Geometrical guidance of stem cell fate : elucidation studies and implication in tissue engineering

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**GEOMETRICAL GUIDANCE OF STEM CELL FATE:
ELUCIDATION STUDIES AND IMPLICATION IN
TISSUE ENGINEERING**

TAY CHOR YONG

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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ELUCIDATION STUDIES AND IMPLICATION IN
TISSUE ENGINEERING**

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A thesis submitted to the Nanyang Technological University
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2012

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“I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.”

-Isaac Newton

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Tay Chor Yong

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*I dedicate this thesis to
my family and my wife, Yu Li, for their relentless
support and unconditional love.*

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Abstract

Tissue engineering and regenerative medicine aims to construct functional tissues replacement through precise control of local environment that promotes proliferation and differentiation of cells. Widespread attention has been focused on using stem cells as the primary source for tissue engineering due to its self-renewal capability and multipotency. Stem cells are highly responsive to the microenvironment signals and given the right condition, it is possible to direct stem cell differentiation into a particular cell type in the body. Recent findings have underscored the important role of mechanical environment in directing stem cell lineage commitment. For instance it has been shown that materials compliance, applied mechanical stimulus (e.g. stretch, strain, and shear) and regulation of cell shape can exert significant influences in regulating tissue specific gene expression and protein translation.

The objective of this project is to employ a simple and versatile technique to control the spatial arrangement, orientation and geometry of stem cell for the purpose of elucidating the basis of cell shape distortion, cytoskeleton rearrangement and focal adhesions distribution on bone marrow derived human mesenchymal stem cells (hMSCs) lineage commitment in the absence of exogenous soluble factors. To achieve this, microcontact printing was employed to control cell spreading on extracellular matrix protein defined regions. It was found that hMSCs cultured on 20 μ m wide fibronectin lanes printed on poly (lactide-co-glycolide) substrate adopted a highly elongated morphology with concomitant upregulation of myogenic mRNA transcripts and protein, indicative of muscle differentiation. Further elucidation studies revealed that repression of the FAK-ERK signaling events may serve as a molecular basis for the cell shape induced muscle lineage commitment hMSCs.

However, when the cells were patterned as single cells on polyacrylamide hydrogels with physiological relevant stiffness, only the optimal cell shape-matrix rigidity combination were able to induce MHC expression in hMSCs. Most significantly, a critical stiffness threshold is required for MHC expression, suggesting that cytoskeletal tension may act downstream of the shape mechanotransduced events. Our findings underscored the complexity involving the interplay of multiple extrinsic biophysical cues to direct stem cell behaviours. Furthermore, novel insights have been gained into the mechanism by which physical distortion of the stem cells can regulate stem cell differentiation and contribute extensively to the advancements of stem cell research.

Introduction

1.1 Background

1.1.1 Stem cell and regenerative medicine

A recent statistics released by World Health Organization in 2008 has identified cardiovascular related diseases to be the leading cause of death worldwide (<http://apps.who.int/ghodata/>). This has placed a heavy burden on the health care sector and it was estimated that by year 2030, the total number of cardiovascular diseases related death can reach a staggering figure of 23.3 million globally [1]. Under this category of death, ischemia heart diseases resulted from myocardial infarction (MI), contributed to 12.8% of total cases of morbidity in 2008 alone. (<http://apps.who.int/ghodata/>). In MI, myocytes within the vicinity of the damaged region is deprived of oxygen due to reduced blood supply to the heart and thus leads to the eventual death of the tissue. As a result of the sudden loss in the number of functional myocytes, the myocardium undergoes a pathological remodelling process that ultimately leads to the deterioration of the contractile function [2]. Presently, the available therapeutic measures to manage post MI ventricular remodelling are limited to the use of drug therapy and mesh devices that aim to mechanically restraint ventricular enlargement. Even so, these methods do not fully revive the damaged tissue and thus only serves to delay the deterioration. Till date, heart transplantation remains the only viable option to fully restore the myocardial performance to a pre infarction state. However, due to the chronic shortage of available heart donors and an ever increasing waiting list of applicants requesting for donated heart, alternatives are

much needed. The aforementioned painted picture is very much relevant to other types of diseases characterized by irreversible death of differentiated cells that impairs the proper functioning of a tissue/ organs. Regenerative medicine has the potential to rise up to this challenge. The working paradigm of regenerative medicine is to “*replace or regenerate human cells, tissue or organs, to restore or established normal function*” [3]. While the underlying principle of regenerative medicine can be elegantly spelt out in just one statement, the execution is by no means trivial due to the number of technical challenges that have to be addressed.

Over the last two decades, the field of regenerative medicine has evolved into a multidisciplinary subject incorporating materials engineering, biology and medicine with the aim of assembling and/ or regenerating biological substitutes for replacing and repairing damaged tissue due to disease or traumatic insult [4, 5]. It is widely believed that regenerative medicine holds tremendous clinical potential for the treatment chronic and incurable diseases [6]. *De novo* reconstitution of functional tissue or organs primarily focuses on the optimal combination of the three main components namely the (i) *cell source*, (ii) *scaffold* and (iii) *biological signals* [4, 7]. In the context of regenerative medicine, stem cells represent the primary cell source and are characterized by two main unique features: Self-renewability and potency.

Self-renewability refers to the cells ability to undergo multiple cycles of cell divisions while retaining its uncommitted state. Potency can be defined as the cells’ competency to differentiate into specialized cells that constitutes a particular tissue lineage. Broadly speaking, stem cells can be classified as embryonic stem cells

(ESCs), adult stem cells (ASCs) and induced pluripotent stem cells (iPSCs) depending on their source of origin (**Figure 1.1**).

Figure 1.1. Classification and types of stem cells can be categorized based on their origin. Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocysts (a), induced pluripotent stem cells (iPSCs) are genetically modified differentiated cells (example: skin fibroblast) to attain an ESCs like state (b) and naturally occurring adult stem cells (ASCs) that can be found in various tissues such as bone marrow and muscle (c). Figure adapted with permission.[8] Copyright 2009 Macmillan Publishers Limited.

ESCs are derived from the inner cell mass of the mammalian blastocysts, 4-5 days post fertilization, and are considered pluripotent (i.e. possess the ability to differentiate into cells belonging to the three germ layers). ESCs display high level of telomerase and thus are able to proliferate extensively *in vitro*, while maintaining their potency for up to 4-5 months [9]. Strikingly, when injected into severe combined immunodeficient (SCID) mice, teratomas were developed [9]. Clearly, its ability to differentiate virtually into any kind of cell lineages can potentially serve as a double edged sword if the differentiation process is not well managed. Typically, to attain extensive ESCs self-renewal while retaining the pluripotency of the cells lies in the use of complex mix of exogenous soluble factors such as Leukaemia inhibitory factor

(LIF) and either serum or bone morphogenetic protein (BMP) to manage the intrinsic signalling pathways that acts to balance cell propagation and differentiation [10]. Recently, in a series of authoritative works conducted by Austin Smith's group, it was documented that the use of chemically defined inhibitors targeting the mitogen-activated kinase (ERK1/2) and the glycogen synthase kinase-3 (GSK3) pathways is able to maintain long term pluripotency of ESCs[11, 12]. These studies not only highlighted a newly defined platform for large scale expansion of ESCs but also have delineated the critical signalling pathways that control pluripotent state of stem cells.

In a breakthrough study conducted by Takahashi et al in 2006, it was demonstrated that fully differentiated cells such as an adult fibroblast can be induced to attain an embryonic-cell like state, by simply introducing 4 essential factors, namely, Oct3/4, Sox2, c-Myc and Klf4 [13]. They then coined these ESCs like cells as iPSCs. iPSCs not only exhibited ESCs like morphology *in vitro*, but also possess similar proliferative and differentiating characteristics. The discovery of iPSCs is an important milestone in regenerative medicine as it can circumvent the ethical issues commonly associated with ESCs. However, there are still several obstacles that impede their therapeutic usage in the near future. Among them is the need to eradicate genetically and epigenetically introduced errors during the reprogramming process and reduce the risk for teratome formation [14, 15].

In contrast, ASCs are multipotent, with the capacity to differentiate into multiple but limited lineages. Perhaps the most well studied and characterized type of ASCs is the mesenchymal stem cells (MSCs). Although the physiological role of MSCs *in vivo* has yet to be fully elucidated, it is widely believed that they play an active role in the maintenance and regeneration of tissue [16]. MSCs have been isolated from various tissue sources such as the bone marrow [17], skeletal muscle [18], adipose tissue[19]

and umbilical cord[20]. The use of MSCs for the treatment of disease is attractive because they may be autologous, multipotent, exhibit robust self-renewability and is more acceptable than ESCs from an ethical standpoint. Furthermore, MSCs have been reported to promote angiogenesis [21-23], exhibit hypoimmunogenic property and anti inflammation capability [24, 25], making them particularly well suited for cell-based therapies to restore damaged tissues.

Earlier studies involving the use of MSCs to treat chronic diseases in animal models such as myocardial infarction [22, 23, 26], and spinal cord injuries [27], have yielded very encouraging results, generating sparks of optimism and excitement in the medical community. For example, implantation of autologous MSCs in swine MI model resulted in substantial MSCs engraftment at MI site and expression of several myocardial protein markers can be detected in the implanted MSCs. Furthermore, the MSCs treated specimens exhibited significant attenuation of end diastolic wall thickness 4-5 weeks post infarction compared to the control group [26]. Other groups have also observed significant improvements in several key cardiac parameters such as reduced fibrosis and apoptotic events at the MI site, increased injection volume of the left ventricle and enhanced compliance of scarred tissue [22, 28, 29]. Significant progress has been made over the years and currently, there are several ongoing phase I-III clinical trials that utilize adult MSCs as the principle cell source for the treatment of a multitude of noncommutable diseases such as cardiovascular related diseases, bone imperfecta, brain and spinal cord injuries, stroke, diabetes and graft versus host diseases (GvHD) [6]. (**Refer to table 1.1**). Importantly, of all the clinical trials conducted so far, there have not been any reports of undesirable post treatment effects (e.g. formation of tumours) and this further reinforces the notion that MSCs are safe

to be used for therapeutic applications. At present MSCs appears to be an excellent cell source for regenerative medicine.

Table 1.1. Update on the progress of MSCs based clinical trials. Adapted with permission [6]. Copyright 2010 Elsevier Inc

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Condition by Organ System	Trials ^a (Patients)	Allogeneic	Auto geneic	Trophic ^b	Differentiate ^b	IV ^c	Local ^c	IA ^c		
Multiple Systems	18 (1067)									
GvHD	16 (1027)	15	1	16		16				
Sjgren's Syndrome	1 (20)	1		1		1				
SLE(Lupus)	1 (20)	1		1		1				
Bone/Cartilage	26 (1487)									
Arthritis-Foot Fusion	1 (100)	1			1		1			
Bone Fracture	2 (210)		2		2		2			
Bone Neoplasms	1 (50)	1			1		1			
Cartilage Defects	4 (185)	1	3		4		4			
Meniscectomy	2 (110)	2			2		2			
Osteodysplasia	2 (58)	1	1		2	1	1			
Osteogenesis Imperfecta	3 (35)	3			3	3				
Osteonecrosis	2 (51)	1	1		2	1	1			
Periodontitis	1 (10)	1			1		1			
Spinal Fusion	8 (678)	8			8		8			
Cardiovascular	19 (951)									
Dilated Cardiomyopathy	2 (80)		2	2			2			
Heart Failure	3 (200)	2	1	2	1		3			
Ischemic Heart Disease	3 (160)		3	2	1		3			
Myocardial Infarction	7 (428)	4	3	6	1	3	4			
Limb Ischemia	4 (83)	3	1	4			3	1		
Gastrointestinal	3 (480)									
Crohn's	3 (480)	3		3		3				
Kidney	6 (136)									
Acute Kidney Injury	1 (15)	1		1				1		
Kidney Transplant	4 (101)	2	2	4		4				
Lupus Nephritis	1 (20)		1	1		1				
Liver	7 (204)									
Cirrhosis	6 (203)		6	3	3	2	4			
Fam. Hypercholesterolemia	1 (1)	1			1		1			
Lung	1 (60)									
COPD	1 (60)	1		1		1				
Nervous	12 (294)									
Multiple System Atrophy	1 (NA)		1	1				1		
Neuroblastoma	1 (15)	1		1		1				
Spinal Cord Injury	2 (103)		2	2			2			
Multiple Sclerosis	4 (84)	1	3	4		3	1			
Parkinson's Disease	1 (5)		1		1		1			
ALS	1 (24)		1	1			1			
Stroke	2 (63)		2	1	1	2				
Pancreas	4 (210)									
Type 1 Diabetes	3 (110)	2	1	3		3				
Type 2 Diabetes	1 (100)		1	1				1		
Skin	5 (455)									
Diabetic Wounds	3 (360)		3	2	1		3	1		
Systemic Sclerosis	1 (20)	1		1		1				
Epidermolysis Bullosa	1 (75)	1		1		1				
Total	101 (5,344)	59 (3,385)	42 (1,959)	65 (3,588)	36 (1,756)	48 (2,495)	49 (2,683)	5 (166)		
Completed trials (n=21)		Scheduled for completion (n = 63)								
2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	Not specified/other
1	1	2	8	8	21	20	13	5	1	17/4

^aData were collected from ClinicalTrials.gov as on 13 March 2010. Searches were made with the following keywords: 'Mesenchymal Stem Cells', 'Mesenchymal Stromal Cells', 'Multipotent stromal cells', 'Stem cells for Spinal fusion', 'Prochymal', and 'connective tissue progenitor'. 85% of trials utilize culture-expanded cells. Rationale for study can be classified as either ^btropic or ^bdifferentiation driven. Modes of delivery: ^cIV(intravenous); IA (intra-arterial); local delivery by means of scaffold or direct injection into target tissue.

However recent reports have documented deleterious calcification at the MI site where undifferentiated MSCs was implanted, raising concerns whether MSCs should be pre differentiated prior to implantation [30, 31]. It has been shown that

transplantation of allogenic fetal neural stem cells in human can lead to the development of brain tumour [32]. Further analysis suggests that the tumour was developed from the introduced undifferentiated fetal stem cells [32]. Thus, it is imperative that stem cell differentiation *in vivo* is regulated with sufficient extracellular stimuli from their surrounding environment. Because stem cells are not like drugs, the likelihood of the cells “misbehaving” may result in detrimental outcomes. Clearly, further measures to effectively engineer the behaviours of stem cells in a more reproducible and predictable manner is essential to fully harness its potential for regenerative medicine. One approach is to manage the differentiation process through the design of materials [8, 33].

1.1.2 Materials science and stem cell biology: A strengthening interface

To unlock the full potential of stem cells will require a holistic approach that encompasses the existing cell therapy protocols and integration of materials based platform that aims to: (i) provide fundamental structural support (scaffolding material) to house the stem cells, (ii) improve engraftment efficacy at the diseased or injured tissue, and (iii) provide the cells with a carefully engineered microenvironment to modulate its behaviours [34].

In vivo, stem cells are located at tissue specific microenvironment termed “niches” that possess a complex set of regulatory factors orchestrating cell growth, differentiation and homeostasis [35]. Within the niche of residences, stem cells receive extrinsic biochemical signals via membrane bound receptors. Concurrently, the cells constantly survey their immediate biophysical surrounding, receiving and assimilating information that initiates vital signalling cascades, modulating the behaviour. Central to the development of stem cell based therapeutics will require the need to appreciate

how stem cells interrogate the interplay of biochemical and biophysical regulatory factors, leading to proper stem cell development, maturation and differentiation.

Over the past decades, enormous efforts have been placed on identifying and understanding the use of soluble factors such as small molecules and cytokines to direct stem cell fates. Standard chemical concoctions have been established to expand and home stem cell differentiation along distinct tissue lineages [36]. However, in recent years, with the advent of “smart petri-dish” platforms, there are mounting evidences, suggesting that the biophysical cues are as fundamental an element as soluble signals, capable of regulating key stem cell activities. The common physical stimuli included substrate mechanical properties, small scale surface features and external mechanical stimuli, which will be reviewed in Chapter 2. Emergences of these enabling technologies are results of the interdisciplinary, concerted efforts involving tissue engineers, materials scientists, clinicians and biologist bringing new ideas and innovative platforms to solve issues associated with traditional stem cell biology. Employments of these platforms have gained novel findings and valuable insights into how biophysical spatial and temporal cues presented at the cell-materials interface can direct targeted differentiation and remodelling of stem cells.

By employing an interdisciplinary approach involving materials science and engineering concepts and techniques, this study aims to shed light on the fundamental questions:

“How do mechanical forces regulate cellular growth and form?”

Although recent works emerging from Chen’s lab [37, 38] have shown that cell shape can serve as a pretext to direct hMSCs to differentiate along a particular lineage under the influence of appropriate biochemical cues (interplay of biomechanical and

biochemical cues), it is not known how cell shape alone can impact stem cell differentiation in the absence of exogenous soluble factors. Progress in this study will provide a novel insight into the mechanism of how physical distortion of stem cell shape can regulate stem cell differentiation and contribute extensively to the advancement of stem cell research and tissue engineering.

1.2 Research objectives

The general objective of this study is to engineer a series of *ex vivo* artificial stem cell niche to control the spatial organization and spreading of cells on 2-dimensional substrates for the purpose of gaining insights into mechanotransduced lineage commitment of human bone marrow derived mesenchymal stem cells (hMSCs). This can be achieved by employing micro contact printing (μ CP) [39], a highly versatile technique capable of printing ECM proteins with micro scale precision, to the substrates of interest and conferring cell adhesion exclusively within the printed regions [40].

The specific objectives are:

1. Develop and evaluate biomaterials based strategies to invoke stem cell responsiveness to geometrical cues at the cell-materials interface (Engineering the stem cell niche)
2. Elucidate the role the canonical FAK-ERK signalling pathway in cell shape regulated stem cell lineage commitment.
3. Develop novel platform to examine the interplay of multiple biophysical signals and its implication in directing stem cell lineage commitment

1.3 Research programme

The framework of the proposed research programme is based on the listed specific objectives presented in the previous section. The study can be sub divided into 3 phases (**Figure 1.2**):

1.3.1 Phase 1: Examine influence of cell elongation on hMSCs differentiation

Recent studies have employed nanofeatures such as nanogratings and nanofibers to induce myogenic and neurogenic activity in hMSCs [41, 42]. Furthermore, when cultured on these engineered substrates, cells are coerced to grow and elongate. Such consistent correlation between cell elongation and induction of stem cell differentiation towards the soft tissue lineages raises an intriguing question:

“Is cell elongation a regulator of myogenesis and/ or neurogenesis of hMSCs? Or is cell shape simply a manifestation of the differentiation process induced by the nanofeatures?”

Using μ CP, ECM protein was transfer printed onto biodegradable substrates for the purpose of controlling spatial attachment of hMSCs. Against a non-bio fouling background, hMSCs were forced to elongate and migrate exclusively along micron sized ECM protein defined lanes (20 μ m wide). Subsequently, cell lineage commitments of these elongated cells were analyzed both at the mRNA and protein level.

1.3.2 Phase 2: Elucidate downstream signalling events as a result of coerced hMSCs elongation

As an extension to the study conducted in phase 1, this part of the study aims to dissect the molecular machinery that may be involved under the condition of hMSCs elongation. Critically, we seek to understand:

“How do hMSCs translate geometrical cues into intracellular signalling events? What are the potential mechanisms by which signalling activities are triggered to direct specification along a particular lineage?”

Early dynamic spreading of hMSCs was monitored and adhesion dependent signalling pathway was assessed at the protein level. The involvement of the signalling pathway will be validated by use of specific inhibitors to block the pathway and examine its effect on hMSCs differentiation.

1.3.3 Phase 3: Examine interdependence of both cell shape and matrix rigidity on stem cell behaviour and lineage specification

In vivo, hMSCs are subjected to a multitude of biophysical cues and collectively, these cues regulate its behaviour. Due to a lack of suitable platforms, the effects of biophysical cues such as cell shape and matrix rigidity are often being examined in isolation. Therefore in phase 3, we present hMSCs to multiple biophysical signals and examine the interplay of these sets of signals on hMSCs behaviour. Using this novel platform we hope to address the following question:

“How do hMSCs response to multiple biophysical cues and does one biophysical cue dominates the others?”

Recent findings have also advocated the use of hydrogel-based platforms as cell culture surface, because the substrate stiffness can be engineered to mimic physiological relevant stiffness normally encountered by the cells *in vivo* [40]. In this manner, it would be of interest to examine the combined effects of coerced cell shape and matrix rigidity on hMSCs responses.

Figure 1.2. Overview of the proposed research program.

1.4 Thesis outline

To realize the therapeutic application of hMSCs, it is imperative to understand the factors governing stem cell differentiation. In chapter 2 we review the concept of mechanotransduced stem cell responses. Various *in vitro* methods that exploit mechanical forces to control stem cell fate and the affected intracellular signalling activities will be discussed. We then illustrate how the use of micro-fabrication techniques can be employed to modulate stem cell morphology and its eventual fate. The general materials and methods utilized in this thesis will be elaborated in chapter 3. Content presented in chapter 4 will encompass the use of the μ CP technique on bioresorbable substrates to engineering stem cell morphology and alignment. We will examine the influence of coercing stem cells to adhere and grow on these spatially predefined patterns on lineage specification along various cells types that arose from the mesodermal layer. In chapter 5, we seek to understand the underlying molecular mechanisms that govern the eventual fate of hMSCs on the micropatterned bioresorbable platform. Then, in chapter 6, we design and develop a novel micropatterned platform that allows us to examine influences of multiple biophysical forces on hMSCs cytoskeletal and focal adhesion development, cellular contraction force and lineage commitment simultaneously. Finally, conclusion and future work will be presented in chapter 7.

Literature review

2.1. Introduction

Stem cells play a pivotal role in the development and maintenance of tissue function. *Ex vivo*, these cells exhibit extensive self-renewal and multipotent differentiation capabilities, making them an attractive cell source for regenerative medicine. *In vivo*, stem cells are located in tissue-specific microenvironments, termed “niches”, that possess a complex set of regulatory factors orchestrating cell growth, differentiation and homeostasis [35]. Within the niche of residences, stem cells receive extrinsic biochemical signals via membrane-bound receptors. Concurrently, the cells constantly survey their immediate biophysical surroundings and receive and assimilate information that initiates vital signalling cascades, which modulate the behaviours of the cells. Development of stem cell-based therapeutics will require understanding how stem cells interrogate the interplay of biochemical and biophysical regulatory factors to ensure proper stem cell development, maturation and differentiation.

Over the past few decades, enormous efforts have focused on identifying and understanding the use of soluble factors, such as small molecules and cytokines, to direct stem cell fates. Standard chemical concoctions have been established to expand and direct stem cell differentiation along distinct tissue lineages. However, in recent years, with the advent of “smart petri dish” platforms, mounting evidence has suggested that biophysical cues are as fundamental an element as soluble signals are and regulate key stem cell activities (**Figure 2.1**). The emergence of these enabling

technologies is the result of interdisciplinary, concerted efforts involving tissue engineers, materials scientists, clinicians and biologists who bring new ideas and innovative platforms to solve issues associated with traditional stem cell biology. The use of these platforms has generated novel findings and valuable insights into how biophysical spatial and temporal cues presented at the cell-material interface can direct the targeted differentiation and remodelling of stem cells.

Figure 2.1. Beyond the flat and dull surfaces of conventional petri dish. “Smart petri dish” surfaces that confer precise stem cells-materials interaction are useful tools to manage stem cells behaviours. Biophysical parameters such as micro-nanotextured surfaces, tuneable matrix stiffness, mechanical stimuli and small-scale patterning can be incorporated to recreate *in vivo* like microenvironment for regenerative medicine.

Central to the mechano-regulation of stem cell responses is the concept of mechanotransduction. By definition, this term refers to the intrinsic capability of the cell to sense external physical cues and translate them into molecular signalling, which leads to biological responses [44]. Mechanotransduction is gaining importance as researchers are starting to appreciate the influence it exerts on stem cells [45, 46]. Stem cells physically engage onto a given substrate via discrete attachment points composed of integrins, which are heterobifunctional transmembrane proteins that link

the intracellular cytoskeletal components to the extracellular microenvironment. Through these focal points, the cells mechano-sense and regulate force transmission to the underlying substrates. Material properties, such as substrate rigidity, applied mechanical loading, patterned surfaces and intricate small-scaled features where the cells interface with the materials, have been shown to contribute to the feedback loop and regulate stem cell fates. In some cases, the mechanical microenvironment is also crucial to set the appropriate context before stem cells can respond effectively to biochemical cues.

In this section, an overview of the various *in vitro* platforms that have been developed over the years to investigate, elucidate and regulate stem cell response to biomechanical signals will be provided. Particular emphasis will be placed on the interaction of stem cells with engineered materials that offer controllable bulk and surface properties to control stem cell adhesion and signalling. Involvement of the focal adhesions and the cytoskeleton systems as a common biophysical impetus through which these mechanical signals are transduced will also be discussed. These insights are envisioned to provide the basis for the rational design of future biocompatible materials and may inspire alternative drug-free therapeutic strategies to manage diseased sites via biomechanical management.

2.2. *In vitro* mechano-regulation of stem cell fate

In the continual pursuit to attain adequate control over the fate of stem cells, biologists have turned to innovative *in vitro* models that are capable of recapitulating distinct aspects of the *in vivo* microenvironment. Systematic manipulation of the surface properties, which spans from the macro- to the nanoscale, offers new insights

into cell-material interactions and unprecedented control over stem cell fate. We will highlight recent developments in the use of biophysical methods at the cell-material interface to drive the self-renewability and the lineage commitment of stem cells.

2.2.1. Matrix compliance: Adopting the softer approach

Economics in scalability and ease of fabrication have made tissue culture plates by far the most widely used platform for basic cell culture. Nonetheless, such plastic surfaces are of several magnitudes stiffer than most *in vivo* tissues [47, 48]. The elastic modulus, E , of a given material is its inherent ability to deform elastically and serves as a useful measure of how “hard” a material is. Mathematically, it can be defined as the stress experienced by the material per unit change in dimension without sustaining any permanent/ plastic deformation. The commonly used plasma treated tissue culture plates (TCPS) typically have an elastic modulus in the range of 10^9 Pa, while at the cellular level, the range of tissue stiffness the cells experience are drastically softer than a hard slab of plastic. Such disparity raises question on the widely accepted viewpoint such as the appearance of “normal” adherent cells and the possible accompanied deviation of its unadulterated *in vivo* phenotype. For instance, cells grown on much softer substrates were documented to display drastic differences in a diverse set of cellular behaviours such as cell spreading, motility, differentiation and growth compared to conventional TCPS surfaces [49]. Furthermore, a biomimetic stiffness that emulates the mammary gland (75-120Pa) favours production of β -casein in primary mammary epithelial cells compared to a stiffer microenvironment, suggesting *in vivo* mimicking environment is essential to impart functionality [50]. Consequently, these considerations have promoted many researchers to employ compliant hydrogels as replacement substrates for both 2D and 3D *in vitro* cultures in an attempt to recapture *in vivo* like cellular responses.

Figure 2.2. Schematics of in vitro techniques to regulate stem cell fate by means of modulating substrates compliance. hMSCs displayed phenotype resembling cells located at native tissue on Polyacrylamide hydrogels with different elasticity (i.e. brain:0.1-1kPa, muscle: 8-17kPa, bone: 25-40kPa). (panel A). hMSCs spreading on 1kPa polyacrylamide hydrogels was shown to be a function of gel thickness. Below the threshold thickness of 5μm, the cell began to spread suggesting that cells can feel and respond to the rigid support. (panel B) hMSCs osteo-adipogenic differentiation as a function of pillar height. Cells were stained for alkaline phosphatase (osteo marker) and oil red (adipo marker). (panel C) Figures adapted with permission [43],[51] and [52] Copyright 2006 Cell Press, 2010 Institute of Physics, and 2010 Nature Publishing Group.

Systematic studies that probe the influences of matrix stiffness on a diverse set of cellular behaviours will require a mechanically tuneable platform over an acceptable range of E while maintaining the same ligand type and density presentation. Till date, there are several methods to modulate the cells' perception of stiffness namely: changing (i) **cross-linking density** or (ii) **thickness** of hydrogels and (iii) **geometrical parameters** of microscaled post arrays on which the stem cells are cultured on (Figure 2.2).

The most commonly used method to control hydrogel stiffness is to modulate the cross-linking density. When presented with an aqueous environment, the hydrogels will swell due to the difference in osmotic pressure, until equilibrium is reached. The degree of cross-linking for a given hydrogel will determine the swelling ratio of the polymeric network. In general, the higher the swelling ratio the more compliance the

material is. There are several synthetic and natural occurring hydrogels and their derivatives that are well suited for this purpose, such as polyacrylamide (PA), polyethylene glycol (PEG), chitosan, alginate, matrigels and collagen gels. Naturally derived hydrogels with its inherent biochemical cues can facilitate cell adhesion and growth. However, the use of synthetic hydrogels is also appealing, as it enables the end users to tailor its mechanical, chemical and biological properties in a reproducible manner.

In a landmark work conducted by Engler et al., it was shown that hMSCs cultured on collagen I coated PA hydrogels of stiffness that mimics the brain (0.1-1kPa), muscle (8-17kPa) and bone (25-40kPa) tissue, were coax to differentiate into cells belonging to the respective tissue lineages in the absence of any exogenous factors (**Figure 2.2A**) [43]. Following this study, several other labs have reported such substrate stiffness regulated stem cell differentiation effect independently; further reinforcing the idea that biomechanical cue is anything but merely a passive cue in a biological setting [53-56]. However, recent studies have also highlighted that stiffness alone is not sufficient to trigger lineage specific differentiation and that the complex interdependency of surface biochemistry and materials elasticity needs to be considered to direct stem cell differentiation [50].

When cultured on hard surfaces, cells adhere and flatten extensively and eventually undergo cell senescence [57]. Several groups have also reported spontaneous differentiation of both embryonic and adult tissue derived stem cells when cultured in typical tissue culture plate setting with increasing passage number [58, 59]. Both observations bear significant consequences in the bench-bedside translation potential

of stem cells and may perhaps be useful to explain why *in vivo* hMSCs do not readily differentiate in the soft bone marrow niche where it resides, having a low storage modulus of $220\pm 50\text{Pa}$ [60]. Indeed, when hMSCs were grown on PA hydrogels with stiffness of 250Pa , the cells remained quiescent but preserved its ability to respond positively to osteoinductive and adipogenic medium when re-plated on glass substrates [60].

Presently, several expensive optimal soluble cocktails to attain adequate balance between stem cell dormancy, renewability and its intrinsic capability to self differentiate have been devised. For example, LIF is often used in mouse embryonic stem cell culture to suppress spontaneous differentiation and promote pluripotent cell proliferation [13]. Recently, Chowdhury et al. demonstrated that by culturing mESCs on compliant PA hydrogel of stiffness 0.6kPa , mESCs was able to proliferate and retain the expression of major stemness markers such as Oct3/4 and Nanog upon withdrawal of LIF from the culture medium up to a culture period of 5 days [61]. Whereas, mESCs cultured on rigid TCPS or harder PA hydrogel acquired aberrant phenotype with extremely low expression of stemness markers. Similarly, muscle stem cells isolated from mouse cultured on compliant PEG hydrogels with muscle like stiffness (12kPa) displayed efficient self-renewal and retained its stemness after multiple divisions [62]. Findings presented herein advocate the relevance and importance of bio mimicking stiffness as a critical parameter for homeostatic regulation of cell and tissue.

In most cases, the hydrogels are firmly attached to rigid substrate such as glass coverslips to facilitate handling. Under such circumstance, it is critical to control the

thickness of the hydrogels to eliminate any stiffness interference from the underlying rigid support. hMSCs spreading on 1kPa PA hydrogels was shown to be a function of gel thickness (**Figure 2.2B**) [51]. Cell on the thick gels remained round and spreading was restricted. Below the threshold thickness of 5 μ m, the cell began to spread suggesting that cells can feel and respond to the rigid support [51]. The authors then made a fitting analogy on the ability of the cells to feel the rigid support, to the story of the princess being able to feel the pea beneath soft mattress. Can the princess then feel the pea when another material is used? In other words, is such phenomenon material dependent? Leong et al. demonstrated by culturing hMSCs on 1440 μ m thick rat-tail derived type I collagen gel (intrinsic shear modulus of $\sim$ 100Pa), cell proliferation was halted and the cells remained in its quiescent state [63]. When the thickness of the gel was reduced to 130 μ m, cell spreading increase, indicative that the cells are sensing a higher effective modulus due to the interference effect. The disparity between the threshold thickness of synthetic (i.e. polyacrylamide) and biopolymer (i.e. collagen gel) before the cells can sense the rigid support may be explained, in part by the strain stiffening effect commonly exhibited by biopolymers [64]. Accordingly, cells will experience higher net localized materials stiffness on naturally occurring polymer as it exerts a force onto the substrate. Both studies revealed that gel type and thickness are important design criteria that should be considered when used as a surface coating for tissue engineering purposes.

An alternate strategy to engineer the “apparent stiffness” perceived by the cells, is to culture the cells on an array of micromolded elastomeric pillars with adjustable parameters (**Figure 2.2C**) [52, 65]. The resistance to the bending motion of the micropillars is characterized by the spring constant, k and is directly proportional to

the radius and inversely proportional to the length of the micropillars [66]. (i.e. $k \propto r^4/L^3$) Therefore, by changing the height and/ or radius of the micro-post, the degree of bending will be altered for a given lateral force. An added advantage of this approach is that the bending of the micro-posts can be easily detected and its associated cell traction force (CTF) exerted by the cells can then be measured at the same time [52, 65]. CTF is a measure of the cell-generated tension via actomyosin interaction and is transmitted to the underlying substrate via the focal adhesion sites. CTF has been implicated in key cellular activities such as adhesion, migration, homeostasis and stem cell differentiation, serving as a pretext for the cells to respond effectively to biochemical induction medium. CTF, spreading and adipogenic/osteogenic differentiation of hMSCs was modulated as a function of micropillars height [52]. Cells cultured on a sea of short (rigid) micropillars developed high CTF and favours osteogenic differentiation while cells on tall (soft) micropost displayed reduced spreading, low CTF and seemingly favours adipogenic differentiation [52].

2.2.2. Micro-nano surface architecture: Introduction of textured surfaces

The push to miniaturize electronic devices has yielded numerous small-scaled technologies in the semi-conductor industry. Coincidentally, these technologies are readily translatable in many *in vitro* settings and have been proven useful from a stem cell biologist standpoint to reconstruct the micro-nano (10^{-6} - 10^{-9} m) features cells usually interact *in vivo* [8, 33, 46, 67, 68]. Advances in micro-nanoscaled fabrication methods have enabled the development of numerous novel platforms to probe stem cells interactions with its biophysical microenvironment [45]. Application of micro-nano textured surface in stem cell biology is primarily driven by 2 motivations. Firstly, it offers researchers the tools to systematically examine the responses of stem cells on these biosynthetic surfaces. In such cases, the types of micro-nano features

usually consist of regular motifs of defined geometries in which the associated spatial-temporal parameters can be engineered to a great precision in a reproducible manner. Secondly, tissue engineers can employ small-scaled techniques to reconstruct textures that mimic the native extracellular matrix, thus conferring some form of biomimetic characteristics to the engineered constructs. Biomimetic surfaces will be extremely useful for stem cell based therapies that requires an engineered graft to enhance cell engraftment competence, integrate efficiently with the host tissue or induce tissue like alignment [46].

Broadly speaking, micro-nano biosynthetic surfaces can be categorized in terms of their regularity. Some examples of surfaces with regular micro-nano sized topologies include grooves/ ridges [41, 69, 70], vertically standing tubes [71-75], arrays of pits [76, 77] and pillars [52, 78]. On the other hand, examples of irregular configurations include mesh-like network [79], disordered arrangements of nanopits [76, 77] or even wrinkles [80].

A consistent and interesting phenomenon commonly observed when cells are cultured on micro-nano grooves is that the cells typically elongate and aligned along the axis parallel to the features. Harrison first observed this phenomenon of “contact guidance” in 1911 when he noticed that cells derived from the embryo of *Rana Palustris* (pickerele frog) orientated and migrated along micron thick spider webs [81]. Likewise, when ESCs and hMSCs were cultured on micro and nanoridges, the cells were coerced to elongate substantially and underwent neurogenesis without the aid of any induction medium [41, 69, 70]. When compared to biochemical neuronal induction medium supplemented with retinoic acid, hMSCs on nanogrooves displayed comparable neuronal differentiation efficiency. hMSCs neuronal differentiation was further enhanced as assessed by the gene expression of Sox1 and Sox2 under the

influence of both retinoic acid and nanogrooves, implying that the use of biophysical cue complements targeted neurogenic differentiation using biochemical means [41]. Furthermore, these results suggest that nanofeatures induced cellular elongation may provide a biophysical impetus that may target a common biochemical pathway that initiate neuronal differentiation in embryo and mesenchymal derived progenitor cells.

While regular structures that induce cell elongation and alignment can induce stem cells to differentiate along soft tissue lineages, it appears that nano features arranged in a random manner favours osteodifferentiation. For instance, Dalby et al. explored the possibility of inducing osteogenic differentiation of hMSCs cultured on Polymethylmethacrylate (PMMA) surface decorated with nanopits of 120nm in diameter and 100nm depth without the aid of osteoinductive medium [76]. The nanopits were arranged with an average centre-to-centre distance of 300nm with 5 different configurations namely, (i) Square array (SQ), (ii) hexagonal array (HEX), (iii) disordered array where the pits were placed randomly with mean displacement of 20nm in both axes with respect to the neighbouring pits (DSQ20), (iv) disordered array where the pits were placed randomly with mean displacement of 50nm in both axes with respect to the neighbouring pits (DSQ50) and (v) pit placed randomly over a 150µm by 150µm field (RAND). After 21 days of culture, of all the configurations examined, only hMSCs cultured on DSQ50 formed dense aggregate suggestive of bone nodule formation. Indeed, via immunocytochemical assay, hallmark osteomarkers such as osteopontin and osteocalcin were highly expressed for cells grown on DSQ50 [76]. Recently, using the same nanofeatures but on another polymeric substrate, polycaprolactone (PCL), it was shown that SQ configuration can maintain the stemness (assessed by immunodetection of STRO-1 and ALCAM) and multipotential differentiation of hMSCs (assessed using classical osteoinductive and

adipoinductive medium) up to eight the weeks in culture [77]. Although details regarding the mechanism of such nano-features induced maintenance of stem cell stemness and pluripotency remains to be elucidated, the authors proposed that it might take effect in part by regulating the metabolomic profile of hMSCs [77]. Collectively, findings presented by the author highlighted the competency of stem cells to reciprocate to biophysical cues at length scale much smaller than the cell itself. It would be of interest to investigate if other sources of stem cells would respond similarly to the substrates as it can potentially be considered as an alternative culture strategy for long-term culture of stem cells.

Several authors have also reported nanotube-inspired culturing platform for stem cells culture. The use of carbon nanotube is attractive because of its reported biocompatibility, long-term stability and ease of fabrication and surface functionalization to meet its needs [82-84]. An immediate consequence of introducing these small-scale features is increasing the surface roughness and hence the surface area made available for protein adsorption [85, 86]. As protein adsorption constitutes the first of a multifarious sequence of events that mediates cellular adhesion and behavior, it should be regarded as an important consideration on nanotextured surfaces. Deposition of single wall carbon nanotubes (SW-CNTs) as a mesh like network (<100nm in height) on coverslips promoted hMSCs adhesion and spreading [79]. Interestingly, high resolution field emission scanning microscopy images revealed high occurrence of filopodia and cellular process interacting with the underlying nanocues, suggesting that nanoroughness can enhance cell-material interaction. Furthermore, screening of several osteogenic and neurogenic gene markers indicated a transient upregulation of neuronal markers after one week of culture in normal DMEM.[79] Oxygen plasma treated carbon nanotubes (CNTs) thin

film can also greatly enhance osteogenic differentiation of hMSCs without any biochemical treatment [87]. Recently, thick layer ($5\mu\text{g}/\text{cm}^2$) of multi walled carbon nanotubes (MW-CNTs) has also been proposed as a substitute feeder layer for the expansion of mouse induced pluripotent stem cells (iPSCs). iPSCs cells on the synthetic feeder layer were able to retain their stemness markers expression over a period of 5 days in culture [88]. CNTs can also be micropatterned on surfaces to control the morphology and spatial organization of hMSCs [89]. Recently, the same method has been extended to modulate neuronal differentiation and structural polarization of neural stem cells [90].

Vertically standing nanotubes constitutes yet another class of nanofeatures frequently used to control stem cell behaviours. Diameter of vertically aligned TiO_2 nanotubes form by anodization of titanium in suitable electrolyte was reported to be an important parameter effecting cell adhesion behaviours, induce osteogenic differentiation and dictate viability of hMSCs [71, 72, 74, 75]. On 15nm TiO_2 nanotubes, integrin clustering, cell spreading was enhanced whereas cell adhesion and the canonical FAK-ERK signalling activity were severely impaired for MSCs grown on 100nm TiO_2 . Consequently, majority of MSCs underwent apoptosis/ anikis as nanotube $>70\text{nm}$ does not permit formation of cellular focal contacts (**Figure 2.3**) [72, 74]. Smooth muscle and endothelial differentiation of MSCs on TiO_2 in biochemically-defined medium was also favoured for 15nm TiO_2 nanotubes compared to 100nm TiO_2 nanotubes [75].

Figure 2.3. **TiO₂ nanotube pore size regulated focal adhesion development and hMSCs fate.** Top panel: hMSCs counterstained for the focal adhesion protein paxillin (red) filamentous actin (green) and nucleus (blue). Cellular spreading and focal adhesion formation of hMSCs on 15nm nanotube (A,C), and on 100nm nanotube (B,D). Scale bars=50 μ m (A,B); =100 μ m (C,D). Bottom panel: Hypothetical model put forward by the authors, suggesting that the optimal spacing of 15nm supported integrin clustering and induced the FAK-ERK signalling cascade which plays a critical role to ensure adherent cell survival. Adapted with permission [72]. Copyright 2007 American Chemical Society.

2.2.3. Applied mechanical loading: Stressing stem cells to work

Biomechanical forces play a pivotal role in the physiological maintenance and development of tissues [91]. Forces derived from cell division and apoptosis of the aminoserosa cells was reported to contribute significantly to the cell-sheet closure during *Drosophila* embryogenesis [92]. Likewise, in mature tissues, biomechanical forces continue to play a critical role in the physiological maintenance and tissue development that persist throughout adulthood. This is particularly true as in the case of the musculoskeletal system, in which its primary function is to enable locomotion and yet tissue movement itself regulates the growth and development of the system. Observations dated back to the nineteenth century by Julius Wolff demonstrated that the bone display an adaptive remodelling capability in response to mechanical loading and changes its form accordingly [93]. Application of quasistatic axial tensile stress to chick rudiments also encouraged bone formation *in vitro* [94]. In contrast, lack of

mechanical loading will lead to net resorption of bone mass and osteoporosis [95]. At the cellular level, bone marrow derived osteoprogenitor cells located in the intramedullary canal are constantly subjected to fluid flow and hydrostatic pressure which are essential for bone generation [96-98]. Despite its apparent involvement, how mechanical forces regulate the behaviours of stem cells both *in vivo* and *ex vivo* is not well understood.

In vivo stem cells may experience different types of mechanical stresses depending on the anatomical location and physiological function of the tissues in which they reside in. Broadly speaking, the type of mechanical stress cells normally experience *in vivo* can be (i) tensile, (ii) compressive or (iii) shear (**Figure 2.4**). Each class of force can be applied readily *in vitro* using specialty bioreactors, thereby allowing researchers to systematically examine the force-induced cell responses. In recent years, there are increasing evidences and growing consensus that specific stress type have been consistently observed to induce stem cells to develop towards a particular phenotype and will be discussed in this section.

Figure 2.4. Schematics depicting representative mechanical forces normally experience by cells in organs such as heart, knee joints and blood vessels and the corresponding *in vitro* techniques to micmic these forces. [99-116]

Mechanical tensile strain has been utilize to direct smooth muscle differentiation of MSCs. Smooth muscle cells (SMCs) that are aligned around the blood vessels circumferentially to regulate blood flow are constantly subjected to cyclic tensile loading due to the pulsatile nature of the blood flow. Employment of cyclic stretching to induce SMCs differentiation have been well documented in various types of progenitor cells such as, mESCs [99, 103], hMSCs [100, 101], rat bone marrow derived progenitor cells [102]. Apart from the typical stretching parameters such as strain magnitude, duration and frequency that have routinely been investigated for SMCs induction, it was demonstrated that depending on the direction of mechanical strain, cells response could be vastly different. For instance, Park et al. reported uniaxial but not equiaxial mechanical strain upregulated SMCs gene expression in hMSCs and also enhances cellular proliferation [101]. However it was noted that the stretch-induced upregulation of SMCs genes was transient and further stretching resulted in basal level expression of SMCs genes that is comparable to the un-stretched control group, after the cells reoriented and aligned perpendicular to the stretching axis [101]. This phenomenon of adherent cells reorienting themselves away from the main stretching axis beyond a certain threshold strain is common among other cell types and may indicate an intrinsic mechanism of the cells to counter excessive stress [117-119]. With the results obtained by Park et al. in mind, it does raise the possibility that if long term straining of the cells parallel to the stretch vector can be attained, the propensity for hMSCs to undergo SMCs differentiation could be enhanced. To achieve this, cells were cultured on parallel microgrooves to first control cell alignment via “contact guidance” before it was subjected to stretch [100, 120, 121]. This is a facile way to attain precise control over the direction of force that

is to be applied to the cells and examine the corresponding responses. Kurpinski et al., adopted this approach to investigate anisotropic sensing of hMSCs. After 2-4 days of cyclic uniaxial stretching at 1 Hz, for cells strained in the direction parallel to the stretching axis, cell proliferation and expression of calponin 1 (SMC marker) was significantly enhanced relative to the un-stretched samples; while cells coerced to stretch perpendicular to the stretch axis represses expression of SMC genes [100]. The findings corroborated observations made by Park et al.[101]. and also in earlier work that shows high expression of α -SMC protein in fibroblast when stretched in the direction parallel to the applied load [120]. Although the underlying mechanism for this anisotropic stretching cellular response is largely unknown, common observations such as stretch-induced enhanced stress fibers formation, does indicate microenvironmental conditions promoting cytoskeletal assembly may serve as a prerequisite for smooth muscle differentiation of hMSCs.

In vitro chondrogenesis of MSCs is a standard regime employed by many researchers to validate the multipotential differentiation of MSCs. The most commonly employed biochemical factor to induce chondrogenic differentiation of stem cells entails the use of the transforming growth factor beta (TGF- β) superfamily members [104, 122, 123]. In 2004, Huang et al. compared the chondrogenic potential of rabbit bone marrow derived mesenchymal stem cells using different induction techniques namely (i) stimulation with TGF- β 1, (ii) intermittent compressive strain of 15% at 1Hz and (iii) strain + compressive loading [106]. Remarkably, it was found that mechanical stimulation alone was able to achieve comparable MSCs chondrogenesis compared to TGF- β 1 stimulation and combination of both loading and TFG- β 1 treatment induced higher expression of collagen 2 and aggrecan relative to the TGF- β 1 control group [106]. Additionally, compressive loading was found to increase TFG- β and its

corresponding receptor gene expressions, thus providing a probable mechanism for this compression-induced chondrogenic differentiation [106, 107]. Since then, several other groups have reported compressive stress either in the form of dynamic cyclic loading [106, 107, 109, 124, 125] or hydrostatic pressure [108, 126] in 3D constructs can greatly enhance tenogenesis and chondrogenesis of MSCs. However, conflicting data have emerged from other labs documenting applied continuous cyclic loading to agarose-MSCs construct may potentially inhibit chondrogenesis of MSCs if the loading regime is not optimized. Concomitant application of TGF- β 3 and compressive strain impair chondrogenic differentiation in MSCs and bovine cells, while priming the cells with TGF- β 3 prior to the application of compressive stress can yield beneficial chondroinductive influences [124, 127, 128]. Studies conducted by Terraciano et al. yielded similar results depicting the need to delay application of compressive strain to embryonic but not adult stem cells for chondrogenic differentiation in the presence of TGF- β 1 [105]. Recently, cyclic compressive loading of hMSCs in polyurethane 3D foam alone was shown to stimulate mineralized matrix deposition, achieving similar effects by use of the synthetic glucocorticoid, dexamethasone [116].

Shear stress constitutes another class of mechanical force cells normally experiences *in vivo*. Endothelial progenitor cells (EPCs) respond to blood/ tissue flow by elongating and aligning themselves in the direction of flow.[113] Additionally, at the functional level, proliferation was enhanced, expression of several hallmark markers of vascular endothelial cells were detected and were able to form tube like structures in collagen gels indicative of tubulogenesis [113]. Interestingly, majority of the current literatures suggest that stem cells can also respond to fluid flow by differentiating toward an endothelial fate [110]. Murine embryonic mesenchymal

progenitor cell line, C3H/10T1/2 exposed to a steady fluid flow of 15dyn/cm^2 was induced along the endothelial cell lineage, as shown by expression of several mature endothelial cell makers such as CD31, von Willebrand factor and vascular endothelial-cadherin [109]. In addition, RT-PCR analysis of angiogenic and SMC growth factors indicated that shear stress favours endothelial but not SMCs differentiation [111]. Similar results were also attained by Yamamoto et al. using FLK-1-positive (FLK-1+) mESCs that shows shear stress stimulated cell growth and directed its differentiation specifically towards the endothelial cell lineage compared to the static control [112]. Within one day of shear stress exposure at 1.5dyn/cm^2 , the cells were seeded onto collagen gel and extensive tubular network formation was prominent as a function of culture time [112].

Intermittent fluid flow induced shear stress have also been reported to promote osteogenic differentiation of MSCs by several authors [114, 129, 130]. In 2004, Li et al. found that MSCs subjected to oscillatory fluid flow (OFF) displayed 53% higher proliferation, ~10 fold higher Ca^{2+} activity and promoted gene expression of osteopontin and osteocalcin [130]. Because Ca^{2+} can function as a second messenger in the mechanotransductive pathway, it supports the notion that fluid flow can heighten the mechanosensitivity of MSCs and thus initiates a series downstream signalling cascades such as the ERK1/2 pathway influencing stem cell fates [114]. Another strategy to apply shear stress to stem cells in a 3D context is by use of perfusion chambers. For instance, hMSCs grown in 3D Ti/ ECM fiber mesh subjected to 1ml /min of fluid flow for 16 days significantly increase calcium deposition and osteopontin expression with or without the aid of dexamethasone [131].

2.2.4. Engineered cell size and geometry: Shaping the future of stem cells

Form and function are foundational tenets of cell and tissue physiology. Spindle-shaped vascular smooth muscle cells, wrapped itself circumferentially around the blood vessels to regulate blood flow; neurons with extensive networks of fine cellular processes (dendrites) to form multiple synaptic points to transmit chemical-electrical signals; adipocytes assumes a rounded morphology that gives the highest volume to surface ratio to store fat as a source of reserve energy. Due to the intertwined relationship between cell shape and function, an interesting question thus arises: *is (cell) shape a manifestation of cellular functions or could shape itself modulate cellular function?*

Earlier works using engineered surfaces provided strong indication that cell shape can function as a compelling regulator of cell fate. Folkman et al. controlled degree of cell spreading using substratum coated with varying concentrations of poly (2-hydroxyethyl methacrylate) and observed a tight correlation between cell spreading and DNA synthesis [132]. In another study, it was demonstrated that myogenesis of murine embryonic mesenchymal cells is highly dependent on cellular elongation [133]. Polycarbonate membranes with 10 μ m perforated pores hindered cell spreading and myogenesis, while cells on surfaces with 20 μ m pores elongated with concomitant expression of several hallmark myogenic markers [133].

In the 1990s, Whitesides et al. devised a novel technique termed microcontact printing (μ CP), capable of transferring “ink” patterns of definite size and geometry onto a given substrate via conformal contact [39]. Since its introduction, numerous researchers have exploited this technique to attain precise 2D control of cell shape on micropatterned ECM proteins “islands”. Using this method, Chen et al. patterned human and bovine endothelial cells on fibronectin islands of varying sizes and

observed apoptosis progressively decline with increasing cell size [134]. Conversely, cell growth was promoted for cells on larger fibronectin islands in agreement with observations made by Folkman et al.[134]. Smooth muscle cell proliferation and human tendon fibroblast collagen type 1 expression was also observed to depend on cellular elongation as demonstrated using μ CP [135, 136]. Collectively, patterned surfaces to control cellular morphology offer a powerful tool for researchers to delineate the precise role of cellular morphology in regulating a myriad of cellular behaviours [137]. It is anticipated that micropatterned platforms will continue to gain prominence in the field of tissue engineering and extend its usefulness into 3D culture in the near future with more advanced photo or laser based patterning techniques.

A

Cell type	Conditions		Cell fate	References
	ECM micropattern LOW contractility	HIGH contractility Biochemical cues		
Mesenchymal stem cells		Mixed medium inducing adipogenic and osteogenic differentiation	 Adipocytes Osteoblasts	(McBeath et al., 2004) (Kilian et al., 2010)
		TGF- β	 Chondrocytes Myocytes	(Gao et al., 2010)

Figure 2.5. Studies suggested cell shape can effectively serve a potent modulator of stem cell differentiation in the presence of established soluble factors. Stem cells spreading are restricted within the micropatterned regions and assume different intricate shape at single cell level (A). Patterning multi cellular aggregates revealed region-traction force dependent differentiation of hMSCs (B). Adapted with permission [68, 137]. Copyright 2010 Journal of Cell Science. Copyright 2010 The Royal Society of Chemistry.

In the context of stem cells, μ CP have been used to probe the possibility of guiding stem cells differentiation by controlling the shape it assumes in the presence of biochemical factors (**Figure 2.5**). Motivation for such studies stems from the requirements, set forth by standard *in vitro* protocols to direct hMSCs towards the osteogenic, adipogenic, chondrogenic and smooth muscle lineage. For instance, in the case of osteogenic differentiation, it was recommended that differentiation medium should be introduced to sub confluent cells, whereas higher cell confluence is recommend before cells are exposed to adipo-inductive medium. Similarly, hMSCs in pellet culture is a prerequisite for chondrogenic induction while sub confluent adherent hMSCs is needed for smooth muscle differentiation, even though both protocols use the same primary cytokine (i.e. TGF- β). As cells spreading and cell-cell contact very much depend on the state of confluency, one may postulate that the responsiveness to the biochemical cues can be tweaked by modulating either one of the parameter. To isolate the effect of cell shape and cell-cell contact, Macbeath et al. employed μ CP to pattern single isolated hMSCs on fibronectin square adhesive islands of different sizes. It was revealed that hMSCs under the influence of 1:1 osteoinductive and adipogenic induction medium, majority of cells micropatterned on large fibronectin island ($10,000\mu\text{m}^2$) underwent osteogenesis while cells on smaller fibronectin islands ($1,024\mu\text{m}^2$) became adipocytes [37]. Cells on large fibronectin islands spread better and expressed higher RhoA activity, relative to cells on smaller islands suggesting that a well developed cytoskeletal system provides the cells with a

bias to become osteocytes. Treatment with Cytochalasin D (CytoD) that disrupted the integrity of the cytoskeleton abrogates osteogenic differentiation and instead the cells were programmed to become adipocytes. Involvement of RhoA activity in this shape-dependent phenomenon was further confirmed when hMSCs infected with constitutively active RhoA were stained positive with alkaline phosphatase (indicative of osteodifferentiation) after 1-week of culture in normal growth medium [37]. Subtle differences such as the curvatures of a given shape with comparable printed area have also been shown to regulate cytoskeletal development and modulate osteogenic and adipogenic differentiation of hMSCs [138]. More recently, using the same patterning platform as Macbeath et al., Gao et al. demonstrated that hMSCs on large fibronectin islands became smooth muscle cells while cells on small fibronectin islands became chondrocytes in the presence of TFG- β 1 [38]. However, in this case, it is the differential expression of Rac1 that contributes shape directed differentiation [38]. Taken together, the gathered findings emphasize the need to provide hMSCs with the appropriate context before it can respond effectively to the biochemical cues.

Micropatterning multicellular aggregates such as the embryoid bodies (EBs) of embryonic stem cells is a simple yet powerful biophysical parameter that can be employed to direct lineage specification [139]. Size of EBs can be controlled by use of microwells and has been shown that cardiogenesis was enhanced in larger EBs (450 μ m) while smaller EBs (150 μ m) promoted endothelial cell formation via differential expression of WNT5a and WNT11 [139]. On 2D flat surface, Ruiz et al. observed spatial patterning of hMSCs towards the osteogenic and adipogenic fate within micropatterned multicellular aggregates [140]. When incubated with 1:1 adipogenic and osteogenic medium, majority of the cells located at the outer or convex edges of the micropatterns became bone cells while cells positioned at the

centre or concave edges of the pattern became fat cells independent of the geometry of the micropattern. CTF analysis revealed that cells at the convex edges exhibited 3-fold higher traction force compared to the cells at the concave side of the pattern suggesting this segregation of cell lineage differentiation is through a cell-tension dependent pathway [140]. In a similar study, it was reported that the rate of hMSCs adipogenesis is not dependent on the size but rather the shape of the micropattern. Recently, understanding of this cell-shape-tension dependent switch between osteogenic and adipogenic fate have been extended to hyaluronic acid based 3D constructs [141].

2.3. Convergence of biophysically regulated signalling events at the cell-material interface

Engler et al. showed that matrix stiffness could determine stem cell fates [43]. Therein, the treatment of human MSCs with blebbistatin, which is a potent non-muscle myosin IIa (NMMIIa) inhibitor, negates the matrix stiffness-dependent differentiation program, suggesting that the cytoskeletal apparatus is vital for the cells to sense and elicit the corresponding response to the underlying substrate [43]. Clearly, cell surface ‘mechanosensors’ and their ability to transduce and to alter cellular cytoskeletal architecture, which are determinant of cell shape and size, are major players in biomechanically-induced stem cell differentiation. (**Figure 2.6**)

One major protein complex at the matrix-cell interface is the integrin complex. The integrins are the first interactors with the ECM. The integrin complex is a heterodimer consisting of α and β subunits. There are 18 α and 8 β subunits in vertebrates [142]. Each of these integrins consists of a large extracellular domain (700-110 residues long) and a smaller cytoplasmic domain (30-50 residues long) that provides

intracellular-extracellular connectivity and enables bidirectional-signalling crosstalk (outside-in and inside-out) [143, 144]. These transmembrane proteins are in the nanometer size range and are approximated to be 10nm in diameter and project 20nm extracellularly [145, 146]. Both α and β integrins interact with different permutations conferring substrate specificity to ECM proteins such as collagen, fibronectin and laminin mediating cell adhesions. Matrix elasticity has also been shown to affect integrin activity (conformational change upon binding to ECM) and internalization [147]. Using a specific antibody that recognizes the active form of β 1 integrin, it was found that bone-marrow-derived MSCs that were plated on a softer matrix apparently demonstrated higher β 1 integrin activity but had few integrin complexes at the cell surface. Similar cells plated on a stiffer matrix exhibited more integrin receptors at the cell surface. The inhibition of integrin internalization prevented the MSCs from adopting the neuronal cell fate when plated on soft matrices, which suggests that the internalization and trafficking of integrin receptors are important in cell fate determination. One prominent group of proteins that acts downstream of the integrins are the focal adhesions (FAs). FAs are formed at sites of integrin clustering. They also act to connect the ECM to the actin cytoskeleton via their interaction with the integrins. It has been reported that plating cells on a stiff substrate or applying force to the integrin complex will increase the size of the FAs [148-151]. Several focal adhesion proteins exhibit the ability to stretch when they are under mechanical forces, thereby exposing sites for phosphorylation or domains for interaction with other proteins. Talin and tensin are prime examples. Talin is a focal adhesion protein, which has a unique role in the activation of integrin as well as binding to actin and serving as a force sensor. There are several proposed models in which Talin can activate integrins [152]. (i) Cytosolic talin binds to the tail-end of the β integrin, exposing the

basic segments on talin to electrostatically interact with the phospholipid head groups on the cellular membrane. (ii) Engagement of talin to membrane-proximal $\beta 3$ integrin tail stabilizes the entire transmembrane helical structure, allowing talin to modulate the crossing angle on the β -integrin transmembrane domain. (iii) Talin might directly form a salt bridge with the β -integrin Asp and thus disrupt the highly conserved α -integrin ARG and β -integrin Arg interaction. Stretching talin under forces also exposes binding sites to vinculin [153]. In contrast, stretching p130CAS exposes sites for tyrosine phosphorylation by kinases from the Src family [154]. Phosphorylated CAS proteins can subsequently bind to other proteins, such as Crk, Nck and Ship2 [155-157]. Taken together, integrins and FAs are capable of functioning as mechanosensors to relay the mechanical cues from the microenvironment to the cell, and other downstream mediators, such as p130CAS, can act as a scaffold to transduce integrin-mediated signalling.

The activation of integrin also leads to tyrosine phosphorylation of the focal adhesion kinase (FAK). FAK is a non-receptor tyrosine kinase that is recruited to the focal adhesions [158]. It can interact with both guanine nucleotide exchange factors (GEFs) e.g., p190RhoGEF and GAPs e.g., p190RhoGAP [159, 160]. Thus, FAK can act as a transducer of signals from the integrin complex to activate the Rho GTPase signalling pathways. The recruitment of FAK to the FAs and the activation of FAK are also dependent on the stress experienced by the cell [161]. Surprisingly, FAK^{-/-} cells are able to form strong FAs [162]. However, the turnover and disassembly of the FAs are affected in these cells. FAK-deficient fibroblasts showed no preference regarding substrate stiffness, whereas control fibroblasts preferentially migrated toward stiffer substrates, suggesting that FAK can act as a mechanotransducer [163]. Aside from

focal adhesion proteins, many other proteins or enzymes can become activated or inactivated by mechanical stimuli. One example is the small GTPases from the Rho family. The Rho GTPases have been found to participate in many cellular activities that require the re-organization of the actin cytoskeleton [164]. The group regulates cell morphology and motility and is also implicated in the control of neuronal development [165], transcription [166] and cell cycle regulation [167]. RhoA is activated by fluid shear [168], whereas myosin-mediated cell contraction and cell stretching down regulate Rac activity [169]. hMSCs on large fibronectin islands spread better and expressed higher RhoA activity relative to cells on smaller islands, which suggests that a well-developed cytoskeletal system provides the cells with a bias to become osteocytes [37]. Mesenchymal stem cells adopt an osteogenic cell fate when RhoA is activated [37]. In contrast, when RhoA is inhibited, MSCs develop into adipocytes. Epidermal stem cells with Rac1 depleted show impairment in cell migration and differentiate prematurely [170]. Interestingly, Rho GTPases have been implicated in regulating the Wnt pathway and being regulated by Wnt signalling.[171] Wnt signalling has long been established to maintain stem cell self-renewal. The convergence of Wnt and Rho GTPase signalling confirms the importance of Rho GTPases in the regulation of ES cell proliferation and differentiation.

The ERK signalling pathway is yet another FAK associated signalling cascades that has been shown to play a vital role in regulating stem cell lineage commitment [172] and renewal [173]. Auto phosphorylation of FAK, leads to binding of the Grb2/ Sos complex that can activate the Ras-Raf-MEK-ERK transduction pathway [174]. Recently, evidences have illustrated that the signalling activity of ERK can be mechanically regulated. Human bone marrow-derived MSCs subjected to equiaxial

mechanical strains enhance matrix mineralization via the concomitant activation of ERK signalling in osteogenic medium [115, 175]. Conversely, cyclic mechanical stretching hinders adipogenesis by downregulating PPAR γ 2 expression [176].

Another protein often associated with cell-matrix sensing is myosin, more specifically, NMMII. Myosin provides the contractility of cells as a result of pulling and interaction with the ECM. The contractility of the actin-myosin system is regulated by the activities downstream of RhoA. RhoA activates ROCK and mDia, which in turn modulate the state of the actin cytoskeleton in the cell. ROCK is instrumental in increasing myosin light chain phosphorylation and inhibiting the activity of myosin light chain phosphatases. The inhibition of ROCK has been reported to block cell proliferation in many cell types and the activation of ROCK has been shown to be necessary for G1-S cell cycle progression [177]. However, ROCK inhibitor has been used in the maintenance of human ESCs, presumably to inhibit apoptosis [178]. Indeed, it has been shown that the inhibition of acto-myosin contractility by either blebbistatin to inhibit the myosin heavy chain or the ROCK inhibitor to block the phosphorylation of the myosin light chain in addition to the use of siRNA to knockdown the subunits of myosin, all lead to improved survival of human ESCs [179]. Ohgushi et al. went further to demonstrate the underlying mechanism [180]. They reported that the activation of ROCK leads to hyperactivation of NMMII during the dissociation of human ESCs. The cell-cell adhesion force serves to counteract the acto-myosin contracting force. When this balance is lost due to the activation of myosin, apoptosis ensues. It is this increased acto-myosin contractility that causes the apoptosis of human ESC upon dissociation [180].

Figure 2.6. Biophysical regulation of signaling events at the cell-material interface. Materials characteristics such as substrate rigidity, topography and patterns influence the activities of integrins and focal adhesion (FA) complexes (Talin, Tensin, Paxillin, Vinculin α -actinin and FAK, etc). These complexes function as cell surface mechanosensors to detect changes in the ECM and respond to these changes via modification of downstream signaling cascades resulting in cytoskeletal adaptation. These downstream signalling ultimately modulate the cell motility, adhesion and genes expression to determine stem cell fate.

2.4. Summary

Looking forward, through the numerous examples highlighted in this section, the regulatory role of biophysical factors is expected to gain continual prominence in stem cell biology. Coupled with the rapid advances made in the field of biomaterials, micro-nanofabrication methods and specialty bioreactors have created unique opportunities for regenerative medicine. As stem cell differentiation and renewal are dynamic and complex processes, future development and the use of smart materials to attain precise spatial and temporal control is imperative. Although much effort have been invested to examine the interplay of biochemical and biomechanical cues, the interplay of multiple biomechanical cues dictating stem cells behaviours remains elusive.

Materials and Methods

A broad overview of the experimental materials and techniques are presented in this chapter. Further details pertaining to specific experiments will be detailed in subsequent chapters.

3.1 Cell culture and reagent

Bone marrow derived human mesenchymal stem cells, harvested from an 18 year old male donor were purchased from Lonza (Cambrex). Flow cytometry analysis conducted by the company revealed expression of the surface antigens CD 105/+, CD166/+, CD 29/+, CD 44/+, CD 14/-, CD34/- and CD45/-. The cells were routinely expanded in mesenchymal stem cell basal medium (Lonza) for optimal expansion and sub-cultured at a density of 4000-6000 cells /cm² as recommended by the company's data sheet. Media was changed every 2-3 days. Low glucose Dulbecco's Modified Eagle Medium (DMEM) containing L-glutamine (Sigma) supplemented with 1% antimycotic antibiotic solution and 10% Fetal Bovine Serum (FBS) (FBS Gold, PAA) [denoted as: LD10] or 1% FBS [denoted as: LD01] were employed as the standard growth medium for the experimental studies since a low glucose (1000mg/L) culture condition better represents physiological glucose concentration. The FBS chosen for the studies ensures that there is minimal batch to batch consistency as documented by the manufacturer. Only early passage hMSCs [passage 4-6] were employed for experimental studies since it was previously reported that proliferation rates and other properties of hMSCs will change gradually with higher passage number. The cells

were maintained at 37°C in a humidified atmosphere of 5% CO₂. Phosphate Buffered Saline (PBS) (PAA) and 0.05% trypsin-EDTA (Gibco) were used for washing and cell detachment purposes respectively.

3.2 Multi lineage differentiation of hMSCs

The multipotency of the hMSCs (passage 6) was ascertained by conditioning the cells to differentiate towards the osteo, adipo and myo lineage using established chemical concoctions. (**Figure 3.1**)

3.2.1 Osteogenic differentiation

Cells were seeded in 6 well plates with an initial density of 2×10^4 cells/ cm² and cultured in high glucose DMEM supplemented with 10% FBS, 1% L-glutamine and 1% antibiotics/ antimycotic solution [denoted as: HD10]. Following which, the cells were allowed to grow to 70% confluence before they were exposed to osteoinductive medium consisting of HD10 supplemented with dexamethasone (10nM), ascorbic acid (0.2mM) and β -glycerophosphate (10mM) over a period of 21 days. Medium was changed every 3-4 days. Cells grown in HD10 alone serve as negative control. By the end of the experiment, the cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) for 15 min at 4°C. This was followed by 3 washes with PBS and counter stained for calcium deposit using Alizarin Red S (Sigma) as an indication of mineralization normally associated with osteogenesis.

3.2.2 Adipogenic differentiation

Adipogenesis was achieved by incubating a fully confluent layer of cell in 6 well plates to adipo-inductive medium consisting of HD10 supplemented with dexamethasone (1 μ M), insulin (50 μ M) and indomethacin (200 μ M) for 21 days. Medium was changed every 3-4 days. Cells cultured in HD10 alone serve as negative

control. After 21 days of incubation with the adipo-inductive medium, cells were washed with PBS and fixed with 10% formalin for 15min at 4°C. Cells were subsequently washed 3 times with PBS and counter stained with Oil red O (Sigma) to detect formation of oil droplets, indicative of adipogenesis.

3.2.3 Smooth muscle differentiation

Briefly, cells were seeded (2×10^4 cells/ cm²) on a 22 x 22mm coverslip pre-sterilized with 70% ethanol and cultured in LD10. After 24hours, the culture medium was aspirated and was replenished with smooth muscle differentiating medium containing 10ng/ml of TGF β_1 (Sigma) and allowed to be cultured for a further 7 days. Medium was changed every 2-3 days. Cells cultured in LD10 alone serve as negative control. After 7 days of cell culture, cells were fixed and stained with α -smooth muscle actin indicative of smooth muscle differentiation as per lined out in section 3.4.

Figure 3.1. Bone marrow derived hMSCs were biochemically induced to differentiate into osteocytes (A), adipocytes (B) and smooth muscle cells (C) that originates from the mesodermal layer in order to examine its multilineage potential. Cells were stained with Alizarin Red S (A), Oil Red O (B) and α -smooth muscle actin (green) (C). Panel D, E and F depict undifferentiated cells cultured in normal growth medium and serves as negative controls. Scale bar= 100 μ m.

3.3 Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA (tRNA) was extracted using RNeasy mini kit (Qiagen) according to the

protocol provided by the manufacturer. The concentration and quality of the extracted tRNA was determined spectrophotometrically (Nano drop-N100, Thermo Scientific). First strand complementary DNA (cDNA) was synthesized either with ImProm-II™ reverse transcription kit (Promega) or iscript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc, USA). qRT-PCR was performed on a CFX96 real time PCR detection system (Bio-Rad Laboratories, Inc, USA). The reaction mix comprises of 10µl of KAPA SYBR FAST master mix (2x) universal, 0.4µl of forward and reverse primers (10 µM) respectively, 1µl of diluted cDNA and PCR grade water to make up a final volume of 20µl. The following cycling thermal profile was employed: enzyme activation at 95 °C for 3min, 45 cycles of 95 °C for 3s and 60 °C for 20s followed by a dissociation step to analysis the melt curve of the amplified product. Primers specific to the targeted genes (**Table 3.1**) were obtained from primerbank (<http://pga.mgh.harvard.edu/primerbank/>) an online public resource for PCR primers. Specificity of the chosen primers to the gene of interest was examined by performing a BLAST (Basic Local Alignment Search Tool) search. Melt curve analysis was included to ensure that only one PCR product was formed. Relative quantification of gene expression was analyzed with the Relative Expression Software Tool (REST) software [181]. Results presented are fold change expression normalized against the calibrators: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β-actin as the endogenous housekeeping genes.

Table 3.1. Compiled genes targets and primer sequences obtained via primerbank [182].

Genebank accession number	Gene target	Sequence (5'-3')	Amplicon length (bp)
NM 001101	β -actin	CATGTACGTTGCTATCCAGGC CTCCTTAATGTCACGCACGAT	250
NM 002046	GAPDH	CATGAGAAGTATGACAACAGCCT AGTCCTTCCACGATACCAAAGT	113
NM 004348	Runt related transcription factor 2 (RUNX2)	TCCTATGACCAGTCTTACCCCT GGCTCTTCTTACTGAGAGTGGAA	190
BC 021289	Alkaline phosphatase (ALPL)	CTCTCCAAGACGTACAACACC AATGCCCACAGATTTCCAGC	201
NM 003118	Secreted protein, acidic, cysteine-rich (SPARC)(osteonectin)	AGCACCCCATTTGACGGGTA GGTCACAGGTCTCGAAAAAGC	105
NM 000711	Osteocalcin (OCal)	CACTCCTCGCCCTATTGGC GCCTGGGTCTCTTCACTACCT	138
NM 002052	GATA-4	CCCAGACGTTCTCAGTCAGTG GCTGTTCCAAGAGTCCTGCT	146
NM 002478	Myoblast Differentiation Protein 1 (MyoD1)	CGGCGGAAGTGTACGAAG GCGACTCAGAAGGCACGTC	172
NM 000257	β -Myosin Heavy Chain (MHC)	CACTGATAACGCTTTTGATGTGC TAGGCAGACTTGTACGCCTCT	165
NM 000364	Cardiac Troponin T (cTnT)	TCTCCGAAACAGGATCAACGA GCCCGGTGACTTTAGCCTT	68
NM 000363	Cardiac Troponin I (cTnI)	TCCAGGGACTCCTTAGCCC TTTGACCTTCGAGGCAAGTTT	107
X65964	Nestin (NES)	CAACAGCGACGGAGGTCTC CCTCTACGCTCTCTTCTTTGAGT	163
NM 002500	Neurogenic differentiation 1 (NeuroD1)	GCCTTGCTATTCTAAGACGCA GTGGGTTGGGATAAGCCCTT	156
NM 002374	Microtubule Associated Protein (MAP2)	CAGGAATTGACTCCCTCTACAGC TCTTACCAGGCTTACTTTGC	80
NM 002055	Glial Fibrillary Acidic Protein (GFAP)	ATCGAGAAGGTTTCGCTTCCTG TGTTGGCGGTGAGTTGATCG	151

3.4 Immunocytochemical staining

Cells at predetermined time points were fixed with 4% paraformaldehyde (PFA). Following which, the cells were washed 3 times and 0.1-0.2% of triton-X100 in PBS was added and allowed to stand at room temperature for 10min to improve antibody penetration. Triton X-100 was then aspirated washed 3 times before the samples were blocked with either 5% bovine serum albumin (BSA) or 5% normal goat serum (NGS) for 45-60min at room temperature. The respective primary antibodies as listed in **Table 3.2** were then added to the sample overnight at 4°C. Samples were washed 3 times with PBS and the corresponding secondary bodies (**Table 3.2**) was added and allowed to stand at room temperature for 1 hour in the dark before it was aspirated and washed with PBS 3 times. The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to reveal the cell nucleus and in some cases the filamentous actin (F-actin) of the cells were counterstained with Tetramethylrhodamine B isothiocyanate (TRITC) conjugated –Phalloidin before they were mounted on a glass slide (DAKO mounting agent) and imaged with an upright fluorescence microscope (Nikon, 80i eclipse).

Table 3.2. Compiled list of antibodies and florescent probes utilized in this project.

Primary antibodies					
Target	Source	Species reactivity	Cat no.	Host	Dilution
Fibronectin	Sigma	Hu	F3648	Rb (polyclonal)	1:400
Vinculin	Chemicon	B, Hu, Pig, Rb	FAK100	Ms (monoclonal)	1:400
α -Smooth muscle actin	Sigma	Hu, Ms, R, Rb, Gp, Ch, S, G, S, Fg, Canine, B	A2547	Ms (monoclonal)	1:200
Cardiac myosin heavy chain (MHC)	Abcam	Hu, Ms, R, C, D, P, Rb	ab15	Ms (monoclonal)	1:100-200
Microtubule Associated Protein 2 (MAP2)	Abcam	Hu, Ms, R, Cow, CynMk	ab5392	G (polyclonal)	1:10000
FAK (A-17)	Santa Cruz	Hu, Ms, R, Ch, Xenopus	Sc-557-G	G (polyclonal)	1:200
p-FAK (Tyr 925)	Santa Cruz	Hu, Ms, Rb	sc-11766-R	Rb (polyclonal)	1:200
p44/42 MAPK (ERK1/2)	Cell Signalling	Hu, Ms, Rat, Hm Mk, Mi, Z, B, Pg, Sc	9102	Rb (polyclonal)	1:1000
P-p44/42 MAPK (ERK1/2)	Cell Signalling	Hu, Ms, Rat, Hm, Mk, Mi, Z, B, Pg	9106S	Ms (monoclonal)	1:1000
Secondary antibodies					
Alexa Fluor 488	Molecular Probes	Ms	A11001	G (IgG)	1:400
Alexa Fluor 568	Molecular Probes	Ch	A11041	G (IgG)	1:400
Other fluorescence probes					
DAPI	Chemicon	-	FAK100	-	1:1000
TRITC conjugated Phalloidin	Chemicon	-	FAK100	-	1:400

Reactivity key: Hu=Human; Ms=Mouse; R=Rat; Hm=Hamster; Mk=Monkey; Mi=Mink; Z=Zebrafish; B=Bovine; Pg=Pig; Gp=Guinea Pig; Ch= Chicken; S=Snake; G=Goat; Fg=Frog Sc=S. cerevisiae; CynMk= Cynomolgus Monkey

3.5 Micro contact printing (μ CP) of ECM protein

3.5.1 Preparation of micropatterned elastomeric stamps

Elastomeric stamps made of *polydimethylsiloxane* (PDMS) were used to transfer print the ECM protein, human plasma derived fibronectin (BD, Biosciences) onto the substrates of interest. Briefly, silicon (Si) master template bearing the desired microfeatures was developed (Bonda Tek) and subsequently function as a mould for the fabrication of the elastomeric stamps. Treatment of the Si master template with octadecyl trichlorosilane (OTS) was essential to facilitate easy peeling of the cured PDMS from the Si surface. To achieve this, the master template is first cleaned and hydroxylated with Piranha solution for 2h at 60°C. Subsequently, the master template was incubated with 1% OTS (Sigma) in anhydrous toluene (Sigma) for 1 week and washed thoroughly with toluene and ethanol before it was dried with purified nitrogen gas. Use of anhydrous toluene is essential since OTS is hygroscopic and would rapidly oxidize in the presence of water. Liquid PDMS (1: 10 elastomeric base: curing agent, Sylgard 184, Dow Corning) was poured over the master, cured at 100°C for 2 hours and peeled away by hand.

3.5.2 Transfer print of ECM to substrate of interest

The PDMS stamp was then inked with fibronectin dissolved in phosphate buffer saline (PBS) solution (50 μ g/ml) for 1 hour, blown dry with purified nitrogen gas and placed in conformal contact with the substrate for ~ 10 seconds. Additional step of placing a stainless steel weight (25g) can also be placed on top of the stamp to hold it in place on the substrate for a further 30min if desired (optional step). The unprinted region was then passivated with either polypropylene (PPO)-poly ethylene oxide (PEO)-poly propylene (PPO) (Pluronic F 127, BASF) or 2% BSA for 1 hour at 37°C

to yield a non biofouling surface. This step is essential to allow exclusive adherence of the cells on the micropatterned surface. The substrate was then washed several times with PBS to remove excess blocking agent that is not adsorbed on the substrate prior to cell seeding. Printed fibronectin patterns were immuno-labeled with rabbit polyclonal anti fibronectin antibody (Sigma) and counter stained with anti rabbit Cy3 dye (Molecular probes).

Figure 3.2. Generic procedure adapted to micro-print ECM protein onto the desired substrates.

3.6 Statistical analysis

All data are presented as mean±standard deviation error. One-way ANOVA followed by Turkey means analysis, and student t-tests were employed to determine statistical significance differences between experimental groups. OriginPro 8 (OriginLab, Northampton, MA) was used to plot graphs and conduct all statistical analysis. Unless otherwise stated, $p < 0.5$ was considered significant.

Regulation of cell shape on biodegradable scaffold modulates stem cell lineage commitment

4.1 Abstract

Encouraged by several recent documented works illustrating how cell shape can exert a significant impact on stem cell fate, in this chapter, we examined the influence of cell morphology on stem lineage commitment by micropatterning bone marrow derived hMSCs on biodegradable substrate. Fibronectin lanes (~20µm wide) were printed on solvent cast poly (*lactide-co-glycolide*) thin film. hMSCs that were allowed to culture on unpatterned substrata attached and spread extensively (~10,000µm²) while cells grown on the micropatterned adhesive strips adopted a highly elongated morphology with much smaller cell spreading coverage. Gene expression analysis revealed several hallmark markers associated to neurogenesis and myogenesis for cells that were highly elongated were upregulated while osteogenic markers were down-regulated or remained at its basal level. While there is clearly upregulated levels of both neuronal and myogenic lineages, the myogenic lineage is dominant within the time scale studied as determined by the exclusive immuno-detection of cardiac myosin heavy chain for the micropatterned cells. These results demonstrated that control of cellular shape in the absence of any induction factors may be a viable strategy to coax lineage-specific differentiation of stem cells. This novel finding may find its potential to be integrated with autologous cell graft aiding lineage commitment of hMSCs into myocyte like cells.

4.2 Introduction

Over the past years, ASCs have emerged as an ideal cell source in the tissue engineering and regenerative medicine [183]. Their rise to prominence is primarily due to its self-renewal and multipotential differentiation capability. Furthermore, its usage does not implicate the ethical issues that are normally associated with embryonic stem cells. The key to fully exploit stem cells is to be able to direct its differentiation along targeted lineages. Stem cell fate is modulated by a wide spectrum of signals found within its microenvironment [8, 184]. These signals may be presented in the form of soluble factors, cell-matrix, cell-cell interactions and biophysical inputs. All these signals act in concert to instruct whether the stem cells should undergo apoptosis, proliferation, differentiation or remain quiescent [8].

In vitro, stem cell responses can be modulated by a multitude of exogenous chemicals, topological and/ or mechanical physiochemical cues. Among them, the use of soluble factors is perhaps the most well studied strategy to direct stem cells differentiation. Established chemical or cytokine concoctions to differentiate bone marrow derived stem cells into osteocytes, chondrocytes, adipocytes and smooth muscles cells are readily available. The general view of using biochemical molecules is that they will bind to surface receptors located at the cell membrane activating second messenger such as cyclic adenosine monophosphate, or produce kinases leading to initiation of a cascade of signalling pathways influencing the eventual cellular responses [185-187]. Nonetheless, in recent years, there are an increasing number of publications that document the importance of biophysical cues within the stem cell niche as a potent regulator of stem cell fate [45]. Coupled with the advancements in micro-nanotechnologies, novel engineered platforms using

mechanically tunable substrates and micro-nanoscaled features have been shown to direct stem cell lineage commitment in a soluble factor free manner [46].

In a seminal work conducted by Engler and colleagues, it was demonstrated that lineage commitment of bone marrow derived mesenchymal stem cells (hMSCs) can be controlled solely based on the mechanical property of the substrates [43]. hMSCs grown on collagen 1 coated polyacrylamide hydrogels with stiffness that mimic the brain (0.1-1kPa), muscle (8-17kPa) and bone (25-40kPa) tissues became neurons, myocytes and osteocytes respectively [43]. *in vitro* cultivation of embryonic stem cells on multi-perforated polycarbonate (PC) with micro-scale holes with 10 μ m or 20 μ m in diameter, revealed that only cells that were elongated underwent myogenesis while rounded cells remained undifferentiated [133]. In another work involving the use of nanoscaled cell-matrix interactions, myogenic activity of hMSCs were shown to be enhanced when cultured on electro-spun hydroxybutyl chitosan nanofibers [42]. The cells elongated and aligned along the fibers. Significant upregulated expression of several hallmark myogenic transcripts such as myogenin desmin, PAX3, and PAX 7 was also observed, compared to cells cultured on 2D flat surfaces. Yim et al. also independently showed that hMSCs cultured on 350nm wide PDMS nanogratings can effectively coerced hMSCs to elongate and expresses several myogenic and neurogenic markers [41]. Strikingly, the neurogenic inductive capability of the nanogratings is comparable (better) to the use of retinoic acid suggesting that such biophysical cues can function as potent regulator of stem cell fate [41]. It is worth mentioning that the aforementioned examples consistently show that myogenic and neurogenic activities of hMSCs are often accompanied by cell elongation. This brings about an interesting question as to whether the observed cellular elongation is a

manifestation of the myogenic/ neurogenic program or if cell elongations can function as a mechanoregulatory signal to drive hMSCs differentiation?

In this chapter, we explore the possibility to direct stem cell lineage commitment by culturing hMSCs on micropatterned fibronectin lanes (20 μ m wide) on *poly (Lactic-co-glycolic acid)* (PLGA) thin film. It was shown that engineered cell shape and its associated cytoskeletal and focal adhesions (FAs) development could exert significant effects on early stages of stem cell differentiation. Elongated hMSCs expressed higher level of mRNAs that are usually associated with neuronal and myogenic differentiation, but specifically downregulated several osteogenic genes. Filamentous actin (F-actin) arrangement, FAs distribution and cell nuclei of the elongated cells differed starkly from the unpatterned cells. Collectively, the results presented in this study suggested that solely by manipulation of hMSCs morphology is sufficient to steer lineage commitment towards the myocardial lineage. This method can potentially be used to improve graft take of many engineered tissues.

4.3 Materials and methods

4.3.1 Solvent cast biodegradable ultra thin film

Poly (*lactide-co-glycolide*) (80:20 PLGA) (Boehringer, Resomer ® LG824 S) was dissolved in dichloromethane (DCM) with a constant weight ratio of 1:12-15 and solvent cast using an industrial film applicator over 13mm coverslips. Residual solvent in the films was removed by drying in vacuum oven (37°C) for 5 days and confirmed with thermogravimetric analysis (TGA Q500, TA instruments). Experiment was conducted in nitrogen environment and ramped up to 800°C at a rate of 10°C/min. Complete mass loss was observed beyond 370°C and decomposition occurs in a single step implying that residual solvent has completely been removed.

Figure 4.1. Representative TGA thermogram of solvent cast PLGA thin film after being placed in the vacuum oven (37°C) for 5 days.

Thickness of the solvent cast PLGA thin film was characterized using a thickness gauge (elcometer) and surface wettability was characterized using static sessile drop method. Surface topography was further characterized with atomic force microscopy (AFM) (Digital Instruments, Nanoscope III) using tapping mode. The AFM probe (Pointprobe, Nanoworld) used in this instance is made of silicon and it is shaped like a pyramid with a polygon base. The base radius is 10-15nm and the tip of radius is typically less than 8nm. A scan size of 10 μ m by 10 μ m was employed to attain the AFM image.

4.4 Results and Discussion

4.4.1 Surface characterization of pristine solvent cast PLGA thin film and micropatterned PLGA

In this study, micro patterning technique was employed to control the shape of hMSCs cultured on transparent PLGA(80/20) thin film. Unlike most studies that employed glass, tissue culture polystyrene and PDMS surfaces as common substrates for micropatterning experiments, few studies have examined the effects of micropatterning hMSCs on biodegradable platforms. This is of particular interest since findings of clinical relevance can readily be translated to applications as in the case of engineered biodegradable thin films. Depending on the targeted application, the choice of the type of biodegradable material may vary accordingly. The aim of this study was to establish patterned cells on biodegradable platform as a viable strategy to direct stem cell differentiation. PLGA was chosen as the model substrate due to its widespread usage. The solvent cast PLGA is optically transparent, thus facilitating the use of conventional phase contrast microscope to monitor cell behaviours. Furthermore, because of its biocompatibility and biodegradable properties making them suitable for various tissue engineering applications [188-190].

Additionally, the degradation profile of the co polymers can easily be tailored by altering the ratio of the co polymers component [191], thus offering the flexibility to customize the performance of the substrate.

Table 4.1. Characterization of solvent cast PLGA thin film.

PLGA thin film properties		Characterization tools/ techniques
Thickness	$15.8 \pm 2.4 \mu\text{m}$	Thickness gauge (elcometer)
Static contact angle	$82.6 \pm 2.8^\circ$	Static Sensile Drop
σ_{RMS}	0.51nm	Atomic Force Microcopy
Opticality	Transparent	Visual inspection
Residual solvent	-	Thermogravimetric Analysis
Patterned surface	Uniform	Immuno-fluorescence staining

Table 4.1 shows the list of properties of the solvent cast PLGA film and the corresponding employed characterization techniques. The prepared PLGA substrates are optically transparent which facilitated *in situ* observations. The film has a final dry thickness of $15.8 \pm 2.4 \mu\text{m}$ ($n=6$) as measured using an elcometer thickness gauge and has a static contact angle of $82.6 \pm 2.8^\circ$ ($n=6$). As the aim of our study is to examine the effect of cell shape on stem cell differentiation, smooth surface or with minimal nanoroughness is essential to eliminate any interference from nano-scaled induced effects on the cells. Such exquisite sensitivity of hMSCs to nanoroughness (10-100nm in height) was previously established in our lab [79]. Furthermore, Yim et al. had also clearly demonstrated that hMSCs grown on synthetic surfaces decorated with nano-grooves could drive differentiation of hMSCs along the neuronal lineage [41]. AFM analysis of the PLGA film revealed a smooth surface (σ_{rms} : 0.5096nm) with sporadic small sub micron sized pits (**Figure 4.2**). Both our contact angle measurements and AFM image of the solvent cast PLGA agree well with results obtained by Vargha-Butler et al.[192].

Figure 4.2. Representative 2D (A) and 3D (B) AFM images of solvent cast PLGA thin film obtained via tapping mode. Images were processed and analyzed using the WSxM 5.0 software.[193]

Fabrication of the PDMS elastomeric stamp constitutes the first step of μ CP technique. Micro features on the elastomeric PDMS stamps were validated and characterized using phase contrast microscopy. As shown, the fabricated micro features on the PDMS stamp surface comprises of $\sim 40\mu\text{m}$ wide and $1\mu\text{m}$ deep grooves, separated by $\sim 20\mu\text{m}$ wide elevated lanes (**Figure 4.3A and 4.3B**). Fidelity of the transferred fibronectin pattern was ascertained using immunostaining showing uniform distribution of the printed saturated amount of ECM protein with regular spacing of $\sim 40\mu\text{m}$ (**Figure 4.3C**). Measured fluorescence intensity across section x-y indicated marginal difference in the concentration of the printed ECM protein across the PLGA scaffold. Comparable gray value (fluorescence intensity) observed for the unpatterned sample (Inset) suggested that the cells were exposed to a consistent surface ligand presentation. Unlike conventional micropatterning substrates such as glass, poly(*dimethyl siloxane*) or silicon wafer, transfer printed fibronectin onto PLGA did not require any additional surface treatments.

Figure 4.3. Soft lithography technique was employed to transfer microscale patterns onto the **PLGA scaffold**. Top (A) and cross sectional (B) view of PDMS elastomeric stamp consisting of 20 μm wide features separated by grooves of 40 μm in width. The height of the elevated lanes is approximately 1-2 μm (A). (Scale bar= 25 μm) Immunolabelling of transfer printed fibronectin (50 μg/ml) with goat anti rabbit Cy3 fluorescence probe revealed uniformity and regularity of the transferred ECM protein (c). (Scale bar=100 μm)

4.3.2 Bone marrow MSCs adopted drastically different morphology and cytoskeletal configuration when plated on cell-shape defining substrates

Figure 4.4. Passivation of PLGA substrate with F127 is required for long term retention of the imposed micropatterned. Cell adhesion and spreading were monitored on PLGA substrates with different surface configuration. By 2 hours after cell seeding, cells attached rapidly on PLGA that was uniformly coated with fibronectin (A), patterned fibronectin without passivation (B) and patterned fibronectin with passivation (C). When cultured overnight on the engineered surfaces, extensive cell spreading was observed on the unpatterned surface (D), while cells cultured on the patterned but unpassivated grew out of the printed fibronectin lanes. Cells grown on patterned and passivated region constitute the optimal condition to retain cellular elongation over extended culture duration (F). (Scale bar=200 μ m)

Control over the spatial arrangement and cell morphology requires defined regions that promote and prevent cell adhesions accordingly. The former is mediated by proteins containing integrin binding motifs that are present on a given surface and the latter can be achieved on surfaces that repel protein adsorption. Integrin binding is known to be mediated by specific molecular epitopes of ECM proteins. Among them, fibronectin is an established and potent attachment factor as it possesses several integrin recognition sites such as Arg-Gly-Asp (RGD) [194], Pro-His-Ser-Arg-Asn (PHSRN) [195], Leu-Asp-Val (LDV) [196] and Arg-Glu-Asp-Val (REDV) [197]. Furthermore, it has been used extensively to micropattern cells [37, 38, 134, 138]. Therefore, the choice of fibronectin to be transfer printed onto the solvent cast PLGA

is ideal for the purpose of controlling hMSCs morphology. By coating the surface of PLGA with a saturated amount of fibronectin, cell adhesion was facilitated. Cells cultured on the unpatterned control surface attached and spread at random as shown in **figures 4.4A** and **4.4D**. When regions of cell attachment were defined, cell responded to the printed ECM cues by adopting an elongated morphology on the lanes 2 hours post cell seeding (**Figure 4.4B** and **4.4C**). However, 20 hours after cell seeding, cells grown on the unpassivated surface grew out of the patterned lane (**Figure 4.4E**), even though we were still able to observe retention of residual cell alignment due to the micropatterned lanes (black arrows). Physi-sorption of the FDA approved anti biofouling agent (Pluronic® F127) on the non-printed region resulted in exclusive adherence and migration of hMSCs on the ECM printed lanes. Physi-sorption of the surfactant Pluronic® F127 was achieved by the hydrophobic interaction between the PPO segment and the hydrophobic surface, resulting in the extension of the PEO segment into the medium yielding a non-biofouling surface [40, 198]. Surface bounded PEO greatly enhances the hydrophilicity of the surface and causes the polymer coil to be hydrated by water molecules from the surrounding. Under such circumstance, adsorption of protein onto the highly hydrated surface will be impeded, as it will require the extended PEO coil to be compressed, which is thermodynamically unfavourable [199]. Attachment and spreading of hMSCs on the printed ECM protein can be observed 45min after cell seeding by use of time lapse video microscope (not shown) and the cells adopted a bipolarized shape exclusively within the printed regions (**Figure 4.4F**).

In addition to the apparent differences in terms of the cell morphology, cytoskeletal arrangement, FAs distribution (as represented by localization of vinculin) and nuclei were immuno-stained to examine how the enforced cellular shape influenced the

aforementioned subcellular components. Representative immuno-labelled cells are shown in **Figure 4.5**. In general, the unpatterned cells were well spread with prominent display of well-defined thick bundles of stress fibers. (**panel 4.5A**). Arrangement of stress fibers can broadly be categorized as either aligned (arrow A) or arranged circumferentially around the cell nucleus (arrow B). Furthermore, vinculin staining revealed distinct positioning of the FAs at the ends of stress fibers, cell peripheral and ventral to the cell nucleus. DAPI staining showed that cell nuclei of the unpatterned cells remained predominately circular in shape (arrow C).

Figure 4.5. Modulated FAs and cytoskeletal development of hMSCs cultured on micropatterned PLGA substrates. hMSCs seeded on unpatterned (A) and patterned (B and C) substrate were immuno-labeled for F-actin (red), vinculin (green) and cell nucleus (blue). Cells were cultured for 4 days prior to fixation and staining. All images were taken with a 20× objective lens. (Scale bar=100μm).

In stark contrast, the micropatterned cells displayed an elongated morphology (**panel 4.5B and 4.5C**). The F-actin aligned parallel to the longitudinal axis of the cells and were less developed compared to the unpatterned cells. Additionally, FAs appeared to be less defined compared to the unpatterned control. Particularly, the cell nuclei were

highly elliptical (arrow D). Cells can either conform perfectly to the printed fibronectin strips of width approximately $20\mu\text{m}$ (arrow E) or adopt a highly stretched and thin morphology as indicated by arrow F. Taken together, on the engineered substrate, hMSCs were coerced to adopt a highly elongated morphology that conformed to the micropatterned fibronectin strips.

Figure 4.6. hMSCs seeded on unpatterned (A) and patterned (B and C) substrate were immuno-labeled for F-actin (red), vinculin (green) and cell nucleus (blue). Cells were cultured for 14 days prior to fixation and staining. All images were taken with a $20\times$ objective lens. (Scale bar= $100\mu\text{m}$).

By day 14, the cells had grown to confluence as shown in **Figure 4.6**. hMSCs seeded on the unpatterned substrate exhibited an increase in cell spreading (relative to day 4 samples) and the stress fibers are well-developed (**Panel 4.6A** and **4.6B**). Notably, regions of localized hMSCs alignment can be observed on the unpatterned surface as shown in panel 4A. In contrast, the micropatterned cells exhibited higher degree of large-scale alignment and were denser in the transverse direction, thus giving rise to higher cell numbers per unit area (**panel 4.6C**).

4.3.3 Cellular morphological indices analysis

Morphological differences between the cells cultured on the engineered and control substrates were quantified. Measurable cellular parameters such as the cell spreading area, bipolarity index (BI), 2D projection of cell nucleus area and nucleus shape index (NSI) were processed and analyzed with imageJ (rsbweb.nih.gov/ij/). The BI and NSI can be determined using the following expressions.

$$BI = \frac{\text{Cell length}}{\text{Cell width}} \quad (4.1)$$

[A higher BI value indicate a more elongated cell]

$$NSI = \sqrt{1 - \left(\frac{AB}{CD}\right)^2} \quad (0 \leq NSI < 1) \quad (4.2)$$

Where AB and CD refer to the lengths of the minor and major axes of the cell nucleus, respectively.

[NSI closer to 0 indicates a rounder nucleus; NSI closer to 1 indicates a more elliptical nucleus]

Altogether, 40 cells/ experimental group taken from 3 separate images of each group were analyzed to compute the mean and standard deviation of the respective morphological indexes.

The measured morphological differences between the patterned and unpatterned hMSCs were subjected to statistical analysis. Cellular spreading area, BI, 2D projection of the cell nucleus area and the NSI were examined. Samples retrived on day 4 of culture revealed that cells that were grown on substrate with a uniform

coating of fibronectin displayed larger mean spreading area of $11212.5 \pm 5494.7 \mu\text{m}^2$, a BI value of ~ 2.1 , suggestive of a weak biopolarized configuration (**Figure 4.7A** and **4.7B**), a large nucleus area of $415.0 \pm 150.5 \mu\text{m}^2$ and a NSI value of approximately 0.65 (**Figure 4.7C** and **4.7D**). On the contrary, the morphological indices for the patterned cells differed significantly to that of the unpatterned cells. The micropatterned cells exhibited a much smaller spreading area of $2167.1 \pm 945.1 \mu\text{m}^2$, which corresponded to 80% reduction in cell area relative to the unpatterned cells, a BI value of ~ 13.6 indicative that the cells adopted a highly elongated form, a projected cell nucleus area of $236.3 \pm 81.8 \mu\text{m}^2$ and NSI ~ 0.80 , signifying that cell nuclei were well elliptical. Morphological indices obtained on day 14 of cell culture revealed a similar trend. Altogether, hMSCs cultured on micropatterned surface displayed reduced cell spreading area, lower CSI value and smaller elliptical cell nuclei when compared with the unpatterned hMSCs cultured on the fibronectin coated PLGA surface.

Figure 4.7. Measured morphological parameters. Cell spreading area (A), BI (B), 2D projection of cell nucleus area (C) and NSI were examined from immuno-stained images of hMSCs cultured under two different *in vitro* conditions (Unpatterned cells : white bars, patterned cells : Shaded bars) for 4 and 14 days. Bars represent mean $\pm$ standard deviation derived from 40 data points (n=40) per experimental group. Data were collected from at least two experiments. Single and double asterisk indicates statistical significance difference of $p<0.01$ and $p<0.05$, respectively, between the two experimental groups.

4.3.3 Enforced hMSCs elongation promotes myogenic and neurogenic gene expression

Next, we examined whether the engineered hMSCs morphology was capable to modulate stem cell differentiation by performing qRT-PCR of selected genes that are commonly associated to osteogenic, neurogenic and myogenic differentiation.

4.3.2.1 Reduced osteogenic-related genes in hMSCs cultured on patterned surface

Alkaline phosphatase, a tissue non-specific isozyme encoded by the ALPL gene is believed to play a vital role in bone matrix mineralization [200]. For samples retrieved 4 days post cell seeding, patterned hMSCs ALPL transcription level was significantly reduced by 7.3 times and remained at its diminished level till the 14th day of culture (**Figure 4.8A**). During the onset of osteogenesis, osteocalcin is expressed in high levels, which in turn is regulated by the basic helix loop helix (bHLH) transcription factor, RUNX2 [201-203]. At day 4, expression of the RUNX2 transcript in the patterned hMSCs was approximately 4.7 times lower than the basal level while differential expression of osteocalcin between the patterned and unpatterned samples was marginal. By the 14th day of cell culture, the patterned cells displayed a slight increase in RUNX2 expression. For other osteogenic markers, there are either no or not statistically significant transcripts upregulation relative to the control sample. Taken together, expression of osteogenic genes were either down modulated or maintained near nominal level for cells that adopts an elongated or spindle like form.

4.3.3.2 Upregulation of neurogenic and myogenic genes

NeuroD1 and nestin are both well-established markers during neural development. NeuroD1 belongs to the NeuroD family (D1-D3) and is the first to be expressed during neural development [204], while nestin is a transitory neurogenic marker for

multipotent neural stem cell [205]. Remarkably, both the selected early neuronal markers were upregulated on the 4th and 14th day of culture, with the NeuroD1 gene showing the greatest increase at day 14 (~3.2 fold). Similarly, upregulation of nestin was observed by approximately 2 times on both days (**Figure 4.8B**). GFAP is a type III intermediate filament expressed in mature astrocytes in the central nervous system [205] and the MAP2 gene encodes the microtubule associated protein believed to be critical for the development and maintenance of neuritis [206]. Both GFAP and MAP2 genes were observed to be consistently higher for cells that take on an elongated/ spindle configuration (**Figure 4.7B**). These observations are in agreement with work conducted by Yim et al. showing elongated hMSCs on nanogratings promotes neurogenesis [41]. Our results suggest that controlling the cell shape can modulate genes associated with neurogenesis.

GATA4 and MyoD1 are major early transcription factors regulating the process of cardiomyogenesis [207-209]. As shown in **figure 4.8C**, both early myocardiogenic genes were upregulated in the micropatterned hMSCs. The products of β -MHC and cTnT genes are essential to impart functionality to the contractile machinery of skeletal and cardiac muscle [210]. Furthermore, expression of the β -MHC gene is in part regulated by GATA4 during cardiomyogenesis [211]. In concordance, the mRNA expression level of β -MHC and cTnT for the micropatterned cells were increased after 14 days in culture compared to unpatterned control (**Figure 4.8C**). Collectively, gene transcripts that are usually associated with myogenesis were all upregulated for the micropatterned cells.

Figure 4.8. Relative gene level expression analysis of osteogenic (A), neurogenic (B) and myogenic (C) markers. White bar and shaded bar represent samples retrieved on the 4th and 14th day of culture respectively. Bars represent mean $\pm$ standard deviation. Relative gene expression levels (I.e. $\Delta\Delta C_T$) were normalized to the control samples indicated by the dashed line in red. The asterisks indicate statistical significance ($p < 0.05$) difference between the two experimental groups (patterned and unpatterned samples).

4.3.4 Micropattern directs hMSCs differentiation into myocyte like cells

Since upregulation of both neuronal and myocardial mRNA transcripts level were observed for the micropatterned cells relative to the control samples, it will be of interest to determine if:

- (i) the increase in mRNA transcripts level is significant enough to steer differentiation towards a particular tissue lineage at the protein level
- (ii) and whether the hMSCs can co-express differentiation markers belonging to different tissue lineages.

To gain insights into the aforementioned questions, cells were co-stained immunocytochemically with both myogenic and neurogenic markers.

Mature markers indicative of myogenesis, cardiac MHC and neurogenesis MAP2 were chosen to be examined, because expression of either marker is specific and distinct to the respective tissue lineage (**Figure 4.9 panel A to D**). Interestingly, at the protein level, only significant expression of cardiac MHC for the micropatterned cells but not the unpatterned cells at day 4 of cell culture was observed. Furthermore, traces of MAP2 expression were undetectable regardless of the cell configuration. By day 14, weak cardiac MHC expression can be observed for a sub population of the cells adopting elongated morphology on the unpatterned substrates. Taken together, concomitant expression of early and late molecular signatures associated with myogenesis strongly suggests that the micropatterned cells on the PLGA substrates were directed uniquely towards the myogenic lineage at the time scale being examined.

Figure 4.9. Dual immuno-detection of myogenic and neurogenic differentiation markers. Cells were co-stained with distinctive and mature markers of myogenesis and neurogenesis using mouse monoclonal anti heavy chain cardiac myosin (**green**) and chicken polyclonal anti MAP2 (**red**) (panels A–D). Positive controls were used to verify the specificity of the primary antibodies. C2C12 were induced to differentiate in 2% horse serum and stained for cardiac MHC (panel E). Neural stem cells were induced to differentiate in DMEM F-12 and stained for MAP2 (panel F). (Scale bar=100 μ m).

The multipotential differentiation of hMSCs into cells belonging to the three germ layers makes them an important cell source in regenerative medicine. Due to its clinical relevance, concerted efforts have been placed in recent years to employ a multitude of approaches that utilize chemicals, biological factors and nanotechnologies to regulate stem cell lineage commitment. Interestingly, regardless employed methods, cell shape alteration is often observed, leading up to the resultant cell fate. This raises an intriguing possibility cell shape itself may be a driving factor in stem cell differentiation. Indeed, our study herein demonstrated that hMSCs coerced to elongate and proliferate on a micropatterned fibronectin-PLGA surface favoured upregulation of genes normally associated with neurogenesis and myocardiogenesis, even when cultured in non-differentiating condition.

When seeded on shape defining substrate, hMSCs adopted a highly aligned and stretched morphology, and also displayed much smaller cell spreading area relative to the cells cultured on surface uniformly coated with fibronectin. Within minutes of cell seeding, hMSCs attaches on the fibronectin coated surface and form FAs. Microprinted fibronectin lanes dictated the spatial distribution of these FAs, confining the cells to attach and grow exclusively within these regions and thus resulting in the global deformation of the cells. Such distortion at the cellular level is then conveyed to the entire cellular architecture as a result of mechanical continuity between the FAs, cytoskeleton and cell nucleus scaffold based on the cellular tensegrity model proposed by Inger et al.[212, 213]. As it is well established that the cellular cytoskeletal assembly plays an integral role in signal transduction, changes made to the FAs spatial distribution or cytoskeletal re-organization can thus provide a physical impetus by which mechanical deformation (of the cell) is translated into biochemical responses [214]. Although little is known on the exact mechanism that converts these biophysical inputs into biochemical signalling activities, one possible mechanism for this is via the deformation of the cell nucleus, which is mechanically linked to the cytoskeleton by intermediate filaments. Nucleus deformations have been shown to alter gene transcription profiles and thereby regulate cellular functions [215]. Moreover, progress in stem cell differentiation can also be inferred from the biomechanics of nucleus, suggesting that the nucleus can potentially function as a potent mechanosensor or modulator of cell differentiation [216]. At present, several mechanisms of nuclear mechanotransduction have been suggested [215]. Mechanical tugging of the nucleus membrane can directly affect gene activity by regulating accessibility of the transcriptional factors to the genes and also modulating the nuclear pore size thus influencing regulatory molecules (e.g. mRNA, DNA) or ion flux.

However, based on our observations, it appears that nuclei shape alteration might play a smaller role in guiding hMSCs differentiation along the soft tissue lineages, since similarities in the NSI did not correlate well with the upregulation of gene expression at day 14. As cells proliferation commences secretion of endogenous ECM proteins and desorption of F127 (anti fouling agent) over time may occur. Additionally, cell confluence may also limit the elongation of cells, consequently, the computed NSI is significantly less elliptical as compared to the cells on the 4th day of cell culture.

The ability of the micropatterned substrate to direct hMSCs commitment along the neuronal and myocardial lineages was further ascertained by immuno-detection of lineage-specific differentiation markers such as cardiac MHC (myogenic) as well MAP2 (neurogenic). While negligible expression was observed for expression of MAP2 protein, significant expression of the myogenic mature marker cardiac MHC was detected only for the micropatterned cells. Taken together, with results attained from the qRT-PCR assay, we reasoned that the stretched and longitudinal cell shape appears to be conducive for both myogenic and neurogenic differentiation mRNA transcripts expression. However, it is also intuitive to conceive that the inherent differentiation wiring of these stem cells will prevent both myogenic and neurogenic differentiation pathways to occur till maturity. Even though there are clearly upregulated levels of both lineages but at the functionally relevant level of protein expression, the myogenic lineage is dominant within the time scale studied. This area opens up a very interesting extension to our project that post transcriptional control drives hMSCs in our patterning system towards a predominant myogenic lineage at the time scale we looked at. Indeed, the unique mechanism by which shape can induce the differentiation of stem cells is an area that warrants future studies.

4.4 Conclusion

Collectively, via a series of experiments, our result strongly support the notion that cellular shape modulation without the use of induction media may be a feasible strategy to coax lineage-specific differentiation of hMSCs. Immunocytochemical analysis revealed that cells grown on the micropatterned substrates promoted differentiation of hMSCs into myocyte-like-cells as determined by significant expression of cardiac MHC detected at the protein level. More importantly this novel approach that drives lineage specification of hMSCs not only complements present stem cell differentiation procedure, the biocompatibility and biodegradable properties of PLGA also makes the engineered platform suitable for tissue engineering applications [188-190]. The molecular events implicated for the observations made in this study would undoubtedly be of interest for future studies and will be addressed in the subsequent chapter. From an application standpoint, micropatterning hMSCs on PLGA scaffold could enable large scale alignment of cells required for certain applications like cardiac muscle tissue engineering, among others.

Involvement of the FAK-ERK canonical signalling cascade in cell shape regulated myogenesis of hMSCs

5.1 Abstract

In the previous chapter, we have demonstrated that hMSCs coerced to adopt an elongated morphology is able to modulate stem cell lineage commitment, in particular, towards the myogenic lineage. However, the underlying mechanism of this mechanotransduced response remains elusive. Employing the same technique as outlined in chapter 4 and implementing a new set of experimental designs, the present chapter seeks to elucidate the molecular basis of cell elongation induced myogenesis of hMSCs. Early dynamic events (8h-120h) upon cell attachment on the engineered substrates were monitored. The patterned cells consistently exhibited reduced occurrence as well as the size of the FAs. Expectably, accompanied with this altered morphological form, numerous myogenic transcripts were observed to be upregulated as a function of culture time, consistent with the observations made in the previous chapter. Furthermore, myogenic activity was also ascertained via immunodetection of myosin heavy chain. Proximity Ligation Assay revealed diminished phosphorylation activity of FAK and its downstream target ERK of the micropatterned cells by a factor of ~1.6 and 1.4 relative to the unpatterned control group after 120h of culture, suggesting that the cell shape driven myogenesis may implicate this canonical signalling pathway. Treatment of hMSCs with PD98059 (50 μ M), a potent MEK1

inhibitor not only enhanced MyoD and Myogenin expression but also induced significant expression of myosin heavy chain as assessed by immunocytochemical staining. Taken together, our data suggest that the canonical FAK-ERK signalling cascade may play an important role in this stem cell elongation mediated myogenesis.

5.2 Introduction

Form and function are one of the foundational tenets of cell and tissue physiology. Form is largely determined by cells but brought about by intracellular structures and extracellular material properties. Learning from this important intertwined relationship between form and function, we showed in chapter 4 that myogenic activity of hMSCs can be driven via a mechanoregulated process. However, the molecular basis of this mechanotransduced process remains ill defined. Elucidating the regulatory mechanistic cascades that direct stem cell behavior in response to these biophysical cues is important because it will bring about rational designs of novel biomaterials and configurations.

The term mechanotransduction refers to the process of converting mechanical cues from the microenvironment into a series of biochemical signalling cascades/epigenetic programs that modulates stem cell fates [44, 214, 215, 217]. Such exquisite ability of the cells to reciprocate to its immediate environment is highly dependent on the cells to (i) sense its surrounding, (ii) transduce these biophysical signals into signalling cascades that governs and elicit an appropriate (ii) response accordingly [217]. A large body of evidences have supported the notion that the process of mechanotransduction plays a pivotal role in tissue development, cell growth, migration and stem cell differentiation.

Mechano-sensing comprises the first step of mechanotransduction at which the cells survey its immediate surroundings. Till this end, the actual mechanosensors of a given mammalian cells have yet to be defined. Nevertheless, based on accounts made by a large body of published works, several “usual suspects” have been identified as playing a pivotal role in the initial sensing process. Among them is the integrin, a

heterodimeric transmembrane protein that function as a molecular bridge facilitating communication between the intracellular machinery of the cell and its ECM.

Integrins belong to a family of heterodimeric transmembrane proteins composed of 2 sub units: α and β . Interactions between a cell and the ECM initiates integrin clustering that subsequently leads to the formation of large FAs via a positive feedback mechanism [145]. At the cytoplasmic tail domain, the integrin associates itself with the cytoskeleton. In this circumstance, the integrin recruits a large number of active signalling kinases that initiate signal transduction to the cell's nucleus through a series of phosphorylation events, thus influencing the transcriptional activity of the cell [145, 218]. This system allows a cell to react to its surface, altering its gene expression profile to suite the requirement of the environment.

Similarly, adherent MSCs forms focal contacts which are attachment sites containing a multitude of signalling proteins with the underlying ECM. FAK a 120 kDa non-receptor tyrosine kinase localizes at these focal adhesion sites has been implicated in regulating adhesion dependent phosphorylation events [145]. Above all, a downstream target of FAK, the ERK, part of the mitogen associated protein kinase (MAPK) signalling cascade, can be activated via phosphorylation of FAK at Tyr-925 creating a Src homology domain 2 (SH2) site for the Grb2/SOS complex [174, 219]. Notably, activation of ERK has been shown to play a pivotal role in guiding MSCs differentiation. hMSCs subjected to mechanical strains enhances matrix mineralization via concomitant activation of ERK activity [115, 175]. Inhibition of ERK activity abrogates osteodifferentiation of hMSCs and instead promotes adipodifferentiation when cultured in an osteoinductive medium.[172] Knock down of FAK inhibited activation of ERK (1/2), resulted in blockade of ECM protein induced osteogenesis of hMSCs, establishing the role of canonical FAK-ERK signalling

cascade mediating osteodifferentiation [220, 221]. Similarly, hMSCs coerced to adopt a star shape that exhibited elevated expression several genes associated with the MAPK pathway was shown to favour osteodifferentiation [138]. Conversely, ERK activity was shown to exert an inhibitory effect on myogenic transcription in murine myoblast during the early phase of myogenesis [222]. Blockade of ERK and GSK3 pathways using a 3 inhibitors (3i) system with SU5402, PD184352 and CHIR99021 was also shown to suppress differentiation and maintain long term pluripotency of ESCs [11, 12] Recently, it was also shown that when treated with PD98059, a potent inhibitor of ERK, hMSCs were driven to differentiate towards the muscle lineage [223]. These examples suggest that ERK may play a pivotal role mediating and directing hMSCs to differentiate into different cells belonging to the mesodermal lineage.

In the present study, we examined the involvement of the FAK-MEK/ERK canonical signalling pathway in directing stem cell lineage commitment towards the myogenic lineage for hMSCs cultured on micropatterned adhesive strips adopting highly bipolarized shape. Hallmark transcripts of myogenesis were monitored for hMSCs cultured on fibronectin coated PLGA substrata with and without micropatterns. Cellular proliferation and FAs development contrast starkly between the two experimental groups. Directed polymerization of the F-actin towards the apical ends resulted in formation of smaller, diminished expression of FAs and less developed cytoskeletal stress bundles. Apart from the obvious morphological differences, highly bipolarized morphology seemingly favours upregulation of several myogenic transcripts expression in line with previous observations made by our and other research groups [42, 224]. Yet, a systematic account for the underlying molecular mechanisms involved in this intriguing observation remained largely unexplained. We

postulate that differential FAs development may be the initiation point to differentially activate a cascade of signalling pathways regulating hMSCs to undergo myogenesis. We examined the activity of the signalling molecule that is closely associated to cellular FAs namely the FAK and its downstream target ERK.

5.3 Materials and methods

5.3.1 Cell proliferation assay

Proliferating cells were counter stained with 5-bromo-2'-deoxyuridine (BrdU), a synthetic nucleoside that gets incorporated into newly synthesized DNA during the S phase of the cell cycle. 7 hours post labelling the cells were fixed with 70% ethanol (4°C) and BrdU incorporation was detected with the Zymed BrdU kit (Invitrogen). More than 100 cells from 20 different regions were imaged and counted with an inverted bright field microscope (Nikon) on each sample. Technical replicate: 4 for each experimental group.

5.3.2 In situ proximity ligation assay

hMSCs cultured on PLGA film were fixed with PFA (4%) for 5 min at room temperature. Samples were washed thrice with PBS, blocked for 1 h at room temperature with NGS (5%) in PBS containing Triton X-100 (0.1%) followed by incubation with antibody pair overnight at 4°C: FAK and phospho-FAK (Tyr925) (Santa Cruz Biotechnology); ERK1/2 & phospho-p44/42 MAPK (ERK1/2) (Cell Signaling). Samples were washed with PBS as mentioned above. Duolink^{TM} *in situ* PLA was performed in accordance to the manufacturer's instructions (OLink Biosciences). Samples with no antibody served as negative controls. n = 20 image fields with ~5 cells per image per sample type, each sample run in duplicate.

5.3.3 *ERK inhibition and western blot assay*

hMSCs (10^5 cells/ well) were cultured overnight with FBS (3%) culture medium on fibronectin coated 6 well tissue culture plate. Cells were then exposed to PD98059 (50 μ M) (Sigma), a specific and potent MEK1 inhibitor for 24h. For western blotting, protein extracts were isolated in ice-cold lysis buffer (20mM Na₂H₂ PO₄, 250mM NaCl, 1% Triton X-100, and 0.1 % SDS). Equal amounts of protein extracts (50 μ g) were resolved by SDS-PAGE and electrotransferred onto PVDF membranes. Membranes were processed as described by the manufacturer of antibodies, and proteins were detected by chemoluminescence (Millipore). For immuno-detection of MHC, hMSCs were continuously exposed to PD98059 (50 μ M) for 120h before the cells were fixed and counter stained for MHC as described earlier in chapter 4.

5.4 Results and Discussion

5.4.1 *Micropatterned matrix modulates dynamics of focal adhesion and F-actin polymerization of human mesenchymal stem cells as a function of time*

Immunocytochemical staining provided insights into how the micropattern affected the dynamics of cytoskeleton arrangement as well as FAs development. hMSCs were labeled with TRITC-conjugated phalloidin (**red**), Alexa Fluor488-labeled FAs plaque protein, vinculin (**green**) as well as DAPI for the nucleus (**blue**). Growth of the cells was monitored over a course of 120h after cell seeding. In the case of the unpatterned cells, at 8h, majority of the cells adopted relatively small spreading area and ‘circular’ morphotype with distinct ruffles of lamellipodia outgrowth in an unsystematic manner. FAs were located at the ends of the F-actin and localized at the cell peripheral region. By 24h onwards, formation of distinct stress fibers bundles and exaggerated expression of elongated dash like FAs could be observed at the cell periphery and perinuclear regions of the cells cultured on uniform fibronectin coated PLGA. In

contrast, F-actin of the micropatterned cells were aligned in parallel with the fibronectin lanes and appeared to be less developed. The directed polymerization of the F-actin along the micro sized lanes was accompanied by small dot-like FAs at the apical ends of the cells when compared to the unpatterned cells (**Figure 5.1 bottom panel insert**). Mean number/ cell, size and density of FAs as represented by the immunodetection of vinculin, was measured and quantified. Results obtained corroborated with the visual observations made. As shown in **Figure 5.1A**, in the case of the cells cultured on the unpatterned substrate, we observed a relatively high number of FAs per cell during the early phase of cell culture (i.e. 8 and 24h) with ~150 FAs per cell. The FAs was observed to increase by ~ 2.6 fold and ~3.3 fold at 72h and 120h of cell culture respectively. In contrast, the number of FAs for the patterned experimental group was consistently lower compared to the unpatterned cells, presumably due to the guided directional outgrowth of the lamellipodia along the fibronectin adhesive strips with a mean FAs per cell of ~50 throughout the course of our study. Comparatively, the unpatterned cells exhibited approximately 8 fold higher FA expressions by the end of the 120h culture duration. Unpatterned hMSCs consistently displayed higher FAs density and larger size as shown in **Figure 5.1B and 5.1C**, respectively. Comparing pattern cells across the examined time period, we did not observed any significant difference in relation to the FA number and FA size. However, statistical significance ($p<0.01$) was observed when comparing the FA density of the patterned cells as a function of culture duration. Specifically, FA density measured at 8h was significantly different from FA density measured at 72h, 8h and 24h. These results suggest that the micropatterned matrix has profound impact on the cytoskeletal and FA development of hMSCs.

Figure 5.1. Cell adopting bipolarized morphology suppresses formation of FAs and cytoskeletal development. Fluorescence images of hMSCs depicting cellular morphological evolution as a function of culture duration. Focal adhesion adapter protein, vinculin (green), F-actin (red) and cell nucleus (blue) are immuno-detected as shown. (Refer to inserts; scale bar= 20μm) Number of FA (A), FA density (B) and size (C) were quantified as a function of culturing time for both experimental groups. Bars represent means $\pm$ standard error of mean derived from 30 data points ($n = 30$) per experimental group. All data shown exhibited statistical significance at $p < 0.01$ (comparing patterned cells with unpatterned controls) Scale bar= 100μm.

5.4.2 Enforced cellular bipolarity significantly modulates hMSCs proliferation activity and myogenesis

Adhesion to the substrate is mandatory for survival and cell cycle progression. Such adhesion-dependent responses are in part mediated by integrin clustering which can be affected by the mechanical properties of the underlying substrate [43, 63]. Recently, it was shown that proliferative capability of human SMCs was highly dependent on the degree of cellular elongation [135]. Similarly, we observed that elongated hMSCs exhibited lower proliferation activity as indicated by reduced

percentage of cells stained positive for 5-bromo-2-deoxyuridine (BrdU). Aligned hMSCs cultured on the patterned substrate consistently exhibited statistically significant difference proliferative activity relative to the unpatterned control throughout the course of our study as shown in **Figure 5.2A**. Peak difference for the BrdU labeling was observed at 24h with the unpatterned control and micropatterned cells exhibiting ~35% and ~17% of BrdU positive cells respectively. The gradual decrease in the number of BrdU incorporated cells on the control substrate with culture duration is presumably due to the effect of contact inhibition. Conversely, the micropatterned cells exhibited a fairly constant proliferation rate of ~ 20% from 24h onwards.

Bone marrow derived hMSCs have been shown to possess the propensity to differentiate into muscle cells. Endogenous molecular signatures associated with myogenesis were identified both at the mRNA and protein levels even when the cells were not stimulated under the influence of soluble factors [58]. Myogenesis is a coordinated program involving activation of myogenic regulatory factors (MRFs) such as MyoD, MYF5, Myogenin and MRF-4 [225]. The process of myogenesis is typified by cell cycle withdrawal and expression and upregulation of muscle-specific proteins. Furthermore, the cells will undergo cellular elongation and fusion of the myoblast into multinucleated myotubes/myofibers [226]. We have previously shown that the expression of myogenic genes and MHC protein were upregulated in micropatterned cells adopting an elongated morphology. To examine the kinetics of this cell shape-driven myogenic lineage activation, real time-PCR experiments were designed to track the gene expression profiles at various time points (i.e. 8, 24, 72 and 120h) after cell seeding and expression of the MHC in hMSCs cultured on fibronectin coated substrates. As shown in **Figure 5.2B**, significant upregulation of myogenic

related genes were observed after 72h of culture on the micropatterned surface. Statistically significant peak myogenic gene expression was observed after 120h with GATA4, NKX2-5, MyoD, Myf5, MHC and cTnI exhibiting 3.7, 3.0, 4.2, 3.9, 3.3 and 2.9 fold higher expression respectively (relative to the unpatterned cells). In contrast, expression of ALPL, an early osteogenic marker was observed to be downregulated with culture time while osteonectin expression level remained largely unchanged and exhibited no significance difference compared to the unpatterned cells. RUNX2 gene which encodes the bHLH transcription factor displayed a modest increase ~2 fold and remained elevated. Since, elevated gene expression was not observed for osteogenic related genes, this suggests that myogenic activation was unique to hMSCs coerced to adopt an elongated morphology. Additionally, as shown in **Figure 5.2C**, significant MHC expression was immunodetected for the micropatterned cells, whereas weak or negligible staining was observed for the unpatterned controls after 120 h of cell culture (**Figure 5.2D**).

Next, we examine if a lowered cell proliferation can account for the activation of myogenic differentiation in hMSCs. hMSCs were cultured in reduced serum condition (1%FBS) and stained for MHC after 5 days of culture. Under 1%FBS culturing condition, number of cells labelled positive with BrdU was significantly reduced to approximately 6.2%. Furthermore, expression of MHC was non-existence. Our results suggest that under reduced serum condition, myogenic events were not promoted. As serum contains a rich mixture of growth factors and cytokines, reduction of the proliferative capacity may not be the sole cellular response that is influenced. We cannot exclude the possibility that a wide spectrum of signalling networks, including those that support myogenic events could be suppressed/ in-activated. (**Figure 5.2E**). Taken together, our results revealed that coerced cellular elongation in soluble factor

free condition drastically modulated cellular responses in terms of cellular lineage commitment and proliferative activity of hMSCs. Both mRNA and protein level analyses strongly suggested that the micropatterned matrix promoted myogenic activity of hMSCs.

Figure 5.2. Activation of myogenic specific transcript expression as a result of enforced cell elongation. Expression of mRNA transcript fold change analyzed with RT PCR is expressed in the form of heat map (A). Gene expressions for the elongated cells were normalized to that of the unpatterned controls. BrdU incorporation assay revealed that the micropatterned cells displayed lower proliferation activity (B). Significant protein hallmark myogenic marker MHC was immunodetected in the micropatterned group (C) but not for the unpatterned control cells (D). hMSCs cultured under reduced serum shows absence of MHC staining (E). Bars represent means $\pm$ standard deviation. $**p < 0.05$; $*p < 0.01$. Scale bar = 100 μ m. Insert scale bar = 15 μ m

5.4.3 Translation of cell shape regulated intracellular signalling activities via the FAK-ERK pathway

Although it has become increasingly evident that many of the cellular responses can be influenced by the mechanical or surface physicochemical properties of the

materials, the series of targeted intracellular events remains elusive. From the standpoint of a materials scientist, such understanding can, in principle, further aid to develop suitable and novel biomaterials for the targeted activation of specific molecular signalling pathways to bring about desired cellular responses. The vast difference in terms of FAs development observed in the earlier section led us to postulate that FAK, a potent signalling molecule closely associated to the development of cellular focal contacts might be implicated [145, 219, 227]. To validate our hypothesis, we measured and monitored phosphorylation of the non-receptor protein kinase FAK (phospho-FAK Y925) using *in situ* proximity ligation assay (**Figure. 5.3A**). We observed a time-dependent increase in phospho-FAK Y925 for the unpatterned controls whereas suppressed expression of phospho-FAK Y925 for the micropatterned cells was observed to be ~1.6 fold lower by the end of 120h culture period. Representative images of detected phospho-FAK Y925 for the patterned and unpatterned hMSCs are shown in **Figure 5.3C** and **5.3D** respectively.

Following which, we assessed the impact of morphological differences on ERK activation, a downstream target of FAK. Activation of Ras-ERK pathway is facilitated by formation of docking site for growth factor receptor-bound-2 adapter protein (Grb2) via phosphorylation of FAK Y925 [219]. Moreover, the ERK pathway has also been shown to be involved in mesenchymal stem cell differentiation, thus making the ERK pathway a potential regulatory linkage in translating differential FAs development to regulation of cell lineage commitment in hMSCs. Notably, regulation of ERK activity has been shown to play a pivotal role in guiding MSCs differentiation. Recently, it was shown that when treated with PD98059, a potent inhibitor of ERK, hMSCs were driven to differentiate towards the smooth muscle lineage [223]. Therefore, we measured the expression of phospho-ERK1/2 (pERK)

and as expected, the micropatterned cells consistently exhibited lower pERK activity (~1.5 fold) throughout the course of this study (**Figure 5.3B**). Furthermore, as ERK signalling activity is also known as a positive regulator of cell proliferation, our current findings may in part be useful to explain why a lowered proliferative activity was observed as in the case of the micropatterned hMSCs (**Figure 5.2A**). These results imply that FAK-ERK activity was repressed for hMSCs cultured on the engineered PLGA scaffold.

Figure 5.3. Regulation of FAK-ERK signaling activities via enforced cell elongation. Expression levels of pFAK(Y925) (A) and pERK (B) were quantified using proximity ligation assay technique. Representative images of detected rolling circle product for pFAK (C,D) and pERK(1/2) (E,F) are shown. Bars represent means $\pm$ standard deviation. ** $p < 0.05$; * $p < 0.01$ Scale bar=100 μ m.

Involvement of ERK1/2 mediated hMSCs responses was further examined via pharmacological inhibition of phospho-ERK1/2 activity by exposing hMSCs with PD98059, a potent ERK kinase 1 (MEK1) inhibitor for 24h. Negligible inhibition was observed when we conducted the study in 10% FBS culturing condition that is consistent with observation made by Tamama et al.[223]. (**Figure 5.4A**) Western blot

analysis (**Figure 5.4B**) revealed significant blockade of pERK1/2 activity when hMSCs were exposed to 50 μ M of PD98059 under reduced serum condition (3% FBS). Interestingly, concomitant increase in early transcriptional skeletal myogenic markers, MyoD and Myogenin was modestly increased (**Figure 5.4B**). A plausible explanation for the increased expression of these myogenic markers could be due to the anti-myogenic action of the MEK1/ERK complex. Nuclear translocation of the activated cytoplasmic MEK1/ERK complex binds to the active domain of MyoD inhibiting the transcriptional activity of MyoD:E protein dimer thus hampering myogenesis [228]. Expression of MHC (a mature myogenic marker) was also detected for hMSCs after being exposed to 50 μ M of PD98059 for 120h (**Figure 5.4C**). Observations made from our ERK inhibition complements and extend findings made by Tamama et al. that showed pharmacological intervention using PD98059 directed hMSCs to differentiate along the smooth muscle lineage [223]. In this case, we show that the pro-myogenic ERK activity may also be regulated by cell shape modulation (**Figure 5.5**). To investigate if the interplay of ERK inhibition using PD98059 and cellular elongation can bring about synergistic influences to further push hMSCs myodifferentiation, hMSCs were grown on micropatterned matrix, exposed to PD98059 under reduced serum condition for 5 days and immunostained for expression of MHC. Unfortunately, majority of the cells did not attach, presumably due to excessive suppression of the ERK activity. (data not shown) Collectively, our results suggest that the mechanistic regulation of hMSCs towards the myogenic lineage may potentially effect via the differential activation of the FAK-ERK signalling transduction pathway activity resulting from the significant alteration of cytoskeletal and FAs development of the cells cultured on the engineered substrates.

Figure 5.4. Expression of myogenic markers modulated by MEK1 inhibitor. hMSCs were treated with PD98059 for 24 hours with the respective concentrations to. Whole cell lysates from untreated (-) or PD98059-treated cells (+) were analysed by immunoblotting with specific antibodies for the proteins indicated. In 10%FBS (A) and 3% FBS culture condition (B). β -tubulin expression shows equal loading (C) MHC staining for PD980590 treated cells (C) and cells treated with the diluent (D). Scale bar=100 μ m.

5.5 Conclusion

In summary, our results shows that cell shape can function as a potent regulator that steer hMSCs to undergo myogenesis when coerced to adopt an elongated morphology. (Figure 5.5) The directed cell growth and migration along the narrow micropatterned adhesive strips hampered formation of large mature FAs and suppressed FAK-ERK signalling activity. Inhibiting phospho-ERK activity with PD98059 resulted in upregulation of hMSCs myogenic differentiation events, suggesting that ERK may potentially play a crucial role in driving hMSCs towards the muscle lineage. In translating our novel findings to clinical relevance, one may envisage designing and engineering a biomaterial-cell inspired platform, capable of evoking the deliberate

involvement signalling pathways that may enhance our ability to direct stem cell differentiation with/ without the aid of other potential microenvironmental cues.

Figure 5.5. Hypothetical model depicting directed cell lineage commitment by means of induced bipolarity. Induced cell elongation resulted in directed FAs formation at the apical end of the cells. Coupled with the lack of stress bundles formation, consequently activate the “inside-out” signaling resulting in formation of small focal adhesion. The inability to form large number and size of focal adhesions regulated the FAK-ERK pathway, thus modulating stem cell lineage commitment.

Mechano-regulation of stem cell shape, cytoskeletal tension and lineage commitment on micropatterned mechanically tuneable polyacrylamide hydrogel

6.1 Abstract

Control of cellular contractile force and maintenance of cell shape are mutually complementary events, fundamental in the regulation of a diverse set of cellular behaviours. Intracellular generated cell traction force (CTF) by adherent cells have been shown to exert profound influences on a variety of fundamental cell responses such as migration, proliferation, differentiation and can be modulated as a function of both cell shape and/ or matrix elasticity. Hitherto, due to the lack of a suitable platform, the respective cues (i.e. cell shape and matrix stiffness) have only been studied independently; undeniably the interdependence of the two cues is still at an exploratory stage. In this chapter we continue to examine how cell shape can regulate myodifferentiation of hMSCs under the influence of multiple biophysical cues. A library of 15 different permutations of cell shape (aspect ratio 1:1 to 15:1)-matrix stiffness (1.6kPa, 15kPa, 37kPa) was examined. It has been shown that both matrix rigidity and cell shape significantly affects cells responsiveness in terms of cytoskeletal arrangement, FAs placement and CTF generation. Regardless of the stiffness, the cytoskeletal network aligned parallel to the cell body with increasing aspect ratio. On PA hydrogels with intermediate and highest stiffness, cells coerced to adopt shapes of aspect ratio 1:1 exhibited organized cytoskeletal architecture while cytoskeletal regularity was absent in cells on the softest substrates. Importantly, we define conditions under which one factor can dominate the other. Expression of MHC

was found to be closely associated to a critical CTF level. Our results provided insights into how hMSCs are able to perceive and process various biophysical cues simultaneously and the responses elicited via generation of CTF.

6.2 Introduction

Mechanical forces play a critical role in many cellular activities. Mechano-regulation of cell behaviours can be viewed from two different standpoints. As an indicator, it has been shown that strong correlations exist between cell/ tissue mechanics with progression of diseases and differentiation of stem cells. For instance, it was reported that red blood cells (RBCs) infected by plasmodium falciparum, a protozoan parasite that causes malaria, was 50 times stiffer than healthy RBCs [229]. Stiffening of ESCs nucleus was also reported during the course of neuronal differentiation by 6 fold [216]. As a modulator, mechanical forces are employed as extrinsic cues to control cell responses. Some of the classical examples include the use of passive mechanical stimuli (e.g. matrix rigidity, patterned surface) and applied mechanical forces (e.g. tensile, compressive and shear strain) as were discussed in chapter 2. When cells mechano-sense these extrinsic signals, the cells in turn fine-tune its internal mechanical property (i.e. intracellular cellular tension) by activating a feedback loop involving regulation of focal adhesion and actin polymerization. Such intracellular generated tension can manifest in the form of cell traction forces (CTFs) via interaction of the NMMII with the actin network leading to cell contractility.

Regulation of cell contractility plays a crucial role in stem cell lineage commitment. For instance, stem cell spreading and substrate mechanics can profoundly influence lineage commitment. Using micropatterning technique, it was shown that increasing cellular spreading area and contractility promotes myogenic and osteogenic differentiation of hMSCs under the influence of appropriate biochemical cues [37, 38]. On the other hand, substrate stiffness has been documented by several authors to guide stem cell differentiation with or without the addition of exogenous soluble factors, presumably via regulation of myosin contractility [43, 53-55]. Engler et al.

showed that culturing hMSCs on polyacrylamide hydrogel with muscle-like stiffness assumed spindle morphology and promoted myogenic events [43]. Yet, substrate stiffness is also a known mediator of cell spreading [49]. Increasing matrix stiffness from 14 to 30kPa causes a 25% increase in cell spreading area [230]. In view of the interdependency between cell spreading and matrix mechanics and their involvement in the mechanotransduction of stem cell behaviours, relevant questions thus arises: Is cell spreading regulated by the underlying biomechanics that in turn direct stem cell response or whether cell shape and matrix rigidity can act independently? Under what circumstance will one biophysical cue dominates the other? Understanding the interplay between these biophysical cues is important as it can potentially contribute to the rational design of scaffolding materials for regenerative medicine.

Herein, we developed and advocate the employment of micropatterned hydrogel platforms that allow us to examine the interdependency of cell shape, cell spreading and matrix mechanics simultaneously on hMSCs responses (**Figure 6.1**). Fibronectin defined adhesive micropatterns with fixed area of $\sim 7000\mu\text{m}^2$ were transfer printed on polyacrylamide hydrogels with stiffness that mimics the fat (1.6kPa), muscle (15kPa) and bone tissue (37kPa) respectively. Since we have previously shown that cell elongation (mean aspect ratio of 10) can induce muscle differentiation of hMSCs, cell elongation was systemically increased (i.e. from aspect ratio =1 to 15). We ask whether cell elongation and matrix stiffness induced myodifferentiation of hMSCs are mutually exclusive events. Remarkably, it was shown that these biophysical factors could collectively regulate cytoskeleton assembly, FAs development, CTFs and expression of the myogenic contractile marker (myosin heavy chain, MHC) on a single cell level. Cytochalasin D treatment that disrupts actin polymerization and CTFs, negates MHC expression suggesting that a threshold level of CTFs is essential

to induce MHC expression. Our results revealed a complex interplay between cell shape and matrix rigidity driven myodifferentiation of hMSCs.

Figure 6.1. Schematic of developed micropatterned hydrogel platform to examine the interplay of multiple biophysical cues on stem cell behavior

6.3 Materials and methods

6.3.1 Preparation of PA hydrogel with physiological relevant stiffness

The protocol to fabricate PA coated coverslips was adopted from Tse et al. with slight modifications [231]. Briefly, 400 μ l of a 0.1M NaOH solution was added to cover the entire surface of each circular coverslips (22mm in diameter). The coverslips were then heated to approximately 80°C to fully evaporate the NaOH leaving behind a thin layer of dried NaOH. Next, 350 μ L of aminopropyltrimethoxysilane (APTES) (3%) purchased from Sigma, was added to the coverslips and allowed to stand at room temperature for 5-10min. Subsequently, the cover slips were thoroughly rinsed with de-ionized water to remove any excess APTES solution. 400 μ l of 0.5% glutaraldehyde was then pipetted onto the rinsed cover slips and allowed to stand for 20min. Following that, the cover slips were dried with purified nitrogen gas. By varying the respective concentration of acrylamide and bis-acrylamide in the aqueous solution that affects the degree of crosslinking in the solution, will result in different elasticity modulus of the PA hydrogel. Given a 10ml polymerized acrylamide solution of different elasticity modulus each, the respective mixing ratio is shown in **Figure**

6.2

In order to polymerize the acrylamide and bis-acrylamide, tetramethylethylenediamine (1:1000) (Bio-rad laboratories) and 10% ammonium persulfate (1:100) were added along with the acrylamide and bis-acrylamide mixture and vortexed briefly for about 5s. Then, 35 μ l of the mixed solution was pipette immediately onto a transparency sheet and cover polyacrylamide solution with the amino-silanated cover slip on top. Allow it to stand for 30min to complete polymerization and remove the top cover slip with the PA hydrogel attached to it

slowly, placing each of them into in a 6-well cell culture plate and stored in PBS. Viscoelastic behavior of the prepared PA hydrogels was characterized with an automated rotational rheometer (Anton Paar, physical MCR 501) at ambient condition. PBS was constantly being added to prevent drying of samples.

6.3.2 *Surface activation of PA gel*

In order for cells to attach properly onto the PA hydrogel, ECM protein coating is necessary. However, PA hydrogel is not adhesive enough for the coating to take place. Therefore, a heterobifunctional protein cross-linker sulfo-succinimidyl-6-(4-azido-2-nitrophenylamino)-hexanoate (sulfo-SANPAH) purchased from Proteochem, was required to cross-link the ECM protein on the PA hydrogel. 250 μ l of sulfo-SANPAH (0.2mg/ml) diluted in 50mM HEPES with pH 8.5 was added onto the PA hydrogel surface and exposed to ultraviolet(UV) radiation (365nm) using an UV lamp for 15 min, that darkens the sulfo-SANPAH solution considerably. The PA hydrogels were then rinsed three times with HEPES to remove any unreacted sulfo-SANPAH. Subsequently, the rinsed PA hydrogels were dried in the oven at 60°C for 30min before the substrates were to be microprinted with fibronectin as outlined in chapter 3. The unprinted region was passivated with 2% BSA.

6.3.3 *Cell culture*

Pilot cell culture experiments using the engineered substrates were carried out to affirm its efficacy. We noted that hMSCs grown in LD10 developed aberrant phenotype was unable to adapt satisfactorily onto the micropatterns (not shown). Furthermore, by 120h of cell culture, multiple cell nuclei were observed within a single fibronectin island, indicative of excessive cell proliferation and thus hinder further examination at single cell level. In contrast, when cells were cultured under

reduced serum condition (i.e. 1% FBS) (LD01), the cells attached and conformed to the micropatterns, with majority of the cell population remaining as single cells within the pattern after 120h of cell culture. Therefore subsequent experiments were conducted using LD01.

6.3.4 Construction of vinculin heat map

Protocol to construct the average vinculin heat map was adopted and modified from Kilian et al..[139] hMSCs at predetermined time point were fixed and labelled for the focal adhesion plaque protein, vinculin. Images were captured using the Nikon eclipse 80i, fluorescent microscope using a 20x objective lens. 50 separated images for each of the 15 different permutation of cell shape and matrix stiffness were digitally aligned with imageJ and stacked to attain the average vinculin map. Experiments were duplicated for each cell shape-matrix stiffness permutation.

6.3.5 Cell traction force measurement

To measure cell contraction forces, hMSCs were cultured on PA hydrogels embedded with fluorescent beads. Briefly, glass coverslips were cleaned by use of oxygen plasma before it is placed in a vacuum dessicator containing a small drop of 1H,1H,2H,2H-perfluorooctyltrichlorosilane for 30min. Following which, the surface treated coverslips were removed and 100 μ L of diluted yellow-green FluoSpheres® microspheres (0.2 μ m, carboxylate-modified, 0.1vol%, Molecular Probes) was added to the coverslips with the treated surface facing upwards. This step forms a monolayer of fluorescent beads on the coverslips. The acrylamide and bis acrylamide solution was then polymerized, sandwiched between the coverslip and another amino silanized coverslip as per described in the section 6.3.1. Only samples with well-dispersed beads were used in the study. Preliminary studies that compared CTF measured at 24h

post seeding and 120h post seeding for a few given cell-shape-matrix stiffness combination did not reveal any significant differences and thus subsequent CTF was measured 24h post cell seeding.

Calculation of CTFs, was based on the elastic moduli of PA substrate and fluorescent beads displacements, which was similar to the technique described by Dembo and Wang [232]. Spatial displacement of the beads was tracked by taking images of the fluorescent beads before and after cell trypsinization. The cell traction mapping was based on the Differential Interference Contrast (DIC) image of hMSCs on PA substrate and the corresponding traction stress mapping in pseudocolor, which indicated regions of traction stresses (dark blue to light pink according to stresses from low to high). Briefly, the traction force at discrete point $\vec{f}_i$, located at the position (x_i, y_i) was calculated based on the following formulation: $\vec{u}_t(x, y) = \sum_{i=1}^n \vec{G}(x - x_i, y - y_i) \vec{f}_i$, where $\vec{G}$ denoted the Greens' tensor and $\vec{u}_t$ denoted the experimental 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, $F = \int \int \sqrt{T_x^2(x, y) + T_y^2(x, y)} dx dy$, where $T(x, y) = [T_x(x, y) + T_y(x, y)]$ is the continuous field of traction vectors defined at any spatial position (x, y) within the cell.

6.4 Results and discussion

6.4.1 Preparation and characterization of polyacrylamide hydrogels coated coverslips

Protocol for the preparation of the PA hydrogel was adopted based on work by Tse et al.[228] with slight modifications to reduce fabrication cost and shorten the production time. Typically, a drop of pre-formulated acrylamide-biacrylamide solution is to be sandwiched between the aminosilanized coverslip and a flat hydrophobic surface. The aminosilanized coverslips function as a base on which the polyacrylamide is firmly attached to and the hydrophobic flat surface is essential to attain gel of uniform thickness and to ensure the polymerized polyacrylamide does not stick. Surface hydrophobicity can be rendered by use of several hydrophobic agent such as dichlorodimethylsilane [231, 233], allyltrimethylchlorosilane [51] or commercially available chemical agents such as Rain-X [234]. Herein, we report that the use of transparency can effectively serve as an ideal substitute for the non-stick hydrophobic surface for only a fraction of the cost and significantly reduce the preparation time compared to the aforementioned methods. Additionally, the flexibility of the thin transparency film facilitated easy peeling of the cross-linked PA gel from the transparency.

The mechanical properties of the PA hydrogels can be easily tailored through careful formulation of the acrylamide and bis-acrylamide composition. The moduli of the PA hydrogels were intentionally selected to mimic stiffness corresponding to fat, muscle and bone tissue since it was previously shown that lineage commitment of hMSCs can be modulated by matrix stiffness alone [43]. Formulation was adopted from Tse et al.[231] as shown in **Figure 6.2B**. The bulk elastic moduli of the PA hydrogels were measured and validated using the rheometer. Firstly, an amplitude sweep was

conducted to determine the range of strain at which the gel remained predominately elastic. Then, a constant strain of 0.5% was applied to the PA hydrogel to obtain the frequency sweep graph as shown in **Figure 6.2A**. Noticeably, the measured complex storage modulus did not vary significantly over the range of applied strain rate. The average complex modulus was then computed and the corresponding Young's modulus within the elastic constraints was derived using equation (6.1).

$$E = 2G^*(1 + \nu) \quad (6.1)$$

Where E is the Young's modulus, G^* is the measured average complex shear modulus and ν is the Poisson ratio of polyacrylamide. Poisson ratio for polyacrylamide ($\nu_{\text{polyacrylamide}}=0.45$) was used in our calculation [235]. As shown in **Figure 6.2B**, our computed PA gel elastic moduli agrees well with the Young's modulus reported by Tse et al. and is within the range of stiffness that correspond to the fat, muscle and bone tissue we are interested in.

Since pristine PA hydrogel surface does not permit protein adsorption, therefore, surface activation using the heterobifunctional crosslinker sulfo-SANPAH is required before the fibronectin protein can covalently bind onto its surface [231]. Fidelity of the transferred micropatterns was assessed by means of immunofluorescence imaging and we did not observed any significant differences between the patterns on PA hydrogels of differing matrix stiffness (not shown). Thickness of the PA gels with different stiffness was approximated by measuring the Z-axis distance between the coverslips and the patterned surface using the confocal laser scanning microscope (Leica). As shown in **Figure 6.2C** the average thickness of the prepared PA hydrogel is approximately 50 μm independent of the PA hydrogel rigidity. Importantly, the

measured thickness is well beyond the 5 μ m effective PA hydrogel thickness defined by Buxboim et al. at which hMSCs will start to respond to the underlying glass coverslips [51]. hMSCs were cultured on these engineered hydrogels to ascertain the long term performance of the engineered hydrogels. 120h post cell seeding, cell attachment and spreading remained restricted to the spatially fibronectin-defined adhesive islands (**Figure 6.2D**).

Figure 6.2. Characterization of micropatterned PA hydrogels. Frequency sweep of 4%/0.15% (*Group A*), 10%/0.1% (*Group B*) and 8%/0.48% (*Group C*) PA hydrogels with fixed amplitude of 0.5% (**A**). Comparison of computed Young's modulus with the Young's modulus reported by Tse et al. for the polyacrylamide hydrogels (**B**). Thickness of polyacrylamide hydrogels were approximated by use of confocal microscopy. Polyacrylamide hydrogels of differing stiffness (i.e. 1.6, 15 and 37kPa) were micropatterned with fibronectin patterns and immuno-labeled with Cy3 probe that denotes the surface of the hydrogel (**C**). Phase contrast pictograph of micropatterned hMSCs on 37kPa hydrogel adopting the 1:1 pattern (**D**). Image was taken 120h post cell seeding cultured in LD01. Scale bar=100 μ m.

6.4.2 Modulated cytoskeletal arrangement and focal adhesion development as a function of cell shape and matrix rigidity

Cell spreading was controlled by micropatterning fibronectin defined geometries on mechanically tunable PA hydrogels. Since previous findings made by others and preceding chapters had indicated cell elongation can induce myogenesis of hMSCs, the adopted geometries were designed to systemically increase the aspect ratios with a fixed constant surface area of $\sim 7000\mu\text{m}^2$. Upon seeding the cells onto the patterned hydrogels, hMSCs responded to the surface biochemical cues and attached exclusively within the printed region regardless of the substrate stiffness. After 120h of culture in LD01, the cells were fixed and stained for F-actin and vinculin containing FAs to examine the cytoskeletal arrangement and development as a function of both matrix rigidity and geometry.

Figure 6.3. Effects of cell shape and matrix rigidity on actin cytoskeletal and FAs organization. Representative F-actin and vinculin staining images of micropatterned hMSCs on PA hydrogels of varying stiffness. hMSCs were cultured in LD01 for 120h and subsequently fixed with 4% PFA. F-actin (red), vinculin (green) and nucleus (blue) were labelled with TRITC-conjugated phalloidin, goat anti mouse Alexa flour 488 secondary antibodies and DAPI respectively. Scale bar= 25 μm .

As shown in **figure 6.3**, physically constrained cells exhibited distinct cell spreading and F-actin arrangement. We observed that the cells were able to adopt the gross imposed geometry regardless of the matrix stiffness. However, comparing hMSCs on the circular and 1:1 micropatterns seeded on hydrogels with different rigidity, cells on the softest substrate (1.6kPa) exhibited disorganized thin F-actin and irregular spatial distribution of FAs, whereas cells grown on PA hydrogels with 15 and 37kPa stiffness displayed organized F-actin arrangement with prominent transverse arc stress fibers (tSF) [236] that follows the outline of the shape. Furthermore, on the 15 and 37kPa PA hydrogels, occurrences of elongated FAs were limited to the cell peripheral for the circular cells and at the four corners of the square pattern, which are linked to the dorsal stress fibers (dSF) [236]. dSF are stress fibers connected to the substrate via the focal adhesion at one end while the other end is frequently linked to the transverse arc [236]. Higher occurrence of ruffles along the cell periphery can be observed for cells micropatterned on the 15kPa substrate with intermediate stiffness as compared to cells on the 37kPa substrate. In general, increasing cellular aspect ratio causes parallel alignment of the actin network. This observation holds true for cells with aspect ratios of 5:1, 10:1 and 15:1, independent of the matrix stiffness. In addition, we noted a tight coupling between cell body and nuclear elongation. Cell nucleus became more elliptical with increasing aspect ratio, consistent with the findings made in chapter 4 and 5. In contrast, we did not observe any significant differences in terms of nucleus shape as we vary the substrate stiffness. Average vinculin heat maps were constructed in order to examine the distribution of the focal adhesions of hMSCs using the ImageJ software. 50 separated individual patterned cells for each cell shape-matrix stiffness permutation were imaged and digitally aligned. Background fluorescence was subtracted and stacked upon each other. The average heat map is colour coded with

the maximal frequency of vinculin spatial localization indicated by red and the least number of hits denoted by dark blue (please refer to colour scale bar provided in **Figure 6.4**).

FAs for cells cultured on the 1.6kPa substrate were randomly distributed regardless of the shape it takes on. In contrast, comparing the circular and 1:1 pattern on the 15kPa and 37kPa substrates, we are able to identify a consistent patterning of the FAs around the periphery of the cells adopting the circular morphology, while FAs was observed to be concentrated at the four corners of the square-patterned cells. Such preferential localization of FAs at the 4 corners of the square micropatterns was previously reported, suggesting that cells are able to respond exquisitely to subtle geometrical factors [237, 238]. One possible explanation by which the cells can discriminate these “geometrical stress points” is the preferential actin assembly along the long axis of the square (i.e. diagonal) via an actomyosin generated tension dependent process [237].

Figure 6.4. Computed average vinculin heat map of micropatterned hMSCs on PA hydrogels with different stiffness. hMSCs were cultured in LD01 for 120h and subsequently fixed with 4% PFA. Vinculin was immuno-detected and labelled with Alexa flour 488 secondary antibodies. n=50 for each experimental permutation.

Next, we evaluated the effect of cellular elongation on the distribution of FAs as a function of matrix stiffness. Remarkably, increasing the cell aspect ratio on 15kPa substrate seemingly has a drastic influence on redistributing the FAs from the apical ends (5:1 shape) to the entire cell body (10:1 and 15:1). This contrast starkly to the FAs distribution of the cells on the 37kPa substrate, in which the majority of the FAs were consistently located at the apical ends for cells grown on shapes with aspect ratio 5:1 and 10:1. However this trend subsequently disappears when the aspect ratio reaches 15:1. Instead, we observed a reduction in FAs occurrence and a lack of regularity in terms of its placement within the cells.

Adherent cells feel and respond to extracellular cues via an intricate feedback mechanism. In this instance, the cells were subjected to two independent biophysical cues namely (i) stiffness and (ii) geometry. The printed fibronectin islands determine the initial attachment of the cells on the substrate through integrin binding motifs and subsequently recruit a multitude of adhesion proteins at the FAs site. Once attachment is initiated, the cells will spread and search for available attachment sites demarcated by the pattern boundary. However, the cells' ability to navigate effectively to the edge of the pattern will require an intricate balance between the contractile force that tends to keep the cell in a compressed state and the protrusive force that allows the cells to flatten and spread [239]. The former is largely dependent on the non-myosin II activity and the latter typically involves a feedback mechanism that controls dynamics of actin polymerization and remodelling to eventually assume the micro-printed shape [239]. This process of cell spreading is in turn highly dependent on the matrix stiffness. It is through this feedback mechanism that permits the cells to constantly survey and responds to their biophysical environment and adjusts the cytoskeletal and FAs assembly accordingly.

Our results strongly indicated that both cell shape and matrix modulus can profoundly influence cell spreading. In some cases, the cells may be presented with conflicting extracellular signals that do not permit the cells to respond effectively to one cue under the influence of another. This is aptly illustrated for cells that are exposed to fixed fibronectin patterns but were unable to form well established FAs and actin network on the 1.6kPa substrate. A simple analogy seems fitting to explain our observation: The cell membrane can be viewed as a tent characterized by the cell membrane tension and its structural integrity will require cytoskeletal poles that impinges onto the ECM via FAs, providing the necessary traction force (inward force) in order to counter the cell membrane tension [230, 240]. On the soft (1.6kPa) hydrogel, due to the lack of underlying mechanical support, the cytoskeletal poles slips and loses traction. Consequently, FAs and F-actin development are impaired. On the other hand, cells on the 15kPa substrate coerced to elongate, redistribute its anchorage points (i.e. FAs) across the entire cell body to compensate for the lack of matrix rigidity in order to respond (spread) to the micropatterns. The novel finding presented herein suggests an inherent adaptive mechanism for the cells to react to surface ligands beyond a critical stiffness threshold.

6.4.3 *Semi quantification of cellular responsivity to multiple biophysical cues*

Can we quantify cells' responsiveness to multiple sets of biochemical and biophysical cues? Being able to do so can lend important insights into the interdependence and dominance of extracellular inputs on cellular behaviours. Herein, we introduce the concept of conformity index (C.I.) that measures cells' responsiveness to the printed fibronectin as a function of matrix rigidity. C.I. can be computed using expression 6.2.

$$\text{Conformity index (C.I.)} = \frac{\text{Cell area (measured)}}{\text{ECM area (printed)}} \quad (6.2)$$

C.I. is simply defined by the measured cell area normalized by the area of the printed FN-adhesive islands. Cell area is derived from the F-actin staining of the cells while the printed ECM area is measured from the immunostained fibronectin pattern.

As shown in **Figure 6.5**, maximal C.I. values of approximately 0.7 were observed for cells cultured on the 15 and 37kPa hydrogels. Based on the immunostained cytoskeletal images of the hMSCs on the 15 and the 37kPa substrates, one would expect to attain higher C.I. values. This is because the edges of the patterned cells clearly follow the outline of the fibronectin islands, indicative of a well conformed cell. One possible reason for the lower than expected C.I. values may be due to the fact that this formulation failed to take into account of Z-axis displacement of the cell into the compliant hydrogel. Consequently, this will lead to a decrease in the measured cell area taken in the x-y focal plane. Furthermore, the computed C.I. strongly suggested the cells' ability to respond to the surface biochemical cues decreases dramatically by about 20% when the matrix rigidity transitioned from 15kPa to 1.6kPa.

Figure 6.5. Semi quantification of cells responsiveness to ECM pattern under the influence of matrix rigidity. n=50 per experimental group. $p < 0.05$

6.4.4. Modulated traction force as a function of cell shape and matrix rigidity

Cellular communication with the ECM can manifest itself in the form of CTF through myosin-actin interaction [163, 241]. The involvement of traction force in stem cell differentiation is prevalent. Stem cells on micropost that confers high traction force promote osteodifferentiation but suppress adipodifferentiation of hMSCs [49]. In contrast, impaired cellular contractility by use of actin disrupting drug drive precursor cells to differentiate into adipocytes and chondrocytes [242, 243]. CTF is quantitatively determined by tracking the PA hydrogel-embedded fluorescent beads displacement before and after cell trypsinization from the fibronectin islands. While regulation of CTF by cell spreading, cell shape and matrix stiffness is well established; the interplay of the aforementioned biophysical parameters remains largely unknown.

Referring to **Figure 6.6**, we observed a strong coupling effect of both substrate stiffness and cell shape mediated CTF profile. Also, the spatial positioning of the traction force corresponded well with the vinculin heat map. This is expected since intracellular tension is transmitted via these anchorage points. Maximal CTF values for each cell shape were measured on the stiffest (i.e. 37kPa) substrate with the circle, 1:1, 5:1, 10:1 and 15:1 shaped cells displaying mean CTF values of $10.96 \pm 0.84 \mu\text{N}$, $12.01 \pm 2.00 \mu\text{N}$, $8.08 \pm 1.63 \mu\text{N}$, $6.93 \pm 1.89 \mu\text{N}$ and $7.08 \pm 2.20 \mu\text{N}$ respectively. Except for the transition from the circle to the 1:1 shape, this generic trend of decreasing CTF with increasing aspect ratio is also observed on the 15kPa substrate. Previous work by Rape et al. also showed such cell-elongation dependent CTF generation [238].

For a given cell shape, increasing the rigidity of the PA hydrogels increases the CTF by about 1.8-3.6 fold for each incremental increase in matrix stiffness (i.e. 1.6kPa-15kPa-36kPa). Linear regression analysis revealed that the CTF values scale linearly with matrix rigidity with a high coefficient of determinant value (i.e. >0.95) but a lack of correlation was found between cell shape and CTF. Instead, it appears that that measured CTF decreases in a stepwise manner for shapes with aspect ratio of 1:1 to an elongated form as depicted in **Figure 6.6**. However, cells on 1.6kPa substrate displayed a lack of dependence of CTF on cell shape as a narrow range of CTF values between 1-1.8nN was observed irrespective of the shape it assumes. Interestingly, Tee et al. recently also demonstrated that both substrate stiffness and cell shape can regulate cortical stiffness of hMSCs, but the effect of cell shape on the cortical stiffness became less influential on softer substrate [244]. Taken together, these observations underpin the existence of signal threshold defined by the microenvironmental rigidity before the hMSCs can respond effectively to other biophysical cues. Under such condition, the effect of matrix rigidity dominates that of

cell shape. Such hierarchical processing of multiple biophysical cues by hMSCs as demonstrated in this study sheds an important design consideration for scaffolding materials, especially in cases where the material is geared to modulate the mechanoreponse of hMSCs.

6.4.5 Cell shape-matrix stiffness regulated cell tension drives expression of contractile protein in hMSCs

Numerous studies have demonstrated CTF to be a key regulator of stem cell differentiation. Since our previous findings suggested that elongated morphology promotes myodifferentiation, we ask whether there exist an optimal combination of cell shape and substrate stiffness that can further enhance expression of contractile markers (MHC) in hMSCs. Based on results obtained by Engler et al. that showed

micropatterned C2C12 myofibroblasts on substrate with muscle like stiffness developed myosin/ actin striations [245], therefore, one might expect to see a higher myogenic activity for elongated hMSCs on the 15kPa substrate. Expression of MHC protein was assessed immunocytochemically. Surprisingly, of all the permutations being examined, significant expression of MHC was limited to approximately 10% and 39% of cells adopting the circular and 1:1 pattern cultured on the stiffest (i.e. 37kPa) hydrogels respectively (**Figure 6.7**). In addition, both of these cell-shape and stiffness combination yielded the highest CTF, suggesting that CTF may be implicated in the expression of MHC in hMSCs. To further examine the involvement of cell tension as a regulator of MHC expression in hMSCs, the actin network was disrupted using actin-depolymerizing drug. Cytochalasin D (CytoD) treatment abrogated MHC expression (**Figure 6.7 B and C**) and impaired F-actin assembly as shown in **Figure 6.7 E and F**. Furthermore, CTF measured for the CytoD treated cells decreased significantly to values of $2.39 \pm 0.20 \mu\text{N}$ and $3.52 \pm 0.21 \mu\text{N}$ for the circular and 1:1 cells which corresponded to approximately 80% and 70% decrease in CTF for the circular and 1:1 cells. Taken together, it appears that expression of MHC requires a threshold CTF to be attained.

Figure 6.7. Threshold CTF is required to promote expression of MHC in hMSCs on compliant hydrogels. Significant expression of MHC was observed for a sub population of cells cultured on the 37kPa hydrogel adopting the circular (A) and 1:1 morphology (B). hMSCs were treated with 0.02 μ M of cytoD for 120h and remarkably, expression of MHC was negated (C and D). Effect of cytoD on F-actin polymerization inhibition was ascertained via F-actin staining of cytoD treated cells (E and F). Concomitant decrease in CTF was also observed for cytoD treated hMSCs. Scale bar= 25 μ m.

Caution should be exercised when comparing the favourable effect of cell elongation on hMSCs myogenesis as shown in previous chapters and the observations made in this chapter. There are several reasons that make both systems (micropatterned PLGA and PA hydrogel) incomparable since the culturing conditions for the micropatterned PLGA substrate and PA hydrogel are fundamentally different on several folds.

The elastic modulus of PLGA is significantly stiffer than the hardest PA hydrogel utilized in this study by approximately 5 orders of magnitude. Having shown how stiffness can drastically affect hMSCs behaviours, the large disparity in the matrix stiffness makes it a compelling consideration when making comparison between the two systems. In addition, the FBS content was also decreased by 10 folds to improve the micropatterning of hMSCs. Since FBS contains a multitude of growth factors, changes in FBS content can drastically alter the metabolic profile of hMSCs as indicated by a reduction in cell proliferation that was documented in chapter 5. By the same argument, it is highly possible then that lineage specification towards the muscle

lineage (in this context) will require a new set of factors to take effect in reduced FBS culture.

hMSCs grown on the PLGA film was allowed to migrate freely on the lanes while the cells on the PA hydrogel were confined to a fixed and static spatial position. This point opens up an interesting possibility suggesting that hMSCs migration and FAs turnover rate may be as important an inherited biophysical cue to direct stem cell lineage commitment. hMSCs on PA hydrogel (free surface) with muscle like stiffness became spindle shape and myogenic activity was enhanced [43]. Coincidentally, such bipolarized morphology is a hallmark phenotype for highly migratory cells. For instance, it was shown that fibroblast forced to move on straight lines displayed 3 fold faster migration on flat 2D substrate [246]. In addition, PA hydrogels with intermediate stiffness was also reported to promote cell motility [244]. Extending these findings to the observations made by Engler et al., it does exhibit some coherency that link cell migration to myogenesis. Nonetheless, whether cell migration may be essential to generate sufficient contractile force to express contractile markers such as MHC remains to be elucidated.

Significantly, our current findings does suggest that endogenous cell contractility may act downstream of cell shape mediated differentiation. Goffin et al. also documented critical level of cell tension is essential to recruit α -SMA to the actin of myofibroblast [248]. Furthermore, hMSCs treated with the Rho associated kinase (ROCK) inhibitor Y-27632, a potent drug that attenuates cell contraction, failed to undergo osteogenesis even when cultured in osteogenic medium [37].

6.5 Conclusion

In this chapter, a novel approach has been devised to examine the interdependency of cell shape and matrix rigidity on hMSCs behaviour. Uniquely, the micropatterned hydrogel system can be used to measure CTF simultaneously. A library of 15 different permutations of cell shape-matrix stiffness combination was examined. Several stiffness thresholds were observed that critically regulated cell spreading, cytoskeleton orientation, focal adhesion development, traction force generation and MHC expression. On the softest PA hydrogels, hMSCs were unable to respond effectively to the patterned ECM as indicated by having a low C.I. value of in the range of 0.38-0.5. Beyond this stiffness threshold, the effect of cell shape became more apparent in terms of directing the localization of focal contact points and generation of cell contractility. This is well reflected by the constructed average vinculin heat map that exhibited a distinct spatial patterning of focal contacts. Traction force generation was found to be influenced by both cell shape and matrix rigidity beyond the first level of stiffness threshold (1.6kPa). Traction force generation scale linearly with matrix stiffness. In contrast, the effect of cell shape on traction force generation appears to take on a stepwise pattern, with a marked decrease in measured traction force as the shape with aspect ratio of 1:1 transits to an elongated form. Remarkably, significant MHC expression was only detected for cell-shape and matrix stiffness combination that gave the highest traction force. CytoD treatment caused CTF to be significantly reduced and negated expression of MHC, suggesting the existence of a threshold intracellular generated tension required for the expression of MHC.

Conclusion and recommendation

7.1 Conclusion

The aim of this dissertation was to regulate stem cell shape and understand the underlying mechanism by which lineage specification can be directed. By adopting an interdisciplinary approach involving the technical knowhow of materials science and engineering, as well as the use of molecular biology techniques, this project was geared towards the exploration of novel platforms that can steer stem cell differentiation without the aid of exogenous soluble factors. Understanding the molecular mechanisms implicated using biophysical cues to drive differentiation is equally important as it is expected to contribute significantly to the rational design of future biomaterials. Due to its clinical relevance, hMSCs was chosen as an ideal model primary stem cell line for this study. The objectives were achieved using μ CP as the main technique to confine hMSCs attachment and growth on geometrically defined fibronectin micropatterns. Each phase of the study was aimed to progressively examine the effects of micropatterning on hMSCs shape modulation and function. The results attained in this study have yielded valuable insights into the biophysical parameters that regulate stem cellular interactions with engineering biomaterials, and may lead to the development of novel biomaterials for stem cell based therapies.

In phase 1, we examined the influence of cell elongation on lineage commitment of hMSCs. hMSCs were patterned onto PLGA substrates decorated with 20 μ m wide fibronectin lanes. To prevent hMSCs from growing out of the patterned surface, the

unprinted regions were backfilled with pluronic F 127. On the engineered substrates, hMSCs grew in elongated form and large-scale alignment was obtained after 2 week of culture. Therefore, such micropatterning technique can be used for other applications that require cells to be aligned, for instance, to match the anisotropic arrangement of tissues such as the myocardium. It was found that cell elongation could significantly modulate stem cell differentiation, especially towards the soft tissue lineages (i.e. myocardial and neural) and the mRNA level. However, at the protein level, myogenic differentiation seems to take precedence as indicated by immunodetection of MHC.

After having established that the micropatterned PLGA platform was capable to direct hMSCs differentiation, the study progressed to elucidate the underlying molecular mechanisms that may be involved. Mechanistic studies were designed to investigate the effect of cell elongation on the canonical FAK-ERK signaling pathway. Myogenic transcripts level were upregulated (relative to unpatterned cells control) as a function of cell culture duration for cells on the micropatterned surface. We noted that within the first 2 time points (8h and 24h), there were minimal differences in terms of expression of myogenic transcripts relative to the unpatterned control. By 72h, statistically significant upregulation of myogenic transcripts (> 1.5 fold) was detected and this upregulated state of myogenic transcripts persisted and were heightened 120h into the cell culture duration (> 3 fold). Additionally, micropatterned cells displayed lower proliferative activity compared to the unpatterned cells. Noteworthy, focal adhesion development was significantly different for cell on the micropatterned and cells that were allowed to spread on a free surface. An elongated morphology repressed formation of large FAs and its associated phosphorylated FAK and ERK (1/2) (downstream target of FAK) signaling. Coupled with recent studies

that showed high phosphorylated ERK(1/2) activity may inhibit myogenesis [223], our observations does indicate that shape induced lowering of phosphorylated ERK(1/2) activity may potentially serve as a molecular basis to promote hMSCs myogenesis. Our hypothesis was later confirmed with MEK1 inhibition assay. Cells treated with PD98059 enhanced endogenous expression of MyoD and Myogenin as well as MHC expression. Therefore, future biomaterials designs employed to target the FAK-ERK signaling may find useful applications not restricted to stem cell biology, but also in areas such as cancer research, given the strong involvement of the FAK-ERK signaling in cancer research [249].

A major drawback of the micropatterned PLGA system was that cell-cell contact and cell shape could not be studied independently. Furthermore, hMSCs on the patterned lanes exhibited various degree of cell elongation and such heterogeneity may potentially further complicate the experimental analysis. These limitations were acknowledged and improvements in terms of pattern designs had been made in phase 3 of the study. hMSCs were cultured on fibronectin adhesive islands of different aspect ratio with a constant area primed to confine single hMSC. Additionally, cells were micropatterned on hydrogels with different physiological stiffness (i.e. 1.6kPa, 15kPa, 37kPa) to investigate the interplay of both cell shape and matrix elasticity and its effect on hMSCs behaviors. Spatial organization of FAs was significantly influenced by cell shape. Critical signal thresholds defined by the substrate stiffness is crucial for the cells to respond effectively to the printed ECM protein. Combination of both cell shape and substrate stiffness that confers the highest traction force promoted significant MHC expression as assayed by immunostaining. Importantly, when CTF was artificially reduced using cytoD, MHC expression was abolished, implying that a minimal traction force threshold is essential for MHC expression.

Taken as a whole, it is clear that adherent hMSCs constantly probe its immediate biophysical environment for vital cues in order to carry out key cellular process such as cell adhesion, migration, renewal and differentiation. Physical engagement of the cells onto a substrate rapidly recruits large number of proteins at the point of contacts, forming small nascent focal complexes and via a feedback loop; maturation of FAs and actin assembly is regulated. It is through this iterative process that allows cells to mechano-sense the geometrical cues and rigidity of the underlying substrates. These biophysical parameters work in concert to control stem cell fate (**Figure 7.1**). For instance, although cardiac troponin T expression was detected in hMSCs engrafted at the infarcted site in rat models, the cells fall short of expressing functional sarcomeric contractile units, presumably due to the mismatch in matrix stiffness of a damaged and healthy myocardial [29, 245]. Solid tumors typically have a stiffer desmoplastic stroma than its immediate surrounding and may also lead to excessive self-renewal and *in vitro* migration of mammary stem cells (MaSCs) via the FAK signaling axis [250, 251]. Given the clinical relevance of biophysical regulation of stem cells fate, our studies lend insights into how stem cells process these inputs. We have shown that engineered cell shape can modulate the density and length of FAs and in turn can manipulate transcriptional events through adhesion dependent phosphorylation of signaling molecules such as FAK and ERK. At the same time, the developments of FAs in turn regulate actin polymerization and the overall cytoskeletal tension of the cell. However in the event that the substrate mechanics does not allow sufficient traction to be established, cytoskeletal tension and FAs development will be impaired regardless of the shape it assumes. Optimal combination of these biophysical signals is critical to bring about targeted stem cell responses.

Figure 7.1. Interdependence of cell shape, focal adhesions and mechanical forces in regulating stem cell behavior.

7.2 Recommendation

There are several directions in which the current study and findings can be extended.

7.2.1 Improving myogenic differentiation of hMSCs

The use of micropatterning by itself might be too unspecific on its own to direct hMSCs towards a specific lineage since upregulation of both myogenic and neurogenic transcripts were observed. hMSCs differentiation must be well controlled in order to realize its full therapeutic potential. One possible way is to enhance directed differentiation of micropatterned hMSCs in conjunction with known soluble concoctions. Alternatively, hMSCs may be transfected with myogenic lineage specific genes. Different techniques that complement micropatterning of hMSCs should be examined to determine the optimal condition for efficient myodifferentiation.

7.2.2 Existence of a range of CTF “window” for optimal myodifferentiation of hMSCs?

In chapter 6 of this thesis, through a series of experiments, we have highlighted the importance of providing hMSCs with the right biophysical context in order to modulate its behaviors. Notably, it appears that a minimal threshold CTF is required for expression of MHC. However, it is also highly possible that promotion of MHC expression will occur within a given optimal range of CTF as supported by works by Engler et al.[43, 245] Using the current micropatterned hydrogel system, this range of pro-myogenic CTF “window” can be determined by expanding the library of cell-shape-matrix stiffness combination. Since MHC expression was detected in the round and square hMSCs grown on the 37kPa hydrogel, CTF can be further fine tuned by changing the size of the micropatterns while retaining the aforementioned geometries. Additionally, using a series of soluble agents to artificially increase or decrease contractile force can also be implemented to further validate involvement of CTF regulated myodifferentiation of hMSCs. Such comprehensive approach has the potential to identify the optimal contractility that confers myogenesis in hMSCs, which is beyond the scope of the current work.

7.2.3 Development of myocardial patch

The ability to promote myogenesis and attain large-scale cell alignment of the micropatterned platform makes it a potential candidate to be used as a scaffolding material for a myocardial patch. However, because the number of cells may be limited on a single sheet of PLGA to bring about appreciable therapeutic effect, a layer-by-layer construct will be a more suitable configuration for this application. As the construct becomes thicker, mass transport of nutrients and metabolic wastes must be considered. Future efforts should also be placed in areas to develop 3D

micropatterned hydrogel platforms to induce myogenesis. Further optimization in terms of culture condition (static vs. dynamic), method to stack the layers of micropatterned hMSCs in place, coupled with *in vivo* analysis of myocardial patch performance will be essential to validate its efficacy for the treatment of MI.

7.2.4 Applying the concept of “threshold CTF” in the context of biochemically induced smooth muscle differentiation using TGF- β 1

hMSCs prevented from spreading were unable to undergo smooth muscle differentiation even when exposed to known cytokine that promotes smooth muscle differentiation [38]. As an extended findings made by Gao et al. [38], it will be interesting to apply the experimental design (as was conducted in reference 38), in the context of the engineered platform presented in Chapter 6. The established library allows one to systematically screen through the different permutations (cell shape-matrix stiffness) and determine if the concept of “threshold CTF” can also be applied to culture medium containing pro-myogenic factor such as TGF- β 1, which is known to induce smooth muscle differentiation. In practical terms, our system will allow one to determine the optimal combination of cell-shape and matrix stiffness that can induce or negate smooth muscle differentiation under the influence of TGF- β 1. Demonstration of biophysical environment that does not permit smooth muscle differentiation even when presented in a permissible condition will strengthen the notion of “Optimal biophysical context” for effective stem cell differentiation and should constitute as an imperative consideration when designing advanced scaffolds for regenerative medicine.

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Peer reviewed publications

1. **C.Y. Tay**, M. Pal, H. Yu, W.S. Leong, N.S Tan, K.W Ng, S. Venkatraman, F. Boey, D.T Leong, L.P Tan (2011). Bio-inspired micropatterned platform to steer stem cell fate. *Small*, 7(10), 1416-1421. (Impact Factor: 7.33)
2. **C.Y. Tay**, S.A. Irvine, F. Boey, L.P. Tan, S. Venkatraman (2011). Micro-nanoengineered cellular responses for soft tissue engineering and biomedical applications. *Small*, 7(10), 1361-1378. (Impact Factor: 7.33)
3. D.Ang, **C.Y.Tay**, P.R.Presier, L.P Tan, R.V. Ramanujan (2011). In Vitro Studies of Magnetically Enhanced Transfection in COS-7 Cells. *Materials Science and Engineering C*, 33(7), 1445-1457 (Impact factor: 2.178)
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5. **C.Y Tay**, H. Yu, M. Pal, W.S Leong, N.S Tan, K.W Ng, D.T Leong, L.P Tan. (2010). Micropatterned matrix directs differentiation of human mesenchymal stem cells towards myocardial lineage. *Experimental Cell Research*, 316(7), 1159-1168. (Impact Factor: 3.609)
6. **C.Y Tay**, H. Gu, W.S Leong, H. Yu, H.Q Li, B.C Heng, H. Tantang, S.Y.C Loo, L.J Li, L.P Tan. (2010). Cellular behavior of human mesenchymal stem cells cultured on single-walled carbon nanotube film. *Carbon*, 48(4), 1095-1104. (Impact Factor: 4.893)
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9. J.C. Wu, Y.K. Wong, K.W. Chang, **C.Y. Tay**, Y. Chow, M.M. Talukder, W.J. Choi. (2007). Immobilization of *Mucor javanicus* lipase by entrapping in alginate-silica hybrid gel beads with simultaneous cross-linking with glutaraldehyde. *Biocatalysis and Biotransformation*, 25(6), 459-463. (Impact Factor: 1.275)

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1. W.S Leong, **C.Y Tay**, L.P Tan. (2011) Engineering stem cell niche: Regulation of cellular morphology and function. *Stem cells and cancer stem cells*, 3(5), 345-355. © Springer Science + Bussiness Media

International and Local Conferences Presentations:

1. Oral presentation: 2009 Asia-Oceania Top University League on Engineering , National Taiwan University, Taipei, Taiwan. 2nd 3rd December 2009.
2. Oral Presentation: Directed growth of stem cells on micropatterned fibronectin adhesive strips mediates osteogenic and myogenic lineage commitment via the FAK-MEK/ERK signalling pathway. 8th International Conference on Cell and Stem Cell Engineering , Trinity College, Dublin, Ireland . 11th-12th June 2010.
3. Poster Presentation: Cellular behavior of human mesenchymal stem cells cultured on single-walled carbon nanotube film. Joint Symposium on Materials Science and Engineering for the 21st Century . KAIST, Daejon, Korea. 27th-30th June 2010.

Symposia Related Recognition

1. Organizing committee : Joint Symposium on Materials Science and Engineering for the 21st Century (2011) 19th-21st June 2011

Teaching experience

1. Modules taught:
  - a. Organic Molecules: Geometry and Bonding (FE1071)
  - b. Materials Science (FE1005)
  - c. Polymers and composites (MS2010)