Modulating stem cell differentiation via cell-material interaction

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**MODULATING STEM CELL DIFFERENTIATION VIA
CELL-MATERIAL INTERACTION**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2016

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Abstract

Human mesenchymal stem cells (hMSCs) have become the popular candidate in the field of regenerative medicines due to unique properties like multipotency, ease of availability and nonimmunogenicity. In the past, various biochemical methods have been employed to regulate the stem cell differentiation but shortcomings such as tumorigenesis and cell death are associated with these methods. Therefore, in recent years, biophysical induction methods are emerging rapidly to control the stem cell lineage commitment. In biophysical induction methods, stem cell behavior can be modulated by regulating the cell-material interactions but detailed study of these cell-material interactions is still limited to date. Here, in the first part, we studied the cell-material interactions by investigating the spatial distribution of integrin- β_1 receptors (ITG- β_1) at micro- and nanoscale level and systematic study of the relationship between spatial distribution of ITG- β_1 and stem cell differentiation (cardiomyogenesis) was performed. We observed the distinct recruitment of ITG- β_1 in hMSCs when hMSCs were committed to myocardial lineage induced by cell patterning. We investigated the spatial distribution of ITG- β_1 using super resolution imaging in those committed hMSCs. Aligned and elongated ITG- β_1 focal adhesions (ITG- β_1 FAs) were found in those committed patterned hMSCs in contrast to short and nonaligned ITG- β_1 FAs in unpatterned hMSCs. Nanoscale distribution study of integrins revealed that ITG- β_1 clusters were uniformly spread within FAs of patterned hMSCs, whereas ITG- β_1 clusters were expressed at the periphery of FAs of unpatterned hMSCs.

Further, we deciphered the decisive role of cell patterning in generating the optimal cytoskeletal tension in hMSCs to induce cardiomyogenic differentiation via mechanotransduction pathways. The cell's mechanical properties (cell stiffness and traction forces) which are indicator of cell cytoskeletal tension were drastically reduced in the committed hMSCs as compared to the non-committed unpatterned hMSCs. This fact suggested the positive correlation between the cell patterning-triggered myocardial differentiation and actomyosin-generated optimal cytoskeletal tension within patterned cells.

In the next part, we utilized the same dimensions and spatial distribution data of ITG- β_1 FAs to design the unique biofunctionalized gold micropatterned platform and reverse engineer the hMSCs differentiation process. The platform was fabricated by following standard photolithography, bioinert polyethylene glycol (PEG) passivation and precise immobilization of ITG- β_1 antibodies to gold pattern lanes. Aligned and elongated morphology was shown by hMSCs cultured on this platform and later these patterned hMSCs displayed end to end fusion to form multinucleated myotubes with continuous actin cytoskeleton after two weeks of culture. Aforementioned results illustrated that cell patterning and ITG- β_1 mediated signaling synergistically promoted the myotubes formation from patterned hMSCs. This ITG- β_1 antibody immobilized micropatterned platform together with hMSCs is a tissue engineered construct and in future, may find use for a wide range of applications, right from muscle tissue engineering to the investigation of stem cell-material interactions to gain insights into signaling pathways involved in stem cell myogenesis.

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Figure 6.4 Biofunctionalized gold micropatterned substrate induces myotubes generation from hMSCs **(a)** hMSCs cultured on gold micropatterned substrate showed aligned and elongated morphology and exhibited end to end connections on day 7. These connected hMSCs fused with each other to form myotubes after two weeks of culture. On the other hand, no such cell-cell connection/fused morphology was observed in unpatterned cells. Double arrow red line indicates pattern direction. Single arrow white line indicates cell nucleus position. **(b)** Aspect ratio is the ratio of cell length to cell width. Patterned cells showed higher aspect ratio than unpatterned cells after 7 days of culture. (n=3), $P < 0.01$. **(c)** Cell nuclei orientation was used to calculate the cell alignment after 7 days of culture. Cell with nucleus orientation less than 20° was considered as aligned cell (n=3). **(d)**

C2C12 murine myoblasts were used as a positive control. Myoblasts fused to form myotubes after three days of culture. Single arrow yellow line denotes myotubes formed. Immunofluorescent image of C2C12 myoblasts illustrated a positive expression of β -MHC (green), a mature myogenic marker which confirmed the myotubes formation from myoblasts. Scale bar: 100 μ m

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Figure 6.6 Investigation of myogenic lineage commitment of cells cultured on biofunctionalized micropatterned substrate. Immunofluorescence imaging confirmed the upregulation of muscle transcription factor (MyoD) in patterned cells after 7 days. C2C12 myoblasts distinctly expressed MyoD after 3 days and used as a positive control. Double arrow white line indicates pattern direction. Scale bar: 100 μ m

Figure 6.7 Cells cultured on FN functionalized substrates experienced more adhesion and high proliferation rate **(a)** Optical images showed nearly confluent cells on FN functionalized substrates as compared to ITG- β_1 antibody functionalized substrates after one week of culture Double arrow white line indicates pattern direction. **(b)** Cells cultured on ITG- β_1 antibody immobilized substrates consistently showed lower cell density than that of FN immobilized substrates after two weeks of culture ($n=3$), $P<0.05$. Scale bar: 200 μ m.

Figure 6.8 Comparative study of cells cultured on ITG- β_1 antibody and ITG- β_3 antibody functionalized substrates. By day 2, many cells attached and showed elongated morphology along with pattern direction on ITG- β_1 antibody immobilized patterned substrate whereas, cells completely failed to recognize pattern direction on ITG- β_3 antibody immobilized patterned substrate. Even more cells attached and retained their characteristic spindle shaped MSCs-like morphology on both ITG- β_1 antibody immobilized surfaces in comparison to ITG- β_3 antibody immobilized surfaces. Double arrow white line indicates pattern direction. Scale bar: 200 μm .

Figure 6.9 Micropatterning induces the formation of elongated ITG- β_1 FAs and aligned actin cytoskeleton and in turn upregulates myogenesis. **(a)** Expression of elongated ITG- β_1 FAs along with aligned actin cytoskeleton was observed in cells from patterned group (first row) after two weeks of culture while, unpatterned cells expressed dot-like ITG- β_1 FAs and nonaligned actin cytoskeleton (second row). Double arrow white line indicates pattern direction. **(b)** Large population of cells cultured on patterned surface followed pattern direction and exhibited upregulation of β -MHC and generation of aligned actin cytoskeleton (first row). On the contrary, few cells that did not follow pattern direction demonstrated downregulation of β -MHC and randomly organized actin cytoskeleton (second row). Double arrow white line indicates pattern direction. Scale bar: 100 μm .

Figure 6.10 Proposed model recapitulating the myotubes generation on biofunctionalized gold micropatterned substrate. **(a)** Biofunctionalized micropattern induces the formation of elongated ITG- β_1 FAs and aligned F-actin stress fibers. Both ITG- β_1 FA mediated signaling and optimal cytoskeletal tension generated by aligned stress fibers synergistically promotes upregulation of myogenesis and in turn myotubes formation from patterned hMSCs. **(b)** In contrast, hMSCs cultured on biofunctionalized plain gold coated substrate expressed dot-like ITG- β_1 FAs along with dense cross-linked network of F-actin stress fibers. Generation of high cytoskeletal tension due cross-linked network of F-actin stress fibers and diminished ITG- β_1 FA mediated signaling lead to failure of myotubes formation from unpatterned hMSCs.

Abbreviations

AFM	Atomic force microscopy
BSA	Bovine serum albumin
β -MHC	Myosin heavy chain
CSK	Cytoskeleton
CTF	Cell traction force
DMEM	Dulbecco's modified Eagle's medium
dSTORM	Direct stochastic optical reconstruction microscopy
ECM	Extracellular matrix
FA	Focal adhesion
FAK	Focal Adhesion Kinase
FN	Fibronectin
hMSC	Human mesenchymal stem cell
ITG- β 1	Integrin β 1
ILK	Integrin Linked Kinase
NM II	Non-muscle myosin II
PA gel	Polyacrylamide gel
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PEG	Poly ethylene glycol
PLGA	Poly (lactic-co-glycolic acid)
TIRF	Total internal reflection fluorescence

Chapter 1

Introduction

The first chapter provides a brief introduction about rationale for the research and outlines the objectives and scope of the thesis. Main objective of this work is to inspect the spatial distribution of ITG- β_1 receptor in cardiomyogenic micropatterned hMSCs for the purpose of gaining insight into the role of cell-material interactions at integrin receptor level during stem cell differentiation as well as to implement this ITG- β_1 spatial distribution data to engineer novel biofunctionalized platform to induce stem cell differentiation maturity. This chapter further briefly presents an overview of dissertation content and novelty of this work. Novelty of this work is present in exploring the spatial distribution of ITG- β_1 in myocardial lineage committed patterned hMSCs using advanced super-resolution imaging technique (dSTORM).

1.1 Hypothesis

Focal adhesions are dynamic protein complex that connect cell cytoskeleton to extracellular matrix proteins.¹ In literature, the central role of FAs in cell-material interaction has been well documented. FAs sense the cell microenvironment, convert mechanical signals into biochemical signals and transduce these signals to nucleus via stress fibres to alter cell activities.^{2,3} Goffin et al. proved that micropatterned supermature FAs are responsible for alpha-smooth muscle actin recruitment to the stress fibres in differentiated myofibroblasts.⁴ Dugina et al. observed an increase in FA size during myofibroblasts differentiation and anchorage proteins (vinculin and paxillin) recruitment from cytoplasm to FA site.⁵ It is reported that focal adhesion kinase (FAK), one of a key components of FA, regulates adhesive forces, vinculin recruitment and plays key role in embryonic stem cell cardiogenesis.^{6,7} Recently, Yu et al. proved that by controlling FA expression through micropatterning, it subsequently modulates the cell cytoskeleton arrangement and induces myogenic lineage commitment in hMSCs.^{8,9} From these previous works, it is hypothesized that the interaction could be further modulated by regulating the spatial distribution of specific type of integrin receptor and consequent in more specific differentiation. Here, a systematic study of the relationship between modulation of spatial distribution of integrins and stem cell differentiation (especially cardiomyogenesis) will be performed.

1.2 Objectives and Scope

The general objective of project is to explore the spatial distribution of ITG- β_1 receptors in patterned hMSCs that are committed to myocardial lineage for the purpose of gaining insight into the role of cell-material interactions at integrin receptor level during hMSC differentiation. In this study, ITG- β_1 receptor is chosen as from our previous work it was shown to be expressed significantly in the patterned cells that committed to myogenesis.

The specific objectives are

- To explore the spatial distribution of ITG- β_1 at micro- and nanoscale level in the myocardial lineage committed patterned hMSCs

- To elucidate the mechanism behind the myocardial lineage commitment of patterned hMSCs
- To engineer the biofunctionalized gold micropatterned platform using the same micro scale dimensions and spatial distribution data of ITG- β_1 FAs observed above

1.3 Dissertation Overview

The first chapter provides the rationale for this research and outlines the goals and scope of this cell-material interaction study. Chapter 2 reviews the literature concerning implication of mesenchymal stem cell in the tissue engineering especially cardiac tissue engineering. In addition, we emphasized the role of cell-material interactions, stem cell niche and integrin signaling in stem cell differentiation in the same chapter. In Chapter 3, the experimental methods and techniques were discussed in detail along with the principles of techniques employed. Chapter 4 covered the investigation of the spatial distribution of integrin β_1 in stem cell differentiation at micro- and nanoscale level. Chapter 5 elaborated the role of cell mechanotransduction pathways behind the stem cell differentiation and presented the hypothetical model of regulation of stem cell differentiation. In Chapter 6, we reported the fabrication of novel biofunctionalized gold micropatterned platform that can be used to induce the mature stem cell differentiation. Chapter 7 illustrated the general discussion of this cell-material interaction study and proposed the strategies for the future to improve stem cell differentiation in the context of myogenesis.

1.4 Novelty and Significance

In the past, the role of integrins in various cell activities has widely been studied.¹⁰⁻¹³ However, its specific role in directing the stem cell differentiation especially in cardiomyogenesis/myogenesis has not extensively been studied yet. Even in previous studies, there has been no deliberate intention to regulate the integrin patterning to affect cellular behavior. Those studies were merely showing that integrins were involved in different cellular behavior. Using the knowledge that integrins are closely related to cellular behavior especially when physically induced hMSCs differentiation were

involved, we hypothesized that not only the integrin type but also its spatial distribution is critical in inducing specific stem cell differentiation. To the best of our knowledge, this is the first study on deliberate spatial distribution of ITG- β_1 in myocardial lineage committed hMSCs using super resolution imaging technique. The revelation of the distinct difference in the spatial distribution integrins of committed hMSCs and the control could help to investigate and understand the cell-material interactions and subsequent mechanotransduction signaling going on at cell-material interface.

Further, the reverse engineering strategy of using the similar dimensions and spatial distribution data of ITG- β_1 FAs to design and fabricate the novel biofunctionalized gold micropatterned platform is itself an innovative idea and has never been exploited before. More importantly, we observed the more mature myogenesis i.e. generation of myotubes from hMSCs on this platform. In future, thus, this unique approach will contribute significantly to stem cell-based muscle tissue engineering.

1.5 Thesis Outline

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Chapter 2

Literature Review

In this chapter, we firstly reviewed the literature concerning about different cell lines used for cardiac tissue engineering earlier and especially, the implication of mesenchymal stem cells in the cardiac tissue engineering. In the next part, we focused on the decisive role of stem cell-material interactions, stem cell niche and material properties in modulating several stem cell behaviors. Moreover, we elaborated the extensive use of microcontact printing technique done by researchers for manipulating the stem cell differentiation. The important role of transmembrane receptors, integrins and its signaling pathways in controlling stem cell behaviors (especially stem cell differentiation) was covered at the end of chapter.

2.1 Human Mesenchymal Stem Cells and Tissue Engineering

MSCs have been widely accepted in tissue engineering and regenerative medicines due to their multi-lineage differentiation potential and self renewability.^{1,2} In addition, MSCs are nonimmunogenic and thus can be obtained from autologous or allogenic sources without any risk of immune rejection. MSCs could be harvested from human bone marrow or adipose tissue, making them easily accessible. Since hMSCs are multipotent cells, they can differentiate into several tissue lineages based on environmental conditions e.g. cardiac tissue, muscle, fat, bone, cartilage and neuron (Figure 2.1).

Previous literature studies have shown that hMSCs are very sensitive to their surrounding microenvironment and stem cell behavior can be controlled by manipulating this microenvironment.^{3,4,5} Based on these findings, in the field of stem cell and tissue engineering, many biomaterials and techniques have emerged that can be used as physical impetus to induce stem cell differentiation into the desired tissue lineage. Unlike biochemical induction methods, biophysical induction methods are devoid of severe side effects such as uncontrolled cell growth, tumor and apoptosis.⁶ Therefore, in recent time, researchers are more inclined to use the biophysical induction methods such as surface topography, micro- and nano-patterning, substrate stiffness and mechanical stimulation to investigate stem cell behavior and the stem cell differentiation in particular.⁷⁻¹⁰ In the biophysical induction methods, interaction between the cell and material is of utmost importance but systematic study of this interaction is still limited to date. We believe that this study will provide insight for better biomaterial design and tissue scaffold engineering. The implication of hMSCs in cardiac tissue engineering will be elaborated in the next part.

2.1.1 Implication of Mesenchymal Stem Cells in Myocardial Tissue Engineering

Heart diseases are one of the major reasons of human death worldwide. Myocardial infarction (MI) causes death of cardiomyocytes as well as it damages the highly organized architecture of ECM present in heart.¹¹ Thus, in natural healing process of MI, it becomes difficult to regenerate the damaged ECM architecture. Heart transplantation is the only standard therapy present for heart failure so far. However, the biggest hurdle in

Figure 2.1 Multipotent human mesenchymal stem cells. [Adapted from reference 58]

the success of heart transplantation is the lack of organ donation. Recently significant progress has been made in the field of cardiac tissue engineering using stem cells-based therapies. Figure 2.2 depicts the types of cells that can be implemented in heart disease treatment for the purpose of cardiac tissue regeneration.

The first study demonstrating the treatment of damaged heart ventricle using bone marrow cells was done by Tomita et al. in 1999.¹² In this study, bone marrow cells were injected into the heart ventricular scar tissue of rat and after 8 weeks of myocardial injury, improved angiogenesis and myocardial function were observed. It has been reported that a subset of MSCs can differentiate into cardiomyocytes *in vitro* under controlled environment.¹³ More importantly, MSCs can support other cells present in injured heart by offering paracrine growth factor signaling.¹⁴ Researchers used biochemical factors like

5-azacytidine to induce bone marrow stroma-derived MSCs fusion to form myotubes. Interestingly, these myotubes showed synchronous beating after 3 weeks.¹⁵ Promoting the cell-cell contacts is also another method to induce MSC differentiation into myocardial tissue. Previously, it was observed that the co-culture of MSCs with myocytes encouraged the generation of cardiac like cells from MSCs.¹⁶ These cardiac like cells also expressed the cardiomyogenic markers like GATA-4 and myocyte enhancer factor-2, indicating their myocardial lineage commitment. All these studies highlight the various ways to use the MSC potential in the treatment of heart diseases. However, improving the efficiency and functionality of these cardiac like cells is still big challenge. Also, chemical induction methods create unknown side effects to stem cells. In cardiac tissue engineering, understanding the basics about stem cell-material interactions is prerequisite to engineer the bio-scaffold that will enhance the myocardial differentiation as well as improve cardiac maturity. In the following part, we will discuss about biophysical induction methods to induce the myocardial differentiation.

Figure 2.2 Cell types for cardiac tissue regeneration and their potential mechanisms. [adapted from reference 59]

2.2 Stem Cell Niche and Cell-Material Interaction

In vivo, stem cell activities are greatly influenced by stem cell microenvironment (niche). In addition, it has been widely reported that stem cell activities can be influenced by regulating the cell-material interactions. In the field of stem cell and tissue engineering, biomaterial based strategies are successfully implemented to mimic stem cell niche and to control cell-material interactions to regulate the stem cell activities. In this chapter, we highlight the effect of stem cell niche and cell-material interactions that affect the stem cell behavior.

2.2.1 Stem Cell Niche

Stem cell ‘niche’ concept was first proposed long time back to describe the specialized microenvironment surrounding the stem cell.¹⁷ Stem cell niche is a multidimensional microenvironment surrounding the cell that provides essential chemical and physical factors for stem cell growth and development (Figure 2.3). Stem cell niche consists of complex mixture of biochemical and biophysical cues. Generally, these cues are generated by supporting cells (niche cells) and ECM (fibronectin, glycosaminoglycans). Cell-cell junction between stem cell and supporting cell facilitates communication in between these cells via soluble biochemical factors or membrane bound factors. Moreover, there have been several reports stating that stem cell niche is also equipped with nervous system supply.^{18,19}

In the past, researchers specifically worked to elucidate the contribution of wide range of biophysical and biochemical cues like substrate stiffness, topography, ECM proteins, growth factors, peptides, cell patterning and mechanical force in creating the stem cell niche and in turn its effect on stem cell behavior.²⁰⁻²³ Collectively, all these studies emphasize the fact that understanding the role of stem cell niche components and architecture is critical for fabrication of biomaterials that can ultimately control the stem cell lineage commitment.

Figure 2.3 Illustration of stem cell niche. [Adapted from reference 60]

2.2.2 Cell-Material Interactions

Cell interaction with underlying substrate triggers the cascades of intracellular signaling pathways to dictate the cell response. Thus, it is highly important to understand the fundamentals of cell-material interactions to control stem cell fate. From figure 2.4, it is understandable that different material properties (surface chemistry, topography, stiffness, coating proteins) can affect the cell behavior in various ways. Since such materials may experience deformation and degradation under *in vivo* condition, there has always been risk of biomaterials in triggering unwanted cell/tissue behavior. Currently, more than a hundred of biomaterials have been used for *in vivo* application. Such biomaterials following *in vivo* implantation trigger host reaction to repair damaged tissue. Sometimes, cascade of unpredictable events can be initiated by host-implant interaction. These host reactions include scar formation, fibrosis, acute or chronic inflammation,

swelling, granulous tissue formation, blood-material interaction and severe allergies.²⁴ It has been demonstrated that changes in biomaterial properties can impact the cell activities/behavior and quality of tissue that is produced. It was reported that neural cell proliferation was improved in PEG hydrogels prepared with increasing degradable macromer content.²⁵ However, no sign of neural differentiation was observed. Bone substitutes like calcium sulfate dihydrate (gypsum) have been extensively utilized to repair bone defects. But, due to its poor bioactivity in pure form, it fails to bond covalently with newly generated bone during early stage of healing.²⁶ Studies have also reported that local tissue reacts to biomaterial scaffold even in the absence of immune-mediated reaction to non-autologous cellular material. For instance, polymers like α -hydroxy acids induce the inflammatory reaction in addition to acidosis happened due to their breakdown and cause bone destruction and draining fistulae formation.²⁷ Biomaterial's lack of cytotoxicity doesn't necessarily assures its biocompatibility. For example, the application of UV-crosslinked chitosan for intraperitoneal use was assessed previously and surprisingly the substrate was found to trigger granulomatous reaction along with adhesion formation.²⁸ In many cases, biomaterials are nontoxic compounds, but their breakdown products or cross-linking agents may result in toxicity. For example, dialdehyde cross-linked dextran-gelatin hydrogel subcutaneous implant triggered foreign body reaction in tissue surrounding the implant.²⁹ Fragmented hyaluronan was involved in activating the expression of inflammatory genes by immune cells at injured site.³⁰ Polylactic acid (PLA), poly(L-lactide) (PLLA) and poly(D,L-lactide) (PDLA) are widely used thermoplastic polyesters for *in vivo* bioscaffold fabrication. However, a severe inflammatory reaction was triggered by acidic byproducts generated after their implantation.³¹ Similarly, subcutaneous implantation of polyvinyl acetate-PLGA microsphere in rats activated a series of host response to implant including acute inflammation, chronic inflammation, fibrosis and subsequently mineralization around the implant.³² Such numerous studies suggest that biomaterial biocompatibility should be prime focus while exploring and implementing new biomaterial as the implant. But no matter what the materials properties are, cells interact with material surface using transmembrane receptors 'integrins'. In our study, we choose the 'integrin' as a model protein and the focus will be on the spatial distribution of specific type of integrin due to

its important role in cell-material interaction as well as in the upregulation of cardiomyogenic gene expression of physically induced cells. In the next subsection, we have explained in detail the diverse role of integrins in controlling the stem cell behavior.

Figure 2.4 Graphical illustration of the cell-material interactions. [Adapted from reference 61]

2.2.2.1 Modulating Cell-Material Interactions using Micropatterning Technique

It is increasingly clear that inherent properties of materials can decide the lineage specific stem cell differentiation (Figure 2.5). At the same time, researchers developed several biophysical induction techniques to use these inherent properties with some modifications to drive stem cell differentiation (figure 2.6). Regulating stem cells differentiation by modulating the cell shape through ECM patterning is well accepted in stem cell research.^{3,4} Micropatterning is one of the types of ECM patterning techniques that is used to control the cell shape and to study cell-material interactions. Cell micropatterning is done by either standard photolithography followed by covalent protein binding or protein transfer from protein inked patterned stamp to desire substrate. Microcontact printing is a top-down surface patterning technique and it is immensely popular method due to its

simplicity and wide range of applications in the field of stem cell research and biomaterials.

Figure 2.5 Schematic of inherent properties of materials that can affect the stem cell fate decisions such as nanotopography, stiffness, chemical functionality, cell adhesivity to material, its binding affinity for soluble factors, its cell-mediated degradability and its degradation by-products. [Adapted from reference 62]

McBeath et al. used microcontact patterning method to control the size and shape of hMSCs and demonstrated that osteogenic and adipogenic differentiation of hMSCs can be coaxed in part by cell shape and cytoskeletal tension.³³ Similarly, Tay et al. applied microcontact patterning technique to induce cell elongation and found that these elongated hMSCs showed myogenic lineage commitment.³⁴ Human tendon fibroblast cultured on adhesive islands of varying aspect ratios displayed an increase in collagen I expression in cells with elongated morphology, suggesting the influence of cell shape on specific cellular functions.³⁵ In general, microcontact patterning technique is a simple as

well as effective tool to regulate the cell-material interactions and by implementing this technique we can effectively control the spatial distribution of integrins at micro- and nanoscale to manipulate cell-material interactions and can control the stem cell lineage commitment.

Figure 2.6 Diagram representing the different biophysical induction techniques to regulate stem cell differentiation via modulating cell-material interactions. **(A)** Modulation of substrate stiffness, **(B)** Cell shape distortion by ECM patterning, **(C)** surface topographical modifications, **(D)** and application of mechanical forces. [Adapted from reference 8]

2.3 Integrins in Cell-Material Interaction

2.3.1 Integrins and its Signaling Machinery

Integrins are heterodimeric transmembrane receptors that are used by cells for cell-ECM interaction and signaling.³⁶ Integrins are present in several animal species including mammals. It is a dimer of two subunits, α and β subunits and these subunits combine to form 24 types of different integrins.³⁷ All these $\alpha\beta$ combinations differ in their ECM binding specificity and signal transduction pathways. Integrins transmit signals at cell-material interface in either direction i.e. inside-out signaling and outside-in signaling.³⁷ These transmembrane receptors also act as the linker between cell cytoskeleton (CSK) and ECM. Inside the cell, CSK is connected to the nucleus.¹⁰ Thus integrins effectively transmit the signals from ECM to nucleus. At the same time, cells sense the microenvironment and convey the traction forces generated by CSK to ECM via integrins.

Short cytoplasmic tails of integrins interact with many adaptor proteins and cytoplasmic kinases to establish the connection with CSK. Initially, integrin receptor coupling to ECM protein results in the integrin clustering. This promotes the recruitment of several anchorage proteins (talin, vinculin, paxillin), cytoplasmic kinases (FAK, ILK) and actin filaments from cytoplasm to the site of integrin-ECM conjugation. Actin filaments reorganize to form the actomyosin stress fibres which in turn induce more integrin clustering and strengthen the integrin-ECM binding. As a result, ECM protein, integrins and cytoskeletal proteins develop an aggregate at the cell membrane and it is known as focal adhesion (FA).³⁷ FAs can be easily seen by immunofluorescence imaging techniques. Cells use these FAs to exert the traction forces on underlying substrates.³⁸ At the same time, formation of these FAs needs actomyosin-generated cytoskeletal tension.³⁹

Figure 2.7 Schematic of integrin signaling complex. [Adapted from reference 63]

2.3.2 Role of Integrins in Regulating Cell Behavior

There have been mounting evidences to demonstrate the decisive role of integrins in regulating the various cell activities like cell adhesion, spreading, migration, proliferation, differentiation and apoptosis. The cell-ECM binding through integrin is necessary for cell survival. Failure to do so causes apoptosis in many cell types.^{40,41} This phenomenon is known as an ‘anoikis’. There have been many reports which stated that anoikis can be controlled by ECM-integrin specific binding. For instance, $\alpha_5\beta_1$ integrin binding to fibronectin protein triggers the expression of Bcl-2 (anti-apoptotic protein) that protects cells from stress-induced apoptosis.⁴² Similarly, $\alpha_v\beta_3$ integrin helps for survival of endothelial and melanoma cells by down regulating p53 pathway and activating the nuclear factor kappa B transcription factor.⁴³ The regulatory role of integrins along with other soluble mitogen receptors in cell spreading and migration has been reported before and it was regulated through the activation of Rho family of guanine nucleotide-binding proteins.⁴⁴ In many primary cell lines, it was observed that the synergistic approach of

integrins and growth factor is necessary for cell proliferation. Shc-linked integrin conjugation with ECM allows cells to progress through G₁ phase in presence of growth factor. On the contrary, cell binding to ECM through integrins that failed to activate Shc pathway, experiences the cell growth arrest even in the presence of growth factors.^{45,46} Previously, it was shown that activation of integrin mediated ERK pathway can control the cell migration by inducing myosin light chain kinase activity which in turn phosphorylates the myosin light chain.⁴⁷ Broadly speaking, all these studies clearly indicate the diverse and critical role of integrins in regulating several cellular activities.

2.3.3 Role of Integrins in Stem Cell Differentiation

Integrin's specificity towards cell growth and cell cycle regulation has been observed before. In such cases, ECM composition also plays important role in controlling the cell growth. For instance, myoblasts demonstrated proliferation on fibronectin surface but experienced growth inhibition on laminin surface and differentiated to form myotubes. Mammary epithelial cells were also observed to be differentiated on laminin surface.^{48,49} These studies proved that integrins differ in their ability to promote cell growth as well as it regulates cell differentiation too. It is assumed that cell adhesion via integrin-ECM binding facilitates exit from the cell cycle and provides signals for differentiation. The cell cycle withdrawal is necessary for differentiation and interestingly, integrins may facilitate differentiation by inducing exit from the cell cycle.³⁷

Figure 2.8 Schematic of integrin downstream signaling pathways and regulation of cell activities. [Adapted from reference 64]

In the past, several studies were published which suggested the direct involvement of integrins in stem cell differentiation. Damsky et al. showed that there was change in surface expression of ITG- β_1 when myoblasts fused to form myotubes.⁵⁰ Fassler et al. demonstrated the importance of ITG- β_1 in cardiac muscle cell differentiation and specialization (Figure 2.9).³⁵ To prove it, he used the integrin-deficient embryonic stem cells to test their ability to differentiate into cardiac muscle cells.⁵¹ Watt et al. used chemical agents to induce differentiation of multipotent embryonic stem cells and embryonic carcinoma cells into multiple lineages and attempted to correlate it with the changes in the types of integrin expressed.⁵² Similarly, Dedhar et al. observed up-regulation of integrin $\alpha v \beta_1$ in retinoic acid induced neural differentiation of P19 cells.⁵³ G Tan et al. investigated the role of ECM protein (collagen) and integrin in hMSC

differentiation into cardiomyocytes like cells using myogenic differentiation medium.⁵⁴ Kundu et al. illustrated the importance of ECM and integrins in osteogenic and chondrogenic differentiation of hMSCs.⁵⁵ Brafman et al. found that fibronectin and vitronectin promote endoderm differentiation of human embryonic stem cells via interaction with integrin α_5 and integrin α_v respectively and ECM-integrin interaction is crucial for differentiation of human embryonic stem cells into mature cells.⁵⁶ Researchers also used other strategies such as surface topography to regulate stem cell differentiation by modulating integrin expression and FAs. For instance, Yim et al. showed that nanotopography can influence hMSC behavior by changing the integrin clustering, integrins expression and FA assembly and in turn steer the stem cell differentiation.⁵⁷

Altogether, above studies show the importance of integrins in regulating the stem cell behavior and more importantly in stem cell differentiation regulation. But integrin's specific role in stem cell cardiomyogenesis/myogenesis has not been extensively investigated yet. In aforementioned studies, nobody has deliberately tried to regulate the integrin patterning to affect cellular behavior. Those studies merely observed the integrin expression and stated that integrins were involved in different cellular behavior. More importantly, we are interested to investigate the role of integrin patterning on stem cell differentiation because we assume that development of elongated integrin FAs via micropatterning promotes the formation of aligned actin CSK. Since actin CSK plays crucial role in the signal transmission from FAs to the cell nucleus, changes in stem cell niche or remodeling of actin CSK can alter the gene expression profile of stem cell. Subsequently, both elongated FAs and aligned actin stress fibers may trigger the upregulation of myocardial genes by developing the optimal cytoskeletal tension. Hence, in this project, we will study the specific type of integrin and its spatial distribution and show that both could be engineered to induce specific stem cell differentiation (especially cardiomyogenesis). Therefore, this study will fill the research gap by providing a new approach to regulate stem cell differentiation by controlling the spatial distribution of specific type of integrins.

Figure 2.9 Immunofluorescent images of sarcomeric myosin heavy chain (a,d), sarcomeric actin (b,e) and α -actinin (c,f) from cardiomyocytes of wild-type D3 embryonic stem cells (a-c) and ITG- β_1 null embryonic stem cell clone G201 (d-f). Scale bar 10 μ m. [Adapted from reference 35]

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Chapter 3

Experimental Methodology

The purpose of this chapter is to offer the detailed information about the methods and techniques that we used for the experimental study. In this chapter, we discussed the principle and methodology of several advanced microscopic techniques that have been applied for super resolution imaging of integrin spatial distribution and the measurement of cell mechanical properties (cell stiffness and cell traction forces). Further, the methods of PDMS stamp fabrication, PA gel synthesis and PA gel substrate fabrication were elaborated in this section. The details of cell culture as well as immunofluorescence staining to check the presence of tissue protein markers were illustrated here. At the end, we briefly mentioned the statistical analysis methods used to show the statistical significance between sample and control.

3.1 Substrate Fabrication

3.1.1 Fabrication of Elastomeric PDMS Stamps

Polydimethylsiloxane (PDMS) was used to prepare the elastomeric stamps for printing the extracellular matrix (ECM) protein onto substrates. The standard photolithography technique was employed to fabricate the silicon master templates bearing the desired topographic features (Figure 3.1). Briefly, photoresist layer was deposited onto silicon wafer by spin coating and later exposed to UV light through a patterned photomask. Silicon wafer coated photoresist was then developed using developer solution to dissolve the non-cross linked polymer. Eventually, 1:10 (base: curing agent) liquid PDMS (Sylgard 184, Dow Corning) was added over the silicon master template, cured at 100 °C for 2 h and peeled away manually.

3.1.2 Fabrication and activation of PA Gel Substrate

PA (polyacrylamide) gel was used for substrate fabrication due to following advantages. Firstly, it is easy to handle because of its non tackiness. It can be easily cross linked to glass coverslips and more importantly, it permits fabrication of substrate with wide range of stiffness. To prepare PA gel with different stiffness, acrylamide and bis-acrylamide with different concentrations were mixed as reported before (Table 3.1).² The scheme of PA gel activation and fibronectin protein patterning is shown in figure 3.3. Briefly, acrylamide and bis-acrylamide solutions were mixed with 1/100 (v/v) of 10 % ammonium persulfate and 1/1000 (v/v) of N,N,N',N'-tetramethylethylenediamine (Bio-Rad). The mixture (25 μ L) was then quickly poured onto the glass coverslips coated with red fluorescent latex beads (100 nm diameter) and subsequently, amino-silanized glass coverslips were placed on the drop of mixture and allowed to dry for an hour to polymerize the gel solution. To improve the ECM protein binding to PA gel, it was treated with 0.2mg/mL N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (Sulfo-SANPAH) (Pierce) in 50 mM HEPES buffer (pH 8.5) followed by irradiation with UV light (365 nm) for 10 min. Finally substrates were washed with HEPES buffer to remove excess Sulfo-SANPAH.

Figure 3.1 Schematics showing PDMS stamp fabrication using standard lithography technique [adapted from reference 1]

Table 3.1 Elasticity modulus of PA gel (expected) after crosslinking of Acrylamide and Bis-Acrylamide concentrations [adapted from reference 2]

Acrylamide %	Bis-acrylamide%	Acrylamide from 40% stock solution (ml)	Bis-acrylamide from 2% stock solution (ml)	Water (ml)	$E \pm \text{St. Dev. (kPa)}$
3	0.03	0.75	0.15	9.1	0.20 ± 0.03
3	0.06	0.75	0.3	8.95	0.48 ± 0.16
3	0.1	0.75	0.5	8.75	1.10 ± 0.34
3	0.15	0.75	0.75	8.5	1.37 ± 0.22
3	0.225	0.75	1.125	8.125	1.67 ± 0.14
3	0.3	0.75	1.5	7.75	1.78 ± 0.19
4	0.03	1	0.15	8.85	0.71 ± 0.24
4	0.06	1	0.3	8.7	1.16 ± 0.54
4	0.1	1	0.5	8.5	2.01 ± 0.75
4	0.15	1	0.75	8.25	2.55 ± 0.17
4	0.225	1	1.125	7.875	3.13 ± 0.42
4	0.3	1	1.5	7.5	3.24 ± 0.58
5	0.03	1.25	0.15	8.6	1.00 ± 0.31
5	0.06	1.25	0.3	8.45	1.80 ± 0.44
5	0.1	1.25	0.5	8.25	3.15 ± 0.85
5	0.15	1.25	0.75	8	4.47 ± 1.19
5	0.225	1.25	1.125	7.625	8.44 ± 0.82
5	0.3	1.25	1.5	7.25	8.73 ± 0.79
8	0.048	2	0.24	7.76	2.61 ± 0.82
8	0.264	2	1.32	6.68	19.66 ± 1.19
8	0.48	2	2.4	5.6	40.40 ± 2.39
10	0.03	2.5	0.15	7.35	2.83
10	0.06	2.5	0.3	7.2	7.43
10	0.1	2.5	0.5	7	10.61
10	0.15	2.5	0.75	6.75	16.70
10	0.225	2.5	1.125	6.375	23.43
10	0.3	2.5	1.5	6	34.88

Figure 3.2 Synthesis of polyacrylamide gel using Acrylamide and Bis-Acrylamide monomers
[adapted from reference 2]

Figure 3.3 Schematic illustrating the activation of polyacrylamide gel and fibronectin patterning on activated gel surface.

3.2 Characterization

3.2.1 Direct Stochastic Optical Reconstruction Microscopy (dSTORM) Setup

Super resolution imaging technique i.e. direct stochastic optical reconstruction microscopy (dSTORM) is used to map the nanoscale distribution of intergrin clusters within FAs. The principle of super resolution imaging technique is based on the examination of the individual fluorophores emission by acquiring the single-molecule localization randomly (Figure 3.4). This is accomplished by shifting the large portion of fluorophores to a reversible non fluorescent OFF state followed by stochastic activation

of individual fluorophores. At the beginning of the experiment, fluorophores are irradiated with the laser of appropriate wavelength and intensity and transferred to the reversible non fluorescent OFF state. A sparse portion of fluorophores which is in non fluorescent OFF state is then reactivated either spontaneously or by irradiating with a second laser wavelength. If the probability of fluorophore reactivation is adequately low, then the reactivated fluorophores with their fluorescent ON state are statistically spaced further apart than the resolution limit and their exact locations can be determined by fitting a point spread function (PSF) to the measured photon distributions.³ Eventually, such repetitive activation, localization and deactivation of fluorophores allow the temporal separation of spatially unresolved structures in a reconstructed image.⁴

Single molecule imaging for super resolution microscopy was performed in a custom made free space coupled TIRF imaging system. Briefly, the lasers are coupled to an inverted microscope (Olympus IX-71, Olympus, Japan). A translating mirror was conjugated to the back focal plane of the objective lens to achieve total internal reflection fluorescence (TIRF) configuration. A 100x/1.49 Olympus objective lens was used for TIRF imaging. Lasers include LD 405 nm (50 mW) Cube laser, LD 488 nm (100 mW) coherent laser, LD 561 nm (150 mW) coherent laser and LD 647 nm (150mW) coherent lasers. The fluorescent light was filtered with a Semrock Quadband dichroic and emission filter before imaging with a high sensitive liquid cooled Photometrics Evolve Electron Multiplying CCD camera (Andor Ixon DV897; Belfast, UK). Further an intermediate zoom lens of 1.6x was used in the light path to generate wide field TIRF images with a resolution of 100 nm per pixel. Reconstructed super resolution images were generated and analyzed by rapidSTORM software. Distribution of integrin clusters were analyzed by using the Quick PALM ImageJ software. Reconstructed images of active integrin B1 clusters were obtained by direct iterative stochastic activation of subsets of Alexa Fluor 647 molecules and subsequent position determinations applying simultaneous excitation at 647 and 405 nm. Typically, we recorded 10,000 frames at frame rates of 10 Hz, resulting in acquisition times of approximately five minutes for single image.

Figure 3.4 Graphic representing the principle of dSTORM imaging (left side picture) and reconstructed image displaying the cell microtubules (right side picture) (adapted from reference 4)

3.2.2 Atomic Force Microscopy Indentation

Currently, AFM indentation is widely used tool to determine the elastic properties of biological samples such as elastic modulus of cells and tissues (Figure 3.5). Different models can be applied to calculate the elastic properties of interest. However, Hertz model is the most popular model among them. In hertz model, sample is considered as

linear and isotropic elastic solid which occupies infinitely extending half space. It is also considered that indenter is non deformable and no additional interactions are present between indenter and sample. These conditions are prerequisite to calculate the Young's modulus using hertz model. To calculate elastic properties of sample, several properties of sample and indentation probe must be specified accurately.

Here, cell stiffness was measured using Nanowizard II BioAFM (JPK instruments AG, Germany). The samples were submerged in culture medium during measurement study. The probe is made up of polystyrene bead (diameter: $4.5\ \mu\text{m}$) connected to a silicon nitride cantilever (Novascan Technologies, Inc., Ames, IA). The spring constant of the cantilever was typically about $0.03 \pm 0.003\ \text{N/m}$. A maximum force of $3\ \text{nN}$ and loading rate of $5\ \mu\text{m/s}$ were applied. Indentation depth was around $500\ \text{nm}$. JPK data processing software (JPK instruments AG, Germany) was used to calculate the Young's modulus values for recorded curve. It uses a Hertz contact model for spherical indenters (diameter $4.5\ \mu\text{m}$; Poisson ratio 0.5) fitted to the extend curves.⁵

Figure 3.5 (A) Illustration of cell stiffness measurement with AFM indentation technique, δ represents indentation. (B) SEM image showing beaded tip used for indentation purpose. (C & D) Figures illustrate single cell stiffness measurement (adapted and modified from reference 6)

3.2.3 Traction Force Microscopy

Cell Traction Force (CTF) is the force generated by actomyosin stress fibres interaction and exerted on underlying substrate via focal adhesion (Figure 3.6). CTF microscopy was carried out on polyacrylamide gel film embedded with fluorescent microspheres prepared as explained before.⁷ To record the cell traction force applied on the gel substrates, the $\vec{u}_t(x, y) = \sum_{i=1}^n \vec{G}(x - x_i, y - y_i) \vec{f}_i$, deformation field of the gel substrate was initially calculated by tracking fluorescent microbeads. The obtained deformation field was then translated into traction stress field according to Green's function. Briefly, the traction force at discrete point f_i , located at the position (x_i, y_i) was calculated based on formulation: where G denoted the Green's tensor and μ_t denoted the experimental $F = \iint \sqrt{T_x^2(x, y) + T_y^2(x, y)} dx dy$ displacements of fluorescent beads at position (x_i, y_i) . The overall force of the cell F is an integral of the traction field magnitude over the area, where, $T(x, y) = [T_x(x, y) + T_y(x, y)]$ is the continuous field of traction vectors defined at any spatial position (x, y) within the cell. The cell traction force map was then imposed with the DIC image of cells, to construct the corresponding traction force mapping in pseudocolor (dark blue to light pink corresponding to traction stresses of lower to higher levels)

Figure 3.6 Depiction of CTF exerted by cells on underlying substrate. Actomyosin interactions are responsible for traction force generation and integrin receptors serve as a mean to apply CTF on surface. [Adapted from reference 8]

3.3 Cell Culture

Cryopreserved adult human bone marrow derived hMSCs were purchased from Lonza (Cambrex) and were expanded in MSC growth medium (Lonza) according to the vendor's instruction. hMSCs were subcultured at a density of $2-3 \times 10^3$ cells cm^{-2} . For the experimental purpose, hMSCs were cultured in low glucose Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine (Sigma Aldrich) supplemented with 10% FBS (PAA) and 1% antibiotic/antimycotic solution (PAA) at 37 °C in a humidified atmosphere of 5% CO_2 . Early passage hMSCs (passage 4-6) were used for all experimental work. The medium was changed every 2-3 days of culture. Trypsin-EDTA (0.25%) (Invitrogen) was used for cell detachment purpose.

3.4 Immunostaining and Imaging

Immunostaining is a powerful technique to detect specific types of proteins/antigens using fluorescent-labeled antibodies. Generally, it is categorized into two types i.e. direct immunofluorescence and indirect immunofluorescence. The first type is one-step procedure in which fluorescent-tagged antibodies are directly employed to conjugate with target protein. On the other hand, indirect immunofluorescence is two-step procedure in which primary antibodies (unlabeled) are allowed to conjugate with target protein and then secondary antibodies (fluorophore-labeled) are added to bind specifically with primary antibody (Figure 3.7).

hMSCs were fixed with paraformaldehyde (4%) solution for 10 min followed by addition of Triton X-100 (0.1%) for 5 min for permeabilization purpose. Bovine serum albumin (BSA) in PBS (5%) was then added for 1 h to passivate surface before incubating with antibodies. Samples were incubated with following primary antibodies solution overnight at 4 °C: anti human cardiac myosin heavy chain (β -MHC, 1:400, Abcam), polyclonal rabbit anti MYH 7 IgG (1:200, Aviva Systems Biology), active integrin β_1 (1:100, Millipore), MyoD (1:100, Santa Cruz), Ki67 (1:100, Life Technologies), rabbit polyclonal anti osteocalcin IgG (1:200, Santa Cruz Biotechnology) and polyclonal rabbit anti-fibronectin (1:400, Sigma). After washing several times with PBS, samples were the incubated with Alexa Fluor 488 goat anti mouse (1:400, Invitrogen) or Alexa Fluor 647 goat anti mouse (1:100, Invitrogen) or Alexa Fluor 568 goat anti rabbit (1:400, Invitrogen) or Alexa Fluor 488 goat anti-rabbit (1:400, Invitrogen) secondary antibodies for 1h at room temperature. Cell nuclei were counter stained with 4'-6-Diamidino-2-phenylindole (DAPI) (1:400, Chemicon). For F-actin staining, cells were incubated with Tetramethyl Rhodamine Iso-Thiocyanate (TRITC) conjugated-phalloidin (1:400, Chemicon). Immunofluorescent images were captured with Eclipse 80i upright microscope (Nikon) or confocal microscope (Leica TCS SP8) or inverted microscope (Olympus IX-71, Olympus, Japan) equipped with dSTORM setup.

Figure 3.7 Picture of IgG antibody, a commonly used antibody for the purpose of immunostaining (left) and the depiction antigen-antibodies interaction that occurs during immunostaining procedure (adapted from reference 9)

3.5 Statistical Analysis

All results are presented as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) or two-sample t-test was performed to calculate the *p-value*. A *p-value* of less than 0.05 was considered statistically significant.

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Chapter 4

Modulation of Spatial Distribution of Integrins to Regulate Stem Cell Differentiation

The purpose of this study is to investigate the spatial distribution of integrin β_1 present in the myocardial lineage committed patterned hMSCs and the non-committed unpatterned hMSCs at micro- and nano-scale level using super-resolution imaging. In this chapter, we mentioned the hypothesis behind the exploration of integrin distribution, detailed methodology and techniques and finally the outcome along with the possible reasons behind these outcomes. In conclusion, we demonstrated that cell micropatterning induced the generation of aligned, elongated and long ITG- β_1 FAs and these FAs are involved in cardiomyogenic differentiation of patterned hMSCs. In addition, the spatial distribution of ITG- β_1 at micro- and nano-scale level is varied widely.

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4.1 Introduction

In recent years, hMSCs are extremely popular in the field of tissue engineering, due to their ability to differentiate into different cell types arising from different tissue lineages, their vascular progenitor property and hypoinmunogenicity.^{1,2} Targeted lineage-specific differentiation can be achieved by several means, such as the use of classical soluble factors, application of physical stimulation as well as culturing the cells on biomimetic surfaces.³⁻⁵ In the past, biochemical methods were widely used for stem cell differentiation but numerous shortcomings such as uncontrolled cell growth, tumorigenesis and unintended cell death have been associated with such methods.⁶ In contrast, methods that relied on biophysical means to induce stem cell differentiation could potentially avoid these side effects. Among the several classes of biophysical signals, the use of micropatterned ECM surface is a widely employed technique to induce substantial distortion to the cell shape and in turn, cellular adhesion and generation of intracellular actomyosin driven tension can be modulated.⁷⁻⁹ Cell adheres to ECM via transmembrane receptor called integrins.¹⁰⁻¹³ More than just an adhesion protein that pins the cells down to the surface, integrin-ECM interaction can also trigger the intracellular mechanotransduction signaling that recruits over the hundreds of anchorage proteins, linking the actin cytoskeleton to ECM.¹³⁻¹⁵ Collectively, the aggregates of ECM proteins, integrins and cytoskeletal proteins are known as focal adhesions.^{10,16}

Focal adhesion is the site at which cells exerts traction forces on underlying substrate.¹⁷ In the past, the crucial role of integrins in several cell activities has widely been highlighted.^{10,14} Damsky et al. illustrated that during the event of myoblasts fusion, ITG- β_1 surface expressions were changed.¹⁸ Fassler et al. demonstrated that ITG- β_1 integrin-deficient embryonic stem cells have failed to differentiate into cardiac muscle cells.¹⁹ Researchers also demonstrated that nanotopography can persuade hMSCs differentiation by modulating the integrin clustering and focal adhesion formation.²⁰ Dedhar et al. observed up-regulation of integrin $\alpha\beta_1$ in retinoic acid induced neural differentiation of P19 cells.²¹ G Tan et al. investigated the role of ECM protein and integrin in hMSC differentiation into cardiomyocytes like cells using myogenic differentiation medium.²² Kundu et al. reported the role of ECM matrix and integrin in osteogenic and

chondrogenic differentiation of hMSC.²³ Overall, these findings established a strong correlation between integrin-substrate interaction and cell fate.²⁴ While the use of micropatterned ECM to direct stem cells fate have been extensively demonstrated^{7,9,25}, a systematic study of cell-ECM interaction at integrin receptor level has not been well demonstrated yet. In the past, because of diffraction limit of conventional fluorescence microscopy, researchers were not able to visualize the nanosized sub-cellular structures. But, recent progress in the field of imaging made it possible to investigate the sub-cellular structures with a size of few tens of nanometers and super resolution imaging techniques like direct stochastic optical reconstruction microscopy (dSTORM)²⁶, interferometric photo-activated localization microscopy (iPALM),²⁷ single particle tracking-PALM (sptPALM)²⁸ have become popular tool in the cell imaging area. The principle behind super resolution imaging is the precise position localization of single fluorophores. In dSTORM, stochastic fluorophore photoswitching provides temporal separation of fluorophore emission and in turn, aids to examine the structures at molecular level with spatial resolution well beyond the diffraction limit. Presently, these super resolution imaging techniques are well adapted in the research field to map the nanoscale organization of the complex cell structures.^{27,28} Previously, we demonstrated that cell patterning-induced cell shape distortion triggers the myocardial lineage commitment in hMSCs.^{7,9,25,29} In addition, we observed that myogenic commitment of hMSCs can be regulated by modulating the FAs.^{8,30} Here, we briefly summarize our previous results as follows to improve the understanding regarding current findings. hMSCs grown on 20 μm fibronectin strip pattern printed on PLGA film were highly elongated and showed myogenic commitment.⁷ Further study revealed that focal adhesion kinase (FAK) and mitogen-activated protein kinase (MAPK) pathways were involved in hMSC elongation-mediated myogenesis.²⁵ In another study, our group fabricated 3-D microchannels of PLLA-PCL polymer (30 μm width, 30 μm interval and 20 μm depth (30–30–20)) and observed that hMSCs grown on such microchannels were aligned, elongated and consequently committed to myogenic lineage.²⁹ In the next stage, to highlight the effect of FA modulation, hMSCs were grown on square and rectangular patterns of 12.6 kPa PDMS and it was found that dense FA failed to induce myogenesis, whereas elongated FA promoted myogenesis.⁸ In this project, in order to get down to the

sub-micron and nano level to map the spatial distribution of integrin clusters, conventional fluorescence microscopy will not be adequate to provide such information. Therefore, super-resolution imaging technique was employed to gather the necessary information of integrins at nanoscale level.

Based on the relationship between integrin and cell fate, we hypothesize that the spatial distribution of integrins in the myocardial lineage committed patterned hMSCs and the non-committed unpatterned hMSCs will differ significantly. We thus employed the super resolution imaging technique (dSTORM) to investigate the spatial distribution of integrin in myocardial lineage committed hMSCs.

4.2 Experimental Methods

4.2.1 ECM Inking and Characterization

Phase contrast microscopy was used to image the fabricated PDMS micropatterned stamp showing elevated 20 μm strip pattern and 40 μm spacing among the lanes (Figure 4.1). To ink the ECM on substrate, stamps were dipped in fibronectin dissolved PBS solution (PAA) (50 $\mu\text{g}/\text{ml}$) for 1 h and then blow dried with pressurized purified nitrogen gas. Efficiency of the fibronectin inking on substrate was assessed by immunofluorescent staining (Figure 4.1).

Figure 4.1 Characterization of PDMS stamps and efficiency of the fibronectin inking. (a) PDMS micropatterned stamp showing 20 μm strip pattern was captured with phase contrast microscope. (b) Efficiency of transferred fibronectin lanes on glass coverslip was confirmed by

labeling with rabbit polyclonal anti-fibronectin primary antibodies (Sigma) and Alexa Fluor 488 secondary antibodies. The scale bar is 100 μm .

4.2.2 Micropatterning of hMSCs

Customized microcontact printing method from the procedure described before was applied³¹ and illustrated in Figure 4.2a. Lab Tek two well chambered cover glasses were washed with freshly prepared piranha solution and then treated with APTES (3%, Sigma) for an hour. Afterwards, substrates were rinsed with deionized water several times. Glutaraldehyde solution (2.5%) was then added to treat APTES linked substrates for an hour followed by washing with deionized water. Fibronectin coated stamps were pressed against the above treated substrates for a min and kept in conformal contact with treated substrates for 1 h. The unpatterned region was then blocked with freshly prepared BSA (2%) for 1 h at 37 °C to avoid nonspecific cell adhesion. Previously, it was found that BSA could successfully passivate PDMS surface until the 9th day of cell culture.⁸ Substrates were then rinsed thrice with PBS to remove the excess BSA prior to the cell seeding. UV sterilization was done for 15 min before cell seeding. Eventually, hMSCs were seeded at a density of $2-3 \times 10^3$ cells cm^{-2} on the substrates. Unattached cells were discarded after 20-30 min of cell seeding.. Culture medium was changed every 2–3 days.

4.2.3 Controls for Immunostaining

To assess the β -MHC antibodies performance, C2C12 murine myoblasts (ATCC) were used as a positive control and samples without β -MHC antibodies were treated as a negative control. C2C12 myoblasts were cultured in DMEM containing 2% horse serum to induce myogenic differentiate. After 5 days of culture, cells were stained to check the β -MHC expression.

4.2.4 Image Analysis and Statistical Study

A total of 20 cells were analyzed each from both experimental groups to study the spatial microscale distribution of integrins. To determine the statistical significance, a total of 200 ITG- β_1 FAs from three samples of each experimental group were considered. 10-12

ITG- β_1 FAs were selected from each cell and 7-8 cells were selected per sample from each experiment group. FA length and width were calculated by 'Measure' function of ImageJ software. Six cells were analyzed in each experimental group to examine the nanoscale distribution of integrin clusters. Individual integrin cluster area and total number of integrin clusters per FA were measured using ImageJ software. Briefly, the FA area was marked and the remaining portion of image was eliminated. Then, image was converted to 8 bit image and 'Adjust threshold' function was used to adjust the threshold of image automatically. Analyze particles' function of ImageJ was then used to calculate the individual integrin cluster area and to count the number of integrin clusters. The 'plot profile' function was used to check the fluorescence intensity of integrin clusters.

Figure 4.2 Micropatterning procedure. (a) Flow chart showing the cell patterning procedure. (b) Cells cultured on fibronectin patterned substrate showed elongated morphology. Image was taken 3 days after cell seeding. The scale bar is 200 μm .

4.3 Results and Discussion

Microcontact printing method has been extensively implemented to understand the cell-material interactions.^{8,32,33} Herein, the micropattern used to modulate cellular adhesion consist of an array of 20 μm wide fibronectin coated strips, which was previously shown to promote differentiation of hMSCs along the myocardial lineage.⁷ The efficacy of our devised patterning technique was validated with immunostaining method (Figure 4.1). Within a few minutes after the cells were seeded onto the micropatterned surface, hMSCs were observed to adhere exclusively onto the 20 μm wide fibronectin strips and elongated substantially with cell culture duration (Figure 4.2b).

Patterned cells were fixed and examined for β -MHC expression after 5 days of cell culture. β -MHC (isoform of MHC) is commonly used mature marker of cardiomyogenesis and thus it was chosen in our experiment to confirm myocardial lineage commitment of patterned cells. β -MHC immunostaining results revealed that expression of β -MHC was restricted exclusively in the patterned cells in comparison with the unpatterned cells (Figure 4.3). Our findings are in accordance with previous reports in which hMSCs were cultured on poly (lactic-co-glycolic acid) (PLGA) films with 20 μm wide patterned fibronectin strips and it displayed similar trend of elongated morphology with much smaller area of coverage.⁷ In addition, real-time polymerase chain reaction (PCR) results from the same study showed significant upregulation of cardiomyogenic associated genes expression such as MyoD, MyF5, GATA4, NKX2-5, MHC and cTnI, compared to the unpatterned hMSCs.

Having established that the micropatterned platform is capable of inducing hMSCs to acquire a myocardial-like phenotype, we studied the integrin involved in the interaction with the substrate. We stained for various types of integrin β and then chose the ITG- β_1 for the experimental study as we found out that activated form of ITG- β_1 was expressed significantly in hMSCs committed to myocardial lineage (Figure 4.4) and ITG- β_1 blocking study revealed the promising role of ITG- β_1 in myocardial differentiation of hMSCs (Figure 4.5). After ascertaining that ITG- β_1 is highly involved in the interaction,

Figure 4.3 Assessment of β -MHC expression. After 5 days of culture, β -MHC (green) was significantly expressed in the patterned cell group (a), while the unpatterned cell group did not exhibit positive β -MHC staining (b). β -MHC primary antibody specificity was assessed using C2C12 murine myoblasts (positive control). C2C12 myoblasts were cultured for 5 days in the presence of 2% horse serum (c). Negative control was the cell sample without β -MHC primary antibodies to inspect the secondary antibody specificity (d). The scale bar is 100 μ m.

we seek to understand the molecular basis of this phenomenon by examining the spatial distribution of the ITG- β_1 . Even in literatures, there are several lines of evidence to suggest that ITG- β_1 is heavily implicated in the maintenance of cardiomyocyte functions.^{19,34} However, the role of ITG- β_1 in hMSCs cardiomyogenic lineage commitment is currently unclear. To inspect the spatial distribution of ITG- β_1 in myocardial lineage committed hMSCs, we firstly, located the myogenic cells with TIRF imaging followed by examining the spatial distribution of ITG- β_1 with super-resolution imaging technique.

Figure 4.4 Immunostaining of different types of β integrin expressed in patterned cells. Fluorescent images showing the expressions of (a) ITG- β_1 , (b) ITG- β_3 , (c) ITG- β_4 , (d) ITG- β_5 and (e) ITG- β_7 in patterned cells after 5 days of culture. Significant expression of ITG- β_1 FAs was observed in the patterned cells, while ITG- β_3 was expressed poorly in those patterned cells. No expression for ITG- β_4 , ITG- β_5 and ITG- β_7 was found in patterned cell sample. The scale bar is 100 μm .

Figure 4.5 ITG- β_1 blocking study. (a) Optical image of ITG- β_1 blocked patterned hMSCs was captured after 5 days of cell culture. Immunostaining results showed severely down-regulated expression of active ITG- β_1 (b) and β -MHC (c) in ITG- β_1 blocked patterned hMSCs after 5 days. The scale bar is 200 μm in (a) and 100 μm in (b) and (c)

Double immunostaining method was carried out to check the expression of β -MHC and ITG- β_1 after 5 days of culture (Figure 4.6). The sequential approach of immunostaining was employed here³⁵ because the cross-reactivity of antibodies can be minimized using the sequential staining. TIRF images of MHC (coded by MYH-7 gene) (Figure 4.7a) and ITG- β_1 FAs (Figure 4.7b) from the same field of view in patterned cells were captured.

Figure 4.6 Schematic presentation of double immunostaining method.

Interestingly, long and aligned active ITG- β_1 FAs were observed exclusively in the patterned cells. In contrast, unpatterned cells that were not observed to undergo cardiomyogenesis, (Figure 4.7e) exhibited short and less aligned ITG- β_1 FAs (Figure 4.7f). Moreover, the patterned cells developed elongated ITG- β_1 FAs as well as well organized actin filament network (Figure 4.8) whereas dot like ITG- β_1 FAs along with randomly organized actin filament network were observed in the unpatterned cells. Our findings are in agreement with literature findings in which predominant expression of long and elongated ITG- β_3 FAs were found in patterned hMSCs cultured on micropatterned PDMS substrate with intermediate stiffness (12.6 kPa). We thus assume that the ECM pattern geometry is responsible for regulating the development of integrin FAs. Further, we propose that ECM pattern would control the spatial distribution of

integrins in a certain way that might be responsible for the mechanotransduction event of cell differentiation towards the myocardial lineage.

After that, super-resolution imaging technique was used to elucidate the nanoscale distribution of ITG- β_1 clusters within FAs because of its ability to resolve structures with lateral resolution down to 20 nm.^{29,36} dSTORM images of ITG- β_1 FAs from same locations in patterned and unpatterned hMSCs were shown in Figure 4.7d & 4.7h respectively. Moreover, to study the microscale distribution of ITG- β_1 FAs, dSTORM images (Figure 4.9) of ITG- β_1 FAs were considered. Several FA morphological parameters (length, width, aspect ratio and alignment) were scrutinized using ImageJ software. A higher AR value denotes a more elongated ITG- β_1 FAs. It was observed that long ITG- β_1 FAs were expressed in patterned hMSCs after 5 days of culture (Figure 4.10a). Most of the FAs were 4-8 μm in length and few FAs recorded the length up to

Figure 4.7 Localization of MYH-7 expression and ITG- β_1 FAs from same cell location using TIRF imaging. MYH-7 (cardiac marker) expression was observed in patterned cell (a), while it was absent in unpatterned cell (e). TIRF images of spatial distribution of ITG- β_1 FAs from respective cell locations were taken and illustrated in panels (b, f). Panels (c, g) denote the merged images of MYH-7 expression and ITG- β_1 FAs. Reconstructed dSTORM images of ITG-

β_1 FAs were shown in panels (d) and (h). The scale bar is 5 μm in panels (a), (b), (c), (e), (f) and (g), and 2 μm in panels (d) and (h).

15 μm . On the other hand, large number of unpatterned cells displayed short ITG- β_1 FAs with length ranging from 1 μm to 5 μm . In addition, quantification of aspect ratio proved that ITG- β_1 FAs in patterned cells were more elongated than that of ITG- β_1 FAs in unpatterned cells (Figure 4.10c). No significant difference was found in the case of ITG- β_1 FAs width for both experimental groups and most of the FAs were lying in the range of 200-500 nm (Figure 4.10b). ITG- β_1 FAs orientation study was performed on day 5 of

Figure 4.8 Evaluation of spatial distribution of ITG- β_1 FAs and cell cytoskeleton using TIRF imaging. Predominant expression of elongated and aligned ITG- β_1 FAs as well as parallel and aligned F-actin filaments were observed in patterned cell (a, b). On the other hand, punctate and randomly distributed ITG- β_1 FAs as well as dense network of cross linked F-actin filaments were developed in unpatterned cell (c & d). The scale bar is 10 μm .

cell culture. ITG- β_1 FAs from unpatterned cells were randomly expressed (0° to 90° alignment), while ITG- β_1 FAs from patterned cells were better aligned with maximum angular distribution of around 5° – 10° (Figure 4.11a). To further confirm the angular distribution, ITG- β_1 FAs from both experiment groups were characterized by 2-D Fast Fourier Transform (FFT) analysis which converts spatial information into mathematically defined optical data. FFT analysis provides a tool for the morphological quantification of microscopic structural alignment, for instance cell alignment.³⁷ Briefly, the information of the image pixel is transformed into Fourier space (power spectra). The radial sum intensities (constrained within the ring analysis) are formed for 360 radii around the center of the FFT image. Pixel intensity values are calculated for each point (angle) around the circumference of the ring centered on the FFT image. The 2-D FFT

Figure 4.9 Investigation of nanoscale distribution of ITG- β_1 clusters. dSTORM imaging was used to capture the reconstructed super-resolution images of ITG- β_1 FAs from both groups (**a-c** & **d-f**). The magnified areas illustrated in panels (**b**) and (**e**) were shown in panels (**c**) and (**f**) respectively. Histograms of integrin clusters distribution inside marked FA area highlighted the difference of cluster distribution from unpatterned (**g**) and patterned (**h**) cell. Scale bar is 2 μm in panels (**a**), (**b**), (**d**) (**e**), (**g**), (**h**), 500 nm in (**c**), (**f**) and 250 nm in magnified panels of (**g**, **h**).

frequency plot reflects the degree of integrin FAs alignment by depicting the grayscale pixels distributed in patterns around the origin. The 2-D FFT images (Figure 4.11d & Figure 4.11e) revealed that ITG- β_1 FAs in patterned cells were much more aligned than their unpatterned counterparts.

Collectively, the patterned cells illustrated the long, aligned and elongated ITG- β_1 FAs in comparison to the unpatterned cells. Myocardial lineage commitment of patterned cells might be the outcome of activation of ITG- β_1 and subsequently triggered ITG- β_1 -mediated downstream signaling pathways. Previously, long supermature FAs (8-30 μm long) consisting integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ along with particular anchorage proteins were observed in cultured myofibroblasts.³⁸ The formation of supermature FAs which control the recruitment of α -smooth muscle actin (α -SMA) to preexisting stress fibers and the recruitment process was driven by specific tension generated by super mature FAs

Figure 4.10 Quantification of ITG- β_1 FA using morphological parameters. (a-c) Morphological parameters like length, width and aspect ratio of FAs were chosen to highlight the difference in FAs from both groups. Bars denote standard deviation among 3 samples of each group. (n=20).

itself.³⁹ Cells sense the external mechanical force via FAs and transmit it to nucleus through actomyosin stress fibres.⁴⁰ It has been widely reported that these mechanical forces are able to control gene expression and stem cell fate ultimately. In the past, we emphasized the crucial role of elongated FAs in the development of aligned stress fibers and generation of myosin light chain kinase (MLCK).⁸ We assumed that both MLCK and actomyosin-IIA complex together helps to organize actomyosin stress fibres to generate optimal cytoskeletal tension and in turn induces cardiomyogenic differentiation in patterned cells. In accordance with these observations, here we also observed an elongated ITG- β_1 FA formation in patterned cells that may modulate cytoskeletal tension by regulating the development of actin cytoskeleton and consequently steers myocardial lineage commitment of patterned cells.

Figure 4.11 Assessment of ITG- β_1 FAs alignment. (a) ITG- β_1 FAs from patterned group were more aligned than that of ITG- β_1 FAs from unpatterned group (n=5). ITG- β_1 FAs were manually marked (b & c) and FFT analysis (d & e) was performed to confirm FA alignment from patterned and unpatterned groups respectively. The scale bar is 2 μ m in panels (b) and (c).

The super resolution imaging revealed the fact that ITG- β_1 FA is the aggregate of thousands of tiny ITG- β_1 clusters (Figure 4.9) and made it possible to inspect the nanoscale distribution of ITG- β_1 clusters inside the FAs. Surprisingly, ITG- β_1 clusters were uniformly spread within FAs of patterned hMSCs, whereas ITG- β_1 clusters were expressed at the periphery of FAs of unpatterned hMSCs. Spatial distribution of integrin clusters of single FA from unpatterned cell was examined (Figure 4.9g) with adjacent clusters being found to be spaced apart by more than 200 nm. On the contrary, spatial distribution of integrin clusters of FA from patterned cell demonstrated the uniform distribution (Figure 4.9h). Previous findings showed that FAs were developed at the edges of ECM patterns or ECM coated biophysical cues.^{32,41,42} Researchers termed it as the ‘edge effect’ phenomenon and proved that it was the outcome of equilibrium between the available potential adhesion sites and cell traction forces.⁴³ Also, it was noticed that FAs interacting with these biophysical cues or patterns were frequently connected to each other by the network of actin bundles.^{32,42,43} Actin bundle network helps to withstand traction forces and distribute the cytoskeletal tension exerted by the cell.⁴⁴ It has been illustrated that integrins and anchorage proteins located directly at the edge of biophysical cues can tolerate the maximum cytoskeletal tension.⁴³ We assume that the traction forces exerted by unpatterned cells on underlying substrate could be higher than that of patterned cells, as evident by a higher spreading area. Therefore, to counter traction forces, unpatterned cells may further recruit more FAs with focal adhesion components at their periphery. This notion is in line with a previous study that demonstrated concomitant increase in the recruitment of focal adhesion components (integrins and anchorage proteins) and traction force when the FAs are mechanically perturbed.⁴⁵ We thus quantified the spatial distribution of integrin clusters of FAs from both experimental groups. A higher number of integrin clusters were consistently observed for most of the FAs in the patterned cells compared to the unpatterned group (Figure 4.12a). Total

number of integrin clusters in patterned FAs was 5219 ± 461 , whereas it decreased to 2830 ± 276 in unpatterned FAs. The estimated value of density of integrin clusters in patterned FA and unpatterned FA was found to be 27 ± 2 and 18 ± 4 clusters per μm^2 respectively. It was obvious that the total area occupied by integrin clusters per individual FA was larger for FAs from patterned cells (Figure 4.12b). Most of the patterned FAs displayed a total area of integrin clusters per FA in the range of $0.2\text{-}0.7 \mu\text{m}^2$, while the unpatterned FAs were lying in the range of $0.1\text{-}0.3 \mu\text{m}^2$. Briefly, this study proved that the nanoscale distribution of ITG- β_1 clusters varied largely in FAs from both groups.

Figure 4.12 ITG- β_1 quantification studies at nanoscale level. (a) Higher number of integrin clusters per FA was found consistently in patterned cells in comparison to unpatterned cells. **(b)** Similarly, total area of integrin clusters per FA was found to be higher for patterned group. (n=6).

4.4 Conclusion

In summary, we investigated the cell-material interactions at integrin receptor level when hMSCs were engineered to undergo cardiomyogenesis on our micropatterned platform. TIRF system as well as super-resolution imaging was utilized to capture sub-micron and nano level differences. We confirmed the decisive role of ITG- β_1 and its spatial distribution in hMSC cardiomyogenic differentiation. We also confirmed that cells micropatterned on the 20 μm wide fibronectin strips were coerced to express elongated ITG- β_1 containing FAs, whereas the unpatterned cells displayed shorter and less elongated active ITG- β_1 FAs. Further, we revealed the importance of long and aligned FAs in cardiomyogenic differentiation of patterned hMSCs. Super-resolution imaging revealed that ITG- β_1 clusters were distributed uniformly in FAs of patterned cells. In stark contrast, ITG- β_1 clusters were expressed at the periphery of FAs of unpatterned cells. Collectively, these results may help us to explore the cell-material interactions in depth and subsequent signaling pathways at cell-material interface.

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Chapter 5

Elucidation of Cell Mechanotransduction Pathways behind Stem Cell Differentiation

The purpose of this chapter is to decipher the mechanism behind the cardiomyogenic differentiation of patterned cells. Basically, here we attempted to correlate the changes occurred in cell mechanical properties after cell shape distortion to hMSCs differentiation fate. We mentioned the assessment of cell mechanical properties such as cell stiffness and cell traction forces using AFM indentation and Cell Traction Force Microscopy respectively. At the end, we presented the model of regulation of cardiomyogenic differentiation in patterned hMSCs which stated that optimal cytoskeletal tension generated in patterned cell is responsible for myocardial lineage commitment of patterned cells.

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5.1 Introduction

In chapter 4, we reported the distinct difference in the spatial distribution of ITG- β_1 FAs of the control and myocardial lineage committed cells at micro- as well as nanoscale level. Based on this finding, we hypothesized that the different distribution pattern of ITG- β_1 FAs from these experimental groups would consequent in significantly different traction forces and in turn, trigger the different mechanotransduction signaling pathways. In short, our previous work paved the way into deeper understanding of cell-material interactions and encouraged us to delve deeper into subsequent mechanotransduction pathways occurring at cell-material interface.

The regulatory role of the stem cell niche in governing stem cell fate has been widely documented in numerous studies.^{1,2} A complicated but organized process of mechanotransduction pathways taking place in the cellular environment could regulate cell activities such as cell adhesion, spreading and differentiation.^{3,4} Unlike the biochemical cues, the role of biophysical cues such as topography, stiffness and ECM patterning as well as the biophysical signaling triggered by them in steering stem cell differentiation has not been extensively studied. The mechanical force-induced alteration in the extracellular biophysical cues can be used to regulate the biophysical signals and in turn to activate intracellular biochemical signaling pathways to determine cell fate.^{2,5-7} The process of conversion of biophysical signals into biochemical signals at cell-material interface is known as cell mechanotransduction.^{8,9} There are mounting evidences to suggest a combined role of integrins, FAs and actin stress fibres as being integral in various cell signaling pathways.¹⁰⁻¹³ Integrin-ECM binding at cell-material interface allows cells to sense the surrounding and triggers the mechanotransduction pathways to initiate the highly coordinated series of cell responses such as integrin clustering, FA formation, recruitment of actin stress fibres, modulation of actin CSK and regulation of genes expression.^{9,14} It has been reported that the cells treated with contractile agonist experienced an increase in the cell mechanical properties (cell stiffness and traction forces).¹⁵ In another study Roca-Cusachs et al. used micropatterning technique to control the endothelial cell proliferation rate via regulating the cell cytoskeletal mechanics and nuclear volume.¹⁶ A recently published studies proved that cell fate can be determined by

substrate topography via modulating actomyosin contractility.¹⁷⁻²⁰ Collectively, aforementioned studies emphasize on a distinctive role of actomyosin-generated cytoskeletal tension as an important biophysical impetus to stem cells behavior.

Here, we hypothesize that biophysically induced-stem cell differentiation observed in hMSCs is an outcome of optimal cytoskeletal tension development via mechanotransduction pathways.¹⁷⁻²⁰ In similar context of biophysically induced-stem cell differentiation, readers are encouraged to refer to some recent excellent reviews that have elaborated the effect of material properties like stiffness, nanotopography, surface chemistry, surface degradability and external forces on stem cell differentiation.^{4,7,8} The aim of this work was to correlate the changes occurred in cell mechanical properties after cell shape distortion to hMSCs differentiation fate. To prove our hypothesis, the evaluation of two complementary mechanical properties i.e. cell stiffness and cell traction forces were done. Cell stiffness and cell traction forces are reliable indicators of cytoskeletal tension and determined using AFM indentation and traction microscopy respectively.

5.2 Experimental Methods

5.2.1 Cell Micropatterning

For cell stiffness measurement, cells were cultured onto fibronectin inked amino-silanized glass coverslips. The detailed procedures of preparation of fibronectin inked coverslips and the cell micropatterning were mentioned in chapter 3. Briefly, fibronectin inked PDMS stamps were placed onto amino-silanized glass coverslips. BSA solution was used to block the nonspecific cell attachment. Cells were then seeded on UV sterilized substrates at a density of $2-3 \times 10^3$ cells per cm^2 . Unattached cells were discarded by changing culture medium after 30 min. After every 2–3 days, culture medium was replaced. For cell traction forces measurement, cells were cultured on PA gel coated glass coverslips. Before cell seeding, PA gel coated substrates were dried at 60°C for 30 min and later cell micropatterning procedure was done. Cells were seeded at a density of 100-200 cells/well.

5.2.2 Characterization of Polyacrylamide Gel Substrate

The elastic modulus of PA gel was measured using rheometer (Physica MCR 501, Anton Paar) with parallel plates. Table 5.1 displayed the measured values of elastic modulus.

Table 5.1 Measurement of elastic modulus of PA gel

Acrylamide %	Bisacrylamide %	E $\pm$ St. Dev. (kPa) (Reported)	E $\pm$ St. Dev. (kPa) (Measured)
4	0.15	2.55 $\pm$ 0.17	1.1 $\pm$ 0.19
10	0.1	10.61	9.65 $\pm$ 0.5
8	0.48	40.40 $\pm$ 2.39	38.44 $\pm$ 3.4

5.2.3 Non-muscle Myosin II Inhibition Study

Blebbistatin (50 μ M) (Tocris Bioscience) containing cell culture medium was used from second day of culture to inhibit the non-muscle myosin II (NM II). Blebbistatin containing culture medium was replaced every 2 days. Experiments involving blebbistatin were conducted in the dark.

5.2.4 Image Analysis

A total of 50 cells grown on 40 kPa stiff substrates were considered to calculate cell area and FA analysis. Actin stained cell images were considered to measure the cell area. The cell outline was outlined and the ‘Measure’ function of ImageJ was used for cell area measurement. The ITG- β 1 FA stained cell images were considered for FA quantification study. Briefly, background fluorescence was reduced by using the “Subtract Background” function. Image was converted into an 8-bit image. Area of single cell was then chosen and remaining image portion was deleted. The ‘Adjust threshold’ function was used to adjust the image threshold automatically. Lastly, ‘Analyze Particles’ function was used to quantify the total FA area per cell and FA count per cell.

5.3 Results and Discussion

5.3.1 Micropatterned ECM Lanes Induces Cell Elongation and Upregulates Cardiomyogenic Differentiation

A conventional microcontact printing technique was used to print the fibronectin lanes onto amino-silanized glass coverslips as mentioned in the previous chapter 4 (**Figure 5.1A**). Cells were immunostained with different hallmark protein markers to confirm the lineage commitment at predetermined time intervals. In the later stage of culture, patterned cells displayed a prominent upregulation of β -MHC (coded by MYH-7 gene), whereas osteocalcin (an osteogenic marker) expression was downregulated (Figure 5.1). On the contrary, unpatterned cells exhibited distinct expression of osteocalcin and failed to show any sign of β -MHC expression after 7 and 14 days of culture. These results are in accordance with previous RT-PCR results in which hMSCs with similar elongated morphology grown on poly (lactic-co-glycolic acid) (PLGA) films illustrated a distinct upregulation of several cardiomyogenic genes expression and drastic decrease in the osteogenic genes expression.²¹ Moreover, these findings support the fact that cell spreading can trigger the RhoA signaling pathway which is known to exert a pro-osteogenic effect.²²

5.3.2 NM II Generated Cytoskeletal Tension is Required for Shape Driven Myogenic Activity on Micropatterned Platform

F-actin stained cell images were used to understand the cell patterning effect on the F-actin filaments arrangement (**Figure 5.2A**). After 1 day, patterned cells displayed dense and parallel F-actin bundles that spread all over the cell, while unpatterned cells demonstrated randomly organized F-actin bundles present mainly at the cell edges. After 7 days, we observed the aligned F-actin bundles in patterned cells. In contrast, isotropic network of F-actin bundles was found in unpatterned cells. After 14 days, a visible change of remodeling of F-actin bundle mesh network into aligned F-actin bundles was seen in unpatterned cells. Unpatterned cells remodeled the actin CSK into thick and aligned F-actin bundles perhaps due to the engineered spreading restriction. Conversely, this remodeling effect was not evident in patterned cells after two weeks of culture. A drastic variation in between the development of actin CSK of both groups indicates the role of actomyosin driven contractility which may act as biophysical force to regulate stem cell fate.^{23,24} NM II is one of the mediators of RhoA pathway and responsible for

generation of actomyosin contractility.²⁵ To check the crucial role of actomyosin-generated cytoskeletal tension in hMSCs differentiation, cells were treated with blebbistatin (NM II inhibitor) up to a week. NM II blocking results in the disruption of actin CSK formation and the complete loss of elongated morphology in patterned cells (Figure 5.2B). Immunofluorescence staining results confirmed a severe downregulation in β -MHC expression in those blebbistatin-treated patterned cells suggesting that an intact actin CSK network and actomyosin generated cytoskeletal tension are essential to steer the myocardial differentiation in patterned cells.

Figure 5.1 Micropattern characterization, cell micropatterning and immunofluorescence staining. (Ai) Phase contrast image of top view of PDMS stamp bearing 20 μm elevated lanes. (Aii) Fibronectin pattern transfer efficiency on amino-silanized coverslip was validated using rabbit polyclonal antifibronectin antibodies. (Aiii,Aiv) Optical images showing hMSCs morphology on fibronectin patterned and unpatterned substrates after 3 days of culture. (B) Patterned cells showed positive β -MHC staining, whereas unpatterned cells displayed positive osteocalcin staining at different time intervals. Pattern direction was indicated by double arrow red line. The scale bar is 100 μm in (Ai,Aii,B) and 200 μm in (Aiii, Aiv).

5.3.3 Elastic Modulus Characterization Shows Cell Stiffness Increases with Development of Dense Network of the Actin CSK

Cell stiffness measurement was done at predetermined time intervals (**Figure 5.3**) and it revealed the fact that increase in cell spreading induces cell stiffness since cell spreading is strongly coupled with the development of F-actin filaments network.^{16,17,26} Immunofluorescent images of actin CSK showed the formation of aligned and elongated F-actin bundles in patterned cells with less spreading area in comparison to unpatterned cells (Figure 5.2A). In addition, these images suggested that the cell patterning was involved in the development of parallel F-actin filaments without crosslinking, while such cell patterning-induced effect was missing in unpatterned cells. As a result, we found a significantly lower stiffness in patterned cells as compared to unpatterned cells. Moreover, these images suggested that micropatterning plays a key role in the formation of parallel F-actin filaments without crosslinking and such effect was absent in unpatterned cells. Therefore, patterned cells displayed a significantly lower stiffness compared to unpatterned cells. Surprisingly, after day one, the opposite trend of stiffness was observed where patterned cells exhibited higher stiffness in comparison to the unpatterned cells. We believe that the stark difference in the distribution of F-actin filaments mentioned above was responsible for the opposite trend of stiffness after day one.

5.3.4 Traction Forces and ITG- β_1 FAs Maturation Increase with Cytoskeletal Tension

The actin CSK is linked to the ECM via transmembrane receptors known as integrins which assist to anchor the cell to underlying substrate.²⁷ The actin CSK contractility thus

can be judged by the traction forces that are exerted on the underlying substrate via FAs. Previously CTF microscopy was used to measure the traction forces.²⁸ It was observed that with increase in substrate stiffness, cytoskeletal tension was increased due to the formation of prominent actin CSK.^{29,30} These findings imply that cell traction forces can be regulated by changing the substrate stiffness via altering the actomyosin-generated cytoskeletal tension. In accordance to the previous studies, the traction force map clearly

Figure 5.2 F-actin immunofluorescence staining and NM II blocking study. (A) Actin CSK morphology from both cell groups were observed at different time intervals. Magnified images demonstrated the extent of F-actin filaments cross-linking from patterned and unpatterned cells. (B) After blebbistatin treatment, actin CSK was severely degraded in cells from both groups as well as β -MHC was downregulated in patterned cells after 7 days of treatment. Pattern direction was indicated by double arrow white line. The scale bar is 100 μ m in (A) and (B).

Figure 5.3 Young's modulus measurement. AFM indentation technique was applied to measure the elastic modulus of cells. A significant difference was observed in the elastic modulus of patterned and unpatterned cells at different time intervals. (n=20)

indicated that traction forces increased with substrate stiffness in both cell groups at different time intervals (**Figure 5.4**). Also, traction forces were found to be concentrated at the cell leading edges. Further, traction force map showed that traction forces increased with time in both cell groups but surprisingly, this trend was absent in the cells cultured on soft substrate (2.5 kPa). After 7 days, cells grown on moderate stiffness (10 kPa & 40 kPa) showed spread-out morphology while, cells failed to show such morphology on soft substrate (2.5 kPa). This fact could be attributed to the inability of soft substrate to apply sufficient force to counter the traction forces exerted by the cells. In our recently published work, we mentioned about the predominant expression of active ITG- β_1 FAs in hMSCs cultured on fibronectin coated substrates in comparison to other types of ITG- β .³¹ Another recent study demonstrated that activation of ITG- β_1 receptors through increased fibronectin density promotes the cell traction forces.³² Therefore, we decided to investigate the spatial distribution of ITG- β_1 FAs as well as its exact role in exerting traction forces on substrates with varying stiffness. Immunofluorescence images of ITG- β_1 FAs revealed the development of distinct ITG- β_1 FAs in both cell groups with an increase in substrate stiffness (Figure 5.4). After day 1, we found the few distinct ITG- β_1

FAs at leading edges of the cells cultured on stiff substrates (10 kPa & 40 kPa). These FAs grew in size and after 7 days, prominent and elongated ITG- β_1 FAs were observed in the cell corners. However, cells cultured on soft substrate (2 kPa) expressed dot-like ITG- β_1 FAs after the same time interval of day 1 and day 7. It could be due to the cell's inability to adhere and form stable FAs on soft substrate. These observations are in agreement with past studies which illustrated that stiff substrate induces recruitment of FAs protein and FAs formation.^{24,33}

Figure 5.4 Cell traction forces measurement and ITG- β_1 FAs immunofluorescent imaging.

Traction forces were observed to be higher on substrate with higher stiffness irrespective of cell group. Predominant expression of ITG- β_1 FAs was found on stiff substrates in cells from both groups. The scale bar is 50 μ m and 100 μ m in traction force map images and ITG- β_1 FA images respectively.

5.3.5 Micropatterning Suppresses Cell Spreading and Limits Traction Force by Decreasing FA Area and FA Population

During CTF measurement study, it was found that traction forces declined in patterned cells as compared to unpatterned cells on substrates with varying stiffness and this downward trend remained constant after 1 day as well as 7 days (**Figure 5.5**). Also, CTF data suggested that cells exerted higher traction forces with increase in substrate stiffness despite the cell groups. As per our expectation, a downtrend was observed on soft substrate in traction forces measurement for both cell groups (Figure 5.5a). These CTF values were in agreement with above illustrated traction force map and FA distribution data where we noticed weak traction forces and sparse FA expression in the cells cultured on soft substrate (Figure 5.4). Both cell groups were found to exert higher traction forces on stiff substrates than that of cells grown on the soft substrate. However, unlike the cells on soft substrate, cells grown on stiffer substrates displayed uptrend of traction forces with time (Figure 5.5b & 5.5c). Patterned cells cultured on both moderate stiffness substrates (10 kPa & 40 kPa) displayed up-regulation of β -MHC (Figure 5.6). Hence, we chose the cells cultured on 40 kPa substrates to analyze the cell area and FA quantification study. Interestingly, cell area and FA quantification study also reinforced our traction forces measurement data. Previous study demonstrated that cell traction stress increased with cell spreading as total FA area/cell also enlarged with cell spreading.³⁴ Likewise, a significantly higher cell spreading area was found for unpatterned cell in contrast to patterned cell (Figure 5.5d). Further, FA analysis suggested a significant increment in both total FA area and FA count in unpatterned cells as compared to patterned cells (Figure 5.5e & 5.5f). These FA quantification results are in agreement with previous reports where it was observed that the local contact forces were increased with FA area.³⁵

Stem cell fate can be controlled by force-mediated changes in the biophysical signals via altering the external biophysical cues.^{8,36} However, the exact mechanism by which biophysical induction methods control stem cell differentiation remains elusive till date. In this study, cell patterning technique was applied to promote cell elongation and in turn to regulate the hMSCs differentiation. The myocardial lineage commitment of patterned

Figure 5.5 Assessment of total traction forces, cell spreading area and ITG- β_1 FA morphology. Significant decline in total traction forces exerted by patterned cells on substrates with different stiffness was observed (A, B & C) ($\#p < 0.05$, $*p < 0.05$, $n=12$). Likewise, after 7 days, patterned cells experienced reduction in cell area (D), total FA area (E) and FA number (F) on 40 kPa stiff substrate ($*p < 0.05$, $n=50$).

cells were confirmed after observing the upregulation of mature cardiomyogenic markers (β -MHC & cardiac troponin T) and downregulation of osteogenic markers (osteocalcin & RUNX2) (Figure 5.1 & Figure 5.7). We believe that it is possible to control the myocardial lineage commitment of patterned cell via actomyosin-generated cytoskeletal tension regulation. F-actin stained images clearly illustrated that cell patterning controls the structural reorganization of actin CSK. The actin CSK is mechanically coupled to ECM and to cell nucleus as well as it is actively involved in cell signal transduction. Therefore, it can transmit signals from ECM to nucleus to control the genes expression,^{37,38} Cell shape distortion triggers reorganization of actin CSK and in turn generates optimal cytoskeletal tension to induce myocardial differentiation.^{39,40}

Figure 5.6 After 7 days, cells cultured on 10 kPa and 40 kPa stiff substrates showed positive β -MHC staining. The scale bar is 100 μ m

Blebbistatin treated patterned cells displayed the downregulation of β -MHC expression which highlighted the role of actomyosin-generated cytoskeletal tension in stem cell differentiation. Blebbistatin particularly inhibits NM II, one of the important mediators of RhoA pathway. The RhoA pathway is responsible for the generation of actomyosin contractility in cell and NM II blocking thus diminish the actomyosin contractility. In general, cells sense and interact with the matrix using the actomyosin contractility.^{9,12} Depending on the external biophysical impetus, cells then dynamically restructure the actin CSK which resulted in the change in cell mechanical properties.⁶ Ideally, this restructuring of actin CSK changes the cell mechanics by altering the development of cytoskeletal tension. Hence, blebbistatin treated patterned cells were unsuccessful in developing the optimal cytoskeletal tension to steer stem cell differentiation due to impaired RhoA pathway.

The spatial distribution of the actin CSK mostly influence the cell mechanics.¹⁶ The cell mechanical properties like cell stiffness and traction forces are reliable indicators of the changes occurred in actin CSK. Therefore, to evaluate such changes that occurred in the actin CSK due to cell patterning, cell mechanical properties (cell stiffness and traction forces) were examined. For that purpose, AFM indentation and CTF microscopy were used respectively. We observed that both stiffness and traction forces declined in the

patterned cells. These findings proved the fact that cell shape distortion influences the cell mechanical properties by remodeling the actin CSK. Similarly, previous reports also observed a downward trend of cell contractility in elongated cell.^{41,42} Previous independent findings revealed that cell stiffness was drastically decreased due to actin filaments alignment in elongated cells.^{16,42} The reason behind a decrease in stiffness in elongated cell was assumed to be the reduction in F-actin microfilament crosslinking due to cell patterning which eventually resulted in cell softening. In support of this reason, we also found the reduction in F-actin filament crosslinking in the F-actin stained images of patterned cells (Figure 5.2A).

Figure 5.7 Patterned cells displayed positive expression of cardiac troponin T (cTnT), while unpatterned cells showed distinct RUNX2 expression after two weeks of cell culture. Primary antibodies, mouse monoclonal cardiac troponin T (1:400, Abcam) and rabbit polyclonal RUNX2 (1:50, Santa Cruz Biotech) were used. Scale bar is 100 μ m.

Cells exert traction forces on the substrate via FAs.^{11,33} The traction force is defined as the net force per unit area applied by cell on underlying substrate.¹⁵ Several previous reports showed that the actomyosin contractility increases with substrate stiffness and

these actomyosin-generated contractile forces encourage FA maturation.^{12,24} Consistent to these reports, our ITG- β_1 FA immunostaining results suggested an increment in FA development with stiffness and consequently, magnitude of cell traction forces was also increased on stiff substrates. Interestingly, when we tried to examine a resemblance between the magnitude of traction force and overall distribution of ITG- β_1 FAs, only partial correspondence was noticed (figure 5. 4). It indicated that only a fraction of ITG- β_1 FAs participated in exerting the traction forces. In accordance to our results, it was already shown that small and nascent FAs generated at leading cell edges exerted more traction forces in migrating cells, while mature FAs were involved in anchoring the cell and exerted weak traction forces.⁴³

Based on our findings, here we presented the model of regulation of cardiomyogenic differentiation in patterned hMSCs (**Figure 5.8**). In the past, researchers observed an elongated FAs in myogenic cells.^{40,44} Moreover, we proved the decisive role of elongated ITG- β_1 FA in cardiomyogenic differentiation of similar patterned hMSCs.³¹ Therefore, based on these collective evidences, we proposed that cell patterning stimulated the development of elongated ITG- β_1 FA and subsequently triggered the development of aligned actin CSK without crosslinking. Since the actin CSK plays crucial role in the signal transmission from FAs to the cell nucleus, changes in stem cell niche or remodeling of actin CSK can alter the gene expression profile of stem cell. Previously, researchers found the well aligned stress fibers in the skeletal myotubes that involved in creating the anisotropic contractility.⁴⁵ In agreement to these findings, we also observed both elongated ITG- β_1 FAs and aligned actin stress fibers in patterned cells that may trigger the upregulation of myocardial genes by developing the optimal cytoskeletal tension.

Figure 5.8 Proposed model elucidating the decisive role of ITG- β_1 FA and optimal cellular tension in upregulating cardiomyogenic gene expression in patterned cells.

5.4 Conclusion

We concluded that cell patterning steers the cardiomyogenic differentiation of hMSCs by controlling the generation of actomyosin-mediated cytoskeletal tension through remodeling the actin CSK. Blebbistatin inhibition study reinforced the dynamic role of actomyosin-generated cytoskeletal tension in stem cell differentiation. Assessment of cell mechanical properties (cell stiffness and traction forces) strongly indicated that there is a tight correlation between cell mechanical properties and stem cell differentiation since these properties were drastically declined in patterned hMSCs committed to myocardial lineage. In addition, we illustrated the involvement of cytoskeletal tension generated on stiff substrate in the development of mature ITG- β_1 FAs. We revealed that magnitude of traction forces increased with ITG- β_1 FA density on PA gel substrates with varying stiffness. In near future, it would be interesting to investigate the specific role of ITG- β_1 FAs mediated signaling pathways in coaxing the stem cell differentiation.

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Chapter 6

Fabrication of Biofunctionalized Gold Micropatterned Platform to Induce Mature Stem Cell Differentiation

In this chapter we reported the fabrication of novel biofunctionalized gold micropatterned platform that can be used to generate skeletal myotubes from hMSCs. Here, we mentioned the stepwise procedure to fabricate the biofunctionalized gold micropatterned substrate that includes photolithography, bioinert polyethylene glycol (PEG) deposition on glass and immobilization of ITG- β_1 antibodies to gold micropattern. The crucial role of elongated ITG- β_1 FAs and aligned cytoskeleton in the induction of myogenesis was highlighted. At the end, we showed the formation of myotubes using several myogenic markers and actin cytoskeleton uniformity.

6.1 Introduction

In chapter 4, we investigated the spatial distribution of ITG- β_1 FAs in myocardial lineage committed hMSCs and further performed the morphological studies to measure ITG- β_1 FAs length and width (Figure 4.10). Based on this quantification study, here, we designed the unique gold micropatterned platform by retrieving the data of dimensions and spatial distribution of ITG- β_1 FAs expressed in those myocardial lineage committed hMSCs (Figure 6.1). This novel platform was used to generate the skeletal myotubes from hMSCs by synergizing the role of cell patterning and ITG- β_1 mediated signaling pathways.

Skeletal muscle makes up nearly half of human body mass (40-50%) and it plays important role in different physical activities like locomotion, breathing and homeostasis. Skeletal muscle has incredible regeneration capacity and can cure minor injuries, for example exercise-induced muscle injuries.^{1,2} However, muscle wasting caused by severe injuries, muscular dystrophies, sarcopenia and neuromuscular disorders cannot be alleviated by skeletal muscle system itself.³⁻⁵ Also, current surgical reconstruction therapies do not repair injured muscle fully. In the past, several strategies were implemented to treat skeletal muscle disorders/injuries which consist of therapies based on drugs, genes and cells.⁶⁻⁹ One of the therapies was intramuscular myoblasts injection which failed to regenerate muscle at injury sites due to poor migration, low survival rate and immune rejection of injected cells.^{10,11} In addition, development of fibrotic tissue in degenerative muscle disorders stiffens the ECM, mimicking the formation of bone-like environment at damaged site. It leads to formation of the calcified lesion upon intramuscular cell injection into injured muscle, partly due to aberrant stiff muscle environment.¹²

Thus, currently, development of potentially useful stem cell-based therapies to treat muscle injuries/disorders is extensively sought-after area in regenerative medicines. Due to the following characteristics, MSCs have already been the popular candidate in the field of stem cell-based regenerative therapies.¹³ MSCs are multipotent i.e. they can differentiate into several tissue lineages. MSCs can be derived from bone marrow or

adipose tissues, making them easily available than other stem cells. These cells are nonimmunogenic which solves the common problem of the risk of immune rejection of cell sources in tissue engineering.^{14,15} Cell patterning is a commonly used technique to regulate stem cell lineage commitment via cell shape modulation.¹⁶⁻¹⁸ Previous reports demonstrated the potential use of cell patterning to improve cell-cell coupling and electrical coupling among cardiomyocytes by accelerating the formation of gap junctions.¹⁹ Cell patterning was also observed to be involved in the development of myotubes by encouraging end to end fusion of myoblasts.²⁰ Similarly, patterned hydrogel substrate with stripes of different stiffness was used to steer stem cell myogenesis and induce myotubes formation using adipose-derived stem cells.²¹ However, observed cell-cell fusion rate was not substantial. Thus, there is tremendous scope to develop the biomaterial scaffold that can synergize the effect of biophysical and biochemical cues to mimic skeletal muscle cell niche and in turn induce the skeletal muscle generation from stem cells.

Figure 6.1 Schematic depicting an idea of reverse engineered gold micropatterned substrate. Gold micropattern features were determined using the dimension and spatial distribution of ITG- β_1 FAs expressed in patterned hMSCs grown on 20 μm wide strip pattern

Based on the aforementioned studies and our previous findings, here we fabricated the biofunctionalized gold micropatterned platform to combine the individual role of cell patterning and integrin- β_1 mediated signaling in the process of stem cell myogenesis to attain higher maturity. This novel platform ensured the ITG- β_1 receptor mediated cell-substrate binding in order to trigger the ITG- β_1 regulated intracellular signaling cascades. Further, micropattern features of this platform controlled the cell alignment to encourage end to end fusion of elongated cells. Stem cell fusion was assessed in depth to validate the myotubes formation on the biofunctionalized gold micropatterned platform.

6.2 Experimental Methods

6.2.1 Fabrication of Gold Micropatterned Substrate

The gold micropatterned substrate fabrication was done using standard photolithography method. Briefly, positive photoresist solution was spin coated onto 13 mm round coverslips. Photoresist coated substrates were then exposed with UV light through photomask followed by substrate washing with developer solution to dissolve the exposed photoresist. A gold layer was deposited into empty regions of photoresist coated substrate by vapor deposition technique. A titanium layer (10 nm thick) was sputtered on the substrate before gold layer deposition to improve the gold-glass bonding. Substrates were eventually washed with acetone to remove remaining photoresist residue. Phase contrast imaging was employed to examine the gold micropatterned substrates using a 100x objective lens. (Eclipse 80i upright microscope, Nikon).

6.2.2 Measurement of Gold Pattern Thickness

The surface topography of micropatterned surface was evaluated by AFM system (NX-Bio, Park Systems, South Korea). Briefly, common AFM contact mode cantilever (spring constant 1 N/m, PPP FMR, NANOSENSORS, Switzerland) was used to image the 30 μm x 30 μm size of sample surface. The scan rate and the maximum loading force were set at 0.5 Hz and 30 nN, respectively. All experiments were performed at room temperature. The AFM cantilevers were calibrated by the thermal method which checked thermal vibration of the AFM cantilever.

6.2.3 PEG Passivation and Gold Micropattern Biofunctionalization

PEG-silane was used to create non adhesive background to prevent cell-glass surface attachment and to facilitate specific cell adhesion to the biofunctionalized gold micropattern. Briefly, 3 mM PEG-silane (1000 MW, Laysan Bio Inc.) was dissolved in anhydrous toluene. After that, samples were immersed in above toluene solution. Triethylamine (1%) was added into toluene solution and reaction was run for 48 h at 60°C under inert atmospheric condition created by continuous supply of nitrogen. Finally, samples were washed with toluene, ethanol and distilled water respectively followed by gentle drying with pressurized nitrogen. For ITG- β_1 antibodies (or ITG- β_3 antibodies or fibronectin) functionalization, PEG passivated samples were immersed with 5 mM 3-mercaptopropionic acid solution for 12 h at room temperature. Samples were then treated with 10 mM 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and 5 mM N-Hydroxysulfosuccinimide solution for 1 h at pH 4-6. Subsequently, ITG- β_1 antibodies solution (10 μ g/mL) was added on chemically conjugated samples after washing with DI water and incubated at 37°C for 1 h. Finally, biofunctionalized gold samples were rinsed with PBS.

6.2.4 Cell Fusion Study

Myotube formation was validated by observing the continuous cytoskeletal morphology and multinucleated state of cell. To check the continuous cytoskeletal structure, F-actin staining was used. Ki67 marker was used to distinguish between differentiated myotubes and multinucleated proliferating cells. During myotube quantification study, cells showing continuous actin cytoskeletal structure, multinucleated state and absence of Ki67 marker were considered as differentiated myotubes.

6.2.5 Image Analysis

Actin stained images were used to calculate the cell aspect ratio which is a ratio of cell length to cell width. Two hundred cells from each of the cell groups were considered to calculate the aspect ratio using ImageJ software. DAPI stained cell nuclei (150 cells from three samples) were considered to measure the nucleus orientation using ImageJ

software. It was quantified by measuring the angle between the major elliptic axis of cell nucleus and the gold micropattern axis. The cell density on different biofunctionalized sample was calculated by counting the cell nuclei numbers automatically using ImageJ software. Five individual images each from both cell groups were used to count the cell nuclei density. More than 400 nuclei were observed from three independent samples each from both groups to calculate the fusion index. Fusion index is defined as the number of nuclei involved in myotubes formation divided by the total number of nuclei.

6.3 Results and Discussion

6.3.1 Fabrication and Characterization of Biofunctionalized Gold Micropatterned Platform

We designed the unique gold micropatterned platform by retrieving the data of dimensions and spatial distribution of ITG- β_1 FAs expressed in hMSCs grown on 20 μm wide strip pattern (Figure 6.1) and the details are mentioned before.²² Phase contrast imaging revealed the formation of desired gold micropattern features on glass substrate (Figure 6.2c). AFM imaging was performed to check the height of gold pattern lanes and average height observed was ~ 600 nm (Figure 6.2d). Fabricated substrates were then passivated with bioinert PEG-silane to avoid nonspecific cell attachment to the substrate. PEG passivation is to ensure specific cell adhesion to gold pattern lanes via integrin receptor-antibody coupling. Water contact angle measurement was done to check the effect of PEG passivation on different surfaces (Figure 6.3b). PEG treated glass ($34^\circ \pm 2.2$) indicated an increase in contact angle as compared to very hydrophilic piranha treated glass ($6^\circ \pm 0.89$). The contact angle value of PEG treated glass is similar to the previous literature values.^{23,24} Contact angle for hydrophobic gold micropatterned surface was observed to be $87^\circ \pm 1.8$ and it decreased to $64^\circ \pm 3.5$ after PEG treatment. Immobilization of ITG- β_1 antibodies to PEG treated gold pattern resulted in drop of contact angle from $64^\circ \pm 3.5$ to $50^\circ \pm 2.8$ and such downward trend in the contact angle values is stated previously.²⁵ To determine the effectiveness of PEG passivation, hMSCs were cultured on the PEG passivated and nonpassivated surfaces. Cells cultured on PEG passivated surfaces were not able to attach well and did not spread even on day 2 (Figure 6.3c). In

contrast, cells cultured on nonpassivated surface demonstrated well-spread morphology. In the next step, functionalization of gold substrate was carried out by immobilizing ITG- β_1 antibodies to the gold pattern and the immobilization was corroborated using immunofluorescence staining (Figure 6.3d).

Figure 6.2 Fabrication and characterization of gold micropatterned substrate. (a) Flow diagram illustrating the fabrication of gold micropatterned substrate by standard photolithography technique. (b) Drawings of top- and cross-section view of gold micropatterned substrate

representing its dimensions. (c) Phase contrast image of fabricated gold micropatterned substrate (Scale bar: 10 μm). (d) Line profile illustrated the height of gold micropatterned substrate. 3D reconstruction of 30 μm x 30 μm micropatterned surface showing the AFM indented area by dotted red line.

Figure 6.3 Biofunctionalization of gold micropatterned substrate. (a) Scheme showing PEG passivation and immobilization of ITG- β_1 antibodies to the gold micropattern lanes. This strategy enhanced specific cell adhesion to the gold micropattern lanes via ITG- β_1 receptor-antibody binding. (b) Water contact angle measurement was done to check the effect of PEG passivation on surface hydrophilicity of different surfaces. (c) Effect of PEG passivation on cell adhesion to different surfaces. Cells were failed to adhere and spread on the surface passivated with PEG even on day 2 (Scale bar: 200 μm). (d) Confocal image of PEG passivated gold micropatterned substrate displaying precise immobilization of ITG- β_1 antibodies to the gold micropattern lanes. Alexa Fluor 488 secondary antibodies used for labeling purpose (Scale bar: 20 μm).

6.3.2 ITG- β_1 Antibody Functionalized Gold Micropatterned Platform Induces Myotubes Formation

ITG- β_1 antibody functionalized platform was used to regulate hMSC's lineage commitment by synergizing the effect of cell patterning and ITG- β_1 mediated specific cell signaling. Cell morphology was monitored over the period of two weeks after cell seeding. Cells exhibited an aligned and elongated morphology on this biofunctionalized patterned substrate (Figure 6.4a), whereas cells cultured on the flat biofunctionalized gold coated substrate demonstrated a randomly-spread morphology. After one week of culture, neighboring patterned cells demonstrated end to end connections. Interestingly, these connected cells went ahead to fuse to form a myotube-like morphology over the course of two weeks (Figure 6.4a). On the other hand, unpatterned cells did not exhibit any end to end connections and/or fused morphology. To quantify such visible morphological differences in between two groups, various cell morphological parameters were assessed. Firstly, the aspect ratio was measured to evaluate the cell elongation which was considerably high for the patterned group (Figure 6.4b). A higher aspect ratio means a more elongated cell. More than 50% patterned cells were found to be aligned along with pattern direction (Figure 6.4c).

Figure 6.4 Biofunctionalized gold micropatterned substrate induces myotubes generation from hMSCs

(a) hMSCs cultured on gold micropatterned substrate showed aligned and elongated morphology and exhibited end to end connections on day 7. These connected hMSCs fused with each other to form myotubes after two weeks of culture. On the other hand, no such cell-cell connection/fused morphology was observed in unpatterned cells. Double arrow red line indicates pattern direction. Single arrow white line indicates cell nucleus position. (b) Aspect ratio is the ratio of cell length to cell width. Patterned cells showed higher aspect ratio than unpatterned cells after 7 days of culture. ($n=3$), $P<0.01$. (c) Cell nuclei orientation was used to calculate the cell alignment after 7 days of culture. Cell with nucleus orientation less than 20° was considered as aligned cell ($n=3$). (d) C2C12 murine myoblasts were used as a positive control. Myoblasts fused to form myotubes after three days of culture. Single arrow yellow line denotes myotubes formed. Immunofluorescent image of C2C12 myoblasts illustrated a positive expression of β -MHC (green), a mature myogenic marker which confirmed the myotubes formation from myoblasts. Scale bar: 100 μm

Now, we interested to see if there are any morphological and phenotypic similarities in between the fused patterned cells and native myotubes. For that purpose, C2C12 myoblasts cultured in 2% horse serum were used as a positive control. One can easily detect the newly formed multinucleated C2C12 myotubes after three days of culture as well as distinct expression of β -MHC (mature myogenic marker) in those myotubes (Figure 6.4d). After close inspection of fused patterned cells and C2C12 myotube, morphological similarities like multinucleated state and continuous cytoskeletal structure were revealed. Multinucleated cell with continuous CSK is a hallmark of skeletal myotube.²⁶ Therefore, F-actin and DAPI staining was performed to verify the cytoskeletal continuity and multinucleated state of cells respectively. Validation of continuous actin CSK along with bi-nucleated state deciphered the fact that myotubes were generated from the cells cultured on patterned substrate (Figure 6.5a). At the same time, we hardly observed any cells from unpatterned group indicating the myotube-like morphology. Further, positive expression of β -MHC and the muscle transcription factor (MyoD) validated the myogenic lineage commitment of patterned cells (Figure 6.5a, Figure 6.6). Absence of proliferation marker, Ki67, suggested that multinucleated patterned cells were differentiated myotubes and not the multinucleated proliferating cells

(Figure 6.5b, first row). Multinucleated cell expressing Ki67 was considered as a proliferating cell and is excluded while counting the differentiated myotube population (Figure 6.5b, second and third row). Significantly high fusion index was detected for the patterned group (13%) in contrast to the unpatterned group (3%) (Figure 6.5c).

Figure 6.5 Validation of myotubes formation (a) Immunofluorescent staining of F-actin (red) and β -MHC (green) from both cell groups validated the myotubes formation on patterned substrate. Double arrow white line indicates pattern direction. (b) To distinguish between differentiated myotubes and proliferating cells, Ki67 dye (proliferation marker) was used. First row represents multinucleated myotube with continuous actin CSK and negative Ki67 staining

from patterned group. Second row displays multinucleated proliferating cell with continuous actin CSK and positive Ki67 staining from patterned group. Third row illustrates multinucleated proliferating cell with continuous actin CSK and positive Ki67 staining from unpatterned group. (c) Patterned cells demonstrated higher fusion index in comparison to unpatterned cells ($n=3$), $P<0.05$. Scale bar: 100 μm .

Figure 6.6 Investigation of myogenic lineage commitment of cells cultured on biofunctionalized micropatterned substrate. Immunofluorescence imaging confirmed the upregulation of muscle transcription factor (MyoD) in patterned cells after 7 days. C2C12 myoblasts distinctly expressed MyoD after 3 days and used as a positive control. Double arrow white line indicates pattern direction. Scale bar: 100 μm

In order to find out the effect of substrate functionalization with other proteins on the myotubes formation, we immobilized both substrates (patterned and unpatterned) with fibronectin (FN), a commonly used ECM. Densely packed cells were observed on both FN functionalized substrates after 7 days of culture which made it nearly impossible to inspect if any cell-cell connections were formed (Figure 6.7a). On the other hand, comparatively fewer cells were observed on both ITG- β_1 immobilized substrates and the cell-cell connections formed on ITG- β_1 biofunctionalized micropatterned substrates were

easily identified. After two weeks, cells cultured on patterned and unpatterned FN functionalized substrates demonstrated five folds and two folds increase in cell density than that of patterned and unpatterned ITG- β_1 functionalized substrates respectively (Figure 6.7b). Again, we failed to observe cell-cell fusion on FN functionalized substrates mainly because of exceedingly tightly packed cells. In another experiment, cells were cultured on ITG- β_3 functionalized substrates to check if it supports cell adhesion and cell-cell fusion. Unfortunately, comparatively few cells attached to ITG- β_3 antibody immobilized substrates. Moreover, cells were unable to identify pattern direction on ITG- β_3 antibody immobilized patterned substrate (Figure 6.8). hMSCs failed to show their characteristic spread and elongated morphology on ITG- β_3 antibody immobilized substrates. Altogether, these experiments clearly underline the importance of the ITG- β_1 biofunctionalized micropatterned substrate in the induction of myotubes formation.

Figure 6.7 Cells cultured on FN functionalized substrates experienced more adhesion and high proliferation rate (a) Optical images showed nearly confluent cells on FN functionalized substrates as compared to ITG- β_1 antibody functionalized substrates after one week of culture. Double arrow white line indicates pattern direction. (b) Cells cultured on ITG- β_1 antibody immobilized substrates consistently showed lower cell density than that of FN immobilized substrates after two weeks of culture (n=3), $P < 0.05$. Scale bar: 200 μm .

Figure 6.8 Comparative study of cells cultured on ITG- β_1 antibody and ITG- β_3 antibody functionalized substrates. By day 2, many cells attached and showed elongated morphology along with pattern direction on ITG- β_1 antibody immobilized patterned substrate whereas, cells completely failed to recognize pattern direction on ITG- β_3 antibody immobilized patterned substrate. Even more cells attached and retained their characteristic spindle shaped MSCs-like morphology on both ITG- β_1 antibody immobilized surfaces in comparison to ITG- β_3 antibody immobilized surfaces. Double arrow white line indicates pattern direction. Scale bar: 200 μm .

6.3.3 Cell Patterning Triggers Elongated ITG- β_1 FAs Expression and Actin Cytoskeleton Modulation

We selected ITG- β_1 receptor for our current study because we previously observed the profound expression of ITG- β_1 FAs in hMSCs as compared to other types of ITG- β FAs and its role in influencing the cell mechanical properties.^{22,27} ITG- β_1 FAs stained images clearly indicated the expression of elongated ITG- β_1 FAs in patterned cells (Figure 6.9a). In stark contrast, dot-like ITG- β_1 FAs structures were developed in unpatterned cells. These results are in agreement with our previous findings where we reported the

development of elongated ITG- β_1 FAs and punctate ITG- β_1 FAs in patterned and unpatterned hMSCs respectively²². Similarly, actin stained images also highlighted the changes occurred in the actin CSK modulation due to the micropatterning. Cell patterning triggered the development of parallel F-actin bundles while, the isotropic network of F-actin bundles were formed in unpatterned cells (Figure 6.9a). Since, the integrins are transmembrane receptors that connect the extracellular matrix to actin CSK, cell patterning can directly affect the actin CSK modulation via controlling the spatial distribution of integrins.^{22,27,28} Therefore, elongated ITG- β_1 FAs and subsequently formed aligned actin CSK can clearly be seen in patterned cells.

Figure 6.9 Micropatterning induces the formation of elongated ITG- β_1 FAs and aligned actin CSK and in turn upregulates myogenesis. (a) Expression of elongated ITG- β_1 FAs along with aligned actin CSK was observed in cells from patterned group (first row) after two weeks of culture while, unpatterned cells expressed dot-like ITG- β_1 FAs and nonaligned actin CSK (second row). Double arrow white line indicates pattern direction. (b) Large population of cells cultured on patterned surface followed pattern direction and exhibited upregulation of β -MHC and generation of aligned actin CSK (first row). On the contrary, few cells that did not follow pattern direction demonstrated downregulation of β -MHC and randomly organized actin CSK (second row). Double arrow white line indicates pattern direction. Scale bar: 100 μ m.

As we mentioned above, cells cultured on patterned substrate apparently showed parallel growth along with pattern direction. However, very few cells (7%) cultured on patterned substrate managed to grow in perpendicular direction. In accordance to above stated results, cells that followed pattern direction exhibited well aligned actin CSK morphology and upregulation of myogenesis (Figure 6.9b, first row), while perpendicularly grown cells showed randomly organized actin CSK and downregulation of myogenesis (Figure 6.9b, second row). A clearly visible difference of actin CSK morphology in between patterned cells and unpatterned cells/perpendicularly grown cells pointed out the role of actomyosin-generated cytoskeletal tension in the induction of myogenesis. These findings are in agreement with our previous results where we proved that the optimal cytoskeletal tension generated in patterned cell steered its myogenic lineage commitment.²⁷

The important role of nano-/micropatterning technique in coaxing stem cell's lineage commitment has well been documented.²⁹⁻³¹ Similarly, in the past, we ascertained the positive effect of coerced cell shape and FA modulation on myogenic lineage commitment of cells.³²⁻³⁴ However, these strategies were not robust enough to persuade terminally differentiated myotubes formation from hMSCs. Thus, we followed a reverse engineering approach as portrayed in figure 6.1 to construct the novel platform garnered with both biophysical cue (pattern) and biochemical cue (ITG- β_1 antibody) to synergistically induce and attain greater maturity in the context of myogenesis. As per our expectation, cells cultured on this platform displayed an elongated morphology. Cell shape modulation is an indirect indicator of phenotypic changes for adult stem cells.³⁵

Hence, patterned cell's phenotypic behavior was evaluated using myogenic markers which confirmed patterned cell's myogenic lineage commitment. Moreover, cell patterning encourages end-to-end cell fusion to generate multinucleated myotubes with continuous actin CSK.

In previous studies, an elongated FAs and aligned actin CSK were identified in myogenic cells.^{22,27,36} Likewise, in the current study, similar features were observed in the myotubes generated on ITG- β_1 antibody functionalized micropatterned substrate. In another study, Damsky et.al. illustrated that ITG- β_1 surface expression was altered during myotubes formation.³⁷ Based on these findings, we propose the hypothesis for generation of myotubes on biofunctionalized micropatterned substrate (Figure 6.10). ITG- β_1 antibody functionalized patterned surface promotes ITG- β_1 receptor mediated cell adhesion and elongated ITG- β_1 FAs formation which in turn stimulates ITG- β_1 regulated specific intracellular signaling cascades. At the same time, this micropatterned surface encourages the development of aligned actomyosin stress fibres. These aligned stress fibres serve as a linkage in between elongated ITG- β_1 FAs and cell nucleus and transmit ITG- β_1 mediated specific signals from FA site to nucleus. Additionally, generation of aligned stress fibres leads to development of optimal cytoskeletal tension that might trigger the upregulation of myogenic genes expression.²⁷ In support of our interpretation, previously well-aligned actomyosin stress fibres generated in the skeletal myotubes were observed to be involved in the development of anisotropic contractility.³⁸ Eventually, integrin- β_1 mediated specific signaling and actomyosin-generated optimal cytoskeletal tension synergistically trigger the formation of myotubes on biofunctionalized micropatterned surface.

Figure 6.10 Proposed model recapitulating the myotubes generation on biofunctionalized gold micropatterned substrate. (a) Biofunctionalized micropattern induces the formation of elongated ITG- β_1 FAs and aligned F-actin stress fibers. Both ITG- β_1 FA mediated signaling and optimal cytoskeletal tension generated by aligned stress fibers synergistically promotes upregulation of myogenesis and in turn myotubes formation from patterned hMSCs. (b) In contrast, hMSCs cultured on biofunctionalized plain gold coated substrate expressed dot-like ITG- β_1 FAs along with dense cross-linked network of F-actin stress fibers. Generation of high cytoskeletal tension due cross-linked network of F-actin stress fibers and diminished ITG- β_1 FA mediated signaling lead to failure of myotubes formation from unpatterned hMSCs.

6.4 Conclusion

Our results thus illustrate the potential of novel biofunctionalized micropatterned platform in inducing the skeletal myotubes formation from hMSCs. Combining the effect of other biophysical and biochemical cues like material compliance and morphogens/cytokines respectively with this novel platform will certainly improve the maturity and population of skeletal myotubes generated. This scaffold will be used not only to engineer the muscle fibres for treatment of severe muscle injuries/disorders but also to investigate stem cell-material interactions to gain insight into signaling pathways involved in stem cell myogenesis. Therefore, in future, this new approach will open a promising therapeutic avenue in muscle tissue engineering and restorative applications.

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Chapter 7

Discussion and Future Work

In this chapter, we briefly discussed and summarized the results of this study and then explained the strategies for future work. This chapter highlighted the major findings of the spatial distribution of ITG- β_1 FAs study, study of mechanism elucidation behind stem cell differentiation and fabrication study of novel platform to improve stem cell myogenic maturity. In future work, we mentioned to explore the specific integrin- β_1 mediated signaling pathways at molecular level that involved in stem cell myogenesis. Such fundamental study will provide essential information for fabrication of biomaterials to trigger specific pathways and manipulate stem cell fate. In addition, we propose the unique strategy to combine material compliance with our novel platform to further accomplish greater myogenic maturity since the incorporation of different biophysical and biochemical cues in bio-scaffold has been shown to be involved in triggering an additive effect to enhance the stem cell differentiation maturity.

7.1 General Discussion and Conclusion

Previous studies from our group and literature emphasized that FA plays important role in cell-material interaction and stem cell fate can be modulated by FA modulation via cell patterning. Based on these findings we hypothesized that it could be possible to modulate the cell-material interactions by regulating the spatial distribution of specific type of integrin receptor to steer stem cell differentiation. A highly advanced super resolution imaging technique known as direct stochastic optical reconstruction microscopy (dSTORM) was employed to explore the micro- and nanoscale spatial distribution of ITG- β_1 in myocardial lineage committed hMSCs. Super resolution imaging technique allowed us to view the ITG- β_1 clusters with size of few tens of nanometers and to investigate their spatial distribution inside ITG- β_1 FAs. The investigation of microscale distribution of ITG- β_1 FAs showed that patterned cells expressed aligned, long and elongated ITG- β_1 FAs, whereas the unpatterned cells displayed nonaligned and short ITG- β_1 FAs. Moreover, ITG- β_1 blocking study demonstrated the decisive role of long and aligned ITG- β_1 FAs in myocardial differentiation of hMSCs. Nanoscale distribution study of ITG- β_1 FAs revealed for first time that in patterned cells, ITG- β_1 clusters were distributed uniformly inside FAs while, in unpatterned cells, ITG- β_1 clusters were mainly spread at the periphery of FAs. Altogether, study of spatial distribution of ITG- β_1 FAs paved the way into deeper understanding of cell-material interaction and consequent mechanotransduction of signals at cell-material interface.

On the basis of above findings, we decided to elucidate the cell mechanotransduction pathways behind the cardiomyogenic differentiation of patterned cells. To do that, initially, we attempted to correlate the changes occurred in cell mechanical properties (cell stiffness and cell traction forces) to cell fate after cell shape distortion. Cell stiffness and cell traction forces were chosen for assessment as both are reliable indicators of actomyosin mediated cytoskeletal tension, a main driving force behind patterned cell's myocardial lineage commitment. Cell mechanical properties were found to be decreased considerably in myocardial lineage committed patterned cells. Blebbistatin inhibition study further strengthened the active role of actomyosin mediated cytoskeletal tension in patterned cell's cardiomyogenic differentiation. We also observed the distinct and mature

ITG- β_1 FAs formation on stiff PA gel substrate due to generation of higher cytoskeletal tension. Magnitude of cell traction forces were found to be increased with PA gel substrate stiffness since ITG- β_1 FA density increased with substrate stiffness. Collectively, these results further pushed us to investigate the specific role of ITG- β_1 FAs mediated signaling pathways in stem cell myogenesis.

In the next project, based on the aforementioned results of dimensions and spatial distribution of ITG- β_1 FAs, we applied a reverse engineering approach to fabricate the novel biofunctionalized gold micropatterned platform to synergize the role of cell patterning and integrin- β_1 mediated signaling to improve stem cell myogenic maturity. This platform induced cell-cell fusion to form the myotubes. In future, such platform after further improvement like combining the effect of other biophysical and/or biochemical cues can be used to generate the muscle fibres for the purpose of chronic muscle injury healing as well as for fundamental study of stem cell-material interactions to understand the signaling pathways involved in stem cell myogenesis.

The specific objectives such as to explore the spatial distribution of ITG- β_1 at micro- and nanoscale level, to elucidate the mechanism behind the myocardial lineage commitment of patterned cells and to engineer the novel platform to induce mature stem cell myogenesis are well achieved. All these studies suggest that stem cell differentiation can be manipulated via cell-material interaction modulation at integrin receptor level. The induction of myotubes formation on novel biofunctionalized gold micropatterned platform indicated that in future, the strategies to integrate other biophysical and biochemical cues should be considered to improve the stem cell differentiation in the context myogenesis.

7.2 Future Work

In chapter 6, we demonstrated the formation of myotubes on ITG- β_1 antibody functionalized micropatterned platform. We believe that ITG- β_1 antibody functionalized micropatterned platform encourages ITG- β_1 receptor mediated cell adhesion and promoted an elongated ITG- β_1 FA formation. These elongated ITG- β_1 FAs then trigger

specific intracellular signaling cascades to induce upregulation of myogenic genes and in turn myotubes formation. Hence, we propose to explore the specific integrin- β_1 mediated signaling pathways at molecular level that involved in stem cell myogenesis. Such investigation study will assist researchers to design the scaffold to induce specific integrin- β_1 mediated signaling pathways to improve myogenic maturity.

Further we propose using biocompatible material with tunable material stiffness coupled with existing micropattern features to promote myogenesis in stem cells. The proposed biocompatible scaffold will be having intermediate elastic modulus (10-12 kPa) to mimic the muscle tissue stiffness since the previous studies stated the regulatory role of material stiffness in stem cell differentiation.¹ ITG- β_1 antibodies will be immobilized on the scaffold to enhance the ITG- β_1 receptor-mediated cell adhesion to the surface. The synergistic effect of material stiffness, cell patterning and ITG- β_1 receptor-mediated cell signaling is hypothesized to promote mature stem cell myogenesis, leading to formation of terminally differentiated functional myotubes.

This reverse engineered platform strategy can be implemented to develop a biocompatible muscle cell patch to enhance myogenesis and dystrophin expression in animal model of muscular dystrophy disease. Muscular dystrophy is a group of muscle disease that marked by progressive weakening and wasting of the muscles. Similar muscle cell patch can be used to treat the muscle wasting caused by severe injuries, sarcopenia and neuromuscular disorders that can't be alleviated by skeletal muscle system itself.

Moreover, reverse engineering strategy can be applied to fabricate novel micro/nanopatterns to stimulate other mature tissue lineage formation e.g. neural tissue, bone tissue, cartilage, adipose tissue etc. Also, similar strategy can be utilized for induced pluripotent stem cells (iPSCs) differentiation into other tissue lineages that cannot be achieved using multipotent stem cells because of its limited potency.

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APPENDIX

Publications

- (1) **Tijore, A.**; Cai, P.; Nai, M. H.; Zhuyun, L.; Yu, W.; Tay, C. Y.; Lim, C. T.; Chen, X.; Tan, L. P., Role of Cytoskeletal Tension in the Induction of Cardiomyogenic Differentiation in Micropatterned Human Mesenchymal Stem Cell. *Adv. Healthc. Mater* **2015**, *4*, 1399-1407.
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