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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Cell-matrix Interactions Regulate Adult Stem Cell Migration and
Differentiation**

A dissertation submitted in partial satisfaction of the
requirements for the degree
Doctor of Philosophy

in

Bioengineering

by

Ludovic Guillaume Jean-Marc Vincent

Committee in charge:

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2014

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Chair

University of California, San Diego

2014

DEDICATION

Pour Lauren et mes parents, Benoît et Liliane Vincent

EPIGRAPH

Real knowledge is to know the extent of one's ignorance.

—Confucius

TABLE OF CONTENTS

Signature Page	iii
Dedication	iv
Epigraph	v
Table of Contents	vi
List of Figures	x
List of Tables	xii
Acknowledgements	xiii
Vita	xv
Abstract of the Dissertation	xvii
Chapter 1 Effect of Substrate Modulus on Cell Function and Differentia- tion	1
1.1 Synopsis	1
1.2 Introduction	2
1.3 Young's Modulus	2
1.3.1 Definition and Measurement	2
1.3.2 Microspheres Under Gravitational Forces	4
1.3.3 Microindenters	6
1.3.4 Atomic Force Microscopy	6
1.3.5 Additional Considerations for Measurement Tech- niques	7
1.4 Development, Maintenance, and Variation of Young's Modulus in Biology	8
1.4.1 Development and Maintenance of Tissue Stiffness	8
1.4.2 Ageing and Disease	9
1.5 Fabrication of Substrates with Defined Young's Modulus	10
1.5.1 Natural ECM Components	10
1.5.2 Synthetic Hydrogels	13
1.6 Cell Functions and Differentiation	19
1.6.1 Neutrophils	21
1.6.2 Fibroblasts	21
1.6.3 Neural cells	22
1.6.4 Myoblasts	23
1.6.5 Stem Cells	24

	1.6.6 Cancer Cells	26
	1.6.7 Other Cell Types	27
	1.7 Conclusion	29
	1.8 References	30
Chapter 2	Mesenchymal Stem Cell Durotaxis Depends on Substrate Stiffness Gradient Strength	43
	2.1 Abstract	43
	2.2 Introduction	44
	2.3 Materials and Methods	46
	2.3.1 Polyacrylamide hydrogels	46
	2.3.2 Fabrication of Substrates with Step Gradients	47
	2.3.3 Fabrication of Substrates with Pathological Stiffness Gradients	47
	2.3.4 Fabrication of Substrates with Physiological Stiffness Gradients	49
	2.3.5 Material Stiffness and Surface Topography	50
	2.3.6 Cell Culture	50
	2.3.7 Immunofluorescent Staining and Imaging	50
	2.3.8 Traction Force Microscopy	51
	2.3.9 Statistical Analyses	52
	2.4 Results	52
	2.4.1 Fabrication of Hydrogels with Stiffness Gradients	52
	2.4.2 Directed Cell Migration on Stiffness Gradients	63
	2.4.3 Regulating Directed Cell Migration	67
	2.5 Discussion	70
	2.5.1 Challenges of Fabricating Reproducible Gradient Hydrogel Systems of Varying Gradient Strength and Stiffness Range	70
	2.5.2 The Origins of Durotaxis	72
	2.6 References	75
Chapter 3	The Alignment and Fusion Assembly of Adipose-Derived Stem Cells on Mechanically Patterned Matrices	80
	3.1 Abstract	80
	3.2 Introduction	81
	3.3 Materials and Methods	83
	3.3.1 Photolithography and Fabrication of Mechanically-Patterned Hydrogels	83
	3.3.2 Microcontact Printed (μ CP) Hydrogel	84
	3.3.3 Mechanical Force Spectroscopy Mapping by Atomic Force Microscopy	85
	3.3.4 Cell Isolation and Culture	85

	3.3.5 Immunocytochemistry, Fusion, and Function Blocking Assays	87
	3.3.6 Statistical Analysis	88
3.4	Results	88
	3.4.1 Mechanically-Patterned Hydrogel Fabrication and Characterization	88
	3.4.2 Cell adhesion and migration based on mechanical pattern	95
	3.4.3 Improved Cell Maturation on Mechanically Pat- terned Matrix	100
3.5	Discussion	105
	3.5.1 Matrix Considerations when Micropatterning . .	105
	3.5.2 Improving Cell Fate and Mimicking Disease . .	106
	3.5.3 Differential Cell Sorting for Organoid Culture .	107
3.6	Conclusions	108
3.7	References	109
Chapter 4	Modulating Cell Contractility by Elongated Stem Cell Mor- phology and Matrix Stiffness	114
	4.1 Introduction	114
	4.2 Methods	115
	4.2.1 Polyacrylamide Gels	115
	4.2.2 Microfabrication	116
	4.2.3 Protein Microcontact Printing	116
	4.2.4 Cell Culture	117
	4.2.5 Immunofluorescence Staining	117
	4.2.6 Atomic Force Microscopy	118
	4.2.7 Traction Force Microscopy	118
	4.3 Results	118
	4.4 Discussion	132
	4.5 Conclusion	134
	4.6 References	134
Chapter 5	Interplay of Matrix Stiffness and Protein Tethering in Stem Cell Differentiation	137
	5.1 Abstract	137
	5.2 Introduction	138
	5.3 Results	139
	5.3.1 Tuning Hydrogel Porosity Independent of Stiffness	139
	5.3.2 Differentiation and Deformations are Independent of Porosity	144
	5.3.3 Modulating Protein Tethering by Changing Linker Density	149

5.3.4	Differentiation and Deformations do not Depend on Tethering	155
5.3.5	Differentiation Occurs in the Absence of Tethering	161
5.3.6	Cell Spread Area on PA and PDMS Substrates .	166
5.3.7	Measuring PDMS Mechanical Properties on a Cell-Sensing Scale	168
5.3.8	PDMS Substrates do not Support Protein Tethering	173
5.4	Summary	178
5.5	Methods	179
5.5.1	Polyacrylamide Gels	179
5.5.2	PA-PEG-RGD Gels	180
5.5.3	PDMS Substrates	180
5.5.4	Scanning Electron Microscopy	180
5.5.5	DNA Gel Electrophoresis	181
5.5.6	Stem Cell Culture	181
5.5.7	Immunofluorescence	181
5.5.8	Differentiation Assays	182
5.5.9	Atomic Force Microscopy	182
5.5.10	Traction Force Microscopy	184
5.5.11	Förster Resonance Energy Transfer	184
5.5.12	Statistics	185
5.6	References	186
Chapter 6	Conclusions	190
6.1	Introduction	190
6.2	Mechanical Gradients in Migration: a Target for Therapy	190
6.3	Traction Forces: Where and When to Pull Next?	191
6.4	Matrix Biology in the Third Dimension: Where Will the Forces Take Us?	193
6.5	References	196

LIST OF FIGURES

Figure 1.1:	Techniques used to determine the Young's Modulus of Materials	5
Figure 1.2:	Range of Elastic Modulus of Polyacrylamide Gels	15
Figure 1.3:	Methods to Generate Stiffness Gradients	16
Figure 1.4:	Natural Variation in Tissue Elasticity and Resulting Cell Behavior	20
Figure 2.1:	Polyacrylamide Hydrogels with Stiffness Gradients	53
Figure 2.2:	Mechanical Characterization of Polyacrylamide on Polyacrylamide	56
Figure 2.3:	Stiffness Gradients Fabricated using Microfluidics	58
Figure 2.4:	Challenges of Polyacrylamide Polymerization in PDMS Microchannels	60
Figure 2.5:	Polyacrylamide Gradients Generated Using Photomasks . . .	62
Figure 2.6:	Protein Coating on Gradient Hydrogels	64
Figure 2.7:	MSC Migration of Gradient Hydrogels	66
Figure 2.8:	Inhibited MSC Migration and MSC Force Generation on Gradient Hydrogels	69
Figure 3.1:	Mechanically-Patterned Hydrogel Fabrication and Characterization	90
Figure 3.2:	Ligand composition does not affect cell adhesion to mechanically-patterned hydrogel	93
Figure 3.3:	Cell adhesion is independent of mechanically-patterned hydrogel pattern dimension	94
Figure 3.4:	Cell Adhesion Preference	96
Figure 3.5:	Attachment and Alignment of Cardiomyocytes on Neurogenic-Myogenic Mechanically-Patterned Hydrogel	97
Figure 3.6:	ASCs on Fibronectin-Microcontact Printing	99
Figure 3.7:	Myotube Formation is Independent of Mechanically-Patterned Hydrogel Pattern Size but is Improved by Cell Alignment . .	102
Figure 3.8:	Mechanically-Patterned Hydrogel Enhances Myotube Fusion of ASCs	103
Figure 4.1:	Protein Microcontact Printing	119
Figure 4.2:	Fibronectin Protein Islands	120
Figure 4.3:	MSCs on Protein Islands of Different Shape	122
Figure 4.4:	Heat Maps and Quantification of Vinculin from MSCs on Patterns	123
Figure 4.5:	Mathematical Determination of Gel Surface for TFM	126
Figure 4.6:	Patterned MSC Tangential Forces on a Deformed Substrate .	128
Figure 4.7:	Patterned MSC Normal Forces on a Deformed Substrate . . .	129

Figure 4.8: Tangential Strain Energy of MSCs on Patterns	131
Figure 5.1: Influence of Substrate Porosity on ASC Differentiation	141
Figure 5.2: Characterization of Porosity on PA Gels Made with Various Monomer to Crosslinker Ratios	143
Figure 5.3: Osteogenic and Adipogenic Differentiation as a Function of Porosity	145
Figure 5.4: Effect of Confluence on ASC Osteogenesis	146
Figure 5.5: Displacement Maps Computed from Traction Force Microscopy	148
Figure 5.6: Visualization of surface protein coating on PA substrates	151
Figure 5.7: Atomic Force Spectroscopy Validation	152
Figure 5.8: Influence of Protein Tethering on ASC Differentiation	153
Figure 5.9: Osteogenic Differentiation as a function of sulfo-SANPAH Con- centration	156
Figure 5.10: ASC Adipogenic Differentiation as a Function of sulfo-SANPAH Concentration	158
Figure 5.11: FRET Characterization of Dual-Labeled Fibronectin	160
Figure 5.12: Direct Incorporation of a Short Adhesive Peptide to the PA Substrate	163
Figure 5.13: MSC Osteogenic Differentiation on RGD Hydrogels	165
Figure 5.14: ASC Spread Area on Tether-less Hydrogel Systems	167
Figure 5.15: Mechanical Characterization of PDMS Substrates by Atomic Force Microscopy	169
Figure 5.16: Cells Sense by Contracting Against their Substrates	171
Figure 5.17: Atomic Force Spectrography Analysis of Ligand Coated PDMS Substrates	174
Figure 5.18: Diagram for the Covalent Attachment of Protein to the Surface of PDMS Substrates	176

LIST OF TABLES

Table 1.1: Studies on the effects of substrate compliance on cell behavior	28
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2. Wen J H*, **Vincent L G***, Choi Y S, Fuhrmann A, Hribar K, Taylor-Weiner H, Chen S, Engler A J. Interplay of Matrix Stiffness and Protein Tethering in Mechanically Based Differentiation. Nature Materials. 2014 (*In Press*)
*Equal contribution
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ABSTRACT OF THE DISSERTATION

Cell-matrix Interactions Regulate Adult Stem Cell Migration and Differentiation

by

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Doctor of Philosophy in Bioengineering

University of California, San Diego, 2014

Professor Adam J. Engler, Chair

Stem cell therapies have shown promise in the treatment of musculoskeletal diseases by integrating with host tissue, remodeling diseased environments, and recruiting other cells through paracrine signaling. However, clinical trials have yielded limited success in part because the role of the extracellular matrix (ECM) has been underappreciated. The physiochemical properties of this protein network, notably the density, composition, and degree of crosslinking, all influence the Young's modulus, i.e. 'stiffness,' of this matrix. Cells 'feel' changes in their local environment by contracting against this material, integrating physical cues that guide cell function. In this thesis, we aim to elucidate how cell-matrix mechanosensing guides stem cell migration and differentiation. Our studies have

employed synthetic hydrogels to decouple specific ECM properties, allowing us to study how each component independently regulates cell behavior. After fabricating hydrogels with mechanical gradients, we observed that stem cell migration velocity scaled with gradient strength over a given stiffness range as long as the cytoskeleton remained intact. This mechanical regulation of migration in normal and disease pathologies suggests that stem cells may better contribute to repairs in stiffer regions of tissues where they may preferentially accumulate. Mechanical step gradients provided a means to mechanically confine stem cells, which readily aligned and robustly underwent myogenic differentiation and fused, and could serve as a synthetic platform for micro tissue engineering constructs. Subsequent studies focused on cell-sensing in geometrically constrained environments. Tuning cell shape revealed that focal adhesion formation and cell-generated traction stresses follow opposing trends over a range of stiffness. Our contractility-based observations suggest that differentiation may be possible in non-permissive environments and could prove beneficial to treat fibrotic diseases. Further investigation on a microscopic scale of how cells interact with synthetic materials *in vitro* revealed that the substrate stiffness of our systems, and not the attached cell-adhesive ligands nor their configuration, was the most important factor to determine differentiation of stem cells. Taken together, these data imply that the mechanics of the environment drive biased cell migration and differentiation and that stem cell clinical trials may have higher chances of success if ECM mechanics are factored into the therapy design.

Chapter 1

Effect of Substrate Modulus on Cell Function and Differentiation

1.1 Synopsis

During development, tissue-specific stiffness arises from changes in the extracellular environment, notably the density, composition, and crosslinking of the protein network that surrounds cells called the extracellular matrix. These parameters all influence the Young's modulus, i.e. 'stiffness,' of this matrix, and cells 'feel' these changes by contracting against the material. Importantly, mechanosensitive proteins in the cell cause it to respond and adapt to matrix stiffness affecting important cell processes and functions. Here we provide a succinct review of how one measures and manipulates stiffness in both biological and synthetic materials. We also provide a description of how different types of cells respond to stiffness, e.g. stem cells can differentiate into a specific cell type when matrix stiffness closely mimics the appropriate physiological stiffness for that lineage. We conclude by giving some perspective on the how the stiffness-related findings from tradition 2D cultures are now applied to more physiologically appropriate, 3D materials.

1.2 Introduction

A cell's surrounding environment is composed of a variety of different components: adjacent cells to which they adhere via cadherins proteins, interstitial fluid containing soluble growth factors, and extracellular matrix (ECM), a 3-dimension (3D) protein scaffold to which cell adhere via heterodimers of α and β integrins. While ECM comes in many different forms and can be composed of many different proteins, cells can use the ECM to actively 'feel' their surroundings by pulling against it to transmit forces to adjacent cells. Neighboring cells can then use a series of mechanosensitive pathways to sense and respond to these forces via mechanical crosstalk. The nature of this communication between cells and their environment is regulated by several important properties of the ECM. One notable observation over the last decade has been that the ECM's Young's modulus, which is commonly referred to as 'stiffness' in biological literature, can direct cell response and drive specific function. Studies examining how ECM properties affect cells often have employed synthetic hydrogels, which serve as an underlying substrate where one can decouple many of the chemical and physical properties that native ECM cannot. By studying each component independently, one can determine how a specific ECM property, e.g. stiffness, composition, etc., regulates cell behaviors, e.g. spreading, migration, and differentiation, over a range of physiological conditions. Here we first outline the techniques and materials used to modulate substrate modulus before describing how changes in modulus affect cell function and differentiation in widely studied 2-dimensional (2D) culture systems. In a concluding discussion, we contrast behavioral differences on 2D and 3D cultures.

1.3 Young's Modulus

1.3.1 Definition and Measurement

Young's modulus (abbreviated as E) is an intrinsic material parameter that describes the relationship between the unitless percent elongation of a material,

i.e. strain (ϵ), and the force applied per area, i.e. stress (σ). Young's modulus can often be described by the linear relationship $E = \epsilon/\sigma$, especially at low strain and is measured in units of Pascal (Pa) or Newton per meter squared. Materials that are palpably stiffer will exhibit higher elastic moduli compared to softer materials. For biological tissues, most range between the two extremes of bone and brain: the modulus of calcified bone is on the order of ~ 5 GPa [1], though bone cells live within the non-mineralized 'osteoid,' while that of brain tissue is on the order of 1 kPa [2]. Such numbers are determined from small ($< 20\%$) strain measurements where many tissue and biomaterials exhibit a constant modulus [3]. At larger deformations, these materials tend to stiffen in a process called strain stiffening as materials begin to irreversibly deform, i.e. fibrils within ECM permanently rearrange causing a non-linear modulus [4, 5]. To account for this behavior, numerous non-linear models have been developed [4], but for cells in general, the strains generated do not exceed the 20% threshold [5]. Thus our discussion here will be limited to techniques that determine Young's moduli at small strains.

The modulus of materials is most easily determined by applying a small force to a sample and measuring the resulting strain. Since many biological tissue and cells experience smaller forces on the order of nano- or pico-Newtons, advanced methods have been developed to characterize the mechanics of cells and their surrounding tissue. Three of these techniques, microsphere indentation, microindenters, and atomic force microscopy (AFM), are summarized below. While we focus on micro- and nano-indentation methods here due to the length scale of cells and the magnitude of forces that they generate, it is important to note that there is a correlation between bulk mechanical properties and microscopic properties for many standard materials, e.g. polyacrylamide [6]. However, there are important deviations from this correlation, such as with poly(vinyl pyrrolidone) hydrogels [4, 7]. In subsequent sections, we will extend our description of these techniques to the materials of interest, e.g. synthetic hydrogels and natural materials where their mechanical properties depend on chemical composition and material structure.

1.3.2 Microspheres Under Gravitational Forces

Microsphere indentation determines a material's Young's modulus by measuring the indentation depth of a metal microsphere on top of a substrate and comparing it to the gravitational force of the sphere [8] (Fig. 1.1a). Beads typically a few hundred nanometers in diameter are confined in the substrate and images of their positions before and after microspheres are placed on the substrate enable one to compute indentation depth or displacement. The Young's modulus is then calculated from the sphere indentation model developed by Hertz (Eqn 1.1) [9]:

$$\frac{3(1 - \nu^2)\mathbf{f}^2}{4\delta^{3/2}\mathbf{r}^{1/2}} \quad (1.1)$$

where ν is the Poisson's ratio, $\mathbf{f}$ is the buoyancy-corrected weight, δ is the depth of the indentation created by the ball, and $\mathbf{r}$ is the radius of the ball. This simple technique works with a high degree of reproducibility for $E \sim 2.2\text{--}33$ kPa on both polyacrylamide and gelatin substrates where indentation is small but measurable [10]. However determining Young's modulus of relatively thin (<100 μm) or exceedingly soft substrates, which are optimal for microscopy, requires a correction factor as the vertical displacement of the beads is affected by the underlying glass substrate. Moreover, this measurement technique requires confocal microscopy to accurately determine 3D bead positions, and with moderately small displacements and beads, it can be prone to overestimate moduli relative to more sensitive techniques, e.g. atomic force microscopy (AFM) or microindenters. This technique also applies a single force determined by ball density and volume and thus cannot determine if the magnitude of indentation falls within the small strain linear range of the material or not. Despite such drawbacks, this measurement method is relatively straightforward and easy to perform.

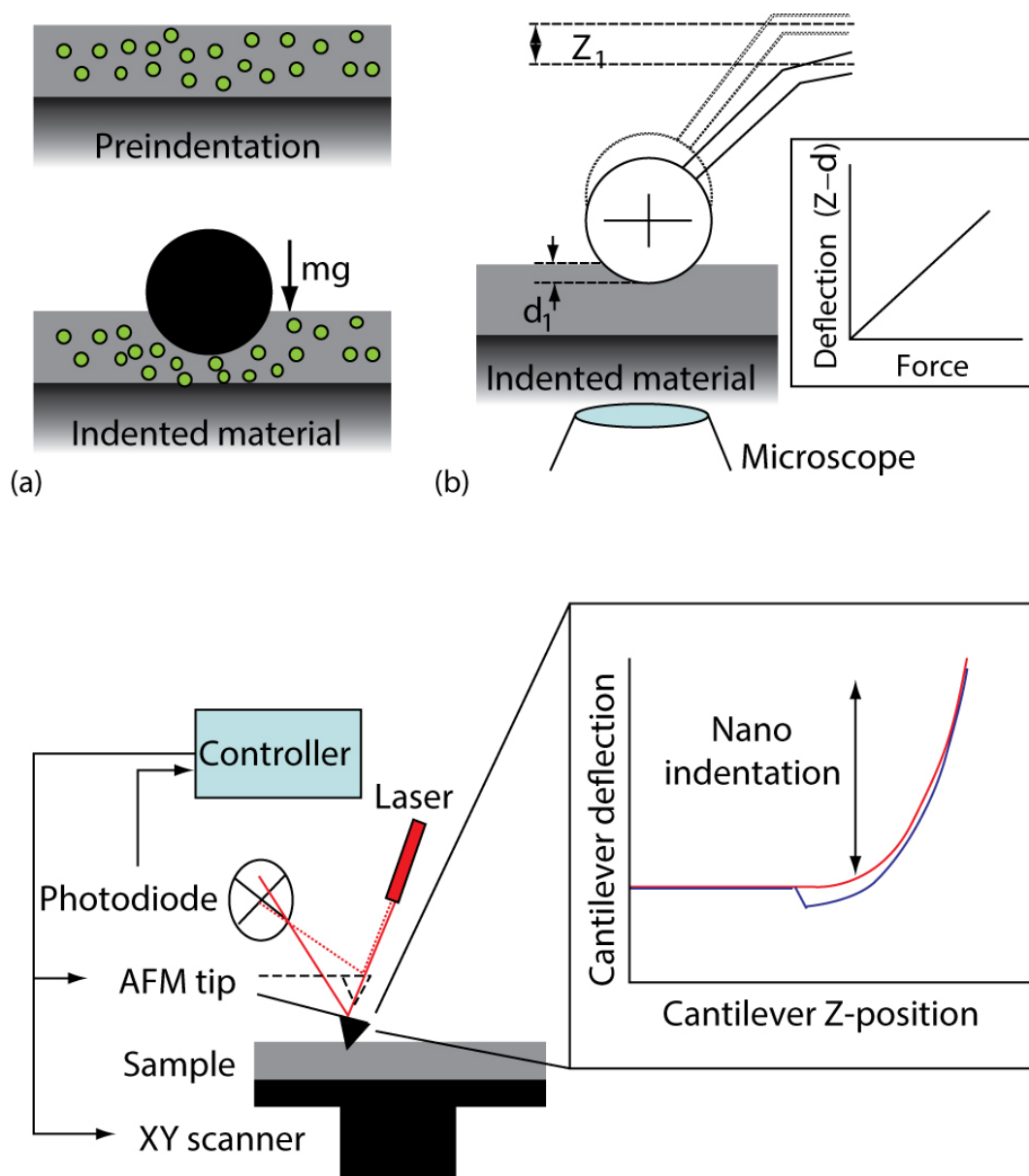


Figure 1.1: Techniques used to determine the Young's Modulus of Materials. Schematic representation of the experimental setup for microspheres (a), microindenters (b), and atomic force microscopy (c) to measure the Young's modulus of substrates.

1.3.3 Microindenters

Microindenters, originally developed to measure the Young's modulus of very stiff materials [11], have recently been adapted to measure the Young's modulus of soft materials with biologically relevant stiffness on the range of kilo- and mega-Pascals [12]. Microindentation for soft materials typically uses a spherical tip with a radius of several microns that is attached to the end of flexible microneedle to probe the substrate (Fig. 1.1b). Deformation of the tip is measured by optical microscopy and the resulting displacement compared to a standard curve to obtain the modulus as determined by the Hertz model [9]. By tuning the stiffness of the indenter arm, varying ranges of substrate moduli can be measured. As such, microindentation measurements of soft hydrated materials such as polyacrylamide substrates have been shown to correlate well with AFM measurements over the range ~ 2.5 -40 kPa [13]. As with the microsphere technique, microindenters yield accurate moduli measurements when strains are kept small and when sample thickness is in the range of ~ 100 -150 μm . This technique relies on visual determination of tip position, and thus error from inaccurate vertical measurements can dramatically affect the modulus determined from the experiment. However, microindentation is performed on a microscope stage, and therefore offers an economical measuring technique for tissues over a wide range of sample geometries.

1.3.4 Atomic Force Microscopy

AFM was developed for imaging applications at microscale resolution but has been extensively used to assess the mechanical properties of hydrated substrates [14-17]. This technique uses a pre-calibrated flexible cantilever with a pyramidal or spherical tip mounted on a piezoelectric device to indent the sample through a controlled amount of tip deflection (Fig. 1.1c). A laser reflects off the backside of the tip, which is typically fabricated out of silicon nitride with a reflective gold coating on the top, onto a position-sensitive photodetector that measures the tip deflection during indentation. Since the tip behaves similarly to a Hookean spring, the indentation depth can be converted to force (f) using the tip spring constant, much like for microindenters. By separately comparing tip

deflection when pressing on a hard material (where deflection scales perfectly to the piezomotor position), e.g. glass, to the same result when pressing into a soft material, it is possible to determine the actual indentation (δ) into the material. Using indentation models of a pyramid/cone developed by Sneddon (Eqn 1.2) [18] or a sphere developed by Hertz (Eqn 1.1), it is possible to determine the Young's modulus, E :

$$f = \left[\frac{2 \tan(\alpha)}{\pi} \right] \left[\frac{E}{1 - \nu^2} \delta^2 \right] \quad (1.2)$$

where α is the half opening angle of the pyramidal or conical tip. A thin film correction can be introduced to accurately determine the Young's modulus of substrates for sufficiently thin samples where the underlying support could dominate the mechanics absent this correction [19]. While the degree of correction is material-specific, for near linearly elastic materials, e.g. polyacrylamide gels, the correction enables one to measure Young's moduli of a sample independent of its thickness provided that sample thickness is a known quantity. For polyacrylamide substrates, the correction has been shown to be necessary for gels below 20 μm thick independent of the material's actual bulk stiffness [16]. With these advantages, AFM is a preferred measurement technique over the other indentation methods to perform repeated measurements over small sample areas with high accuracy. However, instrument cost, limited longevity of cantilever tips, and adhesive interactions between the tip and the gel may make AFM difficult to implement for the average user [20].

1.3.5 Additional Considerations for Measurement Techniques

While many synthetic materials used in biological applications are hydrogels that are nearly linearly elastic, natural ECM is often not. Thus it is important to note that materials are often anisotropic, meaning that their Young's modulus for the measured axis of indentation may not be identical to another axis of the material. In such instances, one must not rely solely on one indentation method but

instead employ measurement techniques that probe different mechanical modes, e.g. rheology. Though we will not go into great detail, rheology measures a shear modulus as it applies a force parallel to the top surface of a material rather than perpendicular as with the indentation techniques previously described. By converting shear to Young's moduli, direct comparison from multiple force modes is possible. So far, comparisons of AFM- and rheology-determined Young's moduli have shown good agreement for hydrogels [21].

1.4 Development, Maintenance, and Variation of Young's Modulus in Biology

Cell and tissue modulus changes drastically during development and establishes the wide variation of elasticity in the body. Before producing materials on which to culture cells, it is important to develop an understanding for the physiological context in which the cells of interest live, from development through ageing, and ultimately disease.

1.4.1 Development and Maintenance of Tissue Stiffness

In early development, the embryo is a conglomerate of cells with little architecture and is very compliant, i.e. soft [22, 23]. Further development into the three germ layers that comprise all tissues requires cell migration into the appropriate spatial configuration [22, 24]. The ability for these cells to self-segregate into cells of similar type has been shown to depend on expression of particular markers such as E-cadherin and β -catenin involved in cell adhesion [25], which has led to the development of the idea of 'tissue affinity' or topobiology as coined by Edelman and colleagues [26]. However, cell sorting *in vivo* has also been attributed to the mechanics of the ECM [24, 27]. For example, the stiffness of mesoderm dorsal tissue is orders of magnitude stiffer than endoderm in early amphibian embryos [28]. Additionally, amphibian embryos show a ~ 2 fold increase in stiffness during gastrulation [22] that is specific to the germ layer in question as shown in zebrafish

[29]. Young’s modulus may therefore be a driving force in the development of tissues at the embryonic stage and not simply a consequence of development. Yet once fully developed, biological tissues exhibit tremendous stiffness variation. Appreciating this variation and designing materials to meet the needs of the cells of interest is critical for therapeutic applications and will be discussed further later. However, it is easy to appreciate this variation by considering the two extremes of bone and brain again: the bulk modulus of calcified bone is on the order of ~ 5 GPa [1] while that of brain tissue is on the order of 1 kPa or less [2]. While this variation is six orders of magnitude, it may represent a common misconception; the actual environment that osteoblasts inhabit is actually more representative of a highly crosslinked, collagenous environment with a modulus of 30-40 kPa [30]. Osteoblasts do not typically inhabit calcified regions of bone [31]. Nevertheless, it is absolutely critical to appropriately mimic one’s *in vivo* surrounding *in vitro* (Fig. 1.4a) to obtain meaning cell behavior in culture.

1.4.2 Ageing and Disease

It should also be noted that the modulus of many tissues changes naturally with time and after the onset of certain diseases. For example, as we age our skin becomes less elastic [32], and blood vessels [33] and articular cartilage [34] become progressively stiffer. At the root of these mechanical changes are protein level changes in ECM composition and crosslinking of adjacent matrix fibers. However, abnormal variations in modulus can also play a role in the development of many diseases [33, 35]. Arthritis patients exhibit cartilage softening from progressive breakdown of the cartilage collagen meshwork [34] whereas patients with liver disease or myocardial infarction see enhanced stiffness due to the onset of fibrosis [35, 36], i.e. the abnormal deposition of ECM. These changes impact the function and behavior of cells, owing to the constant crosstalk between cells and their microenvironment and result in disease progression. With myocardial infarction, stiffening of the myocardial wall is accompanied by cell apoptosis, wall thinning, and a decrease in cardiac output as measured by ejection fraction [36].

1.5 Fabrication of Substrates with Defined Young's Modulus

To mimic the properties mentioned and measured above, we will profile a variety of natural and hydrogel-based supports that are often used in cell culture. For the former, close biomimicry provides a distinct advantage over all other materials. On the other hand, the latter provides materials with high water content that closely mimic physiological environments while at the same time allowing one to decouple variables, e.g. ECM chemistry and mechanics, and observe how one matrix property in particular may influence cell behavior. Mechanical properties of both natural and synthetic matrices are based on a) pore size, b) polymer concentration, c) polymer molecular weight, and d) the degree of crosslinking among other parameters and can be varied by changing any one or a number of these factors. Below we offer material-specific discussions of how Young's Modulus is changed using these parameters starting with natural matrices and concluding with synthetic ones.

1.5.1 Natural ECM Components

With the complexity of the natural cellular environment, we will focus on popular examples of several classes of biopolymers: collagen — representing the class of large network-forming matrix proteins including fibronectin and laminin [37], hyaluronic acid and alginate — representing the large carbohydrate polymers called glycosaminoglycans [38], and Matrigel — a commercially available reconstituted basement membrane product. Though these matrices have shown particularly interesting results in how they regulate cell behavior, our discussion here will be limited to how these materials can be controlled to display a range of stiffness for cells.

Collagen

Fibrillar collagen is the most abundant ECM protein and comprises a large portion of our connective tissue. There are a number of readily available sources,

e.g. rat tail, where collagen can be easily isolated [37]. By simply adjusting the pH of the solution [39] or collagen density [37], polymerized matrices can be produced in the presence of cells (as a 3D matrix) or they can subsequently be added to the culture to provide a flat culture substrate (using collagen as a 2D coating). Given the relative ease with which one can produce these gels and the flexibility one has when using it, collagen has become a widely used cell scaffold. However, there are important mechanical considerations when using collagen. Altering collagen polymerization conditions or pre-treating with pepsin can alter the pore size in the final product [40]. Changing the collagen concentration from ~ 0.1 -3 mg/mL will also increase material modulus [37, 41], and these changes can also be presented in concentration gradients that also yield stiffness gradients [42]. Yet collagen gels have inherently coupled collagen density and stiffness: the more collagen ligand present, the stiffer the gel. Non-adhesive 'stiffening agents' can be introduced to try to decouple these components [43], but as with many 3D materials, smaller pores and ligand accessibility may alter cell adhesion and overall behavior in 3D [44, 45], making a link between stiffness and cell behavior more challenging. That said a number of applications of 2D and 3D collagen gels have been realized. Collagen has long been used as a surface coating, and can dictate heart fibroblast behavior in 2D [46]. Collagen gels also have been engineered into sheet-based 3D models to study cell behavior [47] and compressed around cells to provide high-stiffness 3D environments similar to native tissue [48]. Overall, collagen gels exhibit good cytocompatibility, but given their mechanochemical coupling, they also possess drawbacks compared to more tunable synthetic systems, e.g. polyacrylamide, when examining *in vitro* cell characteristics [49].

Hyaluronic Acid

Hyaluronic acid (HA) is a negatively charged linear polysaccharide made up of D-glucuronic and D-N-acetylglucosamine commonly found in the ECM and connective tissue. Unique physical and chemical properties make HA an attractive biological substrate, but due to suboptimal mechanical properties and rapid degradation, chemical modification of the HA backbone is often performed to in-

duce hydrogel formation and to build in specific functionality. For example, HA hydrogels can be formed by modifying the HA backbone and adding in crosslinkers, e.g. thiolation to form disulfide bonds [50] or addition of polyethylene glycol (PEG)-diacrylate to the thiols [51], or via direct conjugation of synthetic polymers, e.g. polyethylene glycol (PEG), poly(L-lysine) acid, poly(*N*-isopropylacrylamide), etc. [52]. Copolymerization to form an interpenetrating network using collagen, alginate, or gelatin also yields stiffer hydrogels [53, 54]. Most single chemical modifications provide gels that are best suited to mimic the mechanics of soft tissues ~ 10 -3500 Pa, such as a brain [53, 55]. HA can also readily undergo multiple, sequential modifications, such as being methacrylated for dithiol crosslinking via Michael-type addition and then undergoing radical polymerization via the free methacrylated sites (similar to photolithography techniques outlined in Fig. 1.3b*ii*). This process enables multiple crosslinking steps to provide maximal flexibility and produce moduli ranging from 1-100 kPa [56]. As a natural polymer, HA is often used as an injectable material, and it has been shown to maintain left ventricle wall thickness during the normal remodeling process post-myocardial infarction [57]. While it is not certain if HA restores normal ventricle mechanical properties altered by the infarct [36], it certainly implies that HA could be a promising natural material for regenerative use.

Alginate

Alginate is a derivative of sea algae that is often used for drug delivery, to encapsulate cells, or as a cell culture support [58]. To form a hydrogel, mannuronic (M) and guluronic acids (G) are interspersed along the alginate backbone and divalent ions, e.g. Ca^{2+} , crosslink alginate monomers into a reversible hydrogel. The M/G ratio has been shown to regulate how tightly crosslinked the hydrogel can become: gels with a low M/G ratio swell less than gels with a higher M/G ratio and thus yield stiffer hydrogels [59]. Alginate hydrogel stiffness can also be modulated by alginate concentration and chemical composition as well as the cationic species used. Increasing alginate and divalent ion concentration allows for higher crosslink density, yielding more rigid gels with moduli ranging from ~ 1 -120

kPa [60, 61]. Use of these gels in cell culture, however, reduces their stiffness up to 10-fold as the Na^+ and Ca^{2+} present in culture media can interfere with crosslinks [62], but this can be overcome in part by using high molecular weight alginate and introducing covalent crosslinks [63]. Gels composed of alginate derivatives such as poly(aldehyde guluronate) have been reported to achieve compressive modulus up to 1 MPa [64].

Matrigel

Matrigel is a preparation of solubilized basement membrane proteins extracted from mouse sarcoma [65], which typically contains varying amounts of laminin, collagen IV, heparan sulfate proteoglycans, entactin, and nidogen. To vary the mechanical properties of the material as with collagen, one must change the Matrigel concentration and stiffness has been reported to range from 10 to 50 Pa depending on concentration [66]. Co-polymerization with type I collagen as an inner penetrating polymer network can further increase the composite's Young's modulus but again over a limited range [67, 68]. The lack of stiffness variation is an important consideration but Matrigel is often used as a ligand coating or supporting matrix in conjunction with a synthetic material that independently varies stiffness, e.g. mammary acinar cultures with Matrigel and polyacrylamide hydrogels [69]. In this capacity, it is more often used in studies that want to ensure that the correct matrix composition is present, e.g. during self-renewal of human embryonic stem cells pluripotency in tissue culture [70]. Matrigel is not without its distinct advantages though; it is easily able to be cast into channels and shapes to pose questions about mechanics in pseudo-3D or 3D environments [71] without requiring functionalization.

1.5.2 Synthetic Hydrogels

Polyacrylamide

Polyacrylamide (PA) gels are inert, synthetic hydrogels most commonly used for protein separation in gel electrophoresis. Since PA gels are easy to fab-

ricate, have widely tunable mechanical properties, and are easily functionalized with adhesive ligands, these substrates are also well suited to study the effects of substrate modulus on a variety of cellular functions detailed in the next section. Polymerization of PA hydrogels occurs through a free radical-driven reaction where acrylamide and bis-acrylamide solutions in varying concentrations are mixed with a free radical source, most commonly ammonium persulfate [72]. The mechanical properties of the synthesized gels vary from ~ 0.1 -100 kPa (Fig. 1.2) [16, 73], where moduli values are based on common principles of condensed matter physics [74, 75]; higher concentrations of acrylamide monomer yield stiffer gels (insets *iii* and *iv*) due to increased chain entanglement. Increasing crosslinker concentration, on the other hand, generates more physical tethers between the polymer backbone, thereby reducing individual chain flexibility and increasing the Young's modulus of the material (insets *ii* and *iv*). Uniform distribution of the free radical source or even mixing of the monomer and crosslinker generate static gels [7], which are most commonly used for cell culture as they generate a roughly even cell response, making them suitable for many biochemical assays. However, PA gels can also be micropatterned with step or smooth gradients of Young's modulus made by polymerizing adjacent gels of different moduli (Fig. 1.3a) [8, 76] or by photoactivation of the free radical initiator Irgacure 2959 via gradients of UV initiation (Fig. 1.3b*i*) [77, 78] or crosslinker concentration established in microchannels (Fig. 1.3b*ii*) [79], respectively. Ultimately, these gradient study a cell's ability to undergo durotaxis ('duro' being latin for hard), i.e. the characteristic movement of a cell along a stiffness gradient, and each of these techniques yield different gradient strengths, which has recently been shown to be important for cell behavior [80]. However, one of the main drawbacks to using polyacrylamide substrates is acrylamide and initiator cytotoxicity. Gels must often be soaked in buffer before use to allow unreacted species to diffuse out of the substrates, thus limiting these polymers to 2D *in vitro* studies.

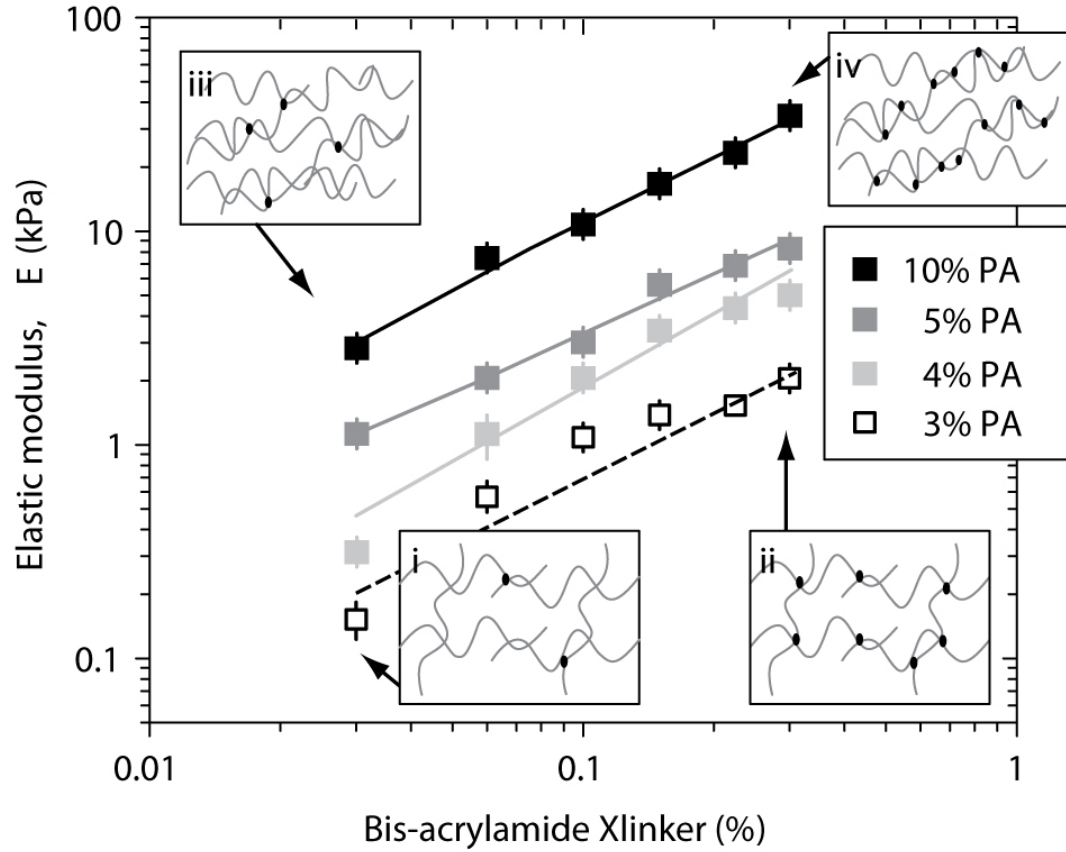


Figure 1.2: Range of Elastic Modulus of Polyacrylamide Gels. Elastic modulus of polyacrylamide substrates for gels with different concentrations of acrylamide monomer and bis-acrylamide crosslinker. Substrates polymerized in solutions with higher concentrations of acrylamide monomer yield stiffer gels (insets *iii* and *iv*) due to increased chain entanglement and non-specific interactions between polymer chains. Adding increasing amounts of crosslinker in the polymerization mixture generates more physical tethers between the backbone of polymers and increases the Young's modulus of the material (insets *ii* and *iv*).

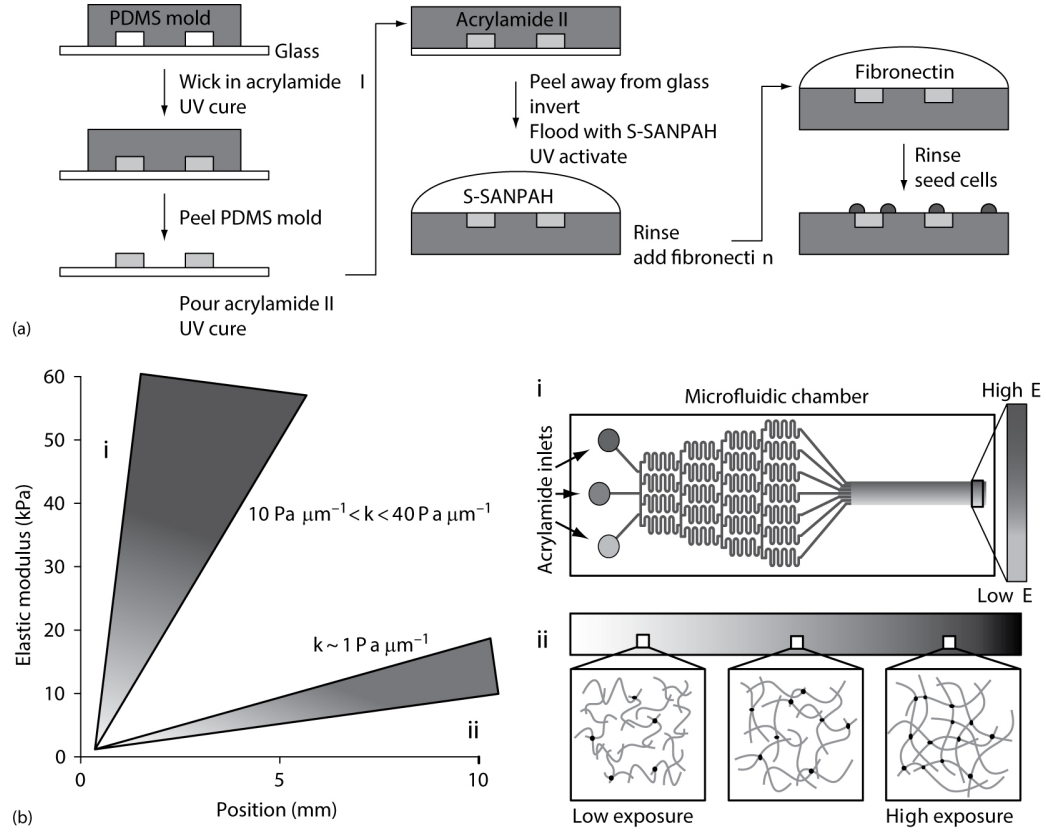


Figure 1.3: Methods to Generate Stiffness Gradients. Several photolithographic methods have been used in synthetic systems to generate spatial gradients of stiffness, including (a) using sequential polymerization to generate step gradients and (b) photoactivateable methods where gradients are formed either by spatially controlling solution exposure to UV (i) or by employing microfluidic mixing to have crosslinker gradients with uniform activation (ii). Each of the latter cases can yield moduli of different ranges, which is critical for cell sensing.

To facilitate cell attachment to the non-adhesive polyacrylamide, proteins are usually covalently attached to the PA gel surface. Incubation with N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH), a UV-activatable cross-linker [81], or hydrazine hydrate [82] allows for covalent tethering of ECM proteins such as fibronectin [83], collagen [84], or combinations of proteins [85] onto PA gels. In addition, spatial distribution of proteins has been achieved on PA gels using micropatterning [86, 87] and microcontact printing [85]. Varying the concentration of solubilized protein added to these synthetic matrices allows for more or less cell attachment sites, allowing investigators to control the adhesion strength of cells on these matrices [6].

Polydimethylsiloxane

Polydimethylsiloxane (PDMS) is a silicone elastomer most often used in microfluidic or lab-on-a-chip applications to form devices with defined microstructures. Many formulations of PDMS exist with Sylgard 184 being the most commonly used in biological-based research [88]. Fabrication of polymer substrates involves mixing of a 10:1 ratio of elastomer to curing agent, degassing, and baking for a few hours at 90°C. This polymerization process provides good strength, optical clarity, and ability to irreversibly bond glass, making PDMS an ideal substrate for studies that require both microscopy and defined physical gel features. Since the elastomer maintains its shape after curing, PDMS is often poured over micropatterned substrates fabricated using soft-photolithography to obtain gels with defined topographical features down to $\sim 10 \mu\text{m}$. By varying the ratio of the curing agent to the PDMS or baking time, substrates with a Young's modulus between ~ 12 -2500 kPa can be achieved [76, 89], making it a suitable system for more contractile cells where the microenvironment is often < 10 kPa [5]. Pouring the curing PDMS solution over micropatterned surfaces made by photolithography can also create useful surface topologies, such as with high-density pillar arrays where posts can be engineered to have an effectively lower apparent substrate stiffness of ~ 2 -130 kPa simply by changing the physical dimensions of the pillars on which the cells pull [90]. Deformable pillars are useful not only to generate compliant substrates

but also to quantify substrate deformation and traction forces exerted by crawling cells. Photolithography also has been used to impart spatially varying mechanical properties using PDMS master molds, e.g. step (Fig. 1.3a) [76] or smooth 1-D gradient (Fig. 1.3b*i*) [79] PA hydrogels have been made via micropatterned using PDMS microchannels to study durotaxis. As with PA hydrogels, the surface of PDMS gels is easily modified to absorb proteins to facilitate cell attachment [91]. While this system can generate polymer substrates with considerably higher substrate moduli than the other systems discussed, it is not well suited to fabricate gels within a small range of moduli.

Other Systems

A number of other synthetic systems have been used to investigate the role of stiffness on cellular function, each having a unique property not easily produced by the previously covered materials. Semi-interpenetrating polymer networks consisting of poly(*N*-isopropylacrylamide-*co*-acrylic acid) modified with synthetic peptides provide a different stiffness range and are more easily modified by cell adhesion ligand. In this context, they have been used to study the effects of matrix stiffness and ligand density of human embryonic stem cells (hESCs) [92]. Interpenetrating polymer networks purposefully mix polymers to create domains on one polymer within the other and photolithography can segregate solutions to yield domains [76], but incomplete free radical polymerization can also result in the formation of soft and stiff polymer domains within a material. For example, the nano-domains that form in bulk poly(vinyl pyrrolidone) hydrogels are due to nano-scale incomplete free radical polymerization [7], though it is not clear if cells can sense those differences on such a small length scale. Biosynthetic hydrogels, particularly composed of poly(ethylene glycol) (PEG), are now more readily in use due to their controlled chemical and mechanical properties not to mention their lack of cytotoxicity which enables them to be used to encapsulate cells in a 3D environment [45]. Purely synthetic PEG hydrogels also have the additional functionality of being able to undergo photoactivated degradation, effectively softening the hydrogel [93], and the addition of diacrylate groups provide spatially tunable

stiffening [94] as with HA hydrogels [56]. As with the biosynthetic hydrogels, PEG hydrogels can be polymerized into a 3D shape enabling encapsulation. However none of the systems highlighted above are without limitations. While some hydrogels provide better physical characteristics than others, careful consideration of each system and the specifics of the study should drive the choice of material.

1.6 Cell Functions and Differentiation

Though we have limited our discussion thus far to how matrix stiffness varies throughout the body (Fig. 1.4a) and how one can generate environments of varying mechanical properties, treatment of subject would be incomplete without discussing the impact of stiffness on various cell functions, e.g. migration [8], adhesion [95], morphology [73], and differentiation [30], and its interplay with extracellular matrix composition [85, 96]. While the molecular mechanism(s) behind how substrate compliance drives cellular function are still debated, it should become apparent that the functions previously mentioned are optimal in environments that most closely match the *in vivo* environment and suggest that potential sensing mechanisms must be tuned to such an optimum. While we provide a description of several cell types and tabulate additional cell types (Table 1.1), this is by no means an exhaustive list and other reviews on this area should also be consulted for cell types not covered [5, 14, 97-100].

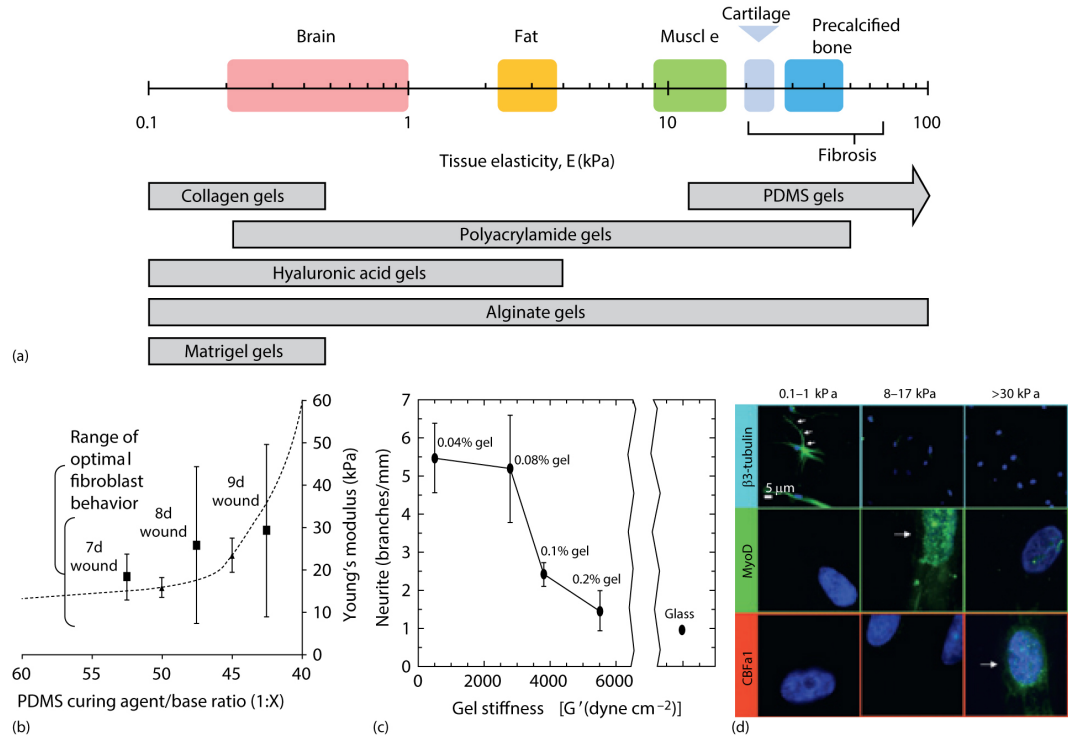


Figure 1.4: Natural Variation in Tissue Elasticity and Resulting Cell Behavior. (a) Schematic depicting the normal variation in elasticity of the indicated tissues and the hydrogel systems that span these ranges. (b) Stiffness of the wound environment of a fibroblast. (c) Neural cell behavior is optimal on matrices that have a stiffness that mimics brain. (d) Mesenchymal stem cell differentiation is specific to the stiffness of the original tissue.

1.6.1 Neutrophils

Neutrophils are short-lived phagocytic cells that circulate in the blood stream before becoming active and migrating through the vascular endothelium and surrounding matrix to the site of infection. Given that neutrophils must move through tissues of varying stiffness, they have a variety of atypical behaviors not present in cells that are adherent throughout their lives. Upon activation, neutrophils adhere to substrates and spread, even on soft hydrogels [95]. Though the degree of spreading is uncertain, investigations all have reported that as with normally adherent cells, neutrophils noticeably spread more on stiffer substrates [101, 102] and may even display optimal spreading [102]. Given the highly migratory state of these cells, cell migration is also a critical metric to follow. On soft substrates, neutrophils cannot generate sufficient traction forces, which hinders their ability to migrate while on stiff substrates, strong adhesion to the hydrogel prevents detachment and hence movement. Neutrophils thus exhibit a biphasic motility profile with peak velocities occurring on 4-7 kPa hydrogels depending on fibronectin coating density [102]. The shift in optimal stiffness may be explained by the ligand coating modulating adhesion strength, a trend also observed with smooth muscle cell spreading [6]. Since traction stress scales with hydrogel stiffness [101], it would appear that as with cardiomyocytes [103] there is an optimal amount of work done by adhesions to propel the neutrophil forward [102]. Though there is an optimal speed, the persistence time of migrating neutrophils is higher on substrates with increasing stiffness [102]. Thus regardless of net migration distance, the c of neutrophils is similar on substrates of varying moduli [102]. Given that these observations have been performed on static hydrogels and most tissue naturally varies within itself [101], gradient hydrogels provide an interesting system to resolve these components in the future.

1.6.2 Fibroblasts

Fibroblasts play an important role in wound healing, synthesizing and depositing extracellular matrix proteins such as collagen and fibronectin at the site of injury. To do so, they migrate through connective tissue to the site of injury in a

mesenchymal mode by spreading and assembling highly adhesive focal interactions with the ECM [104]; fibroblasts durotax towards stiffer matrix [8] and assemble focal adhesions in a highly stiffness-dependent process that regulates the tension generated within these maturing adhesions [105]. While fibroblasts can form actin microfilaments on substrates as soft as $\sim 3\text{-}4$ kPa [95] and remain spindle-shaped [73], they cannot maintain those behaviors on even softer hydrogels [106]. Fibroblasts exhibit drastically reduced proliferation on soft hydrogels and the cell itself is less stiff, presumably from its inability to assemble a cytoskeleton [106]. What results is optimal behavior on substrates that most closely mimic the *in vivo* wound environment (Fig. 1.4b) [105], where fibroblasts exhibit stronger traction forces than on softer hydrogels [107] and show an increase in fibroblast tyrosine phosphorylation [73]. These cells may therefore sense matrix rigidity through Src family kinases to activate downstream signaling effects [108] such as controlling the retrograde flux of actin filaments at focal adhesions in the lamella region [109]. As with other cell types, fibroblast traction forces are inversely correlated with cell migration speed [73, 106] to the point where on much stiffer >500 kPa substrates, migration favors not the typical protrusive model but rather a form of amoeboid migration [89]. However, such measurements were made using PDMS, which also showed markedly less spreading on stiffer substrates [89] than did PA hydrogels [8], further emphasizing the importance of material and ligand choice.

1.6.3 Neural cells

Neural cells, including glia, sensory neurons, dorsal root ganglia, motor neurons, and interneurons, make up a large portion of the human nervous system. Neurons are known for sending out cell projections, or neurites, to form networks of processes that extend large distances to innervate muscles [110]. In light of the soft nature of brain tissue, i.e. $\ll 1$ kPa [2, 112, 112] neurons had a significant 3-fold decrease in branch number, suggesting that neuronal morphology is acutely sensitive to substrate mechanical properties (Fig. 1.4c) [113]. Similar results were obtained with chick dorsal root ganglia cultured in 3D agarose hydrogels where the elongation rate of neurites was found to be decreased on hydrogels with increasing

stiffness [114]. Astrocyte and glial cell behavior was also found to depend on substrate mechanics, but in a way that opposes the behavior of neurons: astrocytes on soft PA hydrogels display small, round morphologies that lack stress fibers in comparison with highly spread morphologies on harder hydrogels. While neurons grow well in mixed cultures on laminin-coated soft hydrogels, astrocytes do not proliferate and exhibit reduced adhesion [111]. Even glial cells failed to proliferate and did not survive on soft, deformable substrates [113]. In contrast, astrocytes and glial cells overgrow post-mitotic neurons on stiffer hydrogels [111, 113]. These findings suggest that various neuronal cell types respond differentially to matrix stiffness: neurons prefer to live in soft matrices of 0.1-1 kPa while astrocytes and glial cells, like fibroblasts, appear to prefer stiffer matrices to spread and proliferate.

1.6.4 Myoblasts

Skeletal muscle formation involves the activation, proliferation, and differentiation of myoblasts into myocytes and subsequent fusion to form multinucleated myofibers [115]. While skeletal myoblast proliferation and differentiation have been shown to depend on surface ligand density [116], matrix mechanical properties play an important role in the higher order functions of muscle, e.g. striation and contraction. For example, C2C12 skeletal myoblasts, regardless of substrate stiffness, will fuse into myotubes after 48 hours in culture, but myosin striations were maximal on hydrogels that exhibited muscle-like stiffness, e.g. ~ 10 kPa [117, 118]. Multi-nucleated myotubes on soft (≤ 5 kPa) and stiff (≥ 23 kPa) hydrogels showed little to no striations after 4 weeks of culture [117]. Similar reports have indicated that myoblasts line could spread and proliferate but not form myotubes on ≤ 1 kPa hydrogels [60], and though subtle differences in behavior exist, they may be the result of different ligand type: fibronectin versus type I collagen. This again hints at the coupling of matrix chemistry and mechanics. Myoblasts seeded atop a layer of confluent myoblasts of the appropriate stiffness also differentiated into myotubes, forming myosin striations after 4 weeks in culture, but when plated on top of cells with suboptimal stiffness, striation was not apparent [117]. Together these data support the claim that hydrogels that mimic the stiffness of native tissue are opti-

mum for myotube differentiation. Freshly isolated primary myoblasts also exhibit a stiffness-dependent behavior, forming myotubes on 45 kPa hydrogels [60]. Given that these cells were allowed to form syncytially whereas normal muscle is aligned, studies using micropatterned collagen, where fusion and striation are tightly controlled by muscle-like stiffness, are perhaps more mimetic [117]. This implies that both mechanical and geometrical cues from the matrix are involved in myoblast function.

In addition to skeletal myocytes, cardiomyocytes have been studied significantly in terms of their stiffness sensitivity. Embryonic quail cardiomyocytes produce optimal work on their substrates and maintain rhythmic contraction [103] at matrix stiffness that mimics normal myocardium [36]. Neonatal rat ventricular myocytes were also found to generate the largest forces and beat most effectively as measured by large calcium transients present on muscle-like ~ 10 kPa PA substrates [119]. Rhythmic contraction of these cells seeded on a 3D PEG-containing fibrinogen matrix showed a dependence on substrate elasticity but for hydrogels that were only 25-150 Pa. Increasing fibrinogen concentration, and thus Young's modulus, drastically reduced contractile activity [120], but since ligand type and concentration significantly affects myoblast function [116], this difference in behavior may be attributed to the large difference in ligand presentation in addition to the differences in dimensionality.

1.6.5 Stem Cells

Stem cells are broad class of progenitor-type cells that have the ability to undergo self-renewal and can differentiate into many mature lineages when sufficiently stimulated to do so [121]. Adult bone-marrow-derived mesenchymal stem cells (MSCs) and embryonic stem cells (ESCs) are two commonly-studied stem cell types whose differentiation is typically controlled by soluble factors. Having not yet committed to a lineage, stem cells have the ultimate ability to respond to local matrix cues such as stiffness [122]. True progenitor cells, those that are not fully committed to a lineage but that are not stem cells, also express some ability to mature in response to stiffness. For the latter case, MC3T3-E1 pre-osteoblast

cells have increased growth rates on hydrogels of increasing stiffness with moduli ranging from 20-110 kPa, but exhibit optimal differentiation on substrates mimicking osteoid stiffness [30, 123]. This observation has been noted in other bone precursors [124] and in murine mesenchymal stem cells (D1). Similar behavior has been observed when cells are co-induced into an osteogenic lineage by growth factors [125]. MSCs plated on 34 kPa type I collagen- and fibronectin-coated PA hydrogels differentiated optimally down the osteogenic lineage as evident by the expression of a number of osteogenic markers (Fig. 1.4d, bottom) [30, 96].

MSCs have also been differentiated down myo-, neuro-, and adipogenic lineages by tuning substrate compliance. For instance, MSCs encapsulated in RGD-presenting alginate hydrogels differentiated down the adipogenic lineage best in 2.5-5 kPa hydrogels [44], in agreement with the mechanical properties of adipose tissue *in vivo* [126]. On softer 0.1-1 kPa type I collagen-coated hydrogels that mimic the mechanical properties of brain tissue [2, 112], MSC morphologically resembles branched neuron-like structures, expressing neuron-specific markers such as nestin, β 3 tubulin, and neurofilament light chain among others [30]. Similar neural induction has been obtained with rat adult neural-stem cells where cells preferentially differentiate into neurons on soft hydrogels (100-500 Pa) and into glial cells on hydrogels of intermediate stiffness (1-10 kPa) [127]. When plated on hydrogels of intermediate stiffness, MSCs expressed myoD and desmin, indicating myogenic commitment [30]. It is interesting to note that MSC myogenic differentiation was maximal on type I collagen-coated PA hydrogels where the stiffness was strikingly similar to the optimum stiffness for myoblast fusion and striation formation, indicating that early and late differentiation towards mature myofibrils occurs on matrices with nearly identical mechanical properties as those reported for muscle tissue [30, 117]. It is also important to note that all of these expression data are dependent on the ligand used for cell attachment; type I collagen and fibronectin permit stiffness-dependent differentiation but type IV collagen and laminin are not supportive, indicating again that the coupling of matrix-related cues may be more subtle than previously appreciated [96].

In contrast to MSCs, little work has been done to determine to what extent

ESC differentiation is stiffness dependent. Osteogenic differentiation of ESCs has been reported on PDMS substrates, but only with moduli ≥ 1 MPa [128]. While most of these moduli are supraphysiologically, these data at least suggest that expression of pluripotency and early differentiation genes may be modulated by substrate mechanics and certainly warrants further exploration.

1.6.6 Cancer Cells

The hallmarks of cancer include limitless replicative potential, sustained angiogenesis, loss of contact inhibition, and the ability form new tumor foci in foreign tissues [129]. Cancer progression has previously been linked to differential integrin expression [130], which in turn has been implicated in the maintenance of both the normal and transformed phenotype of mammary epithelial cells [131]. One factor that more recently has been proposed to contribute to the transformed phenotype is modification of the mechanical properties of the extracellular milieu, which can change how a cell 'feels' and reacts to this changing environment. Indeed, ECM stiffening has been implicated as a plausible prerequisite for carcinogenesis due both to the onset of fibrosis [132] and the more tactile sensation one gets by feeling the stiff lump associated with a tumor mass, most notably in breast cancer [133]. Numerous examples now have emerged in the literature; neuroblastomas cultured on hydrogels ≥ 1 kPa grew longer neurites than on soft hydrogels [134], in marked contrast to the previous section on neurons. However, these cells still retain compliance sensitivity despite their transformed state [111] unlike other soft tumors [133], illustrating the complexity of cancer.

In an effort to understand why cancers would prefer a stiffer environment, breast mammary epithelial cancers have been linked to increased tension and compressive forces as a result ECM stiffening, implicating both mechanotransduction and tensional homeostasis as regulators of cancer development [135]. Accordingly, ECM stiffening disrupts mammary epithelial organization and acini formation in 3D Matrigel-polyacrylamide hydrogels, forming gradients of stiffness in the affected stroma surrounding the tumor [133]. Since cancer cell motility is stiffness-sensitive [136] but often erratic [107], cells may possibly see these gradients as durotac-

tic cues for directed migration to new tumor sites. Again, migration is perhaps even more complex than these data may indicate as ligand density couples with this modulation; DU-145 human prostate carcinoma cells migrate fastest in 3D Matrigel gels with storage moduli $G' \sim 30$ Pa but on hydrogels with lower concentrations of ligand, maximal migration speeds were observed on softer hydrogels [66].

Increased cytoskeletal tension and extracellular signal-regulated kinase (ERK) activation due to the less compliant environment also influences cellular proliferation, and is a first step towards a cancerous phenotype. However, a partial normal phenotype can be restored in malignant mammary epithelial cells if the mechanosensing pathways, such as ERK activity and Rho signaling, are disrupted, which links cytoskeleton tension and focal adhesion assembly with mechanosignaling and repression of the malignant phenotype [133]. These studies suggest that there is perhaps an additional angle for cancer therapies: controlling the compliance of the environment or how cancer cells 'feel' their environment may be an avenue to prevent or delay cancer progression or even restore a normal phenotype.

1.6.7 Other Cell Types

Substrate compliance modulates behavior — adhesion, proliferation, migration, contraction, and extracellular matrix production — of many other cell types such smooth muscle cells (SMCs), chondrocytes, endothelial cells, and epithelial cells. While too numerous to describe in detail, we have summarized the findings of a number of studies involving matrix stiffness and these cell types in the following table (Table 1.1), and we would refer the reader to those works cited for additional information.

Table 1.1: Studies on the effects of substrate compliance on cell behavior.

Cell Type	Substrate	Modulus	Findings
Vascular SMCs	Polyacrylamide, type I collagen	15, 28 kPa and ~ 1 Pa/ μm gradient	Faster motility on intermediately stiff gels, preferential cell accumulation on stiff portion of gradient (durotaxis) [77]
Vascular SMCs	Polyacrylamide, type I collagen	1-80 kPa, 10-40 Pa/ μm	Cellular durotaxis increases on steeper stiffness gradients irrespective of absolute values of substrate elasticity [80]
SMCs	poly(L-lysine) and hyaluronan with 20-60 layers	40-300 kPa	SMC spreading generally increases with increasing elastic modulus [140]
Aortic SMCs	Polyacrylamide, type I collagen, (PLL/HA) ₂₀ / type I collagen	1-35 kPa (PA), 85 kPa (PLL/HA) ₂₀	SMC spreading increases with increasing elastic modulus according to a hyperbolic fit, biphasic dependence of spreading on collagen density [16]
Bovine chondrocytes	RGD-functionalized alginate	12-127 kPa	Adhesion scales with matrix stiffness, rounded morphology on soft substrates compared to flat with stress fibers on stiffer gels [61]
Bovine chondrocytes	Poly(ethylene glycol) dimethacrylate	34-1370 kPa	Type II collagen synthesis in observed intermediate stiffness gels (360 kPa) and glycosaminoglycans diffuse through gels with large equilibrium swelling ratios [141]
MC3T3-E1 osteoblasts	Polyacrylamide, type I collagen	11-40 kPa	Maximum cells speeds on gels of intermediate stiffness, higher proliferation rates and actin stress fiber formation on stiff substrates [84]
Bovine aortic endothelial cells	Polyacrylamide/RGD	0.5-33 kPa	MSD increases with gel stiffness for single cells, smaller increase for cell pairs. Cell pairs remain in contact on 5.5 kPa and dissociate on stiffer substrates [142]
Mouse aortic endothelial cells	collagen/PA/glass 'sandwich gel'	0.45, 14 kPa	Spindle morphology with more pseudopodial branches on soft gels than on stiff substrates [143]
Human umbilical vein endothelial cells (HUVECS)	Matrigel, copolymerization with collagen, polyacrylamide		Tube-like structures formed on soft substrata [68]
Human endothelial cells, HUVECS	Type I collagen gels	6-10 kPa	Compliant substrates support capillary morphogenesis but this effect is cell-type dependent within specific endothelial cell populations based on force generation capabilities [144]
MDCK epithelial cells	PDMS micropillars	1-100 kPa	Higher forces generated on stiffer gels, constant substrate deformation [90]

1.7 Conclusion

From the discussion here it is hopefully clear that there are various materials to test stiffness responses in cells, various means of measuring stiffness, and that cell types are affected by mechanical signals in different ways, ultimately changing behaviors including migration, spreading, and differentiation. Thus it is reasonable to conclude that substrate compliance may play an important role in embryonic development, angiogenesis, homeostasis, cancer, and disease progression. As a final consideration, it should be noted that our discussion was largely limited to 2D tissue culture results, and that while investigations performed in 2D are convenient to study cellular responses, they do not accurately mimic the 3D environment of tissues that surround cells *in vivo*. In fact, important differences exist between 2D and 3D studies; for instance, fibroblasts alignment is perpendicular to strain direction in 3D but parallel in 2D [137, 138] and human MSC morphology in 3D is independent of matrix stiffness unlike in 2D [44]. More striking is the observation that matrix stiffness has little effect on SMC proliferation in 3D, possibly due to the differential assembly of focal adhesions in 3D hydrogels compared to 2D hydrogels of comparable stiffness [45, 139]. Taken together, these data in nascent 3D systems [43-45, 122] strongly motivate the further refinement of mechanically tunable 3D models with similar flexibility to current 2D systems to gain a better perspective on the effect of substrate compliance on cellular functions. These new material models as well as a greater understanding of the molecular mechanics behind cellular mechanosensing will be instrumental in revolutionizing the current paradigms of cellular mechanobiology.

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Chapter 2

Mesenchymal Stem Cell Durotaxis Depends on Substrate Stiffness Gradient Strength

2.1 Abstract

Mesenchymal stem cells (MSCs) respond to niche elasticity, which varies between and within tissues. Stiffness gradients result from pathological conditions but also occur through normal variation, e.g. muscle. MSCs undergo directed migration even in response to shallow stiffness gradients before differentiating. More refined gradients of both stiffness range and strength are needed to better understand mechanical regulation of migration in normal and disease pathologies. We describe polyacrylamide stiffness gradient fabrication using three distinct systems that generate stiffness gradients of physiological ($1 \text{ Pa}/\mu\text{m}$), pathological ($10 \text{ Pa}/\mu\text{m}$), and step ($\geq 100 \text{ Pa}/\mu\text{m}$) strength spanning physiologically relevant stiffness for most soft tissue, i.e. 1-12 kPa. MSCs migrated to the stiffest region for each gradient. Time-lapse microscopy revealed that migration velocity scaled directly with gradient strength. Directed migration was reduced in the presence of the contractile agonist lysophosphatidic acid (LPA) and cytoskeletal-perturbing drugs nocodazole and cytochalasin; LPA- and nocodazole-treated cells remained

spread and protrusive, while cytochalasin-treated cells did not. Untreated and nocodazole-treated cells spread in a similar manner, but nocodazole-treated cells had greatly diminished traction forces. These data suggest that actin is required for migration whereas microtubules are required for directed migration. The data also imply that *in vivo*, MSCs may have a more significant contribution to repairs in stiffer regions where they may preferentially accumulate.

2.2 Introduction

In their native environment, cells are surrounded by extracellular matrix (ECM) which provides complex biochemical signals to cells as well as biophysical ones [1-3]. One example of a biophysical cue is the elastic modulus of the ECM, which varies dramatically between and within tissues [4, 5] (Fig. 2.1A). By deforming their surroundings through cell-generated forces, cells can 'feel' or sense this elastic modulus, often referred to as 'stiffness' in the biological literature (measured in Pascals, or Pa). In the past two decades, hydrogel systems have been developed to more closely approximate the native ECM stiffness [6] in order to investigate mechanically coupled cellular functions such as cell morphology, spreading, maturation, and differentiation [6-14]. The majority of work to date has focused on cell responses to substrates of uniform stiffness, but cells may encounter dynamic environments where stiffness varies spatially, either naturally within tissues or as the result of a pathological condition such as the fibrotic lesions that develop after a myocardial infarct [15].

Durotaxis, the directed migration of cells up a stiffness gradient, was originally observed in fibroblasts migrating across a soft-to-stiff interface of two juxtaposed polyacrylamide hydrogels [16]. While such sharp transitions clearly illustrate this behavior, most pathological conditions create gradients that are much less steep, e.g. myocardial infarction establishes gradients $\sim 8 \text{ kPa}/\mu\text{m}$ [15]. Indeed, preferential migration of vascular smooth muscle cells (VSMC) but not valvular interstitial cells has been documented on substrates ranging from ~ 2 to 40 kPa [17-19]. Both the range and the strength, or change in elastic modulus per unit

length, of these mechanical gradients varies between studies, thus making it unclear as to how each parameter contributes to durotaxis. While VSMC polarization was found to increase with increasing gradient strength, differences in the gradient range require producing mechanical gradients of varying strength while keeping the stiffness value within a defined range to better understand the durotactic process [20]. Here, we explore this issue by using a defined range of physiological relevance.

Mesenchymal stem cells (MSCs) are also very migratory as they must egress from bone marrow, migrate through tissue, and hone in on an injury site, but unlike VSMCs, they also differentiate in response to stimuli, making their behavior more complex and difficult to predict. To migrate *in vivo*, local fibrotic tissue may serve as a homing signal encourage cells to preferentially accumulate, and while chemical signals are commonly implicated, ECM stiffness gradients may serve as a guide to MSC migration to ensure that the right cells can differentiate in the right place as part of the healing process [8, 21-23]. Such durotactic homing does occur for MSCs as we have previously observed MSCs undergoing directed migration even in response to shallow, physiological ($1 \text{ Pa}/\mu\text{m}$) stiffness gradients. This process precedes differentiation [5], making its understanding more relevant to better address migration in therapeutic niches. Given the existence of stiffness variation between and within tissues, it remains to be seen whether MSC homing or durotaxis is influenced by the stiffness range or gradient strength. Such knowledge could help determine how stiffness gradients may be most effective at directing the therapeutic accumulation of MSCs.

Here we sought to understand the response of MSCs on hydrogels that mimic natural tissue stiffness variations ($1 \text{ Pa}/\mu\text{m}$), pathological conditions ($10 \text{ Pa}/\mu\text{m}$), and tissue interfaces that present step changes in stiffness ($>100 \text{ Pa}/\mu\text{m}$). By focusing on gradient ranges between 1-12 kPa, we can better determine how stiffness gradient strength dictates MSC migration versus differentiation. To this end, we first fabricated polyacrylamide (PA) hydrogels with gradients using three separate techniques (Fig. 2.1B), each suited to create a specific gradient strength. Photopolymerization through a photomask yields shallow gradients due to polymer diffusion over the time scale required for polymerization [5, 17]. Microfluidic

mixing chambers can create steeper gradients than photomasks, given that the input solutions and degree of mixing govern gradient strength [14, 24]. Two-step reverse cast polymerization techniques mirror previous juxtaposed polyacrylamide hydrogels [16] in such a way as to create reproducible, defined gradients. For example, Marklein and Burdick have recently created 500 μm -wide stripes of alternating stiffness in hyaluronic acid hydrogels [25] while Choi et al. developed 100 and 500 μm -wide stripes of alternating stiffness in PA hydrogels [26]. We cultured MSCs on these gradients to ask whether stiffness-directed migration is influenced by gradient strength, and we provide the first evidence for MSC durotaxis as a function of gradient strength over a specified physiological range. We also suggest a potential cytoskeletal mechanism that could regulate durotaxis but not necessarily migration in general.

2.3 Materials and Methods

2.3.1 Polyacrylamide hydrogels

Polyacrylamide (PA) hydrogels were prepared from acrylamide monomers and the crosslinker N,N'-methylene-bis-acrylamide (Fisher Scientific). PA stiffness gradients of approximately 1, 10, and 100 Pa per μm corresponding to physiological, pathological, and step gradients, respectively, were created using three distinct systems described below. To facilitate cell attachment, human plasma fibronectin was covalently attached to the hydrogel surface. Substrates were incubated in 0.2 mM sulfo-SANPAH (Pierce) in sterile 50 mM HEPES pH 8.5, treated with 4mW/cm² 350 nm UV light for 10 min, washed three times with HEPES, and incubated with 10 $\mu\text{g}/\text{mL}$ human fibronectin overnight at 37°C. Samples were stored in PBS at 4°C and UV sterilized prior to use. All chemicals were obtained from Sigma unless otherwise noted.

2.3.2 Fabrication of Substrates with Step Gradients

To create hydrogels with very steep stiffness gradients, we employed a two-step polymerization scheme recently developed by Choi and co-workers [26]. First, master Si wafers were patterned with 25 mm long by 100 μm wide by 20 μm high cuboids spaced 500 μm apart using soft photolithography like described above. SU-8 2015 was used instead of SU-8 2050, and the exposure, development, and pre- and post-bake times were adjusted according to manufacturer specification. To covalently attach substrates to glass, glass coverslips (Fisher) were cleaned of organics and oxidized by exposing both sides for 60 sec to UV/ozone (BioForce). Samples were immediately functionalized with 20 mM 3-(trimethoxysilyl)propyl methacrylate in ethanol, washed with ethanol, and dried. 20 μL of a polymer solution consisting of 4% acrylamide and 0.4% bis-acrylamide 1/100 volume of 10% ammonium persulfate (APS) and 1/1000 volume of N,N,N',N'-tetramethylethylenediamine (TEMED) was pipetted onto the wafer and covered with a methacrylated coverslip and the solution allowed to polymerize for 15 min. The hydrogel was released from the wafer and placed face down onto a 20 μL drop of polymer solution consisting of 3.2% acrylamide and 0.4% bis-acrylamide, 1/100 volume of 10% APS, and 1/1000 volume of TEMED on top of a dichlorodimethylsilane treated glass slide. The second solution was allowed to polymerize for 15 minutes before soaking the resulting inter-penetrating hydrogel network in DI (deionized) water. Many gradients were fabricated simultaneously from the same polymer solutions by using multiple master wafers to limit batch to batch variability. Two hydrogels from each polymerization batch were checked by atomic force microscopy to verify substrate mechanical properties.

2.3.3 Fabrication of Substrates with Pathological Stiffness Gradients

Graded photoactivation is of insufficient resolution to achieve pathological stiffness gradients. Using the microfluidic mixing device developed by Zaari and coworkers [18] and detailed by Byfield and coworkers [27], we created gradients

steep enough to mimic pathological stiffness variations [15]. Briefly, silicon wafers were cleaned with acetone, methanol, and ethanol prior to processing. Approximately 100 μm of SU-8 2050 negative photoresist (Microchem) was spin coated onto the wafer, prebaked at 65°C for 5 min and then 95°C for 20 min. The substrates were allowed to cool at room temperature before exposing with 300 mJ of 365 nm light through the transparency photomask designed in AutoCad depicting the microfluidic channels (CAD/Art Services). Exposure was performed on an MA-6 mask aligner (SUSS MicroTec). A postbake was performed for 1 min at 65°C followed 5 min at 95°C. The samples were once again allowed to cool to room temperature before submerging for 10 min in SU-8 developer to remove the uncrosslinked regions. Samples were washed with isopropanol and dried with ultrapure nitrogen. Feature dimensions were verified using a Dektak profilometer (Veeco). Master wafers were treated overnight with the fumes of (Tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies) to promote the subsequent polymer release. A 10:1 polydimethylsiloxane (PDMS) elastomer:curing agent solution (Sylgard 184, Dow Corning) was thoroughly mixed and degassed under vacuum for 1 hour before pouring over the masters, baked at 60°C for an hour in an oven, and then released from the wafer. The resulting channels were prepped, treated for 30 sec under UV ozone along with methacrylate functionalized glass slides, and immediately reversibly bonded together. The microfluidic channels were then further processed before use by selectively wicking in a 10% solution bis(3-Triethoxysilylpropyl)disulfide (SCA 985, Struktol) in acetone into the outlet portion of the channel for 30 sec as indicated. The solution was suctioned out the outlet channel with vacuum and the photoinitiator solution consisting of 10% 2,2-diethoxyacetophenone (acetophenone) in acetone was wicked three times at 30 sec intervals into the outlet channel to remove residual SCA 985. The acetone swells the PDMS and the water insoluble hydrophobic initiator binds to the elastomer surface. Finally, the mixture was suctioned out and the channels used within the hour. Polymer solutions consisting of 10% acrylamide and either 0.05% (low) or 0.5% (high) bis-acrylamide in DI water were injected with a syringe pump (KD Scientific) at 30 $\mu\text{L}/\text{min}$ into the three inlets in this order:

low-high-low. The solutions split and recombined and after reaching steady state in the outlet portion of the channel the flow was turned off and the polymerization initiated by turning on the UV transilluminator for 6 min located directly beneath the outlet portion of the microchannel. After polymerization, the PDMS was gently removed and the resulting hydrogel stuck to the methacrylated glass, 1.7 mm in width and ~ 2 cm in length, immediately immersed in DI water.

2.3.4 Fabrication of Substrates with Physiological Stiffness Gradients

A strategy for making physiological stiffness gradients was modified from Tse and Engler [28]. Photomasks decreasing in transparency from 100 to 30% were designed in Photoshop (Adobe) over different lengths and were printed on transparency sheets using a 600 DPI printer. A stock polymer solution consisting of 10% acrylamide and 0.1% bis-acrylamide in DI water was prepared, stored at 4°C, and used for all experiments. Small aliquots of the polymer solution were mixed with 2,2'-azobis(2-methylpropionamidine) dihydrochloride (azobis), a photoinitiator, to a final concentration of 0.5% w/v. 20 μ L of the polymer solution was sandwiched between 25 mm square methacrylate- and dichlorodimethylsilane-treated slides (the former for attachment and the latter for release). The glass-polymer-glass sandwich was aligned on top of the photomasks and the whole apparatus placed on the surface of a benchtop UV transilluminator equipped with 1 mW/cm² 305 nm tubes. A cutout was placed around and below the photomask to prevent stray light from influencing polymerization. After polymerization for 6 min, the substrate was removed and immediately immersed in water to remove unreacted species. Samples were created from the same polymer solution mixture, and two hydrogels from each polymerization batch were checked by atomic force microscopy to verify consistent substrate mechanical properties.

2.3.5 Material Stiffness and Surface Topography

Force-mode Atomic Force Microscope (AFM) was performed to determine the mechanical properties of the various hydrogels. Samples were mounted on glass slides using vacuum grease and then on the AFM stage (3DBio; Asylum Research). Samples were indented 300 nm using gold-coated, pyramid-shape SiN cantilevers (TR400PB; Olympus) with ~ 25 pN/nm nominal spring constants as determined from indentations on a silicon surface and thermal calibration. Measurements were taken every 500-1000 μm , 250-500 μm , and 10-50 μm for stiffness gradients of 1, 10, and ≥ 100 Pa/ μm , respectively. Force curves were analyzed in Igor using a linearized Hertz model to determine the Young's modulus [29]. For 100 Pa/ μm gradients, the soft-stiff transition topography and modulus were obtained with 90x90 μm force maps.

2.3.6 Cell Culture

Mesenchymal stem cells (MSCs, Lonza Walkersville) were cultured in low glucose DMEM supplemented 10% fetal bovine serum and 1% Penn/Strep (Gibco). For culture maintenance, media was changed every three days and cells passaged before reaching confluence to maintain multipotency. Stem cells between passages 4-8 were used for all experiments, seeded at 5×10^3 to 2.5×10^4 cells/ml and media changed every 2-3 days. For inhibitor studies, lysophosphatidic acid (LPA, Enzo Life Sciences), nocodazole and cytochalasin D were used at a final concentration of 20 μM , 0.5 μM , and 2 μM , respectively. Inhibitors were dissolved in DMSO, stored at -20° and the final concentration of DMSO in the media did not exceed 0.1% v/v.

2.3.7 Immunofluorescent Staining and Imaging

Cells were fixed with 10% formalin for 15 min at room temperature. Actin cytoskeleton was stained with 1:500 rhodamine phalloidin (Invitrogen, Carlsbad, CA) in 1% bovine serum albumin and a wash buffer (1 mM MgCl_2 in phosphate buffered saline) for 30 min at 37°C . After rinsing thrice with wash buffer, nuclei

were stained with 1:5000 Hoechst 33342 for 10 min at room temperature. For surface protein visualization, fibronectin-coated hydrogels were incubated with 1:500 R457 rabbit polyclonal anti-rat antiserum against the amino-terminal 70 kDa fragment of fibronectin in staining solution for 30 min at 37°C, washed thrice with buffer, and then incubated with 1:500 Alexa fluor 488-conjugated secondary antibody (1:500; Invitrogen) for 30 min at 37°C. All samples were washed with DI water and mounted using Fluoromount-G (SouthernBiotech). Samples were imaged by a CARV II confocal (BD Biosciences) Nikon Eclipse Ti microscope equipped with a TE2000-U motorized, programmable stage using a Cool-Snap HQ camera (Photometrics) and controlled by Metamorph 7.6 (Molecular Devices). For time-lapse measurements, cells were placed inside a temperature, CO₂, and humidity controlled LiveCell chamber (Pathology devices) and custom Metamorph journals acquired, reconstructed, and processed multi-positional scan slides images. Post-processing was performed in Methamorph 7.6 and ImageJ (NIH).

2.3.8 Traction Force Microscopy

Traction Force Microscopy (TFM) was performed as described elsewhere using in-house Matlab (Mathworks) routines [30]. 2% v/v of 0.5 μ m diameter Fluoresbrite YG Microspheres (Polysciences, Inc) were added to the polymer solutions prior to gelation and the positions of the beads acquired over time using fluorescent and brightfield time-lapse microscopy. After cell trypsinization, bead positions were acquired again and displacement maps were generated using image correlation algorithms similar to particle image velocimetry [31]. Traction stress maps were determined from the measured displacement maps by solving the equation of elastic equilibrium for the substrate. The hydrogel's spatially-varying mechanical properties were considered by performing a perturbation expansion of the solution in terms of the stiffness gradient [32]. Samples were again imaged by a CARV II confocal (BD Biosciences) Nikon Eclipse Ti microscope equipped with a TE2000-U motorized, programmable stage using a Cool-Snap HQ camera (Photometrics) and controlled by Metamorph 7.6 (Molecular Devices). Cell velocities were computed with ImageJ and only migration along the gradient direction

was measured. The error in migration velocities measurements due to the spatial resolution of the images is on the order of $1\ \mu\text{m/hr}$.

2.3.9 Statistical Analyses

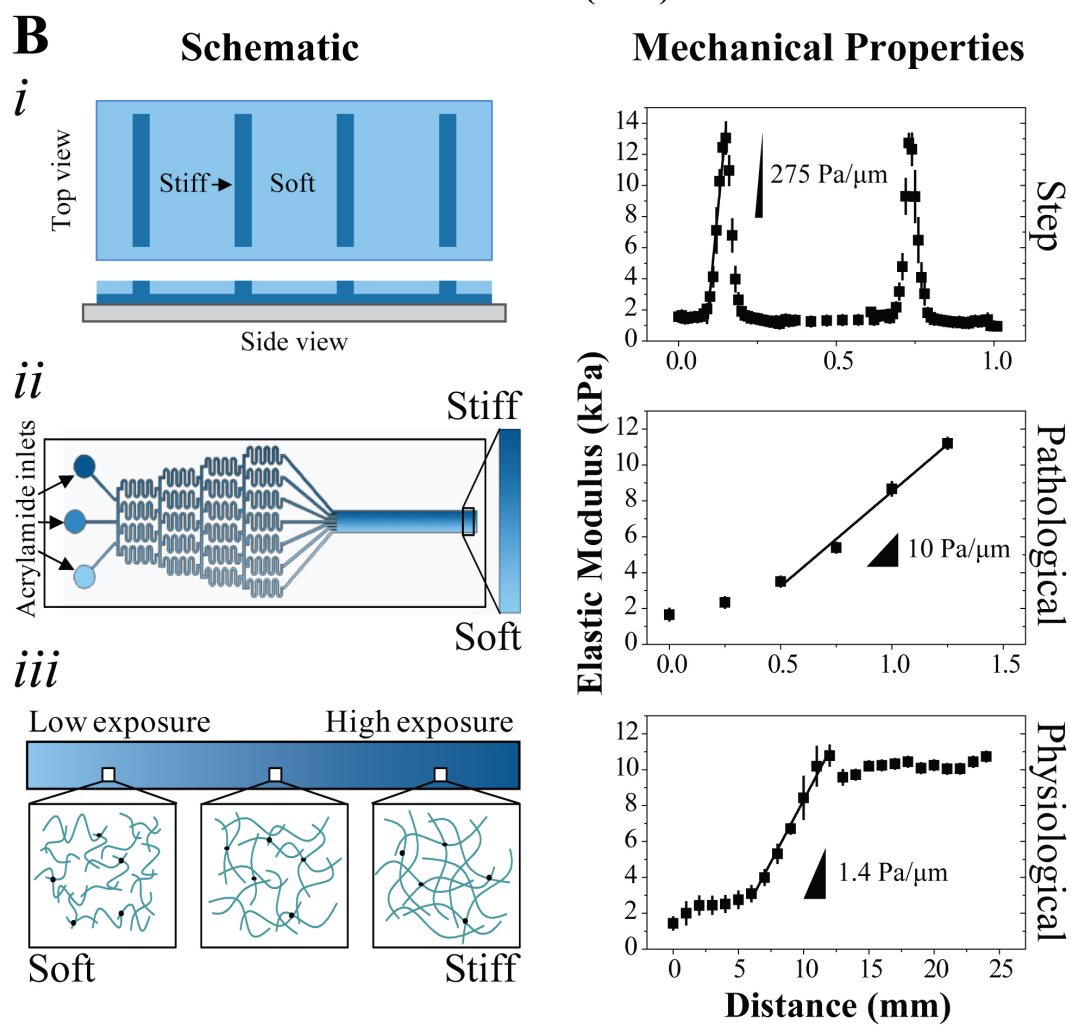
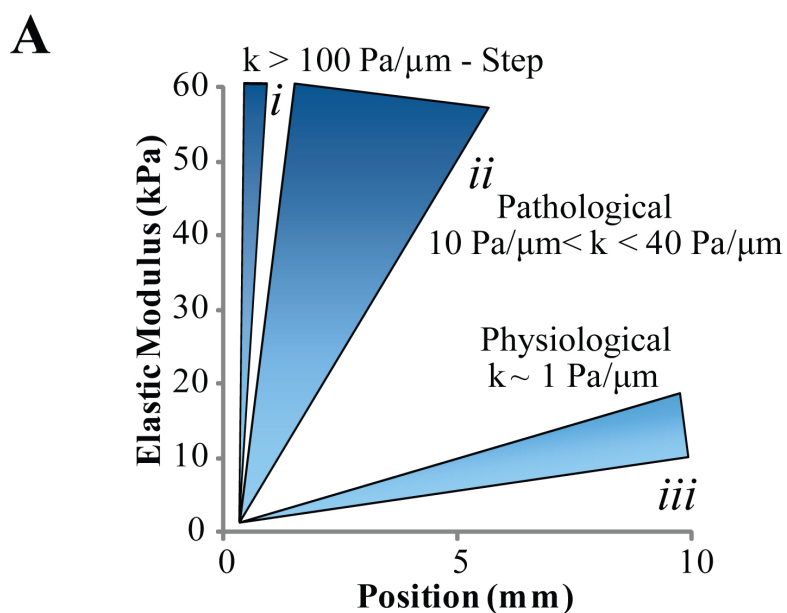
All data are expressed as mean $\pm$ standard deviation of experiments unless otherwise noted. Non-parametric Wilcoxon rank-sum tests were used to perform all statistical analysis. Differences were considered significant when $p < 0.005$ and indicated for all comparisons. All experiments were performed in triplicate unless otherwise noted, and in such cases, the number of cells used in the measurement has been stated.

2.4 Results

2.4.1 Fabrication of Hydrogels with Stiffness Gradients

To generate physiological, pathological, and step stiffness gradients of defined range corresponding to approximately $1\ \text{Pa}/\mu\text{m}$, $10\ \text{Pa}/\mu\text{m}$, and $100\ \text{Pa}/\mu\text{m}$ respectively (Fig. 2.1A), we utilized three distinct systems (Fig. 2.1B). Since our goal was to investigate the migration behavior of human mesenchymal stem cells (MSCs) solely based on gradient strength, system parameters were chosen such that the stiffness range was identical across all systems, spanning 1 to 12 kPa.

Figure 2.1: Polyacrylamide Hydrogels with Stiffness Gradients. (A) Schematic representation of step (i), pathological (ii), and physiological (i) stiffness gradients. (B) Three separate PA systems were developed to generate mechanical gradients of varying strength and of defined range spanning ~ 1 -10 kPa. (i) At left is a schematic of a two-stiffness hydrogel where $500\text{ }\mu\text{m}$ wide regions of soft PA alternate with $\sim 100\text{ }\mu\text{m}$ wide strips of stiff hydrogel producing a stripped stiffness profile. At right, there is a plot of stiffness with position, indicating that the sharp transitions between soft and stiff regions create gradients of $\sim 100\text{ Pa}/\mu\text{m}$, $n=4$ gels. (ii) At left is a schematic of a microfluidic mixer that splits and recombines polymer solutions to generate a smooth gradient from discrete inputs. Photopolymerization of the solution in the outlet channel yields a PA hydrogel with a uniform, one-dimensional pathological stiffness gradient of $10\text{ Pa}/\mu\text{m}$ as indicated at right, $n=3$ gels. (iii) A photomask decreasing in transparency from 100 to 30% modulates the intensity of UV that reaches the polymer solution, leading to changes in polymer chain length as illustrated at left resulting in a UV transmission gradient. This results in PA substrate with a $\sim 1\text{ Pa}/\mu\text{m}$ mechanical gradient as shown at right, $n=3$ gels.



For step gradients, a two-step polymerization mechanism was used resulting in mechanically-patterned matrix. The stiff, bottom hydrogel was first polymerized on top of a micropatterned Si wafer; a second polymer solution was subsequently added on top of the first where it could selectively polymerize into the 'fingers' of the first layer, making them even stiffer, or it could be retained in grooves where it formed a softer hydrogel (Fig. 2.1Bi) [26]. To highlight this, top and bottom hydrogel composition was set to be relatively similar — 3.2% vs. 4.0% acrylamide, respectively — but the bottom hydrogel's stiffness after a second polymerization was roughly 13 kPa (Fig. 2.1Bi), four times the expected value when polymerized as a thin film (Fig. 2.2). Changing the bottom hydrogel's composition by increasing the acrylamide percentage lead to stiffer bottom hydrogels (Fig. 2.2A), but also magnified the stiffening effect during the second polymerization step. For instance, increasing the bottom hydrogel's stiffness to 7.4 kPa using a 6% acrylamide solution (Fig. 2.2A) resulted in a mechanically-patterned matrix whose stiff regions consistently exceeded 100 kPa (Fig. 2.2B). The transition distance from soft to stiff stripes occurred over approximately $40\text{ }\mu\text{m}$, effectively creating a stiffness gradient of $275\text{ Pa}/\mu\text{m}$ whose range spans an order of magnitude, i.e. 1 to 13 kPa (Fig. 2.1Bi). It is important to note that these hydrogels have a continuous top layer made with a high crosslinker-containing solution, i.e. 0.4% bis-acrylamide, which minimizes topographical differences [26].

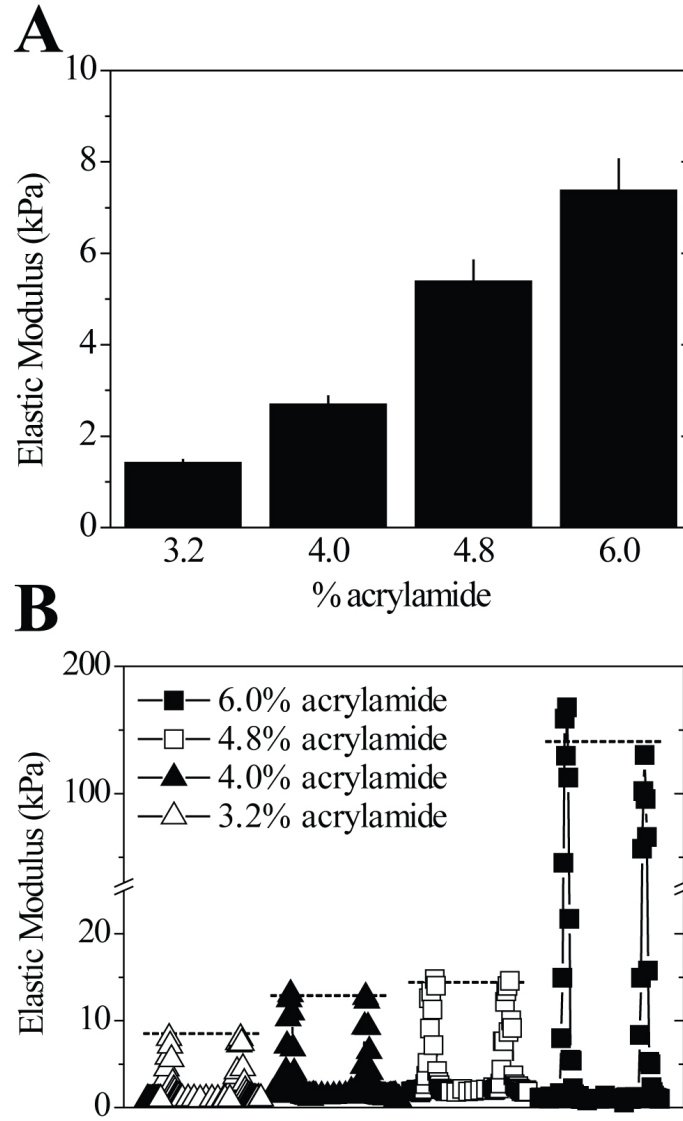


Figure 2.2: Mechanical Characterization of Polyacrylamide on Polyacrylamide. (A) Mechanical properties of 40 μm thick substrates made from the solutions used for the 'bottom' hydrogels in part (B). Data is shown as average $\pm$ standard deviation. Bisacrylamide concentration is constant at 0.4% for all hydrogels. (B) Representative stiffness profiles of step gradient hydrogels made with the indicated percentages of acrylamide and 0.4% bis-acrylamide for the 'bottom' hydrogel. All soft regions between stiff peaks are approximately 1 kPa. Peak-to-peak distance is 600 μm .

For pathological stiffness gradients, a microfluidic mixing device made out of polydimethylsiloxane (PDMS) was used to generate hydrogels with varying mechanical properties [18, 27]. Solutions with different concentrations of bis-acrylamide crosslinker but similar acrylamide concentrations are flowed in the inlets of the microchannel (Fig. 2.1B*ii*) after the outlets had been functionalized with SCA 985 to promote later release of the hydrogel from the mold and the photoinitiator acetophenone to induce polymerization only in the outlet channel (Fig. 2.3A). As the solutions reach the branch points, they split and recombine, mixing in the process and generating a gradient of crosslinker in the outlet portion of the channel (Fig. 2.3B and 2.3C). Photopolymerization of the solution produced a hydrogel with a defined stiffness gradient up to $10 \text{ Pa}/\mu\text{m}$ (Fig. 2.1B*ii*). Modulating crosslinker concentration in microchannel inlets changed the stiffness profile of the resulting hydrogel including the gradient range and magnitude (data not shown).

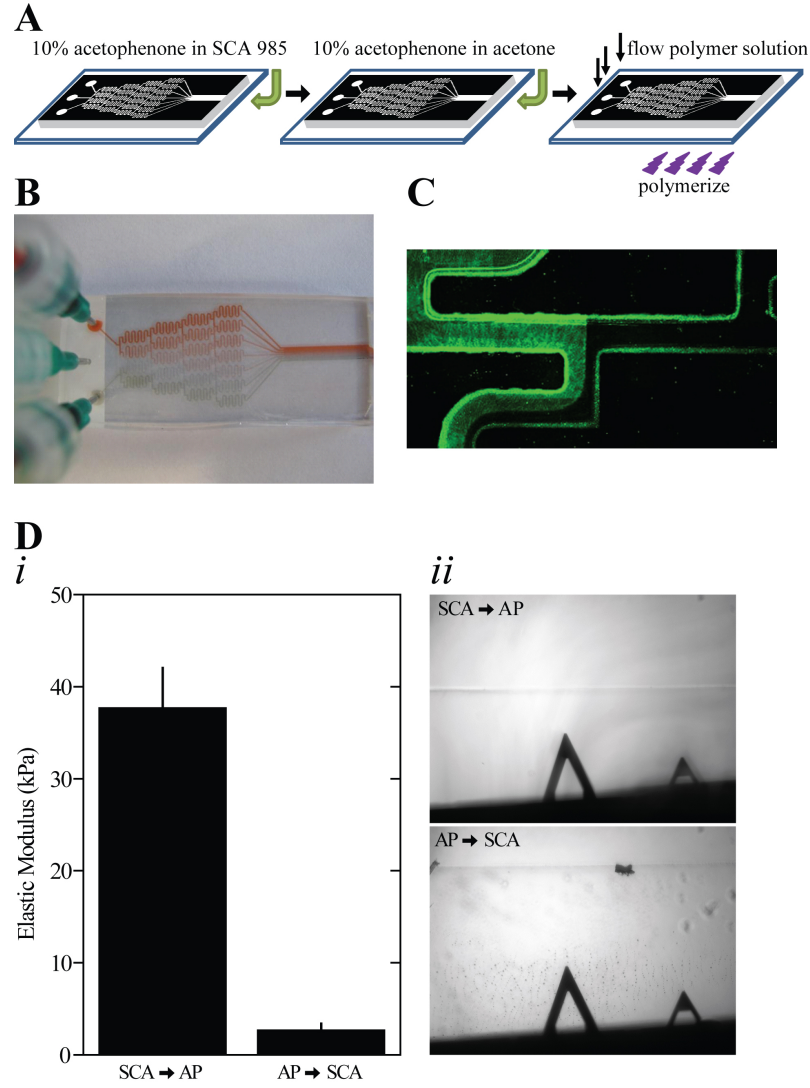


Figure 2.3: Stiffness Gradients Fabricated using Microfluidics. (A) Schematic of the microfluidic gradient generator preparation process. (B) Image of the microfluidic gradient generator with inlets containing red, green, or no food coloring in water to visualize mixing in the channel. (C) Magnified fluorescent image of active flow in the microfluidic gradient generator at a branch point. The left inlet contains EosinY. (D) A solution was polymerized in the outlet of the gradient generator with acetophenone dissolved in SCA 985 or acetophenone dissolved in acetone and SCA 985 subsequently added. (i) Hydrogel stiffness is shown for the indicated order of adding acetophenone and SCA 985 to the gradient generator output channel. Error bars depict standard deviation. Measurements in triplicate were made at 6 distinct positions, $n=3$ gels. (ii) Phase contrast images are shown of the hydrogel edge when either SCA 985 or acetophenone was added first in the preparation process.

Several design changes from previous PDMS microfluidic mixing devices [18, 24, 33] were also introduced to ensure a consistent gradient with minimal swelling, limited topographical features, and a range of approximately 1 to 12 kPa, which were not controlled for in other methods and may have limited polymerization. For instance, using prior methods and a variety of UV wavelengths and exposure times, polymer solutions that normally yield 40 kPa substrates using APS/TEMED initiators produced less than 2 kPa here (Fig. 2.4A) [18]. Moreover, a range of polymer concentrations and photoinitiators including irgacure 2959 and azobis when polymerized inside the PDMS microchannel could not create a gradient. Polymerization through fused quartz, which has superior UV transmission properties than glass [34], did not significantly increase gradient slope in the PDMS device either (Fig. 2.4B). To address initiator concerns, acetophenone was adsorbed onto the PDMS surface to deliver it locally to the polymer solution [35], and photopolymerization of static hydrogels inside the outlet portion produced substrates with the same mechanical properties as thin films, e.g. 40 kPa (Fig. 2.3Di). In contrast, acetophenone flowed through the channel prior to the releasing agent SCA 985 resulted in softer hydrogels with numerous surface defects that may interfere with cell adhesion (Fig. 2.3Dii).

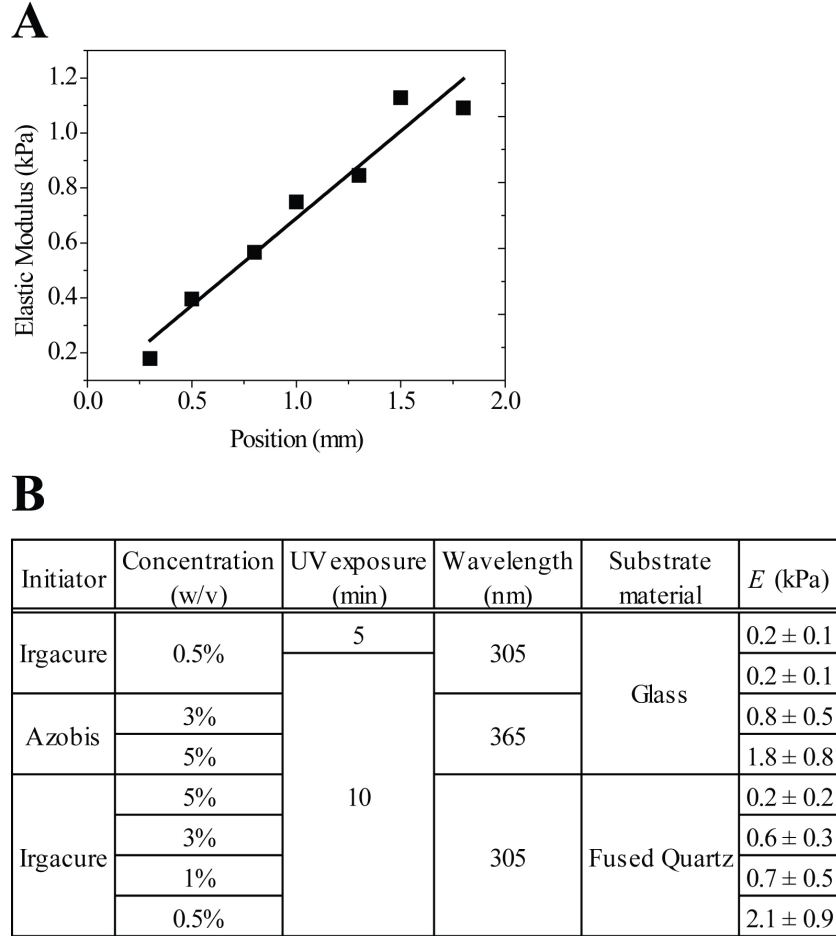


Figure 2.4: Challenges of Polyacrylamide Polymerization in PDMS Microchannels. (A) Representative mechanical properties of hydrogels made using the microfluidic gradient generator and three inlets consisting of 10% acrylamide, 0.5% (w/v) irgacure 2959 as the initiator, and from right to left 0.05%, 0.275%, and 0.5% bis-acrylamide, respectively. (B) Polymerization conditions attempted to polymerize PA hydrogels inside the outlet region of the microfluidic gradient generator and resulting mechanical properties. For all hydrogels, the polymer solution consisted of 10% acrylamide and 0.5% bis-acrylamide.

Photomasks allow one to spatially control the amount of UV light reaching the PA solution to modulate photopolymerization kinetics (Fig. 2.1B*iii*). To achieve physiological gradients, we adapted a photoactivated polymerization procedure used by Tse and Engler where irgacure and a radial photomask are used to generate a shallow gradient (Fig. 2.5A) [5]. Substituting in a linear photomask to more easily change gradient distance and using the more water-soluble initiator azobis, we were able to modulate gradient strength 10-fold, e.g. from 0.38 to 3.46 Pa/ μm , even for gradient hydrogels with narrow stiffness ranges, i.e. 1-5 kPa (Fig. 2.5B). For any given photomask, changing solution concentration, and thus the gradient hydrogel's stiffness range, also changes the gradient strength from ~ 0.4 to 4 Pa/ μm (Fig. 2.5C). Tailoring this system to our specifications, we are able to span the 1 to 12 kPa range at a gradient strength of ~ 1 Pa/ μm (Fig. 2.1B*iii*) using a 12 mm opacity gradient photomask and a 10% acrylamide/0.1% bis-acrylamide solution.

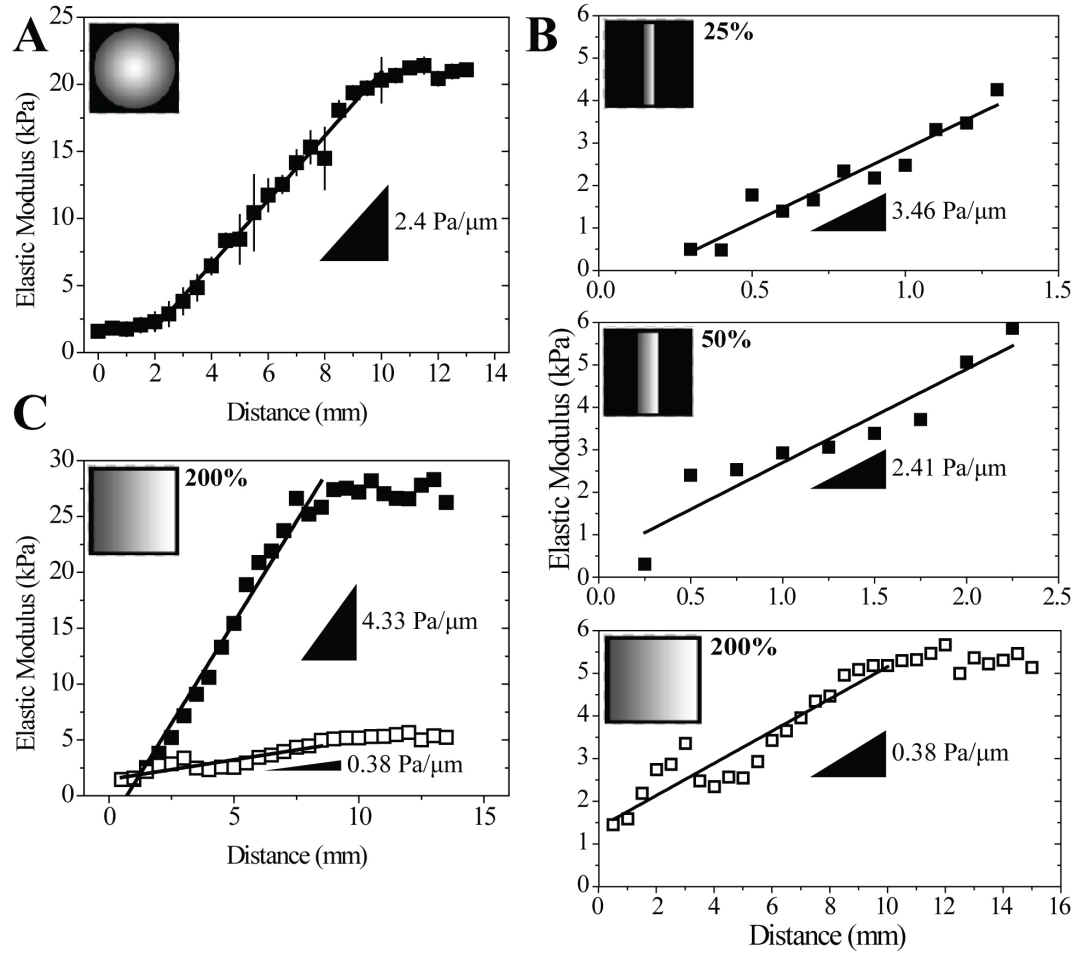


Figure 2.5: Polyacrylamide Gradients Generated Using Photomasks.

(A) Gradients generated using a radially symmetric mask and a solution containing 10% acrylamide, 0.3% bis-acrylamide, and 0.5% irgacure as the initiator. $n=4$ gels (B) Gradients produced with the same polymer solution (10% acrylamide, 0.1% bis-acrylamide) but using photomasks where the opacity gradient distance was scaled to 25%, 50%, or 200%, $n=1$. (C) Two different gradients made with the same photomask but different polymer solutions. Closed squares: 10% acrylamide and 0.3% bis-acrylamide, open squares: 10% acrylamide, 0.1% bis-acrylamide, $n=1$. Insets (A)-(C): Photomask images used for gradient fabrication with indicated relative photomask gradient distance.

2.4.2 Directed Cell Migration on Stiffness Gradients

Prior to cell adhesion, each PA hydrogel was functionalized with fibronectin to promote cell attachment to an otherwise inert substrate. With different PA hydrogel concentrations made by different methods, it was important to verify that the protein coating was consistent across individual gels, i.e. independent of substrate stiffness. Confocal cross-sections of fluorescently-labeled fibronectin indicated that there were no qualitative differences in protein attachment as a function of fabrication method, gradient strength, or absolute stiffness (Fig. 2.6). Thus subsequent cell behavior differences should not be the result of spatial changes in surface ligand density.

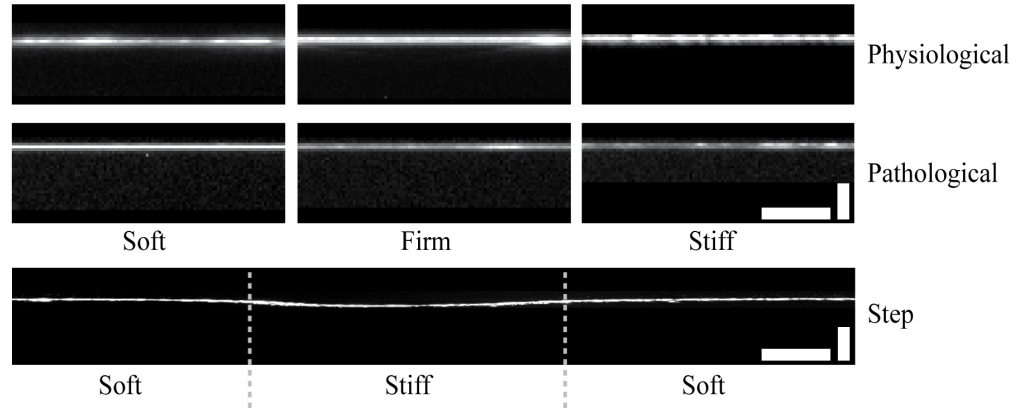


Figure 2.6: Protein Coating on Gradient Hydrogels. Representative confocal cross-sections of each hydrogel system with fluorescently-labeled human plasma fibronectin. Each physiological gradient and pathological gradient image was averaged over 13 overlapped fluorescent cross-sections and repeated at least twice. Scale bars are $25\ \mu\text{m}$ (horizontal) and $5\ \mu\text{m}$ (vertical) for the physiological and pathological gradients and $25\ \mu\text{m}$ (horizontal) and $20\ \mu\text{m}$ (vertical) for step gradient.

MSCs attached and spread independent of gradient strength or stiffness within hours of seeding, and after 3 days, cells migrated to stiffer portions of the substrates. Migration was most evident on step and pathological gradients (Fig. 2.7A). It is important to note that MSCs durotax on physiological gradients although this is less evident in short-term experiments [28]. To verify that cells durotax and that the spatial differences in cell density were due to migration and not preferential proliferation on stiffer regions [13], durotactic migration velocity, i.e. migration in the direction of the gradient, was measured from time-lapse video microscopy. The distribution of instantaneous migration speeds was broad for cells on physiological and pathological gradients (Fig. 2.7B), yet the population average indicated a net biased migration in the direction of the gradient at a rate of 3.0 ± 0.7 and $6.2 \pm 0.6 \mu\text{m/hr}$ for physiological and pathological gradients, respectively (Fig. 2.7C). Cells on step gradients migrated at $18.0 \pm 0.7 \mu\text{m/hr}$, more than 6-fold faster than on other gradient strengths, and it should be noted that negative velocities were not observed for cells migrating up step gradients. These data imply that biased migration velocity scales directly with gradient strength over two orders of magnitude, i.e. 1 to $275 \text{ Pa}/\mu\text{m}$ (Fig. 2.7C inset).

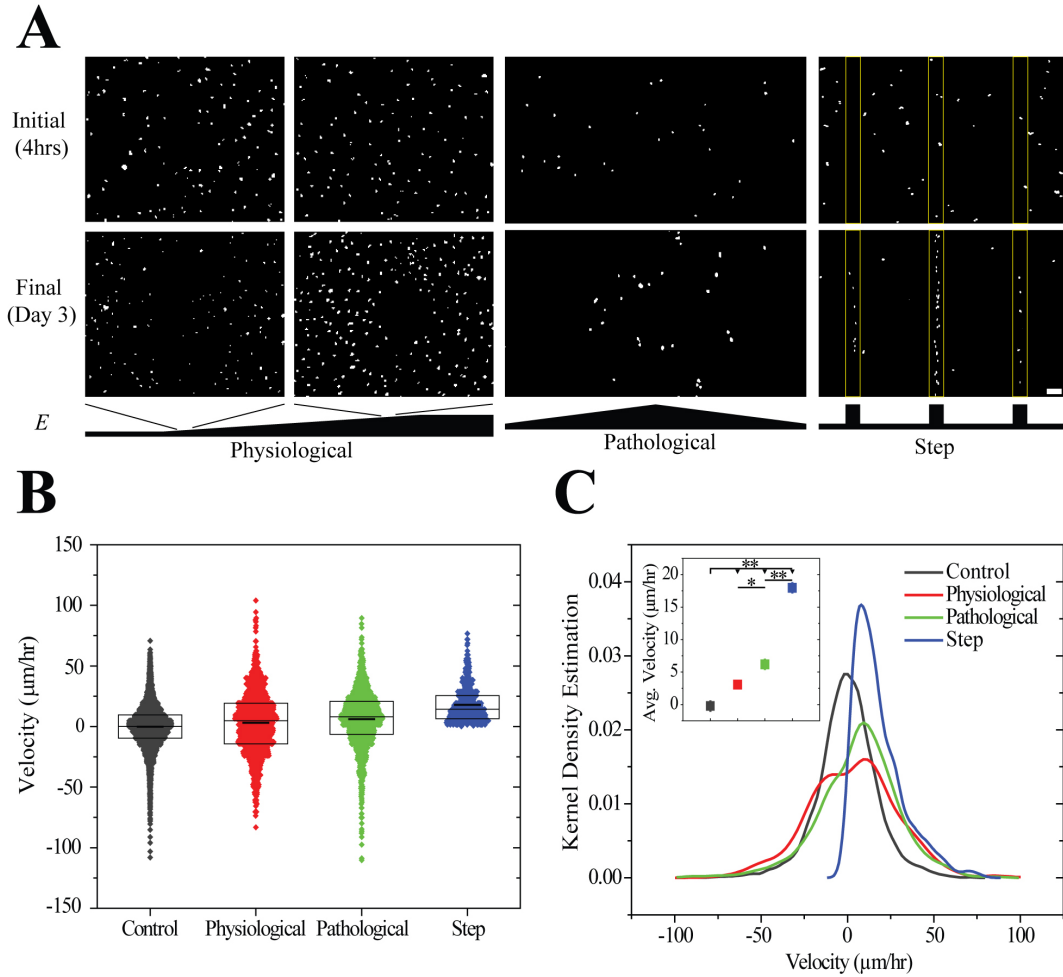


Figure 2.7: MSC Migration of Gradient Hydrogels. (A) Thresholded images of Hoechst-stained MSCs on physiological ($1.4 \text{ Pa}/\mu\text{m}$), pathological ($10 \text{ Pa}/\mu\text{m}$), and step gradients ($275 \text{ Pa}/\mu\text{m}$) 4 hours and 3 days after plating. Scale bar is $100 \mu\text{m}$. (B) Velocities of migrating MSCs in the direction of the gradient determined from tracking live cells using time-lapse microscopy on physiological, pathological, and step gradients. Boxes indicate median, 25^{th} , and 75^{th} percentile and the thicker line indicates the average. (C) Kernel density estimation of cell velocities on the three gradient systems and average $\pm$ standard error of cell migration velocity for each system (inset). * p-value $\leq 10^{-2}$, ** p-value $\leq 10^{-5}$. For step gradient, $n=450$ independent velocities. For physiological and pathological gradients, $n \leq 1300$ independent velocities. Data were obtained from three biological replicates.

2.4.3 Regulating Directed Cell Migration

Since cell migration results from the coordination of cytoskeleton assembly and disassembly in both space and time, we sought to understand which elements were crucial for durotaxis. Untreated cells were less spread than cells treated with lysophosphatidic acid (LPA), a contractile agonist, equally spread compared to nocodazole-treated cells that cannot form stable microtubules, and more spread than cytochalasin D-treated cells which cannot polymerize an actin cytoskeleton (Fig. 2.8A). Untreated cells on gradient substrates did not differ in spread area compared to cells on static matrices ($3570 \pm 340 \mu\text{m}^2$ vs. $3400 \pm 260 \mu\text{m}^2$). After 3 days in culture, untreated cells polarized their actin cytoskeleton in the direction of a pathological gradient, e.g. $8.7 \pm 1.9 \text{ Pa}/\mu\text{m}$, whereas LPA-treated cells were randomly polarized and nocodazole- and cytochalasin D-treated cells maintained a rounded morphology that could not polarize (Fig. 2.8B). All treatments impaired durotaxis significantly (Fig. 2.8C), but while cytochalasin D- and nocodazole-treated MSCs remained largely stationary, LPA-treated MSCs remained spread, protrusive and migrated randomly. Together these data suggest that a stable actin cytoskeleton under appropriate tension is essential for MSC spreading but that microtubules are required for MSC polarization to initiate directed migration.

To determine if the directed migration of MSCs was due to contractile differences as a result of cytoskeletal changes caused by drug treatments, we performed traction force microscopy (TFM) on durotaxing MSCs plated on these gradients. Since MSCs were plated onto gradients, TFM software used to calculate traction stresses was specifically modified to adjust for a spatial stiffness gradient, and these changes were detailed elsewhere [32]. Over time, deformations on the stiffer, right side of the image for an untreated cell increased while cell deformations on the softer side decreased, resulting in MSC directed migration. In contrast, nocodazole-treated cells only slightly deformed the hydrogel despite changing morphology (Fig. 2.8D). Converting displacements to traction forces and taking into account the graded mechanics of the hydrogel, untreated migrating MSCs are also better at forming force-generating protrusions than nocodazole-treated cells over time. These data indicate that stable microtubules are crucial to generate di-

rected traction forces that encourage durotaxis but not essential for some modes of spreading.

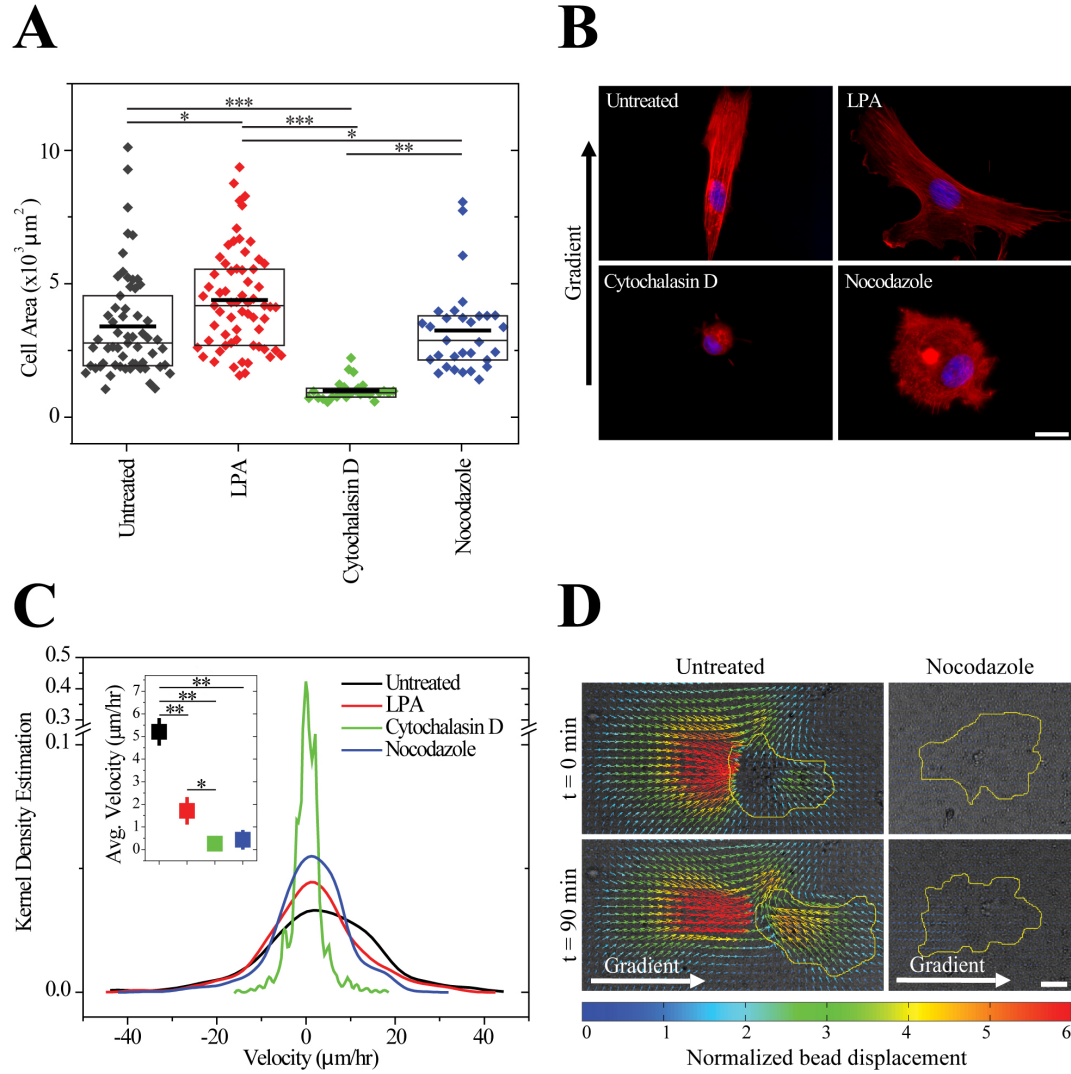


Figure 2.8: Inhibited MSC Migration and MSC Force Generation on Gradient Hydrogels. (A) Spread area of MSCs on gradients either untreated (gray) or treated with lysophosphatidic acid (red), cytochalasin D (green), or nocodazole (blue) after 3 days. Boxes indicate median, 25th, and 75th percentile and the thicker line indicates the average. (B) Nuclei (blue) and actin (red) of MSCs stained after 3 days in culture with inhibitors. Scale bar is 20 μm . (C) Migration of untreated and inhibitor treated MSCs on hydrogel with a pathological gradient of $8.7 \pm 1.9 \text{ Pa}/\mu\text{m}$ and range of 1 to 12 kPa. Inset depicts average $\pm$ standard error of cell migration velocity for each condition. (D) Displacement maps of fluorescent particles embedded in the hydrogel obtained using particle image velocimetry for untreated and nocodazole-treated cells. Brightfield images and cells contours in yellow are overlaid with the displacement maps. Gradient is from left to right. Scale bar is 30 μm . * p-value $\leq 10^{-2}$, ** p-value $\leq 10^{-5}$.

2.5 Discussion

To study durotaxis over a range of gradient strengths, i.e. 1 to $100 \text{ Pa}/\mu\text{m}$, but defined stiffness range, i.e. 1 to 12 kPa, we developed three individual hydrogel systems, each of which was optimally suited for a specific gradient strength. Within that context, it is also critical to appreciate basic mechanistic reasons why cells would undergo directed migration. Thus here we put these systems, their troubleshooting, and the subsequent MSC behavior and mechanism in a broader context.

2.5.1 Challenges of Fabricating Reproducible Gradient Hydrogel Systems of Varying Gradient Strength and Stiffness Range

A variety of methods have been used to achieve spatial stiffness gradients, but each has a limited range of stiffness and gradient strength. Each method also requires standardizing methods to ensure that the gradient is reproducible and that there are no other mitigating factors that could unduly influence cell behavior. We have provided an overview of three gradient hydrogel systems noting their range and strength, and here describe their similarities and differences with similar systems used previously to fabricate stiffness gradients. For photolithographically patterned gradients, modulating light intensity to change radical polymerization kinetics has previously been used with photomasks [5, 17] or a sliding mask to vary hydrogel UV exposure time [36, 37]. This technique is well suited to generate physiological to pathological gradients, and we show its versatility in precisely controlling both the range and strength of gradients between 0.4 and $8.7 \text{ Pa}/\mu\text{m}$ and 1 to 12 kPa. Here azobis was used as the photoinitiator due to its increased solubility in aqueous solutions and activation with long wave UV. Silica glass does not transmit below 300 nm, and since irgacure's peak absorption occurs at 276 nm, its reaction is always suboptimal [34, 38]. Light diffraction and lateral diffusion of radicals also limit gradient resolution, but photomasks with high resolution have been used to control stiffness at the micron lengthscale of pre-polymerized

hydrogels [25].

To achieve higher resolution and gradient strength, microfluidic channels more tightly constrain hydrogel dimensions and provide superior control over stiffness at the micron lengthscale based on channel design. In this type of system, stiffness was modulated by changing crosslinker concentration instead of modulating initiator activation along the width of the hydrogel [18, 24, 27]. Changing input solutions is simple, and allows for systematic spatial control over the mechanics in the outlet channel. However, PDMS is the common material used in these devices; it is porous and allows molecular oxygen to diffuse through the surface which reacts with radicals generated by the photoinitiator, effectively terminating the polymerization reaction [39, 40]. This effect has been minimal in quickly polymerizing systems such as poly(ethylene glycol) and even advantageous as the non-polymerized layer at the interface of the hydrogel and PDMS serves as a lubricating layer and promotes hydrogel release [24, 40]. For radical-based polymerization that takes several minutes, e.g. PA, oxygen radical scavengers in PDMS prevented the synthesis of hydrogels above 2 kPa over a range of water-soluble initiators, initiator concentrations, and UV treatments here despite studies showing otherwise [18, 20]. Absorbing hydrophobic acetophenone to the surface of PDMS allows for the polymerization of stiff PA hydrogels [27, 35], presumably because the initiator-generated radicals saturate at the PDMS surface and the rest diffuse into the polymer solution. However, PA polymerization with this method lacks the lubricating layer, and thus the PA grafts into the PDMS [27]. This prevents release and may cause the substrate to rip. SCA 985 covalently bound to the PDMS surface likely maintains the lubricating layer, and by coating it prior to absorbing acetophenone, stiff hydrogels were released from the PDMS mold without damaging the substrate’s surface.

There are also several challenges associated with step stiffness gradients from mechanically-patterned substrates. For example, the original method of polymerizing adjacent droplets of distinct acrylamide concentrations [16] forms a gradient from uncontrolled mixing, and while microfluidic gradients are well controlled, they often cannot achieve the steepest gradients, i.e. >100 Pa/ μm [18, 24, 27].

Controlling the transition from soft to stiff was recently described by using a 2-step polymerization method to make mechanically-patterned hydrogels [25, 26]; here, by increasing the soft region width, we allowed cells to spread and randomly migrate before feeling the gradient near the interface and undergoing directed migration to the stiffer region. Though we used this system to understand migration up an exceedingly steep gradient, this platform has also been used to study how aligning cells improves their function, e.g. muscle cell fusion [26]. There are specific technical challenges worth noting; differential swelling between layers in microfabricated hydrogels have been reported and could introduce contract guidance cues similar to topographical patterns. By using substrates with high crosslinker content however [41], polymer chains become less free to slide across each other, dramatically reducing differential swelling between layers, i.e. $\sim 2 \mu\text{m}$, as well as roughness changes between stripes on the hydrogel, i.e. $\sim 200 \text{ nm}$ [26]. Polymer depletion effects in the soft stripes formed for the second layer’s polymerization also confounds predicting layer stiffness. For example the small changes in bottom hydrogel stiffness dramatically changed the final stiffness after the second acrylamide solution was polymerized and significantly deviated from monolayer hydrogel stiffness [5]. Materials that do not undergo such mixing may form more predictable layered materials, e.g. PDMS [42]. Despite both of these challenges, it should be noted that across all three systems, protein coating appeared uniform and initial cell adhesion was similar independent of polymer concentration and crosslink density.

2.5.2 The Origins of Durotaxis

Cells plated on the gradient systems migrated to the stiffer end of the hydrogels at different speeds, indicating that MSC durotaxis velocity depends on gradient strength as previously suggested with other cell types [20] but over a wider range of gradients here. It is important to note that the migration velocities reported here were obtained over the same physiological stiffness range of 1 to 12 kPa, indicating that gradient strength and not absolute stiffness drove directed MSC migration on gradient substrates. While these velocities were time-averaged,

MSCs did not always feel the gradient, as with step gradients for the mechanically-patterned hydrogels; MSCs that approached the interface migrated faster than the time-averaging used here and thus their 18 $\mu\text{m/hr}$ speed could be an underestimation. Despite that consideration, it is clear that durotactic speed increases but is not linear with respect to gradient strength. In addition to speed, cell traction forces enable MSCs to feel stiffness gradients and then migrate, but as of yet, this process remains unclear. Neutrophils, which undergo amoeboid-like migration, form rearward contractile centers, which subsequently squeeze the cell forward in the direction of a chemotactic gradient [43, 44]. This process, though different from the multi-step mesenchymal migration of MSCs [45], also requires myosin II [46], and interestingly, MSC traction distribution in cells migrating up the gradient seemingly mirror the rearward contractile stresses observed in neutrophils. One plausible explanation may be that cells feel their environment by sensing the strain they can impart on their matrix [47]. When oriented into a gradient, displacements were highest in the more compliant rear of the cells, where strain sensors could be most active and positively feeding signals to the cell. A variety of strain sensors have been proposed [48-50] and likely require some minimum level of signaling to encourage cell behaviors such as spreading and migration [51, 52].

The sensors required for this process, and thus their signaling capabilities, are likely connected to the cells' cytoskeleton, since altering the cell's ability to assemble a stable cytoskeleton significantly reduced MSC migration though these observations did not necessarily correlate with changes in cell spread area. The loss of microtubule architecture inhibited cell polarity with the cells remaining spread yet non-migratory. Similar to a previous report on nocodazole-treated fibroblasts [53], MSCs appear to require assembled microtubules to transmit forces to their surrounding environment but an actin cytoskeleton is necessary to adopt a spread morphology. Interestingly, cells treated with the multifunctional phospholipid messenger LPA, which promotes activation of the Rho and Ras GTP-ases [54] and thus cellular tension [55], did not undergo directed migration despite their heightened ability to 'feel' matrix stiffness [56]. LPA signaling is thought to contribute to cancer initiation, progression, and metastasis [57] though our results also

suggest the inhibition of durotaxis. This observation is particularly relevant since the migration of untreated cells up the gradient relies on their ability to deform the matrix and generate traction stresses on the order of a few hundred Pascals.

Together these data imply that durotaxis mechanism(s) are force dependent, require an assembled microtubule network, and also require precise coordination of contractility in time and space since both increased and decreased cell contractility abolished directed migration. More importantly through the development of these three platforms, it was possible to fabricate gradients of the necessary range and strength to answer these questions.

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Chapter 3

The Alignment and Fusion Assembly of Adipose-Derived Stem Cells on Mechanically Patterned Matrices

3.1 Abstract

Cell patterning is typically accomplished by selectively depositing proteins for cell adhesion only on patterned regions; however in tissues, cells are also influenced by mechanical stimuli, which can also result in patterned arrangements of cells. We developed a mechanically-patterned hydrogel to observe and compare it to extracellular matrix (ECM) ligand patterns to determine how to best regulate and improve cell type-specific behaviors. Ligand-based patterning on hydrogels was not robust over prolonged culture, but cells on mechanically-patterned hydrogels differentially sorted based on stiffness preference: myocytes and adipose-derived stem cells (ASCs) underwent stiffness-mediated migration, i.e. durotaxis, and remained on myogenic hydrogel regions. Myocytes developed aligned striations and fused on myogenic stripes of the mechanically-patterned hydrogel. ASCs aligned and underwent myogenesis, but their fusion rate increased, as did the number of

cells fusing into a myotube as a result of their alignment. Conversely, neuronal cells did not exhibit durotaxis and could be seen on soft regions of the hydrogel for prolonged culture time. These results suggest that mechanically-patterned hydrogels could provide a platform to create tissue engineered, innervated micro-muscles of neural and muscle phenotypes juxtaposed next to each other in order better re-create a muscle niche.

3.2 Introduction

Regulation of stem cell fate has traditionally relied on presenting small molecules such as growth factors and cytokines in developmentally appropriate ways [1,2], but such a view omits other important niche characteristics. Biomaterials have recently been used to reproducibly control stem cell differentiation by directly mimicking the niche of the injured or diseased tissues [3-5]; niche mimicry to drive differentiation includes intrinsic extracellular matrix (ECM) cues such as composition [6] and elastic modulus, E , i.e. 'stiffness' (measured in kiloPascals, kPa) [7]. A reductionist approach has been used to appreciate the control that each of these properties has on fate, but more recently, properties have been examined in combination. Often cues do not appear additive [8] or at least their combination may be context specific: some ligands support stiffness-induced differentiation whereas others do not [9]. These cues are also rarely static and often presented in spatial or temporal gradients. Stem cells can respond to surprisingly shallow ligand [10] and stiffness gradients [11], emphasizing the need for appropriate spatial control of ECM properties.

Spatial control has often been performed via cell patterning by selectively depositing ECM proteins to allow cell adhesion only on protein-patterned regions, e.g. microcontact printing (μ CP) [12]. μ CP has been used to control cell area and shape, which can regulate cell membrane tension and ultimately stem cell fate [13,14]. It can also encourage end-to-end fusion and striation assembly in myoblasts on substrates of myogenic stiffness [15]. However, μ CP fidelity relies on maintaining the integrity of the non-fouling region; proteins in serum-containing media,

which are often required for stem cells, may deposit over time on soft substrates and decrease pattern fidelity [16]. On the other hand, stiffness alone has been used to show that mesenchymal stem cells become myogenic [17], and specifically for mesenchymal stem cells originating from adipose tissue, i.e. adipose-derived stem cells (ASCs), 2% of cells can even fuse into bi-nucleated myotubes that have lost lineage plasticity [7]. To mechanically regulate where cells polymerization [18] or photocleavable hydrogels post-polymerization [19] have been used, but these methods have not been applied to study how spatially-controlled stiffness can regulate stem cell fate.

In tissues, cell position and fate may be the result of stiffness sorting or other mechanisms [20] with the net result being layers of juxtaposed cell layers with different stiffness [21]. In skeletal muscle specifically, cells reside in an aligned ECM comprised of regions with muscle-like stiffness, ~ 10 kPa [15,17,22], juxtaposed with regions where softer neurons innervate the firm muscle [23]. Thus, a mechanically patterned substrate with alternating neural and muscle stiffness may be a more physiologically appropriate environment in which to study stem cell myogenesis and encourage cell alignment and end-to-end fusion.

There is also mounting biological evidence that aligning stem cells may promote a more terminally differentiated muscle phenotype. On compliant matrix, cell tractions can propagate over some distance [24] and preferentially align myoblasts over hundreds of microns [15]. Cell-cell adhesion proteins, including M- and N-cadherin, aid cell end-to-end association; function blocking antibodies for these proteins reduce myoblast fusion [25,26]. Their expression is also regulated by RhoA [26], implying that spatially regulating cell attachment and alignment could direct expression of these fusion markers and promote fusion by directing traction forces versus unpatterned matrix. With these data in mind, we designed mechanically patterned hydrogels with stripes of alternating stiffness, as well as μ CP substrates with the same pattern dimensions. These substrates were used to assess the degree to which different cell types, e.g. neurons, muscle cells, and stem cells, adhered to and sorted on patterned matrices. Stemcell myogenesis was also examined in detail to determine if alignment improved fusion due to a change in

fusion-supportive cadherin expression.

3.3 Materials and Methods

3.3.1 Photolithography and Fabrication of Mechanically-Patterned Hydrogels

Mechanically-patterned hydrogel photomasks and molds were created using photolithography. To briefly outline the process illustrated in Fig. 3.1A left, photoresist polymer SU-8 2015 (Microchem) was spin-coated onto a silicone wafer generating $\sim 27 \mu\text{m}$ thin film. Photomask with alternating black and clear stripes (Zebra pattern) of $100 \mu\text{m}$ width was placed on top of the photoresist film to selectively polymerize regions of the film, and the wafer was exposed to UV light. The exposed regions remained as a positive feature ($\sim 27 \mu\text{m}$ in height) after it was developed in a buffer, which washed away non-polymerized photoresist from the wafer.

Mechanically-patterned hydrogel fabrication consisted of a two-step polyacrylamide (PA) hydrogel polymerization process illustrated in Fig. 3.1A right. Acrylamide was polymerized on aminosilanized, 25 mm diameter coverslips (functionalized coverslip) according to the method modified from previously established protocol [27]. Briefly, a gel solution containing the crosslinker N,N'-methylene-bis-acrylamide, acrylamide, 1/100 volume of 10% Ammonium Persulfate and 1/1000 volume of N,N,N',N'-Tetramethylethylenediamine was mixed. $20 \mu\text{l}$ of the polymerizing hydrogel solution was sandwiched between the aminosilanized coverslip and a dichlorodimethylsilane (DCDMS)-treated silicone mold made in Fig. 1A left to ensure easy detachment. Initial acrylamide/bis-acrylamide solutions were 4%/0.4% and 4.8%/0.4% for neuro-/myogenic and myo-/osteogenic mechanically-patterned hydrogel, respectively. After 15 min to allow for polymerization, the hydrogel was detached from the mold, and $15 \mu\text{l}$ of a second hydrogel solution was added on top of the polymerized hydrogel, covered by a DCDMS-treated coverslip, and polymerized again for 15 min. Input acrylamide/bis-acrylamide solutions

for the second layer were 3.2%/0.4% for neuro-/myogenic and 6%/0.4% for myo-/osteogenic mechanically-patterned hydrogel, respectively. After detaching the composite hydrogel from the DCDMS-treated coverslip, mechanically-patterned hydrogel was kept in phosphate-buffered saline (PBS). 10 $\mu\text{g}/\text{ml}$ fibronectin [28] or 100 $\mu\text{g}/\text{ml}$ type I collagen [29] was chemically crosslinked using a photoactivating crosslinker, Sulfo-SANPAH (Pierce), as indicated. Alternatively, other concentrations of monomer and crosslinker concentrations for the initial and second layers were used, but only when specifically indicated.

3.3.2 Microcontact Printed (μCP) Hydrogel

To compare mechanical and protein-based matrix patterns, microcontact printing was used to create alternating protein pattern on static 10 kPa gels. PDMS stamps were made from a degassed mixture of 10:1 elastomer base to curing agent mixture of Sylgard 184 (Dow Corning) that was poured onto the patterned hydrogel silicon wafers described above and baked for 1 h at 60°C on a hotplate. Following release from the wafer, the stamps were incubated with a thin film of a 100 $\mu\text{g}/\text{ml}$ human plasma fibronectin solution sandwiched between a coverslip and the stamp for 20 min. Meanwhile, 10 kPa polyacrylamide gels prepared as described above were incubated in a solution of 1 mg/ml Sulfo-SANPAH in 50 mM HEPES pH 8.5 and placed for 10 min under a 4 mW/cm² 350 nm UV source. Following three washes in 50 mM HEPES pH 8.5, the gels were placed on a hotplate set to 60°C until all remaining solvent had evaporated. PDMS stamps were brought in direct contact with the gels for 10 min and a small weight was placed on top of the stamp to ensure good contact between the PDMS microprinting tool and the PA substrate. Hydrogels were peeled from the stamps using tweezers and the resulting substrates immediately immersed in 50 mM HEPES pH 8.5 overnight at 37°C.

3.3.3 Mechanical Force Spectroscopy Mapping by Atomic Force Microscopy

Matrix stiffness was confirmed by atomic force microscopy (AFM; MFP3D, Asylum Research) as detailed previously [27,30]. Briefly, a pyramidal probe, 0.02 N/m spring constant with a 35° half angle (TR400PB, Olympus), was used to indent a substrate every 25 μm in triplicate over two repeats of the low/high acrylamide-based pattern stripes. Probe indentation velocity was fixed at 2 $\mu\text{m/s}$ with the trigger force of 2 nN. Force spectroscopy was performed over a regular array of spatial coordinates in order to map substrate stiffness over a defined region using the AFM scanning stage (scan size limit of 90 μm x 90 μm); mapping down to 20 nm resolution with this technique is possible as previously shown [31]. Elastic modulus maps were determined by the Hertz cone model with a sample Poisson ratio of 0.5 fit over a range of 10%-90% indentation force [30]. Surface height and roughness are also simultaneously computed based on when probe deflection occurs as it indents the material. Topographical images were modified using a flattening function to eliminate overall slope from imperfect sample mounting. AFM software (Igor pro 6.22) was applied to generate the force maps, analyze height data, and perform 3D rendering. When applicable, three adjacent maps were assembled together to cover at least two stripes of the alternating pattern.

3.3.4 Cell Isolation and Culture

Human ASCs were isolated from freshly aspirated human subcutaneous adipose tissue (donor age between 26 and 31 years) according to the method described previously [32] with approval of UCSD human research protections program (Project#101878). Liposuction samples (300 ml) were washed extensively with equal volumes of phosphate-buffered saline (PBS), and then incubated at 37°C for 45-60 min in 0.1% type I collagenase (Worthington Biochemical). Enzyme activity was neutralized with Dulbecco's modified Eagle's medium (DMEM)-low glucose (Invitrogen), containing 10% fetal bovine serum (FBS; Thermo Scientific) and 1% antibiotic/antimycotic (Invitrogen). Cells were centrifuged at 1200 rpm for 10

min to remove adipocytes. The pellet was resuspended in 0.16 M NH_4Cl and incubated at room temperature for 5 min to lyse red blood cells. Cells were collected by centrifugation at 1200 rpm for 5 min, filtered through a 100 μm cell strainer (BD Falcon) to remove fissile debris, and incubated overnight on tissue culture plastic in complete medium at 37°C and 5% CO_2 . Plates were then washed extensively with PBS to remove residual non-adherent cells. To reduce donor-to-donor variation, cells from three different donors were pooled. ASCs were used at low passage numbers between 4 and 8. Morphology was assessed and analyzed using ImageJ software (NIH).

C2C12 skeletal myoblasts (ATCC) were cultured in high glucose DMEM (Invitrogen), 10% FBS and 1% antibiotic/antimycotic unless cell fusion was induced in which case serum concentration was reduced to 2%. PC12 cells (ATCC) were cultured in high glucose DMEM, 10% FBS and 1% antibiotic/antimycotic.

Chicken embryonic hearts and heart cells were obtained by isolation at 240 h post fertilization. Animals received humane care in compliance with University of California, San Diego's Institutional Animal Care and Use Committee (protocol #S09200). As detailed elsewhere [33], hearts were obtained by dissection and digested for cell isolation. Isolated hearts were minced using sterile razor blades, washed in PBS and collected with 10 ml of 0.05% trypsin-EDTA (Invitrogen) and incubated in a sterile humidified incubator at 37°C and 5% CO_2 for 15 min. In order to remove red blood cells, the tube was inverted and tissue was allowed to settle prior to a change of solution to 10 ml of fresh trypsin. After 15 min incubation, the sample was centrifuged and the pellet was carefully triturated with normal heart medium containing aMEM (Invitrogen), 10% FBS, and 1% antibiotic/antimycotic. The cell solution was passed through a 70 μm cell strainer and pre-plated on a sterile tissue culture dish for 1 h at 37°C and 5% CO_2 in order to remove fibroblasts from the solution. The unattached cells were collected, counted, and plated at a density of 10^6 cells/cm². Media changes were performed every 2 days.

3.3.5 Immunocytochemistry, Fusion, and Function Blocking Assays

Cells were fixed with 3.7% formaldehyde at room temperature for 15 min. Samples were treated with PBS containing 1% Triton X-100 for 15 min and washed with a washing solution of PBS containing 1 mM MgCl_2 . The primary antibodies listed below with their indicated dilutions in a staining solution containing 2% bovine serum albumin were then added to samples: α -actinin (1:500; Sigma), MyoD (1:100; Santa Cruz), skeletal myosin (1:100; Sigma), β tubulin (1:100; Developmental Studies Hybridoma Bank), Ki67 (1:100; eBioscience), and Desmin (1:100; Sigma). Antibodies were incubated with samples for 30 min at 37°C. After washing three times with the washing solution, samples were incubated with Alexa fluor 488- or 568-conjugated secondary antibody (1:400; Invitrogen) for 30 min at 37°C. After washing three times with washing solution, rhodamine-phalloidin (1:1000; Invitrogen) stained F-actin for 30 min at 37°C if needed and nuclei were stained by Hoechst 33342 (1:1000; Invitrogen) for 2 min. All samples were examined by a CARV II confocal microscope (BD Biosciences) mounted on a Nikon Eclipse TE2000-U microscope with a motorized, programmable stage using a Cool-Snap HQ camera controlled by Metamorph 7.6 software.

Alternatively, the rabbit polyclonal GC-4 antibody (Sigma) was used to block the function as well as stain N-cadherin depending on whether it was applied chronically to cultures every 48 h at 40 μL or during normal staining procedures. For chronic treatment, cell fusion was assessed as described below. ASCs' myotube fusion was confirmed by continuous cytoskeletal structure by staining for β -tubulin and proliferation marker Ki67 to identify and exclude proliferating cells with two nuclei as described previously [7]. To quantify myotube formation frequency, more than 500 nuclei were observed from four independent samples of mechanically patterned hydrogels. Only multi-nucleated cells with continuous cytoskeleton but lack of Ki67 were considered as myotubes. Fusion rate was expressed as the total number of fusion events divided by the total number of nuclei. ASC fusion rate on mechanically-patterned hydrogels was compared to the fusion rate on static 10 kPa hydrogels [7] as well as protein patterned ASCs.

3.3.6 Statistical Analysis

All data are expressed as mean $\pm$ standard deviation of experiments from at least four independent repeats. Statistical analysis was performed using student *t*-test on Graphpad Prism software. P-values ≤ 0.05 were considered to indicate statistical significance.

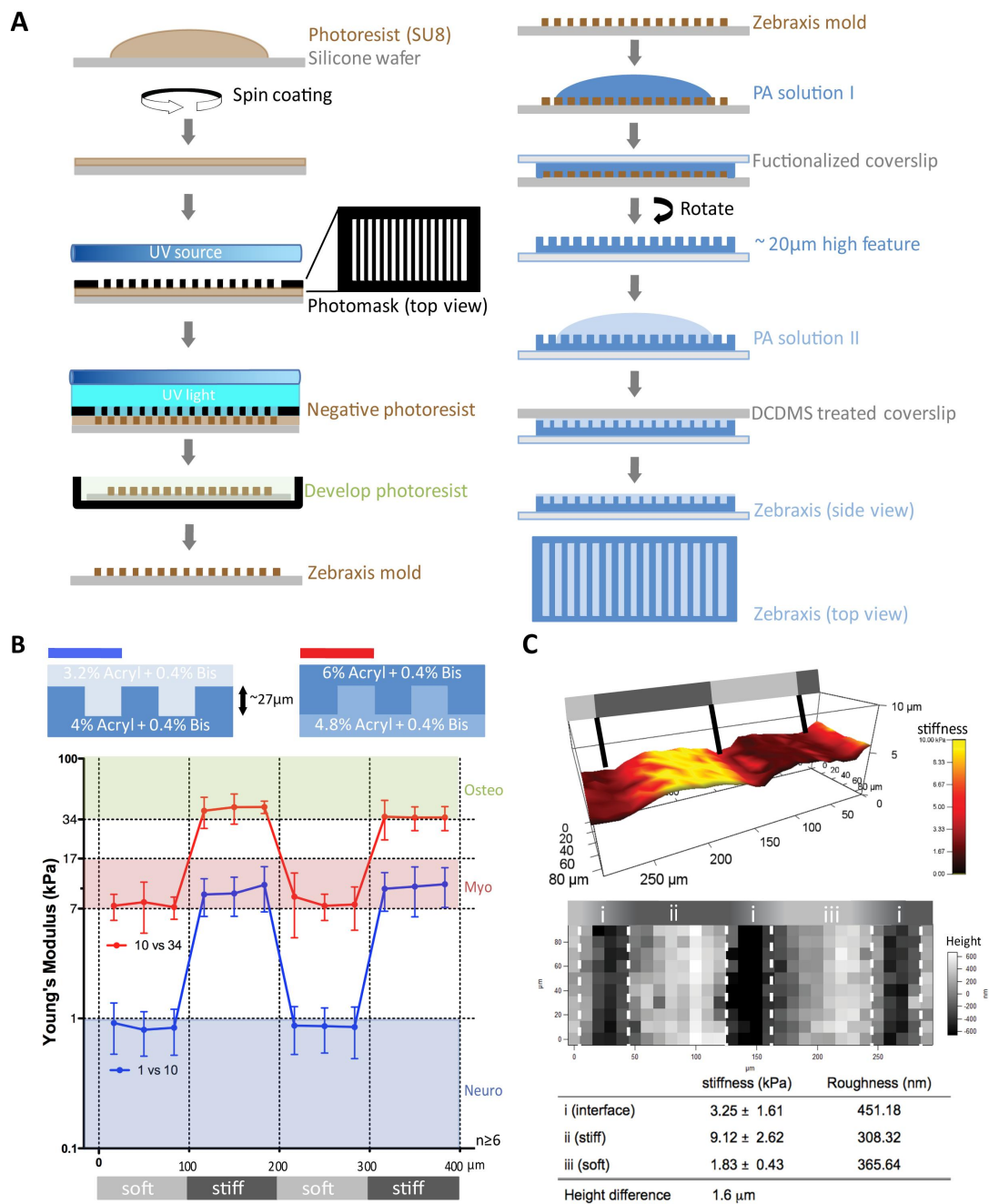
3.4 Results

3.4.1 Mechanically-Patterned Hydrogel Fabrication and Characterization

Two different acrylamide concentrations were sequentially polymerized with the first hydrogel polymerized in a micromold and the subsequent one layered on top of the first (Fig. 3.1A). This method created a spatially varying stiffness profile of 100 μm wide stripes in hydrogels; 1 and 10 kPa stripes were produced to make a neurogenic-myogenic mechanically-patterned hydrogel using a small 3.2-4% variation in acrylamide concentration with relatively high 0.4% crosslinking bis-acrylamide (Fig. 3.1B, blue). Stripes approximately of 10 and 34 kPa were made for a myogenicosteogenic mechanically-patterned hydrogel (Fig. 3.1B, red) using a 4.8-6% variation in acrylamide concentration. However, layering hydrogels of different concentration could introduce differential swelling. To assess substrate height across a neurogenic-myogenic mechanically-patterned hydrogel, both height and stiffness were measured simultaneously by AFM force mapping. Over a 270 μm x 90 μm map, the maximum height range was less than 2 μm , spanning both a soft (dark red) and stiff stripe (Fig. 3.1C, top; yellow). Mapping indicated that the height difference was most pronounced in a small interfacial region between stripes, but this did not create any appreciable change in surface roughness in this zone or any other, regardless of the stiffness of the pattern (Fig. 3.1C, bottom). Soft and stiff regions were 80 μm -wide with a 40 μm -wide interface, effectively creating a step gradient in stiffness, since the gradient is 2 orders of magnitude higher than physiological [11]. Compared to controlling hydrogel stiff-

ness by manipulating crosslinker density [34,35], strategies like the one used here, which employ varying bulk monomer concentration to modulate stiffness in the presence of a high crosslinker concentration minimize PA hydrogel swelling [36]. Using a lower crosslinker concentration, e.g. 0.1%, resulted in greater differential swelling between layers and created height differences ranging between 8 and 10 μm for 4% and 10% acrylamide layers, respectively, as assessed by AFM force mapping. Larger height differences may induce significant topographical signaling, so only high crosslinker mechanically patterned hydrogel were investigated further.

Figure 3.1: Mechanically-Patterned Hydrogel Fabrication and Characterization. (A) Schematic of photolithography and 2-step polyacrylamide gel fabrication. (B) Matrix stiffness (bottom) was measured for neurogenic-myogenic (blue) and myogenic-osteogenic (red) mechanically-patterned hydrogels ($n \geq 6$) fabricated using the indicated polyacrylamide concentrations for top and bottom hydrogels (top). Light and dark gray regions in the bottom schematic represent soft and stiff regions, respectively. (C) Matrix stiffness, height, and surface roughness of mechanically-patterned hydrogels were visualized with three adjacent $90 \mu\text{m} \times 90 \mu\text{m}$ force maps. Matrix stiffness of adjacent soft and stiff strips was overlaid with surface height in a 3D reconstruction of the surface features (top). 2D height topography is shown individually (middle). Values for stiffness and roughness are shown for stiff and soft stripes as well as the interface. Overall height difference was also computed and shown. Light and dark gray regions in the schematics represent soft (1 kPa) and stiff (10 kPa) regions, respectively.



To ensure that mechanically-patterned hydrogel stiffness differences did not preclude uniform protein attachment and cell adhesion, both human plasma-derived fibronectin and type I collagen were coated onto the substrates and C2C12 myoblasts were allowed to adhere. No differences in cell adhesion, spreading, or morphology were observed after 48 h in culture (Fig. 3.2). Stripe pattern was also manipulated to ensure that pattern recognition was independent of pattern geometry. C2C12 myoblasts as well as adipose-derived stem cells were allowed to adhere to mechanically-patterned hydrogels containing 100 μm stiff (10 kPa) and 500 μm soft (1 kPa) stripes or 500 μm stiff (10 kPa) and 100 μm soft (1 kPa) stripes. Both ASCs and C2C12 cells were able to detect the stiffness gradient at the stripe interface and undergo directed migration, i.e. durotaxis [37], toward the stiffer stripes even when cells were up to 250 μm away from the interface (Fig. 3.3). As cells can only sense stiffness and strain differences over tens of microns [15], this phenomenon is likely not due to sensing the interface from such a distance [24] but is rather due to random migration toward the interface prior to the onset of durotaxis.

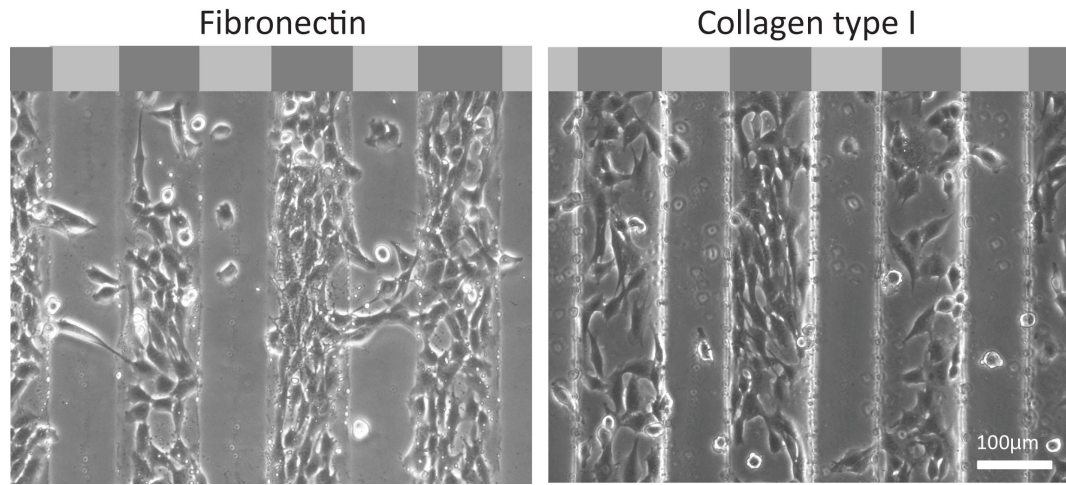


Figure 3.2: Ligand composition does not affect cell adhesion to mechanically-patterned hydrogel. Fibronectin (left) and type I collagen (right), covalently attached to mechanically-patterned hydrogel substrate at the requisite concentrations for cell adhesion [28, 29], did not show significant differences in C2C12 myoblast attachment, migration, or morphology after 2 days in culture. Light and dark gray regions in the schematic at the top of the figure indicates soft (1 kPa) and stiff (10 kPa) regions, respectively.

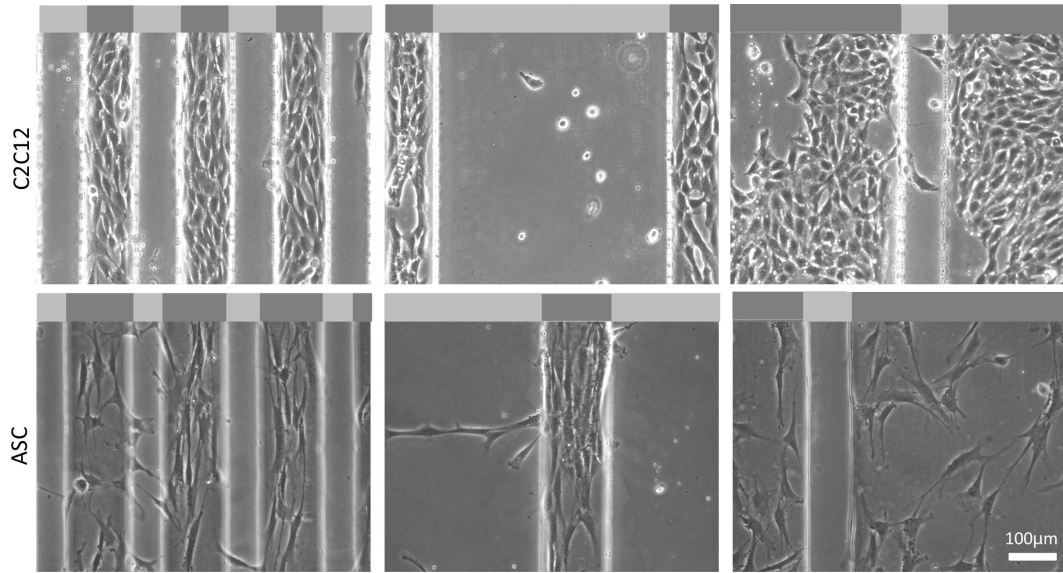


Figure 3.3: Cell adhesion is independent of mechanically-patterned hydrogel pattern dimension. C2C12 myoblasts (top) and ASCs (bottom) after 48 hours in cultured were found only adhered to the stiff regions of mechanically-patterned hydrogels independent of the spacing pattern of soft (light gray) and stiff regions (dark gray). Left: alternating stripes of soft and stiff $100\ \mu\text{m}$ regions. Center: $500\ \mu\text{m}$ (soft) and $100\ \mu\text{m}$ (stiff) alternating stripes. Right: $500\ \mu\text{m}$ (stiff) and $100\ \mu\text{m}$ (soft) alternating stripes.

3.4.2 Cell adhesion and migration based on mechanical pattern

To assess if the cell clustering on stiffer regions of the substrate observed above was due to preferential adhesion or durotactic migration, C2C12 myoblasts were monitored by time-lapse video microscopy for 24 h after initial seeding. Cells settled uniformly and attached to the mechanically-patterned hydrogel, but after 1 h, cell spreading occurred preferentially on the stiffer regions, and by 6 h, cells began to undergo durotaxis toward stiffer regions (Fig. 3.5). Given the variety of contractile cell types that undergo durotaxis, ASCs and cardiomyocytes were also monitored for preferential adhesion and pattern recognition; all three cell types were observed after 48 h to preferentially attach to stiffer regions of the pattern and, for ASCs and C2C12 myoblasts, align in the direction of the long axis of the pattern (Fig. 3.4, top and bottom right). Yet again though, cell patterning was not the result of preferential adhesion as cardiomyocytes were evenly distributed and then migrated onto the stripes (Fig. 3.5A). However for less contractile lineages such as neurons [17], PC12 cells showed no preferential adhesion or migration on either stiffness stripes (Fig. 3.4, bottom left), consistent with previous observations that these cells have a less spread, more neural phenotype on softer matrix [38].

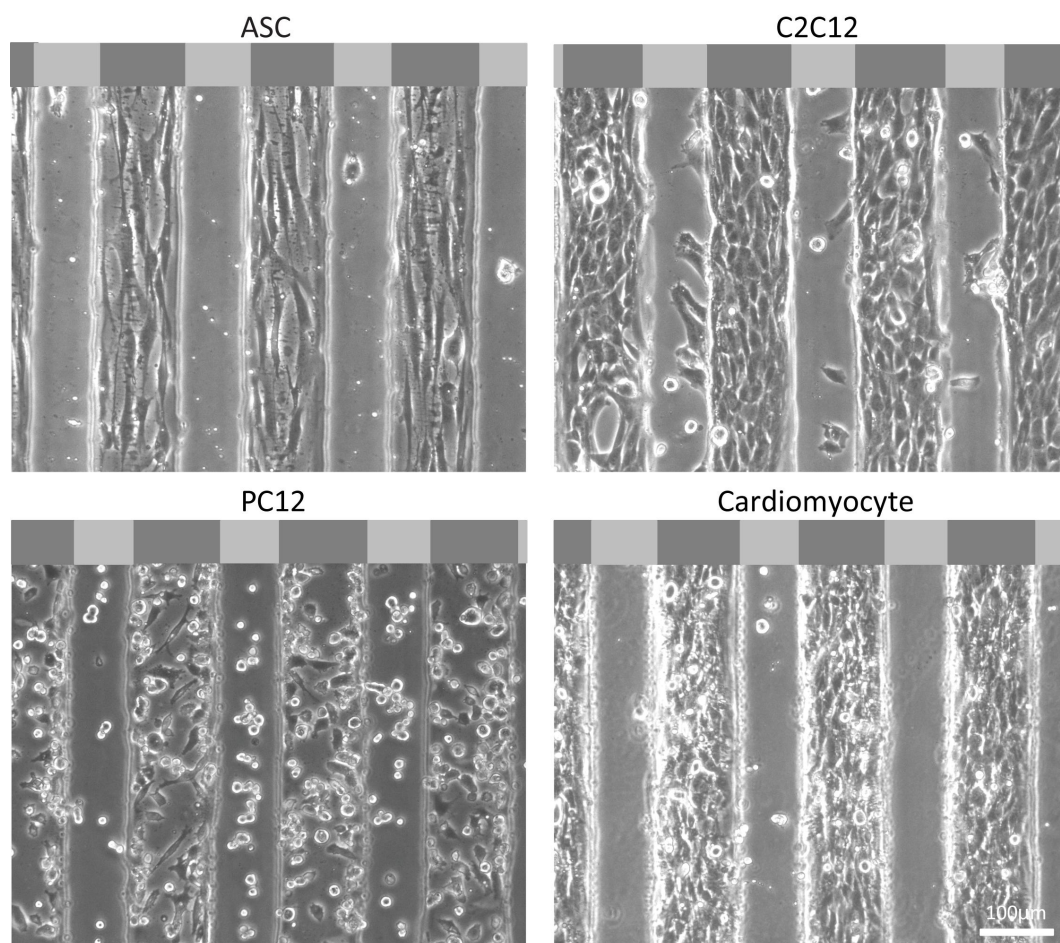


Figure 3.4: Cell Adhesion Preference. Different cell types preferentially localized to the stiffer regions of the neurogenic-myogenic mechanically-patterned hydrogels after 48 h in culture. Phase contrast images show representative images with very confluent cells on the stiffer myogenic lanes for ASC, C2C12, and chicken cardiomyocytes. A portion of the PC12s remained less well-spread and adherent on the softer neurogenic stripes. Light and dark gray regions in the schematic at the top of each image represent soft (1 kPa) and stiff (10 kPa) regions, respectively.

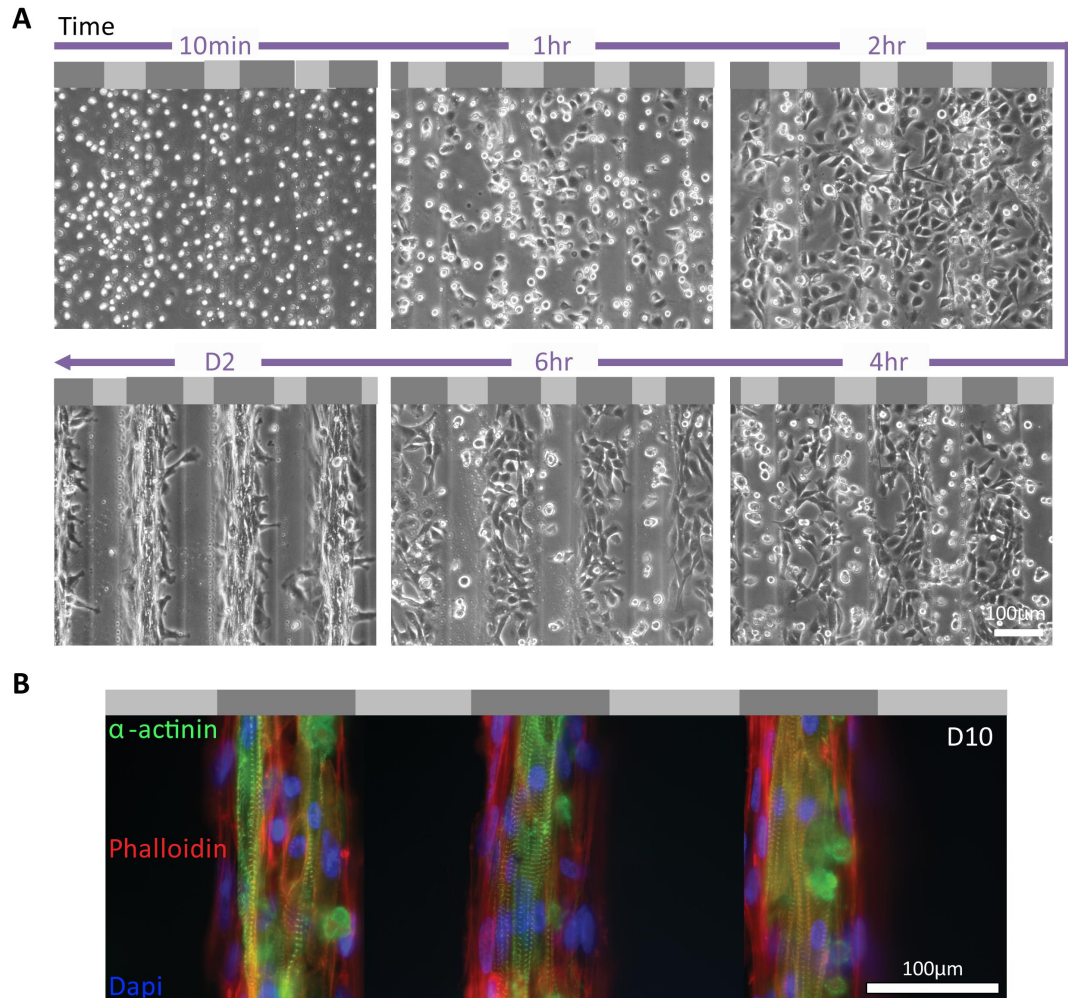


Figure 3.5: Attachment and Alignment of Cardiomyocytes on Neurogenic-Myogenic Mechanically-Patterned Hydrogel. (A) Time lapse images of chicken cardiomyocyte attachment to neurogenic-myogenic mechanically-patterned hydrogel showed adhesion and preferential migration and spreading on stiffer myogenic stripes. Light and dark gray regions in the schematic at the top of each image represent soft (1 kPa) and stiff (10 kPa) regions, respectively. (B) Three consecutive myogenic stripes were fluorescently stained by rhodamine-phalloidin (red), α -actinin (green), and DAPI (blue), revealing an aligned network of cardiomyocytes on myogenic stripes of mechanically-patterned hydrogel at day 10 with adjacent myofibroblasts that lack α -actinin.

While pattern fidelity appears to be robust for contractile cell types, it is not clear to what extent mechanical patterning may improve cell patterning versus more commonly used microcontact printing (μ CP) methods. Using the same pattern, fibronectin was printed onto 10 kPa hydrogels that lacked the mechanical pattern, and its localization was confirmed immunofluorescently (Fig. 3.6, left). ASCs seeded onto the μ CP pattern initially recognized and attached to pattern, but cells failed to remain on the pattern over time when cultured in serum-containing media (Fig. μ , right). It should be noted that local cell density when pattern recognition became compromised for the μ CP pattern was significantly lower than for mechanically-patterned hydrogel (Figs. 3.4 and 3.5 versus Fig. 3.6).

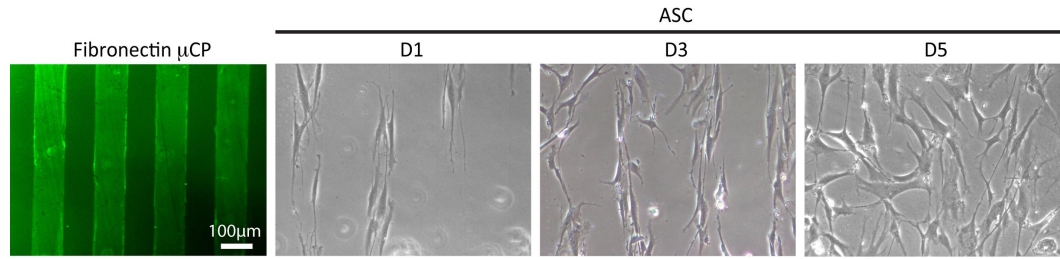


Figure 3.6: ASCs on Fibronectin-Microcontact Printing. μ CP was confirmed by anti-fibronectin staining (right). ASCs recognized pattern initially at day 1 and proliferated and aligned on pattern at day 3, however, pattern was failed from day 5 and cells attached on both printed and non-printed area.

3.4.3 Improved Cell Maturation on Mechanically Patterned Matrix

Primary isolated chicken cardiomyocytes were plated onto neurogenic-myogenic mechanically-patterned hydrogel to determine if the pattern would enhance cell alignment and improve cytoskeletal organization. As a result of mechanical pattern of substrate underneath the cells by day 10, cardiomyocytes were aligned along the long axis of stripes and that allowed cell-cell adhesion with alignment; striations were also well aligned along the stripes, with mature striation length of $1.88 \pm 0.15 \mu\text{m}$ found in almost all cells, better than previous reports using unpatterned myocytes [15]. Myofibroblasts (red actin staining only) were also observed by always at the cardiomyocyte periphery (Fig. 3.5B).

Though relatively mature cardiac cells may reassemble their cytoskeleton on these patterns, it was not clear if undifferentiated stem cells can undergo the same process via lineage specification. Adult stem cells typically undergo a morphology change early in the differentiation process [17], so we first assessed whether cell morphology on mechanically-patterned hydrogels versus stiffness-matched controls was altered. ASCs on mechanically-patterned hydrogel were more elongated with a spindle shape versus cells on unpatterned stiffness-matched hydrogels regardless of culture time (Fig. 3.8C). Since morphology is only an early and indirect indicator of phenotypic changes, ASCmyogenesis was evaluated on neurogenic-myogenic mechanically-patterned hydrogel versus control C2C12 myoblasts. Again both cell types preferentially adhered on stiffer, myogenic stripes at day 1 (Fig. 3.8A, left). C2C12 myoblasts showed long, fused myotubes at day 7 expressing myoD and skeletal fast myosin (Fig. 3.8A, right). Myoblast fusion was independent of pattern geometry as fused, desmin positive myotubes could be observed on larger patterns, though alignment was less robust at larger pattern spacing (Fig. 3.7). ASCs also underwent myogenesis, expressing MyoD by day 7 (Fig. 3.8A, right), despite myotube formation not being as apparent when observed in brightfield (Fig. 3.8A). Careful inspection with cytoskeletal proteins, however, indicated that ASCs on stripes of myogenic stiffness did in fact form fused, multi-nucleated cells that were negative for the proliferation marker Ki67 (Fig. 3.8B), consistent with

previous reports on unpatterned, myogenic matrix [7]. However with the enhanced alignment of cells on patterned versus unpatterned, especially at day 7 (Fig. 3.8D), we noted that the fusion rate on mechanically-patterned hydrogel was almost twice ($3.8 \pm 0.3\%$) as the rate on unpatterned 10 kPa gels ($2.0 \pm 0.3\%$) (Fig. 3.8E, top). Only binucleated myotubes were previously observed from matrix stiffness induced differentiation, but with the enhanced alignment from mechanically-patterned hydrogel, ASC-derived myotubes with more than 3 nuclei were observed (Fig. 3.8E, bottom).

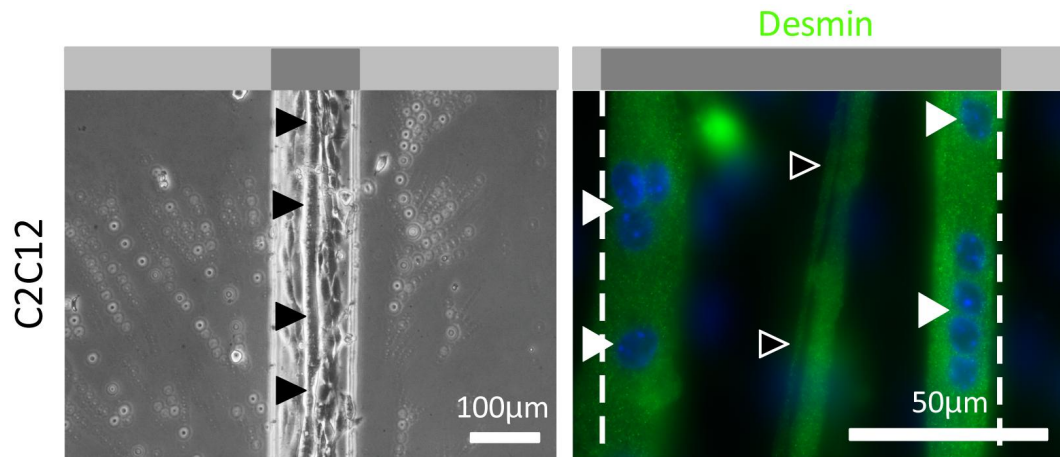
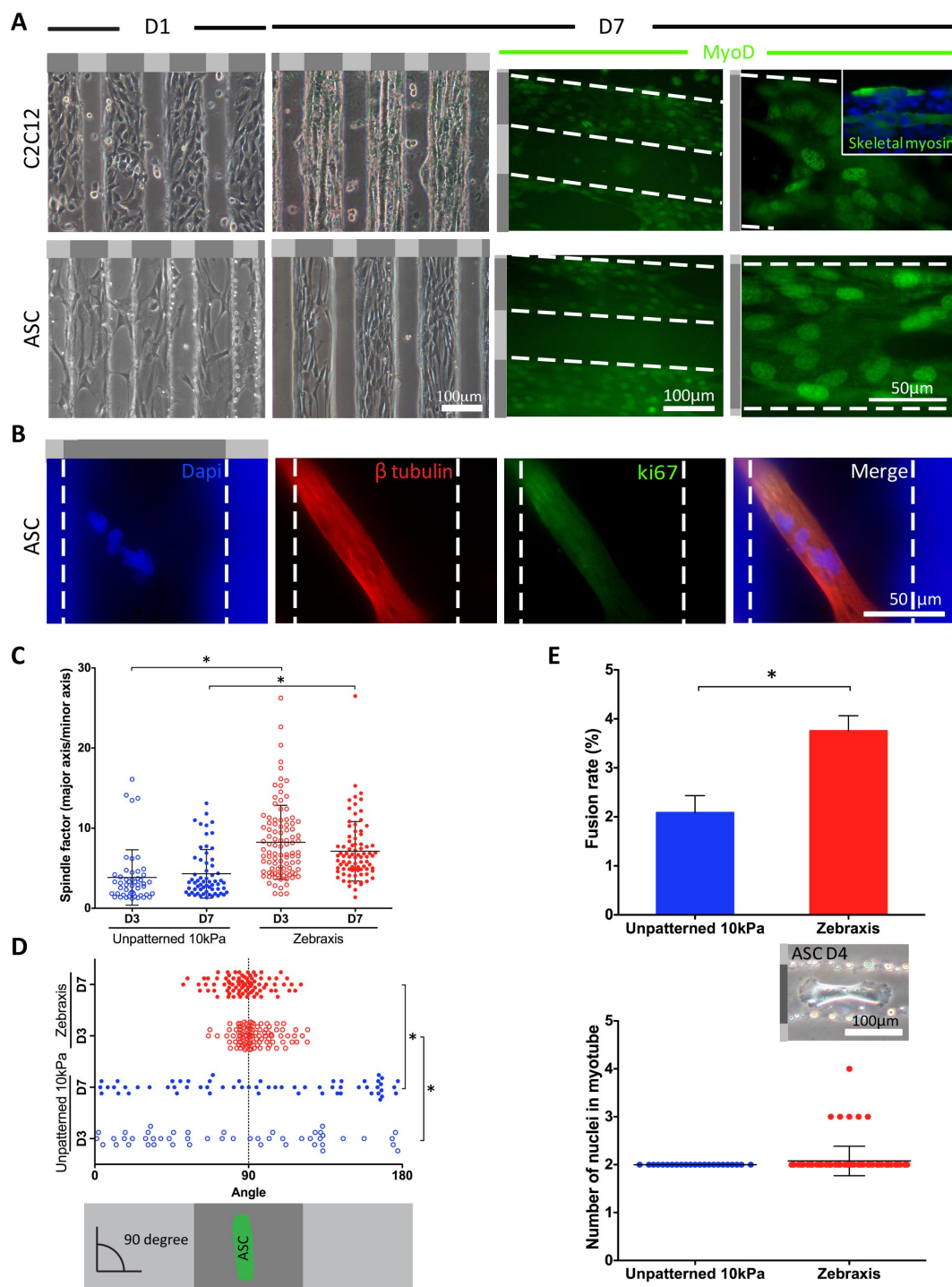


Figure 3.7: Myotube Formation is Independent of Mechanically-Patterned Hydrogel Pattern Size but is Improved by Cell Alignment. (Left) Brightfield image of C2C12 myoblasts fusing into highly aligned myotubes after 7 days on mechanically-patterned hydrogel with 500 μm (soft) and 100 μm (stiff) alternating stripes. (Right) On mechanically-patterned hydrogel with 500 μm (stiff) and 100 μm (soft) alternating stripes, C2C12 myoblasts fused into myotubes expressing desmin but alignment with mechanically-patterned hydrogel was diminished. Closed and open arrowheads indicate highly and less well-aligned myotubes, respectively. Light and dark gray regions in the top schematic represent soft (1 kPa) and stiff (10 kPa) regions, respectively. White dashed lines indicate the edges of each region of mechanically-patterned hydrogel.

Figure 3.8: Mechanically-Patterned Hydrogel Enhances Myotube Fusion of ASCs. (A) Representative cell alignment on neurogenic-myogenic Zebraxis hydrogels at day 1 and 7 for both C2C12 and ASCs shown in phase contrast (top) and with MyoD staining at day 7 (bottom). Inset image shows C2C12 myoblasts, which further differentiated into skeletal fast myosin-expressing myotubes. White dashed lines indicate the edges of each region of the Zebraxis hydrogel. (B) Representative multi-nucleated myotube stained with Dapi that was identified with β -tubulin (red) and negative for ki-67 staining (green), which is indicative of quiescent cells. (C) Spindle factor from ASCs on Zebraxis were significantly greater than those on unpatterned 10 kPa PA hydrogels at both day 3 and 7. (D) To show the alignment of ASCs on gels, cell angle was measured by phalloidin staining (90° — perfectly aligned on mechanically-patterned hydrogels). ASCs on Zebraxis showed higher level of alignment than those on unpatterned 10 kPa PA hydrogels at day 7. (E) A higher fusion rate was observed on the mechanically-patterned hydrogels compared to unpatterned 10 kPa PA gels (top) [7]. The number of nuclei per mechanically-patterned hydrogels or unpatterned hydrogel myotube was also quantified (bottom). The inset image shows characteristic multi-nucleated myotube in phase contrast. Light and dark gray regions in the schematic next to or on top of each image represent soft (1 kPa) and stiff (10 kPa) regions, respectively. * $p < 0.05$ for all indicated comparisons.



3.5 Discussion

Our ability to regulate cell fate via matrix chemistry and/or mechanics has been well documented [3,4,13,14,21], and while stem cell hapto- [10] and durotaxis [11] has been observed via gradients, the effects of precisely patterned matrix on stem cell behavior has not been completely explored. Such precise patterns are commonly found *in vivo*, e.g. skeletal muscle contains layers of aligned myotubes [15,17,22] juxtaposed with regions where softer neurons innervate the firm muscle [23]; thus our objective was to develop a hydrogel platform that mimicked *in vivo* ECM stiffness of normal muscle to examine stiffness-based micropatterning's influence on stem cell fate. Relative to μ CP patterns, myogenic stiffness patterns remain robust over time and produce cultures of aligned stem cells that fuse at higher rates and with greater numbers of fusing cells than unpatterned matrices. These data implicate matrix-based patterns as means of enhancing ECM-based cell cues, but closer examination of these data is warranted in the broader context of ECM and micropatterning.

3.5.1 Matrix Considerations when Micropatterning

While the fidelity of longer-term μ CP patterns on compliant hydrogels may be difficult, mechanically-patterned hydrogels also have a unique set of considerations that must be overcome. For example, differential swelling has been reported in microfabricated hydrogels but can be minimized when using substrates with high crosslinker content [36]; here we report the same observation where mechanically-patterned hydrogels can be fabricated with minimal differential swelling, i.e. $\pm 2 \mu\text{m}$, and with minimal roughness difference, i.e. $\pm 200 \text{ nm}$, between soft and firm regions when the crosslinker fraction is higher than previously used [11]. The 2-step soft lithography process used in creating the mechanically-patterned hydrogel also makes predicting hydrogel stiffness inherently difficult due to layer diffusion during polymerization. In hydrogels with wider spaced stripes, e.g. the 500 to 100 μm stripe width mechanically-patterned hydrogel, and/or when sequentially polymerized within a single layer [18], stiffness changes from monolayer hydrogels

were less appreciable; the amount of monomer and crosslinker from the large second layer was not significantly depleted. Yet with sequentially polymerized small stripes of equal spacing, significant deviations from monolayer hydrogels [11] were evident as depletion may cause noticeable loss of monomer from the second layer. As a result, small changes in monomer, e.g. 4% (bottom) versus 3.2% (top), produce regions with an order of magnitude difference in stiffness. Materials that lack mixing issues, e.g. polydimethylsiloxane (PDMS), may have a more predictable but often limited stiffness range when creating spatial stiffness patterns [39]. However all of these materials appear to have similar ligand binding independent of stiffness, as was observed here with similar cell densities on fibronectin- and type I collagen-coated substrates.

Aside from other materials, stiffness gradients have been created using photoinitiators and masks [11,40,41], but significant diffusion and UV diffraction prohibited the sharp stiffness gradient present at the stripe interface of mechanically-patterned hydrogels, e.g. ~ 200 kPa/mm; only chemical initiators could create such transitions. Another common method used to create molds similar to the photore-sist mold here is to etch the pattern into a glass substrate. However deep etching with hydrofluoric acid may compromise pattern sidewalls [15], thus obtaining sharp feature transitions in the resulting hydrogel cast from the mold is likely to be difficult. On the other hand once the mold is fabricated, the 2-step polymerization method used to make mechanically-patterned hydrogels can be performed rapidly and within almost any setting, aside from tuning the acrylamide concentrations appropriately for the desired stiffness.

3.5.2 Improving Cell Fate and Mimicking Disease

Mechanical patterns encouraged ASC elongation and alignment, perhaps in part by directing how cell tractions, which are required for fusion [7], propagated through the matrix to align myoblasts [15,24]. The end-to-end cell orientation likely increased the probability of M- and N-cadherin contact between cells, which is required for fusion and is regulated by RhoA [26]. It has been suggested that other fusion related proteins have contractility-dependent expression, including

the Rho-GTPase-activating protein (GRAF1) [42] and myoferlin [43]. Though they do not fuse, striation assembly in cardiomyocytes is also directly regulated by N-cadherin-containing structures [44], whose disruption or misalignment in an unpatterned matrix could impair assembly. Further molecular evidence supporting mechanically-patterned hydrogel mechanism is that loss of talin, which itself is a mechano-sensor [45], impairs both myoblast fusion and sarcomere assembly [46].

Mechanical patterns do not just encourage alignment, they can be used to mimic the diseased niche. Muscle related diseases, while they increase global tissue stiffness, have shown dramatic changes in local ECM stiffness from the fibrosis that they induce [15,47,48]. A myogenic-osteogenic mechanically-patterned hydrogel mimics the stiffer, fibrotic but heterogenous environment of dystrophic muscle [15]. Prior to *in vivo* studies with injected stem cells, sequentially culturing muscle and ASCs on a mechanically-patterned hydrogel could allow one to examine how ASCs might behave in the diseased state *in vivo*. Mechanically-patterned hydrogel may also be suitable for studies examining mechanisms behind impaired fusion with fibrotic muscle diseases. For example, it is not certain if the traction-mediated M-cadherin expression mentioned above, which likely becomes altered in the diseased state, is responsible for M-cadherin's down- and up-regulation in caveolin-3 transgenic and null cells, respectively [49]. Taken together, mechanically patterned hydrogel is likely a suitable platform to pose a variety of muscle function questions.

3.5.3 Differential Cell Sorting for Organoid Culture

Differential adhesion did not occur on mechanically-patterned hydrogels, though it has been reported on other durotactic substrates [39]. However all of these substrates take advantage of the well-established observation that contractile cells exhibit a 'normal' phenotype on stiffer substrates [15,28,29,50]. The lack of differential adhesion here can simply be explained by our durotactic observation with C2C12 myoblasts or from the accumulation of cells treated with mitomycin C in stiffer regions of a gradient hydrogel [11]. Yet mechanically-patterned hydrogel can also use cells which prefer softer niches, e.g. neurons [38], to pattern cells

of different stiffness preference. On the other hand, Tien and coworkers have used μ CP to pattern different ligands where a stamp is used to selectively block protein adsorption when the substrate is bathed in a ligand [51]. While they used the stamp to also selectively place cells on their patterns, two model cell lines expressing specific integrin heterodimers may recognize a differential ligand pattern and sort themselves once plated onto the substrate; such behavior in stem cells, which undergo stiffness-mediated differentiation only on certain ligands [9], is not likely. Yet differential sorting does occur during development [20], and these substrates may serve as a strategy to recapitulate that process *in vitro* and build cultures where neurons and muscle cells can be juxtaposed to encourage intercellular signaling.

3.6 Conclusions

We have demonstrated here that mechanically patterned cell culture substrates, which mimic some of the spatial parameters of ECM *in vivo*, can be used to orient muscle and stem cells into favorable positions for cell fusion and striation assembly, not by preferential adhesion but rather by their ability to durotax to a prefer set of environmental conditions. Opposing this behavior are cells such as neurons, which prefer a softer niche, and these data support the development of cultures where muscle and neuron are juxtaposed with each other to form nascent junctions. Longer-term patterning required for these observations was not possible via conventional μ CP on compliant hydrogels, which suggests mechanically-patterned hydrogel as an alternative when substrate stiffness is a concern for patterned cells. Mechanically-patterned hydrogels may also serve as a potential platform in which to study mechanically induced cell behaviors in diseased muscle, which may have juxtaposed regions of firm and stiff tissue, e.g. the myogenic-osteogenic mechanically-patterned hydrogel. These data support the importance of physiologically relevant mechanical guidance cues that matrix presents to cells in regulating higher order cell functions, e.g. striation and fusion.

Chapter 3 in a reprint of the material *Choi Y S, Vincent L G, Lee A R, Kretchmer K C, Chirasatitsin S, Dobke M K, Engler A J. The alignment, fusion and striation assembly of adipose-derived stem cells on mechanically patterned hydrogel matrices. Biomaterials. 2012; 29: 6943-6951.* The dissertation author was the second author.

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Chapter 4

Modulating Cell Contractility by Elongated Stem Cell Morphology and Matrix Stiffness

4.1 Introduction

Human adult mesenchymal stem cells (MSCs) are multipotent stem cells that have the potential to become various cell types including bone, muscle and fat. Previous work in mechanobiology has shown that MSC differentiation towards a specific lineage is optimal on substrates that match the mechanical properties of that tissue in vivo, i.e. myogenesis on muscle-like stiffness [1]. Similarly, the fate of MSCs depends on both cell shape [2] and the area over which cells can spread [3]. MSCs thus 'feel' the properties of their surrounding extracellular matrix (ECM) via myosin and differentiate in response to them, e.g. stiffness, porosity, cell shape, ligand composition, etc.

As evident from these studies and others, cell force generation is crucial in driving differentiation. Mechanotransduction, the conversion of cell-generated traction forces into biochemical signals, is thought to be responsible for guiding cellular processes like spreading, migration, and differentiation [4]. Disrupting or tampering with the contractile mechanisms using inhibitors changes the archi-

texture of the cytoskeleton, as well as the physical properties of cells such as cell stiffness spreading [5], not to mention cell-generated traction forces and as a result, numerous biochemical signals .

Many degenerative diseases, such as muscular dystrophies, cause abnormal stiffening of the local environment, or niche, as a result of fibrosis [6]. Aberrant changes in ECM composition are sensed by cells through mechanical interactions. Changes in the mechanical properties of the ECM also changes the expression of myosin isoforms, and therefore the ability of cells to contract against their environment [1]. These cues alter the cytoskeletal structures that over time creates variability in cell behavior and misdirect stem cell fate [7, 8].

Since mechanical interactions with the external environment alter the cytoskeleton and cell behavior, we sought to modulate the physical properties of the environment to guide cell behavior. That is, by controlling cell shape, cell spread area, and matrix stiffness in a disparate fashion, we hypothesized that a myosin contraction-dependent balance could induce a subset of MSCs to become myogenic despite residing in a dystrophic-like stiff niche. For instance, elongated MSCs on an abnormally stiff microenvironment would maintain myosin activity similar to cells on normal ECM. Dialing in multiple disparate cues that alter cell contractility independently, and therefore traction forces, is one approach to decouple the intracellular signal pathways that ultimately control cell fate. Careful tuning of cell shape and cell spread area could commit MSCs down the myogenic pathway in a stiffness-independent manner.

4.2 Methods

4.2.1 Polyacrylamide Gels

To covalently attach hydrogel substrates to glass, glass coverslips (Fisher) were cleaned of organics and oxidized by exposing both sides for 3 minutes to UV/ozone (BioForce). Samples were immediately functionalized with 20 mM 3-(trimethoxysilyl)propyl methacrylate (Sigma) in ethanol, washed with ethanol, and dried. A polymer solution containing acrylamide monomers, crosslinker N,N

methylene-bis-acrylamide, 1/100 volume of 10% ammonium persulfate (APS), and 1/1000 volume of N,N,N',N'-Tetramethylethylenediamine (TEMED) was prepared. 25 μ L of polymerizing hydrogel solution was sandwiched between a functionalized coverslip and a dichlorodimethylsilane (DCDMS)-treated glass slide to ensure easy detachment of hydrogels subsequent to polymerization. Hydrogels were allowed to polymerize for 15 minutes then soaked in water prior to use as a cell culture substrate. The ratio of acrylamide%/bis-acrylamide% was varied in order to control hydrogel stiffness.

4.2.2 Microfabrication

A high resolution photomask was designed in AutoCAD (Autodesk) and transferred in chrome on a fused silica substrate (Advanced Reprographics). Molds for patterns were created using photolithography. Photoresist SU-8 2015 (Microchem) was spin-coated onto a silicon wafer generating a ~ 20 μ m thin film. The photomask was placed in hard contact on top of the photoresist and the sandwich exposed to UV light using a mask aligner (MA6, SUSS Microtech). The exposed regions remained as a positive feature after the wafer was developed to strip unpolymerized regions. Wafers were washed with isopropanol and dried with ultrapure nitrogen. A Dektak profilometer (Veeco) was used to characterize the height of the features. Prior to use, the master wafers were treated overnight with the fumes of (Tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies) under vacuum to promote subsequent release of elastomer.

4.2.3 Protein Microcontact Printing

A 10:1 polydimethylsiloxane (PDMS) elastomer:curing agent solution (Sylgard 184, Dow Corning) was thoroughly mixed and degased under vacuum for 1 hour before pouring over the master wafers, baked at 60°C for an hour in an oven, and then released from the wafer. Individual sections of the PDMS mold containing the target shape were cut and stored in acetone. PDMS stencils were incubated with a thin film of a 50 μ g/ml human plasma fibronectin solution sandwiched

between a coverslip and the stamp for 20 minutes. Meanwhile, polyacrylamide gels prepared as described above were incubated in a solution of 0.2 mg/ml sulfo-SANPAH in 50mM HEPES pH 8.5 and placed for 10 minutes under a 1 mW/cm² 350nm UV source. Following three washes in 50mM HEPES pH 8.5, the gels were placed on a hotplate set to 60°C until all remaining solvent had evaporated. PDMS stencils were brought in direct contact with the gels for 5 min and a small weight was placed on top of the stamp to ensure good contact between the stencil tool and the PA substrate. Hydrogels were peeled from the stamps using tweezers and the resulting substrates were stored in phosphate buffered saline (PBS) prior to use for cell culture.

4.2.4 Cell Culture

Human ASCs were isolated from freshly aspirated human subcutaneous adipose tissue according to the method described elsewhere [9]. MSCs were commercially purchased from a reputable source (Lonza). MSCs and ASCs were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum and 1% v/v antibiotics. For experiments, MSCs and ASCs were seeded on PA substrates at a density of 500 cells/cm² and the medium was changed to fibronectin free-serum at either 1% or 10% final concentration in DMEM with antibiotics.

4.2.5 Immunofluorescence Staining

Cells were fixed with 3.7% formaldehyde in PBS with 1 mM MgCl₂ at room temperature for 15 min. Samples were permeabilized with PBS containing 1% Triton X-100 for 15 min and washed. Cells were stained with anti-vinculin primary antibody (1:400) in a staining solution containing 2% bovine serum albumin for 30 min at 37°C. After washing, samples were incubated with Alexa fluor 488-secondary antibody (1:400; Invitrogen), rhodamine phalloidin (1:1000; Invitrogen), and by Hoechst 33342 (1:1000; Invitrogen) for 30 min at room temperature. Samples were examined with an epifluorescent microscope and imaged. Data analysis was performed in ImageJ using the FIJI suite.

4.2.6 Atomic Force Microscopy

Substrate stiffness was confirmed by atomic force microscopy (AFM; MFP3D, Asylum Research) as detailed previously [10, 11]. Briefly, a pyramidal probe, 0.02 N/m spring constant with a 35° half angle (TR400PB, Olympus), was used to indent the substrates. Indentations were conducted at $2 \mu\text{m/s}$ with a trigger force of 2 nN. Analysis to determine the mechanical properties of substrates was performed in IgorPro (Wavemetrics) using a linearized Hertz cone model with a sample poisson ratio of 0.5 [10].

4.2.7 Traction Force Microscopy

Traction force microscopy was performed as previously described [12]. Briefly, fluorescent $0.2 \mu\text{m}$ microspheres were added to the pre-polymer solution. Instead of glass coverslips, 35 mm glass bottom dishes (MatTek) were utilized for live cell imaging. Microspheres underneath selected live cells were imaged at with a 40x dry confocal objective (Zeiss) using the CV1000 high-speed laser confocal imaging system equipped with an EM-CCD camera and a stage-top incubation system (Yokogawa). Traction force microscopy was performed as previously described [12] using a particle image velocimetry script in MATLAB (MathWorks). All reported forces generated during cell contraction are relative to the resting state of the gel after cells were released with trypsin.

4.3 Results

To control cell shape and spread area, polydimethylsiloxane (PDMS) substrates were fabricated using conventional soft photolithography. The height of the features were $5 \mu\text{m}$, and easily controlled by tuning the spin speed during the photoresist deposition step of the fabrication process (see methods). The different shapes designed on the photomask were nicely replicated both on the master wafers and the elastomeric stamps (Fig. 4.1).

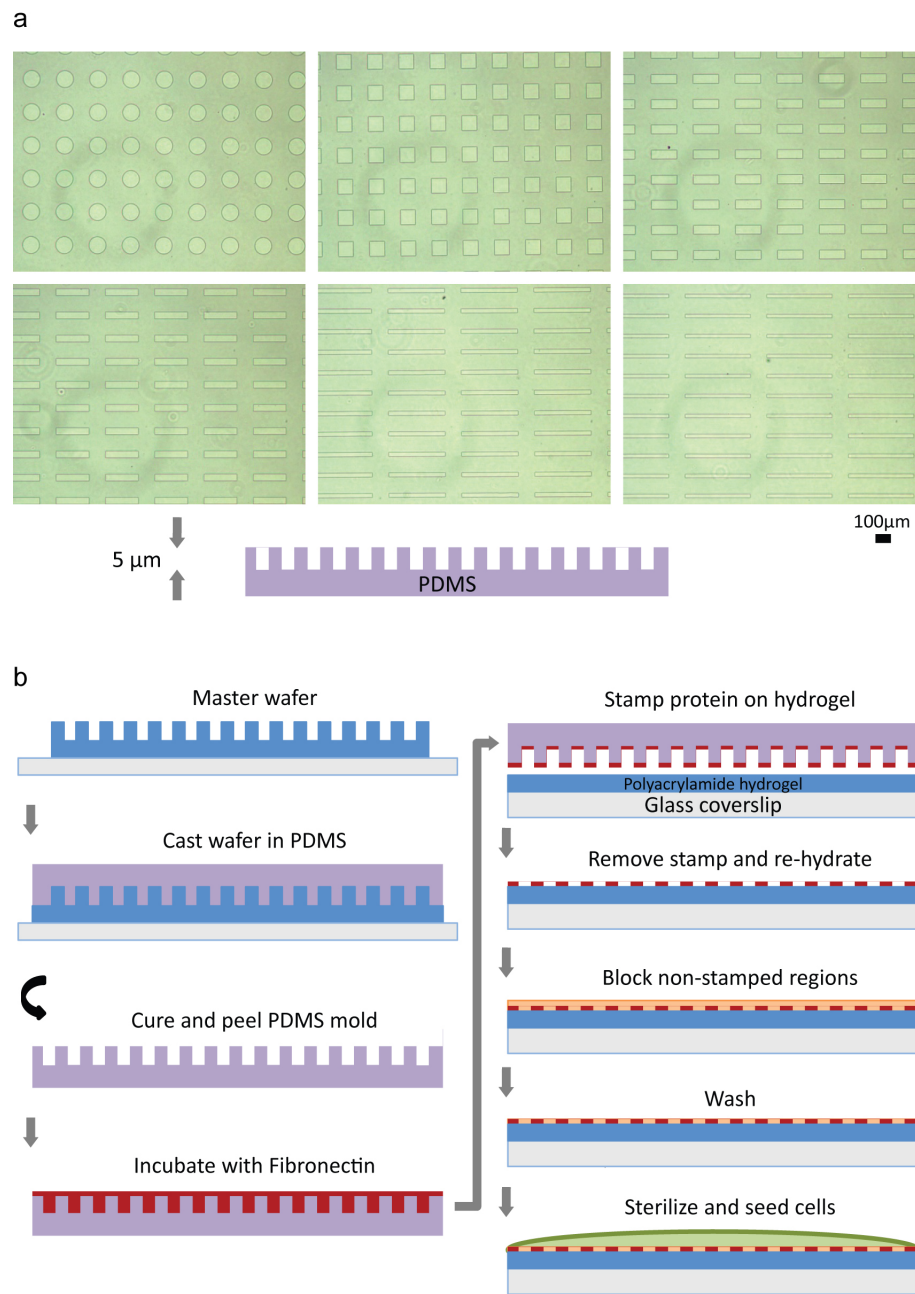


Figure 4.1: Protein Microcontact Printing. (a) PDMS stamps with different shapes and constant area of $7000\mu\text{m}^2$. (b) Illustration depicting the microcontact printing process.

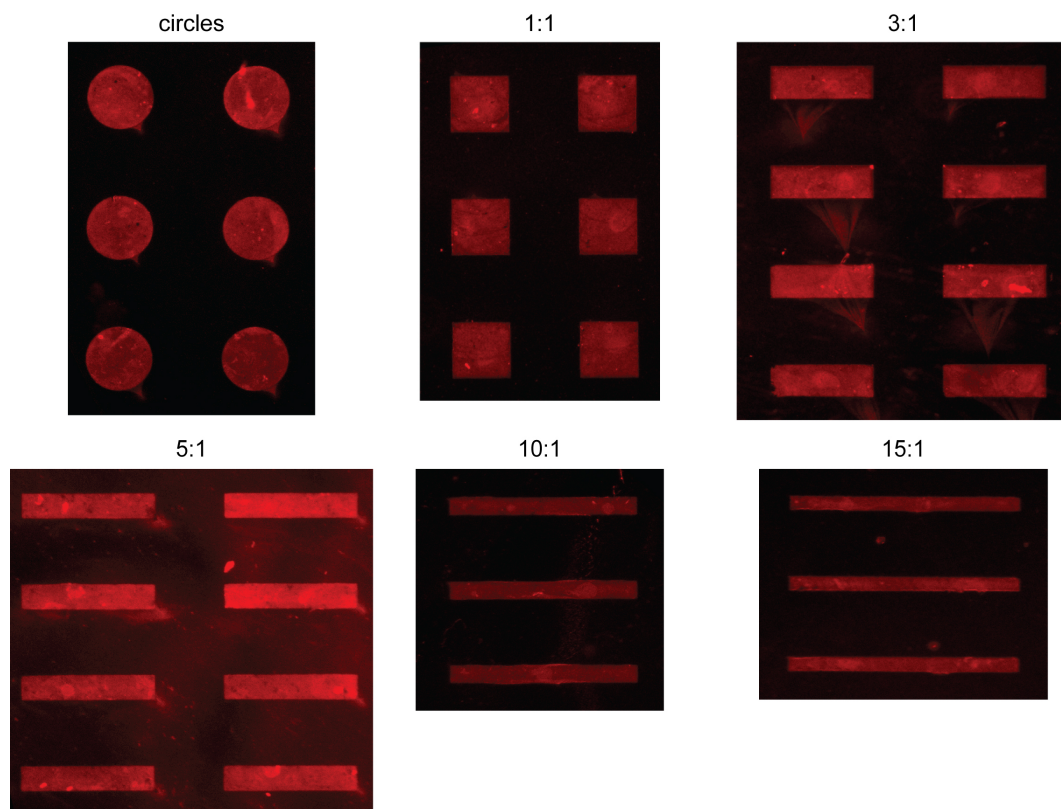


Figure 4.2: Fibronectin Protein Islands. Fibronectin protein islands printed on 10 kPa polyacrylamide substrates stained with an anti-fibronectin antibody and red fluorescent secondary antibody. Microcontact printing allows for deposition of protein on the substrate with high spatial accuracy and little bleed.

Stamps incubated in the protein fibronectin according the process depicted in Figure 1 were able to faithfully transfer the protein onto a thin activated hydrogel. This stamping step patterns only the protruding features onto the hydrogel, providing spatial control over where the protein is localized on the substrate (Fig. 4.2).

After blocking to prevent non-specific cell attachment, MSCs plated on the protein island responded to these patterns, spread to defined shape, and maintained the shape for up to five days when cultured in 1% fetal bovine serum (FBS) depleted of fibronectin (Fig. 4.3a). Culturing cells in complete serum at 1-10% was not sufficient to prevent the majority of cells to divide or migrate out of the confines of the protein island (data not shown).

Cells on patterns in fibronectin-depleted low serum conditions aligned cytoskeletal elements, notably actin fibers, in the direction of the long axis of the pattern. This effect was more pronounced on higher aspect ratio islands — 5:1, 10:1, and 15:1. Cells on isometric square patterns preferentially assembled actin fibers along the edges of the pattern while cells on circle patterns had circumferential stress fibers connecting radially oriented fibers (Fig. 4.3a).

The localization of vinculin, a protein that localizes to stabilize focal adhesions (FAs), was used to determine where cells connected to the underlying matrix on 10 kPa (kilo Pascal, unit of stiffness) substrates (Fig. 4.3b). Immunofluorescence images revealed that vinculin clustered at the periphery of cells on protein islands. On circular isometric shapes, cells assembled FA uniformly at the periphery of the shape. On isometric square shapes, FA were concentrated at the apex of any two edges. On more highly elongated patterns, FAs were found throughout the spread area of the cell but did tend to concentrated along the edge of the short axis (Fig. 4.3 and 4.4b). These images imply that cell shape directs the spatial arrangement and differential clustering of FAs.

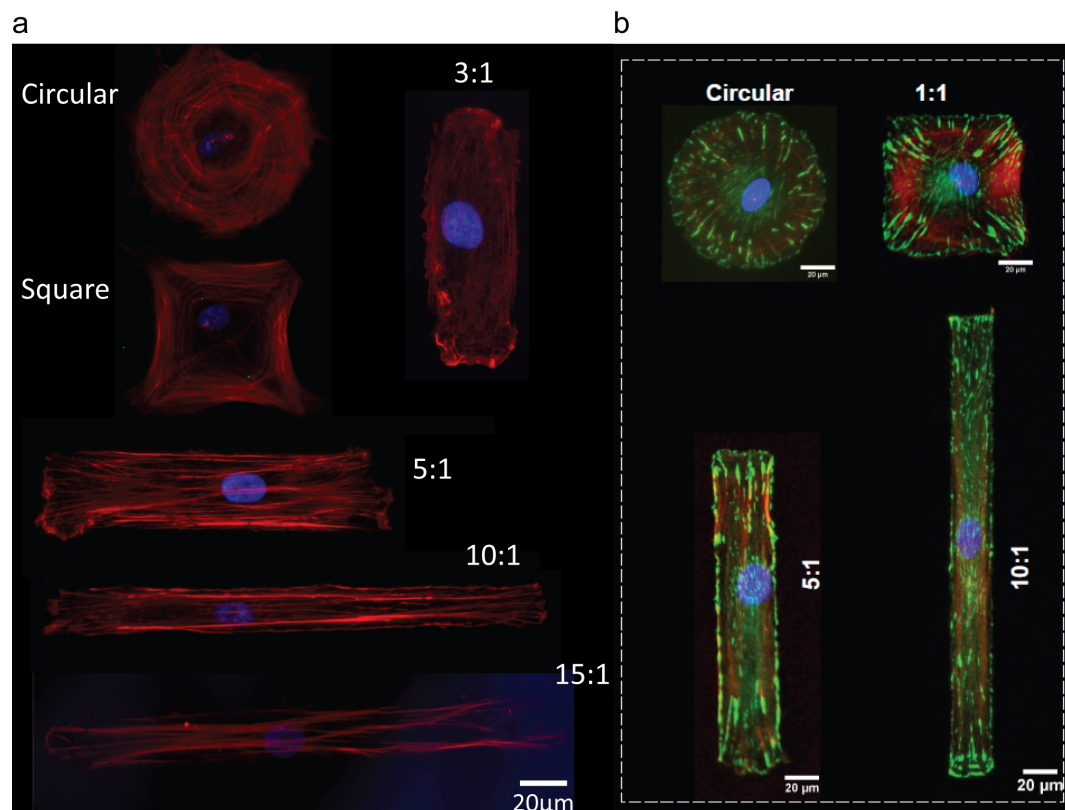


Figure 4.3: MSCs on Protein Islands of Different Shape. (a) MSCs attached and spread to the protein islands after 5 days in culture in low serum fibronectin-depleted culture conditions on 10 kPa hydrogels. Red: Actin (rhodamine phalloidin), Blue: Nucleus (Hoescht). (b) Vinculin localizes to focal adhesions and is regulated by cell shape on 10 kPa polyacrylamide hydrogels. Red: Actin (rhodamine phalloidin), Blue: Nucleus (Hoescht), Green: Vinculin.

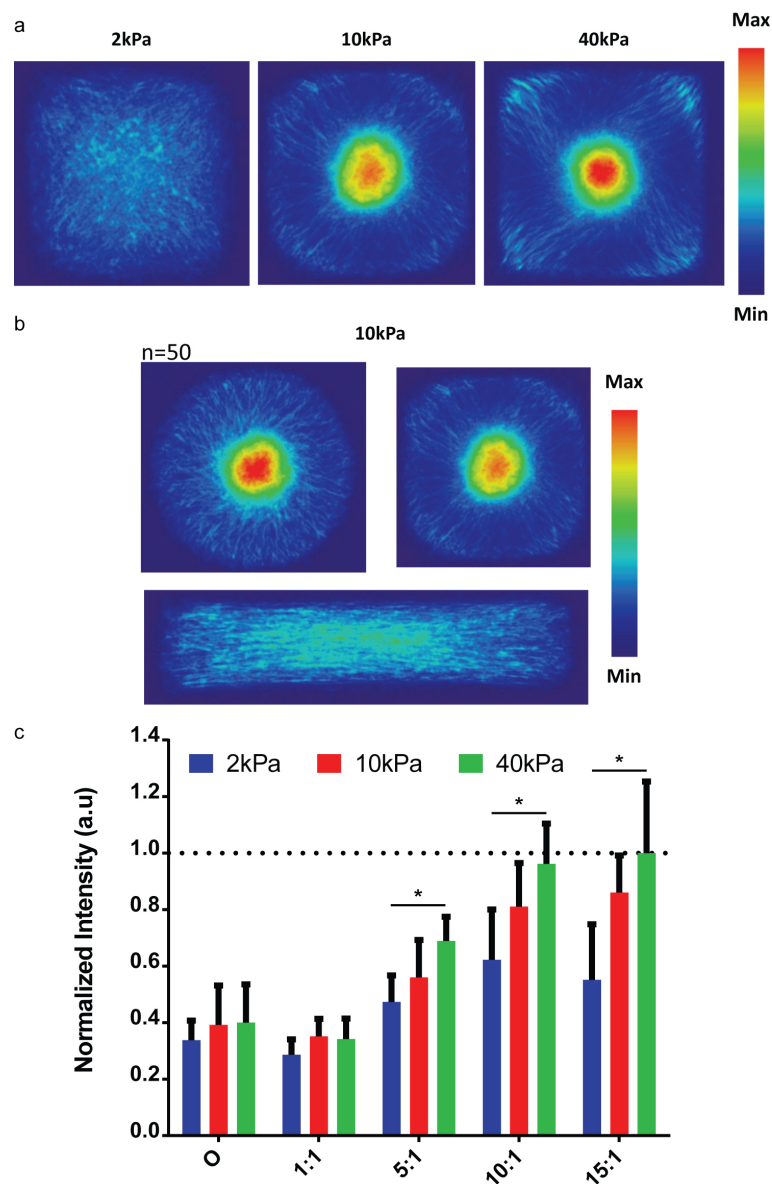


Figure 4.4: Heat Maps and Quantification of Vinculin from MSCs on Patterns. (a) Vinculin heat maps of MSCs on square patterns and various underlying substrate stiffness. (b) Vinculin heat maps of MSCs on different patterns and similar underlying substrate stiffness. (c) Quantification of vinculin expression of cells various patterns and various underlying substrate stiffness.

To determine if cell shape and substrate stiffness altered vinculin expression and localization, heat maps of immunofluorescence image of cells on patterns stained with a vinculin antibody were generated. The aspect ratio trends observed on 10 kPa hydrogels were similar on soft (2 kPa) and stiff (40 kPa) substrates. On soft substrates, vinculin loosely clustered to the edges of cells on square patterns whereas on stiff substrates robust vinculin localization to the corners was evident (Fig. 4.4a), suggesting that substrate stiffness modulated spatial arrangement of FAs.

Since myosin isoform expression was previously shown to depend on substrate stiffness [1, 13], we wanted to establish if vinculin expression also scaled with substrate stiffness. Immunofluorescence quantification of vinculin heat maps suggests that substrate stiffness does not affect the total amount of vinculin per cell only for cells on isometric shapes (circles and squares). On more highly elongated patterns (5:1, 10:1, 15:1) vinculin expression scaled with substrate stiffness and trended with increasing aspect ratio (Fig. 4.4b and c). Despite maintaining a constant spread area across all shapes, vinculin expression was greatest for highly elongated cells on stiff substrates (Fig. 4.4c). These data suggest that cell shape and substrate stiffness act together to govern vinculin levels in MSCs.

Traction force microscopy was employed to measure cell-generate traction forces. This analysis requires the user to acquire the position of fiduciary tracking particles embedded inside the substrate and then to mathematically correlate the bead position in space between a reference and initial frame. While the details have been worked out elsewhere [12], one particularly time intensive step is to define the top slice of each z-stack for each cell for both the initial and reference stacks. To define the initial slice from which to conduct our mathematical analysis, we plotted the integrated intensity of the bead signal as a function of the z-axis. Beads fluoresce over a finite distance in three dimensions according to a point-spread function, and the integrated signal for any given slice is the result of the convolution of all the fluorescent bead signals at that given plane. At the top slice, there will be no signal contribution due to the lack of beads in the positive z-direction while the bead-containing slices below (-z direction, within the substrate) will still

contribute to the measured signal. Thus, the surface of the substrate is defined as the point of inflection of the sigmoid-shaped curve of this integrated signal profile in z . The inflection point in the fluorescence signal was easily and automatically determined using a MATLAB script which found the minimum of the derivative of the integrated density with respect to slice number using numerical methods, greatly increasing the output of the software while decreasing user dependence (Fig. 4.5). This technical improvement allowed for increased efficiency and consistency in the analysis for the various experiments, while removing the potential for an otherwise user-introduced uncertainty.

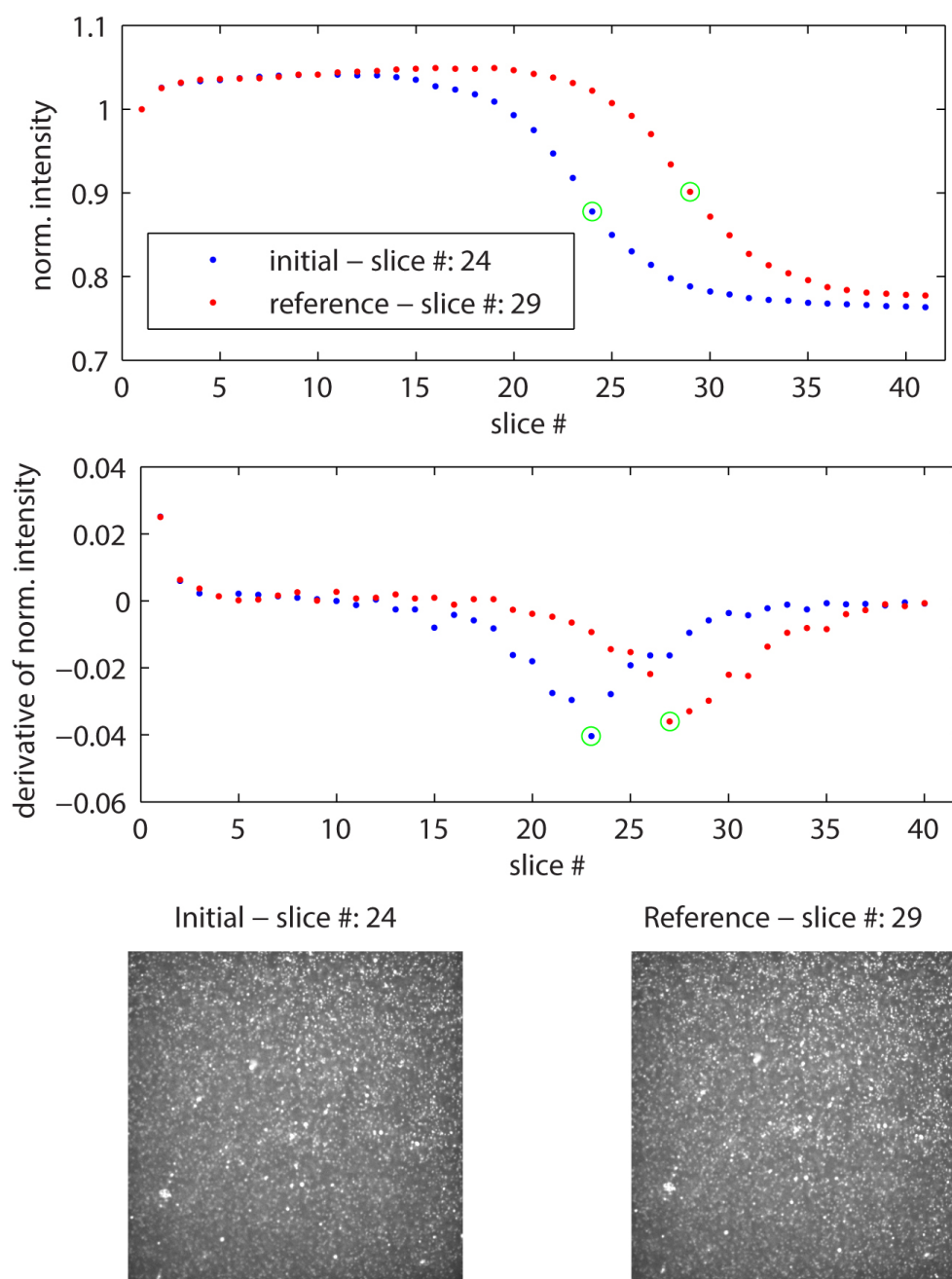


Figure 4.5: Mathematical Determination of Gel Surface for TFM. To account for changes in gel height due to swelling or movement of the dish from the holder, the integrated density of the signal from each stack slice is plotted and the index at which the inflection point occurs mathematically determined by finding the minimum of the first derivative (green circles). The index or slice determined as the substrate surface for the initial and reference stack are shown.

We next wanted to determine if cell-generated traction forces localized to FAs. Traction force microscopy of cells on isometric shapes indicated that force generation co-localized with vinculin, as expected, for both tangential and normal stresses (Fig. 4.6 and Fig. 4.7). Because cells reside just above the plane of the substrate, tangential forces create torques around focal adhesions that are counteracted by a vertical resistance from the substrate. These normal forces are spatially depicted in figure 7 and localize with FAs. Since this mathematical problem is (essentially) static, the resulting downward normal forces are spread somewhat uniformly over the rest of the cell spread area (Fig. 4.7, blue areas).

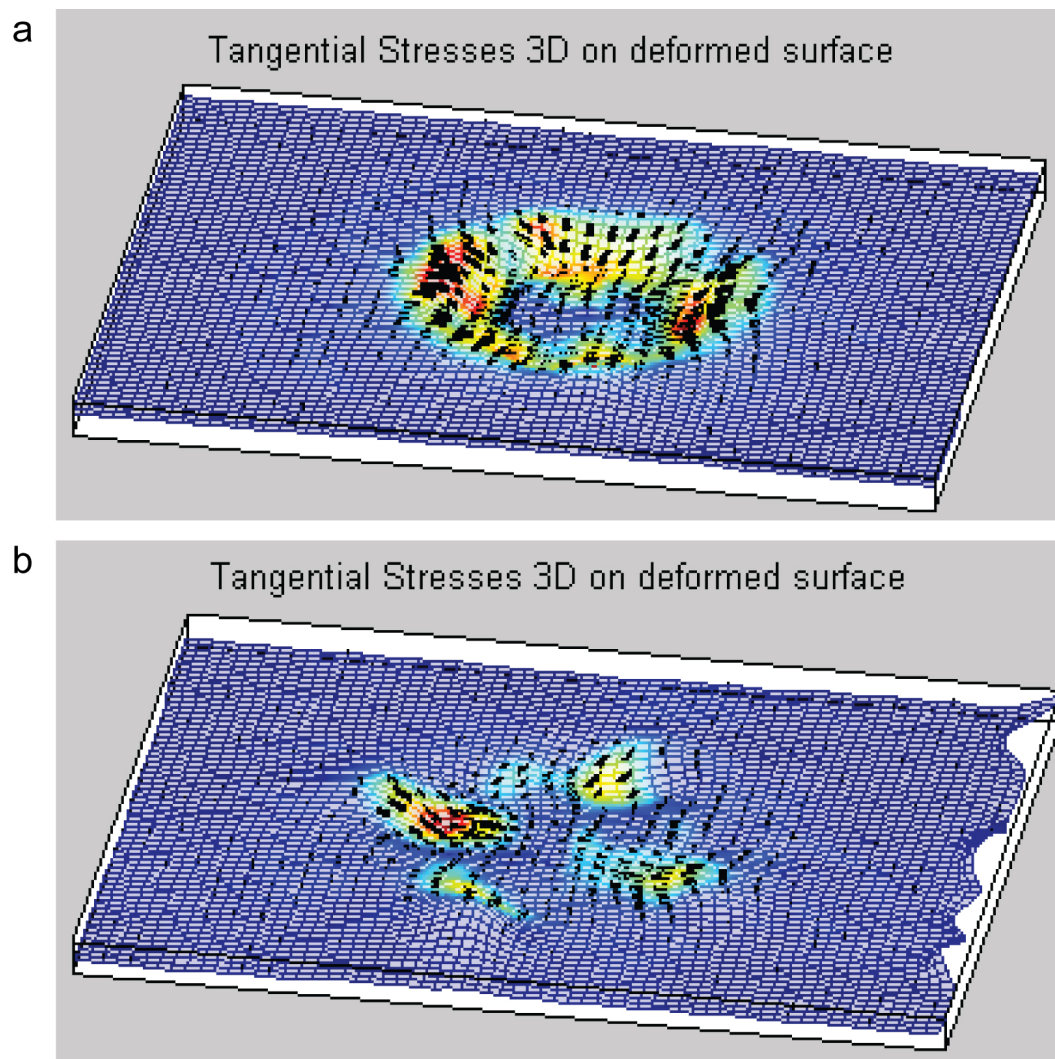


Figure 4.6: Patterned MSC Tangential Forces on a Deformed Substrate. Representative computations of tangential forces for MSC patterned on circle and square protein islands.

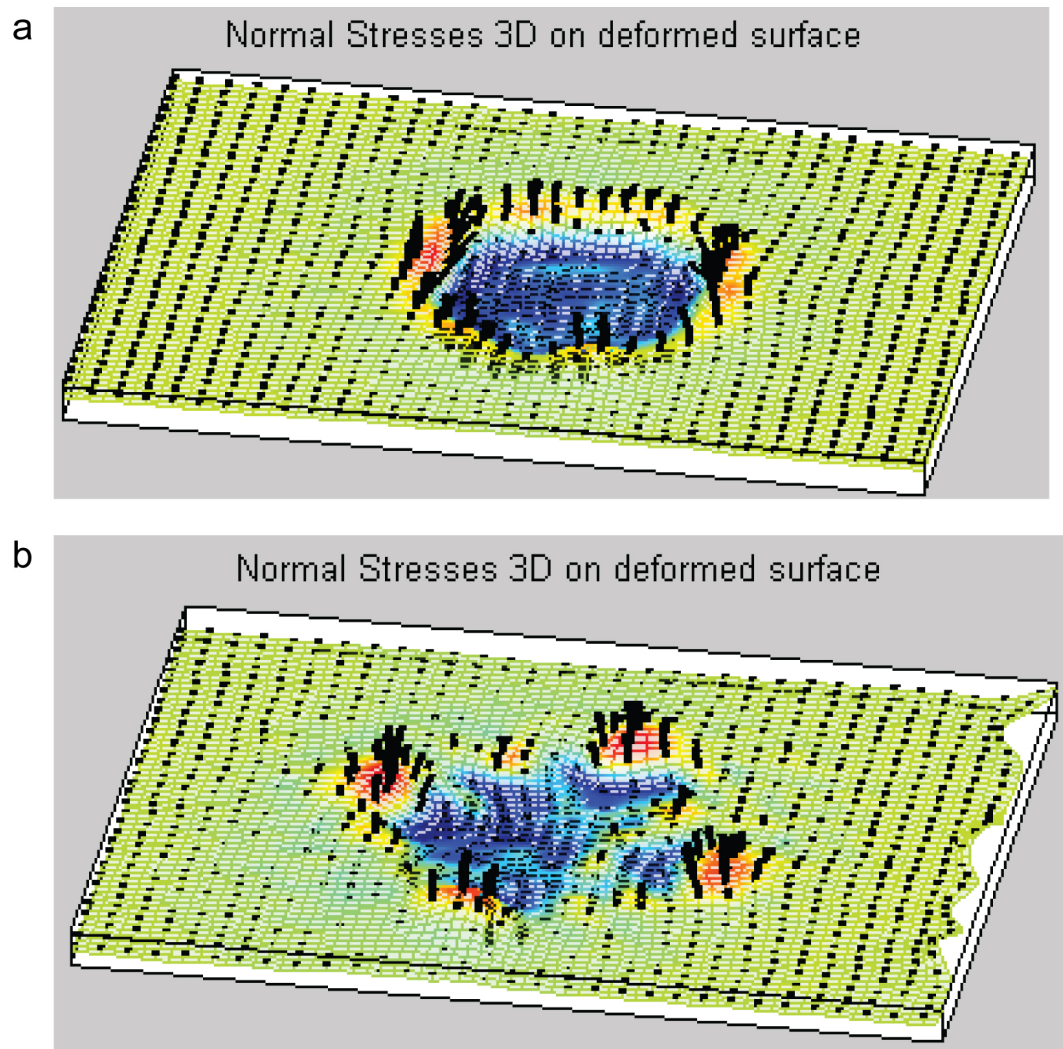


Figure 4.7: Patterned MSC Normal Forces on a Deformed Substrate. Representative computations of normal forces for MSC patterned on circle and square protein islands.

Given our vinculin data, we sought to establish if cell forces scaled with FA abundance. Quantifying tangential, normal, or total strain energy by integrating the forces over discrete areas provided a metric to compare individual cells. Tangential strain energy of cells on firm, 10 kPa substrates did not change with increasing aspect ratio, except for the most elongated 15:1 patterned cells (Fig. 4.8). Normal and total strain energy computations also followed suite. These observations suggest that though more vinculin is available on more highly elongated substrates, the average force across assembled FA is lower than on cells patterned on isometric and slightly elongated shapes.

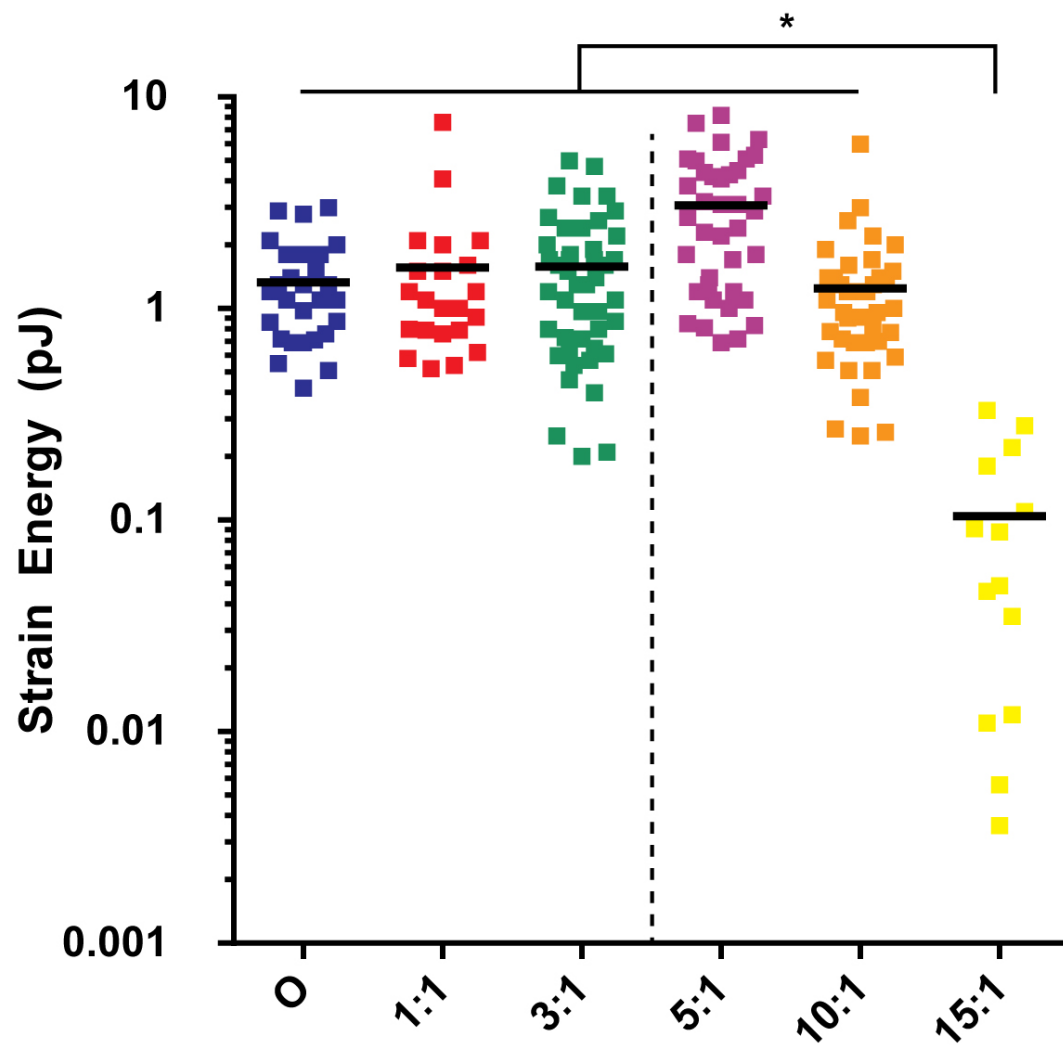


Figure 4.8: Tangential Strain Energy of MSCs on Patterns. Tangential cell work, or strain energy, of MSCs on protein islands of various aspect ratios on 10 kPa polyacrylamide substrates. Cells to the left of the dashed line fit within one field of view during image acquisition while cells to the right of the dashed line required 2 fields of view and 3D stack stitching to determine traction forces. * $p < 0.05$.

4.4 Discussion

Patterned protein islands have been used in a number of studies to test combinatorial arrays of matrix proteins and subsequent cell behavior [14, 15], and ECM arrays are now commercially available for high throughput screening. However, one major drawback is that the spatial resolution of such arrays is limited to the size of the spot printed, usually in the hundreds of microns in diameter, which is much larger than the size of single cells.

To circumvent the spatial limitation of arrays, elastomeric molds have been developed to increase the spatial resolution down to $1\ \mu\text{m}$, allowing for single cell patterning. Microcontact printing on hard surfaces like glass has been widely used to confine cells using various chemistries to elicit particular cell responses [2, 3]. These substrates, however, do not recapitulate the mechanical properties of the native ECM.

The stiffness of the PDMS mold itself is orders of magnitude softer than tissue culture plastic or glass. Cleverly, microfabricated post-array-detectors (mPADs) of different height were developed to modulate the bending stiffness of the post and provide a platform to measure cell forces and cell-cell tugging forces without the need to release the cell from the substrates [16, 17]. Microcontact printing onto the PDMS posts allows for simultaneous control of cell shape and the measurement of cell forces [18]. Though these mPADs present a convenient method to measure cell traction forces, the lack of continuity of the substrates alters where cells can form FAs and disturbs any coupling effects from neighboring FAs.

Microcontact printing on soft materials like polyacrylamide (PA) resolves many of the limitations listed above, though this technique is not without its own limitation. Stamping on glass and subsequently polymerizing the PA gel [19], or stamping on a polymerized hydrogel as in this study, are both valid techniques to pattern the substrate surface to control cell shape and spread area. Unfortunately, blocking the surface after stamping only transiently prevents cell escape from the pattern. Cell proliferation, protein synthesis, and reorganization of soluble matrix proteins all contribute to the ability of cells to break out of the geometrical confines

imposed. Long culture times are not conducive on this material, unlike what has been previously reported on glass and PDMS substrates. This limitation precludes the use of microcontact printing on PA to study cell-shape effects on differentiation.

Promising advances using deep UV activation to pattern cells on glass [20] have been adapted to soft materials like PA [21]. Deep UV activation may be a challenge to combine with fluorescent beads as the technique bleaches the tracker particles, though unpublished reports have reported success using this technique. Regardless of the technique used, cell force measurements are normally performed 24 hours after initial cell seeding after cells spread over the entire protein island but prior to initial cell division or migration out of the island.

Our results confirm that cell forces are localized where focal adhesions typically assemble for cells constrained into specific shapes. However, it was striking that traction stresses did not scale with vinculin expression, suggesting that more focal adhesions do not necessarily indicate greater force generation. In fact, cells that expressed the most vinculin on 15:1 patterns had the lowest overall force generation, contrary to our hypothesis. These data suggest that mechanical perturbations influence cellular signaling through more complex mechanisms [22], and that the sum of traction forces is not an appropriate metric to predict cell behavior. Indeed, recent work has shown that individual FAs have distinct force transmission profiles over time [23], implying that intracellular signaling is FA specific. The structures responsible for mechanotransduction function would thus act as filters, selectively transmitting certain signals given the right amount of force [24, 25].

Furthermore, previous studies have reported that vinculin recruitment to the FAs and force transmission are regulated separately [8, 24], implying that there is perhaps little correlation between vinculin expression and force generation. Nevertheless, patterning of cells does influence protein expression and traction forces at the extreme aspect ratios. It is possible that MSCs try to maintain a constant overall contraction and that cells can easily compensate for changes in cell shape up to a point. Further studies are required to elucidate the interplay of substrate stiffness, cell shape, and differentiation.

4.5 Conclusion

We sought to elucidate the dynamics of cellular mechanotransduction of controlling physical parameters of the cell niche including cell spread area, cell shape, and substrate stiffness. Substrates patterned with protein to confine mesenchymal stem cells spreading modulated vinculin expression and traction forces on the most elongated patterns. Interestingly, substrate stiffness had little effect on vinculin expression on isometric shapes but robustly controlled spatial assembly of focal adhesions. Unfortunately, microcontact printing on polyacrylamide substrates was not conducive for differentiation studies due to conflicting cell functions such as migration and proliferation. The implications of altering the cytoskeleton organization in a lasting way using mechanical interactions with the external environment requires further investigation.

The material of Chapter 4 is original. The author would like to thank Chor Yong (Dalton) Tay and Professor Lay Poh Tan from Nanyang Technological University in Singapore and the rest of the Tan lab for their assistance with micropatterning and for welcoming me into the Tan lab over the summer of 2012. The author would also like to thank Dr. Juan Carlos del Alamo for help with the traction force microscopy code. The dissertation author was the primary author.

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Chapter 5

Interplay of Matrix Stiffness and Protein Tethering in Stem Cell Differentiation

5.1 Abstract

Stem cells regulate their fate by binding to, and contracting against, the extracellular matrix. Recently, it has been proposed that in addition to matrix stiffness and ligand type, the degree of coupling of fibrous protein to the surface of the underlying substrate, i.e. tethering and matrix porosity, also regulates stem cell differentiation. By modulating substrate porosity without altering stiffness in polyacrylamide gels, we show that varying substrate porosity did not significantly change protein tethering, substrate deformations, or the osteogenic and adipogenic differentiation of human adipose-derived stromal cells and marrow-derived mesenchymal stromal cells. Varying protein-substrate linker density up to 50-fold changed tethering, but did not affect osteogenesis, adipogenesis, surface-protein unfolding, or underlying substrate deformations. Differentiation was also unaffected by the absence of protein tethering. Our findings imply that the stiffness of planar matrices regulates stem cell differentiation independently of protein tethering and porosity.

5.2 Introduction

The stiffness of ECM has been shown to regulate both shorter- and longer-term cell functions such as cell spreading [1] and stem and progenitor cell phenotype changes on planar substrates [2-7], respectively. For example, many types of adult stromal cells grown on substrates of stiffness similar to that of the osteoid or muscle express lineage markers of terminally differentiated cells found in those tissues [3, 4, 6]. Common myosin-based contractile mechanisms are required for matrix-induced differentiation in two-dimensions (2D) [3, 8-10]. However in three-dimensions (3D), a labile [11] or degradable matrix [12], which permits cells to first spread and then adhere to the ECM, is required. Similarly, force-mediated protein unfolding of the ECM *in vivo* regulates cell responses as a function of stiffness [13, 14]. While creating 3D matrices has become a widespread approach towards understanding how the matrix affects cell fate, the regulatory role of substrate-anchored fibrous protein deformations on stem cell fate in 2D is still unclear.

Recent literature suggests that the mechanical resistance provided by ECM, which opposes myosin-based contractility and results in cell signaling and differentiation, could be due to protein tethers rather than substrate stiffness for planar cultures [15]. Since most synthetic planar matrices are not normally cell adhesive, an adhesive layer of matrix protein is attached to the hydrogel surface and covalently 'tethered' to the substrate surface at distinct anchoring points. Thus, changing protein-substrate linker density or substrate porosity can vary the length of the fiber segment between two adjacent anchoring points. When a load is applied perpendicularly to the fiber segment, the deflection of the fiber segment is directly related to the load applied, fiber stiffness, and the length of the fiber segment cubed [15, 16]. If enough resistance were present in tethers, stem cells could differentiate independent of substrate stiffness. However, it is unclear what length these tethers may be and how they compare to substrate deformations [17], which have been implicated in mechanotransduction and hence stem cell differentiation [18]. Thus it is critical to decouple protein tethering and substrate stiffness and to determine if and how these factors collectively regulate stem cell differentiation.

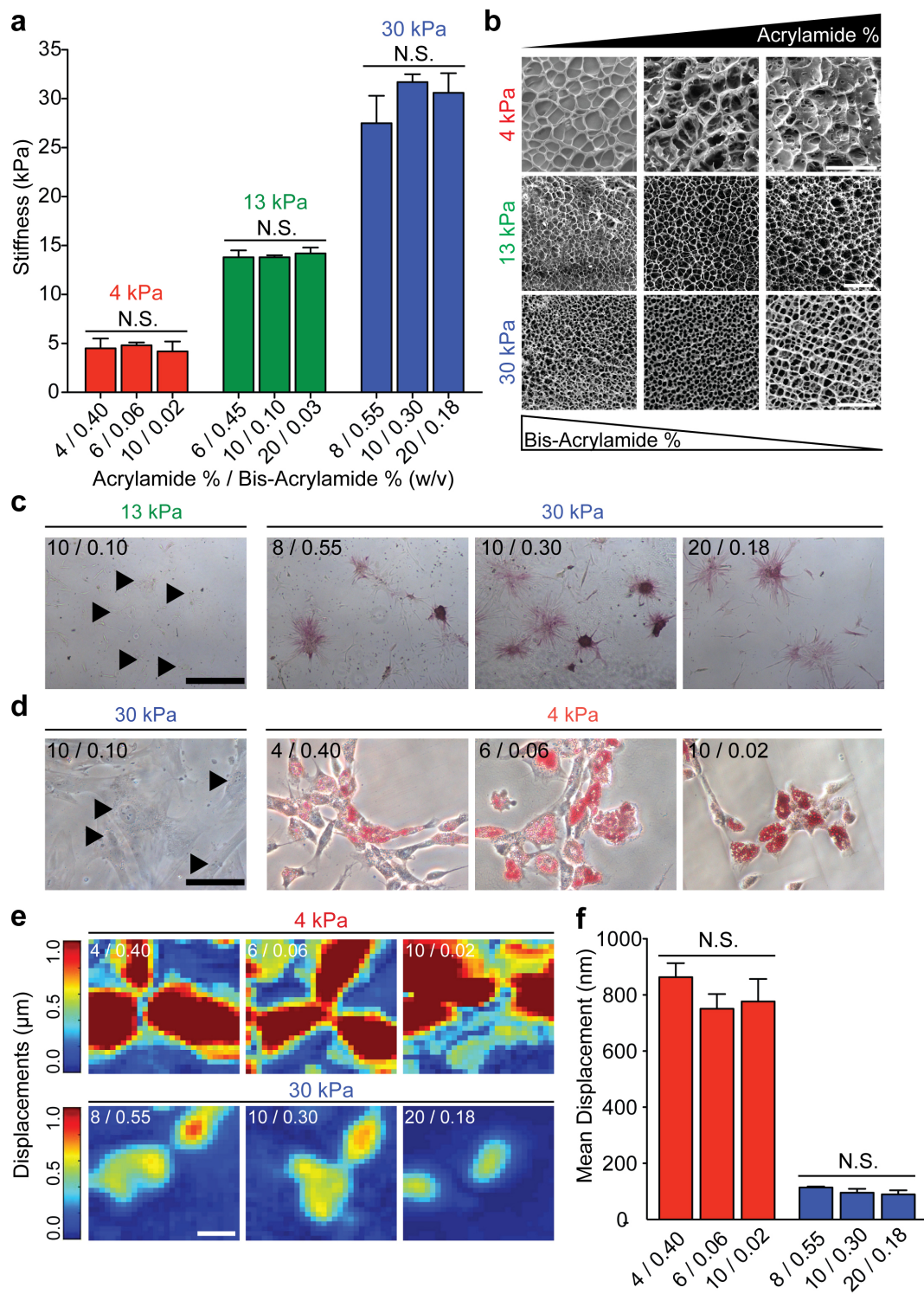
5.3 Results

5.3.1 Tuning Hydrogel Porosity Independent of Stiffness

Tuning the acrylamide monomer and bis-acrylamide crosslinker ratio can change polyacrylamide (PA) hydrogel porosity, i.e. the distance between tethering points, independently of stiffness. To accomplish this, three separate acrylamide/bis-acrylamide formulations were polymerized to yield hydrogels of ~ 4 , ~ 13 , and, ~ 30 kiloPascals (kPa; Fig. 5.1a), which correspond to the stiffness of adipose tissue, muscle, and osteoid [2, 3, 6, 19-21], respectively. Differences in volume and mass swelling ratios between each of the hydrogels with similar stiffness suggest significant differences in porosity among each substrate subgroup (Fig. 5.2a and 5.2b). The radius of gyration of extended DNA may be used to estimate the effective maximum pore size of the hydrogel [22]. DNA size standards were exposed to an electrophoretic gradient in swollen and unconfined 4 and 30 kPa PA hydrogels to further quantify hydrated pore size. For 30 kPa hydrogels, a 45 nm DNA fragment failed to migrate through the 8/0.55 formulation indicating that the maximum pore size of this formulation is between 23 and 45 nm. Larger DNA fragments migrated through the 10/0.3 and 20/0.15 gel formulations indicating that the approximate pore sizes are between 88 and 166 nm for both formulations; differences in DNA mobility suggest that the two gels have pore sizes that differ within this range. Similarly, differences in DNA mobility suggest that the three 4 kPa formulations yield hydrogels with different pore sizes (Fig. 5.2c). Scanning electron microscopy (SEM) of dried PA hydrogels showed increasing pore sizes with increasing acrylamide and decreasing bis-acrylamide concentrations for 4, 13, and 30 kPa hydrogel formulations (Fig. 5.2b); these data are consistent with pore size trends in hydrated measurements and together demonstrate that increasing the bis-acrylamide crosslinker concentration decreases relative pore size without substantially changing hydrogel modulus. However, it is important to note here that pore sizes derived from SEM images of freeze-dried hydrogels are likely not representative of actual substrate pore sizes in a hydrated state. Cells interact with hydrated substrates *in vitro*, and thus SEM images are only provided for relative

comparison of pore sizes for the hydrogel formulations reported.

Figure 5.1: Influence of Substrate Porosity on ASC Differentiation. (a), Elastic modulus measured via AFM ($n = 3$) for the indicated acrylamide:bis-acrylamide ratios. (b), SEM images of PA hydrogels made with varying monomer to crosslinker ratios as indicated (scale bars, $50\ \mu\text{m}$ [top and bottom], $10\ \mu\text{m}$ [middle]). (c), Alkaline phosphatase staining of ASCs on 13 and 30 kPa hydrogels of the indicated compositions after 14 days of culture in normal media. Arrowheads indicate stained but yet negative cells (scale bar, $500\ \mu\text{m}$). (d), Oil Red O staining of ASCs on 4 kPa and 30 kPa hydrogels of the indicated compositions after 7 days of culture in adipogenic induction media. Arrowheads indicate stained but yet negative cells (scale bar, $100\ \mu\text{m}$). (e), Displacement maps of embedded fluorescent particles resulting from ASC traction forces on 4 kPa and 30 kPa hydrogels of the indicated compositions (scale bar, $50\ \mu\text{m}$). (f), Quantification of mean displacement was plotted for hydrogels of the indicated composition and stiffness range ($n \geq 20$; mean $\pm$ S.E.M.; N.S. = not significant).



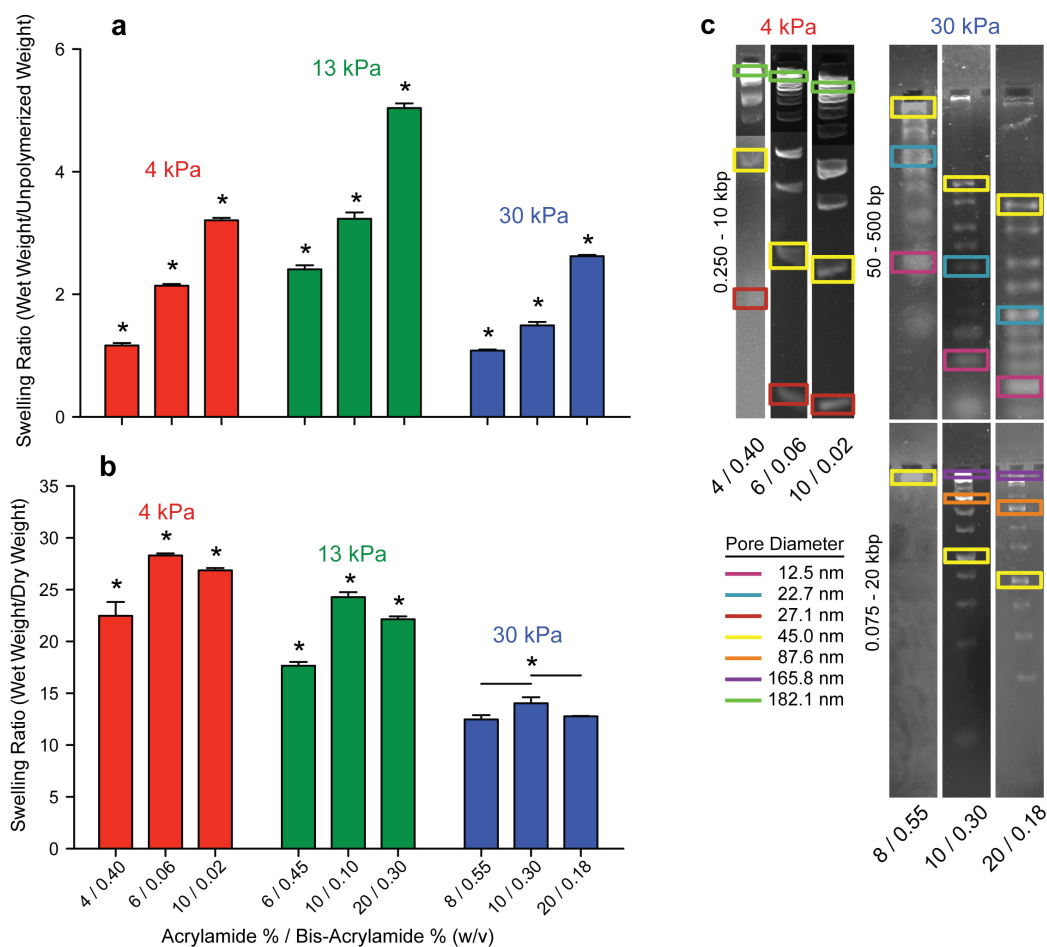
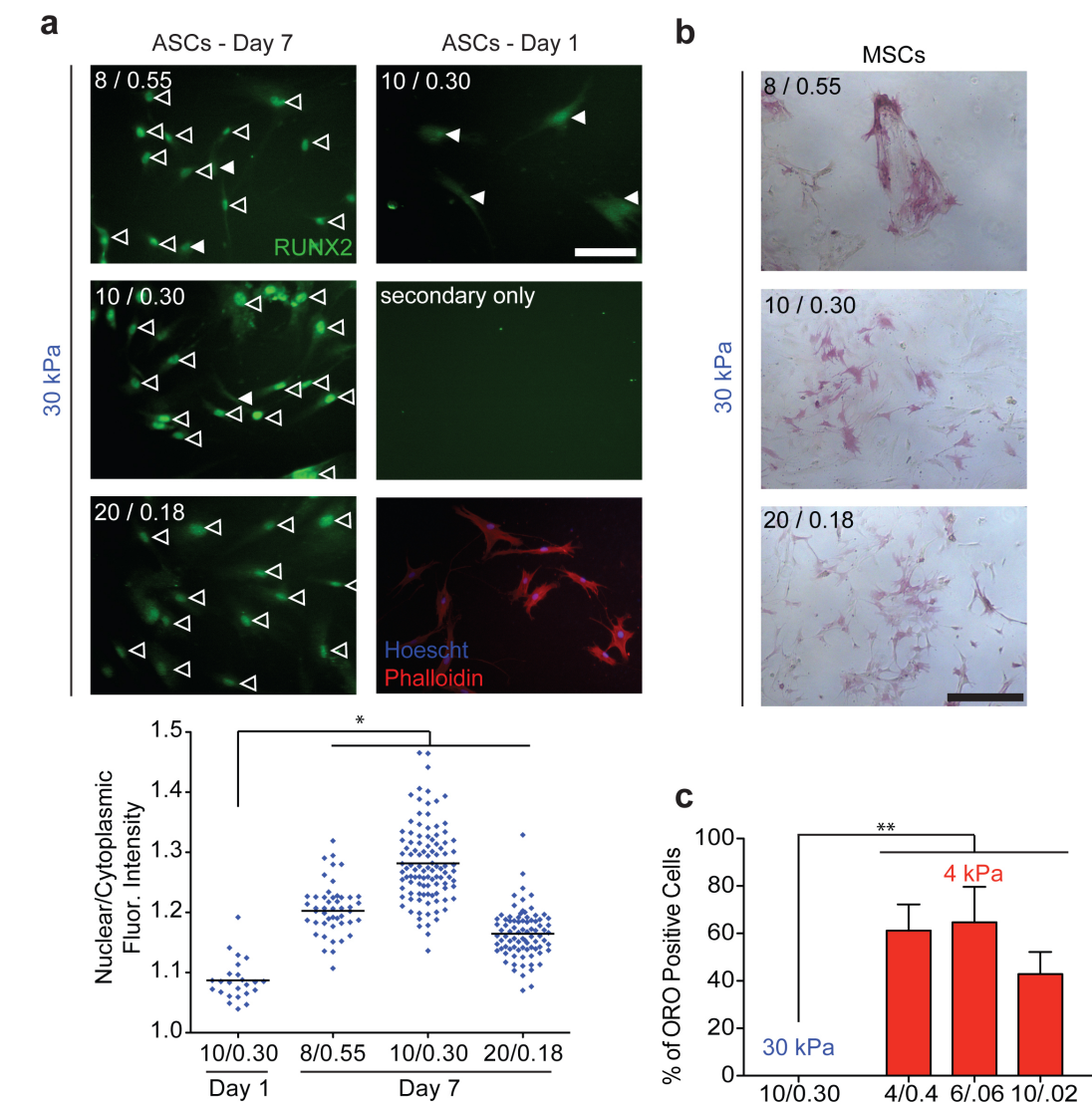


Figure 5.2: Characterization of Porosity on PA Gels Made with Various Monomer to Crosslinker Ratios. (a), Volume swelling ratio - polymerized wet weight/unpolymerized wet weight - and (b), mass swelling ratio - polymerized wet weight/dry weight ($n = 3$; mean $\pm$ S.D.; * $p < 0.05$). (c), Ethidium bromide stained DNA fragments that were subjected to electrophoresis through the hydrated hydrogels of indicated compositions. For 4 kPa hydrogels (left), the DNA ladder ranged from 250-10,000 base pairs (bp). For 30 kPa hydrogels (right), the DNA ladders ranged from 50-500 bp (top) and 75-20,000 bp (bottom).

5.3.2 Differentiation and Deformations are Independent of Porosity

Human ASCs were plated onto 13 and 30 kPa PA hydrogels of the formulations indicated in Fig. 5.1c. After 14 days of culture in normal growth media, osteogenic differentiation was evident by positive alkaline phosphatase (ALP) staining in sub-confluent cells independent of hydrogel formulation but directly dependent on substrate stiffness, as 13 kPa substrates were negative for ALP (Fig. 5.1c). Further confirmation of this is demonstrated by positive and nuclear localized RUNX2 immunofluorescence (IF) staining after 7 days in culture on all 30 kPa hydrogels (Fig. 5.3a). The expression of early osteogenic markers ALP and RUNX2 suggest that changes in porosity independent of stiffness have no noticeable effects on differentiation for the range of hydrogel formulations tested. However, allowing cells to reach confluence in normal media on any hydrogel formulation was sufficient to override substrate stiffness-mediated differentiation and induce osteogenesis as previously observed [15], most likely due to other factors including cell-cell signaling and secreted paracrine factors (Fig. 5.43). To avoid complications arising from confluent monolayers and to focus on cell-ECM signaling only, osteogenic differentiation studies were conducted at low cell densities. MSCs, another commonly used cell type in differentiation experiments, also stained positive for ALP after 14 days in culture on the three 30 kPa hydrogel formulations (Fig. 5.4b), implying that substrate porosity has little effect on multiple stem cell types. Additionally, after 14 days in culture in adipogenic induction media, adipogenic differentiation, as assessed by Oil Red O (ORO) presence, was found in over 40% of ASCs on all 4 kPa substrates independent of hydrogel formulation but directly dependent on substrate stiffness as 30 kPa substrates were negative for ORO (Fig. 5.1d and Fig. 5.3c).



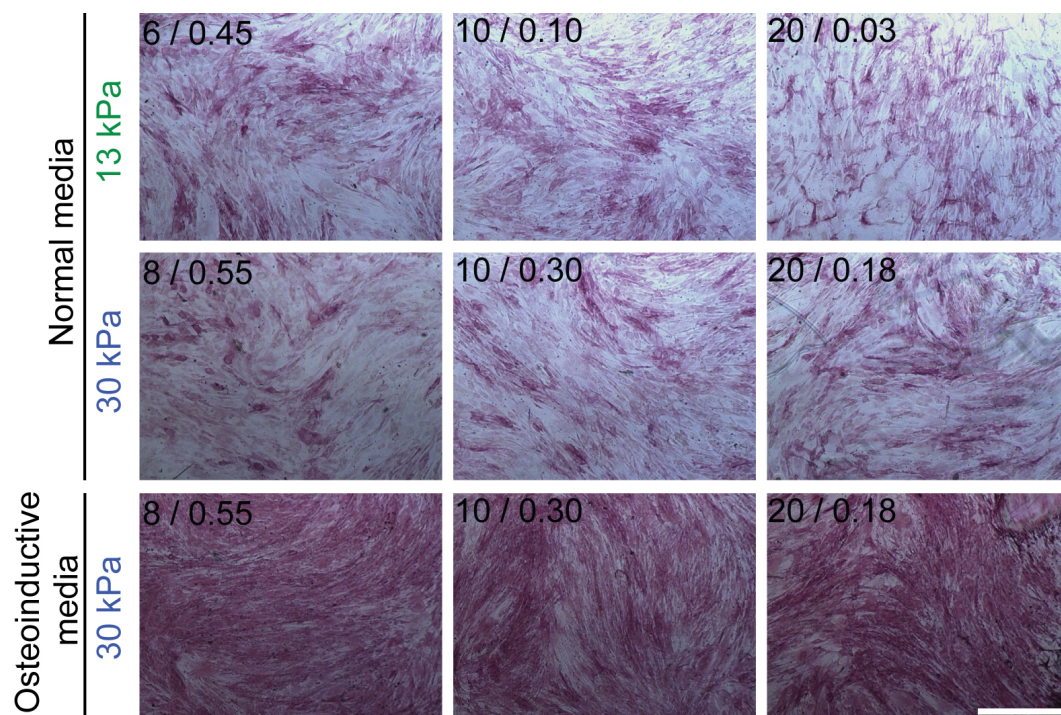


Figure 5.4: Effect of Confluence on ASC Osteogenesis. Images show representative ALP staining of ASCs grown to confluence on 13 and 30 kPa PA hydrogels of varying compositions in either normal or osteogenic induction media after 14 days (scale bar, 500 μm).

As cell-ECM signaling depends on contractility, and differences in contractility have been shown to regulate differentiation [3, 8-10], displacement maps of embedded fluorescent particles resulting from ASC traction forces on all 4 and 30 kPa hydrogel formulations were computed (Fig. 5.1e, Fig. 5.5) using traction force microscopy (TFM) [23]. Mean displacements were similar between all formulations of 4 and 30 kPa hydrogels, but different between hydrogels of different stiffness (Fig. 5.1f). These data indicate that over the range of formulations tested, hydrogel deformations due to cell contractions are similar regardless of porosity but dependent on stiffness (Figs. 5.1e and 5.1f). Taken together, these data show that varying porosity alone does not appear to be sufficient to alter the fate of two different adult stem cell sources.

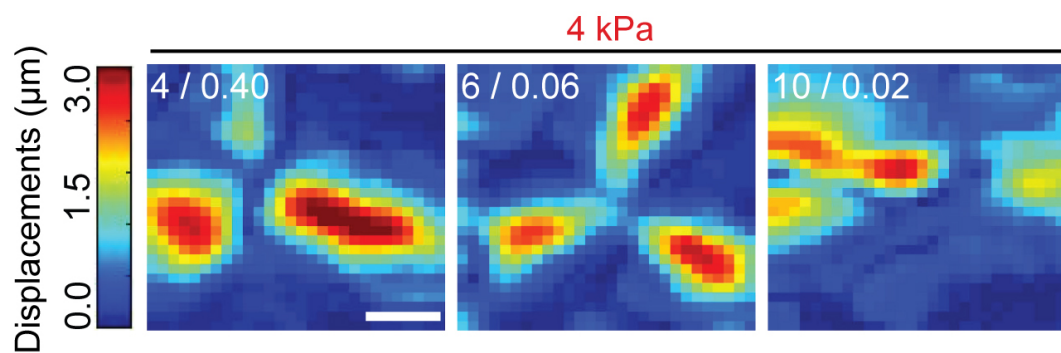


Figure 5.5: Displacement Maps Computed from Traction Force Microscopy Representative displacement maps of embedded fluorescent particles resulting from ASC traction forces on 4 kPa hydrogels of varying porosity. Color bar spans particle displacements ranging from 0-3 μm . (scale bar, 50 μm).

5.3.3 Modulating Protein Tethering by Changing Linker Density

Culturing cells on synthetic hydrogels requires the covalent coupling of a cell-adhesive matrix protein, such as collagen type I, to the hydrogel surface using a protein-substrate linker, such as sulfo-SANPAH [1]. Changing the concentration of such linker has been proposed to modulate protein tethering [15]. To modulate the tethering of fibrous collagen to PA hydrogels, we tuned the surface density of anchoring points by varying the concentration of sulfo-SANPAH, thus varying the average distance between adjacent anchoring points. To assess possible differences in the physical structure or total amount of bound protein, immunofluorescence staining of collagen covalently coupled to PA substrates activated with varying concentrations of sulfo-SANPAH was performed. Images revealed noticeable surface heterogeneity making quantification of absolute protein amount difficult (Fig. 5.6a); this was further illustrated by collagen pixel intensity histograms for 13 and 30 kPa hydrogels over a range of sulfo-SANPAH concentrations (Fig. 5.6b). Fluorescent detection was unable to quantify surface-bound protein as previously suggested [15]. To directly quantify collagen tethering, we obtained individual force spectrograms (Fig. 5.7a) from microindentations of collagen coated PA hydrogels. Substrates were activated with a range of sulfo-SANPAH concentrations using a probe functionalized with an anti-collagen type I antibody (Fig. 5.8a). As the tip retracts from the surface, the collagen unfolds and/or stretches until the antibody-protein bonds rupture (Fig. 5.7a). Force spectrograms were analyzed to locate rupture events and to determine the force at rupture, i.e., the force required to break a protein-antibody bond, and the rupture length, i.e., the deflection of the collagen fiber segment at rupture. Larger rupture forces and a greater number of rupture events were detected in the presence of collagen I (Fig. 5.8b, left; Fig. 5.7b) and indicate that the antibody was specifically binding and loading collagen. Decreasing rupture length with increasing sulfo-SANPAH concentration (Fig. 5.8b, right) confirmed that the number of protein anchoring points scaled with sulfo-SANPAH concentration without substantial changes in rupture force (Fig. 5.8b). This trend held for all 30 kPa formulations tested despite significant

changes in the number of available protein anchoring sites, which is proportional to acrylamide concentration. We observed differences in rupture length between sulfo-SANPAH concentrations across hydrogel formulations (Fig. 5.8c, gray vs. white bars) indicating that anchoring sites must not be saturated. Furthermore, for a given sulfo-SANPAH concentration, although small differences in average rupture length were detected between the three 30 kPa hydrogel formulations, i.e. ± 40 nm, these differences were smaller than the changes in pore size, which were up to 120 nm (Fig. 5.2c). Thus differences in rupture lengths between the hydrogel formulations are not likely due to porosity changes.

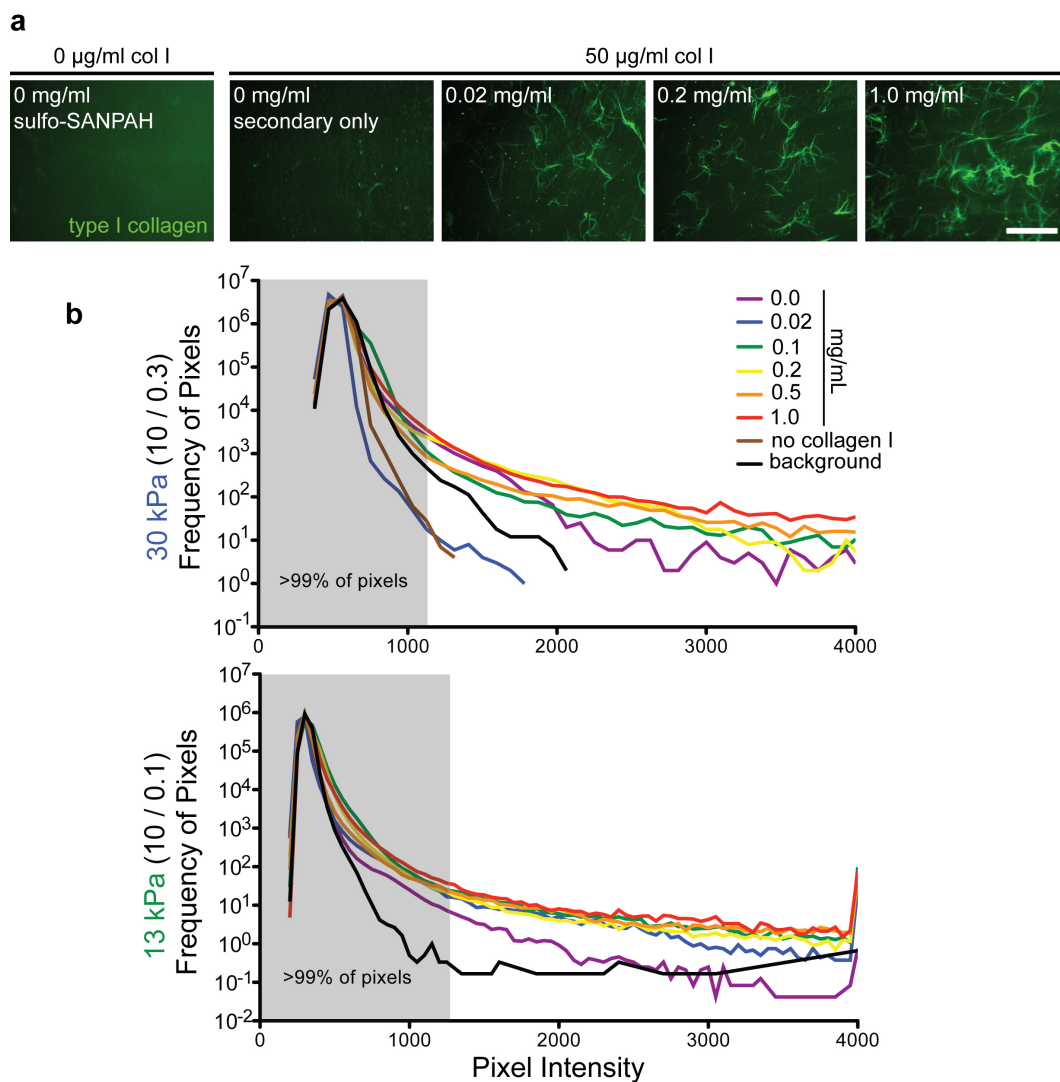


Figure 5.6: Visualization of surface protein coating on PA substrates. (a), Collagen staining of 30 kPa hydrogels activated with 0, 0.02, 0.2, and 1 mg/ml sulfo-SANPAH and subsequently coupled with collagen type I (scale bar, 500 μm). (b), Histograms of image pixel intensities for 13 kPa (bottom, $n = 8$) and 30 kPa hydrogels (top, $n = 5$) for a range of sulfo-SANPAH concentrations.

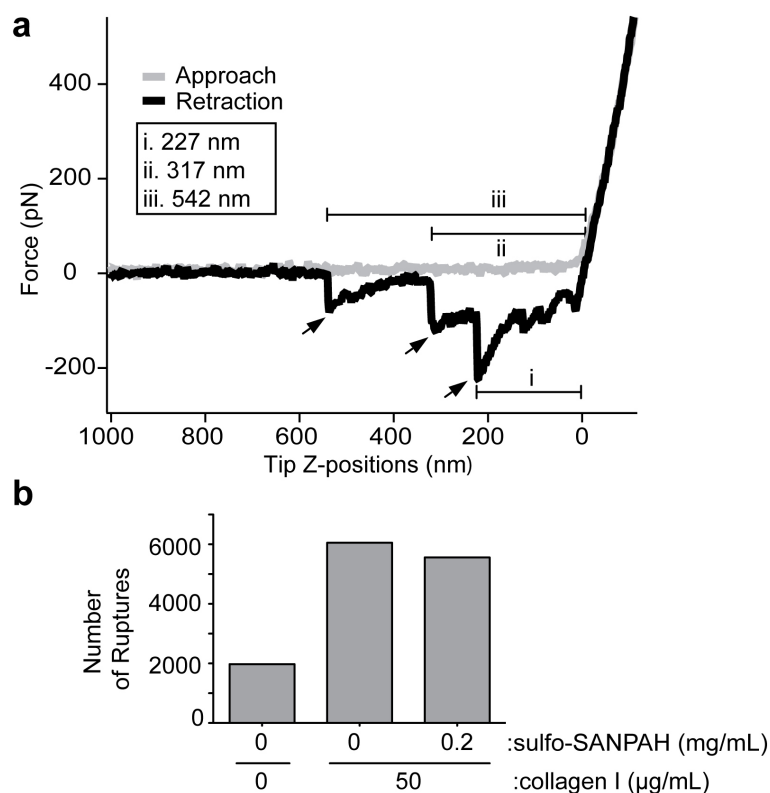
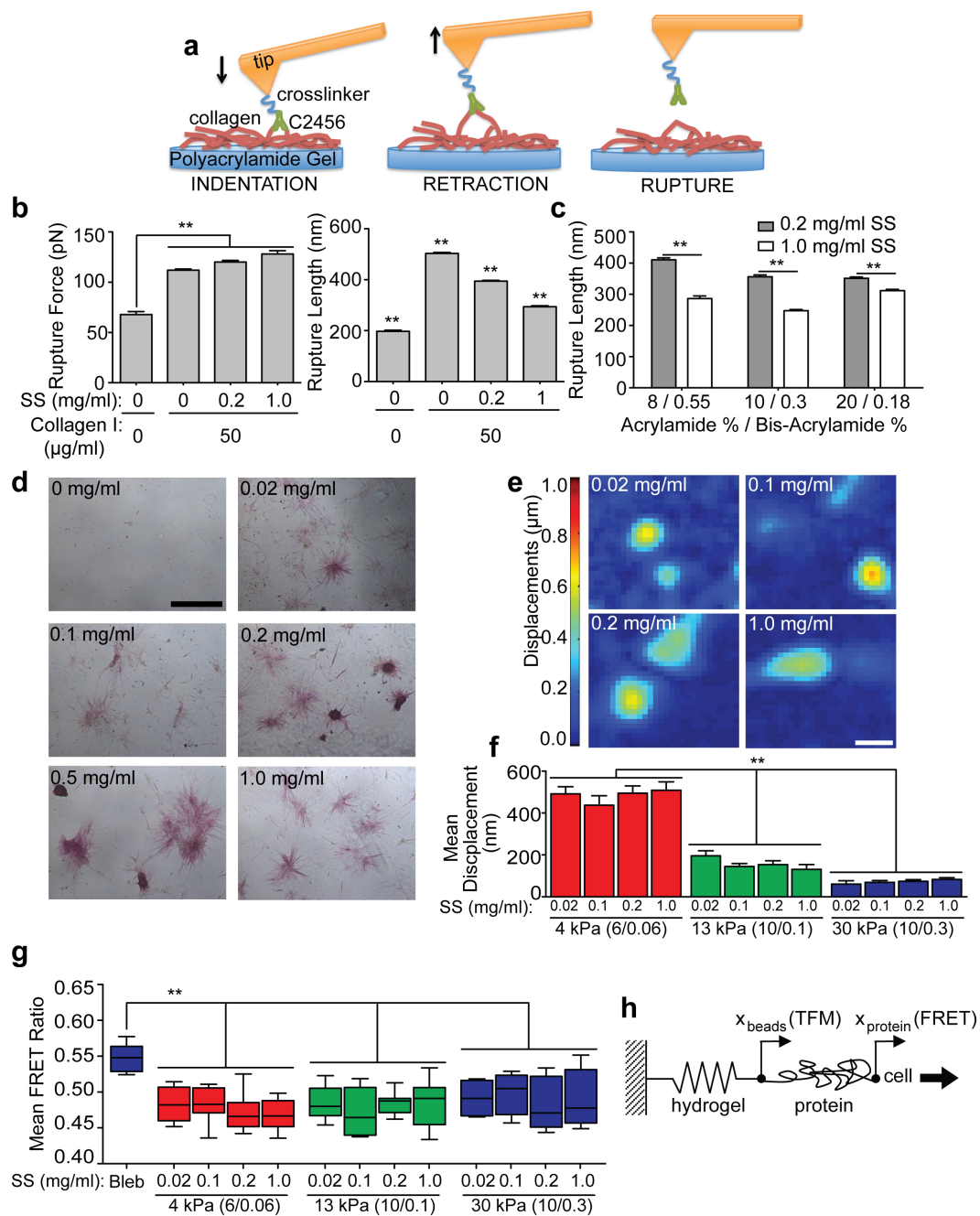


Figure 5.7: Atomic Force Spectroscopy Validation. (a), Representative force vs tip-sample separation spectrogram of the collagen antibody-functionalized tip approaching (grey) and retracting (black) from a collagen I functionalized hydrogel. Rupture events between the antibody functionalized tip and collagen (arrows) are indicated with their distances from the substrate. (b), The number of rupture events detected for PA hydrogels with and without collagen and sulfo-SANPAH as indicated ($n = 500$).

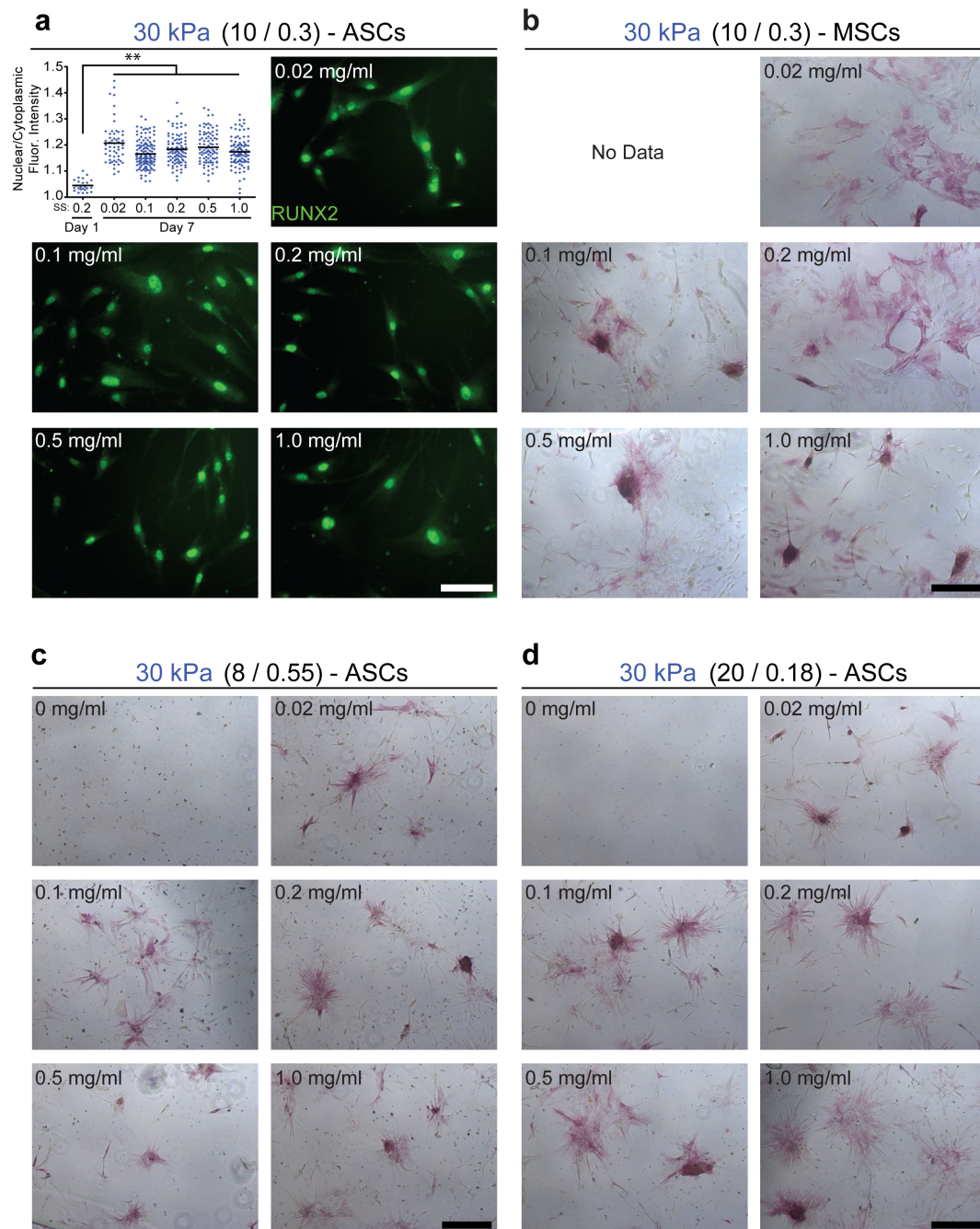
Figure 5.8: Influence of Protein Tethering on ASC Differentiation. (a), Schematic depicting the interaction between an AFM tip (orange) functionalized with a collagen I antibody (C2456; green) and the hydrogel (blue) functionalized with bound collagen I (red). The black arrow indicates the direction of motion. A rupture event occurs following retraction of the tip from the surface. (b), Measured rupture force (left) and rupture length (right) for rupture events that occurred on 10/0.3 30 kPa hydrogels activated with the indicated sulfo-SANPAH (SS) and collagen I concentrations ($n = 500$; mean $\pm$ S.E.M.; $**p < 0.0001$). (c), Rupture length was measured for rupture events that occurred on 30 kPa hydrogels with indicated monomer to crosslinker ratios. Hydrogels were activated with either 0.2 mg/ml or 1 mg/ml sulfo-SANPAH. ($n = 500$; mean $\pm$ S.E.M.; $**p < 0.0001$). (d), Images of ASCs stained for ALP expression on 10/0.3 hydrogels as a function of sulfo-SANPAH concentration after 14 days of culture in normal media (scale bar, 500 μm). (e), Displacement maps of embedded fluorescent particles resulting from ASC traction forces on 10/0.3 hydrogels for a range of indicated sulfo-SANPAH concentrations (scale bar, 50 μm). (f), Quantification of mean bead displacement for the indicated hydrogel stiffness and composition as well as sulfo-SANPAH (SS) concentration ($n = 20$; mean $\pm$ S.E.M; $**p < 0.0001$). (g), Measured fibronectin FRET intensity ratio for ASCs on 4, 14, and 30 kPa hydrogels activated with the indicated concentrations of sulfo-SANPAH ($n = 8$; $*p < 0.05$). (h), Proposed model of a cell on a protein coated substrate attached to a rigid base (glass coverslip) where cell forces are translated through the protein and through the substrate. Deformations of the substrate are measured via TFM and deformations of the protein are measured via FRET.



5.3.4 Differentiation and Deformations do not Depend on Tethering

To investigate whether or not tethering impacts stem cell fate, subconfluent ASCs and MSCs were cultured in normal growth medium on 30 kPa hydrogels over a range of sulfo-SANPAH concentrations and assessed for osteogenic differentiation. Positive ALP and RUNX2 staining was observed on all 30 kPa hydrogels regardless of sulfo-SANPAH concentration, hydrogel formulation, and cell type (Fig. 5.8d and Fig. 5.9). ASCs were also cultured on 4 kPa hydrogels over a range of sulfo-SANPAH concentrations and ORO expression was observed in over 30% of ASCs regardless of sulfo-SANPAH concentration (Fig. 5.10). Together these data indicate that the degree of collagen tethering to the substrate surface had no observable effect on stem cell fate unlike what has been suggested [15]. Myosin contractility deforms the ECM and is required for matrix-induced differentiation [3, 8-10]; thus to confirm differentiation results, substrate displacements for hydrogels across a range of sulfo-SANPAH concentrations were mapped using TFM (Fig. 5.8e). Average displacements of beads embedded in hydrogels were independent of sulfo-SANPAH concentration and only dependent on substrate modulus (Fig. 5.8f), suggesting that for the range of protein-substrate linker concentrations used in this study, the surface density of collagen fiber covalent anchoring points has no impact on how cells deform the underlying substrate.

Figure 5.9: Osteogenic Differentiation as a function of sulfo-SANPAH Concentration. (a), ASCs stained for RUNX2 on 10/0.3 30 kPa hydrogels as a function of sulfo-SANPAH concentration after 7 days (scale bar, 100 μm). RUNX2 nuclear to cytoplasmic mean fluorescent intensity of ASCs was plotted for the indicated time points and sulfo-SANPAH concentrations ($n \geq 20$ cells; $**p \leq 0.0001$). (b), MSCs stained for ALP on 10/0.3 hydrogels as a function of sulfo-SANPAH concentration after 14 days (scale bar, 500 μm). (c), ASCs stained for ALP on 8/0.55 30 kPa hydrogels and d, 20/0.18 30 kPa hydrogels as functions of sulfo-SANPAH concentration after 14 days (scale bar, 250 μm). Sulfo-SANPAH concentrations range from 0 to 1 mg/ml as indicated.



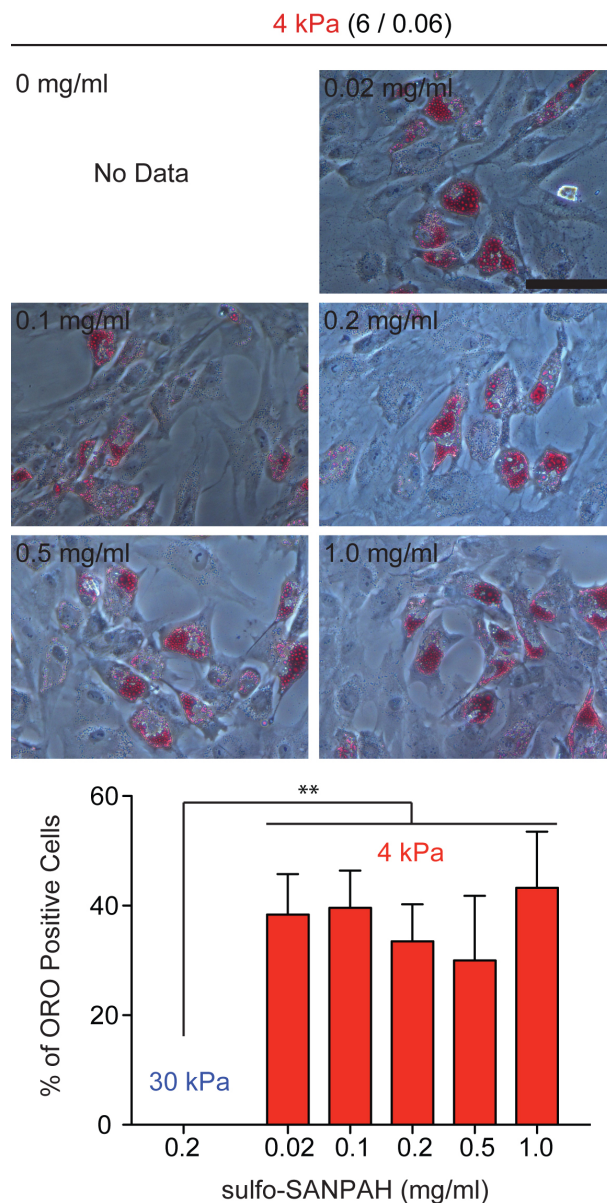


Figure 5.10: ASC Adipogenic Differentiation as a Function of sulfo-SANPAH Concentration. ORO staining of ASCs on 4 kPa 6/0.06 hydrogels for sulfo-SANPAH concentrations ranging from 0 to 1 mg/ml (scale bar, 250 μ m) (top). Percent of ASCs expressing ORO on the indicated formulations of 4 and 30 kPa hydrogels after 7 days of culture in adipogenic induction media (bottom). Data represents an average and standard deviation per field of view. (n $\geq$ 50 cells; **p $\leq$ 0.0001).

To determine whether or not differences in rupture lengths, i.e. tethering, detected via force spectrograms could be felt by cells on a molecular scale, a fibronectin FRET sensor [14] was covalently attached to hydrogels in place of collagen. Cell-generated forces unfold the protein thus increasing the distance between paired fluorescent probes, which results in a decrease in the FRET ratio (Fig. 5.11a) that can also be shown via chemical denaturation (Fig. 5.11b-c). Changing sulfo-SANPAH concentration has no statistical effect on the FRET ratio of fibronectin underneath spread ASCs regardless of hydrogel formulation, while perturbing myosin contractility using blebbistatin caused a significant increase in the FRET ratio (Fig. 5.8g and Fig. 5.11d). Thus, molecular conformational changes in protein caused by ASC traction forces are similar regardless of protein-substrate linker concentration implying that ASCs deform the surface protein similarly on all sulfo-SANPAH activated hydrogels. Based on these findings, we propose that cells deform both the adhesive protein on the hydrogel surface well as the underlying polyacrylamide substrate according to the model depicted in Fig. 5.8h. Cell forces are translated sequentially through the protein layer and the hydrogel. However, our findings suggest that the degree of coupling of the protein to the substrate does not influence substrate deformation and thus differentiation; therefore it was not depicted in Fig. 5.8h.

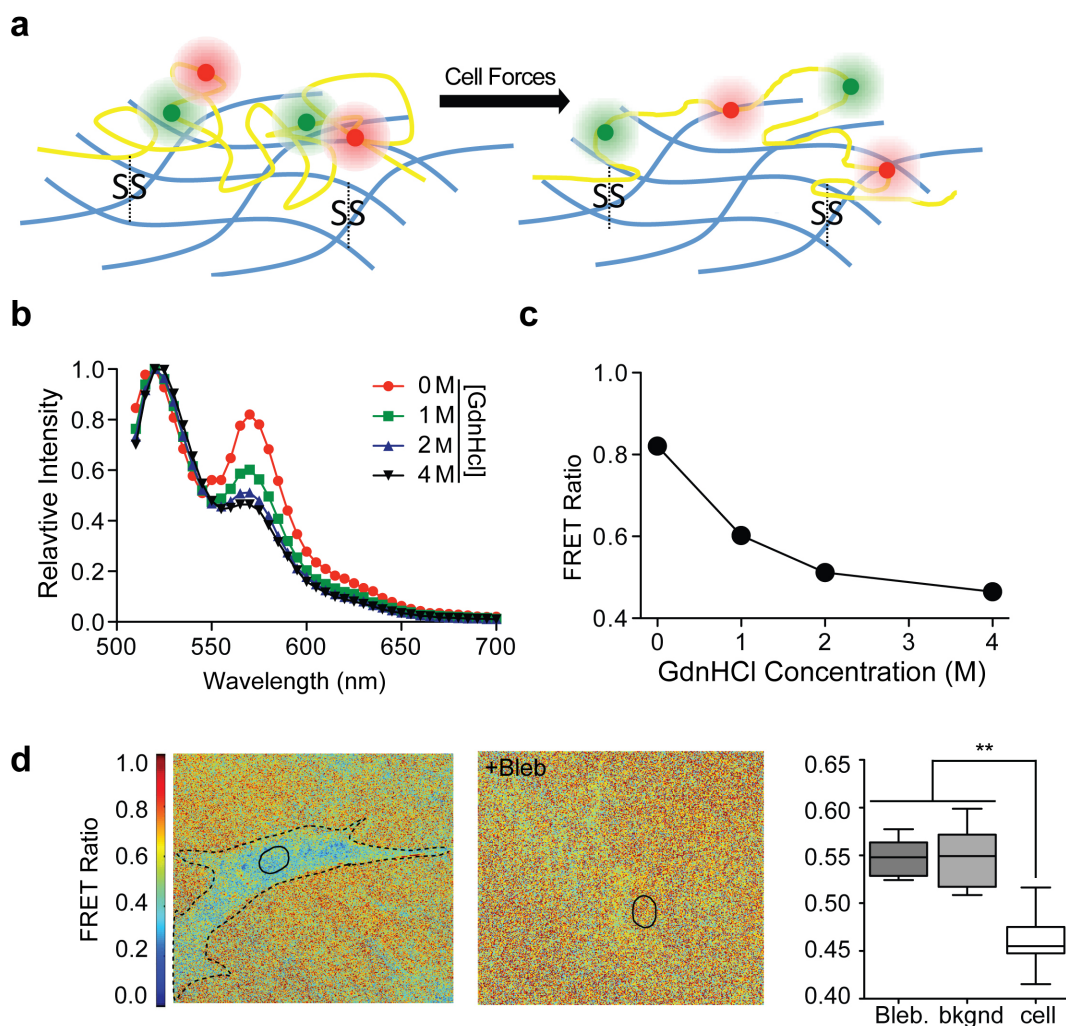


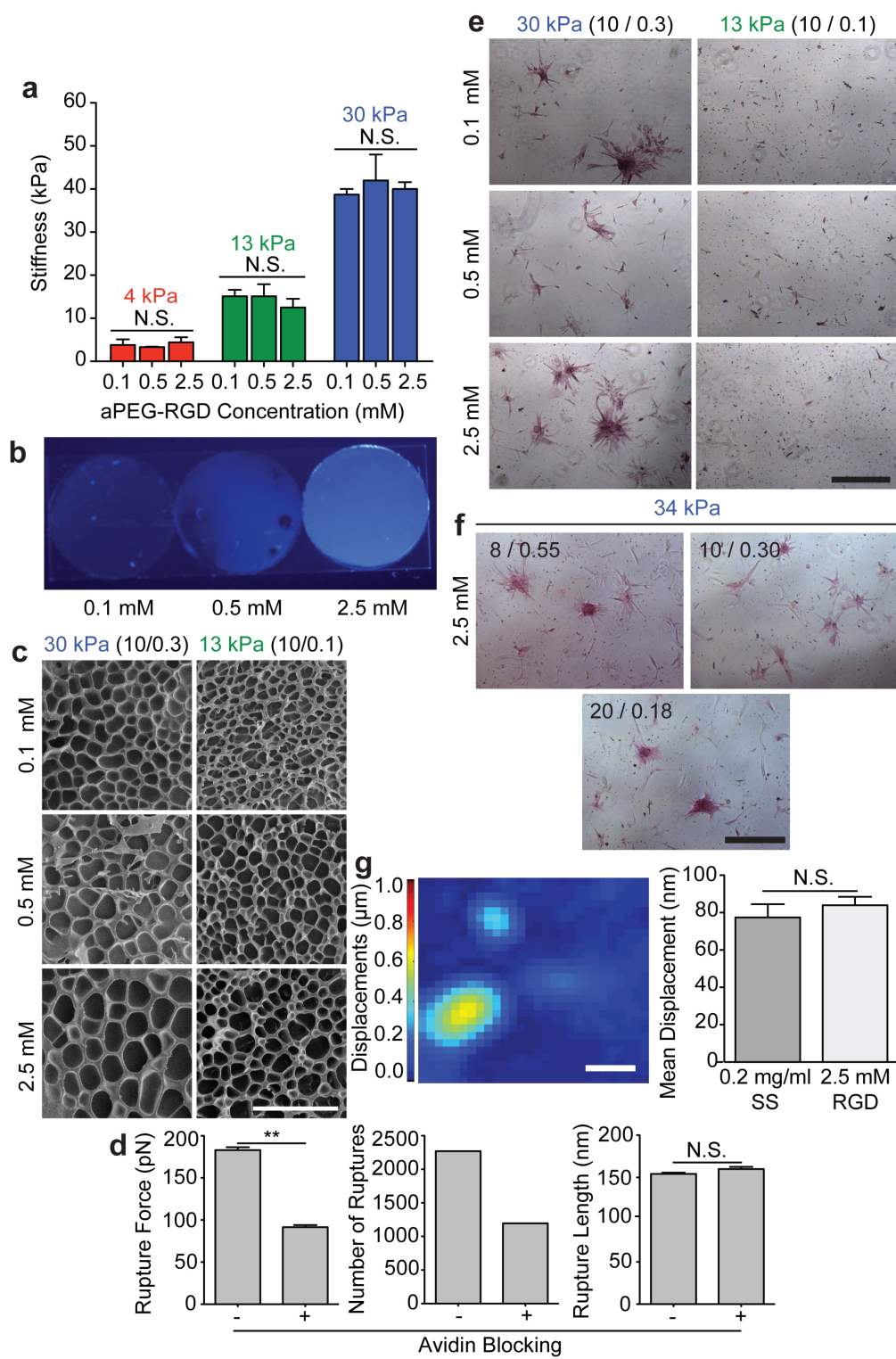
Figure 5.11: FRET Characterization of Dual-Labeled Fibronectin. (a), Cell forces are applied to the dual-labeled fibronectin (left to right), causing the fibronectin to unfold, and fluorophores to move apart, resulting in a decrease in FRET intensity ratio. (b), Emission spectrum of dual-labeled fibronectin denatured in 0-4 M GdnHCl and excited at 484 nm. (c), Resultant FRET intensity ratios. (d), Fibronectin FRET intensity ratio map of an untreated ASC (left) and a blebbistatin treated ASC (center) on a 10/0.3 hydrogels activated with 0.2 mg/ml sulfo-SANPAH (left), and measured FRET intensity ratio for the undeformed protein surrounding a cell, the deformed protein underneath a blebbistatin treated cell and an untreated cell on 10/0.3 hydrogels (right) ($n = 8$; $**p < 0.0001$).

5.3.5 Differentiation Occurs in the Absence of Tethering

To demonstrate that stiffness-induced differentiation is possible in the absence of fibrous protein tethering, RGD, a short cell-adhesive peptide from fibronectin [24], was directly incorporated into the polyacrylamide backbone by including acrylated-PEG-RGD during polymerization rather than tethering an adhesive protein to the substrate. Three separate hydrogel formulations with 0.1, 0.5, and 2.5 mM RGD yielding the same gel stiffness were made for 4, 13, and 30 kPa substrates using the acrylamide/bis-acrylamide ratios listed above (Fig. 5.12a). A hydroxycoumarin dye-conjugated acrylated-PEG-RGD confirmed that the peptide was incorporated in a dose-dependent manner (Fig. 5.12b). SEM images of dried hydrogels show similar pore sizes regardless of the concentration of acrylated-PEG-RGD incorporated within each substrate (Fig. 5.12c). This ensures that differentiation effects can be attributed to changes in adhesive-peptide density and not porosity. Furthermore to ensure that the PEG moiety does not act as a tether, individual force spectrograms were obtained from biotin terminated PEG-coated PA hydrogels and an avidin functionalized probe. Larger rupture forces and a greater number of rupture events were detected on substrates prior to blocking with excess avidin in solution (Fig. 5.12d, left and middle), which indicate that the avidin functionalized probe was specifically bound to the biotin coated surface. Rupture lengths prior and subsequent to blocking were not statistically different (Fig. 5.12d, right) and were similar to rupture lengths measured on control PA substrates with no surface coating (Fig. 5.8b, right). In contrast to collagen coated PA substrates that exhibited significantly greater rupture lengths, the deformations of the PEG moiety are minimal. Thus, PA-PEG-RGD substrates are a valid culture platform absent of protein tethering for the given concentration range and size of PEG tested. ASCs were then cultured for 14 days in normal growth media to determine if differentiation was possible without tethering over the range of peptide concentrations tested. ASCs underwent osteogenic differentiation on 10/0.3 30 kPa hydrogels independent of RGD concentration (Fig. 5.12e). Furthermore, osteogenic differentiation was seen in ASCs and MSCs cultured on all 30 kPa hydrogel formulations with 2.5 mM RGD (Fig. 5.12f and Fig.

5.13). Together, these data suggest that differentiation occurs in the absence of fibrous protein tethering over the range of peptide concentrations tested. Cell generated substrate displacements were similar to that of collagen coated hydrogels (Fig. 5.12g) lending further evidence that matrix-induced differentiation operates through common myosin-based contractile mechanisms given that differentiated cells on these and collagen coated hydrogels were similar.

Figure 5.12: Direct Incorporation of a Short Adhesive Peptide to the PA Substrate. (a), Elastic modulus measured via AFM ($n = 3$; N.S. = not significant). (b), aPEG-RGD-dye incorporation is detected under UV light. (c), SEM images of PA hydrogels of indicated stiffness made with varying RGD concentration (scale bar, $50\ \mu\text{m}$). (d), Measured rupture force (left), number of events (middle), and rupture length (right) for rupture events that occurred on 10/0.3 30 kPa hydrogels coated with PEG-biotin ($n = 1000$; mean $\pm$ S.E.M.; N. S. = not significant; $**p < 0.0001$). (e), ALP staining of ASCs on 13 kPa and 30 kPa hydrogels with low, medium, and high concentrations of RGD (scale bar, $500\ \mu\text{m}$). (f), ALP staining of ASCs on 30 kPa hydrogels of varying monomer to crosslinker ratio and constant high concentration of RGD after 14 days of culture in normal media (scale bar, $500\ \mu\text{m}$). (g), Representative displacement map (left) of embedded fluorescent particles resulting from ASC traction forces on a 30 kPa hydrogel with 2.5 mM RGD. Mean displacement is shown (right) for a collagen coated hydrogel (0.2 mg/ml sulfo-SANPAH and $50\ \mu\text{g/mL}$ collagen I) and a PA-PEG-RGD hydrogel (2.5 mM RGD). ($n = 30$; mean $\pm$ S.E.M; N.S. = not significant; scale bar, $50\ \mu\text{m}$).



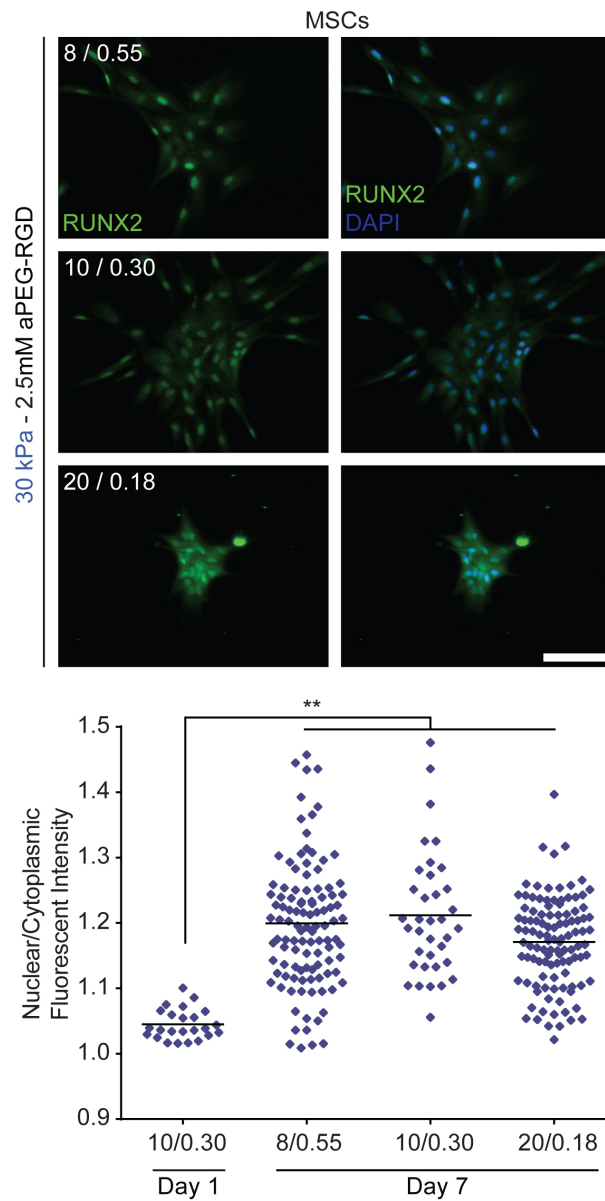


Figure 5.13: MSC Osteogenic Differentiation on RGD Hydrogels. RUNX2 staining after 7 days of culture of MSCs in normal media (top) and their corresponding RUNX2 nuclear to cytoplasmic mean fluorescent intensity ratio (bottom) are shown for different formulations of 30 kPa hydrogels and constant 2.5 mM RGD. For the ratio, day 1 cells are shown as a reference (scale bar, 100 μm ; $n = 35$ cells; $**p < 0.0001$).

5.3.6 Cell Spread Area on PA and PDMS Substrates

To further support the claim that stiffness mediates cell functions generally, we observed the basic behavior of cell spreading on PA-PEG-RGD hydrogels in the absence of protein tethers. ASC spread area 24 hours after seeding scaled with increasing hydrogel stiffness (Fig. 5.14a). This suggests that stiffness is an important physical factor independent of how adhesive ligands are presented (but not independent of concentration [25]). In order to determine whether or not this phenomenon is specific to acrylamide-based systems, polydimethylsiloxane (PDMS) substrates were fabricated with base-to-curing ratios of 100:1, 75:1, and 50:1 to modulate stiffness as noted previously [15]. These substrates were not functionalized with adhesive protein. Without covalently attaching or tethering ligands to the surface, cell adhesion and spreading was still possible for all substrates due to the well-known fouling properties of PDMS, and furthermore, cell spread area was similar on all substrates (Fig. 5.14b). This observation is in agreement with previous observations that imply stiffness independent cell spreading on PDMS substrates [15], suggesting that cells may sense similar mechanical cues on the three PDMS formulations.

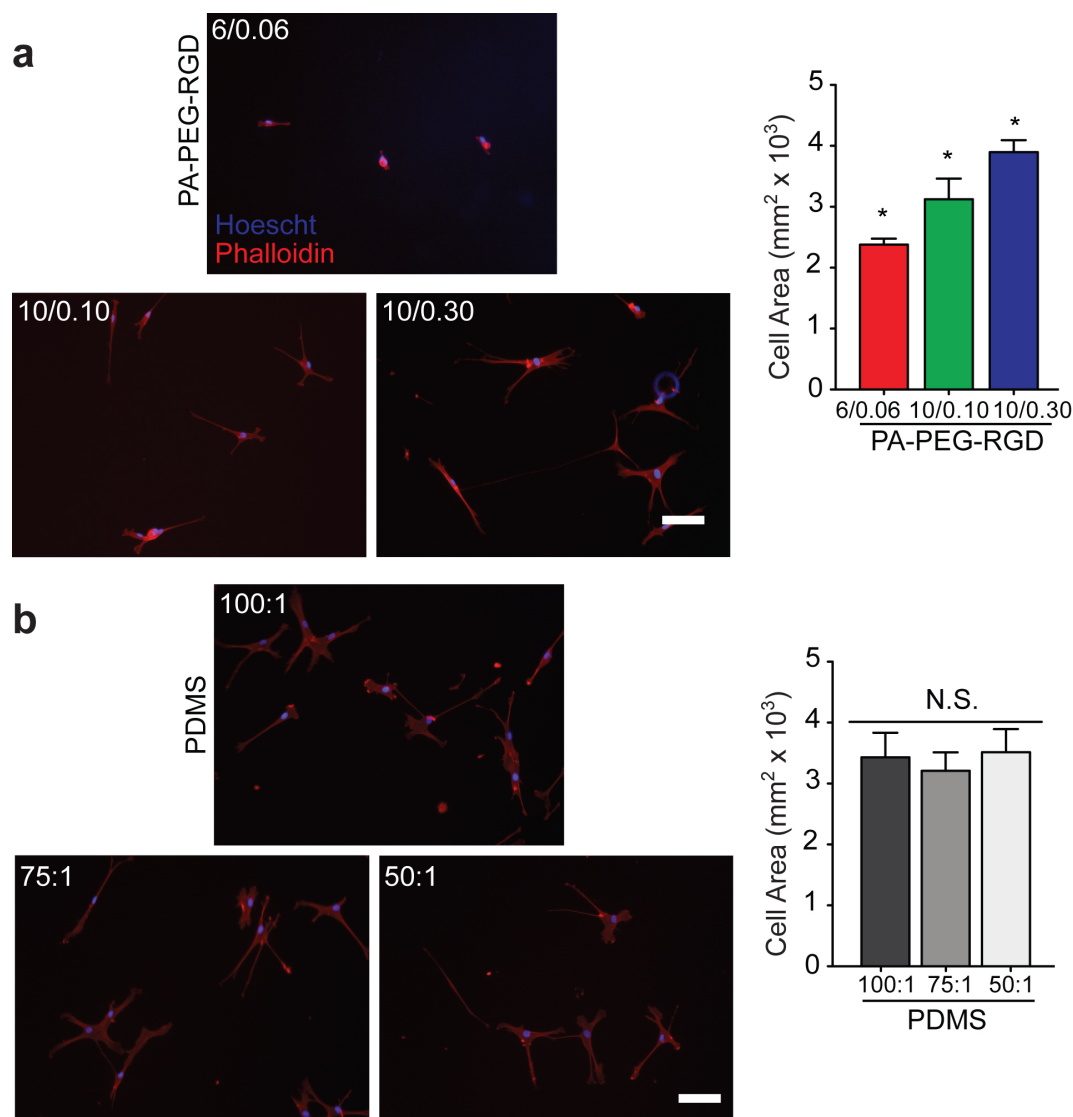


Figure 5.14: ASC Spread Area on Tether-less Hydrogel Systems. (a), Images show ASCs on PA-PEG-RGD (2.5 mM aPEG-RGD) 24 hours after seeding (phalloidin staining, red; nuclei staining, blue). At right is the quantification of cell spread area on PA-PEG-RGD substrates 24 hours after seeding (scale bar, 100 μm). (b), Images show ASCs on PDMS substrates 24 hours after seeding (phalloidin staining, red; nuclei staining, blue). At right is the quantification of cell spread area on PDMS substrates 24 hours after seeding (scale bar, 100 μm). (n $\geq$ 40; mean $\pm$ S.E.M; *p $\leq$ 0.05).

5.3.7 Measuring PDMS Mechanical Properties on a Cell-Sensing Scale

Because of a lack of correlation between cell spread area and PDMS base-to-curing ratio, we independently measured PDMS stiffness by AFM microindentation. The stiffnesses of 50:1 and 100:1 PDMS were found to be 250 and 550 kPa (Fig. 5.15a) — orders of magnitude greater than previously reported [15]. Since PDMS has previously been shown to be viscoelastic at higher base-to-curing ratios [26], substrates were instead indented using different indenter geometries and at different indentation speeds. Using two different probes and a wide range of indentation speeds confirmed the viscoelastic behavior of PDMS (Fig. 5.15b) and suggests that different methods of characterization may account for discrepancies in reported values of PDMS stiffness.

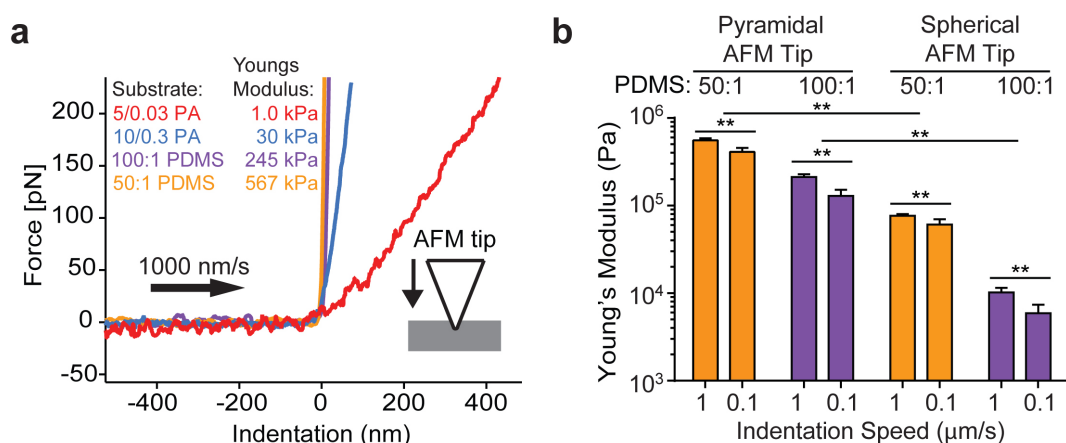
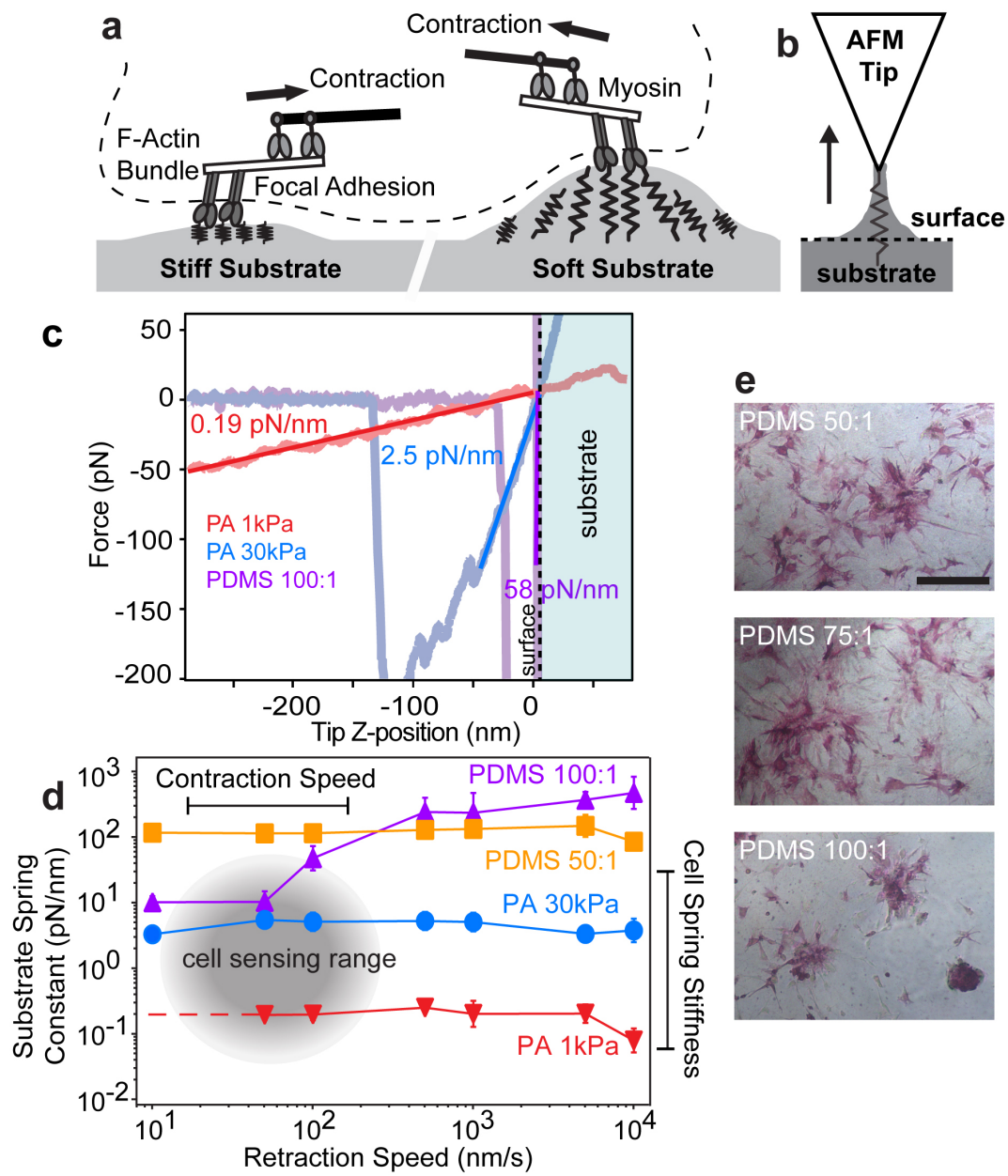


Figure 5.15: Mechanical Characterization of PDMS Substrates by Atomic Force Microscopy. (a), Force spectrograms from indentations of PDMS substrates fabricated from 50:1 and 100:1 base to curing agent ratio (yellow and purple, respectively) and 1 and 30 kPa PA hydrogels (red and blue, respectively) performed with a pyramidal cantilever. (b), Calculated Young's modulus from indentations on 50:1 and 100:1 PDMS substrates with either pyramidal (yellow) or spherical tips (purple) at different indentation speeds (** $p < 0.0001$).

Given the lack of consensus on measuring the mechanical properties of PDMS, it is important to use the most appropriate technique to closely mimic cell-substrate interactions. Cells pull against substrates at 20 to 120 nm/s resulting in deformations that scale inversely with stiffness [17, 27] (Fig. 5.16a). We can match AFM tip retraction velocity to the pulling velocity and size of focal adhesions. Consequently, we can simulate these dynamically fluctuating pulling events by analyzing the retraction curves (as opposed to indentation curves) obtained by AFM where the tip has pulled and deformed the material above the surface (Fig. 5.16b). The substrate stiffness is determined by fitting the linear region beginning at the contact point with the (undeformed) surface ($F = 0$ pN) to where the force reaches -100 pN (Fig. 5.16c). 1 and 30 kPa PA hydrogels demonstrated little variation in stiffness over a range of cell relevant strains [26] and retraction speeds [17, 27]. The stiffness of 50:1 and 100:1 PDMS were both significantly higher than the PA hydrogels, and the stiffness of 100:1 PDMS increases 50 fold over the range of retraction velocities tested (Fig. 5.16d). These data confirm that 100:1 PDMS is highly viscoelastic and 50:1 PDMS is predominantly elastic but both are stiff over cell relevant strains in agreement with prior data [26]. Though previous studies have noted lower stiffness values of PDMS for the same cure ratios [15], it is well known that the mechanical properties of PDMS are different at the cellular mechano-sensing scale than at the macroscopic scale [27]. At the scale at which a cell mechano-senses [17, 27], both 50:1 and 100:1 PDMS substrates are stiffer than 30 kPa PA hydrogels (Fig. 5.16d). This provides a reasonable explanation as to why cell spreading (Fig. 5.14b) and osteogenic differentiation (Fig. 5.16e), neither of which changed with cure ratio, were previously reported to be stiffness independent [15]. We note here however, that it is possible to decrease the effective stiffness of PDMS by fabricating microposts of identical cure ratios but different heights. Fu et al. found that MSC contractility and differentiation towards adipogenic or osteogenic lineages scaled as a function of effective stiffness pillar height [28]. Thus, even in PDMS systems where cure ratio is not directly modulated, effectively modulating stiffness can still yield mechanically-driven differentiation.

Figure 5.16: Cells Sense by Contracting Against their Substrates. (a), Schematic depicting how cells dynamically deform soft and stiff substrates by pulling (and not pushing) via myosin contractions. Softer substrates are deformed to a greater extent than stiffer substrates. (b), Schematic depicting the interaction between an AFM tip and the surface of a substrate. The arrow indicates the direction of motion of the tip during retraction. (c), Representative retraction curves for 1 and 30 kPa PA hydrogels and a 100:1 PDMS substrate. The dashed line indicates the point at which the tip is no longer indenting into the substrate (shaded light blue). Substrate stiffness is calculated by fitting the linear region of the retraction curve starting at the (undeformed) surface. (d), Substrate spring constant determined by the method depicted in c for 1 and 30 kPa PA hydrogels and 50:1 and 100:1 PDMS substrates as a function of AFM tip retraction speed. Cells are sensitive to substrate stiffnesses of 1-100 kPa. Previously measured myosin contraction speeds range from 10-100 nm/s (gray). (e), Alkaline phosphatase staining of ASCs on PDMS substrates after 7 days of culture in normal media (scale bar, 500 μm).



5.3.8 PDMS Substrates do not Support Protein Tethering

To address the possibility of fibrous protein tethering on PDMS, 50:1 PDMS substrates were examined using force spectroscopy. When 50:1 PDMS substrates were pre-incubated in a collagen solution, rupture events with lengths and forces much greater than that of PA substrates were detected (Fig. 5.17a) confirming that collagen non-specifically adsorbs to PDMS. Attempting to functionalize PDMS with sulfo-SANPAH prior to collagen incubation [15] did not alter rupture force (Fig. 5.17b). However, rupture length drastically increased from 450 nm to 1.5 μm , which is larger than cell deformations on stiff PA substrates (Fig. 5.8g and 5.17a). This observation is opposite of what was seen with PA; treating PA with sulfo-SANPAH increases fibrous collagen tethering to PA, consequently decreasing rupture length (Fig. 5.8b).

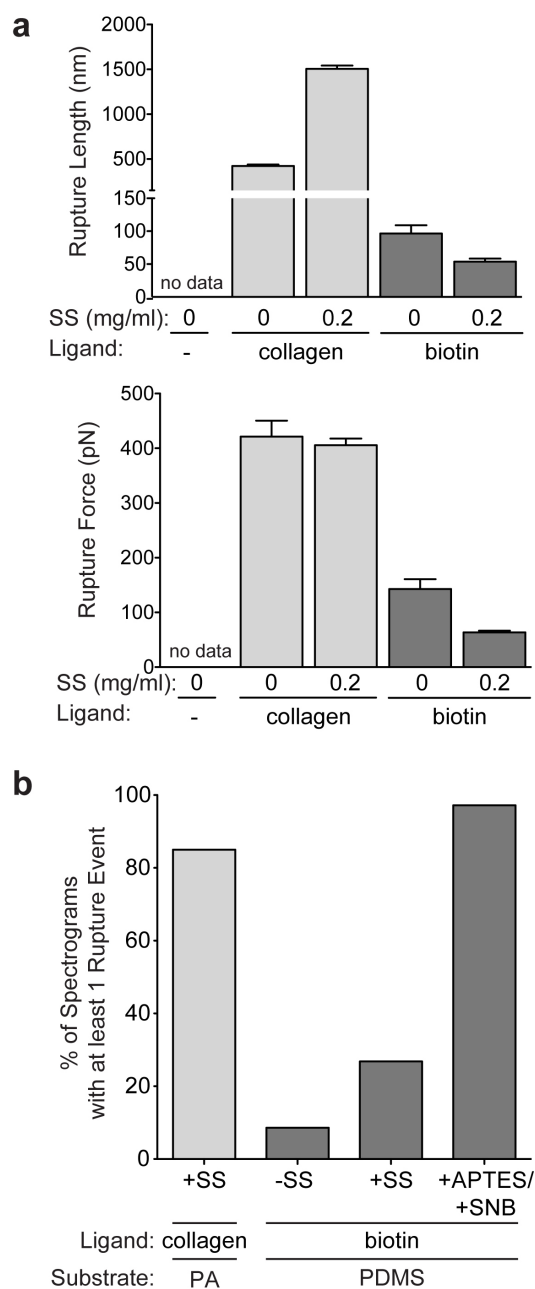
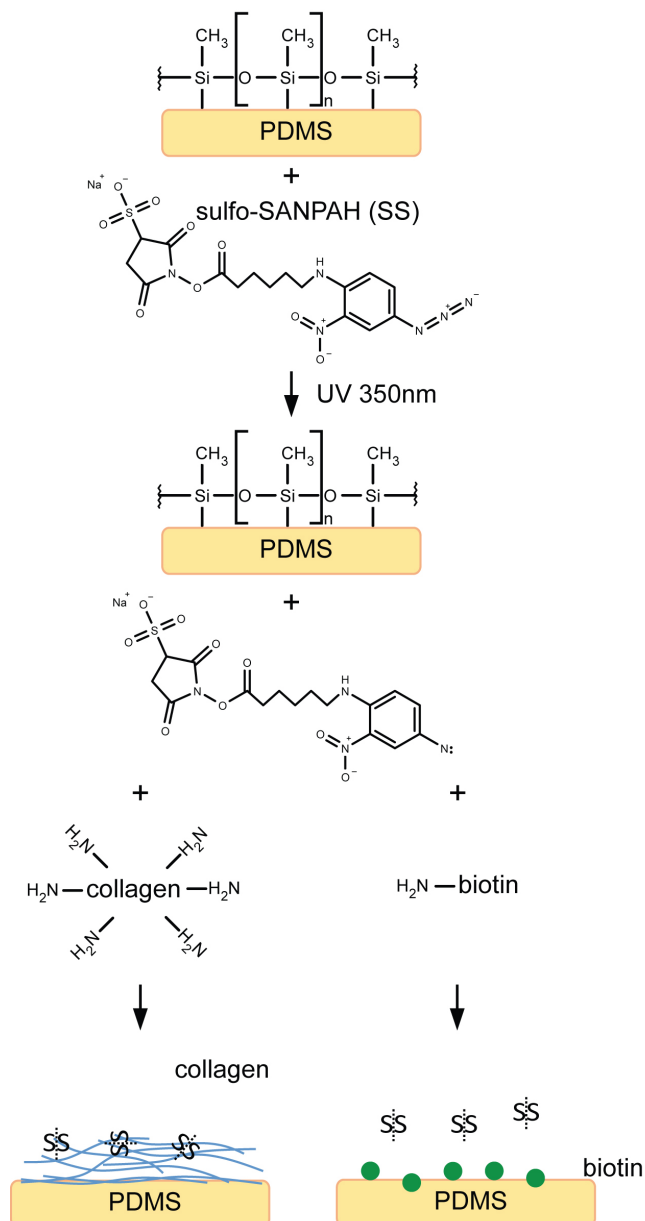
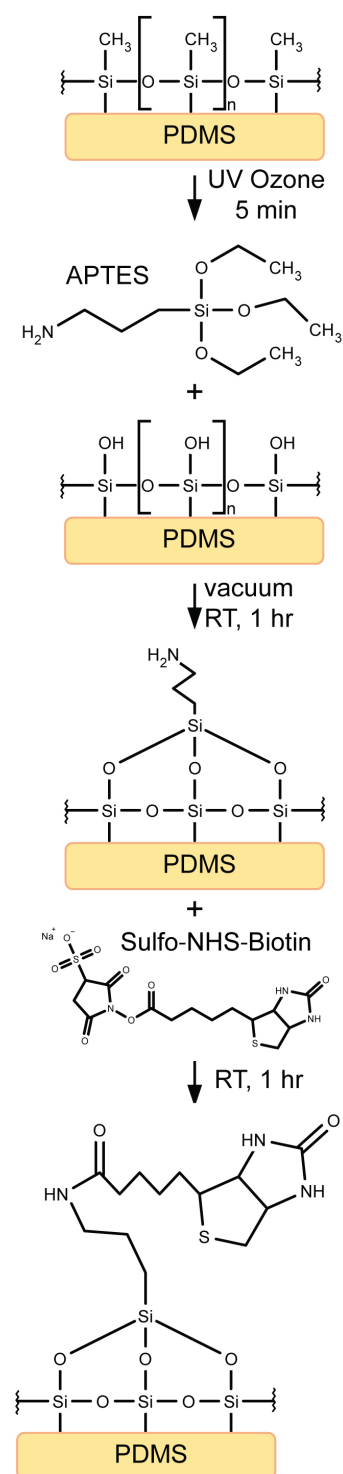


Figure 5.17: Atomic Force Spectroscopy Analysis of Ligand Coated PDMS Substrates. (a), Measured rupture length (top) and rupture force (bottom) for rupture events detected on 50:1 PDMS substrates activated with the indicated sulfo-SANPAH (SS) and collagen or NH₂-PEG-biotin concentrations. No data indicates that no rupture events were detected. (b), Percentage of spectrograms with at least one rupture event on PA and PDMS substrates activated with the linkers and ligands shown.

The increased rupture lengths seen in PDMS may be attributed to the formation of long chains of collagen forming on the PDMS surface as collagen contains many primary amines for sulfo-SANPAH to crosslink, whereas PDMS is void of amines (Fig. 5.18a, left). In this hypothesis, sulfo-SANPAH is not directly coupled to the PDMS surface, but rather only to collagen chains. To test this hypothesis, substrates were functionalized with sulfo-SANPAH prior to incubation in NH_2 -PEG-biotin, which has only one free primary amine (Fig. 5.18a, right). Rupture lengths and forces obtained from force spectrograms using avidin tips were similar on biotin-coated substrates functionalized with and without sulfo-SANPAH (Fig. 5.17a).

Figure 5.18: Diagram for the Covalent Attachment of Protein to the Surface of PDMS Substrates. (a), Sulfo-SANPAH does not react with the methyl groups of untreated PDMS upon UV activation, but may interact non-specifically with the PDMS surface. Collagen and biotin-NH₂ adsorb to the PDMS surface. Collagen amine-containing residues may react with sulfo-SANPAH molecules leading to an accumulation of crosslinked collagen at the surface. Biotin-NH₂ one contains a single free amine, and thus cannot aggregate at the PDMS surface. (b), Activation of the PDMS surface with UV ozone and APTES coats the PDMS surface with primary amines, which react with the sulfo-succinimidyl functional group of sulfo-NHS-biotin, covalently attaching a single biotin molecule.

a**b**

To further confirm that sulfo-SANPAH does not react with PDMS, amines were covalently bound to PDMS substrate surfaces using the chemistry outlined in Fig. 5.18b. At least one rupture event was detected in more than 90% of force spectrograms obtained from biotin-coated samples. In contrast, rupture events were detected in only 30% of force spectrograms obtained from biotin-coated but not amine-functionalized PDMS samples independent of sulfo-SANPAH (Fig. 5.17b). Thus it is clear that the sulfo-succinimidyl group requires amines in order to form a covalent bond. Regardless of UV treatment, PDMS surfaces do not display free amines, and thus protein cannot be covalently bound to the surface via sulfo-SANPAH. Prior efforts do not appear to have amine-functionalized PDMS [15], and thus it is difficult to attribute fibrous protein tethering on PDMS to cell spreading and differentiation. These results in conjunction with cure ratio-independent stem cell spreading (Fig. 5.14b) and differentiation (Fig. 5.16e) emphasize the shortcomings of PDMS as a model system to investigate stiffness-dependent behavior over a relevant cell-sensing range [27]. Elastic 2D hydrogel systems with controlled stiffness such as polyacrylamide, poly-ethylene glycol [29], hyaluronic acid [30, 31], and alginate [32] are better suited to investigate these cell behaviors.

5.4 Summary

The commonly used PA hydrogel system is easily tuned to modulate substrate porosity, and in combination with different concentrations of sulfo-SANPAH, provides a platform to investigate how substrate stiffness, porosity, and ligand tethering affect stem cell fate. The data presented here provide direct evidence that the mechanical feedback provided by hydrogel deformations on planar matrices regulate osteogenic and adipogenic differentiation of ASCs and MSCs independent of protein tethering and substrate porosity. Furthermore, these data indicate that substrates have fibrous protein tethers as previously hypothesized [15]; however, these tethers are not essential for the osteogenic and adipogenic differentiation of ASCs and MSCs. This work further highlights the importance of bulk matrix stiffness as the major mechanical regulator of stem cell differentiation.

5.5 Methods

5.5.1 Polyacrylamide Gels

To covalently attach hydrogel substrates to glass, glass coverslips (Fisher) were cleaned of organics and oxidized by exposing both sides for 3 minutes to UV/ozone (BioForce). Samples were immediately functionalized with 20 mM 3-(trimethoxysilyl)propyl methacrylate (Sigma) in ethanol, washed with ethanol, and dried. A polymer solution containing acrylamide monomers, crosslinker N,N-methylene-bis-acrylamide, 1/100 volume of 10% Ammonium Persulfate (APS), and 1/1000 volume of N,N,N',N'-Tetramethylethylenediamine (TEMED) was prepared. 25 μ L of polymerizing hydrogel solution was sandwiched between a functionalized coverslip and a dichlorodimethylsilane (DCDMS)-treated glass slide to ensure easy detachment of hydrogels subsequent to polymerization. Hydrogels were allowed to polymerize for 15 minutes then soaked in water prior to use as a cell culture substrate. The ratio of acrylamide%/bis-acrylamide% was varied in order to control hydrogel stiffness and porosity. For 4 kPa gels, ratios of 4/0.4, 6/0.06, and 10/0.02 were used. For 13 kPa gels, ratios of 6/0.45, 10/0.1, and 20/0.03 were used. For 30 kPa gels, ratios of 8/0.55, 10/0.3, and 20/0.15 were used.

To allow for cell adhesion and fibrous protein tethering, collagen type I (BD Biosciences) was coupled to the surface using the heterobifunctional linker N-sulphosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH) as a protein-substrate linker. Substrates were incubated in 0.02, 0.1, 0.2, 0.5, or 1 mg/ml sulfo-SANPAH (Pierce) in sterile 50 mM HEPES pH 8.5, activated with UV light (wavelength 350 nm, intensity 4 mW/cm²) for 10 minutes, washed three times in HEPES, then incubated in 50 μ g/ml rat tail collagen type I in DI H₂O (BD Biosciences) overnight at room temperature. For AFM experiments, 0.5 mg/ml amine-PEG₃₄₀₀-biotin (Laysan Bio) was used in place of collagen type I. Coated hydrogels were stored in PBS at 4°C and UV sterilized prior to use in cell culture.

5.5.2 PA-PEG-RGD Gels

PA-PEG-RGD hydrogels were fabricated by incorporating acrylated-PEG₃₄₀₀-GRGD-amide (aPEG-RGD; 21st Century Biochemicals) into the polymerizing hydrogel solution with acrylamide monomers, N,N'-methylene-bis-acrylamide (Fisher Scientific), APS, and TEMED prior to polymerization. Acrylamide/bis-acrylamide ratios were as described in the previous section. Hydrogels were allowed to polymerize for 15 minutes then soaked in water prior to use as a cell culture substrate. Concentrations of 0.1 mM, 0.5 mM, or 2.5 mM of aPEG-RGD were incorporated into the hydrogels. In order to visualize RGD concentration differences, a fluorescent hydroxycoumarin dye (excitation 350 nm) was conjugated to the peptide to yield acrylated-PEG₃₄₀₀-[K-7-hydroxycoumarin-3-OH]GGGRGD-amide (21st Century Biochemicals).

5.5.3 PDMS Substrates

PDMS (Sylgard 184, Dow Corning) was mixed at various elastomer base:curing agent ratios (50:1, 75:1, 90:1, 100:1), thoroughly mixed, and degassed under vacuum for 1 hour before pouring directly into multi-well plates or onto coverslips and baked at 60°C overnight. In certain instances, substrates were functionalized with sulfo-SANPAH and ligand (see Polyacrylamide Gels) (Fig. 5.18a). For covalent attachment of moieties to the surface, PDMS substrates were treated with UV/Ozone for 5 minutes (BioForce) following a 1 hour incubation under vacuum in the presence of (3-aminopropyl)triethoxysilane (APTES). Surfaces were then incubated in a 1 mg/mL solution of sulfo-NHS-biotin in water (G-Biosciences) for one hour at room temperature (Fig. 5.18b).

5.5.4 Scanning Electron Microscopy

PA and PA-PEG-RGD solutions were polymerized in 1.7 mL microtubes or on glass coverslips. Hydrogels were swelled in water overnight, flashed frozen in liquid nitrogen, then lyophilized over night. Lyophilized samples were sputter coated for 6 seconds with iridium. SEM (FEI XL30 and Zeiss Sigma VP) images

were taken at 200x, 1000x, or 5000x at 5 kV.

5.5.5 DNA Gel Electrophoresis

GeneRuler 1 kb (250-10,000 bp) and 1kb Plus (75-20,000 bp) DNA ladders (Thermo Scientific) and HyperLadder V (50 to 500 bp; Bioline) were run through polyacrylamide electrophoresis gels in TAE buffer with ethidium bromide at 30 V for 14 hours. Images were taken using a ChemiDoc MP system (Bio Rad). DNA fragment lengths were converted to radius of gyration described elsewhere [22].

5.5.6 Stem Cell Culture

Human ASCs were isolated from freshly aspirated human subcutaneous adipose tissue (donor age between 26 and 31 years) according to the method described elsewhere [33]. To reduce the effects of donor-to-donor variation, cells from three different donors were pooled. Commercially available MSCs were purchased from Lonza. MSCs and ASCs (P4-P7) were cultured in low glucose Dulbecco's modified eagle medium (DMEM) (Gibco) with 10% fetal bovine serum (Gemini) and 1% antibiotics (Corning). For differentiation experiments, MSCs and ASCs were seeded on PA and PDMS substrates at a density of 1,000 cells/cm² and on PA-PEG-RGD gels at a density of 2,000 cells/cm² and cultured for 7 or 14 days; the medium was changed every 2-3 days. For studies conducted with osteogenic inductive media, growth media was supplemented with 100 nM dexamethasone (Sigma), 200 μ M ascorbic acid (Sigma) and 10 mM glycerol 2-phosphate (Sigma). For studies conducted with adipogenic inductive media, growth media supplemented with 0.5 mM isobutylmethylxanthine (IBMX), 100 nM dexamethasone, 10 μ g/ml insulin (Sigma), and 10 μ M indomethacin (Sigma).

5.5.7 Immunofluorescence

Cells were fixed in 10% formalin for 15 minutes at room temperature, rinsed in PBS, and permeabilized with 1% triton-X in PBS for 10 minutes at room temperature. Cells were stained with 1:500 rhodamine phalloidin (Life Technologies)

and 1:5000 DAPI in 2% bovine serum albumin in PBS for 30 minutes. Samples were washed with DI water and mounted with fluoromount-G (SouthernBiotech). Imaging was performed using a Nikon Eclipse TI microscope equipped with a CARV II confocal system (BD Biosciences), motorized stage and MS-2000 controller (Applied Scientific Instrumentation), and a Cool-Snap HQ camera (Photometrics) and controlled by Metamorph (Molecular Devices). For osteogenic differentiation studies, cells were stained with 1:100 RUNX2 (ab-23981; Abcam). To quantify RUNX2 expression, CellProfiler [34] (Broad Institute) was used to measure cytoplasmic and nuclear fluorescent intensities using the nuclei and cell outlines as masks to define these regions of interest in the RUNX2 fluorescent channel.

5.5.8 Differentiation Assays

ASCs and MSCs were stained for alkaline phosphatase (ALP), an osteogenic marker, using a Millipore ALP detection kit (SCR004; Millipore) according to the manufacturer's instructions. Briefly, cells were fixed in 10% formalin for 2 minutes at room temperature, rinsed in PBS, then incubated in staining solution composed of a 2:1:1 ratio of Fast Red Violet, naphthol AS-BI phosphate, and DI H₂O at room temperature in the dark for 15 minutes. Subsequent to staining, samples were rinsed in PBS then imaged.

ASCs were stained for Oil Red O (ORO), an adipogenic marker. Briefly, cells were fixed in 10% formalin for 15 minutes at room temperature, rinsed in PBS, incubated in 60% isopropanol for 5 minutes, then incubated in a filtered staining solution composed of 1.8 mg/ml ORO in 60% isopropanol in DI H₂O for 20 minutes. Subsequent to staining, samples were rinsed in DI H₂O then imaged.

5.5.9 Atomic Force Microscopy

To determine the mechanical properties of the various PA hydrogels by indentation and to quantify protein tethering by force spectroscopy, a MFP-3D-Bio (Asylum Research) atomic force microscope (AFM) was used. Chromium/gold-coated, silicon nitride (SiN) cantilevers with pyramid-shaped tips (PNP-TR;

NanoWorld) with ~ 50 pN/nm nominal spring constants as determined from the MFP-3D's built-in calibration function were used for both methods. All AFM data was analyzed using custom written code in Igor Pro (Wavemetrics). For indentation, samples were mounted on glass slides using vacuum grease and then placed on the AFM stage. Samples were placed in PBS and indented at a velocity of $2 \mu\text{m/s}$ until a trigger of 2 nN was detected using. Indentation curves were analyzed to determine the Young's modulus as previously described [35].

PDMS substrates were indented with the same cantilevers mentioned above. Additionally a cantilever with a $45 \mu\text{m}$ diameter polystyrene bead tip (Novascan) with 0.03 N/m nominal spring constant was used. Prior to indentation, all PDMS substrates were pretreated with a solution of 1% bovine serum albumin in PBS to limit non-specific adhesive interactions between the tip and the PDMS surface. Indentation curves were obtained and analyzed as described above. For retraction experiments, samples were indented with approach and retraction velocities ranging from 1 nm/s to $10 \mu\text{m/s}$. The substrate spring constants were determined by fitting the linear portion of the retraction curve starting at the undeformed surface.

For force spectroscopy, cantilevers were functionalized (Fig. 5.12a) with the antibody C2456, a mouse monoclonal anti-collagen type I antibody (Sigma), or avidin (PROSPEC) using a previously established method [36,37]. Briefly, the cantilevers were cleaned with chloroform for 30 seconds and incubated overnight immersed in 5 M ethanolamine-HCl in dimethylsulfoxide at room temperature resulting in amine group functionalization on the cantilever tips. After rinsing with phosphate-buffered saline (PBS), tips were incubated in 25 mM bis[sulfosuccinimidyl] suberate (BS₃; Pierce) for 30 minutes. After rinsing again in PBS, tips were immersed in 200 $\mu\text{g/ml}$ C2456 or 1 mg/mL avidin for 30 minutes to crosslink the protein to the tip. Functionalized cantilevers were rinsed, dried, and kept in 4°C until use. Samples were placed in PBS and force curves were taken in a regular 10×10 array of points spaced $10 \mu\text{m}$ apart using a functionalized SiN cantilever at $2 \mu\text{m/s}$. To promote binding of the antibody to collagen or avidin to biotin, a dwell time of 1 second was added between approach and retraction cycles. Force curves were converted to force vs. tip Z-position curves (Fig. 5.7a)

and then analyzed for rupture events using a previously described algorithm [38]; rupture lengths and forces were determined.

5.5.10 Traction Force Microscopy

Traction force microscopy was performed as previously described [23]. Briefly, fluorescent 580/605 0.2 μm microspheres (Invitrogen) were added to the pre-polymer solution to a final concentration of 1% v/v. Substrates were functionalized and treated as described above. The microspheres underneath selected live cells were imaged with a 40x dry confocal objective (Zeiss) using the CV1000 high-speed laser confocal imaging system equipped with an EM-CCD camera and a stage-top incubation system (Yokogawa). Cells were released with 2.5% trypsin and the same confocal stacks were acquired. Bead displacements were determined using a particle image velocimetry script in Matlab (MathWorks).

5.5.11 Förster Resonance Energy Transfer

Fibronectin was isolated from human plasma using gelatin-sepharose binding and eluted with 6 M urea. Isolated fibronectin was concentrated to ~ 3 mg/ml using an Amicon Ultra Centrifugal Filter (10 kDa NMWL) (Millipore), according to manufacturer's instructions, and denatured for 15 minutes in 4 M guanidine hydrochloride (GdnHCl). Denatured fibronectin was dual-labeled with Alexa Fluor 488 (donor) and Alexa Fluor 546 (acceptor) fluorophores, as previously described [13]. Briefly, denatured fibronectin was incubated with a 30-fold molar excess of Alexa Fluor 546 C5 Maleimide (Life Technologies) for 2 hours to label cysteine residues within the III₇ and III₅ domains of fibronectin. The single-labeled fibronectin was buffer exchanged into 0.1 M sodium bicarbonate pH 8.3 and separated from unreacted acceptor fluorophores using a spin desalting column (Thermo Scientific), according to manufacturer's instructions. The single-labeled fibronectin was then incubated with a 40-fold molar excess of Alexa Fluor 488 succinimidyl ester (Life Technologies) for 1 hour to label amine residues throughout fibronectin. Unreacted donor fluorophores were removed using a spin desalting column and

dual-labeled fibronectin was stored with 10% glycerol at -20°C . The average number of acceptor and donor fluorophores per fibronectin dimer was 3.6 and 8.8, respectively, and was determined using published extinction coefficients and the absorbances of the dual-labeled fibronectin at 280, 498, and 556 nm.

The emission spectrum of the dual-labeled fibronectin was characterized in varying concentrations of denaturant by fluorescence spectroscopy using a Synergy 4 Microplate Reader (BioTek). Briefly, 100 $\mu\text{g}/\text{ml}$ dual-labeled fibronectin in PBS was denatured in 0 to 4 M GdnHCl and excited at 484 nm. The resulting emission spectrum was measured from 510 to 700 nm (Fig. 5.11b) and the ratio of the maximum acceptor emission (~ 570 nm) to the maximum donor emission (~ 520 nm) was determined at each concentration of GdnHCl (Fig. 5.11c).

Images of the dual-labeled fibronectin were acquired on a Zeiss LSM 780 Confocal Microscope and analyzed using a custom MATLAB script, as previously described [14]. Briefly, images were averaged with a 3 x 3 pixel sliding block and perinuclear regions of DAPI stained cells were manually selected for analysis. The FRET ratio for each pixel within a selected region was calculated by dividing that pixel's intensity in the acceptor image by its corresponding intensity in the donor image. FRET ratios less than 0.05 and greater than 1.0 were excluded from analysis. The mean FRET ratio within the selected regions was calculated for each cell and then averaged over all the cells in each condition ($n = 16$ cells per condition) (Fig. 5.8g).

5.5.12 Statistics

Data are expressed as mean $\pm$ standard deviation or standard error of the mean as indicated. For statistical analyses, student's t -test were performed on the data presented in Fig. 5.11d, Fig. 5.8c, Fig. 5.12d, and Fig. 5.15b. 2-way ANOVAs were performed on the data in Fig. 5.8f and Fig. 5.8g. 1-way ANOVAs were performed on all other data where significance is shown. Multiple comparisons Tukey post tests were performed where appropriate. Differences were considered significant when $*p < 0.05$ and $**p < 0.0001$. All experiments were performed in triplicate unless otherwise noted, and in such cases, the number of

cells used in the measurement has been stated.

The material of Chapter 5 has been accepted for publication and is displayed as it may appear in *Wen J H*, Vincent L G*, Choi Y S, Fuhrmann A, Hribar K, Taylor-Weiner H, Chen S, Engler A J. Interplay of Matrix Stiffness and Protein Tethering in Mechanically Based Differentiation. Nature Materials. 2014 (In Press)*. The dissertation author was the primary co-author.

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Chapter 6

Conclusions

6.1 Introduction

In their native environment adult stem cells are surrounded by a matrix, which provides biochemical signals and a structure for physical cell-matrix interactions to occur. In fact, cells actively deform and remodel their extracellular matrix (ECM), probe its rigidity and porosity, and undergo lineage-specific differentiation in part by integrating various biophysical signals. Although the underlying signaling pathways are still a matter of debate, it is clear that cell-generated traction forces play an integral role in detecting biophysical cues and in both guiding cell migration and inducing subsequent differentiation.

6.2 Mechanical Gradients in Migration: a Target for Therapy

A number of age-related diseases are characterized by fibrosis progression, characterized by aberrant protein deposition. The local ECM composition changes, which leads to increased ECM stiffness. The mechanism involved in driving tissue rigidity progression have yet to be fully elucidated. Furthermore, research has suggested that local stiffening may be a cause, as well as a consequence, of disease progression [1].

Recent work with collaborators has looked further into the spatiotemporal changes in matrix rigidity. One key finding of *in vivo* fibrosis models of skin, lung, kidney, and joints is that matrix stiffness rises and falls over time, forming spatial gradients much like the ones fabricated for the studies depicted in chapter 2.

It is also well known that fibroblasts can be locally activated to become myofibroblasts. Under certain scenarios, this process can be initiated by a chemical response and propagated locally [2]. This could lead to an initial increase in stiffness that would then act as a positive feedback loop and activate more fibroblasts. While the mechanisms of fibrosis onset are out of the scope of this work, suffice it to say that the resulting stiffness gradients would then be amplified by fibroblast durotaxis to these stiffer areas as suggested by the findings in chapter 2.

Indeed, fibroblasts aligned in the direction of the gradient and durotaxed in the same manner as mesenchymal stromal cells in the absence of a chemo-attractive gradient, recapitulating observations of fibroblast accumulation in lung tissues biopsies of patients suffering from idiopathic pulmonary fibrosis [2].

These findings suggest that targeting durotaxis of various cell types has potential to a new therapeutic strategy for the treatment of a broad range of fibrotic diseases [3]. At the very least, some of the tools required to study the effects of treatments on durotaxis are presented within our body of work.

6.3 Traction Forces: Where and When to Pull Next?

The evolution of traction force microscopy (TFM) has allowed researchers to measure cell forces in increasingly complex environments with higher spatiotemporal precision. We can now measure the traction forces of individual focal adhesions [4], track the tangential and normal forces of migrating cells [5-7], and even measure the forces of cells without releasing them from the substrate by using a reference frame [8]. All of these applications of TFM aim to elucidate how cells interact with their materials, similar to what is presented in many of the chapters of this thesis.

One area that where TFM can be particularly useful is disease-in-the-dish models. For instance, dilated cardiomyopathy (DCM) has been modeled by human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). While most techniques used in studies have focused on protein, gene expression, and various -omic characterization, few have been functional tests of the living cells, with the exception of calcium handling using live fluorescence microscopy. Since cardiomyocytes are contractile in nature, traction force microscopy is well suited to study the force generation in time and space of these cells. Using a rapid acquisition camera and advanced optics, we can capture with high spatial accuracy the positions of beads embedded in hydrogels underneath beating cardiomyocytes while simultaneously imaging the intensity of cell-permeable dyes. This allows us to quantify both the calcium handling and force generation of beating cells in real time at up to 100 frames per second. The high temporal resolution is crucial in this instance as the beating frequency of human cardiomyocytes *in vitro* is ~ 50 Hz at ~ 60 beats per minute.

In DCM, the beta adrenergic signaling and pathological mechanisms are not well understood. In a recent collaboration of ours, we found that up-regulation of phosphodiesterases subtypes lead to impaired cAMP generation, blunted beta adrenergic signaling, and weakened contractility in DCM iPSC-CMs compared to iPSC-CMs derived from healthy patients. Our findings provide new insight into beta adrenergic signaling development in iPSC-CMs and identify a novel epigenetic mechanism of DCM pathogenesis, which may serve as a new therapeutic target for DCM.

In summary, traction force microscopy may complement a number of cell studies and shed insights on mechanosensitive pathways by providing real-time information of live cell forces.

6.4 Matrix Biology in the Third Dimension: Where Will the Forces Take Us?

The focus of our body of work has been confined to planar substrates and our analysis mostly limited to two dimensions. It is worth re-iterating from chapter 1 that investigations performed in 2D are convenient to study cellular responses. However, these environments do not accurately mimic the 3D environment of tissues that surround cells *in vivo* (see chapter 1 conclusions for additional comments).

As previously mentioned, it has been demonstrated that stem cells on 2D substrates respond to mechanical cues, and recent experiments with mesenchymal stem cells (MSCs) encapsulated in non-degradable, ionically crosslinked alginate hydrogels have implicated matrix rigidity as a regulator of cell fate in three dimensions [9].

Still, substrate stiffness is not sufficient to guide cell differentiation in 3D. MSCs encapsulated within non-degradable methacrylated hyaluronic hydrogels maintained a rounded morphology and differentiated into adipocytes independently of matrix rigidity, unlike in previous observations where stem cells were deposited on substrates of varying rigidity [10].

When situated within a degradable hydrogel, local degradability was needed for entrapped cells to be able to rearrange their cytoskeletal structure and undergo osteogenesis. Moreover, traction force microscopy determined that osteogenic differentiation in these degradable hydrogels was independent of cell morphology and dependent on cell-generated traction forces [10].

On the other hand, restricting cell rearrangement with contractile inhibitors, or introducing non-degradable crosslinks either right after initial seeding or a week after the cells were allowed to spread and migrate, attenuated osteogenesis and favoured adipogenesis. These findings imply that cell fate decisions in three-dimensional hydrogels are directed by force-dependent, cell-substrate interactions that require a degradable matrix in which MSCs can spread.

Importantly, these findings support the notion that, unlike in two dimensions, in three dimensions matrix-driven cell tension drives cell fate irrespective of

cell morphology and spread area. Similar conclusions were reached in a study involving MSCs encapsulated in ionically crosslinked alginate hydrogels, but in that case matrix rigidity directly, rather than indirectly, regulated cell fate. It thus seems that cell tractions and ultimately differentiation are directly influenced by the cells' ability to deform rigid substrates or indirectly affected by the cells first degrading the matrix and then contracting it. Indeed, it is the type of crosslink — covalent versus ionic — that affects the behaviour of the hydrogel matrix at the molecular scale: whereas ionic crosslinks enable cells to locally reorganize their focal adhesions without undergoing large morphological changes, cells encapsulated in relatively rigid hyaluronic hydrogels must alter their cytoskeletal structure by locally degrading the surrounding matrix to reach otherwise unavailable adhesive ligands. Therefore, the crosslinking nature of the matrix modulates the mechanical feedback of encapsulated cell-matrix interactions.

Yet, what are the underpinnings of particular matrix interactions that drive stem cell differentiation in both ionic and covalent hydrogels? Interestingly, delayed restriction of cell-mediated degradation greatly diminishes traction forces and favours adipogenesis, suggesting that cell-generated forces are dynamic in time and space, and require active cytoskeletal rearrangements to both maintain appreciable traction stresses over time and promote osteogenesis. Instead, MSCs in relatively rigid ionically crosslinked alginate gels were able to reorganize local matrix-bound ligands within a limited distance from the cell despite remaining poorly spread. Thus, it is likely that interactions between ligands and integrins (transmembrane proteins that mediate cell attachment) act as active molecular-force sensors of substrate mechanics [9].

In this regard, recent work with cells cultured on gel substrates identified that traction dynamics at individual focal adhesions act autonomously, and that adhesions exist either in a stable state involving a constant low force, or exhibit repeated tugging with an increased average force [4]. Adhesion dynamics also depended on substrate stiffness, with stiffer ECMs promoting more stable tractions, and soft substrates favouring tugging traction dynamics. Hence, dynamic rigidity sensing occurs at a resolution determined by the density of integrin ligands. These

considerations could be particularly important in cellular processes such as durotaxis (directed cell migration due to a gradient in substrate stiffness); in fact, a shift in the ratio of tugging to stable adhesions changes at the leading and trailing edges of the cell as a result of differences in substrate stiffness. Durotaxis is thus magnified by increasing gradient strength [11].

In addition to changes related to adhesion and matrix crosslinking, force-mediated stem cell differentiation depends on the spatiotemporal recruitment of load-bearing structural proteins and on the activation of downstream biochemical signalling pathways. For instance, interactions between paxillin (a protein involved in signal transduction) and vinculin (a cytoskeletal protein involved in the formation of focal adhesions) promote force transmission and focal-adhesion dynamics on substrates of similar stiffness to those previously used [10]. In fact, the ability of vinculin to bear a mechanical load has been identified as a regulatory process that stabilizes focal-adhesion dynamics [12], most likely preceded by the force-mediated unfolding of the cytoskeleton protein talin during adhesion maturation and the subsequent exposure of its cryptic binding sites for vinculin [13].

Assuming that signalling at individual adhesions scales with global, cell-generated forces and is sufficient to drive human MSC differentiation, it is surprising that osteogenesis has not been observed for cells either on or within very rigid substrates. Perhaps the maintenance of a certain force threshold over time would be sufficient to drive osteogenic commitment of isolated human MSCs; this could actually be predicted by monitoring the temporal evolution of cell-generated tractions [14]. Still, osteogenic commitment of MSCs scales initially with increasing stiffness (and presumably force) and peaks (or reaches an optimum) for cells on or within not-too-rigid substrates and three-dimensional matrices, as previously reported [9, 15]. Such a biphasic response could result from either MSCs sensing a change in the average bond lifetime of integrin ligands with rigid substrates as a consequence of frequent bond rupture, or from a varying number of integrin ligands under tension, which has been shown to peak on stiffer substrates [9].

Novel material platforms allow for the fate of encapsulated stem cells to be investigated as a function of ligand type and density, substrate stiff-

ness, and the presence or absence of covalent crosslinks that can restrict cell motility and modulate cell morphology. It is indeed the ability to alter the crosslinking density at will without drastically changing matrix mechanics that allowed researchers to discover the importance of hydrogel degradability on cell commitment. Although the fine details of force transmission will require close investigation of integrin/ECM ligand dynamics at the level of individual focal adhesions, traction-force measurements emphasize contractility as a general driver for cellular differentiation.

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