

**DIFFERENTIAL CELLULAR RESPONSE TO LINEAR AND STRAIN
STIFFENING HYDROGEL SUBSTRATES**

Jessamine Winer

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Paul A. Janmey, PhD

Professor of Physiology

Supervisor of Dissertation

Susan Margulies, PhD

Professor of Bioengineering

Graduate Group Chairperson

Dissertation Committee

Dennis Discher, PhD, Professor of Chemical and Biomolecular Engineering

John Weisel, PhD, Professor of Cell and Developmental Biology

Rebecca Wells, MD, Assistant Professor of Medicine

Beth Winkelstein, PhD, Professor of Bioengineering

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May the lab fairy smile upon you

ABSTRACT

DIFFERENTIAL CELLULAR RESPONSE TO LINEAR AND STRAIN STIFFENING HYDROGEL SUBSTRATES

Jessamine P. Winer

Supervisor: Paul A. Janmey

The mechanical properties of the substrate upon which cells are cultured have been shown to influence a variety of cell properties including cell adhesion, spreading, protein expression and differentiation. The work presented here examines how the nonlinear mechanical properties of biopolymer gels affect the cellular responses to substrate stiffness. Cell spread area decreases with decreasing substrate stiffness when cells are cultured on linearly elastic polyacrylamide gels but display no spread area sensitivity when cultured on fibrin gels of various moduli. Fibrin gels, and other semiflexible biopolymer networks, exhibit strain stiffening, whereby the elastic modulus of the gel increases with increasing applied strain. Mechanosensitive cells and strain stiffening gels engage in a mechanical feedback loop with cells increasing their applied force and the gel modulus increasing as a result until the cells reach their maximum spread area. Cell applied forces locally induce anisotropy in an initially isotropic matrix providing a mechanism for cell/cell communication over a distance of ~ 5 cell lengths. This results in alignment of adjacent cells and formation of ring-like multicellular patterns. Finally, due in part to its mechanical properties, fibrin is an appealing scaffold for neural tissue repair. Initial animal studies confirm that salmon derived fibrin mitigates pain and inflammation after injury to the central nervous system.

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CHAPTER 1

Introduction

1.1 Introduction

Until recently, with the exception of sensory nerves, the general consensus was that cell signaling was achieved solely through chemical messengers such as insulin and calcium. As cells were perceived to be insensitive to mechanical cues, the vast majority of cell culture was and is performed on glass coverslips or polystyrene dishes. While some cell types such as keratinocytes and fibroblasts flourish on these substrates, others such as endothelial cells adopt an aberrant phenotype or, like neurons, generally fail to thrive. Two main techniques are employed when cells can not be cultured on glass. In some cases cells are cultured on gels made from components of the extracellular matrix (ECM) (Schor et al. 1983) and in other cases they are grown on a feeder layer of supporting cells (Huettnner and Baughman 1986). In keeping with the central dogma of the field, these methods were hypothesized to work by providing the necessary chemical environment such as ligands in the case of ECM gels and soluble factors in the case of cell feeder layers. Over the past decade it has been shown that in many cases a physiologic phenotype can be achieved simply by culturing cells on a more compliant matrix (Engler et al. 2004; Georges et al. 2006) suggesting that it is proper mechanical signals not just chemical signals that the alternative culture methods provide.

Numerous studies in the previous several decades have overturned the conventional wisdom that chemical signals alone drive cell physiology. Mechanisms for

sensing the application of external force have been shown to be critical for many processes such as vasodilation (Naruse and Sokabe 1993) and bone remodeling (Misra and Samanta 1987). Yet cells are not just receivers of force, they can also actively apply force on their surroundings (Hellam and Podolsky 1969). This is true not just of muscle cells but for all motile cells because movement requires force. These traction forces have been quantified by measuring the ability of a cell to deform a substrate with known mechanical properties (Harris et al. 1980). To measure these traction forces new cell culture substrates had to be developed that were compliant enough to be deformed by cells (Pelham and Wang 1997). It was quickly recognized that cells behaved quite differently on these soft matrices than on traditional rigid substrates. They displayed altered motility, morphology and protein expression in response to matrix mechanical properties. While not all cell types exhibit substrate mechanosensing, most tissue forming cells including fibroblasts, mesenchymal stem cells, endothelial cells and epithelial cells exhibit increased spreading, increased traction forces and decreased motility on stiffer substrates (Dembo and Wang 1999; Engler et al. 2006; Pelham and Wang 1997; Pelham and Wang 1998; Yeung et al. 2005).

Though the features of the phenomenon have been fairly well described, the mechanism by which cells sense the mechanical properties of their matrix is unknown. What is known are many of the proteins and structures required for the cell to apply force upon its substrate and execute responses to the measured mechanical properties. Of key importance to the application of force by a cell upon its matrix are the proteins actin, myosin and integrins (Fig. 1.1) (Galbraith et al. 2002). In this system, non muscle

myosin II is the force generating component (Wakatsuki et al. 2003). Myosin utilizes the energy from ATP to exert force on filamentous actin, with a single myosin motor exerting 3-4 pN of force (Finer et al. 1994). The actin network serves as the scaffold upon which these forces are exerted and transmitted. A complex of proteins connects the actin filaments to clustered transmembrane integrin dimers which transiently bind to the extracellular matrix. This system works together to exert localized forces on the order of 1-10 nN/ μm^2 at distinct locations predominantly at the cells periphery. In one paper it was reported that cells adjusted the force they applied over two orders of magnitude as the stiffness of the substrate was varied from 1 - 100 kPa to maintain a constant applied strain of approximately 130 μm (Saez et al. 2005). This raises a critical question. Why do non muscle cells have the capacity to apply such significant forces?

The literature is full of incidental reports of *in vitro* cellular remodeling of ECM gels. A classic example is clot retraction by platelets (Quick and Hussey 1950), whereby the progression of the clotting cascade not only initiates polymerization of fibrinogen to fibrin but also activates local platelets which aggregate and are incorporated into the clot. The platelets apply contractile force upon the fibrin fibers, leading to gel compaction, water loss and stiffening of the matrix. Without proper clot retraction bleeding cannot be controlled because the mechanical properties of the native fibrin gel are insufficient to resist the force of blood flow (Weiss 1973). Fibroblasts, endothelial cells and other contractile cells have also been shown to compact fibrin and collagen gels (Barbieri et al. 1981; Guidry and Grinnell 1985; Niewiarowski and Goldstein 1976). These cell/gel constructs exhibit a high degree of fiber and cell alignment (Bell et al. 1979; Elsdale and

Bard 1972).

It has been proposed that the purpose of the high forces that non muscle cells exert is to compact and align the fibers of the ECM, creating tissues with the necessary structural design to resist the forces nature applies (Harris et al. 1981). This dissertation begins the process of combining the existing knowledge of cell traction forces, cell substrate mechanosensing and the mechanics of biopolymer networks to construct a theory explaining the mechanical interactions between contractile cells and mechanosensitive gels.

To start, a new role for substrate mechanosensing in regulating cell cycle progression of human mesenchymal stem cells (hMSC) is described in Chapter 2. Culturing hMSCs on gels with a shear modulus below 200 Pa, the approximate modulus of bone marrow, leads to almost complete cessation of proliferation without compromising the ability of these cells to subsequently re-enter the cell cycle and/or terminally differentiate. This is consistent with a recent report of decreased proliferation of dermal fibroblasts in less dense, more compliant 3D collagen matrices compared to denser, stiffer gels (Hadjipanayi et al. 2008). Work subsequent to that published in this dissertation has shown that FAK dependent activation of Rac and induction of cyclin D1 is suppressed on soft matrices indicating a stiffness dependent G1 cell cycle checkpoint (Klein et al. 2009).

The study in Chapter 2, like most studies of substrate mechanosensing, was executed using ligand coated gels made from a synthetic polymer such as polyacrylamide. These gels are ideal for such studies because they are inherently non

adhesive so the researcher can select and control the ligand type and density (Reinhart-King et al. 2003). Additionally, for studies of traction forces, gels made from these materials are linearly elastic making it mathematically tractable to determine the amount of force required to produce the observed strain. The problem, as Chapter 3 reveals, is that the substrate mechanosensing behavior observed on synthetic gels can not always be reproduced on biopolymer gels such as fibrin.

The strength and elasticity of fibrin gels allow them to resist external forces without tearing. These properties come from the monomer, fiber and bulk structure. At the monomer level it has been shown that each coiled coil domain can reversibly unfold, stretching 23 nm, when a force of approximately 94 pN (Brown et al. 2007) or 20 myosin powerstrokes (Ishijima et al. 1996) is applied. AFM data indicates that fibrin protofibrils are linearly elastic (Lim et al. 2008) with a modulus on the order of 1 MPa. As the fibrils laterally aggregate the structures increase in stiffness in the range of 10s of MPa (Collet et al. 2005) but the elastic modulus remains independent of strain. The low strain, less than 15%, elastic modulus of the gels formed by these fibers is 5 orders of magnitude lower than that of the individual fibers but the bulk modulus increases several orders of magnitude as the strain increases. This nonlinear elasticity occurs in gels made from many biopolymers and is thought to result from an open mesh network being formed by fibers of intermediate stiffness such that their persistence length is on the order of the distance between crosslinks (Storm et al. 2005).

Chapter 3 investigates and confirms the hypothesis that it is the nonlinear mechanical properties of the fibrin matrix that dictate the degree of deformation that

fibroblasts and hMSCs apply to fibrin networks. Fibroblasts and hMSCs are shown to deform the fibrin matrix up to 5 cell lengths away from their periphery. This deformation not only leads to fiber alignment but also stiffening of the adjacent fibrin network. By applying sufficient force to engage the strain stiffening regime of the biopolymer networks, cells create a degree of mechanical anisotropy that cannot be obtained or explained by fiber orientation alone (Thomopoulos et al. 2007).

Chapter 4 investigates a role for this single cell induced local mechanical and structural anisotropy in driving multicellular pattern formation. Pattern formation has been observed in cells cultured in a variety of biopolymer networks including fibrin, collagen and matrigel (Nicosia and Ottinetti 1990; Vernon et al. 1992). Endothelial cells, which form non physiologic, cobblestone monolayers when cultured on tissue culture plastic, will form branching networks with interconnected lumen when cultured on or within collagen gels (Bach et al. 1998). Their ability to form these networks depends on the collagen concentration and the gel's internal tension (Sieminski et al. 2004). The data in Chapter 4 correlates the deformability of the gel with the degree of pattern formation demonstrating how the ability of cells to sense the mechanical properties of their matrix and the nonlinear mechanical properties of biopolymer networks combine to facilitate formation of multicellular patterns.

In Chapter 5 the actin crosslinking protein filamin A is investigated as a critical element in the ability of cells to deform collagen but not fibrin gels. Cells which lack filamin A display unstable cortical actin (Cunningham et al. 1992) and are unable to mechanosense on collagen coated substrates but retain that capacity on fibronectin coated

substrates (Byfield et al. 2009). Thus it is filamin's role in the collagen binding focal complex not its role as an actin crosslinker which is critical to substrate mechanosensing on collagen. The work done here suggests that this is also true for the ability of cells to compact their matrices since cells lacking filamin A retain the ability to deform fibrin but not collagen gels. Additionally, the data suggest that melanoma cells and HEK 293 cells apply smaller forces on collagen matrices than fibrin matrices.

These studies on cellular interactions with fibrin gels were supplemented by *in vivo* studies investigating fibrin's potential as a scaffold for neural tissue repair. Fibrin has been shown to be conducive to neuron cell culture (Dubey et al. 2001; Herbert et al. 1998). Neurite extension and branching is further enhanced by using non mammalian fibrin such as that derived for salmon (Ju et al. 2007). Additionally, salmon fibrinogen lacks the binding site on mammalian fibrinogen for macrophages and microglia which has been shown to exacerbate inflammation (Adams et al. 2007; Akassoglou et al. 2004). For these reasons it was hypothesized that salmon fibrin would enhance recovery after traumatic brain injury or nerve root compression. The results for facilitating traumatic brain injury recovery were mixed but there was significant improvement of behavioral allodynia and suppression of inflammation after nerve root compression making salmon fibrin a novel candidate for treatment of inflammation after injury to the central nervous system.

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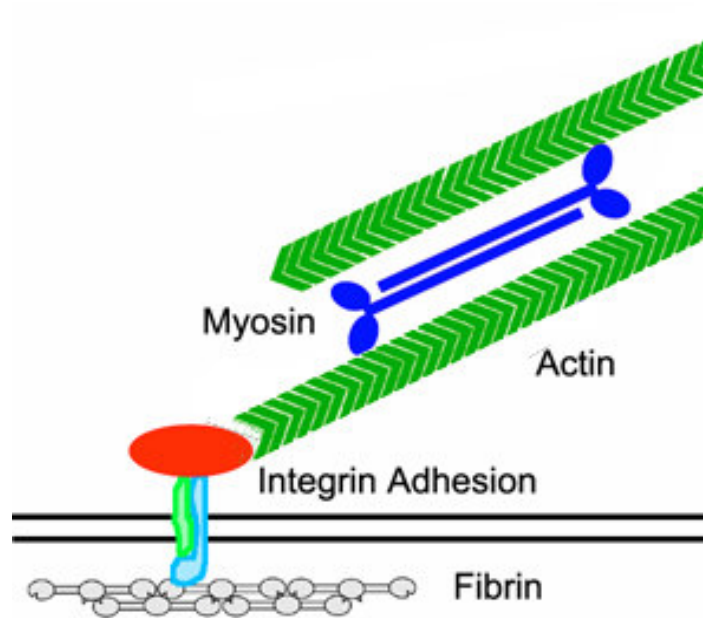
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Figure 1.1 Cartoon of a focal complex



A simplified diagram of a force transmitting focal complex.

CHAPTER 2

Substrate stiffness regulates cell cycle progression in human mesenchymal stem cells

(Adapted from Winer JP, Janmey PA, McCormick ME, Funaki M. 2009 *Tissue*

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1.1 Abstract.

Self-renewal and differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs) are strictly regulated by their microenvironment. Culturing these cells *ex vivo* leads to a rapid expansion followed by senescence, which is characterized by a lack of proliferation and differentiation. In this study, 250 Pa polyacrylamide gels were coated with a mixture of type 1 collagen and fibronectin, which mimics the elasticity of bone marrow and fat tissues. When hMSCs were seeded sparsely on these gels they halted progression through the cell cycle despite the presence of serum; however, when presented with a stiff substrate these non-proliferative cells reentered the cell cycle. Non-proliferative hMSCs on 250 Pa gels also exhibited the capability to differentiate into adipocytes when cultured in adipogenic induction medium or into osteoblasts if transferred to a stiff substrate and incubated with osteoblast induction media. These results demonstrate that hMSCs on 250 Pa gels are quiescent, but competent to resume proliferation or initiate terminal differentiation with appropriate cues.

2.2 Introduction.

Many adult tissues contain a small population of stem cells that are capable of producing identical daughter cells and differentiating into multiple, although not all types of, cell lineages. These cells serve as reservoirs when tissues are turned over or are damaged by trauma, disease, aging, etc (Tarnowski and Sieron 2006). Mesenchymal tissues, such as bone marrow, adipose tissue and cartilage, have been shown to contain mesenchymal stem cells that can differentiate into not only cells in the tissue they originate from but also cells in other mesenchymal tissues (Pittenger et al. 1999). For instance, mesenchymal stem cells isolated from adult bone marrow can differentiate and regenerate fat, bone, cartilage, muscle, ligament, tendon and stroma (Beyer Nardi and da Silva Meirelles 2006; Friedenstein et al. 1970).

Due to their multipotency and capacity for self-renewal, adult somatic stem cells have received significant attention for use in clinical applications such as regenerative tissue engineering and gene therapy (Kassem 2006). Among adult somatic stem cells, bone marrow-derived human mesenchymal stem cells (hMSCs) are one of the most promising target populations, due to the relative ease with which they can be isolated from the bulk of the cells in bone marrow and their significant expansion capacity *in vitro* (Baxter et al. 2004; Bonab et al. 2006; Liu et al. 2004; Reiser et al. 2005). One limitation that hampers clinical use of hMSCs is that once isolated and cultured *in vitro*, they age quickly and lose their potency for proliferation and differentiation [8-10]. To function as a reservoir, proliferation and differentiation of hMSCs must be suppressed during their resting period but have the capacity for upregulation upon stimulation. Signals from the

bone marrow microenvironment such as the presence of soluble factors, cell-cell interactions and cell-matrix interactions help to regulate stem cell behavior (Minguell et al. 2001). For instance, extracellular matrix proteins have been shown to regulate differentiation of MSC into chondrocytes and osteoblasts (Bosnakovski et al. 2006; Kundu and Putnam 2006). Many of the precise features of the extracellular matrix required for regulating the pool of undifferentiated stem cells remain unclear.

Over the last 10 years, the importance of substrate stiffness as a mechanism for modulating cell shape and phenotype has become increasingly studied. Work on differentiated tissue-forming cell types suggests that each responds to a different and specific range of substrate stiffness and often exhibits the most *in vivo* like morphology when the gel stiffness matches their native tissue compliance (Georges and Janmey 2005). For instance neurons exhibit more neurite branching on gels mimicking the stiffness of brain tissue and myotubes exhibit striation only on gels whose compliance is comparable to muscle tissue (Engler et al. 2004; Flanagan et al. 2002). Fibroblasts adopt a round shape on a soft matrix, and stress fibers are apparent only when they are plated on matrices stiffer than 3600 Pa (Yeung et al. 2005). In multipotent hMSC matrix elasticity can drive morphological and protein expression changes toward neurogenic, myogenic or osteogenic profiles in a myosin II-dependent fashion (Engler et al. 2006). Most *ex vivo* studies on hMSCs have been carried out by seeding cells on plastic or glass surfaces, which are drastically stiffer than any non calcified adult tissue. Polyacrylamide offers a well characterized system for modulating substrate stiffness while maintaining control over adhesive ligand density and composition (Georges and Janmey 2005).

In this report we provide evidence that bone marrow-derived hMSCs become quiescent on soft polyacrylamide gels that mimic the elasticity of bone marrow. Quiescent hMSCs on soft gels remained competent to resume proliferation or initiate terminal differentiation when additional cues were delivered. Thus, the elasticity of bone marrow may contribute to the ability of the bone marrow microenvironment to maintain quiescent multipotent mesenchymal stem cells.

2.3 Materials and Methods.

2.3.1 Cell Culture

Bone marrow-derived human mesenchymal stem cells were maintained in growth medium (GM), DMEM with 1 g/L D-glucose, 0.3 mg/ml L-glutamine and 100 mg/L sodium pyruvate and 10% heat inactivated fetal bovine serum on tissue culture plastic prior to seeding on gels. For adipocyte differentiation, cells were exposed to two chemical induction cycles consisting of 3 days in adipogenic induction media (GM, 1 μ M dexamethasone, 200 μ M indomethacin, 10 μ g/ml insulin and 0.5 mM 3-Isobutyl-1-methylxanthine) and 1 day in adipogenic maintenance media (GM and 10 μ g/ml insulin). Undifferentiated cells were kept in GM and re-fed every 3 days. For osteoblast differentiation cells were incubated with osteoblast induction media (GM, 50 μ M ascorbic acid-2-phosphate, 10 mM β -glycerophosphate and 100 nM dexamethasone) for 24 days changing the media every 3-4 days. Cells were plated at a density of 5×10^4 cells per 4.9 cm^2 on gels, or 1×10^5 cells per 4.9 cm^2 (confluent) or 1×10^4 cells per 4.9 cm^2 (sparse) on glass.

2.3.2 1D polyacrylamide gels

Polyacrylamide gels for cell culture were prepared using a protocol derived from the seminal Pelham and Wang experiments (Wang et al. 2001; Wang and Pelham 1998). Solutions of 3.0% acrylamide and 0.2% bisacrylamide (for 250 Pa gel) or 7.5% acrylamide and 0.5% bisacrylamide (7500 Pa gel) were prepared in phosphate buffered saline (PBS), pH 7.4. Polymerization was initiated with N,N,N',N'-tetramethylethylenediamine and ammonium persulfate. A droplet of 150 μ l was deposited on a 25 mm glass coverslip previously modified with 3-aminopropyltrimethoxysilane and glutaraldehyde. 15 μ l of 2% acrylic acid N-hydroxysuccinimide ester in toluene was applied to the droplet and a 25 mm chlorosilanized coverslip was placed on top of the droplet and removed after polymerization is completed. The gels were placed into an agarose coated six well polystyrene tissue culture plate and washed with 50 mM HEPES, pH 8.2. The N-succinimide crosslinker on the top of the gel was reacted for 1 hour with the ligand of interest diluted in HEPES buffer. Gels were washed and cells were seeded within 24 hours. Ligands used were either a mixture of 0.1 mg/ml rat tail collagen and 0.01 mg/ml human fibronectin.

2.3.3 Rheology of Tissues and Gels

To measure the dynamic shear storage modulus (G') of polyacrylamide gels, 500 μ l of monomer solution was polymerized between two 25 mm steel parallel plates in a hydration chamber. The shear modulus was calculated from the in-phase shear stress on a strain-controlled RFS III fluids spectrometer rheometer (Rheometrics, Piscataway, NJ) using a 2% oscillatory shear strain at a frequency of 10 radians per second. These

parameters were chosen to probe the elastic response of the tissues in the region where G' is independent of strain. To measure the G' of animal tissues, either 8 mm diameter, 2 mm thick samples (for rat tissues) or 25 mm diameter, 2 mm thick samples (for bovine tissues) were punched out. The shear moduli of the hydrated tissue samples were measured at 37°C using parallel plates with geometries matching the sample. Rat tissues were dissected from the animal, kept hydrated in PBS for no longer than 1 hour prior to the measurements. Bovine tissues were purchased from a commercial slaughterhouse, transported on ice, hydrated and warmed to 37°C before measurements were made. Three samples of each tissue were measured.

2.3.4 Imaging and Analysis

Immunostaining was performed as described previously (Funaki et al. 2004). Fatty acid rich vesicles were stained with Oil red o after 2 cycles of adipogenic induction or 8 days in growth media as described previously by Shao and Lazar (Shao and Lazar 1997). Calcium rich extracellular matrix was stained with Alizarin Red S after 24 days in osteoblast induction media according to the manufacturer's instructions. BrdU incorporation was measured by incubating the cells in BrdU-containing medium overnight. Cells were fixed and stained for both BrdU and DAPI according to the manufacture's instructions. In all cases, cells were imaged on a Leica DM-IRBE microscope (Wetzlar, Germany) with a Hamamatsu ORCA camera (Hamamatsu, Japan).

Images of phalloidin staining were used to measure the projected cell area, the images were converted to 8 bit, thresholded and the projected area was analyzed using Image J software. To verify that there is no overlapping of cells, cells were also stained

with DAPI. Only isolated cells were counted.

2.3.5 Quasi 3D polyacrylamide gels

Substrate sandwiches to mimic a 3D environment were composed as described by Beningo et al.(Beningo et al. 2004) with some minor modifications. Cells were seeded onto a 25 mm gel and allowed to adhere for 48 hours. Then a second coverslip, with or without a 250 Pa polyacrylamide gel on the bottom side, was placed on top of the seeded gel. To bring the cells into close proximity with a second coverslip, a sterilized 35 gram weight was placed on top of the sandwich for 30 seconds. The media was reintroduced after which the weight will be removed and the cells were left in the sandwiches for 2 days. The cells were imaged just before the top coverslip is applied and 2 days afterward.

2.4 Results.

2.4.1 The elasticity of bone marrow and a differentiated tissue with comparable stiffness.

To determine the gel stiffness appropriate for studying the role of substrate compliance in maintaining a reservoir of resting hMSCs, rheological testing was conducted on bone marrow. The shear modulus was measured because the lateral movement seen in traction force microscopy indicates that cells cultured in a 2D environment are applying predominantly shear forces to the substrate (Balaban et al. 2001). Bovine bone marrow exhibited an oscillatory storage modulus (G') of 220 Pa (Table 2.1). Adipose tissue was identified as a differentiated tissue with an elastic modulus comparable to that of bone marrow. Rat adipose tissues exhibited a slightly lower G' of approximately 150 Pa with no statistical difference between visceral and subcutaneous fat. Based on these measurements a polyacrylamide solution of 3.0%

acrylamide and 0.1% bisacrylamide was adopted, which has a polymerized G' of 250 ± 25 Pa, to mimic the elasticity of their natural environment. In some experiments a second substrate of 7.5% acrylamide and 0.5% bisacrylamide with a storage modulus of 7500 ± 250 Pa, which is in the range of muscle tissue, served as a control (Engler et al. 2004).

2.4.2 Soft gels induce a round shape and cell cycle arrest in hMSCs.

After two days on 250 Pa gels coated with a mixture of fibronectin and collagen I, hMSC had small, rounded morphologies with a disorganized F-actin cytoskeleton (Fig 2.1 A and G). They had an average projected cell area of only $650 \pm 250 \mu\text{m}^2$ compared to a spread area of $3500 \pm 1100 \mu\text{m}^2$ on glass coated with the same adhesion proteins. A similar phenotype has been observed in fibroblasts, endothelial cells and astrocytes on 250 Pa gels (Georges et al. 2006; Pelham and Wang 1997; Yeung et al. 2005). Extending the culture time to seven days resulted in some of the cells strengthening their adhesion to the compliant matrix and beginning to spread; however, there was no noticeable increase in cell number (Fig 2.1 D).

In comparison hMSC kept on 7500 Pa gels for two days were partially spread, with a projected cell area of $2600 \pm 1500 \mu\text{m}^2$ and exhibited some actin organization but few stress fibers (Fig 2.1 B and E). After seven days they apparently increased their numbers and became increasingly spread upon cell-cell contact (Fig 2.1 H). On glass, hMSC exhibited a spread morphology, had abundant stress fibers and proliferated rapidly (Fig 2.1 C, F and I).

To investigate the qualitative observation that matrix elasticity may alter the proliferative behavior of hMSC, a 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdU)

incorporation assay was performed on cells cultured on various substrates (Fig 2.2). After an overnight incubation with BrdU, virtually no hMSCs adhering to 250 Pa gels incorporated BrdU. Thus, despite the presence of serum, DNA replication, a step required for hMSC cell division, is suppressed. On 7500 Pa gels approximately half of the cells replicated their DNA in 24 hours and when sparsely seeded on glass over 80% of the cells exhibited nuclear BrdU staining. To check that the quiescent phenotype was not a donor specific phenomenon, the results were reproduced using a second batch of hMSCs. These results imply that substrate compliance can significantly affect progression of the cell cycle in hMSCs.

2.4.3 Quiescent cells competent to resume proliferation and maintain multipotency

Damaged viability of hMSCs on a soft substrate could explain the observations that cells on 250 Pa gels round up and cease proliferating despite the presence of serum; however, hMSCs on 250 Pa gels did not incorporate trypan blue or annexin V (data not shown), indicating these cells are not dead or apoptotic. To determine whether the soft gel-induced cell cycle arrest is reversible, the cells were put in a quasi 3D environment (Fig 2.3) This consisted of placing either a 250 Pa gel or a glass coverslip, both of which are coated with the same matrix ligand as the bottom gels on top of round non-proliferating hMSCs on 250 Pa gels. Cells sandwiched by two 250 Pa gels either stayed round or spread to a small degree, but no apparent increase in their number was observed (Fig 2.3 C). On the other hand, cells facing glass coverslips on top became spindle shaped and proliferated (Fig 2.3 D). Once the hMSCs made firm adhesions to the upper glass surface they detached from the soft substrate. This response was confirmed with

cells from a second donor to ensure it was not a donor specific response. Therefore, hMSCs on 250 Pa gels are competent to resume proliferation, once they receive a mechanical signal from a stiffer substrate.

Terminal differentiation is achieved through a highly coordinated set of events that includes growth arrest and expression of cell lineage restricted phenotypes. Thus, the temporary growth arrest of hMSCs on 250 Pa gels motivated this investigation into whether these cells are competent to differentiate. Since substrate elasticity is reported to specify the lineage into which hMSC differentiate, adipocyte differentiation of hMSCs was tested on 250 Pa gels, which is an elasticity comparable to fat tissues (Engler et al. 2006). Morphological changes such as a round shape and disorganized F-actin observed in hMSCs on 250 Pa gels, have been associated with adipogenic differentiation (Koutnikova and Auwerx 2001; Spiegelman and Ginty 1983).

When cells on matrices with various stiffnesses were treated with adipogenic differentiation medium, there was an inverse relationship between the percentage of cells that incorporated BrdU into their nuclei and the percentage of cells that accumulated lipid droplets detected by Oil red O staining. Approximately 75% of cells sparsely seeded on 250 Pa gels accumulated lipid droplets, whereas only slightly more than 20% of cells sparsely seeded on 7500 Pa gels or 2% of cells sparsely seeded on glass coverslips responded to adipogenic differentiation medium by accumulating lipid droplets (Fig 2.4 A). Notably without incubation in adipogenic differentiation medium hMSCs on 250 Pa gels did not accumulate lipid droplets suggesting that a soft matrix facilitates but is not sufficient to induce adipogenic differentiation of hMSCs..

Efficient adipogenic differentiation of growth-arrested hMSCs on 250 Pa gels raises the possibility that these non-proliferative cells are differentiated into preadipocytes and committed to an adipogenic lineage, instead of staying quiescent as stem cells. To rule out this possibility, hMSCs were sparsely seeded on a soft gel for two days, then transferred to a glass coverslip using the quasi 3D method and finally cultured in osteoblast induction media for 24 days. As mentioned earlier cells on 250 Pa gels that came in contact with a stiff surface transferred to that substrate, resumed proliferation and took on the spindle phenotype typical of hMSCs on tissue culture plastic. After 24 days in osteoblast induction media calcium rich extracellular matrix was clearly visible in the culture dish and stained positive with Alizarin Red S (Fig 2.4 B) matrix mineralization is a late stage marker of osteoblast differentiation (Kundu and Putnam 2006). Without stimulation by osteoblast induction media the cells did not deposit calcified extracellular matrix (data not shown). These results demonstrate that hMSCs on 250 Pa gels maintain their multilineage potential, supporting the hypothesis that hMSCs seeded on soft gels stay quiescent as stem cells.

2.5 Discussion.

The results presented here suggest that hMSC on 250 Pa gels are in a quiescent state awaiting a further signal to determine their fate. Three fates explored in this study are adipogenic differentiation, driven by the presence of chemical factors, a return to the cell cycle, driven by the availability of a stiff surface on which to attach, or osteogenic differentiation which requires both chemical induction and a stiff substrate. Stimulating cells cultured on soft gels with adipogenic differentiation factors results in a remarkably

high number of cells accumulating lipid droplets, yet without chemical induction there was no lipid accumulation. Chemical induction was also required for osteoblast differentiation. This requirement for synchronized mechanical and chemical stimulation provides an explanation for how hMSCs can be compartmentalized into compliant tissues such as bone marrow and yet resist spontaneous differentiation.

In this study the polyacrylamide gel system developed by Pelham and Wang was employed to modify substrate elasticity without changing the chemical environment to which the cells are exposed (Pelham and Wang 1997). In addition to matrix elasticity, the choice of extracellular ligand can also strongly affect hMSC adhesion and differentiation (Salasznyk et al. 2004). Collagen type 1 is found in a variety of tissues including bone and adipose and is regularly used as a substrate for cell adhesion experiments; however, on the 250 Pa gel collagen alone was not sufficient to ensure adhesion of a majority of cells (unpublished observation). Fibronectin alone has been shown to inhibit adipogenesis by strengthening the actin cytoskeleton; however, a round shape, such as that induced by a compliant substrate, negates this inhibition (Spiegelman and Ginty 1983). A mixture of collagen type I and fibronectin (10:1) provided the best adhesion of cells to the 250 Pa gels without affecting differentiation potential. HMSCs have the capacity to remodel their microenvironment by altering the expression of matrix metalloproteases, which might help to promote efficient terminal differentiation after an initial strong adhesion is achieved (Neth et al. 2006; Urs et al. 2004).

DNA synthesis in hMSCs was shown to decrease almost completely when hMSCs were cultured on soft gels and took on a round phenotype. This is in contrast

with other proliferating cell types such as NIH 3T3 fibroblasts, bovine aortic endothelial cells and normal rat kidney epithelial cells which all continue to divide when cultured on gels soft enough to promote a round phenotype (Guo et al. 2006; Yeung et al. 2005). Thus stem cell quiescence on 250 Pa gels is not a general shape induced failure of cytokinesis but a specific sensitivity of hMSCs to substrate compliance.

When nonproliferating hMSCs were presented with a protein-coated glass substrate, the cells developed a spindle morphology and reentered the cell cycle. In addition to showing that the round cells on soft matrices were only quiescent and not senescent, this experiment also demonstrated that the presence of a stiff substrate overrides the physical cues from a compliant matrix. Thus, in agreement with experiments using NIH 3T3 fibroblasts on stiffness gradients, hMSCs select a stiffer substrate over a more compliant one (Lo et al. 2000). Further experiments will be required to show if this dominant signal from a stiff matrix true also occurs in a fully 3D and not just a quasi 3D environment.

Much research has gone into understanding the requirements for adipogenesis including cytoskeletal reorganization and the induction of transcription factors such as C/EBP α and PPAR γ (Chawla et al. 1994; El-Jack et al. 1999; Freytag et al. 1994; Tontonoz et al. 1994). Clonal expansion and confluence were also thought to be required for adipogenesis until recent work by McBeath et al. demonstrated that at least in hMSCs, a round shape could replace the need for cell crowding (McBeath et al. 2004). McBeath et al. generated round hMSCs by confining their attachment area to 1024 μm^2 . These confined cells are fundamentally different than round cells on soft gels because they are

still proliferating and had a 30% lower percent adipocyte differentiation after 1 week in differentiation media. A role for substrate stiffness in priming cells for adipogenesis has not previously been considered. The enhanced conversion of hMSCs on soft gels into adipocytes upon a chemical signal may help to explain why bone marrow becomes fatty with age (Gurevitch et al. 2007).

On the soft 250 Pa gels without any chemical induction, there was not a significant population of cells exhibiting a neuronal phenotype with neurite-like protrusions such as those described by Engler et al. on gels of similar compliances (Engler et al. 2006). On stiff 7500 Pa gels, cells did exhibit the elongated morphology described in that study (Engler et al. 2006). Although their conditions were quite similar to the ones used here, two key differences likely led to a round adipocyte-like shape over a neuronal phenotype. First, fibronectin, which made up 10% of the adhesion mixture used in this study, has been shown to decrease the differentiation of neural stem/precursor cells to neurons and those neurons that did commit on a fibronectin-coated substrate extended shorter neurites (Flanagan et al. 2006). Second, bone marrow donor variability might contribute to a predisposition toward adipocyte or neuronal differentiation as donor age and sex have been shown to effect osteoblast and myoblast differentiation capacity (Deasy et al. 2007; Siddappa et al. 2007). The results presented here indicate that soft gel induced quiescence is not strongly donor dependent because two independent batches produced similar BrdU incorporation profiles.

Substrate compliance appears to regulate differentiation of cells with specific phenotypes, but three lines of evidence show that mechanical properties alone cannot

direct terminal stem cell differentiation. First, several tissues in the body have similar compliances; for example brain, fat and bone marrow tissues all have a storage modulus of approximately 200 Pa, yet all maintain unique populations of cells. Second, *in vivo* hMSCs are stored in an individual's bone marrow for decades and yet retain multipotency. Third, hMSCs are typically cultured *ex vivo* on stiff tissue culture plastic and retain multipotency for several passages. Evidence from this and several previous studies strongly suggest that mechanical and chemical stimuli are integrated by the cell to determine its response, and whereas in some cases chemical stimuli can override the influences of substrate mechanics, in other cases an inappropriate mechanical environment prevents a normal cellular response to chemical agonists (Engler et al. 2006; McBeath et al. 2004). This hypothesis was confirmed by the finding that quiescent cells differentiate into osteoblasts only as the result of changing both their physical and chemical environments to those that stimulate osteogenesis. Thus a compliant matrix has the capability to maintain a quiescent population of multipotent bone marrow mesenchymal stem cells that respond to both mechanical and chemical stimuli which drive proliferation and differentiation.

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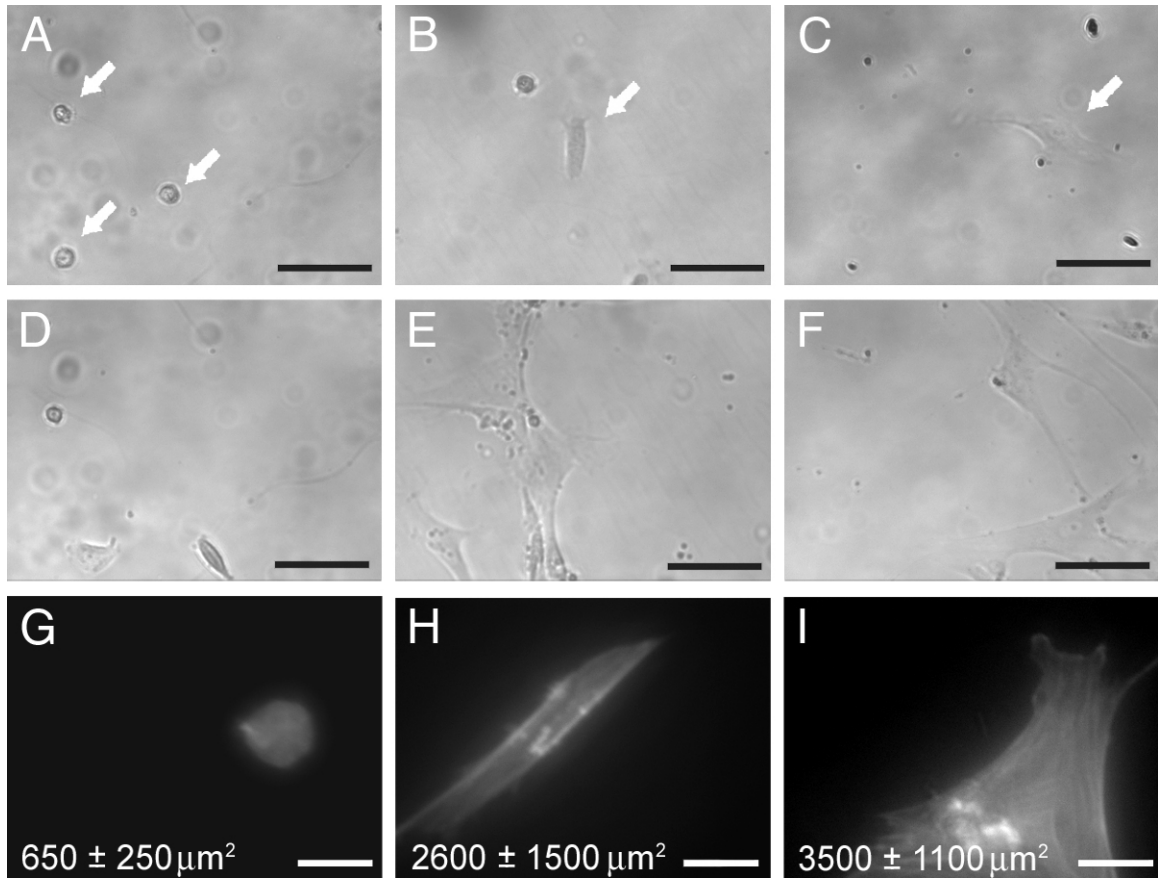
Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, Zahir N, Ming W, Weaver V, Janmey PA. 2005. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. Cell Motil Cytoskeleton 60(1):24-34.

Table 2.1 Rheological properties of bone marrow and fat tissue

Species	Bovine	Rat	Rat
Tissue	Bone Marrow	Visceral Fat	Subcutaneous Fat
Storage Modulus (mean $\pm$ SD)	220 $\pm$ 50 Pa	130 $\pm$ 70 Pa	160 $\pm$ 70 Pa

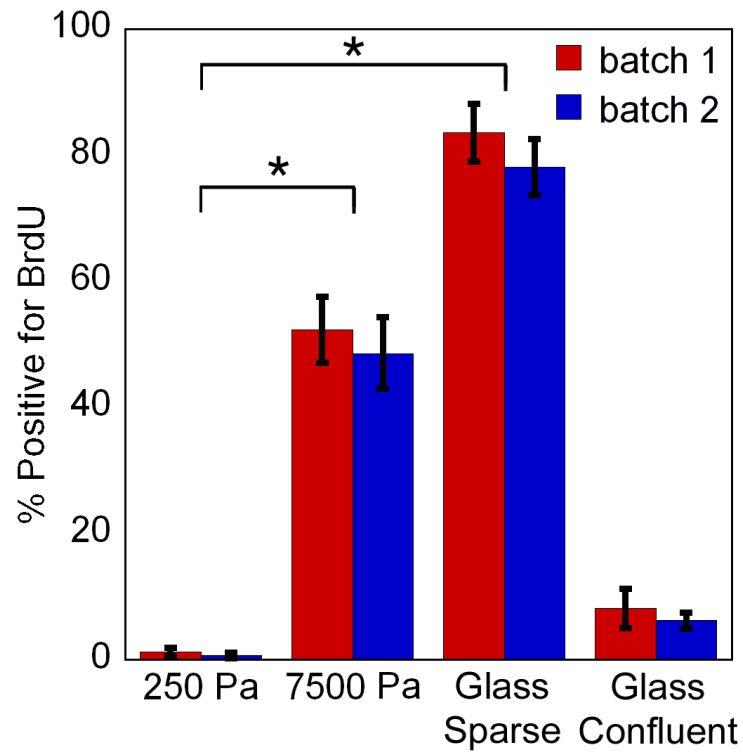
Animal tissues were freshly harvested and their shear storage modulus was measured on a strain controlled rheometer. At least three samples of each tissue type were measured and the mean $\pm$ SD is reported.

Figure 2.1 Substrate stiffness regulates hMSC shape, spread area and proliferation



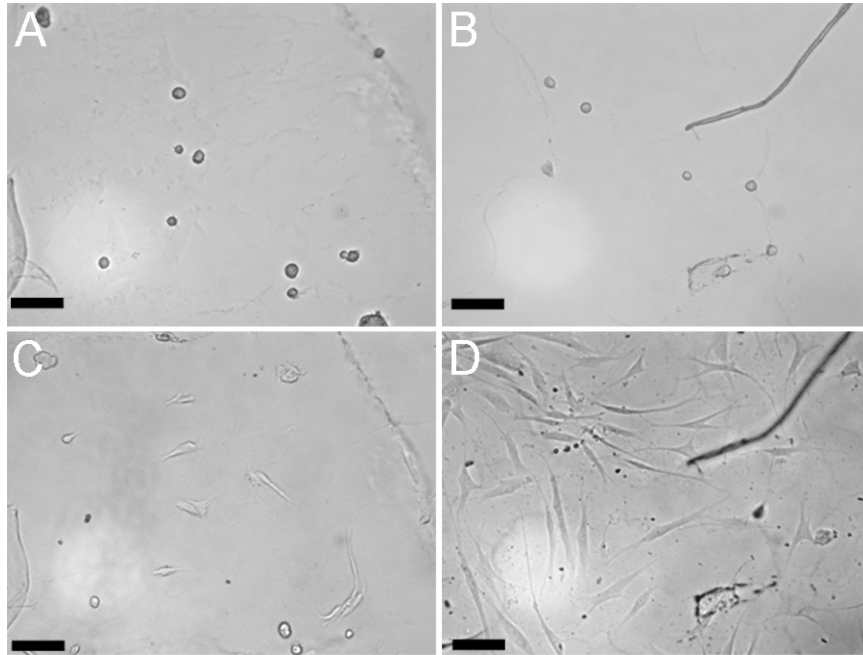
A-C: cells were plated on collagen type I and fibronectin-coated substrates and imaged in bright field after 24 hours of incubation in growth media (GM). Arrows indicate location of cells. D-F: Images at the same locations after 7 days of incubation in GM. Cells were kept at 37°C during imaging. Representative images from 2 independent experiments are shown. Scale bar = 100 μm . G-I: F-actin in hMSC was labeled with FITC conjugated phalloidin two days after seeding. Also reported is the mean projected area $\pm$ standard deviation. At least 40 cells in seven randomly chosen fields were counted for each condition. Scale bar = 20 μm .

Figure 2.2 A soft substrate suppresses Bromodeoxyuridine uptake by hMSCs



Cells were plated on substrates for 48 hours before incubating the cells in media containing BrdU overnight. Cells were kept in serum-containing media for the entire period, except for the Glass Confluent, which was serum starved for 24 hours before the BrdU was added in serum free media. Cells were stained with anti-BrdU and the nuclei were counterstained with DAPI. At least 40 cells were counted on each substrate and data is expressed as mean $\pm$ SE of three independent experiments. * $p < 0.01$

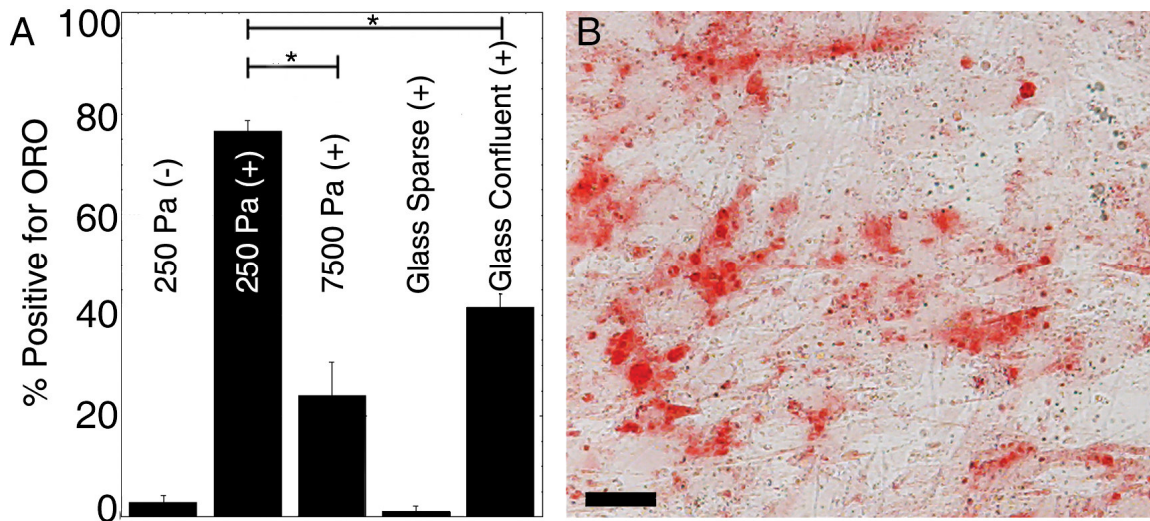
Figure 2.3 Presentation of a hard substrate to cells on a soft gel stimulates spreading and cycle re-entry



Cells were seeded onto 250 Pa gels and imaged after two days in serum containing media (A,B). Then a top substrate of either another 250 Pa gel (C) or a glass coverslip (D) was used to sandwich the cells in a quasi 3D environment. Two days later the cells were imaged again at the same locations. Results were repeated in an independent experiment.

Scale bar = 100 μm .

Figure 2.4 Cells retain differentiation potential



A: Oil red O staining for lipid accumulation in hMSCs. Cells were seeded on to 250 Pa, 7500 Pa or glass substrates (in either sparse or confluent conditions) and subjected to either 8 days of growth media (-), or two cycles of adipogenic induction (+). * $p < 0.02$.

B: Cells were cultured on a soft gel for two days then transferred to a glass coverslip using the quasi 3D method and then incubated with osteoinduction media for 24 days. Calcium deposition was seen in two independent experiments. Scale bar = 200 μm

CHAPTER 3

Contractile cells respond to the high strain modulus of strain stiffening biopolymer gels

(Adapted from Winer JP, Oake S, Janmey PA.2009. *PLoS One* 4: e6382)

3.1 Abstract

Most tissue cells grown in sparse cultures on linearly elastic substrates typically display a small, round phenotype on soft substrates and become increasingly spread as the modulus of the substrate increases until their spread area reaches a maximum value. As cell density increases, individual cells retain the same stiffness-dependent differences unless they are very close or in molecular contact. On nonlinear strain-stiffening fibrin gels, the same cell types become maximally spread even when the low strain elastic modulus would predict a round morphology. Displacement microscopy reveals that fibroblasts and human mesenchymal stem cells on fibrin deform the substrate by several microns up to five cell lengths away from their plasma membrane through a force limited and integrin dependent mechanism. Atomic force microscopy and rheology confirm that these strains locally and globally stiffen the gel in a density dependent manner. Thus cells are acutely responsive to the nonlinear elasticity of their substrates and apply forces strong enough to measurably contract their substrates.

3.2 Introduction

Over the last decade it has been demonstrated that a variety of tissue-forming cells can both sense the stiffness of their substrate and apply a controlled force onto that substrate. Not all cell types respond to stiffness changes in the same way, but many including endothelial cells (Yeung et al. 2005), fibroblasts (Pelham and Wang 1998), mammary epithelial cells (Paszek et al. 2005) astrocytes (Georges et al. 2006), macrophages (Fereol et al. 2006) and mesenchymal stem cells (Engler et al. 2006), exhibit increased spreading and adhesion on stiffer substrates compared to softer ones. Numerous proteins and protein complexes required for responding to substrate stiffness, such as the actomyosin network, small GTPases, protein phosphatases, and integrin adhesion sites have been identified, but the mechanism by which forces govern the interactions among these proteins is not yet defined.

Mechanically compliant materials for studying cellular responses to substrate stiffness are typically made from synthetic polymers whose elastic moduli are independent of applied strain and are determined by polymer and crosslinker density. The polyacrylamide gel system developed by Pelham and Wang allows the substrate's physical properties to be manipulated without affecting its chemical properties (Wang and Pelham 1998). Adhesion molecules are covalently attached to the gel's surface after polymerization, resulting in a uniform coverage regardless of gel stiffness. Similar gels have been adapted for traction microscopy to quantify the forces that cells exert on compliant substrates (Reinhart-King et al. 2003), and the linearity of the elastic response is essential to the algorithms that permit forces to be calculated from the measured

displacement fields. Other synthetic systems with linear elasticity include silicone films (Harris et al. 1980) and flexible PDMS micropillars (Tan et al. 2003).

Studies done on synthetic gels have been used to understand how cells respond to the mechanical properties of the tissue microenvironment; however, extracellular matrix proteins such as collagen type I and fibrin display nonlinear mechanical properties such as strain stiffening (Storm et al. 2005) and negative normal stress (Janmey et al. 2007). In these materials the elastic modulus of the gel increases orders of magnitude as the applied strain increases such that the resistance that a cell feels would be a strong function of the strain that it applies. Many cell types also modulate the force they apply according to the stiffness of the gel, applying smaller forces when cultured on softer gels (Pelham and Wang 1999). Using responses to linearly elastic materials to predict a cell's behavior on nonlinear gels is further complicated since it is not known what property the cell's mechanosensor is measuring. For example, whether cells attempt to exert a constant deformation and monitor the required stress, or whether they exert a constant stress and respond to the degree of strain remains an open question (Saez et al. 2005).

Strain stiffening is a property rarely seen in synthetic polymers but is common among gels made from filamentous biological polymers such as fibrin, collagen and actin. As it is a property prevalent in extracellular matrix proteins, it has been postulated that strain stiffening evolved to protect tissues from tearing under large stresses but it could also play a role in tissue development, homeostasis and repair. The results presented here show that contractile, durotactic cells such as NIH 3T3 fibroblasts and hMSCs are acutely responsive to the nonlinear properties of their substrate and respond

to the material's high strain modulus. On fibrin with a low strain modulus of 100 Pa the cells spread as if on a much stiffer gel using actomyosin contraction to strain the fibrin gel, locally increase its modulus, and achieve optimal spreading through a force-limited mechanism.

3.3 Materials and Methods

3.3.1 Cell Culture

Bone marrow-derived human mesenchymal stem cells (Cambrex) were maintained in DMEM (GIBCO) with 1 g/L D-glucose, 0.3 mg/ml L-glutamine and 100 mg/L sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% heat inactivated fetal bovine serum (GIBCO) on tissue culture plastic prior to seeding on gels. NIH 3T3 fibroblast's (ATCC) culture media was identical except it contained 4.5 g/L D-glucose and 10% calf serum (GIBCO) instead of fetal bovine serum. All cells were maintained at 37°C and 5% CO₂. Unless otherwise specified all reagents are analytical grade and purchased from Sigma.

3.3.2 Fibrin Gel Preparation

Lyophilized salmon fibrinogen (Wang et al. 2000) and thrombin (Michaud et al. 2002) were provided by Sea Run Holdings. Fibrin gels were prepared by diluting the stock solution of fibrinogen with T7 buffer (50 mM Tris, 150 mM NaCl at a pH of 7.4) to make a working solution of desired concentration. Polymerization was initiated in a 24 well tissue culture plate by adding 5 µl of thrombin (activity = 100 NIH units/ml) to 250 µl of the fibrinogen solution. Gels were allowed to polymerize for 30 minutes before cell culture media was added to the wells. Gel thickness was measured using the z control of

the rheometer and found to be approximately 1 mm at the thinnest point. In some cases 0.15 mM glycine-proline-arginine-proline (GPRP) was added to the working solution or the already polymerized gel was incubated in a 0.01 mg/ml solution of type 1 rat tail collagen for four hours at 4°C

3.3.3 Collagen Gel Preparation

Acid solubilized Type I rat tail collagen was purchased from BDBioscience. Collagen gels were prepared by adding a 1:10 dilution of 10x PBS to the stock solution of collagen and then bringing the volume to the desired concentration with 1x PBS. Gelation was initiated by adding NaOH until the solution reached pH 7.5. 250 µl of the working solution was added to each well of a 24 well tissue culture plate. The plate was then placed at 37°C to accelerate gelation. After 1 hr, 1ml PBS was added to each of the wells. In some case the already polymerized gel was incubated with a 0.01 mg/ml fibrinogen solution for 1 hour at 4°C, washed with PBS and then incubated with 1 U/ml of salmon thrombin for 10 min at room temperature.

3.3.4 Polyacrylamide Gel Preparation

Polyacrylamide gels of varying stiffness were prepared as reported previously (Pelham and Wang 1997) with the modifications described in (Winer et al. 2009). The ligand attached to the surface was one of the following: fibrinogen monomers, fibrin monomers, thin fibrin fibrils, thick fibrin fibrils, a network of thin fibers or a network of thick fibers. Fibrinogen monomers were ligated to the gel at a concentration of 0.1 mg/ml for 1.5 hours. To achieve the coating of fibrin monomers, the gel was first coated with fibrinogen monomers, washed with T7 buffer and then immersed in a solution of 1

U/ml thrombin for 30 minutes. For the two network coated substrates, the polyacrylamide was first ligated to fibrinogen monomers which act as nucleation sites and then coated with either 0.1 mg/ml fibrinogen and 0.5 U/ml thrombin for 30 minutes or with 0.5 mg/ml fibrinogen and 0.5 U/ml thrombin for 30 minutes. The networks were 5-10 fibers high and less than 2 microns thick. The thin fibers were produced by preparing a solution of 0.1 mg/ml fibrinogen and 0.1 U/ml thrombin 1 hour in advance and then allowing it to react with the gel for 1.5 hours. The thick fibers were prepared by allowing a solution of 0.5 mg/ml fibrinogen to react with 0.2 U/ml thrombin for 2 minutes and then adding excess of the thrombin inhibitor p-nitrophenyl-p'-guanidinobenzoate. This solution of fibers was then reacted with the surface of the polyacrylamide for 1.5 hours.

3.3.5 Displacement Microscopy

Untreated fibrinogen was spiked with biotinylated fibrinogen (1:50), the gels were polymerized as usual and then the surface was coated with streptavidin-coated red fluorescent beads (1000 beads/ μm^2 , Invitrogen). Biotin-NHS (Sigma) was conjugated to salmon fibrinogen using the manufacturer's protocol. In the case of collagen gels Biotin-maleimide was conjugated after polymerization and before addition of the beads due to the low pH of the soluble collagen inhibiting conjugation. Cells were seeded on fluorescent bead-coated fibrin gels (100 cells/well) and allowed to adhere and spread for 18 hours. For imaging, the cells were kept in serum-free media buffered with 50 mM HEPES and the microscope stage was heated to 37°C. Cells were imaged in phase contrast mode and the beads were imaged in fluorescence before the treatment (1%

DMSO, 5 μ M blebbistatin, 20 μ M Cytochalasin D or 10 μ M Nocodazole) was added. After 30 minutes the beads were imaged again. Image J (NIH) and Adobe Photoshop were used to generate a map of bead displacements for each pair of images. Due to the low magnification needed to capture the scale of the gel deformation the pixel resolution resulted in a error of $\pm 0.25 \mu\text{m}$. To minimize the effect of cells other than the one of interest, only 50-100 cells were seeded per well of the 24 well dish. In addition the imaged cells were selected because they were at least 1 mm from the nearest cell.

3.3.6 Rheology

To measure the dynamic shear storage modulus (G') of fibrin gels, 300 μl of fibrin with or without cells were prepared in a 24 well plate. These gels were prepared identically to the microscopy gels except that the fibrinogen was diluted to 2 mg/ml in a cell suspension or just cell type specific media. The gels were cultured overnight at 37°C to allow sufficient time for gel contraction. The shear modulus was calculated from the in-phase shear stress on a strain-controlled RFS III fluids spectrometer rheometer (Rheometrics, Piscataway, NJ) with an 8 mm parallel plate geometry on a stage heated to 37°C using a 2% oscillatory shear strain at a frequency of 5 radians per second. These parameters were chosen to probe the elastic response of the gels in the low strain region where G' is independent of strain. To measure the strain stiffening response, gels were polymerized between 25mm parallel plates for 10 minutes at which time silicone oil was added to the perimeter to prevent drying and the gels were allowed to polymerize for another 50 minutes on a Bowlin Gemini rheometer. The strain was then ramped from 0 to 100% strain at 5 radians per second and 10 points per decade. The resisting stress and

estimated elastic modulus were measured at each point.

3.3.7 Atomic Force Microscopy

Force indentation curves of fibrin gels were performed as previously described (Solon et al. 2007) on a Veeco Bioscope I using a silicon nitride probe with a cantilever spring constant of 0.01 N/m and a 5 μ m polystyrene particle attached. Relative stiffnesses of different points on the gel are estimated by fitting the first 500 nm of the indentation curves to the Hertz model (Hertz 1882).

3.3.8 Image Analysis Scanning Electron Microscopy

For scanning electron microscopy samples were fixed with 2% glutaraldehyde in sodium cacodylate buffer, dehydrated with ethanol, critical point dried and coated with gold/palladium as described previously (Langer et al. 1988). The images were taken on a Philips XL20 scanning electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) in the electron microscopy facility at the Department of Cell Biology University of Pennsylvania, PA, USA.

3.3.9 Image Analysis

All light imaging was done on a Leica DMIRE microscope using a Hamamatsu ORCA-ER camera and a 10x air lens with a numerical aperture of 0.30 or a 40x air lens with a numerical aperture of 0.60. With the exception of the displacement images, analysis was done on images of cells fixed and stained with phalloidin and DAPI. Focal adhesion staining was done using 3T3 fibroblasts transfected with GFP-paxillin. Image J software was used to convert images to 8 bit, and then threshold and analyze the cells' projected area, alignment and distance to the nearest cell. Cell projected area was

determined using Image J's analyze particles command.

3.3.10 Statistics

All statistics was preformed using Kaleidagraph software and unless otherwise mentioned significance was determined using a one way ANOVA and then applying a Tukey HSD post test with an α threshold of 0.05

3.4 Results

3.4.1 Cells cultured on soft fibrin spread as though on a stiff substrate

Consistent with previous reports (Yeung et al. 2005) fibroblasts are round on fibrinogen-coated polyacrylamide gels with a shear modulus of 100 Pa and become increasingly spread as the gel stiffness increases until they reach a spread area of approximately $2200 \mu\text{m}^2$ on 16 kPa polyacrylamide gels (Fig. 3.1 A). In contrast, on 1 mm thick fibrin gels of 1, 2, 4 and 8 mg/ml, with low strain shear moduli of 30, 60, 140 and 350 Pa respectively, the fibroblasts spread to areas statistically similar to each other and to the average spread area on the stiffest fibrinogen-coated polyacrylamide gel. Similar results were obtained using human mesenchymal stem cells (hMSCs) on the two kinds of substrates. Cells on fibrin displayed actin stress fibers, which are noticeably absent in cells cultured on soft polyacrylamide (Fig. 3.1 B-D). Cells on fibrin also had large focal adhesions consistent with a contractile phenotype (Fig. 3.1 E).

One possible explanation for this response is that the cells deform the fibrin gels enough to sense the high strain modulus rather than the low strain modulus of the substrates. The shear modulus of polyacrylamide is independent of strain whereas the modulus of fibrin increases as the applied strain increases (Fig. 3.1 F) (Storm et al. 2005).

Therefore, if application of a small strain to the substrate is sufficient for the cells to respond to substrate mechanics, then 2 mg/ml fibrin and 70 Pa polyacrylamide gels will appear equally soft, but if the cells apply strains large enough to enter the strain-stiffening regime of fibrin elasticity, then they will respond to fibrin as a stiffer matrix than 70 Pa polyacrylamide. The latter hypothesis is consistent with the data, for figure 3.1 E shows that the modulus of 2 mg/ml fibrin at 80% strain is 3.7 kPa and the average spread area of cells on these gels is $\sim 1900 \mu\text{m}^2$; this is comparable to cells on a 3.2 kPa fibrinogen-coated polyacrylamide gel which have an average spread area of $\sim 1800 \mu\text{m}^2$.

3.4.2 A network of fibers is required for cells to spread on fibrin

In addition to the network mechanical properties there are several other differences between fibrinogen-coated polyacrylamide and fibrin gels: ligand conformation, ligand density, ligand orientation and individual polymer mechanical properties. To test if these factors account for the different morphologies on fibrin gels and fibrinogen-coated polyacrylamide, the average spread area of cells was measured after incubation on 100 Pa polyacrylamide gels coated with fibrinogen monomers, fibrin monomers, a non contiguous coating of thin fibrin fibers, a non contiguous coating of thick fibrin fibers, a continuous network of thin fibers, or a continuous network of thick fibers. Only the gels coated with a fibrin network induced cell spreading, and the same trend occurred regardless of cell type (Fig. 3.2 A and B). Since the fibrin monomers and filaments all have higher tensile moduli than the gel but are linearly elastic (Brown et al. 2007; Collet et al. 2005; Lim et al. 2008) this result bolsters the argument that the fibrin gel's nonlinear mechanical properties, rather than their shape or chemical properties,

induce cell spreading.

Another factor that could affect cell morphology is ligand density, which could not be independently controlled under the experimental conditions that alter fibrin stiffness; however, analysis of scanning electron micrographs (Fig. 3.2 C-H) of the substrate surface does not support this hypothesis. These images were used to estimate the fractional surface coverage of the gel and indicated that monomer-coated substrates were 30% covered, filament-coated substrates were 50% covered and network-coated substrates were 100% covered. If ligand density were the controlling factor, then one would expect that the filament substrates would have displayed an intermediate projected cell area. They have both an increased local linear density from the presence of fibers and an increased global density. Since the spread area on the filament-coated substrates is not significantly different from that on the monomer-coated substrates, it is unlikely that ligand density or activation of a wound healing response is responsible for the dramatic increase of spread area upon the gels coated with a network.

3.4.3 Cell applied displacements are cell type, distance, gel stiffness and integrin type dependent.

To study the relationship between cell adhesion forces and network structure, cells were cultured on fibrin gels coated with fluorescent beads, and the contraction or relaxation of the gel was monitored by tracking bead displacements in response to drug treatments. On 2 mg/ml fibrin, fibroblasts, which are approximately 50 μm in diameter when spread, displaced beads an average of 1.5 μm up to 250 μm away from the cell's centroid (Fig. 3.3 A and B), and the 100 μm diameter hMSCs displaced beads up to 450

μm away (Fig. 3.4 A). This displacement was determined by treating the cells with either blebbistatin, to inactivate force generation by non muscle myosin II, or with cytochalasin D, which disassembles the actin fibers that myosin acts upon. Both treatments resulted in a similar pattern of bead displacements, consistent with an effect on acto-myosin contractility. Treating fibroblasts with nocodazole, a microtubule destabilizing drug, resulted in the cells increasing the applied force and drawing closer most beads in the field of view (Fig. 3.3 A and C). This nocodazole-induced contraction is consistent with previous reports that depolymerization of microtubules stimulates myosin light chain phosphorylation and increased contractility (Kolodney and Elson 1995). The DMSO control had no significant effect on the position of the beads. The responses to drug treatments reported here are consistent with previous traction force microscopy on synthetic gels (Dembo and Wang 1999; Reinhart-King et al. 2008).

The hMSCs applied stronger active tractions, possibly because their larger spread area allowed them to engage a larger number of integrins, allowing for a more dynamic range. Figure 3.4 A shows that the concentration of fibrin also affected the degree of bead displacement, with displacements increasing as fibrin concentration decreases. Since both stiffness and ligand density increase with increasing fibrinogen concentration, the polymerization inhibitor GPRP was added to a 2 mg/ml and 4 mg/ml gels to lower the low strain modulus without changing the ligand density (Schindlauer et al. 1986). Under these conditions the hMSCs produced even larger average displacements, confirming that stiffness and not ligand density plays the dominant role in determining the size of the displacements (Fig. 3.4 B). This result fits with the hypothesis that the cells exploit the

strain-stiffening properties of the material to achieve a specific substrate resistance, since the lower the initial modulus, the more the cell must displace the beads in order to achieve the same final stiffness.

To examine the effect of other ECM proteins that engage different integrins on the cell's applied forces gels were incubated with monomeric type 1 collagen prior to the addition of cells. Figure 3.5 A shows that adsorbing collagen monomers to the fibrin gel has no effect on the displacement of beads close to the cell boundary, but it increases the displacement of beads farther than 250 μm from the cell center. One interpretation of these results is that adding collagen increases the stress threshold that the hMSCs achieve either by chemically stimulating an increase in applied force, or by engaging a larger number of integrins, since fibrin and collagen bind separate populations of integrins. Type I collagen is engaged through the $\alpha 1/\beta 1$ and $\alpha 2/\beta 1$ integrin pairs (Leitinger and Hohenester 2007) whereas fibrin gels are reportedly engaged through the $\alpha v/\beta 3$ integrin pair (Hong and Stegemann 2008; Weisel 2005). Due to fibrin's strain stiffening, stress and strain are not linearly related, and in regions where the gel is already highly stressed increases in applied stress result in negligible strain increases but in regions where the gel was only slightly prestressed the strain increases can be significant (Fig. 3.5 B).

When hMSCs are cultured on 2 mg/ml collagen gels, whose modulus is comparable to a 4 mg/ml fibrin gel, the resulting displacement are smaller than those on a 4mg/ml fibrin gel. When additional fibrin monomers are adsorbed to the collagen gel's surface a jump in bead displacements is seen over all distances, though only significant in

the 250 μm bin. This suggests that on 2 mg/ml collagen gels the hMSCs are not able to contract the gel enough to reach the plateau region of the stress/strain curve, thus applying additional traction force can further contract the gel in each of the three regions.

3.4.4 Strains applied by cells stiffen the gel globally and locally

To determine if the cell-applied strains are sufficient to globally stiffen a 2 mg/ml fibrin gel, cells were suspended in polymerizing fibrin and allowed to spread for 18 hours before the low strain shear modulus of gels was measured. Both fibroblasts and hMSCs stiffened the gels in a cell density dependent manner, and at low concentrations hMSC's stiffened the gel to a greater degree than the smaller fibroblasts (Fig.3.6 A). This result is consistent with the finding in figure 3 that hMSC's apply larger forces than fibroblasts. One anomaly in the data is that gels with 500,000 hMSCs/ml had a lower modulus than those with 100,000 hMSCs/ml, possibly because hMSCs are large cells compared to fibroblasts and at such a high density they may disrupt the fibrin network structure. As a control, melanoma cells, which bind to but do not spread well in 3D fibrin, did not increase the gel's stiffness when embedded and cultured within fibrin gels. This result shows that the cells must actively contract the matrix to stiffen it.

To examine local effects on gel stiffness by cell-applied strains, the atomic force microscope was employed to measure the gel stiffness both adjacent to the cells periphery and far from the cells periphery (Fig. 3.6 B). Far from the cell the gel stiffness measured by AFM matched the low strain modulus measured by rheology. The gel stiffness measured within 50 μm of the cell's membrane was significantly higher than that of uncontracted gel.

A stiffness map of a 2 mg/ml fibrin gel around a spread fibroblast was generated with the AFM (Fig. 3.6 C). The gel is clearly stiffer closer to the cell than at the periphery. At the map's periphery the measured Young's modulus is slightly above 100 Pa and is comparable to the low strain modulus measured by conventional rheology. After the cell is treated with blebbistatin, both the modulus of the cell and the modulus of the surrounding gel drop significantly (Fig. 3.6 C and D). This drop in gel modulus with inactivation of non-muscle myosin confirms that the cellular forces are responsible for locally stiffening the gel.

3.4.5 Cell stiffness is independent of fibrin gel stiffness

Another cell parameter that usually correlates with substrate stiffness is cell stiffness. The cortical stiffness of hMSCs cultured on polyacrylamide, glass or fibrin gels was as measured with AFM (Fig. 3.7). The stiffness of the cells cultured on polyacrylamide increased with increasing stiffness of the substrate until they reached a maximum tensile modulus of ~7 kPa on gels of 48 kPa. The average stiffness observed on stiff polyacrylamide matched the average stiffness of hMSCs on glass. In contrast, hMSCs cultured on fibrin gels exhibited a cortical stiffness which correlated with the low strain modulus of the fibrin gels. This suggests that on fibrin gels the cell stiffness is independent spread area.

3.5 Discussion

The mechanical interactions between cells and their substrates have primarily been investigated using synthetic, linearly elastic materials. When studies have been done using biological materials the results have been interpreted assuming that these

materials behave similarly to their synthetic counterparts (Bischofs and Schwarz 2003). Some cell types, such as neurons, do appear to respond similarly to the low strain elastic modulus of PA and fibrin gels (Georges et al. 2006). This similar response is likely to occur because neurons apply very small forces to their substrate (Bridgman et al. 2001; Chan and Odde 2008) and therefore sense only the low-strain linear elastic modulus. The work presented here demonstrates that highly contractile mesenchymal cells are acutely responsive to the nonlinear properties of their substrates. Both NIH 3T3 fibroblasts and human mesenchymal stem cells strongly contract fibrin gels to locally stiffen them enough so that they can achieve optimal spreading through a force-limited mechanism.

Many studies of mechanosensing have been performed using NIH 3T3 fibroblasts because this cell line is robust, immortalized, simple to culture and reasonably contractile. Recent studies, however, indicate that immortalized cells may not be representative of how primary cells respond to mechanical stimuli since the expression of cytoskeletal remodeling proteins changes with immortalization (Alge et al. 2006). Fewer studies have examined hMSCs but due to their multipotency and large expansion potential (Kassem 2004) they are appealing for tissue engineering and since they display differentiation sensitivity to substrate stiffness (Engler et al. 2006), understanding their response to the mechanical properties of biological gels is essential for rational biomaterial design. Since both cell types displayed the same trends on fibrin, this sensitivity to nonlinearity may be common in durotactic cells. The capacity of cells to sense and manipulate the nonlinear elasticity of their substrate appears to depend on the degree to which the material strain-stiffens, the inherent contractility of the cell and the

engagement of appropriate integrins.

To use fibrin or collagen as a substrate for traction microscopy several challenges had to be overcome. First, these biopolymer gels have a mesh size on the order of 1 μm , the same scale as the diameter of the beads typically used in these experiments (Dembo and Wang 1999). If 0.1 – 1 μm beads are used, they may be able to diffuse throughout the gel. If larger 2 – 5 μm beads are used they may be too large to measure the displacements of interest. Instead 40 nm streptavidin coated fluorescent beads were attached to fibrin or collagen gels which had been dilutely conjugated to biotin. The 40 nm diameter beads are small enough that they should not, at this low density, greatly hinder the fibers which have an average diameter of 80 – 200 nm.

Another obstacle was selecting a releasing agent that will not alter the gel's properties. Several labs use trypsin or EDTA as the treatment to detach the cells however trypsin will enzymatically digest the gel and EDTA may interact with fibrin bound calcium. Instead more specific inhibitors were used: 20 μM cytochalasin D to disrupt F actin structures, 5 μM blebbistatin to inhibit non-muscle myosin 2 activity, or 10 μM nocodazole to disrupt microtubule polymerization. Also, these experiments only reveal the recoverable, elastic component of the cell-applied strains and not any permanent rearrangements due to mechanical creep or chemical modifications of the matrix that occur during the prolonged application of force.

Though the displacements were not converted to strains due to the lack of an applicable theoretical model, two experiments support the hypothesis that these displacements are straining the gels enough to cross the threshold into fibrin's strain

stiffening regime. First, the stiffness of fibrin gels increased if there were contractile cells embedded within them. This was seen before in fibrin gels containing activated platelets (Shah and Janmey 1997). Second, AFM stiffness measurements confirmed that cells locally stiffen the gel and the modulus drops back down to baseline if the cell's tractions are disabled by inhibition of non muscle myosin II.

In past experiments on linearly elastic materials, cells applied weaker tractions on softer substrates, and stronger tractions corresponded to larger spread areas (Lo et al. 2000). As cells appear to reach their maximum spread area on fibrin gels regardless of initial gel stiffness, it appears that the cell and gel engage in a mechanical “tug-of-war” until one or the other can no longer increase resistance. In the case of polyacrylamide gels the resistance is constant so if the cell can match the gel’s resistance, as 3T3 cells can on a gel of 1 kPa, the cell applies that much resistance but no more. This response is indicated by a smaller than maximum spread area and cortical stiffness measured by AFM (Solon et al. 2007). In contrast to linear materials, a fibrin gel will increase resistance as the cell applies greater force resulting in a feedback loop that will continue until either the cell or the gel can no longer increase resistance. In all the conditions of fibrin gels tested so far, fibroblasts and hMSCs spread to their maximal areas, suggesting that the gel can stiffen beyond the cell’s ability to contract. Figure 5B shows that, as predicted by the tug-of-war hypothesis, the cells applied larger displacements on gels with a lower initial modulus, because cells must apply larger strains to a softer gel to achieve the same final stiffness. This result also indicates that the cell's mechanosensor is stress limited not strain limited which is consistent with a previous study on a collagen

substrate (Freyman et al. 2002).

One incongruity is that though cells contract and spread as though they are on a stiff matrix their stiffness correlates with that of cells on a soft matrix. Several studies have shown that the stiffness of cells on collagen or fibronectin coated polyacrylamide gels increase as the modulus of the gel increases until the cell stiffness reaches a maximum value (Engler et al. 2006; Solon et al. 2007). These results were replicated in figure 7 using fibrinogen coated polyacrylamide gels. This trend was also shown on endothelial cells recovered from a 3D collagen matrix (Byfield et al. 2009a), but these cells had been replated on glass so it's unclear if the collagen stiffness was the cause of increased cell stiffness. It was recently shown that cell stiffness can be decoupled from cell spreading in cells that lack a critical actin crosslinker (Byfield et al. 2009b), so it may be that the relationship between cell stiffness, cell spreading and gel stiffness is coincident and not causal.

The results presented here may help to understand the progression of diseases correlated with changes in tissue stiffness such as cancer (Paszek et al. 2005) and fibrosis (Li et al. 2007). It was recently reported that early stiffening of liver tissue is likely due to an increase in lysyl oxidase mediated crosslinking of the collagen ECM and that this stiffness increase precedes myofibroblast activation and fibrosis in chronic liver disease (Georges et al. 2007). Crosslinking not only increases the low strain modulus but, by decreasing the contour length between network junctions, it can also increase the degree of strain stiffening, so the ultimate stiffness felt by the cell is likely higher than reported from low-strain rheology. Further studies need to be done but as differentiation from

portal fibroblast to myofibroblast requires both TGF-beta and a stiff substrate (Li et al. 2007) an increase in local strain stiffening may be a key step in chronic liver disease.

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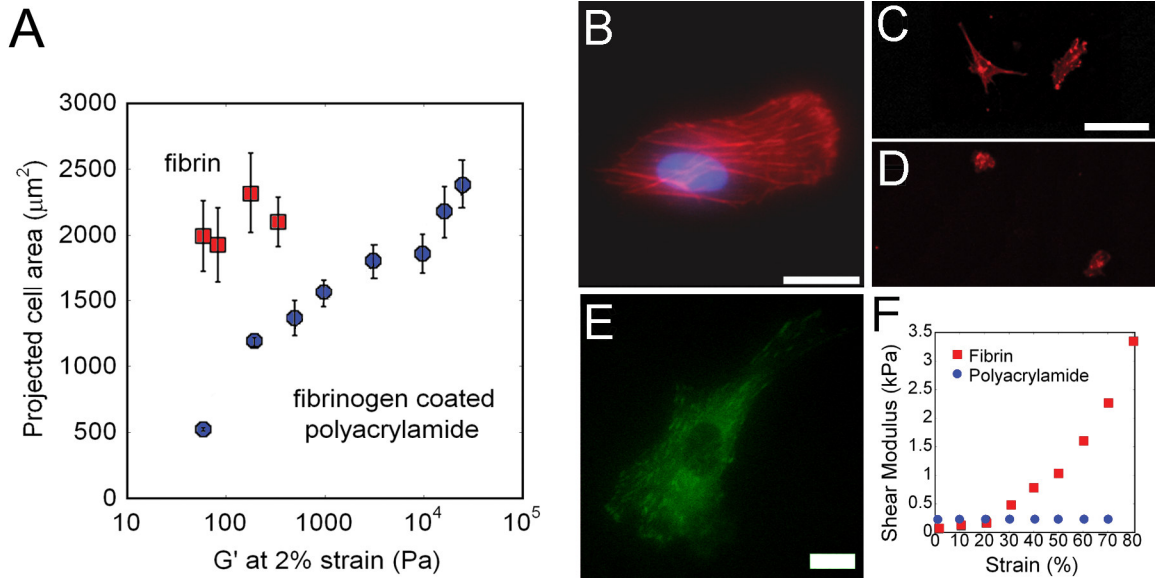
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Figure 3.1 Fibroblast spreading on fibrin is comparable to spreading on stiff polyacrylamide



Fibroblast's spread area on fibrin or fibrinogen coated polyacrylamide gels of varying stiffnesses after 18 hours on substrate. Fibrin is red and polyacrylamide is blue.

Reported as mean $\pm$ SE, $n = 3$, at least 50 cells analyzed in each run (A) Fluorescence

image of actin (red) structures of fibroblasts on 2 mg/ml fibrin Scale bar = 20 μm ,

Nucleus in blue.(B), 10 kPa fibrinogen coated polyacrylamide Scale bar = 100 μm (C)

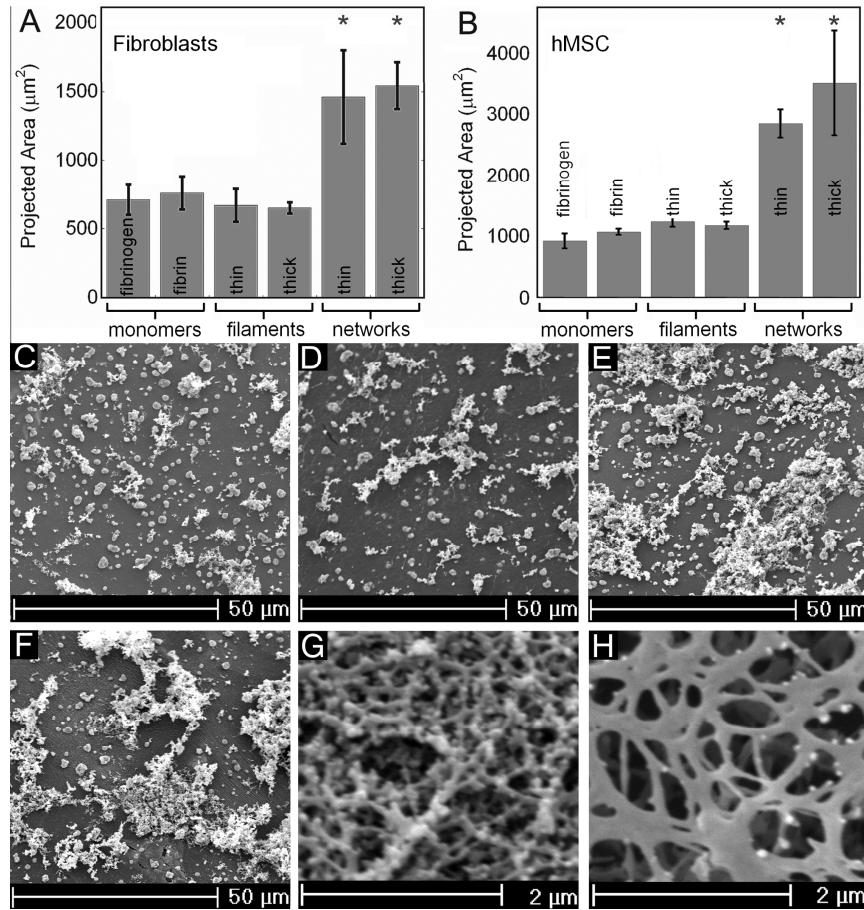
and 0.1 kPa fibrinogen coated polyacrylamide Scale bar = 100 μm (D). Focal adhesions

of hMSC stained with paxillin (green) (E). Strain versus shear modulus, measured by

oscillating rheology, of 2 mg/ml fibrin (■) or 7% acrylamide, 0.05 % bisacrylamide gels

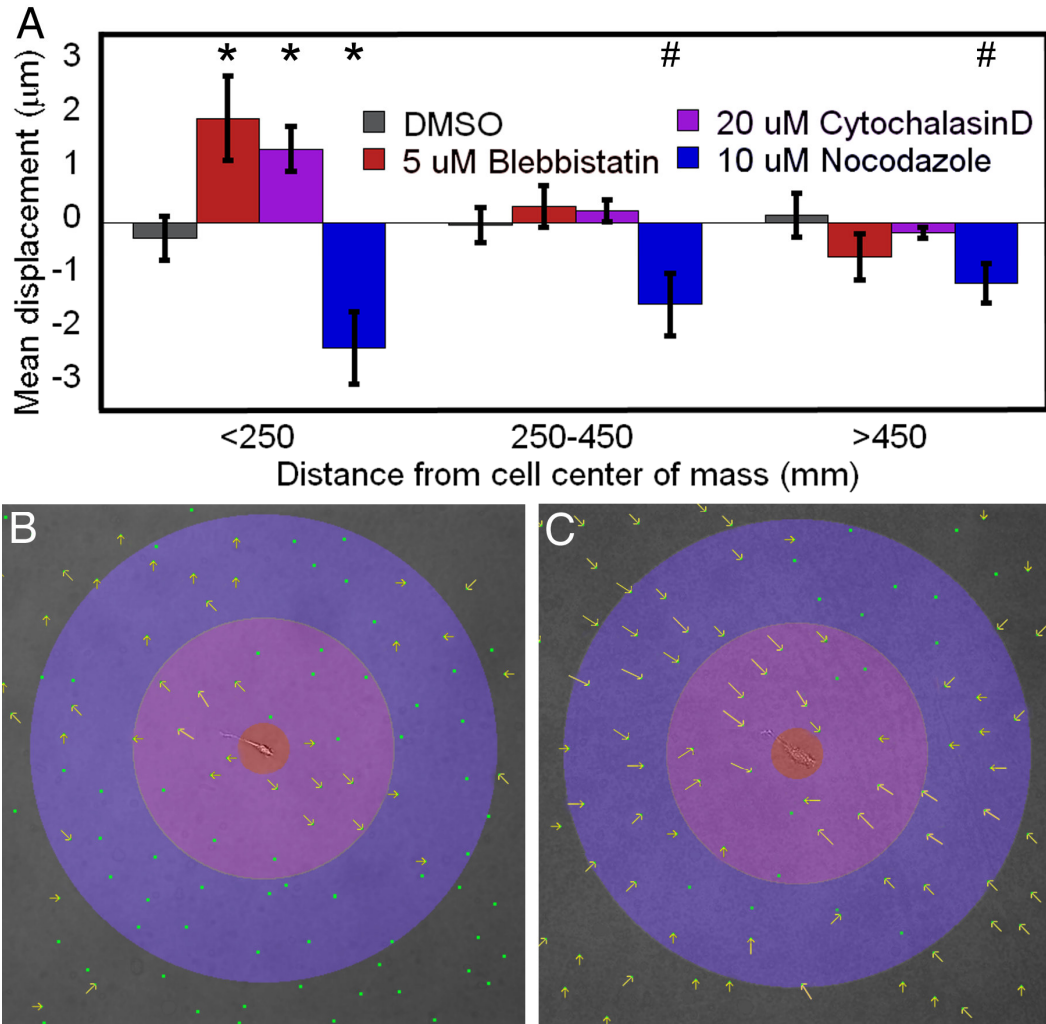
(●)(F).

Figure 3.2 Cell spreading on “soft” polyacrylamide requires a continuous network of fibrin



Spread area of fibroblasts (A) or hMSCs (B) cultured for 18 hours on 100 Pa polyacrylamide gels coated with fibrinogen or fibrin monomers, thin or thick fibers, or networks of thin or thick fibers. Reported as mean $\pm$ SE, $n = 3$, at least 50 cells analyzed in each run, * $p < 0.01$ compared to fibrinogen coated polyacrylamide. Representative scanning electron micrographs of coated polyacrylamide surfaces: (B) fibrinogen monomers, (C) fibrin monomers, (D) non contiguous thin fibers, (E) non contiguous thick fibers, (F) a network of thin fibers, or (F) a network of thick fibers.

Figure 3.3 Cells actively contract fibrin gels over several cell lengths

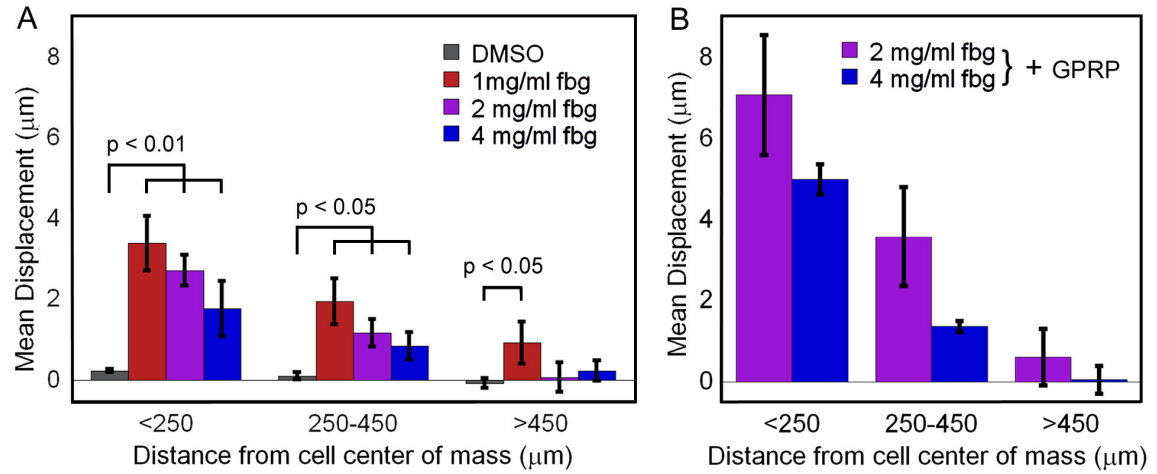


Average displacement applied by fibroblasts (A) as a function of distance from the cell's center. Reported as mean $\pm$ SE, $n = 5$, at least 80 beads counted in each zone, * $p < 0.01$,

$p < 0.01$ one way ANOVA with Dunnet post test compared to DMSO control.

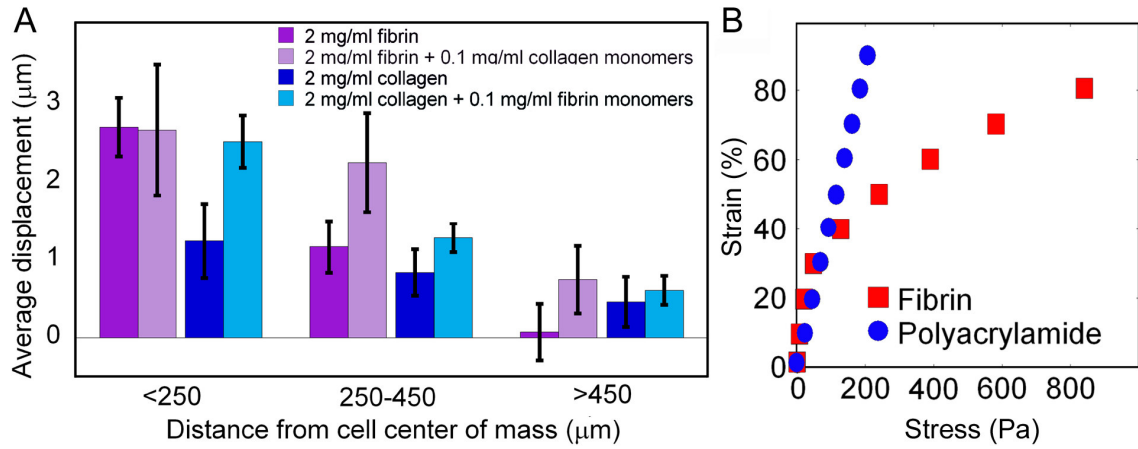
Example displacement plots of fibroblasts on 2 mg/ml fibrin gels treated with either blebbistatin (A) or nocodazole (B). Only $1/10^{\text{th}}$ of all beads are shown and displacements are magnified 5x and arrows indicate direction. The red circle has a radius of 50 μm . The radius of the purple circle is 250 μm and the radius of the blue circle is 450 μm .

Figure 3.4 Cellular restructuring of fibrin gels is cell type, distance and gel stiffness dependent



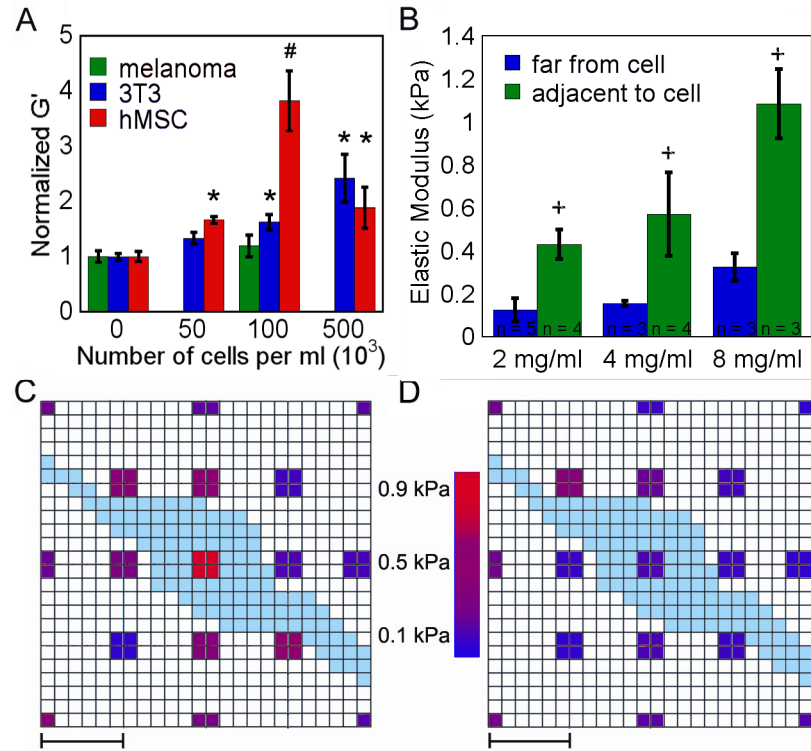
Average bead displacement, as a function of distance from the cell's center, applied by hMSCs on fibrin gels of increasing concentration (A). Within zone one way ANOVA with Dunnet post test compared to DMSO control. GPRP was added to fibrin to modify the matrix stiffness without changing the ligand density (B). Cytochalasin D was used to relax hMSCs. Reported as mean $\pm$ SE, $n = 5$ cells per condition, at least 80 beads counted in each zone.

Figure 3.5 Integrin type mediates restructuring of fibrin gels



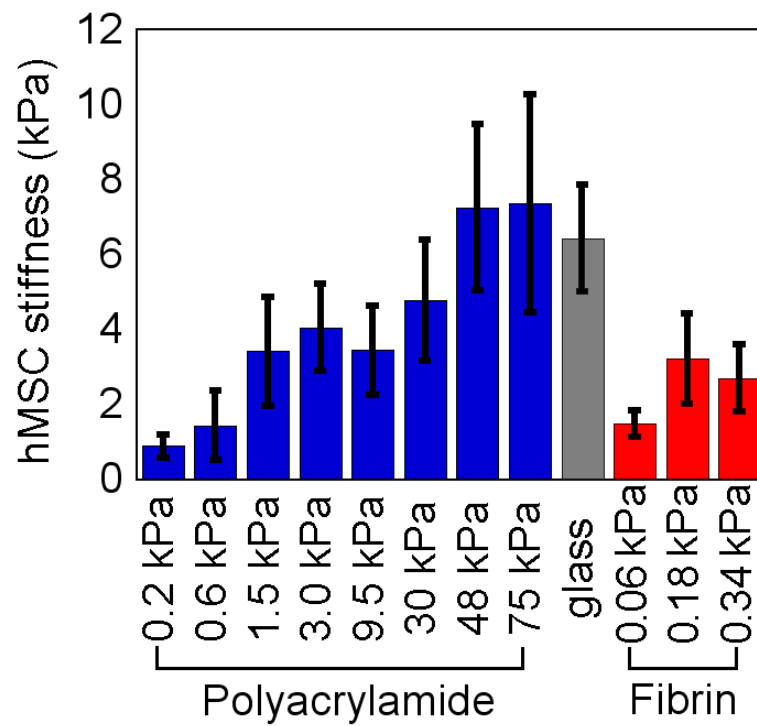
Average bead displacements applied by hMSCs on fibrin or collagen gels uncoated or pretreated with monomers of the opposite type (A). Reported as mean $\pm$ SE, $n = 5$ cells per condition, at least 80 beads counted in each zone. Stress versus strain, measured by oscillating rheology, of 2 mg/ml fibrin (■) or 7% acrylamide, 0.05 % bisacrylamide gels (●) (B).

Figure 3.6 Contractile cells strain gels sufficiently to stiffen fibrin gels both in 2D and 3D



Bulk shear modulus, measured by rheology, of fibrin gels cultured for 18 hours with varying densities of cells (A). Reported as mean $\pm$ SE, $n = 3$, 8 measurements per sample, * $p < 0.05$, # $p < 0.01$ Within group one way ANOVA with Dunnet post test compared to cell free control. Average elastic modulus of the fibrin gel far from or adjacent to a fibroblast (B). Reported as mean $\pm$ SE, $n = 3$. + $p < 0.01$ using Student's T-test. AFM generated stiffness maps of a fibroblast (in blue) on a 2mg/ml fibrin gel before (A) and after (B) blebbistatin treatment. Scale bar = 30 μ m. The center measurement is of the cell stiffness not the gel stiffness. (C).

Figure 3.7 Cell stiffness correlates with modulus of relaxed gel



Cell stiffness measured by AFM on polyacrylamide (blue), glass (grey) or fibrin (red) substrates. Reported as mean $\pm$ SE, 3 measurements per cell, 3 cells per data point.

CHAPTER 4

Cell applied forces promote asymmetry in nonlinear gels that in turn promotes pattern formation

(Adapted from Winer JP, Oake S, Janmey PA.2009. *PLoS One* 4: e6382)

4.1 Abstract.

While the early embryo is isotropic it quickly develops into an increasingly anisotropic organism. At the tissue level, extracellular matrix proteins often display patterning which is not recapitulated when these proteins are gelled *ex vivo*; however, the application of external force induces alignment similar to that found in tissues which must resist high stresses like tendons and the aortic valve. Contractile cells apply significant forces to biopolymer gels and these stresses are sufficient to locally induce anisotropy in an initially isotropic matrix. This local anisotropy in structural and mechanical properties of the fibrous substrate provides a mechanism for cell/cell communication over a distance of approximately 5 cell lengths. This results in alignment of adjacent cells and formation of ring like multicellular patterns providing a mechanical mechanism for cellular pattern formation *in vivo*.

4.2 Introduction.

Without anisotropy, gradients, patterns and polarity in signals it would be impossible to derive a structure as complex as the human body from an isotropic egg. While stochastic events initiate early embryo polarity (Rossant and Tam 2009), something as seemingly minor as a reversal of cilia rotation in an 8 day old mouse embryo leads to changes to the flow of chemical factors and left/right inversion of organ position (Nonaka et al. 2002; Okada et al. 1999). At the tissue level, paracrine signaling induces directed capillary growth in both normal (Peters et al. 1993) and pathological (Connolly et al. 1989) development and at the single cell level leukocytes will follow the chemical tracks left by bacteria and ultimately engulf the invaders (Harris 1960).

The processes described so far are mediated by chemical factors but other types of variable signals such as mechanical (Ando et al. 1987), structural (Rosenberg 1963) and electromagnetic (Blakemore 1975) can also influence cell behavior. For instance, fibroblasts will align with grooves in a substrate (Walboomers et al. 1999) and neurite outgrowth can be guided by substrate curvature (Smeal et al. 2005). Mechanical signals such as shear flow (Ando et al. 1987) or sharp gradients in substrate stiffness (Lo et al. 2000) can direct cell migration. There is not yet enough data to fully characterize which types of signals are dominant, but for instance if endothelial cells are aligned in grooves perpendicular to applied shear flow, they will continue to migrate back and forth along the grooves at moderate flow rates but at high flow rates they will begin to migrate downstream (Uttayarat et al. 2008).

Strain stiffening gels, such as fibrin and collagen, are inherently mechanically and

structurally anisotropic under the application of strain. This is because in networks of semiflexible filaments the phenomenon of strain stiffening results in part from the alignment of fibers parallel to the applied strain (Kang et al. 2009; Storm et al. 2005). In the previous chapter, it was shown that cell applied strains are sufficient to induce strain stiffening in fibrin gels. The data presented in this chapter will demonstrate that cell induced local strain stiffening allows an initially isotropic matrix to reinforce cell-applied mechanical anisotropy and transmit forces between cells up to half a millimeter apart. In this way isolated cells can create far-reaching mechanical gradients and produce a global pattern, a phenomenon potentially related to pattern formation during wound healing or tissue development.

4.3 Materials and Methods.

3.3.1 Cell Culture

Bone marrow-derived human mesenchymal stem cells (Cambrex) were maintained in DMEM (GIBCO) with 1 g/L D-glucose, 0.3 mg/ml L-glutamine and 100 mg/L sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% heat inactivated fetal bovine serum (GIBCO) on tissue culture plastic prior to seeding on gels. Unless otherwise specified all reagents are analytical grade and purchased from Sigma.

3.3.2 Fibrin Gel Preparation

Lyophilized salmon fibrinogen (Wang et al. 2000) and thrombin (Michaud et al. 2002) were provided by Sea Run Holdings. Fibrin gels were prepared by diluting the stock solution of fibrinogen with T7 buffer (50 mM Tris, 150 mM NaCl at a pH of 7.4) to make a working solution of desired concentration. Polymerization was initiated in a 24

well tissue culture plate by adding 5 μ l of thrombin (activity = 100 NIH units/ml) to 250 μ l of the fibrinogen solution. Gels were allowed to polymerize for 30 minutes before cell culture media was added to the wells. Gel thickness was measured using the z control of the rheometer and found to be approximately 1 mm at the thinnest point. In some cases 0.15 mM glycine-proline-arginine-proline (GPRP) was added to the working solution or the already polymerized gel was incubated in a 0.01 mg/ml solution of type 1 rat tail collagen for four hours at 4°C

3.3.3 Collagen Gel Preparation

Acid solubilized Type I rat tail collagen was purchased from BDBioscience. Collagen gels were prepared by adding a 1:10 dilution of 10x PBS to the stock solution of collagen and then bringing the volume to the desired concentration with 1x PBS. Gelation was initiated by adding NaOH until the solution reached pH 7.5. 250 μ l of the working solution was added to each well of a 24 well tissue culture plate. The plate was then placed at 37°C to accelerate gelation. After 1 hr, 1ml PBS was added to each of the wells. In some case the already polymerized gel was incubated with a 0.01 mg/ml fibrinogen solution for 1 hour at 4°C, washed with PBS and then incubated with 1 U/ml of salmon thrombin for 10 min at room temperature.

3.3.4 Polyacrylamide Gel Preparation

Polyacrylamide gels of varying stiffness were prepared as reported previously (Pelham and Wang 1997) with the modifications described in (Winer et al. 2009). Fibrinogen monomers were ligated to the gel by first reacting the top of the gel with sulfo-SANPAH (Pierce) using UV light (320 nm) and then reacting the remaining NHS

ester with fibrinogen monomers at a concentration of 0.1 mg/ml at room temperature for 1 hours.

3. 3.5 Atomic Force Microscopy

For imaging the samples were fixed with 2% glutaraldehyde in sodium cacodylate buffer, and dehydrated with ethanol. The samples were then immersed in 50% ethanol, 50% hexamethyldisilazane (HMDS) for 10 minutes and then immersed in 100% HMDS for 10 minutes. The excess solution was removed and the samples were placed in a vacuum chamber for 1 hr to facilitate evaporation of the HMDS. HMDS dried samples were then imaged in tapping mode on a Veeco multimode AFM with pyramid tip silicon probes with a cantilever spring constant of 3 N/m.

3.3.6 Image Analysis

All light imaging was done on a Leica DMIRE microscope using a Hamamatsu ORCA-ER camera and a 10x air lens with a numerical aperture of 0.30 or a 40x air lens with a numerical aperture of 0.60. Cells were fixed and stained with phalloidin and DAPI. Image J software was used to convert images to 8 bit, and then threshold and analyze the cells' projected area, axial ratio, circularity, alignment, orientation and distance to the nearest cell. Cell projected area was determined using Image J's analyze particles command. Cell alignment was determined by using Image J to fit an ellipse to a cell and then using the angle tool to measure the angle between the cell's long axis and the membrane of the nearest cell with the vertex at the cell's center of mass. Axial ratio was determined by using ImageJ to fit an ellipse to a cell and then taking the ratio of the long axis to the short axis. Distance from membrane to membrane of the nearest cell was

determined using the ruler tool. DAPI staining allowed verification that only individual non-dividing cells were counted.

Radial displacement analysis was done by summing bead displacements in 30° bins, starting with 0° - 30°, where 15° is aligned with the long axis of the cell. These bins were then sorted by their relationship to the long axis of the cell and averaged. For instance angle 45 would contain the bins 30 – 60, 150 – 180, 210 – 240 and 330 – 360.

3.3.7 Statistics

All statistics was preformed using Kaleidagraph software. Significance was determined using a one way ANOVA and then applying a Tukey HSD post test with an α threshold of 0.05 or using Student's t-test where p values of less than 0.05 are considered significant.

4.4 Results

4.4.1 Cell applied forces induce local anisotropy in fibrin gels

In Chapter 3 bead displacement was reported as an average over all points within a certain distance from the hMSC; however, this analysis does not fully describe displacements applied by the cell. When the displacements within a defined distance and are summed radially in 30° bins, it becomes clear that the displacement field is anisotropic and approximately the same shape as the cell (Fig. 4.1 A). This anisotropy in substrate deformation was quantified and found to be highest along the long axis of the cell and smallest perpendicular to the long axis of the cell (Fig. 4.1 B). Consistent with the displacement analysis in Chapter 3, the displacements decrease significantly as you move away from the cell. Statistical analysis is provided in table 4.1.

This anisotropy in cell applied forces results in alignment of local fibrin fibers. AFM reveals that while prior to cell attachment the gel is an isotropic mesh of thin fibers that vary from 40-90 nm in diameter (Fig. 4.2 A), the high strains applied by the cells appear to bundle and align the fibers perpendicular to the cell's membrane (Fig. 4.2 B) creating a zone that is structurally anisotropic. This alignment is enhanced in the space between two opposing cells (Fig. 4.2 C). Alignment and bundling of fibers also leads to mechanical anisotropy since once the local gel is aligned the cell is more likely to engage the stretching modulus than the bending modulus which is at least 10 fold more compliant.

4.4.2 Nonlinearly elastic substrates induce elongation and patterning of cells.

Cells have been shown to be sensitive to chemical, mechanical and structural anisotropy (Clark et al. 1991; Lo et al. 2000; Postlethwaite et al. 1976). To determine if the gel anisotropy induced by one cell was affecting the structure of a neighboring cell several common shape parameters (cell area, circularity and axial ratio) were analyzed by distance to the nearest cell. If induced anisotropy was affecting these parameters then they should increase or decrease as a function of the distance between the cells.

Membrane to membrane distance was measured because previous experiments have shown that the strongest cell forces, and thus greatest strains, are applied through focal adhesions at the cell's periphery (Balaban et al. 2001). Cells on polyacrylamide gels of comparable low strain modulus to fibrin are round and apply minimal forces to their substrate, thus soft linear elastic gels are an unsuitable control. As the cells on fibrin gels apply large forces and appear to spread in accordance with a stiffer modulus, a 16

kPa fibrinogen-coated polyacrylamide gel was chosen as the control because hMSCs on this substrate had the same average spread area as those on fibrin.

None of the three shape parameters change as a function of the distance to the nearest cell; however, unlike the cell spread area both the axial ratio and the circularity of the cell change with fibrin gel concentration (Fig. 4.3). Cells on fibrin display a decrease in circularity (Fig. 4.3 B), a compactness shape factor defined as $4\pi (\text{area})/(\text{perimeter})^2$, with a decrease in fibrinogen concentration. The circularity is 1 for a circle and decreases toward 0 as the cell becomes more elongated or stellate in morphology. For 2 and 8 mg/ml fibrin gels the axial ratio of the cells is comparable to that of cells on stiff polyacrylamide gels; however, on 1 mg/ml gels the axial ratio doubles and some cells had an axial ratio as high as 20:1 (Fig. 4.3 C). The circularity and axial ratio data indicate that on 1 mg/ml gels the cells pull at two polar points and become elongated as indicated by a high axial ratio and low circularity. On 2 mg/ml gels the lower circularity but baseline axial ratio suggests that these cells are adopting a stellate morphology whereas those on 8 mg/ml gels with a baseline circularity and axial ratio have a polygonal morphology,

4.4.3 Pattern formation of cells seen on fibrin but not polyacrylamide gels

Though hMSC shape did not appear to be dependent on distance to the nearest neighbor visual inspection of the cells suggested that the fibrin gels were inducing multicellular pattern formation. On fibrinogen coated polyacrylamide gels, dilutely seeded hMSCs appear randomly oriented (Fig. 4.4 A). In contrast, hMSCs dilutely seeded on a 2mg/ml fibrin gel form ring like structures (Fig 4.4 B). In 3D the cells appear to be

forming a continuous network (Fig. 4.4 C). Sectioning the 3D gels and staining them for the gap junction protein connexin 43, reveals punctate staining at the cell/cell junction indicating that the cells are forming a chemically connected network.

To characterize this pattern formation and to determine if it was dependent on distance to the nearest cell two possible parameters were analyzed: cell alignment and orientation (Fig. 4.5 A). Cell alignment, defined as the angle between the long axis of a cell and the long axis of its nearest neighbor, was independent of the distance between cells and the fibrinogen concentration (Fig. 4.5 B). For cells cultured on 1 and 2 mg/ml fibrin gels, cell orientation angle, defined as the angle between the long axis of the cell and the shortest line to the nearest cell, decreased as the distance between the two cells decreased (Fig 4.5 C). The range over which this effect occurs corresponds to the range over which hMSCs displaced beads in the earlier experiments for 2 but not 1 mg/ml gels. Cells on 8 mg/ml fibrin display a similar trend, but the populations are not statistically different from each other, although at all distances they are significantly more oriented to each other than cells on polyacrylamide.

To confirm that this distance dependent cell/cell patterning was due to the nonlinearity of the fibrin substrate, cells were cultured on another strain stiffening substrate: 2 mg/ml collagen gels (Janmey et al. 2007). Cells on these gels were fully spread and had an axial ratio between 1 mg/ml and 2 mg/ml fibrin gels, but they displayed an orientation pattern similar to that of hMSCs on 2 mg/l fibrin gels. This distance dependent cell/cell orientation confirms that pattern formation is due to the nonlinear strain stiffening of the substrate.

4.5 Discussion.

It has long been known that chemical gradients can induce anisotropic behavior in single cells (Harris 1953) and cell clusters (Gerisch 1982). More recently it has been shown that externally induced mechanical (Ando et al. 1987; Lo et al. 2000) and structural (Rosenberg 1963) anisotropy can also induce anisotropic responses from cultured cells. The data presented in this chapter demonstrate that on strain stiffening gels, cells themselves can induce mechanical and structural anisotropy in the substrate. This substrate anisotropy induces patterning in the distribution of cells on the matrix.

There are two distinct processes that contribute to the mechanisms by which a gel formed of filamentous biopolymers strain-stiffens. For some biological materials, like fibrin protofibril gels or crosslinked F-actin where the polymer persistence length is on the order of the distance between crosslinks, nonlinear elasticity is thought to result from the intrinsically non-linear force-extension relations of the network strands (Storm et al. 2005). For networks with stiffer polymers, like collagen or thick fibrin fibers an additional phenomenon associated with strain stiffening is an increase in local fiber alignment, and a transition from filament bending to filament stretching (Huisman et al. 2007; Onck et al. 2005).

It has been shown that straining networks of fibrin fibers results in filament alignment (Kang et al. 2009) and bundling (Brown et al. 2009). Culturing cells in these mechanically aligned gels leads to pattern formation along the axis of high strain (Matsumoto et al. 2007). As shown in this chapter, individual cells can induce local anisotropy in fibrin gels. This anisotropy induces pattern formation in nearby cells as a

function of the distance between the cells. However unlike the externally applied strains which induce linear pattern formation, the cell applied strains induce a ring like patterns. This is likely because the cells aren't perfect dipoles and the curvature of the cell results in curvature in the pattern.

Cells on fibrin gels adopted one of three dominant morphologies. On 1 mg/ml gels the cells become elongated. On 2 mg/ml gels they took on a more stellate structure and on 8 mg/ml gels they adopted the polygonal structure of cells on stiff polyacrylamide gels. On soft gels it would take a significant amount of force to strain the gel sufficiently to reach a stiffness high enough to drive cell spreading. Cell traction forces are smaller in the initial phase of spreading (Dubin-Thaler et al. 2008) compared to later stages and as a result the probability of sufficiently straining the gel is low. Once a region of the gel is stiffened the cell would elongate along these stiffened regions applying stronger tractions at the same time. As the gel adjacent to the short axis of the cell remains soft the probability of sufficiently straining these regions of the cell remains low. On stiff 8 mg/ml fibrin gels it takes less force to achieve that critical strain and this may increase the probability of multiple spreading axes resulting in less elongated cells. On transitional 2 mg/ml gels the cells spread along multiple axes but don't broaden the membrane between the narrow protrusions resulting in more stellate cells with a lower circularity but no increase in axial ratio.

The elongated morphology of cells on soft gels suggests an explanation for the reduced pattern formation of cells on 1 mg/ml gel compared to 2 mg/ml gels. Cells that are highly elongated would have a very narrow radial strain field. This would result in

reducing the probability that a second cell would encounter the deformed region of the gel and thus a reduced probability of pattern formation

The displacement fields also predict a sphere of influence that an individual cell can use to communicate its position and orientation to other cells. Experimental data confirms that hMSCs become increasingly aligned as the distance between them decreases. At low concentrations in 2D and 3D gels, the cells orient to form a network structure similar to that seen during endothelial cell tubulation (Deroanne et al. 2001) and may help to explain how hMSCs stabilize functional engineered vasculature (Au et al. 2008). In this way, durotactic cells and strain stiffening gels may have evolved to facilitate patterning of tissues.

In this system it is difficult to separate the response to mechanical properties of the gel from responses to structural properties. In the case of initial cell spreading, prior to cell attachment the gel is an isotropic mesh and the onset of alignment in fibrin occurs later than the onset of strain stiffening (Kang et al. 2009); therefore initial cell spreading is likely driven by the gel's mechanical properties. The contribution of the fiber alignment observed at the cell's periphery to the orientation phenomena is unknown, but the tendency for cells to align with grooves on a substrate correlates more with the depth than the width of the grooves, suggesting that enhanced contact area, which simulates a 3D environment, rather than a preference for aligned substrates is the driving force (Clark et al. 1991). The filaments and grooves that have been shown to effectively induce contact guidance are 5 – 20 times larger than the fibrin fibers used in this study (Walboomers et al. 1999; Xu et al. 2004; Yim et al. 2005). If the topography is too fine it

does not supply the increased contact area which promotes contact guidance.

Making the system more complex is that structural anisotropy also alters the subjective stiffness of the gel from the perspective of a cell. When the fibers are randomly oriented the focal adhesions, which are 5 – 10 times larger than the individual fibers are likely to engage a mixed population of fiber orientations; however, once the fibers are aligned the adhesions are likely to engage only the bending modulus or the stretching modulus. Since the stretching modulus is higher than the bending modulus the mechanosensitive cells would preferentially move along the direction of alignment. Regardless, it is probable that both the mechanical and structural anisotropy contribute to the observed orientation effect and both of these features result from the underlying nonlinear response of the fibrin gels to cell-applied strains.

Recent experimental and computational studies reported that cells on moderately compliant synthetic matrices altered the behavior of neighboring cells through traction forces transmitted through the compliant gel (Reinhart-King et al. 2008; Sen et al. 2009). Since the experiments were done using polyacrylamide substrates, where the cell contraction scales with gel stiffness, the reported mechanical communication only occurred if the cells were within 50 microns and large scale pattern formation such as was seen in the system reported here was not observed. This difference underscores why linearly elastic materials might be an incomplete model system for predicting how cells will respond in biological matrices.

The results presented here have implications for smart design of tissue engineering constructs. One significant hurdle limiting clinical viability of tissue

engineering is finding a way to stably vascularize the construct. A recent study reported that hMSCs stabilized functional engineered vasculature composed of human umbilical cord vein endothelial cells suspended in a gel of fibronectin and collagen (Au et al. 2008). Although mechanics was not explicitly considered, the results from this work suggest that in addition to providing paracrine support, the hMSCs may also facilitate the network formation required for capillary development by dynamically restructuring the gel.

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Table 4.1 Statistical analysis of figure 4.1 B

A		50-250			
		15	45	75	105
	250-450	15	<0.0001		
		45		<0.01	
		75			N/S
		105			N/S
B	50-250	15	45	75	105
	15		<0.03	<0.0001	<0.0001
	45			<0.001	<0.002
	75				N/S
	105				
C	250-450	15	45	75	105
	15		N/S	<0.02	<0.002
	45			N/S	<0.04
	75				N/S
	105				

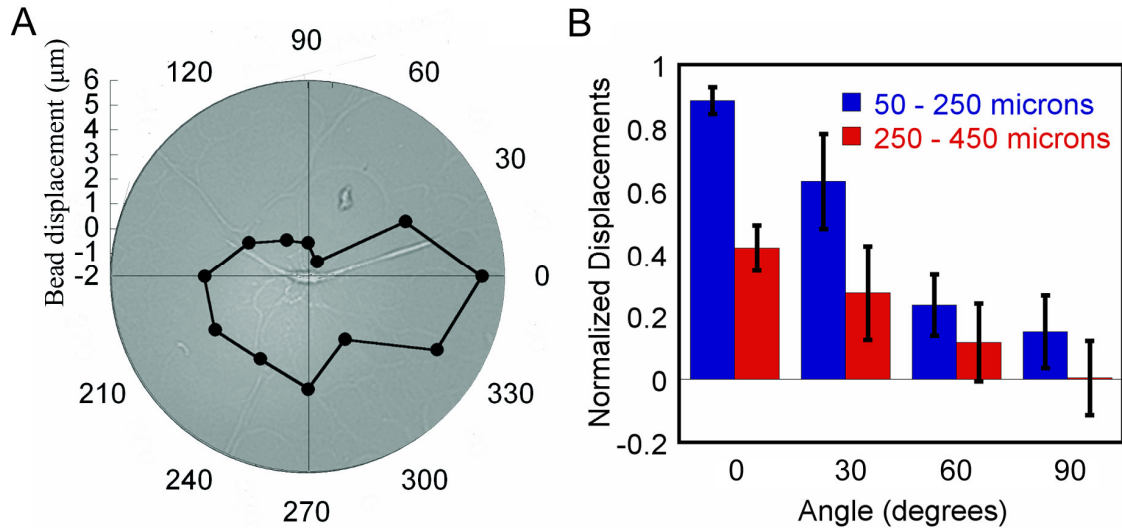
P values of radial displacement (average angle) data of inner circle (50 – 250 microns) compared to the outer circle (250 – 450 microns) using a one tailed Student t-test (A) for each pair. P values of data within the inner circle (B) or outer circle (C) using ANOVA analysis with $\alpha = 0.05$ and a Tukey post test.

Table 4.2 Stiffness of biopolymer gels

Fibrin (mg/ml)	G' (Pa)
1	30
2	60
8	320
Collagen (mg/ml)	
2	110

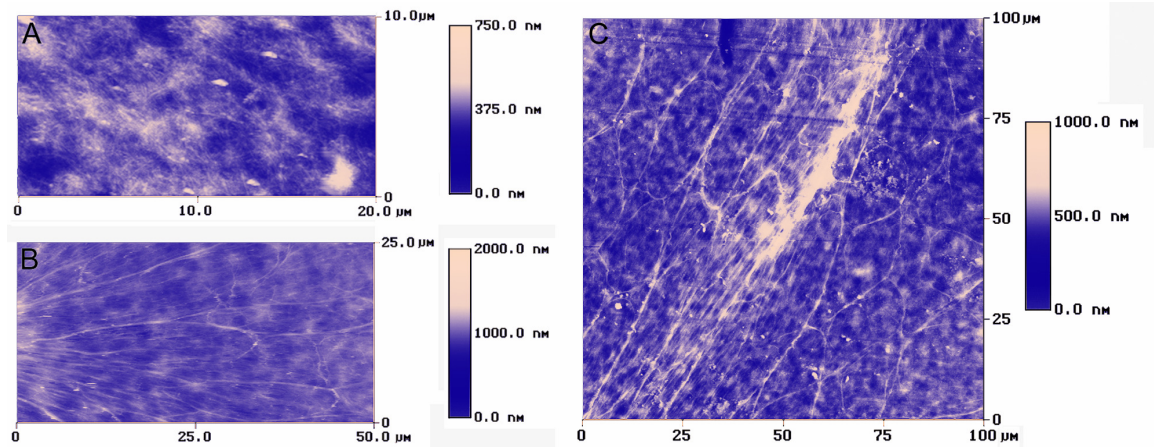
Approximate low (2%) strain elastic shear modulus (G') of fibrin and collagen gels used
in this chapter.

Figure 4.1 Displacement fields are anisotropic and correlate with cell shape



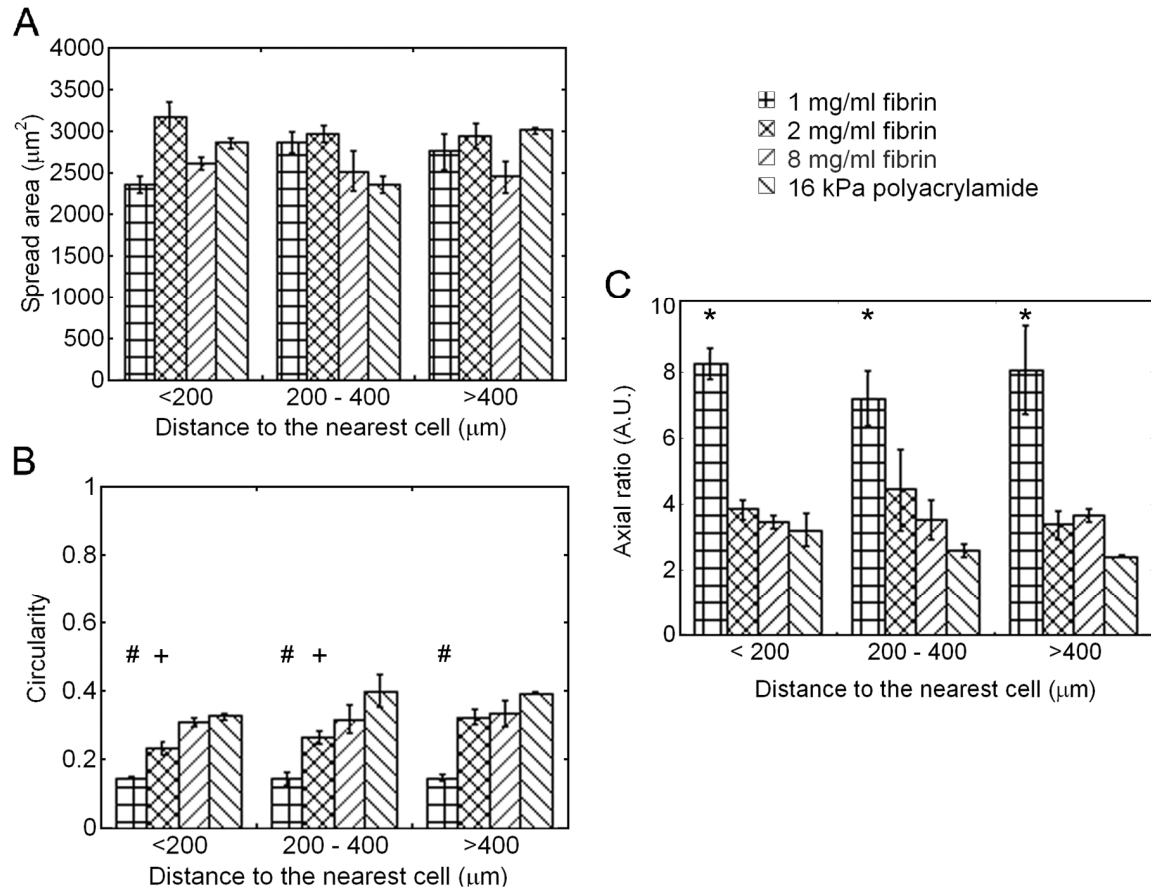
Radial plot of bead displacements created by a hMSC on a 2 mg/ml fibrin gel averaged over 30° bins (A). The cell is not to scale with the bead displacements. Displacements were then summed and averaged in 30° bins with the long axis parallel to 15° and perpendicular to 105° (B). $n = 4$.

Figure 4.2 Cellular contraction aligns adjacent filaments



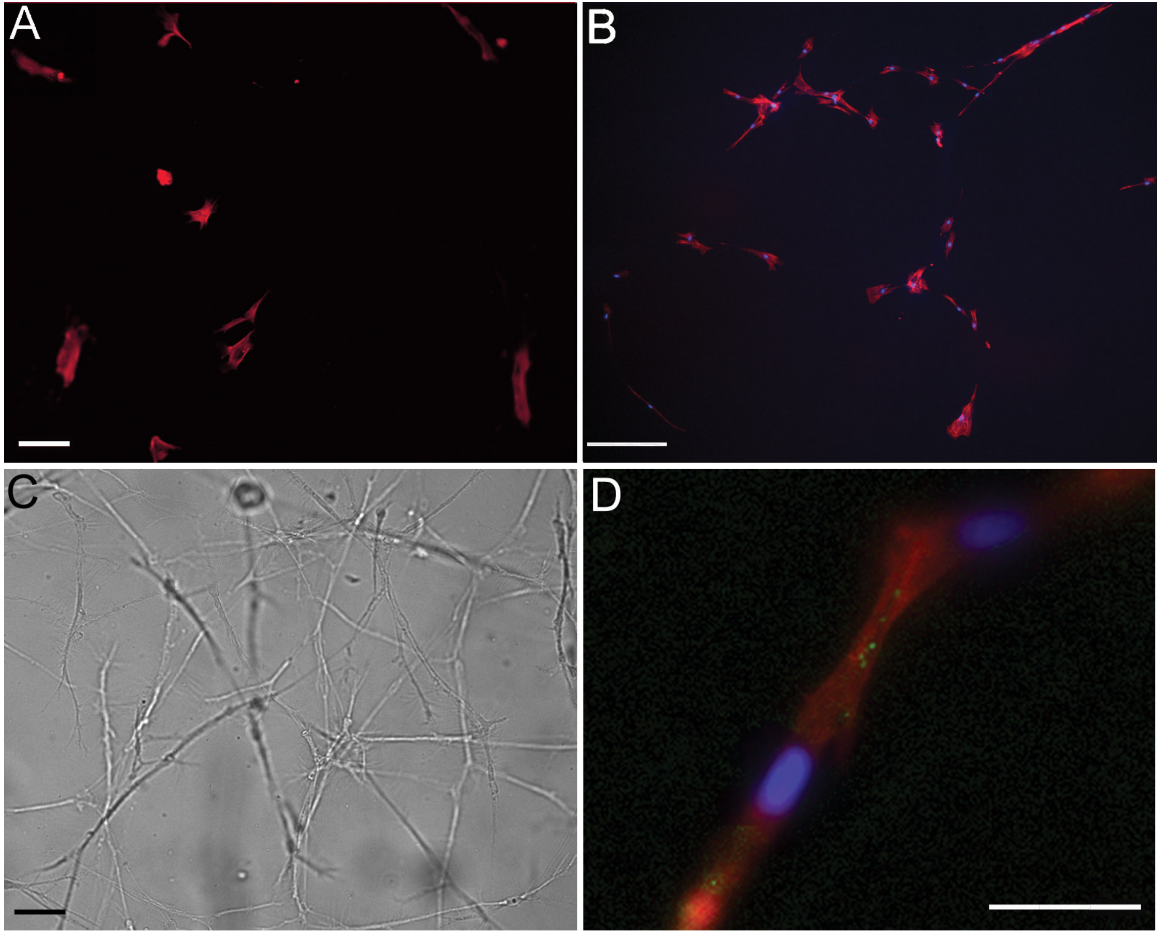
Tapping mode AFM images of a cell free 2 mg/ml fibrin gel (A), a section of gel adjacent to a spread hMSC which is adjacent to the left edge of the AFM image (B) and fibrin between two cells: one below the lower left corner and the other above the upper right corner (C). The gradient scale bar reports the sample height.

Figure 4.3 Fibrinogen concentration and distance to the nearest cell regulates cell shape but not spread area



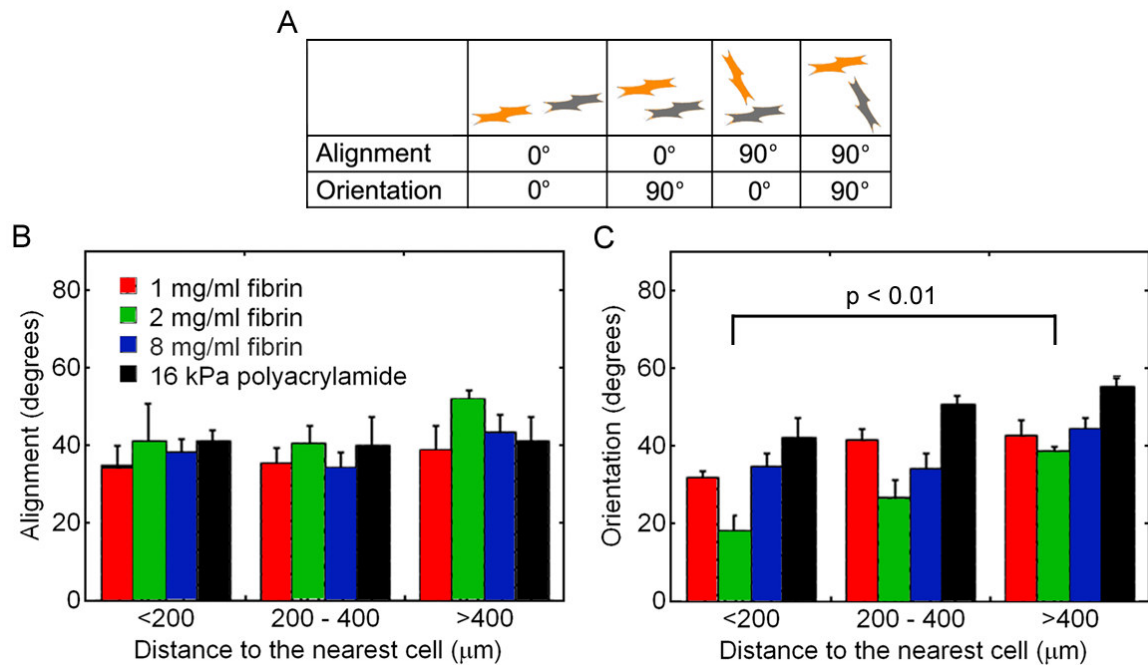
The projected cell area (A), circularity (B) and axial ratio (C) of neighboring hMSCs were analyzed as a function of distance to the nearest cell on four substrates: 1, 2, and 8 mg/ml fibrin as well as 16 kPa fibrinogen coated polyacrylamide. Reported as mean $\pm$ SE, $n = 3$, at least 10 cells per group. , * $p < 0.001$, # $p < 0.01$, + $p < 0.05$. Within zone one way ANOVA with Dunnet post test compared to 16 kPa polyacrylamide control.

Figure 4.4 Low density seeding of hMSCs on fibrin promotes formation of ring structures



Characteristic fluorescence image of F-actin structures in hMSCs cultured for 4 days on 16 kPa fibrinogen coated polyacrylamide (A) or 2 mg/ml fibrin (B). C) A bright field image of hMSCs cultured in 3D for 7 days in a 2 mg/ml gel. D) A fluorescently stained gap junction between two hMSCs in a 3D fibrin gel. For all images F-actin is labeled in red, the nucleus in blue and connexin 43 in green. Scale bar = 100 μ m for all images.

Figure 4.5 Pattern formation is characterized by cell / cell orientation not alignment

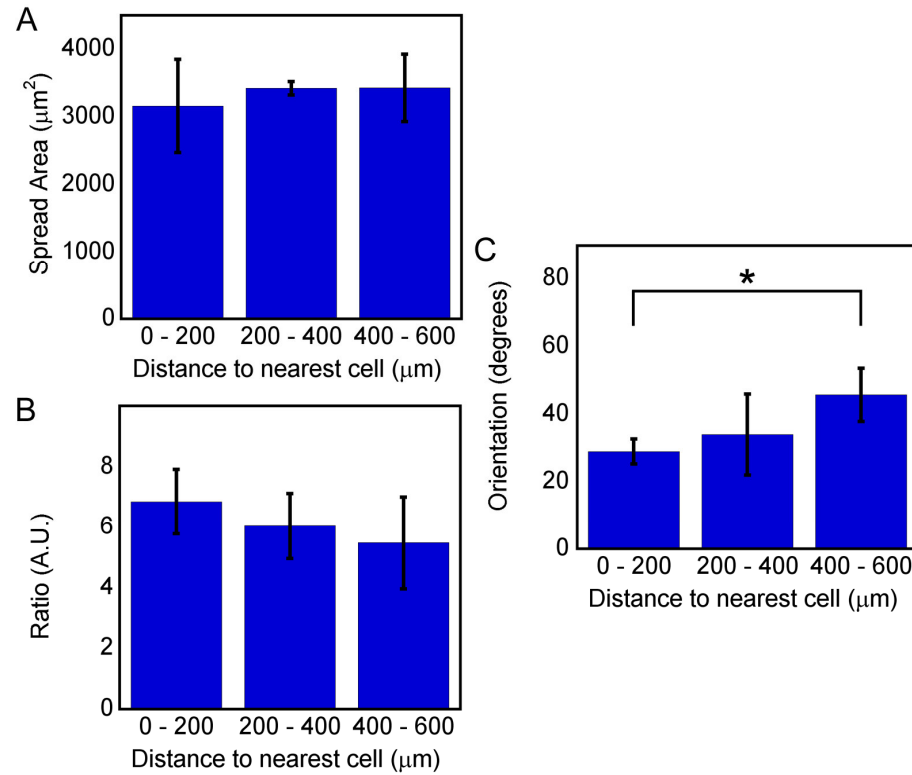


A cartoon demonstrating how the alignment and orientation are determined for the orange cell with respect to the grey cell (A). The alignment (B) and orientation (C) of neighboring hMSCs were analyzed as a function of distance to the nearest cell on four substrates: 1, 2, and 8 mg/ml fibrin as well as 16 kPa fibrinogen coated polyacrylamide.

Reported as mean $\pm$ SE, $n = 3$, at least 10 cells per group. Within group one way

ANOVA with Tukey post test.

Figure 4.6 HMSCs exhibit high spread area, high axial ratio and strong orientation on collagen gels



The projected cell area (A), axial ratio (B) and orientation (C) of neighboring hMSCs were analyzed as a function of distance to the nearest cell on four substrates: 1, 2, and 8 mg/ml fibrin as well as 16 kPa fibrinogen coated polyacrylamide. Reported as mean $\pm$ SE, n = 3, at least 10 cells per group. * = p < 0.03 one way ANOVA with Tukey post test.

CHAPTER 5

Filamin A is required for mechanical interactions between contractile cells and collagen but not fibrin gels

5.1 Abstract.

Filamin A is a Y shaped dimer with actin binding domains at the tip of each branch and a $\beta 3$ integrin binding domain at the stem. Filamin A is critical for collagen but not fibrin or fibronectin mediated substrate mechanosensing. Using two cell pairs, M2 (filamin A null) and A7 (filamin A positive) melanoma cells as well as HEK 293 wild type and filamin A knockdown cells, we show that filamin A expression is critical for actomyosin based compaction of collagen but not fibrin networks. Though A7 cells spread more on 100 Pa fibrin gels than collagen gels, in both cases the range over which A7 cells are manipulating the fibers is approximately 5x the long axis of the cell. Thus, filamin's role as a component of the $\beta 1$ focal adhesions, but not its role as an actin crosslinker is critical for compaction and alignment of collagen fibers.

5.2 Introduction

Formation of a dynamic actin network from individual filaments is essential to the functioning of contractile and motile cells. Filamin A was the earliest identified actin binding protein after myosin and tropomyosin (Hartwig and Stossel 1975; Wang et al. 1975) and was shortly identified as an actin crosslinker (Wang and Singer 1977). Cells which lack filamin A are capable of surviving and replicating but they have impaired locomotion and cortical actin stability (Cunningham et al. 1992).

Filamin A is a Y shaped homodimer with actin binding domains at the end of each branch and flexible linkers connecting each branch to the central stem (Fig. 5.1) (Hartwig and Stossel 1981). This structure facilitates formation of branching actin networks in purified (Hartwig et al. 1980) and cellular (Hartwig and Shevlin 1986) systems. Many other proteins also bind to filamin A. These include extracellular calcium receptors (Awata et al. 2001), Smad (Sasaki et al. 2001) and caveolin-1 (Sverdlov et al. 2009). Loss of filamin A leads to reduced caveolae trafficking which may explain the requirement of filamin A for internalization of the μ -opioid receptor and attenuation of the signal (Onoprishvili et al. 2003).

Discovery of the role of filamin in these and other pathways has been greatly facilitated by a pair of melanoma cell lines known as M2 and A7 cells. M2 cells were one of three melanoma cell lines which were identified as lacking filamin A (Byers et al. 1991) and displayed continuous blebbing without undergoing apoptosis. Filamin A was subsequently stably transfected into M2 cells, creating the A7 cell line (Cunningham et al. 1992). Expression of filamin A eliminated the blebbing phenomenon and restored

normal cell motility and functioning. Recently a second pair of cells was established consisting of wild type human embryonic kidney (HEK) 293 cells and HEK 293 cells transfected with shRNA specific for filamin A (Kim et al. 2008).

In the field of cell mechanobiology of greatest interest is the binding of the stem region of filamin to beta integrins creating an additional link between the actin cytoskeleton and the extracellular matrix (Loo et al. 1998). Although in purified systems filamin A binds to both $\beta 1$ and $\beta 3$ integrins, in cells talin outcompetes filamin A for binding to $\beta 3$ integrins (Goldmann 2000) making $\beta 1$ binding more physiologically relevant. This $\beta 1$ /filamin/actin interaction is critical for mechanoprotection of cells in response to otherwise lethal external forces (Glogauer et al. 1998; Kainulainen et al. 2002). Specifically, application of force through $\beta 1$ containing integrin dimers leads to upregulation of filamin A and increased recruitment of F-actin to the site of applied force (D'Addario et al. 2001).

In previous chapters it was shown that cells spread on strain stiffening biopolymer gels, such as collagen, as though they were on stiff linearly elastic gels. In agreement with data showing that cell mechanosensing on collagen gels is dependent on expression of filamin A (Byfield et al. 2009), the data in this chapter show that filamin expression regulates the cell's ability to contract and align the fibers within soft collagen gels. In contrast filamin A is not required for cells to exert force upon fibrin gels which are engaged through $\beta 3$ integrins (Weisel 2005). These results confirm that filamin A is a critical mediator of mechanical interactions between cells and collagen but not fibrin gels.

5.3 Materials and Methods.

5.3.1 Cell Culture

M2 (filamin A deficient) and A7 (M2 cells transfected stably with a full-length filamin cDNA) (Cunningham et al. 1992) melanoma cells were cultured in DMEM (BioWhittaker) supplemented with 10 % fetal bovine serum (Hyclone), 100 units/ml penicillin and 100 µg/ml streptomycin. Human kidney (HEK-293) cells wild type and shRNA filamin A knockdown cells were a gift from Dr. Chris McCulloch. Briefly, a filamin A-specific short hairpin RNA was constructed from two inverted 21-base sequences (5'GGGCTGACAACAGTGTGGTGC3') of the filamin A cDNA and incorporated into a plasmid with the U6 promoter for shRNA expression and the pPUR vector for puromycin resistance. HEK-293 cells were cultured in DMEM with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin (Kim et al. 2008). For filamin A-knockdown cells, 1 µg/mL of puromycin dihydrochloride (Sigma) was added to the culture medium. All cells were maintained at 37°C and 5% CO₂. Unless otherwise specified all reagents are analytical grade and purchased from Sigma.

5.3.2 Fibrin Gel Preparation

Lyophilized salmon fibrinogen (Wang et al. 2000) and thrombin (Michaud et al. 2002) were provided by Sea Run Holdings. Fibrin gels were prepared by diluting the stock solution of fibrinogen with T7 buffer (50 mM Tris, 150 mM NaCl at a pH of 7.4) to make a working solution of desired concentration. Polymerization was initiated in a 24 well tissue culture plate by adding 5 µl of thrombin (activity = 100 NIH units/ml) to 250 µl of the fibrinogen solution. Gels were allowed to polymerize for 30 minutes before cell

culture media was added to the wells. Gel thickness was measured using the z control of the rheometer and found to be approximately 1 mm at the thinnest point.

5.3.3 Collagen Gel Preparation

Acid solubilized Type I rat tail collagen was purchased from BD Bioscience. Collagen gels were prepared by adding a 1:10 dilution of 10x PBS to the stock solution of collagen and then bringing the volume to the desired concentration with 1x PBS. Gelation was initiated by adding NaOH until the solution reached pH 7.5. 250 μ l of the working solution was added to each well of a 24 well tissue culture plate. The plate was then placed at 37°C to accelerate gelation. After 1 hr, 1ml PBS was added to each of the wells.

5.3.4 Atomic Force Microscopy

The samples were fixed with 2% glutaraldehyde in sodium cacodylate buffer, and dehydrated with ethanol. The samples were then immersed in 50% ethanol, 50% hexamethyldisilazane (HMDS) for 10 minutes and then immersed in 100% HMDS for 10 minutes. The excess solution was removed and the samples were placed in a vacuum chamber for 1 hr to facilitate evaporation of the HMDS. HMDS dried samples were then imaged in tapping mode on a Veeco multimode AFM with pyramid tip silicon probes with a cantilever spring constant of 3 N/m.

To determine the degree of alignment of collagen gels fibers, 25 by 25 μ m square AFM images of the collagen network,. Matlab software was used to enhance image features, apply a two-dimensional Fast Fourier Transform from these images, collect radial intensity line scans, plot intensity as a function of angle, and fit a Gaussian curve to

the major peak. Standard deviation of the Gaussian fit was used as a parameter of the orientational order. Qi Wen assisted in optimization of this code.

5.3.5 Displacement Microscopy

Untreated fibrinogen was spiked with biotinylated fibrinogen (1:50), the gels were polymerized as usual and then the surface was coated with streptavidin-coated red fluorescent beads (1000 beads/ μm^2 , Invitrogen). Biotin-NHS (Sigma) was conjugated to salmon fibrinogen using the manufacturer's protocol. In the case of collagen gels Biotin-maleimide was conjugated after polymerization and before addition of the beads due to the low pH of the soluble collagen inhibiting conjugation. Cells were seeded on fluorescent bead-coated gels (100 cells/well) and allowed to adhere and spread for 18 hours. For imaging, the cells were kept in serum-free media buffered with 50 mM HEPES and the microscope stage was heated to 37°C. Cells were imaged in phase contrast mode and the beads were imaged in fluorescence before 5 μM blebbistatin was added. After 30 minutes the beads were imaged again. Image J (NIH) and Adobe Photoshop were used to generate a map of bead displacements for each pair of images. Due to the low magnification needed to capture the scale of the gel deformation the pixel resolution resulted in a error of $\pm 0.25 \mu\text{m}$. To minimize the effect of cells other than the one of interest, only 50-100 cells were seeded per well of the 24 well dish. In addition the imaged cells were selected because they were at least 1 mm from the nearest cell.

5.3.6 Image Analysis

All light imaging was done on a Leica DMIRE microscope using a Hamamatsu ORCA-ER camera and a 10x air lens with a numerical aperture of 0.30 or a 40x air lens

with a numerical aperture of 0.60. Image J software was used to convert images to 8 bit, and then threshold and analyze the cells' projected area and circularity. Cell projected area was determined using Image J's analyze particles command. Circularity was determined using the equation $4\pi (\text{area})/(\text{perimeter})^2$. The circularity is 1 for a circle and decreases toward 0 as the cell becomes more elongated or stellate in morphology.

5.4 Results

5.4.1 Filamin A required for force induced alignment of collagen fibers

Atomic force microscopy (AFM) reveals that collagen fibers adjacent to melanoma cells which lack filamin A (M2 cells) are less oriented (Fig. 5.1 B) than those adjacent to A7 cells which are M2 cells stably transfected with filamin A cDNA (Fig. 5.1 A). Quantification of the AFM images reveals that under static conditions collagen polymerization results in randomly arranged fibers. A7 cells significantly aligned the fibers perpendicular to the cell's membrane at least 25 microns from the cells periphery, whereas M2 cells only significantly aligned the gel within 12.5 microns from the cell (Fig. 5.2 C). Additionally the A7 cells more strongly align the collagen fibers than the M2 cells at both distances.

5.4.2 Filamin A is required for cell induced contraction of collagen

HEK 293 wild type cells and A7 melanoma cells contract collagen gels up to 125 μm or 4 cell lengths from their periphery. M2 cells which are filamin A null did not contract the gel significantly at any distance whereas HEK filamin A knockdown were able to contract the gel somewhat within 75 μm (Fig. 5.3). As the knockdown of filamin A in HEK 293 cells is not 100% these cells retained some ability to contract the matrix.

In the melanoma A7 and M2 cells, the A7 cells which express filamin A were less circular and had a larger spread area than their filamin null counterparts (Fig. 5.4). This was not true for the HEK 293 cells which had higher variability in both average displacement and spread area. Again this difference is likely due to the incomplete knockdown of filamin A in the HEK cells.

5.4.3 Filamin A is not required for cell induced contraction of fibrin

Unlike on collagen substrates, filamin A was not required for attachment and contraction of fibrin gels. M2 cells on collagen gels are as effective at contracting fibrin gels as A7 cells. The range over which the melanoma cells contract the fibrin gels is twice as far as that on collagen gels and the average displacements are 20 times higher on fibrin gels (Fig. 5.5 A). Analysis of the spread area and circularity indicates that while circularity is a poor indicator of contractility, the spread area of melanoma cells correlates with contractility (Fig 5.5 B).

5.5 Discussion.

Filamin A an actin binding protein found in tissues throughout the body (Wang et al. 1975). In addition to binding and crosslinking actin, filamin A interacts with a variety of other proteins (Popowicz et al. 2006) which likely contributes to the changes in phenotype upon loss of filamin A. A lack of filamin A is embryonic lethal and this lethality has been attributed to a disruption in cell motility and incomplete development of adherens junctions (Feng et al. 2006). At the cellular level, loss of filamin A is not lethal and thus filamin null cells can be used to study the functions of filamin A including its role in substrate mechanosensing. The data presented in this chapter suggest that

filamin A's role in the $\beta 1$ focal adhesion and not its role as a stabilizer of cortical actin is responsible for the inability of filamin null cells to contract collagen gels.

In addition to actin, filamin A also binds β integrins. Integrins, the primary transmembrane links between the extracellular matrix and the actin cytoskeleton, are heterodimers composed of one α and one β subunit. The ability of an integrin to bind a particular substrate depends on the composition of the dimer. For instance collagen is primarily bound by $\alpha 1\beta 1$ and $\alpha 2\beta 1$ dimers (Kramer and Marks 1989) whereas cells bind to fibrin and fibronectin through $\alpha 5\beta 1$ and $\alpha v\beta 3$ dimers (Dejana et al. 1990). Thus if binding through $\beta 1$ containing dimers is compromised cells cannot interact with collagen substrates but can still interact with fibrin substrates because $\beta 3$ containing dimers can compensate.

Force transduction through integrins is required for cells to apply traction forces to a substrate. When external force is applied to collagen gels the fibers align and increase resistance to the applied force (Vader et al. 2009). AFM showed that collagen fibers adjacent to A7 cells are aligned better and over a greater distance than the fibers adjacent to M2 cells which lack filamin A. Displacement microscopy confirmed that filamin A expression regulates the ability of cells to contract collagen gels but not fibrin. Thus filamin A is required for cells to apply forces to a collagen substrate.

One contradiction with published literature is that the cells are more spread and more contractile on fibrin gels compared to collagen gels. On ligand coated polyacrylamide gels A7 cells spread equally well when the ligand is collagen or fibronectin. Though M2 cells do not spread on collagen coated gels, on fibronectin

coated polyacrylamide they have the same average spread area as A7 cells (Byfield et al. 2009). On fibrin gels the average spread area of both cell types was equivalent to the spread area on stiff polyacrylamide gels; however, on collagen gels A7 cells are slightly more spread than M2 cells but significantly less spread than cells on fibrin gels or stiff polyacrylamide. The reason for this isn't clear but possibly has to do with the amount of initial applied force required to strain stiffen the matrix being achieved on fibrin gels but not on collagen gels. This would be due either to differences in the nonlinear properties of the gels or differences in the amount of force applied through the different types of focal adhesions. Further work must be done to distinguish between these two possibilities.

As M2 cells spread and apply force equivalently to A7 cells when cultured on fibronectin coated polyacrylamide or fibrin gels, filamin's role as a crosslinker which stabilizes cortical actin is not critical to the cell's ability to substrate mechanosense through $\beta 3$ integrins. The question then arises as to whether or not filamin's actin crosslinking ability is critical to its role in collagen mechanosensing. A recently generated library of filamin A fragments (Nakamura et al. 2007) could be utilized to study this and other questions regarding coupling of filamin's crosslinking ability to its interactions with other binding partners.

5.5 References

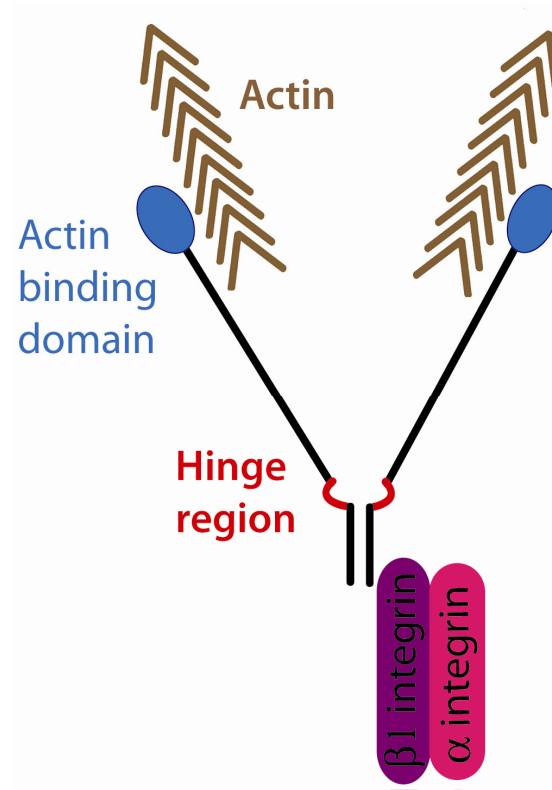
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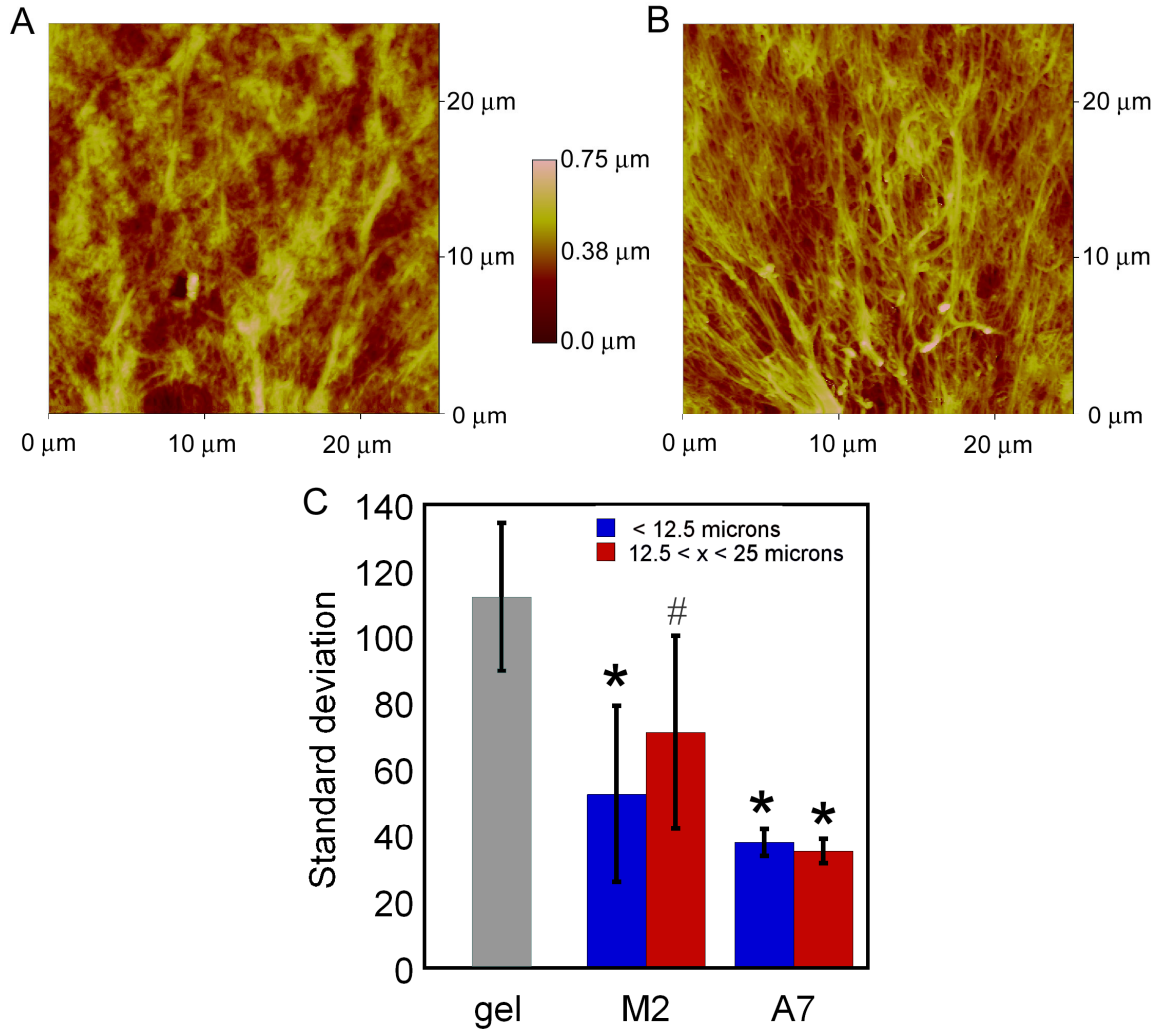
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Figure 5.1 Cartoon of filamin A and its relevant binding domains



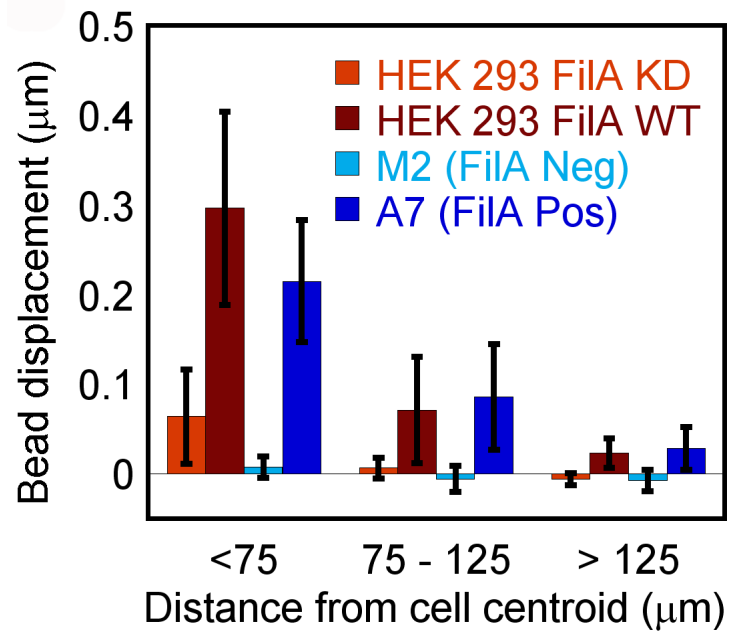
Cartoon of relevant structural and binding elements of filamin A.

Figure 5.2 Filamin A is critical for alignment of local collagen fibrils



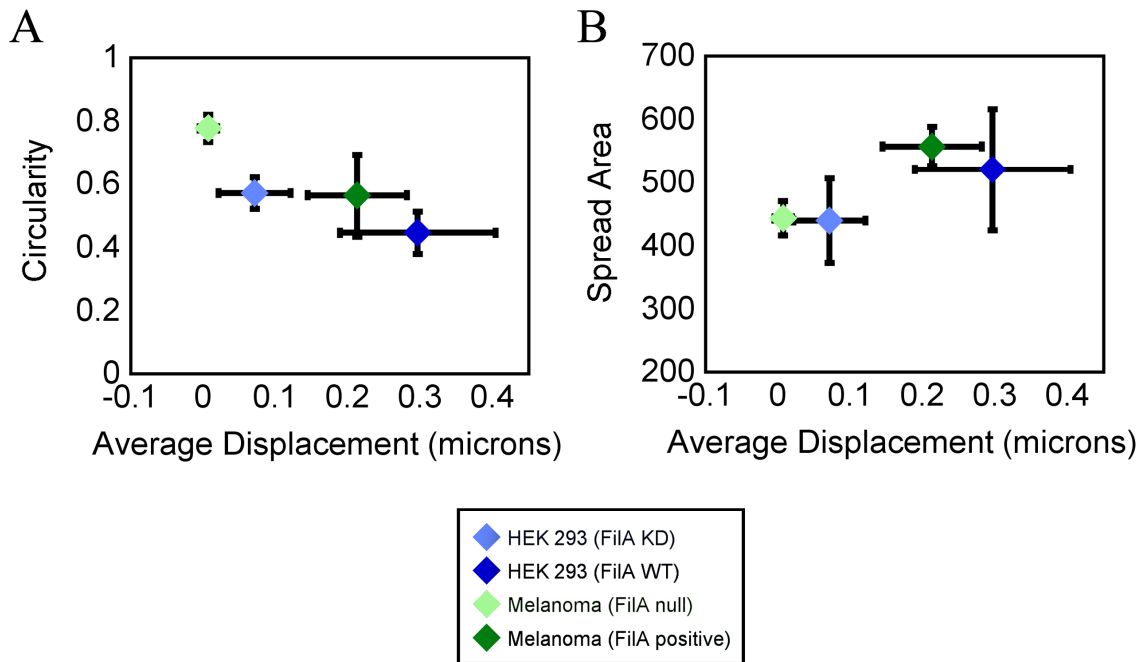
Typical AFM image of the collagen network adjacent to an M2 cell (A) or an A7 cell (B). Cell is located below the bottom edge of the images. Standard deviation of the Gaussian fit to Fourier transform of AFM images of the collagen gel (C). Reported as mean $\pm$ SD, $n = 5$. # = $p < 0.5$ using paired Student's T-test between the two M2 populations. All populations except the fibrin gel more that 12.5 microns from M2 cells are significantly different ($p < 0.1$) than the fibrin gel control using an ANOVA ($* < 0.1$) and a Dunnet post test.

**Figure 5.3 Cells lacking filamin A do not apply active forces to collagen but can on
fibrin**



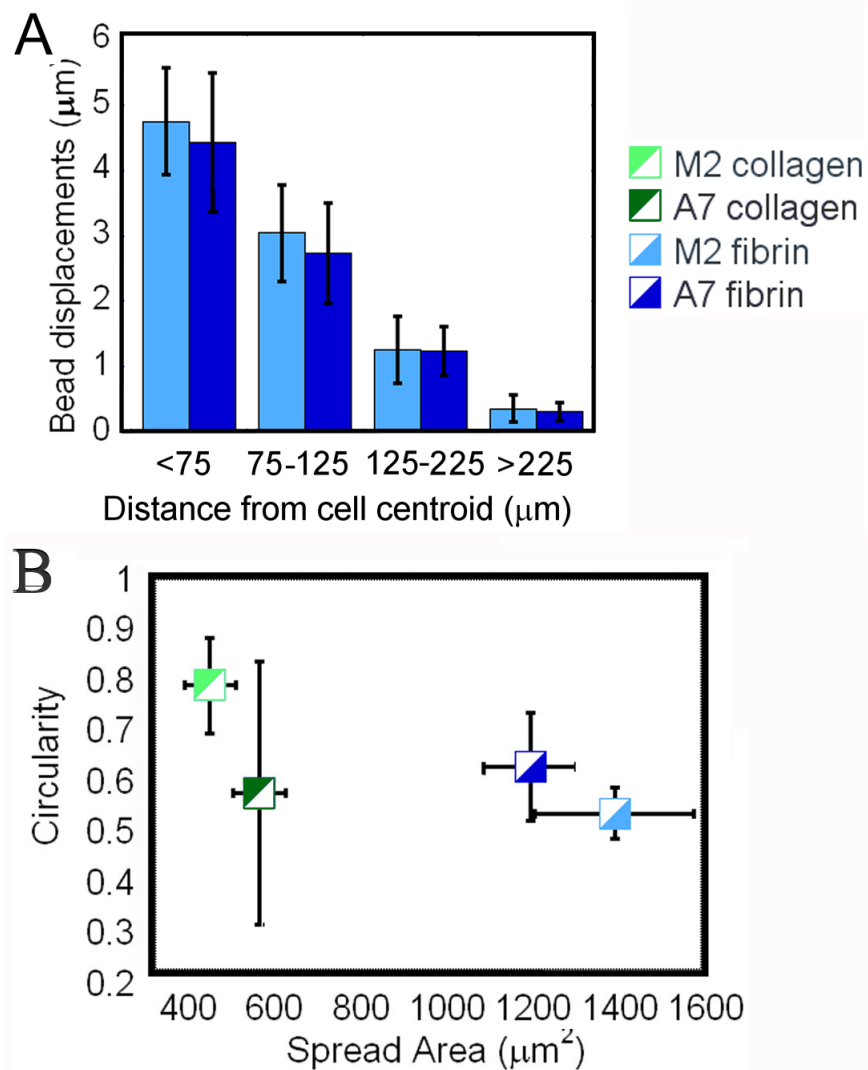
Average bead displacements as a function of distance from the cell centroid by cells on 2 mg/ml collagen gels. At least 50 beads per zone. Reported as mean $\pm$ SE, n = at least 5.

Figure 5.4 Active contractility does not correlate with spread area or circularity



Average bead displacement within the first 75 microns of the cell's centroid plotted versus average circularity (A) and average spread area (B) in μm^2 . Reported as mean $\pm$ SE, n = at least 5.

Figure 5.5 Melanoma cells spread better on fibrin gels than collagen gels



Average bead displacements as a function of distance from the cell centroid by cells on 2 mg/ml collagen gels (A). Reported as mean $\pm$ SE, $n = 5$. Spread area versus circularity of melanoma cells on collagen or fibrin substrates (B). Reported as mean $\pm$ SD, $n = 5$.

CHAPTER 6

Salmon fibrin as a tissue engineering construct for nervous system repair

6.1 Abstract.

Nerve damage comes in many forms from a localized pinched nerve to extensive penetrating brain injuries. After the initial traumatic event, there is often a prolonged inflammatory response including activation of local glial cells which can further damage the neurons and inhibit neural tissue repair. There is great need for therapies which can protect neurons from reactive gliosis and persistent inflammation. Treatment of cortical ablation injuries with injections of salmon fibrin suggests that fibrin is a neutral scaffold which can be used as a carrier for drug and cell therapies after traumatic brain injury. When used to treat painful nerve compression injuries, salmon fibrin reduced behavioral sensitivity and inflammation. These results indicate that salmon fibrin has potential as a treatment for nerve tissue damage.

6.2 Introduction

While many tissues, such as bone, skin and liver, show a high potential for regeneration after injury, damage to the nervous system is often not reparable. Damage to the central nervous system from a traumatic brain injury results in astrocyte activation and the formation of a glial scar. This scar prevents recovery by mechanically and chemically blocking neuronal invasion into the damaged area (Saha et al. 2008). In the spine, nerve root compression can lead to glial activation, sustained inflammation and persistent pain (Winkelstein et al. 2001). Due to its *in vitro* ability to promote neurite growth and inhibit astrocyte activation, salmon fibrin is being investigated as a therapy for nerve damage.

Fibrin is the insoluble filamentous form of the soluble blood protein fibrinogen. Fibrinogen self assembles by polycondensation into a branching network upon cleavage by the serine protease thrombin (Janmey 1982). This occurs rapidly after an injury to the endothelium and the resulting clot serves first to restore hemostasis and later as a scaffold for fibroblast invasion during wound healing (Brown et al. 1993; Laurens et al. 2006). Current clinical uses include stemming bleeding, mesh fixation for inguinal hernia repair, reattachment of severed sciatic nerves and improvement of skin graft adhesion (Martins et al. 2005; Mittermayr et al. 2006). Fibrin is an attractive scaffold for tissue engineering due to its inherent cell, ECM and growth factor binding domains as well as its ability to stimulate a wound healing response. It is currently being investigated as a scaffold for bone (Kneser et al. 2005), cartilage (Eyrich et al. 2007), vascular (Mol et al. 2005) and neural (Galla et al. 2004) tissue engineering.

For clinical use there are currently three FDA approved fibrin sealant products that are all manufactured by Baxter Healthcare. The most widely used is Tisseel which contains both human and bovine components (Buchta et al. 2004). Use of human and bovine components carries a risk of viral or prion transmission even though extreme purification methods are taken. This intense purification adds to the cost of these products. The risk of prion transmission is heightened due to mammalian fibrinogen's capacity to selectively bind the infectious form of the prion protein (Fischer et al. 2000). These risks can be minimized by the use of non-mammalian clotting proteins such as those derived from salmon.

Due to the large evolutionary distance between fish and humans disease transmission between species is unlikely. While salmon express one isoform of the prion protein, it has a very different structure from the mammalian protein and there is no evidence of prion disease in fish. Purification methods have been developed to isolate salmon clotting factors (Michaud et al. 2002; Wang et al. 2000) and large quantities of salmon blood are readily available from the salmon farming industry. An additional clinical advantage of salmon thrombin is its ability to clot at hypothermic temperatures (Laidmae et al. 2006) and thus could prove useful in controlling bleeding during the low temperature surgeries which are shown to improve patient outlook after nervous system injury (Davies 2005; Shibuya et al. 2004).

Salmon clotting factors are currently being investigated both *in vivo* and *in vitro* to determine their therapeutic efficacy. A hemostatic bandage composed of salmon thrombin and fibrinogen has been used to successfully to arrest bleeding in a swine

aortotomy model (Rothwell et al. 2005). Rats or swine injected with salmon clotting factors did not develop antibodies which cross-react with their own clotting factors nor did they display symptoms of clotting or autoimmune disorders after injection (Laidmae et al. 2006; Rothwell et al. 2009). In vitro work has shown that salmon fibrin facilitates neurite outgrowth and branching in both 2D and 3D (Ju et al. 2007). Preliminary work has shown that treatment of spinal cord injury with salmon fibrin improves functional recovery and reduces glial scarring (Flanagan et al. 2007).

In this chapter two animal models were employed to investigate salmon fibrin's potential as a treatment for nervous system repair given that salmon fibrin may have properties that both promote neuronal repair and inhibit inflammation. Fibrin's effectiveness as a scaffold for treatment of traumatic brain injury was tested using a rat cortical ablation model. Rats treated with fibrin showed no difference in either their behavioral recovery or inflammatory response compared to untreated rats, indicating the fibrin could be used as a neutral scaffold for delivering growth factors and/or neural stem cells. The second study investigated salmon fibrin's potential to treat painful mechanically-induced cervical radiculopathy, using a rat model of transient dorsal root compression. In this model salmon fibrin reduced behavioral hypersensitivity and inflammation by 7 days post injury and salmon thrombin alone was sufficient to achieve these results. These studies demonstrate the potential usefulness of salmon fibrin to treat nervous system injuries.

6.3 Materials and Methods.

6.3.1 Animal Care

All experimental procedures have been approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Experiments were performed using male Holtzman rats, weighing 275–350 g at the start of the study. Animals were housed under USDA and AAALAC-approved conditions with free access to food and water.

6.3.2 Cranial Ablation

Rats were anesthetized with sodium pentobarbital and placed in a stereotaxic instrument to immobilize head position. A trephine was used to remove a 5 mm diameter piece of skull above the left motor cortex. A 2 mm diameter, 3 mm long cylinder of dural and CNS tissue was removed with a custom tool (Fig. 6.1 A). Immediately after tissue ablation, 10 μ l of either saline or salmon fibrin (3 mg/ml salmon fibrinogen, 2 U/ml salmon thrombin in 10 μ l neuralbasal (NB) media) was injected into the lesion (Fig. 6.1 B) resulting in a fibrin plug (Fig. 6.1 C) of either rat (due to natural clotting) or salmon fibrin. Injury was then sutured closed and rats were allowed to recover on a heated pad before being returned to their cage.

6.3.3 Nerve Root Compression Injury

This surgery was performed under inhalation anesthesia (4% halothane for induction, 2% for maintenance) and follows procedures developed in the Winkelstein lab (Hubbard and Winkelstein 2005; Rothman et al. 2005). Rats were placed in a prone position, and the C7 dorsal root was surgically exposed. The C7 dorsal nerve root was compressed for 15 minutes using a 10gf microvascular clip (World Precision Instruments, Inc., Sarasota, FL). After compression 20 μ l of one of the following treatments was

applied directly to the nerve root: saline, neurobasal media (NB), 2 NIH U/ml salmon thrombin in NB, 2 NIH U/ml human thrombin in NB or 2mg/ml salmon fibrinogen clotted with 2 NIH U/ml salmon thrombin in NB. The sham surgery followed the same protocol but without compression of the C7 dorsal root. Rats recovered in room air and were monitored continuously.

6.3.4 Histology

Animals received an overdose of sodium pentobarbital and were perfused through the heart with saline followed by 4% paraformaldehyde. Then either the brain or DRG were resected and stored in 4% paraformaldehyde before flash freezing and sectioning by a freezing microtome. Tissue slices were stained with hematoxylin and eosin or immunostained for GFAP or ED1. Images were analyzed using custom code written in Matlab.

6.3.5 Behavior Testing

Motor function recovery was tested by measuring contraflexion (Fox et al. 1998), cylinder rearing (Schallert et al. 2000) and Morris water maze (Morris 1984). All scoring was performed by blinding testers. Forelimb contraflexion was assessed by suspending the rat by its tail and assigning a score from 1-5 according to the following criteria: 1 – rat makes no effort to use contralateral limb to break a perceived fall. 2 – rat exhibits unilateral turning with contralateral limb flexion of greater than 90° from outstretched position. 3 – rat exhibits unilateral turning with contralateral limb flexion of less than 90° from outstretched position. 4 – rat exhibits preferential turning while suspended. 5 – rat exhibits normal response characterized by outstretching forelimbs to reach for flat surface.

In rearing tests two rats were placed in a transparent cylinder 9-in diameter and 15 in high and videotaped during the test. Two mirrors were angled behind the cylinder to enable tester to view forelimb movements even when rat was turned away from the camera. The rat's rearing behavior was taped for 15 minutes and forelimb use was scored by the following criteria: (1) The first forelimb to contact the wall during a full rear was recorded as an independent wall placement for that limb. (2) Simultaneous use of both the left and right forelimb by contacting the wall of the cylinder during a full rear and for lateral movements along the wall was recorded as "both" movement. The final score = (impaired forelimb movement) / (nonimpaired forelimb movement + impaired forelimb movement + both movement) normalized by the baseline data. Animals which failed to rear at least 10 times within 10 minutes were excluded.

For the Morris water maze, rats were trained to locate a submerged platform using extramaze visual cues. The rats were randomly placed in a large water drum filled with 12 inches of water and an isolated, submerged platform. The sides of the drum are painted white except for specially placed brightly colored shapes. These provide the visual cues by which the rats locate the platform. The reported value is the average time in seconds to reach the platform.

6.3.6 Mechanical Allodynia

Mechanical allodynia was performed according to standard protocols (Lee et al. 2004). Bilateral allodynia was measured for each rat prior to surgery to establish baseline values. Rats were given 20 minutes to acclimate and then allodynia was measured rats by stimulating the plantar surface of each forepaw using 3 von Frey filaments (1.4, 2, and

4g; Stoelting, Wood Dale, IL). Each testing session had 3 rounds of 10 stimulations, separated by 10 minutes. Total withdrawals were recorded for each forepaw of each rat and averaged for each group. A single tester performed all measurements.

6.3.7 Statistics

Statistical significance ($P < 0.05$) was determined using a one-way ANOVA with Student-Newman-Keuls post test (cranial ablation) or the Bonferroni correction (DRG crush). Statistical methods represent preferences of collaborating laboratories.

6.4 Results

6.4.1 Salmon fibrin is a neutral scaffold for CNS repair after cortical ablation

Three tests were used to gauge motor function after cortical ablation: Morris water maze, contraflexion and forelimb usage during rearing. The Morris water maze tests coordination and memory formation. The time to find the platform decreased for the first 3 weeks of testing and then leveled off (Fig. 6.2 A). There was no difference between the fibrin group and the saline control group. Similarly, contraflexion, which measures the ability of rats to utilize the contralateral forelimb to break a simulated fall, increased to baseline gradually post injury and there was no difference between the fibrin and control groups (Fig. 6.2 B). There was trend toward improved contralateral forelimb usage during rearing in the fibrin group suggesting a possible benefit from the fibrin treatment. The animals treated with fibrin recovered faster than those treated with saline and they achieved a return to baseline function by 4 weeks (Fig. 6.2 C).

The response to fibrin was also characterized by staining for GFAP, a marker of activated astrocytes as well as ED1, a marker of activated microglia and macrophages.

Activated astrocytes are responsible for the formation of the glial scar after injury whereas activated microglia and macrophages are key components of the inflammatory response after injury. Both of these responses negatively affect the ability of neurons to recover after injury. Neither GFAP nor ED1 staining increased with fibrin treatment indicating that fibrin treatment does not exacerbate astrocyte activation or inflammation (Fig. 6.3). GFAP staining is filamentous whereas ED1 staining is punctate and there is little overlap in staining which indicates low background staining. This is consistent with their role as an intermediate filament and a lysosomal marker respectively. Overall these results suggest that salmon fibrin is a neutral scaffold for CNS repair and can be used as a carrier for drug delivery after traumatic brain injury.

6.4.2 Salmon fibrin mitigates pain and inflammation after nerve root compression

Compression of the C7 DRG results in persistent neuropathy in the ipsilateral forelimb. Mechanical allodynia of the forepaw provides a measurement of behavioral sensitivity. Mechanical allodynia in the ipsilateral forepaw following the compression injury was elevated and significantly greater than sham throughout the 7 day study (Fig. 6.4 A). These results are consistent with previous studies using this pain model (Hubbard and Winkelstein 2005; Rothman et al. 2005). Treatment with vehicle control (NB media) did not significantly reduce allodynia and was significantly greater than fibrin at day 7 ($p < 0.05$) (Fig. 6.4 A). Salmon fibrin treatment significantly reduced allodynia compared to both controls by day 5 and recovered baseline sensitivity by day 7. None of the treatments increased sensitivity in the contralateral forepaw above sham levels at any time point.

Randomly selected C7 nerve root sections were collected on day 7 for analysis of ED1 positive cells. Strong ED1 staining was evident 7 days after injury, but not after a sham procedure. Treatment with salmon fibrin reduced the level of ED1 staining compared to injury indicating a role for salmon fibrin in mitigating inflammation after DRG compression injury.

6.4.3 Salmon thrombin is sufficient to mitigate pain after nerve root compression

The salmon fibrin treatment contained two proteins: salmon fibrinogen and salmon thrombin. To test whether one or both were critical for reducing sensitivity after nerve compression, salmon thrombin in NB media was tested without salmon fibrinogen. Salmon thrombin alone was as effective as salmon fibrin at reducing mechanical allodynia after injury. This response was specific to salmon thrombin as treatment with human thrombin had no effect on forepaw sensitivity compared to control.

6.5 Discussion.

Upon traumatic injury the clotting cascade is initiated resulting in activation of thrombin which cleaves two polypeptides from the central domain of soluble fibrinogen dimers converting them into insoluble fibrin dimers which then polymerize into fibrin fibrils forming a branching network. This network forms the primary structural component of a blood clot which first serves to restore hemostasis and later serves as a provisional scaffold for tissue repair. Fibrin's role as a native provisional scaffold with inherent binding sites for an array of cells (Cheresh et al. 1989), growth factors (Sahni and Francis 2000; Sahni et al. 1998) and extracellular matrix proteins (Weisel 2005) makes it a promising candidate as a tissue engineering construct. In this study salmon

fibrin was investigated as treatment after damage to central or peripheral nerves.

To study salmon fibrin's potential as a scaffold for CNS repair rats underwent a cortical ablation of the left motor cortex to simulate the wound after debridement of a penetrating traumatic brain injury. Immediately after ablation, the lesion was filled with either saline or a solution of 3 mg/ml salmon fibrinogen and 2 U/ml salmon thrombin. The solution began to gel upon mixing and had to be injected within 30 seconds of mixing. As polymerization occurred rapidly and was initiated prior to injection it did not flow away from the injury site after application. The fibrin plug was visible in the injury site up to 7 days after application.

The presence of salmon fibrin in the wound had no significant effect on behavior recovery, astrocyte activation or local inflammation. This is in contrast to previous experiments using human fibrin which has been shown to exacerbate inflammation in the CNS (Akassoglou et al. 2004). Human fibrinogen has a binding site on the gamma chain for MAC-1 integrins which are expressed primarily by macrophages and microglia (Adams et al. 2007). Zebrafish fibrinogen, the closest sequenced evolutionary predecessor to salmon fibrinogen, has poor homology in the region identified as the MAC-1 binding domain suggesting that salmon fibrinogen would not have the proinflammatory effects of human fibrinogen. This was confirmed by ED1 staining of traumatic brain injury lesions treated with salmon fibrin.

As a neutral scaffold, salmon fibrin could be used as a delivery mechanism for therapeutic treatments such as neurotrophic factors or neural stem cells. Mammalian fibrinogen is already being explored as a drug delivery vehicle for bioactive proteins

including nerve growth factor (Bhang et al. 2007). Controlled release has been achieved by screening for peptides with the desired binding coefficient for the protein of interest, fusing the peptide to a transglutaminase substrate domain which can then be covalently incorporated into polymerizing fibrin by factor XIIIa (Willerth et al. 2007). Alternatively, mammalian fibrin has been shown to be a permissive 3D substrate for differentiation of embryonic stem cells into neurons (Willerth et al. 2006) and could be used as a carrier to deliver cells to the affected area, enhance retention of cells within the injury and serve as a scaffold for tissue regrowth. Salmon fibrin may provide additional enhancement of neural recovery because neurite outgrowth and branching is enhanced on salmon fibrin compared to human or bovine fibrin (Ju et al. 2007; Uibo et al. 2009).

While salmon fibrin turned out to be a neutral scaffold for treatment of traumatic brain injury, it showed significant potential as a treatment of behavioral sensitivity and inflammation following a painful nerve root compression. Compression injury induces an inflammatory response which leads to local swelling, macrophage recruitment and cytokine release (Rothman et al. 2009). Inflammation leads to additional damage to the nerve root and persistent pain. Suppression of this inflammatory response can be achieved with administration of steroids or nonsteroidal anti-inflammatory agents (NSAIDs). Both these treatments cause undesirable side effects such as suppression of the immune system or liver damage. Fibrin treatment is a promising alternative to steroids and NSAIDs because, as a natural component of the wound healing response, it has a minimal side effect profile.

Treatment of nerve root compression with salmon fibrin significantly reduced

post injury pain and inflammation. The initial hypothesis was that this was due to the mechanical properties of soft fibrin gel. Native blood clots contain large numbers of activated platelets which quickly contract the fibrin filaments, increasing the compliance of the clot by strain stiffening and gel compaction. The injected fibrin treatment does not contain platelets, so while some may be incorporated in the polymerizing gel in situ, the total number of platelets within the gel will be much less resulting in a more mechanically dynamic matrix. Previous in vitro work has shown that soft materials inhibit the activation of astrocytes potentially reducing cytokine production and release as well as inhibiting formation of a glial scar (Georges et al. 2006).

Subsequent tests showed that salmon thrombin alone was sufficient to reduce pain after nerve compression. This challenges the hypothesis that gel compliance is responsible for the positive effects of salmon fibrin treatment. Salmon thrombin can clot mammalian fibrinogen and activate platelets (Michaud et al. 2002), a cell type fish lack, so thrombin's role in clotting is unlikely to be responsible for the significant benefits of treatment with salmon thrombin. The time course of platelet activation by salmon thrombin is slightly different than activation by human thrombin suggesting that salmon thrombin may not bind all the cellular targets that human thrombin does. Mammalian thrombin acts as an agonist of acute and chronic inflammation through activation of PAR receptors on several cell types including monocytes and t-lymphocytes (Strukova 2001). If salmon thrombin does not activate PAR receptors and other proinflammatory targets, then a possible mechanism of action would be that the salmon thrombin competes with the native thrombin and limits its proinflammatory activity.

Salmon fibrin shows significant promise for treatment of nervous system injuries. Additional studies are required to determine the optimal fibrinogen and thrombin concentration for each application. Incorporation of cells or small molecules should be considered in future tests. Further work is required to determine the key differences between salmon clotting factors and their human orthologues as relates to inflammation.

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Figure 6.1 Cortical ablation technique

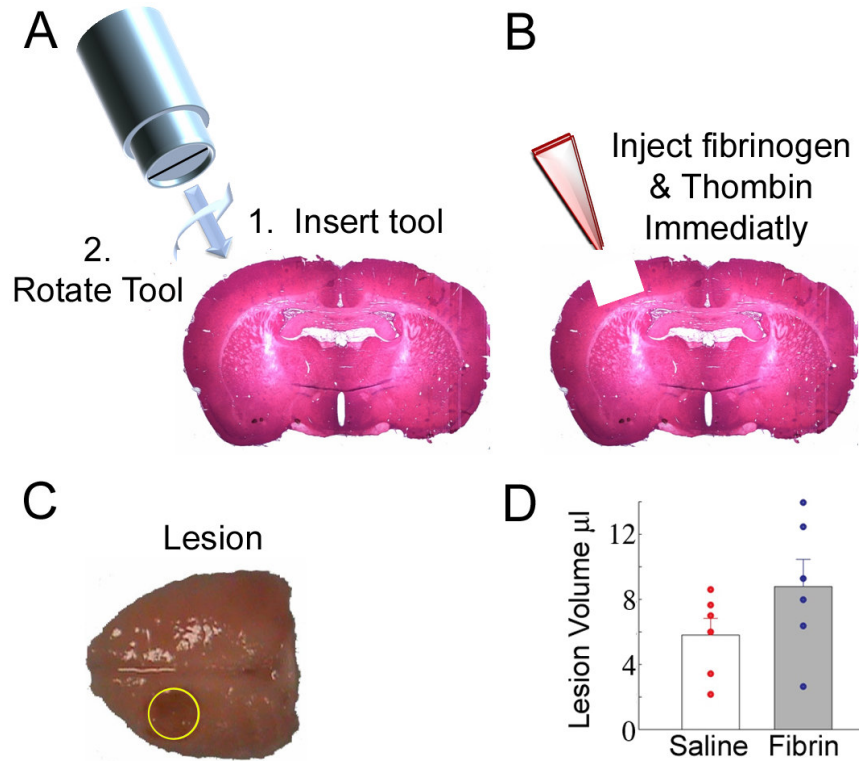
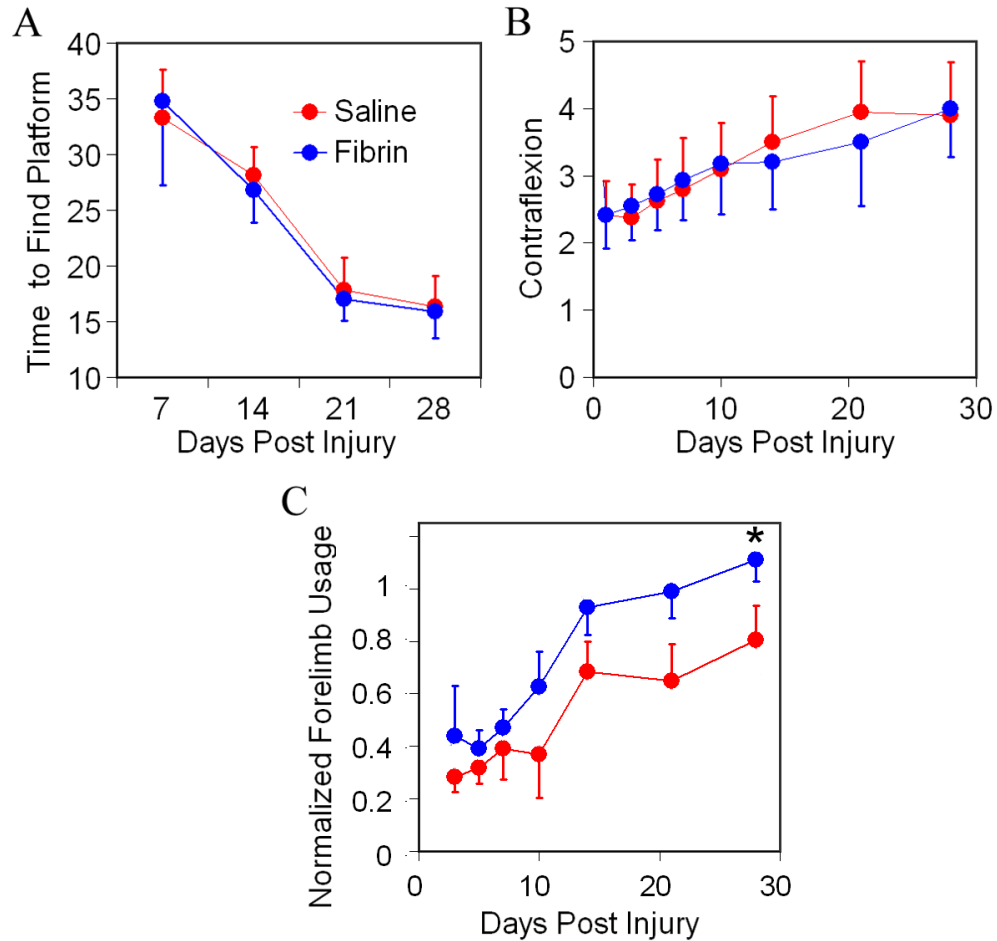


Diagram of method for cortical ablation (A) and treatment (B). Typical brain after harvesting 7 days after surgery (C) yellow circle indicates lesion position. Average lesion volume measured 24 hours after surgery (D). Reported as mean $\pm$ SEM, $n = 6$.

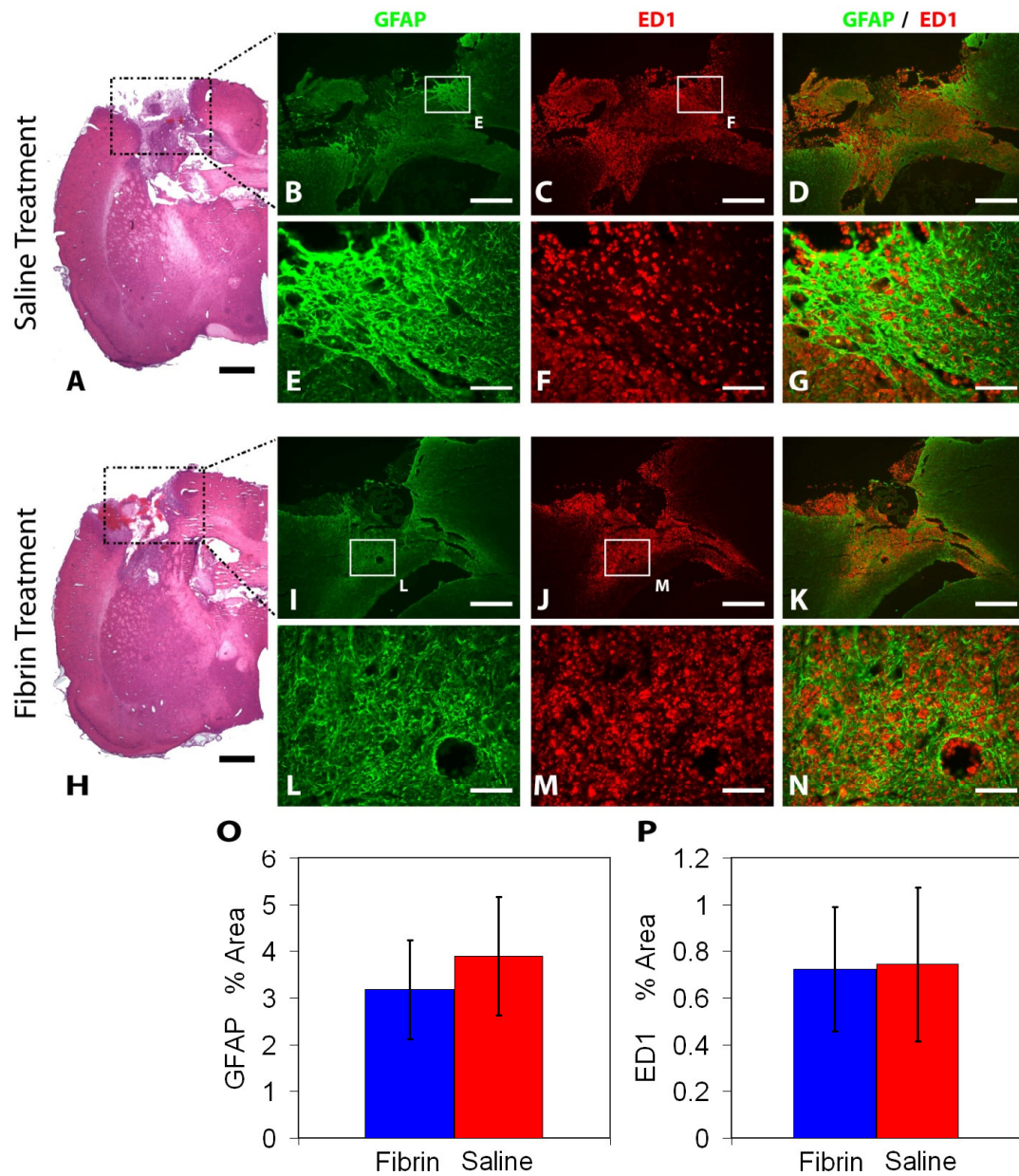
Figure 6.2 Fibrin scaffold does not adversely affect behavior recovery after ablation of motor cortex.



Recovery of motor function after cranial ablation was monitored by seconds to finish Morris water maze (A), contraflexion score (B) and normalized forelimb usage during rearing (C). Reported as mean $\pm$ SEM, water maze $n = 9$, contraflexion $n = 20$ and cylinder rearing $n = 14$, at least 10 rears per test. Significance determined by one-way

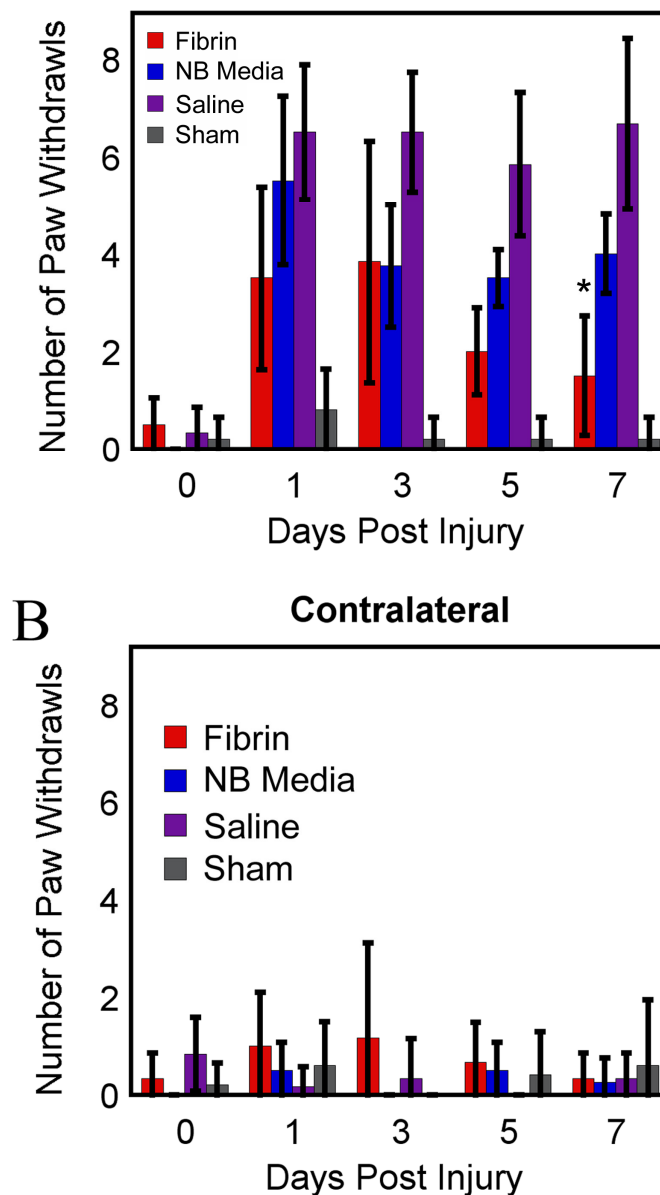
ANOVA with Student-Newman-Keuls post test, * $p < 0.05$

Figure 6.3 Fibrin scaffold does not adversely affect gliosis or inflammation after ablation of motor cortex.



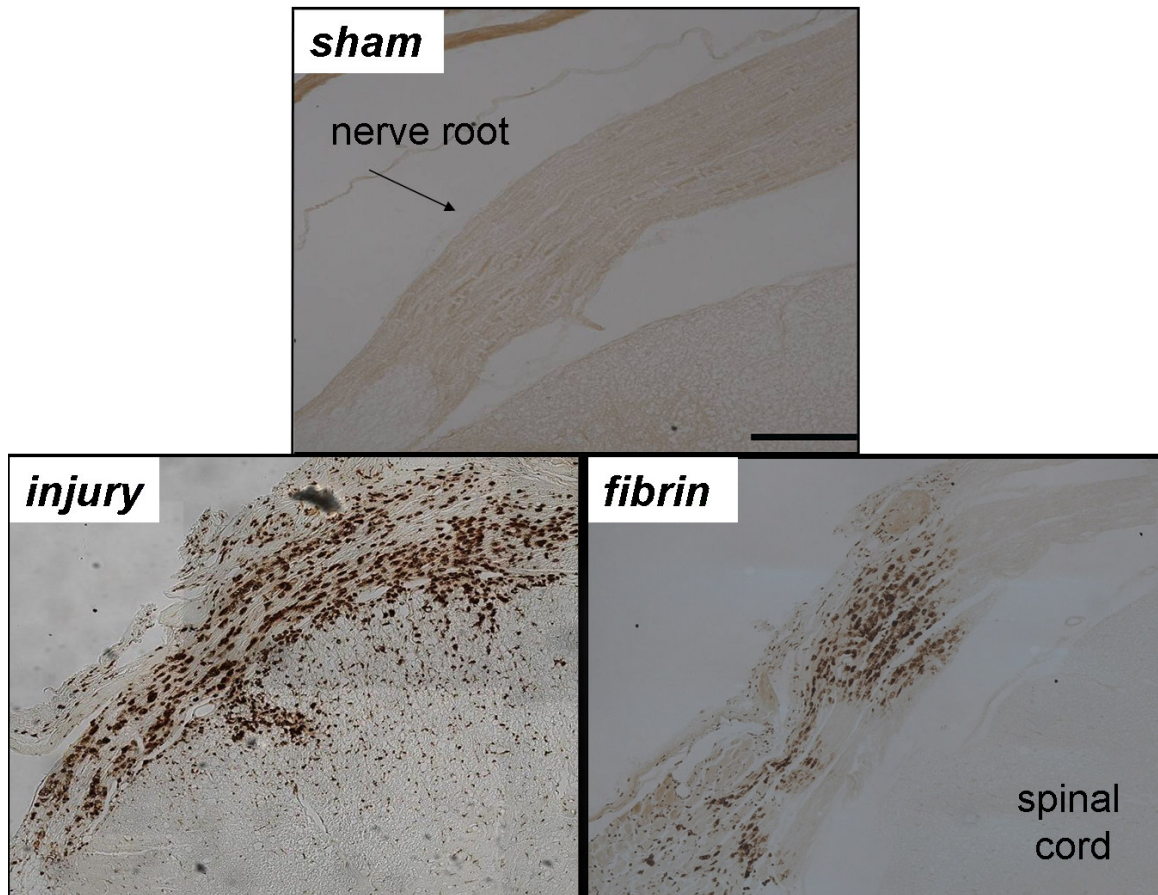
GFAP and ED1 staining of saline and fibrin treated cortical ablations. Scale bars: 1mm in A & H, 500μm in B – D and I - K, and 100μm in E – G and L - N. O-P reported as mean $\pm$ SE, 6 slices per group, three zones per slice. Each slice was from a different animal.

Figure 6.4 Salmon fibrin reduces pain following a nerve root injury



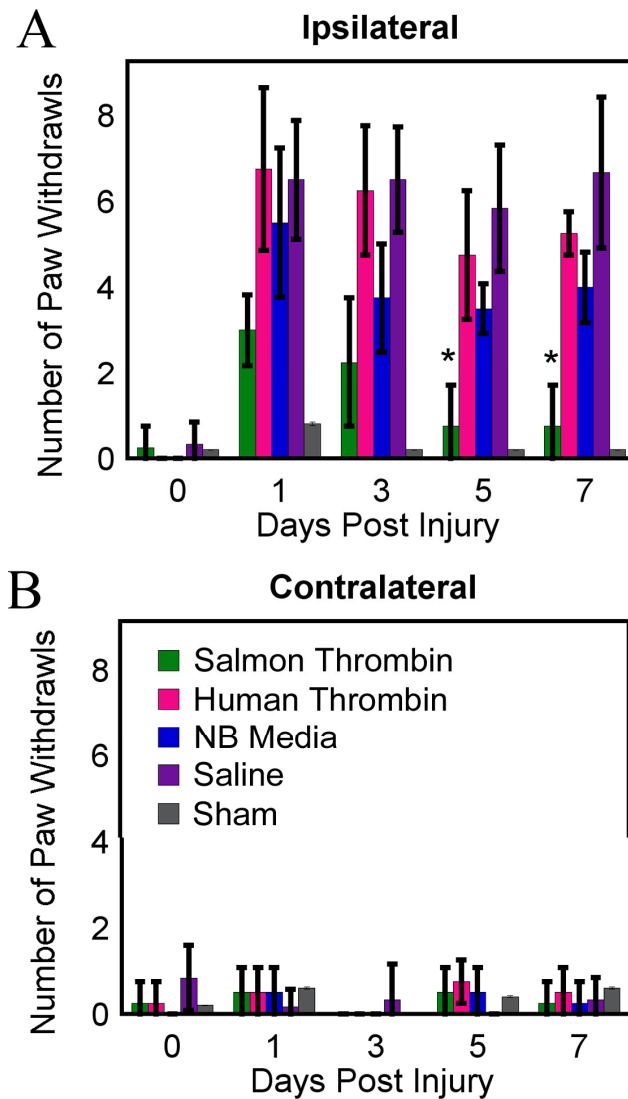
Mechanical allodynia in the ipsilateral (A) and contralateral (B) forepaw during the 7-day postoperative period following sham or compression injury treated with salmon fibrin, neurobasal (NB) media or saline. mean $\pm$ SD, n = 4, * p < 0.001 within group one way ANOVA using Bonferroni correction.

Figure 6.5 Salmon fibrin reduces inflammation following a nerve root injury



Representative micrographs showing ED1 staining of the C7 ipsilateral nerve root at day 7 after surgery for sham, injury and injury with fibrin treatment. Scale bar is 100 μ m.

Figure 6.6 Salmon thrombin is a critical component in reducing pain after nerve root injury



Mechanical allodynia in the ipsilateral (A) and contralateral (B) forepaw during the 7-day postoperative period following sham or compression injury treated with salmon thrombin, human thrombin, neurobasal (NB) media or saline. mean $\pm$ SD, n = 4 * p < 0.001 within group one way ANOVA using Bonferroni correction.

CHAPTER 7

Conclusions and Future Directions

7.1 Summary of Results

The central theme of this dissertation is the characterization of the complex mechanical interactions between strain stiffening biopolymer gels and mechanosensitive tissue forming cells. These interactions were studied using several conventional techniques including fluorescence microscopy, bulk rheology and atomic force microscopy. In addition a novel imaging method was developed to visualize the deformation of fibrin or collagen gels by cell applied forces. These techniques revealed a mechanical mechanism for cell-cell communication through biopolymer networks that may facilitate understanding of tissue morphogenesis and development of advanced biomaterials.

The ability of a soft matrix to maintain a population of quiescent human mesenchymal stem cells (hMSC) was tested in Chapter 2. Spreading and proliferation of hMSCs was characterized as a function of the stiffness of the polyacrylamide substrate on which they were cultured. Both cell spread area and proliferation rate decreased with decreasing stiffness until on gels below 200 Pa the cells adopted a round morphology and entered a non proliferative state. Cells cultured on 200 Pa gels retained their ability to differentiate into adipocytes and when presented with a stiff substrate re-entered the cell cycle and were competent to differentiate into osteoblasts.

On fibrin gels with a modulus below 200 Pa the spread area of fibroblasts and hMSCs was equivalent to their spread area on fibrinogen coated polyacrylamide with a modulus of 16,000 Pa. Chapter 3 was dedicated to elucidating the property of fibrin that induced this spreading response. A network of fibers was required to induce cell spreading which led to the hypothesis that the cells were responding to the nonlinear, strain stiffening property of the fibrin substrates. The degree to which hMSCs deformed the adjacent fibrin network was dependent on the low strain modulus of the gel and the type of integrin through which the cell engaged the matrix. The force applied by the cells was sufficient to locally and globally stiffen the gel in a myosin II dependent manner confirming that the cell applied forces are strong enough to engage the strain stiffening regime of the fibrin network.

Chapter 4 dealt with the implications of this long range mechanical reconstruction of the fibrin gel by contractile cells. The deformation of the fibrin gel was anisotropic and correlated with the shape of the contracting cell with fibers aligning perpendicular to the cell's contracting edge. The deformations decreased as the distance from the contracting cell increased. This created a gradient in deformation and fiber alignment which adjacent cells would sense and elongate towards resulting in multicellular linear and ring like patterns. These results could be recapitulated on collagen gels but not polyacrylamide gels suggesting that this response required a substrate capable of strain stiffening.

The role of filamin A in this mechanical interaction of contractile cells with collagen gels was investigated in Chapter 5. Filamin A expression levels regulated the

ability of cells to contract and align the fibers of collagen gel substrates. There was poor correlation of filamin A expression with either cell circularity or spread area on collagen gels. Filamin A expression had no effect on the ability of melanoma cells to contract fibrin gels.

Salmon fibrin's potential to facilitate repair of damage to neural tissue was investigated in Chapter 6. First, salmon fibrin was investigated as a scaffold for repair in a cortical ablation model of traumatic brain injury. Rats treated with fibrin showed no increase in the inflammatory response and significant improvement in only one of the three behavioral recovery tests indicating that salmon fibrin could be used as a neutral scaffold for delivering therapeutic agents. Salmon fibrin showed greater promise as a treatment of pain and inflammation that occurs after compression of the C7 nerve root. Salmon thrombin, but not human thrombin, was sufficient to achieve the improvement in behavioral sensitivity after nerve root injury.

7.2 Future Work

7.2.1 Contribution of enzymatic degradation of fibrin to cell spreading

Fibrin is a 3D matrix which cells can penetrate and invade during the incubation period prior to the displacement measurements. Cell invasion into a fibrin network requires production of proteases capable of degrading fibrin such as plasminogen and matrix metalloproteases (Hotary et al. 2002; Knox et al. 1987). It is possible that the contractile behavior observed in chapter 3 was a result of the cells encountering a three dimensional environment

HMSCs cultured on top of a 2mg/ml fibrin in the presence of GM6001, a broad

spectrum matrix metalloprotease inhibitor, had narrower protrusions than those cultured without GM6001 but there was no significant difference in the ability of the cells to contract fibrin (Fig. 7.1). This suggests that fibrin degradation is not critical for cell contraction of fibrin gels but confirmation of this requires the inhibition of plasminogen, the other major fibrin protease. To test this, cells will be cultured with trans ϵ -aminocaproic acid (TEA), which inhibits proteins with kringle domains such as plasminogen, or with a combination of TEA and GM6001. It is expected that neither of these treatments will significantly affect the ability of hMSCs to contract fibrin in 2D culture.

This may not hold true when cells are cultured within 3D fibrin networks. A 2 mg/ml fibrin gel has a mesh size of approximately 1 μm making the network too dense for cells to extend protrusions (Baradet et al. 1995; Weisel 1996). It has been shown that proteolytic degradation is required for endothelial cell extension into and migration through 3D fibrin gels (Hiraoka et al. 1998). Without the ability to extend protrusions into the fibrin matrix it is likely that encapsulated cells will be unable to assemble the actin and myosin structures required for cell contraction and gel reconstruction. To confirm this hypothesis cells will be cultured within a fibrin matrix in the presence of GM6001 and/or TEA. Tracking beads in 3D is prohibitively complex so cell contraction will be analyzed by quantifying the degree of gel compaction after 24 hours.

7.2.2 Time lapse imaging of fibrin deformation during cell spreading and migration

In Chapter 3 cells were allowed to spread on fibrin for 18 hours before the contraction of the gel by the cells were measured. This method captures only the active

contraction of the cells and the recoverable elastic strain of the gels. In future studies time lapse microscopy will be employed to capture the dynamic restructuring on the fibrin substrates.

Initial time lapse experiments revealed three interesting features which should be further explored and quantified. First, in Chapter 3 the largest measured bead displacements were on the order of several microns whereas during initial cell spreading gel features were observed moving over 100 μm . This suggests that the amount of unrecoverable strain is much higher than initially anticipated. Second, as cells contracted the fibrin gel, they moved further and further out of focus. Over the 18 hour incubation, some cells were observed to move 20 microns down (data not shown). One explanation for this behavior is a second mechanical phenomenon observed in gels made from semiflexible polymers: negative normal force. When fibrin and other biopolymer gels are strained not only do they exert a resisting force in the plane of applied strain but they also exert a negative force normal to the applied strain (Janmey et al. 2007). Further analysis must be done to determine if the force applied by the cell and the resulting negative normal force by the gel is large enough to explain the downward motion of the cells.

Finally, in Chapter 4 multicellular patterns on fibrin gels were observed and quantified. It was hypothesized that this pattern formation occurred by transmission of mechanical and structural cues through the fibrin network by neighboring cells. An alternative explanation is that the pattern formation occurs as two cells migrate away from each other after cell division. Time lapse microscopy reveals that the patterns form

as a result of cells extending towards each other rather than from cells moving away from each other (Fig. 7.2). Further experiments will reveal the dynamics involved in pattern formation including whether cells migrate faster or slower on portions of the gel already contracted by other cells.

7.2.3 Development of an in vitro model of initial liver fibrosis

Numerous studies have shown that cells adopt their physiologically relevant morphology when cultured on a substrate the same stiffness as their native tissue environment (Engler et al. 2004; Sieminski et al. 2004; Yeung et al. 2005). As the matrix stiffness deviates from the healthy tissue stiffness cells begin to behave abnormally. In vivo, changes in adult tissue stiffness usually occur by one of two mechanisms: injury or disease. In the case of injury a provisional fibrin matrix is deposited and contracted first by platelets (Burstein and Lewi 1952) and later by myofibroblasts (Majno et al. 1971). As a key step in wound healing is degradation and replacement of the provisional matrix with a more integrated scar tissue it is unsurprising that several recent papers have reported increased release of matrix metalloproteases (Karamichos et al. 2008) and ECM proteins (Schlunck et al. 2008) on stiff substrates compared with softer ones.

Stiffening of the tissue is associated with several pathological conditions including atherosclerosis, tumorigenesis and liver disease. Often these conditions can be diagnosed early by monitoring changes in the tissues mechanical properties (Farrar et al. 1982; Nightingale et al. 2000; Shi et al. 1999) Though in most cases it is not yet clear whether tissue stiffening is causal or coincident to the progression of the disease, new results from the Wells lab have begun to deconstruct this process in liver fibrosis. Their work is

suggestive of a two stage process for stiffening, which begins with the existing collagen matrix being stiffened by lysyl oxidase crosslinking (Georges et al. 2007). In culture, a stiff substrate in combination with TGF beta signaling drives the transdifferentiation of portal fibroblasts into myofibroblasts (Li et al. 2007) which have been shown to deposit a fibrotic matrix (Friedman 2000; Wells 2008) and thus may contribute to later stage stiffening. This demonstrates how changes in matrix stiffness can facilitate differentiation from a quiescent phenotype to a remodeling phenotype which then furthers stiffens the matrix creating a positive feedback loop that furthers disease progression.

Better understanding and treatment of this condition will require an *in vitro* system which better mimics the early disease state. Transdifferentiation of hepatic stellate cells into myofibroblast occurs within 10 days of being cultured on a stiff substrate such as tissue culture plastic (Brenzel and Gressner 1996); however, differentiation into myofibroblasts does not occur when cells are cultured on a matrix with the compliance of healthy liver tissue (unpublished data from PC Georges and RG Wells). To mimic to initial disease progression hepatic stellate cells will be cultured on collagen gels with an initial stiffness of 300 Pa. After several days the collagen crosslinker genipin will be added to stiffen the matrix underneath the cells to 600 Pa. Collagen and genipin concentrations will be optimized to achieve these modulus values. Cells will be monitored for loss of vitamin A droplets, increased spread area and expression of α smooth muscle actin, which are all signs of a myofibroblast phenotype. Initial studies indicate that hepatic stellate cells cultured on collagen gels with a shear

modulus of 100 Pa for 20 days maintain a round phenotype (Fig. 7.3 A) whereas cells cultured on 100 Pa gels that were stiffened to 200 Pa using genipin adopted a more stellate phenotype consistent with a more physiologic substrate stiffness (Fig. 7.3 B). If the initial 2D cultures are promising the model will be extended to 3D culture of hepatic stellate cells and eventually whole liver extracts.

7.2.4 Salmon fibrin as a regulator of cytokine production

In Chapter 6 salmon fibrin was used as a successful treatment of allodynia and inflammation after a compression injury of the C7 nerve root. The mechanism by which salmon fibrin mitigates pain is currently unknown. One possibility is that mammalian fibrin exacerbates the inflammatory response while salmon fibrin inhibits this response by outcompeting for the same binding sites. This hypothesis will be tested by measuring the ability of salmon and human fibrin to stimulate cytokine release by astrocytes and microglia..

In healthy central nervous system tissue astrocytes and microglia play supportive roles in maintaining normal functioning. Astrocytes provide supportive chemical factors to neurons and microglia clean up cellular debris. After damage to the central nervous system both cell type release proinflammatory cytokines such as IL-6 and IL-1 β (Hanisch 2002; Lee et al. 2000). Expression of these cytokines correlates with pain and inflammation (Eliav et al. 2009; Komatsu et al. 2009).

To isolate how salmon fibrin might be mitigating inflammation after nerve root compression, target cells will be cultured on fibrin gels made from salmon or human clotting factors. Astrocytes and microglia will be isolated from whole cortical cultures.

Each cell type will be cultured on salmon or human fibrin gels and the media will be collected after 4, 24 and 48 hours. Enzyme linked immunosorbent assays (ELISA) will be used to quantify release of cytokines such as IL-6 and IL-1 β by each cell type. Results from these experiments may suggest a mechanism for salmon fibrin's anti inflammatory activity and could provide a new strategy for treating pain and inflammation.

7.3 Materials and Methods

7.3.1 Cell Culture

Bone marrow-derived human mesenchymal stem cells (Cambrex) were maintained in DMEM (GIBCO) with 1 g/L D-glucose, 0.3 mg/ml L-glutamine and 100 mg/L sodium pyruvate, 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% heat inactivated fetal bovine serum (GIBCO) on tissue culture plastic prior to seeding on gels. Primary hepatic stellate cells were a gift from the lab of Dr. Rebecca Wells. They were plated directly on to collagen gels and cultured with MED199 earls media, 10% fetal bovine serum ,100 units/ml penicillin, 100 μ g/ml streptomycin and 1% fungizone. All cells were maintained at 37°C and 5% CO₂. Unless otherwise specified all reagents are analytical grade and purchased from Sigma.

7.3.2 Fibrin Gel Preparation

The stock solution of fibrinogen was diluted with T7 buffer (50 mM Tris, 150 mM NaCl at a pH of 7.4) to make a working solution of desired concentration. Polymerization was initiated in a 24 well tissue culture plate by adding 5 μ l of thrombin (activity = 100 NIH units/ml) to 250 μ l of the fibrinogen solution. Gels were allowed to polymerize for 30 minutes before cell culture media was added to the wells. Salmon

fibrinogen and thrombin were provided by SeaRun Holdings.

7.3.3 Displacement Microscopy

Untreated fibrinogen was spiked with biotinylated fibrinogen (1:50), the gels were polymerized as usual and then the surface was coated with streptavidin-coated red fluorescent beads (1000 beads/ μm^2 , Invitrogen). Biotin-NHS (Sigma) was conjugated to salmon fibrinogen using the manufacturer's protocol. Cells were seeded on fluorescent bead-coated fibrin gels (100 cells/well) and allowed to adhere and spread for 18 hours. The broad spectrum matrix metalloproteases inhibitor GM 6001 (Calbiochem) was added to the cell culture media prior to seeding the cells at a concentration of 20 μM . For imaging, the cells were kept in serum-free media buffered with 50 mM HEPES and the microscope stage was heated to 37°C. Cells were imaged in phase contrast mode and the beads were imaged in fluorescence before blebbistatin was added. After 30 minutes the beads were imaged again. Image J (NIH) and Adobe Photoshop were used to generate a map of bead displacements for each pair of images. Due to the low magnification needed to capture the scale of the gel deformation the pixel resolution resulted in a error of $\pm 0.25 \mu\text{m}$. To minimize the effect of cells other than the one of interest, the imaged cells were selected because they were at least 1 mm from the nearest cell.

7.3.4 Collagen Gel Preparation

Collagen gels were prepared by adding a 1:10 dilution of 10x PBS to the stock solution of collagen (BD Bioscience) and then bringing the volume to the desired concentration with 1x PBS. Gelation was initiated by adding NaOH until the solution reached pH 7.5. 250 μl of the working solution was added to each well of a 24 well

tissue culture plate. The plate was then placed at 37°C to accelerate gelation. After 1 hr, 1ml PBS was added to each of the wells. Hepatic stellate cells extracted by the Wells lab were plated directly onto collagen gels and cultured in either normal media or media supplemented with 0.5 mM genipin.

7.3.5 Time Lapse Microscopy

Cells were seeded onto a fibrin gel and allowed to attach for 30 minutes before they were placed in a sterile environmental imaging chamber (TOKAI Hit Co. Ltd.) with controlled CO₂ (5%) and temperature (37°C). Images were taken on an Axio-observer Z1 (Carl Zeiss) every 10 minutes for 18 hours on a Coolsnap HQ (Roper Scientific) camera.

7.4 References

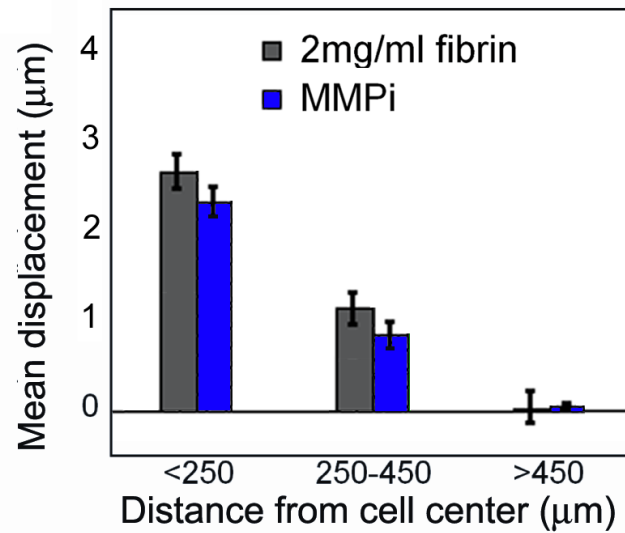
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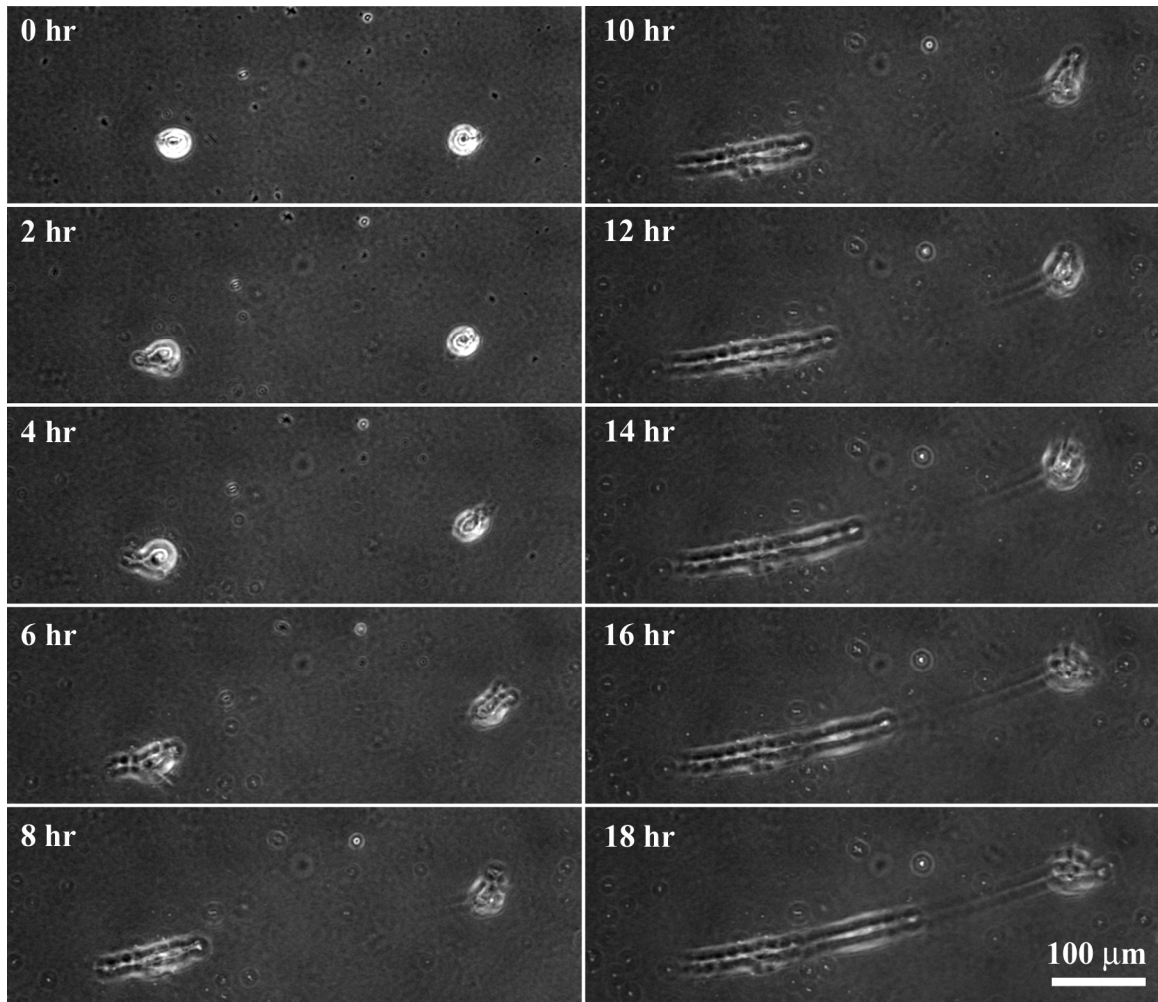
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Figure 7.1 Cellular contraction of fibrin gels does not require MMP activity



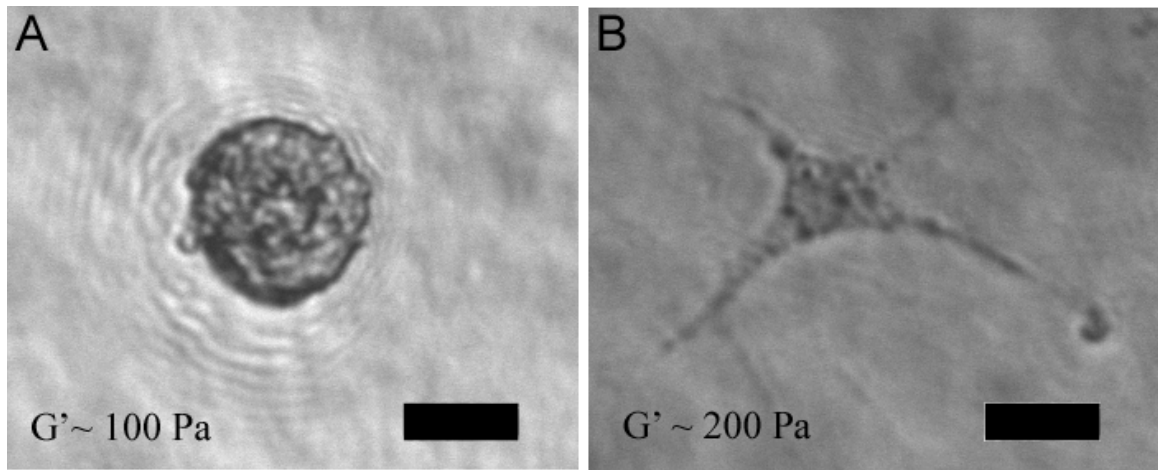
Average bead displacement, as a function of distance from the cell's center, applied by hMSCs on 2 mg/ml fibrin gels with or without addition of GM6001. Reported as mean $\pm$ SE, n = 5 cells per condition, at least 80 beads counted in each zone.

Figure 7.2 Time lapse imaging confirms coordinated cell movement on fibrin



Mesenchymal stem cells were seeded onto a 2 mg/ml fibrin gel and imaged every 10 minutes for 18 hours.

Figure 7.3 Collagen gel stiffness influences morphology of hepatic stellate cells



Hepatic stellate cells were cultured on 2 mg/ml collagen gels for 20 days with (B) or without (A) addition of genipin to the media. Scale bar is 20 μm .