The effect of topological pattern on human mesenchymal stem cells and its implication in tissue engineering

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**NANYANG
TECHNOLOGICAL
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**The Effect of Topological Pattern on Human
Mesenchymal Stem Cells and Its Implication in Tissue
Engineering**

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SCHOOL OF MATERIALS SCIENCE & ENGINEERING

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List of Abbreviation

2-D	two-dimensional
3-D	three-dimensional
ALPL	alkaline phosphatase
ATR-FTIR	attenuated total reflectance fourier transform infrared
bHLH	basic helix-loop-helix
BSA	bovine serum albumin
cTnI	cardiac troponin I
DAPI	4'-6-diamidino-2-phenylindole
ECM	extracellular matrix
F-actin	filamentous actin
FFT	fast fourier transform
fs	Femtosecond
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAPs	GTPase-activating proteins
GATA4	GATA binding protein 4
GEFs	guanine nucleotide exchange factors
HBC	hydroxybutyl chitosan
hMSCs	human mesenchymal stem cells
LbL	layer-by-layer
MAP2	microtubule-associated protein 2
MHC7	myosin heavy chain 7
MHC	myosin heavy chain
MyoD1	myogenic differentiation 1
NeuroD1	neurogenic differentiation 1
Nkx2.5	NK2 transcription factor related, locus 5
OCN	Osteocalcin

ON	Osteonectin
PLLA	poly(<i>L</i> -lactide acid)
PCL	poly(caprolactone)
PDMS	Polydimethylsiloxane
PLC	poly(<i>L</i> -lactide- <i>co</i> - ϵ -caprolactone)
PLGA	poly(D, <i>L</i> -lactide-co-glycolide) acid
PMMA	poly(methyl methacrylate)
qRT-PCR	quantitative real time polymerase chain reaction
ROCK	Rho-associated kinase
RUNX2	runt-related transcription factor 2
SEM	scanning electronic microscope
SMC	smooth muscle cells
TGF β	transforming growth factor β
TRITC	tetramethyl rhodamine iso-thiocyanate
XPS	X- ray photoelectron spectroscopy

Abstract

Human mesenchymal stem cells (hMSCs) have the potential to differentiate into numerous cell types in vivo and in vitro. The ability to control hMSCs lineage commitment would bring great benefits to regenerative medicine. Many efforts have been made to use biochemical method to regulate hMSCs growth, whereas recently more progress contributes to the effort of physical cues on hMSCs behavior. In this work, highly controlled substrate topography was utilized to influence cell behavior including adhesion, proliferation and differentiation. In the first part, hMSCs were cultured on femtosecond laser machined polymer substrates including three sizes of microchannels (width-interval-depth: 20-20-20, 30-30-20 and 80-30-20; μm). Cytoskeleton of hMSCs was better aligned and elongated along the narrow laser induced microchannels (20-20-20 and 30-30-20). Meanwhile, cells on these scaffold also showed significant up-regulation of several hallmark myogenic markers. It was also suggested that RhoA/ROCK signaling is involved in myogenesis of hMSCs on these scaffolds. Then, laser machining and electrospinning techniques were utilized to obtain a bi-layered hybrid scaffold with hierarchical topographical features. HMSCs were cultured in vitro in the bi-layered hybrid scaffolds, and cell orientation, proliferation and differentiation were evaluated. The results proved that, without appearance of bio-chemical factors, these hierarchical scaffolds could offer a conducive microenvironment for enhancing hMSCs proliferation, viability, orientation and myogenesis in vitro.

Chapter 1 Introduction

1.1 Background

In native tissue, cells are enclosed by multi-functional extracellular matrix (ECM) and they receive numerous chemical and physical cues from their local microenvironment. These cues incorporate connections with neighboring cells, binding molecular ligands, and interactions with extracellular matrix (ECM) [1]. Understanding the interactions between cells and their cellular microenvironment is essential for the scaffold materials development, as well as tissue engineering applications. Tissue engineering is a multi-disciplinary field that incorporates engineering, material science and life science, aims to understand structure-function relationships of healthy and pathological tissues, and develops biological alternatives for tissue restoring, maintaining and function improving [1-4].

Intrinsic to this achievement is a demand for readily usable cells which can provide appropriate functions. Therefore, stem cell, which are unspecialized precursor cells with the capability to self-renew and differentiate to various cell types, are becoming more and more important in tissue engineering applications [5]. Adult human mesenchymal stem cells (hMSCs) isolated from bone-marrow, which is one of the popular stem cell types, have the potential to treat a wide range of diseases [6, 7], as well as can be used in a variety of screening applications [8]. HMSCs also have been proven to self-renew and differentiate into a wide range of lineages in response to suitable cues [9-11]. The cues

consist of immobilized/soluble factors, chemical and physical stimuli from extracellular matrix (ECM) [12]. Numerous studies have been done on using genetic and molecular regulators (e.g., growth factors, transcription factors) to regulate stem cell fate. However, increasing evidence has shown that the diverse physical environment factors also can control the stem cell activities [13], and meanwhile can avoid the side effects brought from biological or chemical additions, such as uncontrolled cell growth, oncogenesis and even cell death.

1.2 Hypothesis

Topography has not figured prominently in the design of scaffolds, although increasing advanced fabrication technologies provided by biomedical scientists were used to show the potential importance of topography in affecting cell behavior, and in particular, controllable nanoscale topography [14]. The mechanisms of how topography can influence cell growth are not well studied but appear to comprise the changes of cytoskeleton, which is potentially in response to the underlying geometrical features [13]. A recent landmark study done by CS Chen et al[15] and the work in our group [16, 17] demonstrated that cell shape can impact stem cell differentiation, especially towards myogenic lineage with elongated cell shape.

The hypothesis of this project is that lined topography in scaffolds would influence stem cell lineage differentiation through the changes in cell shape. It is also very interesting to elucidate how stem cells sense the micro-environment. Progress in this study will provide a novel insight into the mechanism of how topography can regulate stem cell

differentiation through cytoskeleton rearrangement, as well as contribute extensively to the advancement of stem cell research and especially soft tissue engineering.

1.3 Research objectives and significance of study

The ultimate goal of tissue engineering is to generate 3-D artificial matrix with natural ECM-like features that can replace damaged tissue in human body. The general objective is to apply and understand how surface topography of biopolymeric matrices in a 3-D scaffold modulates stem cell behavior for tissue engineering application. The microchannels can be achieved by directly employing femtosecond laser, a highly versatile fast technique capable of micromachining any shape on biopolymers. This technique is independent of the substrate and can avoid any contact contamination. Electrospinning is another versatile technique to incorporate micro- and nano- features on the surface.

To achieve the ultimate goal, the specific objectives laid down are:

- Develop the technique of femtosecond laser to create micropatterns on surface of biopolymer substrates
- Hierarchical control in a 3-D scaffold ranging from nano-, micro- to macro- using electrospinning and laser machining
- Study hMSCs behavior (cell proliferation, viability, alignment and differentiation) on the laser machined scaffolds
- Explore the molecular signaling mechanisms associated with hMSCs lineage commitment.

Recent progress revealed that designed micro- and nano- topography could influence cell behavior and steer stem cell differentiation towards desired phenotypes [18, 19]. Topographic structures with micro and nano-scale features were specifically designed to interact with cells. However, few studies were carried out to investigate cell behavior in complete absence of biological or chemical induction mediums [20]. Out of the few studies that demonstrated stem cell differentiation without induction medium, all of them were cultured on 2-D models. Here, we would like to introduce a 3-D model that has all the biomimetic features of macro-, micro- and nano-scale topography in one system. This model is not only significantly more practical, it also allows for fundamental studies of cell-material interaction in a more realistic environment. The systematic investigation on stem cell behavior response to microscale features (especially microchannels) and the mechanism behind, in the absence of induction medium, have not been reported yet.

Besides that, many studies have been done on how the electrospun fibers affect cell behavior, including stem cell. However, to the best of my knowledge, laser patterning on electrospun fiber mesh to regulate stem cell behavior has never been attempted. Moreover, the combination of soluble regulators with physical factors in this Nano, Micro and Macro defined 3-D multiscale architecture will provide a new insight into the regulation of stem cell fate.

The strategy involving the use of femtosecond laser for the fabrication of micro-patterned (Two- or Three-Dimensional) scaffolds, presents many strong advantages over traditional fabrication methods. For instance, conventional photolithographical technique requires the use of templates and specialized equipment and facilities (*e.g.*, spin coater, an

appropriate photoresist, plasma or chemical etching of the surface, removal of photoresist and each of the step needs special systems). Using femtosecond laser, templates are not required and hence it is very versatile and we can rapidly fabricate & create patterns directly on the biodegradable polymer surface. The precisely defined 3-D biomimetic structures fabricated using femtosecond laser on fiber mesh also allow good mass transportation (*e.g.*, oxygen and nutrition) apart from providing the required surface topography. The mass perfusion throughout the whole construct can support high density of viable cells. This will have significant implications on stem cell tissue engineering, *e.g.*, fabrication of myocardial tissue patch to treat heart infarction.

1.4 Outline

The first Chapter of this thesis shows basic overview of stem cell tissue engineering, and most importantly presents the objectives and significance of the project. Chapter Two gives a detailed literature review on bio-mimicking ECM directing stem cells growth and advanced techniques used for scaffold fabrication. Topics discussed here are about three commonly applied physical cues (*i.e.* matrix stiffness, cell shape and topography) control the stem cells fate. Additionally, two popular and well-known scaffold fabrication methods which are electrospinning and laser machining, adopted in the thesis, are highlighted for their specific application in bioengineering. Chapter Three introduces typical approaches utilized in this thesis, *i.e.* material fabrication, laser machining, as well as basic cell culture technique.

Chapter Four and Chapter Five present the study of hMSCs behavior on the laser machined scaffolds in details. In Chapter Four, the femtosecond laser is employed to machine highly-organized microchannels on solvent casting film. Stem cell proliferation, alignment and differentiation on the film scaffold are shown. For the exploration of molecular signaling mechanisms associated with hMSCs lineage commitment and phenotypic change, the role of Rho-GTPases in stem cell myogenic differentiation is examined. Chapter Five emphasizes on the rational design of new scaffolds based on electrospun fiber mesh. Fibers with nano- and micro- scale diameters can be generated to adjust the mechanical property and permeability of the 3-D electrospun scaffold, which is essential for mass transportation to influence cell survival and fate. With combination of electrospinning and laser machining technologies, hierarchical control in 3-D scaffold ranging from nano-, micro- to macro-scale is achieved. Not only cell proliferation, organization and differentiation were positive, but cell viability is also highly attained after stem cells were cultured on the multilayer scaffolds. Chapter Six gives a brief conclusion to the whole project and provides an outlook on several topics to be further explored in near future with improvement and deeper studies in real tissue engineering applications.

Chapter 2 Literature Review

2.1 Introduction

The cells in living organisms are enclosed with different type of cells and extracellular matrix (ECM), which contains water, numerous soluble and insoluble biomolecules. The composition, mechanical properties and topography of the ECM will regulate the cell function in the development of tissues. With recent rapid progress in stem cell biology, the interests of tissue engineering are trying to biomimic the ECM microenvironment *in vitro*, providing necessary biochemical and structural cues for stem cell growth, differentiation and functionality (**Figure 2.1**). Numerous studies have been done on the optimization of biomaterials with appropriate biochemical additives to promote cells to develop into a functional tissue. Those can be achieved by controlling the delivery of soluble growth factor (*e.g.*, chemokines and cytokines), as well as the location of insoluble receptor ligands to direct cell behavior. However, recent work revealed that non-biochemical control of the ECM, such as elasticity and topography, can modulate the stem cell lineage commitment. Here the first section of this literature reviews briefly how to direct stem cell fate by physical interaction with the ECM (*e.g.* matrix stiffness, cell shape and topography). The second part reviews the role of Rho-GTPases in stem cell differentiation. And finally two fabrication techniques (electrospinning and laser machining) and their application on bio-related field will be introduced.

Figure 2.1. Schematic of controllable parameters (e.g., matrix property, culture environment, soluble and matrix-bound factors) to direct stem cell fate. Adapted from [21].

2.2 Physical cues of stem cell differentiation

2.2.1 Matrix elasticity as a regulator of stem cell fate

Whether *in vitro* or *in vivo*, cells attached to the substrate are often exposed to forces, inducing stretching or relaxation of the cytoskeleton [22]. These matrix stiffness caused forces and ECM can affect the cell migration [23], apoptosis [24], proliferation [25] and differentiation [26, 27]. Focal adhesions are what cause the ECM stiffness to have effect on stem cell differentiation. As with the theory behind cell adhesion, traction is applied by the cell on the matrix. This traction in return determines the force exerted through the focal adhesions and sends specific signals through the cell to direct differentiation. Hence, the amount of stress to be exerted on the matrix, which is in turn determined by the matrix stiffness, is directly related to the differentiation pathways of stem cells. The role of elasticity on stem cell differentiation was best illustrated by Engler and co-workers [28], in which hMSCs were grown on polyacrylamide gels with three stiffnesses which mimic three different mechanical properties of tissues in the body. In the absence of any knowing inducing soluble factors, MSCs grown on gels with three different types

of elastic moduli would differentiate into three different lineages. As shown in **Figure 2.2**, the representative immunostaining of neural ($\beta 3$ Tubulin), myogenic (MyoD) and osteogenic (CBF $\alpha 1$) transcription factors were observed on gels at elastic moduli of 0.1~1 kPa, 8~17 kPa, and 34~40 kPa respectively. One further study reported that neurogenesis of stem cell behavior can be regulated by the underlying substrate with varied elasticity range from 10 to 10,000 Pa [29]. Soft substrates with 10Pa elastic moduli inhibited cell spreading and differentiation, while cells cultured on 100Pa substrates having closer elasticity to brain tissue revealed a peak level expression of neuronal marker. Meanwhile softer substrates (~0.1-0.5 kPa) improved neuronal differentiation, stiffer substrates (~1-10 kPa) gave rise to glial differentiation. More interestingly, hMSCs can maintain their quiescent state through growing them sparsely on polyacrylamide gels that mimick the bone marrow niche [30]. hMSCs could be induced to adipogenesis or osteogenesis when treated with relevant induction cocktails, which means that the soft matrix mimicking the bone marrow niche can be used to preserve their multilineage potential. The mechanism behind this regulation is widely accepted as the influence of the contract tension inside the cell. This tension will regulate phosphorylation of proteins, assembly of stress fiber, activation of signaling pathways and so on [31]. However, there are some different types of tissues having similar stiffness. Thus, we can see that it might not be easy to control the stem cell fate using only a single regulator such as mechanical property.

Figure 2.2. Matrix mechanics dictates MSC differentiation. Human MSCs stained for β 3-tubulin, MyoD1 and CBFA1 as markers of neurogenic, myogenic and osteogenic differentiation, respectively. MSCs differentiation correlated with the *in vivo* matrix stiffness. Adapted from [28].

2.2.2 Cell shape as a regulator of stem cell

In addition to the effect that elasticity of an artificial ECM may have, there is another physical property of ECM might also direct stem cell fate. Cell shape is a powerful regulator of cell growth and physiology [32-35]. Cell shape modulation involves in the many processes, like embryonic development and stem cell differentiation [13, 36, 37]. Regardless of what kind of stimuli applied to cells, cellular response is always accompanied by alteration in cell shape. This observation led us to postulate that cell shape may act as a functional nodal point capable of instructing how the cell should behave. Many studies have revealed that stem cell fate can be affected by the shape of the

artificial ECM. McBeath et al showed that MSCs cultured on various patterned islands coated with fibronectin can control lineage commitment of MSCs towards adipogenesis (on smaller islands) or osteogenesis (on larger islands) [15]. This work demonstrated that cell shape, cytoskeletal tension and RhoA pathways, are essential to the stem cell lineage commitment. While active RhoA cause osteogenesis in spread hMSCs, inactive RhoA commit round hMSCs towards adipogenic lineage [15]. And more recently, the results from the same group revealed that cell shape acted as an important microenvironmental cue to drive hMSC differentiation towards different lineages [38]. A switch between chondrocytes and smooth muscle cells (SMC) can be achieved by cell shape modulation, with the introduction of transforming growth factor β (TGF β). In spreading cells, upregulation of N-cadherin by TGF β 3 and Rac1 signaling leads to SMC differentiation. Otherwise, the restriction of cell spreading will drive induction of chondrogenic genes. These results implicated that cells have progressed an elaborate mechanism to integrate various soluble/immobilized, mechanical physical, and structural factors in the microenvironment [38]. In another work, Kilian and colleagues indicated that even subtle shape cues (*e.g.*, aspect ratio and subcellular curvature) can significantly promote MSCs differentiation (**Figure 2.3**) [39]. The regulatory mechanism of the differentiation is contractile cells promote osteogenesis by MAP kinase pathways and wingless-type signaling [39]. At the same time, the results also suggested that combination of small molecules and geometric cues can attain a more specific outcome.

Figure 2.3. (A) Percentage of cells captured on rectangles of varying aspect ratio differentiating to adipocyte or osteoblast lineage. (B) Percentage of cells differentiating to either lineage when captured on fivefold symmetric shapes. (C) Comparison of color intensity histograms generated by measuring the red and purple channels of cells in flower and star shapes ($n = 393$). Adapted from [39].

2.2.3 Topography as a regulator of stem cell

As discussed above, cell shape can be a potent regulator of cell growth and differentiation. Cells have the capability to sense and respond to the underlying micro- and nano- scale geometric features, which can lead to obvious, macroscopic alteration in cell shapes [13, 14, 18, 19, 40-42]. Such features may refer to differences in molecular chain arrangement, surface roughness or topology, fiber diameter, or other parameters. The dependency of cell behavior on substrate topography is due to mechanical stresses of the pattern walls on cell nucleus, inhibiting the morphology of the nucleus, especially for nanotopographical patterns with comparable sizes to that of cell nuclei, and in turn reorganizes the growth of actin filaments into their cytoskeleton structures and hence differentiates in specific pathway. For instance, cells cultured on the surface with artificially formed micro- and

nano-channels elongated their shape and orientated along the direction of the channels, although the change of cell morphology is cell type dependent, as well as closely related to channel depth and width [43]. Another study shown the vital role of fiber topography, especially the fiber diameter, in guiding differentiation and proliferation of rat hippocampus-derived adult NSCs in culture [44]. And more importantly, recent studies revealed that the topographical cues alone, may direct the differentiation of stem cells without adding any inductive biochemical factors in the culture medium. One study done by Leong et al [45], demonstrate that aligned electrospun hydroxybutyl chitosan (HBC) fibers can direct hMSCs differentiation towards myogenic lineage commitment. In the work done by Dalby et al, the osteoprogenitor cells and hMSCs were cultured on PMMA with five different nanopit arrays [46]. The expression of osteopontin (OPN) and osteocalcin (OCN) in hMSCs cannot be observed on substrate with completely ordered or completely random nanopits, while slightly irregular nanoarrays did exhibit significant amount of those two proteins and form with increased bone nodule. In another study, dimension changes of titanium oxide nanotubulars improved hMSCs adhesion and directed specific differentiation into osteoblasts by using only the geometric cues [47]. One interesting work done by Yim et al. presented that even a stronger effect of neurogenesis was shown on nanograting compared to biochemical cue along on unpatterned control [48]. They also found that the combination of nanotopography and biomolecular cues would further enhanced MSCs neurogenesis. The mechanism by topographic cues affect stem cell behavior are still not well studied but appear to involve changes in cytoskeleton organization and structure, potentially in response to the

geometry and size of the underlying features of ECM [13]. These recent studies highlighted that topographical cues can influence the differentiation of adult stem cells.

2.3 Role of Rho-GTPases in stem cell differentiation

Many features of cell functions are regulated by Rho family GTPases [49]. One of most insightful effects of these small G-Proteins is on the actin cytoskeleton. Rho proteins have also been reported to regulate many other cellular activities, such as cell adhesion, cell polarity, endocytosis, vesicle trafficking, cell differentiation and gene transcription [50]. There are more than 20 Rho family proteins that have been described to date, including three best studied proteins: RhoA, Rac1 and Cdc42. The RhoA mainly contributes to contractility and formation of stress fibers and focal adhesions. Rac1 was first described to stimulate the formation of lamellipodia and membrane ruffles. Cdc42 was originally explained to motivate the formation of filopodia. In this report, only small G-proteins Rho and Rac, which play a pivotal role in actin cytoskeleton, are discussed (**Figure 2.4**). It has been demonstrated that RhoA and Rac1 GTPases, were two of the most vital molecular signals, which would make signals transductions from extracellular to nuclear, and then influence stem cell differentiation [15]. Rac1 is a small G-protein in the Rho family that drives polymerization of actin and formation of lamellipodia [51]. Rac1 is activated by Guanine Nucleotide Exchange Factors (GEFs) and repressed by GTPase-Activating Proteins (GAPs). Activation of Rho is required for the formation of stress fibers, through one of its downstream effector, Rho Kinase (ROCK), to phosphorylate myosin light chain kinase, then activate myosin driven cell contractility. ROCK was discovered as the first effectors of Rho. And ROCK is one the major

regulator of cytoskeleton to induce the formation of stress fibers and focal adhesions by phosphorylating MLC (myosin light chain) [52]. Established work were done to show myosin contractility resulted tension drives the formation of stress fibers[51]. Recent studies have led to interesting conclusion that the Rho GTPases especially the RhoA as a key molecular regulator in hMSCs differentiation induced by both mechanical (cell spreading, intracellular tension) and soluble (insulin/IGF1) factors [15, 53]. For example, MSCs with an undifferentiated state need high RhoA activity to maintain, while down-regulation of RhoA lead to myogenesis [54]. Some reports already characterized the functionality of Rho family proteins in cell myogenesis [55-57]. However, much is unknown, on the function of Rac1 in this process [55]. One report from Christopher S. Chen demonstrated that activation of Rac1 inhibited chondrogenesis, but which was also needed for smooth muscle cell differentiation [38]. Another paper reported that Rac1 activation acted an important role in myogenesis inhibition [58]. Activated Rac1 suppressed the activity of MyoD in promoting muscle differentiation. Similar results were demonstrated that active Rac1 would inhibit muscle-specific protein expressions [59]. One interesting paper shown Rac1 plays a dual role in stem cell cardiac lineage differentiation [60]. At early stages, Rac GTPase inhibited cardiomyocytes differentiation, and then promoted cardiomyocytes proliferation and myofibrillogenesis. It is also very interesting to understand the crucial complex cross-talk between RhoA and Rac1 during stem cell differentiation [61]. RhoA can suppress Rac1 and vice versa, which is well-known as Rho-Rac antagonism phenomenon during many cellular processes, such as cell polarization, cell-cell and cell-ECM interaction, cell proliferation and cell migration [62, 63].

Figure 2.4. Rho & Rac Signaling pathways. Adapted from[51]

2.4 Electrospun scaffolds with ECM bio-mimicking topography

A current challenge is taking place in medicine from using synthetic implants and tissue grafts to a tissue engineering approach that uses degradable porous material scaffolds integrated with biological cells or molecules to regenerate tissues [64]. One reason to build up this porous scaffold is to provide the cell growth and tissue regeneration with mass transportation (*e.g.*, oxygen and nutrition). Electrospinning, one of the most

attractive processes to mimic the structure of natural ECM by means of producing fibrous scaffolds, has been employed extensively in tissue engineering. Electrospinning, a textile manufacturing process, has been widely used to convey micro- or nano- topographic features to biomaterials for tissue engineering applications in the past decades [65-73]. This method uses a high electrical field created between a metallic capillary of a syringe and a grounded collector to draw a thin stream of polymer solution towards the collector, forming continuous fibers. Many different materials including poly(*L*-lactide acid) (PLLA), poly(D, *L*-lactide-co-glycolide) (PLGA), poly(caprolactone) (PCL) collagen, gelatin, elastin and blends have been successfully electrospun into nanofibers.

This technique has offered many appealing features as compared to the other conventional methods such as ease of processing, greater control over fiber geometry, flexible selection of many different polymers, possibility of scale-up manufacture and high surface area for cell attachment. In addition, wide-range of the electrospinning assembly in different manners can be utilized to develop scaffolds for different applications. For instance, many native musculoskeletal tissues show anisotropic property. Aligned PCL/collagen based nanofibers fabricated by electrospinning showed that a unidirectional fiber orientation can provide better guidance to human skeletal muscle cells alignment and significantly enhance myotube formation as compared to the random fiber arrangement [74]. In another study by Li et al., the authors found that comparing with microfibrillar PLLA scaffold, nanofibrillar PLLA scaffold had better support for chondrocytes proliferation and cell morphology maintaining, demonstrating

the importance of topological cues and the biological activities of the cell varied with the dimensionality of the fibers [75].

However, there are also limitations of using this technique. Disadvantages of this technique include the toxicity of residual solvent and cross-linking agents, which could harmfully influence the cellular behavior if not completely removed. Also, a lack of in-depth cell penetration has been reported due to the small pore size of fibrous scaffolds. Modified electrospinning techniques have been developed to resolve the issues. For example, water is used as an alternative to toxic organic solvents for electrospinning. Chen et al. have electrospun silk fibroin using water as the solvent [76].

In another study, layer-by-layer (LbL) or sequential electrospinning technique has been developed to produce hierarchically ordered architecture with multi-scales topologies ranging from nano-, micro- to macro-scale. In this process, each individual layer is fabricated by electrospinning one type of polymer solution, resulting in scaffold composed of alternating layers of fibers with various types of materials and fiber dimensions [65, 72, 77]. Taken one step further, Yang et al. improved this method by using electrospun PCL/collagen nanofibers (with average fiber diameter of 454.5 ± 84.9 nm) and human dermal fibroblasts to form a 3-D tissue with cells and fibrous architecture, which is called on-site LbL cell assembly [67]. In this approach, the 3-D multilayered cell-nanofiber constructs were built with alternating cell seeding and fiber collection on site of electrospinning. This technique provides a homogeneous cell distribution and penetration throughout the matrix with the flexibility to vary cell type

and to control the fiber diameter and orientation for each layer, facilitating the tissue formation.

2.5 Laser processing of polymers to induce topography

Recently, the treatment of materials by lasers was carried out much more widely due to better and well developed technology of laser. Laser processing offers distinct advantages, when comparing with traditional alternative techniques. It is versatile that a wide range of materials can be applied for laser processing. It is fast, easily adaptable, scalable and non-contact that other techniques may not offer. Most important, high resolution features even down to the nano-scale may be achieved by laser. The advance of laser-assisted surface modification of polymer and its application on bio-related field has won extensive interests in the past decade.

UV lasers, excimer and frequency-converted Nd:YAG lasers, were used most often to induce nano- and micro-topography by researchers [78-84]. The UV KrF excimer laser were used to create well-defined micro-topography at micron and sub-micron scale to check osteoprogenitor cells adhesion and growth [78]. ArF excimer laser was used to generate micro- and nano-scale surface features on polyamide to examine their effects on cytoskeleton organization of osteoblast-like cells [79]. Wang and co-workers [80] found that sub-micrometer laser-ablated periodic topographic features on the polystyrene, induced by a *p*-polarized Nd:YAG laser, would help the cell migration and oriented division. These long-pulse laser systems can only process polymers with strong absorption at excimer wavelengths, may modify the thermal and mechanical properties of the materials in a non-homogeneous manner and also have low material removal rate.

Although many different types of laser were used for biomedical applications, a big concern is the thermal effect during scaffold processing by those lasers mentioned above. Femtosecond laser, as a new class of ultrafast laser, can modify transparent materials at wavelengths at which they are normally transparent and also reduce the heat-affected zone. Femtosecond laser has been applied to surface modification based on polymer [82, 83, 85-88], however, only recently few groups started to carry out some studies using femtosecond laser induced topography on electrospun mat to check cell behavior[89-91]. For instance, femtosecond laser was applied to cause perforation in acellular matrix grafts that is used for small-bore vascular implant. When transplanted, the perforated implants exhibited faster recellularization with endothelial cells than contralateral controls [92]. Another study of micropatterning on collagen showed viable growth of rat mesenchymal stem cells and fibroblasts in the ablated patterns, but without illustrating any further studies like differentiation of stem cells [93]. The use of femtosecond laser to ablate electrospun scaffolds can minimize the thermal and physical stress with clean edges[90]. There is a study that applied femtosecond laser to tackle the small pore size problem of electrospun scaffolds. Femtosecond was used to create desired micro-topography on poly(L-lactide) nanofibrous mat, and the scaffold was then evaluated for cell attachment, cell morphology and cell proliferation *in vitro*, as well as cell infiltration *in vivo*[89]. Another paper focused primarily on different kinds of femtosecond laser ablated micropatterns but with limited biological characterizations, such as the cell spreading and proliferation on the ablated surface of scaffolds [91]. To the best of our knowledge, there has been no report on utilizing femtosecond laser machined micropattern on

electrospun fiber mat and then applying this hybrid scaffold in inducing myogenesis of stem cells .

Electrospun fiber mesh has been widely utilized as a tissue engineering scaffold to support cell growth [94]. But, neither the 2-D flat film nor the 3-D microporous structure of fiber mesh can spatially control cell growth. Cells were grown on the exterior surface or randomly distributed. Direct-writing femtosecond laser can provide us a rapid and flexible method to precisely control surface topography then influence the cell behavior.

Chapter 3 Materials and Methods

3.1 Femtosecond laser

The micropatterning of the biopolymer film was carried out using a Ti-sapphire (Clark-MXR CPA 2001, Dexter, MI) infrared solid-state laser with a chirped pulse amplifier to produce high-intensity pulses, with the following technical specifications: pulse duration = 150 fs; central wavelength = 800 nm; maximum power = 800 mW; fixed repetition rate = 1 kHz. The laser pulse energy is adjusted by an attenuator. The femtosecond laser beam is focused by an optical focus lens. With the right choice of pulse energy, the laser beam can directly focused on the substrate surface and induce the polymer ablation. A scheme of the femtosecond laser illumination facility is presented in **Figure 3.1**. The pulses that is attenuated using neutral density filter and is shutter controlled.

Figure 3.1. Experimental set-up of the femtosecond-laser irradiation facility with the scanning unit of the sample holders (scanner and xyz-stage).

3.2 Surface characterization

The surface morphology of the microchannel scaffold was observed using SEM performed on gold-sputtered samples (Jeol JSM-5800, Tokyo, Japan). After coating, the samples were viewed under Jeol JSM-6360LV scanning electron microscope.

Alicona InfiniteFocus was employed to generate 3-D structure for surface analysis. The sample was placed onto the motorized stage and was illuminated with light. Objective lens with 10X magnification was selected. The distance between the sample and the objective was adjusted to focus the image of the structure. Once the image was focused, upper limit and lower limit of the structure surface (in the software) were set to generate the 3-D structure surface. The profile of the surface and its particular roughness could be obtained via the 3-D structure.

3.3 Cell culture

3.3.1 Culturing

Human mesenchymal stem cells (hMSCs) from an 18 years old male donor and cell culture medium were both obtained from Lonza (Cambrex, UK). hMSCs were expanded and cultured in mesenchymal stem cell basal medium according to the vendor's instruction. For the experiments, the cells were cultured with low-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing *L*-glutamine (Sigma Aldrich, USA) supplemented with 10% FBS (PAA, Pasching, Austria) and 1% antibiotic/antimycotic solution (PAA, Pasching, Austria), at 37 °C in a humidified atmosphere of 5 % CO₂. Culture medium was changed every 2-3 days. PBS and 0.05% trypsin-EDTA obtained from Invitrogen were used for washing and cell detachment purposes respectively.

Scaffolds were sterilized using 70% ethanol for 30min. Then scaffolds were washed with deionized water and PBS three times for 15 min each time.

3.3.2 Immunocytochemistry and microscopy

Briefly, cells were fixed with 4% paraformaldehyde for 15-20 minutes and then permeabilized with 0.1% Triton X-100 for 5 min and followed by blocking with 5% bovine serum albumin (BSA) or 5% goat serum. The primary antibodies used to target interested proteins were vinculin, MAP2, MHC and fibronectin. Secondary antibodies were Alexa Fluor® 488 goat anti-mouse, Alexa Fluor® 568 goat anti-chicken, and Cy3 goat anti rabbit IgG (1:400, Millipore). C2C12 myoblast and neural stem cell were used as positive controls for MHC and MAP2, respectively. Negative controls in the absence of primary antibodies were also performed. Filamentous actin (F-actin) was stained with Tetramethyl Rhodamine Iso-Thiocyanate (TRITC) conjugated-phalloidin (1:400, Chemicon) and cell nucleus were counter stained with 4'-6-Diamidino-2-phenylindole (DAPI) respectively (1:500, Chemicon). Fluorescence images were visualized with a Nikon 80i eclipse (Nikon, Japan) upright microscope and captured with the Nikon DS-Fi1 (Nikon, Japan) using two 20X and 40X objective lens. The confocal laser scanning microscope system (Leica TCS SP5) was also used to capture 3-D images using a 40X objective lens (oil).

3.3.3 PicoGreen assay for assessing cell proliferation

The scaffolds with cells were washed three times in PBS, prior to being lysed with 0.1% (v/v) Triton X-100 solution with gentle pipetting for a duration of 30 min (0.5 mL per

well of 12-well plate). The PicoGreen working solution was then prepared by diluting the concentrated PicoGreen reagent in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) at a dilution ratio of 1:200 according to the manufacturer's instructions. Subsequently, the cell-lysate was mixed with the PicoGreen working solution at a 1:1 ratio (100 μ l within each well of a 96-well plate), incubated for at least 5 min at room temperature, prior to reading the fluorescence emission at 520 nm under an excitation wavelength of 480 nm, with an Infinite200[®] microplate reader (Tecan Inc., Maennedorf, Switzerland).

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

Total RNA was extracted with RNeasy mini kit (Qiagen, Valencia, CA). The concentration and quality of the extracted total RNA was determined spectrophotometrically (Nano drop-N100, Thermo Scientific). Subsequently, cDNA was obtained by reverse transcription of total RNA with random hexamers and superscript III reverse transcriptase (invitrogen). Quantitative real time RT PCR was performed on a CFX96 real time PCR detection system (Bio-Rad Laboratories, Inc, USA) for 40 cycles. Primers specific to the targeted genes (listed in Table 1) were obtained from primerbank [95]. Relative quantification of gene expression was determined by comparing the target-amplified product against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin (both were used as internal standard). The results were analyzed with the Relative Expression Software Tool (REST) software [95].

3.4 Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was carried out using one way ANOVA and the values are considered significantly different when $p < 0.05$.

Table 1. Compiled list of gene targets probed in this study. Adapted from [96].

Genebank accession number	Gene target	Sequence (5'-3')	Amplicon length (bp)
NM 001101	β -actin	CATGTACGTTGCTATCCAGGC CTCCTTAATGTACGCACGAT	250
NM 002046	GAPDH	CATGAGAAGTATGACAACAGCCT AGTCCTTCCACGATACCAAAGT	113
NM 004348	RUNX2	TCCTATGACCAGTCTTACCCCT GGCTCTTCTTACTGAGAGTGGAA	190
BC 021289	ALPL	CTCTCCAAGACGTACAACACC AATGCCACAGATTTCCCAGC	201
NM 003118	Osteonectin (ON)	AGCACCCCATTTGACGGGTA GGTCACAGGTCTCGAAAAAGC	138
NM 002052	GATA-4	CCCAGACGTTCTCAGTCAGTG GCTGTTCCAAGAGTCCTGCT	146
NM 002478	MyoD1	CGGCGGAAGTCTACGAAG GCGACTCAGAAGGCACGTC	172
NM 000257	β -Myosin Heavy Chain (β -MHC)	CACTGATAACGCTTTTGATGTGC TAGGCAGACTTGTACGCCTCT	165
NM 002374	Microtubule Associated Protein (MAP2)	CAGGAATTGACTCCCTCTACAGC TCTTCACCAGGCTTACTTTGC	80
X65964	Nestin (NES)	CAACAGCGACGGAGGTCTC CCTCTACGCTCTCTTCTTTGAGT	163
NM 002500	NeuroD1	GCCTTGCTATTCTAAGACGCA GTGGGTTGGGATAAGCCCTT	156
NM 000363	Cardiac Troponin I (cTnI)	TTTGACCTTCGAGGCAAGTT TCCAGGGACTCCTTAGCCC	107

Chapter 4 Laser Induced Micropattern on Film Substrate and Its Effect on hMSCs Behavior

4.1 Introduction

It has been shown that the unspecialized precursor stem cells are attracting more and more attention due to their multipotent properties and potential application in tissue engineering [5]. Bone-marrow derived adult human mesenchymal stem cells (hMSCs), which is currently one of the most studied stem cell type, has been proven capable of self-renewal and differentiation into a wide variety of lineages in response to proper cues [9-11]. As reviewed in Chapter Two, the cues include soluble/immobilized factors, chemical and physical signals from the extracellular matrix (ECM) [12]. Increasing evidence has shown that the diverse physical environment factors can also control the stem cell activities [13], and meanwhile can avoid the side effects associated with biological or chemical additions. Hereby, this chapter will demonstrate that topography has great potential in regulating cell behavior even in the absence of any biological or chemical induction factors.

As reviewed in Chapter Two, although femtosecond laser, as a new class of laser, has been applied widely for surface modification of polymer [82, 85-88, 92, 93, 97, 98], the application in biomaterial is still very limited. In the previous study from our group, we have developed a technique using femtosecond laser to create scaffolds and investigated the effect on mouse myoblast cell (C2C12) alignment [99]. Here, extensive studies were carried out on using the femtosecond laser to create highly uniform microchannels

directly on the selected biodegradable polymer, PLC (poly(*L*-lactide-*co*- ϵ -caprolactone)) substrate, for human mesenchymal stem cell growth and behavior. The channel dimensions were optimized to cause the cells to align and elongate and consequently induce myogenic differentiation. The differentiation occurred in the absence of any induction medium. Other cellular behaviors such as proliferation and cellular organization were also investigated.

Meanwhile, similar laser micropatterns on another polymer PLGA substrate with different chemical composition were also shown to induce myogenesis of hMSCs. This demonstrated that induction of hMSCs differentiation towards myogenic lineage by femtosecond laser formed microchannels is independent of polymer substrate chemical and mechanical properties. Furthermore, activity of Rho GTPases within microtopography induced stem cell myogenic differentiation was studied to elucidate the molecular mechanism of this action.

4.2 Materials and methods

4.2.1 Preparation of film substrate

Poly(*L*-lactide-*co*- ϵ -caprolactone) (PLC) film were fabricated by the solvent casting method. The block copolymer PLC (70:30), comprised of 70% *L*-lactide and 30% ϵ -caprolactone, was used in this study (Purac Biomaterials, Lincolnshire, IL). The copolymer PLC granules were dissolved in dichloromethane (Tedia, Fairfield, OH) in 1:5 mass/volume ratio to achieve an optimal viscosity that is suitable for solvent casting process. Poly(D, *L*-lactide-*co*-glycolide) (PLGA, 80/20) films were also prepared by solvent casting method. The copolymer PLGA (Purac Biomaterials, Lincolnshire, IL) granules were dissolved in dichloromethane (Tedia, Fairfield, OH) in 1:7 mass/volume ratio for subsequent solvent casting. The films were cast using an industrial film applicator (Paul N. Gardner Company, Inc., Sheen automatic film applicator, FL, USA). The polymer films were dried in a 37 °C vacuum oven for 7 days. The thickness of the dry cast films were approximately 100 μm .

4.2.2 Femtosecond laser machining on film substrate

Figure 4.1 shows the scaffold design on a 100- μm -thick polymer film. The microchannels were machined onto a 10 mm $\times$ 10 mm polymer film. Three feature sizes were produced. The length of each channel is 8mm, with a targeted channel width W_i of 80, 30 or 20 μm . The femtosecond laser scanning speed was 10 mm·s⁻¹. The channels

with 20 μm and 30 μm widths were created by scanning two passes on the toolpath at the laser power of 50 mW and 100 mW, respectively. The wider channels with 80 μm width only need one pass at the laser power of 450 mW, to get the same depth as that of narrower channel.

Figure 4.1. Design of laser machined scaffolds with two kinds of microchannels.

4.2.3 Characterization of polymer films

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopic analysis of PLC substrates before and after laser machining were performed on PerkinElmer Spectrum GX at a range of 600–4000 cm^{-1} . All the spectra were measured with 2 cm^{-1} resolution and 8 scans with correction for atmospheric water and carbon dioxide. The surface chemical composition of substrates was measured by X-ray photoelectron spectroscopy using a VG ESCALAB 250 spectrometer (Thermo Electron, U.K.). All the binding energies were corrected by referencing the binding energy of C 1s in the C-H group to 285.0 eV.

4.2.4 G-Lisa kit

The RhoA and Rac1 expression was checked by RhoA and Rac1 G-LISA specific Activation Assay (luminescence, Cat. BK121 and BK126, Cytoskeleton, Inc) according to the vendor's instructions as reported in the literatures [100, 101]. The signals were read by measuring absorbance at 490 nm using a microplate spectrophotometer (Infinite200[®] microplate reader, Tecan Inc., Maennedorf, Switzerland).

To evaluate if RhoA regulates the microchannel induced myogenesis, hMSCs were treated with 10 μ M ROCK inhibitor, Y27632 (Millipore, USA). Y27632 was dissolved in dimethyl sulfoxide (DMSO) to make a 10 mM stock solution. 10 mM Y27632 was diluted in a complete DMEM to a final concentration of 10 μ M to make the block medium. After 7 days culture of cells on micropatterned scaffolds, the normal DMEM was replaced with block medium and changing of block medium was done every 24hours. Three biological samples were prepared for each type of characterization.

4.3 Results and discussion

4.3.1 Characterization of polymer surface

PLC is one of the commonly used biodegradable materials in tissue engineering and regenerative medicine. In this thesis, it was chosen because of its mechanical property which is elastic and less rigid as compared to most other aliphatic polyesters. Moreover, a relatively mild degradation, which avoids an abrupt drop in pH value during degradation, similar to PLA and PGA scaffolds [102, 103]. Femtosecond laser is able to apply highly focused energy for the removal of materials. By changing the laser power, scan speed and scan pass, the microchannels with three desirable dimensions were machined onto a 10 mm × 10 mm PLC film. The channels were fabricated at a predetermined width, depth, as well as interval distance. When the distance between two microchannels is much narrower than the cell width, there is possibility that cells may migrate from one track to another. However, the occurrence is also dependant on depth (height) of microchannels. Endothelial cells adhered smoothly on surface with 10 µm depth grooves, while fibroblast cells encapsulated in grooves with 10 µm [104]. The depth of microchannels was first selected as 20µm because the deeper the microchannels, the less chances for cells to jump over, and the more pronounced contact guidance could occur [105]. The microchannel 30-30-20 (channel/interval ratio = 1) was selected because those channels and intervals have close widths with cell width, whereby cells can elongate efficiently. The microchannel 80-30-20 was selected because it has higher channel/interval ratio than 30-30-20. The microchannel 20-20-20 was selected to study if channels and intervals with narrower width have more significant effect on stem cell behavior.

The feature pitches (the combination of groove and interval widths) were uniform in each sample. As shown in **Figure 4.2**, 3-D microchannel topography with lined structure on the top view (SEM images) and sinusoidal surface on the cross section (3-D profiler images) was achieved on the flat 2-D surface of raw material through femtosecond laser machining. 3-D profiler was used to measure the roughness in and outside the microchannels. Two parameters, average roughness (Ra) and root-mean-square (Rq), were utilized to characterize the roughness of surfaces. The roughness in the channels for 20-20-20, 30-30-20 and 80-30-20, were Ra=0.78 μm , and Rq=0.95 μm , Ra=0.80 μm and Rq=0.98 μm , as well as Ra=0.83 μm and Rq=1.05 μm respectively. For the intervals (outside the channels), the roughness for all three groups of microchannels was approximately, Ra=0.35 μm , Rq=0.42 μm , which was evidently smaller than the roughness in the microchannels. Therefore, all types of channels were considered to have similar roughness.

Figure 4.2. Topography of microchannels fabricated directly using femtosecond laser on biodegradable PLLA-PCL film, with sinusoidal surface topography inside the microchannels (SEM & 3-D profiler). Partially Adapted from [96].

The substrates with and without laser machining were examined by ATR-FTIR and XPS. As shown in the ATR-FTIR spectra (**Figure 4.3**), there were almost no differences between laser machined substrates and controls. All spectra revealed the characteristic peaks of pure PLC copolymer. The bands between 2870 and 2995 cm^{-1} were present due to C–H stretching vibrations of alkyl CH_3 , CH_2 and CH groups. The bands from 1350 to 1460 cm^{-1} were assigned to alkyl C–H bending vibrations [106]. The characteristic ester absorption peak of PLC was observed at 1744 cm^{-1} , and the C–O–C stretching vibrations were observed at 1084 , 1128 and 1182 cm^{-1} [107]. The results of XPS spectra (**Figure 4.4**) showed us the elemental and chemical state of specific atoms on the material surface. Identical carbon and oxygen signals were detected by XPS survey scan on the substrates surface before and after laser machining. The C 1s core-level detailed spectra revealed

three major peaks with similar binding energies in each high-resolution XPS spectrum (C-H, C-C: 285.0 ± 0.1 eV; C-O: 287.0 ± 0.1 eV; C=O: 289.1 ± 0.1 eV) [108] again indicating that there was no significant chemical change after laser machining.

Figure 4.3. ATR-FTIR spectra of unpatterned polymer substrate (control) and the laser machined substrates at range of 600-4000 cm⁻¹. Partially Adapted from [96].

Figure 4.4. XPS spectra of unpatterned polymer substrate (control) and the laser machined substrates. Survey scan spectra for each C 1s and O 1s, as well as narrow scan spectra for the C 1s core level are shown. Partially Adapted from [96].

4.3.2. Cellular organization

4.3.2.1 Cell elongation and orientation along the micropatterns

hMSCs were cultured on the respective substrates (laser-machined substrates with dimensions 20-20-20, 30-30-20, 80-30-20 and control without laser machining) for 1 day, 4 days, 7 days, 11 days and 14 days. To examine the cell morphology and microfilament arrangement, scaffolds with hMSCs were stained with phalloidin for F-actin and DAPI

for nucleus. When hMSCs were cultured on the PLC scaffolds with laser machined microchannels, all channels aided the cells to elongate and align along the grating axis. In contrast, hMSCs cultured on controls showed no elongation or alignment in any specific direction. The quantitative and qualitative analysis of cell alignment on different substrates is shown in **Figure 4.5**. For cells cultured on control, the cells were randomly oriented with degrees of alignment from 0-90° even when the cells were becoming confluent. The cells cultured on scaffolds with narrow microchannels, 20-20-20 and 30-30-20, have better alignment than that on the wider channels, 80-30-20. This was observed from the beginning of cell growth on day 1 (**Figure 4.5A**). As seen in **Fig 4.5**, for the narrower channel system, the angular distribution of the cells changed from a maximum at around 10-19 degree on day 4 (**Figure 4.5B**) to a maximum around 0-5 degree on day 7(**Figure 4.5C**), clearly indicating progressively greater alignment of the cells along the grooved direction. This is not as evident in the wider channel substrate (80-30-20), where the preferred alignments continued to be in the 10-19 degree range regardless of the time of incubation. The cell alignment distribution on different surfaces after 7 days was also characterized by 2-D FFT analysis (**Figure 4.5D**), which converts spatial information into mathematically defined optical data. The 2-D FFT frequency plot depicting this data, represents the grayscale pixels distributed in patterns around the origin to reflect the degree of cell alignment.

Figure 4.5. Alignment of hMSCs on unpatterned (control) and laser micropatterned substrates. Quantitative study of cell orientation was done at Day 1, Day 4 and Day 7; Qualitative analysis using 2-D FFT analysis was done at Day 7. (panel A: Day 1; panel B: Day 4; panel C: Day 7; panel D: 2-D FFT analysis of Day 7.) Partially Adapted from [96].

From the results of phalloidin stained F-actin (**Figure 4.6**), the unpatterned cells were well spread, with well-defined F-actin arranging circumferentially around the cell nucleus. Cell nucleus remained round after 11 days. Cells cultured on both laser micropatterned scaffolds experienced increased cellular alignment. The F-actin stress fibers, which were highly organized in bundles on the scaffolds with narrow microchannels, were stretched along the long axis of cells. The orientation of the stress fibers and the polarized elongated cell morphology on the scaffold with narrow microchannels, demonstrated good contact guidance. Contact guidance is a phenomenon that anisotropic topographies could induce in different cell types to align and migrate along the direction of the anisotropy [105]. The formation and alignment of mature focal adhesion (FA) complex, which have a direct relationship with cellular cytoskeleton reorganization, has been proposed to have a better understanding of contact guidance phenomenon [109]. The possible reason of the more specific cell alignment on the narrow microchannels is that more FA complexes are located in the laser micromachined grooves (on the bottoms or walls) where cells would like to attach. When the width of microchannels is similar or

smaller to cell size, a greater cell shape index will be exhibited, comparing with the much wider microchannels or flat surfaces [110].

Figure 4.6. Phalloidin labeled **F-actin** (red), **DAPI** nuclear staining (blue) and merged fluorescent image for the non-patterned (left column) and micropatterned hMSCs (middle and right column). Samples were cultured in DMEM for 1 day and 11 days before they were fixed and stained. All images were taken with a 63X objective lens (oil). (Scale bar, 50 μ m) (top panel A: Day 1; bottom panel B: Day 11). Partially Adapted from [96].

4.3.2.2 Cell distribution on the micropatterned scaffolds

The aim of our study was to investigate whether laser etched 3-D microchannels can regulate hMSCs lineage differentiation. Our hypothesis was that the femtosecond laser-formed channels together with its macro and micropatterns would influence cell alignment and in turn affect cell proliferation and differentiation. We observed that hMSCs were able to align themselves along the direction of the microchannels. The morphological alterations and cytoskeleton reorganization caused by microtopography were consistent with previous observations in other cell types [105, 109, 111]. Much more interestingly, we observed that there were more cells found inside the microchannels (on the bottom or attached to the wall of microchannels) than on the intervals especially when it is not passivated. This might be due to the surface modifications created by laser machining. Immunofluorescent staining of fibronectin on the laser patterned scaffolds without hMSCs after 12 hours of incubation in culture media with 10% FBS is shown in **Figure 4.7**. This biased fibronectin patterning may explain why more hMSCs were found inside the laser patterned microchannels compared to the intervals. It was observed that there were many aggregated dots of fibronectin (only some aggregates were shown by arrows) clearly located inside the laser scanned microchannels (**Figure 4.7**). On the other hand, less fibronectin dots were found on the intervals and this may be due to the fact that the roughness on the channel is much greater than that on the interval. In the same logic, proteins from culture media serum will adsorb onto the surface and act as cell adhesion nodes [47]. Consequently, this changes cell distribution between the intervals and channels as well as that in the controls. To exclude the possibility that the chemical changes of polymer surface after laser machining had caused

the differential attachment pattern, ATR-FTIR and XPS spectra showed no notable differences in functional groups, chemical states of carbon and oxygen between the laser machined substrates and the control group. It can be concluded that the surface topography created by the laser machining is the dominant factor in affecting protein adsorption and consequentially cell adhesion.

Figure 4.7. Bovine fibronectin immunofluorescent staining (*red*) was performed on laser patterned scaffolds (without cell culturing) to identify protein adsorption on the surface (C = channel, I = interval). Fibronectin “aggregates” were detected on all surfaces that were incubated in media for 12 h. Less fibronectin was detected on the intervals, than the laser-machined microchannels; labeled as “C”. (Scale bar, 50 μ m) Partially Adapted from [96].

4.3.3. Cell proliferation

Proliferation of hMSCs on biodegradable polymer scaffold with and without laser machining was examined using PicoGreen DNA quantification. **Figure 4.8** shows absolute cell number per scaffold at day 1, day 4, day 7, day 11 and day 14 after seeding at an initial cell seeding density of 5000 cells/cm². The control group without laser machining had higher cell number compared with the laser machined scaffolds. Cells in the control group reached 100% confluence after 11 days. Differences in cell numbers of all three laser machined scaffolds were not statistically different ($p>0.05$).

Figure 4.8. Cell number of hMSCs 1 day, 4, 7, 11 and 14 days after seeding at an initial seeding density of 5000 cells/cm² on scaffolds without laser machining, scaffolds with narrow channel width and wide channel width. There was no significant difference in the cell number between those two groups with laser machining ($p>0.05$). Partially Adapted from [96].

Many studies have been done to investigate the effects of surface topography features on cell attachment, spreading and proliferation [112-114]. It was reported that nano-roughness plays a role in regulating cell physiology, which is perhaps not surprising since cells are surrounded by nano-scale structures in their native environment [115]. One study demonstrated that the levels of short-term cell attachment were higher on rough surfaces compared to smooth surfaces, and cell morphology was directly related to the nature of underlying substrate [116]. Another *in vitro* study also demonstrated that osteoblasts exhibited greater initial attachment to rough titanium surfaces [117]. On the other hand, it is known that micro-topography features may impede cell migration and

proliferation due to physical constraints [118]. When compared to the scaffolds with wide microchannels, the scaffolds with narrow microchannels have similar surface roughness inside the channels but would show greater impedance to cell proliferation in the perpendicular direction of channels, due to the narrower channel width.

4.3.4. Stem cell lineage commitment on micropatterned scaffolds

4.3.4.1 Gene expression analysis

Figure 4.9 shows the profile of gene expression by hMSCs cultured on the laser micropatterned scaffolds after 7, 11 and 14 days, with cells cultured on unpatterned scaffolds as the control. Alkaline phosphatase (ALPL), Osteonectin (ON) and runt-related transcription factor 2 (RUNX2) were chosen as markers of osteogenesis. Cardiac Troponin I (cTnI), GATA binding protein 4 (GATA4), myosin heavy chain 7 (MHC7), myogenic differentiation 1 (MyoD1) and NK2 transcription factor related, locus 5 (Nkx2.5) were used to confirm myogenesis, while microtubule-associated protein 2 (MAP2), Nestin and neurogenic differentiation 1 (NeuroD1) were taken as the markers of neurogenesis.

Figure 4.9. Relative gene level expression analyses of osteogenic, myogenic and neurogenic markers on day 7, 11 and 14 of cell culture. Bars represent mean $\pm$ standard deviation. Relative gene expression levels (*i.e.* $\Delta\Delta C_T$) were normalized to the control samples ($t=7$ days time-point) without laser machining. * represents statistical difference between patterned and unpatterned samples ($p<0.05$, $n=6$). Partially Adapted from [96].

Osteogenic genes did not show any statistically significant difference ($p>0.05$). Alkaline phosphatase (ALPL) is essential for the process of mineralization [119, 120]. Osteonectin (ON) is a glycoprotein in the bone that binds calcium, which was detected during bone formation and initiating mineralization [121]. Osteocalcin (OCN) is often used as a marker for the bone formation process [122]. RUNX2 is a key transcription factor involved in bone mineralization maintenance [123, 124]. At day 7 and 11 of cell culture, RUNX2 levels were high but decreased by day 14. For the other osteogenic markers,

ALPL and osteonectin, there were no statistically significant down-regulation as compared to the control at the earlier time points with a dip at day 14.

MyoD1 is one of the basic helix-loop-helix (bHLH) transcription factors capable of regulating myogenesis [125]. The other four selected myogenic genes cTnI, GATA4, MHC7 and Nkx2.5 are widely known as the important regulators of cardiomyogenesis. The tissue-restricted GATA-4 transcription factor and Nkx2.5 homeodomain protein are two early markers of precardiac cells [126]. The MYH7 gene encodes a protein known as the cardiac beta (β)-myosin heavy chain, which plays a critical role on the skeletal and cardiac muscle functionality of the contractile machinery [127]. The gene expression of hMSCs cultured on the scaffolds with narrow microchannels showed a definitive upregulation of myogenesis and was found to gradually increase from day 7 to day 14 when compared to control samples. The result can be taken to imply that hMSCs cultured on the laser patterned scaffolds with narrow microchannels have differentiated towards the myogenic lineage as a result of elongated cell shape [16]. After 11 days of cell culture, cell-cell contact caused by confluence may lead to elongated cell shape formation. But this effect may not be sufficient; unlike the elongated cell shape caused by narrow channels resulting in the unstable myogenic genes up-regulation. This is supported by the fact that hMSCs cultured on the laser patterned scaffolds with wider microchannels only showed upregulation of myogenesis at a later time point of 11th day.

Nestin is a transient neurogenic marker during early stage of development in neural stem/progenitor cells [128]. NeuroD1 is essential for the survival and maturation of adult-born neurons [129], while MAP2 gene involved in the process of microtubule assembly,

which is critical for the formation and maintenance of neuritis [130]. For cells cultured on the narrow microchannels, nestin, the chosen early stage marker for neurogenesis, was statistically significantly upregulated on the 7th and 11th day but then it gradually decreased, and eventually down to nearly the same level as control on the 14th day. The bHLH transcription factor NeuroD1 and late neurogenic markers MAP2 both showed higher upregulation of gene expression than nestin at days 7 and 11, however, they were then reduced to a similar level as nestin on the 14th day of cell culture. Meanwhile, for the laser patterned scaffolds with wide microchannels, only NeuroD1 gene showed significant upregulation on the 11th day culture of hMSCs.

4.3.4.2 Immunostaining of myogenic and neurogenic markers

In order to confirm the phenotypes and differentiation capacities of hMSCs cultured on the laser micropatterned scaffold, immunostainings for mesenchymal lineage-specific markers (mature markers of myogenesis, cardiac MHC and neurogenesis MAP2) were performed. Dual labelling with antibodies against MHC and MAP2 detected MHC staining on the laser patterned scaffolds with microchannels on the 11th and 14th day of cell culture, but no observable MAP2 staining on both groups of laser patterned scaffolds at all time points (**Figure 4.10**). Surmising from the qRT-PCR data and immunostaining data suggest that our scaffold with microchannels help drive the MSCs towards myogenic lineage.

Figure 4.10. Representative images showing immunostaining of myogenic and neurogenic markers (C = channel, I = interval). Cells were costained with distinctive and mature markers of myogenesis and neurogenesis using mouse monoclonal anti heavy chain cardiac myosin (*green*) and chicken polyclonal anti MAP2 (*red*). (Scale bar, 50 μ m)

It is well known that cell morphology alteration, cytoskeleton organization and nuclear shape variation play a critical role in signal transduction [18]. Macroscopic change of cell shape reveals that cells are able to sense micro- and nanoscale cues from the environment, such as surface topography or roughness. The topographical features used in cell culture are mainly basic shapes (*e.g.*, lines, pillars or wells), among which line-shaped structures were commonly used to align cells and influence cellular behaviour. It is suggested that the mechanical tension caused deformation of the nucleus; which is physically linked to the cytoskeleton by intermediate filaments, to alter the gene expression and then cellular behaviour [16, 131]. In this reported work, femtosecond laser was used to fabricate a system of micropatterns consisting of continuous sinusoidal surface topography and micro-roughness, both of which proved to enhance cell adhesion and defined cell orientation. Compared to conventional 2-dimensional (2-D) cell culture systems, our laser machined scaffolds could partly provide a more 3-D like environment that is essential for the maintenance of cellular function and tissue formation *in vivo*. The difference between those two laser machined scaffolds is the surface curvature produced inside the microchannels, which has been shown to affect cell morphology and then tissue growth [132, 133]. Cells cultured on laser machined scaffold took on consistent morphologies; cells on 20 and 30 μm width versus those on 80 μm took on a more elongated shape and appeared relatively smaller. This could be caused by the physical constraint induced by narrower channels in the perpendicular direction, which have relatively shorter radii of curvature. Thus, cells in the narrow channels would elongate more in the direction of microchannels leading to cell alignment. In contrast, the hMSCs, grown on the control substrates, grew in random directions, and were 30-50 μm wide and more than 100 μm

long. Therefore, microchannels induce cell elongation by generating cytoskeletal tension and that may direct myogenic differentiation [16, 17]. The narrower 20 and 30 μm microchannels with smaller radii of curvatures would give rise to higher intracellular tension force than the wider 80 μm microchannels. This high intracellular tension recruits α -smooth muscle actin to stress fibers [134, 135]. Micropatterning of even narrower 20 μm wide fibronectin strips on PLGA film could induce hMSCs towards the myocardial lineage [16, 17]. Micropatterned matrix imposed cell shape restrictions and led to cytoskeletal rearrangement and nucleus shape alteration; these changes translated into biochemical response and effected cell differentiation [16]. Compared to 2-D surfaces, the laser machined 3-D scaffolds was able to increase the extent of myogenic genes upregulation in the cells (MyoD1 gene up-regulation ~ 5.1 v.s. ~ 3.4 fold; GATA4 gene up-regulation ~ 4.1 v.s. ~ 3.0 fold). Since cell proliferation and differentiation are known to have an inverse relationship [136], the directed myogenic differentiation of hMSCs might be one possible reason for the observation of lower cell numbers in laser micropatterned scaffolds compared to the control.

4.3.5 Assessing hMSCs differentiation on a laser micropatterned polymer surface with different chemical composition and stiffness

In order to confirm that laser machining is a versatile method as well as the myogenic differentiation of hMSCs was induced by the topography and is independent on substrate property, another polymer, with different chemical composition and different mechanical property, poly(D, L-lactide-co-glycolide) acid (PLGA, 80/20) was used as substrate for similar study. Based on the previous results, both narrow channels with 20 and 30 μm width can induce myogenic differentiation of hMSCs. Hereby, only microchannel

dimension with 30-30-20 and 80-30-20 will be produced to check cellular behavior. In the literature review part, matrix stiffness, one of the biophysical cues has been proven to guide cell growth. It also has been reported that surface chemistry of biomaterials could influence cell function and determine stem cell differentiations [137-141]. There is a firm correlation between protein adsorptions or focal adhesion formation on the surface and stem cell differentiation which is directly related to surface chemistry [142]. The polymer surface functionalities including charges, hydrophobicities and matrix elasticity can affect cell spreading, and then have a significant effect on stem cell fate [140].

To quantify the efficiency of hMSCs differentiation into osteogenic, myogenic and neurogenic lineage, qPCR was applied to detect the marker genes of stem cell differentiation. **Figure 4.11** shows the alteration of gene expression by the hMSCs cultured on the laser micropatterned PLGA scaffolds after 7, 11 and 14 days, using unpatterned scaffolds as the control. Same gene markers were chosen: ALPL, Osteonectin and RUNX2 expression were chosen as markers of osteogenesis; GATA4, MHC7, MyoD1 and Nkx2.5 were used to confirm myogenesis; MAP2, Nestin, NeuroD1 and Tubulin were taken as the markers of neurogenesis.

Figure 4.11. Relative gene level expression analyses of osteogenic, myogenic and neurogenic markers on day 7, 11 and 14 of cell culture. Bars represent mean $\pm$ standard deviation. Relative gene expression levels (i.e. $\Delta\Delta CT$) were normalized to the control samples (t=7 days time-point) without laser machining. * represents statistical difference between patterned and unpatterned samples ($p < 0.05$, $n = 6$).

From the qPCR results of osteogenic differentiation, there was no significant upregulation between the laser micropatterned scaffolds and unpatterned control samples. Instead, both wide and narrow microchannels would cause downregulation of osteogenesis after a culture of 14 days (**Figure 4.11**). This result is similar to that of laser micropatterned PLC substrates shown previously in **Figure 4.9**. Although there was a transient upregulation of neurogenesis in hMSCs on laser machined PLC substrates from

day 7 to day 11, it was downregulated to a normal level eventually at day 14. Here, for the neurogenic differentiation of hMSCs cultured on PLGA substrate, four chosen neuro-markers (MAP2, Nestin, NeuroD1 and Tubulin) were all downregulated after 14 days for both kinds of microchannels, with statistically significant downregulation of the MAP2, Nestin and NeuroD1 for all collected time-points for cells culture on the laser patterned scaffold with narrow microchannels (30-30-20).

And most importantly, similar to the trend on PLC substrate, a definitive upregulation of myogenesis of hMSCs on PLGA substrate with narrow microchannels (30-30-20) was revealed and found to gradually increase from day 7 to day 14. The expression of these genes can be taken to imply that hMSCs cultured on the laser patterned scaffolds with narrow microchannels have been induced into the myogenic lineage as a result of elongated cell shape [16, 17, 96]. On the other hand, hMSCs cultured on the laser micropatterned PLGA scaffolds with wider microchannels (80-30-20) only showed upregulation of myogenesis at 11th day, which probably may be due to the delayed elongation of the cells as compared to the 30 μ m width intervals. Again these qPCR results from cells on PLGA substrates showed consistent trends with that of cells on PLC substrates.

In order to confirm the phenotypes and differentiation capacities of hMSCs cultured on the laser micropatterned PLGA scaffolds, immunostainings for mesenchymal lineage-specific markers (mature markers of myogenesis, cardiac MHC) were performed just as with the PLC scaffold. Immunostaining with antibodies against MHC detected MHC

staining on the laser patterned PLGA scaffolds with microchannels on the 14th day of cell culture (**Figure 4.12**). Combined with the results of qPCR on upregulation gene expression, it demonstrated that the commitment to myogenic lineage is also induced specifically by laser machined microchannels on PLGA scaffolds as with the PLC scaffolds

Therefore, it is concluded that the myogenic induction is primarily triggered by the microchannels and is less dependent on substrate property (chemistry and stiffness) in this study.

Figure 4.12. Representative images showing f-actin staining (red) of hMSCs cultured at day 1 and day 11; and immunostaining of myogenic marker (Myosin Heavy Chain, MHC, green) on hMSCs cultured after 11 days. (Scale bar, 50 μ m)

4.3.6 Role of Rho-GTPase in stem cell differentiation

4.3.6.1 GTPases expression on laser micropatterned substrates

A mechanistic study is performed here to elucidate the potential molecular basis of femtosecond laser machined topography of microchannels modulated stem cell differentiation through the elongation of cell shape. It has been demonstrated that Rho

family of small GTPases (including three mainly studied proteins, RhoA, Rac1 and CDC 42) can regulate cell shape through cytoskeleton reorganizations [50]. They act as transducers which are responsive for translating extracellular signals to cell movement and spatial dynamics of cytoskeleton [143]. One interesting report presented RhoA activity mediated cell-shape dependant modulation of hMSCs differentiation toward osteoblast or adipocytes, through the cell shape regulation by restricting adhesive areas [15]. More recent evidence show that small Rho family GTPases monitor corneal epithelial cell alignment on substrate with anisotropic nano-topographies [144]. However, the role of Rho family GTPases in modulation of hMSCs contact guidance, shape elongation and lineage commitment on laser micropatterned surface still remain to be explored.

G-Lisa assay was applied to investigate whether hMSCs myogenic differentiation caused by laser machined micropattern involved the activation of RhoA or Rac1 GTPases. The G-Lisa assay results in **Figure 4.13** showed that there is a significant increase of RhoA GTPases in hMSCs on scaffolds with narrow microchannels (20-20-20 & 30-30-20) compared with that on scaffolds with wide microchannels (80-30-20) after 7, 11 and 14 days of cell culture. There was a gradual increase of RhoA GTPase activity in cells on 20-20-20 and 30-30-20, with the culture time, from 28% at day 7 to 42% at day 11 and 51% at day 14. However, no significant differences in the activation of RhoA GTPase were exhibited for hMSCs on wide microchannels (80-30-20).

As for Rac1, there was no obvious difference in the activities presented in hMSCs on all three micropatterned groups, comparing with that of unpatterned control group. Based on the results showed previously in Part 4.3.4 and G-Lisa assay results here, activation of RhoA, but not Rac1, might be involved in the myogenic differentiation of hMSCs on laser machined substrate.

Figure 4.13. RhoA and Rac1 GTPases activation in hMSCs on control PLC films and laser micropatterned PLC scaffolds as measured by GLISA. Each reaction was performed in triplicate.

4.3.6.2 The role of Rho-family GTPases on topography induced stem cell differentiation

As reviewed in Chapter 2, the small G-protein Rho leads to the reorganization of actin filaments as well as assembly of contractile stress fiber formations, which subsequently influence stem cell differentiation. Myogenic differentiation is associated with induction of a group of muscle-specific genes, many of which encode cytoskeletal proteins [145]. Contractility in cytoskeleton is essential for stem cell myogenic differentiation [146]. It has been shown that the existence of cytoskeletal tension resulted in the differentiation of fibroblasts into myofibroblasts [147]. Through the manipulation of cell adhesion area, cytoskeleton rearrangement always stems from activation of RhoA/ROCK, which is important to detect biophysical stimuli and direct stem cell differentiation. One well-known report by McBeath revealed that the changes of RhoA/ROCK activity and cytoskeletal tension influence hMSCs lineage commitment through the control of cell shape [15]. Another report showed that the micropatterns induced changes in cell shape alter RhoA-mediated cell contractility [148]. Chapter 2 already reviews surface topography would affect the reorganization of cytoskeleton and formation of cellular tension. Two more recent studies also showed RhoA/ROCK pathway is involved in topography induced actin cytoskeleton organization [149, 150]. Therefore, based on the literatures presented above, demonstrating the important role of RhoA/ROCK in regulating cytoskeletal organization in general and subsequently myogenesis, this specific pathway is also deemed to be involved in our microchannel committed stem cell myogenic differentiation. This is based on the observation where there was obvious cytoskeleton re-organization when cells were cultured in the microchannels. Here, hMSCs were treated with Rho-associated kinase (ROCK) inhibitor (Y27632) after cells

were cultured for 7 days, to check if the activation of RhoA is involved in the microchannel directed myogenic differentiation of hMSCs.

To characterize the effect of Y27632 drug treatment on changes in cell morphology and microfilament formation, the cell morphology of each group was observed under confocal fluorescent microscope (**Figure 4.14**). Cells on the control group without exposure to ROCK inhibitor (10 μ M Y27632) were mostly well spread out with randomly distributed actin fibers. Cells on the laser micropatterned scaffolds treated with ROCK inhibitor lose their elongated cell shapes, became stellate-like cells, without clearly formed microfilaments. Despite of that, it is noted that the inhibition of ROCK induced lamellipodia outgrowth accompanied with dendritic structures. From the images, more dendritic structures were found in cells cultured on scaffolds with wider microchannels (80-30-20) than those structures found in cells on scaffolds with narrow microchannels (20-20-20 and 30-30-20). qPCR results (**Figure 4.15**) revealed that neuro markers of MAP2, Nestin and NeuroD1 were upregulated in cells on micropatterned scaffolds with the treatment of ROCK inhibitor. Most of osteo markers remained unchanged as compare to control, while myo markers were even downregulated after ROCK inhibition. Immunostaining of MHC in cells on all scaffolds after drug treatment was also found to be negative. Therefore, presence of ROCK inhibitor not only disrupted the formation of actin filament and elongated cell shape, interrupted myogenic differentiation of hMSCs, but also stimulated surface protrusions (lamellipodia). This was accompanied by upregulation of neurogenic markers especially for wider channels (80-30-20).

Figure 4.14, Fluorescent images of **f-actin** (red) and immunostained myogenic marker **MHC** (Green) for the non-patterned (0.1%DMSO) and micropatterned hMSCs after ROCK inhibition (10 μ M Y27632).

Figure 4.15. Relative gene level expression analyses of neurogenic, osteogenic and myogenic markers after 14 days of cell culture with ROCK inhibition (10 μ M Y27632).

It is well known that RhoA and Rac1 GTPases have antagonistic functions during many processes, like dendritic branching in neurons [151], cell-cell contact formation [152], epithelial morphogenesis [153], *etc.* Based on the Rac1 G-Lisa assay results shown above (Figure 4.13), a slight up-regulation of Rac1 activity was revealed on scaffolds with wide

microchannels (80-30-20), and a down-regulation of Rac1 activity was appeared on scaffolds with narrow microchannels (20-20-20 and 30-30-20). Although the decrease in Rac1 activity was not significant, these results showed that RhoA and Rac1 might have opposite effect on cell functions. Similar results were reported on the promotion of myogenic differentiation through RhoA positive and Rac1 negative regulation triggered by N-cadherin [154]. Hence, the possible mechanism was that RhoA activation in cells on scaffolds with narrow microchannels (20-20-20 and 30-30-20), initiated clustering of focal adhesion and polymerization of stress fibers which caused increase in cellular tension, and promoted myogenic differentiation [13, 101]. On the other hand, activation of Rac1 in cells on scaffolds with wide microchannels (80-30-20) might encourage the lamellipodia formation and dentritic branching, which is in agreement with another report [155]. Therefore, these results suggested that activation of RhoA GTPase, but not Rac1, might be engaged in the microchannels induced actin cytoskeleton reorganization and subsequent myogenic lineage commitment.

4.4 Summary

The results presented here show that laser cut microchannels together with the 3-D topographic micropatterns on the polymer substrate affects cell adhesion, proliferation, cytoskeleton reorganization and differentiation. The novelty of this work lies in the application of direct laser machining to create channels with 3-D topographic micropatterns that can coax differentiation of hMSCs towards a defined lineage without any exogenous biochemical factors. Femtosecond laser is a feasible method to fabricate highly regular structures on biodegradable polymer. Substrate with narrower microchannels is favorable for myogenic differentiation of hMSCs due to cell shape elongation caused by laser formed microchannels. Similar trends were observed on two different polymers showing independence of this microchannel induced myogenesis on mechanical property and chemical composition of the substrate. Rho GTPase molecular signaling was examined to elucidate the possible molecular mechanism behind topography of microchannels induced hMSCs myogenesis. RhoA/ROCK pathway is the key regulator for hMSCs elongation on laser micropatterned scaffolds. Inhibition of ROCK suppressed myogenesis of hMSCs, demonstrating that RhoA/ROCK signaling is involved in microchannel directed myogenesis of hMSCs. Moreover lamellipodia structures were observed after ROCK (Y-27632) inhibition.

Chapter 5 Laser Induced Micropattern on Electrospun Scaffold to Promote Cell Viability

5.1 Introduction

Tissue engineering is an interdisciplinary field that encompasses research areas of biology, chemistry, materials science and engineering. The goal of tissue engineering is the construction of artificial tissues or organs to replace the dysfunctional ones. To create a hierarchical structure with defined three dimensional (3-D) architecture biomimicking their unique microenvironments as natural tissues *in vivo* is critical for construction of functional engineered scaffolds [64, 156]. For instance, a tissue-engineered functional cardiac patch should accurately mimic the structure of native myocardium hierarchically from nano-, micro- to macro- scale. At the nanoscale, cells in the engineered cardiac patch must be coupled by functional gap junctions and capable of propagating electrical impulses in order to prevent arrhythmia upon implantation [157]. At the micro- and macro- scale, the elongated myofibers of myocytes with high-cell-density distribution should align in parallel, with the angle changing along the thickness of the ventricle and capability of synchronous contractions [158]. At the centimeter scale, tissue engineered constructs should be mechanically stable. From the biomimetic standpoint of view, the nano- and micro- scale components may provide a proper topography for cell growth as they mimic the natural structures of extracellular matrix (ECM).

As reviewed in Chapter Two, the designed nano- and micro- topography could influence cell behavior [14, 19]. It also has been implied that nano-fibrous features would improve cell adhesion, migration, proliferation, differentiation, and other activities [20, 159]. Moreover, the micro- porous structure would be beneficial for cell infiltration, mass transportation and 3-D tissue formation [160]. In order to successfully integrate hierarchical structures into a scaffold, many attempts have been used, such as electrospinning [94], lithography [161], and gas foaming/particulate leaching [162]. Chapter Two already reviewed that electrospinning, as one of the widely used technique, is gaining much attention because the electrospun fibrous scaffolds would provide desired features mimicking native ECM and therefore improve cell functionalities [163, 164]. It has been suggested that the combination of micro- and nano- scale fibers can promote cell viability, adhesion, proliferation and differentiation [65, 165-167].

In extension to Chapter 4, here, we used similar method but with a base of electrospun fiber mat to integrate hierarchical structures of nano/micro-fiber into the scaffolds. This method combines micropatterning with electrospinning, in which we are able to produce porous structure to support cell viability, as well as microchannels to direct hMSCs biological behaviour. The strategy involves the use of femtosecond laser for the fabrication of multi-scale microchannels on electrospun fiber mats. The precisely defined 3-D biomimetic structures fabricated using laser micropatterning on fiber mat also allow good mass transportation (*e.g.*, oxygen and nutrition) apart from providing the required surface topography. The better mass transportation would directly promote higher cell viability and proliferation in scaffolds. Moreover, designed micro- and nano- topography

could influence cell behaviour and induce commitment of stem cell differentiation to desired phenotypes [16, 17, 19, 96, 159, 168].

Therefore, in this part of the thesis, a multiscale hybrid scaffold has been fabricated to include the concept of both nano-architecture and microenvironment. Microchannels created by direct laser cutting, with dimension that has been proven to upregulate myogenic markers of stem cells from the previous chapter, as well as allowing cell alignment [96]. In addition, the porosity of the electrospun microfiber layer facilitated nutrient transport while the electrospun nanofiber layer encourages cellular adhesion and proliferation. This approach is highly flexible and can be tailored for various cell types by manipulating the compositions/fiber arrangements for each layer as well as incorporation of biomolecules in each layer. This model is not only significantly more practical, it also allows for fundamental studies of cell-material interaction in a more realistic environment. The precisely defined 3-D biomimetic environment will enable us to maintain and regulate cell behaviour, and have significant implications on regenerative medicine tissue engineering, e.g., on fabrication of myocardial tissue patch to treat heart infarction [169].

5.2 Materials and methods

5.2.1 Micropatterned scaffold preparation

Copolymer of L-lactide and ϵ -caprolactone (PLC) in a 70/30 molar ratio (molecular weight 202,000 g/mol, Purac Biomaterials, Lincolnshire, IL) is used as scaffold material. A series of PLC polymer solutions were prepared in solvent mixture and then electrospun using NANON-01A (MECC Co., Ltd., Fukuoka, Japan) under different processing parameters. **Table 2** shows the solution and processing variables used to electrospin the fiber mat of different fiber diameters. The thickness of the electrospun mat was in the range of 100 μm .

Table 2. Electrospinning process conditions and diameter of PLC fiber.

Scaffold type	Mean fiber diameter ^{b)} (μm)	Solvent mixture (by vol)	Concentration (wt %)	Collector distance (cm)	Voltage (kV)	Flow rate (ml/h)	Needle gauge
S1	0.6 ± 0.18	4:1 ^{a)}	10	12	15	1	21
S2	1.2 ± 0.21	7:1	12.5	15	18	5	18
S3	2.0 ± 0.59	7:1	12.5	15	18	10	18

^{a)} Solvent mixture was dichloromethane: *N, N*-dimethylformamide. For the rest of the scaffolds, solvent mixture were chloroform: *N, N*-dimethylformamide at the specified ratio (by volume). ^{b)} Measured from 50 fibers observed under SEM.

Micropatterned scaffolds were fabricated using direct laser writing technique mentioned previously [96, 99]. The laser beam has a wavelength of 800 nm, pulse frequency was fixed at 1 kHz, and the pulse width at 150 fs. For the micropatterned hybrid scaffold, it consists of two layers: a top laser-patterned nanofiber layer and a bottom microfiber layer. The fabrication of the micropatterned scaffold includes a two-step process. In brief, the top layer was firstly fabricated by electrospinning the PLC solution into nano-fiber mat,

which was then being laser cut through with various desired patterns. Subsequently, the laser-cut through patterns will form the base where further electrospinning of a mat is deposited on it. This mat will form the bottom layer. **Figure 5.1** illustrates the scaffold design and **Table 3** presents their specifications (w : channel width, d : channel depth, i : channel interval). For the quantification of fiber diameter, measurements were made from 50 fibers taken randomly in each SEM images.

Figure 5.1. Illustration of the micropatterned scaffold, (a) 3-D view and (b) section view.

Table 3. Specifications of the micropatterned hybrid scaffolds.

Scaffold type	Fiber diameter (Mean) (μm)	Channel width, w (μm)	Channel depth, d (μm)	Channel interval, i (μm)
0.6 $\cup$ 1.2 μm	Top layer: 0.6 Bottom layer: 1.2	30	10	30

5.2.2 Tensile test measurement

Samples for the tensile tests were fabricated according to ASTM D638-99 Type V. The uniaxial tensile properties of the electrospun PLC nano- to micro- fiber mats were determined using an Instron Testing Machine 5548. The stress – strain curves of the samples were obtained from the load-deformation curves recorded at a crosshead speed

of 10mm/min. Young's modulus was calculated from the initial linear region of stress-strain curves.

5.2.3 Porosity and pore size measurements

The porosity of the electrospun scaffolds was measured using liquid intrusion method as mentioned previously [170]. For this method, scaffolds ($n = 10$) were weighted prior to immersion in ethanol, and left overnight on a shaker table to allow 100% ethanol diffusion into the void volume. Then the scaffolds were taken out, gently blotted with Kimwipe and reweighed. The porosity was calculated using the equation below:

$$porosity (\%) = \frac{V_{ethanol}}{V_{ethanol} + V_{PLC}} \quad (1)$$

where

$V_{ethanol}$ is the volume of intruded ethanol, i.e. the ratio between the observed mass change after intrusion and density of ethanol, $\rho_{ethanol} = 0.789 \text{ g/ml}$, and V_{PLC} is the volume of PLC polymeric scaffold (the ratio between the initial scaffold mass before intrusion and density of PLC, $\rho_{PLC} = 1.22 \text{ g/cm}^3$).

The pore size was measured using a capillary flow porometer (CFP-1200-A, Porous Material Inc., USA) in galwick intrusion under an increasing pressure from 0 Psi to 50 Psi. In addition, the maximum pore size was determined as well.

5.2.4 Permeability measurement

Scaffold permeability was measured, following the procedure described in the work of Sell et al [171]. In brief, scaffold was placed in the specimen holder, which was

constructed from two (top and bottom) chambers. The tube extending from the top chamber was then attached to a reservoir of water pressurized with a 1.5 m pressure head. The flow rate of the solution through the scaffold was measured by taking the amount of fluid that passed through the bottom chamber of the permeability device in a given time. The scaffold permeability was calculated according to the Darcy equation:

$$\tau = \frac{Q\eta h_s}{Fp} \quad (2)$$

where τ is the scaffold permeability, Q is the volumetric flow rate (ml/s), η is the viscosity (Pa·s) of the water (8.90×10^{-4} Pa·s at 25°C), h_s is the thickness of scaffold, F is the sample cross-sectional area in the direction of flow, and p is the applied pressure head, which was determined using the following equation:

$$p = \rho gh \quad (3)$$

where ρ is the density of water (1000 kg/m³ at 25°C), g is gravitational force (9.8 m/s²), and h is the total height of the system in meters (1.5 m).

5.2.5 LIVE/DEAD staining assay

To determine cell viability of each layer on the stacked scaffold, a LIVE/DEAD staining assay was performed. Two sheets of micropatterned scaffolds were stacked within each well of the 6 well-plate. After static culturing with hMSCs for 24 hours, individual sheets were incubated for 45 mins with a 4 μ M ethidium homodimer/2 μ M calcein in culture medium (LIVE/DEAD viability/cytotoxicity Kit, Invitrogen). Images were taken by confocal laser scanning microscopy (Olympus FluoView 1000).

5.3 Results and discussion

5.3.1 Fiber mat characterization

Electrospinning is a versatile technique to fabricate porous fibrous scaffolds with large surface area to mass ratio. In general, increasing the flow rate and concentration of polymer solution yields larger fiber diameter. As shown in **Table 2**, by varying the electrospinning solution and processing conditions, scaffolds with fiber diameter ranging from approximately 600 to 2,000 nm were generated in a reproducible manner. **Figure 5.2** shows representative SEM micrographs of the three scaffolds corresponding to the process conditions listed in **Table 2**. These scaffolds consist of randomly oriented fibers, which formed highly interconnected pores within the scaffolds. The physical properties of electrospun scaffold depend on fiber chemical composition, fiber diameter and pore size being generated. As shown in **Table 4**, the difference in fiber diameter among the electrospun scaffolds led to differences in mechanical strength, pore diameter and permeability. As the fiber diameter of fabric scaffolds increases, the mechanical strength in terms of Young's modulus reduces probably due to the decrease of the fiber density as indicated by the increase of porosity. Moreover, the mechanical strength of these scaffolds is much lower than that of PLC solid film ($E = 26.2$ MPa).

Figure 5.2. SEM micrographs of electrospun PLC scaffolds consisting of (a) 0.6 μm , (b) 1.2 μm and (c) 2.0 μm fiber diameters. Scale bar = 10 μm .

Table 4. Physical properties of electrospun PLC fiber scaffolds

Scaffold Type	Mean fiber diameter (μm)	Young modulus, E (MPa)	Porosity (%)	Mean pore diameter (μm)	Max. pore diameter (μm)	Permeability (m^2)
S1	0.6	8.4 ± 0.65	55	1.2	3.7	7.8×10^{-18}
S2	1.2	4.8 ± 0.17	74	1.5	4.5	3.5×10^{-15}
S3	2.0	3.5 ± 0.19	78	2.6	7.4	5.0×10^{-15}

Scaffolds pore size and porosity also play critical roles in determining the scaffold performance as they have a deterministic effect on the cell infiltration, nutrient and waste transfer [172, 173]. Porosities of the electrospun scaffolds were measured using liquid intrusion technique and pore sizes were determined using the capillary flow porometer. It was found that both porosity level and pore size increase with fiber diameter. In this study, extrusion porosimetry was employed for pore size determination instead of mercury intrusion porosimetry, which is commonly used. The rationale behind that is that electrospun materials tend to deform or rupture when high pressures are applied during mercury porosimetry measurement. Moreover, there are reports that mentioned mercury porosimetry is not suitable for pore size measurement when the electrospun scaffolds comprise of fibers with diameter less than $3 \mu\text{m}$ [65, 170]. It should be noted that pore size determination remains a controversial issue for electrospun scaffolds for it assumes cylindrical pore geometry for pore size calculation. In reality, the pores in the electrospun scaffold are not cylindrical, but rather dynamic/polygonal in nature. Therefore, this makes the measurement of scaffold permeability more important in determining the characteristics of electrospun scaffold for tissue engineering. The permeability results are

consistent with our expectation that scaffold permeability increases with increasing pore size and porosity level of the scaffolds. The measured permeabilities of electrospun scaffold made up of both 1.2 and 2 μm diameter fibers were found to be in the order of 10^{-15}m^2 , which is comparable to the permeability values found in the literature for collagen gel and decellularized small-intestinal submucosa (SIS) ($1 \times 10^{-15} - 10^{-16}\text{m}^2$) [174]. From tissue engineering perspective, high scaffold permeability is desirable as high level of metabolic diffusion can be achieved in the scaffold, allowing increased cell proliferation and cell migration into the scaffold.

5.3.2 Fabrication of micropatterned hybrid scaffolds

This work presents a novel and cost-effective method which integrates multi-scale structures within the tissue-engineered scaffold. The fabrication of micropatterned hybrid scaffold consists of three steps that are electrospinning, laser machining and sequential electrospinning. The nanofibers were randomly electrospun on a glass slide to form a fiber mat with 10 μm thickness. After drying, the femtosecond laser was applied to machine channels on the fiber mat. Another layer of fibers ($\sim 80 \mu\text{m}$ thickness) were electrospun on the top of the micropatterned fiber mat. Then all the double-layer hybrid scaffolds were heat treated at 60°C for 30 min in the oven, to prevent delamination of those two electrospun fiber mats. As can be seen from the SEM images (**Figure 5.3**), a hierarchical multiscale topography ranging from nano-, micro- and macro-features, was achieved using femtosecond laser micromachining of the electrospun mat. The top thin layer with laser micropatterns is meant to guide cell orientation through contact guidance,

Figure 5.3. (a,b) SEM micrographs of micropatterned electrospun hybrid scaffolds on top view; (c) Cross section of micropatterned hybrid scaffolds.

as well as the smaller fiber diameter with $0.6\ \mu\text{m}$ would provide more anchor points for cell adhesion thus could maximize cell numbers. The bottom layer provides good structural support. By doing so, the overall scaffold permeability and mechanical property can be tailored by changing the fiber diameter and arrangement of the bottom layer. According to the first part of study, the porosity of electrospun mat increased with increasing fiber diameter [175], and the fiber mat with 1.2 and $2\ \mu\text{m}$ fiber diameter showed similar porosity and permeability, which was higher than the fiber mat with $0.6\ \mu\text{m}$ fiber diameter (**Table 4**). Moreover, the random fibers with a large fiber diameter will produce relatively large pores on the order of tens of microns or larger. Cells could grow into these larger pores and consequently the intended contact guidance of the microchannels to these cells would significantly decrease. Therefore the effect of these channels in inducing cell elongation would be diminished. At those size scales (larger pores on mat with $2.0\ \mu\text{m}$ fiber diameter), cells conform to the porous micro-architecture presented [105] which, does not provide the precise control needed to organize cell shape into the highly organized structures found *in vivo*. Therefore, the randomly electrospun fiber mat with $1.2\ \mu\text{m}$ fiber diameter was used as bottom layer.

5.3.3 Effect of topographical micropatterns on stem cell alignment

To evaluate the effect of hierarchical multiscale topography on stem cell growth and differentiation, we cultured hMSCs on hybrid scaffolds with laser induced micropatterns (Hybird_0.6+1.2 μm), flat solution cast films(cast PLC), electrospun fiber mat with 0.6 μm (Mat_0.6 μm) and 1.2 μm fiber diameter(Mat_1.2 μm). **Figure 5.4** shows

Figure 5.4. Phalloidin labeled F-actin (red), DAPI nuclear staining (blue) and overlaid fluorescent image of immunostained cellular components (merged) for the hMSCs on control groups and hybrid scaffolds. (Scale bar, 50 μm)

representative fluorescence images of hMSCs growing on the respective substrates after 1 day, 7 days and 14 days of culture. The F-actin and nucleus were stained with phalloidin and DAPI respectively to examine cell morphology and microfilament arrangement. Initially, the microfilaments appeared disordered and scattered in all directions when hMSCs were cultured on the planar substrates without laser patterning (cast PLC, Mat_0.6 μm and Mat_1.2 μm). After 14 days of culture, the microfilaments of hMSCs on

those three types of unpatterned substrates showed partial localised alignment without uniform anisotropic cellular organization. As compared with cells cultured on the cast films, cells on fiber mat displayed higher numbers of filopodia-like thin protrusions (**Figure 5.5**) This may be due to the extensive electrospun fiber network that provided

Figure 5.5. Phalloidin labeled F-actin (red), DAPI nuclear staining (blue) and overlaid fluorescent image of immunostained cellular components (merged) for the hMSCs on control three groups. Cell edges with more enhanced filopodia outgrowth on 0.6 μ m and 1.2 μ m fiber mats than Casted PLC. (Scale bar, 50 μ m)

guidance for cells growth [176]. A different cell behaviour and morphology was found on fibers with 0.6 μ m and 1.2 μ m diameter (**Figure 5.6**). The cell spreading area was larger on fiber mat with 0.6 μ m diameter than that of 1.2 μ m diameter. The phenomenon is even more obvious when cells were cultured on the fiber mat with fibers of 2.0 μ m diameter. It was shown that cells cultured on fiber mat with 2.0 μ m diameter would penetrate into the irregular pores, or even maintain the spherical shapes (**Figure 5.6**). This difference might be due to the fact that the diameter of nanofibers (0.6 μ m) are in orders of magnitude smaller than the cells, therefore the cells have more anchor points to support cell spreading [177-179]. In contrast, the hMSCs grown on the Hybrid_0.6+1.2 μ m lasered scaffold showed differing trends. The cells were aligned regularly along the direction of micropatterns from the first day of cell culture, and their microfilaments were uniformly well-ordered. Meanwhile, fluorescent staining revealed elongated cell shape as well as nucleus induced by the micropatterns. Those interval-formed microchannels act as

physical obstacle for cell to grow in the perpendicular direction of micropatterns. Therefore the cells would align and grow in the direction of the channels, which is consistent with the previous Chapter and also other reports done on topography induced cell alignment [150, 180-183].

Figure 5.6. A comparison of hMSCs cultured on 1.2 μm and 2.0 μm fiber mat at day 1 and day 4 to show cell morphology differences and cell penetration on 2.0 μm fiber mat.

5.3.4 Effect of topographical micropatterns on stem cell proliferation and cell viability

The effect of scaffold topography on hMSCs proliferation was evaluated via Picogreen DNA assay (**Figure 5.7**). The cell densities for flat surfaces and laser machined scaffolds were measured after 1, 4, 7, 11 and 14 days. Analysis of cell densities on solution cast film indicated that cell density increased gradually from day 1 through day 14, compared with cell densities on all other electrospun surfaces. Among those two electrospun mat controls, the Mat_0.6 μm exhibited a significantly higher cell density than Mat_1.2 μm at

all time. Interestingly, the Hybrid_0.6+1.2 and Mat_0.6 μm had comparable cell densities. The fiber mat with higher surface area available for cell growth in both cases (Hybrid_0.6+1.2 μm and Mat_0.6 μm), promoted higher levels of cell proliferations. Furthermore, a decreasing cell density with increasing fiber diameter trend was found and this finding is similar to other reports [177, 184, 185]. The smaller fiber diameter would have a higher surface area for the same volume of scaffold, which would bind more proteins from the medium for cell growth. Another possible reason is that cells need less force to migrate through the scaffolds with smaller fiber diameter [186], which is more flexible and pliable. The scaffolds with larger fiber diameter will induce more physical constrain for cell migration [187-189]. The reason why there were more cells obtained on the laser machined hybrid scaffolds might be due to the increased area compared to the flat substrates because of the microchannels. Furthermore cells cultured on the hybrid scaffolds were much more elongated and acquired cell surface area is lower resulting in a higher cell number. These data show that hMSCs proliferated on all substrates tested but the hybrid scaffolds could support the high cell numbers.

Figure 5.7. Cell numbers of hMSCs (1, 4, 7, 11 and 14 days) after seeding at an initial seeding density of 15,000 cells/cm² on scaffolds without laser machining and laser micropatterned hybrid structures (n=6).

In order to verify the hypothesis that the new hybrid scaffold could support better cell viability even with thicker 3-D structure, the laser micropatterned scaffold was stacked up to build a 3-D construct (**Figure 5.8**). In this way, a thick real 3-D scaffold with high cell density could be constructed. To do that, a double-layer stacking of micropatterned hybrid electrospun porous scaffolds and micropatterned solid scaffolds (control) [96] were used for cell culture. Both 3-D constructs have laser induced microchannels to guide cell orientation.

Figure 5.8. Illustration of the micropatterned double-layer stacking 3-D constructs.

A LIVE/DEAD staining assay was used to examine cell viability on both types of 3-D constructs after 48 hours cell culture under static condition. **Figure 5.9** presents the viability results of cells on each individual layer. These microscopy images reveal many dead cells on the bottom layer of the control scaffold as expected and is most probably due to the limited mass transfer of nutrients and oxygen inside the 3-D constructs. A high local waste product concentration by the cells on the bottom layer may cause cell death too. The cells on the top layer with direct contact with medium were able to survive. In contrast, the cells on both top and bottom layers of our hybrid porous 3-D constructs could support cell growth very well. Based on this proof-of-concept study, we were able to show the feasibility of building 3-D constructs out of the hybrid scaffold fabricated.

Figure 5.9. Typical fluorescence microscopy images of viability stained hMSCs-cultured in stacked scaffolds under static conditions after 24 hours. Green indicates living cells and red indicates dead cells.

5.3.5 Influence of topographical micropatterns on stem cell differentiation

In order to ensure that the microchannel effect on myogenesis is positive as in Chapter 4, the gene expression of hMSCs cultured on the hybrid scaffold was assessed by *qPCR* at various time points (*i.e.* 7, 11 and 14 days). The results of the relative mRNA transcript expression levels of the micropatterned cells normalized to the cast PLC control is illustrated in the form of a heat map (**Figure 5.10**). Myogenesis involves the activation of

Figure 5.10. RT-PCR analysis of the relative mRNA transcripts expression levels of the cells on day 7, day 11 and day 14. Relative gene expression levels (i.e. $\Delta\Delta CT$) were normalized to the casted PLC control (t=7 days time-point) without laser machining. The results are illustrated in the form of heat map. * represents statistical difference between experimental and control samples ($p < 0.05$, $n=6$).

myogenic regulatory factors (MRFs) such as MyoD1, myogenic factor 5 (MYF5), Myogenin and MRF-4 [190]. In our previous work, upregulation of myogenic markers and expression of myosin heavy chain (MHC) protein were found in cells cultured on

both scaffolds with micropatterned fibronectin lanes and laser micromachined channels [16, 17, 96]. As shown in **Figure 5.10**, a significant upregulation of myogenic markers (cTnI, GATA4, MHC7, MyoD1 and Nkx2.5) was observed after 11 days of culture on the laser micropatterned hybrid scaffolds as compared to the other two unpatterned electrospun fiber mats. Meanwhile, expression of osteogenic (ALPL, OCN, ON and RUNX2) and neurogenic markers (MAP2, nestin, Tubulin, and NeuroD1), were found to be downregulated or showed no significant difference compared to the unpatterned cells on cast PLC films. To verify whether the topography of electrospun fibers would affect stem cell differentiation, those two fiber mats with different fiber diameters were also chosen as controls. The upregulation of osteogenic-related genes were observed on the substrate of Mat_1.2 μm , which is consistent with other studies done on osteogenesis induction using electrospun fibers [191-194]. However, no elevations were found for both osteo- & neuro-related genes for cells cultured on micropatterned hybrid scaffolds, which implies that specific hMSCs myogenic differentiation was activated by the laser induced micropatterns, rather than the topography of fibers on hybrid scaffolds. In other words, in this study, the microchannels exerted a more dominant effect than topography on differentiation.

Confirmation of qPCR results on gene level is critical for stem cell lineage differentiation study. To supplement the differential expression of lineage specific transcripts we performed immunofluorescent staining to confirm the phenotypes and differentiation capacities of hMSCs cultured on the laser micropatterned hybrid scaffolds. Here, immunofluorescent staining of cardiac beta (β) - myosin heavy chain (MHC, a mature

marker of myogenesis) that was differentially expressed in the *qPCR* data, revealed a comparable trend on the micropatterning directed hMSCs differentiation. As shown in **Figure 5.11**, significant MHC staining was detected for cells on the laser micropatterned hybrid scaffolds, whereas unspecific/ negligible signals were stained for other three unpatterned controls after 14 days of cell culture. Collectively, these results indicated that the laser micropatterned hybrid scaffold would modulate hMSCs myogenic differentiation, even in the absence of soluble inductive factors through cell elongation and alignment, just as that reported in Chapter 4.

Figure 5.11. Representative images showing immunostaining of the myogenic marker on all substrates after 14 days cell culture. Cells were stained with a distinctive and mature marker of myogenesis using mouse monoclonal anti heavy chain cardiac myosin (green).

The laser formed micropatterns provided a “row” topographic induction of cellular organization, subsequently effecting the changes in cytoskeletal reorganization, which in turn would alter gene and protein expression profile, and consequently the fate of cells. Here the upregulation of myogenic markers caused by the hybrid scaffold is in accordance with results demonstrated in the previous chapter, where the laser induced micropatterns on solid substrate was found to alter cell morphology and subsequently induce myogenesis of hMSCs. The possible mechanism of hMSCs myogenic lineage commitment caused by the hybrid scaffold is believed to also include RhoA/ROCK pathway modulated cell contractility changes initiated by laser formed microchannels.

5.4 Summary

In this work we have used a novel hybrid platform to fabricate a scaffold that not only mimics the multiscale structures of ECM but also shown to support cells in a thick 3-D scaffold. This approach combines the strengths of the biomimicking structures of electrospinning fibers and the rapid, versatile direct laser micropatterning of femtosecond laser technique. The multiscale structures together with the optimum microchannels created by laser patterning modulated the stem cell differentiation into cardiac myogenic lineage even; in the absence of myogenic induction medium. The thick 3-D structure was then achieved by stacking the porous hybrid scaffolds and here, we have shown cells viability when two layers were stacked up. This proof of concept could be extended to more layers especially when cultured in a dynamic bioreactor which is our current work in progress. The thickness of each layer could also be modified to acquire the optimum cell-material ratio. In summary, this novel hybrid scaffold not only supports cell growth like traditional scaffolds but also elicit positive responses from the cells like lineage commitment and alignment that are essential features of future scaffolds.

Chapter 6 Conclusions and Future Directions

6.1 Conclusion

Form the literature review and the previous study in our group, it is found that cell shape may regulate stem cell differentiation through changing of underlying ECM features. Based on that, the hypothesis of this report is that it is possible to affect stem cell differentiation via topography modulation. The general objective is to use and understand how topography of microenvironment changes in micro- or nano- scale modulate stem cell growth (and differentiation). These changes were mediated by macroscopic changes in cell shape and cytoskeleton arrangement. The rapid, versatile direct laser micropatterning of femtosecond laser technique was applied to generate topography on biopolymeric matrices. This method can efficiently create micropatterns on a flat 2-D substrate and even 3-D environment as well.

In the first part of this report, intricate microchannels were achieved by direct femtosecond laser method and distributed uniformly on the solvent casted PLC films. The dimensions of laser induced microchannels were optimized to guide stem cell behavior. It has been demonstrated that laser machined microchannels could potentially drive the orientation and differentiation of cells. Myogenic differentiation of hMSCs is favored by laser induced micropatterns on PLC with narrow microchannels (20-20-20 and 30-30-20). The difference on cytoskeleton organization caused by topography of microchannels, observed on different experimental groups suggests that microfilaments rearrangement could serve as a marker during modulation of stem cell fate. To ascertain that the microchannel plays a more dominant role than substrate material type, another

material, PLGA was used. Similar results showing that the femtosecond lasered microchannel induced myogenesis confirmed our hypothesis. Furthermore, the possible molecular mechanism on stem cell myogenesis differentiation via topography of microchannels is also elucidated. The study indicates that microfilament reorganization has close relationship with stem cell myogenic differentiation, which is modulated by RhoA in this process. The RhoA/ROCK pathway is involved in cell shape elongation and myogenic differentiation caused by topography of microchannels (20-20-20 and 30-30-20).

After verifying that the microchannels were able to guide hMSCs into myogenic lineage, the study was extended to fabrication of a real 3-D scaffold that not only acts as a support but more importantly functions to guide specific stem cell differentiation. Laser machining and electrospinning were utilized to integrate macro-, micro- and nano-sized features on the scaffolds which better mimics the in vivo environment of cells. It has been shown in this study that hybrid scaffolds with spatially-patterned regions in a pre-designed fashion could guide stem cell differentiation specifically to a myogenic lineage, with additional large scale cell orientation and cell viability. Cell orientation is critical in engineering anisotropic tissues, e.g. cardiac muscle. The stacking of these micromachined porous scaffolds could provide the means to study stem cell behavior under patterned, complex microenvironments and ultimately aid in building well-designed hybrid organ/tissue constructions, e.g. cardiac patch.

The objectives to explore the critical role of topography on stem cell differentiation and guide cell behavior through incorporating topography in a real 3-D matrix were well achieved. The direct femtosecond laser machining served as a novel, fast and versatile

method to build a potential platform to manipulate stem cell differentiation via topography modulation. Also this study provided an initial understanding of mechanism on topography induced stem cell lineage differentiation. However, more intricate mechanism studies may be provided for an inclusive understanding, as well as the in vivo implantation study of laser induced spatially micropatterned 3-D scaffolds in future.

6.2 Future Directions

The novel use of the femtosecond laser for direct patterning as demonstrated in this thesis could be further improved in its optical hardware, precision motion stage and the machining environment (*e.g.* immerse in water or oil), to achieve nano-size features [195, 196]. The controlled nano-scale resolution will make this platform useful to explain some fundamental phenomenon about cell adhesion, migration, and cell division at the nano-scale level, which is beyond the scope of the thesis.

In the thesis, scaffolds with some different mechanical properties are utilized to study cell behavior. However, it will be very interesting to test this concept on materials with very low modulus (*e.g.* hydrogel) that better mimics the stiffness of natural muscle and neuronal. Using a combination of stiffness and topography, the synergistic effect of these two physical cues on stem cell growth can be explored.

The microenvironment which stem cell resides consists of soluble factor, cell-cell interactions and ECM. More and more studies have shown that physical interaction of cell with ECM may act as a primary regulator of stem cell fate. Cell-cell and cell-ECM interactions are mediated by transmembrane proteins, mainly integrins, as well as other

biomolecules (e.g. cadherins, selectins, and so on). It would be interesting to check the interplay of physical cues and biomolecules (*i.e.* growth factors) on cell behavior. Further modification of micropatterned scaffolds with chemical biomolecules (*e.g.* ligands, growth factors, et al.), as well as changing cell types would be beneficial to engineer functionalized cell/scaffold hybrid construct.

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