UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Vinculin in Mechanosensitive Myogenesis of Mesenchymal Stem Cells

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in

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by

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LIST OF ABBREVIATIONS

APES:	3-Aminopropyl triethoxysilane
APS:	Ammonium persulfate
bp:	Base pair
DCDMS:	Dichlorodimethylsilane
DMEM:	Dulbecco's Modified Eagle Medium
EDTA:	Ethylene diamine tetraacetic acid
EGTA:	Ethylene glycol tetraacetic acid
FBS:	Fetal bovine serum
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kDa:	Kilo Dalton
MOPS:	3-(N-morpholino)propanesulfonic acid
PBS:	Phosphate buffered saline
Pen/Strep:	Penicillin/Streptomycin
PMSF:	phenylmethylsulfonyl fluoride
RT-PCR:	Reverse Transcription-Polymerase Chain Reaction
SDS:	Sodium dodecyl sulfate
TBE:	Tris/Borate/EDTA
TEMED:	N,N,N′,N′-Tetramethylethylenediamine

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ABSTRACT OF THE THESIS

The role of Vinculin in Mechanosensitive Myogenesis of Mesenchymal Stem Cells

by

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Master of Science in Bioengineering

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Mechanical signals have been shown to have an essential role in regulating stem cell fate. Human mesenchymal stem cell (hMSC) differentiation can be regulated by the stiffness of the surrounding extracellular matrix (ECM), via the process of mechanotransduction, in which specific mechanosensors at focal adhesion (FA) convert mechanical cues into intracellular biochemical signaling pathways. However, the

molecular mechanisms underlying mechanotransduction still remain elusive. Vinculin is reported to be capable of undergoing conformational changes exposing a cryptic mitogen-activated protein kinase 1 (MAPK1) binding site on the head domain of vinculin when subjected to physiological forces generated from myosin contraction, suggesting that vinculin, among other FA proteins, may be sensitive to physical ECM properties and thus able to relay information leading to regulation on stem cell differentiation by mechanotransduction. In this thesis, endogenous vinculin in hMSCs was knocked-down by small interfering RNA (siRNA), resulting in a 70% decrease in MyoD muscle differentiation marker expression after four days of growth on matrices with stiffness that mimics muscles (11kPa). Addition of full-length vinculin or just the head domain back into cells was shown to be able to restore MyoD expression; addition of the vinculin tail domain was not able to rescue myogenesis. Together the results suggest that the MAPK1 binding site on vinculin may be important for regulating myogenesis.

Chapter 1. INTRODUCTION

1.1. Overview of Mammalian Stem Cells and Their Characteristics

Stem cells have the remarkable potential to become all of the specialized cell types in the body during early growth and development. The two most notable characteristics that can distinguish stem cells from other types of cells are: first, they are capable of replenishing themselves by long-term cell division and self-renewal; second, they can give rise to highly specialized mature cell types through the process of differentiation (Keller, 2005). There are two main types of stem cells, embryonic stem (ES) cells and adult stem cells. As a fertilized egg divides, it forms a blastocyst over a five-day period, and the inner cell mass of blastocysts is the source of ES cells. Human and mouse ES cells are the two types of ES cells that are widely used in stem cell research to date. Both have the essential stem cell characteristics, but their unspecialized state is maintained under very different growth conditions. Mouse ES cells grow on a layer of gelatin and require the presence of Leukemia Inhibitory Factor (LIF). Human ES cells grow on a layer of mouse embryonic fibroblasts (MEFs) and require the presence of basic Fibroblast Growth Factor (bFGF). Some tissues or organs continue to maintain a population of stem cells after entering adulthood, which are adult stem cells. Unlike ES cells, whose origin has been clearly known to scientists, adult stem cells share no such definitive characterization and their origins in mature tissues are yet to be discovered (Pittenger et al., 1999). Adult stem cells have an essential function in maintaining tissue homeostasis as well as regenerating the tissues to which they home when needed to replace the lost tissues due to apoptosis, injury, or diseases. In order for adult stem cells to maintain this function, they also have the ability to balance between two cell fates: self-renewal and differentiation (Li et al.,

2005). While ES cells are pluripotent and can give rise to all cell types in the body, adult stem cells have restricted differentiation potential and are multipotent in that they are limited in the types of cells that they can become based on their origins. Overall, the remarkable characteristics of stem cells point to their essential role in embryonic development and tissue regeneration.

1.2. Microenvironment Regulates Stem Cell Fate

1.2.1. The Concept of the Microenvironment

The unique ability of stem cells to balance between self-renewal and differentiation also poses a critical question to scientists: how do stem cells decide their cell fate? Intracellular mechanisms of gene regulation at epigenetic, transcriptional, translational and post-translational levels, define stem cell fate and behavior and can be regulated by a variety of means. For example, some microRNAs have been found to promote ES cell differentiation by targeting the coding regions of Nanog and OCT4, reducing the cellular levels of these key proteins involved in maintaining ES cell pluripotency (Gangaraju et al., 2009). In addition, research has also established that numerous exogenous factors in the microenvironments surrounding stem cells, including growth factors, cytokines, extracellular matrix (ECM) proteins, and signals from adjacent cells can also exert strong impacts on the self-renewal and differentiation of stem cells (Keung et al., 2010).

The concept of the extracellular microenvironment's regulatory influence on stem cell fate was first proposed by Schofield in 1978 as 'stem cell niche' to describe the

microenvironment that supports stem cells in a mammalian hematopoietic system (Jin, 2007). More recently, the *in vivo* stem cell niche has been defined as the local environments with specialized cues that regulate stem cell behaviors in embryonic development and tissue regeneration. Stem cell niche is basically composed of different types of accessory supporting cells, ECM components, soluble paracrine factors and metabolic products. The niche nurtures stem cells, saving them from depletion, and regulates tissue generation, maintenance and repair. It can also protects the host from excessive stem cell production, which may induce cancers (Scadden, 2006). Among the niche components, ECM consists of three major structural proteins, collagen, fibronectin and laminin, together with an array of other proteins, including elastin, fibrillin, tenascin and proteoglycans, which surrounds cells in a 3D fibrillar scaffold and provides sites for cell attachment. ECM proteins have functions in specific processes during development, and some are even expressed just before the specification of ES cells, leading to the possibility that the intrinsic properties of ECM can influence stem cell differentiation (Reilly et al., 2009).

1.2.2. The Importance of the Microenvironment

Several interactions within the niche, including interactions between stem cells and each other, adjacent differentiated cells, or ECM adhesion molecules, are also closely involved in controlling stem cell fate (Keung et al., 2010). Stem cells have a characteristic of asymmetric division. When a stem cell divides, one of the daughter cells remain in the niche to continue self-renewing, while the other daughter cell leaves the niche and starts

to differentiate into specialized mature cells. For example, the fate of germline stem cells (GSCs) in *Drosophila* ovary can be controlled by some somatic cells (terminal filament, cap, and inner sheath cells) that form a niche at the tip of the ovariole. After a GSC divides, it can orient its division plane and the daughter cell that lies closer to the terminal filament and cap cells remains a stem cell, while the other daughter cell that more connects to the inner sheath cells differentiates into a cystoblast. The two identical daughter cells receive different signals due to asymmetric contact with the niche, which leads to different cell fates (Xie et al., 2000). Some proteins or supporting cells in the niche have key regulatory functions in maintaining stem cells, loss of which can lead to the depletion of stem cells in multiple tissues. For example, correct positioning of hub cells in the *Drosophila* gonad and their attachment to ECM are closely related to integrin-mediated adhesion. An integrin-defective gonad will result in loss of the hub and GSC population (Tanentzapf et al., 2007). In an individual, adult stem cells function in maintaining tissue homeostasis and repair. However, as the individual ages, extrinsic changes in the stem cell niche have been demonstrated to cause a decrease in stem cell number and activity, which in turn leads to a decline in the ability of stem cells to regenerate and repair damaged tissues during aging (Boyle et al., 2007). Furthermore, deregulation of the signaling pathways in the stem cell niche may transform normal stem cells into cancer stem cells, which leads to tumorigenesis (Li et al., 2006). Due to the importance of stem cell niche and our lack of understanding of it, scientists have paid strong attention and made great efforts into the investigation of stem cell *in vivo* microenvironment (Scadden, 2006).

1.2.3. Depths into Studying the Properties of Microenvironment

In addition to cell-based changes altering the niche, biochemical regulation of stem cells via the niche has also been thoroughly examined. Scientists have long appreciated the roles of some small, soluble protein factors, such as Wnt proteins, insulin, FGFs and cytokines, in regulating stem cell behavior. For example, Wnt signaling has been implicated in the control over various types of stem cells as a niche factor to maintain stem cell proliferation. Cytokines are involved in controlling hematopoietic stem cell fate decisions. Besides the soluble factors and their involvement in paracrine signaling pathways, the solid-state microenvironment, especially intrinsic properties of the ECM, i.e. structure, its components, and how stem cells attach to ECM, have also demonstrated essential roles in regulating stem cell behavior (Reilly et al., 2009). For example, engineered fibronectin with enhanced integrin specificity changes the cellular adhesive interactions with the ECM, and in turn influences stem cell differentiation, as force transmission is enhanced upon binding of integrins (Martino et al., 2009). In addition, the mechanical properties of ECM, such as the variation in its elasticity during natural development may also guide cells as they mature and assemble into tissues. The mechanics of ECM are now recognized as novel regulators for stem cell differentiation, in that they are related to the biophysical/mechanical signals that the cells can sense and respond to. However, it is comparably difficult to study the ECM-related factors since the microenvironment surrounding the stem cells are structurally complex and recapitulating the cell-cell, cell-matrix interactions is a great challenge (Keung et al., 2010).

1.2.4. Tissue Engineering

In recent years, tissue engineering has emerged as the dominant paradigm for recapitulating the developmental process *in vitro*. Tissue engineering has attempted to create 3D scaffolds that functionally mimic the tissue of interest based on the chemical structure and interactions between proteins in the extracellular environment. It has greatly inspired bioengineers to adopt it as a way to create biological constructs that can direct the behavior of stem cells as regenerative medicine used in cell-based therapies to replace or repair damaged tissues or organs. The biochemical, mechanical, topographical, and patterning cues that are primarily used in tissue engineering to direct stem cell behavior reside in ECM, all of which influence how a stem cell behaves and matures into a specific type of cell (Reilly et al., 2009). Therefore, biophysical characteristics of the stem cell microenvironment, such as stiffness, topography, stresses and strains, need to be taken into consideration when engineering the scaffold system *in vitro* to mimic the ECM environment *in vivo* surrounding stem cells. Many natural (e.g., collagen, fibrin, and hyaluronan) and synthetic (e.g., polyacrylamide, alginate, polyethylene glycol, and self-assembling synthetic peptides) gels have been utilized as engineered ECM scaffolds to mimic the natural stem cell microenvironment (Keung et al., 2010).

1.3. Emphasis on the Mechano-Regulation of Stem Cell Fate

1.3.1. Mesenchymal Stem Cells as A Model System

Back in the 1950s, scientists have discovered that bone marrow contains at least two types of adult stem cells: hematopoietic stem cells, which give rise to all types of blood

cells in the body, and mesenchymal stem cells (MSCs) (Kuhn et al., 2010). MSCs are multipotent, spindle-shaped adult stem cells with large nuclei. They can give rise to various hard and soft tissue lineages, including osteoblasts, chondrocytes, adipocytes, skeletal myocytes, smooth muscle cells and even non-mesenchymal lineages such as neurons. Similar to other adult stem cells, MSCs also possess the potential to balance cell fate decision between self-renewal and differentiation, with an essential role in tissue regeneration and repair for cell-based therapies to treat bone, cartilage or muscle disorders or damage. For example, MSCs could be used in repairing connective tissue abnormalities or cardiac infarcts, and in contributing smooth muscle cells to vascular remodeling (Kuhn et al., 2010). As the mechanical stimuli are of greater interest to scientists in studying regulation of stem cell differentiation and the MSC-derived lineages are associated with load-bearing connective tissues with diverse mechanical properties, MSCs have become a particularly appropriate system for studying stem cell behavior regulated by mechanical signals (Keung et al., 2010).

1.3.2. Transduction of Mechanical Signals to Initialize the Regulation

Cells will have restricted functions and receive limited repertoire of information if they only rely on biochemical signaling, such as binding of a molecule to receptors on cell surfaces and intracellular biochemical responses triggered by the binding events. Some biological phenomena, such as different cells having different shapes and different organs having different stiffness, cannot be solely explained by biochemistry (Janmey et al., (2011). During organogenesis, cells can sense the changes in physical forces by

transducing them into intracellular biochemical signals through the process of mechanotransduction. And in turn, through intracellular signaling pathways, these signals can modulate cellular functions and structures like cell shape, polarity, migration, mitosis, differentiation and apoptosis. Stem cells are no exception to the mechanotransduction mechanism. The mechanism takes on an even larger role in stem cells since it has to coordinate the mechanical stimuli sensed and transduced into stem cells, in order for stem cells to form tissues and organs out of large groups of themselves in a highly organized manner (Wolf et al., 2009). It has been shown in recent work that stem cell lineages and fates can be directed by the elasticity of the ECM within which the cells are situated. For example, it has been discovered that MSC specification to different lineages largely depends on the stiffness of their substrates, as determined by analysis of differentiation markers. When MSCs are cultured on soft substrates in the range of 0.1-1.0 kPa, which resemble the stiffness of brain tissues, they undergo neuronal differentiation. However, the use of substrates with intermediate stiffness similar to striated muscle (8-17 kPa), MSCs differentiate into myoblasts and the stiff substrates that mimic bone (25-40 kPa) results in MSCs undergoing osteogenesis (Engler et al., 2006). This example provides a link into studying the mechanotransduction process during mechanical regulation of cell fate and function. However, the exact molecular mechanisms underlying mechanotransduction still remain elusive. Molecular defects in mechanotransduction like mutations in proteins involved in this process can disrupt the chain of mechanical sensing and subsequent intracellular signaling pathways, and therefore, result in disturbance in normal cellular functions and potential development of diverse diseases such as loss of hearing, cardiovascular diseases, muscular dystrophies and cancers (Jaalouk et al., 2009).

It has drawn great attention of scientists to investigating the molecular details underlying mechanotransduction, in order to gain a better understanding in controlling cell behavior and new avenues of therapeutic approaches for mechanotransduction related diseases (Jaalouk et al., 2009).

1.3.3. Two Regimes of Mechanotransduction

The mechanical signals to which cells can respond generally have two forms: externally applied forces that exert on the cells, such as shear stress, extension and compression, and internal forces generated by cell–matrix and cell–cell contacts which enable the cells to measure the mechanical properties of the extracellular environment, for example traction force is generated by cells and applied to cell–substrate adhesions via actin stress fibers to feel changes in stiffness, surface topography, and ligand density (Janmey et al., 2011). The situation that the external forces are detected and transduced upon cells is also known as passive or outside-in mechanosensing (Holle et al., 2011). For example, Shear stress is a fundamental determinant of vascular homeostasis, regulating vascular remodelling and cardiac development. Fluid shear stress can be simulated in a parallel-plate flow chamber to mimic the dynamic blood or extracellular fluid flow on cells in their physiological environment. It has been found that fluid shear stress can be detected by endothelial cells, via a mechanosensory membrane protein complex that bridges the extracellular and intracellular environments (Tzima et al., 2005).

The other situation that cells can generate their own forces to detect changes in their external environment is also known as active or inside-out mechanosensing. It can

be exemplified by directed cell migration in a process of durotaxis, which is induced by gradients in substrate stiffness or surface topology. The traction force generated by cells tends to promote cell movement from soft substrates to stiffer substrates, with substrate stiffness varied by the use of hydrogels such as polyacrylamide or pNIPAAM with different concentrations or components (Holle et al., 2011). In the case mentioned above, MSC differentiation is strongly influenced by substrate stiffness. As cells are deposited onto the substrates, during which the biomechanical sensing and transduction pathway is triggered, cells apply a traction force to sense and measure the mechanical properties of substrates around them and adjust appropriately. Once the values are ascertained and cells would possibly develop in the way of matching their biophysical and biochemical properties to those in their surrounding environment (Wolf et al., 2009).

1.3.4. Focal Adhesion is Important in Mechanotransduction

Regardless of the systems that cells use to respond to mechanical signals, scientists have focused on the sites of cellular sensors that are responsible for converting mechanical stimuli into biochemical signals. In order to carry out mechanotransduction, adhesion of cells to ECM is crucial. The cluster of cell surface adhesion molecules that can enable cell-cell and cell-ECM communications are the prerequisites for sensing and transducing mechanical signals. It has been revealed that cell surface adhesion receptors that link the internal cytoskeleton to ECM and to neighboring cells provide preferred molecular pathways for mechanical signal transfer across the cell surface (Mammoto et al., 2010). The clusters of structural and adaptor proteins in which the mechanosensors typically

reside are focal adhesions (FAs), which are multimolecular complexes that serve as mechanical links between cytoskeleton (and perinuclear cytoskeleton) and ECM (Holle et al., 2011). The research on elucidating the molecular composition of FAs has advanced over the past two decades. It has been identified that FAs consist of more than 50 unique components, which can be separated into three categories: membrane-associated proteins, cytoskeletal proteins and signaling proteins. Integrins are heterodimeric transmembrane proteins, which are required for FA formation and have functions in relaying signals bidirectionally between cells and ECM. The extracellular domains of integrins attach to the extracellular substrate while their intracellular domains provide docking sites for the assembly of a cluster of cytoskeletal proteins inside the cells. The cluster consists of more than 50 different proteins, including talin, α-actinin, filamin, vinculin, paxillin, and tensin, which are known to form the structural scaffold. Several structural proteins directly bridge integrins to actin cytoskeletons, such as talin, α-actinin, filamin and tensin, while some others do not directly attach to integrins, such as vinculin, which interacts with talin, α-actinin and actin (Figure 1). The structural proteins are able to strengthen cell adhesion by stabilizing FA structure and anchoring FAs to actin. There are also proteins that emerge as signaling components at FAs, including focal adhesion kinase (FAK), Src tyrosine kinase and the Rho family of GTPase (Shemesh et al., 2005).

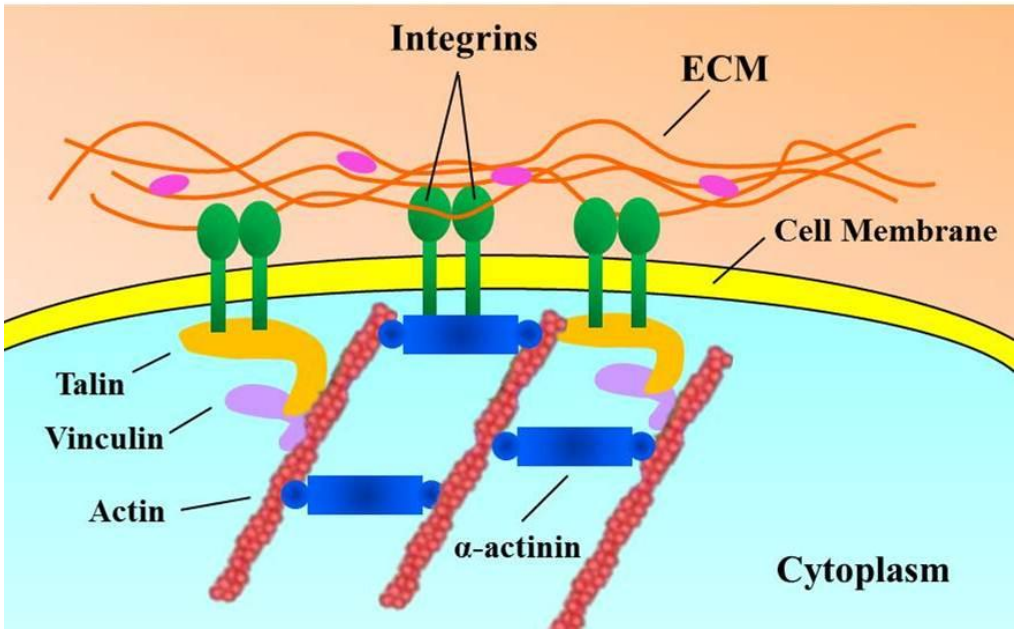

Figure 1. Assembly of some key structural proteins at focal adhesion (FA). Integrins are transmembrane proteins that attach cells to extracellular matrix (ECM). Talin and α-actinin serve as bridges between integrins and actin filaments. Vinculin interacts with talin, α-actinin and actin.

The Mechanosensitivity of FA is manifested in the dependence of their shapes and dimensions on the applied forces. A developing FA usually acquires an elongated shape with a finite length, determined by the direction of force and its magnitude. In most cases, the size of FA is proportional to the applied force (Shemesh et al., 2005). The size of FAs also increased with increasing stiffness, with concomitant changes in the dynamics of FA proteins, such as vinculin, which becomes more stably anchored at the attachment site (Janmey et al., (2011)).

1.3.5. Known Focal Adhesion-Involved Mechanosensing Pathways

1.3.5.1. Rho/ROCK Signaling Pathway

Several FA-based mechanosensing pathways have been extensively studied and clearly known to scientists. The Rho family of small GTPase controls a diverse array of cellular processes and properties, such as cellular contractility, actin assembly, cell shape and gene expression, and coordinating them to further exert influence on FA and actin stress fiber formation (Shemesh et al., 2005). When integrins bind to ECM, some Rho GTPases, such as RhoA and Rac, are catalyzed by guanidine exchange factors (GEFs) from an inactive guanosine diphosphate (GDP)-bound form to an active guanosine triphosphate (GTP)-bound form. The Rho kinase (ROCK), which is in turn activated by RhoA, can therefore regulate cellular contractility via direct phosphorylation of Myosin Light Chain (MLC) and inactivation of MLC Phosphatase. Moreover, activated ROCK is reported to phosphorylate LIM kinase, leading to phosphorylation of the actin-regulatory protein cofilin, which contributes to reorganization of the actin cytoskeleton. Besides ROCK, another RhoA effector, mDia (mammalian homologue *Drosophila* diaphanous) is also responsible for regulating actin dynamics (Keely, 2008). Rho also functions through ROCK and mDia to orient the stress fibers according to the axis of strain.

1.3.5.2. Stretch Activated Channels

Intracellular Ca^{2+} plays a crucial role in the reorganization of the actin cytoskeleton through modulating actin-associating protein activities. During substrate stiffness sensing, traction force is generated inside the cells and transferred onto ECM via stress fiber and FA system. Cells are therefore subjected to stretching, leading to an increase in the permeability of mechanosensitive channels to soluble ions. These channels are referred to

as stretch activated channels (SACs). It has been shown that the mechano-related Ca^{2+} transients can be induced by such intrinsic mechanical stimuli as cell traction force. Ca^{2+} permeable mechanosensitive channels are involved in this process and they are activated intermittently by the traction force in stress fibers. As a result, cells show spontaneous Ca^{2+} oscillations with larger amplitude on stiff substrate than those on soft substrate. It proves that SAC permeability is directed coupled to sensing of the physical microenvironment by cells. However, it is still not clear whether these channels are integral and indispensable components of the mechanosensor itself (Kobayashi et al., 2010). The elasticity of actin network is controlled in response to the mechanical changes in extracellular environment. It has been reported that cells can tune their response to the physical compliance of ECM. This signaling event, which is carried out through integrins, depends on Rho-mediated contractility and can further strengthen cell adhesions, in response to an increase in the 'inside-out' force (Keely, 2008).

1.3.5.3. Force-Sensitive Protein Conformational Changes

Force-inducible protein unfolding is also involved in the FA-based mechanosensing pathway. Some FA proteins have binding sites for other proteins or signaling molecules buried inside. They have shown force-responsive conformational changes, depending on substrate stiffness. Differences in FA protein unfolding would influence the binding of downstream signaling molecules, leading to differential regulation in cell behavior. On soft matrix, the force generated by cellular contractility is relatively weak. When the force is transferred to the ECM through the linker protein at FA, it is not sufficient to

trigger a change in the conformation of the protein to expose the cryptic binding site for signaling molecules (Figure 2(a)). On stiff matrix, the force generated by cells is relatively strong. Due to the incompliance of the matric, nearly the full force focuses on the linker protein. The protein in turn unfolds to a too high degree, causing the binding site to become unfunctional (Figure 2(b)). On optimally firm matrix, appropriate amount of force is generated by cells and applied to the protein at FA, the conformation of the protein is properly changed and the cryptic binding site becomes accessible and functional (Figure 2(c)). Therefore, only under proper physiological force can the cryptic binding sites in FA proteins be properly exposed for other proteins and signaling molecules further down the signaling pathway (Reilly et al., 2009).

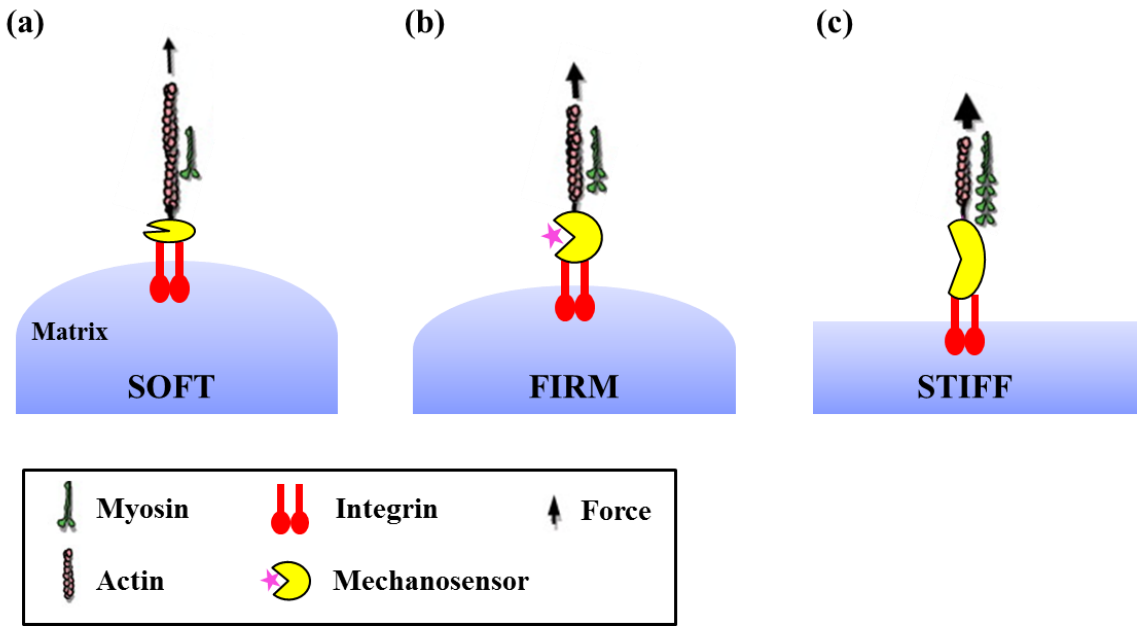

Figure 2. Force- sensitive protein conformational changes. (a) On soft matrix, the force generated by cellular contractility is relatively weak and not sufficient to induce conformational changes in the mechanosensor protein at FA. (b) On firm matrix, appropriate amount of force is generated by cells to induce conformational changes in the protein, allowing for the exposure of a cryptic binding site for intracellular signaling molecules. (c) On stiff matrix, the force generated by cells is relatively strong and the protein unfolds to become unfunctional.

1.3.6. Focuses on Vinculin in Mechanotransduction

It has been previously reported that FA is critical in mechanotransduction in regulating cell behavior. Talin and vinculin, localized at FA site, have key functions as mechanosensors in cell signaling, adhesion, and migration (del Rio et al., 2009). Talin binds to integrin via its head domain. Its tail domain binds to actin filaments both directly and indirectly via vinculin. Talin has 11 vinculin binding sites (VBS) and a number of them are cryptic, which require force-induced activation to bind vinculin (Golji et al., 2011). Vinculin, a 116 kDa cytoplasmic protein, is made up of an N-terminal head domain (domain 1-4 as D1-4) linked to a C-terminal tail domain (Vt) by a short proline-rich sequence, and the intramolecular interaction between the head and tail masks the numerous ligand-binding sites in the protein. While talin directly links integrins to actin cytoskeletons, vinculin does not directly attach to integrins. VBS in talin binds to D1 in vinculin and actin filaments bind Vt (Golji et al., 2011). In the talin-vinculin system, when buried protein or signaling molecule binding sites are exposed under physiological forces, molecular mechanotransduction can occur upon ligand binding (del Rio et al., 2009). Tensile forces that are applied to newly formed adhesion sites can stretch the talin rod in order to activate the vinculin binding sites that are buried within the hydrophobic core of the helical bundles that make up the talin rod (Ziegler et al., 2006). Vinculin has an autoinhibited conformation when in its native cytoplasmic state. The proximity of D1 and Vt in vinculin can prevent its binding partners to bind. Under appropriate physiological forces, vinculin would undergo a conformational change to reduce the proximity of D1 and Vt, allowing for interaction between its binding partners and binding sites in vinculin (Golji et al., 2011). For example, upon physiological stress such as

cellular contraction to feel the matrix stiffness, the talin-vinculin complex undergoes conformational changes to expose a cryptic binding site for mitogen-activated protein kinase 1 (MAPK1) within vinculin (Holle et al., 2011). The MAPK1 signaling cascade can then promote muscle differentiation. Furthermore, vinculin-deficient cells are less stiff than normal cells and the traction forces generated are lower, and they display impaired cell spreading and cell migration. Therefore, vinculin is an ideal candidate for studying mechanotransduction because the binding of vinculin to FA complexes was shown to increase upon the application of force (del Rio et al., 2009).

1.4. Objective of the Thesis

From previous studies, we have known that MSCs are a particularly appropriate system for studying stem cell differentiation regulated by mechanical signals, which are converted into biochemical signals by specific mechanosensors at focal adhesion to regulate downstream intracellular signaling pathways. A systematic analysis of 54 potential mechano-sensitive adhesion proteins yielded 8 that induce MAPK signaling during mechanotransduction. Vinculin is capable of undergoing conformational changes exposing a cryptic MAPK1 site when subjected to physiological force from myosin contraction, suggesting that vinculin, among other focal adhesion proteins, may be sensitive to physical ECM properties and thus able to relay information leading to differentiation of stem cells. Therefore, this thesis focuses on the mechanosensing function of vinculin to investigate the necessity of vinculin in hMSC myogenesis. In this thesis, vinculin was knocked down in hMSCs and different domains of chicken vinculin

were added back individually. The differentiation of MSCs on matrices of a muscle-like stiffness, 11 kPa, was examined based on gene and protein expression of MyoD, a muscle differentiation marker. The objective of this thesis is to experimentally investigate the importance of the focal adhesion-based mechanosensor vinculin in MSC myogenesis when regulated by mechanical signals like matrix elasticity. It in turn leads to the long term goal of elucidating the molecular mechanism underlying the process of mechanotransduction in directing stem cell fate. With advancement in research on the regulating stem cell behavior, scientists are hoping to direct the behavior of stem cells for application in regenerative medicine, making the legendary concept of regeneration into reality by replacing lost, damaged, or aging cells and tissues with the ones from stem cells under directed differentiation in the human body.

Chapter 2. VINCULIN OVER-EXPRESSION

2.1. Introduction

The plasmids obtained are pEGFP vector with chicken vinculin head domain, tail domain and full length genes individually inserted. They are used to rescue the vinculin knock-down hMSCs with different domains of chicken vinculin gene. The ability of the plasmids to survive, replicate, transcribe and translate is first tested by simply introducing them into hMSCs and analyzing their transcription or translation products. In this thesis, after the plasmids were individually over-expressed in hMSCs, Western Blot was first carried out to examine the over-expression result by measuring the levels of vinculin protein domains produced in the cells. However, this method did not provide a conclusive result. An alternative method was then adopted to look at the vinculin gene expression in the cells directly by conventional RT-PCR.

2.2. Materials and Methods

2.2.1. Amplification and Purification of Plasmids

Plasmids encoding full-length chicken vinculin (residues 1-1066), pEGFP-Vf, vinculin head domain (residues 1–851), pEGFP-Vh, and vinculin tail domain (residues 884-1066), pEGFP-Vt were kindly provided by Dr. Susan Craig (John Hopkins University School of Medicine, Baltimore, MD). The plasmids were transformed into Library Efficiency® DH5α Competent Cells (Invitrogen) and purified using QIAGEN Plasmid Midi Kit.

2.2.1.1. Transformation

DH5α competent cells were thawed on ice and three 1.5 ml microcentrifuge tubes were chilled on ice. The cells were mixed and 50 µl of the cell suspension was transferred into each tube. 1 ng of DNA plasmids, pEGFP-Vf, pEGFP-Vh and pEGFP-Vt was mixed with the cells individually and incubated on ice for 30 minutes, at 37°C for 3 minutes, and on ice again for 5 minutes. 150 µl of sterile 20 g/L Lysogeny broth (LB, 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) was added into each tube and the tubes were shaken at 225 rpm at 37°C for 1 hour. The cells were spread plated on kanamycin/LB plates and incubated in a 37°C incubator overnight.

2.2.1.2. Amplification

Bacterial colonies from each transformation were clonally expanded to amplify the plasmids. Briefly, cultures were inoculated in individual sterile culture tubes, each containing 2 ml of sterile LB with 50 µg/ml kanamycin. The tubes were shaken at 225 rpm at 37°C for 8 hours. 1ml of each bacterial culture was then added into individual sterile flasks each containing 200 ml of sterile LB with 50 µg/ml kanamycin. The flasks were shaken at 225 rpm at 37°C overnight.

2.2.1.3. Plasmid Preparation

Competent cells were harvested by centrifuging each 200 ml bacterial culture at 4000 rpm for 30 minutes at 4°C. The pellets were resuspended in 4 ml of Resuspension

Buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA and 100ug/mL RNase A). 4 ml of Lysis Buffer (200 mM NaOH and 1% SDS) was added and each tube was inverted 5 times to mix the components and then incubated at room temperature for 5 minutes. 4 ml of Neutralization Buffer (3.0M potassium acetate, pH 5.5) was then added and each tube was inverted 5 times to mix and incubated on ice for 15 minutes. The tubes were centrifuged at 4000 rpm for 30 minutes at 4°C and each supernatant was collected. The supernatant solutions were then centrifuged again at 4000 rpm for 30 minutes at 4°C and each new supernatant was collected. 4 ml of Buffer Equilibrium Buffer (750mM NaCl, 50mM MOPS, pH7.0, 15% isopropanol and 0.15% Triton X-100) was added into each of the three QIAGEN-tip 100 columns and drained by gravity flow. The supernatants were loaded into individual columns and drained by gravity flow. The columns were washed twice by 10 ml of Wash Buffer (1.0 M NaCl, 50 mM MOPS, pH 7.0 and 15% isopropanol). The DNA plasmids were eluted by 5 ml of Elution Buffer (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5 and 15% isopropanol) from each column and collected into 15 ml Falcon tubes. 3.5 ml of isopropanol was added into each plasmid DNA. The tubes were mixed and centrifuged at 4000 rpm for 1 hour at 4°C. The supernatants were discarded and 2 ml of 70% ethanol was added. The tubes were centrifuged at 4000 rpm for 15 minutes at 4°C. The supernatants were discarded and each pellet was air-dried for 5 minutes and resuspended in 100 µl of TE buffer (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA). The solutions were individually transferred into three 1.5 ml microcentrifuge tubes and their concentrations were determined by Nanodrop 2000 spectrophotometer.

2.2.2. Cell Culture

Bone marrow-derived human mesenchymal stem cells (hMSCs) were obtained from Lonza. Cells were cultured at 37°C and 5% CO_2 in T-150 flasks in MSC growth medium (500 ml low glucose DMEM supplemented with 20% FBS and 1% Pen/Strep). Medium was changed every two to three days until cells became confluent and were then subsequently passaged. Cells were passaged as follows: medium was aspirated and the cells were washed with 10 ml of PBS. Cells were trypsinized with 5 ml of 0.25% trypsin-EDTA at 37°C for 3 minutes. The trypsin was neutralized with 5 ml of MSC growth medium and the cells were centrifuged at 1200 rpm for 2 minutes. Supernatant was aspirated and the cell pellet was resuspended in MSC growth medium. The number of cells was counted using a hemocytometer if needed. Proportions of the cell suspension were transferred to new T-150 flasks for continuing growth.

2.2.3. Vinculin Over-expression in hMSCs

2.2.3.1. Transfection

10^4 cells in 500 µl MSC growth medium were plated per well in a 24-well plate and the cells were incubated at 37°C and 5% CO_2 overnight. Transfection was carried out in the Biosafety Cabinet on the following day. For each well, 2 µl of Lipofectamine 2000 transfection reagent (Invitrogen) was diluted into 50 µl DMEM and incubated at room temperature for 5 minutes. 1 µg of each purified DNA plasmid, pEGFP-Vf, pEGFP-Vh and pEGFP-Vt, was individually diluted into 50 µl DMEM. 50 µl of the diluted Lipofectamine 2000 was added into each 50 µl of diluted DNA and the mix was

incubated at room temperature for 20 minutes. The 24-well plate was removed from the incubator and the MSC growth medium was aspirated. Each 100 µl DNA-Lipofectamine 2000 complex was added into corresponding wells, together with 400 µl of DMEM with 2% FBS and no antibiotics. The plate was placed back to the incubator at 37°C and 5% CO_2 and the medium was changed back to MSC growth medium after 24 hours.

2.2.3.2. Protein Lysates Collection

Protein lysates were collected 24 hours and 72 hours after transfection from cells individually transfected with the three DNA plasmids. MSC growth medium in the wells was aspirated and rinsed twice with PBS. 100 µl of RIPA lysis buffer pH 7.5 (1% Triton-X, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$ and 50 mM HEPES) with protein inhibitors (1 mM PMSF and 1 mM EGTA) was added per well and incubated at room temperature for 5 minutes. The solution was mixed by pipette 20 times and transferred to new 1.5 ml microcentrifuge tubes.

2.2.3.3. Western Blotting

Protein samples were mixed with DTT and sample loading buffer, and heated at 90°C for 2 minutes. They were separated on a 10% resolving polyacrylamide gel with a 5% stacking gel in 1X SDS running buffer (25 mM Tris, 192 mM glycine and 0.1% w/v SDS) at 150 V for 2 hours, until the dye front reached the bottom of the gel. The separated proteins on the gel were transferred to a nitrocellulose membrane in transfer

buffer (25 mM Tris, 192 mM glycine and 20% v/v methanol) at 100 V for 1 hour and 15 minutes. After transfer, the membrane was immersed in blocking buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20 and 1% non-fat milk) overnight in the cold room. On the following day, the membrane was immersed in anti-vinculin antibody (Abcam) (1:2000) in blocking buffer for 1 hour, and washed three times with blocking buffer for 10 minutes each. It was then immersed in goat anti-mouse horseradish peroxidase (HRP) conjugated antibody (Invitrogen) (1:10000) in blocking buffer for 30 minutes, and washed three times with blocking buffer for 10 minutes each. 1 ml of enhanced chemiluminescent substrate solution (Thermo Scientific) was added onto the membrane and incubated for 5 minutes. The membrane was placed in a developing case and exposed to an X-ray film for 8 minutes in the dark room. The film was developed in an X-ray film developer and band intensity was analyzed by ImageJ.

2.2.3.4. Total RNA Extraction

The samples were washed twice with sterile PBS. 1 ml of TRIzol (Invitrogen) was added into each well and pipetted 5 times and then incubated at room temperature for 5 minutes. The samples were transferred into new 1.5 ml microcentrifuge tubes. 200 µl of chloroform was added into each tube. The tubes were shaken vigorously by hand for 30 seconds, incubated at room temperature for 3 minutes, and centrifuged at 14000 rpm for 15 minutes. The upper colorless layer was transferred to new 1.5 ml microcentrifuge tubes and 500 µl of isopropanol was added, mixed and incubated at room temperature for 10 minutes. The tubes were centrifuged at 14000 rpm for 10 minutes. The supernatant

was discarded and 500 µl of 75% ethanol was added. The tubes were centrifuged at 11000 rpm for 5 minutes and the supernatant was discarded. The pellets were air-dried for 5 minutes and resuspended in 10-20 µl DEPC water. RNA concentrations were determined by Nanodrop 2000 spectrophotometer.

2.2.3.5. Conventional Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and DNA Polyacrylamide Gel Electrophoresis

cDNA was synthesized and amplified using the One-step RT-PCR Kit (Qiagen) with 200 ng of extracted RNA, 2 µl of 5X RT-PCR Buffer, 0.4 µl of dNTP mix (10 mM each), 0.6 µl of 10 µM forward primer, 0.6 µl of 10 µM reverse primer, 0.4 µl of Enzyme Mix and DEPC water up to 10 µl per reaction. The one-step RT-PCR was carried out as follows: 30 minutes at 50°C, 15 minutes at 95°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 58°C and 1 minute at 72°C, then another 10 minutes at 72°C and finally stay at 4°C. Polyacrylamide gels were prepared in a mold as follows: 4.33 ml double distilled water (ddH_2O), 1.21 ml of 40% acrylamide, 833 µl of 2% bis-acrylamide, 712 µl of 10X TBE, 36 µl of 10% APS and 13.3 µl of TEMED. The gels were allowed to solidify for 30-40 minutes and 1X TBE was added into the electrophoresis tank. 10 µl RT-PCR product and 1 µl 10X gel loading dye were mixed and loaded into separate wells of the polyacrylamide gel. The gel was run at 60V for 1.5 hours, stained in 0.5 µg/ml ethidium bromide solution in dark for 30 minutes, and imaged under UV light in a transilluminator (UVP).

2.3. Results

2.3.1. Detection of Over-Expressed Vinculin by Western Blot

We first tested the feasibility of introducing different domains of the chicken vinculin gene into hMSCs and the ability of the genes to undergo subsequent transcription or translation events in the host cells. Western Blot was carried out using anti-vinculin antibody to detect the translation products of the over-expressed genes. The result contains two sets of protein samples collected from cells at two time points, one day and three days after transfection. As seen in Figure 3(a), WT represents wild type control sample; H, T and FL represent the samples over-expressed with vinculin head domain, tail domain and full-length gene, respectively. The band intensities for Day 1 and Day 3 over-expression samples (H, T and FL) were similar to the band intensities of the WT sample. It was confirmed by Figure 3(b) in which the vinculin band intensity of each sample was quantified using ImageJ and normalized to the corresponding Gapdh loading control. It showed that the normalized band intensities for the H, T and FL over-expression samples did not have significant increase on either Day 1 or Day 3, comparing with the band intensities for the WT control samples.

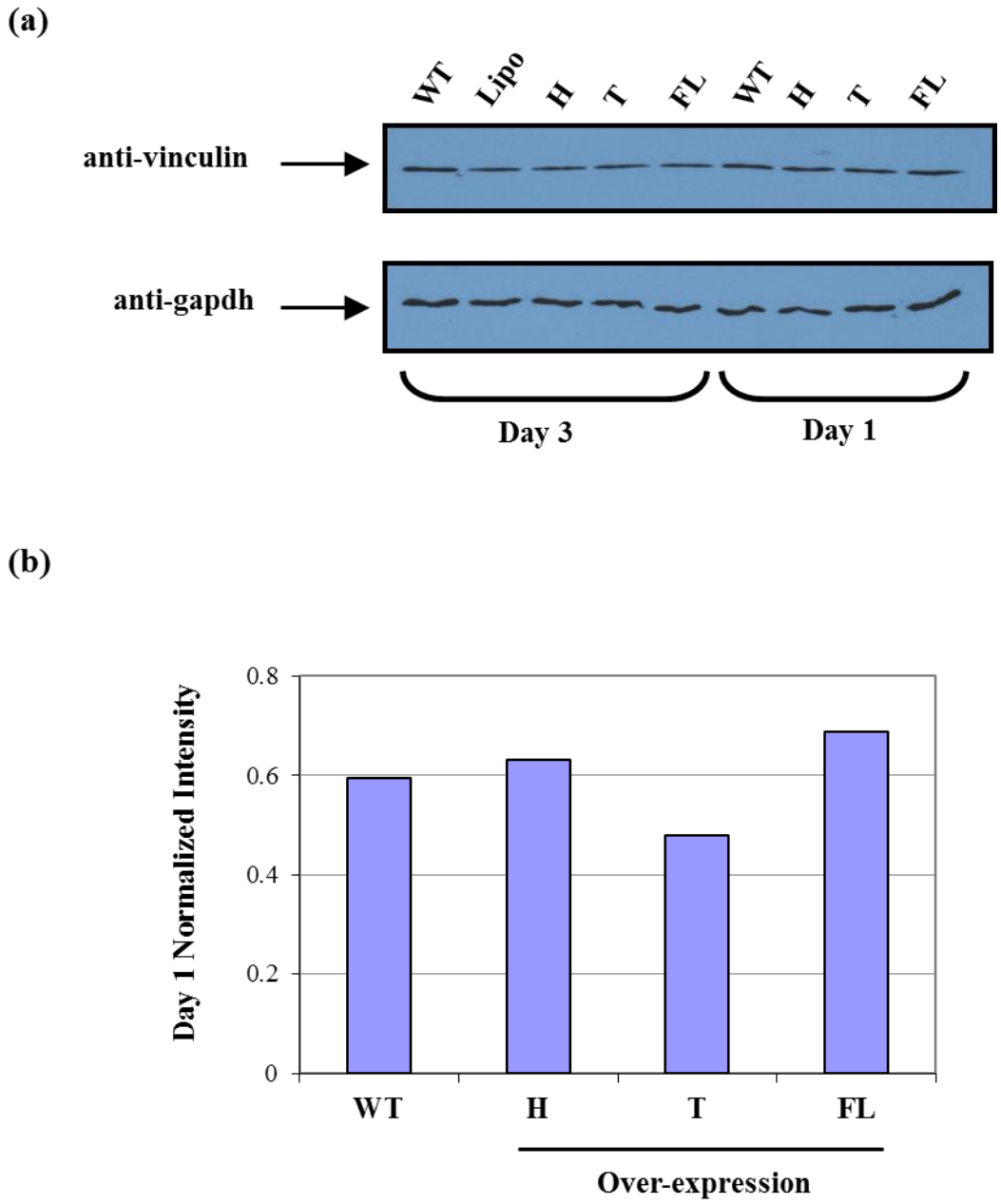

Figure 3. (a) Western Blot results for vinculin over-expression experiment. Antibody against human vinculin was used to detect the transfected vinculin domains. WT is protein extracted from untreated control hMSCs. Lipo sample is from cells treated with Lipofectamine 2000 only with no DNA transfected. H, T and FL samples are from cells transfected with vinculin head domain, tail domain and full-length gene, respectively. Day 1 and Day 3 are two time points to collect the protein lysates from cells, which are one day and three days after transfection. (b) Quantification of band intensity in (a) using ImageJ.

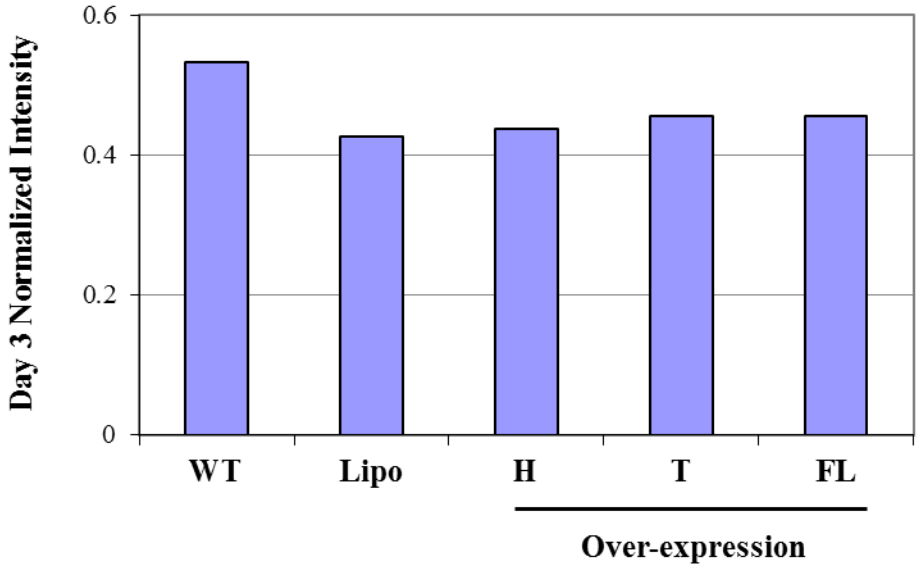

Figure 3. Continued from page 29. Quantification of band intensity in (a) using ImageJ.

2.3.2. Detection of Over-Expressed Vinculin by Conventional RT-PCR

An alternative approach was also used to detect the vinculin genes transfected into hMSCs. Conventional RT-PCR was carried out using primers that can specifically recognize nucleotide sequences in the chicken vinculin head domain (Vh, head primers), tail domain (Vt, tail primers) and the full length gene (Vf, full-length primers). Figure 4(a) showed the RT-PCR result using RNA samples extracted from cells on Day 1, one day after transfection. Lanes 1, 2 and 3 represent the RT-PCR assays where head primers were mixed with RNA samples from cells transfected with pEGFP-Vh, pEGFP-Vt and pEGFP-Vf, respectively. A band migrating at 440 bp showed up in both lanes 1 and 3, but not in lane 2. Lanes 4, 5 and 6 correspond to the RT-PCR assays in which tail primers

were mixed with the same three RNA samples as used in lanes 1, 2 and 3, respectively. Both lanes 5 and 6 have a band at 239 bp, while lane 4 does not have any band showing up. Lanes 7, 8 and 9 stand for the RT-PCR assays that have full-length primers and the three RNA samples mixed individually. There is a band migrating at 440 bp in both lanes 7 and 9, but not in lane 8. Figure 4(b) represents the result of the same combination of reactions as in Figure 4(a) but using RNA samples extracted from cells on Day 3, three days after transfection. It has the same band patterns as in Figure 4(a).

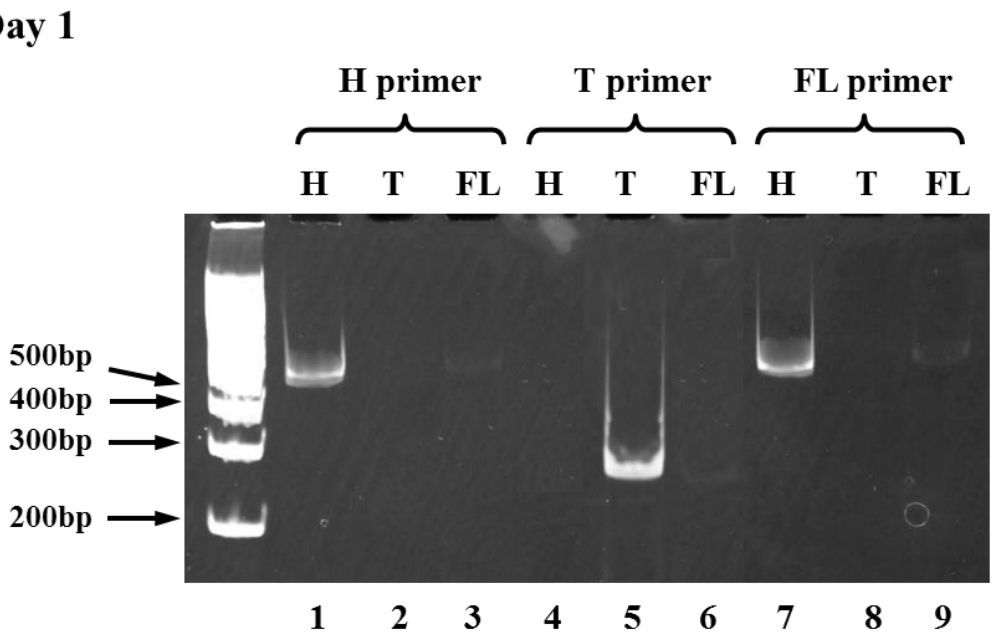

Figure 4. (a) Conventional RT-PCR results for vinculin over-expression experiment using RNA isolated from hMSCs one day after transfection.

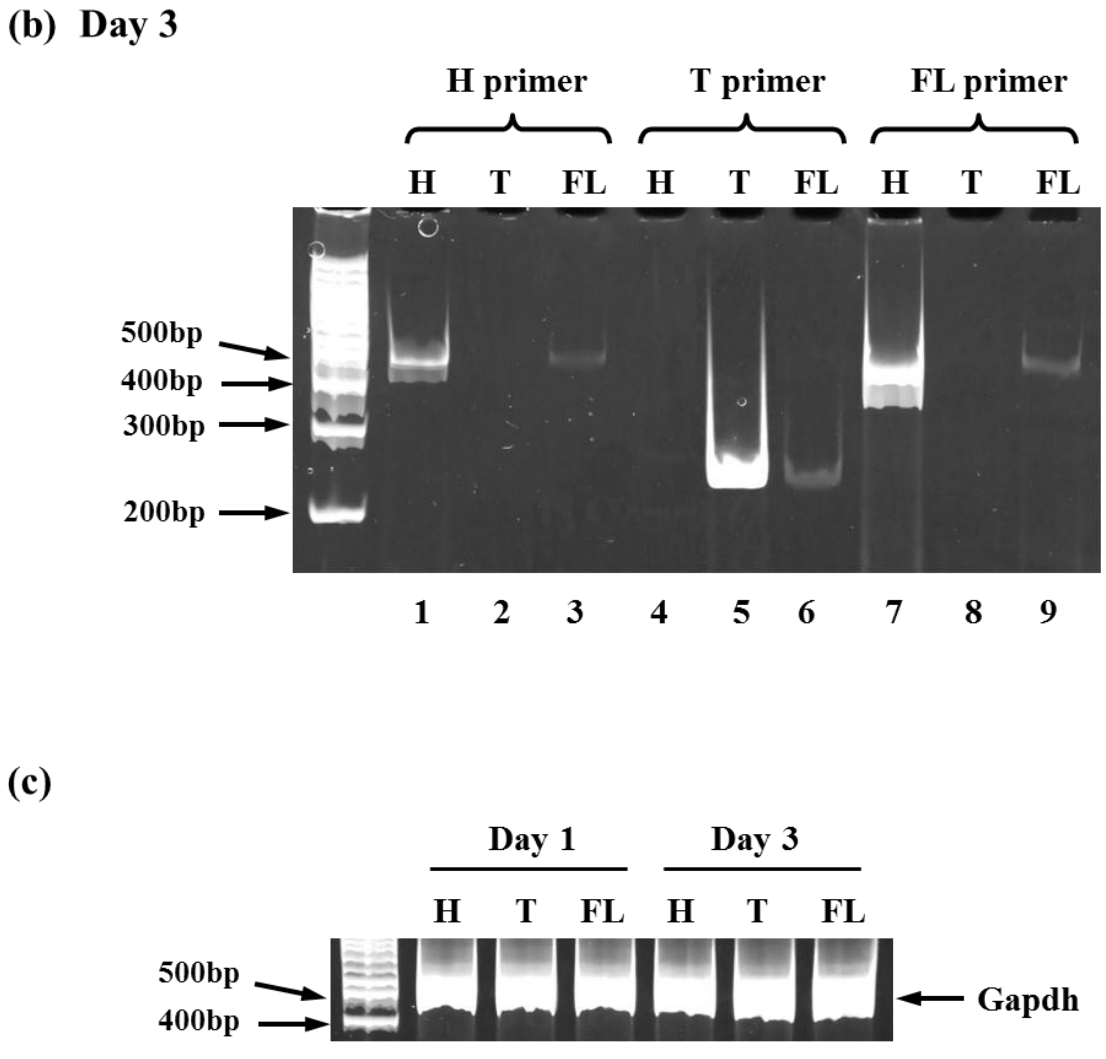

Figure 4. Continued from page 31. (b) Conventional RT-PCR results for vinculin over-expression experiment using RNA isolated from hMSCs three days after transfection. (c) Housekeeping gene Gapdh is used as control.

2.4. Discussion

Western Blot results represent the levels of the proteins of interest in host cells from which the proteins are extracted. The normalized band intensities in Figure 3(b) indicate the vinculin protein levels are similar among the samples, regardless of treated or

not. Therefore, it is not possible to tell if vinculin protein levels rise after different domains of the vinculin gene are individually transfected into hMSCs. Conclusions on the feasibility of vinculin over-expression in hMSCs cannot be drawn from Western Blot results. It might be because the antibody is raised against human vinculin, while the vinculin genes introduced into hMSCs are from chicken. It is not certain that if the antibody can cross-react between human and chicken vinculin genes, therefore, the human vinculin antibody was used as a trial to detect the chicken vinculin over-expressed. However, the results indicated that the antibody mostly only recognize the endogenous human vinculin protein, but fairly the over-expressed chicken vinculin. The similar levels of vinculin protein in all samples are explainable, since they represent the endogenous vinculin protein levels in hMSCs.

The inconclusive results from Western Blot lead to an alternative approach to test the feasibility of transfection. The advantage of conventional RT-PCR is that the primers used can be designed against the different domains of the chicken vinculin genes and the sites in the transfected genes where the primers would bind to are clear and specific. The three primers, head, tail and full-length, are designed to amplify DNA fragments of 440 bp, 239 bp and 440 bp in length, respectively, which correspond to the band sizes in Figure 4. The bands at 440 bp in lanes 1 and 3 show that the head primers recognized the sequences in Vh and Vf and amplified the DNA samples from cells transfected with pEGFP-Vh or pEGFP-Vf. However, the head primers did not amplify the DNA sample from cells transfected with pEGFP-Vt, indicated by no band showing up at 440 bp in lane 2. Similarly, the tail primers that bind specifically to sequences in Vt only amplified the

DNA samples from cells transfected with pEGFP-Vt or pEGFP-Vf, but not the one transfected with pEGFP-Vh. It is implied by the bands at 239 bp in lanes 5 and 6, but no band in lane 4. For the full-length primers, they are designed to bind to sequences in the full-length chicken vinculin gene, and these sequences are located in the head domain of the full-length gene. Therefore, the full-length primers can bind to both transfected Vh and Vf and amplify the DNA samples from cells transfected with pEGFP-Vh or pEGFP-Vf. The bands at 440 bp in lanes 7 and 9 demonstrate the result of recognition by the full-length primers, while no band in lane 8 indicates no primer recognition. The specific amplification or no amplification of DNA fragments in the transfected chicken vinculin genes indicates a successful transfection experiment. The genes transfected into hMSCs successfully replicate and transcribe into corresponding RNA, which is then isolated from the host cells and used in the RT-PCR assays. The plasmid constructs are also specific, since for example, the cells transfected with pEGFP-Vh only specifically express the vinculin head domain gene, and no contamination with other domains. From the results of RT-PCR assays, it can be concluded that the transfection of vinculin domains in plasmid constructs into hMSCs is feasible and specific. Therefore, these constructs with different domains of chicken vinculin genes can be introduced into hMSCs by transfection in future experiment to restore the specific vinculin protein domains in cells.

Chapter 3. VINCULIN KNOCK-DOWN AND ADD-BACK

3.1. Introduction

Vinculin plays an important role in mechanotransduction, via which the mechanical signals can regulate the fate of stem cells. MyoD is a protein that expresses in muscle, which can serve as a marker for the differentiation of hMSCs. The high expression level of MyoD in hMSCs indicates that the stem cells are undergoing differentiation into myoblasts, while low or no MyoD expression indicates that they are still proliferating. In this thesis, hMSCs are growing on PA gels to mimic the ECM microenvironment that hMSCs reside *in vivo*. The stiffness of the PA gels, 11 kPa, is similar to the stiffness of muscles and provides the mechanical cues that are reported to be able to direct the differentiation of hMSCs into muscles. Scientists have long been interested in investigating the molecular mechanism of mechanotransduction in the stem cell fate regulation, and vinculin, as a protein at FA, is actively involved in the process of mechanotransduction. Therefore, in this thesis, endogenous vinculin in hMSCs is knocked down by siRNA to examine the influence of loss of vinculin on hMSC differentiation, which is as a result of impairment in regulation via mechanotransduction. In addition, different domains of chicken vinculin genes are added back into the knock-down hMSCs to examine the restoration of hMSC differentiation.

3.2. Materials and Methods

3.2.1. RNA Interference and Transfection

hMSCs were plated in a 6-well plate as described in 2.3.1 at 1.2×10^5 cells per well and incubated at 37°C and 5% CO_2 overnight. For each well, 10 µl of DharmaFECT transfection reagent (Dharmacon) was diluted into 200 µl DMEM and incubated at room temperature for 5 minutes. 10 µl of 5 µM siRNA (Dharmacon) targeting human vinculin was diluted into 200 µl DMEM. They were mixed and incubated at room temperature for 20 minutes and the 400 µl DNA-DharmaFECT complex was added into the well with hMSCs plated. Then for each well, 10 µl of Lipofectamine 2000 transfection reagent (Invitrogen) was diluted into 250 µl DMEM and incubated at room temperature for 5 minutes. 4 µg of each purified DNA plasmid, pEGFP-Vf, pEGFP-Vh and pEGFP-Vt, was individually diluted into 250 µl DMEM. 250 µl of the diluted Lipofectamine 2000 was added into each 250 µl of diluted DNA and the mix was incubated at room temperature for 20 minutes. Each 500 µl DNA-Lipofectamine 2000 complex was added into corresponding wells. 1.1 ml of DMEM with 2% FBS and no antibiotics was also added to each well. The plate was incubated at 37°C and 5% CO_2 for 24 hours.

3.2.2. Making Polyacrylamide (PA) Hydrogels

The hot plate was turned on to 95°C and cover slips were placed on top. 1 ml of 50 mM NaOH was added to each cover slip until evaporation, followed by 1 ml of ddH_2O until evaporation. The cover slips were moved to fume hood and 300 µl of APES was added, incubated for 5 minutes, and then rinsed three times in ddH_2O. The cover slips were immersed in 0.5% glutaraldehyde for 45 minutes and let dry on a paper towel. Several drops of DCDMS were dripped onto microscope slides and spread around in

fume hood using a 1 ml syringe and an 18 gauge needle. The slides were let stand for 1 minute and wiped down to remove excess DCDMS. 1 ml of 11 kPa PA gel solution was mixed with 10 µl of 10% APS and 1 µl of TEMED. 25 µl of the solution was added onto the slides and covered with a cover slip treated above. The PA gel was allowed to solidify for 15 minutes. The cover slips were then removed from the slides, placed into a sterile 6-well plate. They were rinsed twice with sterile PBS, twice with sterile 50 mM HEPES buffer pH 8.5, and immersed in 1.5 ml of 1X Sulfo-SANPAH. The plate was exposed to UV light for 10 minutes. The cover slips were then rinsed three times with sterile 50 mM HEPES buffer pH 8.5 and immersed in 2 ml of 100 µg/ml collagen type I. The cover slips were incubated at 37°C and 5% CO_2 overnight. They were rinsed twice with sterile PBS on the following day, immersed in 2 ml of sterile PBS and placed in tissue culture hood under UV light for 6 hours to sterilize. hMSCs treated as in 2.4.1 were transferred onto the PA hydrogels with MSC growth medium.

3.2.3. cDNA Synthesis

Total RNA was isolated from the treated cells at three time points: day 2, day 4 and day 6 after the cells were transferred onto the polyacrylamide gels, and in the same way as described in 2.3.4. cDNA was synthesized from the isolated RNA using SuperScript II First-Strand Synthesis System (Invitrogen). For each reaction, 2 µg RNA was mixed with 4 µl of 5X First-Strand Buffer, 1 µl of 0.4 µg/µl random hexamer, 1 µl of dNTP mix (10 mM each), 2 µl of 0.1 M DTT, 1 µl of 200 U/µl SuperScript II Reverse

Transcriptase and DEPC water up to 20 µl. cDNA synthesis was carried out as follows: 60 minutes at 37°C, 5 minutes at 99°C, 5 minutes at 5°C and finally stay at 4°C.

3.2.4. Real-time PCR

Each cDNA sample was run in triplicates for each primer pair with the following 10 µl reaction mix in each well of a 384-well plate: 0.7 µl of cDNA, 5 µl SYBR Green PCR Master Mix (Applied Biosystem), 1 µl of 100 µM forward primer, 1 µl of 100 µM reverse primer and 2.3 µl of DEPC water. The plate was sealed with film and centrifuged at 1000 rpm for 2 minutes. The real-time PCR was carried out as follows: 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

3.2.5. Immunofluorescence Staining

The cover slips were rinsed in PBS and cells were fixed in 3.7% formaldehyde diluted in solution A (500 mM $MgCl_2$ in 1X PBS) for 15 minutes. The cover slips were rinsed three times with solution A and 500 µl of 1% Triton-X in solution A was added, and incubated at 37°C for 30 minutes. They were rinsed three times with solution A. The cover slips were taken out and placed on parafilm. 100 µl of anti-mouse MyoD antibody (Santa Cruz Biotechnology) diluted 1:100 in 2% ovalbumin was added onto each cover slip and incubated at 37°C for 30 minutes, and then rinsed three times with solution A. 100 µl of goat anti-mouse Alexa Fluor 647 tagged antibody (Invitrogen) and Rhodamine Phalloidin (Invitrogen), both diluted 1:1000 in 2% ovalbumin, were added onto each

cover slip and incubated at 37°C for 30 minutes. They were rinsed three times with solution A. 100 µl of Hoechst 33342 (Invitrogen) diluted 1:10000 in ddH_2O was added and incubated at room temperature for 10 minutes. The cover slips were rinsed with ddH_2O and mounted on microscope slides with 2 drops of Fluoromount G (SouthernBiotech). All samples were examined by a CARV II confocal microscope (BD Biosciences) mounted on a Nikon Eclipse TE2000-U microscope with a motorized, programmable stage using a Cool-Snap HQ camera controlled by Metamorph 7.6 software.

3.3. Results

3.3.1. Detection of Vinculin Knock-down and Add-back by Real-time PCR

After testing the feasibility of over-expressing chicken vinculin genes in hMSCs, we then knocked down the endogenous vinculin gene in the host cells and rescued them with chicken vinculin genes. The real-time PCR results are categorized according to the primers used (Figure 5).

WT (wild type) sample is from wild type cells that are not treated with siRNA or transfection, and KD (knock-down), H (head), T (tail) and FL (full-length) samples are from cells that are treated with siRNA against human vinculin gene. In addition, H, T and FL samples have chicken vinculin head domain, tail domain and full-length genes added back into host cells, respectively. As shown in Figure 5(a), two days after the untreated and treated cells grow on 11 kPa PA gels, the hMSC endogenous vinculin level for KD, H, T and FL samples demonstrate a dramatic decrease, which is around 77% - 92%,

comparing with that for the WT sample. The chicken vinculin head domain, which is recognized by the head primers, has a much higher level in H and FL samples than in the other three samples, whose head domain levels are approximately zero. For the chicken vinculin tail domain recognized by the tail primers, its level in WT, KD and H samples are close to zero, while much higher in T and FL samples. The level of chicken vinculin full-length gene is around zero in all except the FL sample, which in contrast shows a much higher level.

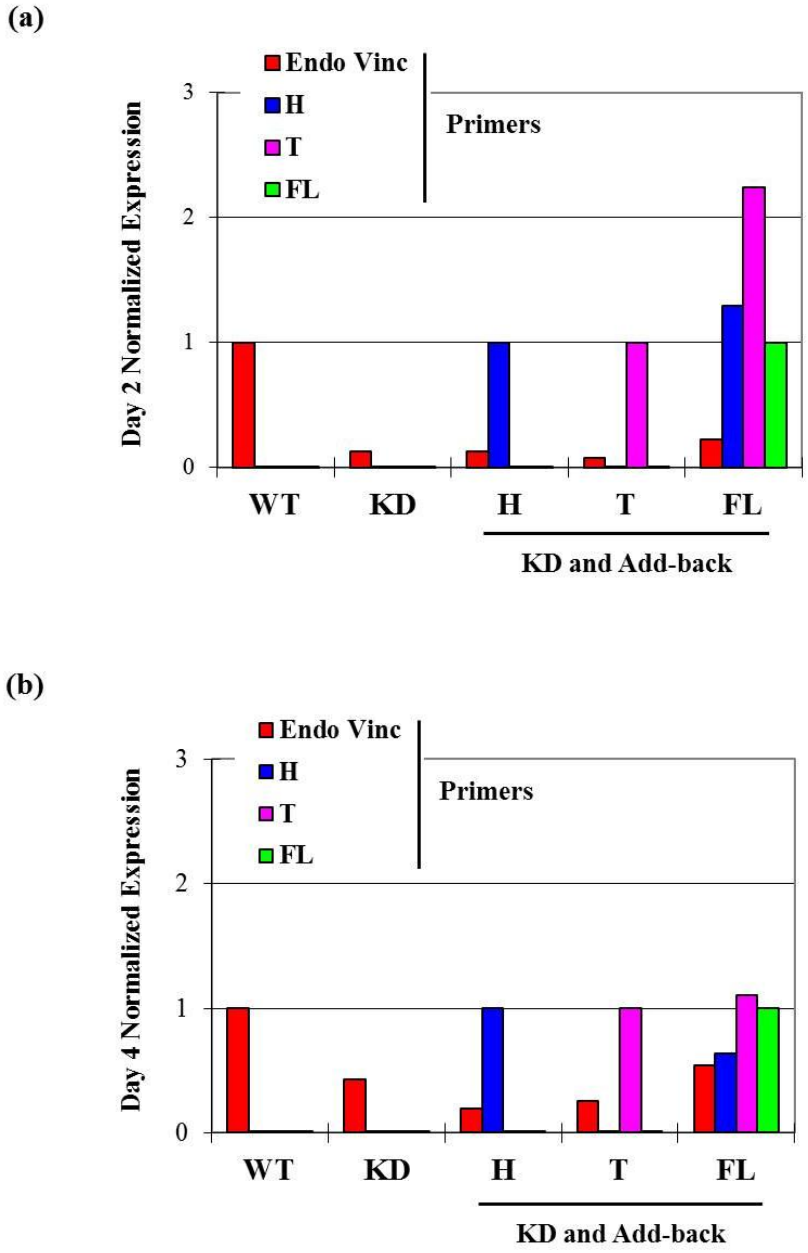

Figure 5. Real-time PCR results for cinvulin knock-down and add-back experiment. WT sample is RNA from untreated control hMSCs. KD, H, T and FL are from cells with endogenous vinculin knock-down by siRNA. H, T and FL also have vinculin head domain, tail domain and full-length gene added back. RNA was isolated two days (a), four days (b) and six days (c) after cells growing on 11 kPa polyacrylamide gels.

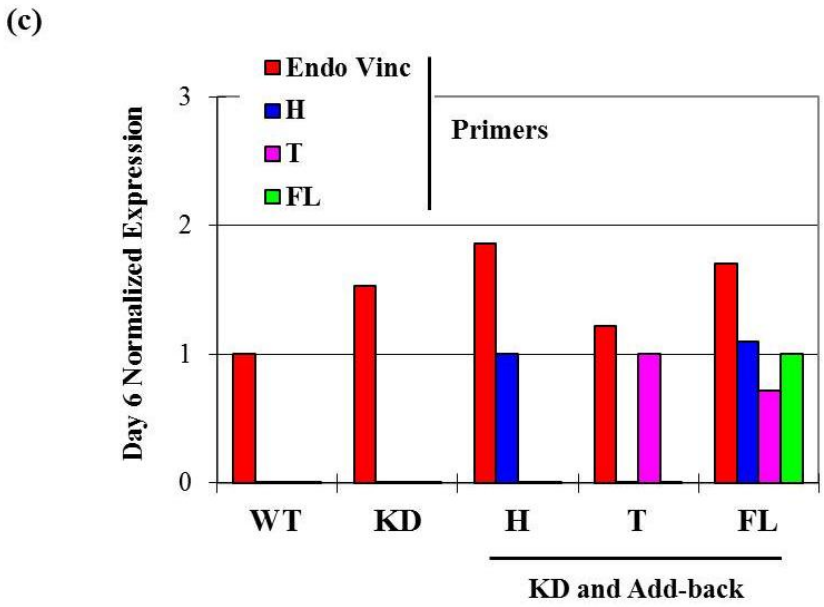

Figure 5. Continued on page 40.

In Figure 5(b), four days after the untreated and treated cells grow on 11 kPa PA gels, the trend of the levels of hMSC endogenous vinculin, chicken vinculin head domain, tail domain and full-length gene for each sample is similar to that for Day 2 samples in Figure 5(a). The level of chicken vinculin head domain is relatively high in H and FL samples, but around zero in WT, KD and T samples. The level of chicken vinculin tail domain is relatively high in T and FL samples, but close to zero in WT, KD and H samples. The level of chicken vinculin full-length gene is relatively high in the FL samples, while around zero in all the other four samples. The hMSC endogenous vinculin level for KD, H, T and FL samples still demonstrated a decent amount of decrease, comparing with that for the WT sample. However, the decrease, which is 45% - 57%, is smaller than that among Day 2 samples. Six days after the untreated and treated cells grow on 11 kPa PA gels, the trend of the levels of chicken vinculin head domain, tail

domain and full-length gene for each sample is similar to that for Day 2 and Day 4 samples. However, the trend of hMSC endogenous vinculin level is different. The level in KD, H, T and FL samples is not lower, but a little higher than that in the WT sample.

3.3.2. Detection of MyoD Expression by Immunofluorescence Staining

IF staining was carried out to determine the expression level of MyoD differentiation marker in hMSCs growing on 11 kPa PA gels under different treatment. As seen in Figure 6(a), four days after the untreated and treated cells grow on 11 kPa PA gels, the MyoD fluorescence is much weaker for the KD sample than for the WT sample. For H and FL samples, MyoD fluorescence is similar to that for the WT sample and stronger than the T sample. It can be confirmed by signal quantification using ImageJ (Figure 6(b)). The intensity of MyoD signal for the KD sample shows a significant decrease, which is around 70%, comparing with that for the WT sample, with $p < 10^{-9}$. The MyoD intensity for the H sample is a little higher than the WT sample. For the T sample, the MyoD intensity is approximately 30% lower than that for the WT sample, with $p < 10^{-4}$. The MyoD signal for the FL sample is not significantly different from the WT sample.

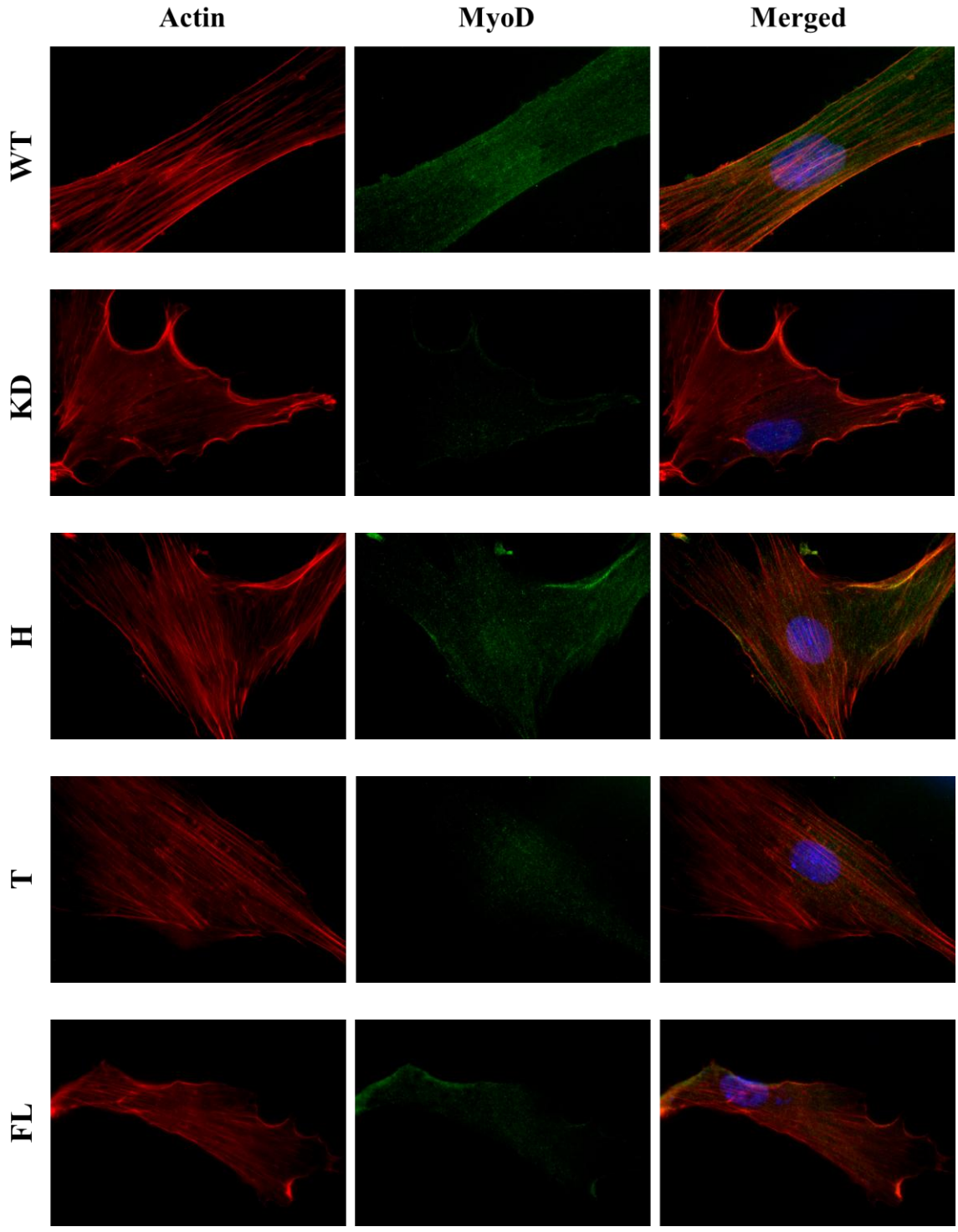

Figure 6. Immunofluorescence (IF) staining results for vinculin knock-down and add-back experiment. (a) IF staining images for WT, KD, H, T and FL samples collected four days after hMSCs growing on 11 kPa PA gels and stained for MyoD (green), actin (red) and nucleus (blue).

(b) Day 4

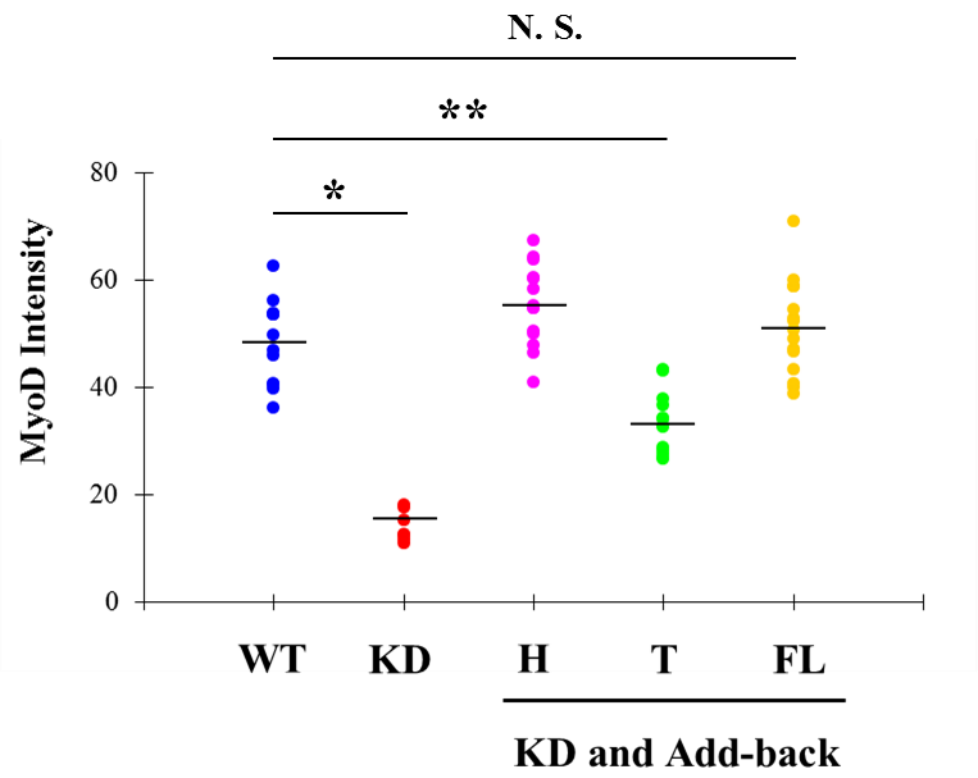

Figure 6. Continued on page 43. (b) Quantification of MyoD signal intensity in (a) using ImageJ. * represents $p < 10^{-9}$, ** represents $p < 10^{-4}$ and N. S. represents Not Significant.

3.4. Discussion

Real-time PCR is used to determine if the endogenous vinculin in hMSCs is knocked down by siRNA against it and if the add-back of chicken vinculin head domain, tail domain or the full-length gene is successful. For Day 2 samples, the 77% - 92% decrease in endogenous vinculin expression in KD, H, T and FL samples comparing with the WT sample indicates that the gene is knocked down by siRNA successfully. However, the decrease in expression for Day 4 samples after siRNA treatment is smaller, and the

expression for Day 6 samples restores to the similar level or even higher than the WT sample. It indicates that siRNA loses its knock-down effect after two to three days of incubation in cells.

The presence of chicken vinculin head domain gene can be detected by the head primers. The close-to-zero expression in WT, KD and T samples shows that there is no chicken vinculin head domain present. The relatively high level in H and FL samples indicates the presence of the head domain gene. The high expression in the H sample is because the head domain gene is added back into the knock-down cells, from which the H sample is extracted. The high expression in the FL sample is because the full-length chicken vinculin gene added back into the knock-down cells also contains the head domain, which is in turn detected by the head primers.

It is the same case for the tail domain gene detected by the tail primers. The gene is only added back into the knock-down cells from which the T sample is extracted. In addition, the full-length gene added back into cells from which the FL samples is extracted also contains the tail domain gene. Therefore, the chicken vinculin tail domain can be detected in both T and FL samples with a relatively high level. The other samples are extracted from cells that do not have the tail domain added back, so they have zero expression of the tail domain gene. The situations correspond to the results in Figure 5.

The full-length primers are designed to recognize the sequences between the dead and tail domain in the full-length chicken vinculin gene, so they can only amplify the full-length gene added back into the knock-down cells. Therefore, the full-length gene expression level is high only in the FL sample, since it is the only sample extracted from

cells rescued with the full-length chicken vinculin gene. The full-length gene expression is close to zero in WT, KD, H and T samples, since they are extracted from cells with no full-length gene added back. The real-time PCR results demonstrate that the H, T and FL samples are from cells whose endogenous vinculin is successfully knocked down and that are specifically rescued with chicken vinculin head domain, tail domain and the full-length gene, respectively.

From Figure 5, it can be seen that the degree of vinculin knock-down is highest on Day 2 and the endogenous vinculin level is restoring after 2-3 days. But for the IF staining results that stained for MyoD, the Day 4 images were chosen to be quantified and analyzed. It is because after vinculin was knocked down, the mechanosensing would take some time to happen and the influence on MyoD level was not immediate. MyoD would be at the lowest level on Day 4. As shown in Figure 6(b), MyoD expression level decreases remarkably in the KD sample and the difference between KD and WT samples is statistically significant ($p < 10^{-9}$), implying that the myogenesis of hMSCs is adversely affected due to vinculin knock-down. The loss of vinculin in these hMSCs impairs the normal signaling pathways in the process of mechanotransduction and the mechanical cues from the 11 kPa PA gels cannot be properly transduced in hMSCs, which in turn impedes the regulation of hMSC differentiation. It reinforces that the normal hMSC differentiation is directed by the mechanical signals via functional mechanotransduction process. When the full-length chicken vinculin gene was added back into the knock-down hMSCs, the results showed restoration of MyoD expression, as the MyoD intensity is at the similar level to the WT sample and the small difference in MyoD intensity between

FL and WT samples is not statistically significant. Therefore, the add-back of the full-length vinculin gene successfully rescued the myogenesis of hMSCs. The results from the KD and FL samples together indicate that vinculin is necessary for MSC myogenesis. When subjected to physiological stress, such as cellular contraction to feel the matrix stiffness, the talin-vinculin adhesion complex would undergo conformational changes to expose a cryptic MAPK1 binding site within vinculin. The MAPK1 signaling cascade can then promote muscle differentiation and the myogenesis of hMSCs can be illustrated by MyoD expression. The add-back of the chicken vinculin tail domain did not successfully rescue the hMSC myogenesis, since the MyoD intensity for the T sample is 30% lower than the WT sample, and the difference is statistically significant ($p < 10^{-4}$). The add-back of the head domain resulted in a MyoD expression higher than the WT sample. We could not conclude if the vinculin head domain itself can rescue MSC myogenesis since the MyoD level is higher than that in the wild type untreated sample. The experiment needs to be repeated for more times to confirm the consistency of the results. It is possible that the higher MyoD level in the H sample is due to the constantly active state of vinculin head domain. The normal full-length vinculin adopts an autoinhibited conformation when in its native cytoplasmic state, with the head and tail domains being in proximity to mask the protein binding sites inside vinculin. It requires force-induced activation to become functional. The head domain gene added back into hMSCs only expresses the head domain of vinculin protein. The protein would no long have the inhibited state due to the lack of the tail domain. It is possible that the constantly active state might have an influence on the function of vinculin that is important for the myogenesis of hMSCs.

Chapter 4. CONCLUSION

Stem cells have the unique ability to decide their fate between self-renewal and differentiation, which is of great significance in embryonic development and tissue regeneration (NIH, n.d.). Intracellular gene regulation mechanisms at epigenetic, transcriptional, translational and post-translational levels have always been known to be involved in defining stem cell fate and behavior (Gangaraju et al., 2009). Research in recent decades has established that exogenous factors in the microenvironments surrounding stem cells also play an essential role in the regulation. Besides the soluble factors, metabolic products and adjacent cells in the stem cell microenvironment, scientists have recently focused on the influence of ECM components that can exert on regulating stem cell fate (Scadden, 2006). In particular, the mechanical properties of ECM are now recognized as novel regulators for stem cell differentiation. MSCs, which have the ability to differentiate into bone, muscle and cartilage cells with diverse mechanical properties, have become a particularly appropriate system for studying stem cell behavior regulated by mechanical signals (Keung et al., 2010). MSCs have shown to be able to differentiate based on the stiffness of their ECM and the mechanical signals regulate MSC differentiation via mechanotransduction. In the process, biophysical/mechanical information from ECM is converted into biochemical signals involved in the downstream intracellular signaling pathways. There are two forms of mechanotransduction: passive or outside-in mechanosensing, in which external forces such as shear stress are detected and transduced upon cells, and active or inside-out mechanosensing, in which cells can generate their own forces to detect mechanical

changes in their external environment (Holle et al., 2011). However, the exact molecular mechanisms underlying mechanotransduction still remain unclear. It has been shown that FA serves as a platform for cell mechanotransduction. Among the key proteins at FA, integrin attaches cells to ECM, talin and α-actinin bridge integrins to actin cytoskeletons, and some others, such as vinculin, interact with talin, α-actinin and actin. They serve as mechanosensors that enable the process of mechanotransduction (Shemesh et al., 2005). Vinculin is an ideal candidate for studying mechanotransduction since it is closely associated with FA mechanosensitivity. Vinculin-deficient cells are less stiff than normal cells and the traction forces generated are lower, and they display impaired cell spreading and cell migration (del Rio et al., 2009). Under appropriate physiological forces, vinculin would undergo a conformational change exposing a crytic MAPK binding site (Holle et al., 2011). hMSC endogenous vinculin knock-down by siRNA resulted in a 70% decrease in MyoD expression after four days growing on matrices with stiffness that mimics muscles (11kPa). MyoD, expressing in muscles, serves as a marker for the differentiation of hMSCs. The chicken vinculin full-length genes with a MAPK1 binding site was shown to be able to restore the MyoD expression level when added back into hMSCs. It is likely that some FA proteins, like vinculin, are sensitive to the mechanical stimuli in the environment surrounding the cells. They can undergo conformational changes induced by cell traction forces that feel the specific matrix stiffness, and can expose some cryptic protein binding sites necessary for the signaling pathways. In this way, mechanical signals are transduced into intracellular biochemical signals and hMSC differentiation can be specifically directed when growing on matrices of specific stiffness..

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