The Effects of Matrix Elasticity, Composition, and Exogenous Growth Factors on the Vascular Differentiation of Mesenchymal Stem Cells

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The Effects of Matrix Elasticity, Composition, and Exogenous Growth Factors on the Vascular Differentiation of Mesenchymal Stem Cells

Thesis directed by Dr. Wei Tan

Cardiovascular diseases are the leading cause of death in the western world. Regeneration of functional vascular tissue remains a critical barrier to successful treatment of these diseases. Attempts to produce functional vascular tissue with autogenous vascular cells have limited success due to the need for invasive surgery. Mesenchymal stem cells (MSCs) are a powerful cellular alternative for vascular regeneration as they are easily obtainable, multipotent, and thrombo-resistant. Currently, the mechanisms that drive MSC differentiation to healthy or diseased vascular phenotypes are not well understood. There is a critical need to define the factors in the cellular microenvironment that guide MSC differentiation. This dissertation examines how matrix elasticity, composition, and exogenous chemicals interact in a 3 dimensional nanofibrous environment to direct the vascular differentiation of MSC. Polymer nanofiber matrices are fabricated with tunable elasticity utilizing electrospinning and photopolymerization techniques. Varying the elasticity of the matrix directs MSC differentiation towards either endothelial or smooth muscle cell lineage, while the addition of exogenous chemicals furthers MSC differentiation to mature vascular phenotypes. The incorporation of peptides such as RGD in these matrices increases MSC adhesion and proliferation. This dissertation highlights the importance of carefully modulating both chemical and mechanical factors when designing cell therapies or tissue engineered grafts for vascular tissue regeneration.

Dedication

So many people in my life have made this journey possible.

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CHAPTER 1

Introduction

1.1 Motivation and Specific Aims

In 2008, cardiovascular diseases resulted in 1 out of every 3 deaths in the United States. (Roger et al., 2012) Regeneration of functional vascular tissue remains a critical barrier to successful treatment of these diseases. Biomaterials that attempt to replicate vascular tissues have limited success *in vivo*; they lack the key vascular cells that provide necessary functionality such as the contractile properties supplied by smooth muscle cells (SMCs) within the media, and anti-thrombogenic characteristics given by endothelial cells (ECs) in the intima. Current attempts to produce functional vascular tissue with autogenous vascular cells have limited success due to the need for invasive surgery and the limited expansion capability of these cells *in vitro*. (Lith and Ameer, 2011)

Mesenchymal Stem Cells (MSCs) are a powerful cellular alternative for vascular tissue regeneration, as they are easily obtained through a bone marrow biopsy, have a large expansion capability *in vitro*, are multipotent, and can be donated from donor to patient with no rejection. (Hodgkinson et al., 2010) The potential role of MSCs in vascular regeneration is becoming increasingly recognized. (Park et al., 2007; Riha et al., 2005a) Hashi et al seeded MSCs on electrospun polymer vascular grafts and found that distinct EC and SMC layers formed on the grafts.(Hashi et al., 2007a) Direct injection of MSCs into the heart resulted in transdifferentiation of MSCs into ECs along the vessel lumen. (Silva et al., 2005a) Currently the local environmental factors that direct vascular MSC differentiation

are poorly understood, which can lead to catastrophic results in treatment. For example, O'Shea et al recently determined bolus delivery of MSCs to injured vasculature can produced a dysfunctional endothelium. (O'Shea et al., 2010a) Furthermore, there has been increased evidence that incorrect differentiation of MSCs may result in progenitor cells involved in vascular pathology.(Xu, 2008a; Yeager et al., 2011) In order to safely utilize MSCs in cardiovascular therapies, it is critical to define the factors in the cellular microenvironment that guide MSCs differentiation to healthy vascular tissue.

Researchers have demonstrated that individually, factors of the cellular microenvironment such as matrix elasticity, matrix composition, and exogenous growth factors such as vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) contribute to MSC differentiation towards vascular phenotypes. (Huang and Li, 2008) However, in the *in vivo* vessel, it is likely that these factors combine to direct MSC differentiation towards specific cellular phenotypes (Figure 1.1).

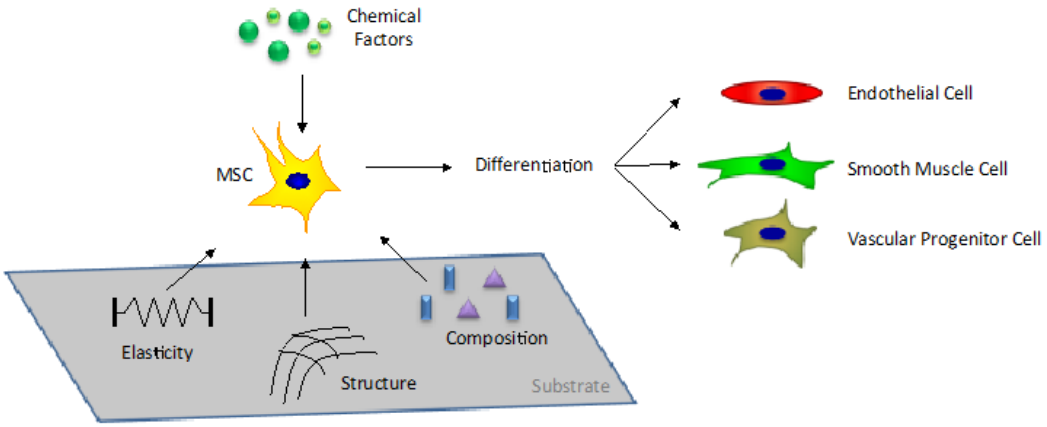


Figure 1.1: Factors in the vascular cellular microenvironment interact to direct MSC towards specific vascular lineages

Currently, it is not well understood how factors in the cellular microenvironment interact to modulate MSC differentiation to healthy vascular phenotypes. Furthermore, many experiments studying MSC differentiation are conducted on flat 2D substrates instead of simulating the 3D nanofiber matrix structure found *in vivo.*

Identifying the influence of biomimetic environments on the vascular differentiation of MSCs in terms of lineage commitment and differentiation stage is critical for cardiovascular research. The mechanisms of vascular MSC differentiation can be utilized to direct healthy vascular tissue regeneration through cell therapies or tissue engineered grafts. We aim to bridge the knowledge gap between the current ability to differentiate MSCs into vascular-like progenitor cells and the need to differentiate MSCs into specific, mature vascular cells. The main goal of this dissertation is to examine how matrix elasticity, matrix composition, and growth factors interact to direct the differentiation of MSCs to specific vascular phenotypes in a 3D nanofiber matrix. Our overarching hypothesis is that specific combinations of matrix elasticity, matrix composition, and exogenous growth factors will act synergistically, guiding MSC differentiation to more mature vascular lineages then the independent use of these factors.

The specific aims for this study are:

Aim 1: Determine the effects of 3D nanofiber matrix elasticity on vascular MSC differentiation.

1.1 Fabricate 3D nanofiber matrices with tunable elasticity that mimic the *in vivo* vascular microenvironments of the intima and media.

1.2 Identify the optimal elasticity values for MSC to EC and MSC to SMC differentiation.

Hypothesis: Utilizing a 3D nanofiber matrix to replicate the elasticity of the mechanical

microenvironments found in the vessel endothelial (2 to 5 kPa) and medial layers (8 to 15 kPa) will induce MSCs to differentiate towards vascular cells with EC or SMC-like characteristics, respectively.

Aim 2: Determine the combined effects of 3D matrix elasticity (2 to 5 kPa) and VEGF on the differentiation of MSCs to endothelial lineage and MSC paracrine signaling capabilities.

2.1 Evaluate MSC differentiation in VEGF, 2 kPa, and 2kPa + VEGF conditions by examining endothelial markers via PCR and western blotting.

2.2 Evaluate MSC paracrine signaling by examining EC migration and angiogenesis abilities in MSC conditioned media from VEGF, 2 kPa, and 2 kPa + VEGF seeding conditions.

Hypothesis: The combined effects of VEGF and a matrix elasticity that mimics that of the vascular intima will increase MSC paracrine signaling abilities as well as induce more complete MSC differentiation into mature endothelial phenotypes compared to the independent use of these factors.

Aim 3: Determine the combined effects of 3D matrix elasticity (8 to 15 kPa), vascular-specific peptides, and TGF-β on the specificity and maturation of MSC-differentiated SMC.

Hypothesis: The combination of TGF-β, a matrix elasticity that mimics that of the vascular media, and vascular-specific peptides will direct more complete MSC differentiation to SMCs that express mature markers.

The overarching hypothesis and aims of this thesis are summarized below in Figure 1.2.

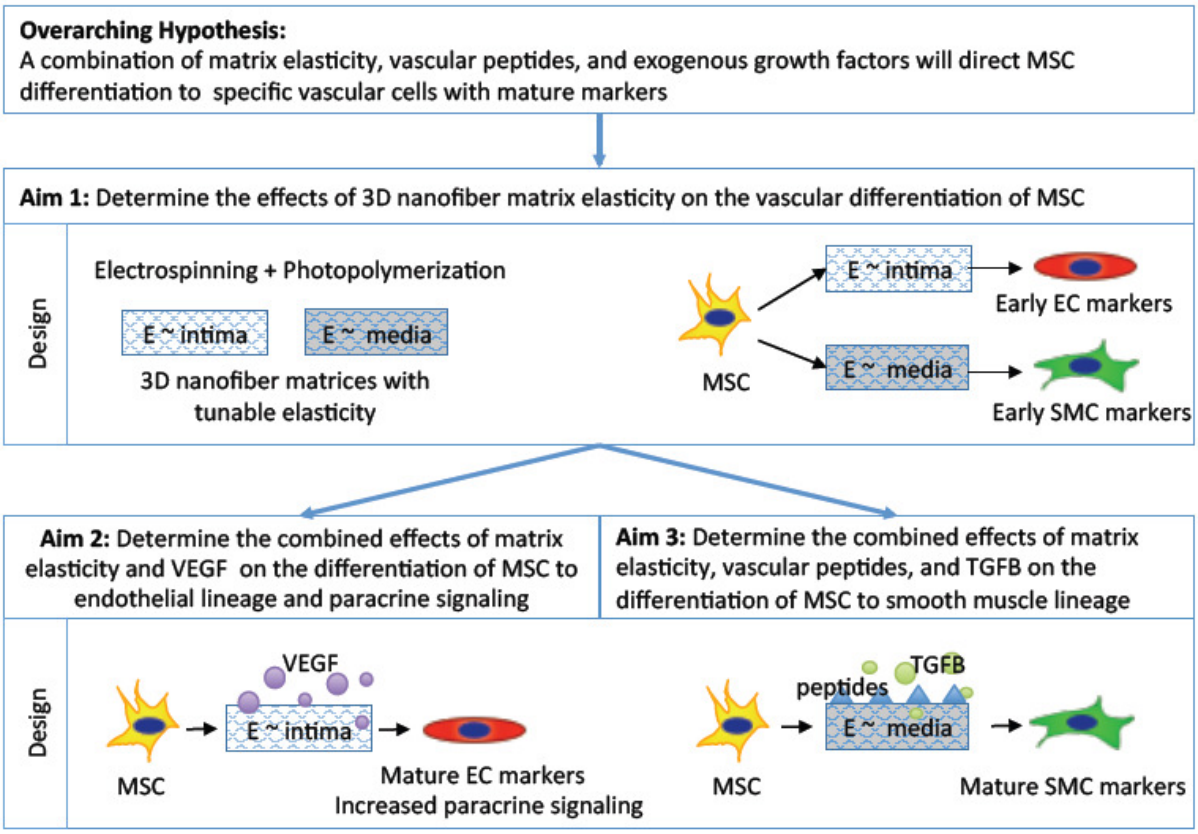


Figure 1.2: Overarching hypothesis, aims, and experimental design of dissertation. E refers to modulus of elasticity.

1.2. Background Information

1.2.1 The Treatment of Cardiovascular Disease

Cardiovascular diseases are the leading cause of death in the western world, resulting in 1 out of every 3 American deaths in 2008. In the year 2008 it is estimated that the treatment of cardiovascular diseases cost the United States over 300 billion dollars. (Roger et al., 2012) The cardiovascular system is comprised of the heart and blood vessels, dynamic conduits that work in synchronous with the heart to control the flow of blood through the body.(Hall, 2010) Blood vessels are composed of vascular tissues that have critical functionalities, such as the anti-

thrombogenic properties of the vascular endothelium and the contractile abilities of the vascular media. Specific vascular cells drive tissue functionality; ECs in the intima secrete anti-thombogenic factors, while SMCs in the media contract, allowing the vessel to regulate blood flow. (Lith and Ameer, 2011; Nerem and Seliktar, 2001) Two of the most prevalent cardiovascular diseases are atherosclerosis, the narrowing of the vessel lumen due to plaque buildup; and myocardial infarctions, the loss of heart function due to interruption of blood supply to the myocardium. In both of these diseases vascular cells are damaged, resulting in the loss of key tissue functionalities, which then reduce cardiovascular capacities (Figure 1.3). (Sima et al., 2008) (Thygesen et al., 2007) To reduce cardiovascular disease morbidity and mortality it is critical to utilize treatments that repair and regenerate functional vascular tissue. In turn, a significant portion of cardiovascular research has focused on developing and improving vascular tissue engineered grafts and cell therapies.(Gnecchi et al., 2008; Lith and Ameer, 2011) Both of these therapies hinge on the ability to supply and sustain healthy vascular cells that can regenerate vascular tissue and restore functionality. (Nerem and Seliktar, 2001) Factors in the cellular microenvironment such as extra-cellular matrix (ECM) composition, elasticity, structure, and exogenous growth factors determine the health and functionality of a cell.(Huang and Li, 2008) *In order to regenerate functional*

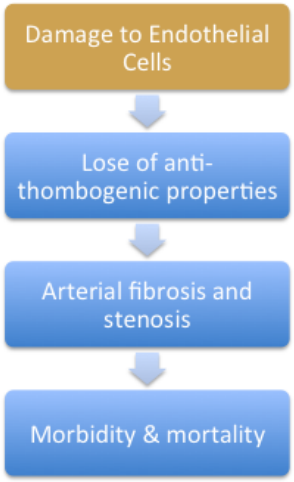


Figure 1.3: The loss of key cellular functionalities results a sequence of events leading to cardiovascular diseases. To treat these diseases, cellular functionality must be restored.

vascular tissue for the treatment of cardiovascular diseases, it is critical to define the cellular microenvironments of healthy vascular cells.

1.2.2 Vascular Cell Microenvironment

The blood vessel is a multilayered structure, composed of the intima, media, and adventitia (Figure 1.4). Each layer of the blood vessel has necessary functionalities that combine synergistically to regulate the flow of blood and transfer of nutrients throughout the body. The innermost layer is the intima, which prevents thrombosis and regulates the transfer of nutrients across the vessel wall.(Bouïs et al., 2001) The media provides compliance and contraction, regulating blood flow in conjunction with the heart.(Hahn and Schwartz, 2009) The adventitia is the outermost layer which provides key mechanical properties that allow the blood vessel to withstand high stresses due to blood pressure. (Wagenseil and Mecham, 2009) The cellular microenvironments of the intima and media will be focused on in this review. Each vessel layer has a unique vascular cell type with specific abilities that drive the tissue's functionality; ECs reside in the intima, while SMCs are located in the media. (Wagenseil and Mecham, 2009) The vascular cell environment is a structured ECM that is composed of collagen type I, III, IV, and V, elastin, and proteoglycans. (Wagenseil and Mecham, 2009) (Herbst et al., 1988) Vascular cells bind to the ECM through integrins, transmembrane receptors that transmit signals from the ECM to the cellular interior. (Alenghat and Ingber, 2002) A feedback loop forms between the

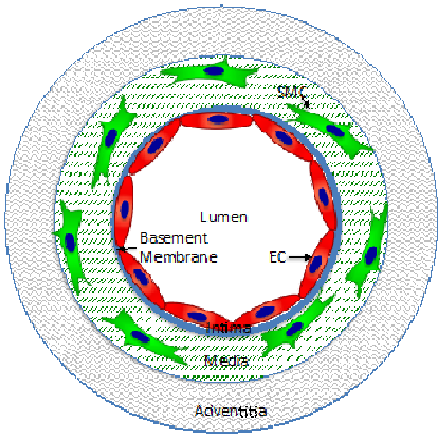


Figure 1.4: Layered structure of the blood vessel.

ECM and cell through the integrin, allowing the cell to sense the composition, elasticity, and structure of the ECM. Simultaneously, exogenous growth chemicals in the vessel environment act other cell receptors. (Discher et al., 2009) These factors combine to direct the cell's phenotype, functionality, and drive cellular remodeling of the ECM. (Lith and Ameer, 2011; Park et al., 2007)

Endothelial Cell: Function, Markers, and Microenvironment

The intima, or the EC layer is considered the 'holy grail' of vascular tissue engineering, as ECs prevent the accumulation of plaque by releasing anti-thombogenic factors. (Lith and Ameer, 2011) ECs control the migration of cells and nutrients across the blood vessel wall, allowing nutrients to enter the tissue. The EC layer has tight junctions that keep blood within the vessel and prevent other cells such as SMCs and fibroblasts from migrating into the vessel lumen. (Bouïs et al., 2001) Further, ECs are critical in angiogenesis, the formation of new blood vessels. (Risau, 1997) ECs are characterized by the Flk-1, Flt-1, VECAD, PECAM, vWF and eNOS protiens, and form a monolayer with cobblestrone morphology along the basement membrane (Figure 1.4). The basement membrane is composed of collage IV and laminins; laminins are believed to provide cell binding sites, while collagen IV is thought to contribute to cellular functionality. (Garlanda and Dejana, 1997) (Ades et al., 1992) (Herbst et al., 1988) While ECs typically reside along the two-dimensional surface of the basement membrane, during angiogenesis ECs will degrade the basement membrane and migrate into the three-dimensional ECM, forming tube-like structures that will eventually become new blood vessels.(Delgado et al., 2011) In the healthy intima, ECs reside in a relatively soft microenvironment; the elasticity of the basement membrane has been measured at 2 to 5 kPa. (Peloquin et al., 2011) (Liliensiek et

al., 2009) VEGF is a prominent chemical in the intima, promoting endothelial cell growth, acting as an endothelial survival factor, and encouraging angiogenesis. (Ferrara, 2004)

Smooth Muscle Cells: Function, Markers, and Microenvironment

SMCs reside in the media, and regulate the contraction of the artery in conjunction with ECs and the sympathetic nervous system. (Deanfield et al., 2007; Hall, 2010) SMCs can proliferate, migrate, and synthesize the components of ECM, changing the structure, compliance, and strength of the vessel due to surrounding environmental cues. (Rensen et al., 2007) SMCs have two phenotypes; a proliferative phenotype utilized in embryogenesis and ECM remodeling, and a contractile phenotype that allows for healthy vessel contraction. The proliferative phenotype is characterized by an epithelial morphology, the smooth muscle alpha actin (SMA) marker, and the loss of myosin heavy chain (MHC) protein. The contractile phenotype has a striated morphology with smooth muscle actin co-located along the f-actin filaments, and the protein markers of SMA, MHC, calponin, and smoothelin. (Rensen et al., 2007) (Wagenseil and Mecham, 2009) The vascular media has a 3D ECM structure, and is located on the opposite side of the basement membrane from intima. The media ECM is composed of collagen I, III, and IV, elastin, and protegolycans. (Stegemann et al., 2005) (Kanie et al., 2012) The media is slightly stiffer then the intima, with an *in vivo* elasticity measured at 5 to 9 kPa. (Richert et al., 2004) TGF-β is found in the vascular media, and is linked to SMC vascular remodeling and contractile phenotypes. (Azhar et al., 2003)

1.2.3 Difficulties of Autogenous ECs and SMCs for Tissue Engineering

While the functionality of SMCs and ECs must be replicated for the successful regeneration of vascular tissue, there are significant hurdles to utilizing these cells for tissue engineering or cell therapies. ECs and SMCs cannot be donated from donor to patient without rejection issues, and therefore the patient's own cells must be utilized. Harvesting ECs and SMCs requires invasive surgery that is prohibitive in sick patients or emergency situations. (Lith and Ameer, 2011) *In vivo,* ECs do not proliferate well, resulting in long wait times prior to treatment, while SMCs de-differentiate to a non-functional phenotypes. (Lith and Ameer, 2011) (Rensen et al., 2007) Further, the use of both ECs and SMCs requires multiple cell seedings on engineered grafts, necessitating complex designs and fabrication processes.(Lith and Ameer, 2011) *In vascular tissue engineering there is a need for an alternative cell source that can replicate both EC and SMC functionalities.*

1.2.4 MSCs for Vascular Tissue Engineering

MSCs are proposed to reside in numerous locations throughout the human body, and thought to home to sites of injury to repair and regenerate tissue. Further, MSCs are multipotent, easily obtained through a bone marrow biopsy, and have nearly unlimited expansion capability given the proper environments *in vitro*.(Parekkadan and Milwid, 2010) Both the natural role of MSCs *in vivo* and their ease of use *in vitro* make them a powerful tool for vascular tissue engineering. (Park et al., 2007) *In vivo*, MSCs

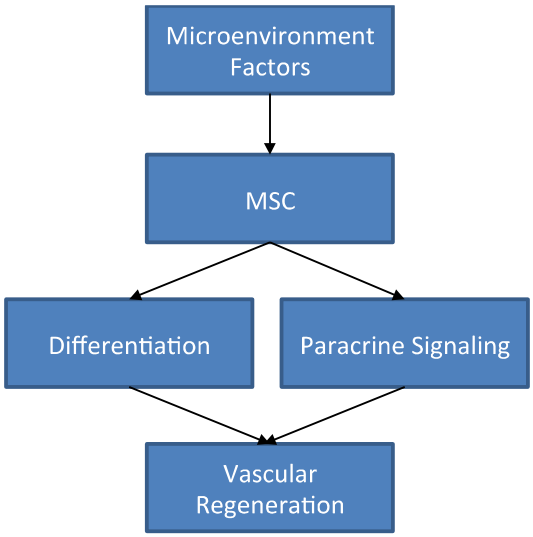


Figure 1.5: MSC regenerate vascular tissue through two mechanisms: differentiation and paracrine signaling. These mechanisms are guided by factors in the MSC microenvironment.

regenerate vascular tissue through two mechanisms, differentiation and paracrine signaling (Figure 1.5).

In paracrine signaling, MSCs release biochemicals that encourage nearby cells to regenerate surrounding tissue. (Hocking and Gibran, 2010) *In vitro,* MSC paracrine signaling has been shown to increase endothelial migration, proliferation, and capillary formation, critical steps in the re-vascularization of damaged tissue. (Caplan and Dennis, 2006; Potente et al., 2011) *In vivo*, it has been demonstrated that sole use of biochemicals released by MSCs can increase vascularization of the myocardium, resulting in recovered heart function. This research has led the FDA to approve clinical human trials of treating myocardial infarctions by the direct injection of MSCs into the damaged myocardium.(National Institutes of Health) However, while it is understood that MSCs promote vascular tissue regeneration through paracrine signaling, the underlying mechanisms that drive MSC paracrine signaling remain uncharacterized. *To improve the safety and efficacy cardiovascular treatments by MSC paracrine signaling, it is critical to understand how factors in the cellular microenvironment drive MSC paracrine signaling.*

Differentiation is the direct change of a MSC from a multipotent cell to a cell with a specific lineage and functionalities. (Angoulvant et al., 2011) Recent studies have shown MSCs have the ability to differentiate towards both SMC and EC lineages *in vitro* and *in vivo.* (Hashi et al., 2007a; Silva et al., 2005a) (Oswald et al., 2004) (Kinner et al., 2002) However, researchers have yet to achieve MSC differentiation into mature, functional vascular cells, a critical step for regenerating healthy vascular tissue. Furthermore, other studies have questioned the safety and efficacy of utilizing MSCs for vascular regeneration. O'Shea et al recently found that injection of MSCs to injured vasculature produced a diseased intima, resulting in a higher rate of vessel occlusion. (O'Shea et al., 2010b) Research indicates a poorly defined vascular microenvironment

for MSC differentiation can result in MSC-derived progenitors, which may be involved in diseased vascular remodeling such as neo-intima hyperplasia. (Tsai et al., 2009; Yeager et al., 2011). Currently, the elements in the cellular microenvironment that guide MSC differentiation into healthy or diseased vascular phenotypes are not well studied. *To unravel the role of MSC in both functional and pathological vascular remodeling, it is essential to understand the factors in the cellular microenvironment that direct MSC differentiation.*

1.2.5 Factors in the Cellular Microenvironment that Impact MSC Paracrine Signaling

Recent studies demonstrate that MSCs release paracrine signaling factors which aid in tissue regeneration. These signaling factors guide ECs to revascularize surrounding tissue through angiogenesis. Recently, Sieb et al found that decreasing substrate elasticity reduced the secretion of an inflammatory chemical by MSCs, suggesting that elasticity can modulate MSC secretory functions. (Seib et al., 2009) Numerous studies have found that VEGF plays a significant role in angiogenesis. (Mirotsou et al., 2011) (Cheng and Yau, 2008) Currently, the mechanisms by which factors in the cellular microenvironment direct MSC paracrine signaling are not well understood. *In aim 2, we demonstrate that VEGF and soft matrix elasticity combine to increase MSC paracrine signaling, improving EC angiogenic abilities.*

1.2.6 Factors in the Cellular Microenvironment that Direct MSC Differentiation

In vivo, factors in the cellular microenvironment such as exogenous chemicals, matrix elasticity, structure, composition, and dynamic mechanical forces interact to guide MSC differentiation. In this dissertation we will focus on the differentiation effects of static factors in the cellular microenvironment, including exogenous growth factors, matrix elasticity,

composition, and structure. Previous studies of MSC differentiation along with studies in this dissertation are summarized in Table 1.

Table 1: Studies examining the vascular differentiation of MSC

Cell Type	First Author	Year	Elasticity	Growth Factor	Composition	Structure	Time Point	Markers and Functionality
EC	Oswald et al	2004	Stiff	VEGF		2D	7 days	vWF, VWCAD, VECAM capillary-like structure formation
	Zhang et al	2011	G' 50 - 200 Pa	-	Fibrin	3D	3 days	Flk-1 PECAM
	Wingate et al	2012	E 2 - 5 kPa	-	Collagen I	3D	1 day	FLK-1
	Wingate et al	2013	E 2 - 5 kPa	VEGF	Collagen I	3D	1 day	FLt-1, vWF, eNOS, capillary-like structure formation
SMC	Kinner et al	2002	Stiff	TGFB		2D	5 days	SMA Contraction
	Xie et al	2011	Stiff		Collagen IV	2D	6 days	SMA
	Park et al	2011	E 15 kPa	TGFB		2D	2 days	SMA, Calponin
	Wingate et al	2012	E 8 - 15 kPa	-	Collagen I	3D	1 day	SMA

Elasticity

The elasticity of the cellular microenvironment plays a critical role in the health, functionality, and differentiation of cells. Kloxin et al demonstrated that fibroblasts could be de-differentiated from a diseased myofibroblast phenotype to a healthy fibroblast phenotype simply be decreasing the matrix stiffness *in vitro*. (Kloxin et al., 2010a) On substrates that mimic the elasticity of a healthy myocardium, cardiomyocytes will beat; when the elasticity of the substrate is increased to that of a post-infart scar, the cardiomyocytes lose the ability to beat. (Engler et al., 2008) MSCs are particularly sensitive to the elasticity of their microenvironment. Past research has shown that cellular substrates with a modulus mimicking that of *in-vivo* neural, muscular, and bone tissues guide MSC differentiation into neural, myogenic, and osteogenic lineages, respectively. (Engler et al., 2006) Recently, studies have examined the effect of elasticity on vascular differentiation in conjunction with other variables in the vascular microenvironment. Zhang et al found that fibronectin-PEG matrices with a storage modulus of 50 to 200 Pa directed MSCs towards endothelial lineage. (Zhang et al., 2010a) Park et al demonstrated that smooth muscle markers SMA and calponin were higher in MSC seeded on 15 kPa gels with TGF-β then

MSC seeded on 2 kPa gels with TGF-β. (Park et al., 2011a) While these studies give some insight into the effect of elasticity on vascular differentiation, it remains unclear if matrix elasticity can independently guide MSC differentiation towards EC and SMC lineages. Further, it is unknown how matrix elasticity affects the vascular functionality and health of the final differentiated MSC phenotype. *In vivo* measurements of the healthy intima and media determine the local compressive modulus of the tissue to be 2-3 kPa (intima) and 5-9 kPa (media), and research indicates that diseased fibrotic vascular tissue can be order of magnitudes stiffer than healthy vascular tissue. (Peloquin et al., 2011) (Liliensiek et al., 2009) (Richert et al., 2004) (Matsumoto et al., 2002) *In aim 1 of this dissertation we demonstrate that replicating the elasticity of the mechanical microenvironments found in the vessel endothelial (2 to 5 kPa) and medial layers (8 to 15 kPa) induce MSCs to differentiate towards vascular cells with EC or SMC-like characteristics, respectively. In aims 2 and 3 we demonstrate that elasticity is an essential factor in guiding MSC differentiation towards functional, healthy vascular phenotypes.*

Exogenous Chemicals

Exogenous chemicals are a key factor in the cellular microenvironment, and have been extensively studied as tools to direct MSC differentiation. VEGF is a prominent chemical in the endothelial environment, linked to EC survival. (Ferrara, 2004) TGF-β is found in the vascular media, and is known to encourage a healthy contractile SMC phenotype. (Azhar et al., 2003) Studies have found that TGF-β directs MSC differentiation into cells with SMC markers, including SMA.(Kinner et al., 2002) Park et al found that stiff substrates combined with TGF-β resulted in high levels of SMA and calponin, 15 kPa substrates combined with TGF-β resulted in medium levels of SMA and calponin, and 1 kPa substrates with TGF-β resulted in low levels of

SMA and calponin. However, the study did not examine mature SMC markers such as MHC. (Park et al., 2011a) VEGF has induced MSC differentiation into endothelial-like cells with endothelial markers, including vWF, VCAM-1 and VE-CAD. However, this differentiation took a long cell culture time (7 days), and did not demonstrate more mature endothelial markers such as Tie 2 and eNOS. (Oswald et al., 2004) While VEGF is an essential chemical in the vascular microenvironment, increased levels of VEGF have been found at the sites of neo-intima hyperplasia, indicating VEGF may play a role in diseased vascular remodeling. (Yeager et al., 2011) (Tanaka et al., 2008) (Inoue et al., 1998) Furthermore, calcified atherosclerotic lesions are magnitudes stiffer then healthy vascular tissue. (Matsumoto et al., 2002) While numerous studies have characterized the independent effects of VEGF on vascular differentiation, the combined effects of VEGF and matrix elasticity on the differentiation of MSC to diseased or healthy endothelial lineages remains unknown. *In aim 2 we demonstrate that a combination of low matrix elasticity and VEGF rapidly direct MSCs towards mature, functional endothelial types, while the combination of VEGF and a stiff 2D substrate directed MSC differentiation to progenitor or diseased vascular phenotypes.*

Composition

The vascular ECM is composed of various proteins, each with different roles in guiding cell function, adhesion, and viability. However, proteins are difficult to incorporate in tissue-engineered grafts due to degradation and orientation issues. Research has turned to the use of peptides, short protein sequences that contain the functionality of proteins without the orientation or degradation issues. SMC reside in the media, contains elastin, fibronectin, and collagen I. (Wagenseil and Mecham, 2009) SMC are also in contact with the basement membrane, which is

primarily composed of collagen IV. (Herbst et al., 1988) Studies have found that while elastin contributes key mechanical properties to the ECM, the sole use of elastin does not induce MSC towards smooth muscle lineage. (Johns, Deirdre, 2003) RGD is short sequence of fibronectin, and is commonly utilized to increase cellular adhesion to scaffolds. (Frith et al., 2012) Additionally, the RGD peptide has been shown to direct SMCs towards a contractile phenotype. (Beamish et al., 2009) Recent studies have found that the collagen IV protein may guide stem cells differentiation into SMC. (Xie et al., 2011) Kanie et al found that SMC selectively adhered to the DGY peptide sequence of collagen IV. Further, EC would not adhere to DGY, suggesting that DGY may play a critical role in SMC adhesion and functionality. (Kanie et al., 2012) *Little work has been done to understand what protein sequences in the vascular ECM guides MSCs towards vascular lineages. In aim 3 of this dissertation, we study the effects of the RGD and DGY peptide sequences on the vascular differentiation of MSCs.*

Structure

Many studies that examine stem cell differentiation in response to variations in elasticity utilize 2D substrates instead of 3D matrices. (Engler et al., 2006) (Park et al., 2011a) (Evans et al., 2009) However, there is mounting evidence that cells behave differently in a flat 2D culture as opposed to a 3D fibrous matrix. Integrins are connection between the cell interior and the ECM, mediating interactions between the cell and its microenvironment. Focal adhesions comprised of numerous molecules for both mechanical and chemical signaling form at the integrin site on the cell cytoskeleton. (Plopper et al., 1995) Interestingly, it has been demonstrated that the composition and morphology of focal adhesions vary on a 2D substrate compared to a 3D nanofiber matrix, resulting in different cellular responses. (Cukierman et al.,

2001) Recent studies indicate MSC exhibit different morphologies when seeded on 2D culture vs in a 3D matrix. (Huebsch et al., 2010) SMCs respond differently to growth factors such as TGF-β in a 3D culture as opposed to a 2D substrate. (Stegemann and Nerem, 2003) Further, SMCs proliferation is greatly reduced in a 3D culture, and gene expression of TGF-β is increased, possibly indicating a healthy contractile phenotype. (Li et al., 2003) Taken together, these results demonstrate that the structure of the cellular microenvironment has a profound effect on cellular differentiation and phenotype. The *in vivo* vascular ECM provides a cellular microenvironment characterized by a 3D nanofiber network with pores that allow for cellular migration and the influx of fluid and nutrients. (Cukierman et al., 2001) *To understand the effects of chemical and mechanical factors on vascular differentiation as it would occur in-vivo, it is critical to utilize a 3D nanofiber matrix. In aims 1-3 we utilize electrospun 3D nanofibrous matrices as culture environments to examine MSC differentiation.*

Underlying Mechanisms

Cells are believed to maintain a constant state of pre-stress, called tensigrity. This form of mechanical homeostasis serves a number of purposes; it allows cells to instantly recognize changes in ECM mechanics, it governs the stiffness of the cell, and regulates the stress the cell applies on the surrounding ECM. Cellular tensigrity is a constant feedback cycle, where cells sense the elasticity of the surrounding ECM, adjust internal actin filaments within the cell, and remodel the ECM to adjust elasticity.(Peyton et al., 2007) The first interaction between the cell and ECM occurs by the cell binding via integrin to a ligand attached to the ECM. During the binding process, the Focal Adhesion Kinase (FAK) pathway is activated, and bundles with Src

tyrosin kinase. This pathway transmits signals from the ECM to the cellular cytoskeleton. (Mammoto and Ingber, 2009) On a stiff substrate or substrate with high adhesivity, FAK is phosphorylated, Rho/Rock signaling increased, the light myosin chain is activated, the formation of actin stress fibers occurs, leading to cell spreading and differentiation to myogenic or

osteogenic lineages. On soft substrates or substrates with low adhesivity, FAK is not phosphyralated and activates a Rho-inhibitor, which prevents light myoscin chain activation and the formation of actin stress fibers. (Mammoto and Ingber, 2009)This results in rounded cells and differentiation to adipogenic or neural lineages. Currently, it is not understood how the Rho/Rock pathway and the cellular cytoskeleton interact with the cell nucleus to trigger differentiation. One possible mechanism is activation of the Rho pathway results in downstream signaling through RAS to the ERK pathway, which is involved in differentiation and gene expression.(Cohen, 2013) *In aim one, we examine the effects of various matrix elasticities on MSC differentiation to specific vascular lineages. It is possibly that a soft matrix will result in down regulation of the Rho/Rock pathway, directing MSC differentiation towards endothelial lineages, while a stiffer matrix will increase Rho/Rock signaling, encouraging MSC differentiation towards smooth muscle lineages.*

Exogenous chemicals also bind to various cell receptors, resulting in activation of numerous cell-signaling pathways. VEGF is known to bind to the VEGF-R2 receptor, which activates the Ca2+/NOS/NO pathways as well as the RAS/RAF/MEK/ERK pathway. Interestingly, it appears that both substrate elasticity and VEGF act on the ERK pathway, which suggests that ERK may be at a critical crossroad for directing MSC differentiation to specific, mature lineages. *In aim 2 we examine the combined effects of VEGF with stiff and soft substrate elasticity on the differentiation of MSC towards endothelial lineage. It is possible that both*

substrate elasticity and VEGF modulate the ERK pathway, which then directs either healthy or diseased endothelial differentiation. However, it is likely that multiple signaling pathways are at play in this complex process.

The interaction between ligands and integrins is a key factor in cell signaling mechanisms. The RGD ligand is known to bind to cellular integrins alphaVbeta3 and alpha1beta5, while a known collagen IV integrins are alpha1beta1.(Barczyk et al., 2010; Xiao et al., 2007) After binding with the integrin, FAK must be phosphorylated to initiate the Rock/Rho pathway, actin polymerization, and cellular entry into S-phase, which allows proliferation. If the cell is inhibited from spreading by blocking the Rock pathway, restricting actin polymerization, or inhibiting FAK phosphorylation, the cell cannot spread, will not enter S-phrase, and proliferation will not occur. (Mammoto and Ingber, 2009)The RGD ligand binding to alphaVbeta3 and/or alpha1beta5 has been shown to increase cell spreading and activate the ROCK pathway.(Gribova et al., 2013) Independently, RGD has been shown to increase cell proliferation. (Davis et al., 2002; Hersel et al., 2003) Collagen IV stimulates the Nox4 and P13K signaling pathways, which have been shown to increase smooth muscle differentiation.(Xiao et al., 2007, 2010) *In aim 3 we hypothesize that RGD ligands will improve MSC spreading and viability, while DGY ligands will encourage differentiation to smooth muscle lineages.*

1.2.7 Fabrication of 3D Biomimetic Scaffolds

To study the effects of mechanical and chemical factors on vascular differentiation as they would occur in-vivo, this study will utilize a 3D nanofiber matrix for cell culture. The 3D nanofiber matrices will be fabricated by electrospinning, a method that can easily form matrices with fibers on the order of those found in the *in vivo* artery. Electrospinning is a fabrication

process that utilizes a high voltage source to stretch a polymer solution into nanofibers, producing 3D nanofiber matrices. The process allows for control over material composition, fiber diameter, and mechanical properties, making it a powerful tool for vascular tissue engineering. (Nisbet et al., 2009) Xu et al found that vascular SMC adhered to a PLCL electrospun nanofiber scaffold and differentiated to a contractile phenotype. (Xu et al., 2004) Vascular ECs that were seeded on collagen coated PLLA scaffolds demonstrated good viability and demonstrated morphology similar to that of EC under flow. (He et al., 2005) Together, these studies suggest electrospun nanofiber scaffolds are good platforms for vascular tissue engineering. Replicating the vascular environment requires nanofibers with a tunable elasticity and composition. Polyethelene glycol dimethacylate (PEGDM) is a material commonly utilized in tissue engineering as it is biocompatiable and the modulus can be adjusted by varying the molecular weight or weight percent. (Bryant and Anseth, 2002; Peyton et al., 2006) (Lynn et al., 2010) PEGDM and PEGDA can be crosslinked by photo-initiated chain polymerization, a process commonly utilized in tissue engineering to fabricate a hydrogel substrate with tunable elasticity. Figure 1.6). Furthermore, peptides can by incorporated into PEGDM or PEGDA scaffolds, allowing for precise control over matrix composition (Figure 1.6).

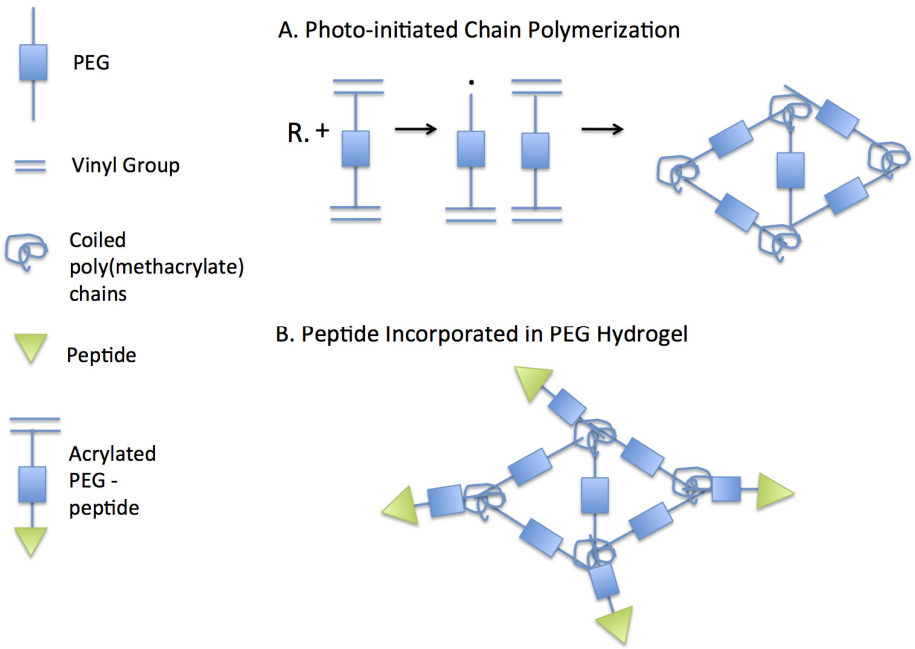


Figure 1.6: Photo-initiated chain polymerization and peptide incorporation in PEG hydrogels. A) Free radicals initiate chain polymerization, which continues to form a PEGDM network. B) Acylate PEG-peptides are incorporated into the PEG hydrogel polyacylate chains.

The modulus of the hydrogel can be tuned by varying the photopolymerization time or light intensity [48] (Kloxin et al., 2010b) (Kloxin et al., 2010a; Wong et al., 2003) Recently, Tan et al developed a process where electrospun scaffolds can be photopolymerized. (Tan et al., 2008) Kanie et al determined that peptides can be incorporated into the electrospinning solution, and then electrospun into 3D nanofiber matrices. (Kanie et al., 2012) PEGDM can be used in conjunction with the electrospinning and photopolymerization processes to develop 3D biomimetic scaffolds with tunable elasticity and composition. *In aim 1, we expand on the process developed by Tan et al to develop PEGDM 3D nanofiber matrices with tunable moduli between 2 to 15 kPa. In aim 2, we electrospin and photopolymerize PEGDM 3D nanofiber matrices with an elasticity mimicking that of the in-vivo intima. And in aim 3, we expand on the process*

developed by Kanie et al and incorporate vascular peptides in PEGDA electrospun 3D nanofiber matrices to study the effects of matrix composition on vascular differentiation.

1.3 Significance

This dissertation provides fundamental understanding of the effects of physiological stimuli on the vascular differentiation of MSCs in terms of the specificity, functionality, and maturity of the final differentiated cells. The results and tunable nanofiber matrices developed in this study will assist in elucidating the roles of MSCs *in vivo* in vascular repair or intima lesion formation. The findings will better define the process of regenerating functional healthy vascular tissue from MSCs through differentiation or paracrine signaling. In summary, studying the combined effects of matrix elasticity, composition, and growth factors on MSCs differentiation in 3D nanofiber culture is critical to improve the safety and efficacy of MSCs in cardiovascular treatments.

CHAPTER 2

Compressive Elasticity of Three-Dimensional Nanofiber Matrix Directs Mesenchymal Stem Cell Differentiation to Vascular Cells with Endothelial or Smooth Muscle Cell Markers

2.1 Abstract

The importance of mesenchymal stem cell (MSC) in vascular regeneration is becoming increasingly recognized. However, few *in vitro* studies have been performed to identify the effects of environmental elasticity on the differentiation of MSC into vascular cell types. We utilized electrospinning and photopolymerization techniques to fabricate a 3D PEGdma nanofiber hydrogel matrix with a tunable elasticity for use as a cellular substrate. Compression testing demonstrated that the elastic modulus of the hydrated 3D matrices ranged from 2 to 15 kPa, similar to the *in-vivo* elasticity of the intima basement membrane and media layer. MSC seeded on rigid matrices (8 -15 kPa) showed an increase in cell area compared to those seeded on soft matrices (2-5 kPa). Furthermore, the matrix elasticity guided the cells to express different vascular-specific phenotypes with high differentiation efficiency. Around 95% of MSC seeded on the 3D matrices with an elasticity of 5 kPa showed Flk-1 endothelial markers within 24 hr, while only 20% of MSC seeded on the matrices with elasticity greater than 8 kPa demonstrated Flk-1 marker. In contrast, around 80% of MSC seeded on 3D matrices with elasticity greater than 8 kPa demonstrated smooth muscle α-actin marker within 24 hr, while less than 10% of MSC seeded on 3D matrices with elasticity less than 5 kPa showed α-actin markers. The ability to control MSC differentiation into either endothelial or smooth muscle-like

cells based purely on the local elasticity of the substrate could be a powerful tool for vascular tissue regeneration.

2.2 Introduction

Vascular diseases affect 1 in 3 Americans (Lith and Ameer, 2011). In 40 % of the cases, the treatment requires surgical replacement of a diseased or dysfunctional blood vessel with a vascular graft. Synthetic vascular grafts always cannot match the efficacy of healthy vessels, leading to short-term or long-term graft failures such as thrombosis or stenosis. These failures may be partly prevented by the development of a robust endothelial layer utilizing the patient's cells along the inner wall of the graft. Recent developments in vascular tissue engineering have shown exciting potentials for using both endothelial cells (EC) and smooth muscle cells (SMC) on a degradable scaffold to regenerate blood vessels (Lith and Ameer, 2011), (Pawlowski et al., 2004). However, obtaining a sufficient number of vascular cells is difficult; as it requires invasive surgery on the patient or donor and these cells have a limited expansion capability *in vitro* (Lith and Ameer, 2011). MSC is an alternative cell source recently employed in vascular graft or tissue engineering (Nieponice et al., 2008),(Park et al., 2007). MSCs are multipotent and thromboresistant, can be easily obtained through a bone marrow biopsy from a patient or a compatible donor, have a large expansion capability given proper environments *in vitro*, and thus are increasingly explored for regenerative medicine (Kan et al., 2005). Studies in the last decade have demonstrated that MSC differentiation and spreading can be controlled by the local mechanical elasticity using a polyacrylamide gel with varied modulus as a two-dimensional (2D) cell substrate (Wong et al., 2003). It has

been demonstrated that the gels that replicated the modulus of neural, muscle and bone tissue directed the differentiation of MSCs towards neural, myogenic, and osteogenic cells, respectively (Engler et al., 2006). Utilizing the local substrate elasticity to control MSC differentiation and activity is an elegant approach to achieve spatial control of cell behaviors.

In contrast to the 2D cell culture employed by most of these studies, the *in-vivo* extracellular matrix provides a cellular microenvironment characterized by a three-dimensional (3D) nanofiber network with pores that allow for cellular migration and the influx of fluid and nutrients. Studies have shown that cellular responses such as morphology, adhesion and differentiation are greatly different between cells seeded on a flat, 2D substrate and those in a 3D porous matrix (Cukierman et al., 2001). Electrospinning is a fabrication process that utilizes a high voltage source to stretch a polymer solution into nanofibers, producing 3D nanofiber matrices. This process allows control over fiber diameter and porosity, producing an ideal 3D substrate for cell scaffolding (Xu et al., 2004). Recently, Tan et al.(Tan et al., 2008) electrospun a photopolymerizable polymer solution of poly□-amino ester and poly(ethylene oxide) (PEO) into a 3D matrix. Their study resulted in a scaffold with a young's modulus of in the range of 5 MPa, good for tendon and ligament tissue engineering (Tan et al., 2008). However, for soft tissues like blood vessels, nanofibers with extremely low elasticity (around 2-15 kPa) will be needed (Richert et al., 2004), (Peloquin et al., 2011). In addition, the artery has a multilayer structure and the elasticity of the layers varies; the basement membrane around the endothelium in the intima layer is the softest, the connective tissue in the adventitia

layer is the most rigid, and the modulus of smooth muscle in the medial layer is in between. EC reside on top of the soft basement membrane while SMC live in the stiffer medial layer (Bou-Gharios et al., 2004), (Wagenseil and Mecham, 2009). The hypothesis underlying this present study is that replicating the elasticity of the mechanical microenvironments found in the vessel basement membrane and medial layers will induce MSC to differentiate towards vascular cells with EC or SMC-like characteristics, respectively.

To test the hypothesis, we have developed 3D photopolymerizable nanofiber grafts (NFG) with low elastic modulus (in the range of 2 to 15 KPa) by electrospinning. The elasticity of these grafts can be dynamically controlled by adjusting the photopolymerization time. These grafts were used to study the effects of modulus on MSC spreading, penetration and differentiation into EC and SMC.

2.3 Methods

2.3.1 Nanofiber Scaffold Fabrication

Polyethylene glycol dimethacrylate (PEGdma) with a molecular weight of 3000 was synthesized utilizing the method developed by Lin Gibson et al (Lin-Gibson et al., 2004). Approximately 90% of the end groups were modified with methacrylates as determined by ^{1}H NMR analysis. An electrospinning solution composed of 3.2% wt PEGdma 3000, 3.4% wt PEO (MW 40,000, Sigma-Aldrich, St Louis, MO), 0.4 % wt of Irgacure 2959 (I2959, 0.6 mg/ml in DI H20, Ciba, Tarrytown, NY) and 93% DI H_2O was mixed for one hr with magnetic stir bar. PEGdma 3000 photopolymerizable nanofiber grafts (NFGs) were fabricated by electrospinning on a custom set up composed of a high

voltage power supply (Gamma High Voltage Research, Ormond Beach, FL), grounded collecting surface, motorized syringe pump, and a 14 mm syringe. The solution (2 ml) was spun at a distance of 26 cm from the stationary collecting surface, a voltage of 22 kV, and a flow rate of 1.10 ml/hr. NFGs with a thickness of around 0.3 mm were cut into 7/8-inch diameter disks and placed in glass vials. Vials were then vacuum-sealed and NFGs were photopolymerized under 365 nm light with an average intensity of 15 mW/cm^2.

2.3.2 FTIR Analysis

NFG double bond conversion was evaluated with mid-range fourier transform infrared spectroscopy (FTIR) (Nicolet 4700, Thermo Fisher Scientific, Waltham, MA) by examining the disappearance of the C=C peak within the acrylate group (~1635 cm^{-1}) on a dry NFG. Three NFGs were photopolymerized for 2, 5 and 30 min respectively, and FTIR was then performed on each sample. To account for sample and background variation, data were normalized with the C=O peak located in the range from 1650 to 1726 cm^{-1}, which is independent of photopolymerization. Data were analyzed using Opus software (Brucker Optics, Billerica MA).

2.3.3 Scanning Electron Microscopy Imaging

Scanning electron microscopy (FESEM, JSM-7401F, Jeol Ltd, Tokyo, Japan) was used to examine the fibrous structure of the NFG samples in dry and hydrated states. For hydrated samples, NFGs were photopolymerized for 5 and 30 min, and submerged in DI H_2O for 24 hr. Then, they were submerged in liquid nitrogen (-195 ^oC), dried in a critical-

point drying chamber for approximately 48 hr, and then imaged with FESEM. The fiber structure was imaged at 400 and 2000 magnifications. Image J was used to analyze changes in fiber diameter and porosity.

2.3.4 Equilibrium Swelling Ratio

The equilibrium swelling ratio, q, for each photopolymerization time have been calculated as

$$q = \frac{equilibrium\ swelling\ mass}{dry\ mass}$$

The 'dry mass' is the mass of the nanofiber grafts after photopolymerization, in a dry state. The samples were then exposed to DI H_2O for 48 hours, and finally weighed to determine the 'equilibrium swelling mass'.

2.3.5 Differential Interference Contrast (DIC) Microscopy Imaging

To examine the changes of fiber structure in the hydrated NFGs and study the effects of crosslinking on the structure, the NFGs were submerged in DI H_2O for 5 min after exposure to UV light. Images were then taken at 20x magnification on a DIC optical microscope (Axioskop 40, Carl Ziess, *Oberkochen, Germany*).

2.3.6 Tensile Testing

Tensile testing was performed using an MTS Insight electromechanical testing system (MTS Systems Corp., Eden Prairie, MN, USA). Five PEGdma NFGs that had not

been photopolymerized were tested in the dry state. Ten PEGdma NFGs that had been photopolymerized for 30 min were prepared; five of the scaffolds were tested in the dry state. The remaining five were submerged in DI H_2O for 24 hr and tested in an environmental chamber submerged in PBS. Four NFGs that had been photopolymerized for 2 minutes and four NFGs that had been photopolymerized for 5 minutes were also submerged in DI H_2O for 24 hr and tested in an environmental chamber submerged in PBS. All scaffolds were cut to 5mm wide by 25 mm length. Sandpaper was attached to the tensile test grips to prevent slipping. A strain rate of 0.03 mm/mm/s was used, following the method used in a previous research (Tan et al., 2008). Uniaxial tensile testing was performed on all samples till failure. Samples with data indicating slippage or excessive noise were not used. The NFGs tested in the dry state showed a linear behavior, and a linear fit of the stress strain curve was used to determine the modulus. The NFGs tested in the hydrated state demonstrated a classic heel toe stress-strain curve. The elastic modulus of hydrated NFGs was determined from the low strain region (10 to 30%) of the curve.

2.3.7 Rheometer Testing

Changes in storage modulus (G') of NFGs due to increased photopolymerization time were characterized using a rheometer for linear viscoelastic regime, ARES TA rheometer, TA Instruments, New Castle, DE). NFG samples that were 0.3 mm thick with 7/8 inch in diameter were photopolymerized for 2, 5, 15, 30 or 60 min, and then submerged in DI H_2O for 24 hr. NFGs were tested with a parallel plate configuration and a temperature-controlled Peltier flat plate at 37 ^{o}C. Adhesive sand paper was attached to

the bottom and top plates to prevent slippage between NFGs and plates. A vertical load of 16 grams was also applied to all samples to prevent slippage. A strain sweep at a frequency of 1 rad/s and a frequency sweep at a strain of 2 % were run on each sample. Data were inspected for slippage or tearing, and only data from the linear visco-elastic region (LVE) in the strain sweep were used to determine the G'. The LVE region was defined as the storage modulus (G') having a change of less than 60 Pa between 1 and 10 % strain, suggesting strain independent behavior.

2.3.8 Compression Testing

The compression modulus of NFGs photopolymerized for 2, 5, 15, 30 and 60 min was characterized using a MTS Synergie 100 (MTS, Eden Prairie, MN) with a parallel plate set up. All tests were completed in a hydrated condition, on a 10 N load cell at a strain rate of 0.50 mm/min up to a maximum strain of 15%. A total of 4 samples per photopolymerization time were tested, and elastic modulus was calculated from the linear elastic region between 10 to 15%.

2.3.9 Cell Culture

NFGs were photopolymerized, submerged in DI H_2O for 24 hr and sterilized with 70% ethanol. Prior to cell seeding, the NFGs were coated with 0.3 % type I rat tail collagen (BD Biosiences, Bedford, MA). Rat MSCs from (Lonza Group Ltd, Switzerland) with passages 2-5 were cultured in Dulbeccos Modified Eagles Media (DMEM, Sigma-Aldrich, St Louis, MO), with 10% defined FBS for MSCs and 1% Penn/Strep (Invitrogen, Carlsbad, CA). To seed cells on the top of NFGs, 300 µl of

150,000 cells/ml media were used for each sample. Cells were seeded in a circular 1.57 mm^2 area defined by a rubber gasket (Grace Biolabs, Bend, OR) on top of the sample. For comparison, primary vascular ECs and SMCs freshly separated from pulmonary blood vessels were cultured DMEM with 10% FBS and 1% Penn/Strep. SMCs were cultured in a collagen gel and ECs were cultured on it.

2.3.10 Measurements of Cell Area

After 20-hr culture, cell assay (LIVE/DEAD kit obtained from Invitrogen Corp., Carlsbad, CA) was performed to evaluate MSC spreading on 2 min, 5 min, 15 min, 30 min and 60 min NFGs. To start the assay, 0.150 ml of the Live/Dead assay solution containing 10 ml PBS, 0.020 ml of 2mM EthD-1 and 0.005 ml of 4mM calcein AM was added on each NFG. NFGs were then incubated in the dark for 30 min, after which cells were imaged on an upright fluorescence microscope (Axiovert S100, Carl Ziess, Oberkochen, Germany) with 20x magnification. The applied stain presented by live cells was green (calcein AM) while the nuclei of dead cells were stained red (EthD-1). Image-J software (NIH, Bethesda, MD) was used to measure the cell area and analyze the cell morphology in the obtained images. For each graft, 10 representative cells were measured for spreading area; only cells that were entirely on a single z-plane were imaged to ensure correct area calculations.

2.3.11 Immunofluorescent Staining

Immunofluorescent staining of cells with FLK1 (endothelial cell markers) and anti-smooth muscle alpha-actin (SMA, a smooth muscle cell marker) was used to

characterize vascular differentiations of MSC. Cells were seeded for 24 hr on 2, 5, 15, 30 and 60 min NFGs. Samples were fixed with methanol at –8 ^{o}C, blocked with 3% BSA, and incubated with primary rabbit polyclonal anti-SMA (Sigma-Aldrich, St Louis, MO), or primary mouse monoclonal anti-FLK1 (Santa Cruz Biotechnology, Santa Cruz, CA). Following primary antibody coupling, samples were washed in PBS and incubated with secondary anti-mouse IgG antibody conjugated with Alexa 488 and anti-rabbit IgG antibody conjugated with Alexa 594. Finally, the samples were mounted with DAPI SlowFade (Invitrogen, Carlsbad, CA) and imaged. Fluorescently labeled cells were evaluated using an epifluorescence and confocal microscope (Zeiss, Peabody, MA). Images from each fluorescence channel were merged using Picassa software (Google, Mountain View, CA).

2.3.12 Real Time RT- PCR

Total cellular RNA from each sample was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Complementary DNA was synthesized from 1 lg of total cellular RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Primers for amplification of Flk-1 and SMA are shown in Table 2.1. The SYBR Green I assay and the iCycler iQ real-time PCR detection system (Bio-Rad MyiQ Real-Time PCR System, Hercules, CA) were used for detecting real-time quantitative PCR products from 2 ng of reverse-transcribed cDNA. PCR thermal profile consisted of 95 C for 10 min followed by 40 cycles of 95 C for 15 s, 60 C for 30 s and 95 C for 1 min. Genes were normalized to the housekeeping gene GADPH and fold change

relative to static condition was calculated using the DCT method 36. Primers shown in Table 2.1.

Table 2.1: Rat primers utilized for real time PCR analysis.

Genes	Forward primer (rat)	Reverse primer (rat)
GADPH	TTGGAGGCCATGTAGGCCAT	CCTCTGGAAAGCTGTGGCGT
Flk-1	ATGGAAGAGGATTCTGGA	CACGGTGGTGTCTGTGTC
SMA	CATCTCCAGAGTCCAGCACA	ACTGGGACGACATGGAAAAG

2.3.13 Statistical Analysis

Statistical testing was performed using MVPstats software (MVP Programs, Vancouver, WA) or SPSS software (IBM, Chicago, IL). For comparing two groups with equal variances, a student's t-test was used. For comparing two groups with unequal variances and unequal sample sizes, Welch's t-test was used. For multiple group comparisons, a one-way ANOVA test was run on both the groups' means and variances. If the groups had equivalent variances, a Tukey Post-Hoc analysis was further performed. If the groups had unequivalent variances, a Games-Howell Post Hoc was further performed. When analyzing the rheological data, due to disproportionate sample sizes, an unweighted means analysis was used to compare groups (Winer et al., 1991).

2.4 Results

2.4.1 Synthesis of nanofiber grafts with varying degrees of crosslinking

PEGdma 3000 was selected for its high elasticity, cytocompatibility, and ability to be photopolymerized (Hwang et al., 2011), (Ifkovits and Burdick, 2007), (LaNasa et al., 2011). As it was difficult to form continuous nanofibers when electrospinning a low molecular weight polymer solution like PEGdma 3000, PEO with a molecular weight of

40,000 was added to the electrospinning solution to act as a carrier polymer. As PEO is water soluble, it acts as a sacrificial polymer that can be washed out of the scaffold over the time in water resulting in a NFG composed of PEGdma 3000. We electrospun a solution with a PEG-to-PEO ratio of 1.0-to-1.1. This was the highest weight ratio of PEG to PEO that resulted in continuous nanofibers; increasing the amount of PEG in solution caused beading and inconsistent nanofibers. The resulting NFGs were composed of 48.35% wt PEGdma 3000, 52.60% wt PEO, and 0.05% wt I2959. Figure 2.1A shows the PEGdma 3000 NFG with consistent and continuous nanofibers characterized by minimal webbing or beading.

The crosslinking of PEGdma 3000 is based on radical chain photopolymerization of its methacrylate groups in the presence of a photoinitiator (I2959) and UV light. PEGdma 3000 NFGs were not sufficiently crosslinked, indicated by dissolution of the fibers in an aqueous environment, when polymerized in the presence of normal atmosphere due to oxygen inhibition. Photopolymerizing NFGs in vacuum resulted in crosslinked scaffolds that maintained a solid, fibrous structure after water submersion. The DIC image of NFGs with no photopolymerization showed loss of fibrous structure after exposure to DI H_2O (Figure 2.1B). The DIC microscopy images of NFGs with photopolymerization times of 5 and 30 min showed that the fibrous structure of both materials remained intact and swollen after hydration in DI H_2O (Figures 2.1C and 2.1D).

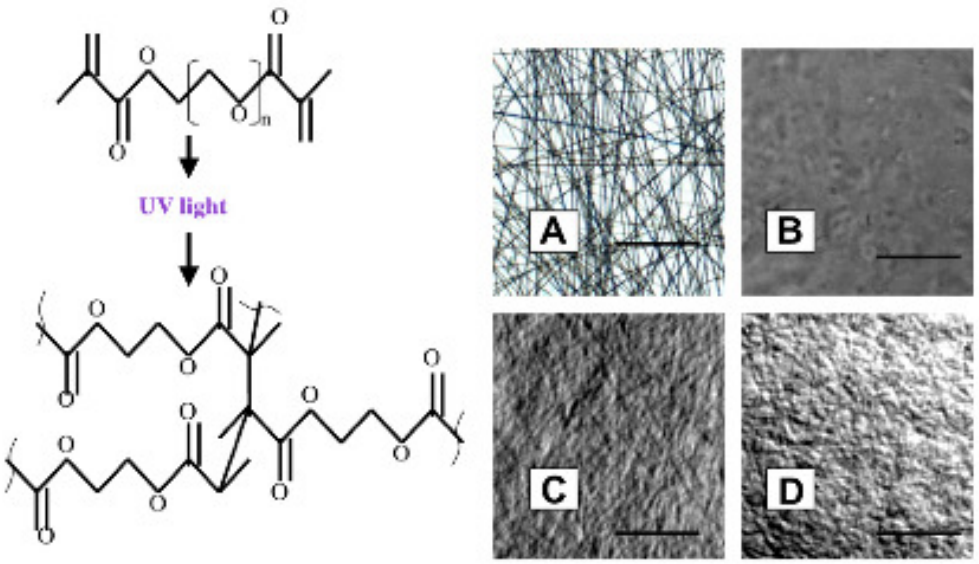


Figure 2.1: PEGdma undergoing photopolymerization. UV light causes the initiator to dissociate into free radicals that react with the vinyl groups, initiating the chain polymerization reaction and propagation until termination. DIC images show the fiber structure of (A) a dry NFG scaffold, (B) a hydrated NFG with 0 min of photopolymerization (no fibers remain), (C) a hydrated NFG with 5 min of photopolymerization (fibers remain), and (D) a hydrated NFG with 30 min of photopolymerization (fibers remain). Images were taken at 40× magnification. Scale bar: 2 mm.

2.4.2 FTIR analysis indicates degree of conversion increases with photopolymerization time

Mid-range FTIR was used to characterize the degree of conversion by monitoring the disappearance of the reactive acylate peak at 1637 cm^{-1}. Due to the low concentration of acrylates present as a result of the high molecular weight of PEGdma 3000, the NFG showed a small acrylate peak. To account for the sample and background variation, data were normalized with the C=O peak located in the range from 1650 to 1726 cm^{-1}, which is independent of photopolymerization. FTIR was performed on the samples photopolymerized for 0, 2 and 30 min. Table 2.2 summarizes the results. Results showed

that the acylate peak in the NFG samples photopolymerized for 30 min decreased by 30% when compared with those with no exposure to UV. The NFG samples photopolymerized for 2 min showed 10 % decrease in acrylate peak compared to those with no exposure to UV.

Table 2.2: Mid-range FTIR data from NFG with various photopolymerization times. Data table shows the calculated peak areas of the C=O and C=C peaks, as well as the ratio of the C=C to C=O peak; the peak area was calculated with Opus software; data was taken in absorbance ode from 400 cm^{-1} to 4000 cm^{-1} wavelengths.

Photopolymerization Time	Peak Area: 1762–1675 cm^{-1}	Peak Area: 1650–1607 cm^{-1}	Ratio (%)
0 min	0.512	0.057	11.11
2 min	0.559	0.056	10.02
30 min	0.637	0.049	7.69

2.4.3 NFGs with varying degrees of photopolymerization have similar fibrous structures after hydration

SEM images of NFGs in the dry state showed a consistent fibrous structure with fibers ranging from 500 to 1000 nm, and minimal beading or webbing at fiber joints (Figure 2.2A). This was consistent to the DIC image results. SEM images were also taken on NFGs which had undergone 24 hr of DI H_2O exposure followed by 48 hr of freeze drying. These images showed intact fiber networks in 5- and 30- min NFGs, demonstrating that fiber networks remained intact after 24 hr of H2O submersion. (Figure 2.2B-C).

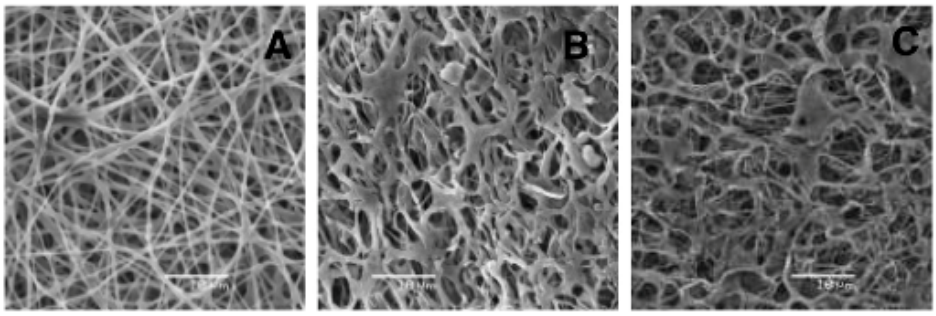


Figure 2.2: SEM images of (A) dry NFG, (B) hydrated NFG photopolymerized for 5 min and (C) hydrated NFG photopolymerized for 30 min. All the images show PEGdma/PEO electrospun NFG. Images were taken at 2000x magnification. Hydrated samples were exposed to DIH2O for 24 hours and freeze dried for 48 hours. Scale bar is 10 microns.

Some fiber coagulation after swelling in the water was found in both NFG samples. Overall, there were minimal differences in porosity and structure noted between 5-min and 30-min NFGs after 24 hr of DI H_2O exposure (Table 2.3). For analysis, 10 fibers on each of the SEM images for dry NFG, 5 min NFG and 30 min NFG were randomly selected and the fiber diameter was measured using Image J software. Assuming material isotropy, comparisons of the volume of dry NFG fiber versus that of hydrated NFG fiber showed a fiber-swelling ratio of $Q = 2.5$ after 24 hr of DI H_2O exposure. No significant differences in NFG fiber diameter or porosity were found between the hydrated NFGs photopolymerized for 5 min and 30 min ($p>0.4$). The average volume swelling ratio of these 3D matrices due to hydration was $Q = 27$, indicating a highly saturated porous structure. Porosity in the matrix gave more area for water to infiltrate, resulting in much higher volume swelling ratio compared to that of a single nanofiber. This was an interesting result as the low nanofiber swelling ratio was closer to the volume swelling ratio of the solid PEGdma gels ($Q = 6.9$), (LaNasa et al., 2011) which did not allow cell movement or penetration over the gel thickness. The high

matrix swelling ratio was an indication of the pores between nanofibers that allowed for cellular adhesion and migration into the 3D matrix.

Finally, the equilibrium mass swelling ratio of the NFGs were found to vary from 41 (5 minute photopolymerization times) to 22.5 (60 minute photopolymerization times). The equilibrium swelling ratio is a function of crosslinking density; as crosslinking density increases, less water can be absorbed into the gel (Ma and Elisseeff, 2005a). Our data reflects this mechanism; low photopolymerization times result in low crosslinking density and therefore a high equilibrium swelling ratio, where as high photopolymerization times resulted in a higher crosslinking density and therefore a lower equilibrium swelling ratio. No statistical differences in equilibrium swelling ratio were found between 15, 30 and 60 minute samples, or between 2 and 5 minute samples.

Table 2.3: Average fiber diameter and average porosity in area fraction for dry and hydrated NFG

Sample	Average fiber diameter (μm) (n = 10)	Average porosity (area fraction) (%)
Dry NFG	0.60 ± 0.14	18.4
Hydrated 5-min-UV NFG	0.80 ± 0.19	18.5
Hydrated 30-min-UV NFG	0.77 ± 0.17	21.0

2.4.4 Mechanical testing shows increase in modulus with increasing photopolymerization time

Tensile tests were performed on NFGs photopolymerized for 0 and 30 min in the dry condition, and on NFGs photopolymerized for 30 min (Wet_30) in the hydrated condition. Results showed that dry NFGs photopolymerized for 30 min (Dry_30) had an elastic modulus of about 350 kPa (Figure 2.3).

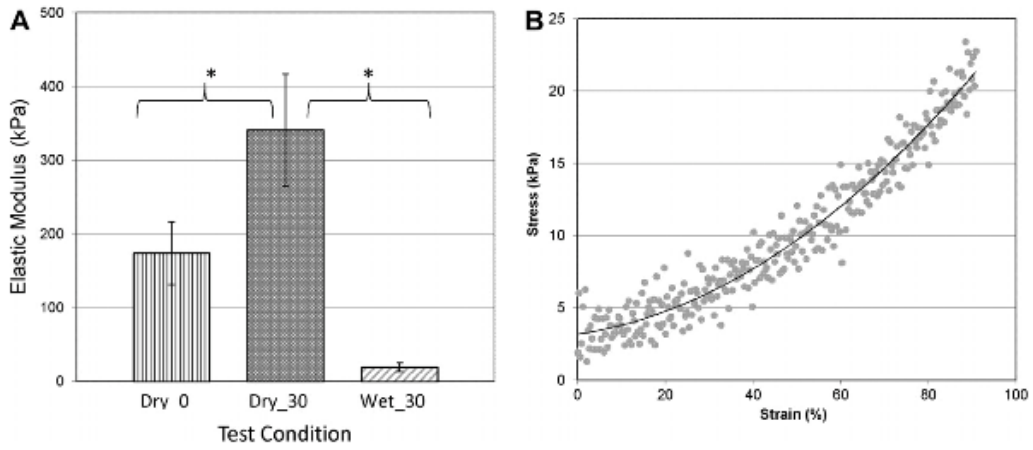


Figure 2.3: The tensile modulus is determined as a function of the photopolymerization time and testing environment. Dry_0 represents the NFG samples tested in the dry atmosphere with no UV exposure. Dry_30 represents the NFG samples that were photopolymerized for 30 min and tested in the dry atmosphere. Wet_ 30 represents the NFG samples that were photopolymerized for 30 min, submerged in DI H_2O for 24 hr and tested in an environmental chamber submerged in PBS. Vertical bars indicate standard deviation. "*" shows statistical differences between the groups, with $p<0.05$.

Statistical analysis using Welches unpaired t-test showed the elastic modulus of Dry_30 samples was significantly higher than that of dry grafts without photopolymerization (Dry_0). The hydrated NFGs (Wet_30) had an elastic modulus of 20±4 kPa in the low strain region from 10 to 30%. This indicates the elastic modulus of 30 min photopolymerized NFGs was reduced by more than 16-fold when exposed to water. Noise levels in testing resulted in variations elastic modulus ranged from ±4 kPa to ± 8 kPa. To compare changes in tensile modulus with photopolymerization time, tensile tests were also performed on NFGs photopolymerized for 2 and 5 min in the hydrated condition. However, due to the high noise levels, no significant variations in elastic modulus were detected with varied polymerization times in wet samples.

Compression tests were performed on NFGs photopolymerized for 2, 5, 15, 30 and 60 min. All samples were tested in a hydrated state. Data shown in Figure 2.4 indicated the modulus of NFGs varied from 2 ±1 kPa using short photopolymerization times (2 or 5 min) to 15 ± 2 kPa using longer photopolymerization times (30 or 60 min).

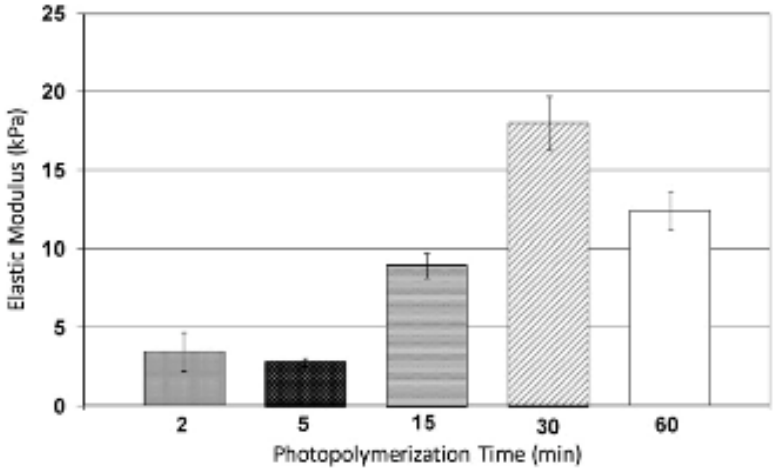


Figure 2.4: Compressive testing of hydrated NFGs with 2, 5, 15, 30 and 60 min of photopolymerization time. NFGs show characteristic heal-toe curve: modulus values were taken from 10 to 15% strain region. n = 3 or 4 for each photopolymerization time. Horizontal lines indicate groups

To characterize how the NFG storage modulus varies with the photopolymerization time, a parallel plate rheometer was used. NFG samples photopolymerized for 2, 5, 15, 30 and 60 min were tested in a hydrated state at 37 ^{o}C. Data were normalized with respect to the average G' value of two min samples to show fold differences. Figure 2.5 demonstrates the storage modulus increases with increasing photopolymerization time. All the NFGs with different photopolymerization times were found to have significantly different storage moduli, indicating that the photopolymerization times of 2, 5, 15, 30, and 60 min produce NFGs with varying stiffness. Storage moduli range from 125 Pa at 2 min to 500 Pa at 60 min.

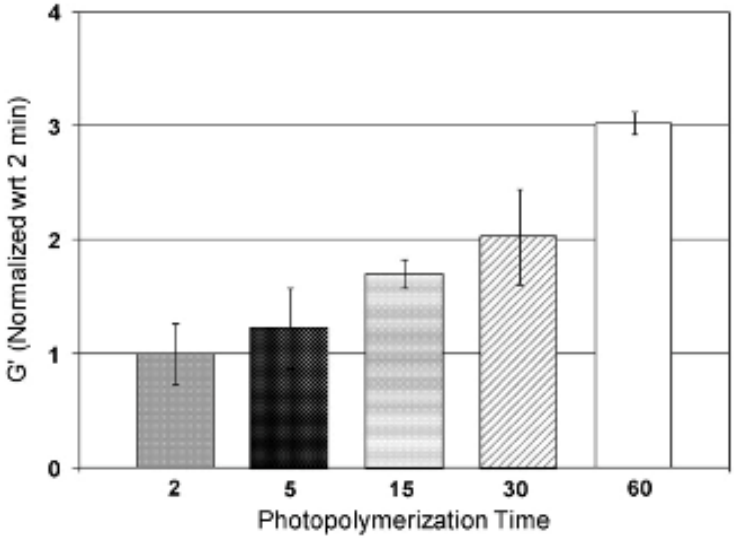


Figure 2.5: Results from rheometer tests for hydrated NFGs photopolymerized for 2, 5, 15, 30 and 60 min. All data have been normalized with respect to 2 min samples. The G' values of all photopolymerization times are statistically different, $p < 0.05$.

2.4.5 Cell penetration into a 3D NFG not a 2D hydrogel

MSCs were seeded on top of the polymerized NFGs and gels, and fixed after 24 hr of seeding. MSCs were found at numerous depths throughout the nanofibrous graft

(Figures 2.6A, 2.6B, 2.6D). In comparison, cells seeded on a solid PEGDA 3000 hydrogels showed no penetration after 24 hr of seeding. (Figure 2.6 C)

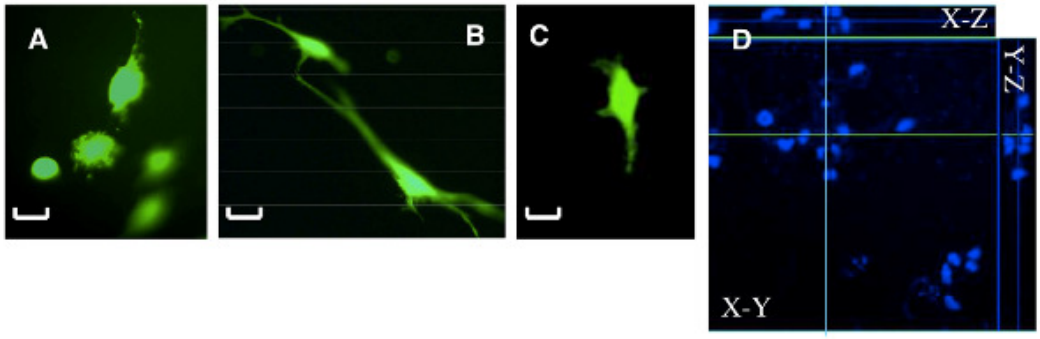


Figure 2.6: MSCs seeded on NFGs photopolymerized for 5 min (A) and 30 min (B). The images show cells at various graft penetration levels with in-focus cells and out-of-focus cells on one z-plane. Cells were only found on the surface of solid PEGdma 3000 gel photopolymerized for 30 min (C). Scale bar = 30 mm. (D) Confocal image of MSC nuclei (DAPI stain) on NFG photopolymerized for 30 min: x-y, x-z and y-z views of the construct. PEGdma nanofibers result in refraction of light, making it difficult to obtain confocal images in the green and red channels

2.4.6 MSC spreading on NFGs is correlated to matrix stiffness

To examine the effects of 3D nanofiber modulus on cell morphology, NFGs were photopolymerized for 2 min, 5 min, 15 min, 30 min and 60 min, respectively. MSCs were seeded on top of each NFG. The cell spreading was analyzed by measuring cell area using Image J software. For each NFG, 10 representative cells were measured and averaged for comparisons. Results demonstrated that MSCs on 2 min, 5 min, and 15 min NFGs exhibited statistically equivalent average cell areas. Also, cells on these matrices demonstrated a less polarized morphology. MSC areas on 30 min and 60 min NFGs also had statistically equivalent cell areas. Cells on these stiffer matrices demonstrated a

striated, elongated morphology. Additionally, MSCs seeded on 30 min and 60 min NFGs were found to have statistically larger areas when compared to MSCs seeded on 5 min and 15 min NFGs. Furthermore, MSCs seeded on the glass were found to be significantly larger than MSCs seeded on any NFG. Cells on the glass demonstrated a multi-polarized morphology. To understand how these differences in MSC spreading area compare to the differences in spreading area of vascular SMC and EC, the areas of EC and SMC were measured. SMC were found to have a significantly larger area than ECs.

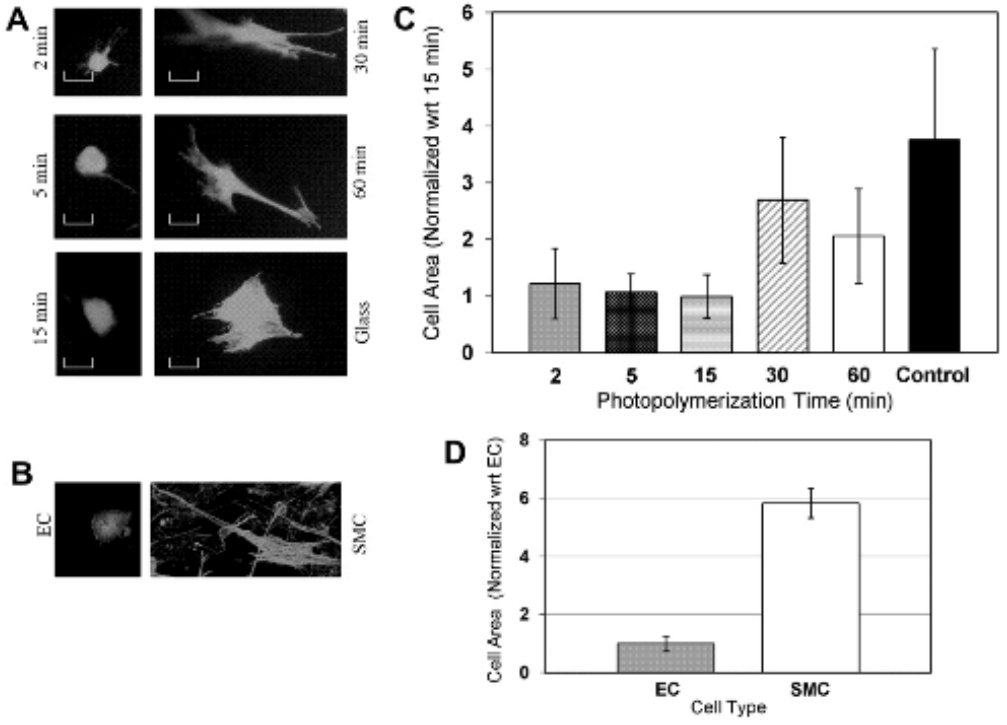


Figure 2.7: MSC spreading increases with nanofiber graft modulus. Normalized to the smallest cell area. (A) Representative images of MSCs seeded on NFG samples photopolymerized for 2, 5, 15, 30 and 60 min, respectively, and image of MSCs seeded on a glass slide. Living cells were stained with calcein and imaged at 20x magnification. Scale bar = 30 mm. (B) Representative images of EC and SMC seeded on/in the collagen gel. (C) Average MSC spreading areas vary with the NFG photopolymerization time. Vertical lines show the standard deviation. Horizontal lines indicate the samples that are statistically equivalent in cell area ($p > 0.05$). (D) The cell areas of EC versus SMC are found to be statistically different ($p < 0.0002$).

2.4.7 Upregulation of smooth muscle cell markers on stiffer NFGs and endothelial markers on softer NFGs

To determine whether MSC differentiations towards EC-like and SMC-like phenotypes were influenced by the 3D nanofiber elasticity, MSCs were cultured on NFGs photopolymerized for 2 min, 5 min, 15 min, 30 min and 60 min, respectively. After 24-hr of culture, the cells were stained with endothelial cell markers (FLK-1) and smooth muscle marker (SMA), and imaged on a fluorescent microscope. Results showed that a minimum of 80% of the cells seeded on 30 min or 60 min NFGs demonstrate SMA markers, which is significantly greater than that of cells demonstrating SMA markers on 2, 5, and 15 min NFGs ($p < 0.01$). Also, 93% of the cells seeded on 5 min NFGs demonstrated FLK-1 markers, significantly greater ($p < 0.01$) than cells seeded on 2 min, 15 min, 30 min or 60 min NFGs. Control testing demonstrated that less than 5% of MSCs seeded on petri dishes for 7 days demonstrated Flk-1 or SMA markers (Figure 2.8).

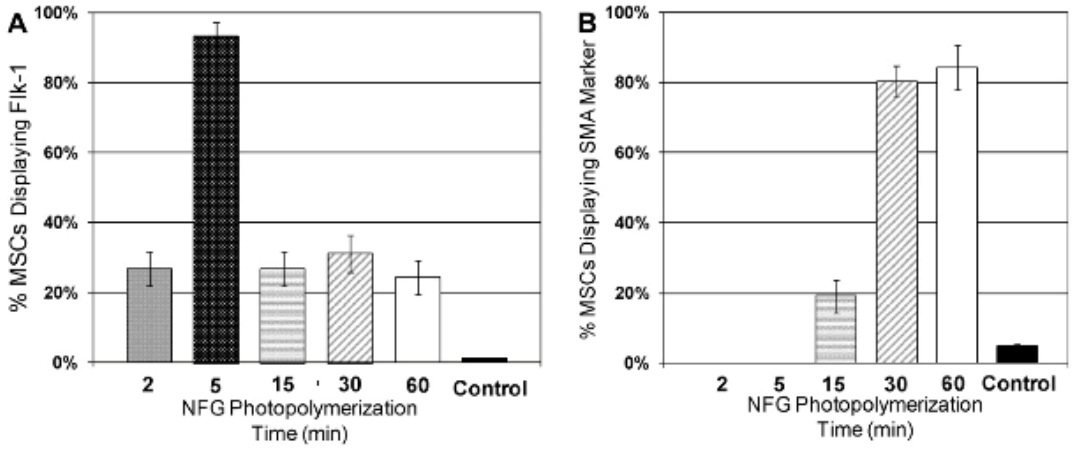


Figure 2.8: A) The percentage of MSCs demonstrating the Flk-1 marker when seeded on NFGs with various photopolymerization times. (B) The percentage of MSCs demonstrating the SMA marker when seeded on NFGs with various photopolymerization times. Vertical bars indicate standard deviation. For control cases, MSCs were seeded on

petri dishes for 7 days. At the end of 7 days, cells were immunostained with Flk-1 and SMA markers.

To further examine gene expression, real time PCR was performed on MSCs cultured for 24 hours on glass slides (control) and NFGs photopolymerized for 5 and 30 min, respectively. MSCs on 5 min NFGs showed a 25 fold increase in Flk-1 mRNA expression, when compared to control. No significant increase or decrease in Flk-1 was found on 30 min NFGs, when compared to control. MSCs on 30 min NFGs show a 2fold increase in SMA mRNA expression, when compared to control. MSCs on 5 min NFGs showed a 50-fold decrease in SMA mRNA expression, when compared to control (Figure 2.9).

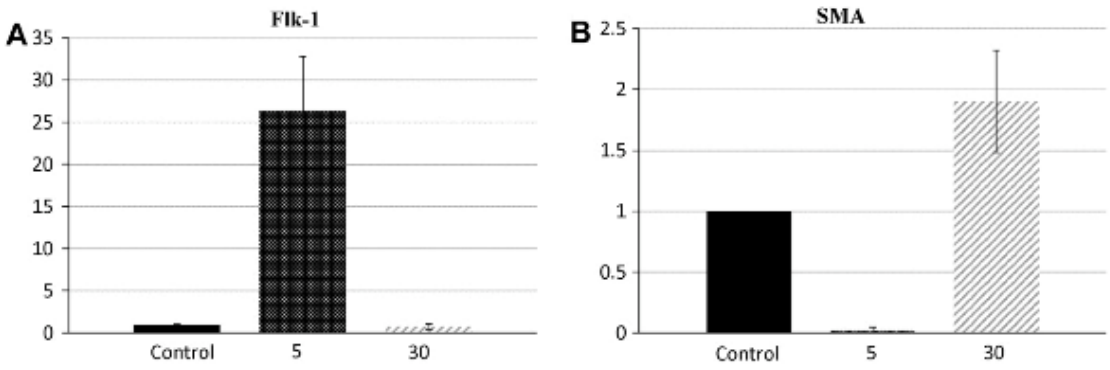


Figure 2.9: PCR data showing fold changes in MSC gene expression. (A) Fold changes in Flk-1 gene expression when MSCs are seeded on glass control dishes, and NFGs with 5 and 30 min photopolymerization times. (B) Fold changes in SMA gene expression when MSCs are seeded on glass control dishes, and NFGs with 5 and 30 min photopolymerization times. Vertical error bars show standard deviation. Flk-1 sample size n=3, SMA sample size n=3.

2.5 Discussion

Recent studies have highlighted the critical impacts of the elasticity of the cellular microenvironment on cell function and differentiation through replication of a native tissue's elasticity (Engler et al., 2006). A majority of the studies were shown with 2D

substrates or 3D gels and related to bone, muscle or neural tissue (Engler et al., 2006), (Sieminski et al., 2004). (Byfield et al., 2009) . By reproducing the modulus and structure of the cellular micro-environment found in the intima basal membrane and media with synthetic polymer matrix (Richert et al., 2004), (Peloquin et al., 2011), this study seeks to direct MSC differentiation into cells with vascular EC and SMC characteristics. We have fabricated a photopolymerizable 3D nanofibrous matrix by electrospinning a solution of PEGdma, PEO, and initiator. The elastic modulus can be controlled by varying the photopolymerization time, producing a matrix with tunable elasticity that ranges from 2 to 15 kPa, close to the modulus of the extracellular matrix in the *in-vivo* intima basement membrane or media layer. MSCs seeded on the nanofiber matrix result in a 3D tissue-like cell culture with the capability of differentiating into EC-like or SMC-like cells.

Cellular response is guided by three main categories of surface characteristics: topography, chemistry and elasticity (Wong et al., 2004). Give the fact that SEM images of NFGs photopolymerized for 5 minutes and 30 minutes showed no significant differences in fiber diameter and matrix porosity, we conclude that there was minimal variation in NFG topography with increasing photopolymerization time. However, at the molecular level, a NFG photopolymerized for 5 minutes should have more uncrosslinked dimethacylite groups than a NFG photopolymerized for 30 minutes, resulting in variations in the molecular structure and surface chemistry between samples. One of the major mechanisms underlying influences of the surface chemistry on cellular responses is ligand density. Interestingly, previous studies (Wong et al., 2004),(Engler et al., 2004) have shown that the ligand density of a polymer surface does not vary significantly with the crosslinking density. Therefore, although it is not possible to eliminate deviations in

the surface chemistry and topography while varying the substrate elasticity, we believe that the variation in elasticity is the dominating contributor to the MSC responses found in this study.

The elastic modulus of biomaterials significantly varies with loading mode, testing, and environmental conditions. To fully characterize the mechanical properties of these newly developed NFGs and to make comparisons between our results and others, several methods were used to analyze the mechanical properties of PEGdma/PEO matrices. As PEG is a highly hydrophilic material, tensile test of NFGs was performed in dry and hydrated conditions in order to understand the change in elasticity due to water exposure.

The blood vessel is characterized by a three-layer hierarchical structure with each layer composed of heterogeneous materials. Wagenseil and Mecham (Wagenseil and Mecham, 2009) utilized mechanical tests to determine the bulk modulus of the vessel wall, in which stiffer layers such as adventitial layer and media layer of the hierarchical structure likely make a major contribution. The purpose of this study is to examine how the elasticity of the local cellular microenvironments in the media and intima direct MSC differentiation into EC and SMC-like cells. Therefore, the local matrix moduli of separated vascular intima and media should be used as our references. For a heterogeneous, anisotropic material like the blood vessel, elasticity changes with testing method and resolution length scale. Thus researchers utilized compression test methods with nano and micro scale resolution, such as AFM indentation, to determine the local matrix elasticity of the cellular microenvironment. Additionally, a recent study has shown cells only sense the mechanical environment around them up to a depth of 5 microns

(Buxboim et al., 2010). Collectively, it is critical for us to replicate the local modulus of the cellular microenvironment found in the intima and media, respectively. Because our material is homogeneous and isotropic, it is reasonable to infer the local compression modulus from bulk compression testing. Compression tests demonstrated the span of NFG elasticity, and indicate the NFG elasticity is in the range of the elasticity of the *in-vivo* intima and media characterized in the literature (Peloquin et al., 2011),(Wong et al., 2003). Our compression and tensile tests did not offer sufficient resolution to fully characterize the differences in elasticity due to variations in photopolymerization time. As a common method to characterize differences in the bulk storage modulus of a hydrogel (Kloxin et al., 2010c), rheology test was further performed. Comparison of the storage moduli in the linear viscoelastic region of nanofiber grafts with 2, 5, 15, 30, and 60 min photopolymerization times showed statistically different storage moduli between samples, revealing the fold differences in modulus with photopolymerization time. Furthermore, this data confirms the expected increase in modulus with increasing photopolymerization time.

In this study, MSCs seeded on NFGs readily penetrated into the graft resulting in cell culture in the 3D fibrous matrix within 24 hr of seeding. It is likely the collagen molecules coating the graft cover the entire 3D fibrous matrix, allowing cellular penetration and adherence to the 3D matrix within 24 hours. Past research has shown cell adhesion, morphology, differentiation and migration vary greatly when seeded on a 2D substrate, a 3D gel, and a 3D fibrous matrix (Lawrence and Madihally, 2008), (Khetan and Burdick, 2010). Our results showed that MSC area increased with increasing NFG modulus. MSCs in softer matrices exhibit less polarized morphology whereas MSCs in

stiffer matrices demonstrate a more striated morphology and larger cell area. All of these elasticity-dependent morphology changes correlated well with MSC spreading results on previous studies on 2D hydrogels by Engler, Evans and Park (Engler et al., 2006), (Evans et al., 2009), (Park et al., 2011b). Recently, it was shown MSCs encapsulated for 14 days in highly crosslinked, less degradable 3D gels were confined, thus forming spherical shapes whereas MSCs in less crosslinked, more degradable 3D gels developed a well-spread morphology (Khetan and Burdick, 2010). The various cellular morphology trends seen in these studies as well as our work highlight the importance of spatial mechanisms on cellular behavior. We believe the high porosity of a 3D nanofiber matrix allows cells to spread. Thus, in highly porous and stable nanofiber matrices, as those presented here and those in the natural vascular matrix environment, cell spreading increases with increasing matrix elasticity. To determine if the morphological changes also resulted in a divergence in cell differentiation, we examined MSCs for the expression of EC marker (Flk-1) and SMC marker (SMA). Immunostaining results demonstrated an upregulation of Flk-1+ cells only on NFGs that have been photopolymerized for 5 min. This data was somewhat surprising, as rheology testing indicated there were small fold differences in elasticity between NFGs photopolymerized for 5 min and those for 2 or 15 min. A recent study by Byfield et al (Byfield et al., 2009) demonstrated that EC seeded in a 3D gel are sensitive to changes in substrate elasticity as low as 400 Pa, responding with variations in actin density and cell morphology. Therefore, it is possible that endothelial markers are only upregulated by a 3D matrix whose elasticity falls in a very narrowed range. Substrates that have been photopolymerized for 5 min have a compressive modulus of roughly 2-3 kPa, in the range of the modulus of the *in-vivo* endothelial basement

membrane (Peloquin et al., 2011). Our results also correlated well with a previous study done by Zhang et al (Zhang et al., 2010b) who found MSC differentiation into vascular cells with endothelial markers on 3D fibrin matrices with a storage modulus of 100 Pa. Furthermore, our results also showed an upregulation of SMA+ cells on NFGs that have been photopolymerized for 30 min and 60 min (compressive modulus of 12-15 kPa), which correlated well with findings by S Park et al on a 2D gel (Park et al., 2011b). Our immunostaining data was further confirmed with real time PCR analysis, where MSCs seeded on 5 min NFGs for 24 hours showed a 22-fold increase of Flk-1 mRNA expression when compared to MSCs seeded on glass controls or 30 min NFGs. MSCs on 30 min NFGs showed a 2-fold increase in SMA mRNA expression when compared to MSCs on glass controls, and a 96-fold increase in SMA mRNA expression when compared to MSCs seeded on 5 min NFGs. After only 24 hr, MSCs showed different responses to 3D fibrous environments with diverse moduli. The upregulation of these early-stage vascular markers indicate that MSC differentiation towards a specific vascular cell or lineage commitment to EC or SMC may be controlled by carefully designing the modulus of the fibrous matrix.

2.6 Conclusions

In conclusion, mechanically tunable 3D nanofibrous matrices have been developed utilizing electrospinning and photopolymerization techniques. Matrices with varying moduli in the range of 2 to 15 kPa were used to determine the preferred mechanical microenvironment for MSC to EC and MSC to SMC differentiation. Variations in MSC spreading and vascular SMA and Flk-1 markers and mRNA

expression in correlation with NFG elasticity were found after only 24 hr of cell seeding. These findings suggest lineage commitment of MSCs towards specific vascular cells can be controlled by carefully designing the substrate modulus.

CHAPTER 3
Synergism of Matrix Stiffness and Growth Factor on Mesenchymal Stem Cells for Vascular Endothelial Regeneration

3.1 Abstract

Mesenchymal stem cells (MSCs) hold tremendous potential for vascular tissue regeneration. Research has demonstrated that individual factors in the cell microenvironment such as matrix elasticity and growth factors regulate MSC differentiation to vascular lineage. However, it is not well understood how matrix elasticity and growth factors combine to direct the MSC fate. This study examines the independent and combined effects of matrix elasticity and VEGF growth factor on both MSC differentiation into endothelial lineage and MSC paracrine signaling. MSCs were seeded in soft nanofibrous matrices with or without VEGF, and in petri dishes with or without VEGF. Only MSCs seeded in 3-dimensional soft matrices with VEGF showed significant increases in the expression of endothelial markers (vWF, eNOS, Flt-1 and Flk-1), while eliminating the expression of smooth muscle marker (SM-α-actin). MSCs cultured in VEGF alone showed increased expression of both early-stage endothelial and smooth muscle markers, indicating immature vascular differentiation. Furthermore, MSCs cultured in soft matrices with VEGF demonstrated faster upregulation of endothelial markers compared to MSCs cultured in VEGF alone. Paracrine signaling studies found that endothelial cells cultured in the conditioned media from MSCs differentiated in the soft matrix and VEGF condition exhibited increased migration and formation of capillary-like structures. These results demonstrate that VEGF and soft matrix elasticity act synergistically to guide MSC differentiation into mature endothelial

phenotype while enhancing paracrine signaling. Therefore, it is critical to control both mechanical and biochemical factors to safely regenerate vascular tissues with MSCs.

3.2 Introduction

In 2008, cardiovascular diseases accounted for 1 in 3 deaths in the United States. (Roger et al., 2012) Regeneration of functional vascular tissues, including capillary network and small arteries, remains a critical barrier to the successful treatment of these diseases. Vascular endothelial cells (ECs) are key building blocks for vascular tissue repair, as they perform vital anti-thombogenic functions and participate in angiogenesis. (Huang and Li, 2008) However, attempts to produce functional vascular tissue with autogenous ECs have limited success due to the need for invasive surgery, poor cell expansion *in vitro,* and inadequate cell proliferation or migration *in vivo.* (Lith and Ameer, 2011) Mesenchymal stem cells (MSCs) are a powerful cellular alternative for vascular tissue regeneration, as they are easily obtainable, multipotent, thrombo-resistant, and low in immunogenicity. (Park et al., 2007) (Riha et al., 2005a)

Previous studies have shown MSCs are capable of regenerating vascular tissues by transdifferentiation or paracrine signaling. (Huang and Li, 2008; Park et al., 2007) (Silva et al., 2005b) (Caplan and Dennis, 2006) Transdifferentiation is the direct differentiation of MSCs into a specific vascular phenotype. (Oswald et al., 2004; Zhang et al., 2010c) Paracrine signaling is the release of molecular mediators from MSCs that aid in the migration and proliferation of surrounding vascular cells, leading to increased capillary formation. (Hocking and Gibran, 2010) Direct injection of MSCs into the heart increased vascular density, and resulted in transdifferentiation of MSCs into ECs along the vessel lumen. (Silva et al., 2005b) Hashi et al cultured MSCs on aligned electrospun

vascular grafts made of poly-L-lactide acid, and found that distinct EC and SMC layers formed on the grafts. (Hashi et al., 2007b) However, other studies have led to concerns about the safety and efficacy of utilizing MSCs for vascular regeneration. O'Shea et al recently found that bolus delivery of MSCs to injured vasculature produced a dysfunctional endothelium, leading to a higher rate of vessel occlusion. (O'Shea et al., 2010b) Research indicates a poorly-defined vascular microenvironment for MSC differentiation can lead to highly heterogeneous cell populations with low vascular lineage commitment and functionality. (Xu, 2008a) In turn, these MSC-derived progenitors may be involved in diseased vascular remodeling. (Sata et al., 2002) The microenviromental factors that guide MSC differentiation into healthy or diseased vascular phenotypes are not well studied. To improve the safety and efficacy of MSCs in cardiovascular therapies, it is critical to define the factors in local vascular microenvironments which regulate MSC differentiation.

In vivo, factors in the cellular microenviroments such as soluble biochemicals and matrix elasticity guide the paracrine signaling and transdifferentiation of MSCs. Studies in the last few years have demonstrated the importance of local matrix elasticity in directing stem cell differentiation. Engler et al demonstrated that gels which replicated the modulus of neural, muscle, and bone tissue directed the differentiation of MSCs towards neural, myogenic, and osteogenic cells, respectively. (Engler et al., 2006) In a healthy vascular microenvironment, ECs reside in the intima tissue, composed of a fibrous matrix with soft elasticity in the low kilopascal (kPa) range. (Peloquin et al., 2011) (Liliensiek et al., 2009) We previously showed that 3-dimensional (3D) fibrous matrices that replicated the intima elasticity directed MSCs toward the endothelial

lineage expressing an early endothelial marker (Flk-1). (Wingate et al., 2012) Substrate elasticity can also play a role in modulating paracrine signaling of MSCs; Sieb et al found MSCs cultured on a soft substrate decreased the release of pro-inflammatory factors, compared to those grown on a stiffer substrate. (Seib et al., 2009) In addition to mechanical properties of matrices, vascular endothelial growth factor (VEGF) is known as one of the most important factors for endothelial differentiation of MSCs. (Oswald et al., 2004) (Cheng and Yau, 2008) (Riha et al., 2005b) VEGF also plays a significant role in paracrine signaling of MSCs to promote angiogenesis and myocardial repair. (Mirotsou et al., 2011) (Cheng and Yau, 2008) Though previous studies have examined the individual effects of biochemical and mechanical factors on vascular differentiation or paracrine signaling of MSCs, the combined effect of these factors as MSCs experience *in vivo* remains unknown. This study aims to elucidate how factors in the local microenvironment interact to modulate both the paracrine signaling and transdifferentiation capabilities of MSCs. We demonstrate that the combined effects of VEGF and soft matrix elasticity result in MSC differentiation into a more mature endothelial lineage and increased MSC paracrine signaling abilities, compared to the individual use of these factors.

3.3. Materials and Methods

3.3.1 Fabrication of 3D Nanofibrous Grafts (NFGs)

Polyethylene glycol dimethacrylate (PEGDM) with a molecular weight of 3000 was synthesized as we previously described (Wingate et al., 2012). Approximately 90% of the end groups were modified with methacrylates as determined by ^{1}H NMR analysis.

An electrospinning solution composed of 3.2% wt PEGdma 3000, 3.4% wt PEO (MW 40 000, Sigma-Aldrich, St Louis, MO), 0.4 % wt of Irgacure 2959 (I2959, 0.6 mg/ml in DI H20, Ciba, Tarrytown, NY) and 93% DI H_2O was mixed for one hour with magnetic stir bar. PEGDM 3000 photo-polymerizable NFGs were fabricated by electrospinning on a custom system composed of a high voltage power supply (Gamma High Voltage Research, Ormond Beach, FL), grounded collecting surface, motorized syringe pump, and a 14 mm syringe. The solution (2 ml) was spun at a distance of 26 cm from the stationary collecting surface, a voltage of 22 kV, and a flow rate of 1.10 ml/hour. NFGs with a thickness of 0.3 mm were cut into 2 inch diameter disks and placed in glass vials. Vials were then vacuum-sealed and NFGs were photopolymerized under 365 nm light with an average intensity of 15mW/cm^2 for 5 minutes. NFGs were submerged in DI H_2O for 24 hours and sterilized with 70% ethanol prior to cell seeding. With random sampling of NFGs, the compressive modulus of ~2kPa was verified using the protocol we previously described. (Wingate et al., 2012)

3.3.2 Cell Culture and Cell Seeding

Rat MSCs from Lonza Group Ltd (Switzerland) with passages 3-8 were cultured in Dulbecco's Modified Eagles Media (DMEM, Sigma-Aldrich Inc, St Louis, MO), with 10% stem cell qualified FBS for MSCs (Atlanta Biologicals, Lawrencdeville, GA) and 1% Penicillin/Streptomycin (Invitrogen Inc, Carlsbad, CA). This media was the standard utilized for all MSC experiments unless otherwise noted. Cells were maintained at 37 ^oC/5% CO_2 and the cell culture medium was changed every second day. To examine the effect of soluble chemical factors on vascular differentiation of MSCs, VEGF-A

(Shenandoah Biotechnology, Warwick, PA) was added to the media. For transdifferentiation experiments, MSCs were seeded in the following 4 experimental conditions: (1) PS – a standard polystyrene cell culture plate; (2) VEGF – a standard PS plate with 10 ng/ml of VEGF; (3) 2kPa – a 3D NFG with compressive elasticity of ~2-3kPa; and (4) 2kPa+VEGF – a 3D NFG with compressive elasticity of ~2-3kPa and 10ng/ml of VEGF. Prior to cell seeding, all substrates were coated with 0.3 % type I rat tail collagen (BD Biosciences, Bedford, MA). NFGs were seeded in ultra-low attachment plates (Sigma Aldrich, St Louis, MO). Our previous studies have shown that MSCs are able to penetrate the NFG scaffolds which provide cells with 3-dimensional adhesion and culture. (Wingate et al., 2012)

3.3.3 Immunofluorescent Staining

Immunofluorescent staining of cells to examine VE-cadherin (VECAD), PECAM, Flk-1, smooth muscle α-actin (SMA), or F-actin was performed to characterize vascular differentiation or MSC morphology. For VECAD and Flk-1 staining, cells were seeded for 168 hours in media with 0, 10 ng/ml, and 50 ng/ml VEGF. For F-actin and SMA staining, $1X10^5$ MSCs were seeded for 168 hours on all the experimental conditions. For PECAM staining, $1X10^6$ MSCs were seeded for 24 hours on the two NFG conditions. For F-actin staining, samples were fixed with 3.7% formaldehyde at room temperature, permeated with 0.1% triton, and blocked with 3% BSA. Then, samples were incubated in Alexa488-phalloidin (Invitrogen Inc, Eugene, OR) in 1% BSA for 1 hour. For immunostaining of vascular biomarkers, samples were first incubated with a primary antibody in 1 % BSA for 2 hours at room temperature. Following primary antibody

coupling, samples were washed in PBS and incubated with secondary antibodies. All samples were finally mounted with DAPI SlowFade (Invitrogen Inc.) and imaged using an epifluorescence microscope (Zeiss, Peabody, MA). Images from each fluorescence channel were merged using ImageJ software (NIH, Bethesda, MA). Primary antibodies were used as follows: rabbit polyclonal anti-SMA (Sigma-Aldrich Inc), mouse monoclonal anti-Flk1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PECAM (Novus Biologicals, Littleton, CO), rabbit polyclonal anti-VECAD (Alexis Biochemicals, San Diego, CA). Secondary antibodies were as follows: anti-mouse IgG antibody conjugated with Alexa 488 and anti-rabbit IgG antibody conjugated with Alexa 594 (Invitrogen).

3.3.4 MSC Formation of Capillary-like Tube Structures on NFGs

MSCs ($2X10^5$) were seeded on NFGs with 4mm radius using a culture medium with or without VEGF (n = 3 for each condition). For comparisons of cell organization, MSCs ($2X10^5$) were also seeded in the PS wells of a 96-well plate using a culture medium with or without VEGF. Cells were incubated for 24 hours before they were fixed, stained with F-actin, and imaged. To quantify the formation of capillary-like tube structures, the total tube length was measured on each sample using ImageJ software. The total tube length per condition was averaged across the three samples used.

3.3.5 Real-Time PCR

Total cellular mRNA from each sample was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Complementary DNA (cDNA) was synthesized from 1 ng of total cellular RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The SYBR Green I assay and the iCycler iQ real-time PCR detection system (Bio-Rad MyiQ Real-Time PCR System, Hercules, CA) were used for detecting real-time quantitative PCR products from 2 ng of reverse-transcribed cDNA. PCR thermal profile consisted of 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds and 95 °C for 1 minute. Genes were normalized to the housekeeping gene GADPH, and the fold change relative to the control (PS) was calculated using the comparative C_t method. All PCR graphs show delta delta Ct fold changes and standard deviations are calculated using the method described previously (Wong and Medrano, 2005). Primer sequences for amplification are shown in Table 3.1.

Table 3.1: Rat primers used in PCR.

Gene	Order	Primer
Flk-1	Forward	5'-AGCTCAGGTTTTGTGGAGGA-3'
	Reverse	5'-CCAAGAACTCCATGCCCTTA-3'
Flt-1	Forward	5'-CTTTCTCAAGTGCAGAGGGG-3'
	Reverse	5'-AGGATTGTATTGGTCTGCCG-3'
vWF	Forward	5'-CACAGGTAGCACACATCACT-3'
	Reverse	5'-CTCAAAGTCTTGGATGAAGA-3'
eMOS	Forward	5'CGACAATCGTGGTGCGTC-3'
	Reverse	5'-GCCTTTTTCCAGTTGTTCCA-3'
GAPDH	Forward	5'-GCCTCGTCTCATAGACAAGATCCT-3'
	Reverse	5'-GAAGGCAGCCCTGGTAACC-3'

3.3.6 Immunoblotting

Immunoblotting or western blotting was used to analyze eNOS protein expressions in MSCs seeded for 168 hours in all 4 experimental conditions. Samples were prepared by first lysing cells in a lysis buffer containing homogenate buffer

containing PBS (pH = 7.5), 0.1% Triton X-100, and a protease inhibitor cocktail. Cells were then centrifuged at 1,000 g, 4°C, for 15 minutes with an Eppendorf centrifuge (model 5417R; Brinkmann Instruments, Westbury, NY). The supernatant was collected, and protein concentrations were analyzed. The supernatant was mixed with an equal volume of sample buffer (100 mM Tris HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, and 10% glycerol). Subsequently, protein samples were run on gradient (4 –20%) minigels (Invitrogen) at 100 V for 2 hours. After transfer, membranes were rinsed with TPBS (PBS containing 0.05% Tween 20) and blocked with 5% nonfat dry milk for 1 hour at room temperature. The blocked membranes were incubated in primary antibodies (diluted to 1:1000 – 1:3000 with TPBS and 5% BSA) at room temperature for 2 hours. After washing with TPBS twice for 5 minutes each time, the membrane was incubated with peroxidase-linked secondary antibodies (diluted to 1:5000 with TPBS and 5% dry milk) at room temperature for 2 hours. Following further washes, ECL solution was added for 5 minutes at room temperature, and then the membrane was exposed on X-ray film. ImageJ software was used to measure the band density. All eNOS bands were normalized to corresponding GAPDH (housekeeping molecule) bands.

3.3.7 Analysis of MSC Paracrine Signaling: Capillary-like tube formation of ECs

MSCs ($5X10^5$) were seeded on at least three samples for each of the 4 experimental conditions. MSCs were cultured for 24 hours in the experimental culture media which were then removed. This was followed by washing the cells in PBS and then adding 1 ml of serum-free DMEM media to each cell sample. After 24 hours, the

conditioned serum-free media from MSCs were extracted from the samples and stored at -80 ^{o}C for future use of EC culture. Following five serum-free medium conditions were used to culture ECs: (1) "PS–CM" is the conditioned media from MSCs seeded in the control "PS" condition as described in Section 2.2; (2) "VEGF–CM" is the conditioned media from MSCs seeded in the "VEGF" condition; (3) "2kPa–CM" the conditioned media from MSCs seeded in the "2kPa" condition; (4) "2kPa+VEGF–CM" the conditioned media from MSCs seeded in the "2kPa+VEGF" condition; and (5) "SFM" is serum-free DMEM which was used as a control. Primary ECs were used for Passage 3-8 and were maintained in the DMEM supplied by 10%FBS. To analyze MSC paracrine signaling to EC tube formation, 0.010 ml of reduced growth factor matrigel (BD, Franklin Lakes, NJ) was placed in each well of a 12 well slide Angiogenesis (ibidi, Martinsried, Germany) and incubated for 1 hour at 37 ^{o}C to allow gelation. For each of the five medium conditions, 0.050 ml of conditioned media was placed in the wells (n=3, from three separate samples), and ~17,000 of ECs were then seeded on top of matrigel. Samples were incubated at 37 ^{o}C for 24 hours, before pictures of tube structure formation were taken on a phase contrast microscope (Nikon, Garden City, NY).

3.3.8 Analysis of MSC Paracrine Signaling: EC Migration Assay

The conditioned media from MSCs cultured in various experimental conditions were obtained with a similar method as described above, but MSCs were cultured for 168 hours before the standard culture media were replaced with the conditioned media for additional 24-hour culture. Transwell plates with 6.5 mm-diameter wells and polycarbonate membrane inserts having 8.0μm pores (Sigma Aldrich Inc) were used for

the migration study. For each of the five medium conditions, 0.75 ml of conditioned media was placed in the wells (n=3, from three separate samples). ECs (~$1X10^5$) in 0.2 ml of serum-free DMEM were seeded on top of the porous polycarbonate membrane inserts before the inserts were placed in the wells. Cells were then incubated for 24 hours. To remove the ECs that did not migrate through the pores, the top side of the polycarbonate membrane inserts were gently cleaned with a cotton swab. Migrated cells on the bottom of the inserts were fixed in methanol and stained with crystal violet. Inserts were imaged using an upright microscope Axiovert S100 (Carl Zeiss, Oberkochen, Germany) at a 20x magnification. For each image, the area fraction was calculated as follows with the areas determined by ImageJ.

$$\text{Area Fraction} = \frac{\text{Cell Covered Area}}{\text{Total Area}}$$

3.3.9 Statistical Analysis

All data is shown as mean ± standard deviation. Statistical analysis was performed using MVPstats software (MVP Programs, Vancouver, WA) or SPSS software (IBM, Chicago, IL). For comparing two groups with equal variances, a student's t-test was used. For comparing two groups with unequal variances and unequal sample sizes, Welch's t-test was used. For multiple parametric group comparisons, a one-way ANOVA test was run on both the groups' means and variances. If the groups had equivalent variances, a Tukey Post-Hoc was further performed. If the groups had unequivalent variances, a Games-Howell Post Hoc was further performed. For non-parametric groups,

a Bonferroni or Kruskal Wallis analysis was used. For PCR, statistics were calculated using the method described previously (Wong and Medrano, 2005).

3.4. Results

3.4.1 Effects of VEGF concentration on MSC differentiation towards endothelial lineage

To determine an appropriate VEGF concentration for this study, MSCs were seeded in media containing 0, 10, or 50 ng/ml of VEGF, and the percentage of MSCs that differentiated towards endothelial lineage was quantified by immunostaining for early endothelial markers (Flk-1 and VECAD). Independent studies have utilized 10 or 50 ng/ml of VEGF to direct MSC differentiation towards endothelial lineage. (Oswald et al., 2004) (Liu et al., 2007) However, the effects of VEGF concentration on the endothelial differentiation of MSCs remain unclear. Therefore, we compared the effectiveness of these VEGF concentrations in terms of differentiating MSCs towards endothelial lineage. Our results showed that both 10 ng/ml and 50 ng/ml VEGF concentrations resulted in roughly 50% of MSCs with VECAD marker, a 500-fold increase compared to the control (Figure 3.1).

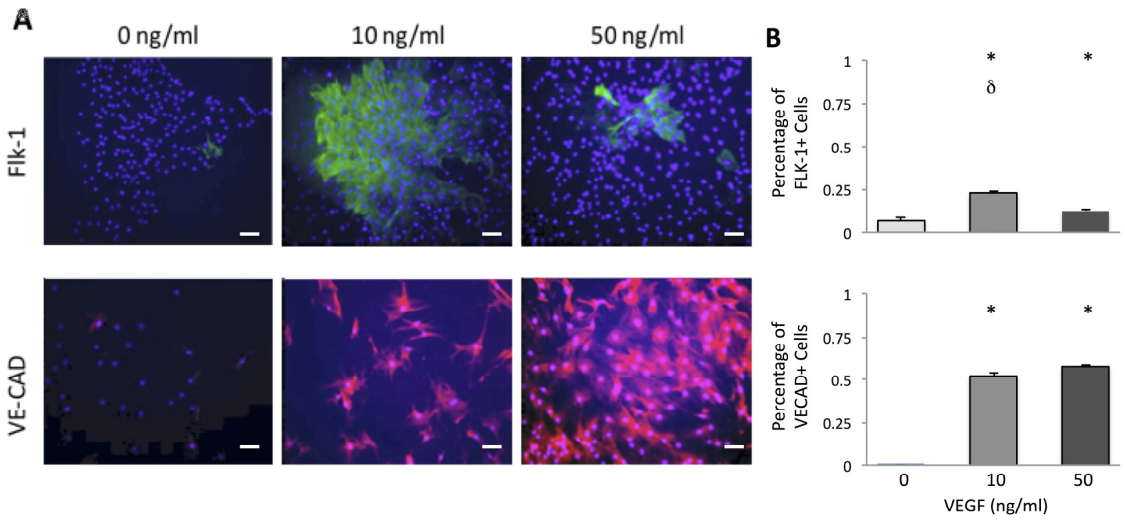


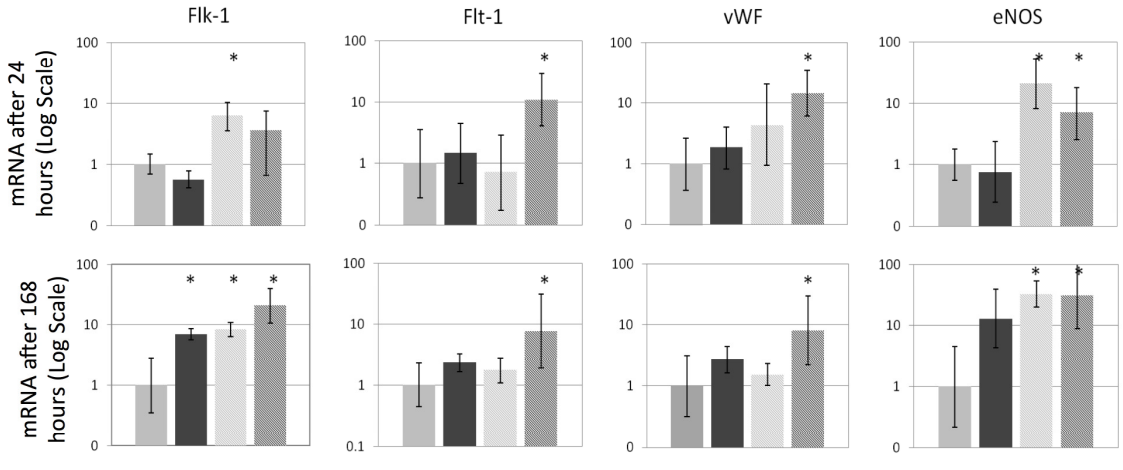
Figure 3.1: Effect of the VEGF concentration on the MSC expression of early endothelial markers. (A) Representative images of MSC stained with endothelial markers (Flk-1 and VECAD) after 168- hour incubation in the media supplied with 0, 10 or 50 ng/ml of VEGF. Scale bar is 10 microns. (B) Percentage of MSCs displaying Flk-1 marker and VE-CAD marker in 0, 10 or 50 ng/ml VEGF. "*": $p < 0.01$ versus 0 ng/ml. "□": $p < 0.05$ versus 50 ng/ml.

MSCs seeded in 50 ng/ml and 10 ng/ml of VEGF resulted in increased percentages of Flk-1^+ cells by 1.8-fold and 3-fold respectively, when compared to the control. As 10 ng/ml of VEGF was highly effective in driving MSCs to endothelial lineage, we continued using this concentration to explore the combined effects of matrix elasticity and VEGF on endothelial differentiation.

3.4.2 The combination of soft matrix and VEGF induces MSCs to upregulate gene expression of matured endothelial markers and to expedite endothelial differentiation

To evaluate the individual and combined effects of soft matrix elasticity and VEGF on the differentiation of MSC to endothelial lineage, the gene expression of MSCs in the PS (control), VEGF, 2 kPa, and 2kPa+VEGF conditions was quantitatively

evaluated after culture for 24 and 168 hours. Recent studies in developmental biology have identified a series of vascular biomarkers characterizing different differentiation stages of stem cells, including lineage commitment, differentiation and maturation. (Luo et al., 2011; Park et al., 2005) To assess the maturity of differentiated MSCs, we examined genes of endothelial markers including Flk-1 (an early endothelial or progenitor marker), vWF and Flt-1 (more mature endothelial differentiation markers), as well as eNOS (an endothelial-specific functional marker). Within 24 hours, MSCs in the 2kPa+VEGF condition showed an 8-fold increase of Flt-1, vWF and eNOS genes compared to the control (Figure 3.2).



Condition	24 hours				168 hours			
	Flk-1	Flt-1	vWF	eNOS	Flk-1	Flt-1	vWF	eNOS
PS								
VEGF					+			
2 kPa	+			+	+			+
2 kPa +VEGF		+	+	+	+	+	+	+

Figure 3.2 The combination of VEGF and 2kPa matrix elasticity upregulates genes showing early and mature endothelial phenotype in MSCs. (A) MSC gene expression of Flk-1, Flt-1, vWF, and eNOS after 24 and 168 hours in seeding conditions. "*": $p < 0.05$ versus PS, $n > 3$ for all conditions. (B) Table summary on MSC expression of endothelial markers after 24 and 168 hours of incubation. "+" indicates > 8-fold increase ($p < 0.05$) compared to the PS control.

After 168 hours, MSCs in the 2kPa+VEGF condition maintained the high increase in Flt-1, vWF, and eNOS gene expression, and Flk-1 gene expression increased by roughly 20 fold compared to the control. For MSCs in the 2kPa condition, Flk-1 and eNOS gene expression significantly increased by 8-fold compared to the control at 24 hours, and this increase was maintained at 168 hours. Finally, MSCs seeded in VEGF showed an 8-fold increase in Flk-1 gene expression at 168 hours compared to the control. Therefore, while the individual use of the biochemical factor (VEGF) or soft matrix (2kPa) induced upregulation of certain endothelial genes, only MSCs in the VEGF+2kPa condition had all four endothelial genes highly upregulated within 168 hours. Furthermore, the combination of VEGF and 2kPa matrix elasticity expedited the endothelial differentiation of MSCs.

3.4.3 The combination of soft matrix and VEGF enhances MSC expression of endothelial-specific functional protein and inhibits development of smooth muscle phenotype

When utilizing MSCs for vascular regeneration, it is critical to direct cell differentiation to a specific, mature vascular phenotype, as opposed to vascular cell progenitor or diseased vascular phenotypes. Though VEGF is an exogenous growth factor present in the arterial microenvironment that is important for directing endothelial differentiation, increased VEGF was also found at the sites of neo-intima hyperplasia where diseased cells simultaneously express both endothelial and SMC markers (e.g. PECAM and SMA) (Yeager et al., 2011) (Tanaka et al., 2008). To determine if these vascular tissue biochemical and mechanical factors guided MSC differentiation into a

highly specific endothelial phenotype, MSC protein expression was evaluated in all seeding conditions by immunostaining or immunoblotting. The protein expression of the functional endothelial marker (eNOS) as well as a smooth muscle biomarker (SMA) characterizing early smooth muscle differentiation was quantified. Results showed that MSCs seeded in the 2kPa+VEGF condition displayed an 8-fold increase in eNOS protein expression compared to the PS control (Figure 3.3).

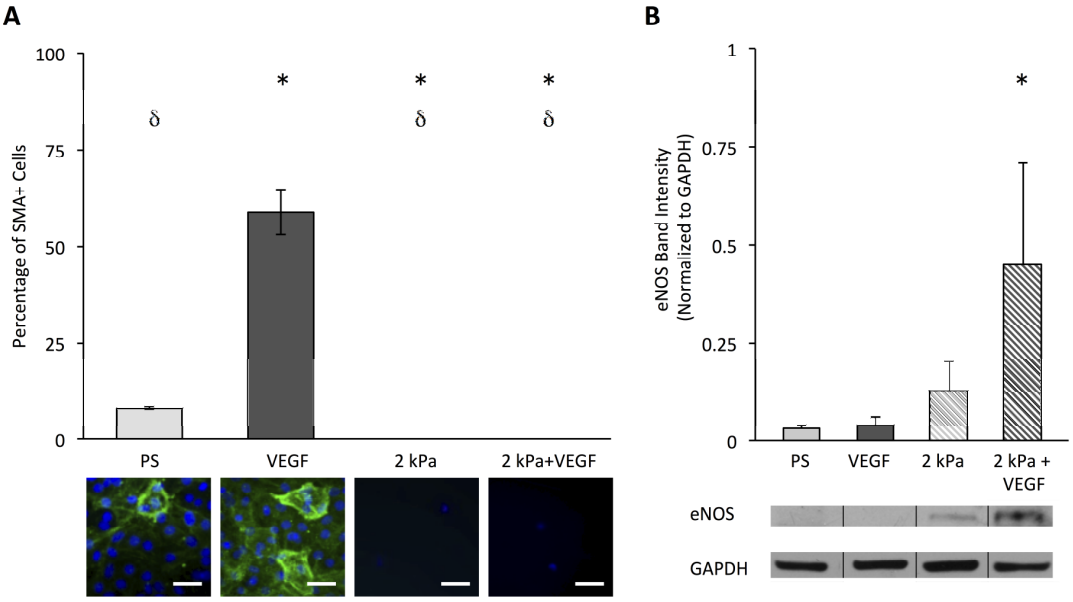


Figure 3.3. The combination of VEGF and 2 kPa matrix elasticity upregulates MSC expression of eNOS protein while downregulating SMA expression. (A) Representative immunostaining images and quantitative measures showing SMA^+ MSCs after cells were cultured in experimental conditions for 168 hours. "*": $p < 0.05$ VEGF. "□": $p < 0.05$ with PS. Scale bar is 5 microns. (B) Western blotting results showing eNOS protein expression in MSCs after cells were cultured in experimental conditions for 24 hours. "*": $p < 0.05$ with PS. Representative eNOS and GAPDH protein bands shown here were obtained from separated lanes on the same gel.

Additionally, MSCs in the 2kPa+VEGF condition did not express SMA protein. For MSCs in the 2kPa condition, the eNOS protein expression was not significantly increased compared to the control, nor was any SMA protein expression found. For MSCs in the

VEGF-conditioned media, eNOS protein was not significantly increased compared to the control. Interestingly, the VEGF condition resulted in roughly 60% of MSCs showing the SMA marker, ~6-fold increase compared to the control. MSCs seeded in both control and VEGF conditions had significantly greater SMA protein compared to MSCs cultured on 2kPa or 2kPA+VEGF conditions, suggesting that soft matrix elasticity may inhibit SMA protein expression. The individual use of VEGF increased both SMA and Flk-1 expression, suggesting that VEGF upregulated both endothelial and smooth muscle markers, promoting MSCs into vascular progenitors or pathological vascular phenotypes. In addition, VEGF significantly increased MSC proliferation by 2 folds within 24 hours, when compared to control (data not shown). Only MSC cultured in the 2kPa+VEGF condition highly upregulated endothelial-specific functional protein while lacking smooth muscle protein, demonstrating that both mechanical and chemical stimuli are required to direct MSC differentiation into specific vascular endothelial lineage.

3.4.4 The combination of soft matrix and VEGF enhances capabilities of MSCs to form capillary-like structures

It is known that functional ECs are capable of undergoing angiogenic process to form capillary-like structures. To determine if MSC differentiation under biomechanical and/or biochemical stimuli exhibited similar angiogenic capability, MSCs were cultured in the PS, VEGF, 2kPa and 2kPa+VEGF conditions and cell organization was examined after 24 hours. MSCs cultured in 2kPa and 2kPa+VEGF conditions formed capillary-like structures (Figure 3.4A), whereas MSCs cultured on PS, VEGF or matrigel with reduced growth factors did not form such structures (Data not shown).

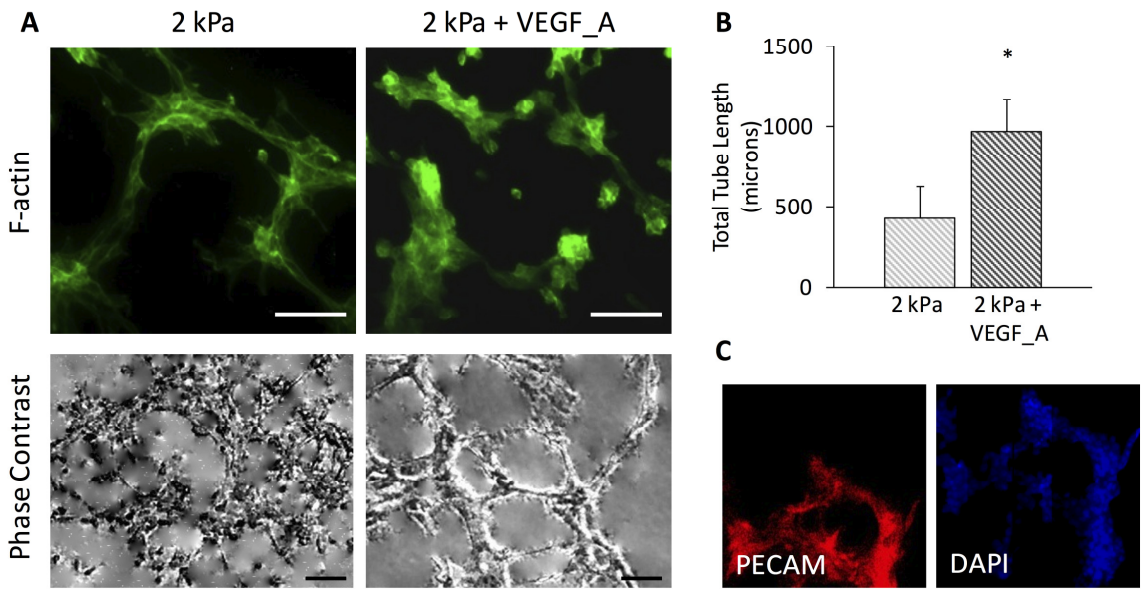


Figure 3.4: MSCs formed capillary-like tube structures in 2kPa and 2kPa+VEGF seeding conditions. (A) Representative fluorescent (F-actin) and phase contrast images of capillary-like structures formed by MSCs in 2kPa and 2kPA+VEGF conditions, after 24-hour culture. Scale bar is 10 microns. (B) Quantitative analysis of the total tube length in capillary-like structures formed on 2kPa and 2kPa+VEGF conditions. "*": $p < 0.05$ versus 2 kPa, n = 3. (C) PECAM and DAPI immunostaining of capillary-like structures formed by MSCs in the 2kPa+VEGF condition.

To determine if the addition of VEGF to 3-dimensional 2kPa matrices improved the angiogenic capacity of differentiated MSCs, the total length of all capillary-like tube structures was determined. Results showed that the tubes formed by MSCs on 2kPa+VEGF matrices exhibited increased total tube length by 2 folds over those formed by MSCs on 2kPa matrices (Figure 3.4B), which indicates enhanced angiogenic capabilities of the differentiated cells. To further confirm if differentiated MSCs in capillary-like structures expressed endothelial markers, MSCs seeded on 2kPa and 2kPa+VEGF conditions were stained with DAPI and PECAM. As illustrated in Figure 3.4C, MSCs in the soft matrix conditions expressed PECAM around capillary-like structures.

3.4.5 Matrix elasticity and VEGF synergistically enhances MSC paracrine signaling to promote EC migration and formation of capillary-like structures

A number of recent *in vivo* studies have found that MSCs improve cardiovascular function by secreting a large reservoir of paracrine signals, which improve the angiogenenic capabilities of ECs. (Gnecchi et al., 2005; Mirotsou et al., 2011) We thus examined how the individual and combined use of chemical and mechanical factors influenced the paracrine signaling capabilities of MSCs. Since both EC migration and formation of capillary-like structures are critical steps in EC angiogenesis, we have performed these assays by culturing ECs in the conditioned media (CM) extracted from the four MSC culture conditions: PS (PS-CM), VEGF (VEGF-CM), 2kPa (2kPa-CM), and 2 kPa+VEGF (2kPa+VEGF-CM). Serum free media (SFM) was used as a negative control. Results showed that ECs cultured in 2kPa+VEGF-CM exhibited significant increases in cell migration, compared to ECs cultured in the SFM and PS-CM conditions by 5 fold and 3 fold, respectively (Figure 3.5A).

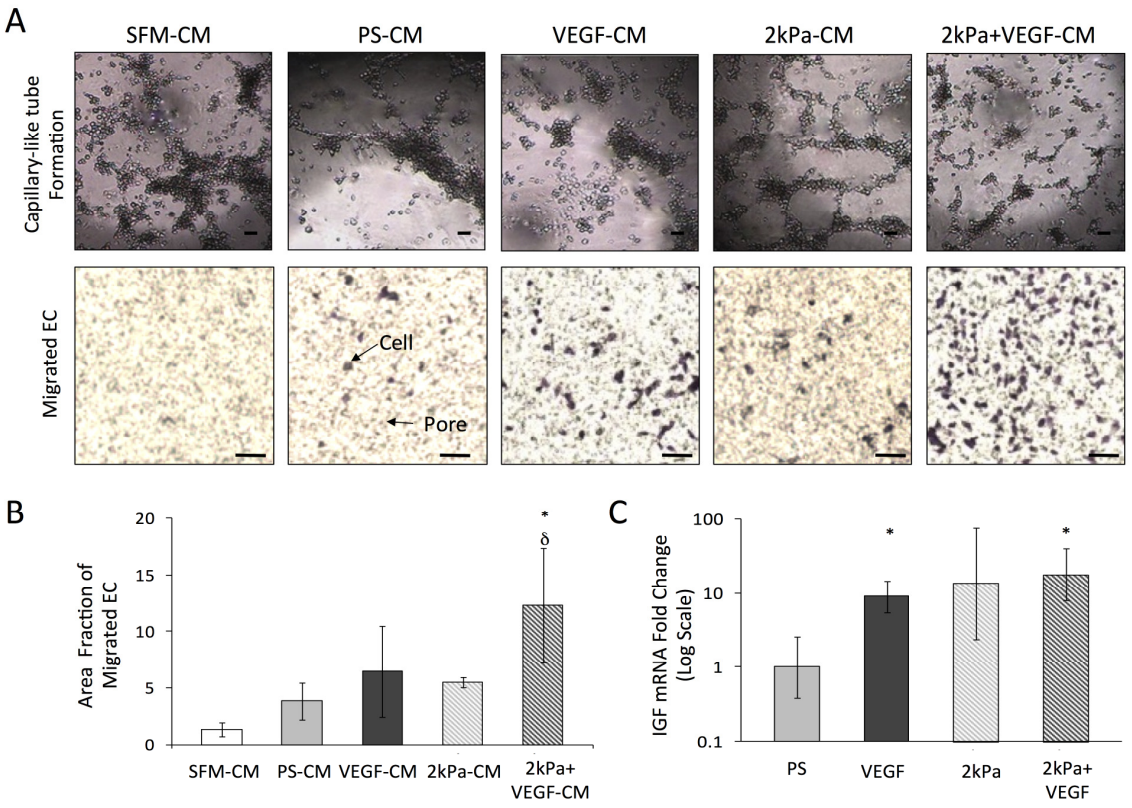


Figure 3.5 MSC–conditioned media from cells differentiated in the 2 kPa+VEGF condition improves paracrine signaling capabilities of MSCs. (A) Top panel: Representative images of migrated ECs on filters. Arrows show pores in a membrane and ECs stained with crystal violet. Bottom panel: Representative images showing EC formation of capillary-like structures on the matrigel with reduced growth factors in various conditioned media (CM). For both panels, the scale bar is 10 microns. (B) Quantitative measure of the area fraction of filter covered by migrated ECs after 24 hours of exposure to MSC-conditioned media. "*": $p < 0.05$ versus SFM. "□": $p < 0.05$ versus PS-CM. $n \geq 3$. (C) Fold change of IGF mRNA in MSCs after 168 hours of seeding on PS, VEGF, 2kPa, and 2 kPa+VEGF conditions. "*": $p < 0.05$ with PS. $n = 3$

Additionally, we found that ECs cultured in 2kPa-CM and 2kPa+VEGF-CM conditions formed capillary-like structures on matrigel with reduced growth factors (Figure 3.5B). These results demonstrate that matrix elasticity can independently enhance MSC paracrine signaling, which in turn increases EC capillary-like tube formation. However, it appears that both matrix elasticity and chemical growth factor are necessary to augment EC migration capabilities via MSC paracrine signaling. To further link

increased EC migration and EC formation of capillary-like structures to the paracrine signaling factors secreted by MSCs, MSC expression of IGF was studied in the PS, VEGF, 2kPa, and 2kPa+VEGF conditions. IGF-1 is one of the most potent natural activators of the AKT signaling, a stimulator of cell growth and proliferation, and recently was recognized as an important factor to improve endothelial function of progenitor cells. (Fleissner and Thum, 2008) MSCs cultured in both VEGF and 2kPa+VEGF conditions showed a 10-fold increase in IGF mRNA expression when compared to the PS control(Figure 3.5C). This suggests that VEGF increases IGF expression in MSCs, partly contributing to enhanced endothelial functions. Other signaling factors we examined did not show significant differences among different conditions. Future work is needed to elucidate the paracrine factors that are released by MSCs co-regulated by the matrix elasticity and VEGF.

3.5 Discussion

The present study has demonstrated that the combined use of VEGF and a soft (~2kPa) matrix synergistically enhances the capability of MSCs to regenerate the vascular endothelium. The synergism occurs through two mechanisms: MSC paracrine signaling to ECs, and MSC transdifferentiation into cells exhibiting matured endothelial phenotype. The paracrine signaling capabilities of MSCs were evaluated by EC migration and formation of capillary-like structures, both of which were increased by conditioned media from MSCs cultured on soft matrices supplied with VEGF. Additionally, the synergistic effects of matrix stiffness and VEGF rapidly drove MSCs to transdifferentiate into cells that expressed several mature endothelial markers after only 24 hours.

Furthermore, our results suggest that the combined use of mechanical and biochemical stimuli enhance the specificity of vascular differentiation. MSCs seeded on NFGs with VEGF showed minimal expression of the early stage smooth muscle marker SMA, while showing significant amounts of the functional endothelial marker eNOS. In contrast, MSCs seeded on rigid substrates with or without VEGF showed a significant amount of SMA expression with low eNOS expression. Taken together, our results have shown that mechanical and biochemical microenvironmental factors act together to guide MSCs to regenerate healthy vascular tissue through transdifferentiation and paracrine signaling mechanisms. The present study also suggested that MSC transdifferentiation into ECs and MSC paracrine signaling to promote activities of ECs might be inherently linked; both MSC transdifferentiation and paracrine signaling are enhanced by the synergistic effects of soft matrix elasticity and a biochemical growth factor.

When utilizing MSCs for vascular regeneration it is critical to direct cell differentiation to a healthy, specific endothelial phenotype. We found that MSCs on soft matrices with VEGF expressed no SMA protein, while expressing several mature endothelial markers. Interestingly, the sole use of VEGF led MSCs to express a significant amount of SMA protein and increase proliferation while upregulating an early endothelial marker. The presence of both endothelial and smooth muscle markers is a sign of a dysfunctional, proliferative cellular phenotype that can be found in diseased vascular tissues such as neointimal hyperplasia (Tanaka et al., 2008; Yeager et al., 2011). Our results are consistent with previous findings *in vivo* and *in vitro*, showing proliferating SMA+ cells were present at the sites of neointimal hyperplasia where a significant amount of exogenous VEGF was found (Bhardwaj et al., 2005). Additionally,

Park et al found that MSCs seeded with TGF-β for 24 hours on polyacrylamide gels with an elasticity of 1 kPa showed significantly less SMA and calponin-1 than those seeded on stiff (> 15kPa) substrates (Park et al., 2011b). Therefore, it is the combination of mechanical and chemical factors that drive MSC transdifferentiation to healthier endothelial phenotypes with increased phenotypic-specific function.

Though a number of studies have explored the individual effects of vascular mechanical and biochemical factors on MSC differentiation, few have attempted to simultaneously modulate both stimuli in a 3D matrix as occurs *in vivo.* To that end, the present study uses a 3D matrix to model the stiffness of the *in vivo* vascular intima and VEGF to model the biochemical environment. The sole use of biochemical factors such as VEGF require at least a 7-day culture time to induce the endothelial differentiation of MSCs (Oswald et al., 2004). Additionally, the independent use of biomechanical factors such as soft matrices may expedite the differentiation process, inducing MSCs to display early to intermediate endothelial markers in 1- to 3- day culture times, as shown by our previous study as well as others (Wingate et al., 2012) (Zhang et al., 2010c). Results from this study demonstrate that the combination of appropriate biomechanical (elasticity of ~2 kPa) and biochemical (a VEGF concentration of 10 ng/ml) stimuli lead to rapid endothelial differentiation of MSCs as well as more mature, specific endothelial phenotype, compared to the independent use of these stimuli. MSC differentiation toward endothelial lineage *in vivo* is regulated by a combination of biochemical and biomechanical factors. We have shown that simultaneous regulation of biochemical and biomechanical factors *in vitro* is an effective way to quickly perpetuate the differentiation

of MSCs into cells that express both mature and functional endothelial markers (Flt, vWF, PECAM and eNOS) of vascular ECs.

Recent studies have highlighted a major biological function of MSCs is their capability to secrete a large number of paracrine signaling factors which in turn affect neighboring cells. The signaling factors include inflammatory factors (Seib et al., 2009), and angiogenic factors that promote surrounding ECs to proliferate, migrate, and organize into capillaries (Mirotsou et al., 2011) (Potente et al., 2011). The injection of MSCs into an ischemic myocardium was shown to improve myocardial function by increasing vascularity (Silva et al., 2005b) (Gnecchi et al., 2005). Furthermore, the sole use of signaling factors released from MSC can improve vascularization (Angoulvant et al., 2011) (Herrmann et al., 2010). Recent studies suggest that microenvironments around MSCs can influence their signal secretory functions. For example, Sieb et al demonstrated that decreasing substrate elasticity reduced MSC secretion of IL8, an inflammatory chemical (Seib et al., 2009). Currently, it is largely unknown how chemical and mechanical factors in the cellular microenvironment influence MSC paracrine signaling. The present study showed that conditioned media from MSCs seeded on soft matrices with VEGF resulted in increased EC migration and formation of capillary-like structures. It seems this combination of chemical and mechanical stimuli increased MSC capabilities of secreting angiogenic factors. Our results also suggest that IGF might partially contribute to the enhanced paracrine signaling from MSCs. Interestingly, Sang et al and Linke et al have also linked the IGF gene to both germ cell and cardiac stem cell migration (Linke et al., 2005; Sang et al., 2008).

3.6 Conclusion

This study has demonstrated that both mechanical and biochemical factors are critical to direct MSC differentiation into healthy endothelial phenotypes. Mechanical and biochemical factors also act synergistically to enhance MSC secretion of paracrine signals, improving EC angiogenesis capabilities. This research highlights the importance of precisely controlling both mechanical and chemical factors when designing cell therapies or tissue engineered constructs.

CHAPTER 4

The Impact of Vascular ECM derived peptides and TGF-β on MSC Adhesion, Proliferation, and Vascular Differentiation in Nanofibrous PEGDA Scaffolds

4.1 Abstract

MSCs hold great promise for vascular tissue regeneration. Research has demonstrated that individual factors in the cell microenvironment such as matrix elasticity, composition, and exogenous chemicals direct MSC differentiation to vascular lineage. However, previous mechanistic understandings are often limited to individual environmental factors on 2D substrates. It is less known how these factors combine to direct MSC fate in a biomimetic nanofibrous environment. Using electrospun matrices with an elasticity matching that of the vascular media, this study examined the independent and combined effects of vascular ECM-derived peptide (fibronectin-derived RGD and/or collagen IV-derived DGY) and growth factor (TGF-β) on MSC adhesion, proliferation, integrin expression, and differentiation towards smooth muscle lineage. RGD, DGY, and a combination of RGD and DGY (RGD+DGY) were incorporated into nanofibrous PEGDA scaffolds. On the RGD+DGY scaffold, a single MSC was shown contact both RGD and DGY peptides. RGD, RGD+DGY, and DGY scaffolds all supported initial MSC adhesion. RGD played a critical role in MSC proliferation on PEGDA peptide scaffolds; at 7 days MSCs seeded in RGD and RGD+DGY scaffolds proliferated, while MSCs seeded in DGY scaffolds had a reduced cell density. MSCs on the soft fibrous PEGDA-peptide scaffolds developed here also demonstrated different responses to TGF-β when compared to MSCs on 2D substrates in terms of smooth

muscle differentiation. The addition of TGF-β did not significantly enhance the protein levels of smooth muscle markers for MSCs seeded on RGD and RGD+DGY scaffolds and it did not change MSC expression of integrins alpha1, alphaV, beta1, or beta 3. Results here suggest the adhesive matrix environment may help to steer the path of growth factor-induced stem cell differentiation.

4.2 Introduction

Cardiovascular disease is the leading cause of death in the western world, resulting in 1 out of every 3 American deaths in 2008.(Roger et al., 2012) Regenerating healthy, functional vascular tissue remains a critical barrier to the treatment of vascular diseases. Smooth muscle cells (SMCs) are a key component of vascular tissue, and play a primary role in the contraction and remodeling of the arteries.(Rensen et al., 2007) These functionalities must be replicated when treating cardiovascular diseases with cell therapies or tissue engineered vessels.(Nerem and Seliktar, 2001) However, regenerating vascular tissue with SMCs proves difficult; these cells require invasive surgery for harvest and differentiate to non-functional phentotypes *in vitro*.(Opitz et al., 2007) (Lith and Ameer, 2011) Mesenchymal stem cells (MSCs) are a powerful tool for vascular tissue regeneration as they are easily obtainable and have the ability to differentiate into multiple vascular lineages, including endothelial and smooth muscle.(Huang and Li, 2008; Riha et al., 2005a)

Recent studies indicate MSCs have the capacity to regenerate vascular tissue through differentiation to smooth muscle lineage.(Huang and Li, 2008; Riha et al., 2005a) *In vivo,* MSCs cultured on a polyurethane vascular prosthesis organized into a layered structure and expressed SMC markers within 2 weeks.(Mirza et al., 2008) *In vitro*, the independent use of either collagen IV or TGF-β on two dimensional (2D) substrates has been shown to induce MSC differentiation to smooth muscle lineage.(Kinner et al., 2002; Xiao et al., 2010) However, numerous studies suggest that incorrect differentiation of MSCs can result in progenitor cells that may be involved in diseased vascular remodeling.(Sata et al., 2002; Xu, 2008b) Currently, it is not well understood how factors in the fibrous cellular microenvironment interact to modulate MSC differentiation to healthy or diseased vascular phenotypes. In order to safely utilize MSCs in cardiovascular therapies, it is critical to define the factors in the cellular microenvironment that guide MSC differentiation to healthy vascular tissue.

In vivo MSC bind to proteins in the extra cellular matrix (ECM) through integrins, which then trigger various signaling pathways guiding cellular behavior and differentiation. SMCs reside in the vascular media and along the basement membrane, which are composed of numerous proteins including collagen IV and fibronectin. (Wagenseil and Mecham, 2009) While these proteins guide cellular behavior, playing an important role in tissue functionality and regeneration, they are difficult to utilize in tissue engineered scaffolds due to degradation and confirmation issues. As short peptides derived from ECM proteins lack these problems, significant research has been done to

replicate the ECM functionality by incorporating short peptides into tissue engineered scaffolds. (Hersel et al., 2003) In this study we examine the impact of two vascular peptides on MSC differentiation: RGD, a commonly utilized peptide that is derived from fibronectin and present in a number of ECM proteins; and DGY, a peptide derived from collagen IV. The RGD peptide has been shown to increase MSC adhesion to tissue engineered scaffolds. (Frith et al., 2012) (Beamish et al., 2009) Recent literature indicates the collagen IV protein directs stem cells towards mature SMC lineage. (Xie et al., 2011) Further, SMCs selectively adhere to the DGY sequence of collagen IV while endothelial cells do not, indicating the DGY sequence may play a critical role in SMC adhesion and functionality. (Kanie et al., 2012) Cells can bind to collagen IV through the alpha1beta1 integrin, and to RGD peptides through numerous integrins including the alphaVbeta3 integrin.(Xiao et al., 2007) (Barczyk et al., 2010) Examining the impact of RGD and DGY peptides on MSC integrin expression, adhesion, proliferation, and differentiation will give further insight into the role of vascular ECM composition on MSC behavior.

Exogenous growth factors are known to direct both cellular differentiation and integrin expression. Transforming growth factor beta (TGF-β) is a prominent chemical in the vascular media, and has been shown to guide MSC differentiation towards smooth muscle cell lineage in 2D environments. (Kurpinski et al., 2010; Park et al., 2011a) Further, recent literature indicates TGF-β increases cellular expression of alpha 2 and beta1 integrins, improving cellular adhesion to stiff 2D substrates coated in collagen I.(Margadant and Sonnenberg, 2010) Therefore, the impact of the vascular ECM derived

peptides RGD and DGY on MSC adhesion and differentiation will be studied in conjunction with TGF-β.

While significant research has been done on the independent effects of peptides and exogenous chemical factors, it remains unclear how these factors combine to modulate MSC differentiation towards smooth muscle lineage in a fibrous environment as seen in the *in vivo* media. This study examines how the RGD and DGY peptide sequences and TGF-β interact to direct MSC differentiation towards smooth muscle lineage. As our group and others have shown the importance of matrix elasticity and structure on healthy MSC differentiation, these peptide and growth factor interactions are studied in a fibrous biomimetic scaffold with an elasticity that replicates the *in vivo* vascular media layer. (Engler et al., 2006; Park et al., 2011a; Wingate et al., 2012) (Richert et al., 2004) In this study, MSC integrin expression, adhesion, and differentiation are examined in RGD, RGD+DGY, and DGY fibrous PEGDA scaffolds with and without TGF-β enhanced media. The hypothesis underlying this study is that vascular ECM derived peptides will increase MSC adhesion and/or differentiation towards SMC lineage, while the addition of TGF-β will further increase these cell activities. In this study soft fibrous PEGDA scaffolds are fabricated with various peptide concentrations and compositions. We demonstrate that the RGD peptides are critical to maintain MSC adhesion and encourage proliferation in these PEGDA scaffolds.

4.3 Materials and Methods

4.3.1 Fabrication of 3D Nanofibrous Grafts (NFGs)

Polyethylene glycol diacrylate (PEGDA) with a molecular weight of 700 was purchased from Sigma (Sigma-Aldrich, St Louis, MO). YRGDS and SDGY peptides were purchases from GenScript (GenScript Piscataway, NJ). Monoacrylated PEG tyrosine-arginine-glycine-aspartic acid-serine [PEG-RGD] and monoacylated PEG serine- aspartic acid- arginine tyrosine [PEG-DGY] was synthesized as described previously. (Lynn et al., 2010) Briefly, 1.1 m YRGDS (GenScript, Piscataway, NJ) and 1.1 m SDGY were respectively reacted with 1.0 m monoacrylated-PEG3400-Succinimidyl Carboxymethyl (Laysan Bio, Arab, AL) in 50 mm sodium bicarbonate buffer (pH 8.4) for 2 h. The monoacrylated PEG-peptide was purified by dialysis, lyophilized and kept under argon at 4 °C. The amount of conjugation of the peptide to monoacrylated PEG was found to be 90% via ^{1}H NMR. To fabricate all scaffolds, a base solution composed of 0.075 ml of PEGDA 700, 0.150 ml of I2959 solution (I2959, 0.6 mg/ml in DI H20, Ciba, Tarrytown, NY), 0.11 g PEO, and 2.85 ml H_2O was mixed for one hour with magnetic stir bar. For RGD scaffolds, 0.059g of monoacylated PEG-RGD was added to the base solution, for RGD+DGY scaffolds, 0.059g of monoacylated PEG-RGD and 0.057g of monoacylated PEG-DGY were added to the base solution, and for DGY scaffolds, 0.057g of the monoacylated PED-DGY was added to the base solution. For control scaffolds, no acylated PEG-peptides were added into the base solution. PEGDA-peptide 700 photo-polymerizable scaffolds were fabricated by electrospinning

on a custom system composed of a high voltage power supply (Gamma High Voltage Research, Ormond Beach, FL), grounded collecting surface, motorized syringe pump, and a 14 mm syringe as described previously.(Wingate et al., 2012) Briefly, respective solutions (4 ml) were spun at a distance of 16 cm from the stationary collecting surface, a voltage of 13 kV, and a flow rate of 1.10 ml/hour. Scaffolds with a thickness of 0.3 mm were cut into 2 inch diameter disks and placed in glass vials. Vials were then vacuum-sealed and NFGs were photopolymerized under 365 nm light with an average intensity of 15mW/cm^2 for 15 minutes. Scaffolds were submerged in DI H_2O for 24 hours and sterilized with 70% ethanol prior to cell seeding.

4.3.2 Fourier transform infared spectroscopy analysis

The PEGDA double bond conversion was evaluated with mid-range Fourier transform infrared spectroscopy (FTIR) (Nicolet 4700; Thermo Fisher Scientific, Waltham, MA) by examining the disappearance of the C=C peak within the acrylate group (~1635 cm^{-1}) on dry scaffolds as described previously.(Wingate et al., 2012) Briefly, three scaffolds were measured prior to UV exposure, were exposed to UV for 15 min, and FTIR was then performed again on each sample at the exact same location. To account for sample and background variation, data were normalized with the C=O peak located in the range 1650–1726 cm^{-1}, which is independent of photopolymerization. Data were analyzed using Opus software (Brucker Optics, Billerica, MA).

4.3.3 Mechanical Testing

Tensile testing was performed using an MTS Insight electromechanical testing system (MTS Systems Corp., Eden Prairie, MN, USA). Scaffolds from all 4 conditions (RGD, RGD-DGY, DGY, and Control) were photopolymerized and hydrated in water for 24 hours prior to testing. All samples were cut to 5 mm wide by 25 mm long. Sandpaper was attached to the tensile test grips to prevent slipping. Samples were tested in a fully hydrated condition, and tests were conducted in less than 2 minutes to prevent evaporation. A strain rate of 0.03 mm mm^{-1} s^{-1} was used, following the method used in previous research. (Wingate et al., 2012) (Tan et al., 2008) Uniaxial tensile testing to 30% strain was performed on all samples. The elastic modulus of hydrated NFG was determined from the low strain region (10–15%) of the curve.

The compression modulus of control scaffolds (PEGDA scaffolds with no peptides incorporated) were characterized using a MTS Synergie 100 (MTS, Eden Prairie, MN) with a parallel plate set up. All tests were completed in a hydrated condition, on a 10 N load cell at a strain rate of 0.50 mm/min up to a maximum strain of 15%. The elastic modulus was calculated from the linear elastic region between 10 to 15%.

4.3.4 Cell Culture and Seeding

Rat MSCs from Lonza Group Ltd (Switzerland) with passages 3-8 were cultured in Dulbecco's Modified Eagles Media (DMEM, Sigma-Aldrich Inc, St Louis, MO), with 10% stem cell qualified FBS for MSCs (Atlanta Biologicals, Lawrencdeville, GA) and 1% Penicillin/Streptomycin (Invitrogen Inc, Carlsbad, CA). Rat aortic SMCs were obtained from the Dr. Stiener's lab, and passages 3-8 were maintained in DMEM media with 10% FBS (Atlanta Biologicals, Lawrencdeville, GA) and 1% Penicillin/Streptomycin. Cells were maintained at 37^{o}C/5% CO_2 and the cell culture medium was changed every second day. To examine the effect of soluble chemical factors on vascular differentiation of MSCs, in some experiments a concentration of 1 ng/ml transforming growth factor (TGF-β1) (GenScript, Piscataway, NJ) was added to the MSC media. Experiments were conducted with MSCs, MSCs in TGF-β enriched media, and SMCs. Cells were seeded at a density of 30,000 cells/mm^2 in the following conditions: (1) PS – a standard polystyrene cell culture plate; (2) RGD – on top of the PEGDA RGD scaffolds; (3) RGD+DGY- on top of the PEGDA RGD+DGY scaffolds; (4) DGY- on top of the PEGDA DGY scaffolds (5) Control- cells seeded on top of plain PEGDA 700 scaffolds. To ensure cells bound to the peptides, for all experiments cells were initially seeded in serum free media for 1 hour, and then appropriate media with serum was added for the remainder of the culture time. Scaffolds were seeded in ultra-low attachment plates (Sigma Aldrich, St Louis, MO).

4.3.5 Measurements of cell area, adhesion, and circularity

After two and seven day cultures cell assays was performed to evaluate MSC and SMC spreading, adhesion, and circularity on RGD, RGD+DGY, DGY, and Control conditions. To do this, cells were stained with F-actin and DAPI using the following protocol: samples were fixed with 3.7% formaldehyde at room temperature, permeated with 0.1% triton, and blocked with 3% BSA. Then, samples were incubated in Alexa 488-phalloidin (Invitrogen Inc, Eugene, OR) in 1% BSA for 1 hour. All samples were washed in PBS, mounted with DAPI SlowFade (Invitrogen Inc.) and imaged using an epifluorescence microscope (Zeiss, Peabody, MA). Image-J software (NIH, Bethesda, MD) was used to count the cells as well as measure the cell area and perimeter in the images obtained. For each condition, the area and perimeter of 30 representative cells over 6 samples were measured. Circularity was calculated as $=\frac{4*\pi*A}{P^2}$ where A is the area of the cell and P is the perimeter of the cell. Only cells that were entirely on a single *z*-plane were imaged to ensure correct area calculations.

4.3.6 Immunofluorescent Staining

Immunofluorescent staining of cells to examine smooth muscle α-actin (SMA) and F-actin was performed to characterize vascular differentiation and actin structure. For F-actin staining, samples were fixed with 3.7% formaldehyde at room temperature, permeated with 0.1% triton, and blocked with 3% BSA. Then, samples were incubated in

Alexa488-phalloidin (Invitrogen Inc, Eugene, OR) in 1% BSA for 1 hour. For immunostaining of SMA, samples were first incubated with the primary antibody monocolonal anti-actin alpha smooth muscle (Sigma-Aldrich Inc) in 1 % BSA for 2 hours at room temperature. Following primary antibody coupling, samples were washed in PBS and incubated with secondary antibody Anti-Mouse IgG Texas Red (Sigma-Aldrich). All samples were finally mounted with DAPI SlowFade (Invitrogen Inc.) and imaged using an epifluorescence microscope (Zeiss, Peabody, MA). Images from each fluorescence channel were merged using ImageJ software (NIH, Bethesda, MA).

4.3.7 Immunoblotting

Immunoblotting or western blotting was used to analyze SMA, MHC, calponin, alphaV, alpha1, beta1, and beta3 protein expressions. Samples were prepared by first lysing cells in a lysis buffer containing homogenate buffer containing PBS (pH = 7.5), 0.1% Triton X-100, and a protease inhibitor cocktail. Cells were then centrifuged at 1,000 g, 4°C, for 15 minutes with an Eppendorf centrifuge (model 5417R; Brinkmann Instruments, Westbury, NY). The supernatant was collected, and protein concentrations were analyzed. The supernatant was mixed with an equal volume of sample buffer (100 mM Tris HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, and 10% glycerol). Subsequently, protein samples were run on gradient (4 –20%) minigels (Invitrogen) at 100 V for 2 hours. After transfer, membranes were rinsed with TPBS (PBS containing

0.05% Tween 20) and blocked with 5% nonfat dry milk for 1 hour at room temperature. The blocked membranes were incubated in primary antibodies (diluted to 1:1000 – 1:3000 with TPBS and 5% BSA) at room temperature for 2 hours. After washing with TPBS twice for 5 minutes each time, the membrane was incubated with peroxidase-linked secondary antibodies (diluted to 1:5000 with TPBS and 5% dry milk) at room temperature for 2 hours. Following further washes, ECL solution was added for 5 minutes at room temperature, and then the membrane was exposed on X-ray film. ImageJ software was used to measure the band density. All protein bands were normalized to corresponding β-actin (housekeeping molecule) bands. Antibodies used are as follows: SMA (Sigma-Aldrich Inc), myosin heavy chain (MHC) (Sigma-Aldrich Inc) , Calponin (Sigma-Aldrich Inc) , alphaV (Cell Signaling, Danvers, MA), alpha1 (Cell Signaling), beta1 (Cell Signaling), beta3 (Cell Signaling).

4.3.8 Statistical Analysis

Statistical analysis was performed using MVPstats software (MVP Programs, Vancouver, WA) or SPSS software (IBM, Chicago, IL). For comparing two groups with equal variances, a student's t-test was used, and when comparing two groups with unequal variances and/or unequal sample sizes, Welch's t-test was used. For multiple parametric group comparisons, a one-way ANOVA test was run on both the groups' means and variances. If the groups were homoscedastic, a Tukey Post-Hoc was further

performed. If groups had heteroscedastic a Games-Howell Post Hoc was further performed. For non-parametric groups, a Bonferroni or Kruskal Wallis analysis was used.

4.3.9 Peptide Density and Spacing Calculations

To calculate the number of reactive acylate groups in moles, N, the following equation was used:

$$N = \frac{W*\%M*2}{M_n} \quad [1]$$

Where W is the weight of PEGDA utilized in grams; %M is the percent methacylation; and M_n is the number average molecular weight of the PEGDA.

To determine the moles of peptides in the scaffolds, P, the following equation was used:

$$P = \frac{W_p*\%M}{M_n + M_{np}} \quad [2]$$

Where W_p is the weight of acylated PEG-peptide in grams and M_{np} is the number average molecular weight of the peptide.

4.4 Results

4.4.1 Characterization of PEGDA-peptide scaffolds.

To incorporate a large number of peptides in the scaffolds, it is critical to ensure that the scaffolds have a high degree of conversion after photopolymerization. FTIR analysis was performed on control PEGDA scaffolds with no UV light exposure, and repeated on the same scaffolds after a 15 minute exposure to UV light. At 15 minutes the C=C peak decreased by 91%, indicating the majority of C=C bonds had broken and that

the scaffolds had a high degree of conversion (Figure 4.1). Consequently, all scaffolds in this study were exposed to UV light for 15 minutes. To verify that the incorporation of different peptides did not change the mechanical properties of the scaffolds, tensile testing was performed. Cells typically act in the low strain regions (up to 20%), and therefore the elastic modulus of all scaffolds was measured at the 10 to 15% strain region. (Sen et al., 2009) Tensile testing did not detect any statistical differences in the elastic modulus across peptide conditions, indicating that the incorporation of various peptide types did not impact the elastic modulus of the scaffold. The average compressive modulus of control scaffolds (scaffolds with no peptide s) was measured to be 15 kPa +/- 5 kPa, which is within the range of elasticity found to direct MSC differentiation towards smooth muscle lineage in our previous work. (Wingate et al., 2012)

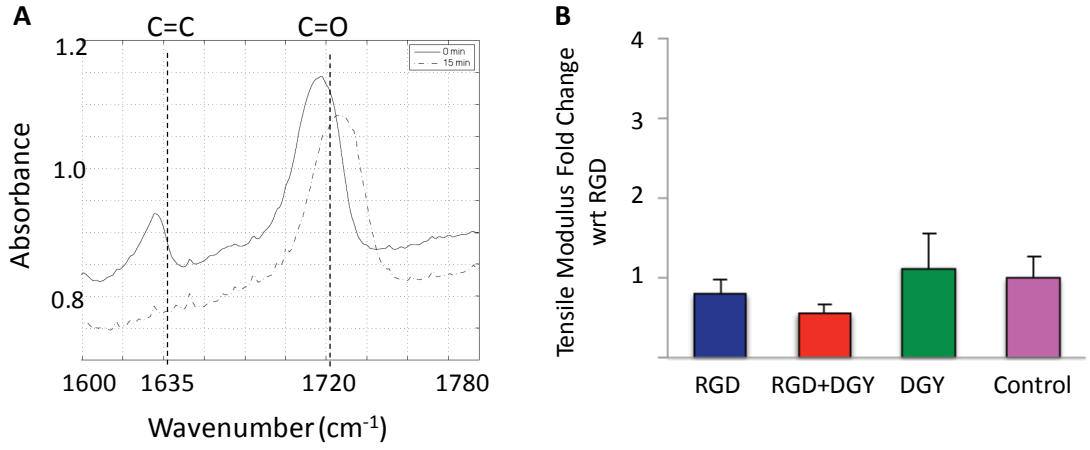


Figure 4.1: Peptide composition does not change tensile modulus of scaffolds. A) FTIR data indicates scaffolds have a high degree of conversion. Blue line show a sample with no UV exposure, dashed line dots shows the same sample after 15 minutes of UV exposure. B) Tensile elastic modulus of RGD, RGD+DGY, DGY, and Control scaffolds, fold change with respect to the RGD condition. No statistical difference found between

scaffolds. Modulus calculated from 10 to 15% strain region, Error bars indicate standard deviation, $n \geq 3$.

4.4.2 Cellular morphology studies demonstrate peptides are incorporated into PEGDA scaffolds, highlights peptide effects.

To validate that the peptides had been successfully incorporated into the PEGDA scaffolds, the spreading and polarity of MSCs and SMCs was quantified in the control, RGD, RGD+DGY, and DGY scaffolds. It is known that PEG-based scaffolds as utilized in the control condition inhibit cell-scaffold interactions, while the incorporation of peptides into these polymer scaffolds is used to increase cell spreading. (Hubbell, 1995; Lin and Anseth, 2009; Sawyer et al., 2005; Shu et al., 2004) Both MSCs and SMCs seeded on the RGD, RGD+DGY, and DGY scaffolds showed statistically greater cell areas and increased polarity when compared to cells on control scaffolds (Figure 4.2). The increase in both cell spreading and polarity on the peptide scaffolds suggests the peptides have been successfully incorporated in the PEGDA scaffolds. We also examined MSC adhesion on other peptide conditions (DGEA, IKVAV, REDV), but all of these peptide scaffolds could not support MSC adhesion without RGD.

Overall, both MSCs and SMCs exhibited statistically greater cell areas and increased polarity on the RGD and RGD+DGY scaffolds compared to the DGY scaffolds. This suggests that the RGD peptide may be more involved in cell spreading mechanisms than the DGY peptide. There were no statistical differences in cell area or circularity between MSCs and SMCs in any of the 4 experimental conditions.

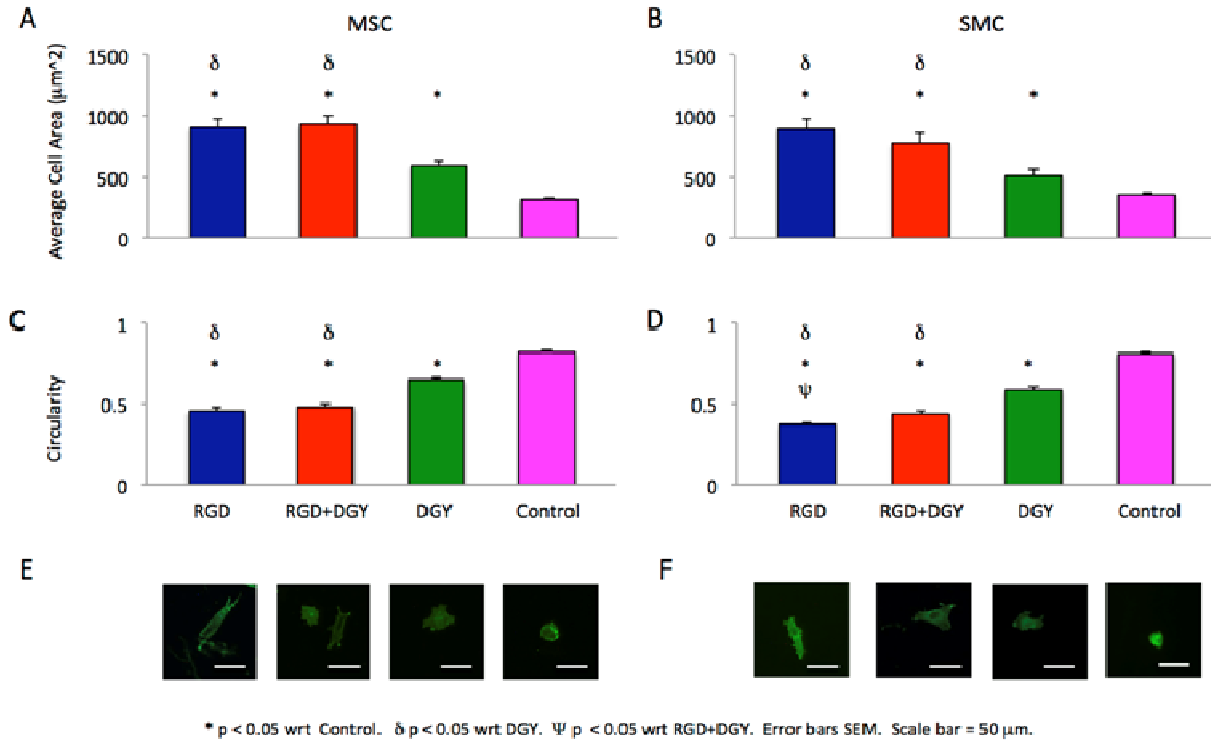


Figure 4.2: The incorporation of RGD and/or DGY peptides increases cellular spreading and decreases circularity. For all panels, 'control' refers to a PEGDA scaffold with no peptides. Top panel: Average cell area for MSC (A) and SMC (B) seeded in all 4 experimental conditions. Middle panel: Circularity for MSC (C) and SMC (D). Bottom panel: Representative photos of MSC (E) and SMC (F) in various peptide conditions.

4.4.3 Initial integrin expression affects MSC and SMC adhesion to the RGD and DGY peptides.

To elucidate the mechanisms underlying cellular adhesion to RGD, RGD+DGY, and DGY peptides, we studied the integrin expression of MSCs and SMCs. Previous studies indicate cells bind to RGD mainly through the alphaVbeta3 integrin, and to collagen IV mainly through the alpha1beta1 integrin. (Xiao et al., 2007) (Barczyk et al., 2010) Therefore, we utilized western blotting to quantitatively examine the protein

expression of alpha1 and alphaV integrins in MSCs and SMCs seeded in plain T74 cell culture flasks. Results found that SMCs had a 15-fold greater expression of alpha1 protein compared to MSCs, while SMCs and MSCs had approximately the same level of alphaV protein expression (Figure 4.3).There was no difference between MSC and SMC adhesion to either the RGD or RGD+DGY scaffolds, which compares well with the similar alphaV integrin expression found in MSCs and SMCs. Interestingly, SMC adhesion to DGY scaffolds was 3.8 fold greater than MSC adhesion, which could be explained by the higher expression of alpha1 protein in SMCs. It seems that MSCs do not initially express high levels of the alpha 1 integrin, which may inhibit the binding of MSCs to DGY scaffolds.

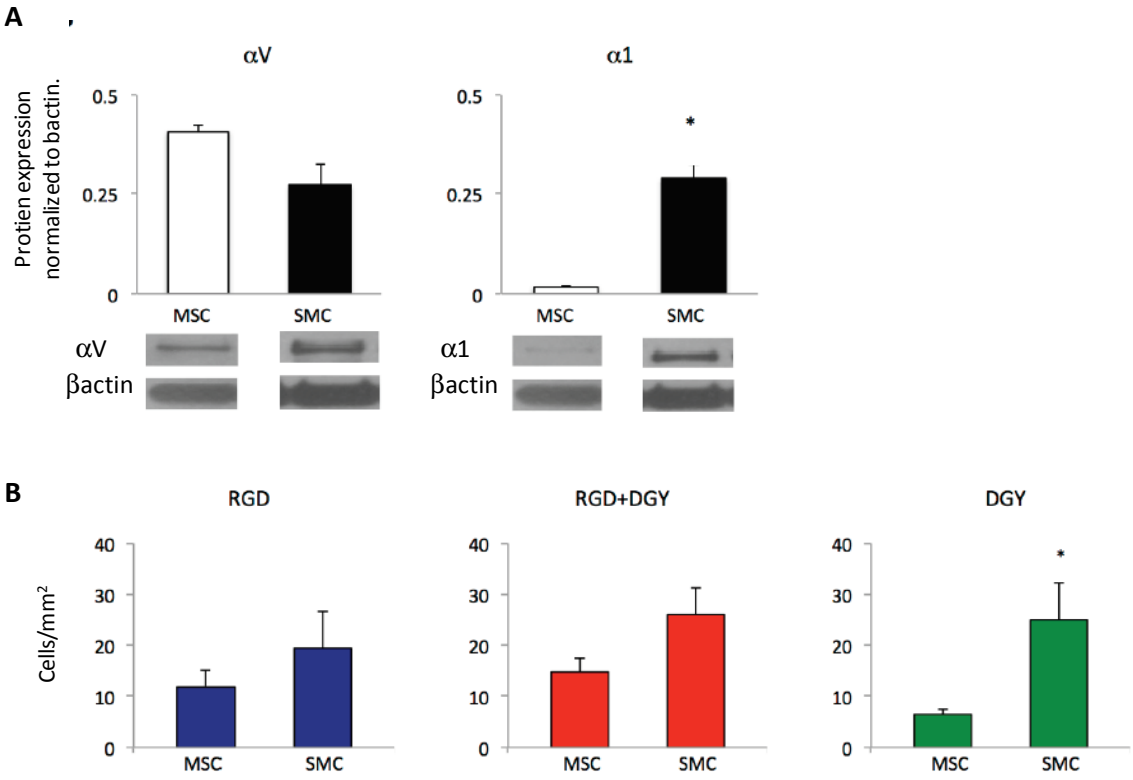


Figure 4.3: Low expression of alpha1 integrin inhibits MSC adhesion to DGY scaffolds. A) Western blot results of MSC and SMC in a T74 flask, examining expression of alphaV and alpha1 integrins. * $p < 0.05$ with respect to MSC, $n = 3$. B) Comparison of MSC and SMC adhesion to RGD, RGD+DGY, and DGY conditions. * $p < 0.05$ with respect to MSC, $n \geq 6$, error bars indicate SEM.

4.4.4 RGD and RGD+DGY scaffolds encourage MSC proliferation while the DGY scaffolds reduces number of MSCs.

To understand the effects of RGD, RGD+DGY, and DGY peptides on MSC proliferation over time, cellular adhesion was studied at 2 and 7 days. MSCs seeded on RGD scaffolds had a 16-fold increase in cellular number at 7 days compared to that at 2 days. MSCs seeded on RGD+DGY scaffolds had an 8-fold increase in cellular number at

7 days compared to that at 2 days. Interestingly, the number of MSCs on DGY scaffolds at 7 days decreased by 12-fold in comparison to adhesion at 2 days (Figure 4.4). Together, these trends suggest that the RGD and RGD+DGY peptides promote MSC proliferation, while the DGY peptide alone may result in a reduced number of MSCs.

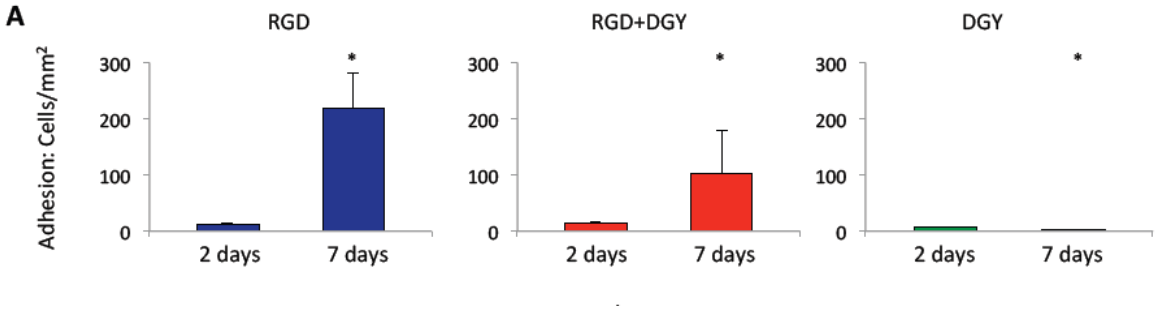


Figure 4.4: RGD and RGD+DGY peptide conditions promote proliferation of MSC. A) MSC density at 2 and 7 days in RGD, RGD+DGY, and DGY peptide conditions. * $p < 0.05$ with respect to 2 days, $n \geq 3$, error bars indicate SEM.

4.4.5 TGF-β does not increase MSC expression of the alpha1 integrin or MSC density on the DGY scaffold.

To study the impact of TGF-β on MSC integrin expression, MSCs were incubated in TGF-β enhanced media for 24 hours, and the expression of the alpha1 and alphaV integrins was examined. To determine if TGF-β could increase the proliferation of MSCs on DGY scaffolds, MSCs were seeded on the scaffolds with TGF-β enhanced media, TGF-β media was refreshed every two days, and the number of MSCs on the scaffolds was analyzed after 7 days. Western blot results demonstrated that 24 hour incubation in TGF-β did not increase MSC expression of the alpha1 integrin, nor did it change the expression of the alphaV integrin (Figure 4.5). At 7 days, MSCs in the TGF-β + DGY

condition showed no increase in cell density compared to MSCs seeded in the DGY condition. As MSC density decreased on the DGY condition with or without TGF-β, MSC differentiation was not examined on the DGY scaffold.

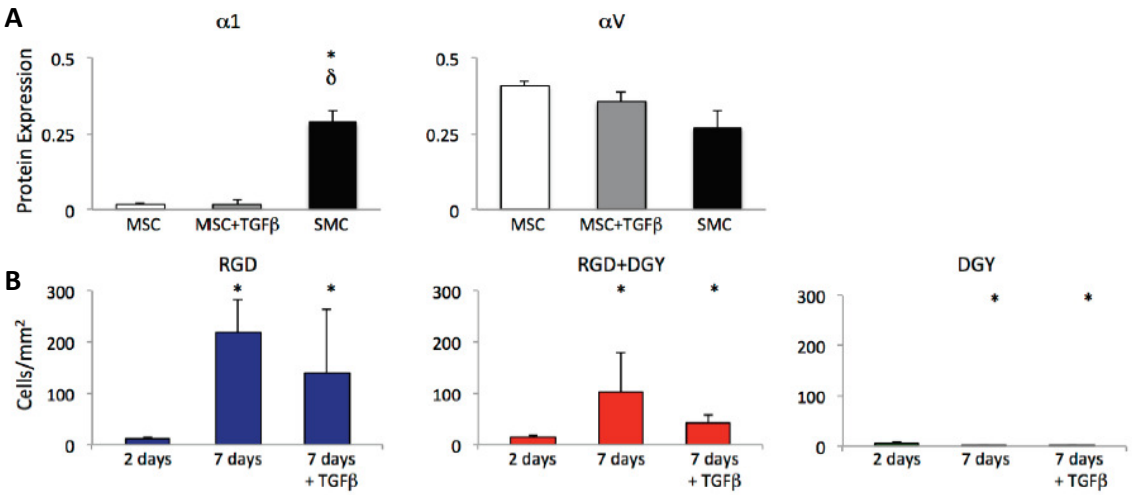


Figure 4.5: TGF-β does not affect expression of alpha 1 and alphaV integrins or adhesion of MSC to DGY peptide scaffolds. A) Western blot data indicates 24-hour exposure to TGF-β does not affect alpha1 or alphaV expression in MSC. Alpha 1 and Alpha V have been normalized to β-actin. Error bars indicate standard deviation, n = 3, * $p < 0.05$ with respect to MSC, δ $p < 0.05$ with respect to MSC+TGF-β. B) TGF-β does not affect MSC density on RGD, RGD+DGY, or DGY peptide scaffolds. Error bars indicate SEM, n ≥ 3, * $p < 0.05$ with respect to 2 days.

4.4.6. A single MSC likely interacts with both RGD and DGY peptides on a RGD+DGY scaffold.

The molar ratio of acylate bonds to peptides was calculated by dividing equation [1] by equation [2]. (Equations and variables are defined in methods section 4.3.9). For the RGD and DGY peptide scaffolds, it was calculated that the molar ratio of acylates

bonds to peptides is 15 to 1, or that there is a peptide every 15 acylate bonds. Therefore, a single MSC contacts numerous peptides, and on a RGD+DGY scaffold will likely contact both RGD and DGY peptides.

4.4.7 MSC integrin expression does not vary with peptide composition or the addition of TGF-β growth factor.

To examine if the peptide composition or the addition of TGF−β growth factor resulted in changes to MSC integrin expression, MSCs were seeded on the RGD and RGD+DGY peptide scaffolds with or without TGF-β . After a 7 day culture, the protein levels of the alpha1, beta1, alphaV, and beta 3 integrins were quantified. Results from immunoblotting showed no changes in alpha1, beta1, alphaV, or beta3 integrin expression in MSCs among any of the 4 seeding conditions (Figure 4.6). These results indicate that the addition of the DGY peptide or TGF-β growth factor does not impact the alpha1, beta1, alphaV, or beta3 integrin expression of MSCs in fibrous RGD scaffolds.

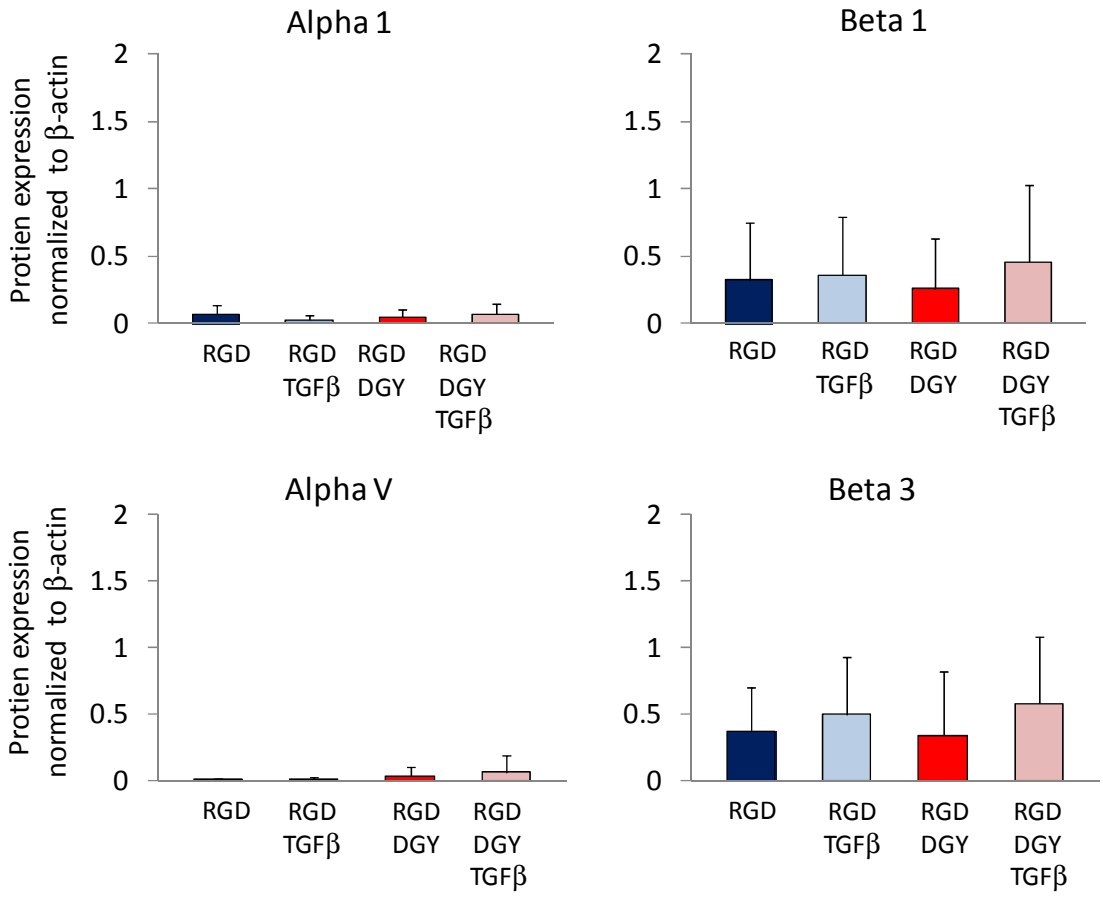


Figure 4.6: MSC integrin expression does not vary with peptide composition or the addition of TGF-β growth factor. Western blot data showing MSC integrin expression after 7 days culture on peptide scaffolds. n ≥ 3 for all conditions.

4.4.8. MSC protein expression indicates the addition of the DGY peptide or TGF-β to soft PEGDA-RGD scaffolds does not induce differentiation toward SMC lineage

To determine if the addition of the DGY peptide and/or TGF-β growth factor impacted MSC differentiation towards SMC lineage, immunostaining and immunoblotting assays were performed. The smooth muscle alpha actin (SMA),

calponin, and MHC proteins are markers that indicate smooth muscle lineage. Therefore, to quantify MSC differentiation towards SMC lineage, these markers were examined on MSC that were seeded on peptide scaffolds for 7 days. Rat aortic SMCs were utilized as a positive control. Immunostaining found that MSCs in the RGD, RGD+TGF-β, RGD+DGY, and RGD+DGY+TGF-β conditions all expressed SMA protein. (Figure 4.7 A)

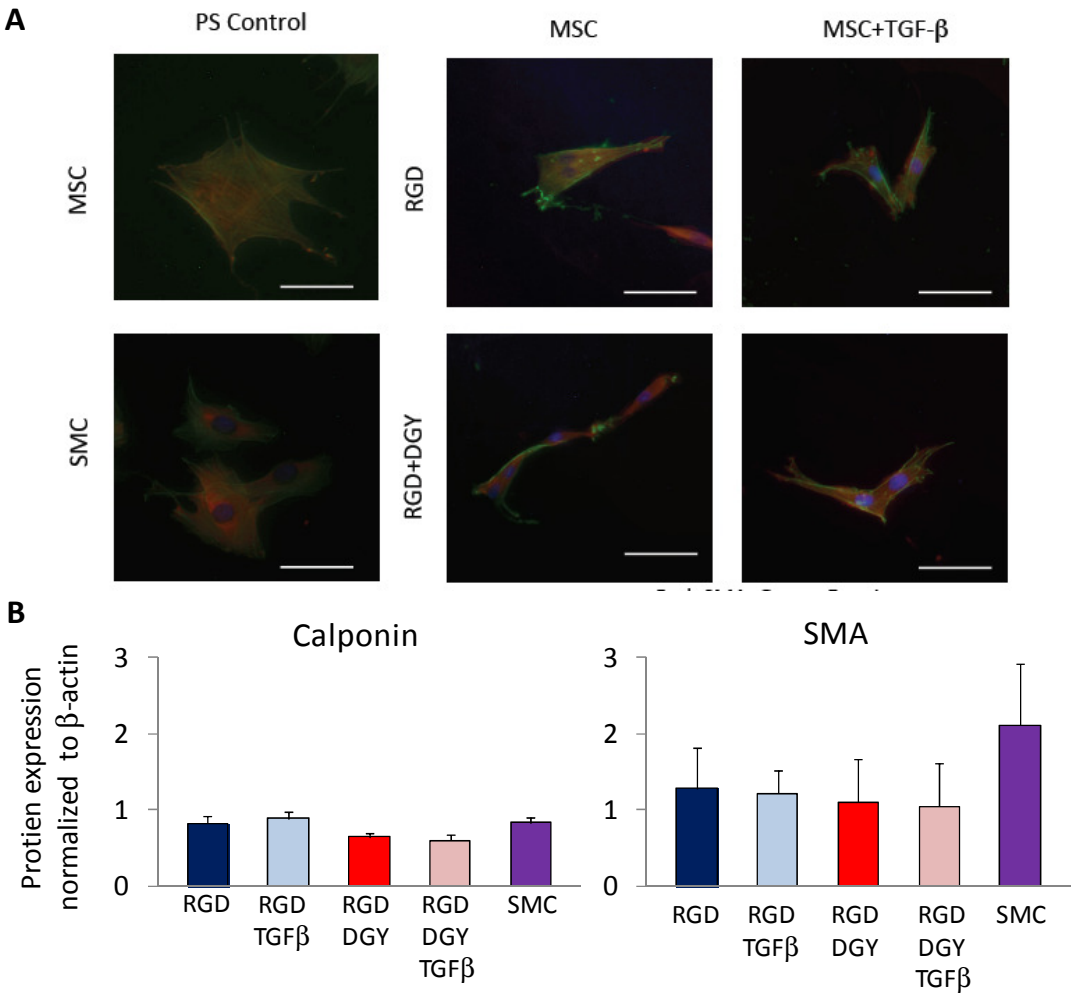


Figure 4.7: The addition of the DGY peptide or TGF-β does not increase MSC differentiation towards SMC lineage. A) Immunostaining results of MSC and SMC stained with SMA (red) and F-actin (green). Scale bar is 50 microns. The PS control condition indicates MSC and SMC seeded on standard polystyrene (PS) petri dish. B) Western blot results showing MSC and SMC expression of smooth muscle cell markers. SMC were seeded in petri dishes. For all conditions, error bars indicate standard deviation and $n \geq 3$.

Immunoblotting indicated that there were no significant differences in the amount of SMA protein found in MSCs seeded in the RGD, RGD+TGF-β, RGD+DGY, and RGD+DGY+TGF-β conditions(Figure 4.7B). Interestingly, MSCs in the RGD condition showed a statistically greater amount of calponin protein compared to MSCs in the RGD+DGY. Furthermore, MSCs in the RGD+TGF-β condition also showed a statistically greater amount of calponin protein compared to MSCs in the RGD+DGY+TGF-β condition ($p < 0.05$). MSCs did not express MHC protein in any of the 4 peptide seeding conditions, suggesting that MSCs have not differentiated into cells with mature SMC lineage. Additionally, there was no change in the MSC expression of Sox9 or RunX2 proteins in any of the 4 seeding conditions, indicating that the addition of TGF-β or DGY did not decrease chrondrogenic or osteogenic markers. Together, this data suggests that the addition of TGF-β or DGY peptides to RGD scaffolds does not increase MSC differentiation towards SMC lineage.

4.5 Discussion

In this study, nanofibrous PEGDA-peptide scaffolds with an elasticity that mimicked that of the *in vivo* media were developed as a platform to study the impact of peptide composition and growth factors on MSC adhesion, proliferation, integrin expression, and vascular differentiation. Specifically, RGD, RGD+DGY, and DGY peptides were incorporated in these fibrous PEGDA scaffolds, and MSC adhered to and spread on these scaffolds within 48 hours. It was found that RGD played a primary role in supporting MSC adhesion and proliferation on these PEGDA-peptide scaffolds. In these soft fibrous RGD-PEGDA scaffolds, the addition of DGY or TGF-β did not increase MSC adhesion or further promote differentiation towards SMC lineage.

When cells bind to ligands through integrins, focal adhesion kinase (FAK) is activated. If FAK is then phosphorylated, cell spreading increases, which in turn allows the cell to enter the S-phase of the cell cycle, resulting in proliferation.(Mammoto and Ingber, 2009) Studies have found that the RGD peptide has been linked to FAK phosphorylation. (Chang and Lo, 1998; Gribova et al., 2013) In our study, at 2 days MSCs on RGD and RGD+DGY scaffolds exhibited greater cell spreading than MSCs on DGY scaffolds. At 7 days, MSCs had clearly proliferated on RGD and RGD+DGY scaffolds, while MSC on DGY scaffold demonstrated a reduced cell density after 2 days. Cells are known to adhere to RGD through the alphaVbeta3 integrin, and western blotting found that MSCs in our study expressed high levels of the alphaV integrin. (Barczyk et al., 2010)Therefore seems likely that on RGD and RGD+DGY scaffolds, MSCs were able to bind to the RGD peptides via the alphaVbeta 3 integrin, likely phosphorylating

FAK and entering S-phase, which then promoted long term adhesion and proliferation. MSCs have been shown to adhere to collagen IV through the alpha1beta1 integrin.(Xiao et al., 2010) While MSCs expressed some levels of alpha1 protein and initially adhered to the DGY scaffolds, it is possible that the limited cell spreading inhibited long term adhesion and viability.

TGF-β has been deemed a powerful chemical factor for inducing MSC differentiation to smooth muscle and chrondrogenic lineages.(Worster et al., 2000; Zhao and Hantash, 2011) Studies have found that MSCs cultured in flat 2D standard petri dishes with TGF-β for 7 days showed an increase in SMA and calponin. (Kinner et al., 2002). Interestingly, Park et al. found that TGF-β increased the expression of SMA and calponin in MSCs seeded for 24 hours on 2D 15 kPa gels. (Park et al., 2011b) In our study, the addition of TGF-β did not increase the expression of SMA, calponin, or MHC in MSCs seeded for 1 week in RGD or RGD+DGY scaffold conditions. It appears that in these soft, fibrous PEGDA-peptide scaffolds, 1 ng/ml of TGF-β does not act synergistically with the RGD or DGY peptides to promote MSC differentiation towards SMC lineage.

TGF-β has been previously utilized to increase adhesion of MSCs to various substrates. Warstat et al. found that incubating MSCs in TGF-β for 24 hours increased their expression of alpha2 and beta1 integrins, thus increasing the adhesion of MSCs to stiff 2D substrates coated in collagen I protein.(Warstat et al., 2010) In our study, a 24 hour incubation of MSCs in TGF-β did not increase the expression of alpha 1 or alpha V

integrins.. Furthermore, a 7 day exposure to TGF-β did not increase MSC adhesion to RGD, RGD+DGY, or DGY fibrous scaffolds or increase expression of the alpha 1, beta1, alphaV, or beta 3 integrins., It is possible that the cell signaling pathways impacted by TGF-β may not increase the expression of the alpha 1, alphaV, beta1, or beta 3 integrins at 7 days. Alternatively, the soft fibrous environment of the scaffolds could result in different cell signaling mechanisms that limit the upregulation of these integrins.

Collagen IV has been shown to be highly efficient in inducing MSC differentiation to mature SMC lineages on stiff 2D substrates via the alpha1beta1 integrin, PI3K pathway, and Nox 4 pathway.(Xiao et al., 2007) Further, the DGY sequence of collagen IV is specific to the vascular basement membrane, and has been shown to increase SMC adhesion while decreasing EC adhesion to polymer substrates.(Kanie et al., 2012) We thus hypothesized that the MSCs would bind to the DGY peptides via the alpha1beta1 integrins, triggering the PI3K and NOX4 signaling pathways to enhance SMC differentiation. Calculations demonstrated that in this study a single MSC contacts numerous peptides in the RGD+DGY scaffolds, suggesting that both peptides types interact with each cell. However, immunoblotting results indicated that the addition of DGY to RGD PEGDA-peptide scaffolds did not increase MSC expression of SMC markers or integrins, indicating that in these conditions DGY does not direct MSC differentiation towards SMC lineage. It is could be that the DGY sequence of collagen IV does not trigger the PI3K or NOX4 pathways. Alternatively, it may be that the soft matrix or fibrous topography resulted in different requirements for

MSC differentiation to SMC lineage. While the DGY peptide sequence did not induce MSC differentiation towards SMC lineage in these experimental conditions, there are numerous other vascular ECM peptide sequences. Considering the powerful impact vascular ECM proteins have on MSC differentiation and behavior, further work examining the effect of other vascular ECM sequences on MSC differentiation is recommended.

4.4 Conclusions

We have developed fibrous PEGDA-peptide scaffolds with an elasticity approximating the *in vivo* media, and the vascular peptides RGD and DGY were incorporated in these matrices. The incorporation of RGD increased MSC proliferation and adhesion, while the addition of DGY or TGF-β to RGD scaffolds did not improve MSC adhesion or direct differentiation towards SMC lineage. These fibrous PEGDA-peptide scaffolds serve as an excellent platform for future work examining the impact of peptide composition on cellular behavior.

CHAPTER 5

Conclusions and Future Studies

5.1 Matrix elasticity is critical factor for vascular tissue engineering

Independently, elasticity can direct MSC towards specific vascular lineages

Past studies have determined that elasticity is an essential factor for directing stem cells towards specific tissue lineages. (Engler et al., 2006) However, these studies have primarily focused on bone, fat, and neural tissues; little work has been done to characterize the effect of matrix elasticity on the vascular differentiation of mesenchymal stem cells (MSCs). In aim 1, we demonstrate that MSCs can be directed towards endothelial or smooth muscle lineages by varying the elasticity of the culture matrix. MSCs seeded on matrices with an elasticity of the *in vivo* intima (2-5 kPa) had upregulation of the EC marker Flk-1, while those seeded on matrices the elasticity of the in *vivo media* (8 -16 kPa) had upregulation of the SMC marker SMA. (Wingate et al., 2012) The use of elasticity as a tool for directing stem cell differentiation has numerous advantages over the more popular use of exogenous chemical factors. Coronary bypasses require a tissue engineered blood vessel with multiple layers, each with a unique microenvironment and cell type. To promote differentiation to multiple vascular cell types, MSCs would require different exogenous vascular chemicals in each graft layer. Spatially controlling the release of different exogenous vascular chemicals in each distinct layer is extremely difficult. However, varying the elasticity of each layer in a tissue engineered blood vessel can easily be accomplished by utilizing different materials,

varying the molecular weight of the same material type, or using photopolymerization processes with the same material. By varying elasticity in a tissue engineered vascular graft it may be possible to seed a single cell type (MSCs) and end up with two distinct EC and SMC layers.

Elasticity rapidly initiates vascular MSC differentiation

Differentiating MSC with exogenous chemical factors requires 5 to 14 days of culture time before cells develop vascular specific markers. (Kinner et al., 2002; Oswald et al., 2004) However, in aim 1 we demonstrate that the use of matrices that mimic the elasticity of the *in vivo* vascular ECM upregulate the expression of vascular markers in MSCs within 24 hours. Recent studies in the literature confirm this trend: Park et al found SMA actin upregulated in MSC within 48 hours of culture on a TGF-β + 15 kPa substrate, while Zhang et al found PECAM upregulated within 72 hours of culture on a fibronectin G' = 100 Pa matrix.(Park et al., 2011a; Zhang et al., 2010a) The rapid differentiation of MSCs by elasticity is particularly powerful in tissue engineered grafts. To utilize exogenous chemicals to differentiate MSCs seeded tissue engineered grafts *in vivo* requires a temporal release over weeks or months, which is difficult to achieve. By controlling the elasticity of the substrate one can achieve rapid differentiation of MSCs, limiting the need for long-term temporal release of exogenous chemicals. In addition, some approaches to vascular tissue regeneration foresee the pre-differentiation of MSCs

in hospital ‘cell banks’ for use in tissue engineered grafts or cell therapies in emergency situations. MSCs are particularly powerful for this application as they can be donated from donor to patient with no rejection. By controlling the elasticity of the substrates in these ‘cell banks’ one could rapidly MSCs in short time periods compared to the use of standard cell culture or exogenous chemicals.

Elasticity is a powerful tool for cellular differentiation that must be taken into consideration when designing tissue-engineered grafts or cell cultures for use in stem cell vascular regeneration. However, significant research remains to fully elucidate the mechanisms by which elasticity directs MSC differentiation. Current research indicates that cells sense elasticity through integrins, trigger signaling pathways through focal adhesion kinase and the Rock/Rho pathway, resulting in changing in cellular morphology, functionality, and differentiation. (Discher et al., 2005; Park et al., 2007) While it is clear that the Rock/Rho pathway plays a role in stiffness induced differentiation, it is unknown how other signaling pathways regulate the Rock/Rho pathway or contribute to stiffness induced differentiation. Further work needs to be done to examine other signaling pathways, particularly on soft substrates where the Rock/Rho pathway appears to be down regulated.

5.2 Both elasticity and chemical growth factors are required for healthy vascular MSC differentiation

Chemical and mechanical factors act synergistically to direct MSC differentiation to mature, functional vascular cells

While elasticity is a critical factor in MSC differentiation, it is not the only factor. Elasticities in the 1 to 5 kPa range have been cited for driving MSC to endothelial, adipogenic, and neural lineages, while elasticities in the 8 to 18 kPa range have been cited for directing MSC to chrondrogenic, myogenic, and smooth muscle lineages. (Engler et al., 2006; Park et al., 2011a; Wingate et al., 2012)Therefore, other factors in the cellular microenvironment must interact with elasticity to direct MSC to specific tissue lineages. In aim 2 we demonstrate that the combination of a 2 kPa matrix elasticity and 10 ng/ml VEGF rapidly directs MSC differentiation to cells with mature endothelial markers such as Flk-1, Flt-1, vWF, and eNOS within 24 hours. In contrast, MSC seeded on 2 kPa matrices only acquired the Flk-1 and eNOS markers, while MSC seeded in 10 ng/ml VEGA on standard petri dishes took one week to develop the Flk-1 marker. MSC seeded on both 2 kPa and 2 kPa+VEGF conditions demonstrated the capacity to form tube like structures, a functional endothelial ability. However, MSC seeded in the 2kPa + VEGF condition were able to form longer tubes then MSC seeded in the 2 kPa condition, suggesting an increase in cellular functionality. These results demonstrate that mechanical and chemical stimuli act synergistically to direct MSC differentiation to cells with more mature markers and increased functionality. These results can be utilized to pre-differentiate MSC for use in vascular cell therapies or tissue engineering. By carefully modulating both chemical and mechanical factors in cell culture, large numbers

of MSCs could be rapidly be differentiated to cells with mature functional endothelial capabilities. Furthermore, the efficacy of vascular tissue engineered grafts can be improved by designs that utilize both elasticity and chemical factors.

Elasticity modulates the effect of chemical factors on MSC differentiation

In aim 2 we found that the correct combination of mechanical and chemical stimuli directed MSC differentiation towards a mature healthy endothelial phenotype, while MSC seeded on stiff 2-D petri dishes with 10 ng/ml VEGF developed both smooth muscle (SMA) and endothelial (eNOS) markers. The expression of both endothelial and smooth muscle markers is a sign of a dysfunctional cellular phenotype that can be found in diseased vascular tissues. (Tanaka et al., 2008; Yeager et al., 2011) Previous research *in vivo* has shown that significant amounts of exogenous VEGF can be found at sights of neointimal hyperplasia. (Bhardwaj et al., 2005) Furthermore, calcified vascular lesions typically have a modulus of elasticity orders of magnitude greater then surrounding vascular tissue. (Peloquin et al., 2011) (Liliensiek et al., 2009) (Richert et al., 2004) (Matsumoto et al., 2002) It seems possible that an elasticity that mimics healthy vascular tissue combined with growth factors results in healthy vascular differentiation, while an elasticity that replicates diseased vascular tissue combined with a growth factor may direct diseased differentiation. Overall, our results suggest that the elasticity of a substrate modulates the effect of exogenous chemical growth factors on MSC differentiation.

Similar conclusions were reached in work done by Dr. Park's lab.(Park et al., 2011a) These finding has a significant impact on the use of cell culture substrates in future research studies, the design of tissue engineered vascular grafts, and the implementation of cell therapies in patients with diseased blood vessels. Cell studies that fail to utilize cell cultures that replicate the elasticity of the *in vivo* environment may yield inaccurate experimental results. Tissue engineered grafts that utilize materials with an elasticity not in the range of healthy vascular tissue may result in the incorrect differentiation of cells. To allow for the regeneration of healthy vascular tissue, the elasticity of the scaffold, culture, or injection site must be carefully designed.

Suggestions for future research examining how matrix elasticity modulates the effect of growth factors on MSC differentiation

Further work needs to be done to understand how elasticity modulates MSC differentiation to diseased and healthy phenotypes. While the cell signaling pathways underlying exogenous growth factors are well studied, the effect of elasticity on these pathways is largely unknown. Critical pathways to study are those that lie between known mechanical and chemical signaling pathways, such as PI3K and MAPK/ERK pathways. Future studies that examine the impact of elasticity on cell signaling pathways known to interact with exogenous growth factors would further the field of cellular mechanobiology. In order to treat cardiovascular diseases such as fibrosis or myocardial infarction with cell therapies, it is critical to understand how cells will respond to the

varying stiffnesses of diseased tissue. Therefore, there is need to study how the stiffening of vascular tissues affects the phenotype, functionality, and differentiation of SMCs and MSCs. Research studying the effects of 3D matrix elastities of 1, 10, 20, 50, and 100 kPa on the phenotype, functionality, and viability of MSC and SMC would be a good preliminary study. Examining addition of VEGF, TGF-β, and platelet derived growth factor (PDGF) to the various culture elasticities on MSC and SMC phenotypes would elucidate the interaction between elasticity and growth factors in diseased and healthy differentiation. Overall, this study would improve knowledge for the design of tissue engineered vascular grafts or cell cultures for the pre-differentiation of MSC. Finally, it would assist with cell therapies; injecting MSC into late stage diseased vascular tissue with stiff calcified lesions may not result in the same differentiation as injecting MSC into early stage diseased vascular tissue where the lesions have not yet become calcified.

5.3 The development of a fibrous PEGDA-peptide scaffold with an elasticity of the *in vivo* media

RGD acts as a critical component in long term MSC adhesion and proliferation

In aim 3 we fabricated a fibrous PEGDA-peptide scaffold with an elasticity that mimics that of the *in vivo* vessel. Initial studies incorporated RGD, IKVAV, REDV, DGEA, VAPG, and DGY peptides into these PEGDA scaffolds. While several labs have had success in MSC adhesion to RGD-polymer systems, few labs have investigated MSC adhesion to other peptide types. (Frith et al., 2012; Park et al., 2010) Initial studies found MSCs had difficulty adhering to DGY, IKVAV, REDV, DGEA, and VAPG peptide-scaffolds. However, MSCs were able to adhere to and proliferate on RGD and

RGD+DGY scaffolds for 7 days. In these PEGDA-peptide scaffolds, DGY was incorporated into the scaffold in conjunction with RGD at a spacing that allows a single cell to interact with both peptide types. This may prove critical for maintaining MSC adhesion while studying the impact of various peptides on differentiation and function.

DGY does not direct MSC differentiation towards SMC lineage

In the vascular ECM, proteins play a key role in vascular cell health and function. Literature has found that proteins can direct MSC differentiation towards numerous lineages, including vascular lineages.(Xiao et al., 2007) However, the use of proteins in tissue engineered scaffolds results in degradation and orientation issues. Peptides lack these issues, making them a powerful tool for tissue engineering. Little work has been done to quantify the impact of vascular peptide sequences on MSC differentiation. Collagen IV has been found to be a powerful factor in inducing MSC differentiation towards SMC lineage.(Xiao et al., 2007) The DGY sequence of collagen IV is specific to the vascular basement membrane, and has been shown to increase SMC adhesion to polymer scaffolds.(Kanie et al., 2012) Therefore, we examined the impact of DGY on MSC differentiation towards SMC lineage. MSCs seeded on RGD+DGY scaffolds showed no increase in the expression of smooth muscle cell markers in comparison to MSCs seeded on RGD scaffolds. It was concluded that the DGY sequence did not induce MSC differentiation to SMC lineage in RGD-PEGDA scaffolds.

Future work examining impact of various vascular peptides on MSC differentiation

Although the DGY peptide sequence did not increase differentiation towards SMC lineage, it is possible that other vascular ECM peptides may do so. The vascular media is composed of collagen I and elastin, while the basement membrane is composed of collagen IV. The elastin VAPG sequence has been shown to be specific to SMC binding, preventing the binding of ECs and fibroblasts.(Gobin and West, 2003) Cells are known to bind to VAPG through the alphaVbeta3 integrin, which was found to be prominently displayed by MSCs in Chapter 4.(Pocza et al., 2008) The collagen IV CAG sequence is specific to the vascular basement membrane, and has also been shown to be exclusive to SMC binding.(Kanie et al., 2012) Finally, the collagen I DGEA sequence has been studied for osteogenic differentiation of MSC, and known to bind through the alpha2beta1 integrin.(Mizuno et al., 2000) Studying the collagen IV CAG, the elastin VAPG, and the collagen I DGEA sequences by incorporating these peptides into the PEGDA-RGD scaffolds would further the understanding of the role of matrix composition on MSC differentiation.

5.4 Concluding Remarks

This dissertation investigates the critical role matrix elasticity plays in the vascular differentiation of MSCs. Independently, matrix elasticity can direct MSC differentiation towards progenitor vascular cells, acting as a switch between endothelial and smooth muscle lineages. Matrix elasticity modulates the impact of exogenous growth factors on MSC differentiation, directing differentiation to either diseased or healthy

vascular phenotypes. To study the impact of various peptides on MSC differentiation in a biomimetic environment, a fibrous peptide scaffold with an elasticity of the *in vivo* artery was developed. The results presented in this thesis demonstrate that it is critical to consider the elasticity of the culture substrate when conducting MSC research, and indicate that matrix elasticity is a powerful tool that should be considered in the design of cell cultures or constructs for vascular tissue engineering.

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