and hence lead to decrease of cell traction force, whereas during relaxation the traction force will increase. Models have also been developed to clarify the traction force dynamics during contraction and relaxation, respectively.

Chapter 4 Oscillation driven reversible migration guided by cellular bridge: the tug-of-war in smooth muscle cell pair

In this chapter, the cellular bridge between a cell pair and its contribution in guiding oscillation induced cell migration has been studied. Why is oscillation essential in living systems? It is crucial for embryonic development, heart beating and circulation, and more specifically, it could contribute to maintenance of tissue integrity.

4.1 Introduction

Reversibility is crucial to ensure sustainability and extend lifetimes for living species, specifically such as cells survival and self-renewing.^[144] Live cells in vivo are mechanically coupled to both extracellular matrix (ECM) and adjacent cells. The mechanical coupling requires maintenance of connection between neighboring cells and is essential for vital activities such as vasoconstriction and heart beating. Based on the importance on maintenance of tissue integrity and function, the crosstalk between cell migration and intercellular adhesion has been investigated since the 1950s in term of ‘contact inhibition locomotion’ (CIL),^[145,146] where cells change direction on contact and separate.^[147] Moreover, its failure could contribute to the invasiveness for malignant cells.^[148,149]

What are already known for the causes of directional migration are gradients of chemical cues, in term of chemotaxis,^[150] and extracellular matrix stiffness or matrix adhesiveness, in term of haptotaxis,^[151] as well as necrotaxis,^[152] electrotaxis,^[153] and

durotaxis.^[154] Most of these causes are focused on how cell-ECM adhesion affects cell migration, whereas the influence from intercellular adhesion is not fully illustrated or elucidated, especially in isolated system, like cell pair.

Here, in our study, an inwards migration of two smooth muscle cells (SMCs) in a pair was achieved by oscillation between contraction and elongation. This is actually a reversible migration to the cells spontaneous outwards migration, which will occur due to CIL and finally separate them. The cell pair was formed with long extended protrusion composed of actomyosin stress fibers in between, where junctional complex connecting these two contractile machineries to each other. We show that the protrusion forms a suspended cellular bridge connecting two cells. Although variable types of intercellular bridges have been reported including cytonemes^[155] and tunneling nanotubes (TNTs)^[44], the function and mechanism presented here are not only limited to intercellular communication and material transport described previously. The bridge between SMC pair has been utilized to guide cell bidirectional migration by its gradual extending or contracting in a controllable way. Specifically, the oscillation induced inwards migration is actually an effective way to inhibit SMC spontaneous migration, which helps minify intimal thickening during in-stent restenosis and prevent vein graft failure.^[156] Additionally, cell-cell contact could be maintained afterwards, which could be contributed to cell survival by protection from apoptosis to cell-ECM contacts.^[157] Moreover, this inwards locomotion of two cells also fulfills similar function as collective cell migration, which regulates cells mobilization to fill the vacancy or repair the defect,^[158] though with different mechanism.

So far, a number of nanotechniques have been integrated to monitor or control mechanical responses of cells. Among them, optical and magnetic tweezers provide remote control of cellular behavior,^[159,160] piezoelectric nanoribbons can precisely detect cellular deformations^[161] and scanning probe-enabled nanocombinatorics achieves statistical study of biological processes with significant sample size.^[162] Here, a mechanosensing platform based on cell-ECM traction with accuracy of nano Newton has been developed by us. Mechanical interaction probing reveals that force transmission through cell-cell junction plays a key role in our cell pair motility since it directs the trajectory for each cell and drives this process by competing against cell traction force.

Our approach may indicate new cues for elucidating collective cell migration since contraction-elongation type of motion has been observed in migrating cell sheets constrained within narrow strips.^[105] Furthermore, combined with those techniques accurately and remotely manipulating filopodial protrusions by particle-applied forces, our approach provides a potential way to guide cell migration and arrange cellular organization.^[163,164]

4.2 Materials and Methods

4.2.1 Cell seeding, Drug stimulus and Microscopy

Human aortic smooth muscle cells (Lonza, Basel, Switzerland) were cultured in SmGM-2 smooth muscle growth medium supplemented with 10% fetal bovine serum (Sigma, St. Louis) at 37 °C in cell incubator with 5% CO₂. Cells were then seeded on polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, Midland, MI) substrate coated with collagen (type I, Rat tail; GIBCO, Gaithersburg, MD). For chemical stimuli loading,

calcium ionophore A23187 (Sigma) was solved in DMSO (Sigma) with concentration at 10^{-5} M. To trigger cell contraction, 0.5 μ L of 10^{-5} M A23187/DMSO solution was added into cell incubator containing 1mL cell culture medium. After repeated stimulus, the cells were detached from substrate by Trypsin (0.05%; GIBCO) to obtain the bead reference images. Cells fully attached on PDMS substrate were imaged on a Leica TCS SP8 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with stage top incubator (37 °C and continuous pumping in of 5 % CO₂; Tokai Hit, INUB-WELS-F1 series). The images were recorded by using a 40 $\times$ /1.40 NA oil-immersion objective lens with scanning resolution at 2048 $\times$ 2048 pixels. The fluorescent beads were excited by 488 nm Argon laser and their emission was collected in the range of 500 nm to 600 nm. Moreover, the differential interference contrast (DIC) images were also recorded for the location and morphology of cells.

4.2.2 Immunostaining and Transfection

Cells were grown on bare PDMS flat substrate. Before staining, the cells were fixed in 4% paraformaldehyde at 25 °C for 15 mins and permeabilized by 0.2% Triton X-100 in PBS for 10 mins. Cells were then incubated with Alexa Fluor 594 phalloidin to stain actin filaments. For myosin visualization, cells were incubated monoclonal anti-myosin (smooth) antibody at 4 °C overnight followed by incubation with Alexa Fluor 488 goat anti-mouse for 1 h at room temperature. To stain the microtubule, control cells and cells treated with Nocodazole (Sigma) for 5 mins before fixation were incubated with anti- α -Tubulin-Alexa 488 for 20 mins, and 4'-6-Diamidino-2-phenylindole (DAPI) was used to stain cells nuclei for 5 mins. Images were captured on Leica confocal microscope

(SP8). For visualizing the actin and microtubule cytoskeleton, green fluorescent protein (GFP) transfection was applied by incubating cells with CellLight Actin-GFP and CellLight Tubulin-GFP (Invitrogen) for 16 h, respectively.

4.3 Results and Discussion

4.3.1 Reverse Spontaneous ‘Outwards’ Migration to ‘Inwards’ Migration by Oscillation Between Contraction and Elongation

Smooth muscle cell (SMC) pairs with long protrusion connecting each other were dynamically investigated and a specific ‘inward’ migration was achieved by their oscillation between contraction and elongation. To form SMC pair, Human aortic SMCs were grown with a suitable density ($\sim 2k/cm^2$) on collagen coated polydimethylsiloxane (PDMS) substrate and allowed for their full attachment. The extended connection between cell pair could be generated either through cytokinesis^[165] or from cellular exploratory machinery like filopodia^[166] (Figure 4.1a). Due to CIL,^[147] this formation of cell pair could only exist for a short time followed by spontaneous ‘outwards’ migration which finally separate them. Cramer has concluded that directed cell migration requires symmetry breaking triggered by forming either cell front or cell rear first.^[167] Here, the ‘outwards’ cell migration was mainly promoted by cell front protrusion.^[168] Conversely, by reversing the sequence of symmetry breaking to forming cell rear first, an ‘inwards’ cell migration was produced (Figure 5.1a).

To induce ‘inwards’ migration, cells were treated with A_{23187} ($\sim 5nM$, overload will lead to severe cell blebbing and even complete detachment from ECM) which triggers an influx of calcium to increase intracellular calcium concentration and hence

result in cell contraction.^[169] The slight detachment at both ends of the cell pair initiate retraction to form cell rear, whereas the maintained junction in between guided both cells to move inwards. This calcium induced cell contraction would not last long and followed by a spontaneous relaxation which elongated both cells and stabilizes their adhesion on ECM. Repeating this oscillation between contraction and elongation further drives the cell migration based on an accumulating effect. Typically, after three or four cycles, the two cells collide with each other by an enlarged contact. Therefore, a similar ‘ratchet’ model that proposed for apical constriction by repeating actin-myosin network contraction and stabilization^[170,171] could be applied to clarify our ‘inwards’ migration, in which the cell contraction acts as propellant whereas relaxation acts as stabilizer.

Figure 4.1 Quantitative analysis of inwards migration of individual SMC pair. (a) An inwards migration completed by four cycles of cell contraction and elongation. The upper images show cells in elongated state, whereas the bottom images show cells in contracted state; stress strength indicated by color bar; scale bar indicates 10 μm . (b) The overall CTF dynamics over the whole inwards migration. CTF decreases during cell contraction and increases during its elongation. (c) CTF polarity (orientation of main axis) dynamics over the whole process. Radius length shows the magnitude of CTF; color bar indicates the time scale. (d) Schematic depicts the cell pair configuration, the cell tugging force (red arrow) and cell-ECM forces (blue arrow) exerted by each cell. (e) Calculated cell tugging force (red column) and measured cell-ECM forces (black column) over the whole

observation period. (f) The correlation between the migration velocity and the force ratio (cell tugging force divided by CTF) for the lower cell shown in (a).

4.3.2 Mechanosensing Platform for measuring Cell-Matrix Traction and Intercellular Tugging Force

In order to elucidate the force regulation for controlled cell migration, the cell traction forces (CTF) were directly measured based on our mechanosensing platform including uniform monolayer distributed fluorescence beads beneath cell-ECM interface and tracker algorithm (Figure 4.2). Briefly, the platform is composed of sequentially arranged glass coverslip, PDMS embedded with fluorescent markers and extracellular proteins like collagen (Figure 4.2a). Combined with confocal microscope imaging, the platform can achieve high-resolution traction force mapping downscale to nano-Newton. For a cell pair grown on this platform, the net force encompasses cell traction force and intercellular tugging force and remains zero. Hence, the tugging force is equal in magnitude but opposite in direction to the net traction force.^[14] Multi-protein complexes (yellow) and integrin (green) facilitate the force transduction from acto-myosin fibers (red) and lead to elastic deformation of PDMS (Figure 4.2b). The deformation are monitored by uniformly distributed fluorescent beads and further turned into traction force mapping by tracking displacement of every bead (Figure 4.2c).

Figure 4.2 Mechanosensing Platform based on Cell-Matrix Traction with accuracy of nano Newton. (a) Scheme of the platform to measure cell traction forces. A thin layer of PDMS is placed on top of glass coverslip, with collagen coated on PDMS surface. Fluorescent nanobeads are embedded in the top of PDMS layer with a uniform distribution. For a cell pair grown on this platform, the net force encompasses cell traction force and intercellular tugging force. (b) Scheme of force transduction. The contractile force generated by acto-myosin fibers (red) transmits to ECM (white) through multi-protein complexes (yellow) and integrin (green). (c) Principle of mechanosensing. Cell traction forces are measured by detecting precise displacement of fluorescence beads induced by elastic deformation of PDMS.

4.3.3 Bidirectional Migration of Two Cells Is Mediated by Tug-of-War Between Cell-ECM Force and Cell Tugging Force

CTF for the overall cell pair exhibited in an oscillating curve over the whole observation period for ‘inwards’ cell migration (Figure 4.1b). A close look into the oscillating curve reveals that the period for every single cycle composed of contraction and elongation is around 20 mins, and the CTF decreased significantly during contraction whereas it increased almost to initial level during elongation. CTF changes are ascribed to cytoskeletal remodeling.^[172] During contraction, the enhanced actomyosin-based contractility shortens cellular length and causes actin stress fibers gradually reduce attachment to focal adhesion sites,^[143] exhibiting the decreased CTF. Conversely, in the following spontaneous relaxation, both cells will become elongated and increase the cytoskeletal tension, measured as the increased CTF. Similar trend of CTF change was also found for oscillation of individual SMC. CTF polarity (defined by the main axis orientation of CTF vector sum) kept parallel to the extended cellular connection only with small fluctuation ($\pm 16^\circ$), illustrating that the ‘inwards’ migration of the cell pair was highly directed towards their junction (Figure 4.1c). Since cells in pair are mechanically coupled to both ECM and the other cell, the coordination between cell-ECM adhesion (determined as CTF) and cell-cell adhesion (determined as cell tugging force) must mediate the migration orientation (Figure 4.1d). To address this correlation, we further calculated cell tugging forces during the whole progress according to equilibrium formula raised by Margaret L. Gardel.^[20] Since cell tugging force is perpendicular to cell-cell contact and only counterbalance the cell-ECM traction in the antiparallel direction (Figure 4.1d), we divided the CTF vectors exerted on each individual cell into $\vec{T}_{\parallel}$

(antiparallel to cell tugging force) and $\vec{T}_{\perp}$ (perpendicular to cell tugging force). Accordingly, the magnitude of cell tugging force is a fraction of cell-ECM traction which equals $\sum \vec{T}_{\parallel,i}$ (Fig. 1E), whereas the $\sum \vec{T}_{\perp,i}$ equals zero due to its internal balance. Consistent changing of CTF over the whole ‘inwards’ migration resulted in the fluctuation for the ratio of $\vec{T}_{\parallel}$ and $\vec{T}_{\perp}$, and thus the ratio between cell tugging force and CTF for each cell (Figure 4.1e). In addition to the cell migration orientation inconsistent with tugging force, the migration velocity over this period were carefully recorded by analysis based on manual tracking technique. Plotting both curves of force ratio and migration velocity exhibits their good correlation (Figure 4.1f), suggesting that cell ‘inwards’ migration is promoted by cell tugging force but impeded by cell-ECM traction. Force analysis also explains cells spontaneous ‘outwards’ migration as a result of cell-ECM traction dominating over cell tugging force (CTF maintains at initial level). Together, we established a mechanical way to regulate cells bidirectional migration by either maintaining cell-ECM tractions or reducing them through contraction.

4.3.4 Suspension of Connection in Cell Pair Reveals the Driving Force and Guiding Route of Migration

Since the bidirectional migration of SMC pair is actually a progress coming with either stretching or shortening of the connected protrusions at middle part, suggesting the significance from its structure and function. Hence, we sought to decipher the intercellular connection structure and distribution of cytoskeleton and motor proteins. Scanning electron microscope (SEM) image of fixed SMC pair showed the overall topographic structure, where the middle connection was suspended above the substrate

(Figure 4.3a). Immunostaining of smooth muscle myosin II revealed its concentrated distribution in the extended connection for SMC pair (Figure 4.3b). Similar enriching of myosin II was also found in cleavage furrow,^[173] which is one precursor to initiate the cell pair forming. Combined with the actin filaments, these acto-myosin bundles demonstrate high capability for generating contractile forces along the connection (Figure 4.3b), originating cell tugging force, and thus establish the intercellular communication by transmitting forces through intercellular adhesion proteins to cytoskeleton (with linkage of cadherin-catenin complex).^[174,175] A close look at immunostaining of cytoskeleton proteins (actin stained in red, whereas microtubule stained in green) through confocal microscopy with Z-stack scanning images, followed by 3D-reconstruction, revealed the extended connection between the SMC pair is suspended above the substrate (clear at sectional view, Figure 4.3c). In fact, the extended connection suspension reveals the tensile stress exerted to it from cell-ECM tractions, similar to the suspended rope in tug-of-war. On the other hand, the cell tugging forces, which counterbalance cell-ECM traction, provide the driving force for cells ‘inwards’ migration once the cell-ECM tractions are reduced.

Figure 4.3 SMC pair with suspended connection and the cytoskeleton distribution. (a) SEM image of fixed SMC pair, again indicates the suspended connection. Scale bar indicates 10 μm . (b) Immunostaining of nucleus (blue), smooth muscle myosin II (green) and actin (red) for individual SMC pair, final picture indicates the overlapped image. Scale bar: 20 μm (c) 3D view of individual SMC pair stained for microtubule (green) and attached on PDMS substrate with monolayer distributed red fluorescence beads beneath cell-ECM interface. Sectional view reveals the extended protrusion based connection is suspended above the substrate. (d) Sectional view of the 3D dynamics for SMC pair migrating inwards and expressing GFP-Actin, on PDMS substrate with monolayer distributed red fluorescence beads beneath cell-ECM interface. Final image shows the cell nuclei stained by Hoechst 33342.

The suspended connection in the SMC pair breaks the symmetry of oscillation between contraction and elongation for each individual cell since no obvious migration could be found during isolated single cell oscillation as it basically retracted and elongated repeatedly by centering on the nucleus. In order to investigate the dynamics of connection suspension during the two cells migrating inwards, we applied transfection technique to obtain GFP-tagged actin for both cells. The continuous fluorescence life cell images exhibited the suspension was maintained until the two cells collided with each other, whereas the intercellular distance was shortened incrementally with every cycle between contraction and elongation, indicating the migration guidance along the route of suspended connection (Figure 4.3d).

4.3.5 Microtubule Depolymerization Enhances Cell-ECM Traction

Because the reversing from cells spontaneous ‘outwards’ migration to ‘inwards’ migration are indeed initiated by reduction of cell-ECM force, we then sought to determine whether enhancement of cell-ECM force before contraction could inhibit the reversing. Actually, traction forces are produced from actin cytoskeleton, in conjunction with myosin II,^[176] and interestingly, could be regulated by the microtubule network.^[177] In addition, both contraction and elongation are consequences from acto-myosin interaction, but the function of microtubule involved in remains unclear, especially for our cell pairs. Hence, we targeted at microtubule to regulate cell-ECM traction and thus its mediation in cells migration. By doing so, we treated cells with nocodazole to induce microtubule depolymerization. Immunostaining of microtubule (green) and actin (red) showed the microtubule was depolymerized completely in the middle connection part and

severely in focal adhesion sites for SMC pair after approximately 5 mins treatment of nocodazole, whereas the actin bundles remained intact. Control cell pair exhibited clear microtubule and actin bundles both in the middle and focal adhesion sites (Figure 4.4a). Quantitative statistic analysis of CTF indicated that the cell pairs upon nocodazole (100nM) treatment for 5mins exhibited ~39% larger cell-ECM tractions (Figure 4.4b and 4.4c). We then measured the CTF dynamics. The overall CTF for three SMC pair all exhibited same tendency: stabilized at a plateau initially, gradually increased after nocodazole (~100nM) treatment and finally dramatically decreased after inducing contraction by A23187 (~5nM) (Figure 4.4d). The signal mechanisms involved in traction increase by microtubule depolymerization has been report by Wang.^[178]

Figure 4.4 CTF increase induced by nocodazole treatment for SMC pair. (a) Top: Immunostaining of nucleus (blue), microtubule (green) and actin (red) for untreated SMC

pair; below: Immunostaining of nucleus (blue), microtubule (green) and actin (red) for SMC pair treated by nocodazole (100nM) for 5 mins. Scale bar: 20 μ m (b) CTF of individual cell pair before and after nocodazole (100nM) treatment for 5 mins. Data indicates the mean value and standard deviation for n=7 cell pairs. (c) CTF of individual cell pair increase around 39% after nocodazole (100nM) treatment for 5 mins. (d) CTF dynamics for three individual cell pairs expressing GFP-microtubule treated with nocodazole (100nM, red arrow), followed by A₂₃₁₈₇ (5nM, blue arrow) treatment.

4.3.6 Role of Compression Forces from Microtubule in Maintaining the Cell Junction During Contraction

Strikingly, the cell junction was broken during contraction after microtubule depolymerization, indicating the key role of microtubule in maintaining intercellular adhesion and facilitating ‘inwards’ migration. In fact, our study provides another evidence to support tensegrity model in Mechanobiology.^[179] This model illustrates the forces balance for cell is based on continuous tension and local compression, and actomyosin apparatus generates tension, microtubule provides compression, respectively. Hence, by inducing nocodazole, the weakened or even lost compression force from microtubule enhanced anchorage between cell and ECM at focal adhesion sites and thus slightly increased CTF, which subsequently pulled two cells apart by imposing the enlarged tension upon cell-cell junction.

Figure 4.5 Schematic of proposed mechanism for regulating maintenance of adherent junction during cell contraction. (a) Relatively low cell tractions exerted on the ECM by resistance from microtubule provided compression force; substrate is slightly deformed. (b) Microtubule depolymerization results in enhanced cell-ECM traction and further junction break.

Despite of forces regulation, the cadherin based cell-cell junction is tightly associated with the microtubule network which transports and facilitates recruitment of cadherin, especially for N-cadherin,^[180] the classical intercellular adhesion protein in SMCs.^[181] Hence, disassembly of microtubule network could result in junction turnover. Together, we proposed a schematic diagram to illustrate the function of compression

forces, which prevent high traction exerted on ECM by cells and thus make cell junction survival to processing ‘inwards’ migration (Figure 4.5a). However, eliminating compression forces will increase substrate traction and weaken cell junction, which eventually lead to cells separation during contraction (Figure 4.5b).

4.4 Conclusion

Our experimental approach, which applies oscillation between contraction and relaxation, complemented by force analysis, allows exploring the role of cell tugging force on generating new cues for directional cell migration. We demonstrate this oscillation induced inwards migration in SMC pair overcome CIL and thus promote the maintenance of tissue integration. The switch from the CIL led spontaneous outwards migration into our inwards migration is actually initiated by contraction, which decreases the cell-ECM tractions and thus retracts both cells, and further stabilized by elongation. Hence, based on the accumulating effect of repeated contractions and following elongations, the reversibility occurs and finally brings two distant cells into colliding, with a significantly enlarged contact. We also observed the cell connection based on pseudopodia protrusion is suspended above the substrate during the whole progress, indicating the tensile force along it promotes and guides this inwards cell migration. Depolymerization of microtubule further reveals the importance of compression force in maintaining cell junction during contraction, as it counteracts with traction force and prevent increased CTF pulling two cells apart. Altogether, our results show a novel method to obtain directed cell migration based on cell communications. This mode of

migration again demonstrates the unique reversibility for living species and provides a practical way to guide cell migration targeted at other cells nearby.

Chapter 5 Matrix Geometry Determines Optimal Collective Cell Migration Strategy

In this chapter, the cellular bridges at multicellular scale are investigated via forming epithelial bridges over large non-adhesive regions. By regulating the geometrical properties of ECM, the epithelial bridges demonstrate variable dynamics for their formation and propagation, which allow us to optimize the cellular migratory strategies for wound healing.

5.1 Introduction

Re-epithelialization, the capability to maintain and restore integrity, distinguishes epithelial cells and enables them to act as a barrier for protection.^[49,52] During wound healing, keratinocytes migrate collectively to establish their cohesion despite the heterogeneities in extracellular matrix (ECM).^[182] The movements of cells that maintain cell-cell junctions yet protrude along or within tissues are ubiquitous for cell positioning in development and regeneration, during which cellular bridges are formed at large scale.^[183,184] However, how to enhance the efficiency of the re-epithelialization and realize quicker healing remains a challenge. Decoding the migratory strategies for keratinocytes, especially attempting to extrapolate findings from the complex matrix environments surrounding keratinocytes, would provide a significant clue for wound healing. In vivo, the both edges of a wound cannot be always geometrically parallel to each other. Along the direction of cellular migration, the both edges would be either convergent or divergent, despite of the parallel cases. Revealing the interplay between the

geometrical aspects of ECM with the formation of epithelial bridges may optimize the migratory strategies for wound healing.

5.2 Materials and Methods

HaCaT cells (spontaneously immortalized primary human keratinocytes) were chosen as the target, which was purchased from Cell Lines Service. They were cultured in DMEM supplemented with 10% fetal bovine serum as well as antibiotics. PDMS (Sylgard 184, Dow Corning) was mixed with a ratio of 1:10, degassed and cured as previous chapters described. A mixture of fibronectin and a relatively small amount of FITC-conjugated fibronectin was chosen to ink the flat stamps. For migration assay, microcontact printing was utilized on the PDMS substrate with designed geometry. Briefly, photolithography was used to fabricate the pattern with raised feature on the PDMS substrate. After performing microcontact printing on the fabricated substrate, the HaCaT cells were seeded into the reservoir to demonstrate a model wound assay. Before initiating the collective cell migration, a portion of the substrate was blocked with a PDMS slab. After reaching sufficient confluence, the PDMS block was removed, allowing the HaCaT cells to collectively migrate into the free space coated with fibronectin.

5.3 Results and Discussion

5.3.1 Bioengineered Substrate for Collective Cell Migration

In order to study how keratinocytes migrate over heterogeneous ECM composed of non-adhesive regions and adherent paths, we developed an *in vitro* platform adapting microcontact printing (μ CP) and soft lithography. Figure 5.1 shows the schematic of the experimental set-up. The substrate was composed of PDMS, with a specifically designed geometry. Basically, the height of the triangle regions is lower than other regions, where Ethylenediaminetetraacetic acid (EDTA) was coated via hydrophobic interaction. A second coating of fibronectin was applied by μ CP so that cells can only attach to the top surface of the substrate, leaving the triangle regions non-adhesive for cells. However, after the cell confluence was high enough to form monolayer, the leading front of the monolayer would overcome the physical constraints of ECM and migrate over the non-adhesive region, in which the suspended cellular bridges at multicellular scale were formed. The designed triangles actually induce anisotropy to our systems.

Thus, when cells collectively migrate from the blunt end of triangle to the sharp end (Figure 5.1b, left pattern), the underlying paths are convergent for cells, whereas for cells migrating in the opposite direction, the paths turn to be divergent. This type of geometrical anisotropy does exist in real system, as the ECM distribution is mostly random, rather than the highly-ordered arrangement like parallel stripes.

Figure 5.1 Surface modification and raised feature of the experimental set-up. (a) EDTA as the link between coated fibronectin and PDMS substrate. (b) Cells collectively migrate from the blunt end of triangle to the sharp end and vice versa.

5.3.2 The Zipper-like Mechanism for Cellular Bridge Formation

During the course of collective migration of HaCaT cells,^[52] we observed the capability of cellular bridge formation is strongly depended on the geometry of ECM. As illustrate in Figure 5.2, the cells were migrating downwards, with the triangle areas as the non-adhesive regions for both patterns. We found that the cellular bridges were easily formed for the right pattern, as the two adherent paths are convergent to the leading front of the migrating cells, whereas for the left pattern, cellular bridges were partially formed when the migrating cells met divergent paths. After around 24 h of migration, the non-

adhesive region in the right pattern was fully occupied by the cellular bridges, with the left one remaining largely unoccupied. Although previous study on epithelial bridges demonstrated that the pulling forces exerted by the cells migrating on fibronectin stripes drove the bridges formation, here we revealed that orientation (determined by ECM geometry) contributed to the tendency and thus the efficiency of re-epithelialization. As the both patterns are geometrically backwards to each other, the components of pulling forces effectively working on bridges formation are theoretically equal for these two patterns. But the yields of cellular bridges are distinguished due to the orientation. Therefore, this orientation-dependent migration assay owns a zipper-like mechanism, with wound sealed in one direction and opened in the other.

Figure 5.2 HaCaT cells were migrating downwards on the substrate with designed geometry. The capability of cellular bridge formation is strongly depended on the geometry of ECM. Scale bar: 120 μm

5.3.3 The Efficiency of Re-epithelialization Relies on the ECM Geometry

Since the orientation is now known to determine whether re-epithelialization can be completed or not, we then wonder how large difference exists between the convergent paths and divergent paths. We thus investigated the effect of included angle between the two adherent paths on the healing capability. The healing capability was quantified through calculating the occupancy of the non-adhesive triangle regions by cellular bridges during the initial collective migration. Noteworthy, regarding the continuous cell proliferation, the backwards migration would occur and fill up those uncovered regions during initial migration, especially for cells moving in divergent paths. Moreover, sometimes the subsequent ‘hole’ could be observed due to cytokinesis, which left the initially occupied regions open. Both of these circumstances are excluded from the calculation as they are not driven by collective cell migration.

For comparison, we chose five different angles: 20°, 30°, 60°, 90° and 120°. By statistic study, we extrapolated the data to Figure 5.3. As distinguished by colors, we found for convergent paths, occupancy by cellular bridge during initial collective cell migration over non-adhesive region was more close to 1 (100%) for almost all the five angles, whereas for divergent paths, the value of occupancy was significantly decreased. Especially, the occupancy difference reached the maximum value (~30%) when the included angle was 60°. This biphasic relationship between occupancy difference and included angle revealed that the aspect ratio of the non-adhesive regions play a role in cellular bridges based re-epithelialization. Since non-adhesive region present in equilateral triangle for included angle of 60°, the aspect ratio of equilateral triangle reach a minimum value as compared to triangle of other angles. Actually, for included angles

larger than 60° , the distance between two neighboring adhesive islands would be further reduced, which facilitates cellular bridge formation along the cell moving direction. On the other hand, for included angles smaller than 60° , the two adhesive paths would be geometrically deformed close to the parallel stripes and thus reduce the anisotropy effect of ECM.

Figure 5.3 The percentage of occupancy (y-axis) by cellular bridge during initial collective cell migration over non-adhesive region for both divergent and convergent patterns. The values on the sector region stand for the included angle for divergent and convergent patterns, respectively.

5.3.4 Cells are Polarized along the Orientations of Collective Migration

Cells comprising a tissue migrate collectively. Unanticipated fluctuations of mechanical stress are raised within the cell sheet. Based on the stress landscape, local cellular migrations should follow orientations of maximal principal stress, especially for endothelial and epithelial monolayers.^[22] Here, in our platform, the HaCaT cells were

constrained in every adhesive patterns and followed the downwards motion as illustrated in Figure 5.4. We observed that the polarity of cells before collective migration was randomly distributed. However, after around 20 hours from we removed the physical block to initiate the migration, the polarity of most cells turned along the orientations of collective migration, as shown in the processed image (right, bottom of the Figure) from edge detection and segmentations. Actually, stress is generated by cytoskeleton and cytoskeleton arrangement and distribution are dependent on the polarity of every cell. Therefore, our results proved that there were corresponded alignment between the orientation of collective cell migration and local maximal principal stress.

Figure 5.4 Before initiating migration, the polarity of cells before collective migration was randomly distributed. In contrast, the polarity of most cells turned along the orientations of collective migration after 20h.

5.3.5 Modelling

Regarding our experimental study, accumulating evidence favours a mechanism based on the tension generated from the actin bundles at the leading edge of bridges that promote the healing over non-adhesive regions. The situation is reminiscent of the physical model where Young-Laplace force induces the pressure difference to overcome resistance for deformable object like liquid or elastic membrane.^[185,186] Considering the geometry of HaCaT cell monolayer, we gave the Young-Laplace formula as $\Delta P = \gamma/r$, where ΔP is the difference in pressure from the front of the leading edge and the tail, γ is the tension, r is the radius of the leading edge for the cellular bridge. By the calculation of differentiation shown in Figure 5.5, the r becomes larger for divergent paths while the cells are moving forward. The subsequent reducing ΔP gradually loses the capability to overcome the resistance to continue bridge formation and eventually leaves an uncompleted cellular bridge over the non-adhesive regions. On the contrary, ΔP is continuously enhanced while cells are moving on convergent paths, demonstrating strong healing capability.

Figure 5.5 Mathematical modeling elaborating the tendency of pressure difference.

5.3.6 The migration velocity is strongly depended on the geometry of ECM

As the healing velocity is most concerned clinically, we further recorded the velocity of the collective migration of HaCaT cells on our migration assay.^[187] As clearly demonstrated in Figure 5.6, for both included angle of 60 °and 30 °(where the occupancy difference between convergent paths and divergent paths is relatively significant), the cells migrated faster on right pattern, where the adherent paths are convergent. Our results revealed that the orientation of collective cell migration on micropatterns played a role in optimizing the migration strategy. Tracking the trajectory of cell motions suggested that backwards motions exist for cells migrating through divergent paths due to the partially formed cellular bridges over non-adhesive regions, which in turn slow down the migration velocity and also reduce the healing efficiency.

Figure 5.6 Comparison of the migration velocity for different patterns. The velocity of the collective migration of HaCaT cells is faster on right pattern, where the adherent paths are convergent.

5.4 Conclusion

In the context of regeneration and tissue engineering, skin repair relied on bioengineered skin models is still lack of full natural properties of uninjured skin. Restoring the skin barrier via cellular bridges formation delights efficient modes of collective cell migration to maintain tissue integrity. Therefore, how to enhance the efficiency of re-epithelialization and realize more efficient migratory strategies for keratinocytes, especially attempting to extrapolate findings from the complex matrix environments surrounding keratinocytes, would provide a significant clue for wound healing, especially for older injured people. Our investigations on the geometry of ECM

and orientation of cell migration provide in-depth understanding of the biophysical mechanism for cellular bridge formation and the corresponding healing efficiency. The whole process is tightly coupled to ECM variables, force development and transmission, and cellular migration trajectory. Decoding their interactions could be highly potential for designing materials to manipulate epithelial cells in various injuries, diseases and also ageing, thereby proving helpful in customizing implantable scaffolds for skin regeneration.

Chapter 6 Conclusions and Recommendations

In this thesis, we discussed the recent developments in the emerging field of mechanobiology and biomaterials. By integrating the micro-fabrication techniques to engineer biomaterials and advanced algorithms to calculate stress field, various cellular behaviors could be modulated and characterized. Due to the essential role of cellular bridge in intercellular communication, which is the basis of all biology in living systems, we systematically investigated the cellular bridges at varied scales and their functions in regulate cell migration and morphology, as well as the potential roles in health and disease.

By forming subcellular bridges on ECM with discrete adhesions and variable rigidity, we first studied how the ECM properties affect cytoskeleton and intercellular adhesion. Enlarged non-adhesive gaps and decreased rigidity would reduce the cell-matrix anchorage, which in turn enhance the intercellular adhesion. Collectively, weakened cell-matrix anchorage would result in unclosed rings even with long-term cell proliferation. The formation of rings can increase the leakiness and thus the permeability of the cell sheet, allowing better delivery of nutrients or therapeutics, while enhanced cell-matrix anchorage would promote the formation of confluent monolayer, serving as a protective barrier which can resist pressure *in vivo*. Consequently, through influencing subcellular bridges, the multicellular morphology and its related protective function determined by the efficiency of cells in maintaining its continuity and integrity can be switched on or off by modifying their environment, thereby suggesting ways in developing implantable materials to specify the assembly of biological structures.

We further established cellular bridge between a smooth muscle cell pair and utilize the cellular bridge to guide cell bidirectional migration, via extending or contracting the cellular bridge in a controllable way. We demonstrated the oscillation between contraction and relaxation of both cells induced the inwards migration, overcoming CIL and thus promote the maintenance of tissue integration. The switch from the CIL led spontaneous outwards migration into our inwards migration is actually initiated by contraction, which decreases the cell-ECM tractions and thus retracts both cells, and further stabilized by elongation. We also observed the cellular bridge based on pseudopodia protrusion is suspended above the substrate during the whole progress, indicating the tensile force along it promotes and guides this inwards cell migration. The results show a novel method to obtain directed cell migration based on cellular bridge. This mode of migration demonstrates the unique reversibility for living species and provides a practical way to guide cell migration targeted at other cells nearby.

Last but not least, by increasing the cellular bridge to multicellular scale, we investigated the formation and regulation of multicellular bridges over large non-adhesive regions. The studies on the geometry of ECM and orientation of cell migration provide in-depth understanding of the biophysical mechanism for cellular bridge formation and the corresponding healing efficiency. The whole process is tightly coupled to ECM variables, force development and transmission, and cellular migration trajectory. Therefore, the interplay between the geometrical aspects of ECM and the epithelial bridges has been revealed, allowing us to optimize the migratory strategies for wound healing.

Recommendation for Future Work

We have demonstrated that cellular bridges at various scales could contribute to the regulation of multicellular morphology and cell migration. The epithelial cells formed in confluent monolayer over discrete focal adhesion sites, the suspended connection between the muscle cell pair and epithelial bridge on heterogeneous ECM are all actually subjected to high mechanical tension. Our findings provide unanticipated modes to engineer artificial, bio-compatible scaffolds, which are then potentially applied for developmental or regenerative biology. In our study, ECM geometry and rigidity are critical properties for regulating cellular behaviors. Therefore, scaffolds designed with advanced 3D printing techniques would further improve the precision of geometry. Moreover, if we can dynamically modulate the geometry and rigidity of artificial scaffolds, these implanted materials would significantly cooperate with our own living systems.

Developing stimulus-responsive materials which could monitor real-time events locally occurred *in vivo* and respond accordingly would open the field for synergistic diagnostics and therapy. The critical capability of structural and bioactive biomaterials in fields ranging from cellular physics to regenerative biology is successful probing the physicochemical and biological microenvironments. In order to detect and record the information throughout the 3D and porous interior, electronics in nano-scales may be a good candidate to be incorporated. Integration of electronics with biocompatible porous 3D scaffolds for localized real-time monitoring of cellular activities and physicochemical change could delight the novel lab-on-a-chip pharmacological platforms, as well for synthetic biology.

List of Publications

1. **B. Hu**, Y. Wu, P. Cai, W. Leow, X. Chen,* “Oscillation driven reversible migration guided by cellular bridge: the tug-of-war in smooth muscle cell pair”, in preparation.
2. **B. Hu**, W. Shi, Y. Wu, W. Leow, P. Cai, S. Li,* X. Chen,* “Orthogonally Engineering Matrix Topography and Rigidity to Regulate Multicellular Morphology”, *Advanced Materials* **2014**, 26, doi:10.1002/adma.201402489.
3. S. Yin, Y. Wu, **B. Hu**, Y. Wang, P. Cai, C. Tan, D. Qi, L. Zheng, W. Leow, N. Tan, S. Wang, X. Chen*, “Three-dimensional Graphene Composite Macroscopic Structures for Capture of Cancer Cells”, *Advanced Materials Interface* **2014**, 1, doi:10.1002/admi.201300043.
4. F. Meng, Y.-M. Hervault, Q. Shao, **B. Hu**, L. Norel, S. Rigault,* X. Chen,* “Orthogonally modulated molecular transport junctions for resettable electronic logic gates”, *Nature Communications* **2014**, 5, 3023, doi: 10.1038/ncomms4023.
(It was highlighted by Nanowerk Spotlight, Materials Views, and ACS Noteworthy Chemistry.)

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