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Topographical cues-guided myogenic differentiation of human embryonic stem cells

A Thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Nanoengineering

by

Timothy Young Nam Seo

Committee in charge:

Shyni Varghese, Chair
Gaurav Arya
Vlado Lubarda

2015

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2015

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ABSTRACT OF THE THESIS

Topographical cues-guided myogenic differentiation of human embryonic stem cells

by

Timothy Young Nam Seo

Master of Science in Nanoengineering

University of California, San Diego, 2015

Shyni Varghese, Chair

Harnessing the matrix topography as biophysical cues has shown to control various cellular organizations, including cell adhesion, proliferation, alignment, and differentiation of stem cells. However, recapitulating topographical and spatial cues in engineering skeletal muscle tissues using human embryonic stem cells (hESCs) remains a challenge. Here, such topographical cues are created by employing the soft lithography techniques to promote topographical cues-guided *in vitro* myogenic

commitment of hESCs. Our findings indicate that hESC-derived myogenic progenitors cells cultured on micropatterned PDMS cell culture substrates could significantly mimic the hierarchically organized characteristics of myoblasts, as evident from their ability to form highly aligned multinucleated myotubes. Myogenic commitment of stem cells could possibly be achieved through the topographical cues-guided actin cytoskeleton changes and their consequent cellular alignment, which promoted the fusion of hESC-derived myogenic progenitors to form multinucleated myotubes. The results described in this study provide a proof-of-principle that matrix topology could effectively control stem cell fate by modulating cell-matrix interaction.

CHAPTER 1: Introduction

1.1 Literature Review

1.1.1 Skeletal muscle

Skeletal muscles are composed of specialized cells called muscle fibers. A single muscle fiber is formed by the fusion of multi-nucleated myoblasts. Hundreds or even thousands of cylindrically shaped muscle fibers are highly aligned and bundled together to form an individual skeletal muscle. Each muscle cell contains myofibrils, which consist of contractile proteins that have a function in generating force for muscle contraction. Muscle contraction not only enables nearly all movement in the body, but also facilitates body to produce heat to maintain body temperature [1].

Satellite cells reside periphery of muscle bundles in quiescent form and become active when muscle either gets injured or needs to be reorganized. Activated satellite cells initially proliferate as a skeletal myoblasts that undergoes dynamic process of cytoskeleton reorganization via cell-cell fusion. Previous studies have shown that the formation and dissolution of F-actin occurs at the distal ends of myoblasts that are capable of fusion, which reveals that myoblast-myoblast fusions are essential in reorganization of the cytoskeleton. Moreover, elongation of the myoblasts during alignment and fusion clarifies that myoblasts fuse in a long, linear fashion rather than into spheroid structures[2]. Clark *et al*. have shown the diameter of myotubes remained constant while the number of myotubes per track increased with track width. Thus, the uniformity of myotubular diameters explains that there is some

sort of mechanism that restricts lateral fusion while promotes fusion between distal ends of myoblasts. Muscle fibers are wrapped around by perimysium and bundles that are wrapped by perimysium are covered by epimysium to provide strength and support [3-5]. Due to highly organized hierarchical formation of muscle, muscle cells alignment in regeneration will be significant to sustain both skeletal muscle shape and function.

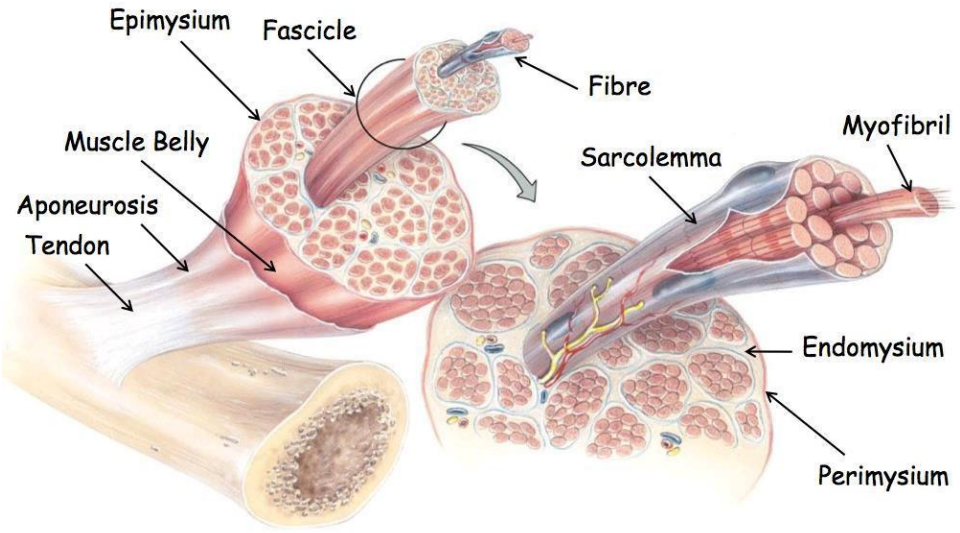

Figure 1: Composition of skeletal muscle [6].

1.1.2 Muscle wasting

As muscle holds great responsibilities of creating movement, protecting organs, pumping out blood, and aiding involuntary digestions, being able to keep healthy skeletal muscle is crucial in our daily lives. However, there are patients who suffer from muscle injuries or muscle degenerative diseases such as cachexia, sacopenia, and muscular dystrophies. Cachexia is a complex metabolic syndrome that causes dramatic loss of skeletal muscle. Cachexia is secondary to pathosis that

associated with increased morbidity and different from age-related muscle loss or starvation[7]. On the other hand, sarcopenia may occur in healthy people and cause gradual decrease in muscle mass and believe to have age-related cause[8]. Lastly, the muscular dystrophies are genetic disorder that cause progressive muscle wasting[9]. Most severe form of muscle degenerative disease is known as Duchenne muscular dystrophy (DMD) which leads to loss of skeletal muscle and eventual death [10]. Usually muscular degenerative diseases cause formation of fibrous tissue, which leads to further damage of muscle tissue by disturbing muscle from precise functioning. Due to diseases that result in severe loss of muscle tissue, studies have been focused to prevent muscle loss, provide healthy muscle cells, and retain its correct functions. In the case of DMD patients, gene therapies have been investigated to allow missing genes to express to rescue the normal phenotype[11]. Also, cell therapies have been spotlighted to rescue regenerative capacity of skeletal muscle. Due to the fact that DMD decrease the regenerative and proliferative potential of muscle satellite cells, delivering healthy myogenically committed cells may be beneficial in the process of muscle regeneration. Although researchers pledge to find complete cure to muscle degenerative diseases, current treatments have only offered to alleviate pain, decelerate symptoms, or partially restore its function [12, 13].

1.1.3 Cell-based therapy

The potential of cell therapy to cure human muscle degenerative diseases has been motivating researchers to find approved cell treatment. The ultimate goal of cell therapy is to replace unhealthy cells with healthy cells, such that newly implanted

healthy cells do adapt to the new environment efficiently and allowing proper function to the body [4, 14, 15]. There are two categories of cell types, autologous and allogeneic, that can be used for cell implantation [14, 16]. Both autologous and allogeneic cells have advantages and disadvantages. Using autologous cells, such as satellite cells, risk of immune systems recognizing implanted cells is decreasing, which may offer higher efficiency in cell implant [17, 18]. However, using autologous cells alert with difficulties in acquiring large number of cells [14, 19]. Using allogeneic cells clear out the main concern that autogenic cells have. While allogeneic cells can be used to resolve a problem associating with acquiring large number of cells, it holds other risks of being recognized by immune system or rejected by native cells and environment. Researchers are also conducting transplantation using genetically modified cells. However, limitation does also exist due to gene silencing that cause gene expressions to gradually turn off over time [17, 19-21]. Due to these problems and limitation, there is no clinically approved cell treatment yet, but studies are being conducted in various different ways to find clinical treatment to ease human suffering from muscle degenerative diseases.

1.1.3.1 Satellite cells

As one of the potential cell sources, muscle satellite cells are mononucleated cells that are located between the sarcolemma and basal membrane of the myofiber [22]. Normally these cells are presented quiescent in muscle, but spontaneously get activated in response to muscle injury and fulfill their roles in repairing and regenerating damaged muscle [22, 23]. Although, many studies have proven satellite

cells to be myogenic progenitor cells, their ability to self-renew has been debatable. Recent study has observed satellite cells supporting many rounds of muscle regeneration on a single myofiber [24]. However, other studies have stated its characteristic of lacking differentiation capacity after several cycles of cell expansion [25], which suggest that different methods in cultivating satellite cells need to be found in order to sustain their differentiation capacity. Also, providing suitable extracellular matrix is pivotal in satellite cell activation. Physical tethering of satellite cell in extracellular matrix allows the transduction of external mechanical force into internal chemical signals that activate satellite cells [26]. Although satellite cells are an ideal cellular candidate for skeletal muscle regeneration and tissue engineering, there are a couple of drawbacks that keep satellite cells from current regenerative medicine application. Resolving its difficulties in isolating satellite cells and loss of differentiation potential in *ex vivo* will contribute tremendous improvement towards regenerative medicine.

1.1.3.2 Mesenchymal stem cell

Mesenchymal stem cells (MSCs) isolated from bone marrow have shown to differentiate into the most of mesodermal lineage-specific cell types, such as bone, cartilage, and fat tissues. It has already been established that they have an ability to differentiate into skeletal muscle cells[27]. Due to its regenerative and multi-potent properties, MSCs have been proposed as a potential candidate for cell-based therapy and various applications in the field of tissue engineering and regenerative medicine. Although different studies concluded conflicting results on whether MSCs have *in*

vivo immunomodulatory functions [28-30], MSC having anti-inflammatory functions in *in vitro* has been consistently reported [31]. Also, having lack of signals that stimulate T-cells makes allogeneic transplantation possible [32]. Despite all these advantageous characteristics of MSCs, studies have pointed out some of the drawbacks of MSCs that clearly show why they can only be suboptimal choice for cell therapy.

Even though MSCs appear to avoid immune recognition, a previous clinical trial has shown immune rejection of MSCs that are cultured and expanded in the presence of FBS [33]. Furthermore, potential risk for spontaneous transformation associates with long term cultured MSC can be raised the possibility of tumor formation [34]. In this regard, use of primary MSCs in clinical trials would be the ideal solution for reducing the risk of cell manipulation; however, from bone marrow isolation, efficient number of MSCs cannot be acquired to perform clinical trials [35]. Lastly, multiple passages in culture has shown to loss its multipotency[36]. In order for MSCs to be an optimal cell source for cell therapies and tissue engineering, some of the current problems need to be resolved.

1.1.3.3 Embryonic stem cells and adult stem cells

Stem cells are a class of undifferentiated cells that have potential to develop into different cell types in the body during early stage of life and growth [37]. In addition, ability to infinitely expand without any phenotypic alteration as well as capacity to differentiate into all specialized cell types propose extensive utility in

applications from cell-based therapies [38, 39] to model to screen new drugs [40, 41]. There are two general types of stem cells exist. While inner cell mass of blastocysts can provide a source to isolate embryonic stem cell, adult stem cell can be isolated from various tissues [37, 42]. In mammalian organisms, ability of stem cells that can be differentiated into specialized cell types serves as an internal repair system for the body. In addition, in some adult tissues, such as muscle and bone marrow, population of adult stem cells provide to replenish other cells that are lost or damaged through daily wear, diseases, or injuries. As unspecialized cells go through stages of differentiation, each stages commits cells more into specialized cell type [43]. Thus, current research continues to advance knowledge in different stages of stem cell differentiation and their properties. Due to their great potential in regenerative abilities, stem cells suggest an ideal treatment for many different diseases, such as heart disease, muscle related disease, and even diabetes [17-19, 44].

Muscle cells or myoblasts are one of the cell types that can be differentiated from embryonic stem cells [45]. Even though embryonic stem cells have ability to proliferate indefinitely, effective use of stem cells requires differentiation of myogenic progenitors in high yield population. Due to the fact that embryonic stem cells have ability to differentiate into all three germ layers, controlling their defined differentiation *in-vitro* may be tricky and difficult. Over the past decade, scientific inquiries have been made to elucidate various efforts to derive such progenitors. Various approaches, such as genetic manipulation, protein and drug treatments, and material cues, have been made to acquire higher results in muscle progenitor cell

population [46-49], detailed mechanics of generating myogenic progenitor cells are still not fully understood. However, such a great potential holds with hESCs in cell therapy regeneration treatment that scientists are making remarkable progress to extend knowledge on hESCs. Boettiger *et al*. have shown the factors that determine between myoblast proliferation and differentiation. In addition, they have demonstrated how muscle cells are being developed and adhered under certain growth factors. Utilizing current knowledge may enhance efficacy of myoblast differentiation *in vitro*. Successfully differentiated myoblast from hESCs will play critical role in engrafting into *in vivo* model. Hwang *et al*. have shown an engraftment of myogenic progenitor cells in a cardiotoxin injury model to develop a proof-of-concept in cell therapy [50]. *In vitro* culture of hESCs seems to play a crucial part in determining efficacy of cell engraftment. Thus, developing the concrete methods to direct hESCs differentiation *in vitro* and engraftment *in vivo* can disclose the huge gap in resolving various muscle diseases.

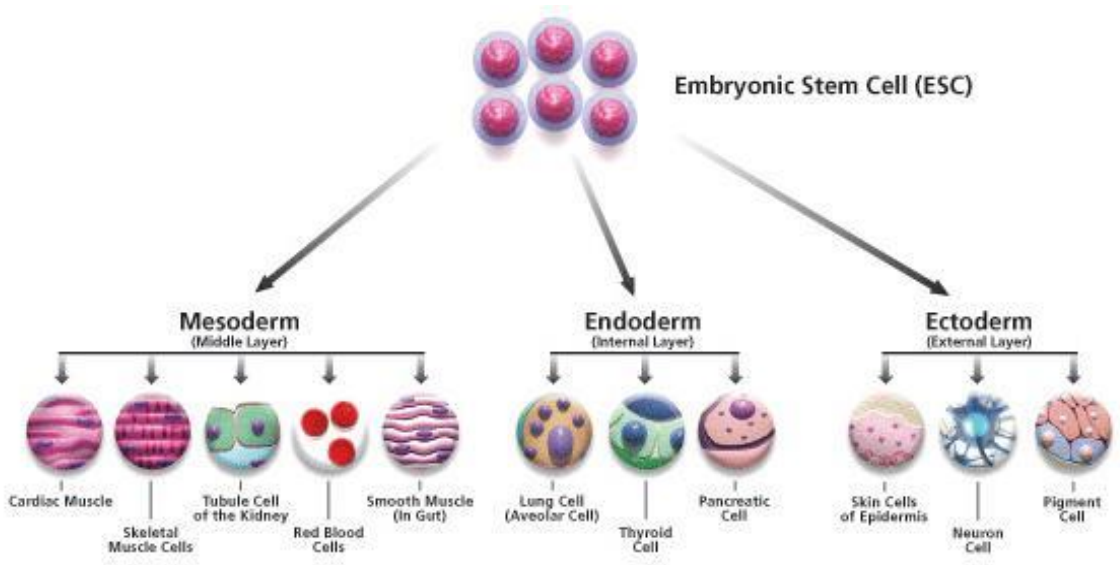

Figure 2: Isolation, cultivation, and differentiation potential of ESCs [51].

1.1.4 Extracellular matrix

As microenvironment is extremely crucial to determine the cell physiology and their fate [52], studies have been conducted to elucidate their relationship. Microenvironments consist of Extracellular matrix, soluble cues, and neighboring cells. Florini *et al*. has shown soluble cues, such as FGF, TGF-β**,** and IGF can promote expression of myogenic regulatory factor genes that advocate myogenic differentiation. In addition, studies have shown that skeletal stem cell from bone marrow can be differentiate into various cell types, including osteoblasts, chondrocytes, adipocytes, and smooth muscle cells, using of certain differentiation factors. There are common soluble factors such as dexamethasone, N2 supplement, and hydrocortisone that influence cell behavior and differentiation [53]. Use of dexamethasone in myoblast culture *in vitro* has been shown to increase in mRNA expression level of myogenic

factors, including MyoD and Myf5 [54, 55]. N2 supplement, more commonly used in neural differentiation, has been shown to promote MyoD, MF20, and MyoG under serum-free conditions [56]. Hydrocortisone is a medication for cortisol, a steroid hormone. Fetal human myoblast treated with Hydrocortisone have shown to shorten their replication time, thus leading to higher proliferation rate in prior to differentiation [57]. Also, another effect has shown to inhibit the bone formation to advocate in controlling myogenic differentiation [58].

Extracellular matrix (ECM) is the complex network of biomolecules residing in the space between cells and tissues. There are numerous studies that show essential roles of extracellular matrix in regulating cell behavior and function. ECM contains proteins, such as collagen, laminin, and fibronectin, which play crucial roles in cell migration and proliferation [59, 60]. For instance, fibronectin and other proteins bind to cell surface receptors called integrins and bridge cell-extracellular matrix interaction [61]. Integrins allow the cell to integrate changes occurring microenvironment and inside the cells by transmitting signals [62]. Extracellular matrix can be modified and adjusted in different arrangements to stimulate cells to trigger different signal pathways, lead changes in protein formations inside the cells, thus result changes in cell function and behavior. Also, embedding the matrix with growth factors enables it to serve as a reservoir. Thus, in the presence of matrix, the cells are allowed to proliferate better with enriched growth factors that are composite within ECM.

Skeletal muscle tissues exceedingly depend on ECM for structural integrity and mechanical function [63]. Hierarchical structures of skeletal muscle are possible to maintain its form due to the ECM components in the muscle. Although many proteins reside in skeletal muscle ECM, majority of ECM proteins is composed of collagen, which accounts for up to 10% of dry weight of muscle mass[64]. Some studies claimed that presence of collagen type I is predominant in perimysium, whereas collagen type III is more evenly distributed between endomysium and epimysium. However, difficulties in isolating certain regions of skeletal muscle ECM make this claim uncertain.

Also, interaction between muscle and ECM is crucial in obtaining mechanical function of skeletal muscle. Mutations in dystrophin-glycoprotein complex, which encodes molecular components involved in linking the intracellular cytoskeleton and ECM, leads to loss of structural integrity and failure in mechanical functions.

In addition to extracellular matrix, topographical cues play a critical role in cellular behavior, especially in modulating differentiation. In the human body, there are numerous different types of specialized cells, including muscle cells, bone cells and adipose cells. As skeletal stem cells can be differentiated into various cell types by conditioning using different induction mediums, there are convincing evidence that show topographical cues alone can accomplish the same effect. Topography-mediated changes in cell adhesion, migration, and proliferation have been shown to affect various cellular processes, thus modulating differentiation via altering cell signaling [65]. In skeletal precursor muscle cell cultivation, topography plays major role in

mimicking the natural skeletal muscle morphology. Although skeletal precursor muscle cells can be cultivated *in vitro* without any topographical cues, it will be differentiated into unorganized manner that leads to nonfunctional myotubes [66]. Topographic modulation is shown to be extremely effective in physical confinement that directs cells into single alignment. The microtopography does not only accommodate initial template for cell alignment, but also supports fusion and differentiation. Cultivating under a microtopographic environment, the cells are shown to adapt to given environment by undergoing a remodeling process. The contact guidance of micropattern leads directional cell growth and permits cells to proliferate along the edge. Spatial confinement that controls cell-cell contact in microtopographic play crucial roles in skeletal development and myogenic commitment. Directing cell alignment results in higher efficiency of singly oriented myotube formation, and it affects functionality of myotubes. Hence, providing microtopographic environment is essential to reach the optimal goal of using *in vitro*-differentiated myogenic committed cells in cell therapy.

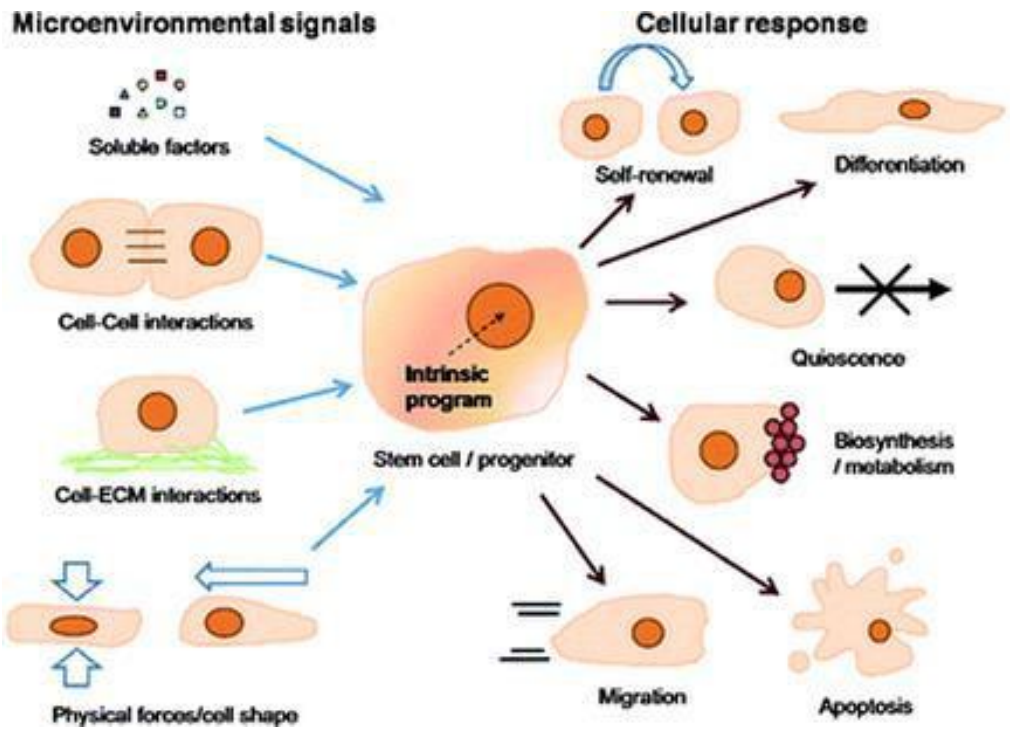

Figure 3: Various cues affecting cellular response [67].

1.1.5 Myogenesis

Skeletal muscle forms from embryonic somites, where various cues from tissues surrounding somites induce the formation of muscle precursor cells. These myogenic-committed cells then migrate to target muscle and initiate muscle growth or regeneration. In myogenic differentiation, as proliferation stops due to cell cycle arrest, genes related muscle function start to increase gradually, which lead to myoblast fusion, giving rise to multinucleated myofibers [68]. Formation of myoblasts and multinucleated myofibers initiate in response to injury or other stimuli, but this process can also be done *in vitro* using well-established models. As myogenic regulatory factors allow a series of transcriptions, it induces non-muscular cells to

commit to myogenic differentiation. MyoD is a basic helix-loop-helix (bHLH) transcription factor that closely associated with Myf5, myogenin, and other MRF proteins [69, 70]. As MyoD and Myf5 determine myogenic lineage of cell commitment, myogenin and Myf5 induce early staged myogenic committed cells into more matured muscle cells. MyoD cooperates with myogenin to regulate gene expression for terminal differentiation [71]. Beyond these general steps, scientific knowledge is somewhat fragmentary. Mal *et al*. has suggested more complex role of MyoD by pointing out this factor being transcriptional repression as well as activation. Knowledge in how functional redundancy occurs within MRF family and how functional impact of binding by individual MRF family member to their target will extensively clarify muscle differentiation as well as muscular regenerative mechanisms in response to injuries or damages.

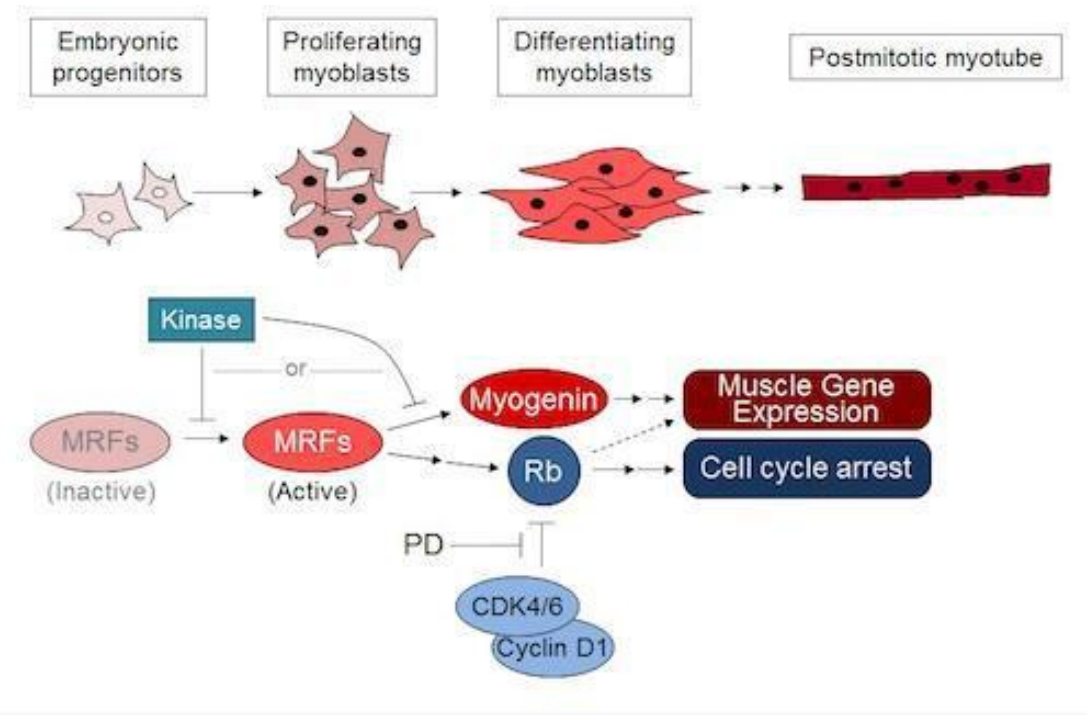

Figure 4: Simplified diagram of myogenesis [6].

1.2 Thesis objective

The main objective of this thesis is to gain knowledge and insights into topographical cues that direct cellular alignment and understand essential effects of cell alignment on proliferation and differentiation.

In this study, we have investigated the effect of topographical cues on myogenic commitment of hESC-derived progenitor cells. Specifically, we hypothesized that by controlling the cellular alignment through the micropatterned PDMS substrates, these mesodermal progenitor cells could be aligned and fused to form multinucleated myotubes, which would consequently enhance *in vitro* myogenic differentiation of hESC-derived mesodermal progenitor cells.

1.3 Experimental Setup

Desired number of cells and pattern molds need to be prepared in order to initiate this experiment.

- hESC-derived progenitor cells, PDGFRá+, were plated and proliferated on the gelatin-coated petri-dish to provide enough cell numbers.
- 3 different dimensions of PDMS molds were made using silicon-wafer pattern and subsequently washed 3 times per day using sterile PBS for a week to ensure the sterile environment for cell culture.
- Prior to cell seeding, PDMS molds were coated with Matrigel to facilitate initial cell attachment and cell proliferation.

- Samples were collected on Day 7, 14, and 21 in order to precede analysis that will determine the effects of topographical cues.

To analyze the efficacy of myogenic differentiation, quantitative PCR and immunofluorescent staining was performed using myospecific primers and myogenic markers.

- Using samples collected on Day 7, 14, and 21, qPCR was performed to compare quantitative date of RNA levels of myogenic differentiation in 3 different dimensioned PDMS mold. Myogenic-expressed primers, such as MyoD, Desmin, MyoG, and MyHC1, were used to determine phase of myogenic commitment of hESC-derived cells.
- Using samples collected on Day 14 and 21, immunofluorescent staining was performed to visualize hESC-derived cells that were committed to differentiate into myogenic lineage. Primary antibodies for myogenic markers, such as MF20 and Desmin, were used to determine between myogenic committed and non-myogenic committed population among the culture. The overall number of cells in culture was determined using nuclear staining (Hoechst).

1.4 Overview of the dissertation

Chapter 1 offers up to current literature reviews on skeletal muscle as well as corresponding diseases and current progress on cell-based clinical trials.

Chapter 2 describes materials that were used in this study as well as various methods that were used to analyze data acquired from different experiments.

Chapter 3 states experimental results as well as the design, development, and characterization of silicon-wafers of varying dimensions and mold using PDMS to define the structure.

Chapter 4 discusses analyzed data and compares the results to initial hypothesis that was made.

Chapter 5 concludes the dissertation and suggests potential future direction of research.

CHAPTER 2: Materials and Methods

2.1 Maintenance of human embryonic stem cells (hESCs)

The HUES9-OCT4-GFP reporter cell line was generated as described earlier [72]. Human embryonic stem cells, HUES9, were maintained on mitotically inactivated MEF (mouse embryonic fibroblast) feeder cells with growth medium [Knockout DMEM supplemented with 10% KSR (knockout serum replacement), 10% human plasmanate (Talecris Biotherapeutics), 1% NEAA (non-essential amino acids), 1% penicillin/streptomycin, 1% Gluta-MAX, and 55 μM 2-mercaptoethanol in the presence of bFGF (30 ng/mL, added daily)] [72]. HUES9 cells were enzymatically dissociated with Accutase (Millipore) and routinely passaged when they were reached to ~80% confluency.

2.2 Derivation of PDGFRA$^+$ mesoderm progenitors from hESCs using FACS

The PDGFRA$^+$ mesoderm progenitors, having *in vitro* myogenic potential, from undifferentiated hESCs were derived according to the previous report [50]. Briefly, undifferentiated colonies of HUES9-OCT4-GFP cells were enzymatically dissociated into single cells by incubating cells with Accutase for 5 mins. Then, 1.0×10^6 of HUES9 cells were seeded into the each well of the ultra low attachment 6-well plates and they were cultured in suspension to form embryoid bodies (EBs) in a 37^oC/5% CO_2 incubator for 9 days. Cells were cultured with the induction medium [high glucose DMEM with 5% FBS, 2 mM L-glutamine, 100 nM dexamethasone, 100 μM hydrocortisone, 1% penicillin/streptomycin, 10 μM transferrin, 860.9 nM recombinant insulin, 20 nM progesterone, 100.1 μM putrescine, and 30.1 nM selenite (Life Technologies)] and the medium was changed every other day. Next, the EBs in each well were transferred to a Matrigel-coated 10 cm dish (1:25 diluted in KnockOut DMEM; BD Biosciences), and they were cultured in the afore-described medium. The attached cells to the Matrigel-coated dishes were cultured for another 7 days to allow a

significant number of cells to be migrated out from the EBs. The cells migrated from the EBs were enzymatically detached with Accutase and they were filtered with a cell strainer having a pore size of 40 μm to achieve $PDGFRA^{+}$/$OCT4\text{-}GFP^{-}$ single cells by fluorescence-activated cell sorting (FACS).

2.3 Fluorescence-activated cell sorting (FACS) analysis

To achieve $PDGFRA^{+}$ mesoderm progenitor cell populations, the hESC-derived cells were enzymatically detached using Accutase (Millipore), resuspended in a buffer solution containing 2% FBS and 0.09% sodium azide in DPBS (BD Biosciences). These cells were stained with either Alexa Fluor 647-conjugated PDGFRA or Alexa Fluor 647-conjugated mouse IgM,κ isotype control antibodies (Biolegend) on ice for 30 minutes. Then, cells were washed with fresh PBS for three times, resuspended in the buffer solution, and followed by cell sorting using BD Biosystems FACSCanto. Data were analyzed with the CellQuest Pro software.

2.4 Fabrication of micropatterned PDMS substrates

The standard soft lithography techniques for fabricating the Si master molds were used to generate micropatterned PDMS substrates (Sylgard 184 silicone elastomer, Dow Corning), which procedures was summarized in Fig. 1A, as previously described [73]. Briefly, the Si wafers were spin-coated with SU-8 2050 negative photoresist (PR) polymer (Microchem Corp.) at 2500 rpm for 60 s to get a thickness of ~ 60 μm, followed by soft-baking at 95°C for 9 mins. To create microgrooved-patterns, the negative PR-coated Si wafer was then exposed to UV at 331W for 15 s under a photomask using a Karl Suss MA6 Mask Aligner. Subsequently, the Si wafer was hard-baked at 95°C for 7 mins, and micropatterns

were developed in SU8 developer solution (Microchem Corp.) for 5 mins. Next, the solution containing SYLGARD 184 Silicon Elastomer Curing Agent and Elastomer Base was mixed in 1:10 ratio, poured onto the Si master mold, and then degassed for approximately 30 mins. Once all the bubbles were disappeared from the solution, Si mold with PDMS solution was placed in 80°C oven for overnight. The micropatterned PDMS substrates were peeled off from the Si wafer, further sterilized in ethanol for 30 minutes, and washed with sterile PBS (2% Pen Strip) 3 times a day for a week.

2.5 Surface characterization of the micropatterned PDMS substrates by SEM

Prior to the SEM imaging, samples were sputter-coated with Ir for 7 s (Emitech K575X Sputter Coater), and the surface topography of non-patterned and micropatterned PDMS substrates were examined using scanning electron microscopy (Philips XL30 ESEM).

2.6 Myogenic differentiation

Prior to the cell seeding on either non-patterned or micropatterned PDMS substrates, these PDMS substrates were cut into the circular shape having a diameter of 22 mm, sterilized with 70% ethanol, thoroughly washed with fresh PBS, and then placed in 12-well tissue culture plates (TCPS). To promote cell adhesion, these PDMS substrates were coated with growth factor-reduced Matrigel (1:25 diluted in DMEM; BD Biosciences) as previously reported [74]. For *in vitro* myogenic differentiation, $PDGFRA^{+}$ cells were plated onto non-patterned and micropatterned PDMS substrates at an initial cell density of 1×10^{4} cells/cm^{2} and cultured in an induction medium (high glucose DMEM supplemented with 2 mM L-glutamine, 100 nM dexamethasone,

100 µM hydrocortisone, 1% penicillin/streptomycin, 10 µM transferrin, 860.9 nM recombinant insulin, 20 nM progesterone, 100.1 µM putrescine, and 30.1 nM selenite with 10% FBS). The induction medium was changed every other day during their differentiation period.

2.7 Immunofluorescence staining

Cells cultured on either non-patterned or micropatterned PDMS substrates were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature, permeabilized, blocked with blocking solution containing 0.1% (v/v) Triton X-100 and 3% (w/v) bovine serum albumin (BSA) in PBS for 1 hour. To evaluate the extent of *in vitro* myogenic differentiation, samples were incubated with following primary antibodies for overnight at 4°C: MF20 (1:200; Developmental Studies Hybridoma Bank) and Desmin (DES) (1:250; Abcam). After washing with fresh PBS 3 times, samples were incubated with following secondary antibodies: goat anti-mouse Alexa 488 (1:250; Life Technologies) and goat anti-rabbit Alexa 546 (1:250; Life Technologies) for 1 hour at room temperature. To assess the cytoskeleton morphology and cellular alignment of cells cultured on either non-patterned or micropatterned PDMS substrates, samples were incubated with phalloidin Alexa-Fluor 488 (1:100; Life Technologies) for 1 hour at room temperature. For staining the nuclei, Hoechst 33342 (2 µg/ml; Life Technologies) was used for 15 mins at room temperature. All images were acquired using a fluorescence microscope (Carl Zeiss; Axio Observer A1).

2.8 2D Fast Fourier Transform (FFT) analysis

Two-dimensional Fast Fourier Transform (FFT) image processing technique was used to characterize the differences in cellular alignments of myogenic progenitors cultured on both the non-patterned and micropatterned substrates, as previously described [75]. Briefly, ImageJ software was used to convert raw images of F-actin staining into the 8-bit greyscale TIFF formats and these images were cropped into 1024 by 1024 pixels. The cropped images were then converted to the frequency domain by FFT transformation, rotated by 90 degree, and analyzed by an oval profile plug-in to conduct a radial summation of the pixel intensities from 0 to 360°.

2.9 Image analysis

Cellular alignment and orientation of $PDGFRA^{+}$ cells cultured on either non-patterned or micropatterned PDMS substrates were determined by using NIH ImageJ software as previously reported [76-78]. Briefly, the alignment of each nucleus was quantified based on the orientation of the major elliptic axis of each nucleus with respect to the horizontal axis. Then, these angles of each nucleus were normalized to the mean orientation angle of all nuclei, and the frequency of alignment angles was calculated in 10 degree increments to their mean orientation. The nuclear shape index was quantified as aspect ratio, defined as the ratio of major axis to minor axis of each nucleus, to evaluate the elongation of nuclei. To evaluate the extent of myogenic differentiation of $PDGFRA^{+}$ cells cultured on either non-patterned or micropatterned PDMS substrates, the fraction of MF20-positive cells to the total number of cells from four random fields of view, defined as the differentiation index, was calculated.

Additionally, the fraction of multinucleated myotubes containing 3 or more nuclei to the total number of MF20-positive cells was presented as the fusion index [79].

2.10 RNA isolation and quantitative PCR

Total RNA was extracted from three biological replicates with TRIzol (Invitrogen), and reverse transcription was carried out by using iScript cDNA synthesis kit (BioRad) following by the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was carried out by using SYBR Select Master Mix (Life Technologies) on ABI Prism 7300 Sequence Detection System (Applied Biosystems). The expression level of target genes was normalized to GAPDH expression as reference and delta Ct values were calculated as $C_t^{target} - C_t^{reference}$, and the relative fold inductions were calculated as $2^{-\Delta\Delta Ct}$ [80]. The primers used in this study are listed in Supplementary Table S1.

2.11 Statistical analysis

All values are presented as mean ± standard deviation and statistical significance was determined by two-tailed unpaired Student's t-test or single-factor analysis of variance (ANOVA) with Tukey's Multiple Comparison Test (*p<0.05, **p<0.01, and ***p<0.001). GraphPad Prism software was used to perform all the statistical analysis.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Professor Shyni Varghese and Dr.Hwang initiated the study, writing, and analysis. The thesis author was the co-author of this material

CHAPTER 3: Results

3.1 Topographical and spatial properties of micropatterned PDMS substrates for cell culture

To control cellular organizations, such as alignment, fusion, and myotube formation, of hESC-derived myogenic progenitor cells, we have fabricated three different topographical features on the PDMS substrates by utilizing the standard soft lithography techniques, and their fabrication schematics is presented in Fig. 1A. Scanning electron micrograph images (Fig. 1B) illustrate these features including non-patterned PDMS substrate (left), microgrooved-patterned PDMS substrates either having a width of 100 μm (middle) or 200 μm (right), while keeping the same depth of 60 μm and ridge/spacing of 50 μm.

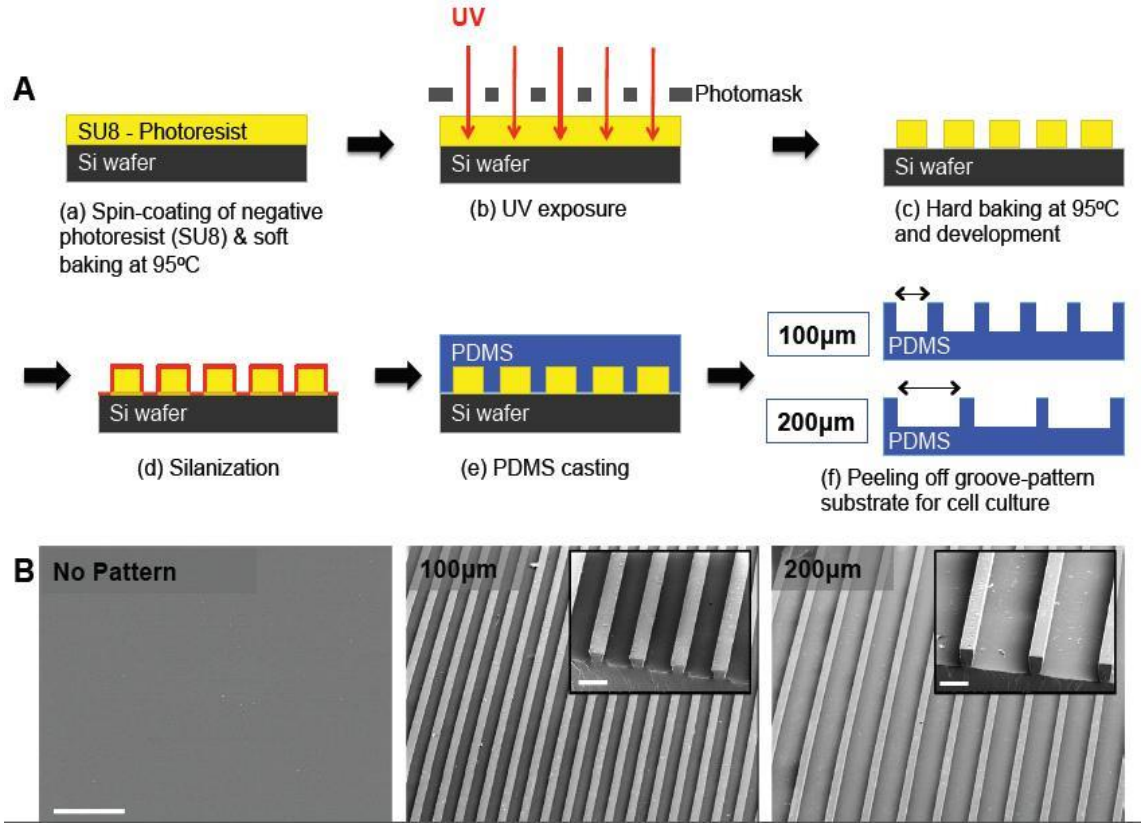

Figure 5: Fabrication and characterization of micropatterned PDMS cell culture substrates using soft lithography techniques. **(A)** Schematic illustration of standard soft lithography procedures for fabrication of micropatterned PDMS cell culture substrates. **(B)** Surface characterization of PDMS cell culture substrates using scanning electron microscopy (SEM). SEM images show non-patterned (left) and micropatterned substrates (100 µm grooves: middle; 200 µm grooves: right). Corresponding higher magnification SEM images were also presented in the insets. Scale bars = 500 µm and 100 µm (inset), respectively.

3.2 Cell adhesion, proliferation, and morphological changes of hESC-derived myogenic progenitor cells on micropatterned PDMS substrates

Previously, we have demonstrated that hESC-derived mesoderm progenitor cells expressing PDGFRA could undergo myogenic differentiation without any genetic modifications [50]. First, to determine whether PDMS substrates with various topographical features could support the cell adhesion, we have assessed their potential as cell culture substrates by seeding hESC-derived myogenic progenitor cells into the matrigel-coated micropatterned PDMS substrates. As shown in Fig. 2, hESC-derived myogenic progenitor cells were well attached to the matrigel-coated PDMS substrates, exhibited spindle-like shapes irrespective to the surface topologies, and their subsequent morphological changes/differences were further evaluated as a function of culture time. Cells cultured on non-patterned PDMS substrates were proliferated and their cellular alignment/elongation showed a randomly oriented manner. On the other hand, cells cultured on micropatterned PDMS substrates were able to proliferate and they became parallelly aligned/elongated along with the direction of microgrooves; especially, the micropatterned surface having a narrow width (~ 100 μm) seems to support better cellular organizations based on the phase-contrast images (2nd row in Fig. 2).

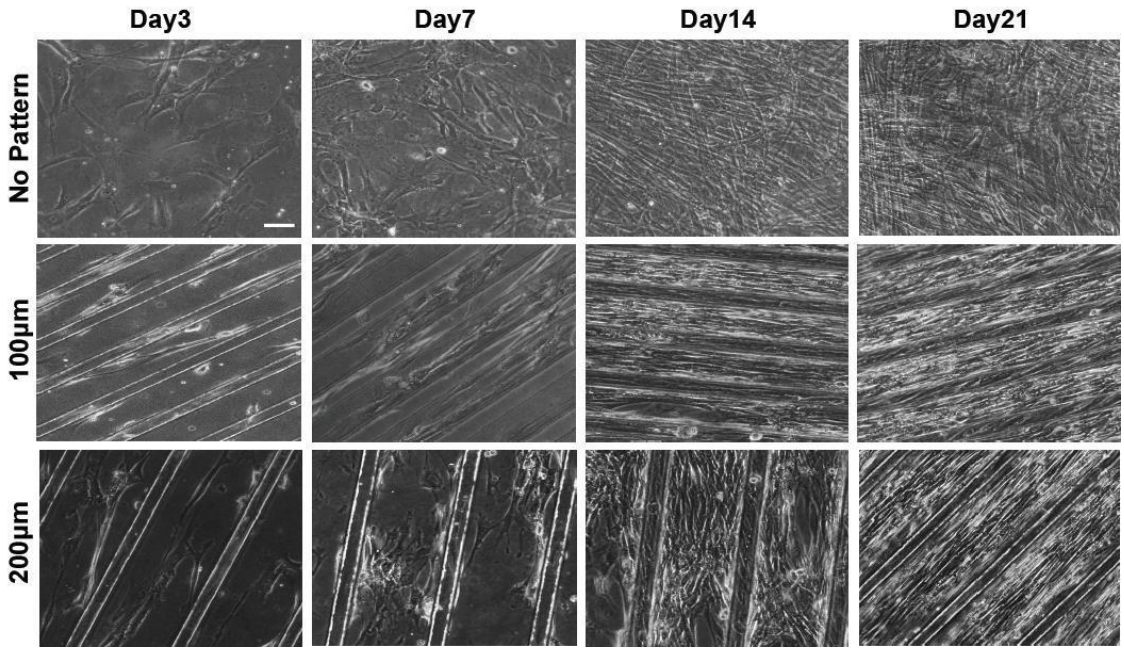

Figure 6: Characterization of cellular morphology of PDGFRA^{+} cells cultured on PDMS cell culture substrates with various topographical features.

Phase contrast images of PDGFRA^{+} myogenic progenitor cells undergoing myogenic differentiation at different time points (top row: non-patterned substrate; middle row: micropatterned substrates having 100 µm grooves; bottom row: micropatterned substrates having 200 µm grooves). Scale bar = 100 µm.

3.3 Matrix topography-mediated cytoskeletal changes and alignments of hESC-derived myogenic progenitor cells

To further explore whether topographical cues could promote the cellular alignment of attached hESC-derived myogenic progenitor cells, their actin cytoskeleton morphology and cellular alignments were examined after 21 days *in vitro* cultivation on both non-patterned and micropatterned PDMS substrates. F-actin staining as shown in Fig. 3 clearly demonstrated that cells cultured on non-patterned PDMS substrate showed randomly oriented and multidirectional actin cytoskeleton with thin stress fibers (1st row in Fig. 3). On the contrary, actin cytoskeleton structures of cells cultured on the micropatterned substrates manifested the parallel, unidirectional organization along with the microgrooves and exhibited actin-rich cytoplasmic polarization (2nd & 3rd rows in Fig. 3).

In addition to the change of actin cytoskeleton morphology, we also performed quantitative analysis of nuclear orientation of cells cultured on both non-patterned and micropatterned PDMS substrates using NIH ImageJ software as previously demonstrated [81]. As shown in Fig. 3B, quantitative analysis revealed that introducing topographical cues, either using 100 μm or 200 μm microgrooves, significantly increased the nuclear alignment of hESC-derived myogenic progenitor cells. In a microgroove with narrow width (100 μm), approximately 88.3% cells were aligned and oriented within 20° of the mean orientation angle of all nuclei. When the width of microgroove was increased from 100 μm to 200 μm, we noticed slight decrease of cellular alignment, however, we have observed the majority (80.7%) of

cells was still aligned to the mean orientation angle. In contrast, cells cultured on non-patterned substrates showed only small amount of cells (32.4%) were aligned and we found most of cells were randomly oriented, showing similar distribution within 0~90° angles.

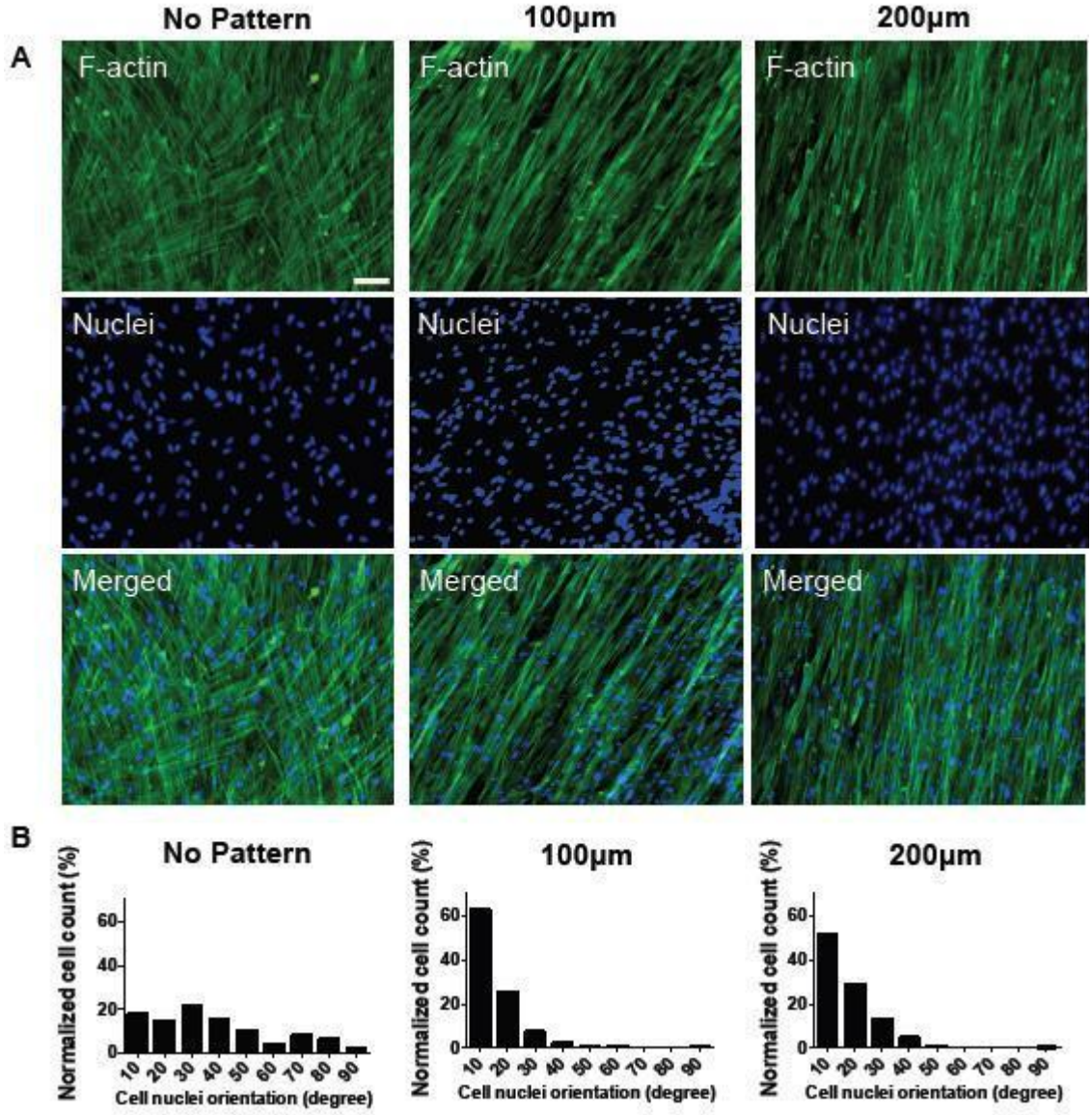

Figure 7: Topological cues-guided cellular alignment of PDGFRA$^+$ cells

(A) F-actin immunofluorescence staining of PDGFRA$^+$ myogenic progenitor cells cultured on PDMS cell culture substrates with various topographical features with an induction medium for 21 days. (left column: non-patterned substrate; middle column: micropatterned substrates having 100 μm grooves; right column: micropatterned substrates having 200 μm grooves). Scale bar = 100 μm. **(B)** Alignment and orientation of PDGFRA$^+$ cells cultured on PDMS cell culture substrates with various topographical features.

The cellular alignment of hESC-derived myogenic progenitors cultured both on the non-patterned and micropatterned substrates was also evaluated by 2D Fast Fourier Transform (FFT) analysis on the F-actin staining images. These F-actin staining images (Fig. 4A) were converted into the FFT frequency plots (Fig. 4B), which showing patterns with grayscale pixels distributed around the origin, and these plots depict the degree of cellular alignment. In case of cells cultured on the non-patterned substrates, their FFT frequency plot showed a symmetrical, circular projection at the origin (Fig. 4B; left), which is typical characteristics of random distribution of pixel intensities, and their corresponding FFT alignment histogram showed many low frequency peaks (Fig. 4C; left) within $0\text{-}360^{o}$. In contrast to the non-patterned substrate, the FFT frequency plots converted from the F-actin staining images of cells cultured on micropatterned substrates showed a uniaxial, concentrated ellipse shape along with the specific axis (Fig. 4B; center and right). In addition, their resulting FFT alignment histograms demonstrated two distinctive peaks with the high frequency, indicating a higher degree of cellular alignment on cells cultured on both micropatterned substrates (Fig. 4B; center and right).

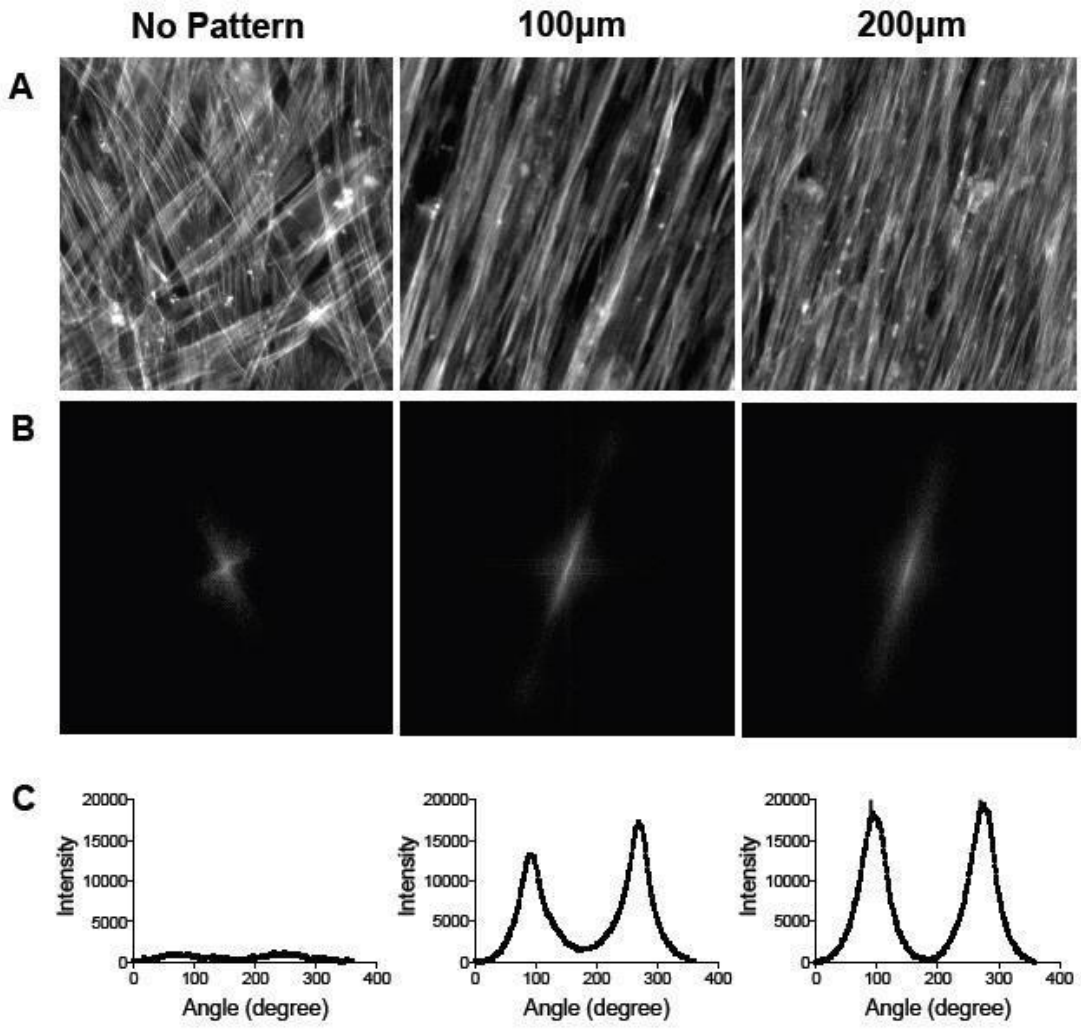

Figure 8: 2D Fast Fourier Transform (FFT) analysis. (A) Representative F-actin staining images of PDGFRA^{+} myogenic progenitor cells cultured on PDMS cell culture substrates with various topographical features (left column: non-patterned substrate; middle column: micropatterned substrates having 100 μm grooves; right column: micropatterned substrates having 200 μm grooves) used for the 2D FFT analysis. **(B)** Frequency domain plots analyzed by the 2D FFT transformation. **(C)** FFT alignment histogram generated by a radial summation of the pixel intensities from 0 to 360°.

3.4 Effect of topographical cues on myogenic differentiation of hESC-derived mesoderm progenitor cells

Next, to examine whether the topographical cues-guided cellular alignment could promote the myogenic differentiation of hESC-derived progenitor cells, the cells were cultured on micropatterned substrates (both 100 and 200 μm grooves) and compared against those cultured on non-patterned substrates. As evident from the upregulated gene expressions of myogenic markers, including MYOD, DES, MYOG, and MYH1, we observed higher degree of myogenic commitment of cells cultured on micropatterned substrates as a function of culture time compared to their counterparts (Fig. 5). These results were also corroborated by the immunofluorescence staining of sarcomeric myosin (MF20) and desmin (DES), which are usually found in terminally differentiated myoblasts, as shown in Fig. 6. The cells cultured on micropatterned substrates for 21 days were highly aligned along axis of microgrooves and we found significantly larger numbers of cells were fused to form multinucleated myotubes. We further examined the various cellular shape analyses on cells cultured on different topographical features and they were presented in Fig. 7. Nuclear shapes were analyzed to calculate the aspect ratio, determined by the ratio of major axis of nuclei to the minor axis of nuclei, and we found that cells cultured on the micropatterned substrates having 100 μm microgrooves became slightly more elongated nuclei compared to their counterparts and the other micropatterned substrates (200 μm microgrooves). The calculated differentiation index, indicating the fraction of differentiated ($MF20^{+}$) cells, also showed that a significantly higher number of cells

on both micropatterned substrates underwent terminal myogenic differentiation and it was much evident on cells grown in the microgrooves with the narrow width (100 μm). In addition, we evaluated the fusion index, which is the representation of $MF20^{+}$ multinucleated myotubes having more than 3 or more nuclei, and we observed most of the cells cultured on micropatterned substrates were fused to form multinucleated myotubes, whereas cells cultured on non-patterned substrates showed randomly oriented and less number of multinucleated myotubes.

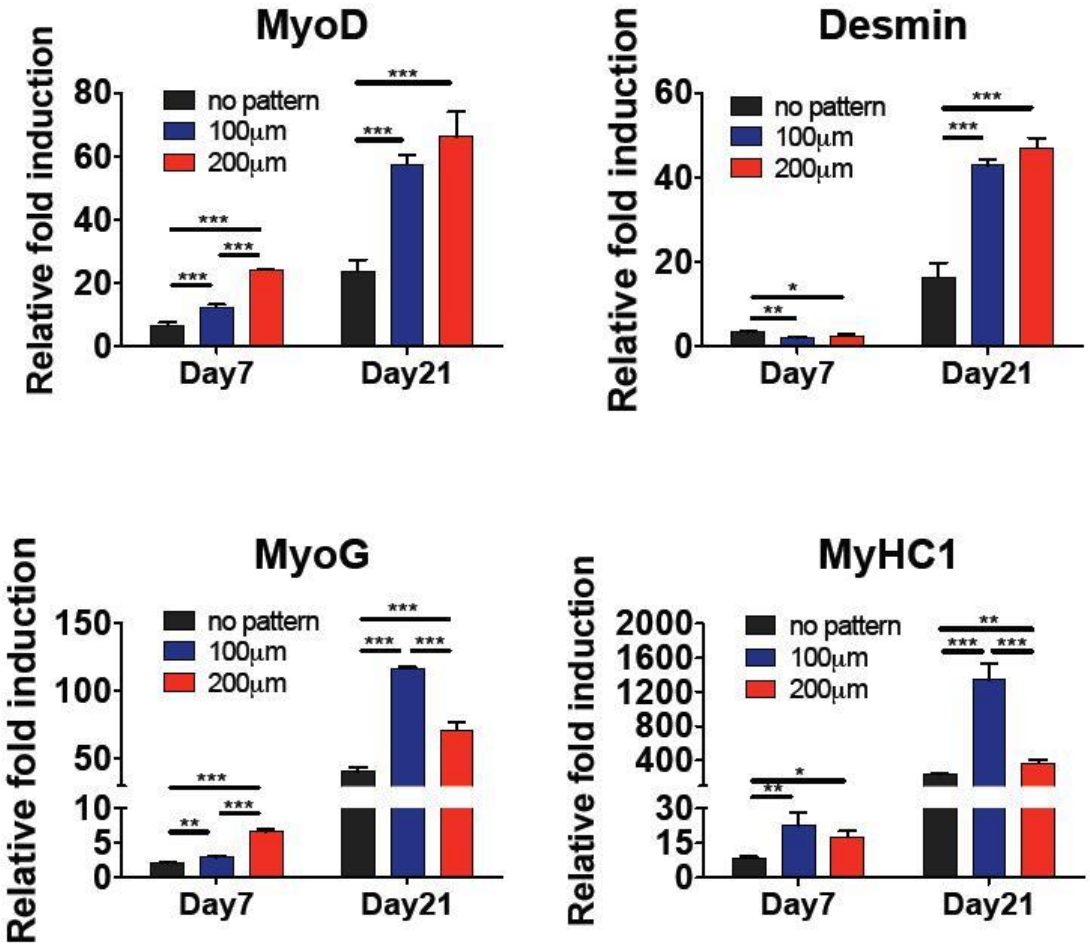

Figure 9: *In vitro* myogenic differentiation of PDGFRA^{+} cells cultured on PDMS cell culture substrates with various topographical features. Gene expression profiles of PDGFRA^{+} cells cultured on PDMS cell culture substrates with various topographical features. Statistical analysis was performed among cells cultured in different cell culture substrates within the same time point. *p<0.05, **p<0.01, and ***p<0.001.

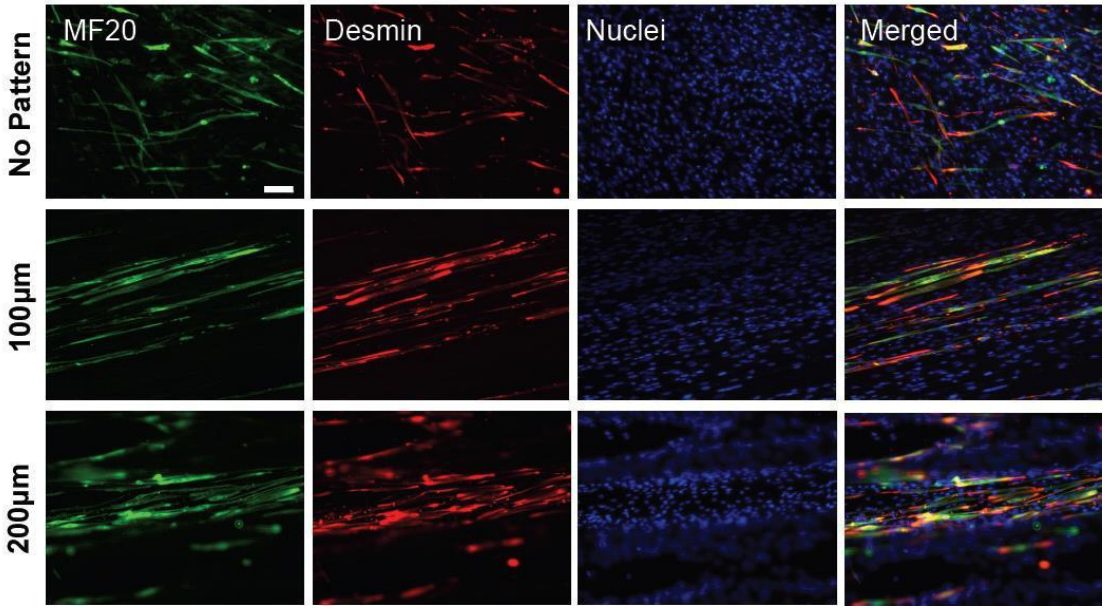

Figure 10: Terminal myogenic differentiation of cells cultured on PDMS cell culture substrates with various topographical features. Immunofluorescence staining for MF20 (green) and DES (red) of PDGFRA^{+} cells cultured on PDMS cell culture substrates with various topographical features. Scale bar = 100 µm.

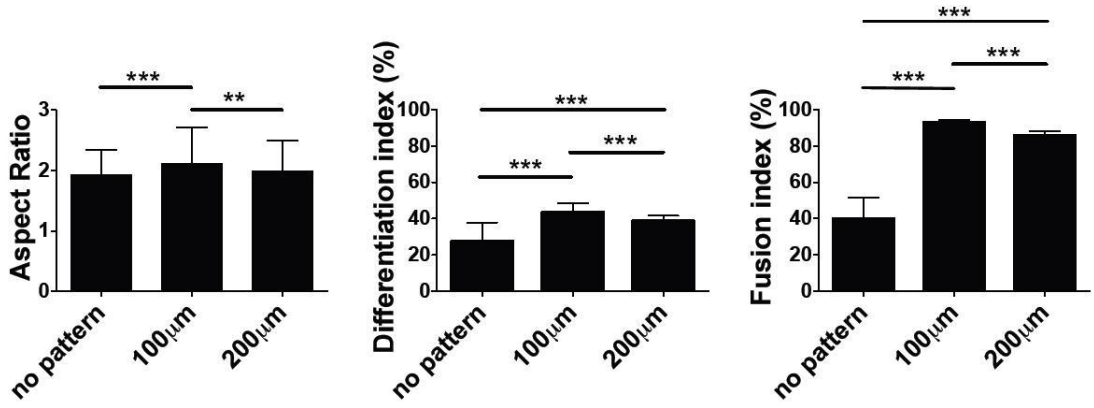

Figure 11: Cell shape analyses of PDGFRA$^+$ cells cultured on PDMS cell culture substrates with various topographical features. **(A)** Shape indices for PDGFRA$^+$ cells while undergoing terminal myogenic differentiation on various PDMS cell culture substrates. n=157, 349, and 379, respectively. **(B)** Estimated differentiation indices of PDGFRA$^+$ in serum and serum-free media. n=209, 325, and 258, respectively. **(C)** Estimated fusion indices of differentiated PDGFRA$^+$ (MF20 positive cells) in serum and serum -free media. n=209, 325, and 258, respectively. $^{**}p<0.01$, and $^{***}p<0.001$.

Chapter 3, in part, is currently being prepared for submission for publication of the material. Professor Shyni Varghese and Dr. Hwang initiated the study, writing, and analysis. The thesis author was the co-author of this material

CHAPTER 4: Discussion

There has been emerging evidence of the roles of topographical and spatial [cues in engineering skeletal muscle tissues by harnessing the matrix topography [82-84]. These attempts include the employment of soft lithographic techniques to regulate microenvironments for myoblasts or satellite cells. For examples, Li and his colleagues have exploited micropatterned PDMS substrates to control various cellular behaviors of C2C12 myoblasts, such as cell adhesion, proliferation, and fusion of cells through the cellular alignment [73, 84, 85]. Given the importance of topographical cues on myoblast behaviors, we here elucidate the role of the topographical cues-guided cellular alignments in myogenic differentiation of hESC-derived myogenic progenitors. Particularly, we have created three different topographical features on the PDMS substrates, including non-patterned surface or microgrooves with 100 μm and 200 μm widths, to recapitulate the cellular organization based upon responses to the matrix topography. Our results indicate that the both non-patterned and micropatterned PDMS substrates could successfully promote the adhesion of hESC-derived myogenitors and their proliferation as a function of cell culture time. However, we have observed that hESC-derived cells cultured on micropatterned PDMS substrates only showed the topographical cues-guided cellular alignments (Fig. 2), which is an essential step to support the fusion of pre-aligned mononucleated myogenic progenitors into the fused multinucleated myotubes [86].

Since it has been well known that the reorganization of actin cytoskeleton upon myogenic differentiation could play a significant role in controlling cellular behaviors

of myoblasts [86, 87], we also evaluated the actin cytoskeleton distribution of cells cultured on both non-patterned and micropatterned PDMS substrates. Our findings clearly demonstrated that reorganization of actin cytoskeleton could be mediated by the topographical cues. For examples, cells cultured on non-patterned substrates showed the randomly oriented actin cytoskeleton structures (Fig. 3A, left). On the other hand, the morphology of actin cytoskeleton of cells cultured on both micropatterned substrates shows the uniaxially elongated/aligned organizations (Fig. 3A, middle and right) parallel to the direction of microgrooves. This is accordance with previous reports that when C2C12 myoblasts were cultured on micropatterned substrates they could undergo a remodeling of actin cytoskeleton [81, 88]. Previously, our group also demonstrated that when human mesenchymal stem cells (hMSCs) seeded onto hydrogel matrix with optimal adhesiveness, cells underwent matrix-mediated reorganization of actin cytoskeleton and this cytoskeletal remodeling process could also promote myogenic differentiation of hMSCs compared to their control, ie. glass cover slip [87]. This would be attributed to the fact that the remodeling of actin cytoskeleton structure is dependent upon a response to cell-matrix or cell-cell interactions [87, 89, 90].

In addition to the significance of remodeling of actin cytoskeleton, alignment of myoblasts has shown to play an important role in regulating various cell behaviors, particularly multinucleated myotube formation, which mimicking the physiologically ordered skeletal muscle tissues *in vivo* [77, 86]. Previously, Ayres *et al.* well described the methods how to utilize the FFT analysis for converting the geometrical

information of a spatial domain into a mathematically assigned frequency domain [75]. Therefore, by using this method, we successfully demonstrated the cellular alignments of cells cultured on both non-patterned and micropatterned PDMS substrates. Our FFT and nuclei shape analyses indicate that cells cultured on micropatterned PDMS substrates showed that majority of cells were uniaxially aligned (Fig. 4). Indeed, in our study, we have strongly observed that cells cultured on micropatterned PDMS substrates underwent much robust *in vitro* myogenic differentiation compared to their counterpart, evident by the higher gene expression levels of late myogenic markers and MF20/DES expression (Fig. 5-6). Furthermore, topographical cues provided by microgrooves could facilitate the fusion of cells to form multinucleated myotubes, and therefore it could also promote *in vitro* myogenesis of hESC-derived myogenic progenitor cells. Taken together, our findings suggest that the topographical cues could support the remodeling of actin cytoskeleton, and consequently it could possibly promote the topographical cues-guided cellular alignment, which eventually improves *in vitro* myogenic differentiation of hESC-derived myogenic progenitors.

CHAPTER 5: Conclusion

This study elucidates the roles of topographical cues in regulating cell adhesion, proliferation, cellular alignment, and differentiation of hESC-derived myogenic progenitor cells. This could be carried out by utilizing soft lithography techniques to create synthetic matrices having micropatterned surfaces. We demonstrated topographical cues-guided cellular alignments could promote formation of multinucleated myotubes and myogenic differentiation of hESC-derived myogenic progenitors. Incorporation of such topographical cues would provide better understanding of muscle function and treating various muscle injuries and degenerative diseases. Also, this finding may be further developed into application that can be used in the *in vitro* cultured muscle patch.

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