Cell-materials interaction and its implications on stem cell fate

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TECHNOLOGICAL
UNIVERSITY**

**CELL-MATERIALS INTERACTION AND ITS
IMPLICATIONS ON STEM CELL FATE**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2012

**CELL-MATERIALS INTERACTION STUDY DURING STEM
CELL DIFFERENTIATION**

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2012

CELL-MATERIALS INTERACTION AND ITS IMPLICATIONS ON STEM CELL FATE

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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List of Abbreviations

Full Name	Abbreviation
Human mesenchymal stem cells	hMSCs
Embryonic stem cell	ESC
Focal adhesion	FA
polydimethylsiloxane	PDMS
Polyacrylamide	PA
Microcontact printing	μ CP
Glutaraldehyde	GA
Myosin light chain kinase	MLCK
Myosin heavy chain	MHC
Collagen type I	COLI
Fibronectin	FN
Dulbecco's Modified Eagle's Medium	DMEM
Extracellular matrix	ECM
Fetal bovine serum	FBS
Phosphate Buffered Saline	PBS

Abstract

Human mesenchymal stem cells (hMSCs) continue to attract prominence in tissue engineering due to their immunosuppressive property, self renew ability, and multi-lineage differentiation potential. While emphasis has always been placed on inducing differentiation of hMSCs into the targeted cells of interest by biological methods in the past, biophysical methods such as mechanical stimulation and cellular morphology are now emerging at a rapid pace. In the physical methods, the interaction between the cell and material is of utmost importance but systematic study of this interaction is still limited to date. In this investigation, cell-materials interaction represented by the development of focal adhesion (FA) was studied systematically and the relationship between FA and stem cell differentiation (especially myogenic differentiation) was investigated. Results indicated that the FA development of hMSCs can be modulated by micropatterning but there was a synergistic effect between FA regulation and matrix stiffness. Elongated FA could be modulated on the substrates with *intermediate stiffness* ((polydimethylsiloxane (PDMS) with a stiffness of 12.6 kPa and polyacrylamide (PA) gel with a stiffness of 10.2 kPa)) and with collagen type I (COLI) as the linking protein. hMSCs with elongated FA showed specific myogenic differentiation at both transcription and translation levels compared with cells with dense FA and those in control group. Mechanistic study showed that elongated FA recruited integrin β_3 clusters, activated RhoA signaling pathway, aligned stress fibers, and increased cellular tension via activation of RhoA signaling pathway and up-regulation of myosin light chain kinase (MLCK). Moreover, the interplay of cell shape and FA on hMSCs differentiation was

investigated and the key role of elongated FA in driving hMSCs myogenic differentiation was confirmed. Similar shaped hMSCs with different FA morphology and distribution were induced using micropatterning technique and it was found that the elongated FA was more supportive of myogenic differentiation.

Collectively, this study demonstrates a novel chemical/biological free and feasible method of inducing myogenic differentiation of hMSCs and improves our understanding of the micropatterning platform in affecting the differentiation.

Chapter 1 Introduction

1.1 Background

Millions of people are suffering from many soft organ diseases like heart attack and skin cancer every year resulting in a rapid development of soft tissue engineering ^[1]. In USA alone, more than 1.5 million of people are suffering from myocardial infarction every year ^[2]. Stem cell based heart therapy provides a new way to overcome the lack of organ donation in treating cardiac disease and now the research in directing stem cell differentiation is highly in demand in tissue engineering ^[3]. Human mesenchymal stem cells (hMSCs), isolated from adult bone marrow and other mesenchymal tissues, have attracted enormous attentions in the past decades due to their multi-pluripotent property and potentials in regenerative medicine ^[4]. It has been shown that stem cells are very easy to be influenced by their micro-environment. Many materials and techniques have been developed as biophysical factors to induce stem cell differentiation into the desired lineage as shown in Fig. 1.1 (Modified from ^[5]). Compared with biological and chemical induction, biophysical method can avoid the side effects brought by the biological or chemical factors, such as uncontrolled cell growth, tumor and cell death ^[6]. Recent reports showed that matrix stiffness played an important part in stem cells differentiation because matrix elasticity was an important cue, which cells could sense from the environment ^[7]. Besides that, surface topography, mechanical stimulation, micro- and nano-patterning have been employed to investigate stem cell behaviors and differentiation ^[8].

Figure 1.1 hMSC based tissue engineering.

While emphasis has been placed on how to induce stem cells differentiation, the mechanism of stem cell-materials interaction is still not clear to date but is definitely and rapidly gaining interest. We believe that such interaction study will help us to understand how materials affect hMSCs behavior and will shed light on biomaterials design especially for tissue engineering.

1.1.1 Stem Cells and Tissue Engineering

Stem cells are cells which have multi-pluripotent ability and can differentiate into various specialized cell types and can self-renew to amplify its population ^[9]. The concept of “plasticity” which implies the capacity for extended self-renewal and multi-lineage

differentiation was firstly observed in blood and has been corroborated by the first bone marrow transplantation in 1956 ^[10]. Tissue engineering is a new technique to combine engineering techniques, cells, and materials properties, and proper biochemical and physical-chemical factors to enhance or take the place of biological functions to treat human disease ^[11]. As mentioned above, cardiovascular diseases are one of the major cause of death in the world, and therapies using stem cells inspired a potential solution to treat heart failure, myocardial infarction (MI), and congenital heart diseases ^[12]. Towards this goal, cardiac tissue engineering cooperated with stem cells is now under investigation as a way to support stem cell-based therapies and improve their efficiency. Pluripotent stem cells such as embryonic stem cell (ESC), adult stem cells, and induced pluripotent stem cell (iPS) own the potential to be differentiated into cardiomyocytes ^[13]. But the use of embryonic stem cell and induced pluripotent cell-based therapies is still under pioneer study. The specificity and efficiency for them to differentiate into cardiomyocytes are still not high. To date, there is only one clinical trial about embryonic stem cell-based therapy for spinal cord injury that has been approved ^[14]. Therefore, there is still a long way to go and biomaterial is involved in tissue engineering to facilitate the progress of cardiac-tissue therapy. Biomimetic synthetic polymers and other biomaterials have been involved to induce specific cellular functions and to influence cell-cell interactions ^[15]. Both the physical and chemical properties of biomaterials used in tissue engineering will affect cellular behaviors such as adherent, migration, proliferation, differentiation, and apoptosis ^[16]. As a consequence, the cell-materials interaction study is needed to understand how the properties of materials will affect cellular behaviors and then make use of these properties to improve stem cell-based tissue engineering.

1.1.2 Cell-materials Interactions

Nowadays, tissue engineering and regenerative medicine are gaining wide attention, especially in the use of stem cells which provide a novel therapeutic option for patients. However, most of these studies were based on biological or chemical induction factors and therefore side effects are inevitable. Physical induction factors are more attractive in that way and numerous biomaterials have been developed to serve as scaffolds or substrates to induce stem cell differentiation or for implantation purpose ^[3, 17]. Since cells can interact with the materials and then trigger cascades responses as shown in Fig. 1.2, it is crucial to understand the mechanism of cell-matrix interactions. From Fig. 1.2 we can see that the features of materials could affect cell behaviors in various ways, but no matter what the materials properties are, the material will interact with cell through focal adhesion (FA). Reports have shown that there is an interplay between matrix compliance and FA development ^[18]. In our study, emphasis has been put on FA due to its central roles in cell-material interaction, while substrates compliance effects will also be taken into consideration.

Figure 1.2 Schematic depicting the interaction between cell and materials.

1.2 Hypothesis, Objectives and Scopes

1.2.1 Hypothesis

Inspired by the work in our group and a recent study by Goffin, J. et al., which showed that large and long FA would recruit α -smooth muscle actin to stress fibers of myofibroblast^[19], we hypothesize that FA plays a key role in cell-materials interactions and in influencing stem cell differentiation. By controlling FA through micropatterning combined with matrix elasticity, we are able to alter the cytoskeleton arrangement, induce stress fiber recruitment and active signaling pathway, which will cause cascade regulations in stem cells and affect their differentiation.

1.2.2 Objectives

The general objective of this study is to control FA morphology and FA density of hMSC by micropatterning technique, in combination with matrix elasticity to modulate stem cell differentiation. The mechanism behind this differentiation was also explored. The specific objectives are:

- Study hMSCs behaviors (FA development and differentiation) on various compliant substrates.
- Design and fabricate micropatterns to control FA, and explore their effects on hMSCs differentiation.
- Explore the mechanisms which cause the changes above and establish a new platform to induce hMSC myogenic differentiation.

The establishment of a novel platform to induce hMSCs differentiation and the understanding of the mechanism will provide a new method to drive myogenic

differentiation of stem cells and contribute better understanding of cell-materials interactions.

1.2.3 Novelty and Significance of Study

FA and observations of FA development on cellular behavior such as adherent and migration, has been widely studied in the past but so far no study has been reported on the use of FA to induce hMSCs differentiation. We hypothesize that the hMSCs differentiation is induced by biophysical method and is triggered by FA. Therefore FA could be used to modulate differentiation. This work has never been reported before and it will contribute significantly to materials design for tissue engineering and regenerative medicine. Besides that, to the best of our knowledge, there have been no reports on the combined effects of matrix elasticity and FA patterning on stem cell differentiation. Systematic study of FA will be performed to gain insights of the phenomena observed. The novel platform established in this work can be extended to other cell types such as iPS, to contribute to cardiac tissue engineering.

1.2.4 Outline of Thesis

Figure 1.3 Scope of the study.

The whole work in this thesis is described in Fig. 1.3. A brief introduction has been given in the first chapter, highlighting the motivation behind and the objectives of this interaction study. In chapter 2, the general trend in bone marrow stem cell research and cardiac tissue engineering has been summarized, followed by a brief discussion on the influence of the biochemical and biophysical properties with examples of works done. In later sections, emphasis will be put on the regulatory role of matrix stiffness and FA in determining cell fate. Chapter 3 covered the general materials and equipments used as well as the experimental procedure in our study. Chapter 4 consisted of two parts: 1) PDMS substrates characterization and optimization of the patterning method; and 2) exploration of both elastic modulus and micropatterning effects in determination of stem cell fate. Chapter 5 studied the FA modulation on PA gel with different stiffness and a depth mechanism exploration. Chapter 6 concluded the findings in this interaction study and proposed recommendations for future works.

Chapter 2 Literature Review

2.1 Bone Marrow Mesenchymal Stem Cells and Tissue Engineering

Bone marrow mesenchymal stem cells (MSCs) are one of the mesenchymal stem cells which can be found in the adherent marrow-derived cells, and have the potential to differentiate into many kinds of cells from mesenchymal origin such as chondrocytes, osteoblasts, adipocytes, and fibroblasts ^[20]. Human mesenchymal stem cells (hMSCs) are multipotent cells, which are mainly present in adult marrow, that own the plasticity to differentiate into various lineages of mesenchymal tissues, such as fat, muscle, fat, bone, cartilage, and marrow stroma as shown in Fig. 2.1 ^[21]. Reports are also available regarding bone marrow stem cells trans-differentiation into cardiac cells ^[22]. hMSCs have been widely used in tissue engineering for bone, neuron, adipose, and muscle reparation combined with biomaterials ^[23].

Figure 2.1 Multi Lineage potential of adult human MSCs. Adapted with permission from ^[20]. Copyright 2003 BioMed Central Ltd.

Compared with the research in embryonic stem cells (ESC) which was mainly performed on mouse since human cell line is just available, hMSCs have been widely used for *in vitro* and *in vivo* study ^[24]. The research on human ESCs also raised a lot of controversies due to ethical issue and U.S.A. government limited federal funding on human ESC research ^[20]. Besides that, the life span of ESC *in vivo* is quite limited; which is around 2 years, as contrast to that of MSCs, which reside much later into adult life ^[13]. The

problem of hMSCs is that they showed a limited life span in *in vitro* environment and go to senescence more rapidly than ESCs ^[25]. However, the techniques invented to maintain the plasticity of MSCs and for sustainable culture expansion already has high yield of MSCs sub-culturing and maintain the plasticity of MSCs, allow sufficient number of MSCs for clinical application ^[26].

Based on the literature review above, we can see that the hMSCs possess the ability to self-renew, and to multi-differentiate into many kinds of cells, all of which promising hMSC as a good source for tissue engineering. The application of hMSCs in cardiac tissue will be reviewed in the following part as below.

2.1.1 Heart Disease and Stem Cell Therapy

Heart failure is the major cause of death worldwide, especially in low-income and middle-income countries which heart failure could cause severe disease burdens in low-income family ^[27]. Ischemic heart disease, which is accompanied by reduced blood supply to heart muscle, is usually due to obstruction of coronary arteries ^[28]. Smoking, hypercholesterolaemia (high cholesterol levels), hypertension (high blood pressure), diabetes, and age could increase the risk of ischemic heart disease and lead to a gradual loss of cardiomyocytes leading to myocardial infarction (MI) ^[29]. The only standard therapy for heart failure so far is cardiac transplantation, but it faces the huge issue of the lack of organ donation ^[30]. Therefore, the regenerative potential of stem cells and progenitor cells for treating cardiac heart disease have attracted a lot of experimental research and led to an explosion in clinical investigation.

Many kinds of stem cells are proposed to be used in cardiac tissue engineering as shown in Fig. 2.2. This chart of cell types reflects the large amount of experimental efforts in

devising cell-based therapies. Based on the literature review above, we can see the importance of stem cell therapy in heart disease treatment and the role of hMSCs, which is one of the main sources in cardiac therapy, will be reviewed in the following part.

Figure 2.2 Proposed cell types and mechanisms for cardiac therapy. Different types of cells have been shown to improve cardiac function through various mechanisms. Adapt with permission from ^[31]. Copyright 2008 Nature Publishing Group.

2.1.2 Bone Marrow Mesenchymal Stem Cells in Cardiac Tissue Engineering

The first use of bone marrow cells for cardiomyoplasty was reported in 1999 by Tomita et al. ^[32]. They transplant bone marrow cells into rats 3 weeks after cryo-injury by injection. Transplanted cells induced angiogenesis and improved myocardial function. Fukuda et al. used 5-azacytidine to treat MSC and found that 30% of the cells, formed myotube-like structures and began spontaneous beating after 2 weeks, and beat synchronously after 3 weeks ^[33]. Besides chemicals treatment, cell-cell contact is also a method to induce MSC to differentiation into cardiac cells. Reports showed that when MSC is co-cultured with myocytes, it can differentiate into cardiac like cells which express cardiac transcription

factors GATA-4 and myocyte enhancer factor-2^[34]. However, the efficiency and reproducibility of these chemical induced cardiac like cells are still a problem. The understanding of the interaction of cell-materials is essential in cardiac tissue engineering and many studies are needed to understand the biomaterials driven stem cell differentiation. Based on the literature review above, we can see that hMSCs have the potential to differentiate into myogenic lineage and even into cardiac like cells. However, most of the induction methods were based on chemicals which might cause unknown side effects to cells. In the following part, we are going to focus on biophysical methods using biomaterials to induce stem cell differentiation.

2.2 Stem Cell Niche and Biomaterials

The properties of biomaterials and the design of scaffold will affect the tissue function and regeneration. Therefore, the understanding of stem cell niche, as well as identify and isolate the potential factors of biomaterials which would affect stem cell behaviors is highly important. Here, we will review the concept of stem cell niche and the potential factors in influencing stem cells behaviors.

2.2.1 Concept of Stem Cell Niche

Schofield first proposed the “niche” hypothesis to describe the specialized microenvironment housing stem cells in 1978 ^[35]. Several models have been reported to validate the evidence for stem cell niche. For example, it has been identified that the intestinal stem cell location was near the crypt base, and the epithelial stem cell location was in the bulge area of hair follicles ^[36]. Generally, a stem cell niche includes three parts: the localized signaling cells and an ECM that controls stem-cell lineage commitment; the effective range covered by the signaling; and the stem cells ^[37]. Identify the niche component, predict the change in cell behavior brought by changes in a niche, and know how to modify stem cell niches are critical knowledge in cardiac tissue engineering. Fig. 2.3 showed the elements identified in stem cells niches and it requires further detailed analysis.

Figure 2.3. Elements identified in stem-cell niches. Adapted with permission from ^[37]. Copyright 2002 Nature Publishing Group.

Understanding of stem cell niche can inspire the biomaterials design and better regulate stem cell function as shown in Figure 2.4. It showed that various signals can affect cell behavior at both 2D and 3D scale. A review of potential factors which will affect stem cell niches will be in the following part.

Figure 2.4 Engineering 2D and 3D artificial stem-cell niches. Adapted and modified from ^[38]. Copyright 2004 Nature Publishing Group.

2.2.2 Soluble Factors and ECM

Growth factors, one of the most important soluble factors secreted by stem cells or nearby niche cells, or added in the culture medium, are shown to affect stem cell lineage commitment. For example, transforming growth factor- β (TGF- β) super-family controls proliferation, cellular differentiation, and other functions in most cells ^[39]. TGF- β 1 enhances the chondrogenic differentiation of bone marrow-derived MSC in a dose-dependent manner ^[40]. TGF- β 1 is also reported to combine with ascorbic acid to stimulate the smooth muscle differentiation of hMSCs in a dose-dependent manner as

well ^[41]. To mimic the soluble niches of stem cell, the growth factor can be immobilized on a substrate, which has been used to regulate certain transcriptional networks in mouse ESCs and promote survival of MSCs ^[42].

By mimicking stem cell niches, cascade of signaling pathways could be triggered through binding of biochemical molecules to the stem cell receptors which situated at the cell membrane and this eventually affects cellular response ^[43]. For instance, the specific interactions between ECM and cells have been identified as important signals to regulate cell fate, other than providing anchoring points and maintaining stem cells within their niches. One evidence is that hMSCs showed osteogenic gene expression when cultured on laminin-5 ^[44]. Breuls et al. found out that Collagen V plays an important role in affecting cell behaviour as it impairs cells spreading, affects fibroblast morphology, attachment, FA formation and actin organization when cultured on relative soft substrate ^[45]. Not only is the type of ECM important, the concentration of protein also plays a part in cell behaviour. Engler et al. found out that the spreading of cells is insensitive to the concentration of collagen on soft substrate and varying the collagen density on any substrate stiffness strongly modulates cell spreading. A higher density of collagen sees no different cell area on the glass substrate but a decrease in cell area on both soft (~1 kPa) and intermediate substrate (~8 kPa) ^[46]. Ranucci demonstrate the differences of cells response and adhesiveness on substrate with and without collagen, and found that cell adhesion increased with collagen presence at intermediate level (5.5ng/cm²) compared to cell seeded on substrate without collagen ^[47].

Besides the attachment site of cells, ECM can act as a platform to allow wide range of bio-molecules such as morphogens to be covalently attached, a procedure which is also known as immobilization of growth factors. This was further supported by successful

diversion of hMSCs toward adipogenic, chondrogenic, osteogenic lineages when tert-butyl, carboxyl groups and phosphate were bound respectively to synthetic scaffolds^[48]. Interestingly, recent reports also revealed that fibronectin promoted more osteogenic differentiation when being absorbed to amine-terminated or hydroxyl surfaces, rather than the absorption to methyl-terminated or carboxyl surfaces^[49]. This suggested that novel active sites formed by altering the ligand conformation may act synergistically with other factors, such as spatial clustering of FA in determining stem cell fate.

2.2.3 Physical Control of Stem Cell Differentiation

Other than responding actively to the previously mentioned conventional soluble factors, cells do sense their microenvironment and have been proven to migrate to substrate with higher rigidity, known as “durotaxis”^[50]. This phenomenon is called “durotaxis” and was observed for the first time by Lo et al. in 2000^[51]. The mechanism is not completely understood but Lo proposed that cells can respond to the mechanical properties of the environment: when the adhesion site occurs on a soft region of the substrate, the FA is weak and unstable, while if the cell lands on a stiff region of the matrix, the FA is more stable and always at the leading part of the cell. This difference will arise a kind of competition between adhesion sites that leads to the preference that gives rise to “durotaxis”, as during wound healing and embryonic development^[52]. Cells can also respond to the force applied on them by fluidizing the cytoskeleton when subjected to mechanical stretch^[53]. Notably, biochemical signals triggered by exogenous force and the subsequent alterations in cell-generated forces may act as a driving force for stem cell differentiation. For instance, MSCs showed chondrogenic expression when subjected to cyclic compressive loading^[54] while application of shear flow is known to promote the endothelial marker expression^[55]. Interestingly, it has been reported that culture of hMSCs on both nanotubes and nanopits with same length scale of about 70-100 nm,

which served as the topographical cues, could induce osteogenesis^[56]. This further suggested that stretching of hMSCs on larger-diameter nanotubes or nanopits would have identical effect with the use of stiff ECM^[57] or broad ECM islets^[58].

In our study, we will explore the cell-materials interaction from two parts. From the view of materials, we focus on the effects of substrate stiffness on hMSCs differentiation based on previous literatures^[7a, 59]. Engler et al. found that hMSCs grown on different compliant polyacrylamide gel differentiated in relation to the stiffness of the substrate (i.e., stiffer substrates induced stiffer cells) as illustrated in Figure 2.5^[57]. The study showed that soft polyacrylamide (PA) gel that mimics brain stiffness would induce neurogenesis, while stiffer PA gel that mimics muscle stiffness induced myogenesis, and comparatively rigid PA gel that mimics collagenous bone stiffness induced osteogenesis. Also, Engler showed that myosin/actin striations emerged only on collagen gels with stiffness typical of normal muscle (around 12 kPa) in C2C12 cells^[7a]. Teixeira et al. demonstrated that PDMS substrate elasticity could drive neuronal maturation of neural stem cells^[60]. The mechanism of this kind of regulation is widely accepted as the influence of the contract tension inside the cell. Guo et al. demonstrated that substrate stiffness regulates the strength of adhesions by forming larger FA where cells exert stronger traction forces^[61]. Cells bear different forces exerted by the matrix through FA. For example, cells on hard substrate have higher contraction compared with relaxed cells on soft matrix^[62]. This tension will regulate protein phosphorylation, stress fiber assembly, signaling pathway activation and growth factors release^[63]. Based on the above review we can see that substrate stiffness is a feasible factor to induce stem cell differentiation, especially into soft tissues.

Figure 2.5 Example of tissue elasticity (A) and matrix compliance induced hMSC differentiation (B). Adapted with permission from ^[57]. Copyright 2006 Elsevier Inc.

In addition to substrate stiffness, the topography of the substrates affects stem cell fate as well. The topography mainly influences stem cell differentiation via affecting FA development and cytoskeleton alignment. For example, in a recent *in vitro* study, small nanotubes (around 30 nm in diameter) promoted cell adhesion without affecting differentiation, whereas larger nanotubes (around 70-100 nm in diameter) promoted a obvious stem cell elongation (about 10 fold increased), which increased cytoskeleton tension and differentiation into osteoblast-like cells of hMSCs ^[64]. To utilize biophysical cues to induce stem cells differentiation, several techniques have been developed and in the following sub-chapter we review the use of micropatterning method.

2.2.4 Micropatterning in Cell-materials Interaction Study

It can be seen that many aspects of materials would affect stem cell fate and as a consequence, many techniques have been developed to modulate stem cell niche and drive stem cell differentiation. On this front, surface patterning is usually required. Micropatterning is one of the surface patterning techniques frequently used in cell-materials interaction study. It includes either photolithography and chemical coupling with saline or transferring from a patterned stamp inked with alkanethiol self-assembled monolayers (SAM) to create patterns on the substrate to modulate cell behaviors ^[65]. Microcontact printing (μ CP), which is a top-down technique first described by Whitesides, remains one of the most popular methods of surface patterning ^[66]. μ CP has gained enormous interests due to its simplicity and wide range of applications in the field of biosensor, cell adhesion, cell differentiation, and biomaterials interaction studies.

McBeath used μ CP to control cell shape and cell size, and found that osteogenic differentiation and adipogenic differentiation have a close relationship with cell shape and cellular tension ^[67]. Tay et al. reported that hMSCs which adopted an elongated cell shape would undergo myogenic differentiation at both transcription and translation level ^[68]. Besides modulating cell shape, μ CP is also used to engineer the FA of cells. Gallant employed μ CP of circular adhesive islands (2, 5, and 10 μ m diameter) to modulate cell attachment and FA development. It was found that adhesion strength exhibited time and adhesive area dependence. The results indicate that FA assembly, independently of cell morphology and redistribution of adhesive structures, contributes significantly to adhesion strengthening ^[69]. However, the conventional μ CP is not able to work well on soft/sticky substrates. To our knowledge, no simple method is available to date to solve

this problem. In our study, we invented a novel μ CP technique to fix this issue which will be discussed in Chapter 4.

In general, μ CP is a powerful tool in cell-materials interaction study and using this method, we can simply modulate cell shape and FA development.

2.3 Focal Adhesion in Cell-materials Interactions

2.3.1 Focal Adhesion

In a cell, FA is the model system and a central regulator because it is a pivotal contact point between the cell and material. FAs are large, dynamic protein complexes through which the cytoskeleton of a cell connects to extra cellular matrix (ECM) as shown in Fig 2.6 ^[70]. In our study we chose vinculin as the typical FA indicator since this adaptor protein is a typical regulator in FA complex ^[71]. For instance, the binding of vinculin to talin triggers the clustering of activated integrins, and then vinculin associates to actin to strengthen actin-integrin link ^[72]. More importantly, although several proteins were reported to sense and feel the mechanical properties of the substrates, only vinculin can regulate mechanical stress in addition to its function as a mechano-coupling protein ^[73].

Figure 2.6 Schematic depicting structure and action of FA in cells. Adapted with permission from ^[74]. Copyright 2007 The Rockefeller University Press.

The FAs sense the environment and then transfer the extracellular information such as mechanical properties and chemical properties of the substrates through the cytoskeleton

into cell nucleus and cause a cascade of changes in the cell. Among the cues which affect FA formation and stress fiber development, RhoA signaling pathway which can increase contractility is one of the most important. Previous reports demonstrated that stimulation of fibroblasts with lysophosphatidic acid, which could activate RhoA signaling pathway, induced myosin light chain phosphorylation ^[75]. This even happened before the formation of stress fibers and FAs and it was accompanied by increased contractility. In addition, the study showed that when contractility was inhibited, integrins dissociated from FAs as stress fibers and FAs disassembled. Therefore, in the beginning of the FAs and stress fiber formation, RhoA signaling pathway and contractility are essential. In the maturation of FAs, Oakes et al. demonstrated that this process was guided by the actin stress fibers that assemble at nascent FAs ^[76]. The contractility is required but stress fiber architecture is more important in FA maturation. Based on the literature we can see that contractility, RhoA, and stress fiber have a close relationship in FAs formation and development. In our study, we are going to explore the role of FAs in cell-materials interactions and make use of it in stem cell differentiation study.

2.3.2 Focal Adhesion in Environment Sensing, Mediating Cell Attachment and Motility

To our knowledge, most of the cell adhesion studies were based on homogeneous coated substrates or micropatterns ranging from 20-500 μm , but few of them explored sub-cellular adhesion using micropatterning of single FA. Lehnert et al. made a study on the geometrical limits of ECM binding sites required for cell spreading using μCP . They found that when the spacing between dots was less than 5 μm , cell morphology was indistinguishable from the non-patterned group, when this spacing exceeded 25 μm , cell was not able to spread ^[77]. Xia et al. found that micropatterning of different shape and

size of ECM islets would affect cell motility and induce cell orientation and elongation, but no differentiation study was performed ^[78]. Chen, C. S et al. micropatterned islets to control cell spreading, and found that the threshold of the space cells could cross was 20 μm for human cells, and cell spreading would increase when spacing was decreased ^[79].

As for the FA function, many reports emphasized their roles in environmental sensing ^[80], signaling regulation via tyrosine phosphorylation, cell shape and cytoskeleton regulation ^[8c]. Fig. 2.7 showed the capacity of cells to respond to different surface properties, including adhesive ligand density, surface compliance, ECM specificity and surface dimensionality. Naturally, all these response is mediated by FA and in Fig. 2.7Ba and Bb, it showed the difference in FA size and distribution when cells were cultured on matrix with different compliance ^[81]. The axes highlighted the diversity of surfaces. For instance, the surfaces can be different in ligand spacing, rigidity, and dimensionality. Cells seeded onto 3D matrix adopt an elongated shape (Aa) that mimicked the shape of MSC *in vivo* niches, whereas cells on 2D matrix always adopt a spread shape (Ab). The α_5 integrin in cells on 2D substrates were different from the integrins of cells in 3D scaffold. The behaviours of human fibroblasts to 100 kPa or soft 10 kPa fibronectin-coated PDMS substrates are shown in B. The organization of GFP-paxillin-labelled FA and phalloidin-labelled F-actin, and overall cell shape, are significantly different in cells seeded onto the two matrices. The FA differs in cells on 2D hard matrices with fibronectin (Ca) or vitronectin (Cb) coating as shown in C. The Paxillin of cells on vitronectin-coated matrix is elongated, whereas classical FAs are observed in cells on fibronectin coated matrix. B16 melanoma cells seeded to nanopatterned surfaces with different spacing between nanodots as shown in D. The cell morphology and the expression of GFP- β_3 integrin (green) are quite different for cells on nano-patterned matrices with different spacing.

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Figure 2.7 A multidimensional space of environmental parameters. Adapted with permission from ^[81]. Copyright 2009 Nature Publishing Group.

Besides environmental sensing, FA also plays an important role in signaling pathway regulation. For vinculin, it regulates FAK by binding to paxillin ^[82] and increases the contractile force inside the cell ^[83]. These studies demonstrate that FA play a pivotal role in stem cell differentiation and it is the direction to gain insight into the roles of physical stimulants of stem cell differentiation.

2.3.3 Role of Focal Adhesion in Stem Cell Differentiation

Goffin et al. induced myofibroblasts differentiation via modulation of elongated FA via micropatterning of small rectangular islets ^[19]. Their study showed that micropatterned 8-30 μm long supermature FAs could significantly enhance the recruitment of α -smooth muscle actin in myofibroblasts. The large, elongated FA also generates higher stress than classic FA as shown in Fig. 2.8. Dugina et al. found that during myofibroblasts

differentiation, the FA size was significantly increased, tensin and FAK were synthesized in FAs and paxillin as well as vinculin were recruited from the cytoplasm into FAs ^[84]. The FA of cells in different cell line is also different. For example, the FAs in neurons are relatively smaller and more sparse than those in osteoblasts ^[57]. In addition to FA morphology and density, FA also plays an important role in the regulation of stem cell differentiation. For instance, focal adhesion kinase (FAK) were observed to regulate adhesive forces and vinculin recruitment and even affected cardiogenesis of embryonic stem cells ^[85]. Another report demonstrates that FAK can regulate osteogenic differentiation of hMSCs via ERK pathway. They found that inhibition of the expression of FAK and phosphorylation of FAK Y397 and ERK1/2 will block osterix activity at gene expression level and the osteogenic differentiation of hMSC ^[86].

Figure 2.8 Super FAs generate higher stress than normal FAs. Adapted with permission from^[19]. Copyright 2006 Rockefeller University Press.

Based on the literature we can see the importance of FA in environment sensing and cell behavior regulation. However, not many reports are available to modulate FA to affect stem cells behaviors. Moreover, no report is available to the best of our knowledge to induce hMSCs differentiation via modulation of FA. Therefore, this study can fill in the gap and provide a novel platform in cell-materials interaction study.

Chapter 3 General Materials and Methods

3.1 Stamp Fabrication and Characterization

The silicon master templates bearing the desired topographic features were fabricated by standard photolithography (Bonda Technology) and were treated with 1% octadecyltrichlorosilane (OTS) (Sigma) before use. 1:10 (cross linker: base) liquid PDMS (Sylgard 184 kit, Corning) was cured against the master at 100 °C for 3 h then the PDMS layer with a dimension of 1.5 cm x 1.5 cm was gently peeled off to be used as the stamp. For no-feature stamp fabrication, mold casted 1:10 PDMS was cut into round shape with a diameter of 22 mm, as the stamp without patterns. The fabricated elastomeric stamps were sputtered coated (SPI-MODULETM coater) for 40 s, and then were examined under a scanning electron microscopy (SEM) (JEOL 6360).

3.2 Cell Culture and Cell Seeding

Cryopreserved adult bone marrow derived hMSC (Lonza) were cultured in MSCGM bullet kit medium (Lonza) at 37 °C, 5% CO₂ before experiment. During the experiment, cells were harvested and cultured in complete medium consist of low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplement with 10% of fetal bovine serum (FBS) and 1% antibiotics (PAA), and the medium was replaced every 2-3 days. The experiments described herein were performed using passage 5 and used a seeding density of 1000 cells/cm². 1 h later after cell seeding, medium was replaced by fresh complete medium to wash away the unattached cells.

3.3 RNA Isolation and Real-time PCR

On selected time point, the RNA of the cell was isolated with RNeasy mini kit (Qiagen) and was synthesized into cDNA (iScript™ cDNA Synthesis Kits, Bio-Rad) according to the manufacture's handbook. The real-time PCR was performed on a CFX96 real time PCR detection system (Bio-Rad) with KAPA SYBR FAST master mix universal (Kapa Biosystems). Primers specific to the targeted genes were obtained from primer bank and were listed in Table 1^[87]. GAPDH and β -actin were used as endogenous housekeeping genes and the heat map was generated using Matrix2png^[88]. The Relative quantification of gene expression was analyzed with the CFX manager software (Bio-Rad Laboratories, Inc.), and the Relative Expression Software Tool 2009 (REST 2009).

Table 3. 1 Primer sequences for quantitative Real Time PCR (qRT-PCR)

Gene target	Primer Sequence (5'-3')	Product size (bp)
β -actin	Sense: CATGTACGTTGCTATCCAGGC	250
	Antisense: CTCCTTAATGTCACGCACGAT	
GAPDH	Sense: CATGAGAAGTATGACAACAGCCT	113
	Antisense: AGTCCTTCCACGATACCAAAGT	
Alkaline phosphatase (ALPL)	Sense: CTCTCCAAGACGTACAACACC	201
	Antisense: AATGCCCACAGATTTCCCAGC	
BSP	Sense: TGGATGAAAACGAACAAGGCA	200
	Antisense: AAACCCACCATTTGGAGAGGT	
Runt related transcription factor 2 (RUNX2)	Sense: TCCTATGACCAGTCTTACCCCT	190
	Antisense: GGCTCTTCTTACTGAGAGTGGAA	
Myoblast differentiation protein 1 (MyoD1)	Sense: CGGCGGCGGAACTGCTACGAA	459
	Antisense: GGGGCGGGGGCGGAAACT	
GATA-4	Sense: GACAAGGGCGTCCAGACTC	140
	Antisense: TGCGAGGAGCAGTAATACTTCTT	
MYH7	Sense: CACTGATAACGCTTTTGATGTGC	165
	Antisense:	

	TAGGCAGACTTGTCAGCCTCT	
	Sense: CAACAGCGACGGAGGTCTC	
Nestin	Antisense: CCTCTACGCTCTCTTCTTTGAGT	163
Microtubule	Sense: CAGGAATTGACTCCCTCTACAGC	
Associated	Antisense: TCTTCACCAGGCTTACTTTGC	80
protein		
(MAP2)		
	Sense: GGCGCCTTTGGACACCTATT	
Tubulin	Antisense: CCTCCGTATAGTGCCCTTTGG	91
	Sense: GAAAACGTGACCCATGAGTTCA	
ITA1	Antisense: TCCTTTCTTGCTGTGTCTATTCC	134
	Sense: CAAGCAGGGCCAAATTGTGG	
ITB1	Antisense: CCTTTGCTACGGTTGGTTACATT	185
	Sense: AGGATGACTGTGTCGTCAGAT	
ITB3	Antisense: GGTAGACGTGGCCTCTTTATACA	281
	Sense: GGCCCTCTTCAGGATGGTG	
MLCK	Antisense: GCACATGCTTTGGTTTTCTC	188
	Sense: CTGTTCCAGCCATCCTTCAT	
SMA	Antisense: GCTGGAAGGTGGACAGAGAG	280
	Sense: CCCATCGAGCATGGTATCATC	
ACTA	Antisense: AGAAGCATACAGGGATAGCACT	228

3.4 Immunostaining and Imaging

Cells were fixed at selected time point with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature and then permeabilized with 0.1% Triton X-100 in PBS for 5 min, followed by blocking with 5% bovine serum albumin (BSA) in PBS or 5% goat serum in PBS for 1 h. After washing with PBS, primary antibodies were applied for 1 h at 37 °C for 1h followed by incubation with secondary antibodies for 1 h. The primary antibodies were mouse anti vinculin (1:400, Millipore), rabbit anti COLI (1:200, Novus), mouse anti integrin β_1 (ITB1, 1:100, Millipore), mouse anti integrin β_3 (ITB3, 1:100, Millipore), mouse anti myosin light chain kinase (MLCK, 1:100, Sigma), mouse anti myosin heavy chain (MHC, 1:100, Abcam), mouse anti human α -sarcomeric actin (1:500, Sigma), RUNX2 (1:100, Santa Cruz Biotechnology), Osteocalcin (Ocal, 1:100, Santa Cruz Biotechnology) and chicken anti MAP2 (1:10000, Abcam). Secondary antibodies were Alexa Fluor® 488 goat anti mouse IgG (1:400, Invitrogen), Alexa Fluor® 488 goat anti chicken IgG (1:500, Invitrogen), and Cy3 goat anti rabbit IgG (1:400, Millipore). F-actin was stained with tetramethyl rhodamine-iso thiocyanate (TRITC)-Phalloidin (1:400, Millipore). Cell nucleus was stained with 40,6-Diamidino-2-phenylindole (DAPI) (1:1000) (Chemicon) for 10 min.

Fluorescence images were captured using an upright fluorescent microscope (Eclipse 80i, Nikon) or confocal microscope ((TCS SP5, Leica). ImageJ 1.44f software was used to analyze focal adhesion and cell morphology^[89]. Briefly, the fluorescent image of vinculin was converted to 8 bit, removed background by “subtract background” function at same level, then use “Adjust Threshold”, followed by “Analyze particles” function. The area parameter to be detected was set to 0.8 μm^2 -40 μm^2 .

3.5 Statistic Study

All assays were done in triplicate for each group and the results were expressed as means $\pm$ standard deviation (SD). Statistical analysis was performed by using a one-way ANOVA followed by Tukey's pair wise comparisons. A value of $p < 0.05$ was considered statistically significant.

Chapter 4 Modulation of Focal Adhesion to Regulate Stem Cell Differentiation

4.1 Introduction

hMSCs have attracted enormous attentions in the past decades due to their multipotent property and potentials in regenerative medicine, especially in heart tissue engineering ^[4]. Many studies have been shown that stem cells are sensitive to their microenvironment, and cell behaviors can be determined by the stem cells niches ^[90]. Amongst several induction factors, biophysical forces regulate important cellular process, such as communication ^[91], migration ^[18], cell attachment and spreading ^[92], and driving stem cell lineage commitment ^[93]. It was shown that soft, intermediately stiff and rigid substrates that respectively mimics brain, muscle and bone stiffness were able to drive the corresponding tissue specific differentiation ^[57]. Despite these promising findings, the actual mechanism of how matrix elasticity can drive hMSCs differentiation is elusive. The main hurdle faced in elucidating this fundamental mechanism is the current inability to experimentally segregate formation of focal adhesions (FAs) morphologies and density from their multi-modal responses to substrate stiffness and cellular morphology. FAs appear to be the connective node in this puzzle. Adherent stem cells sense the underlying substrate environment mainly via FAs; and then translate the “biophysical signals” into cellular responses through the cytoskeleton. This can then result in modulating cell lineage specific gene and protein expression inside the cell ^[94]. Most of the cell-materials interaction studies used homogeneously coated substrates ^[57] or micropatterned (20-500 μm) ^[68] or nanopatterned substrate surface ^[95], but few studies were focused on the function of micropatterning of single FA. In one of these studies, Lehnert et al.

concentrated on the geometrical limits of ECM binding sites which is required for FA development for cell spreading using microcontact printing (μ CP) ^[77]. Xia et al. studied the effects of different shapes and sizes of ECM islets on cell motility and morphology ^[78]. But the use of FA modulation to induce stem cells differentiation is quite limited to date. Goffin et al. investigated on FAs effects on myofibroblast maturation but they did not explore further to see the combined effects of FA patterning and substrates stiffness ^[19]. We reported earlier on the use of μ CP to modulate the shape of hMSCs and found that elongated hMSCs with aligned cytoskeleton increased myogenesis. Moreover, the FA development between elongated hMSCs and normal spread hMSCs were found to be different ^[96]. Implicit in these latter studies is the centrality of the FAs in regulating basic cellular behavior. With this, we set out to investigate the possibilities of modulating FAs specifically to drive stem cell differentiation.

We combined matrix compliance and extracellular enforced adhesion nodes through a novel μ CP technique on PDMS substrates of different compliance to coax the cells to adopt certain cellular shape or FAs configuration. μ CP is a widely used technique to study cell behaviours ^[97] but conventional μ CP is not easy to be performed on soft and/or tacky substrates, such as polydimethylsiloxane (PDMS) with a Young's modulus lower than 12.6 kPa ^[98], on where stamps would adhere to the tacky substrates and distort the patterns as illustrated in Fig. 4.1a-d.

Figure 4.1 Illustrations of the limitations of conventional μ CP on soft and/or tacky substrates. (A) Sagging of the stamp will occur on soft substrates. (B) The stamp adheres to the substrate when printing on tacky substrate. The tacky surface was damaged (D) compared with the surface before patterning (C). (E-G) Immunostaining images of conventional μ CP of FN on different PDMS substrates. Scale bar in (E)-(G) is 100 μ m. Adapt with permission from ^[99]. Copyright 2012 Elsevier Inc.

Therefore, a simple and novel micropatterning technique was developed using polyvinyl alcohol (PVA) as a trans-print media that enabled efficient μ CP on soft and/or tacky substrates ^[100].

We hypothesized that the matrix stiffness, combined with FA development (such as FA morphology and density) and cytoskeleton arrangement would affect hMSCs differentiation. This study has provided insights on the role of FAs and matrix stiffness in cell-materials interactions during hMSCs myogenic differentiation for possible future clinical muscle tissue engineering applications. Moreover, the novel μ CP method expands cell-materials interaction study to soft and tacky surfaces in a simple and cost-effective manner.

4.2 Materials and Methods

4.2.1 Substrate Fabrication and Characterization.

To prepare substrates of different stiffness, PDMS (Sylgard 184, Dow Corning) with three types of cross link ratio (1:10, 1:50 and 1:70 kPa, cross linker: base) were used. The pre-polymer solution was poured into a 35 mm culture dish (1.5 mL each), and then baked in a vacuum oven for 30 min to remove air bubbles. Afterwards, the PDMS was cured in an oven at 65 °C for 24 h. PDMS substrate shear elastic modulus (G') was measured using a rheometer (Physica MCR 501, Anton Paar) according to the instruction provided by the manufacturer. Briefly, PDMS shear elastic modulus was measured at 37 °C using a Physica MCR 501 Rheometer (Anton Paar, Graz, Austria) with a parallel plate geometry (PP25/TG) and Rheoplus software according to the methods previously described ^[101]. PDMS storage shear moduli were determined by oscillatory shear experiments performed with strain amplitude of 1% and oscillation frequency of 10 rad/s. Young's modulus (E') was converted from G' using the relation $E' \sim 3 G'$ ^[102].

4.2.2 Micropatterning of hMSC

The μ CP technique was modified from the method described by Li et al. ^[103] and the procedure was illustrated in Fig. 4.2. Briefly, the PDMS substrates were treated in an oxygen plasma cleaner (PDC-002, HARRICK) with a power of 10.8 W for 1 min at a pressure of 120 mTorr followed by inking with 2 % (3-aminopropyl) triethoxysilane (APTES) (Sigma) in 90 % ethanol for 40 min. After that, substrates were washed with 100 % ethanol and dried in a 60 °C oven for 1 h then immersed in 2.5 wt% glutaraldehyde (GA) (Sigma) in phosphate buffered saline (PBS) for 1 h followed by washing with Milli-Q water for 1h.

To prepare PVA film, 0.5 wt% of PVA (Sigma) was put in deionized (DI) water and left at room temperature overnight, followed by magnetic stirring at 90 °C for 2-3 h to dissolve the PVA particles completely. PVA solution (20 mL) was casted into a glass petri dish (150 × 20 mm), and air dried in a laminar flow hood for 18 h. The dried PVA film was removed from petri dish and was cut into round film by a SCHMIDT® Presses (SCHMIDT Technology, Germany) and used as the trans-print media. The thickness of the film h was determined as:

$$h = \frac{m}{\rho A} \quad (1)$$

where m was the mass of PVA used, ρ was the density of PVA (1.29g mL⁻¹ according to manufacturer's data), and A was the surface area of the Petri-dish. The thickness of the PVA film was about 44 μm in our study.

Figure 4.2 Schematic diagram of μ CP procedure. (A) The conventional μ CP process layout. (B) The novel μ CP process layout. A water-soluble PVA film was used as the trans-print media to avoid the step of removing the PDMS stamp on soft/tacky substrates. Adapt with permission from ^[99]. Copyright 2012 Elsevier Inc.

Inked stamp with a layer of COLI (named non-patterned group) or patterned COLI was placed in conformal contact with PVA film in a laminar flow hood for 30 min instead of directly printing on the PDMS substrate. And then the patterned side of the film was placed onto the PDMS substrate and was left for 30 min. After that, PBS was used to rinse the substrate to dissolve the film completely. Subsequently, 2 % bovine serum albumin (BSA) was used to passivate the un-patterned region. The effect of passivation was validated with a strip pattern and cell was cultured on it until day 9. All the substrates

were UV sterilized for 15 min before use. Finally, hMSCs were seeded on the substrates at a density of 1000 cells/cm².

4.2.3 In Situ Proximity Ligation Assay (PLA)

For PLA, samples were fixed with 4 % paraformaldehyde and then blocked for 1 h with 5% normal goat serum (NGS) in PBS containing 0.1 % Triton-X, followed by incubation overnight at 4 °C with mouse anti-cardiac heavy chain myosin antibody (MHC) (1:100, Abcam) in 5% NGS. Duolink *in situ* PLA was performed as recommended by the manufacturer (OLink Biosciences). The PLA spots were counted by the “Analyze particles” function of ImageJ. The negative control was performed without primary antibody.

4.2.4 Positive Controls for Immunostaining of Differentiation Marker

C2C12 myoblast (ATCC, Manassas, VA) was used as the positive control for MHC immunostaining. It was grown in high-glucose DMEM containing 10% fetal bovine serum, 1% penicillin–streptomycin and 1% L-glutamine. When cells got 70% confluence, the culture medium was replaced with Dulbecco's Modified Eagle Medium (DMEM) containing 2 % horse serum to induce myogenic differentiation. 2 days after induction, cells were fixed and MHC was stained. U2-OS, a kind of human osteosarcoma cell was used as the positive control for Ocal immunostaining. The cells were cultured in low glucose DMEM containing 10% fetal bovine serum, 1% penicillin–streptomycin and 1 % L-glutamine. After cultured for 5 days, cells were fixed and stained with RUNX2 and Ocal antibodies. Neural stem cells were induced to differentiate in DMEM F-12 and stained for MAP2 antibody according to the method described previously ^[68].

4.2.5 Patterned Protein Characterization

The intensity of COLI on PMDS substrates with stiffness of 308 kPa, 12.6 kPa, and 2.1 kPa was characterized by fluorescent microscopy. LUCY® 506 solution (Sigma) was used to stain the coated protein according to manufacture's protocol. Un-patterned PMDS substrates with stiffness of 308 kPa, 12.6 kPa, and 2.1 kPa were used as the blank. Fluorescent images were taken under fluorescect microscopy (Nikon 80i) at the same exposure time, and the mean intensity was measured by ImageJ. The mean fluorescent value was subtracted by the fluorescent value of blank substrate, and was plotted in Fig 4.2.

4.3 Results and Discussion

4.3.1 PDMS Substrates and Stamps Characterization

The average shear storage moduli G' of 1:10, 1:50, and 1:70 PDMS were 102.7 ± 6.9 kPa, 4.2 ± 0.1 kPa, and 0.7 ± 0.1 kPa respectively. For rubber elastic materials, assuming a Poisson ratio of 0.5^[102], the Young's modulus E of 1:10, 1:50, and 1:70 PDMS was about 308 kPa, 12.6 kPa, and 2.1 kPa respectively. PDMS was chosen as the matrix since it has several properties that make it attractive as a common used material for micropatterning and topography modification. The ease of fabrication, non-toxicity, and easy tunable mechanical properties were critical in cell-materials interaction study^[104]. The reason to choose the PDMS substrates with stiffness of 12.6 kPa and 2.1 kPa was that they mimicked muscle and brain environment respectively^[7a, 105] with the 308 kPa group as a non-compliant control group on the other end of the spectrum. The control group we used was 308 kPa hard PDMS, the same material as 12.6 kPa and 2.1 kPa substrates, to eliminate the materials chemistry effects. This stiffness is reminiscent of bone which is biologically very different from muscle and thus the differentiation drivers are different^[106]. μ CP is a widely used technique to study cell behaviours^[97] but conventional μ CP is not easy to be performed on soft and/or tacky substrates, such as polydimethylsiloxane (PDMS) with a Young's modulus lower than 12.6 kPa^[98]. Therefore, a simple and novel micropatterning technique was developed using polyvinyl alcohol (PVA) as a trans-print media which can efficiently be used on soft and/or tacky substrates^[100].

Figure 4.3 Shear modulus of PDMS substrates. Group 1 and group 2 were two batches samples to confirm the PDMS fabrication method was reproducible. Bars represent mean $\pm$ standard deviation derived from 5 samples.

Figure 4.4 Novel microcontact printing (μ CP) could effectively pattern COLI on soft and tacky PDMS substrates. (a): Schematic diagram of novel μ CP procedure which using a PVA film as a trans-print media instead of directly print on the substrate. (b, c): SEM validation of stamp features of S3.6 (b) and L20 (c) patterns respectively. The height of the features on the stamps was 5 μ m. (d, e) Fluorescent images of patterned Cy3 labeled COLI showing novel μ CP method could successfully print protein on soft (2.1 kPa) and tacky substrates. Scale bar in (b) and (c) showed 10 μ m, in (d) and (e) showed 50 μ m. Adapted with permission from ^[107]. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

SEM images (Fig. 4.4b-c) for the surface topographies on PDMS stamps and immunostaining images (Fig. 4.4d-e) of the printed COLI on PDMS substrate were validated as shown in Fig. 4.4. Pattern in Fig. 4.4b (named S3.6) was composed of square islets with the dimension of 3.6 $\times$ 3.6 μ m, which was designed to induce dense FAs development. The pattern in Fig. 4.4c (named L20) consisted of rectangular islets, with the size of 20 $\times$ 2 μ m, was designed to induce elongated FA according to previous report ^[19]. The distance between islets in both of the two patterns was 6 μ m. This was the optimal distance that allows cells to develop FAs across the islets ^[77, 108]. If the distance is

too large, cell may not be able to cross the islets and cell spreading will be restricted. If the distance is too small, cells may not be able to distinguish between the islets. Although the optimal distance may depend on matrix stiffness, we want to decouple the patterning effect from stiffness effect by having a constant islet spacing across all matrix stiffness. The COLI was evenly patterned on PDMS substrates with different compliance and there was no significant difference in protein absorption as shown in Fig. 4.5.

Figure 4.5 Mean fluorescent value of collagen type I on PDMS substrates with different stiffness.

4.3.2 Stiffness Effects on Stem Cell Differentiation

A screening test of matrix elasticity effects on hMSCs differentiation was examined by hMSCs gene expression on day 7 using un-patterned group as shown in Fig. 4.6. Relative gene expression levels of hMSCs cultured on substrates with different stiffnesses were normalized to the control where cells were cultured on stiff substrates (308 kPa PDMS). All substrates were coated with a layer of COLI (200 $\mu\text{g/ml}$).

The results showed that osteogenic markers: ALPL for cells cultured on PDMS with stiffness of 12.6 kPa and 2.1 kPa were maintained at down regulated state. For BSP, it was up regulated on day 7 for 12.6 kPa group but no statistical difference was observed in 2.1 kPa group. For RUNX2, it was up regulated in both of these two experimental groups and showed a higher up regulation in 12.6 kPa group as compared to that in 2.1 kPa group on day 7. In general, PDMS with a stiffness of 12.6 kPa up regulated two osteogenic markers (BSP and RUNX2) while PDMS with a stiffness of 2.1 kPa down regulated ALPL and maintained BSP at the nominal level. Compared with 12.6 kPa group, 2.1 kPa group showed less significant up regulation of RUNX2. Conversely, the hMSCs on 308 kPa PDMS did not show any myogenesis or neurogenesis (data not shown).

Figure 4.6 Matrix stiffness affected on hMSCs FA and cytoskeleton development and differentiation. (a): hMSCs on PDMS with different stiffness showed different FA and cytoskeleton development. Scale bar showed 50 μm . (b): After 7 days of culture, relative gene expression levels (I.e. $\Delta\Delta C_T$) of hMSCs were normalized to those of control group (308 kPa PDMS) indicated by the dashed line. Bars represent mean $\pm$ standard deviation. The asterisks indicate statistic significant difference ($p < 0.05$) compared with control group. Adapted with permission from ^[107]. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

For myogenic markers, PDMS with a stiffness of 12.6 kPa could induce significant up regulation of all these markers on day 7. But cells in 2.1 kPa group showed no myogenesis. These results correspond well with the work reported by Engler *et al.*, which showed 11 kPa matrix would induce myogenesis but no myogenesis was found on 1 kPa substrates ^[57].

These results showed that matrix elasticity could influence hMSCs differentiation. The interaction of hMSCs with the softer 2.1 kPa substrate might not have generated the

required traction force and cellular longitudinal tension for progression into myogenic or osteogenic differentiation. Instead, at such brain tissue-mimicking stiffness, it might initiate the path towards a neuronal lineage ^[109]. This difference translates into signaling pathways activation and molecular changes in a regulated manner (paper in press) and at the transcriptional level, important lineage specific gene expressions are triggered as seen in Fig. 4.6. The mechanism may be related to the fact that cell senses matrix elasticity through FAs while nonmuscle myosin II exerts force on the substrate ^[110]. Different matrix compliance gives different feedback force which will induce changes in cellular tension and cytoskeleton rearrangement ^[111]. Since cells use FAs to sense their immediate mechanical environment and via nuclear membrane spanning connections like vinculin and talin ^[112], we wondered whether there will be differences in lineage commitment through nuclear transcriptional changes with further differences in FA characteristics due to micropatterning. To answer this, we now capitalized on the difference in FA characteristics induced by different micropatterning (S3.6 and L20 configuration) in addition to substrate stiffness comparisons between the 308 kPa and 12.6 kPa PDMS groups.

The screening gene transcription study of the effects of matrix stiffness (308 kPa, 12.6 kPa and 2.1 kPa) indicated that hMSCs were induced into myogenic lineage commitment (albeit not entirely specific) by PDMS substrates with a stiffness of 12.6 kPa, which mimicked muscle environment. Therefore, PDMS substrate with a stiffness of 12.6 kPa could promote myogenesis of hMSCs and was selected in the following studies based on our interest in heart tissue engineering, and hard PDMS with a stiffness of 308 kPa was chosen as a control group.

4.3.3 Micropatterning and Passivation Validation Study

From Fig.4.5, we can see that the FA density and cytoskeleton development decreased when cells were cultured on soft substrates. Since the stress fibers of a cell connect to its niche via FAs, it is reasonable to believe that the extracellular FA patterning would affect the development of FAs and as a consequence, the stress fibers would re-organize into a feedback loop ^[113]. Here, microcontact printing of sub-cell size islets was used to manipulate the FA development. Transfer printed patterns of COLI on PDMS substrates were labeled with Cy3 (red) as shown in Fig. 4.4 d-e which indicated that the novel trans-print μ CP method could efficiently transfer the protein patterns onto soft and tacky PDMS substrates.

A passivation study was performed with a 20 μ m width strip pattern to show 2% BSA was able to passivate the non-patterned region well for at least 9 days (indicated by the fact that cells only grew within the strip pattern in Fig 4.7). The passivation study verified the patterning efficiency of the platform.

Figure 4.7 BSA could successfully passivate the PDMS surface. (a) SEM image showed the strip stamp used to make control patterns. (b) Fluorescent imaging showed Cy3 labeled patterned COLI on 12.6 kPa PDMS. (c) and (d) showed hMSCs aligned well on the patterned COLI until 9th day, few cells grew out of the pattern. Statistical study showed 96.8% of cells strict followed the collagen pattern on 36 h and 93.3% of cells followed the collagen on day 9. 200 cells were studied in each group. Adapted with permission from ^[107]. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

4.3.4 Micropatterning Effect on FA Development

Fig. 4.8 shows the FA development of hMSCs when cultured on the two substrates (12.6 and 308 kPa) with different patterns. On intermediate stiffness (12.6kPa, Fig 4.8a) substrate, the FA size was not significantly affected by different patterns. However, the FA length and FA aspect ratio (AR) were significantly higher in the L20 pattern as compared to S3.6 and the control; indicating the elongation of the FA in the former group. Whereas, high FA density was induced in the S3.6 pattern while the density was significantly lower in the L20 pattern group. This result is amazing since the L20 rectangular pattern is actually denser than the S3.6 square pattern. It indicated that cells

would develop FA not only based on the density of the pattern, but more so in accordance to the shape of the pattern. It is remarkable to observe the dominant effect of patterning morphology on FA development. The statistical result in Figure 4.8 showed that the aim to induce elongated and dense FA was achieved by L20 and S3.6 patterns respectively.

Figure 4.8 Micropatterns affected FA development on PDMS substrate (12.6 kPa) on day 7. (a-c): statistical studies of FA morphology showing that L20 and S3.6 patterns have no effects on FA size while L20 pattern could significantly induced elongated FA

only on 12.6 kPa substrate. (d): S3.6 could increase FA density on substrates with a stiffness of 12.6 kPa. Bars represent mean $\pm$ standard deviation. $*p<0.05$ and $\#p<0.01$ compared with non-patterned groups ($n=3$). (e-j): Fluorescent images showing different vinculin (*green*) and F-actin (*red*) development in cells on PDMS with L20 pattern (e, f), S3.6 pattern (g, h), and without patterns (i, j) respectively. COLI patterns were labeled with Cy3 (*red*) in (e-h) and nuclear was label with DAPI (*blue*). Scale bars showed 50 μm . Adapted with permission from ^[107]. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

On the hard substrate (308 kPa), the result was completely different where there was no apparent trend in the FA size, FA length and FA AR when hMSCs were cultured on different patterns. Both the S3.6 and L20 patterns did not induce significant changes in the FA development; with the exception of FA density; and remained similar to the control. Interestingly, for FA density, just like on intermediate stiffness substrate, L20 pattern induced significantly lesser density as compared to both S3.6 and control groups. This study seems to show that patterning had less impact on FA development when a hard substrate was used.

The reason could be due to the fact that cells would develop a larger and more stable FA when cells cultured on a hard substrate ^[114], and the effect of this matrix stiffness was so dominant that it dictated the behavior of FA development. In this case, the effect of patterns was overshadowed and could not be manifested (Fig. 4.8f, h), reflected by the fact that FAs were not as elongated and cytoskeleton was not aligned as well as those on PDMS substrate with a stiffness of 12.6 kPa. However, on the 12.6 kPa substrate, the effect of stiffness was significantly reduced and this allowed L20 pattern to take effect on the FA development and resulting in aligned cytoskeleton and elongated FA as shown in Fig 4.8e. L20 pattern could significantly increase the FA length by 48 % and FA AR by 38 % as compared to un-patterned group (Fig 4.8b-c) when hMSCs were cultured on 12.6 kPa substrate. This was accompanied by a slight decrease in FA density, same as what we observed in our previous study ^[96] (Fig. 4.8d). On the substrate of the same stiffness, S3.6

pattern increased FA density without altering the morphology (as compared to control) and this resulted in random orientated cytoskeleton (Fig 4.8g). This is an important indication that the FA of hMSCs could be manipulated via this micropatterning platform on tissue compliant substrates.

4.3.5 Effects of FA Modulation via Micropatterning on hMSCs

Differentiation

By assaying for transcript levels of lineage-commitment genes with qPCR, we looked specifically on whether there is any relationship between the geometry of FA in the two micropatterns on hMSCs differentiation at biomolecular level. Then, PLA assay targeting myosin heavy chain (MHC) and conventional immunostaining was used to confirm the observation at protein level. Relative gene expression at 7th day for cells cultured on intermediate and stiff substrates with both patterns was shown in Fig. 4.9a-c and Fig. 4.9d showed the fluorescent images of FA development.

Figure 4.9 The interplay of FA development and matrix stiffness on hMSCs differentiation at transcription level. L20 could induce hMSCs myogenesis as shown in (a, left) while exerting less effects on osteogenic markers and neurogenic markers (b and c, left) when cells were cultured on *intermediate substrate* (12.6 kPa). FA modulation effect was not obvious on stiff substrates (308 kPa) (a, b, c, right). Relative gene expression levels were normalized to non-patterned group indicated by the dashed line. Bars represent mean $\pm$ standard deviation. The asterisks indicate statistic significant difference ($p < 0.05$) compared with non-patterned groups. The small panels in (d) were vinculin (green) and F-actin (red) staining indicating how patterns affect FA development. Scale bar in (d) showed 10 μ m. Adapted with permission from ^[107]. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

On intermediate PDMS (12.6 kPa), S3.6 pattern did not elicit obvious effect on the differentiation of cells. However, the trend was completely different when cells were cultured on the same substrate (12.6 kPa) but with L20 pattern. Up regulation of myogenic markers: MyoD1, GATA-4, MYH7, and cTNT, by 2-3 times was evident but no significant effect on neurogenic and osteogenic lineage commitment was observed. To further confirm that the up-regulated myogenic genes were functionalized at protein level, we employed PLA to quantify the expression of MHC (coded by MYH7).

PLA technology is an advanced immunostaining technique (OLink Biosciences, <http://www.immunoport.com/modules.php?name=News&file=article&sid=205>) makes

it possible to visualize, localize, and quantify individual protein interactions using standard fluorescence microscopy. It shows the MHC in red dots, which enable us to do the quantification instead of continuous stress fiber structure in normal immunostaining. From Fig. 4.10a and 4.10b, there was an apparent increase in the MHC expression (red dots indicted by arrow) for the cells cultured on L20 pattern of 12.6 kPa substrate stiffness as compared to the control. When the expression was quantified using PLA dots density, it showed a dramatic increase by nearly 76% (Fig. 4.10c) as compared to hMSCs on non-patterned PDMS. Conventional fluorescent immunostaining of MHC (Fig. 4.1d), MAP2 (neurogenic marker, Fig.4.10e), RunX2 and Ocal (osteogenic markers, Fig.4.10f-g) was also performed, but only MHC showed significant increased expression, indicating specific myogenesis. The images of positive and negative control in Fig. 4.11 showed the antibodies used worked well.

Figure 4.10 L20 significantly increased MHC expression on PDMS substrates (12.6 kPa) verified by PLA. MHC (red, typical dots indicated by white arrow) of cells on L20 patterned (indicated by the small panel at the upper right) 12.6 kPa PDMS (a) showed a higher expression than that of cells on non-patterned (b) 12.6 kPa PDMS. (c): Statistical study of MHC density. $\#p < 0.01$ ($n = 26$). (d-f) showed conventional immunostaining images of MHC (d), MAP2 (e) RUNX2 (f) and Ocal (g). Scale bar showed 50 μm . Adapted with permission from ^[107]. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Figure 4.11 Negative and positive controls used in immunostaining. Cells in panel a and d are hMSCs, b-c are human osteosarcoma cells. (a) negative control of MHC PLA. Cells were stained without MHC primary antibody. Positive controls of osteo-markers RUNX2 (b) and Ocal (c). (d) positive control of MAP2. Scale bar showed 50 μm . Adapted with permission from ^[107]. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

On stiff PDMS substrate (308 kPa), cells did not significantly change their genes expression of the three lineage markers studied. But all genes were down regulated in both S3.6 and L20 groups. These results clearly showed that there was an interaction between the matrix stiffness and FA patterning on cell differentiation. If these physical conditions were optimally formulated, the differentiation towards a certain lineage could be induced as shown in our studies for myogenesis (elongated FA on substrate with a stiffness of 12.6 kPa). However, if one of the physical cues was too dominant (very stiff substrate in our case), it would suppress the effects of other physical cues (patterning in

our case). In addition, it was interesting to find that in the myogenic differentiation of hMSCs, the morphology of FA is more important than its density. Cells with elongated FA induced by L20 pattern showed higher expression in myogenic markers at both transcript and translation levels while cells with denser FA induced by S3.6 pattern did not show any myogenic differentiation. It corresponds to previous reports showing that the FA density reduced with the myogenic differentiation of hMSCs ^[115].

From above results, we postulated that elongated FA and consequently aligned cytoskeleton were strongly correlated to the stem cell lineage commitment. On 12.6 kPa, L20 patterned PDMS substrate, cells showed elongated FA indicated by statistical studies in Fig.4.8b and c, and immunostaining images in Fig. 4.8e. Since FA was linked to actin filament, elongated FA of cells on L20 patterned PDMS which induced aligned cytoskeleton compared to non-patterned group as shown in Fast Fourier Transform (FFT) images in Figs 4.12b and 4.12d (generated by ImageJ). Previous reports have shown that elongated “supermature” FAs (suFAs) were found during human myofibroblast differentiation ^[116]. These suFAs regulated stress fiber formation and cellular tension ^[117]. In our study, we found that elongated FAs would recruit integrin β_3 (ITB3) to form larger and elongated clusters, and increase the expression of myosin light chain kinase (MLCK), which is a contraction indicator of cells (Figure 4.12e). Therefore, both elongated FA and aligned cytoskeleton were postulated to contribute to up-regulation of myogenic markers as depicted in the model presented in Fig. 4.12f. In the model, elongated FAs could transmit the mechanical signals into the cell nucleus ^[118], which in turn activate cell response such as recruitment of integrin cluster formation, alignment of stress fiber in a particular direction, and also recruiting MLCK to exert the appropriate cellular tension for myogenesis ^[119]. MLCK is actively involved in cellular contraction regulation. It cooperates with myosin-IIA, which would then associate with the actin filament to create contraction along the cytoskeleton and promote stress fiber assembly, and further affect

cell lineage commitment ^[119]. Higher expression of MLCK in cells with elongated FA (Fig. 4.12e) indicated higher contraction inside the cell as compared to that of the un-patterned group. The actin cytoskeleton which was closely associated with cellular tension might also play an important role ^[67]. In native myotubes, cytoskeleton is found to be well organised and aligned which is logical as it helps in the function of anisotropic contractility and tension ^[120]. However, the tension should be in optimal range which may trigger some signalling pathway necessary for myogenesis and matrix stiffness which will affect the cytoskeleton tension and FA development should be within myogenic environment as well. The potential signalling pathway involved in this study will be presented in the next Chapter.

Figure 4.12 Elongated FA and proper cellular tension induced hMSCs myogenesis. (a) F-actin of L20 patterned hMSC on PDMS (12.6 kPa) was manually marked. (b) FFT of L20 showed specific alignment of cytoskeleton. (c) F-actin of non-patterned hMSCs on PDMS (12.6 kPa) show dis-organized arrangement. (d) FFT of (c) showed random distribution of cytoskeleton. (e) showed that hMSCs on L20 patterned 12.6 kPa PDMS exhibited higher expression of MLCK and integrin β_3 (ITB3) compared with cells on 12.6 kPa un-patterned PDMS. Scale bar showed 50 μm . (f) schematic chart showed the interplay between FA modulation, cellular tension, matrix stiffness and cytoskeleton arrangement. Adapted with permission from ^[107]. Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

4.4 Conclusions

In summary, our results showed that elongated FA and aligned cytoskeleton are essential for cell commitment towards myogenesis. By optimally combining FA patterning (to induce elongated FA) with matrix stiffness, we have shown for the first time that there is a synergistic effect on specific myogenic differentiation. This upregulation involved elongated FAs which would recruit integrin β_3 and MLCK, thereby increasing the cellular tensions and promoted myosin heavy chain synthesis. It is necessary to note that this specific lineage commitment took place in the absence of any induction medium but purely driven by manipulation of cell-materials interaction. This work will prove to be a useful tool to induce stem cell differentiation in the future especially in the field of cardiac tissue engineering where scaffolds could have two fold functions of support and at the same time as a platform to modulate stem cell differentiation.

Chapter 5 Insights of Focal Adhesion

Modulation Induced Stem Cell Differentiation

5.1 Introduction

In Chapter 4, we found that the elongated FA could be modulated on PDMS substrate with a stiffness of 12.6 kPa and hMSCs with elongated FA committed to myogenic lineage. However, there are still several drawbacks of the PDMS platform. First, the surface of PDMS substrate is bio-inert and it requires several steps to do surface modification to activate protein binding. The surface modification procedures are complex and the efficiency is low. Secondly, the PDMS polymer is very tacky and not easy to handle. It must be cured in Petri-dish and that will make the following experiments such as immunostaining and imaging very troublesome. Therefore, in this study, we changed to PA gel, a hydrogel substrate which is easy to handle (not tacky and can be cross-linked on cover slips) and can be surface modified efficiently. Using this new substrate, we moved on to explore the role of elongated FA and the mechanistic study.

As reviewed in Chapter 2, the interactions between cell and implant materials are mainly mediated by FA complex ^[80]. The FA complex contains signaling components such as different types of integrins and FA kinase, which sense the microenvironment and initiate signal transduction through downstream effectors such as RhoA and Rac1 ^[121]. FA may activate RhoA signaling pathways and result in the synthesis and re-organization of actin filaments as well as stress fiber formation, which in turn regulates FA assembly ^[122]. To create contraction in the cytoskeleton for myogenic differentiation, it is well known that

myosin light chain kinase (MLCK) is needed to phosphorylate myosin light chain (MLC) and actomyosin II ^[123]. It has also been shown that elongated FA would increase the expression of MLCK and cellular tension ^[19]. In Chapter 4, we found that integrin β_3 was recruited to elongated clusters in the cells with elongated FA. Integrins consist of 24 known sub-units and serve as transmembrane mechanical links from those extracellular contacts to the cytoskeleton inside cells ^[124]. However, each pair of α and β of integrin plays a different role in substrate sensing. For example, $\alpha_2\beta_1$ -integrin modulates epithelial differentiation and β_3 -integrin mediates myogenic differentiation of C2C12 cells ^[125]. Therefore, in the mechanistic study in this Chapter, we mainly focused on RhoA signaling pathway, MLCK, and the role of integrin.

In this work, we developed a novel bio-inspired PA gel platform to combine FA modulation, matrix stiffness, and ECM ligands to synergistically influence hMSCs toward specific myogenic differentiation. Results showed that hMSCs with elongated FA would go to -myogenic differentiation. This was achieved by recruitment of integrin β_3 cluster and activation of RhoA signaling in a tension dependent manner. In order to demonstrate that the myogenic differentiation is primarily driven by elongated FA and not cellular shape, we carried out an experiment to decouple the two effects. Similar shaped hMSCs with different FA morphology and distribution were induced using micropatterning technique and it was found that the elongated FA was more supportive of myogenic differentiation. Collectively, this study demonstrates a novel and feasible method of inducing myogenic differentiation of hMSCs and improves the PDMS platform described in Chapter 4.

5.2 Materials and Methods

5.2.1 Substrate fabrication and surface activation

Polyacrylamide (PA) gel was made according to the method described by Engler et al.^[126]. Briefly, aminosilanated coverslips (diameter was 22 mm) were obtained by treating with 0.1M NaOH, 3% 3-aminopropyltriethoxysilane (APTES) (Sigma) in deionized (DI) water and 0.5% (v/v) glutaraldehyde (Sigma) in phosphate buffer saline (PBS) as described in Figure 5.1a. PA gel premix solution were mixed with 1/100 (v/v) of 10% ammonium persulfate and 1/1000 (v/v) of N,N,N',N'-tetramethylethylenediamine (Bio-rad), and 40 μ l of the mixture was immediately added onto a clean transparency following by placing the aminosilanated cover slip on the drop of solution. The premix solution for PA gel with a stiffness of 40.7 kPa was 8% acrylamide and 0.48% bis-acrylamide. The premix solution for PA gel with a stiffness of 10.2 kPa was 10% acrylamide and 0.1% bis-acrylamide.

Figure 5.1 Schematic diagram showed PA gel fabrication (a) and surface activation process for FN patterning (b) and COLI patterning (c). d) Showed PA thickness characterization image. Typical immunostaining image of patterned COL on 10.2 kPa gel taken by confocal microscopy was shown. Adapted with permission from ^[127]. Copyright 2012 Mary Ann Liebert, Inc.

Thickness of PA gel substrates was assured larger than 20 μm by immunostaining of the coated protein and then using confocal microscope (Leica) to observe the cross section according to the protocol previously described and image was shown in Figure 5.1d ^[128]. The shear elastic modulus of the PA gel was measured at 37 °C using a Rheometer (Physica MCR 501, Anton Paar) with a parallel plate according to the instruction provided by the manufacturer. Frequency sweeps between 0.01 and 10 $rad\ s^{-1}$ were carried out at low strains (0.1-10%) which have been shown to be within the linear

viscoelastic range of the measured samples. The surface activation procedure was shown in Figure S1b-c. To activate PA gel surface for FN patterns, 0.2 mg mL^{-1} N-Sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (Sulfo-SANPAH) (Pierce) in 50 mM HEPES buffer (pH 8.5) was added onto PA gel surface and were irradiated under 365 nm ultra violet (UV) (VL-215 LC, Vilber Lourmat) for 10 min, followed by washing with 50 mM HEPES buffer. To activate PA gel surface for collagen patterns, 80% hydrazine hydrate (N_2H_4 , Sigma) was added onto PA gel surface for 8 h at room temperature followed by washing with 5% acetic acid and deionized (DI) water. N_2H_4 was used instead of Sulfo-SANPAH because COLI would form fiber in HEPES buffer with a pH larger than 7. All activated PA gel substrates were dehydrated at 60°C for 30 min before use.

5.2.2 Microcontact Printing

To pattern fibronectin, human fibronectin (FN) ($50 \text{ }\mu\text{g mL}^{-1}$, BD bioscience) solution was added onto each stamp for 1 h then blown dry with nitrogen gas. To pattern collagen, collagen from rat tail (COLI) ($200 \text{ }\mu\text{g mL}^{-1}$, Invitrogen) was oxidized with 4 mg mL^{-1} sodium periodate in 50 mM sodium acetate buffer for 30 min according to the method described before ^[129]. Afterwards, the COLI solution was added onto stamps for 1 h followed by blowing dry with nitrogen gas. The stamps with FN or COLI were then gently pressed on activated PA gel for 30 min followed by incubation with 2% bovine serum albumin (BSA, Invitrogen) at 37°C for 1 h for blocking. Lastly, PA gel was washed with PBS and UV for 15 min for sterilization before cell culture. The adsorption of COLI and FN on PA gel with different stiffness was tested by LUCY® 506 solution (Sigma) and was analyzed by using ImageJ. The fluorescent intensity data was shown in Figure 5.2a.

5.2.3 G-LISA Test and MLCK Inhibition Assay

hMSCs on patterned and patch 10.2 kPa PA gel with COLI were lysed with 50 μ l of lysis buffer (Cytoskeleton Inc) and cell lysate was collected on ice. Five replicates were collected for each sample type. G-LISA (Cytoskeleton Inc) is a novel small G-protein activation assay and it was used to measure the activation of RhoA and Rac1 on 10.2 kPa COLI patterned groups in this work according to manufacturer's instruction. All the tests were done in triplicate and the absorbance was read with a microplate spectrophotometer (Tecan) at the wavelength of 490 nm. MLCK was blocked by 10 μ M of 1-(5-Iodonaphthalene-1-sulfonyl) homopiperazine (ML-7, Merck). Briefly, ML-7 was dissolved in dimethylsulfoxide (DMSO) to make 10 mM stock solution. On day 3 after cell seeding, 10 mM ML-7 was diluted in complete DMEM culture medium to a final concentration of 10 μ M to make the inhibition medium, and replace the normal DMEM in the culture plate. The blocking medium was replaced every two days. As for control group, cells were incubated with complete medium supplement with 0.1% DMSO. On day 7, cells were fixed for immunostaining.

5.3 Results and Discussion

5.3.1 Polyacrylamide Gel Characterization and Micropatterning Validation

The average shear storage moduli G' of the PA gel fabricated were 13.7 ± 0.93 kPa and 3.43 ± 0.06 kPa, respectively. As the Poisson ratio of polyacrylamide gel is 0.48^[130], so the Young's modulus E of the PA gel was about 40.7 ± 2.8 kPa and 10.2 ± 0.2 kPa, respectively. The patterned proteins on PA gel were immunostained and shown in Fig. 5.2b-e. Fluorescent images showed that proteins were evenly patterned on PA gel with different stiffness.

Figure 5.2 Fluorescent intensity of COLI and FN on 40.7 and 10.2 kPa PA gel showed almost same absorption of protein on PA gel with different stiffness. Experiment was done by LUCY® 506 solution (Sigma) according to manufacturer's protocol and fluorescence intensity was calculated with ImageJ. Fluorescent images of patterns on COLI 10.2 kPa (b), COLI 40.7 kPa (c) PA gel, FN 10.2 kPa (d) and FN 40.7 kPa PA gel indicated that the protein was patterned evenly on PA gel substrates. Scale bar showed 50 μm. Adapted with permission from ^[127]. Copyright 2012 Mary Ann Liebert, Inc.

5.3.2 Elongated FA was successfully modulated cooperatively with substrate stiffness and ECM ligands

The morphologies of the stamps used for patterning were shown in Figure 5.3a-c. Pattern L20 (12, 6) consisting of rectangular islets of size $20 \times 2 \mu\text{m}$, was designed to induce elongated FA according to a previous report ^[19]. In Chapter 4, the spacing between islets is the same (both vertical and horizontal spacing are $6 \mu\text{m}$). Here, we optimized the vertical spacing by changing it from $6 \mu\text{m}$ to $12 \mu\text{m}$ to guide cell growth along the direction of higher ECM density, i.e. denser islet to form elongated FA. The square islets in patterns S6.3 (12, 6) and S6.3 (6, 6) as shown in Figure 1b-c had the same dimension of $6.3 \times 6.3 \mu\text{m}$ while maintaining similar area with the L20 pattern. However, the horizontal spacing between the islets was different (6 and $12 \mu\text{m}$ respectively) and this was designed to explore the effects of spacing on cell behaviors.

Analysis of vinculin and F-actin expression (Figure 5.3d-g) provided insights on how the patterns, stiffness and ECM ligands affected hMSCs FA and cytoskeleton development. In Figure 5.3d-g, elongated FAs were successfully modulated via patterning of collagen type I (COLI) onto PA gel with a stiffness of 10.2 kPa (COLI-L20 (12, 6), Figure 5.3d) and FN onto PA gel with a stiffness of 40.7 kPa (FN-L20 (12, 6), Figure 5.3g) respectively, by using pattern L20 (12, 6). The elongated FA was accompanied with elongated cell shape as well. Relatively sparse FA was observed in L20 (12, 6) patterned groups compared with patch group (PA gel surface was patterned with an even homogeneous layer of protein by a flat PDMS stamp) (Figure 5.3d-g). As expected, densely distributed but not elongated FA was successfully constructed using pattern S6.3 (12, 6) and S6.3 (6, 6). Pattern S6.3 (12, 6) induced densely distributed FA, hMSCs also have adopted elongated cell shape just as those found in cells patterned with L20 (12, 6)

but lower in aspect ratio (AR). This suggested the spacing effects could induce elongated cell shape and align cytoskeleton over the vertical spacing (6 μm) in pattern S6.3 (12, 6), the direction which has higher density of ECM ligand. The observation of similar cell shape but different FA development may suggest difference in lineage commitment. Besides that, micropatterning effect on FA development in hMSC was observed to be dependent on the combination effect of substrate stiffness and ECM ligands. Although elongated FA was observed on PA gel of stiffness 10.2 kPa and surface patterned with COLI-L20 (12,6), this observation is only present on PA gel of 40.7 kPa stiffness when FN was used for patterning; i.e. FN-L20 (12,6). However, the FA AR and length were compromised in FN-L20 (12, 6) group.

Figure 5.3 Different micropatterns and their effects on hMSCs FA development. a) Pattern L20 (12, 6). b) Pattern S6.3 (12, 6). c) Pattern S6.3 (6, 6). The scale bar in SEM images (a-c) showed 10 μm. d-g) Immunostaining images of vinculin (green), F-actin (red), cell nucleus (blue), and pattern (red) showed FA and cytoskeleton development on PA gel with different stiffness and ECMs patterns. The scale bar in (d- g) showed 50 μm. Adapted with permission from ^[127]. Copyright 2012 Mary Ann Liebert, Inc.

To better quantify the observations mentioned above, statistical study of FA area, FA aspect ratio (AR), FA length, and FA density was performed and shown in Figure 5.4. On COLI-L20 (12, 6) patterned group, the FA of hMSCs on PA gel with a stiffness of 10.2 kPa, showed 40% increase in FA AR and 60% increase in length (Figure 2b and 2c, left). COLI-S6.3 (12, 6) and COLI-S6.3 (6, 6) showed less influence on FA shape but 20% increase in FA density (Figure 2d) both on PA gel with stiffness of 10.2 kPa and 40.7 kPa.

On FN-L20 (12, 6) patterned group, successful elongated FA modulation could only occur on PA gel with a stiffness of 40.7 kPa. However, it showed relative lower FA AR and FA length compared with cells on COLI-L20 (12, 6) patterned group.

Figure 5.4 Statistical study of FA development. * $p < 0.05$ between the pattern and patch group with the same kind of pattern protein. # $p < 0.05$ between COLI and FN group with PA gel of same stiffness. 60 cells were analyzed in each group. Adapted with permission from ^[127]. Copyright 2012 Mary Ann Liebert, Inc.

The observations were in line with our previous study which was performed on PDMS in Chapter 4. More importantly, L20 (12, 6) pattern made significant improvement on FA AR and length modulation. This is an important indication showing that the FA could be successfully manipulated via micropatterning with optimal combination of matrix compliance and ligand type.

5.3.3 Elongated FA Influenced Specific Myogenic Differentiation of hMSCs

In Figure 5.5a, COLI-L20 (12, 6) group showed significantly up-regulation of myogenic marker MyoD1 and cardiac myogenic markers GATA4 and MYH7 by 3-4 folds on PA

gel with a stiffness of 10.2 kPa but not on the PA gel with a stiffness of 40.7 kPa. However, neither S6.3 (12, 6) nor S6.3 (6, 6) could induce specific myogenic differentiation on PA gel, regardless of the substrate stiffness. Immunostaining was further employed to determine the above up-regulated genes at translational level. As shown in Figure 5.5c, cardiac myosin heavy chain (MHC), which is coded by MYH7, was only significantly expressed in cells cultured on PA gel with a stiffness of 10.2 kPa patterned with COLI-L20 (12, 6). Beside MHC, immunostaining of α -sarcomeric actin which was specific for α -cardiac muscle actin was also carried out and result showed a high expression in hMSCs with elongated FA (Figure 5.6).

Figure 5.5 Differentiation characterization showed elongated FA could significantly induce myogenic differentiation. Real-time PCR result showed L20 (12, 6) specific up regulated myogenic markers on PA gel with a stiffness of 10.2 kPa in the present of COLI (a) compared with FN (b). The gene expression was normalized to patch group. * showed significant difference in gene expression compared with patch group of same patterning protein. c-f) Immunostaining of cardiac myosin heavy chain (green) and nucleus (blue). Scale bar showed 50 μ m. (c) showed obvious expression of MHC in L20 (12, 6) on PA gel with a stiffness of 10.2 kPa. g) Statistical study of the MHC expression percentage (Cells with positive MHC staining/total cell number) demonstrated a higher expression of MHC in cells with elongated FA. 200 cells were analyzed in each group and the expression was done in triplicate. * $p < 0.01$. Adapted with permission from ^[127]. Copyright 2012 Mary Ann Liebert, Inc.

When FN was used as the ECM ligand (Figure 5.5b), pattern FN-L20 (12, 6) only induced myogenic differentiation on PA gel with a stiffness of 40.7 kPa, but not as obvious as on PA gel with a stiffness of 10.2 kPa and in the presence of COLI in terms of expression fold as well as specificity since up-regulation of osteogenic marker BSP and neurogenic marker MAP2 were also observed. In contrast, pattern FN-S6.3 (12, 6) and FN-S6.3 (6, 6), which induced densely distributed FA, could only up-regulate osteogenic but not myogenic markers. The expression of MHC in immunostaining in Figure 5.5 f confirmed that the above up-regulated myogenic marker could functionalize at protein expression level.

Statistical study in Figure 5.5g semi-quantified the expression of MHC under different culture conditions. COLI-L20 (12, 6) patterned PA gel with a stiffness of 10.2 kPa, showed the highest percentage of MHC expression (cells with positive MHC staining/total cell number). It was 3.6 times higher than that in patch group and also much higher than that in other patterns and FN groups. This further supports that the modulation of FA via micropatterning could potentially induce hMSCs lineage commitment to specific myogenic differentiation at translation level and the results are in line with the PCR data of cells on PDMS substrates in Chapter 4.

Figure 5.6 Immuno-labeled of cardiac α -sarcomeric actin of hMSCs on COL-L20 patterned hMSCs (a) and patch (b) group with a stiffness of 10.2 kPa. (c) and (d) showed negative control of Alexa Fluor® 488 goat anti mouse IgG and Cy3 goat anti rabbit IgG, respectively. Scale bar showed 50 μ m. Adapted with permission from ^[127]. Copyright 2012 Mary Ann Liebert, Inc.

5.3.4 The Roles of Integrin Cluster, RhoA Pathway, and Myosin Light Chain Kinase during Myogenic Differentiation

Mechanistic study has been performed to decipher the potential biomolecular basis of hMSCs differentiation induced through elongated FA, which was constructed under our optimal condition that is COL-L20 (12, 6) patterned on PA gel with a stiffness of 10.2 kPa. In Chapter 4, integrin β_3 (ITB3) in cells with elongated FA on L20 patterned PDMS with a stiffness of 12.6 kPa showed elongated cluster (Figure 4.12). In this study when substrate was changed to PA gel, real-time PCR result highlighted the importance of ITB3 and myosin light chain kinase (MLCK) through their significant up-regulation in

expressions shown in Figure 5.7a. Immunostaining images in Figure 5.7 further indicated higher expression of ITB3 in COLI-L20 (12, 6) patterned group compared with that in the patch control group. Interestingly, ITB3 was recruited to form elongated clusters when hMSC was cultured under our optimal condition which was also found in L20 patterned PDMS substrate with a stiffness of 12.6 kPa as shown in Figure 4.12. RhoA and Rac1 were two of the most important signaling pathways which would transmit the extracellular signals into nucleus and affect hMSC lineage commitment^[67]. According to the G-LISA result shown in Figure 5.7b, the activation of RhoA in hMSC on COLI-L20 (12, 6) patterned PA gel with a stiffness of 10.2 kPa was increased by 80% compared with that in patch group. However, there was no difference in the activation of Rac1 between them. It implied that RhoA was activated by elongated FA and might be responsible for transmitting the sensed niche signal in our optimal condition into the nucleus.

Figure 5.7 Mechanism study of cells on 10.2 kPa PA gel showed elongated FAs induced hMSCs myogenesis was related to ITB3 and MLCK recruitment and RhoA pathway activation. a) Real-time PCR result of hMSCs on 10.2 kPa showed L20 (12, 6) recruits ITB3 and MLCK compared with patch group. The gene expression was normalized to patch group. * showed significant difference in gene expression compared with patch group. b) G-LISA test showed L20 (12, 6) activates RhoA but not Rac1 signaling pathway. c) Immunostaining of ITB3 confirmed the up-regulated ITB3 was expressed at protein level and formed elongated cluster compared with patch group on 10.2 kPa PA gel. d) Control groups for MLCK inhibition study with only 0.1% DMSO but no ML-7 inhibitor. Immunostaining images showed elongated FAs up-regulated MLCK. e) MLCK was inhibited by 10 μ M of ML-7 in DMSO but the vinculin and central stress fiber was not much affected. f) Immunostaining of MHC showed elongated FAs need MLCK to induce myogenesis. Scale bar in (c-f) showed 50 μ m. Adapted with permission from ^[127]. Copyright 2012 Mary Ann Liebert, Inc.

Elongated FA recruited ITB3 clusters and activated RhoA signaling pathway, so it was logical to follow up the response from cell nucleus after receiving the signal. The most obvious outcomes were the cytoskeleton development, as well as MHC maturation shown in Figure 5.3d and 5.5c. Under optimal condition which significantly showed MHC expression, the F-actin was observed to have formed stress fiber and aligned with the cell longitudinal axis, which was also in the same direction with less spaced pattern islets. Such kind of less compact yet aligned and organized F-actin formation was typically seen in fully differentiated myotubes ^[131]. This is in contrast with the F-actin which was randomly distributed when cultured on other patterns.

Here, the gene expression of MLCK, α -smooth muscle actin (SMA) and α -skeletal muscle actin (ACTA1) were examined. Generally, MLCK is known to phosphorylate the serine-19 residue of myosin regulatory light chain which would enhance the actomyosin contraction via RhoA pathway ^[132]. SMA is localized in microfilament bundles, strengthening the contraction of smooth muscle cells, but not in cardiac myocytes ^[133]. ACTA1 is a major constituent of the contractile apparatus which can be found in skeletal muscle but poorly expressed in cardiac myocytes ^[134]. However, as shown in Figure 5.7a, only up-regulation of MLCK was observed. SMA and ACTA1, which were smooth/skeletal muscle markers for smooth/skeletal muscle differentiation ^[135], were not up-regulated. It corresponded well with real-time PCR result in Figure 5.5a, that elongated FA could induce myogenic differentiation and indicated that hMSCs may be able to differentiate into cardiac muscle like cells.

The FA and cytoskeleton development has a direct relationship to the cellular traction ^[136]. To examine our proposed idea that the elongated FA affect cytoskeleton development and arrangement in a tension dependant manner, we used ML-7 to inhibit MLCK function and

reduce cellular tension, while maintaining the central stress fiber structure and FA development (Figure 5.7e lower panel) ^[137]. As a consequence, MLCK expression could be efficiently inhibited by 10 μ M of ML-7 compared to control group (Figure 5.7d and e). Similarly, cells cultured with ML-7 showed lower expression of MHC compared to its control group (normal MHC expression as shown in Figure 5.7f). From this inhibition study we have demonstrated that elongated FA could affect cytoskeleton MHC maturation in a cell tension dependant manner via MLCK.

5.3.5 Elongated FA versus Elongated Cell Shape

Many studies have extensively investigated the modulation of cell shape on hMSCs differentiation, such as using strip patterns to guide myogenic differentiation of hMSCs ^[68, 96]. It was an efficient way to induce myogenic differentiation of stem cells via manipulating cell shape. As the cells in the L20 pattern also adopt a generally elongated cellular morphology, there is a need to demonstrate that the differentiation in this study is indeed caused by elongated FA and not cellular morphology. In order to decouple the two effects on hMSCs differentiation, we designed two micropatterns that could induce similar elongated cellular morphology but with different FA development.

Figure 5.8 The relationship of FA and cell shape in hMSCs myogenic differentiation.

a) Two patterns named *Elongated* and *Dense* induced elongated and dense FA respectively. Scale bar in SEM images showed 10 μm. b) Statistical study confirmed elongated FA and dense FA were modulated in cells with similar strip like cell shape. c) Real-time PCR result showed specific and higher myogenic differentiation by elongated FA. The gene expression was normalized to patch group. * showed significant difference

in gene expression compared with patch group. d-f) showed immune-labeled of MHC in pattern *Elongated*, *Dense* and patch group respectively. Scale bar in all fluorescent images showed 50 μm . Adapted with permission from ^[127]. Copyright 2012 Mary Ann Liebert, Inc.

Both patterns could promote elongated cell shape as shown in Figure 5.8a. The pattern named *Elongated* (upper) was designed to induce elongated FA while pattern named *Dense* (lower) was designed to induce densely distributed (dense) FA. Statistical analysis of immune-labeled vinculin of hMSCs cultured on COLI-patterned PA gel with a stiffness of 10.2 kPa has been employed in order to semi-quantify the distribution of FA and cell area. Figure 5.8b showed 72% increase in FA length and 51% increase in FA AR for pattern *Elongated* compared to patch group. For pattern *Dense*, it mainly increased FA density by 59% but no elongated FA was observed. Similar cell AR indicated both patterns have similar effect on cell shape formation. Hence, cell shape would pose minimum, if any, effect on hMSCs differentiation in this study.

Interestingly, although hMSCs on both patterns have adopted similar cell shape, pattern *Elongated* which induced elongated FA showed significant specific up-regulation in myogenic markers over pattern *Dense* (Figure 5.8c). This suggested that elongated FA tend to have more effect on induction of specific myogenic differentiation over elongated cell shape. The up-regulated expression of MYH7 was confirmed at translation level by immunostaining of MHC as shown in Figure 5.8d. However, no obvious up-regulation of MHC was observed in pattern *Dense* group (Figure 5.8e) compared with patch group (Figure 5f). The expression of MLCK and the inhibition study of cells in these groups were also performed as shown in Figure 5.9. Results showed higher expression of MLCK in *Elongated* patterned cells (Figure 5.9a) and cells with inhibited MLCK did not show expression of MHC (Figure 5.9c). Therefore, the induction of myogenic differentiation by *Elongated* pattern was also in a tension dependent manner.

Figure 5.9 Inhibition study of MLCK. Scale bar showed 50 μm . Adapted with permission from ^[127]. Copyright 2012 Mary Ann Liebert, Inc.

Given their unique regenerative abilities, stem cells offer brilliant potentials for treating diseases such as cardiac failure, muscle damage and neurological disorders, etc. ^[138]. However, much work remains to be done at the bench before real therapy could be realized. One of the critical issues is how to control cell differentiation. In our study, cellular morphology was not restrained but the FA development was manipulated using combination of different patterns, ECM and matrix stiffness. We have successfully modulated elongated FA via micropatterning using COLI-L20 (12, 6) on PA gel with a stiffness of 10.2 kPa. However, this micropatterning effect was only observed on substrate with a stiffness of 40.7 kPa in the presence of FN. Similar phenomenon was observed in previous study showing a shift of the highest myogenic MyoD1 expression level when changing from COLI-coated PA gel (highest myogenic MyoD1 expression on 10 kPa) to FN-coated PA gel (highest myogenic MyoD1 expression on 25 kPa) ^[139]. Distinct surface chemistry has different ability to resist cell traction forces, which would be associated with changes of cell behaviors such as cell attachment, spreading, and differentiation by affecting downstream signaling pathways ^[140]. Through actin stress fiber development, cells exerted higher traction force on collagen coated substrate than on

fibronectin coated substrate ^[141]. This helps to explain our observation as patterning effects would only be realized with the presentation of optimal stiffness and ECM protein. The lineage commitment of hMSC was subjected to optimized condition (COLI-L20 (12, 6)-on PA gel with a stiffness of 10.2 kPa) corroborated our hypothesis. A major function of FA was to transmit the extra cellular signal into nucleus, resulting in proper cellular response to the microenvironment. Elongated FA which was found in mature human myofibroblast was able to increase phosphorylation of FA kinase and regulate stress fiber development in myogenic differentiation ^[117]. Under optimal conditions, recruited FA components such as ITB3 and vinculin formed elongated clusters along the micropatterns as the response to the modulated ECM environment. Thereafter, the elongated FA would bring about the changes in downstream signaling pathways which triggered the myogenic differentiation process ^[142].

Subsequently, the mechanism study revealed that RhoA pathway, instead of Rac1, was activated by the elongated FA. Rac1 activation was responsible for the formation of lamellipodia and membrane ruffles, and the loss of stress fibers ^[142]. In contrast, RhoA could promote the formation of integrin clusters, actin polymerization, and myosin activity ^[143]. More importantly, RhoA signaling pathway activated MLCK and non muscle myosin (NMM) to increase tension in cytoskeleton, which resulted in the myogenic differentiation of hMSC ^[67]. The vital role of MLCK in hMSC myogenic differentiation induced through elongated FA has been determined from immunocytochemical analysis and inhibition study. MLCK could trigger MLC phosphorylation to enhance actin-myosin interaction, which in turn increased the cell tension ^[144]. Without that, cells may maintain the cytoskeleton structure but lose the cellular tension ^[145]. As the result, hMSCs with MLCK inhibited was unable to commit to myogenic differentiation in our study.

The behavior of hMSCs on PA gel and PDMS substrates (Chapter 4) of similar stiffness was comparable. Both substrates with a stiffness of around 11 kPa could support elongated FA development and myogenic differentiation via L20 pattern. The mechanism is also similar which is due to the recruitment of integrin β_3 , activation of RhoA pathway, increase in MLCK expression and contractility when COL1 was used as the ink for patterning. As both surfaces were patterned and passivated with the same ECM and blocking agent respectively, the surface chemistry effect will be minimal if any. Therefore, the functionality of these 2 substrates in this study is similar. This indicates that our micropatterning platform is versatile and PA gel substrate is recommended only because it is easier to prepare and there is no auto-fluorescence issue that interferes with immunostaining results.

Apart from that, we employed another two micropatterns to examine the role of FA and cytoskeleton arrangement in hMSCs differentiation. Even though these micropatterns could induce hMSCs to adopt similar cell shape, the one that induced elongated FA showed higher expression in MLCK and superior cardiac myogenic differentiation over the other which induced densely distributed FA. This is the first study to decouple cellular morphology from FA development in affecting hMSCs differentiation. We understand that this observation was brought about by the insufficient cell tension generated to induce myogenic differentiation in the dense but not elongated FA cells; and this was confirmed by the MLCK mechanistic study. Latest report showed that elongated and larger FA would induce cell polarization and higher traction force compared with small FA ^[146]. The formation of small and densely distributed FA might not provide sufficient signals to activate RhoA pathway which could further trigger stress fiber formation to generate sufficient traction force, which results in insignificant myogenic differentiation.

Figure 5.10 A schematic model showed the mechanism of elongated FA induced tension-dependent myogenic differentiation. a) Elongated FA would promote stress fiber formation and cellular tension in five steps. b-c) L20 (12, 6) with elongated FA and stress fiber showed arc-like structure where was full of actomyosin complex and tension. However, this phenomenon was not obvious in patch group or cells without quite elongated FA. Scale bar in (b-c) showed 50 μm . Adapted with permission from ^[127]. Copyright 2012 Mary Ann Liebert, Inc.

Based on our study on PDMS and PA gel substrates, we propose a model in Figure 5.10a to explain the procedure of elongated FA on induction of myogenic differentiation in hMSCs. Firstly, elongated FA would efficiently generate sufficient traction force to recruit actin monomer and gather myosin ^[147]. RhoA signaling pathway was then activated and further promoted actin polymerization. During the polymerization, myosin II-B would act as the cross linker to cross-link actin monomer ^[148]. Afterwards, myosin II-A and MLCK would associate with the actin filament to create contraction along the actin and promote stress fiber formation and further affect biophysical cellular response to regulate cell lineage commitment ^[119]. Previous result showed that cells would build arc-shape structure over the adherent sites, where there was large tension and lots of

actomyosin complex within ^[119]. In our work, we also observed the arc-like structure and well-developed stress fibers in hMSC with elongated FA as shown in Figure 5.10b. However, only a few of the similar structures and fine actin filaments were observed in other conditions (Figure 5.10c-d). Based on previous study and our observations, these arc-shape structures may be induced by elongated FA, accompanied with actomyosin assembly and contractions. The data also supported that elongated FA regulates hMSCs cytoskeleton development and myogenic differentiation in a tension dependant manner. The tension is essential in FA modulated myogenic differentiation; however, it should be in optimal range. Beyond a certain range, tension would not trigger necessary signaling pathways such as RhoA, and fail to form stress fiber to guide hMSCs to myogenic differentiation. Finally, with myosin II-A, actin and myosin would form contraction flow and assemble stress fiber as well as align the cytoskeleton. The evidence was given in Figure 5.10b-d showing only cells in Figure 6b with elongated FA developed bundle like stress fiber. The model explained the potential mechanism of how elongated FA would induce cellular tension change and myosin associated stress fiber formation, both leading to the myogenic differentiation of hMSCs. The interaction study between each factor (substrate stiffness, ECM ligand, and micropatterning in our case) suggested potentially synergistic effect on cell lineage regulation when certain conditions could be fulfilled. The understanding of these effects working in tandem in inducing stem cell differentiation would contribute significantly to the design and progress of tissue engineered devices.

5.4 Conclusions

In this Chapter, a novel bio-inspired PA gel micropatterning platform to influence specific myogenic differentiation via FA modulation was established. Elongated FA was engineered on PA gel with a stiffness of 10.2 kPa using COLI as the “ink” for L20 (12, 6) pattern. The elongated FA initiated significant recruitment of ITB3 cluster and induced stress fiber alignment. In addition, mechanistic study suggested that elongated FA would activate RhoA signaling pathway and recruit MLCK which indicates an enhancement of the cellular tension. Our findings suggest a new and potential platform to manipulate stem cell differentiation via FA modulation. Just as important, through our study, we provided initial understanding of this platform in influencing stem cell lineage commitment. However, more elaborate mechanistic studies may be required for a comprehensive understanding of the phenomena.

Chapter 6 General Conclusions and Recommendations

6.1 General Conclusions

From the literature review and our previous study, we realized that FA is pivotal in cell-materials interaction. Based on that, we hypothesized that it is possible to influence hMSCs differentiation via modulating of FA development. A novel microcontact printing method using polyvinyl alcohol as a trans-print media was invented to successfully pattern protein on tacky/soft substrates. This method can efficiently create patterns on even extremely soft and tacky PDMS and PA gel with a stiffness of 2 kPa and also can create patterns on 3D scaffolds ^[100].

Using this novel trans-printing technique to pattern COLI, and by optimally combining FA patterning (to induce elongated FA) with the stiffness of PDMS substrates, we have shown for the first time that there is a synergistic effect of FA patterning and substrate stiffness on specific myogenic differentiation. The elongated FA was successfully modulated on PDMS with a stiffness of 12.6 kPa but not on hard PDMS with a stiffness of 308 kPa. hMSCs with elongated FAs showed significant and specific up-regulation in myogenic genes and proteins expression. This upregulation involved elongated FAs which recruited integrin β_3 cluster and MLCK, resulted in increase in cellular tensions and promoting myosin heavy chain synthesis. It is necessary to note that this specific lineage commitment took place in the absence of any induction medium or factors but purely driven by manipulation of cell-materials interactions.

Equipped with this knowledge, we developed another platform which used PA gel as the substrate as it is easier to handle and to conduct surface modification compared with PDMS substrate. Beside COLI, FN was also patterned to study the effects of various coating proteins on stem cell differentiation. Elongated FA was engineered on PA gel with a stiffness of 10.2 kPa using COLI as the “ink” for L20 (12, 6) pattern, but no obviously elongated FA was found when “ink” protein changed to FN. The elongated FA initiated significant recruitment of ITB3 cluster and induced stress fiber alignment, in line with our findings in PDMS work. In addition, a further mechanistic study was made and suggested that elongated FA would activate RhoA signaling pathway and recruit MLCK which indicates an enhancement of the cellular tension.

The objectives to explore a novel platform to modulate hMSCs differentiation and understanding of the mechanism behind are well achieved. The PDMS and PA gel studies suggest a new and potential platform to manipulate stem cell differentiation via FA modulation. Just as important, through our study, we provided initial understanding of this platform on influencing stem cell lineage commitment. However, more elaborate mechanistic studies may be required for a comprehensive understanding in future.

6.2 Recommendations

Our study has shown that FA plays an important role in sensing the environment, transmitting the niche signals into the cells, and other fundamental cellular behaviors, including spreading and differentiation ^[80]. The MLCK was up-regulated inside the cells with elongated FA indicating higher cellular tensions in the cell. Therefore, we propose the use of traction force measurement to confirm the correlation between elongated FA and higher tension in cells for future work.

To complete this study of FA, it is also proposed to extend the work to study integrins at nano scale. Previous reports showed that after binding with its ligand in membrane, the cytoplasmic domain of integrin β subunits interacts with FA proteins, resulting in the activation of focal adhesion kinase (FAK) and Rho GTPases ^[149]. The importance of integrin in cell-materials interactions maybe explored in the future.

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Appendix: List of Patent and Publications

Appendix A U.S. Provisional Patent

1. **Yu H.** Tan LP, Method For Micropatterning A Substrate And A Patterned Substrate Formed Thereof. Patent, WO/2012/057706

Appendix B Publications

2. **Yu H**, Xiong S, Tay CY, Leong WS, Tan LP. A novel and simple microcontact printing technique for tacky, soft substrates and/or complex surfaces in soft tissue engineering. *Acta Biomate* 2012;8:1267-72.
3. **Yu H**, Tay CY, Leong WS, Tan SCW, Liao K, Tan LP. Mechanical behavior of human mesenchymal stem cells during adipogenic and osteogenic differentiation. *Biochem Biophys Res Co* 2010;393:150-5.
4. **Yu H**, Tan LP. Cell-Matrix Interaction Study during Human Mesenchymal Stem Cells Differentiation. *IFMBE Proceedings*, 2011; 30:p. 51
5. **Yu, H**, et al. Insights into the role of focal adhesion modulation in myogenic differentiation of human mesenchymal stem cells, *Stem Cells and Development*, in press
6. **Yu, H**, et al. Modulation of hMSCs Differentiation through Matrix Elasticity and Focal Adhesion Regulation, *Advanced Healthcare Materials*, in press
7. **Yu, H** et al. A novel platform to drive hMSCs myogenic differentiation via integrin modulation, *In preparation*
8. **YU, H** et al. Quantitative Characterization of Cell Morphology: Significance, Challenges and Potentials, *In preparation*
9. Leong WS, Pal. M, Tay CY, **Yu H**, et al. Cyclic tensile loading regulates hMSCs differentiation into neuron-like phenotype, *Journal of Tissue Engineering and Regenerative Medicine*, in press
10. Xiong S, George S, Ji Z, Lin S, **Yu H**, Damoiseaux, R France, B, Ng KW, and Loo SCJ, Size of TiO₂ nanoparticles influences their phototoxicity: an in vitro investigation. *Archives of Toxicology*, In press.

11. Xiong S, George S, Yu H, Damoiseaux R, France B, Ng KW, and Loo JSC, Size influences the cytotoxicity of poly (lactic-co-glycolic acid) (PLGA) and titanium dioxide (TiO₂) nanoparticles. *Archives of Toxicology, In press*.
12. Tay CY, Pal M, Yu H, et al. Bio-inspired Micropatterned Platform to Steer Stem Cell Differentiation. *Small* 2011;7:1416-21.
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14. Tay CY, Yu H, et al. Micropatterned matrix directs differentiation of human mesenchymal stem cells towards myocardial lineage. *Exp. Cell Res.* 2010;316: 1159-1168.
15. Tay CY, Gu H, Leong WS, Yu H, et al. Cellular behavior of human mesenchymal stem cells cultured on single-walled carbon nanotube film. *Carbon* 2010;48: 1095-1104.
16. Yeong WY, Yu H, et al. Multiscale Topological Guidance for Cell Alignment via Direct Laser Writing on Biodegradable Polymer. *Tissue. Eng. Part C: Methods* 2010 16: 1011-1021.
17. Yeong WY, Sudarmadji N, Yu H, et al. Porous polycaprolactone scaffold for cardiac tissue engineering fabricated by selective laser sintering. *Acta Biomater.* 6: 2010; 2028-2034.