

**BIOPHYSICS OF CANCER PROGRESSION AND
HIGH-THROUGHPUT MECHANICAL CHARACTERIZATION OF
BIOMATERIALS**

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ABSTRACT

**LUKAS DYLAN OSBORNE: BIOPHYSICS OF CANCER
PROGRESSION AND HIGH-THROUGHPUT MECHANICAL
CHARACTERIZATION OF BIOMATERIALS.
(Under the direction of Richard Superfine)**

Cancer metastasis involves a series of events known as the metastatic cascade. In this complex progression, cancer cells detach from the primary tumor, invade the surrounding stromal space, transmigrate the vascular system, and establish secondary tumors at distal sites. Specific mechanical phenotypes are likely adopted to enable cells to successfully navigate the mechanical environments encountered during metastasis.

To examine the role of cell mechanics in cancer progression, I employed force-consistent biophysical and biochemical assays to characterize the mechanistic links between stiffness, stiffness response and cell invasion during the epithelial to mesenchymal transition (EMT). EMT is an essential physiological process, whose abnormal reactivation has been implicated in the detachment of cancer cells from epithelial tissue and their subsequent invasion into stromal tissue. I demonstrate that epithelial-state cells respond to force by evoking a stiffening response, and that after EMT, mesenchymal-state cells have reduced stiffness but also lose the ability to increase their stiffness in response to force. Using loss and gain of function studies, two proteins are established as functional connections between attenuated stiffness and stiffness response and the increased invasion capacity acquired after EMT.

To enable larger scale assays to more fully explore the connection between biomechanics and cancer, I discuss the development of an automated array high throughput (AHT) microscope. The AHT system is shown to implement passive microbead rheology to accurately characterize the mechanical properties of biomaterials. Compared to manually performed mechanical characterizations, the AHT system executes experiments in two orders of magnitude less time. Finally, I use the AHT microscope to study the effect of gain of function oncogenic molecules on cell stiffness. I find evidence that our assay can identify alterations in cell stiffness due to constitutive activation of cancer pathways.

ACKNOWLEDGMENTS

While contemplating graduate school, I asked my undergraduate advisor about his opinion on UNC. I received enough initial enthusiasm to keep the school on my list and apply. Later, when a decision was needed, I asked again and showed him a list of faculty members to focus the discussion. “Superfine! With a name like this, *this* guy must be good!” While I can’t say the remark did much to affect any decision making at the time, when I did end up at UNC and saw **Superfine** on the front of a door in Phillips hall, I did wonder.

And as I sit, years later, Rich is the first person I’d like to thank. I sincerely appreciate the time and patience he has given me, the spoken and unspoken lessons, and the opportunities to learn and grow as a professional. “Learn everything you can from life’s lessons. And then move on.” That particular exchange is probably long forgotten by him, yet I applied it countless times over the years. And I like to think things turned out okay. It turns out, Superfine is pretty good.

I’d like to thank my committee. Mike, for showing equal enthusiasm for things in and outside of work. I’ve appreciated all of our discussions – cell mechanics and running. And more running. I respect his insights. And he has been more of a role model than he realizes. Gerald, for giving me my first graduate research project. And then a few years later, having the interest and willingness to learn a bit about cell

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LIST OF ABBREVIATIONS

3DFM	3 dimensional force microscope
AHT	array high throughput
BMP	bone morphogenetic protein
EC	endothelial cell
ECM	extracellular matrix
EMT	epithelial to mesenchymal transition
FN	fibronectin
PBR	passive bead rheology
TGF-β	transforming growth factor beta

Chapter 1: Introduction

1.1 Bird's Eye View of this Work

Cells live in a dynamic mechanical environment. Throughout the body, cells experience external forces. For example, cells of the skeletal system experience compressive stress from body weight and cells of the vascular system experience shear stress due to blood flow. Cells receive these forces via mechanosensitive receptors at the cell surface. In turn, these receptors relay force information throughout the cell by transduction into biochemical signals, a process called *mechanotransduction*. Before transduction, forces can be relayed directly to distant sites (*e.g.* the nucleus or cell-cell junctions) through transmission across cytoskeletal networks. In addition to exposure of external forces, cells can also exert force on other cells or their environment through actomyosin-contraction on mechanosensitive receptors. The deformation of a cell in response to a force depends on the viscoelastic properties of the cell. The schematic in Fig. 1.1 illustrates these transduction concepts.

The investigation of cell mechanical properties is the study of viscoelastic material properties and cell-generated force. Currently, the interdisciplinary field of *cell mechanics* attempts to answer 4 primary questions:

1. How do mechanical properties influence physiological cell functions (*e.g.* metabolism,

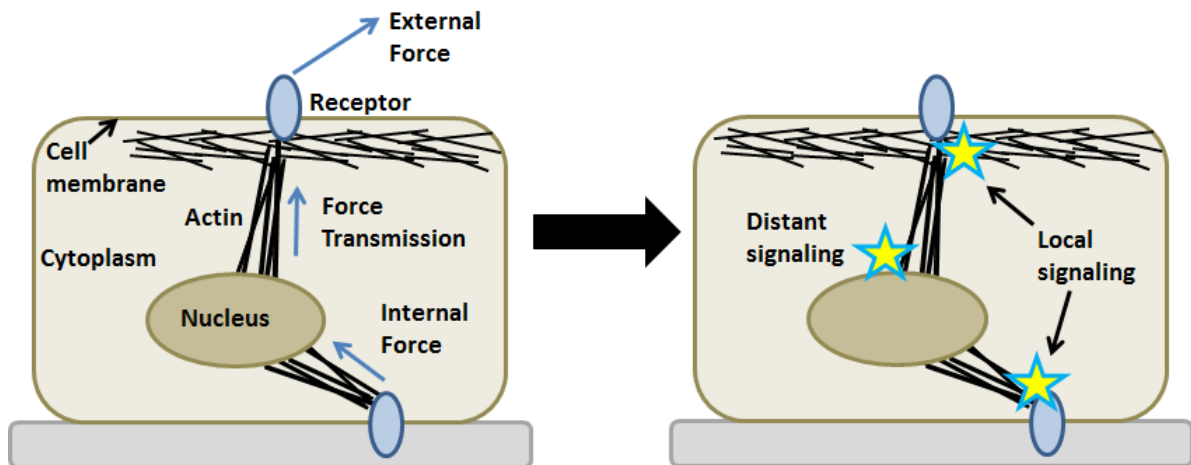


Figure 1.1: **Cellular mechanotransduction of external and cell-generated forces.** The most important physical structures for our considerations are also shown: cell membrane, cytoplasm, nucleus, apical and basal receptors, actin cytoskeleton.

- differentiation, division) and cell behavior (*e.g* morphology, migration)?
2. How do mechanical properties change in response to chemical (*e.g* growth factors) and mechanical signals (*e.g* forces and environment stiffness)?
 3. By what molecular mechanisms are forces transduced? (*i.e.* which molecules are strained, or experience conformation change?)
 4. How are mechanical properties altered during complex disease states such as atherosclerosis or cancer?

The work in this dissertation contributes to the first, second, and fourth questions, mostly in the context of cancer progression. Although our experiments (presumably) induce molecular level strains, our methods probe larger length scales and hence are not designed to contribute the third question above.

In this work, I employ active and passive bead rheology to study the viscoelastic

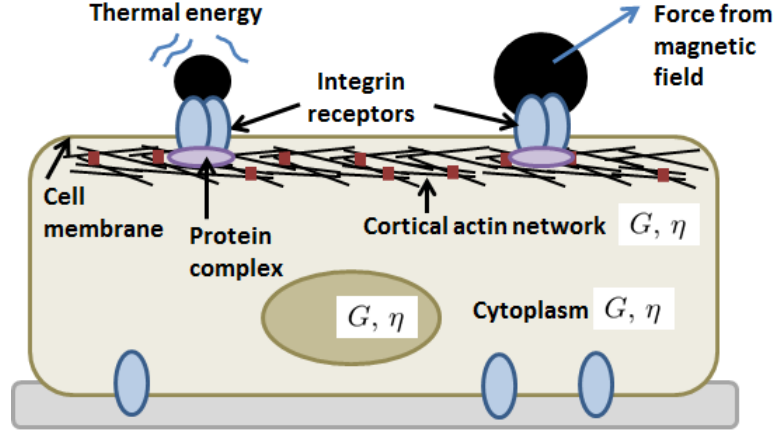


Figure 1.2: Passive bead rheology (PBR) and active bead rheology (magnetic tweezers) probe the cell membrane, adhesions, and cortical actin cytoskeleton through thermal energy and external force, respectively.

properties of cells under different physiological conditions. In the active approach, I apply force to cells using a magnetic field (referred to as magnetic tweezers); in the passive approach, I observe bead motion due to thermal energy (referred to as passive bead rheology, or PBR). Each technique uses micron-sized beads ($1\text{--}4.5\ \mu\text{m}$) that are functionalized with proteins to target specific receptors on the cell surface. The bead functionalization can be changed to form attachments to different cell receptors. Most of the experiments in this work use fibronectin (FN) coated beads that specifically form attachments to integrin receptors; FN is naturally produced by cells and found in the extracellular matrix (ECM) environment. Integrins, perhaps the most well characterized mechanosensor, are anchored directly to the cortical actin cytoskeleton beneath the cell membrane. The physical coupling between integrins and the cytoskeleton occurs through protein complexes (100s of proteins) that serve as physical adhesions to the ECM and as biochemical signaling molecules. The schematic in Fig. 1.2 illustrates

the microbead approaches and the bead to cytoskeleton connection.

For active and passive techniques, bead displacement reflects a composite material consisting of the cell membrane, adhesion-actin linkage, and the cortical actin cytoskeleton (discussed further in Sec. 2.2). Because these bead-based methods average over many receptor connections and are insensitive to molecular level strains (deformations), we parameterize our data with continuum rheological models to determine viscoelastic properties (G, η). We use magnetic tweezers to characterize viscoelastic properties, and to study how these properties change when a cell experiences pulses of force. The low-technology overhead of PBR allows us to characterize viscoelastic properties of cells in automated high throughput experiments.

Note about modulus vs. stiffness: Before moving on, I'd like to make a few comments about language. In general, cell mechanics instruments apply a stress to a cell and measure the resulting strain. Cells are viscoelastic materials, so the resulting deformation has both elastic and viscous contributions. Most studies focus on the elastic response of a cell, parameterizing this quantity with an elastic *modulus*, that depends on the nature of the applied stress (*e.g.* shear stress probes the shear modulus, G). Typically, the community refers to the elastic modulus of a cell as the cell *stiffness*. For our considerations, we also focus on the elastic response of cells, and will generally refer to changes in moduli as changes in cell stiffness (additional discussion in Sec. 2.5.1).

1.2 Motivations and Overview of Chapters

As mentioned above, cellular mechanical properties are increasingly being implicated in the progression of many diseases. However, the molecular mechanisms that regulate or dysregulate disease progression are not well understood. To this end, the primary focus of this work was in elucidating the molecular connection between cellular mechanical properties and cancer cell invasiveness. Previous work by our group and others have shown that, compared to normal or non-invasive cells, highly invasive cancer cells have a reduced stiffness and exert larger forces on their environment. In an attempt to capture both mechanical and biochemical properties during *inducible* cancer progression, I turned my attention to the epithelial to mesenchymal transition (EMT). EMT is a well characterized process in embryogenesis and wound healing, and recently has been implicated as a model for the physical detachment of cancer cells before they begin to metastasize. **Thus, my work was first motivated with the follow questions:**

1. Is there a mechanical phenotype adopted during EMT?
2. If so, what are the biochemical mechanisms responsible for the alternations in cell mechanics?

In preparation for answering these questions, in Chapter 2, I review current efforts in the field of cell mechanics, including discussions of the cytoskeleton, focal adhesions, mechanotransduction, and force-activated biochemical signaling. I then describe

evidence showing cellular mechanical properties have been correlated with cancer invasiveness. I continue in Chapter 2 to discuss continuum models of rheology towards characterization of cell mechanical properties from active and passive microbead rheology experiments.

In Chapter 3 (and related Appendices A, B), I discuss how cell mechanics can be studied using magnetic systems. I briefly review magnetic tweezers as an instrument to apply force to cells. Then, in order to maintain consistency across experiments, I describe improvements to the methodology of magnetic tweezers in the context of cells mechanics (an improved data selection criteria). Finally, I discuss the design and characterization of a novel device aimed to study cellular biochemistry in response to pulsatile force regimens. We examine the effect of pulsatile mechanical force on endothelial cells by applying external force on the mechanosensor platelet endothelial cell adhesion molecule (PECAM)-1.

In Chapter 4, I discuss four applications of passive and active microrheology toward novel projects in cell mechanics. The first application examines how external force on PECAM-1 and extracellular matrix cues regulate the stiffness of aortic endothelial cells *in vitro* and *in vivo*. Second, we examine the effect of force on endothelial intercellular adhesion molecule (ICAM)-1, and the impact that increased endothelial stiffness may have on leukocyte migration and transendothelial migration during the inflammatory response. Third, the stiffness response, and underlying mechanotransduction pathway, of single, isolated nuclei is investigated by applying external force to nesprin-1, a protein of the outer nuclear membrane. Finally, I describe passive and active microrheology

experiments that examine the effect of hypoxia-inducible factor (HIF) on the stiffness and invasiveness of melanoma cancer cells. The work in this chapter references four peer-reviewed publications; my contributions to these studies are described in Chapter 4.

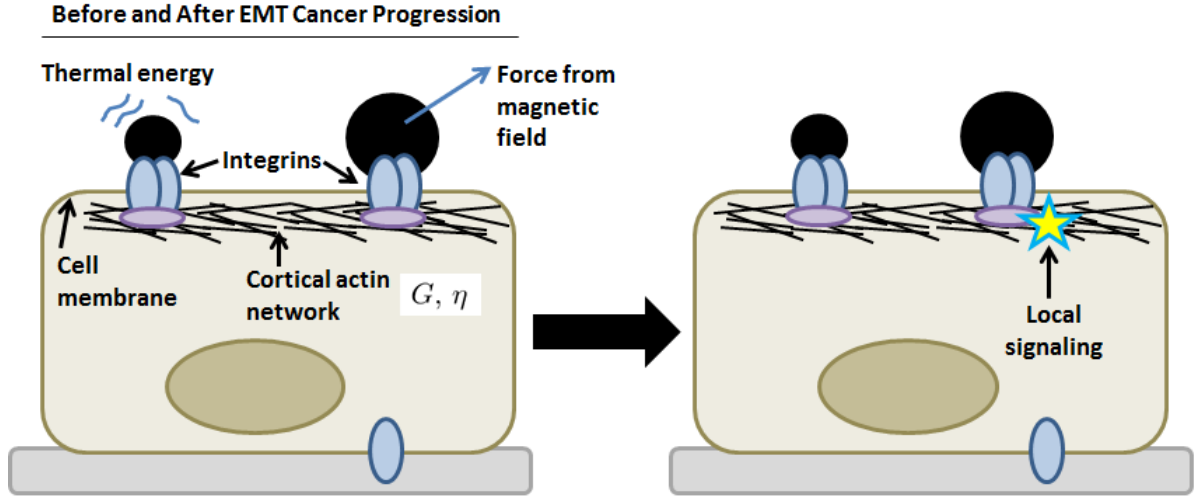


Figure 1.3: (Left) Before and after induced cancer progression, passive and active microbead rheology probe the cell membrane and cortical actin cytoskeleton through thermal energy and external force, respectively. (Right) Force from magnetic tweezers or rotating permanent magnet activates local signaling at the cortical actin cytoskeleton.

In Chapter 5, equipped with the improved selection criteria (discussed in Chapter 3 and implemented in Chapter 4) and the rotating magnet device (Chapter 3), I investigate the two motivating questions stated above. Using force-consistent biophysical and biochemical assays (magnetic tweezers and rotating permanent magnet device, respectively), we examine the mechanical phenotype adopted during EMT and the underlying biochemical mechanisms that regulate this phenotype. Our data suggests that activation of local signaling pathways in the cortical actin cytoskeleton, downstream of integrin receptors, regulate the observed response to force. The schematic in Fig. 1.3

illustrates this transduction concept.

Although significant, results from Chapters 3, 4, and 5 were obtained from a series of low throughput, manually performed experiments testing a small number of environmental conditions or genetic manipulations. In general, the technical overhead and time constraints of manual experiments limit the extent of assayed conditions and the ability to test the ubiquity of findings by examining additional cell lines and model systems. A complete understanding of the connection between cellular mechanical properties and physiology/disease will require advances in instrumentation that enable parallelized, high throughput assays capable of probing complex signaling pathways, studying biology in physiologically relevant conditions, and capturing heterogeneity within cell populations. **To address this motivating instrumentation need**, in Chapter 6, I describe the development and testing of a high throughput microscope designed to characterize the viscoelastic properties of biomaterials and cells.

In Chapter 7, I describe efforts towards a high throughput assay designed to characterize the viscoelastic properties of cells as oncogenic signaling pathways are activated. During assay development, we realized that our power to distinguish between biology conditions would require an advanced analysis procedure capable of identifying subpopulations without our data. In this chapter, I discuss the implementation of a procedure that fits thermally-driven bead trajectories to motion models to enable selection of trajectories relevant to probing cellular viscoelastic properties. Finally, I discuss the preliminary results of several high throughput assays that examine the effect of gain-of-function oncogenes on the stiffness of cells.

1.3 High-Level Observations

In an effort to prepare the reader for ideas and conclusions presented in this work, the following observations are compiled. See links for further discussion.

1. **Cells can distinguish between constant and pulsatile mechanical forces.**

We developed a rotating permanent magnet device that applies pulsatile force to cells before biochemical analysis. Within 2 min of force on apical PECAM-1 (mechanosensor in endothelial cells), we find enhanced ERK1/2 and RhoA activation, and an increase in the number and size of basal focal adhesions. (Ch. 3)

2. **Mechanical response to force depends on the bead-cell attachment.**

Repeated pulses of force on PDL-coated beads (targeting non-specific attachment based on charge) does not increase stiffness. In contrast, repeated pulses of force on FN-coated beads induces a stiffening response within 1 min (FN targets attachment to actin via mechanosensitive proteins). (Fig. 5.6)

3. **Cells integrate ECM biochemistry and mechanical cues to modulate mechanical properties.**

Endothelial cells plated on FN-coated substrates exhibit a stiffening response to force, whereas on CL-coated substrates no response is observed. Evidence of this integration occurring *in vivo* is also observed. (Sec. 4.2)

4. **Pulsatile force on integrins and PECAM-1 active RhoA within 1 min.**

The integrin-mediated response is observed with epithelial cells (Fig. 5.11: 2-fold increase; 50 pN of force at 5 sec on, 10 sec off) and the PECAM-1-mediated response

is observed with endothelial cells (Fig. 3.8: 3-fold increase; 100 pN of force at 2 sec on, 10 sec off). RhoA is a protein that regulates the actin cytoskeleton.

5. Integrin, PECAM-1, ICAM mechanotransduction involve RhoA protein.

Force application on integrins (Fig. 5.5), PECAM-1 (Sec. 3.7), and ICAM-1 (Sec. 4.4) activate RhoA. The activation of RhoA leads to increased cell stiffness.

6. Active and passive microbead approaches reveal similar trends in cell

stiffness. Stiffness measurements determined with magnetic tweezers and PBR have established the same relationship between conditions for cells before and after EMT (Fig. 5.8), and between melanoma cells of varying invasiveness (Fig. 4.8).

7. An altered mechanical phenotype appears to be hardwired into cancer

progression. We have shown that stiffness and stiffness response are decreased after EMT (a model for cancer progression). These mechanical changes are regulated by proteasomal degradation of RhoA activators LARG and GEF-H1. Loss of LARG and GEF-H1 increase cell migration and invasion. (Ch. 5)

8. Our developed high throughput microscope accurately measures viscosity

standards. All 12 channels statistically distinguished between 4 Newtonian fluid standards: water ($\eta = 0.84$ mPa s), 2.04 M sucrose ($\eta = 20.0$ mPa s), 2.5 M sucrose ($\eta = 83.1$ mPa s), and corn syrup ($\eta = 2840$ mPa s). At the plate level, the system measures water, 2.04 M and 2.5 M sucrose with less than 5.5% error. (Sec. 6.4)

9. 95% time is saved for experiments using our high throughput microscope.

Manual execution of 96 well experiments (5 video per well), such as the standards experiment above, would take over 90 hours. On our high throughput microscope this experiment would take 2.2 hours, all of which is unattended. A cell rheology experiment of this size would take 3.2 hours (Ch. 6, Sec. 6.5).

10. **A modeling procedure has been implemented to identify subpopulations in high throughput data.** Towards assay optimization, our goal was to separate subpopulations within MSD data with an approach that penalizes model complexity and enables model competition based on relative probabilities. We implemented a Bayesian analysis procedure and have shown: 1) successful separation of simulated heterogeneous datasets (Fig. 7.10), 2) like-model comparisons between cell conditions within a high throughput cell mechanics assay (Fig. 7.14).
11. **Our high throughput PBR assay has identified 3 oncogenes that decrease cell stiffness.** Our results demonstrate that 3 of 4 tested oncogenes (H-Ras, myr-AKT, and Bcl-2) promote decreased stiffness towards that of a cancer mechanical phenotype define by KRAS transformed cells (Fig. 7.14).

Summary of magnetic tweezer experiments in this work: Fig. 1.4. To compare our forces to physiological stresses in the literature, we estimate the stress: $\sigma = F/(\pi r_c^2)$ where r_c is the contact radius. For 2.8 μm and 4.5 μm beads we assume $r_c = 0.7 \mu\text{m}$ and $r_c = 1 \mu\text{m}$, respectively. While we assume a uniform contact area in estimating the stress, a contact surface that was less uniform – perhaps localized to specific receptors – would generate much larger stresses than what has been approximated here.

Collaborator Experiments discussed in Chapter 4					
	EMT Cancer Study	PECAM-1 Endothelium Study	ICAM-1 Endothelium Study	Melanoma Cancer Study	Isolated Nuclei Study
Cell type	NMuMG and PANC-1	Bovine aortic ECs	HMVECs and HUVECs	A375 human epithelial	Nuclei from Hela, HUVECs
Bead ligand	Fibronectin	PECAM-1	ICAM-1	Fibronectin	Nesprin
Bead size	4.5 μm	2.8 μm	4.5 μm	4.5 μm	2.8 μm
Adhesion structure	Integrins and focal adhesions	PECAM complex	ICAM-1 complex	Integrins and focal adhesions	Nuclear Nesprin of the LINC complex
Physiological mimic	Cell “tugging” on substrate $\sigma = 160 \text{ Pa}$ (Plotnikov 2010)	Shear stress due to blood flow $\sigma = 5 \text{ Pa}$ (Davies 1995)	Leukocyte traction forces $F = 2\text{-}11 \text{ nN}$ (Guilford 1995)	Cell “tugging” on substrate $\sigma = 160 \text{ Pa}$ (Plotnikov 2010)	$\sigma = 160 \text{ Pa}$ (?) (Plotnikov 2010)
Force function	50 pN at 5s ON, 10s OFF	100 pN at 2s ON, 10s OFF	160 pN at 3s ON, 4s OFF	50-100 pN at 5s ON	15-35 pN at 3s ON, 4s OFF
Estimated stress	16 Pa	65 Pa	51 Pa	16-32 Pa	10-23 Pa
Absolute stiffness	NMuMG 1.52 Pa, 0.54 Pa PANC-1 1.71 Pa, 1.22 Pa	BAEC (relative displacement only)	HUVEC + shRNA control: 50 Pa HUVEC + LARG shRNA: 38 Pa	A375 EGFP: 5 Pa HIF1dPA: 1.5 Pa HIF2dPA: 2 Pa	Hela nuclei + shRNA control: 0.61 Pa
Stiffness Response	Decreased after EMT	Increased with PKA inhibition	Decreased with LARG shRNA	Not measured	Decreased with Lamin A/C or Emerin knockdown
Role of RhoA	RhoA levels and activation decreased	RhoA phosphorylation decreases stiffening	RhoA activity increases leukocyte crawling/TEM	Not measured	RhoA does not regulate the stiffening of nuclei
Cell mechanics story	TGFB and BMP induced EMT reduces LARG, GEF-H1 to increase cell migration and invasion and decrease cell stiffness and stiffness response	On collagen (descending), force on PECAM-1 leads to PKA mediated phosphorylation of RhoA. When PKA is inhibited (ascending) cells stiffen	Force on ICAM-1 increases LARG mediated RhoA activation which stiffens the endothelium surface and may facilitate leukocyte crawling and TEM	Stable expression of transcription factors HIF1 α and HIF2 α is enough to individually induce increased cell invasion and decrease cell stiffness. May initiate EMT	Force on nesprin-1 induces nuclear stiffening that required a functional lamina and the nuclear membrane protein emerin, not nuclear actin or DNA

Figure 1.4: **Summary of magnetic tweezer experiments.** EMT cancer study: Ch. 5 (Osborne et al., 2014); PECAM-1 endothelial study, Ch. 4 (Collins et al., 2014); ICAM-1 endothelial study, Ch. 4 (Lessey-Morillon et al., 2014); Melanoma cancer study, Ch. 4 (Hanna et al., 2013); Isolated nuclei study, Ch. 4 (Guilluy et al., 2014). Physiological mimic references: (Plotnikov et al., 2012)(Davies, 1995)(Guilford et al., 1995)

Chapter 2: Background: Cell Mechanics, Cancer, Methods of Measure

2.1 Overview

This chapter will begin with several physical pictures. These pictures will combine findings from different studies in an attempt to visual and explain the measurements we take of microbeads attached to the surface of cells. The hope is that having these schematics upfront will aid in a reader's understanding of the methods and results discussed in this work related to the mechanical properties of cells. Next, we will review cell and cancer cell mechanics, and compare methods with respect to their potential for high throughput implementation. Then, we will review the rheology of elastic, viscous, and viscoelastic materials leading to magnetic tweezer and PBR measurements. We will describe the heterogeneity observed in PBR measurements and discuss the need for an advanced analysis procedure. Finally, we will compare cell modulus measurements derived from magnetic tweezers to other methods of cell mechanics.

2.2 Physical Pictures in this Work

For the majority of experiments in this work (Ch. 5,7), beads are coated with fibronectin (FN) to specifically bind integrin receptors on the cell surface. Inside the cell, integrins are anchored to the cortical actin cytoskeleton through focal adhesion (FA) protein structures (conceptualized in Fig. 2.1).

In the following three subsections, we will describe 1) how microbeads are coupled to the cortical actin cytoskeleton (Sec. 2.2.1), 2) mechanotransduction and a hypothesized physical picture for a cell-attached bead under external force (Sec. 2.2.2), and 3) a hypothesized physical picture for a bead diffusing due thermal energy (Sec. 2.2.3). These physical pictures are developed using a combination of community findings, results from this work, and speculation. The schematics presented in Fig. 2.3, and 2.4 will be used to illustrate magnetic tweezer and PBR experiments in Ch. 5,7.

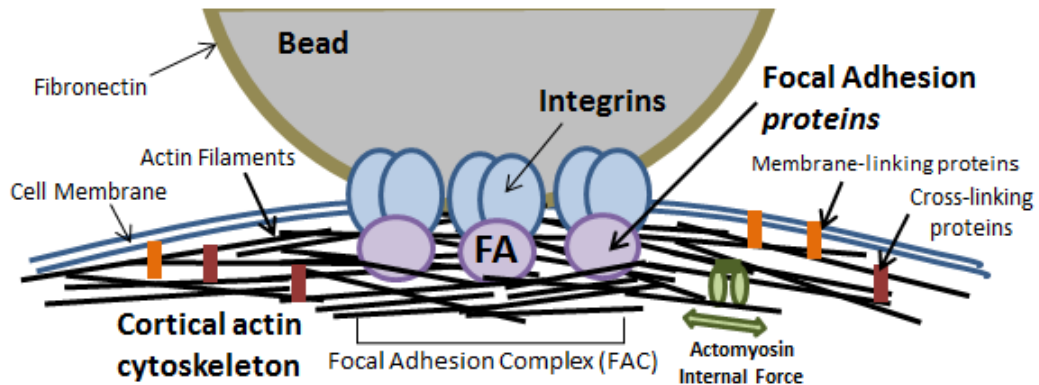


Figure 2.1: Microbead in context with the cortical actin cytoskeleton.

2.2.1 Physical Picture a Bead and the Actin Cortex

Within 10 to 30 min of a FN-coated bead binding to a cell, integrins, focal adhesion (FA) proteins (talin, vinculin, paxillin), and cytoskeleton components (actin and cross-linker α -actinin) are recruited to the site of attachment (Plopper and Ingber, 1993). Directly under the cell membrane is a 2 μm thick polymer network, called the actin cortex or the cortical actin cytoskeleton (Charras et al., 2006). This network is dominated by actin filaments, actin-binding proteins (cross-linkers, membrane-linkers), and force generating myosin-II motors (conceptualized in Fig. 2.1). Although regulation of the cortex and its binding to the membrane are not well known, the cortical actin network is classically understood to determine a cell's ability to resist mechanical deformation.

After 30 min of bead-cell attachment, focal adhesion complexes (FACs) are found to encircle the bead and an increase in local cortical actin is observed (Plopper and Ingber, 1993). However, when beads are coated with acetylated-low density lipoprotein (Ac-LDL), which binds to scavenger transmembrane receptors (responsible for removal of macromolecules from environment), an increase in local cortical actin is observed but there is no recruitment of FA proteins (Plopper and Ingber, 1993). These findings suggest the response of a cell to repeated force (*e.g.* increased stiffness) may depend on the bead coating and the receptor to which the force is applied. We explore this hypothesis in Sec. 2.2.2. Numerous studies have examined proteins that are recruited (bound) to integrin-FACs under external force application using permanent magnets (Glogauer, 1998)(Guilluy et al., 2011)(Osborne et al., 2014). We investigate such FAC

recruitment in the context of cancer in Ch. 5.

2.2.2 Physical Picture of a Bead on a Cell under Force

The leading hypothesis in cellular mechanotransduction is that sensitivity to mechanical stimulation derives from molecular rearrangements. Under force, mechanosensitive proteins are rearranged either by binding-induced conformational changes (allosteric regulation) or strain-induced unfolding (conceptualized in Fig. 2.3 A,B,C). For example, in the context of integrin mechanotransduction, force-dependent unfolding of the FA protein talin (~ 5 pN) results in exposed binding sites for the protein vinculin (Yao et al., 2014). In turn, vinculin regulates the binding of actin filaments to strengthen the FA (Rio et al., 2009)(Ciobanasu et al., 2014). Other studies have shown that vinculin is required for FA stabilization under force, and that a single vinculin protein supports ~ 2.5 pN of tensional force in stable FAs (Grashoff et al., 2010).

Additionally, activators of the protein RhoA – a key regulator of the actin cortex – are known to associate with FA proteins and actin, and are specifically recruited to adhesions during force application (Guilluy et al., 2011)(Osborne et al., 2014). Downstream biochemical signaling of RhoA leads to actin stabilization, polymerization, and actomyosin contractility (Lessey et al., 2012). The end result of mechanical stimulation is a stronger coupling between FAs and actin and a more mature cortical actin network (conceptualized in Fig. 2.3 D). Focal adhesions, mechanotransduction, and the RhoA signaling pathway will be discussed in more detail in Sec. 2.3.

Stress and strain within magnetic tweezers methodology: Magnetic tweezers are used to apply external force to cells, in the form of a shear stress. Resulting bead

displacement reflects the strain of the cell; typical bead displacements are on the order of 0.5-1 μm . This displacement excludes the possibility that the observed strain is due to a single protein ($\sim 1\text{-}10\text{ s nm}$). Additionally, experiments (discussed below) with different bead ligands suggest that the bead “tether” is not contributing to the strain. We suspect the observed strain resulting from a bead under force is likely distributed as deformation of the cell membrane, integrins and FAs, and the cortical actin cytoskeleton. However, the relative strains across each of these components is not known.

Stiffness within magnetic tweezers methodology: Bead displacement from the first pulse of force enables us to determine a stiffness of the cell. The majority of stiffness values determined by magnetic tweezers in this work are $G = 0.5\text{-}2\text{ Pa}$ (FN-coated beads; Ch. 5). This range of stiffnesses is in reasonable agreement with other studies using magnetic tweezers and FN-coated beads to apply force to integrins of many different cell types (Fig. 2.2). We suspect our measurement of stiffness is reflective of a composite material consisting of the cell membrane, integrins and FAs, and the maturation of the actin cortex. To study the effect of the later contribution, Matthews *et.al.* disrupted the actin cortex with 3 modulators which affect actin through distinct mechanisms (ROCK inhibitor, RhoA inhibitor, myosin ATPase inhibitor). These magnetic tweezer experiments revealed a 2-fold increase in bead displacement under force on integrins compared to untreated cells (indicative of a reduced stiffness) (Matthews *et al.*, 2006).

In this work (Ch. ??), to determine the effect of bead ligand on measured stiffness of

Cell Type	Conditions tested	Stiffness, G	Stiffness Response*	Reference
Epithelial cells	Pre- and Post-EMT progression	0.5-2 Pa	20-30%	Osborne et al., 2014
MEF fibroblasts	Vinculin-mediated actin bundling	2-3 Pa	50%	Shen et al., 2011
REF fibroblasts	Knockdown of GEFs and RhoA	0.5-8 Pa	175%	Guilluy et al., 2011
Epithelial cells	varying invasion potential	0.5-5 Pa	not tested	Swaminathan et al., 2011
Epithelial cells	Hypoxia-inducible factors (HIFs)	1.5-5 Pa	not tested	Hanna et al., 2013
Endothelial cells	Disruption of actin cytoskeleton	not computed	30% (relative displacement)	Matthews et al., 2006
MEF fibroblasts	Vinculin-mediated actin bundling	not computed	30-35% (relative displacement)	Tolbert et al., 2014
MEF fibroblasts	Vinculin to actin binding	not computed	23% (relative displacement)	Thompson et al., 2014

Figure 2.2: **Comparison of FN-integrin determined cell stiffness and stiffness response measurements using magnetic tweezers.** All studies used FN-coated beads to attach to integrin receptors (Matthews *et al.* used RGD-coated beads, main sequence in FN). The applied forces were between 40-130 pN.

*The provided stiffness response range is the maximum for control conditions where a response was observed; some of the tested conditions reduced or eliminated the observed stiffness response in the control conditions. The pulse number at which a stiffening response is observed varies between studies. Many studies measured the “relative displacement” of the bead as opposed to the stiffness response; in these cases, “30%” refers to a *reduction* of bead displacement which we approximate here to be an increase in stiffness.

References: (Osborne et al., 2014)(Shen et al., 2011)(Guilluy et al., 2011)(Swaminathan et al., 2011)(Hanna et al., 2013)(Matthews et al., 2006)(Tolbert et al., 2014)(Thompson et al., 2014)

epithelial cells, we assessed cell stiffness using poly-D-lysine (PDL)-coated beads which target non-specific attachment to the cell based on charge. In these experiments, PDL-coated beads yield the same initial stiffness as FN-coated beads (Fig. 5.6 A). However, under repeated pulses of force on PDL beads, no stiffening response is observed; in contrast, repeated pulses of force on FN beads induces an increase in cell stiffness (Fig. 5.6 B). These results suggest PDL beads may probe the actin cortex through membrane-cortex linkages, whereas FN beads probe the cortex through FAs. Additionally, these findings are consistent with Ac-LDL-coated beads (targeting non-integrin cell attachment) evoking enhanced cortical actin localization but not recruiting mechanosensitive FA proteins (Plopper and Ingber, 1993). In this model, the presence of the actin cortex would establish a cell stiffness, but the lack of force-sensitive proteins would prevent a mechanoresponse.

Stiffness Response within magnetic tweezers methodology: We determine the stiffening response of a cell by applying repeated force pulses and normalizing later stiffness values to the stiffness observed during the first pulse. For most of the experiments in this work, epithelial cells exhibit an increase in stiffness of approximately 20-30% (FN-coated beads; Ch. 5). Figure 2.2 compares how the stiffness response to force on integrins varies with cell type.

As a cell transmits pulses of force, strain across talin reveals cryptic binding sites for the head domain of vinculin (Vh) (Yao et al., 2014). Actin binding to the tail domain of vinculin (Vt) is thought to induce conformational changes in vinculin that allow two vinculin tails to bind, resulting in bundling of actin filaments and an enhanced

cytoskeleton (Tolbert et al., 2013). Studies testing the bundling ability of vinculin have shown that a 50% stiffness response observed in cells with wildtype vinculin is completely lost when Vt-Vt binding is prevented (Shen et al., 2011). These results suggest that vinculin-mediated actin bundling is necessary for a stiffness response to force on integrins.

As a cell transduces repeated pulses of force, the RhoA activators LARG and GEF-H1 are specifically recruited to the FA complex (Guilluy et al., 2011)(Osborne et al., 2014). Although experiments have not yet revealed what structures RhoA activators or their upstream regulators bind to, it is possible that these signaling proteins bind to vinculin or actin. Experiments in this work using epithelial cells show that within 1 min of force application, LARG and GEF-H1 are recruited 2-3.5-fold more than without force, and within 2 min, this increase can be as high as 6.5-fold (Fig. 5.11). We suspect LARG and GEF-H1 activate RhoA in regions local to the site of force application. Although our observations of active RhoA in response to force reveal a 2-fold increase over the no force control (limited to non-localized measurements; Fig. 5.11), we suspect active RhoA is even more abundant at the the site of force application. Within this model, RhoA regulates the maturation and organization of the actin cortex to resist deformation from external forces.

Magnetic tweezer data: We observe transduction of repeated pulses of external force as an increase in cell stiffness over time (Fig. 2.3 E).

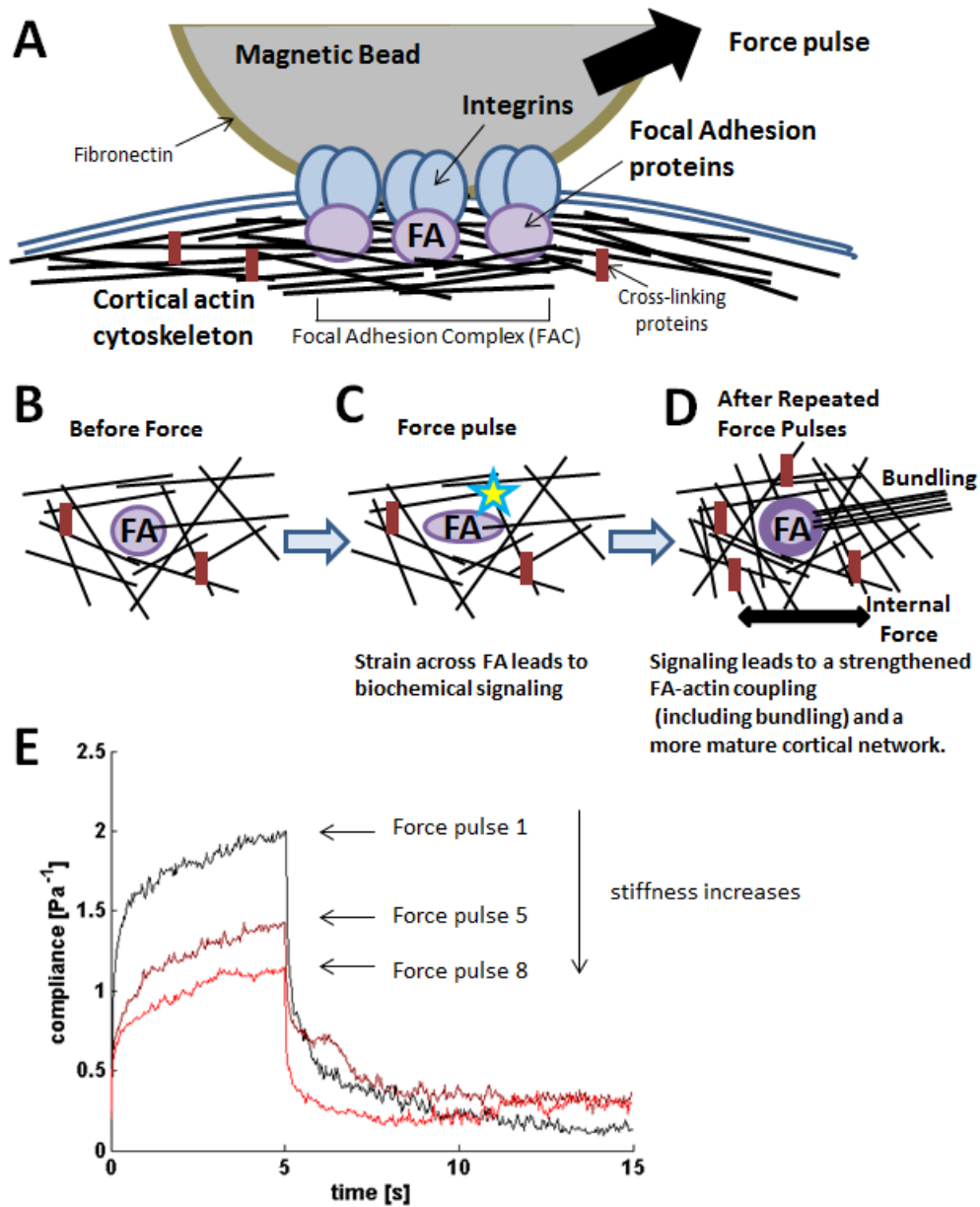


Figure 2.3: **Hypothesized physical picture of an external bead under external force.** (A) Side-view cartoon showing a bead attached to integrin receptors on the cell surface. Inside the cell, integrins are anchored to the cortical actin cytoskeleton through focal adhesion (FA) protein structures. For simplicity, many proteins are not shown. (B) Top-view cartoon showing a single FA within the cortical actin network; attachment to actin is assumed. (C) Force results in strain across FA proteins, leading to biochemical signaling (star). (D) Signaling leads to actin cortex maturation. (E) Representative compliance data from an active microrheology experiment. Stiffness increases (compliance decreases) with repeated pulses of force.

2.2.3 Physical Picture of a Diffusing Bead on a Cell

Stress and strain within PBR methodology: In passive bead rheology (PBR) of cells, thermal energy imparts shear stress to cells (conceptualized in Fig. 2.4 A). Similar to magnetic tweezers, we suspect passive bead displacement reflects the strain of the cell. Although this strain is likely distributed as deformation of the cell membrane, integrins and FAs, and the cortical actin cytoskeleton, the distribution across these components may be different than it is for magnetic tweezers.

Stiffness within PBR methodology: Generally, the measured stiffness for cells using PBR in this work ($G = 0.1\text{-}1\text{ Pa}$) is on the same order as the stiffness determined by magnetic tweezers ($G = 0.5\text{-}2\text{ Pa}$), and in many cases, we identify the same trends between cell conditions using both approaches. This observation is consistent with FN-coated beads inducing the recruitment of integrins, FA proteins, and cortical actin filaments upon attachment (no applied force) (Plopper and Ingber, 1993).

Similar to magnetic tweezers, we suspect our measurement of stiffness is reflective of a composite material consisting of the cell membrane, integrins and FAs, and the maturation of the actin cortex. However, despite also being in the pascal range, PBR derived stiffnesses tend to be moderately lower than stiffnesses observed with magnetic tweezers. We can imagine several possible reasons. First, there may be variations in the bead-cell attachment, in terms of receptor-specificity and binding maturation. Because applied forces are not utilized to “filter” passive data, PBR of cells selects the entire range of bead-cell attachments. In contrast, magnetic tweezers tends to

select only strong bead-cell attachments. Bead-cell binding variability would result in PBR measuring a lower effective stiffness compared to magnetic tweezers. Second, cells are known to exhibit linear and nonlinear mechanical responses (*i.e.* the stress-strain relationship is linear or nonlinear) (Kollmannsberger and Fabry, 2011). Typical RMS displacements of beads in PBR are 30-50 nm, whereas typical bead displacements with magnetic tweezers are on the order of 0.5-1 μm . Therefore it is possible that PBR and magnetic tweezers probe the stiffness of the cell in different rheological regimes and that the observed differences in stiffness are because cells strain-stiffening. As such, PBR may be more sensitive to interactions between FA proteins and actin filaments. In contrast, because of the larger strains involved with magnetic tweezers, beads under a driven force may experience the cross-linking of actin filaments within the cortex and therefore experience a greater stiffness. Third, PBR may not measure biochemically enhanced cell stiffness. Although we currently do not have evidence to suggest the force delivered within a single magnetic tweezer pulse induces biochemical signaling to increase the measured stiffness, if it did and this threshold force was not achieved by thermal energy (or was not achieved as often), one could imagine that PBR would measure a lower effective stiffness.

In Sec. 5.4, we compare the effective stiffness determined by PBR and magnetic tweezers for two cell conditions.

Data for PBR of cells: To gain physical insight of our measurements, we compare MSD data for external PBR of cells from (Hoffman et al., 2006) to our data (Ch. 7). Fig. 2.4 D makes this comparison.

For short timescales, the trajectory of an external bead appears diffusive, potentially reflective of diffusion of integrins within the membrane or FA proteins diffusing within a cage of the actin cortex mesh. The later possibility is conceptualized in Fig. 2.4 B and illustrated by the red region of Fig. 2.4 D. It is important to note that “FA” in Fig. 2.4 A,B,C is meant to describe individual proteins or collections of proteins that compose the total focal adhesion structure. The area of a the total focal adhesion complex can be several microns in size.

For timescales between 0.1 ms and 1 sec, the trajectory of an external bead appears subdiffusive (Fig. 2.4 C and green region of D). Although the origin of this viscoelastic response is not known, a possible contribution is increased interaction of FA proteins with actin filaments, potentially because of binding to or bending of actin filaments. Our PBR data is taken within this timescale range (30 ms to 1 sec) and the observed RMS displacement of beads at the 1 sec timescale is 30-50 nm (Ch. 7). Because the average mesh size of the actin cytoskeleton is 20-50 nm (Wirtz, 2009)(Hale et al., 2009), our data may reflect the interaction of FA proteins with actin filaments (FA proteins are coupled through their respective connection with the bead as in Fig. 2.4 C). Additionally our data is consistent with (scaled) external PBR measurements from Hoffmann *et. al* at the 1 sec timescale. To make this comparison, we performed a volumetric-scaling ($4.5 \mu\text{m} / 2 \mu\text{m}$) of the MSD from (Hoffman et al., 2006) to account for the difference in bead size (Appendix Fig. C.1).

For the PBR assays described in Ch. 7, all comparisons across cell conditions are made at the 1 sec timescale.

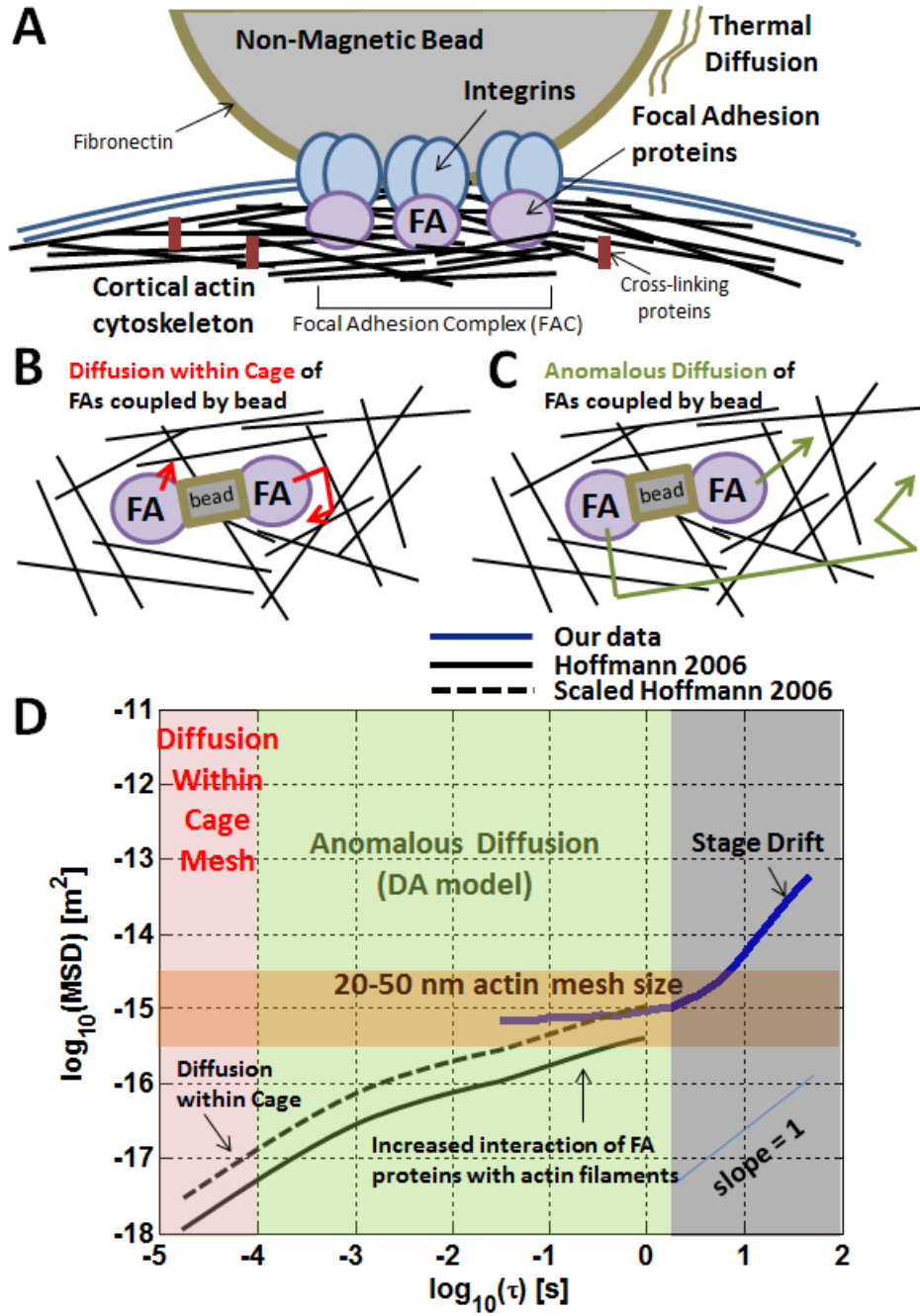


Figure 2.4: Hypothesized physical picture of a passively diffusing bead attached to a cell. (A) Side-view cartoon showing a bead attached to the cortical actin cytoskeleton through integrins and focal adhesion (FA) proteins. For simplicity, many proteins are not shown. (B,C) Top-view cartoon showing FA proteins coupled through their respective connection to the bead (B) diffusing within the cortical actin mesh (red arrow) and (C) anomalously due to interaction with actin (green). Some degree of attachment to actin is assumed. (D) MSD vs τ data for external PBR. Data from $4.5 \mu\text{m}$ beads (Hoffman et al., 2006) are scaled to $2 \mu\text{m}$ beads and compared to our data (blue line) for $2 \mu\text{m}$ beads. See Appendix Fig. C.1 for details of this scaling.

2.3 Review of Cell Mechanics

Cells are a highly complex composite materials consisting of many molecules, proteins, and compartments. Mechanically, cellular structure is provided by the cytoskeleton, which in humans, consists of and is regulated by thousands of different proteins (Venter et al., 2001). The cytoskeleton of eukaryotic (nucleated) cells is composed of three distinct polymer systems: microtubules, intermediate filaments, and actin filaments. These networks give rise to cellular mechanical properties, allow a cell to withstand the impact of external forces, and enable the cell to exert forces on its environment. Despite using a significant portion of its metabolic energy to assemble these networks, cells are constantly disassembling and reassembling these structures. This constant remodeling of the cytoskeleton enables a cell to contraction, move, divide, and change shape. During these processes, the mechanical properties of a cell are altered dynamically. In this section, we will review cell mechanics from the following perspectives:

1. forces in biology: cell experience and exert forces in a variety of contexts
2. physical structure: the actin cytoskeleton and focal adhesions
3. mechanotransduction: mechanotransmission, mechanosensing, mechanoresponse
4. implications of mechanotransduction to disease states

At the end of this section, we will briefly discuss methodologies used to characterize cell mechanics, and their potential for high throughput implementation.

Cells are constantly experiencing and exerting mechanical forces. Endothelial cells lining blood vessels and epithelial cells lining tissue cavities experience shear stresses from the movement of fluid over the cell surface (Jalali, 2001)(Chachisvilis et al., 2006) and tensional force from neighboring cells within the endo- or epithelium (Tzima et al., 2005)(Collins et al., 2012). Osteoblast and chondrocyte cells, which make up the bone and cartilage component of the skeletal system, are constantly under compression forces during body-level activities such as walking or running. Cells also actively exert mechanical forces on their surroundings. Smooth muscle cells generate contractile forces within the vascular system to pump blood and exert force on tendons and bones during general muscle contraction. Fibroblast cells also generate contractile forces on the ECM in order to remodel connective tissue and migrate during wound healing.

The actin cytoskeleton and focal adhesions. While all three cytoskeletal polymer networks contribute to the mechanical properties of cells, most recent attention has been given to the actin cytoskeleton because of its role in cell migration and response to external forces (Nobes and Hall, 1999)(Matthews et al., 2006). The actin cytoskeleton is a highly dynamic structure that can provide extensional and contractile force. Polymerization of actin near the boundary of a cell pushes against the cell membrane through an extensional force. The actin cytoskeleton also provides a scaffolding structure for myosin-II motors which slide actin filaments past one another, and when anchored, this action generates contractile forces within the cell.

Cells are mechanically stabilized to neighboring cells or the ECM through sites of adhesion. These sites, called focal adhesions, are $0.25 - 3 \mu\text{m}^2$ structures that consist of transmembrane integrin receptors and cytoplasmic scaffolding proteins that form a 100 nm thick complex to actin filaments (Bershadsky et al., 2006). Although the identity of many scaffolding proteins already exists, the nanoscale organization of proteins in focal adhesions was only recently revealed using three-dimensional super-resolution fluorescence microscopy (Kanchanawong et al., 2010). It was found that integrins and the actin cytoskeleton are vertically separated by about 40 nm and that three specific protein layers exist within the adhesions: (i) an integrin signaling layer composed of focal adhesion kinase (FAK) and paxillin, (ii) a force transduction layer composed of talin and vinculin, and (iii) an actin regulatory layer containing zyxin, vasodilator-stimulated phosphoprotein (VASP) and α -actinin (Fig. 2.5; (Kanchanawong et al., 2010)). This molecular picture provided the first conceptualization of protein architecture that could be used to understand the functions of focal adhesions. Focal adhesions are multifunctional structures that, in addition to connecting the ECM to the actin cytoskeleton, also serve as conduits for force transmission and biochemical signaling. Although many of the adhesion molecules described above have been implicated in signaling that is initiated in response to force on focal adhesions, a complete picture is not yet known. In the next few paragraphs, we will discuss what transpires during mechanotransduction.

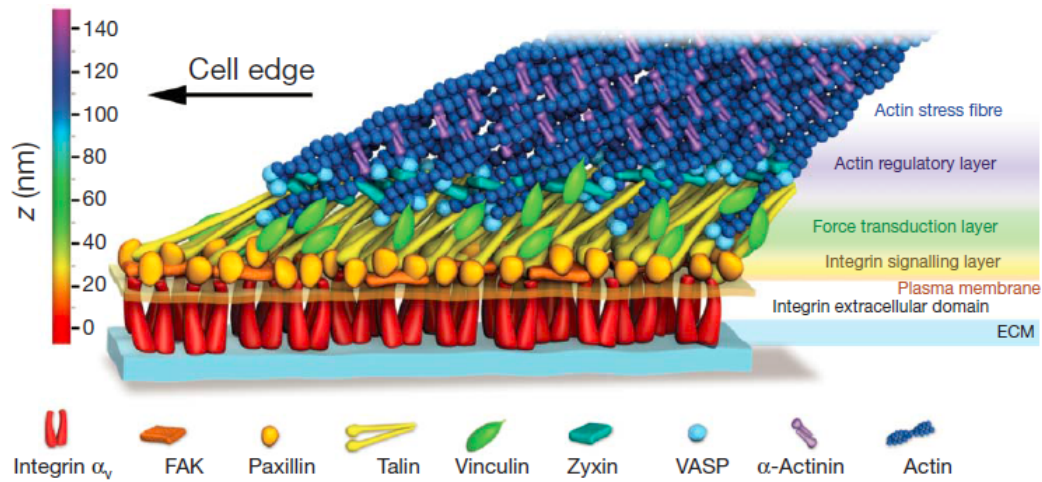


Figure 2.5: **The nanoscale structure of focal adhesions.** Focal adhesions are protein complexes that have structural and signaling roles within a cell. Figure republished from (Kanchanawong et al., 2010) with permission from Nature.

Mechanotransduction is the conversion of physical stimuli into biochemical signals within the cell. As a canonical example, hair cells in the inner ear have mechanosensitive organelles called stereocilia that convert changes in fluid pressure due to sound waves into biochemical signals that ultimately relay information to the auditory nerve (Vollrath et al., 2007). Mechanotransduction can be divided into three subprocesses: 1) mechanotransmission, 2) mechanosensing, and 3) mechanoresponse.

1. **Mechanotransmission:** Before a mechanical stimuli can be sensed or responded to, transmission of the mechanical signal must occur. Transmission of mechanical cues can occur through adhesion proteins or the cytoskeleton (Hoffman and Crocker, 2009). Mechanical propagation allows for cellular responses to force to be on the order of several hundred milliseconds, much faster than what diffusion of soluble factors would allow (Wang et al., 2009).

2. **Mechanosensing:** Transmitted forces ultimately cause strains on mechanosensitive

proteins to induce various biological consequences. For example, tensional forces in the cellular plasma membrane during osmotic swelling cause conformational changes in ion channels that allow passage of ions and enable the cell to survive under dynamic osmotic conditions (Arnadottir and Chalfie, 2010). Another example involves mechanosensing of proteins in focal adhesions. Talin is an adhesion protein in the force transduction layer of focal adhesions (Fig. 2.5). Vinculin serves as the connection between talin and actin filaments. Initially, however, many of the binding sites of vinculin on talin are inaccessible. When force is transmitted to talin, induced strains expose these cryptic binding sites and allow for vinculin recruitment and actin connection (Rio et al., 2009)(Thompson et al., 2014).

3. **Mechanoreponse:** Transmitted and sensed forces induce cellular responses through complex biochemical signaling networks. Mechanoreponses include activation of gene-expression pathways that occur over timescales of hours to days (Olson and Nordheim, 2010), as well as activation of signaling pathways that occur over timescales of minutes. Previous work in our group showed that force application on integrins cause recruitment of two guanine-nucleotide exchange factors (GEFs), LARG and GEF-H1, to the adhesion complex (Guilluy et al., 2011). LARG and GEF-H1 are activators of the small GTPase RhoA, a key regulator of the actin cytoskeleton, and experiments showed that RhoA activation was responsible for increases in cell stiffness in response to force (Fig. 2.6; (Guilluy et al., 2011)).

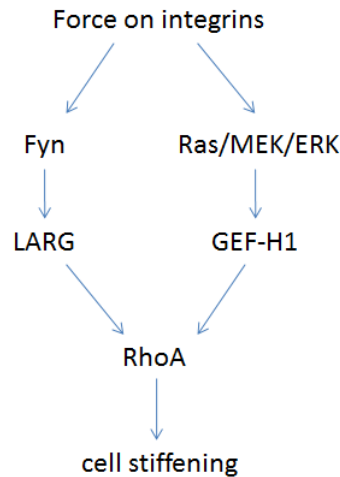


Figure 2.6: **The RhoA mechanoresponse pathway.** A combination of biophysical and biochemical techniques were used by (Guilluy et al., 2011) to reveal two regulatory pathways of stiffness response when cells are subjected to externally applied force on integrins.

Mechanotransduction is implicated in disease states. Because of the ubiquity of mechanical forces in biological systems, it may not be a surprise that mechanotransduction is involved in many disease states (Jaalouk and Lammerding, 2009), including osteoporosis, muscular dystrophy, hypertension, myopathies, and atherosclerosis. For example, in the vascular system, atherosclerosis (chronic inflammation of vessel walls) occurs more frequently in regions where turbulent blood flow cause significantly varied stresses (magnitude and temporally) across cell surfaces compared to laminar flow conditions. We will investigate this phenomena in Chapter 4.

Complications or alternations in normal mechanotransduction processes can also contribute to cancer progression as tension increases in the ECM around growing tumors. Cancer cells adopt several characteristics, including a significantly modified morphology, an increased invasion potential, and varied mechanical properties. A review

of cancer cell mechanics will be given in the next section (Sec. 2.4).

Extending cell mechanics methodologies into high throughput. The next wave of scientific advancement in the cell mechanics community will be in extending our current understanding of how mechanobiology is involved in human diseases, such as cystic fibrosis (Kater et al., 2007)(Rubin, 2007a), blood coagulopathies (Pezold et al., 2012)(Nystrup et al., 2011), and cancer (Swaminathan et al., 2011)(Plodinec et al., 2012). These diseases often involve complex biochemical networks, each comprising of many different proteins or enzymes that must be identified and whose regulatory interactions defined. Current methods to study these systems are often very slow, primarily due to manual data collection and analysis. Future progress will require advances in instrumentation that enable parallelized, high throughput assays capable of probing complex signaling pathways, studying biology in physiologically relevant conditions, and capturing mechanical heterogeneity at the single cell and population level. Recent efforts to increase the throughput of mechanical measurements (Reed et al., 2011)(Wu et al., 2012a)(Gossett et al., 2010) have addressed the need of capturing specimen and mechanical heterogeneity, but still acquire data across experimental conditions serially.

All cell mechanics techniques, however, may not have potential for scaling to high-throughput studies. Fig. 2.7 compares many of the current methods in terms of the physical property the technique measurements, as well as the throughput capacity of the method. Included are the following methods: microplate rheometer (Thoumine and Ott, 1997)(Desprat et al., 2005); optical tweezers (Yamada et al., 2000)(Guck

et al., 2005); magnetic twisting cytometry (Fabry et al., 2001)(Deng et al., 2006); magnetic tweezers ; external/internal passive microbead rheology (PBR) (Hoffman et al., 2006)(Hale et al., 2009)(Wu et al., 2012a); atomic force microscopy (Alcaraz et al., 2003)(Hiratsuka et al., 2009); traction force microscopy (Paszek et al., 2005)(Liu et al., 2013); and hydrodynamic stretching (Dudani et al., 2013)(Gossett et al., 2012).

From Fig. 2.7, we see that most cell mechanics techniques measure the cell membrane, adhesions, and the cortical actin cytoskeleton due to external interaction with the cell. Although Gossett *et.al* have extended measurement throughput to 2000 cells/sec, these experiments are performed on non-adherent cells, and therefore limit the direct biological relevance of their determined mechanical properties to specific situations (*e.g.* circulation within the vascular system in cancer metastasis).

In Ch. 6, we will discuss the development of a high throughput microscope that addresses the above needs. The result is an automated system that can collect external passive bead video data for cells at a rate of 785 cells/hr (assuming a modest 3 cells/video and using the itemized breakdown of assay time shown in Fig. 6.9). Compared to manual collection of the same data (60 cells/hr), our microscope system increases PBR data collection by over an order of magnitude. And, the experiment is completely unattended. For PBR mechanical *measurements* (including analysis), our system characterizes 454 cells/hr. The system characterizes adherent cells, and therefore our measurements are directly relevant to studies that simulate cell migration, cancer cell invasion, intravasation, and extravasation.

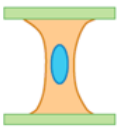
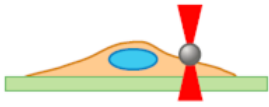
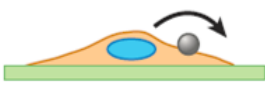
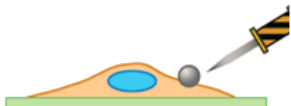
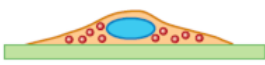
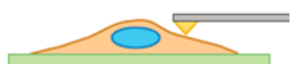
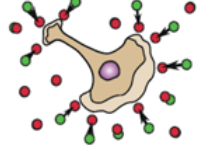
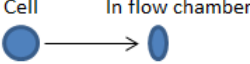
Schematic	Method	Property of cell measured	Measurement throughput
	Microplate rheometer	-- adherent whole cell rheology, no resolution at the subcellular level (Thoumine 1997)	-- low measurement throughput (12 cells per hour) (Desprat 2005)
	Optical Tweezers	-- cell membrane and cortical actin cytoskeleton (Yamada 2000a)	-- average measurement throughput (60 cells per hour) (Guck 2005)
	Magnetic Twisting Cytometry	-- cell membrane, adhesions, and cortical actin cytoskeleton (Fabry 2001)	-- average measurement throughput (30 cells per hr) (Di Carlo 2012)
	Magnetic Tweezers	-- cell membrane, adhesions, and cortical actin cytoskeleton (Fabry 2001)	-- average measurement throughput (30-60 cells per hr) (personal experience)
	External and Internal Passive Microrheology	-- external: cell membrane, adhesions, and actin cortex (Hoffman 2006) -- internal: soft actin network of cytoplasm (Hale 2009)	-- external*: above average throughput (60 cells per hr) (personal experience) *high throughput potential (750 cells per hr; See Ch. 6) -- internal: average measurement throughput (20 cells / hr) (Wu 2012)
	Atomic Force Microscopy	-- cell membrane and cortical actin cytoskeleton (Nawaz 2012)	-- low measurement throughput (5-6 cells per hour) (Di Carlo 2012)
	Traction Force Microscopy	-- actomyosin cell contractile forces (Paszek 2005)	-- low to average measurement throughput (1-20 cells per hour) (Liu 2013)
	Hydrodynamic Stretching	-- solution-suspended whole cell rheology, no resolution at the subcellular level (Dudani 2013)	-- has been extended to very high throughput (2000 cells/s) (Gossett 2012)

Figure 2.7: **Comparison of cell mechanics methodologies in terms of property measured and throughput capacity.** Numbers for measurement throughput reflect only data collection, not analysis. *throughput potential for external PBR is based on 3 cell/FOV and uses the itemized breakdown of assay time shown in Fig. 6.9.

Images republished from (Kollmannsberger and Fabry, 2011) with permission from Annual Reviews and from (Agus et al., 2013) with permission from Nature Publishing Group.

2.4 Review of Cancer Cell Mechanics

The most destructive and deadly aspect of cancer is its ability to spread, or metastasize. The role of physical forces and mechanical properties in this process have begun to change how researchers study cancer progression (Wirtz et al., 2011). In this section, we will review the current state of cancer cell mechanics through the following perspectives:

1. The metastatic cascade: the journey of a cell during metastasis
2. Invasive cells display a reduced mechanical stiffness
3. Invasive cells exert larger tractional forces to their substrate
4. Reduced cell stiffness of cancer cells can be observed at the tissue level
5. The epithelial to mesenchymal transition: a model for cancer progression studies

At the end of this section, we will discuss how these elements of cancer cell mechanics influence and motivate the experiments in this work.

The metastatic cascade: the journey of a cell during metastasis. Although the exact biomolecular mechanisms are not known, cancer progression is hypothesized to involve a series of events known as the metastatic cascade (Fig. 2.8; (Wirtz et al., 2011)). In this process, genetic mutations and uncontrolled cell division lead to the growth of a primary tumor. Cells within the tumor release and begin to respond abnormally to growth factors which leads to sustainability of the tumor (*e.g.* oxygen

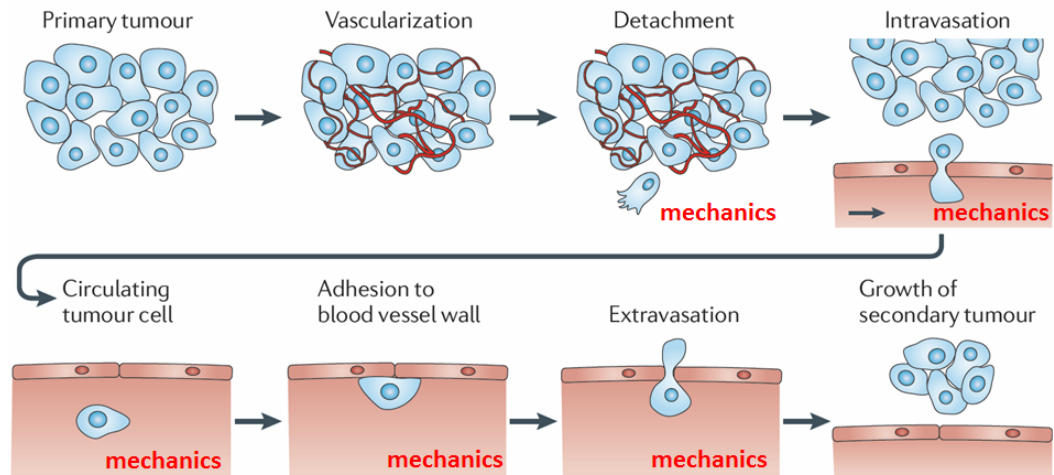


Figure 2.8: **The metastatic cascade.** Cells detach from the primary tumor, invade the surrounding stromal space, transmigrate the vascular system, and establish secondary tumors at distal sites. Physical forces and mechanical properties are likely involved in many of these steps. Figure republished from (Wirtz et al., 2011) with permission from Nature.

and nutrients) through vascularization. Cells then detach from the primary tumor and invade the surrounding stromal space. One proposed model for this process is the epithelial to mesenchymal transition (EMT), which is a biomolecular program that leads to significant changes in biochemical signaling, receptor expression, cell morphology, and invasion potential (EMT will be discussed more later in this section, and at length in Chapter 5). Cancer cells then transmigrate the vascular system by penetrating endothelial and epithelial vessels. Finally a secondary tumor is established at a distal site in the body. Increasing evidence suggests that forces and mechanical properties internal to and external of the cancer cells play a significant role in each step of this process.

Invasive cells display a reduced mechanical stiffness. During metastasis, cancer cells encounter a complex and dynamic, chemical and mechanical microenvironment.

Cell mechanical stiffness has been postulated to play a role in the passage of cancer cells through this constantly evolving environment. The hypothesis is that a certain degree of deformability is required for cells to manipulate through the dense ECM as they travel to the vascular system, and potentially as they invade into and out of the vascular system. Evidence of the invasive cells displaying a reduced mechanical stiffness has been shown in multiple cancer types and using several different techniques (Guck et al., 2005)(Suresh et al., 2005)(Plodinec et al., 2012)(Gossett et al., 2012)(Agus et al., 2013). Work from our lab has shown that mechanical stiffness of cell populations, characterized by active external microbead rheology, inversely correlates with the invasion capacity of ovarian cancer cell lines and primary ovarian cancer cells (Swaminathan et al., 2011). While other groups had previously shown cancer cells to be mechanically less stiff than normal cells, this study was the first evidence that increasingly aggressive or invasive cells are increasingly more compliant than other cancer cells. More recently, another group confirmed the inverse relationship between cell stiffness and invasion using many of the same ovarian cancer cell lines using atomic force microscopy (AFM) (Xu et al., 2012a). In Ch. 4 we will discuss the effect of hypoxia-inducible factors (HIFs) on cell stiffness (Hanna et al., 2013). And in Ch. 5 and 7, we will discuss collaborative projects aimed to elucidate the effect of EMT and oncogenic signaling pathways on cell stiffness.

Invasive cells exert larger tractional forces to their substrate. As cancer cells invade the dense, heterogeneous stromal tissue between the primary tumor and the vascular system, cells must generate force to reorganize their immediate microenvironment to ensure successful passage. Recently, work by Kraning-Rush *et al.* used trac-

tion force microscopy (TFM) to examine the differences in cell-generated force between metastatic and non-metastatic cancer cell lines (Kraning-Rush *et al.*, 2012). Briefly, cells were plated on 500 nm fluorescent bead-loaded polyacrylamide substrates with a known modulus. The beads were imaged before and after removing the cells and the total force was calculated. For breast, prostate and lung cancers, Kraning-Rush *et al.* found that metastatic cells exerted higher force on their underlying substrate compared to non-metastatic cells (Kraning-Rush *et al.*, 2012). This finding was independent of cell area and increasing the density of collagen I on the substrates, which implies that increased cell-generated force is intrinsically tied to the metastatic state and not the cell’s microenvironment. Similar results have been shown by other groups (Agus *et al.*, 2013).

Reduced cell stiffness of cancer cells can be observed at the tissue level. During cancer progression, upregulation of ECM proteins and increased ECM crosslinking cause malignant tissue to be significantly more stiff than surrounding tissue. These findings are consistent with conventional diagnostic practices such as breast palpation, but seemingly inconsistent with measurements of single cancer cells. To address this issue, Plodinec *et al.* performed *ex vivo* AFM stiffness measurements of normal, benign lesion and cancerous tissues. Across several specimens, results from the cancerous tissue showed a dominant peak at a stiffness two-fold less than compared to the peak of the distribution for the normal tissue (Plodinec *et al.*, 2012). The cancerous tissue had two additional peaks in the stiffness distribution, indicating that the tissue had considerable mechanical heterogeneity. The authors additionally found a nearly identical soft

peaks when comparing stiffness distributions of primary (breast) and secondary (lung) tumors from the same mouse (Plodinec et al., 2012). These findings may suggest that the reduced stiffness adopted by cancer cells may persist to the secondary tumor. Overall, this work currently provides the most through evidence that the reduced stiffness of cancer cells can be observed at the tissue level.

The epithelial to mesenchymal transition: a model for cancer progression studies. An intermediate conclusion thus far is that cell mechanics (stiffness and force-generation) is essential to understanding cancer metastasis. However, the studies described above and others in the community have not yet addressed or identified the biomolecular mechanisms behind the altered mechanical properties and cancer. Stated as a question: **What are the mechanistic links between cell mechanics and metastasis** (*i.e.* proteins and signaling pathways)?

Additionally, while incredible informative, the studies described above make mechanical comparisons between different immortalized cells lines, different patient cell line, or tissue samples from different stages of cancer progression. Since there is considerable genetic variation between these specimens, this different-stage approach is not ideally equipped to identify the regulatory pathways (*i.e.* the signaling molecules) behind the altered mechanical properties of cancer cells.

The epithelial to mesenchymal transition (EMT) is an essential physiological process found in development and wound healing that drive adherent, immotile cells to lose polarity and increase migratory ability (Thiery et al., 2009). Recently, abnormal reactivation of EMT has been implicated in the detachment of cancer cells from epithelial

tissue and their subsequent invasion into stromal tissue (Yilmaz and Christofori, 2009). There are several well-known drivers of EMT, including transforming growth factor β (TGF- β) and bone morphogenetic proteins (BMPs) (Gordon et al., 2009). These signaling pathways lead to several classic hallmarks of EMT: 1) altered gene expression (Ranganathan et al., 2007), 2) dramatic changes in cell morphology and the structure of the actin cytoskeleton (Moustakas and Stournaras, 1999)(Hubchak, 2003), 3) down-regulation of cell-cell adhesion molecules such as E-cadherin (Vogelmann et al., 2005), 4) significant increases in cell motility and invasion capacity (Gordon et al., 2009).

These changes in cytoskeletal structure and increased interaction with the ECM implicate a role for altered cell mechanics during EMT (Yilmaz and Christofori, 2009). EMT provides a well characterized biomolecular platform for identifying potential protein mechanisms behind the mechanical changes during cancer progression.

Moving forward, the mechanical phenotype adopted during EMT will be examined in Ch. 5 using magnetic tweezers, PBR, and our rotating magnet device (discussed in Ch. 3). In Ch. 7, we will use our high throughput microscope and PBR to test the effect of multiple oncogenic signaling pathways on cell stiffness.

2.5 Rheology towards Cell Mechanics

Although our experiments (presumably) induce molecular level deformations of many cellular components, our microbead-based methods are insensitive to these individual contributions. Therefore, to parametrize our data, we are going to use continuum models for our magnetic tweezer and passive bead rheology (PBR) assays. Here, we review rheology concepts towards cell mechanics measurements using continuum material models.

Rheology is the study of material deformation under force. More specifically, when you apply a force of a given magnitude in a defined geometry for a certain amount of time to a material, rheology captures these quantities and describes the resulting deformation response of the system. In order to characterize material properties independent of the size or geometry of the system, rheologists use the intensive variables stress (σ) and strain (γ), where

$$\sigma \equiv \frac{\text{force}}{\text{area}} = \frac{F}{A} \quad (2.1)$$

$$\gamma \equiv \text{relative deformation} = \frac{\Delta x}{x} \quad (2.2)$$

In SI units, stress is measured in Pa (N/m²) and strain is dimensionless. Depending on how the stress is applied to the material, three basic deformation geometries can occur: simple shear and extension, and volume deformation (Fig. 2.9). Most biological tissues

$\sigma = M\gamma$		
Stress	Modulus	Strain
σ_K	Bulk, K	$\frac{\Delta V}{V}$
σ_E	Young's, E	$\frac{\Delta L}{L}$
σ_G	Shear, G	$\frac{\Delta x}{h}$

Figure 2.9: **Basic deformation geometries for corresponding stresses.**

are dominated by water, and since water is considered incompressible under typical biological forces, stresses usually result in shear or extensional deformations.

The relationship between stress and strain defines the material response of a system. The limiting cases of mechanical response are elastic behavior of an ideal solid, described by Robert Hooke, and viscous behavior of an ideal liquid, described by Issac Newton. Real materials exhibit both elastic and viscous responses depending on the nature of the applied stress and the timescale over which the stress is applied. Real materials are *viscoelastic* systems. The following three sections will discuss elastic, viscous, and viscoelastic responses.

2.5.1 Ideal Elastic and Viscous Materials

Ideal elastic materials. An ideal elastic system, abstracted as a spring that stores mechanical energy, follows Hooke's law which states that stress is proportional to the strain for small strains. For real materials, as strains become large, the relationship

between stress and strain may become nonlinear. Depending on how the stress or strain are applied or measured, different types of elastic moduli can be defined (Fig. 2.9). For a shear stress σ and a shear strain $\gamma = \Delta x/h$, Hooke's law gives

$$\sigma = G\gamma \quad (2.3)$$

where G is the elastic shear modulus, which is a measure of the rigidity of the material. The elastic shear modulus describes the extent of deformation when a material experiences opposite stresses on opposing surfaces (Fig. 2.9). Under stress, an ideal elastic material will deform immediately and remain in this state until the stress is removed, at which point, the material will return to the undeformed state. Compared to glass ($G \sim 10^{10}$ Pa) and a rubber band ($G \sim 10^6$ Pa), cells typically have a lower elasticity ($G \sim 10^1 - 10^3$ Pa). The different types of elastic moduli are related under specified conditions; for example, the shear (G) and Young's (E) moduli are related through (Benenson et al., 2002):

$$G = \frac{E}{2(1 + \nu)} \quad (2.4)$$

where ν is the Poisson's ratio and a measure of the compressibility of a material. For isotropic, incompressible materials, $\nu = 0.5$ and hence $G = \frac{1}{3}E$.

Note about modulus vs. stiffness: The elastic modulus G of a material is generally not the same as the stiffness of a material. The elastic modulus is an intensive property of the actual material, whereas the stiffness k is an extensive property of the structure

(material plus geometry). For example, for an isotropic, incompressible beam, the on axis stiffness is related to the Young's modulus E through (Benenson et al., 2002):

$$k = \frac{AE}{L} \quad (2.5)$$

where A and L are the cross-sectional area and length of the beam, respectively.

In general, cell mechanics instruments apply a stress to a cell and measure the resulting strain. Cells are viscoelastic materials, so the resulting deformation has both elastic and viscous contributions. Most studies focus on the elastic response of a cell, parameterizing this quantity with an elastic modulus that depends on the nature of the applied stress. For example, compressive forces are often applied in AFM experiments, and by measuring the indentation and making basic assumptions about the probe-cell contact geometry, the Young's modulus E can be calculated. The community, however, often refers to various moduli of a cell as the cell *stiffness*. For our considerations, we also focus on the elastic response of cells, and will generally refer to changes in moduli G as changes in cell stiffness.

Ideal viscous materials. For an ideal viscous system, abstracted as a dashpot that dissipates mechanical energy, the stress is proportional to the strain rate $\dot{\gamma} = \frac{d\gamma}{dt}$, and independent of the strain. These systems are called Newtonian liquids. The stress σ and strain rate $\frac{d\gamma}{dt}$ are related by

$$\sigma = \eta \frac{d\gamma}{dt} \quad (2.6)$$

where η is the viscosity of the liquid (Pa s in SI units). Hence, viscosity is a measure of the resistance to changes in strain due to a stress. Liquid materials continuously deform while the stress is applied and remain in the deformed state after the stress is removed. Depending on how the stress or strain are applied or measured, liquids can be characterized in terms of different quantities, such as bulk or shear viscosities (similar to elastic materials). For orientation, the viscosity of the cytoplasm of a cell ($\eta \sim 0.1$ Pa s) falls between that of water ($\eta \sim 0.001$ Pa s) and honey ($\eta \sim 2 - 10$ Pa s) and is close to the viscosity of a 2.5M sucrose solution ($\eta \sim 0.12$ Pa s), which we use later as a viscosity standard.

2.5.2 Viscoelastic materials

Viscoelastic systems are materials that have characteristics of both elastic and viscous materials. The rheological properties of viscoelastic materials are often measured by applying small-amplitude, oscillatory stresses or strains to the system. As a result, rheologists tend to work in frequency domain rather than the time domain. If we consider a viscoelastic system under an oscillatory shear strain, with amplitude γ_0 and angular frequency $\omega = 2\pi f$ (ω in rad/s; f is the frequency in Hz), we can write

$$\gamma(t) = \gamma_0 \sin \omega t \tag{2.7}$$

The stress σ induced in the material from the strain will also be oscillatory, but will be phase shifted by δ with respect to the strain:

$$\sigma(t) = \sigma_0 \sin(\omega t + \delta) \quad (2.8)$$

where σ_0 is the amplitude of the stress. For ideal elastic materials, the induced stress is in phase with the strain ($\delta = 0$) and for ideal liquids, the induced stress is phase shifted by 90° . Using these boundary conditions for a viscoelastic system, we can write the induced stress as a function of an in-phase component with amplitude $\sigma' = \gamma_0 G'$, and an out of phase component with amplitude $\sigma'' = \gamma_0 G''$ as (Wang and Discher, 2007)

$$\sigma(t) = \gamma_0 (G' \sin \omega t + G'' \cos \omega t) \quad (2.9)$$

where the phase shift is between 0 and 90° . From equation 2.9, the elastic (storage) shear modulus is given by $G' = \frac{\sigma'}{\gamma_0}$ and the loss shear modulus (related to the viscosity) is given by $G'' = \frac{\sigma''}{\gamma_0}$. For an ideal elastic material, $G' = G$ and $G'' = 0$, and for an ideal liquid, $G' = 0$ and $G'' = \omega\eta$. Typical materials in biology, such as cells and tissues, are viscoelastic and therefore have both elastic and loss moduli, each dependent on the rate of deformation ω : $G'(\omega), G''(\omega)$. Additionally, materials that are more elastic than viscous $G'(\omega) > G''(\omega)$ are called viscoelastic solids, whereas materials that are more viscous than elastic $G'(\omega) < G''(\omega)$ are called viscoelastic liquids.

For convenience, the oscillatory strain and induced stress considered above, Eq. 2.8 and Eq. 2.9 respectively, are often expressed as complex quantities: $\gamma^*(\omega)$ and $\sigma^*(\omega)$. In general rheology, the ratio of a given stress to a corresponding strain is referred to as a modulus. When expressed as complex quantities, the shear stress and strain define a

complex shear modulus (Ferry, 1980):

$$\sigma^*(\omega)/\gamma^*(\omega) = G^*(\omega) = G'(\omega) + iG''(\omega) \quad (2.10)$$

with a real component equal to the elastic modulus $G'(\omega)$ and an imaginary component equal to the loss modulus $G''(\omega)$.

The linear relationship between stress and strain (ideal elastic system: equation 2.3) and between stress and strain amplitude (viscoelastic system: equation 2.9) holds for small strains and small strain amplitudes. These conditions define the linear elastic and linear viscoelastic ranges of a material. At larger strains/amplitudes, some materials exhibit nonlinear material responses which may result in reduced G' (strain-softening) or increased G' (strain-stiffening). There is evidence that cells strain-stiffen at high strain (Kollmannsberger and Fabry, 2011).

Creep-recovery experiments probe viscoelastic systems. In this subsection we examined the general, induced stress response from an applied strain for a viscoelastic material. In order to approximate various biological contexts, rheologists often observe the induced strain $\gamma(t)$ of a system under a fixed stress σ_0 , and monitor the recovery after the strain is removed. The creep compliance $J(t)$ is often the quantity to characterize these creep-recovery experiments (Wang and Discher, 2007):

$$J(t) = \frac{\gamma(t)}{\sigma_0} \quad (2.11)$$

A high compliance corresponds to a material unable to resist mechanical deformation

under an applied stress, whereas a material with a low compliance is able to resist deformation under stress. We will conduct creep-recovery measurements during our magnetic tweezer experiments throughout this work.

2.5.3 Physical Considerations in Micro-Rheology

To this point we have discussed rheology principals of ideal and viscoelastic materials without consideration to length scale. To help understand how length scale impacts the study of rheology at the micron scale, we consider applying an external force $F_{external}$ to a point source with mass m that is embedded in a viscous material:

$$F_{external} = \underbrace{ma}_{\text{inertial effects}} + \underbrace{f_v v}_{\text{viscous effects}} \quad (2.12)$$

where f_v is the coefficient of friction. When considering small extended objects a simplification can be made by considering micron length scales and velocities of such particles in a Newtonian fluid. As a microbead moves through a fluid, volumes of the fluid equal to that of the microbead must be displaced. The Reynolds number (Re) is a dimensionless parameter that relates inertial effects of the displaced volume of fluid to that of the internal friction (viscosity) of the fluid. Parameterized in terms of a characteristic length L , the Reynolds number can be written as (Benenson et al., 2002)

$$Re = \frac{\rho v L}{\eta} \quad (2.13)$$

where ρ and η are the density and viscosity of the fluid, and v is the relative velocity between the fluid and the microbead. For a 1 μm microbead in water ($\rho = 1000 \text{ kg/m}^3$,

$\eta = 0.001 \text{ Pa s}$) moving at 1 and 100 $\mu\text{m/s}$ the Re is 10^{-6} and 10^{-4} , respectively. These conditions are within the low Reynolds number regime defined as $Re \ll 1$, indicating that viscous effects dominate inertial effects in the flow of fluid around the microbead. These flow conditions are called laminar flow.

The drag force on a microbead in laminar flow conditions is the sum of the integrated pressure and shear stress imposed on the bead from the fluid. The total drag force on the bead with radius a is given by Stokes law (Benenson et al., 2002)

$$F_D = 6\pi a\eta v \quad (2.14)$$

where η is the viscosity of the fluid and v is the relative velocity. Stokes law assumes spherical particles, homogeneous Newtonian fluid, laminar flow and no particle interaction. Thus, if we apply an external force to a microbead under these conditions, we see that the drag force is equal to the external force

$$\begin{aligned} F_{external} &= F_D \\ &= 6\pi a\eta v \end{aligned} \quad (2.15)$$

The effect of Stokes law will be seen in many ways in this work. To calibrate the forces generated by our magnetic tweezers system, we will apply an external magnetic force to magnetic beads embedded in a fluid of known viscosity (Appendix A). By monitoring the displacement of the beads over time and calculating the velocity, we will be able to determine the magnitude of the applied force using Eqn. 2.15. Additionally, Stokes law will also inspire the friction coefficient used in the Stokes-Einstein relation to relate the diffusion coefficient of particles to the thermal energy.

2.6 Active Microrheology towards Magnetic Tweezer Experiments

Active microrheology uses an external force applied to a microbead to locally deform the material and monitor the resulting strain. Active microrheology is the underlining methodology to several cell mechanics techniques: optical tweezers, magnetic twisting cytometry, and magnetic tweezers. Creep-recovery experiments, as discussed in Sec. 2.5.2, are used to approximate various biological contexts (*e.g.* deformation of a cell under a force applied by another cell) and determine mechanical properties. The creep-recovery methodology will be used in magnetic tweezer experiments discussed in Chapters 4 and 5.

Fig. 2.10 shows data for a typical magnetic tweezer experiment. So we can ask ourselves: how do we capture the non-instantaneous elastic deformation followed by a viscous deformation in our data?

In the following sections we consider a microbead fully embedded in a material (this is an approximation – our bead is attached externally to the cell), and we work towards a response function that is consistent with our data. To this end, we adopt the engineering approach and construct mechanical circuits using Hookean elastic springs (Eq. 2.3) and Newtonian viscous dashpots (Eq. 2.6). After reviewing the basic elements, we will derive analytical solutions for three viscoelastic circuits: the Maxwell model, the Kelvin-Voigt model, and the Jeffreys model. Fig. 2.11 shows a summary of these models and the associated response functions for a step input stress that will be developed below.

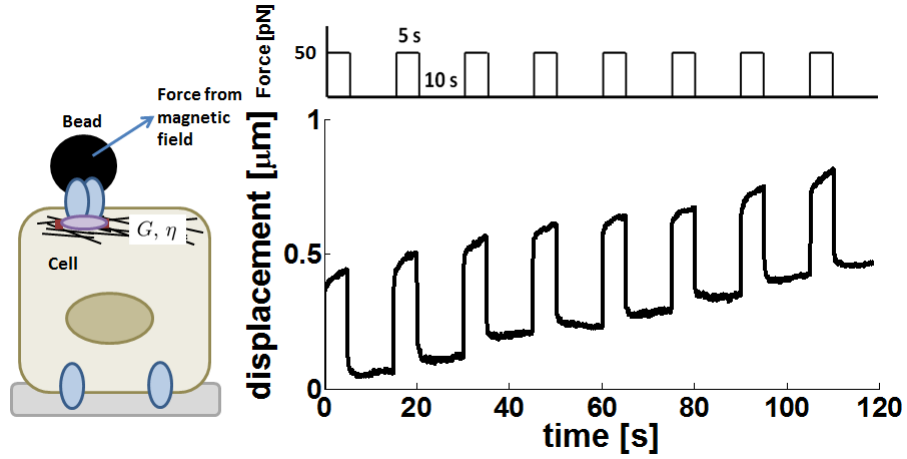


Figure 2.10: **Typical bead displacement in response to a step stress.** We desire a model that captures this functional form under force: a non-instantaneous elastic deformation followed by a viscous deformation. Once we have a model, we intend to fit each bead displacement individually (*e.g.* 8 fits for the experiment above) so that we can determine whether cellular mechanical properties change over time.

2.6.1 Step Response of Ideal Elastic and Viscous Materials

Step response of an ideal elastic material: The response of an ideal elastic material to a step stress is given by rearranging Eq. 2.3 and casting in terms of compliance

$$J(t) = \frac{\gamma(t)}{\sigma_0}:$$

$$J(t) = \frac{1}{G} \quad \text{Hookean Solid} \quad (2.16)$$

Fig. 2.11 shows the response function for an ideal elastic material under a step stress.

Step response of an ideal viscous material: The response of an ideal viscous material to a step stress is given by integrating Eq. 2.6 with respect to time and

casting in terms of compliance $J(t) = \frac{\gamma(t)}{\sigma_0}$:

$$J(t) = \frac{1}{\eta} t \quad \text{Newtonian Liquid} \quad (2.17)$$

Fig. 2.11 shows the response function for an ideal viscous material under a step stress.

2.6.2 Step Response of Viscoelastic Materials

Maxwell Model: Viscoelastic Model 1. The Maxwell model consists of an elastic spring and a viscous dashpot in series. The stress experienced across each element is equal to the total stress (σ_{total}) applied to the system. Also, the total strain of the system (γ_{total}) is equal to the sum of the strain each element allows. We can write this as

$$\sigma_{total} = \sigma_{spring} = \sigma_{dashpot} \quad (2.18)$$

$$\gamma_{total} = \gamma_{spring} + \gamma_{dashpot} \quad (2.19)$$

We take the time-derivative of the total strain

$$\frac{d\gamma}{dt} = \frac{d\gamma_s}{dt} + \frac{d\gamma_d}{dt} \quad (2.20)$$

and rewrite this expression knowing $\sigma_s = G\gamma_s$ and $\sigma_d = \eta \frac{d\gamma_d}{dt}$ and $\sigma = \sigma_s = \sigma_d$:

$$\frac{d\gamma}{dt} = \frac{1}{G} \frac{d\sigma}{dt} + \frac{1}{\eta} \sigma \quad (2.21)$$

Integrating with respect to time for a constant input step stress, $\sigma(t) = \sigma_0$ (this implies $\frac{d\sigma}{dt} = 0$), the strain as a function of time becomes:

$$\gamma(t) = \frac{\sigma_0}{\eta} t + \gamma_0 \quad (2.22)$$

The expression for compliance $J(t) = \frac{\gamma(t)}{\sigma_0}$ for a constant step stress σ_0 is given by

$$J(t) = \frac{1}{\eta} t + \frac{1}{G} \quad \text{Maxwell model} \quad (2.23)$$

Fig. 2.11 shows the step-stress response function for a Maxwell model – a viscoelastic liquid material with instantaneous elastical deformation. This model would approximately our data, but since no real materials deform instantaneously, we will analyze 2 more viscoelastic models.

Kelvin-Voigt Model: Viscoelastic Model 2. The Kelvin-Voigt model consists of an elastic spring and a viscous dashpot in parallel. The total stress applied to the system (σ_{total}) is shared by the two elements, and the total strain of the system (γ_{total}) is equal to the strain each element allows. We can write this as

$$\sigma_{total} = \sigma_{spring} + \sigma_{dashpot} \quad (2.24)$$

$$\gamma_{total} = \gamma_{spring} = \gamma_{dashpot} \quad (2.25)$$

We rewrite the equation for the total stress using the know relations for stress across a spring (Eq. 2.3) and a dashpot (Eq. 2.6):

$$\sigma = G\gamma + \eta \frac{d\gamma}{dt} \quad (2.26)$$

We rearrange terms to cast in the standard form for a first order differential equation:

$$\frac{d\gamma(t)}{dt} + \frac{G}{\eta}\gamma(t) = \frac{1}{\eta}\sigma(t) \quad (2.27)$$

Using the standard solution for a first order differential equation, and assuming a constant input step stress, $\sigma(t) = \sigma_0$, we have:

$$\gamma(t) = Ce^{-\frac{G}{\eta}t} + \frac{\sigma_0}{G} \left(1 - e^{-\frac{G}{\eta}t}\right) \quad (2.28)$$

If we assume zero strain at time zero, $\gamma(t=0) = 0$, then we see that $C = 0$. Thus, we can write the strain response as

$$\gamma(t) = \frac{\sigma_0}{G} \left(1 - e^{-\frac{G}{\eta}t}\right) \quad (2.29)$$

The expression for compliance $J(t) = \frac{\gamma(t)}{\sigma_0}$ for a constant step stress σ_0 is given by

$$J(t) = \frac{1}{G} \left(1 - e^{-\frac{G}{\eta}t} \right) \quad \text{Kelvin-Voigt model} \quad (2.30)$$

Fig. 2.11 shows the step-stress response function for a Kelvin-Voigt model: viscoelastic solid material. This model does not reflect typical response functions of cells under step stresses.

Jeffreys Model: Viscoelastic Model 3. The Jeffreys model consists of a Kelvin-Voigt model with a dashpot in series. The stress experienced across the Kelvin-Voigt element and the dashpot is equal to the total stress σ_{total} applied to the system. Additionally, the total strain of the system γ_{total} is equal to the sum of the strain Kelvin-Voigt element and the dashpot. We can write this as

$$\sigma_{total} = \sigma_{\text{Kelvin-Voigt}} = \sigma_{\text{dashpot}} \quad (2.31)$$

$$\gamma_{total} = \gamma_{\text{Kelvin-Voigt}} + \gamma_{\text{dashpot}} \quad (2.32)$$

Since we already have expressions for the time-dependent strains for the Kelvin-Voigt element (Eq. 2.30) as well as that for a dashpot (Eq. 2.6), we can simply write down the equation for strain as a function of time:

$$\gamma(t) = \frac{\sigma_0}{G} \left(1 - e^{-\frac{G}{\eta_1} t} \right) + \frac{\sigma_0}{\eta_0} t \quad (2.33)$$

The expression for compliance $J(t) = \frac{\gamma(t)}{\sigma_0}$ for a constant step stress σ_0 is given by

$$J(t) = \frac{1}{G} \left(1 - e^{-\frac{G}{\eta_1} t} \right) + \frac{1}{\eta_0} t \quad \text{Jeffreys model} \quad (2.34)$$

Fig. 2.11 shows the step-stress response function for a Jeffreys model – a viscoelastic liquid material with non-instantaneous elastical deformation. This model is capture the features of typical response functions for cells under step stresses.

Calculation of cell compliance from bead displacement. In raw form, magnetic tweezer data are position and time measurements produced by the particle tracking software Video Spot Tracker (VST; cismm.org). The radial displacement of the bead is computed, as seen in the data motivating plot at the beginning of this section, Fig. 2.10. The time-dependent compliance $J(t)$ is then calculated using $r(t)$ and the following expression (Ziemann et al., 1994):

$$J(t) = \frac{6\pi a r(t)}{F(t)} \quad (2.35)$$

where a is the bead radius and $F(t)$ is the applied force. This equation assumes a fully embedded bead. The cell compliance data $J(t)$ is then fit by the Jeffreys model described above in Eqn. 2.34 and shown in Fig. 2.11. This procedure is described in more detail in Appendix. A.

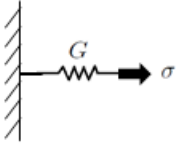
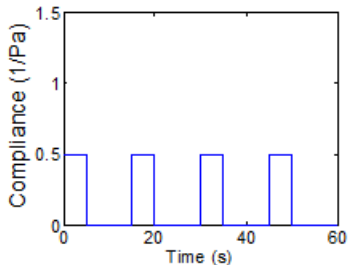
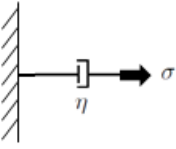
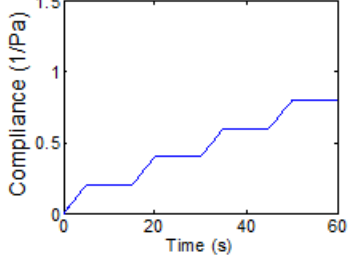
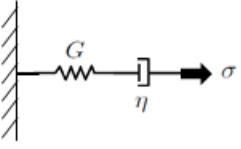
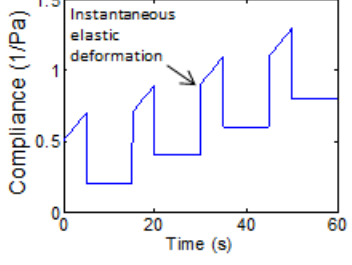
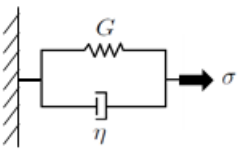
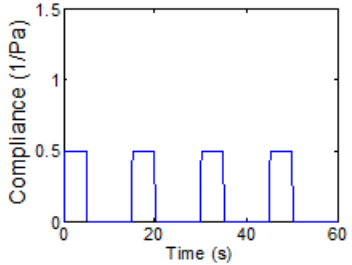
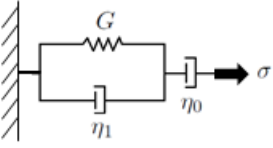
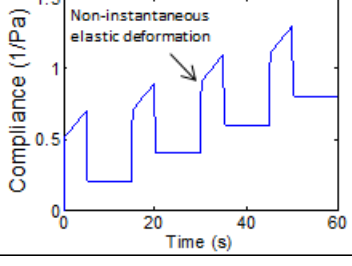
Schematic	Model	Equation	Compliance Response $J(t) = \frac{\gamma(t)}{\sigma_0}$
	Ideal Elastic Solid $\sigma = G\gamma$	$J(t) = \frac{1}{G}$	
	Ideal Viscous Liquid $\sigma = \eta \frac{d\gamma}{dt}$	$J(t) = \frac{1}{\eta} t$	
	(Viscoelastic) Maxwell Model	$J(t) = \frac{1}{\eta} t + \frac{1}{G}$	
	(Viscoelastic) Kelvin-Voigt Model	$J(t) = \frac{1}{G} \left(1 - e^{-\frac{G}{\eta} t} \right)$	
	(Viscoelastic) Jeffreys Model	$J(t) = \frac{1}{G} \left(1 - e^{-\frac{G}{\eta_1} t} \right) + \frac{1}{\eta_0} t$	

Figure 2.11: **Summary of mechanical circuit models.** The response functions are developed in this section and plotted here for an input step stress that is “on” for 5 seconds and “off” for 10 seconds.

2.7 Passive Microrheology towards Cell Rheology Experiments

Unlike active microrheology, passive microrheology uses no external forcing mechanism to drive microbead movement. Instead, in passive microrheology the bead is driven by the spontaneous collisions of particles in the surrounding material produced by the thermal energy kT , where k is the Boltzmann's constant and T is the temperature of the molecular environment. Depending on the nature of the surrounding material, the diffusing bead experiences different counteracting forces.

In the following sections, we will consider single particle diffusion in Newtonian liquids and elastic solids, which serve as limiting cases for single particle diffusion in viscoelastic materials.

2.7.1 Single Particle Diffusion in Newtonian Liquids and Elastic Solids

Single Particle Diffusion in Newtonian Liquids. In a Newtonian liquid, or an ideal viscous material, there are two forces acting on the bead. The first is the small, stochastic driving force generated the thermal energy kT . The second force is a counteracting frictional force that provides resistant to each step the bead takes and is dependent on the radius of the bead a and the viscosity of the liquid η .

In each instant, the thermal energy drives the bead to step in a random direction with a small amplitude. The next step, occuring in the next instant, has an uncoorelated direction and has a different, random step size. Einstein found that for a freely diffusing bead, the mean squared displacement (MSD) $\langle r^2(\tau) \rangle$ is related to the the diffusion

coefficient D by (Einstein, 1905)

$$\langle \Delta r^2(\tau) \rangle = 2nD\tau \quad (2.36)$$

where n defines the dimensionality of the observed process. In this work, we use video microscopy to observe diffusion in two dimensions. Thus, $\langle \Delta r^2(\tau) \rangle = 4D\tau$. The diffusion coefficient is given by the Einstein relation (Einstein, 1905)

$$D = MkT \quad (2.37)$$

where M is the hydrodynamic mobility of the particle. For a spherical particle in a Newtonian fluid, the mobility is given by $M_{sphere} = (6\pi a\eta)^{-1}$, and results in the well-known Stokes-Einstein relation (Rubinstein and Colby, 2003)

$$D = \frac{kT}{6\pi a\eta} \quad (2.38)$$

The Stokes-Einstein relation is powerful to passive microrheology in that it connects the calculated MSD to the viscosity of the liquid η through Eq. 2.36

$$\eta = \frac{2kT}{3\pi a \langle \Delta r^2(\tau) \rangle} \tau \quad (2.39)$$

Single Particle Diffusion in Elastic Solids. In an elastic material, the bead is driven by collisions with the background material, which is generated by thermal energy

kT . However, an ideal elastic material stores all of this energy and instantaneously exerts a restoring force with equal amplitude in the opposite direction. Thus, the MSD of a particle embedded in such a material is constant and independent of time:

$$\langle \Delta r^2(\tau) \rangle_{\text{solid}} = K \quad (2.40)$$

In Section 2.5.2, we wrote the viscoelastic stress response to an oscillatory strain $\gamma(t) = \gamma_0 \sin(\omega t)$ as $\sigma(t) = \gamma_0(G' \sin \omega t + G'' \cos \omega t)$. Evaluating at the limiting cases for an ideal liquid and solid, we have (Wirtz, 2009)

$$\text{Ideal Liquid} \rightarrow G' = 0, G'' = \omega\eta \rightarrow \eta = \frac{\sigma(t)}{\gamma_0 \cos \omega t} \frac{1}{\omega} \leftrightarrow \eta = \frac{2kT}{3\pi a \langle \Delta r^2(\tau) \rangle} \tau \quad (2.41)$$

$$\text{Ideal Solid} \rightarrow G' = G, G'' = 0 \rightarrow G = \frac{\sigma(t)}{\gamma_0 \sin \omega t} \leftrightarrow G = \frac{2kT}{3\pi a \langle \Delta r^2(\tau) \rangle} \quad (2.42)$$

where in Eq. 2.42 we have set $\langle \Delta r^2(\tau) \rangle_{\text{solid}} = K = 2kT/3\pi aG$. Hence, we have arrived at an expression for ideal solids, analogous to Eq. 2.39 for ideal liquids, that can be used to determine the elastic shear modulus by measuring the MSD of embedded beads.

Thus, by measuring the MSD for beads of size a that are stuck to the coverslip (*e.g.* a noise floor sample), these expressions for G and η can be used to estimate the maximum shear modulus and viscosity that our instrumentation is sensitive to. We perform these calculations in Fig. 6.6 for our high throughput microscopes.

2.7.2 Single Particle Diffusion in Viscoelastic Materials

As described above in Eq. 2.10, the general form for the response functions of viscoelastic materials can be written in terms of the complex shear modulus as

$$G^*(\omega) = G'(\omega) + iG''(\omega) \quad (2.43)$$

Recently, a generalization to the Stokes-Einstein relation (Eq. 2.38) was developed to describe these response functions (Mason et al., 1997). The generalized Stokes-Einstein relation (GSER) relates the frequency-dependent complex modulus to a frequency-dependent complex diffusion coefficient, $D^*(\omega)$ through

$$G^*(\omega) = \frac{kT}{6\pi a D^*(\omega)} \quad (2.44)$$

It is conventional in this formulation to use ω as the frequency in units of Hz, such that $\tau = 1/\omega$ is the timescale in units of sec. In terms of the time-scale dependent MSD, $\langle \Delta r^2(\tau) \rangle$, the complex modulus can be written as (Mason, 2000)

$$G^*(\omega) = \frac{2kT}{3\pi a \langle \Delta r^2(1/\omega) \rangle \Gamma[1 + \alpha(\omega)]} \quad (2.45)$$

where Γ is the gamma function and $\alpha(\omega)$ is the logarithmic slope of $\langle \Delta r^2(\tau) \rangle$ evaluated at $\tau = 1/\omega$: $\alpha(\omega) \equiv \left. \frac{d \ln \langle \Delta r^2(\tau) \rangle}{d \ln \tau} \right|_{\tau=1/\omega}$. The shear modulus, $G'(\omega)$, and the loss modulus, $G''(\omega)$, are computed through $G'(\omega) = G^*(\omega) \cos\left(\frac{\pi\alpha(\omega)}{2}\right)$ and $G''(\omega) = G^*(\omega) \sin\left(\frac{\pi\alpha(\omega)}{2}\right)$, respectively. These expressions reproduce the limiting case for an

elastic solid when $\alpha = 0$ and for a Newtonian fluid when $\alpha = 1$ (recall $\eta = G''(\omega)/\omega$).

A note about data for passive microbead rheology of cells in this work. In raw form, passive microbead data are position and time measurements produced by the particle tracking software Video Spot Tracker (VST; cismm.org). The mean-squared displacement (MSD) of the particle trajectory is calculated using

$$\langle \Delta r^2(\tau) \rangle = \langle [x(t + \tau) - x(t)]^2 + [y(t + \tau) - y(t)]^2 \rangle \quad (2.46)$$

for a predetermined vector of timescales $[\tau_1, \tau_2, \tau_3, \dots, \tau_T]$.

The above formulations for passive microbead rheology assume a fully embedded bead in either a Newtonian liquid, an elastic solid, or a viscoelastic material. In Ch. 6, we assess the accuracy of our Array High Throughput microscope to measure the viscosity of standard solutions; in this case, beads are fully embedded in the solutions, and application of Eqn. 2.39 is appropriate. In Ch. 4 and 5 we use Eqn. 2.45 and Eqn. 2.42 to estimate the “effective” shear modulus G' for a bead that is externally attached to the cell surface (Fig. 4.8 and Fig. 5.8, respectively). Because the use of these equations violates the assumption of a fully embedded bead, we limit our implementation in this work to these two instances.

In Ch. 7 we analyze passive bead rheology (PBR) data for cells conditions by comparing the distributions of the MSD at the $\tau = 1$ timescale.

2.7.3 Heterogeneity observed in PBR Cell Experiments

The mechanical properties of cells vary at the single cell level (due to complex and variable microstructures) and at the population level. A consequence of external passive bead rheology (PBR) of cells is that no control over the location of the bead is provided. Washing steps are included in the assay protocol to minimize beads that are in the specimen but are not attached to cells. However, not all unwanted beads are eliminated. In Ch. 6 and 7, we discuss automating the collection of PBR data and therefore inclusion of unwanted beads occurs to some extent. Additionally, PBR of cells adds a layer of heterogeneity due to variability in bead to cell attachment. Fig. 2.12 (left) shows the typical multiple population output of an automated PBR experiment.

During optimization of our PBR assay, we realized that our power to distinguish between biology conditions would require an advanced (and automated) analysis procedure capable of identifying subpopulations within our data. In Ch. 7 we will discuss the implementation of a analysis procedure that identifies subpopulations by fitting data to a number of competing MSD models (Fig. 2.12, right).

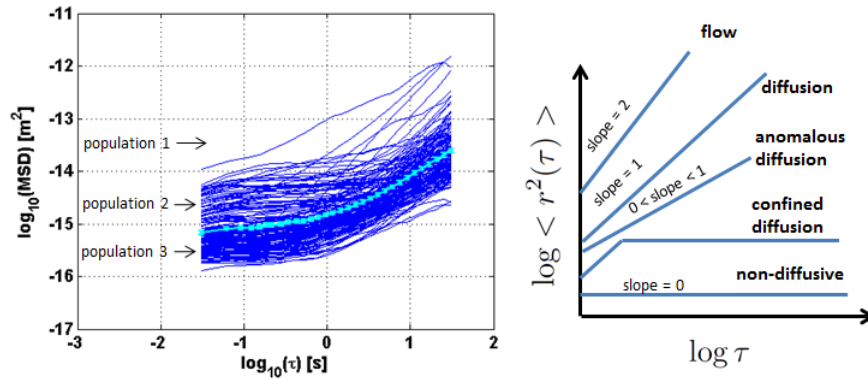


Figure 2.12: Heterogeneity of a typical (automated) PBR experiment.

2.8 Comparison of Moduli Derived from Microrheology and other Methods

An apparent discrepancy has existed between the cell modulus determined from active and passive microrheology – typically in the pascal (Pa) range – and other methods such as atomic force microscopy (AFM), where reported moduli are typically in the kilopascal (kPa) range. Possible sources that contribute to this discrepancy are:

- 1. Methods report different moduli.** AFM typically reports the Young’s modulus E whereas bead microrheology methods output the shear modulus G
- 2. Cells exhibit strain-stiffening.** Cell modulus measurements using AFM are highly dependent on indentation depth into the cell, potentially due to strain-stiffening of the actin cortex (Kollmannsberger and Fabry, 2011). Recently, Agus *et. al* showed that for MCF-10A epithelial cells E increases from 200 Pa to 1.8 kPa as the indentation is varied from 0.1 μm to 0.8 μm . External bead microrheology methods are likely shallow probes of the actin cortex (Hoffman et al., 2006), so comparison to small AFM indentation experiments is most appropriate.
- 3. Different cell types have different moduli.**
- 4. Bead methods underestimate G .** Microbead rheology methods determine G by assuming the bead is completely embedded in the material. Our methodology attaches beads to cell surface and therefore measurements of G likely underestimate material properties.

Here we attempt to mitigate these discrepancies by comparing data from two studies

Method	Bead size	Force	Indentation into cell	Young's Modulus, E	Shear Modulus, G	Cell Type	Reference
Optical tweezers	0.76 μm	10 pN	0.2 μm	100.3 $\pm$ 10.2 Pa	33 Pa	3T3 fibroblast	Nawaz et al., 2012
AFM	2 μm	30 pN	0.2 μm	85.3 $\pm$ 4.5 Pa	28 Pa	3T3 fibroblast	Nawaz et al., 2012
Magnetic tweezers	2.8 μm	40 pN	(shear)	not calculated	8 Pa	REF52 fibroblast	Guilluy et al., 2011

Figure 2.13: **Comparison of shear moduli G across methods.** In rows 1 and 2, G is computed from E using $G = \frac{E}{2(1+\nu)}$, where the Poisson ratio ν is ~ 0.5 (Shih, 2004), and we assume the cell is a homogeneous and isotropic material. In row 3, authors computed G by fitting the compliance J of the cell to the Jeffreys model, given in Eqn. 2.34. References for data: (Nawaz et al., 2012)(Guilluy et al., 2011)

using fibroblasts. Nawaz *et. al* recently showed that the Young's modulus E determined for small indentation AFM measurements (200 nm) with fibroblasts to be consistent with small indentation experiments with optical tweezers (Fig. 2.13; (Nawaz et al., 2012)). When the AFM or optical tweezer determined E are converted into a shear modulus, we see that $G \approx 30$ Pa, which is only factor of 3-4 higher than what has been observed with fibroblasts using magnetic tweezers (Fig. 2.13; (Guilluy et al., 2011)). This difference could reasonably be accounted for by a correction to the cell compliance $J = 6\pi ar(t)/F$ (for bead size a , bead displacement r , and force F) to reflect a boundary condition between the bead and cell, as opposed to assuming the bead is fully embedded in the cell.

Together, these observations suggest that cell moduli determined from various methodologies are consistent. In Sec. 2.2.3 and 5.4 we compare magnetic tweezer and PBR data.

Chapter 3: Advances to Magnetic Tweezer Methods

3.1 Overview

The use of magnetic systems for studying the mechanical properties of cells has a history that dates back almost 100 years. Within the last two decades, magnetic “tweezer” systems have become more sophisticated. These developments have allowed for advances on many biological and biomedical research fronts.

As magnetic tweezers have been increasingly applied to the study of cell mechanics, the need has strengthened for an established data selection criteria procedure. Simultaneously, the cell mechanics community became interested in understanding the biomolecular mechanisms at play in cellular force transduction pathways. However, when performing force transduction biochemical assays, experimenters were unable to mimic the force regimen (magnitude, duty cycle, frequency) offered by systems such as magnetic tweezers.

This chapter will focus on two contributions I made to the use of magnetic systems in cell biology, and will describe the results of an experiment that tested the sensitivity of cells to the nature of an applied force. Specifically we will discuss:

1. Improved selection criteria of cell compliance signatures resulting from magnetic tweezers experiments. A summary of the selection criteria is given in Sec. 3.3,

while in the interest of space, the full criteria is provided in Appendix B.

2. Design and characterization of a rotating magnet device that offers a tunable force regimen for biochemical analysis of force application to cells (Sec. 3.4).
3. Finally, we test whether cells can distinguish between constant and pulsatile forces. We discuss the design of this experiment as well as results.

Notes: Contributions from Ben Rardin, an undergraduate I mentored, in COMSOL simulations, and from Caitlin Collins in performing biochemical assays and data analysis are mentioned in figure legends. The rotating magnet device and results included here are currently described in a in-process manuscript with citation: L.D. Osborne, C. Collins, E. Tim O'Brien III, B. Rardin, E. Tzima, and R. Superfine. Pulsatile Mechanical Force Enhances PECAM-1 Mechanotransduction. (Manuscript *in process*).

3.2 Magnetic Tweezers and Cell Mechanics

The use of magnetic systems for studying the mechanical properties of cells has a long history. The first microrheological measurements of the cellular cytoplasm were performed by Alfred Heilbronn in 1922 using a magnetic tweezer system (Heilbronn, 1922). A couple decades later in the late 1940s, Francis Crick and Arther Hughes used an improved system and reported stiffness values on the order of pascals (Crick and Hughes, 1950). Within the last two decades, magnetic tweezer systems have become more sophisticated (Fisher et al., 2006), the field of microrheology has become more established (Ziemann et al., 1994), and the cell mechanics community is reporting the importance of mechanical forces in cell biology.

Mechanical forces affect many aspects of cell behavior and function (Hoffman and Crocker, 2009), and increasingly, are being shown to regulate many complex disease states (Jaalouk and Lammerding, 2009) such as deafness (Vollrath et al., 2007), osteoporosis (Augat et al., 2005), cardiac and skeletal myopathies (Heydemann and McNally, 2007), and atherosclerosis (Davies, 1995). In the vascular system, for example, blood flow imparts shear stress on endothelial cells (ECs) lining the lumen of blood vessels. This mechanical force is interpreted by ECs into specific biochemical signaling cascades through numerous mechanosensors, including integrins (Jalali, 2001)(Chachisvilis et al., 2006) and platelet endothelial cell adhesion molecule (PECAM)-1 (Tzima et al., 2005)(Collins et al., 2012)(Collins et al., 2014) and define influences vessel physiology and influence pathological states, such as atherosclerosis (Davies, 1995).

Magnetic tweezers are a routine and well documented method of applying exogenous mechanical forces to cells via micron sized magnetic beads (Fisher et al., 2006)(Hoffman et al., 2006)(Kollmannsberger and Fabry, 2007). The success of magnetic tweezers to characterize the physical properties of cell and nuclear mechanics (Matthews et al., 2006)(Guilluy et al., 2011)(Swaminathan et al., 2011)(Guilluy et al., 2014)(Osborne et al., 2014) and numerous biological processes (Collins et al., 2012)(Collins et al., 2014)(Hanna et al., 2013)(Lessey-Morillon et al., 2014)(Shen et al., 2011) has been extensive. Compared to other methods of cell mechanics , magnetic tweezers offer flexibility in force magnitude (pico- to nano-Newton)(Fisher et al., 2006)(Kollmannsberger and Fabry, 2011) and signaling pathway specificity by simply varying the ligand-coating of the magnetic bead.

The magnetic tweezers system used in this work. The magnetic tweezer cell mechanics experiments described in Ch. 4 and 5 use the UNC 3-dimensional force microscope (3DFM) (Fisher et al., 2006). In the interest of space, the 3DFM system, the calibration of forces, and data analysis specific to cell mechanics experiments are discussed in Appendix A.

3.3 Improved Selection Criteria for Cell Mechanics Experiments

As with the use of any technical device and methodology, implementing magnetic tweezers to study cell mechanics involves instrument and experiment overhead, as well as experiment execution and data analysis. In order to maintain consistency across experiments at the point of execution and data analysis, a ‘cell pulling’ selection criteria was established. **The 3 parts of this procedure are discussed in full in Appendix B.** In summary, the criteria are:

1. **Field of view (FOV) selection.** This criteria specifies the FOVs that are appropriate for cell pulling video data collection, including bead location on a cell, bead-to-bead distance, and bead-to-tweezer distance.
2. **Pre- data-collection selection.** The goal of this criteria is to estimate whether the bead-to-cell connection is established and appropriate for probing cell mechanics. This step consists of a passive motion selection and an active motion selection.
3. **Post- data-collection selection.** Once data is collected, the time-dependent displacement $r(t)$ and time-dependent compliance $J(t)$ signatures are selected for viscoelastic responses that suggest the mechanics of the cortical actin cytoskeleton is being probed. Beads are not included in further analyses if they exhibit the criteria described for: stuck beads, beads that detach from cells, directed membrane drift, broken bead-cell attachment, anchorage displacement, or broken

bead-cell attachment and anchorage displacement.

The percentage and number of FOVs and beads that pass each selection step are summarized in Fig. 3.1.

	total # of FOVs in cloning ring sample		2400
2%	# of FOVs that pass FOV selection		48
50%	# of FOVs that pass Pre- data-collection selection		24
33%	# of beads that pass Post- data-collection selection		8

Figure 3.1: **Selection criteria statistics.** After selection criteria, a typical magnetic tweezer experiment yields approximately 8 validated data points (bead trajectories in response to force).

3.4 Can Cells Distinguish between Constant and Pulsatile Forces?

To study the biochemical signaling that results from mechanical force, we and others have used permanent magnets to generate constant forces on cells via magnetic beads attached to various transmembrane receptors (Collins et al., 2012)(Collins et al., 2014)(Guilluy et al., 2011)(Guilluy et al., 2014). Such studies have provided valuable insights into mechanosensitive signaling cascades. However, these experiments often revealed differences in the timescales of significant changes observed for cell mechanics versus biochemical signaling. This led us to ask whether the apparent differences in timescale were due to the nature of the applied forces. In other words, do cells distinguish between constant and pulsatile forces?

To address this question we developed a rotating magnet device with a tunable force regimen (duty cycle, frequency, and magnitude) for biomolecular analysis of cellular response to force. By applying tension to the EC mechanosensor PECAM-1 within a well-characterized endothelial cell model (Collins et al., 2012)(Collins et al., 2014), we find that the timescale of biochemical response depends on the nature of the applied force (constant vs. pulsatile). Specifically, pulsatile forces leads to more rapid activation of ERK and RhoA downstream of force application on PECAM-1. Additionally, we show that this enhancement of PECAM-1 mechanotransduction leads to accelerated growth of focal adhesion number and size. These findings suggest that the nature of mechanical forces not merely their presence is detected by mechanosensitive proteins and influence the dynamics of intracellular signaling cascades which may specifically

be involved in mechanotransduction-related disease progression.

3.5 Design of the Rotating Magnet Device

The goal of this work was to determine if cells could detect the difference between constant and pulsatile mechanical stimulation. To address this question, we designed a rotating permanent magnet device that could apply pulsatile force regimens to adherent cells before biochemical analysis via standard immunoprecipitation and western blotting procedures.

Our intention was to design a device that was compatible with standard 10 cm cell culture dishes, that was compact enough to fit inside a standard cell culture incubator, and that could offer a complex force regimen with tunable duty cycle, frequency, and magnitude. We chose to use arc-shaped permanent magnets of a known angle to define the duty cycle. In practice, we use 2 axially magnetized arc magnets that sandwich an aluminum plate which is used for rotational purposes (Fig. 3.2 A, B). The frequency of the force is controlled by rotating the magnet with a DC motor at a given speed (Fig. 3.2 A, B). For example, a 120° arc magnet generates a force duty cycle of 33% (duty cycle = $100\% * \frac{120 \text{ deg}}{360 \text{ deg}}$), and when rotated at 4 rpm, the frequency of the force is 66.7 mHz. Together, the duty cycle and frequency define the “exposure time” of the force; for the above specifications, the force would be “on” and “off” for 5 and 10 sec, respectively. The magnitude of the force is defined by the magnet to magnetic bead distance, which we control with a linear translation stage (Fig. 3.2 C).

In order to determine force we could apply to cells using the device, we first in-

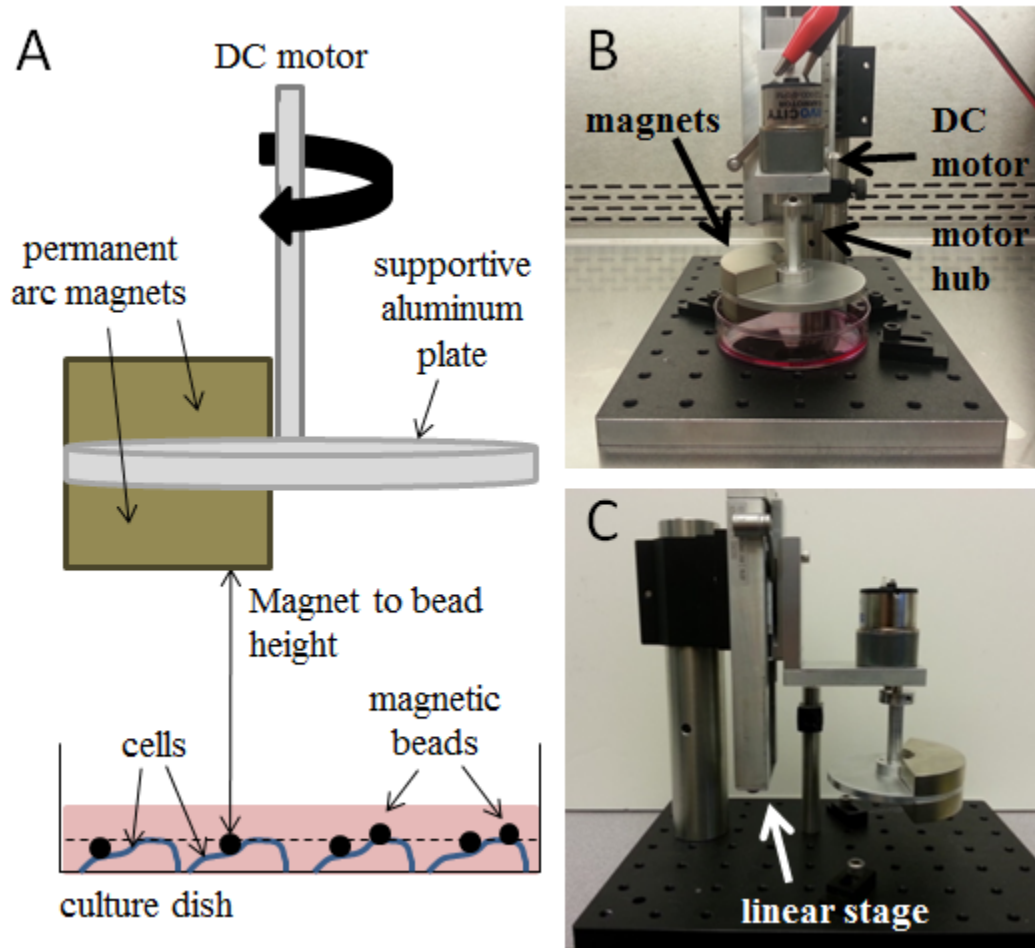


Figure 3.2: **The rotating magnet device generates pulsatile mechanical force.** (A) Schematic of the rotating magnet device, illustrating the position of the magnets relative to the specimen. (B) Plano view shows the DC motor, motor hub, and magnets of the device. The duty cycle of the force can be easily changed by replacing the magnets with those of a difference arc length. The frequency of the force is controlled by the speed of the motor. (C) Side view shows the linear translation stage that allows control over vertical positioning of the magnets. The magnitude of the force is dependent on the magnet to bead (cell) distance.

investigated the strength of the magnetic field generated by the permanent magnets by simulating the magnetic flux density, $\vec{B}$. In the absence of current and electric fields, Maxwell's equations give

$$\begin{aligned}\nabla \times \vec{H} &= 0 \\ \nabla \cdot \vec{B} &= 0\end{aligned}\tag{3.1}$$

where the magnetic field $\vec{H}$ is related to the magnetic flux density $\vec{B}$ and the magnetization $\vec{M}$ through

$$\vec{H} = \frac{1}{\mu_0} \vec{B} - \vec{M}\tag{3.2}$$

with μ_0 being the permeability of free space. Manufacturers of permanent magnets often provide specifications for the remanence flux density $\vec{B}_r$, which characterizes the strength of the magnet, and is related to the magnetization through $\vec{B}_r = \mu_r \vec{M}$, where μ_r is the relative permeability of the material. This allows us to write Eq. 3.2 as

$$\vec{B} = \mu_0 \mu_r \vec{H} + \vec{B}_r\tag{3.3}$$

From 3.1, $\nabla \times \vec{H} = 0$ implies a scalar magnetic potential V_m exists, such that $\vec{H} = -\nabla V_m$. Using this expression, and the fact that $\nabla \cdot \vec{B} = 0$, allows us to write an expression for the scalar potential

$$-\nabla \cdot (\mu_0 \mu_r \nabla V_m - \vec{B}_r) = 0\tag{3.4}$$

Using finite element software (COMSOL Multiphysics; Murlington, MA), we can solve Eq. 3.4 for the scalar magnetic potential V_m . Knowing V_m allows us to determine the magnetic field through $\vec{H} = -\nabla V_m$, and ultimately the magnetic flux density $\vec{B}$ from Eq. 3.3.

Location	B from vendor	B from Hall probe	B from simulation
Center	2.59-2.96 kG	2.64 kG	2.74 kG
Edge	3.76-4.18 kG	3.76 kG	3.69 kG

Figure 3.3: **Simulated magnetic flux density in context.** We compare magnetic flux density simulations of a neodymium cylindrical magnet to vendor specifications and measurements using a Hall probe (DE4, N42 grade, $\frac{7}{8}$ " dia. $\times$ $\frac{1}{4}$ " thick, $\vec{B}_r = 1.48$ kG; K&J Magnetics Inc.). Percent accuracy measurements of the simulation value compared to the vendor were made using the average of the range provided by the vendor (center: 2.78 kG; edge: 3.97 kG). The sensor of the Hall probe is offset approximately 1.8 mm from the outside of the probe, therefore this distance was taken into consideration when obtaining the vendor B range and when performing the simulation.

In order to verify the accuracy of our magnetic flux density simulations, we compared estimates for B at the center and edge of a neodymium cylindrical magnet (K&J Magnetics Inc., Pipersville, PA) to values given by the magnet manufacturer and measurements from a Hall probe (gauss meter) (Fig. 3.3). Compared to the vendor specifications, we find the simulation of B to be 1.4% and 7.1% different at the center and edge, respectively. Compared to the center and edge Hall probe measurements, we find the simulation of B to be 3.8% and 1.9 % different, respectively. The existence of small variability compared to two difference sources serve to validate our simulation procedure of the magnetic flux density B of permanent magnets.

In the presence of a magnetic field, with magnetic flux density $\vec{B}$, the force on a paramagnetic microbead is given by (Spero et al., 2008):

$$\mathbf{F} = \nabla(\mathbf{m} \cdot \mathbf{B}) = \frac{\pi d^3}{4\mu_0} \frac{\mu_r - 1}{\mu_r + 2} \nabla(B^2) \quad (3.5)$$

where $\mathbf{m}$ is the magnetic moment of the microbead and d is the bead diameter. Therefore, by simulating the gradient of the squared magnetic flux density, ∇B^2 , at a distance below the magnet surface, the force on a bead attached to a cell could be evaluated using Eq. 3.5 (Fig. 3.2).

In order to capture how the force varies during a complete rotation of the rotating magnet device, we simulated the the gradient of the squared z-component magnetic flux density, ∇B_z^2 , at an experiment relevant magnet-to-cell distance for circular paths for several radii (Fig. 3.4). Then, by computing the force for each point on the circular path, we were able to observe how the force changes in time for a pulse of force (Fig. 3.4). This method proved to be most efficient as time dependent finite element simulations are computationally expensive.

Therefore, if we, for example, attach 2.8 μm beads to cells, and rotate either a 154° or a 120° arc magnet rotating at 8 or 4 rpm at a magnet-to-bead distance of 2 mm, a force of 70-140 pN is applied to the cells (Fig. 3.4 A, B). Additionally if we attach 4.5 μm beads to cells, and rotate a 60° arc magnet at 5 rpm at a magnet-to-bead distance of 10 mm, a force of 100-130 pN is applied to the cells (Fig. 3.4 B). Although not definitively clear, the variation of the force at different radii is likely due to edge effects. At the edge of the magnet, the gradient of the magnetic flux density (and hence the force) is greater than away from the edge. We will implement the 60 magnet for

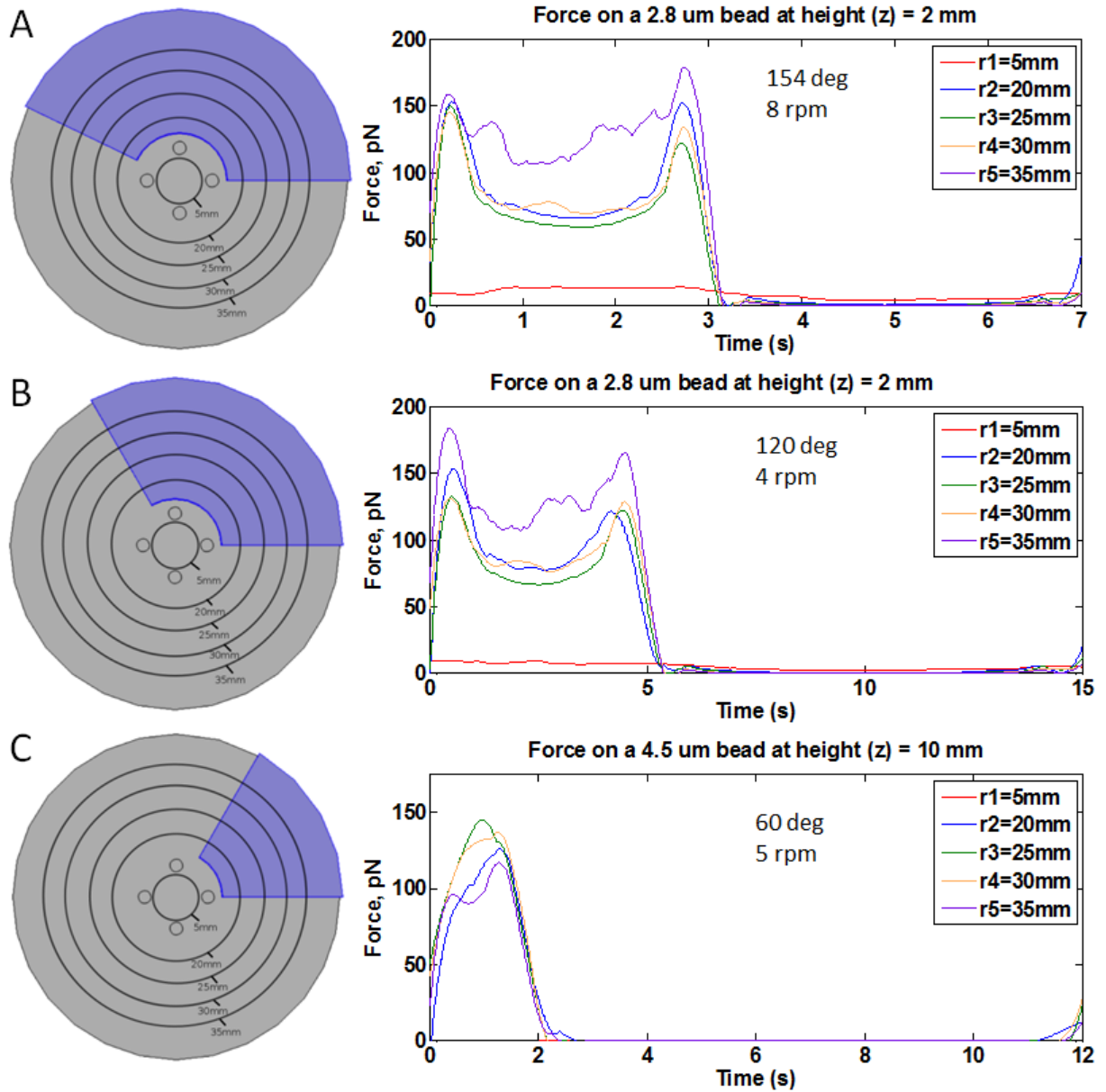


Figure 3.4: Pulsatile force generated by single rotation of permanent magnet. (A) 3.2 sec on, 4.3 sec off force pulse generated by 154° arc magnet for the 5 designated radii. (B) 5 sec on, 10 sec off force pulse generated by 120° arc magnet for the 5 designated radii. (C) 2 sec on, 10 sec off force pulse generated by 60° arc magnet for the 5 designated radii. For each configuration: N52 grade, custom arc magnets, 41 mm O.R. × 11.4 mm I.R. × 12.7 mm thick, $\vec{B}_r = 1.48$ kG; K&J Magnetics Inc. **Data: Ben Rardin**

the application to PECAM-1 signaling explored in Sec. 3.7, 3.8.

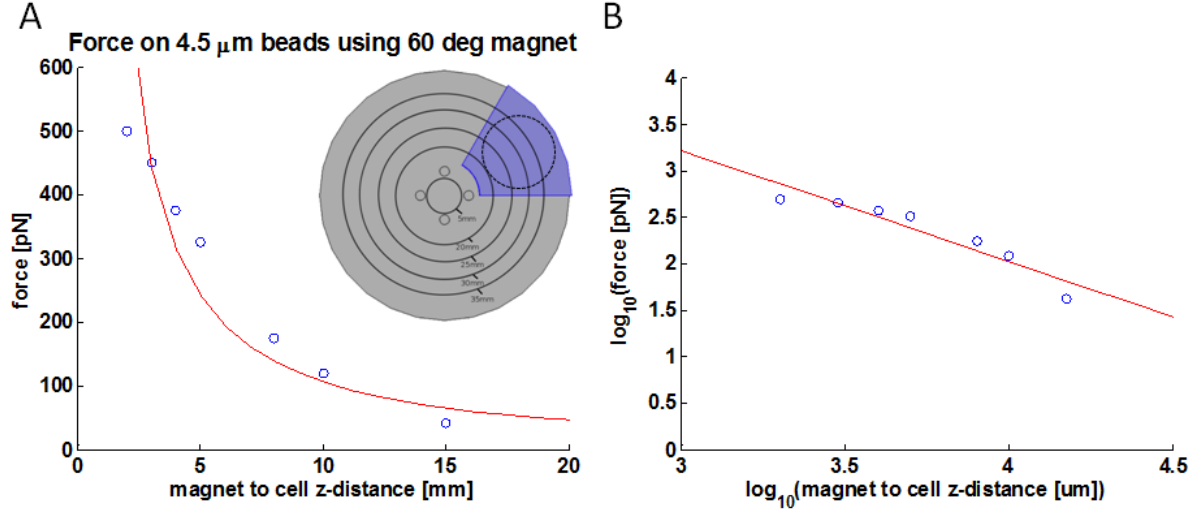


Figure 3.5: **Force versus distance profile for the 60° magnet.** B_z was simulated for variable magnet to cell distances, and F_z was calculated using Eqn. 3.5. B_z and F_z data correspond to the 25mm radius location shown in the inset. Performing a powerlaw fit, we find that $F_z \sim z^{-1.2}$. The force profile is shown in (A) linear and (B) log-log space.

To determine the force profile for the 60° magnet, we simulated the magnetic flux density and calculated the force for several magnet-to-cell distances (Fig. 3.5). We find a powerlaw behavior for the force of $F_z \sim z^{-1.2}$ (Fig. 3.5). We expect the simulated powerlaw for our permanent magnets to underestimate the powerlaw of magnetic tweezers (20 μm -tip; $F_z \sim z^{-2.6}$) (Spero et al., 2008) since permanent magnets has a broader distribution of magnetic material.

3.6 Design of the Constant versus Pulsatile Mechanical Force Experiment

To determine whether cells detect a difference between constant and pulsatile mechanical forces, we designed an experiment where the force magnitude was held constant (120 pN) and the oscillatory nature of the force was varied between a (1) 100% duty cycle, 0 mHz constant force, and a (2) 16.7% duty cycle, 83.3 mHz pulsatile force. Functionally, this was achieved with a 60° arc magnet at a height of 10 mm either in a stationary position (constant force) or rotating at 5 rpm (pulsatile force) (Fig 3.6 A,B). In order to prevent cells in the specimen plate that were not exposed to force (roughly, those outside the 60° arc directly underneath the magnet) from diluting the protein lysate during the biochemical analyses, we created a partition using a rubber mold. Briefly, we removed a 60° section from a 2 mm-thick rubber mold made in an empty 10 cm culture dish, and then plated cells in this section at the same 70% confluence as the rotating condition (Fig 3.6 C). With this consideration, only the cells exposed to the constant force went into constant force cell lysate. During protein-loading in the western blot assay, the protein amount added in the pulsatile force condition lanes were normalized to the amount added in the constant force lanes.

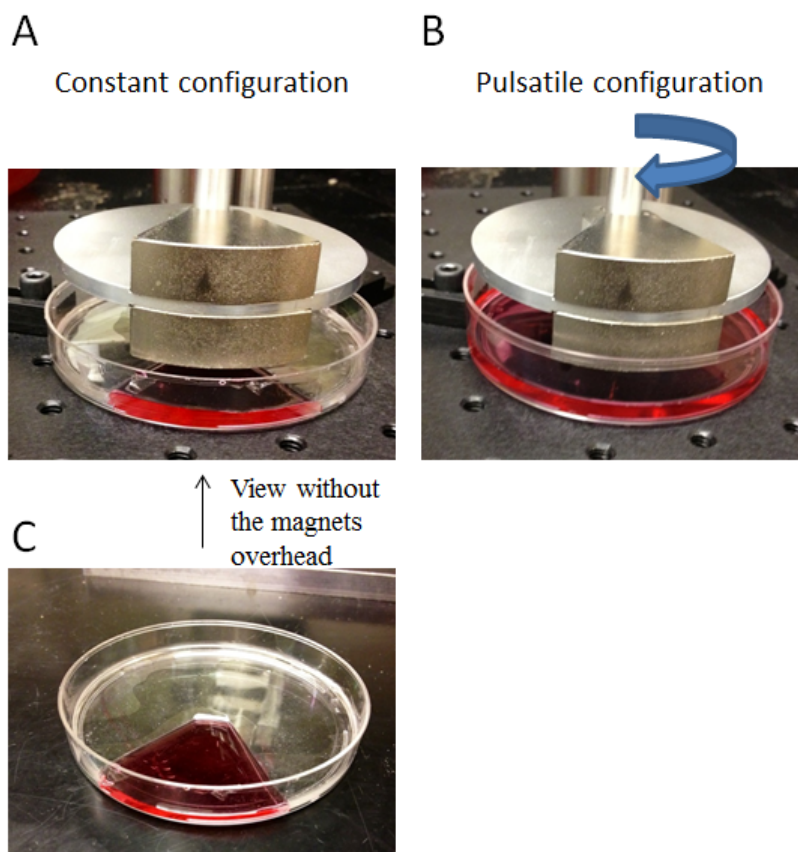


Figure 3.6: **Constant and pulsatile magnet assay configurations.** To detect a possible difference between constant and pulsatile mechanical forces, we held the force magnitude constant at 120 pN (10 mm magnet-to-cell distance). (A) The constant force configuration (100% duty cycle, 0 mHz frequency) is achieved by setting the motor speed to 0 rpm. (B) The pulsatile force configuration (16.7% duty cycle, 83.3 mHz frequency) is achieved by setting the motor speed to 5 rpm. (C) To ensure only the cells exposed to the constant force went into constant force cell lysate, a 60° partition was made using a rubber mold.

3.7 Pulsatile Mechanical Force Enhances ERK and RhoA Activation

In order to assess the biological relevance of this device, we chose to test the system using ECs. ECs are exposed to continuous mechanical stimulation as a result of blood flow, and the nature of the mechanical force can differ (oscillatory vs. laminar) depending on location within the vasculature. Although parallel- and cone-and-plate flow systems have been used to apply laminar or oscillatory shear stress of known magnitudes to ECs, our device offers receptor-pathway specificity by varying the functionalization of the externally attached magnetic beads. Work from our lab and others have indicated that the EC adhesion molecule PECAM-1 plays an essential role in EC mechanotransduction of blood flow (Tzima et al., 2005)(Harry et al., 2008)(Stevens et al., 2008)(Goel et al., 2008). Thus, PECAM-1 is an ideal candidate to study the biological relevance of varying force dynamics.

Mechanical forces trigger the activation of numerous signaling pathways, many of which converge to ultimately regulate the activity of the master regulators of cytoskeletal dynamics, such as the GTPase RhoA (Lessey et al., 2012). Our previous work has shown that force application on PECAM-1 influences effective EC stiffness and that the stiffening response requires the activation of numerous signaling molecules, including ERK and RhoA. Using the magnetic tweezers to apply force on PECAM-1, ECs were observed to significantly increase their effective stiffness within 1 min of force application (Collins et al., 2012), and this stiffening effect required both ERK and RhoA activity. However, when biochemical analyses were performed to investigate

force-dependent activation of these signaling molecules, application of constant force using a permanent magnet did not induce significant activation of ERK or RhoA until later time points (5 or 30 min of force application) (Collins et al., 2012). These data suggested that nature of force application on PECAM-1 (pulsatile vs. constant) might influence the dynamics of downstream signaling. Our device allowed us to directly test this hypothesis by selectively manipulating the duty cycle and frequency of the force, while keeping the force magnitude as well as other experimental variables constant. Here, using a 60° arc magnet that is rotated at 5 rpm (Fig. 3.6 B) and generates a 100 pN force at 10 mm to give a force regimen consistent with the magnetic tweezers in (Collins et al., 2012), we tested the effect of pulsatile force application on dynamics of ERK and RhoA activation.

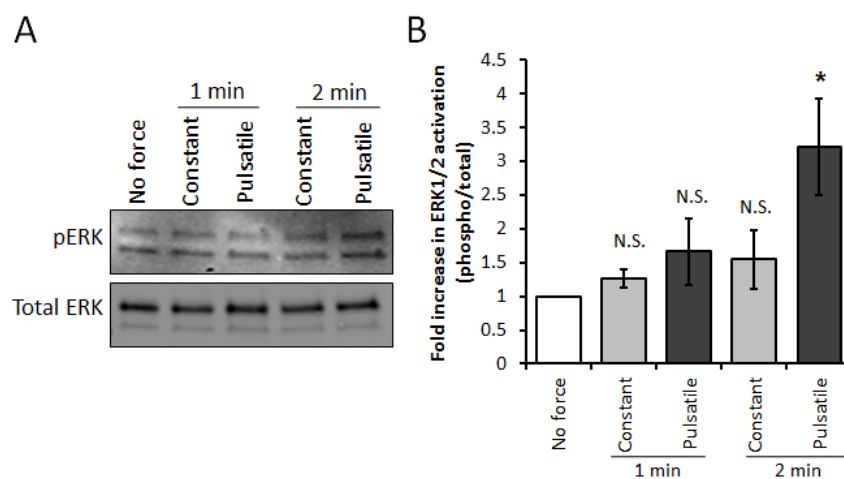


Figure 3.7: Pulsatile mechanical force enhances Erk activation. Adherent ECs on FN were incubated with anti-PECAM-1-coated magnetic beads and subjected to a constant or pulsatile force for either 1 or 2 minutes. (A) Cells were lysed, subjected to SDS-page, and immunoblotted with pERK or ERK antibodies ($n = 3$). (B) Fold increase in activated ERK1/2 was quantified using NIH ImageJ software. Values were normalized to the No Force condition. Bar graphs display averages from at least three independent experiments and error bars indicate s.e.m, $*p < 0.05$. **Data: Caitlin Collins**

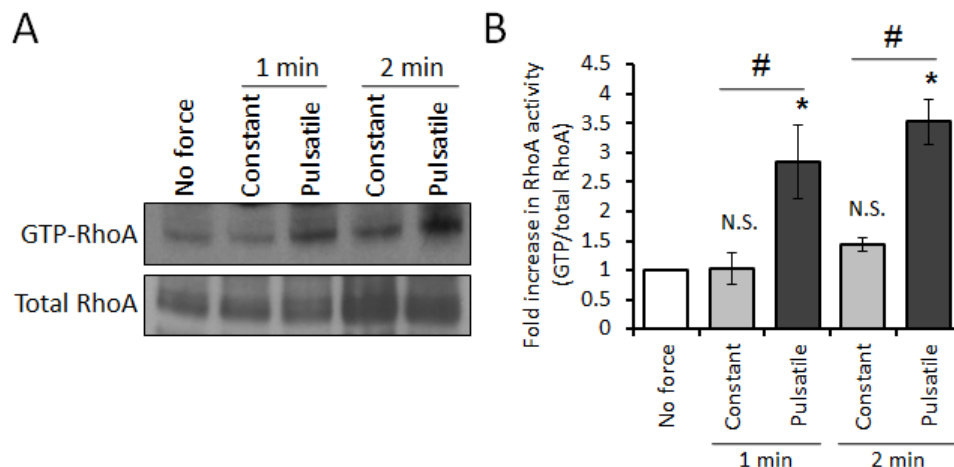


Figure 3.8: Pulsatile mechanical force enhances RhoA activation. Adherent ECs on FN were incubated with anti-PECAM-1-coated magnetic beads and subjected to a constant or pulsatile force for either 1 or 2 minutes. (A) Active RhoA (RhoA-GTP) was isolated with GST-RBD and analyzed by western blot ($n = 3$). Whole cell lysates were subjected to SDS-PAGE to detect Total RhoA protein levels. (B) Fold increase in RhoA activity was quantified using NIH ImageJ software. Values were normalized to the “No Force” condition. Bar graphs display averages from at least three independent experiments and error bars indicate s.e.m, $*p < 0.05$. **Data: Caitlin Collins**

ECs plated were plated on fibronectin (FN)-coated 10cm dishes and incubated with anti-PECAM-1-coated beads for 30 min. Cells were then subjected to constant or pulsatile force using our rotated magnet device. Cells were lysed and processed for immunoblot analysis to assay ERK activation. We did not detect significant activation of ERK activation at 1 or 2 min of force application when the position of the arc magnet was held in a fixed in position to generate a constant force (Fig. 3.7). However, when magnet was rotated in order to produce pulses of forces, ERK was significantly activated after 2 min of force application when compared to the “No force” control (Fig. 3.7).

The small GTPase RhoA is activated in response to force (Collins et al., 2012)(Guiluy et al., 2011)(Osborne et al., 2014) and RhoA activity is required for the cellular

response to tension on PECAM-1 (Collins et al., 2012). In order to test the hypothesis that dynamic force application accelerates activation of RhoA, cells were incubated with anti-PECAM-1-coated beads and subjected to constant (fixed magnet) or pulses (rotating magnet) of force. Rho pulldowns were performed to assess the population of activated, GTP-bound RhoA and lysates were subjected to western blot analysis. Consistent with our previous work, we were unable to detect significant RhoA activation when force was applied to PECAM-1 for 1 or 2 min (Fig. 3.8). In contrast, RhoA activity quickly increased by 3-fold in as little as 1 minute of pulsatile force application, when compared to the “No force” control (Fig. 3.8). Force-dependent ERK activation has been shown to influence RhoA activity by activating the guanine nucleotide exchange factor GEF-H1 (Collins et al., 2012)(Guilluy et al., 2011). Here, we see significant RhoA activation at earlier time points than ERK activation in response to pulsatile force. However, this difference in the timescale of significant Erk phosphorylation (2 minute) and RhoA activation (1 minute) may be due to the fact that other GEFs are known to active RhoA in response to force, such as LARG (Guilluy et al., 2011). These results strongly suggest other proteins in the RhoA pathway are sensitive to pulsatile mechanical forces and may be contributing to early activation of the GTPase.

3.8 Pulsatile Mechanical Force Enhances Basal Focal Adhesions

RhoA activation mediates the adaptive stiffening response by influencing growth of focal adhesions (FAs), which function to resist the applied force (Chachisvilis et al., 2006). Our results indicate that pulsatile force on PECAM-1 resulted in enhanced RhoA activation when compared to constant force application (Fig. 3.8). Therefore, we hypothesized that pulsatile force application would also stimulate rapid assembly and growth of FAs. In order to test this hypothesis, ECs were subjected to constant or pulsatile force using our device for various time points, and subsequently fixed and stained with anti-vinculin antibodies to highlight the FAs (Fig. 3.9). The number of focal adhesions significantly increased compared to the “No force” condition after 1 minute of pulsatile force application (Fig. 3.10 A), and FA size significantly increased after 2 minutes of pulsatile force application (Fig. 3.10 B). Neither FA size nor FA number significantly changed when ECs were subjected to 1 or 2 min of constant force, suggesting that FAs growth is tightly regulated depending on the nature of applied force.

Fig. 3.11 shows a model of the PECAM-1-mediated signaling observed in this study.

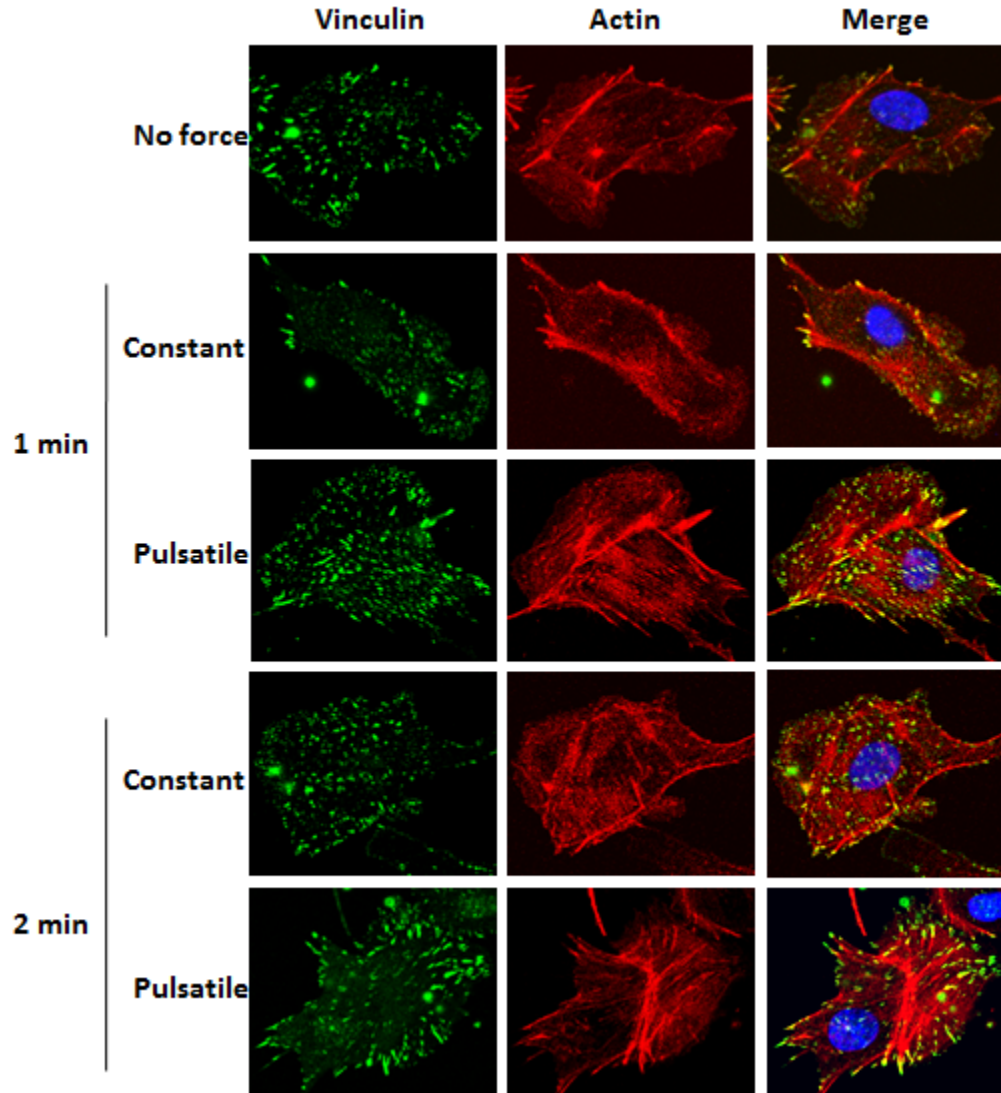


Figure 3.9: **Pulsatile mechanical force enhances growth of focal adhesions.** Adherent ECs on FN or CL were incubated with anti-PECAM-1-coated magnetic beads and subjected to a constant or pulsatile force for either 1 or 2 minutes. ECs were fixed stained with phalloidin and an anti-vinculin antibody to mark focal adhesions. Images are representative of three independent experiments. **Data: Caitlin Collins**

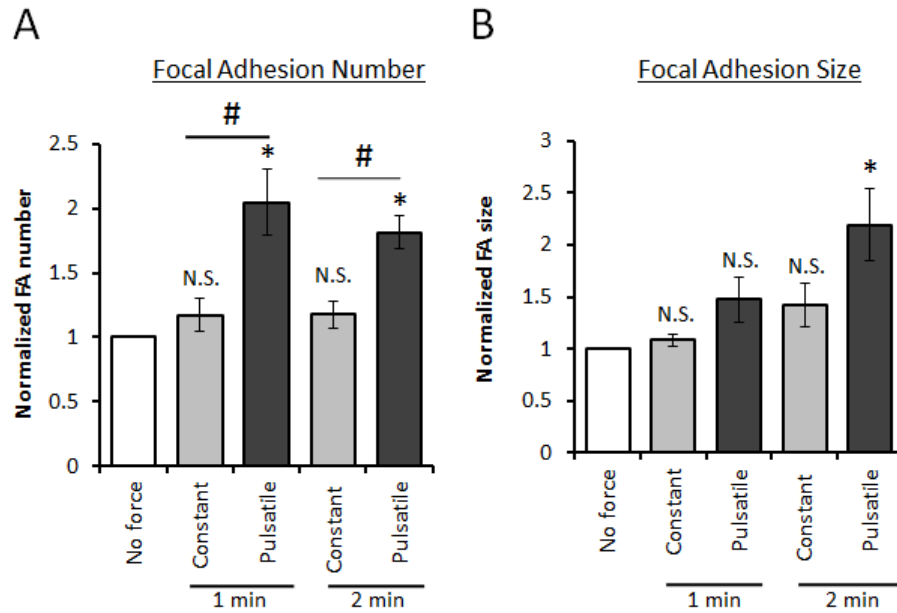


Figure 3.10: **Pulsatile mechanical force increases focal adhesion number and size.** (A) Focal adhesion number and (B) size were quantified using NIH ImageJ software. Values were normalized to the FN "No Force" condition. * $p < 0.05$ (compared to No force). # $p < 0.05$ (compared within the same force time point). Bar graphs display averages from at least three independent experiments and error bars indicate s.e.m, * $p < 0.05$. **Data: Caitlin Collins**

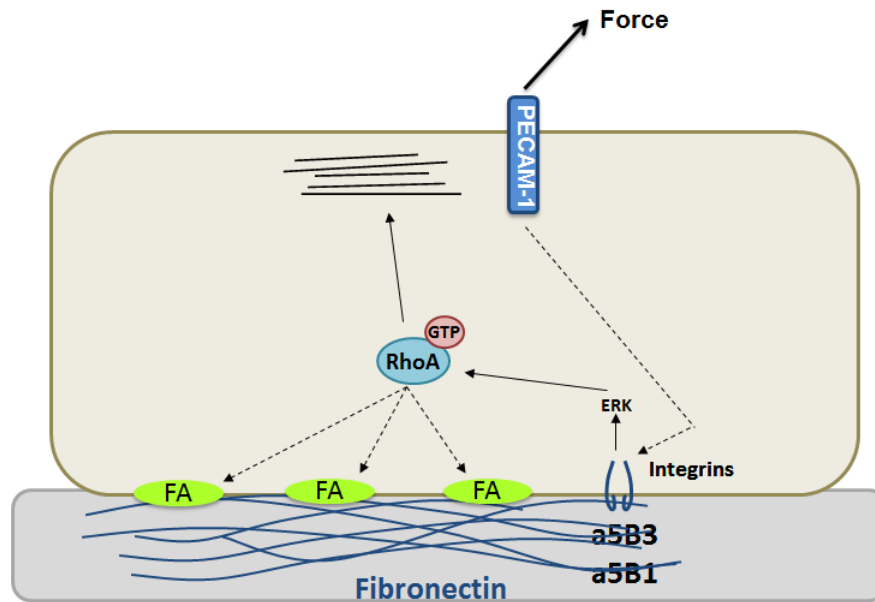


Figure 3.11: **Model of PECAM-1-mediated mechanotransduction.**

3.9 Discussion

Our results illustrate the utility of our simple device to apply realistic force regimes to large populations of cells required for the assessment of biochemical changes related to cell signaling in response to applied forces. We have shown that cells, in this case EC cells, respond much more rapidly to pulsatile forces than to constant forces. If the same phenomenon applies to other cell types, it would help explain the longer times needed to see biochemical changes in other studies (Guilluy et al., 2011). Our observation of cells being able to distinguish between constant and pulsatile forces opens up a large parameter space of investigation:

Force-dependent ERK activation has been shown to influence RhoA activity by activating the guanine nucleotide exchange factor GEF-H1 (Collins et al., 2012)(Guilluy et al., 2011). Here, we see significant RhoA activation at earlier time points than ERK activation in response to pulsatile force. However, this difference in the timescale of significant Erk phosphorylation (2 minute) and RhoA activation (1 minute) may be due to the fact that other GEFs are known to active RhoA in response to force, such as LARG (Guilluy et al., 2011). These results strongly suggest other proteins in the RhoA pathway are sensitive to pulsatile mechanical forces and may be contributing to early activation of the GTPase.

1. Do different force regimens invoke completely different pathways or just influence the activation dynamics of the same pathways?
2. Are all mechanoreceptors on ECs able to distinguish between constant and pul-

satile forces?

3. Here, we see significant RhoA activation at earlier time points than ERK activation in response to pulsatile force. Since RhoA activation downstream of integrin activation has been shown to be regulated by Fyn-LARG and ERK-GEF-H1 (Guilluy et al., 2011), is Fyn also being rapidly activated within 1 min of force on PECAM-1? Are there other GEFs involved in rapid RhoA activation?
4. Are there mechanoreceptors that are universally sensitive or insensitive to constant or pulsatile forces?
5. In Ch. 5, we show that pre-EMT cells respond different to pulsatile force on integrins than post-EMT cells. How do these cells respond to a constant force? Or to pulsatile/constant forces on different mechanoreceptors.

Chapter 4: Novel Applications of Microrheology to Cell Mechanics

4.1 Overview

In this chapter, we discuss several applications of microrheology toward novel projects in cell mechanics. Within the chapter, four peer-reviewed publications I contributed to will be discussed. Data collected by colleagues are acknowledged in figure legends; my contributions to the projects are described below.

1. **PECAM-1 collaboration.** We first examine how external force on PECAM-1 and extracellular matrix cues regulate the stiffness of aortic endothelial cells *in vitro* and *in vivo*; this work studies the same model system that was examined in Ch. 3 for our application of the rotating magnet device. In the aorta project, I designed and performed the *in vivo* passive microrheology experiments and analyzed the data. The citation for this publication is: C. Collins, L.D. Osborne, C. Guilluy, Z. Chen, E.T. O'Brien, III, K. Burridge, R. Superfine, E. Tzima. "Haemodynamic and extracellular matrix cues regulate the mechanical phenotype and stiffness of endothelial cells". Nature Communications. doi: 10.1038/ncomms4984 (2014). Referenced in text as (Collins et al., 2014).
2. **Nuclei collaboration.** We then examine the stiffness response of nuclei that have been isolated from cells. In this project, I conducted training of the magnetic tweezer-

ers methodology (instrument and data selection criteria), performed force calibrations, and provided stiffness values for nuclei under different conditions. The citation for this publication is: C. Guilluy, L.D. Osborne, L. Van Landeghem, L. Sharek, R. Superfine, R. Garcia-Mata, K. Burridge. “Isolated nuclei adapt to force and reveal a mechanotransduction pathway within the nucleus”. *Nature Cell Biology*. doi: 10.1038/ncb2927 (2014). Referenced in text as (Guilluy et al., 2014).

3. **ICAM-1 collaboration.** We then examine inflammation signaling downstream of adhesion molecule ICAM-1. In this project, I assisted with compliance signature selection criteria, performed force calibrations, and provided data analysis. The citation for this publication is: E.C. Lessey, L.D. Osborne, E. Monaghan-Benson, C. Guilluy, E.T. O'Brien, III, R. Superfine, K. Burridge. “The RhoA GEF, LARG, mediates ICAM-1-dependent mechanotransduction in endothelial cells to stimulate transendothelial migration”. *Journal of Immunology*. doi: 10.4049/jimmunol.1302525 (2014). Referenced in text as (Lessey-Morillon et al., 2014).

4. **Melanoma collaboration.** Finally, we examine the role two transcription factors, HIF1 α and HIF2 α , in cancer progression. In this project, I performed passive microrheology experiments and analyzed the data. The citation for this publication is: S.C. Hanna, B. Krishnan, S.T. Bailey, S.J. Moschos, P. Kuan, T. Shimamura, L.D. Osborne, M. Siegel, L. Duncan, E. Tim O'Brien III, R. Superfine, C. Miller, M. Simon, K. Wong, W.Y. Kim. “HIF1 and HIF2 independently activate SRC to promote melanoma metastases”. *Journal of Clinical Investigation*. doi:

10.1172/JCI66715 (2013). Referenced in text as (Hanna et al., 2013).

4.2 Force and ECM matrix cues regulate cellular mechanical phenotype

Here, we examine how external force on platelet endothelial cell adhesion molecule (PECAM)-1 and extracellular matrix cues regulate the mechanical properties of aortic endothelial cells *in vitro* and *in vivo*.

Endothelial cells (ECs) lining the walls of the vascular system sense external forces, such as shear stress due to blood flow and tension due to blood pressure, with mechanosensor proteins such as integrins (Jalali, 2001)(Chachisvilis et al., 2006) and (PECAM)-1 (Tzima et al., 2005)(Collins et al., 2012)(Collins et al., 2014). In addition to mechanical signals, ECs also receive cues from the extracellular matrix (ECM). Although most of the vasculature is dominated by collagen (CL) and laminins, distinct regions have concentrated amounts of fibronectin (FN). In response to force, these variations in ECM composition have been shown to induced ECM-specific intracellular signaling cascades in ECs in different regions of the vascular system. For example, shear stress has been shown to activate protein kinase A (PKA) in ECs adherent to CL, whereas PKA activation is unaffected in ECs under force when adherent to FN (Funk et al., 2010). Here, we examine how ECM composition and external force on PECAM-1 regulate the mechanical phenotype of aortic ECs.

To examine the role of ECM composition on the stiffness response to force on PECAM-1 *in vitro*, we used magnetic tweezers to apply force to anti-PECAM-1 coated beads which were incubated over cells plated on FN or CL. We find that ECs plated on FN exhibit a stiffness response (indicated by decreased bead displacement) after

approximately 40 sec of pulsatile force (Fig. 4.1 A). In contrast, bead displacement of ECs plated on CL were unaffected after pulsatile force (Fig. 4.1 A). These results indicate that ECM composition plays an integral role in mechanoreponse to force on PECAM-1.

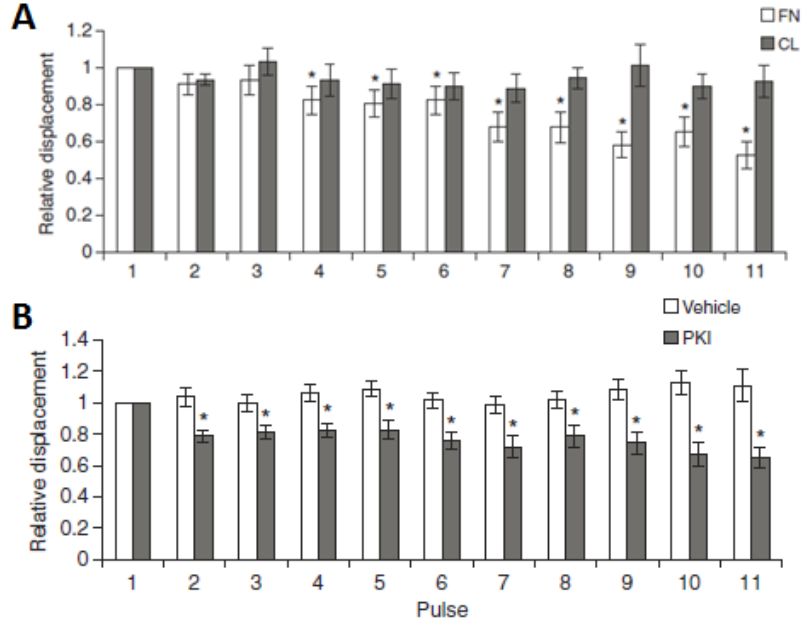


Figure 4.1: **ECM composition determines stiffness response to force on PECAM-1.** (A) Using magnetic tweezers, a 2 sec, 100 pN pulse of force, followed by a 10 sec period of rest, was applied over a 2 min time course. The average relative displacement (from first pulse) was measured for $2.8\mu m$ anti-PECAM-1 coated beads on ECs on either FN or CL ($n > 30$ per condition from 3 independent experiments; Error is SEM, $p < 0.5$). (B) Average relative displacement for anti-PECAM-1 coated beads on ECs pretreated with PKI ($20\mu M$) or vehicle control for 1 hr ($n > 20$ per condition from 3 independent experiments; Error is SEM, $p < 0.5$). **Data: Caitlin Collins.**

Previously, PECAM-1-mediated stiffening response of ECs on FN was shown to be dependent on phosphoinositide 3-kinase (PI3K) regulation of basal integrin activation and activation of RhoA (Collins et al., 2012). Because RhoA is known to be important for the stiffening response to force in other model systems (Matthews et al.,

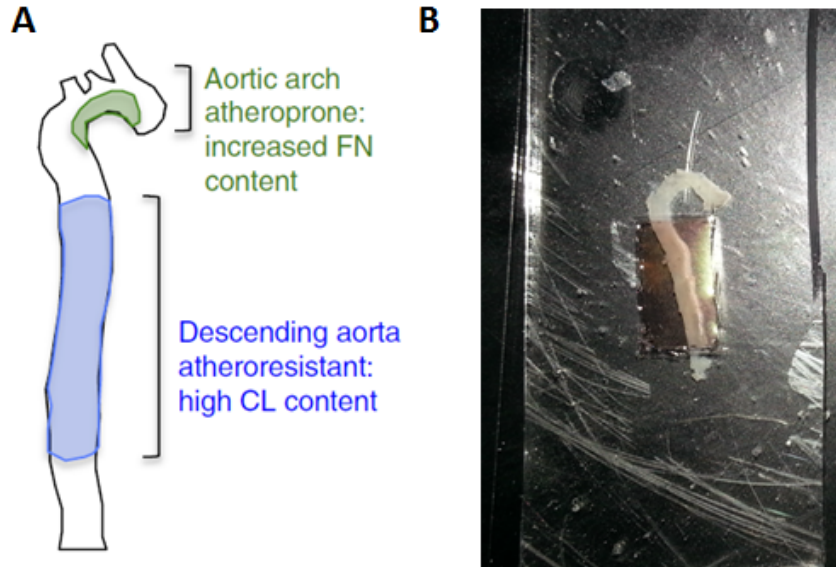


Figure 4.2: **Experimental setup for *en face* mechanical characterization of aortic ECs.** (A) Schematic showing the aortic arch (rich in FN) and descending aorta (dominated by CL) regions of the tissue. Cartoon from (Collins et al., 2014). (B) The aorta was freshly isolated, cut longitudinally and mounted *en face* on a glass cover slip with the endothelium facing up. To anchor the aorta to the cover slip for experiments, a thin sheet (20 x 40 x 2 mm) of polydimethylsiloxane (PDMS) rubber was positioned over the tissue. A small section (3 x 5 mm) of the PDMS sheet was removed before anchorage to serve as a media reservoir for the region of interest (here, the descending aorta). 4.5 μ m FN-coated beads were incubated over the endothelium for 20 min at 37 C.

2006)(Guilluy et al., 2011), we hypothesized RhoA activity may be attenuated when ECs are on CL. Given that RhoA activation is known to negatively regulated by PKA through phosphorylation, we treated ECs on CL with the PKA inhibitor (PKI) and tested for RhoA activation in response to force from a permanent magnet; we found PKI to reverse PKA suppression of RhoA activity (Collins et al., 2014). To test the effect of PKI treatment on stiffness response, we used magnetic tweezers to apply force to anti-PECAM-1 coated beads which were incubated over cells plated on CL. We find that ECs plated on CL exhibit a stiffening response with PKI treatment compared to the vehicle control (Fig. 4.1 B).

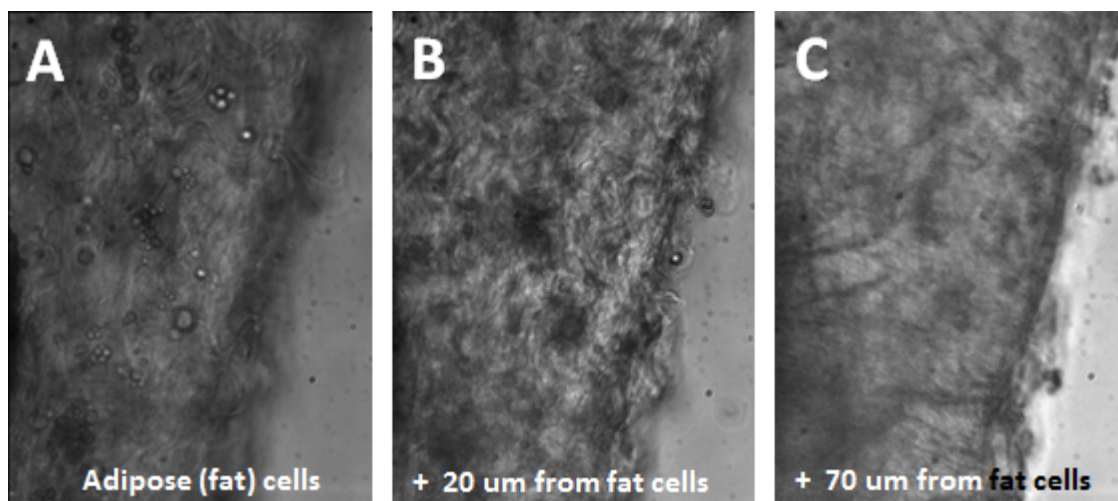


Figure 4.3: **Imaging through layers of aortic tissue.** (A) Adipose cells are found in the outer most layers. (B) Collagen I and IV dominate the ECM composition of the descending aorta, shown here $20\mu\text{m}$ from the adipose cells. Fibroblast cells exist in this layer. (C) Monolayer of endothelial cells over a thin ECM layer and smooth muscle cells, shown here $70\mu\text{m}$ from the adipose cells.

To determine the physiological relevance of these findings, we implemented external PBR to determine whether PKA plays a role in defining EC stiffness *in vivo*. The descending aorta (rich in CL composition) was freshly isolated from control or PKI-

treated mice two hours after injection and prepared *en face* to expose the endothelium for passive microbead rheology measurements (Fig. 4.2 A,B). The descending aorta was incubated with FN-conjugated beads in order to establish integrin-mediated attachments with the cortical actin cytoskeleton. Figure 4.3 shows the monolayer of endothelial cells above a layer of adipose cells and a layer rich in CL. Passive motion of the beads were tracked and the resulting mean-squared displacement (MSD) was calculated for control or PKI-treated aortas (Fig. 4.4 A, B). Because our *in vitro* data showed that force on PECAM-1 resulted in a stiffness response on FN, and that cells did not stiffen in response to force on CL, we hypothesized that ECs located in the aortic arch (rich in FN composition) would exhibit increased stiffness compared to ECs in the descending aorta. Thus, we repeated the PBR measurement for ECs in the aortic arch (Fig. 4.4 D). Ensemble-averaging of the bead populations revealed a significant decrease in the MSD of beads on PKI-treated aortas compared to control aortas and towards that of the MSD of beads on the aortic arch (Fig. 4.4 E,F). These data indicate that ECs of the FN-rich aortic arch are stiffer than ECs of the CL-rich descending aorta, and that PKA plays a role in defining EC compliance in the descending aorta.

The results of this study suggest that cells integrate external mechanical and biochemical cues to modulate their own mechanical properties *in vivo*. Because the FN-rich aortic arch is prone to inflammation and atherosclerosis, and the CL-rich descending aorta is atheroresistant, these results identify PKA as a potential atheroprotective mechanism.

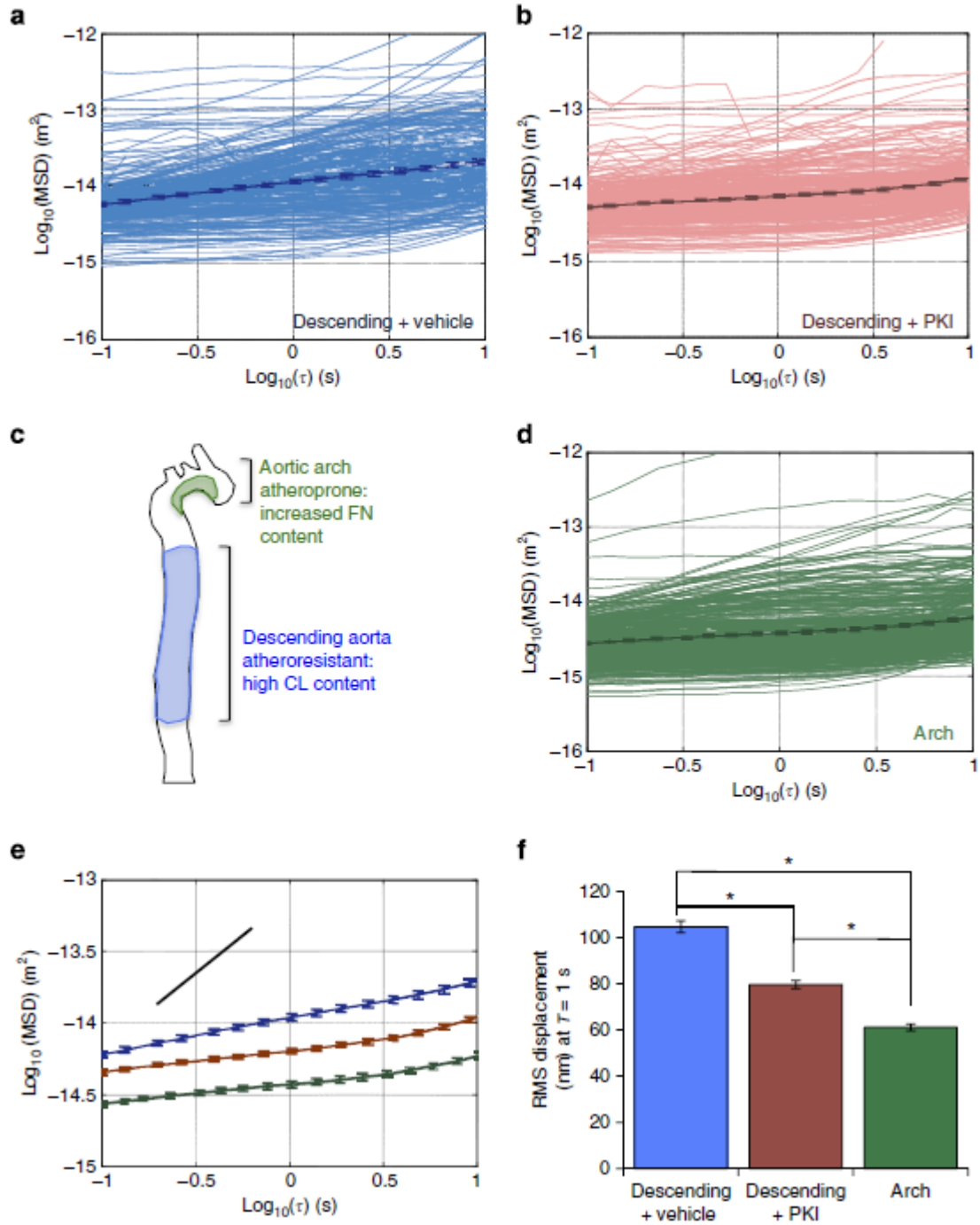


Figure 4.4: **The PKA pathway promotes EC compliance in atheros resistant regions of the aorta.** (A,B,D) MSD trajectories of FN coated, 4.5 μ m beads attached to (A) the atheroresistant/descending region, (B) the descending region from PKI-treated mice, and (D) the atheroprone/aortic arch region of the aorta. MSDs of individual curves ($n > 350$ per condition, aggregated from 3 mice, $p < 0.0001$) are shown in light color and the ensemble average is represented by the dark curve with SEM shown for the indicated timescales. (C) Schematic showing ECM heterogeneity in the aorta. (E) Ensemble-average MSDs of beads attached to the endothelium of aortic preparations. (F) RMS displacement at the 1 sec timescale for the indicated regions.

4.3 Isolated nuclei exhibit a stiffening response to external force

As described in Ch. 1 and Fig. 1.1, mechanical forces can be transduced into biochemical signals at the cell membrane, or before transduction, forces can be transmitted via the cytoskeleton to distant sites in the cell such as cell-cell junctions or the nucleus. Although force is known to be transmitted to the nucleus (Wang et al., 2009), the mechanical response is not known.

To examine the effect of mechanical stimulation on isolated nuclei, we used magnetic tweezers to apply 35 pN pulses of force to anti-nesprin-1 coated beads bound to nuclei of HeLa cells (Fig. 4.5 A,B). Under force application, nuclei exhibited a viscoelastic response (Fig. 4.5 C). Analysis of the average bead displacement over time revealed that nuclei increased their stiffness within 15 sec of pulsatile force application on nesprin-1 (indicated by decreased displacement), whereas force applied to poly-L-lysine bound beads (charge-based attachment) did not show this effect (Fig. 4.5 C). To determine whether the stiffness response was specific to isolated nuclei from HeLa cell, we repeated the magnetic tweezers assay for isolated nuclei from fibroblasts (MRC5) and endothelial cells (HUVEC). Results showed a similar stiffness response in nuclei in these cells.

Investigation into the mechanisms that regulate the nuclear stiffness response to force revealed that emerin, a inner nuclear membrane protein, and nuclear lamina are required (Guilluy et al., 2014). This dependance is shown in Fig. 4.6 A,B,C. This study demonstrated that mechanotransduction occurs at the nucleus.

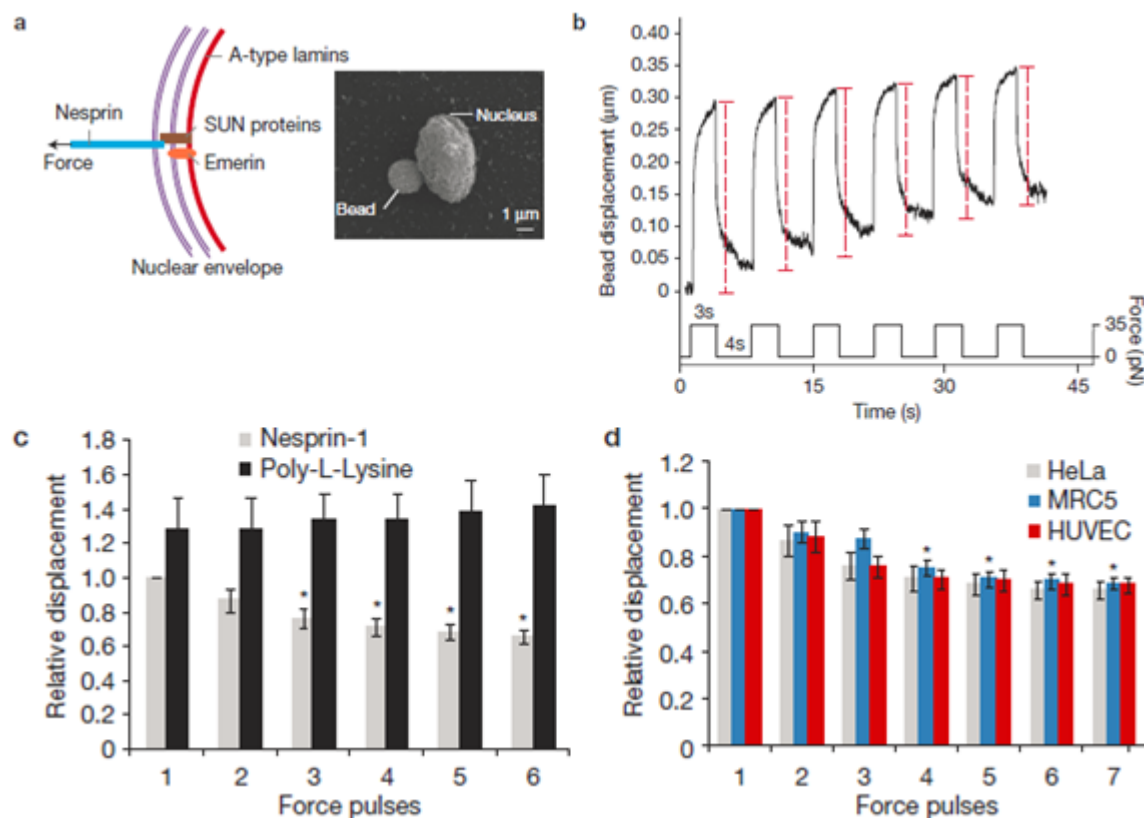


Figure 4.5: Isolated nuclei exhibit a stiffening response to force on nesprin-1. (A) Schematic showing force applied to nesprin-1 of the linker of nucleoskeleton and cytoskeleton (LINC) complex, and a scanning electron micrograph of a bead attached to an isolated nucleus. (B) Magnetic tweezers were used to apply 35 pN pulses of force in a 3 sec on, 4 sec off regimen to 2.8 μm anti-nesprin-1 coated bead attached to an isolated nucleus. Typical displacements exhibited a viscoelastic response. (C) Average relative displacement for anti-nesprin-1 coated beads ($n = 18$) or poly-L-lysine coated beads ($n = 14$) on nuclei isolated from HeLa cells. (data from 3 independent experiments; Error is SEM, $p < 0.05$). (D) Average relative displacement for anti-nesprin-1 coated beads on nuclei isolated from HeLa cells ($n = 18$), MRC5 cells ($n = 21$), and HUVECs ($n = 15$). (data from 3 independent experiments; Error is SEM, $p < 0.05$). **Data: Christophe Guilluy**

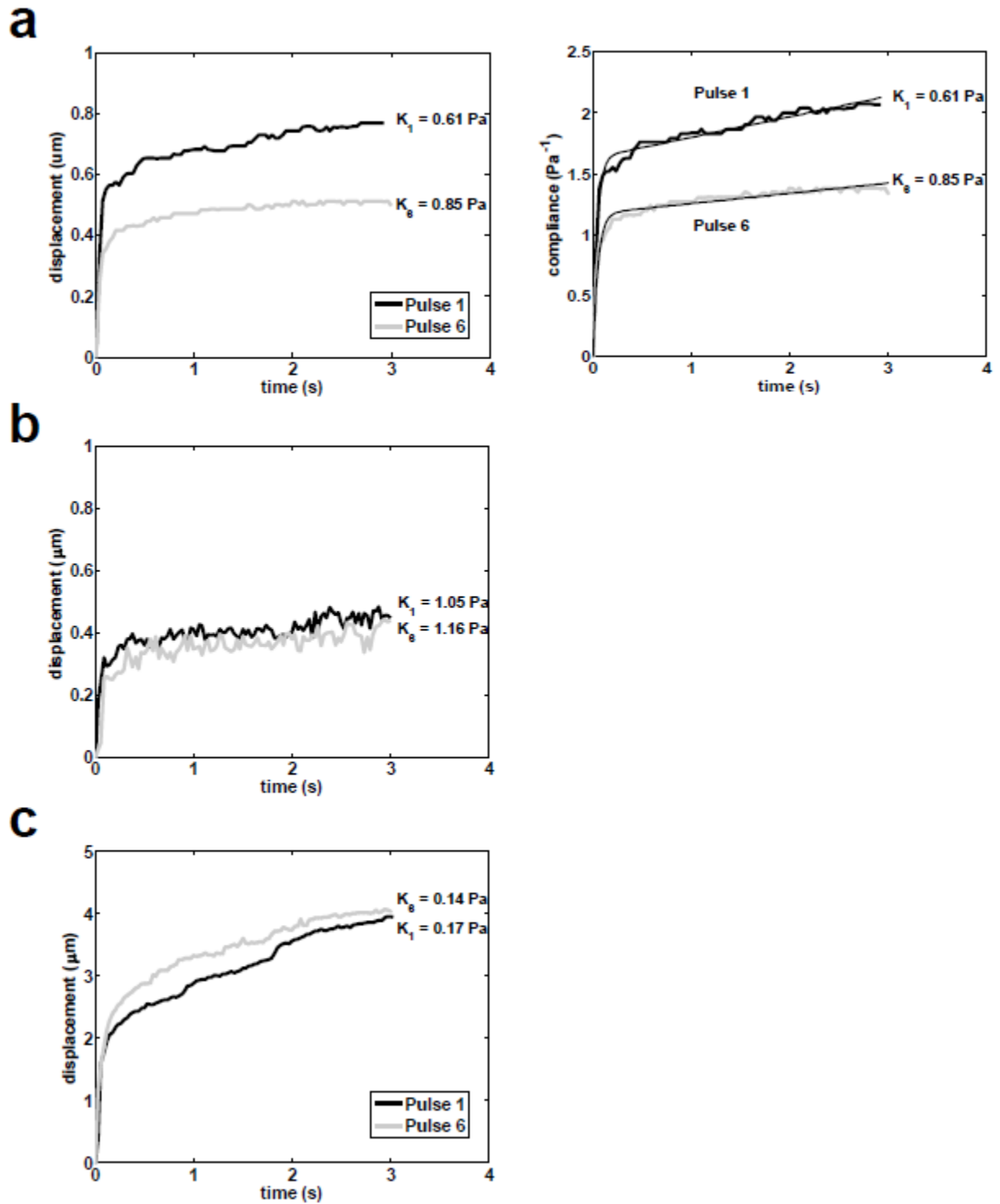


Figure 4.6: **Increased stiffness in response to force depends on emerin and lamin A/C.** Stack plot of typical bead displacements for (A) control shRNA, (B) emerin knockdown, and (C) lamin A/C knockdown. Stiffness, K , determined by Jeffrey model fit to compliance (A, right) is provided for each displacement.

4.4 LARG activates RhoA to affect stiffening response to force on ICAM-1

During the inflammatory response, the expression of many adhesion molecules is increased, including intercellular adhesion molecule (ICAM)-1. Before leukocytes can reach sites of injury or infection they bind and crawl along the endothelium, and then cross via junctions or through the body of the endothelial cells (ECs). This process is called transendothelial migration (TEM). Leukocytes crawl along endothelial cells by applying tractional forces to ICAM-1 receptors (Oh et al., 2007). Although RhoA signaling is known to be activated downstream of force on ICAM-1, little is known about its regulation and the effect of force on ECs.

To examine the effect of mechanical stimulation on neonatal human dermal blood microvascular ECs (HMVECs), we used magnetic tweezers to apply 160 pN pulses of force to anti-ICAM-1 coated beads (Fig. 4.7 A). Analysis of the average bead displacement over time revealed that HMVECs increased their stiffness within 10 sec of pulsatile force application on ICAM-1 (indicated by decreased displacement), and that this stiffness response was diminished by disrupting the actin cytoskeleton or the RhoA pathway with several pharmaceutical interventions (Lessey-Morillon et al., 2014).

Additional experiments revealed that RhoA is activated (within 1 min) in response to force on ICAM-1 (Lessey-Morillon et al., 2014), we sought to determine the GEF mechanisms by which RhoA is activated. Several candidate GEFs were tested but only LARG was observed to mediate RhoA activation downstream of force on ICAM-1 (Lessey-Morillon et al., 2014). To investigate the effect of LARG on stiffness and

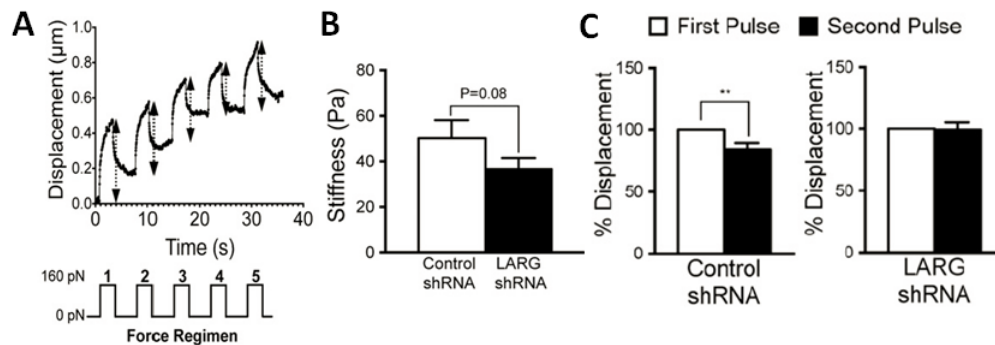


Figure 4.7: The RhoA GEF LARG regulates the stiffness response to force on ICAM-1. (A) Magnetic tweezers were used to apply 160 pN pulses of force in a 3 sec on, 5 sec off regimen to 4.5 μm anti-ICAM-1 coated beads attached to HMVECs. Typical displacements exhibited a viscoelastic response. (B) Average stiffness of HMVECs treated with a control or LARG shRNA lentivirus, and then probed with anti-ICAM-1 coated beads. (C) Relative displacement of anti-ICAM-1 coated beads on control or LARG shRNA treated HMVECs ($n \geq 15$). (Error is SEM, $p \leq 0.01$) **Data: Elizabeth Lessey**

stiffness response, we used a shRNA to decrease LARG expression. We observed that loss of LARG expression reduced HMVEC stiffness ($p = 0.08$; Fig. 4.7 B), and that the significant stiffness response to force was lost after LARG depletion (Fig. 4.7 C). These results suggest that LARG activates RhoA in response to force on ICAM-1 to regulate a stiffening response.

Further experiments revealed that depletion of LARG reduces both neutrophil (a type of leukocyte) migration velocity across a monolayer of HMVECs and passage through the monolayer (TEM decreased $\sim 35\%$) (Lessey-Morillon et al., 2014). Although RhoA activity is known to contribute to TEM by weakening EC junctions (Aghajanian et al., 2008), this work provides the first evidence that LARG activation of RhoA may promote neutrophil TEM by increasing the stiffness of the EC surface.

4.5 HIF1 α and HIF2 α are sufficient to promote cancer phenotypes

During hypoxia (low oxygen conditions), transcription factors called hypoxia-inducible factors (HIFs) upregulate genes to promote cell survival. For example, cells within a growing cancerous tumor often experience hypoxic conditions, and accordingly, HIF activation has been shown in numerous cancers (Semenza, 2003). HIFs promote metastatic progression by transcriptional regulation of genes that mediate ECM degradation, the epithelial to mesenchymal transition (EMT), and cell adhesion and motility (Semenza, 2003). Here, our goal was to study HIFs in melanoma.

While initial results showed that HIF1 α and HIF2 α are necessary for hypoxia-dependent invadopodia formation and cell invasion (Hanna et al., 2013), we sought to determine whether these HIFs were sufficient to drive cell invasion and wanted to examine their effect on cell stiffness. Thus, we tested the invasion of human melanoma A-375 cells that were transfected with control EGFP, HIF1 α (HIF1dPA), or HIF2 α (HIF2dPA). The HIF cells remain stable under normoxia (normal oxygen conditions; typically, HIFs are degraded under these conditions). Assessing invasion revealed significant increases for HIF1dPA and HIF2dPA compared to the EGFP control (Fig. 4.8 A,B). Next, we tested cell stiffness by magnetic tweezers (Fig. 4.8 C) and PBR (Fig. 4.8 D,E) and found that HIF1dPA and HIF2dPA significantly decrease stiffness compared to the EGFP control. Together, these results indicate that HIF1 α and HIF2 α are sufficient to promote cancer phenotypes.

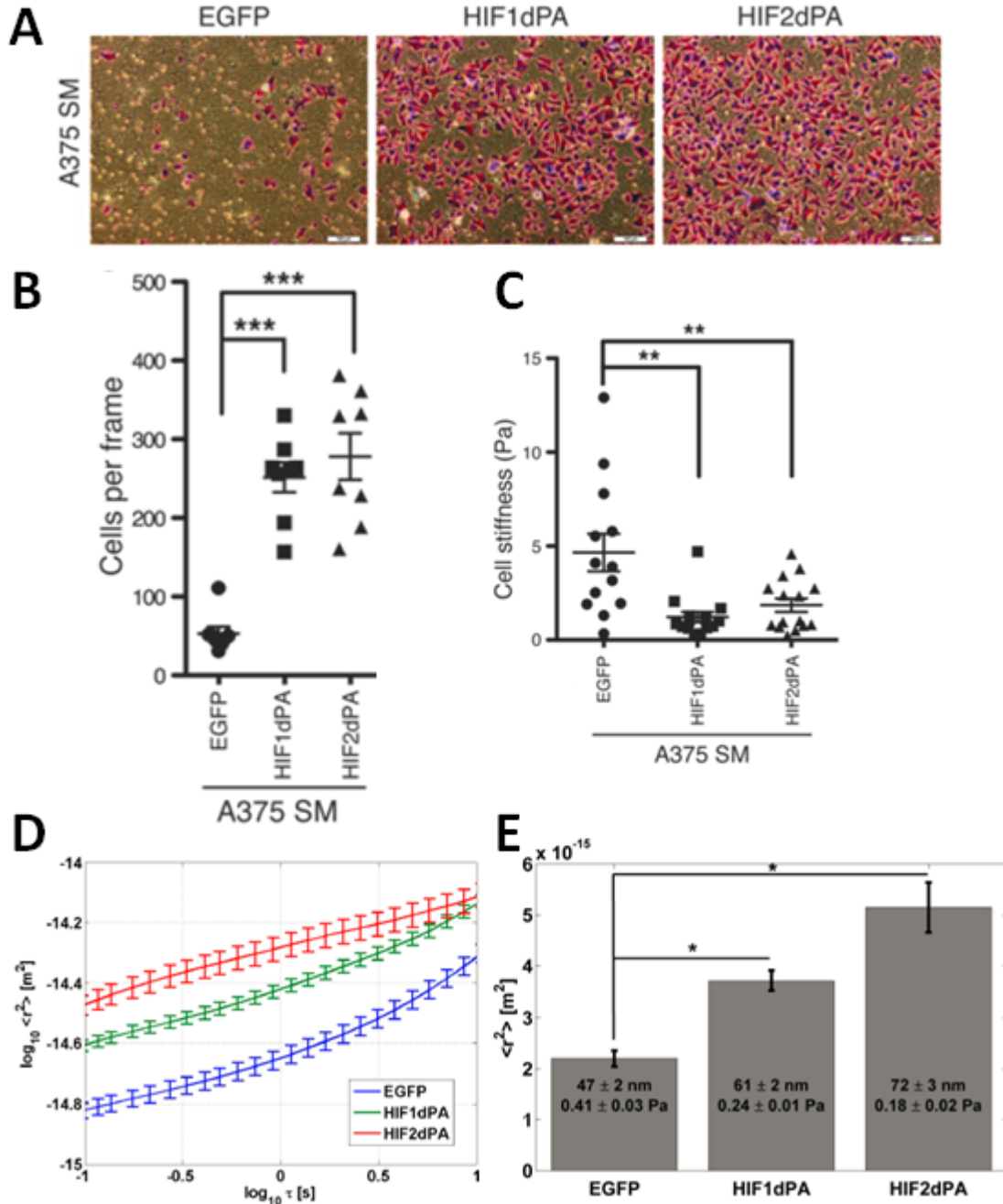


Figure 4.8: **HIF1 α and HIF2 α are sufficient to increase cell invasion and decrease cell stiffness.** (A) Representative images of cells transfected with control EGFP, HIF1dPA, or HIF2dPA after invasion through Matrigel chambers. (B) Quantification of invasion assay. (C) Magnetic tweezers were used to apply a 50-100 pN pulse of force for 5 sec to 4.5 μ m FN-coated beads. (D) Average MSD vs τ for cell populations. (E) Average MSD (inset: RMS displacement, stiffness) at the $\tau = 1$ sec timescale. (Error is SEM, *** $p < 0.0005$, ** $p < 0.005$, * $p < 0.05$) **Data in (A,B): Sara Hanna; Data in (C): Tim O'Brien**

Chapter 5: Cell Mechanics and the Epithelial to Mesenchymal Transition

5.1 Overview

Recent work has shown that invasive cancer cells have a reduced stiffness and exert larger forces on their environment. Although these studies suggest a role for cell mechanics in cancer progression, little is known about the molecular mechanisms that regulate mechanics during cancer. To address this need, we turned our attention to the epithelial to mesenchymal transition (EMT). EMT is a well characterized process in embryogenesis and wound healing, and recently has been implicated as a model for the physical detachment of cancer cells before they begin to metastasize. While classic EMT hallmarks include loss of cell-cell adhesions, morphology changes, and increased invasion capacity, little is known about the associated mechanical changes. Therefore, in this chapter, we ask the following questions:

1. Is there a mechanical phenotype adopted during EMT?
2. If so, what are the biochemical mechanisms responsible for the alternations in cell mechanics?

To answer these questions, we apply a multi-assay approach to investigate, for the first time, the mechanical phenotype associated with growth factor induced EMT. We employ active and passive microrheology assays to characterize cell stiffness and stiffness

response to externally applied force before and after this cancer transition. Using the novel rotating magnet device discussed in Ch. 3, we are able to study the molecular mechanisms behind the mechanical characteristics that cells adopt during EMT. We carefully execute loss and gain of function experiments to reveal a novel, functional connection between cell stiffness and the increased invasion capacity acquired after growth factor induced EMT.

EMT collaboration. In this project, I designed and performed the magnetic tweezer and PBR experiments, and analyzed the data. I developed the rotating magnet device to enable biochemical analysis of force-dependent signaling pathways. George Li managed cell culture, prepared specimens for mechanical experiments, executed biochemical and invasion assays. The work in this chapter has been published: L.D. Osborne, G.Z. Li, T. How, E.T. O'Brien III, G. Blobel, R. Superfine, K. Mythreye. "Altered stiffness and mechanical response to force by the Rho GEFs LARG and GEF-H1 regulate cell invasion during TGF- β induced EMT". *Molecular Biology of the Cell*. doi: 10.1091/mbc.E14-05-1015. (2014). Referenced in text as (Osborne et al., 2014).

5.2 The metastatic cascade and EMT

Cancer metastasis involves a series of events known as the metastatic cascade (Butcher et al., 2009). In this complex progression, cancer cells detach from the primary tumor, invade the surrounding stromal space, transmigrate the vascular system, and establish secondary tumors at distal sites (Fig. 2.8). Specific mechanical phenotypes are likely adopted to enable cells to successfully navigate the mechanical environments encountered during metastasis (Wirtz et al., 2011). The epithelial to mesenchymal transition (EMT) is an essential physiological process that drives adherent, immotile cells to lose polarity and increase migration. Recently, abnormal reactivation of EMT has been implicated in the detachment of cancer cells from epithelial tissue and their subsequent invasion into stromal tissue (Taylor et al., 2010)(Yilmaz and Christofori, 2009). Transforming growth factor β (TGF- β), one of the primary drivers of EMT, initiates this process by altering gene expression (Ranganathan et al., 2007), inducing loss of cell-cell adhesions (Vogelmann et al., 2005), promoting changes to cytoskeletal structure (Moustakas and Stournaras, 1999)(Hubchak, 2003), and increasing motility and invasion (Oft et al., 1998)(Gordon et al., 2009). These changes in cytoskeletal structure and increased interaction with the ECM implicate a role for altered cell mechanics during EMT (Yilmaz and Christofori, 2009).

Changes in the extracellular matrix (ECM) occur in cancer resulting in tumors being stiffer (Paszek et al., 2009) and more heterogeneous than normal tissue (Plodinec et al., 2012). Hence, cancer cells moving through tumor ECM experience stiffness gradients as

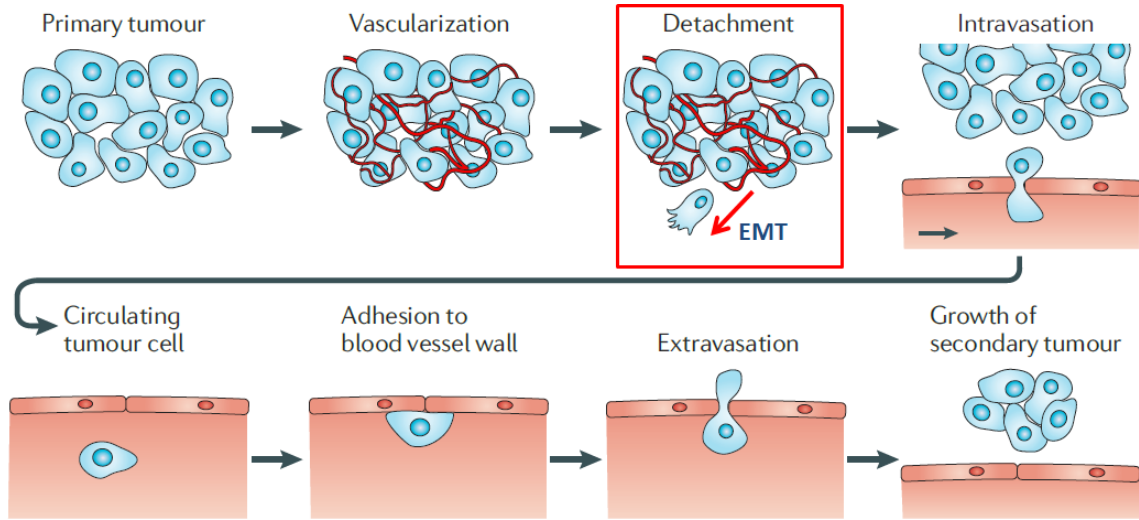


Figure 5.1: **The metastatic cascade.** Cells detach from the primary tumor, invade the surrounding stromal space, transmigrate the vascular system, and establish secondary tumors at distal sites. Figure republished from (Wirtz et al., 2011) with permission from Nature.

well as environmental forces not typically experienced by normal epithelial cells. Integrins are transmembrane, mechanosensitive receptors that provide an essential physical connection between the ECM and the actin cytoskeleton (Wang et al., 1993) during cell adhesion and migration. Studies have shown that cells respond to force on integrins by generating a stiffening response to resist the applied force and maintain mechanical reciprocity (Lessey et al., 2012)(Matthews et al., 2006). We have demonstrated that mechanical response to force is regulated by the activation of the small GTPase RhoA through specific guanine nucleotide exchange factors (GEFs) (Guilluy et al., 2011). Although RhoA and its effectors have been linked to cancer (Lazer and Katzav, 2011), the molecular mechanisms that regulate its activity and involvement in particular steps of the metastatic cascade, including EMT, are not well understood.

Here, we investigate the mechanistic links between cell stiffness and stiffening re-

sponse and the increased invasion capacity acquired after TGF- β -initiated EMT.

5.3 TGF- β induced EMT alters stiffness and stiffness response to force

To determine the effect of EMT on cell stiffness and stiffening response, we induced EMT in normal murine mammary gland (NMuMG) epithelial cells, a well-established TGF- β -induced EMT model (Piek et al., 1999)(Yu et al., 2002)(Xie et al., 2004) (Fig. 5.2). A magnetic tweezers system (Fisher et al., 2006) was then used to apply force via integrins (Matthews et al., 2006)(Guilluy et al., 2011) to the cytoskeleton through externally-attached, paramagnetic beads coated with fibronectin (FN). The viscoelastic response of a cell was observed by monitoring the displacement of a bound bead over time during force application (Fig. 5.3 A).

To quantify the mechanical phenotype in terms of stiffness and stiffening response, the time-dependent compliance of the cell was calculated and fit to a Jeffrey model for viscoelastic liquids (Fig. 5.3 B) (Larson, 1999). The spring constant obtained during the first pulse of force provided a measure of stiffness, and by normalizing the spring constants of subsequent force pulses to the first, the stiffness-response to force, or stiffening response, was obtained. Two classifications of mechanical response were observed: a stiffening response (Fig. 5.3 C), and a softening response (Fig. 5.3 D). TGF- β induced EMT was verified by monitoring reduced E-cadherin levels (Fig. 5.9) and actin reorganization (Fig. 5.2).

Mechanical characterization demonstrated a population-level shift towards lower stiffness in TGF- β treated NMuMG cells compared to untreated cells (Fig. 5.4 A,B). In addition, the average stiffness of mesenchymal cells was 3 fold less than epithelial

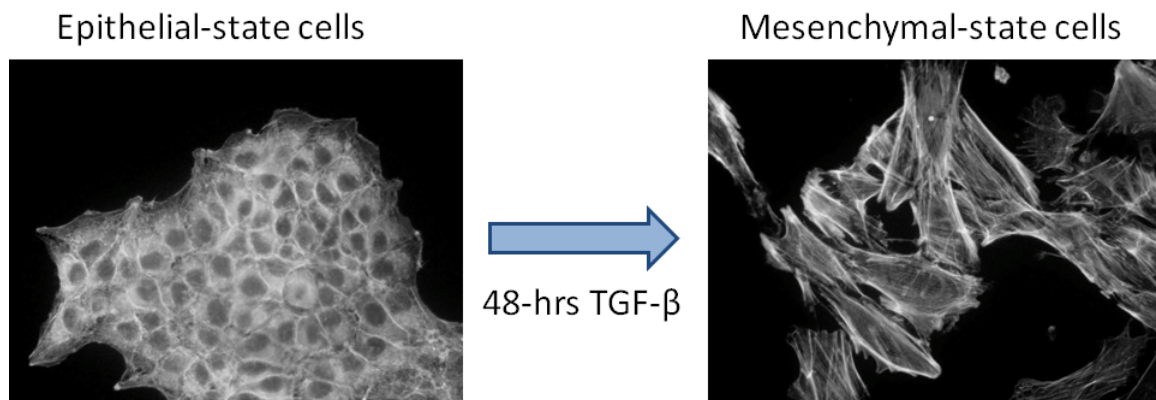


Figure 5.2: **Illustrating the structural change associated with EMT.** NMuMG cells were treated with 100 pM of TGF- β for 48 hours and then stained with phalloidin to show actin organization.

cells (Fig. 5.4 C). In response to successive pulses of force, epithelial cells increased their stiffness (Fig. 5.4 D) significantly after 1 minute or 5 pulses of force (Fig. 5.4 D). After TGF- β induced EMT, this stiffening response to force was lost, indicating that mesenchymal cells are unable to fully adjust their stiffness in response to external force (Fig. 5.4 D). To investigate whether these mechanical changes after EMT are specific to NMuMG cells or to EMT in general, we examined human pancreatic carcinoma (PANC-1) cells, which undergo EMT in response to bone morphogenetic protein-2 (BMP-2) (Gordon et al., 2009) and TGF- β . Similar to post-EMT NMuMG cells, PANC-1 cells exhibited decreased stiffness when treated with BMP-2 (Fig. 5.5 A) and TGF- β (Fig. 5.5 C), and a loss of the normal stiffening response to force on integrins after 1 min (Fig. 5.5 B,D).

To dynamically probe areas of increasing and variable stiffness, as seen in cancer ECM (Butcher et al., 2009), cells use integrin-associated focal adhesions as individual and autonomous stiffness sensors (Plotnikov et al., 2012). As such, to examine whether

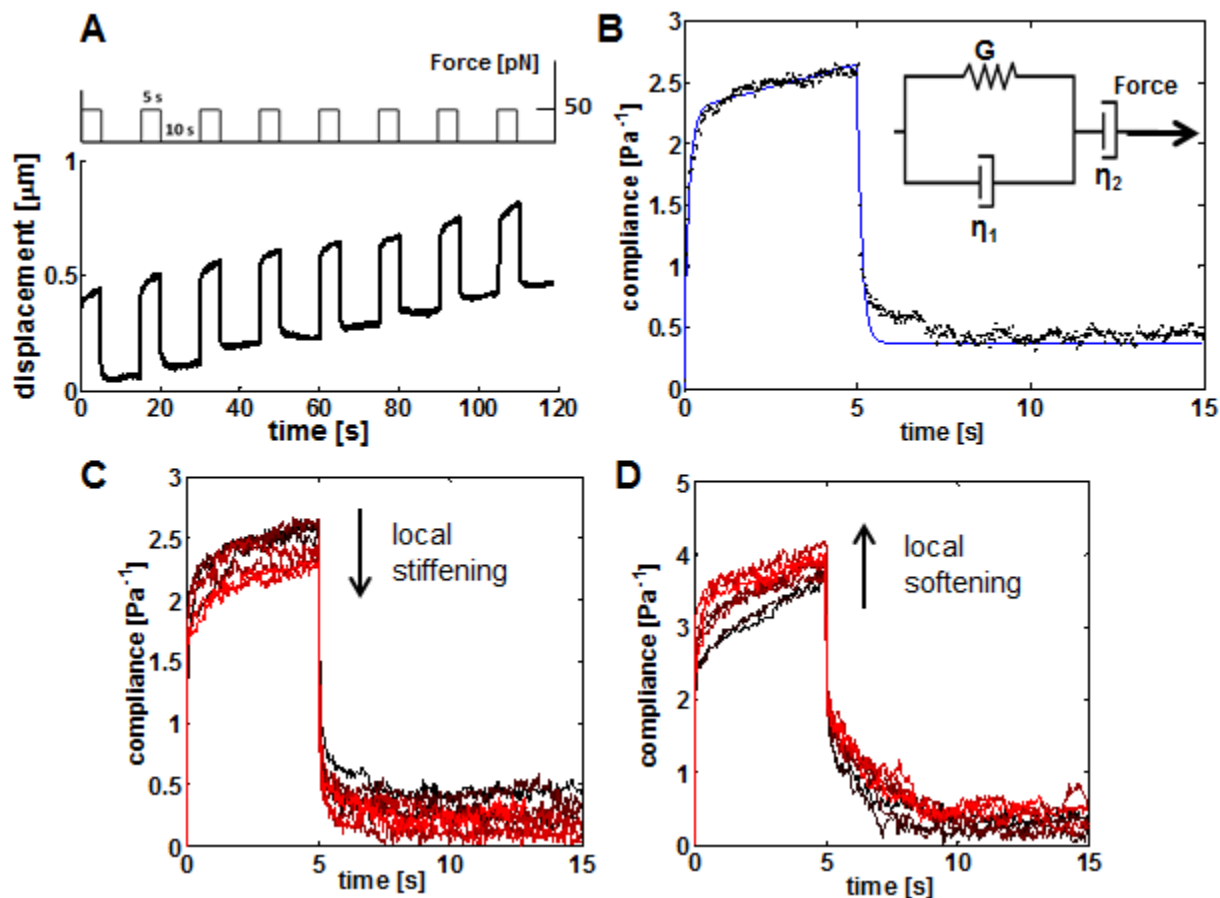


Figure 5.3: Mechanical assay and modeling of cell stiffness. (A) Schematic of the magnetic tweezers experiment: a 50 pN force was applied for 5 seconds, followed by a 10 sec relaxation time, for a total of 8 pulls. The time-dependent displacement for a typical bead is shown below the force regimen. (B) The time-dependent compliance (black data points) is calculated from the displacement of a bead and the applied force. The Jeffreys model (inset) is a mechanical circuit which models the elastic (or stiffness, G) and viscous (η_1 and η_2) responses for a viscoelastic liquid during force application. The Jeffreys model (blue line) was used to quantify the stiffness of the cell as measured during force application. (C,D) Compliance signatures for representative examples of (C) cell stiffening and (D) cell softening. Time is illustrated by progressive shades of red. The initial stiffness of the cell in each example was 0.4 Pa, and the result of the creep recovery experiment was a 30% stiffening (C) or softening (D).

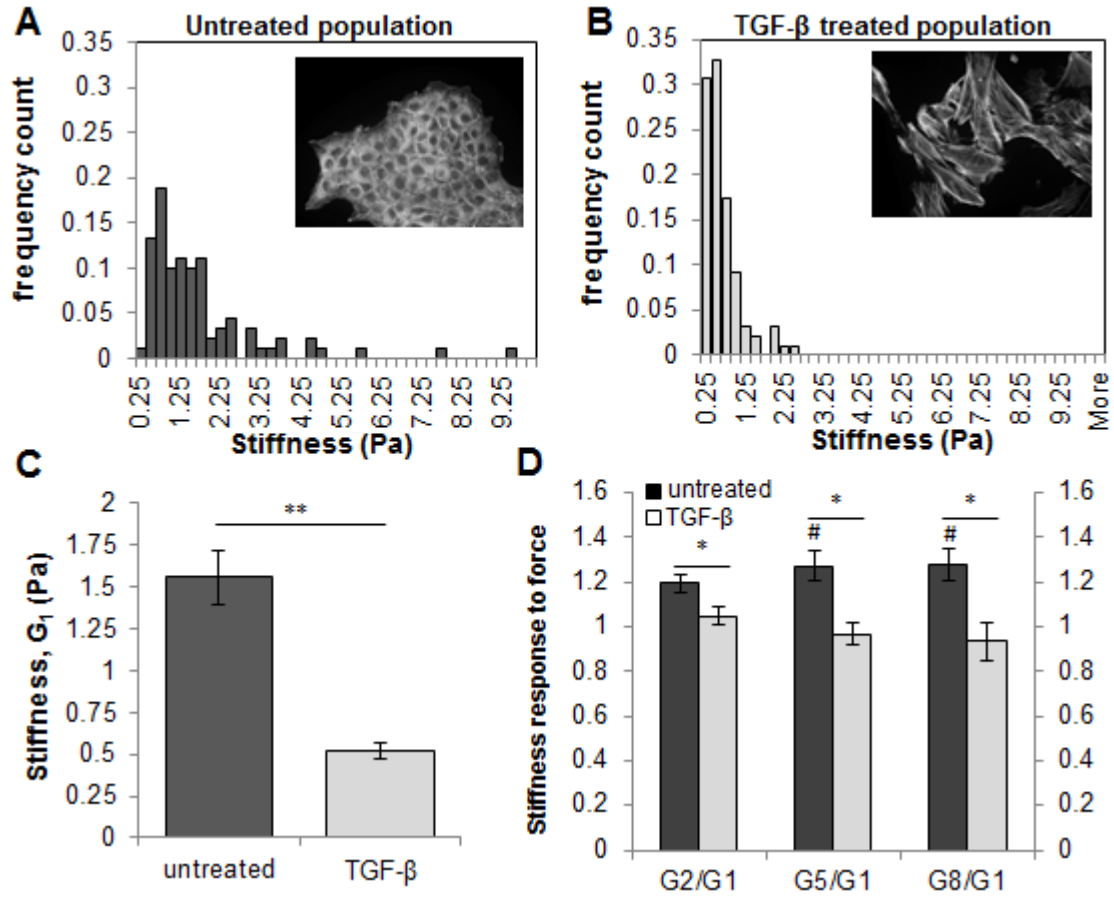


Figure 5.4: **Stiffness and stiffness response to force decrease during TGF-induced EMT.** (A,B) NMuMG cells were treated with 100 pM of TGF- β for 48 hours to induce EMT. Histogram of NMuMG cell stiffness: (A) epithelial-state (untreated) and (B) mesenchymal-state, respectively. (C) Average cell stiffness of NMuMG cells for untreated ($n = 90$) and TGF- β treated ($n = 98$) populations. $**p < 0.001$. (D) Average stiffness response for untreated ($n = 30$) and TGF- treated ($n = 25$) populations. # denotes stiffness difference of G_x from G_1 at the $p < 0.05$ level, and * denotes stiffness response ($\frac{G_x}{G_1}$) difference between conditions at the $p < 0.05$ level.

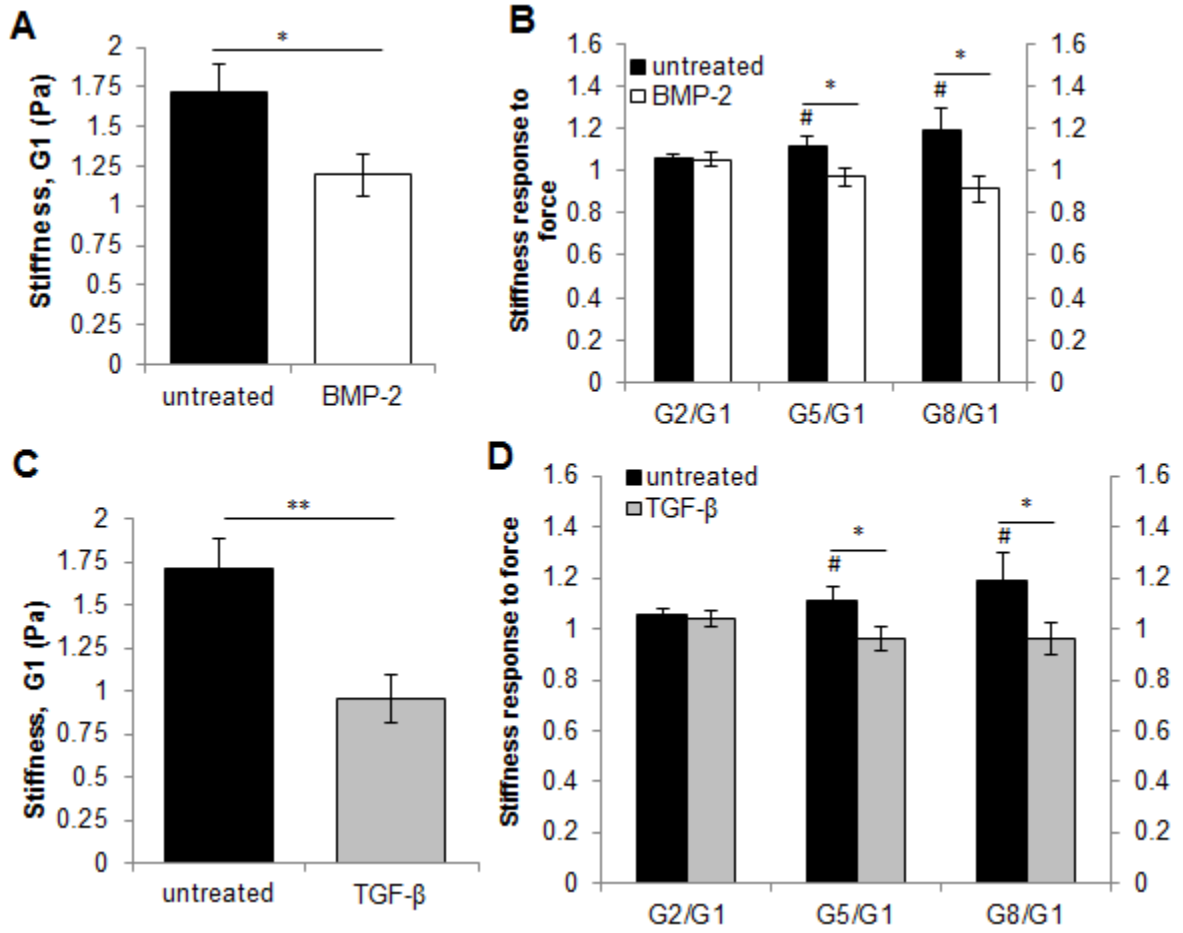


Figure 5.5: **Stiffness and stiffness response to force decrease during BMP-2 and TGF- β induced EMT.** (A) PANC-1 cells were treated with 300 ng/mL BMP-2 for 72 hours to induce EMT. Average PANC-1 cell stiffness for untreated ($n = 86$) and BMP-2 treated cells ($n = 61$). * denotes stiffness difference relative to untreated cells at the $p < 0.05$ level. (B) Average PANC-1 stiffness response for untreated ($n = 20$) and BMP-2 treated ($n = 15$) cells. (C) PANC-1 cells were treated with TGF- β for 72 hours to induce EMT. Average PANC-1 cell stiffness for untreated ($n = 86$) and TGF- β treated cells ($n = 54$). ** denotes stiffness difference relative to untreated cells at the $p < 0.01$ level. (D) Average PANC-1 stiffness response for untreated ($n = 20$) and TGF- β treated ($n = 10$) cells. # denotes stiffness difference of G_x from G_1 at the $p < 0.05$ level. * denotes stiffness response (G_x/G_1) difference between conditions at the $p < 0.05$ level. (Error bars represent SEM, data was collected from 3 independent experiments)

specific binding to integrins was required to elicit a stiffening response during force application, we used poly-D-lysine (PDL)-coated beads, which bind non-specifically to cell surface based on charge. In contrast to the response observed with FN-coated beads, and consistent with findings in endothelial cells (Collins et al., 2012), force applied to PDL-coated beads did not evoke a stiffening response (Fig. 5.6 B). These results suggest the stiffening response to force on FN-coated beads is specific to integrin-mediated attachment to the cytoskeleton.

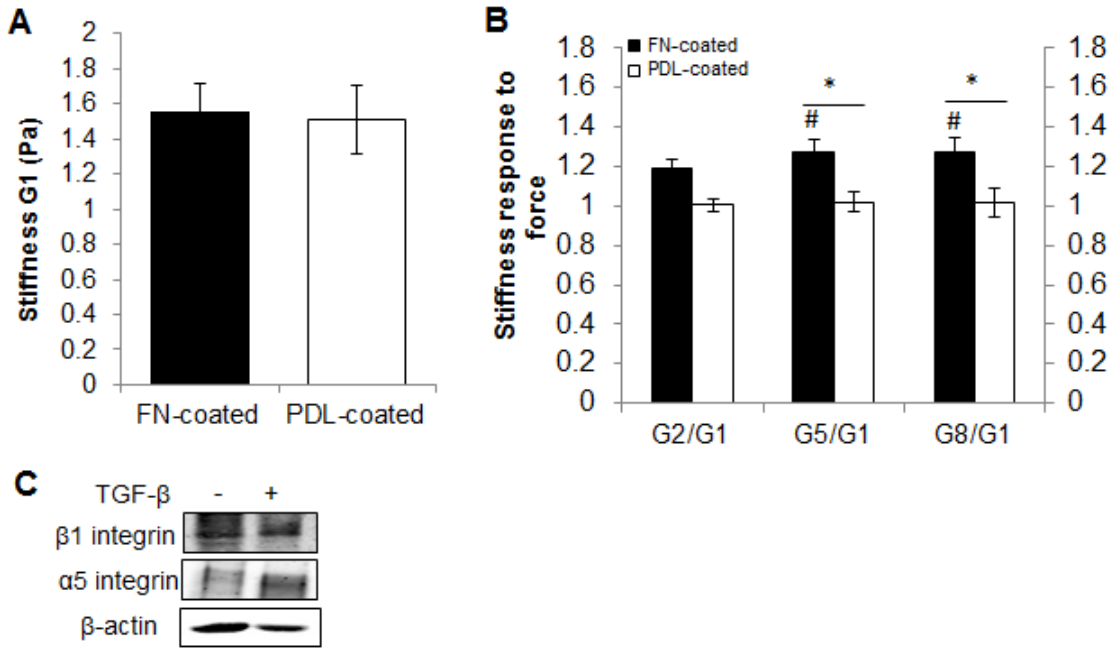


Figure 5.6: **PDL coated beads and integrin expression.** (A) Average stiffness for NMuMG cells incubated with FN-coated ($n = 90$) or PDL-coated ($n = 35$) beads. (B) Average stiffness response for NMuMG cells incubated with FN-coated ($n = 30$) or PDL-coated ($n = 14$) beads. # denotes stiffness difference of G_x from G_1 at the $p < 0.05$ level. * denotes stiffness response $\frac{G_x}{G_1}$ difference between conditions at the $p < 0.05$ level. Error bars represent SEM, data was collected from 3 independent experiments. (C) Protein expression level of $\alpha 5$ and $\beta 1$ integrins in NMuMG cells with or without TGF- β treatment.

In these experiments, PDL-coated beads yield the same initial stiffness as FN-

coated beads (Fig. 5.6 A), suggesting that both bead ligands probe the same material. A potential explanation is that PDL beads probe the actin cortex through membrane-cortex linkages, whereas FN-coated beads probe the cortex through FAs. While it is possible PDL beads attach non-specifically to integrins, the lack of a stiffening response to force makes this unlikely. Future studies should explore the recruitment of structural and signaling proteins attached to PDL-coated beads (as investigated for FN-coated bead in Fig. 5.11 A,B). Within the above model, the presence of the cortical actin cytoskeleton establishes a cell stiffness, but the lack of attachment (or the insufficient attachment) of PDL-bead to force-sensitive molecules prevents a mechanoresponse.

During EMT, cells undergo changes in the expression of many receptors (Ranganathan et al., 2007). To exclude the possibility that reduction in cell stiffness and stiffening response during EMT was due to loss of integrin expression, we examined expression of $\alpha 5 \beta 1$ integrins, the primary receptor for FN. We observed no significant reduction in either $\alpha 5$ or $\beta 1$ levels post-EMT in NMuMGs (Fig. 5.6 C) indicating that the reduction in mechanical properties was not due to reduction of FN receptor expression.

5.4 Comparison of Cell Stiffness from Active and Passive Approaches

To evaluate the stiffness of NMuMG cells before and after TGF- β induced EMT using a different approach, we performed an external passive bead rheology (PBR) experiment with FN-coated beads. We find that the RMS displacement of beads attached to TGF- β treated cells (89.13 nm) was significantly increased from those attached to untreated cells (56.23 nm) (Fig. 5.7). Using Eqn. 2.42 to calculate the stiffness G of each cell condition yields 0.28 Pa for untreated cells and 0.11 Pa for TGF- β treated cells. The table in Fig. 5.8 compares the PBR determined stiffness to that found using magnetic tweezers (Fig. 5.4 C). While there exists a 5-fold difference between the effective stiffnesses determined by each approach, both magnetic tweezers and PBR show a 3-fold decrease in stiffness after TGF- β treatment.

The 3-fold difference in stiffness between cell conditions observed using both approaches suggests that each approach is measuring properties of the same material. This claim is consistent with FN-coated beads inducing the recruitment of integrins, FA proteins, and cortical actin filaments upon attachment – before an external force is applied (Plopper and Ingber, 1993). The difference in stiffness between methods could be due to 1) variability in bead attachment (PBR selects all beads, magnetic tweezer selects the strongest attached), 2) nonlinear mechanical response (bead displacement for magnetic tweezer is ~ 10 times that of PBR), 3) force from magnetic tweezers induces biochemical changes. These sources of difference are discussed more in Sec. 2.2.3.

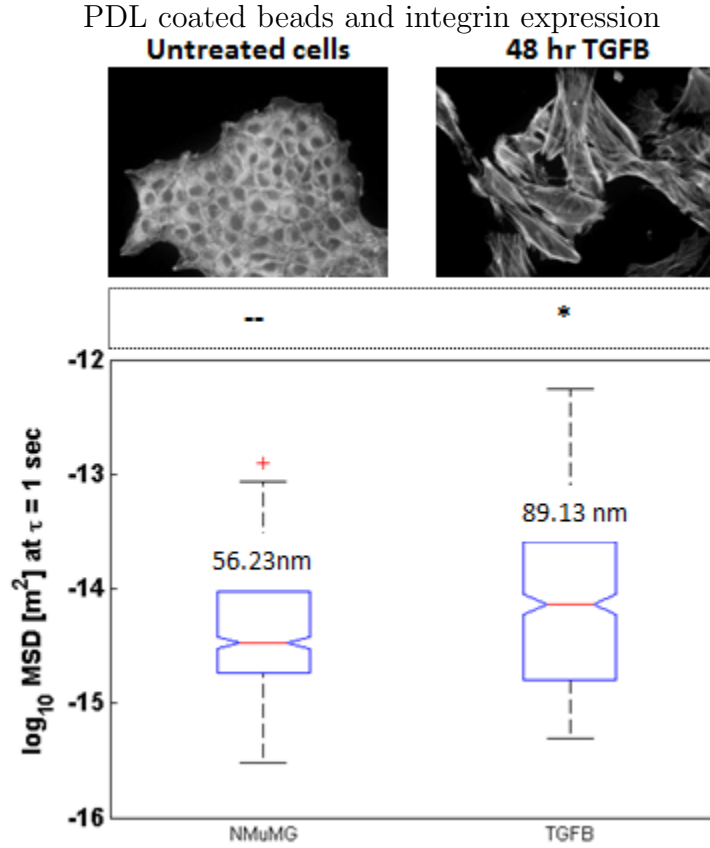


Figure 5.7: **PBR assay of cells before and after TGF- β induced EMT.** MSD distributions (log scale) at the 1 sec timescale are shown for FN-coated beads attached to each cell condition. The median of each distribution is denoted by the red line and the median RMS displacement value is given above the box. Distributions are filtered for anomolous diffusion (DA-model) behavior using an analysis discussed in Sec. 7.5.2. Distributions compared using a Mann-Whitney test (* $p < 0.05$).

	Magnetic Tweezers Stiffness, G' (Pa)	Passive Bead Rheology Stiffness, G' (Pa)
untreated NMuMG cells	1.55 ± 0.16	0.28
TGF-B treated cells	0.52 ± 0.05	0.11

Figure 5.8: **Comparison of cell stiffness determined by magnetic tweezers and PBR.** The magnetic tweezer data are from Fig. 5.4 C. To estimate G for each condition using the median MSD in Fig. 5.7, we assume the cells are completely elastic (reasonable for small α observed in PBR cell data) and use Eqn. 2.42.

5.5 RhoA GEF expression and recruitment is lost after EMT

Forces applied to integrins increase RhoA activity via Rho GEFs, and in turn, induce a stiffening response through reinforcement of adhesion complexes and rearrangement of the cytoskeleton (Guilluy et al., 2011). Based on reduction of stiffness and stiffening response after EMT (Fig. 5.4 C,D and 5.5), we hypothesized that this could be caused by altered RhoA activity through downregulation of specific Rho GEFs. Indeed, expression levels of two Rho GEFs, LARG and GEF-H1, were significantly reduced after EMT, while expression of p114, another Rho GEF, was unchanged (Fig. 5.9 A). Similar results were obtained in TGF- β induced EMT in OVCA420 cells and BMP-2 induced EMT in PANC-1 cells, respectively (Fig. 5.9 B,C). These findings suggested specific roles for LARG and GEF-H1 in cell stiffness and stiffening response.

To test whether EMT affects GEF recruitment to sites of force application, we developed a rotating permanent magnet device (Fig. 5.10 A) to generate a pulsatile force regimen consistent in magnitude, duty cycle, and frequency with that produced by the magnetic tweezers (Fig. 5.10 B) in order to apply forces to cells via externally attached FN-coated beads. After force stimulation, we separated the bead fraction containing adhesion complex proteins from the whole lysate (Guilluy et al., 2011), and found that LARG and GEF-H1 were recruited in a time-dependent manner in epithelial-state NMuMG cells (Fig. 5.11 A). In contrast, TGF- β induced EMT abrogated this time dependent recruitment of LARG and GEF-H1 (Fig. 5.11 A). Examination of p114, another RhoGEF, showed no recruitment to the adhesion complex and was unchanged

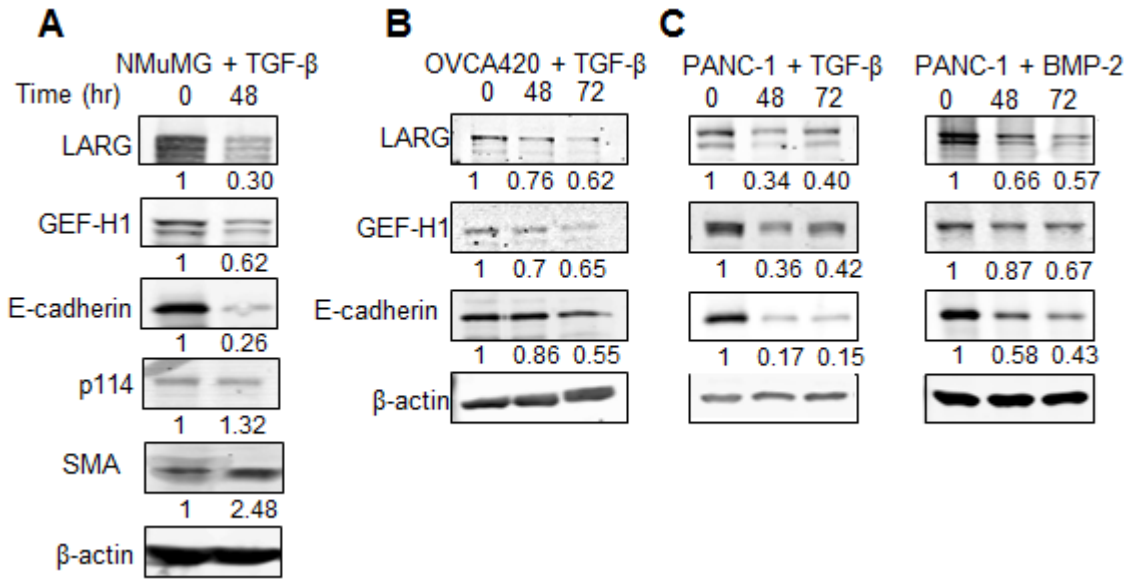


Figure 5.9: **LARG and GEF-H1 expression decreases during EMT (A,B)** Indicated cell lines cells were treated with 100 pM of TGF- β for 48 or 72 hours. **(C)** PANC-1 cells were treated with 300 ng/mL BMP-2 for 48 and 72 hours. Cells were then lysed and protein expression levels were analyzed by western blot. A representative blot of 4 independent experiments is presented. **Data: George Li**

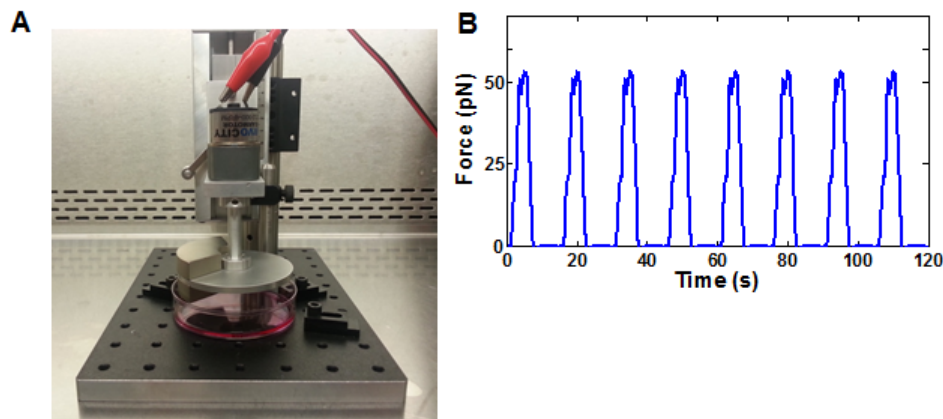


Figure 5.10: **The rotating permanent magnet device is used to generate a consistent force regimen to the magnetic tweezers assay. (A)** Picture and schematic of the rotating magnet device. A DC motor was used to rotate a custom made, axially magnetized, 120-degree arc magnet at 4 revolutions per minute to generate a time varying force of the desired duty cycle and frequency. The footprint of the magnet was designed to lower into a standard 10 cm cell culture dish. **(B)** COMSOL simulation of the force regimen over 2 min for the rotating magnet device at a height of 16 mm and at half-radius in the specimen dish.

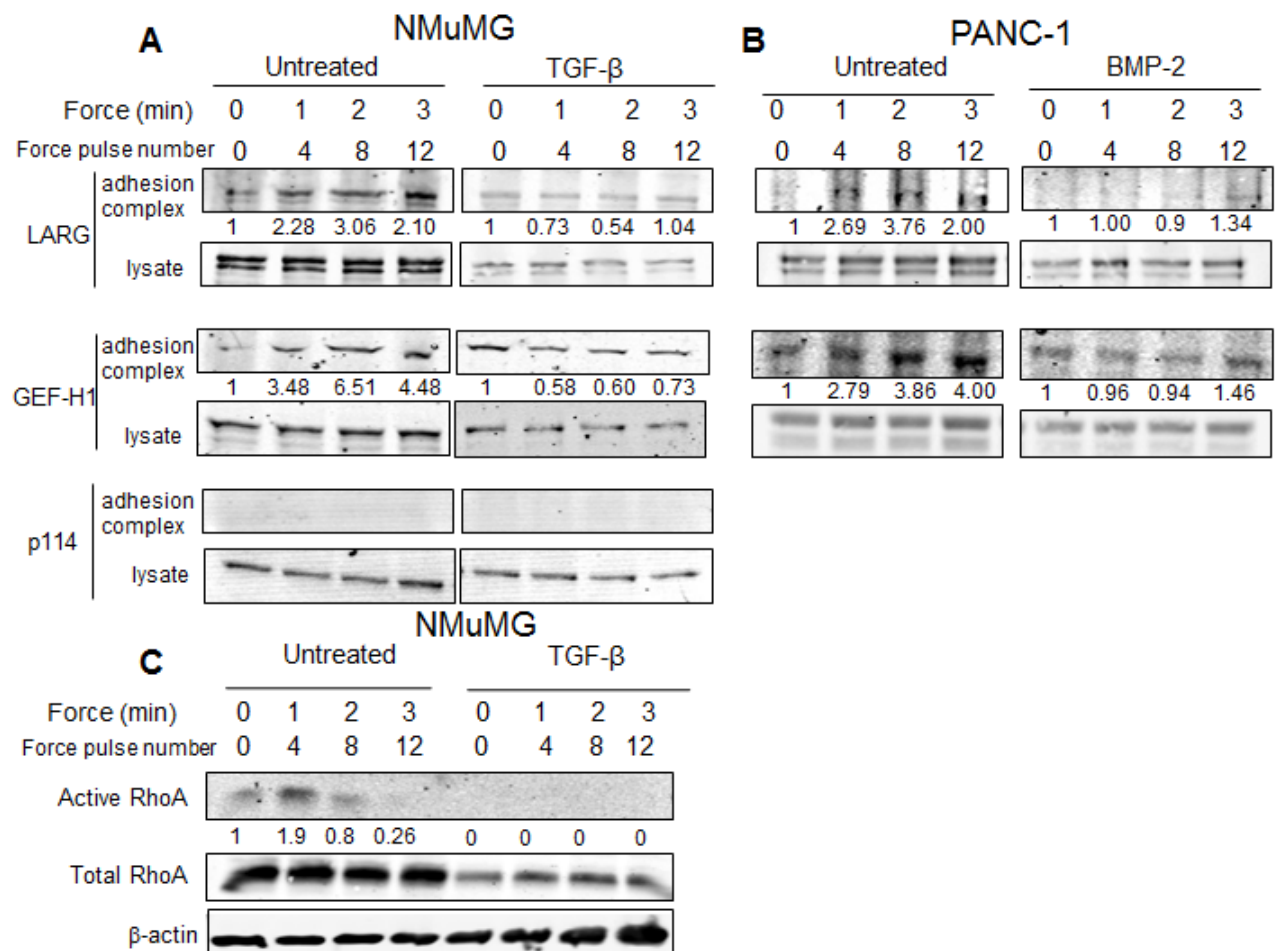


Figure 5.11: **LARG and GEF-H1 recruitment to adhesion complex during force decrease during TGF- β induced EMT.** (A,B) Effect of EMT on RhoA GEF recruitment in either NMuMG (A) or PANC-1(B) cells: Indicated cells were incubated for 30 mins with FN coated beads and stimulated with a force regimen (50 pN; 5 sec force, 10 sec recovery) using a rotating permanent magnet for different amounts of time. Following magnetic separation of the adhesion complex, both the lysate and adhesion complex fractions were analyzed using Western blots. Representative blot of 4 independent experiments is presented. (C) RhoA activity for NMuMG cells before and after EMT. Whole cell RhoA activity was determined as described (Guilluy et al., 2011). Representative blot of 3 experiments is presented. **Data: George Li**

after EMT (Fig. 5.11 A), suggesting that LARG and GEF-H1 have specific roles in force transduction during EMT. Similar loss of force-dependent recruitment of LARG and GEF-H1 was found in PANC-1 cells treated with BMP-2 (Fig. 5.11 B).

Given our observations of post-EMT reduction in LARG and GEF-H1 expression and force-dependent recruitment, we hypothesized that RhoA activity in response to force would also be reduced after EMT. Using GST-RBD-coated beads to pull down active RhoA from cell lysates (Guilluy et al., 2011), we found that epithelial-state NMuMG cells activated RhoA within 1 minute of force application, and that this response was lost after EMT induction (Fig. 5.11 C). Interestingly, this 1-minute timescale was consistent with the point in which the stiffening response in epithelial-state cells becomes significant (Fig. 5.4 D). We also found that RhoA expression was significantly reduced after EMT (Fig. 5.11 C), in line with previous observations (Ozdamar et al., 2005). Taken together, the suppression of force-dependent RhoA activity, as well as the loss of RhoA expression and force-dependent recruitment of LARG and GEF-H1, suggest that disruption of the Rho pathway plays a role in the altered stiffness response to force during EMT.

5.6 TGF- β regulates Rho GEF expression during EMT

TGF- β can regulate activation of the RhoA pathway via canonical (ALK5 dependent) and non-canonical (MAPK dependent) TGF- β signaling mechanisms (Bhowmick et al., 2001). To investigate how TGF- β regulates LARG and GEF-H1 expression during EMT, we used the ALK5 inhibitor SB-431542 (Alk et al., 2002) and find that

ALK5 inhibition partially rescued TGF- β -mediated LARG and GEF-H1 protein down-regulation in both NMuMG and OVCA420 cells, indicating a requirement for ALK5 in maximal regulation of RhoGEF expression (Fig. 5.12 A). In contrast, blocking the MEK/ERK pathway with U0126 (Alk et al., 2002), which can also mediate TGF- β responses (Xu et al., 2009), did not ameliorate TGF- β dependent decreases in LARG and GEF-H1 levels in NMuMG cells (Fig. 5.12 A).

Given that LARG and GEF-H1 have been shown to regulate cell mechanics (Guil-luy et al., 2011), and expression of LARG and GEFH-1 was regulated via an ALK5 dependent mechanism, we hypothesized that the impact of TGF- β on cell mechanics would be dampened upon ALK5 inhibition. Using the ALK5 inhibitor SB-431542 to partially restore LARG and GEF-H1 levels (Fig. 5.12 A), we find that NMuMG cell stiffness (Fig. 5.12 B) and stiffness response (Fig. 5.12 C) were significantly increased compared to cells treated with only TGF- β . These results directly implicate canonical TGF- β signaling mechanisms downstream of ALK5 in regulating cell mechanics during EMT.

RhoA and the RhoA GEF Net1A have been shown to be downregulated during TGF- β induced EMT via a microRNA based mechanism (Kong et al., 2008)(Moustakas and Heldin, 2012) that acts on the 3'- untranslated region (UTR) to induce translational silencing of proteins (He and Hannon, 2004). To test whether microRNAs play a role in TGF- β downregulation of LARG and GEF-H1, we examined the extent of downregulation of exogenously expressed LARG or GEF-H1 cDNA containing an N-terminus GFP tag but lacking a 3'-UTR. We find that exogenous LARG and

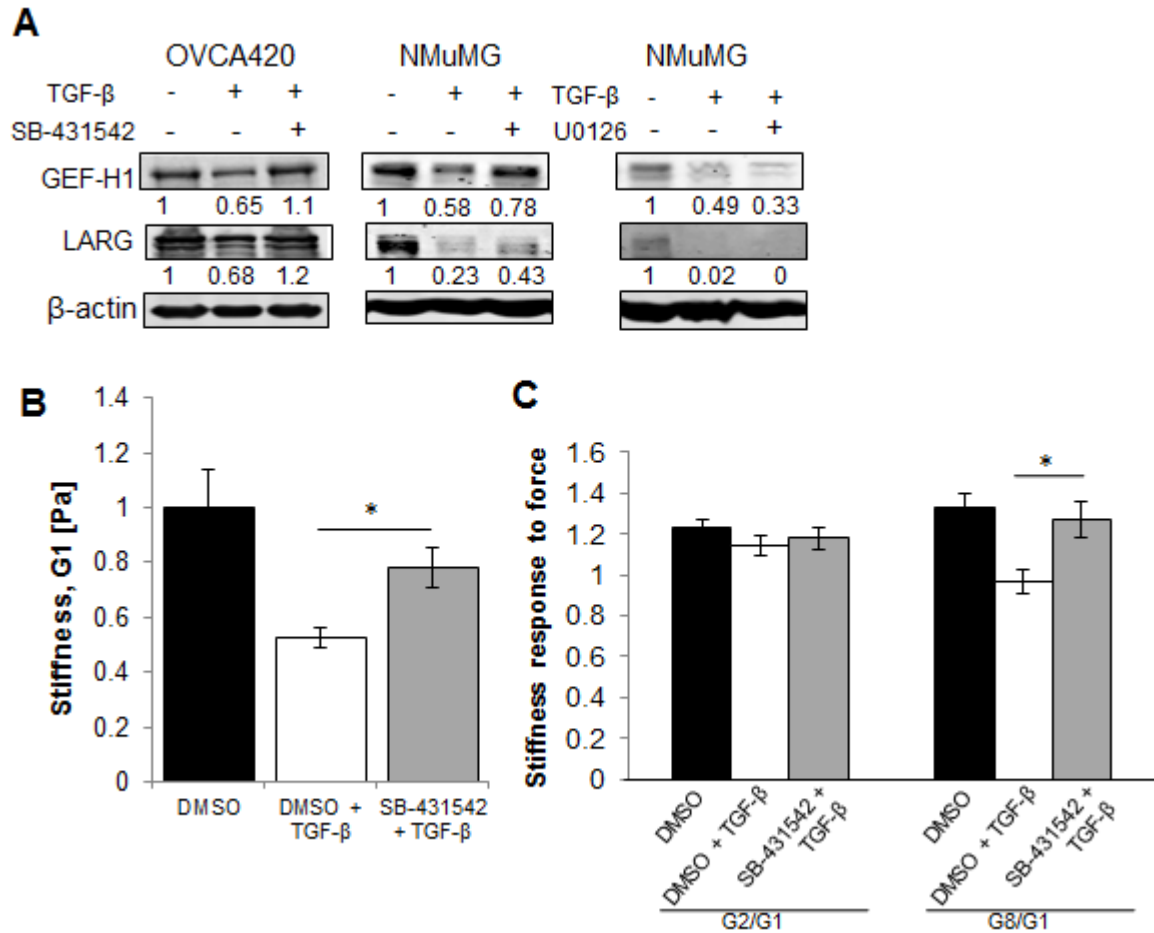


Figure 5.12: **ALK5 inhibitor blocks TGF- β -mediated decrease in GEF expression, stiffness and stiffness response.** (A) Indicated cells were pre-treated for 1 hour with 10 μ M SB-431542 (ALK5 inhibitor) or 10 μ M U0126 (MEK inhibitor), or DMSO (negative control), followed by treatment with 100 pM TGF- β for 48 hours. (B) NMuMG cells were pre-treated for 1 hour with 10 μ M SB-431542 (ALK5 inhibitor) or DMSO, followed by treatment with 100 pM TGF- β for 48 hours, or DMSO. Average cell stiffness for DMSO (n = 55), DMSO and TGF- β (n = 84), and SB-431542 and TGF- β (n = 81) populations. *p < 0.01. Error bars represent SEM, data was collected from 3 independent experiments. (C) NMuMG cells were pre-treated for 1 hour with 10 μ M SB-431542 (ALK5 inhibitor) or DMSO, followed by treatment with 100 pM TGF- β for 48 hours, or DMSO. Average stiffness response for DMSO (n = 31), DMSO and TGF- β (n = 20), and SB-431542 and TGF- β (n = 26) populations. *p < 0.05. Error bars represent SEM, data was collected from 3 independent experiments.

GEF-H1 were downregulated to similar extents as endogenous counterparts (Fig. 5.13 A), suggesting that microRNAs may not play a significant role in the TGF- β -induced decreases of LARG and GEF-H1.

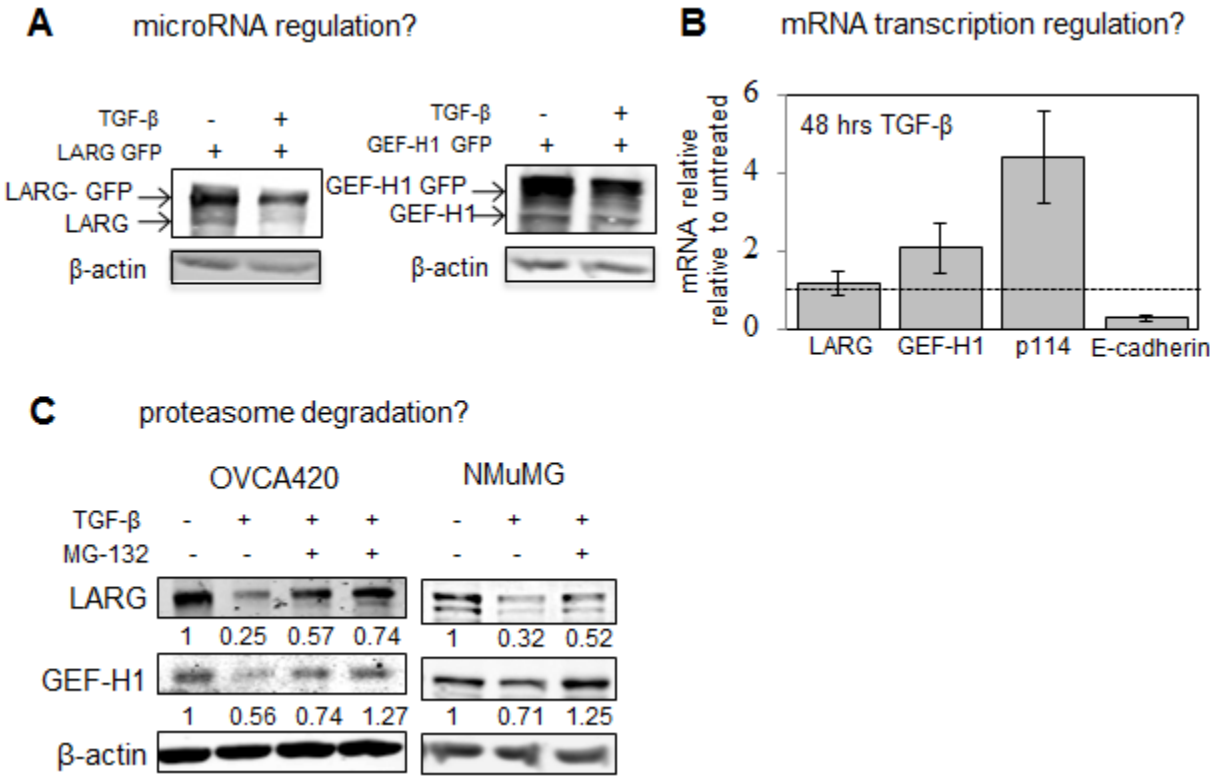


Figure 5.13: TGF- β promotes proteasome degradation of LARG and GEF-H1 during EMT. (A) NMuMG cells transfected with GFP-tagged LARG or GEF-H1 cDNA lacking the 3'-UTR, were treated with TGF- β for 48 hours. Lysates were immunoblotted to determine expression of endogenous and exogenous GFP tagged LARG/GEFH1. (B) TGF- β treatment does not reduce mRNA levels of LARG or GEF-H1. mRNA levels determined by q-PCR were normalized to GAPDH. Error bars represent SEM. The dashed line denotes untreated. (C) TGF- β promotes proteasome degradation of LARG and GEF-H1. OVCA420 cells were treated for 72 hours with TGF- β and with 10 μ M and 20 μ M MG-132 or DMSO as control. NMuMG cells treated with TGF- β for 48 hours and 10 μ M MG-132 or DMSO as control for the last 16 hours of treatment. Representative of at least 3 experiments is presented.

Data: George Li

Since TGF- β regulates the transcription of many genes during EMT (Xu et al., 2009), we examined whether EMT altered transcription of LARG and GEF-H1. We find

that while TGF- β caused downregulation E-cadherin, no significant downregulation of LARG and GEF-H1 at the mRNA level was observed (Fig. 5.13 B). Therefore, we examined the role of the proteasome in regulating LARG and GEF-H1 levels, which has been shown to regulate TGF- β dependent levels of RhoA (Wang et al., 2003). We find that the proteasome inhibitor MG-132 was able to rescue downregulation of LARG and GEF-H1 protein levels after TGF- β induced EMT in both NMuMG and OVCA420 cells (Fig. 5.13 C). These data implicate proteasome-mediated regulation of the RhoGEFs LARG and GEFH-1 during EMT via an ALK5 dependent mechanism.

5.7 Epithelial-state cells partially adopt post-EMT phenotypes

EMT marks the physical initiation of cancer progression as a cell detaches from the primary tumor and invades the surrounding stromal space. Alteration in the RhoA pathway have been implicated in a variety of cancers (Vega et al., 2011)(Simpson et al., 2004). However, while RhoA activation in some cancers is associated with increased invasion (Liao et al., 2012), in others RhoA activation inhibits cell invasion (Bellovin et al., 2006). In addition, RhoA and its associated GEFs LARG and GEF-H1 have been shown to regulate the stiffening response to force applied on integrins (Guilluy et al., 2011).

Based on these and our findings that LARG and GEF-H1 expression and force-dependent recruitment are reduced in multiple EMT models (Fig. 5.9 A-C, 5.11 A,B) and that ALK5 inhibition reverses the TGF- β -mediated reduction in cell mechanics, we examined whether LARG and GEF-H1 downregulation were sufficient for decreased

stiffness and stiffening response to force. Using siRNA to reduce GEF expression in NMuMG epithelial-state cells (Fig. 5.14 A), we find that silencing LARG, GEF-H1, or both, significantly decreased cellular stiffness (Fig. 5.14 B) and stiffening response compared to control siRNA treated cells (Fig. 5.14 C). In contrast, siRNA to p114 had no significant effect on stiffness (Fig. 5.14 B), or suppressing the stiffening response to force (Fig. 5.14 C), suggesting a specific role for LARG and GEF-H1 in determining these mechanical properties.

Given that we and others have previously reported an inverse correlation between cell stiffness and invasion (Swaminathan et al., 2011)(Xu et al., 2012b), we examined the impact of GEF silencing on migration and invasion using siRNA to deplete protein levels in epithelial-state NMuMG cells. We find that silencing LARG, GEF-H1, or both simultaneously, significantly increased cell migration and invasion compared to control siRNA cells (Fig. 5.14 D,E). In contrast, siRNA to p114 did not significantly alter cell migration or invasion (Fig. 5.14 D,E). In line with these results, we find that specific silencing of LARG and GEF-H1 expression in epithelial cells increases invasion capacity towards that of post-EMT mesenchymal cells.

Figure legend for Fig. 5.14 continued.

(C, continued) # $p < 0.05$ denotes stiffness difference from G_1 , * $p < 0.05$ denotes stiffness response difference relative to cells treated with siRNA control. Error bars

represent SEM, data was collected from 3 independent experiments. **(D,E)** Average migration (D) and invasion (E) for NMuMG cells treated with siRNA as indicated: control, GEF-H1, LARG, both, or p114 for 36 hours before plating onto uncoated or Matrigel-coated transwell filters. * $p < 0.05$. Error bars represent SEM, data represent mean of 3 independent experiments. Migration and invasion data taken by George Li.

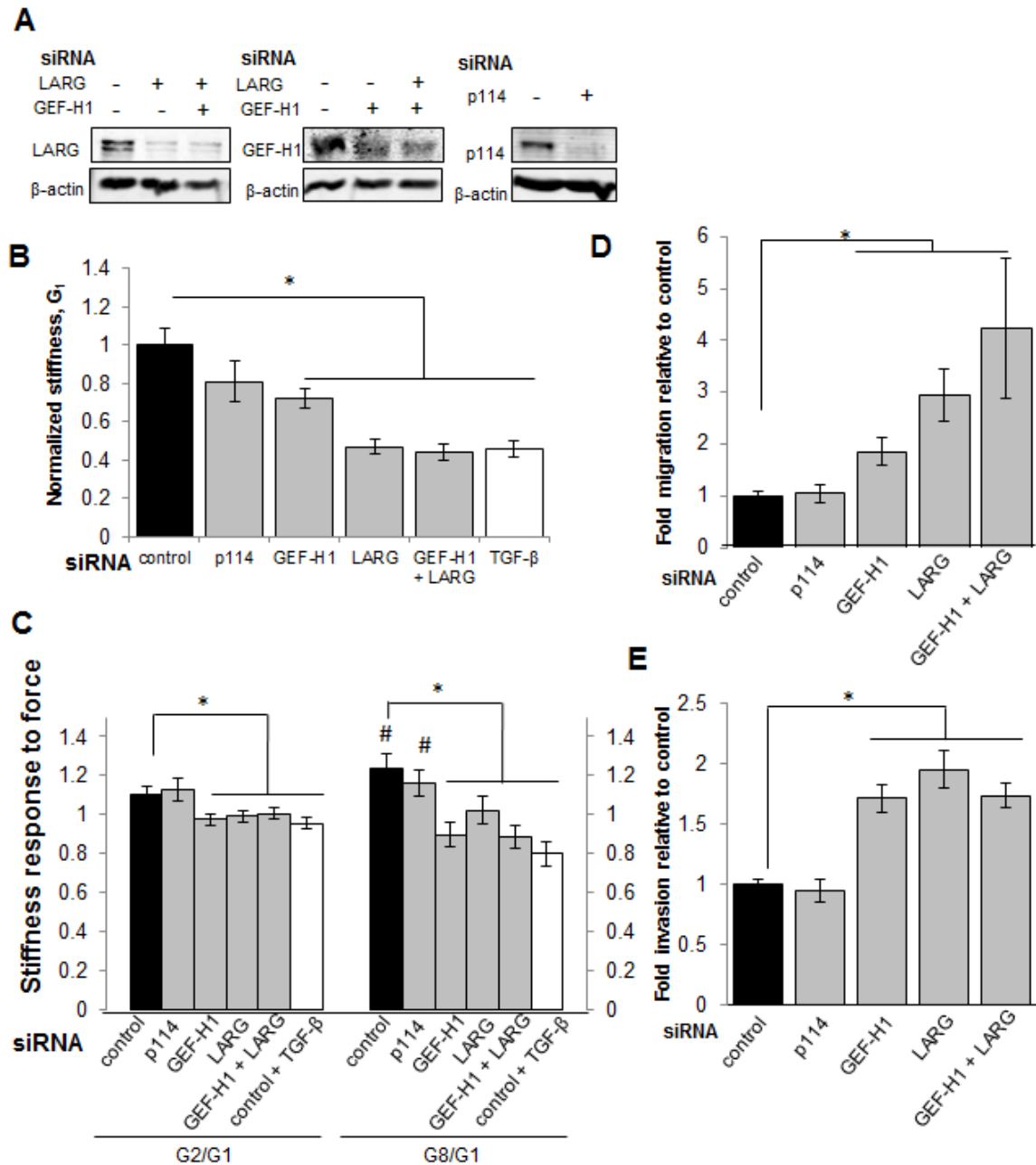


Figure 5.14: LARG and GEF-H1 knockdowns decrease cell stiffness and stiffness response to force and increase cell migration and invasion. (A) NMuMG cells were transfected with siRNA against LARG, GEF-H1, both, or p114 for 48 hours. (B) Average cell stiffness (G_1) for NMuMG cells treated with siRNA control (n = 88), siRNA targeting p114 (n = 70), GEF-H1 (n = 90), LARG (n = 85), GEF-H1 + LARG (n = 100), and NMuMG cells treated with TGF- β and siRNA control (n = 69). *p < 0.01. (C) Average stiffness response at pull 2 ($\frac{G_2}{G_1}$) and pull 8 ($\frac{G_8}{G_1}$) for NMuMG cells treated with siRNA control (n = 20), siRNA targeting p114 (n = 41), GEF-H1 (n = 19), LARG (n = 21), GEF-H1 + LARG (n = 30), and NMuMG cells treated with TGF- β and siRNA control (n = 19).

5.8 Mesenchymal-state cells recover epithelial-state phenotypes

Our results indicate that LARG and GEF-H1 are necessary mediators of stiffness (Fig. 5.14 B) and stiffness response (Fig. 5.14 C). To examine whether downregulation of LARG and GEF-H1 was necessary and sufficient to restore post-EMT loss of stiffness and stiffness response phenotypes, we rescued GEF expression after EMT induction and performed mechanical and invasion assays. Post EMT NMuMG cells were transfected with plasmids containing cDNA to encode GFP-tagged LARG and GEF-H1 to rescue the reduced expression (Fig. 5.15 A). Mechanical measurements were performed only on LARG or GEF-H1 expressing cells, as identified by GFP expression. We found that restoring LARG or GEF-H1 expression rescued the 50% post-EMT reduction in cell stiffness to 70 and 80% of the GFP-control stiffness, respectively (Fig. 5.15 B). Similarly, restoring LARG or GEF-H1 expression rescues the 30% post-EMT reduction in stiffening response to 90% of the response observed in GFP-control cells (Fig. 5.15 C).

We previously observed that loss of LARG and GEF-H1 expression in epithelial cells increased migration and invasion (Fig. 5.14 D,E). To determine whether restoring the expression of these GEFs also impacts cell migration and invasion, we used fluorescence-activated cell sorting (FACS) to isolate GFP expressing cells, and then performed migration and invasion assays with these cells. We found that overexpression of LARG or GEF-H1 fully suppressed EMT-induced increases in migration and invasion (Fig. 5.15 D,E) compared to control GFP cells, thus establishing LARG and GEF-H1 as

being sufficient for the mechanical and invasion phenotypes obtained during TGF- β initiated EMT.

Figure legend for Fig. 5.15 continued.

(C, continued) # $p < 0.05$ denotes stiffness difference from G_1 , * $p < 0.05$ denotes stiffness response difference relative to cells treated with TGF- β and GFP vector control. Error bars represent SEM, data was collected from 3 independent experiments.

(D,E) Average migration (D) and invasion (E) for cells treated with TGF- β and transfected with plasmid containing empty vector control (GFP), LARG-GFP, or GEF-H1 GFP for the final 16-24 hours of treatment and sorted by flow cytometry for GFP expression. Quantifications given as fold migration or invasion relative to control ($n = 3$). * $p < 0.05$. Migration and invasion data taken by George Li.

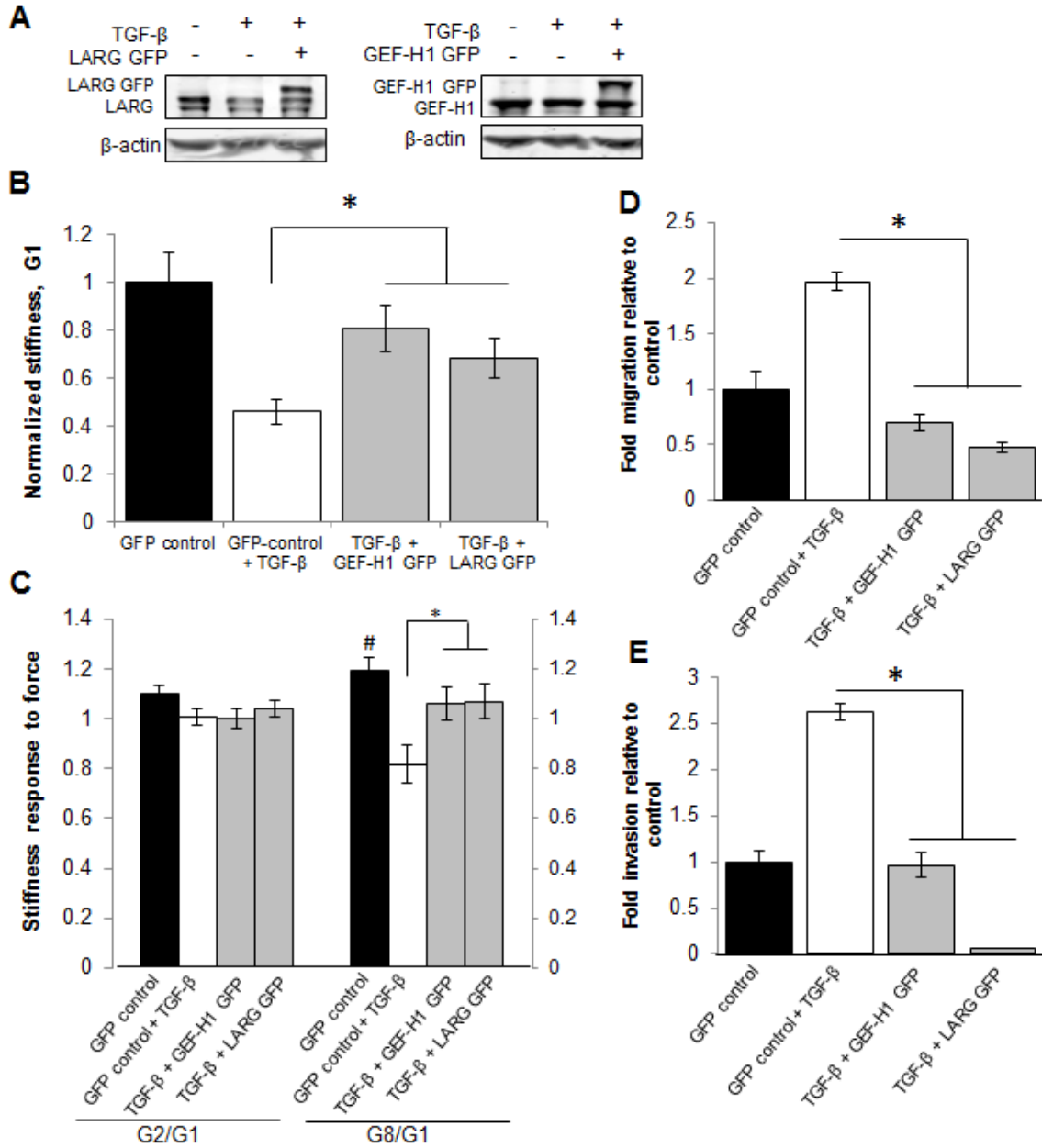


Figure 5.15: **LARG and GEF-H1 overexpression after EMT partially rescues cell stiffness and stiffness response to force and attenuates migration and invasion.** (A) NMuMGs were treated with 100 pM TGF- β for 48 hours. Cells were transfected with plasmid containing LARG-GFP or GEF-H1-GFP for the final 16-24 hours of treatment. (B) Average cell stiffness (G_1) for cells transfected with GFP vector control ($n = 65$), TGF- β and GFP vector control ($n = 55$), and GFP-DNA constructs to overexpress GEF-H1 ($n = 32$) or LARG ($n = 54$). * $p < 0.05$ denotes stiffness difference relative to cells treated with TGF- β and GFP vector control. (C) Average force response at pull 2 (G_2/G_1) and pull 8 (G_8/G_1) for NMuMG cells transfected with GFP vector control ($n = 25$), TGF- β and GFP vector control ($n = 15$), and GFP-DNA constructs to overexpress GEF-H1 ($n = 16$) or LARG ($n = 23$).

5.9 Conclusions and signaling model

To examine the role of cell mechanics in EMT, we employed force-consistent biophysical and biochemical assays to characterize the mechanistic links between stiffness response and cell invasion during EMT. We demonstrate that epithelial-state cells respond to force on integrins by evoking a stiffening response, and that after EMT, mesenchymal-state cells have reduced stiffness but also lose the ability to increase their stiffness in response to force. Using loss and gain of function studies, we establish two RhoA activators, LARG and GEF-H1, as both necessary and sufficient mediators of the effect of EMT on stiffness and stiffness response. We determine that TGF- β mediates proteasome degradation of LARG and GEF-H1 via ALK5, and that reduction of these RhoA activators contribute significantly to the increase in migration and invasion behavior during EMT. Fig. 5.16 shows a signaling model of these findings.

Here, we discuss the significance of GEFs and the RhoA pathway to mesenchymal cell invasion, and TGF- β regulation of the RhoA pathway during EMT.

Significance of GEFs and the RhoA pathway to mesenchymal cell invasion.

EMT marks the physical initiation of cancer progression as a cell detaches from the primary tumor and invades the surrounding stromal space. Invasion is a complex process, however, and it is known that cancer cells can adjust between amoeboid and mesenchymal motility modes depending on the particular ECM environment (Sanz-Moreno et al., 2008)(Sanz-Moreno and Marshall, 2010)(Wolf et al., 2003). During EMT, generally immobile epithelial cells acquire mesenchymal motility, characterized

by protease-dependent degradation of the ECM, Rac1 GTPase-regulated lamellipodial protrusions at the leading edge, and tightly controlled and appropriate RhoA and RhoC-dependent actomyosin contractility at the cell rear that results in disassembly of FAs and retraction of the trailing edge (Yilmaz and Christofori, 2009). Although evidence indicates that the GEF/RhoA pathway is altered in numerous cancers (Vega et al., 2011)(Simpson et al., 2004), its role is complicated, and often conflicting across the community. RhoA activation in some cancers is associated with increased invasion (Liao et al., 2012), while in other models its activation inhibits cell invasion (Bellovin et al., 2006). Studies using HeLa cells (Nalbant et al., 2009) and retinal pigment epithelia cells (Tsapara et al., 2010) showed that GEF-H1 mediates cell migration in wound healing assays and standard invasion assays. Another group used NMuMG cells and found that GEF-H1 is required for invasion across compliant collagen gels (Heck et al., 2012). Interestingly, observations in keratinocytes have shown that the RhoA GEF Net1A is specifically downregulated during TGF- induced EMT (Papadimitriou et al., 2011). Furthermore, work with human breast cancer cells suggest that regulation of RhoA by GEFs alone may initiate EMT invasion as mesenchymal invasion was promoted over amoeboid by knockdown of either Net1A or RhoA (Carr et al., 2013). We find that LARG and GEF-H1 are downregulated during EMT (TGF- β and BMP-2 induced) in multiple epithelial models. In addition, silencing expression of LARG and GEF-H1 increases invasive capacity towards that of post-EMT mesenchymal cells. Our data is consistent with a model in which mesenchymal invasion occurs concurrently with decreased stiffness and is mediated via TGF- β regulation of RhoA activity to enable

passage through the basement membrane and ECM required during EMT.

TGF- β regulation of the RhoA pathway during EMT. TGF- β mediates cellular functions during EMT via both canonical Smad-dependent pathways as well as non-canonical Smad-independent pathways, with canonical pathways typically being downstream of the type I TGF- β receptor (ALK5). Mechanisms of TGF- β regulation include alterations of gene transcription (Massagué, 2012), microRNA-mediated translational silencing (Winter et al., 2009), and enhanced proteasome degradation via increased poly-ubiquitination (Ozdamar et al., 2005). We established that TGF- β enhances proteasomal degradation of LARG and GEF-H1 via an ALK5-dependent pathway, and that microRNAs do not seem to play a prominent role in the regulation of these GEFs. TGF- β has been previously reported to target Net1A, another RhoA GEF, for proteasome degradation, though translational silencing by miR-24 also contributes to its downregulation (Papadimitriou et al., 2011). Interestingly, TGF- β has also been shown to target RhoA for proteasome degradation by activating the ubiquitin ligase Smurf1 via polarity protein Par6 (Ozdamar et al., 2005). Tight spatial and temporal regulation of RhoA and associated GEFs by TGF- β is crucial during the EMT program. Early in EMT, loss of RhoA activation and destabilization of microtubules at the basal surface of epithelial cells causes loss of cell-basement membrane interactions (Nakaya et al., 2008). Interestingly, transient upregulation of Net1A has been shown to be required for EMT initiation, but by 24 hours, Net1A levels are subsequently depleted as the cell progresses through EMT (Papadimitriou et al., 2011), perhaps to allow cells to acquire mesenchymal motility as discussed above.

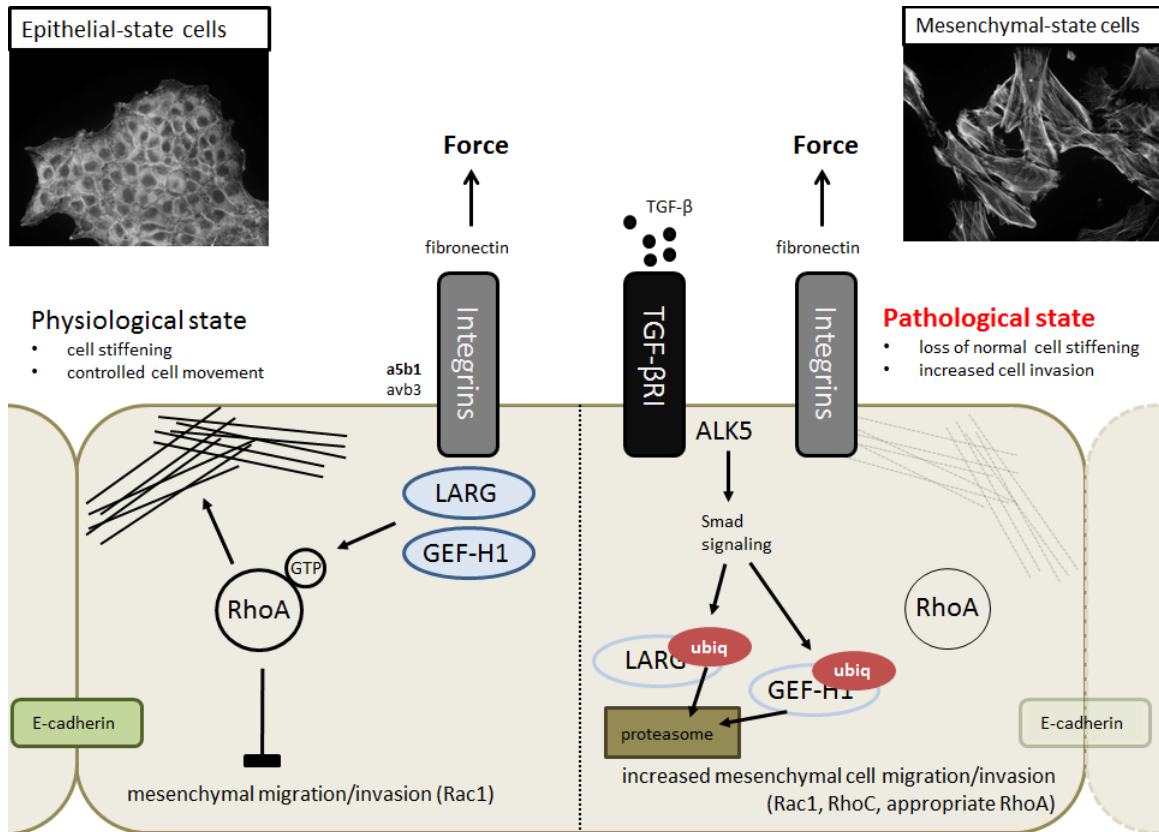


Figure 5.16: Interaction between the TGF- β signaling and RhoA mechanotransduction pathways.

5.10 What is the utility of reduced stiffness and stiffness response?

Compared to normal cells, invasive cancer cells have a reduced stiffness; this observation has been found across instruments and methodologies, across cell types, and at the single-cell and tissue levels (Guck et al., 2005)(Suresh et al., 2005)(Swaminathan et al., 2011)(Hanna et al., 2013)(Xu et al., 2012a)(Plodinec et al., 2012)(Gossett et al., 2012). Additionally, invasive cancer cells have been shown to exert larger tractional forces on their environment (Kraning-Rush et al., 2012). As interest grows around integrated studies – efforts to characterize a single model system in many different assays – the above relationships continue to hold. Recently, a cell mechanics “consortium” study, lead by the Physical Sciences-Oncology Centers Network (physics.cancer.gov), found that as 1D and 3D migration increases, cytoplasmic stiffness decreases (AFM and internal PBR), nuclear and nucleolar stiffness decreases (AFM), and traction forces increase (Agus et al., 2013). In this chapter, we showed evidence to suggest that stiffness and invasion cancer phenotypes are functionally linked through RhoA GEFs, and that these proteins are negatively regulated during EMT.

Here, we discuss the potential utility of altered stiffness and stiffness response in EMT.

1. Reduced stiffness increases ability to traverse narrow passages. Increasing evidence supports the long-standing thought that a certain degree of deformability, or reduced stiffness, is required for metastatic cells to navigate the basement membrane and intra- and extravasate the vascular system (Suresh et al., 2005)(Wirtz et al., 2011).

2. Stiffening near adhesions may negatively effect invasion. These results support a model in which epithelial cells resist external deformation by mounting a stiffening response to maintain mechanical equilibrium and prevent potential injury (Glogauer, 1998)(Matthews et al., 2006). During EMT and associated epithelium detachment, a stiffening response to force may lose its utility to mesenchymal-state cells, as adaptive stiffness near integrins may increase adhesion size and strength, and thus hinder the adhesion turnover required for effective cell migration and invasion.

3. Reduced stiffness may allow for larger contractile forces. An attenuated cortical stiffness and stiffening response to force after EMT may reduce the internal resistance that cell-generated forces act against. Thus, for a given cell-generated force (or stress σ), post-EMT cells could exert higher actomyosin contractile forces to the ECM. Such increased contractile forces have been shown to be correlated with cells of increasing metastatic potential (Kraning-Rush et al., 2012)(Agus et al., 2013), and is specifically required for mesenchymal invasion in certain cancers (Friedl and Wolf, 2003).

4. Reduced stiffness and stiffening response may enable mechanosensing. Another impact of reduced stiffness to EMT may arise in the generation of contractile forces that are tuned for mechanosensing. To dynamically probe areas of increasing and variable stiffness, as seen in cancer ECM (Butcher et al., 2009), cells use integrin-associated focal adhesions as autonomous stiffness sensors (Plotnikov et al., 2012). Migration through these regions is thought to involve appropriate regulation of cell-generated, actomyosin contractile forces (Plotnikov and Waterman, 2013). These

forces are transmitted to the ECM via integrin adhesions and must act through an internal stiffness, which is likely the stiffness of the actin cytoskeleton network and adhesion complexes. Thus, our results suggest that reduced stiffness and stiffening response to external force application (post-EMT) may enable, or increase efficiency of, mechanosensing mechanisms during cancer cell invasion.

Chapter 6: A High Throughput Array Microscope

6.1 Overview

In the last decade, the emergence of high throughput screening (HTS) has enabled the development of novel drug therapies and elucidated many complex cellular processes. Concurrently, the mechanobiology community has developed tools and methods to show that the dysregulation of biophysical properties, and the biochemical mechanisms controlling those properties, contribute significantly to many human diseases. Despite these advances, a complete understanding of the connection between biomechanics and disease will require advances in instrumentation that enable parallelized, high throughput assays capable of probing complex signaling pathways, studying biology in physiologically relevant conditions, and capturing mechanical heterogeneity at single-cell and population levels. Traditional biophysical instruments are unable to meet this need. To address the challenge of large-scale, parallelized biophysical measurements, we have developed an automated array high-throughput (AHT) microscope system that utilizes passive microbead diffusion to characterize mechanical properties of biomaterials.

Panoptes collaboration. The array high throughput (AHT) microscope, internally

referred to as *Panoptes*, was developed by numerous engineers, computer scientists, and physicists over the last several years. I have had the fortune of working with this team during initial testing with our prototype single-channel microscope, during the build of Panoptes, and during the first executed high throughput experiments. Specifically, my contributions included: construction of the Panoptes microscope, XY length scale calibration of the liquid-lens, qualification tests of liquid-lens installed objectives, noise floor measurements, development of data analysis software, and execution of high throughput rheology and cell rheology experiments. As with any collaboration, my contributions were enabled by the hard work and dedication of many colleagues. Work in this chapter is described in a manuscript currently under review, with citation: J. Cribb*, L.D. Osborne*, J. Hsiao, L. Vicci, A. Meshram, E. Tim O'Brien III, R. Taylor II, R. Superfine. A High Throughput Array Microscope for the Mechanical Characterization of Biomaterials. Review of Scientific Instruments. (Manuscript *under review*). (2014). *these authors contributed equally to this work.

6.2 The Need for High Throughput Mechanical Measurements in Biology

Recent advances in imaging technology and computational analysis have facilitated large-scale quantitative biology studies through the development of high throughput screening (HTS). In the last decade, the impact of HTS on biology has been significant (Macarron et al., 2011) in enabling the development of novel drug therapies (Duffy et al., 2001)(Dorr et al., 2005)(Gao et al., 2010), and elucidating complex cellular processes including differentiation (Desbordes et al., 2008), division (Burke et al., 2013), and migration (Simpson et al., 2008). Concurrently, the mechanobiology community has developed tools and methods to show that the dysregulation of biophysical properties, and biochemical mechanisms that control them, contribute significantly to many human diseases such as arthritis (O’Conor et al., 2014)(Sanchez-Adams et al., 2014), atherosclerosis (Collins et al., 2014), cystic fibrosis (Kater et al., 2007)(Rubin, 2007b), blood coagulopathies (Pezold et al., 2012)(Nystrup et al., 2011), and cancer (Swaminathan et al., 2011)(Plodinec et al., 2012)(Osborne et al., 2014). Despite these findings, a complete understanding of the connection between biomechanics and disease will require advances in instrumentation that enable parallelized, high throughput assays capable of probing complex signaling pathways, studying biology in physiologically relevant conditions, and capturing mechanical heterogeneity at the single cell and population level. Such a system would transform the state of the art, placing the onus of experimental design on the targeted hypothesis, rather than on the methodology, workflow, and the time needed to execute such a complicated experiment.

Traditional biophysical instruments and methodologies are unable to provide parallelized, high throughput investigation of biomechanical systems. For example, bulk rheological devices like cone and plate (CAP) require large sample volumes and long duration testing, and therefore lack scalability to parallelized, high throughput studies. Similarly, even small volume techniques such as atomic force microscopy and optical and magnetic tweezers use designs that acquire data on samples in a serial fashion. Thus, although providing great detail, these techniques are limited in the number of treatments or conditions that can be measured before losing specimen viability. Compounding these issues is the uncontrollable heterogeneity commonly found in single cell or material measurements (Kirkham et al., 2002)(O’Callaghan et al., 2011) which demands intellectual attention (Mellnik et al., 2014), but also imposes the challenge of collecting sufficient data for generating statistically sound conclusions. Recent efforts to increase the throughput of mechanical measurements (Reed et al., 2011)(Wu et al., 2012a)(Gossett et al., 2010) have addressed the need of capturing specimen and mechanical heterogeneity, but still acquire data across experimental conditions serially.

In the present work, building on our previous technology (Spero et al., 2008), we present an array high throughput (AHT) microscopy system that enables parallelized, high throughput mechanical measurements of cells and biomaterials. The AHT system implements passive microrheology, a well-established technique that has been successful in characterizing the mechanical properties of a wide range of biological specimens, including mucus (Hill et al., 2014), fibrin (Spero et al., 2011), and cells (Wirtz, 2009)(Massiera et al., 2007)(Daniels et al., 2010). Passive microrheology has been noted

for holding promise as a high throughput methodology (Breedveld and Pine, 2003).

Here we first describe the AHT system design, including discussion of the imaging and mechanical subsystems and associated custom software. We then demonstrate that the ATH system can accurately measure the viscosity of Newtonian fluids.

6.3 Array Microscope: Design, Hardware, and Software

The AHT system was designed to implement passive microrheology, a well-established methodology that uses the thermal deflection of micron-scale particles by energy kT (where k is the Boltzmann constant and T is the temperature of the molecular environment) to estimate the mechanical response functions for the surrounding material (Mason, 2000). By measuring the displacement of fluorescent particles that have been embedded in a material, the mean-squared displacement (MSD) is calculated using (Eqn. 2.46)

$$\langle \Delta r^2(\tau) \rangle = \langle [x(t + \tau) - x(t)]^2 + [y(t + \tau) - y(t)]^2 \rangle \quad (6.1)$$

where τ is the time lag, or timescale. The mechanical response is quantified as the complex, frequency-dependent shear modulus $G^*(\omega) = G'(\omega) + iG''(\omega)$, where $G'(\omega)$ and $G''(\omega)$ correspond, respectively, to the elastic and viscous contributions of the viscoelastic material. Using the ensemble averaged MSD, the linear mechanical response of a material is given by the generalized Stokes-Einstein relation (GSER) in Eqn. 2.45 (Mason, 2000).

Motivated by the need for parallelized, high throughput characterization and time-course mechanical measurements, we engineered the AHT system to comply with the SBS standard geometry for high throughput multiwell plates. To lie within a multiwell plate footprint, twelve independent optical systems were arranged in a 2 by 6 array, each containing a 40 X objective (Universe Kogaku America) with an estimated NA of 0.45 and a maximum working distance of 650 μm , two epi-fluorescence illumination modes, and a remote-head camera (Point Grey, Inc.) (Fig. 6.1 A,B,C). To enable each optics channel to have independent control over its plane of focus at the expense of lower NA, we retrofitted a liquid lens (Parrot Varioptic) into each objective. A commercially-available translation stage (Ludl Electronic Products Ltd.) drives the plate in XY around the stationary optics and imaging subsystem, traversing an entire 96-well plate in seven steps (Fig. 6.1 D). Stepper motors provide z-translation of the multiwell plate, and also serve to neutralize plate tilt and mechanical bow inherent to commercial plates (Fig. 6.1 A). To enable precise control over hardware across the AHT system, a unique and custom-designed operating system manages a multi-functional synchronization framework that coordinates events across multiple hardware domains with $\sim 100 \mu\text{s}$ resolution. Packaged together, the AHT system employs a fully autonomous pipeline that acquires video for a full 96-well plate within 10 minutes and outputs mechanical information for each well within half an hour.

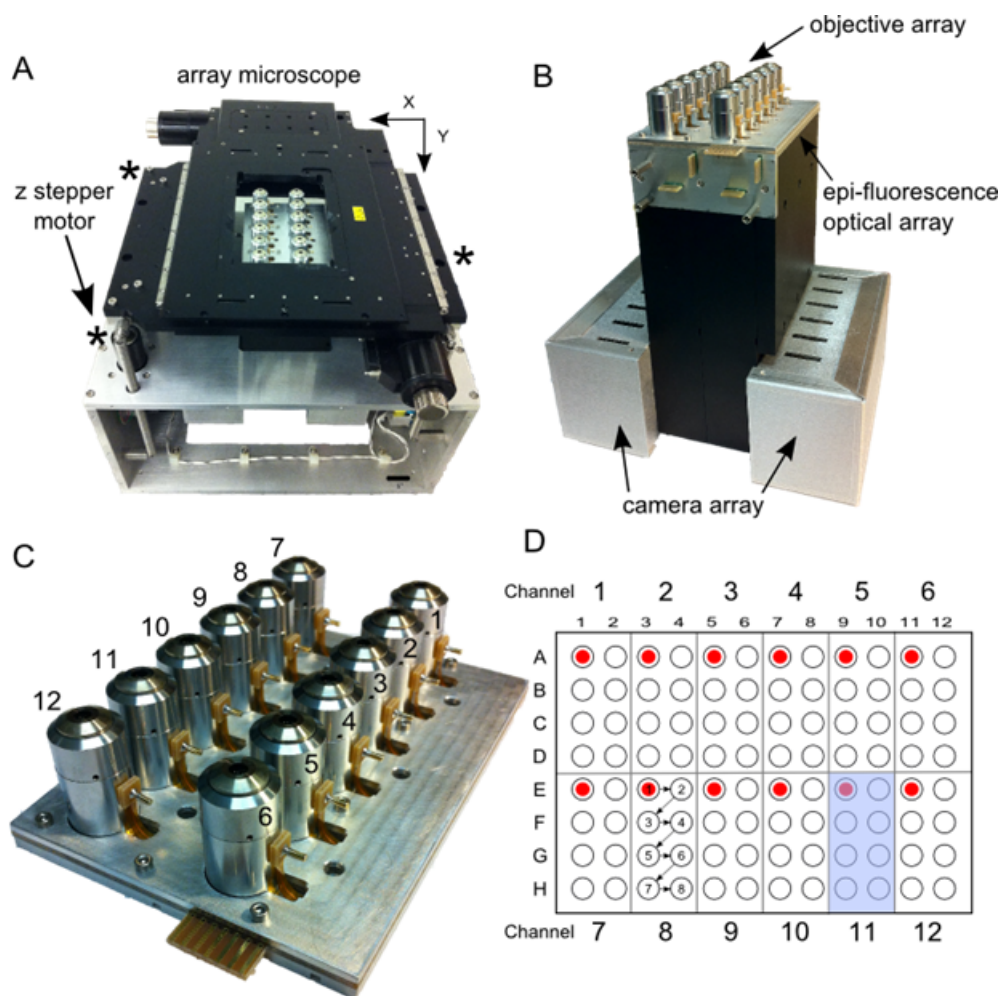


Figure 6.1: **The Array high-throughput (AHT) microscope.** (A) System: In the center of the photo are the 12-objective lenses that sit beneath a 96-well multiwell plate (not shown). The objectives are surrounded by the XY-positioning stage. Three individually-controlled stepper motors (marked by asterisks) drive the XY stage and multiwell plate in Z and compensate for tilt. The entire system is about the size of a breadbox. (B) The AHT imaging block. Beneath the 12-objectives are 5 printed circuit boards that supply voltage to the liquid lenses and current to the amber and blue LEDs that provide epi-fluorescence illumination. At the bottom of the imaging block are the control boards for the 12 cameras. (C) The objective array. The objectives are anchored between a support plate and an objective clamping plate. Each objective has a pin that supplies voltage to its liquid lens. (D) This schematic of a multiwell plate shows the neighborhood of wells visited by each of the 12 imaging channels (example shadowed in blue for channel 11). In a typical experiment each channel starts in the upper-left corner of its neighborhood and follows the sequence shown in the figure for imaging channel 8.

6.3.1 Optics and Imaging Block

The AHT microscope contains twelve independent optical and imaging subsystems. In order to work within the physical footprint of SBS standard multiwell plates, we designed a compact optical hub for epi-fluorescent imaging (Fig. 6.2 A,B). Each optical channel features two LED light sources, each centered at wavelengths that correspond to traditional choices in fluorescence microscopy: the “blue” channel, centered around 490 nm, allows for standard FITC and EGFP illumination, while the “amber” channel, centered around 575 nm, provides rhodamine and Texas red illumination.

The light emitted by both LEDs is gathered by its own collection lens (CL) (Edmund Scientific). To properly filter and transmit the excitation light from both fluorescent LEDs, appropriate excitation filters (EF) and a dichroic filter (DF) were contract manufactured as a custom-designed optical cube (Edmund Scientific) and installed in the optical hub (Fig. 6.2 B). The surface along the diagonal of the optical cube serves as a dichroic, passing the higher wavelength excitation light from the blue channel, and reflecting the lower wavelength excitation light from the amber channel. An anti-reflective (AR) coating applied to the last surface of the optical cube minimizes light scattering within the cube (Fig. 6.2 A). Outside the optical cube, a condensing lens concentrates light which is then sent through the objective to the specimen via reflection from a second dichroic filter (Fig. 6.2 A), designed to block the excitation light from both blue and amber LEDs.

The AHT microscope uses a 40 X objective to collect and focus emission light (Fig.

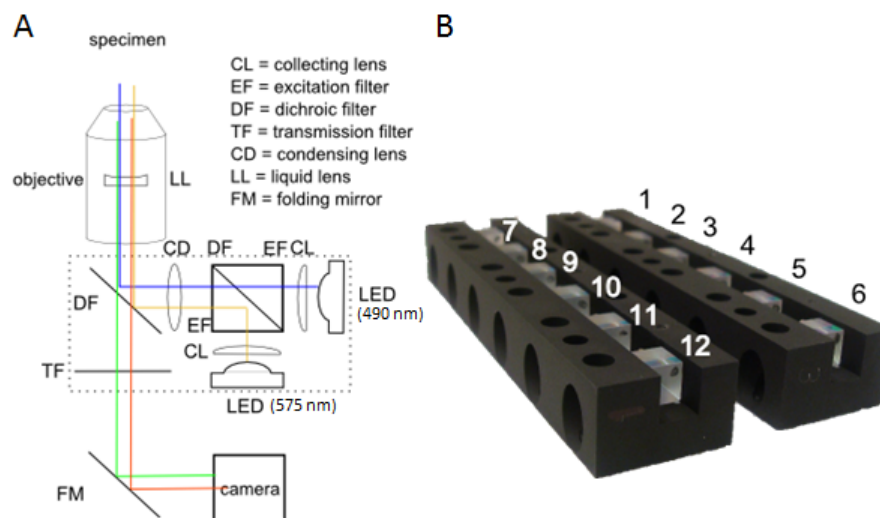


Figure 6.2: **The optical system of the (AHT) microscope.** (A) Schematic of the optical path through the epi-fluorescence optical hub. The “blue” channel emits LED light centered around 490 nm for standard FITC and EGFP illumination. The “amber” channel, emits LED light centered around 575 nm and provides rhodamine and Texas red illumination. (B) Epi-fluorescence optical hubs that direct excitation light for both illumination modes in all 12-channels of the AHT.

6.2 A). The DF and transmission filter (TF) at the end of the optical hub ensures that only emission light passes successfully to the camera (Fig. 6.2 A). Once emission light leaves the optical hub, a folding mirror relays light to the camera (Fig. 6.2 A). In order to meet the space constraints dictated by the footprint of standard multiwell plates, we chose the remote-head version of the Dragonfly camera which offers 50+ frames per second (fps) at VGA resolution, satisfying the desire to collect short timescale mechanical measurements from thermal diffusion of particles. The AHT microscopy system generates sufficient signal to noise to enable successful tracking of 200 nm beads at 15 fps and 500 nm (and larger) beads at 50 fps.

In order to meet the spatial constraints of the SBS plate standard, a significant number of power-dissipating electronic circuits had to share space within the completed

optics subsystem. Due to confined space, the heat generated from these electronics raised the operating temperature of the system from an ambient 23 deg. C to 29 deg. C. Future enhancements to the system include a temperature control subsystem that will allow for measurements at room temperature as well as the more physiologically relevant 37 deg. C.

6.3.2 XY-calibration of Liquid Lens

Cell rheology experiments using the AHT system require autofocus in order to focus on attached beads at the focal plane of the cells. To allow each imaging channel to independently focus in z, we retrofitted the objectives with an electrically-controllable liquid lens (LL; Parrot Varioptic), where applying sufficient voltage to the contact pin alters the electrowetting properties and changes the plane of focus (Fig. 6.3 B, blue). However, the effective magnification of the objective also changes with voltage. To determine the length scaling for variable voltage, the voltage was altered in ~ 5 V steps and the focus was changed manually using a micrometer that translates the entire optics train. An image of a micron-scaled graticule was taken, and the number of $\mu\text{m}/\text{pixels}$ was then measured in ImageJ. A linear fit was made, therefore enabling an estimation of the length scaling for a given voltage across the liquid lens (Fig. 6.3 B, green data).

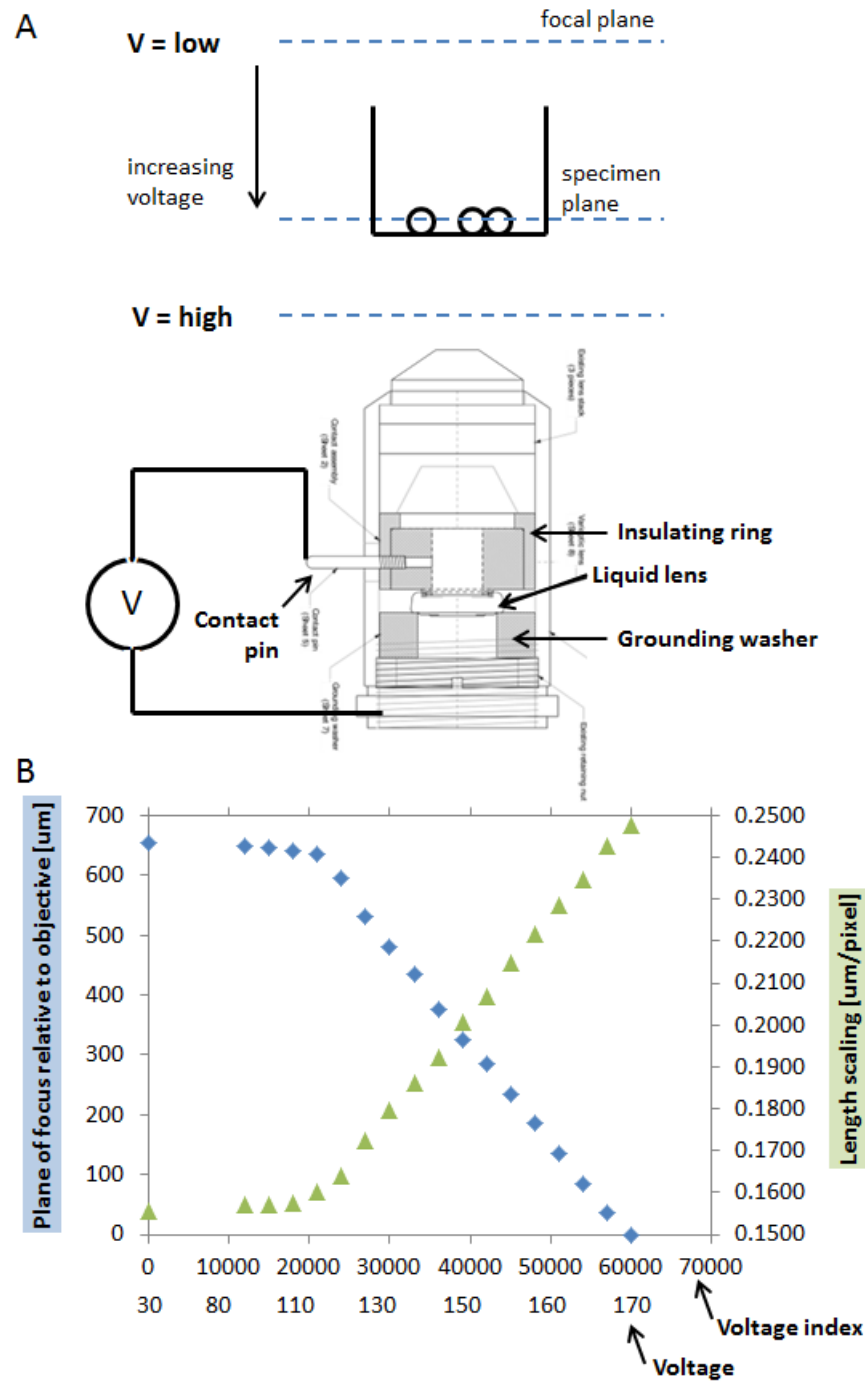


Figure 6.3: **Liquid lens provides voltage tunable z-focus.** (A) Relationship between voltage and focal plane relative to the objective body: as voltage increases, the focal plane is brought closer to the objective body. The maximum working distance is $650 \mu\text{m}$. (B) Plane of focal relative to objective (blue dots) and length scaling in $\mu\text{m}/\text{pixel}$ (green dots) versus voltage.

6.3.3 AHT Data Collection and Analysis

An experiment on the AHT system is initiated on a master control computer as a list of instructions which is distributed to an array of microcontrollers (12, 1 for each channel). Installed on each microcontroller is a custom designed operating system: Launching Events With Optimal Synchronization (LEWOS). LEWOS, designed by Russ Taylor, provides a language for control over LED illumination (for three sets of LEDs), focal plane adjustment via the voltage-tunable liquid lenses, and camera triggering for image acquisition. Each microcontroller has a designated control computer that stores the images that are collected for the channel-specific camera. Once data collection is complete, each control computer uses Video Spot Tracker (VST) software (cismm.org) to, in parallel, rapidly track particle trajectories from the generated videos.

After data collection and tracking, the metadata and tracking data are moved from each control computer to the master control computer for analysis and reporting. A suite of custom MATLAB scripts completely automates the process of: 1. reading in VST-generated tracker position time-series data for each video (one video for each FOV in every well and channel); 2. FOV-specific dead-zone filtering and center-of-mass drift removal; 3. computing the MSD for individual particles in a given video (appropriately scaled by the length scaling for given channel); 4. aggregating MSDs for replicate wells on the plate; 5. plate-wide analyses via heatmaps, and 6. generation of an HTML page for data organization, visualization and reporting.

The workflow of an ATH experiment is shown in Figure 6.4. An analysis procedure

to identify subpopulations within aggregated MSD datasets will be discussed in Ch. 7.

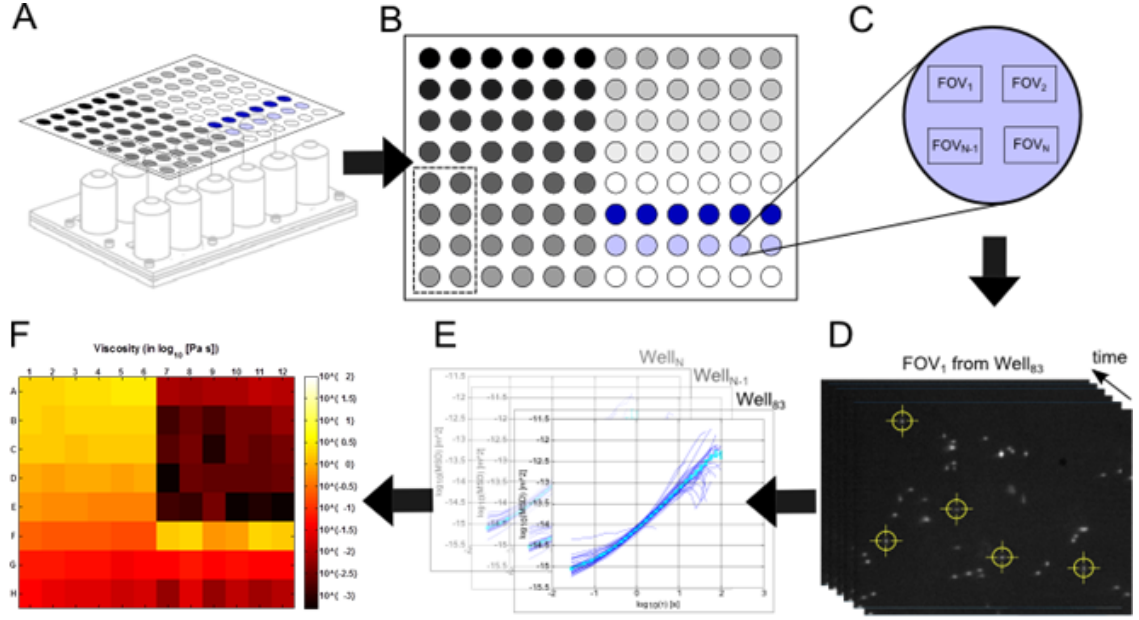


Figure 6.4: **Data collection and analysis workflow for ATH microscope experiment.** (A) Schematic of a specimen-loaded multiwell plate above the 12-channel objective array. (B) Layout of a specimen plate for a “concentration sweep type rheology experiment”. The grey-scale gradient represents increasing concentration of a polymer solution. The dashed rectangle partitions the wells accessed by the 7th objective in the array (see Fig. 6.1 C). (C) Blow-up a well within the multiwell plate. After video is collected in each of the 8 wells accessed by the objective array, additional passes through the wells can be made. This schematic shows multiple locations, or fields of field (FOV), for video collection within the given well. (D) Video data acquisition and single particle tracking. Once data is collected, the video for each FOV is tracked using particle tracking software to generate time-series position measurements. (E) The mean squared displacement (MSD) is computed for each particle (blue curves) using Eqn. 2.45 and the ensemble sample-weighted average is computed (cyan curve). (F) The average MSD can be transformed into complex moduli using Eqn. ?? and visualized in a heatmap at a particular timescale.

6.3.4 Noise floor of the AHT Microscope

To assess the noise floor (NF) of our AHT microscope we measured the effective bead displacement of fixed beads over time. Briefly, beads are suspended in anhydrous ethanol at a designated concentration (*e.g.* 1:50 for 2 μm beads, 3.5 μL delivered to a well of multiwell plate) and allowed to evaporate onto the coverslip.

For our AHT prototype microscope and 1 μm beads, this procedure yields a minimum MSD value of $10^{-16.5} \text{ m}^2$ (flat region of Fig. 6.5 A; blue curve for 1 μm beads), which corresponds to a particle tracking resolution, or NF, of 5.7 nm (Fig. 6.6). This measurement was taken with the liquid lens set to 0 V and focus adjusted manually. Using Eqn. 2.42, $G = \frac{2kT}{3\pi a \langle \Delta r^2(\tau) \rangle}$, we compute the maximum G that the system could measure under these conditions as 55 Pa.

The NF is dependent on the signal to noise (SNR) ratio, and therefore indirectly on bead size a . When we use a smaller, 500 nm bead diameter with a signal to noise ratio (SNR) similar to the 1 μm beads (~ 33), the NF increases modestly to 7.4 nm (Fig. 6.5 A, green curve; an expected result due to a reduced number of pixels). However for 200 nm beads where the SNR is not maintained (~ 4), the NF increases to 22 nm and our maximum G is reduced to 18 Pa despite the reduction in a (Fig. 6.6).

The NF is also dependent on the voltage across the liquid lens. We suspect this is due to reduced SNR (potentially due to reduced numerical aperture). Therefore, for cell rheology experiments which require the liquid lens to autofocus on beads at the focal plane of the cells, the NF is increased to ~ 12 nm on the AHT prototype

microscope (Fig. 6.6).

The lowest observed NF of the AHT microscope (not prototype) is 17 nm (Fig. 7.13), corresponding to a maximum $G = 3$ Pa for 2 μm beads (Fig. 6.6). The NF of the AHT microscope contains all of the above dependencies, and is also dependent on additional factors which may compromise the NF. One factor we suspect significantly effects the NF is the more constrained optical pathes of the AHT microscope compared to the prototype microscope (due to the spatial constriants of the standard multiwell plate imposed on the system). The constrained optical path increases scattered light (which varies channel to channel) and reduces the SNR. A decreased SNR could in turn negatively effect particle tracking and lead to additional error in the measured NF.

Future efforts can be made to improve the noise floor of the system. This can be done by primarily by increasing the SNR. Aside from a more sensitive camera, we can increase the SNR by increasing the bead size. However, since $G_{\text{max}} \sim 1/a$, we desire to increase SNR while using as small a bead as possible. The SNR could be increased by using a higher power LED (increase brightness) and increase exposure time to increase the signal of the beads, while using better filter matches and reducing light scatter to maintain a low background noise. The signal could potentially also be improved by using a liquid lens with a larger numerical aperature.

Figure legend for Fig. 6.5 continued.

(B) The same bead sizes diffusing in water. The guidelines represent the expected MSD for beads in water at the appropriate temperature. Insets: example trajectories for a 1

μm bead diffusing in water or fixed to the plate substrate.

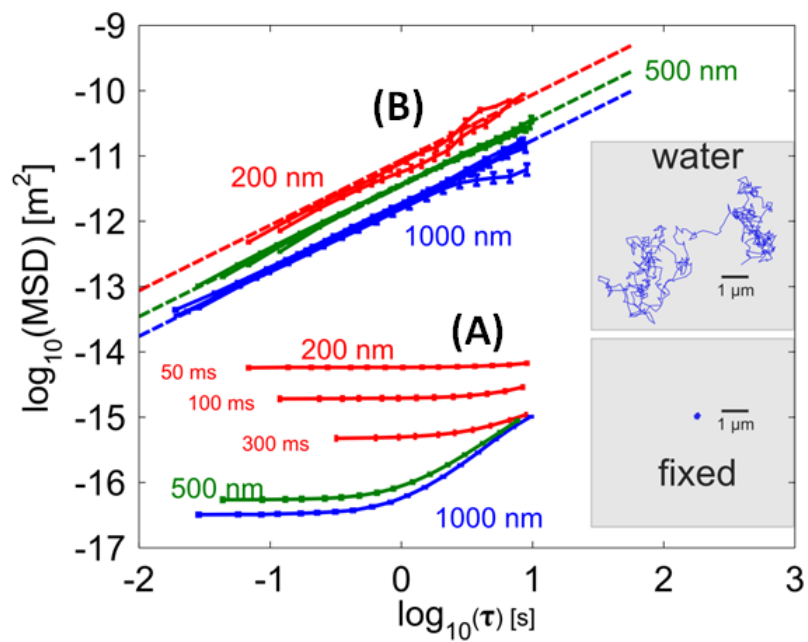


Figure 6.5: **Noise floor for the AHT Prototype Microscope.** See legend on previous page. (A) NF for 200 (red), 500 (green), and 1000 nm (blue) beads.

Microscope	Liquid Lens and Data Collection	Bead size	Noise Floor (RMS disp.)	Max G	Max eta
AHT Prototype "Monoptes"	set to 0 V manual focus/experiment	1 μm	5.6 nm	55 Pa	55 Pa s
AHT Prototype "Monoptes"	set to 0 V manual focus/experiment	500 nm	7.4 nm	64 Pa	64 Pa s
AHT Prototype "Monoptes"	set to 0 V manual focus/experiment	200 nm	22 nm	18 Pa	18 Pa
AHT Prototype "Monoptes"	Linear range*, automated focus/experiment (approx. from Fig 6.7)	2 μm	12 nm	6 Pa	6 Pa
AHT System "Panoptes"	Linear range, automated focus/experiment (From Fig. 7.13)	2 μm	17 nm	3 Pa	3 Pa

Figure 6.6: **Comparison of noise floors and limits of measure.** NF for rows 1,2 have the same SNR (~ 33), and row 3 has lower SNR (~ 4).

*Linear range of liquid lens as seen in Fig. 6.3 B

NF for rows 1,2,3 were taken at $\tau < 1$ sec (the τ independent region of floor). NF for rows 4,5 were taken at 1 sec to be comparable with biology conditions in experiments.

Maximum G and η calculated using $G_{\max} = \frac{2kT}{3\pi a \langle \Delta r^2(\tau) \rangle}$ and $\eta_{\max} = \frac{2kT}{3\pi a \langle \Delta r^2(\tau) \rangle} \tau$ where we use $\tau = 1$ sec for $\eta_{\max}$.

6.3.5 Objective Qualification for the Array Microscope

The varioptic liquid lenses (VOLs) are installed into the objectives to enable electrical control over the plane of focus. To determine whether the VOL-assembled objective performs adequately over the linear range (Fig. 6.3 B, green data), we dry beads onto the bottom of each well of a 96-well plate and run a noise floor experiment (3-pass, 20 sec video assay, according to workflow shown in Fig. 6.4) using the AHT prototype microscope, “Monoptes”. Because SBS standard multiwell plates have an inherent bow of 250 to 300 μm (center lower than edges), by taking noise floor data across the plate, we sample the entire linear range of the VOL.

After the experiment, we aggregate data across passes and plot the RMS displacement versus voltage index (“MCU parameter”). The present threshold for adequate performance is defined as: 1) well sampled MCU parameter range, 2) low-slope linear trend that begins $< 10\mu\text{m}$ at $\text{MCU} = 35,000$ and ends slightly over $10\mu\text{m}$ at $\text{MCU} = 55,000$, and 3) small number of outliers. Figure 6.7 shows successful qualifications for 7 VOL-installed objectives that are currently installed in the AHT system. The rise in RMS displacement is likely due to a decrease in the signal to noise with increasing MCU parameter.

Future work is required to make installation of the VOLs more reproducible. The VOL is held in place with a retaining ring that applies pressure to the grounding washer in Fig. 6.3 A. Currently this installation is done manually with $< 50\%$ efficiency. A custom made torque wrench that would deliver a known torque would make VOL

installation more repeatable.

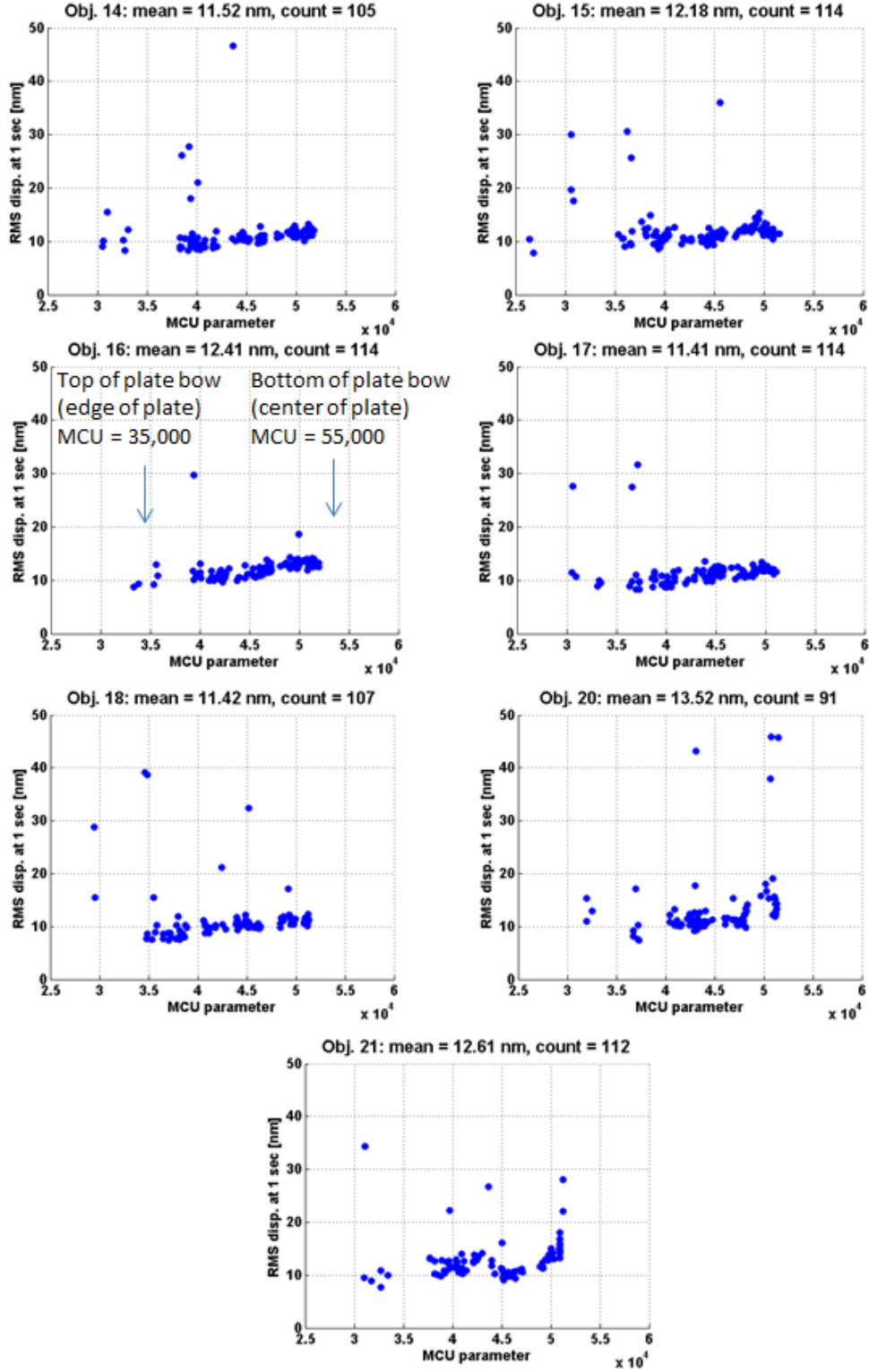


Figure 6.7: Performance tests for liquid lens installed objectives. Each data point represents the average RMS displacement for at $\tau = 1$ for all beads in one video.

6.4 Validation of AHT Microscope: Microrheology of Viscosity Standards

To test AHT system function and accuracy, we evaluated a multiwell plate that contained four Newtonian fluids: water, 2.04M sucrose, 2.5M sucrose, and corn syrup. The materials were dosed with 1 μm fluorescent beads at a concentration of 0.004% (1:500) and characterized on a CAP rheometer at $T = 29\text{ }^{\circ}\text{C}$, the working temperature of the AHT system; The CAP-determined viscosities were: 2.04 M sucrose ($\eta = 20.0\text{ mPa s}$), 2.5 M sucrose ($\eta = 83.1\text{ mPa s}$), and corn syrup ($\eta = 2840\text{ mPa s}$). The viscosity of water at $T = 29\text{ }^{\circ}\text{C}$ was determined as $\eta = 0.84\text{ mPa s}$ from (Lin et al., 2003). The materials were then loaded in alternating rows of the multiwell plate (50 $\mu\text{l/well}$) such that each imaging channel of the AHT system (Fig. 6.1 D) received two replicates of each fluid. The ATH system assayed the test plate, according to the workflow shown in Figure 6.4, yielding viscosity measurements in 90 of 96 wells for timescales τ from 0.03 sec to as long as 120 sec, depending on the material viscosity. These data are shown in Figure 6.8. A plate-wide heatmap of the measured viscosity at the $\tau = 1\text{ sec}$ timescale is shown in Figure 6.8 A.

Distinguishability of the four materials by the ATH system was tested at the channel level by aggregating replicate wells of each fluid within a channel and performing an ANOVA test against the 4 specimens on the value of the MSD at the 1 sec timescale. Results of this analysis showed that each channel measured statistically significant differences ($p < 0.0001$) between the 4 fluids (Fig. 6.8 B).

Accuracy of the AHT system relative to the material characterization by CAP or

(Lin et al., 2003) was then evaluated for each well. To minimize the effect of low sampling at long timescales, the ensemble sample-averaged MSD for each well was contracted by 1 decade at long τ . Since short timescale estimations of corn syrup routinely challenged the system noise floor (indicated by slopes that approached zero in log MSD vs log τ space), timescales before 1 sec were contracted for the aggregated MSD in these wells. The viscosity measurement η for a given fluid in a particular well was then obtained by performing a sampling-weighted fit of the average MSD data to the Stokes-Einstein relation (Eqn. 2.39):

$$\eta = \frac{2kT}{3\pi a \langle \Delta r^2(\tau) \rangle} \tau \quad (6.2)$$

Finally, the plate-level viscosity accuracy of a given fluid was determined by taking a bead-weighted average across wells (and hence across channels or imaging systems) of the given fluid. We find that the AHT system measures water with 4.3% error, 2.04 M sucrose with 5.2% error, 2.5 M sucrose with 3.6% error, and corn syrup with 35.6% error.

Figure legend for Fig. 6.8 continued.

(B, continued) For each distribution, the boxes denote the 25th (Q1) and 75th (Q3) percentiles, whiskers extend to non-outlier data defined by $Q3 + 1.5 * (Q3 - Q1)$ and $Q1 - 1.5 * (Q3 - Q1)$ and symbolized with (+). An ANOVA test was performed on the MSD distributions of each fluid.

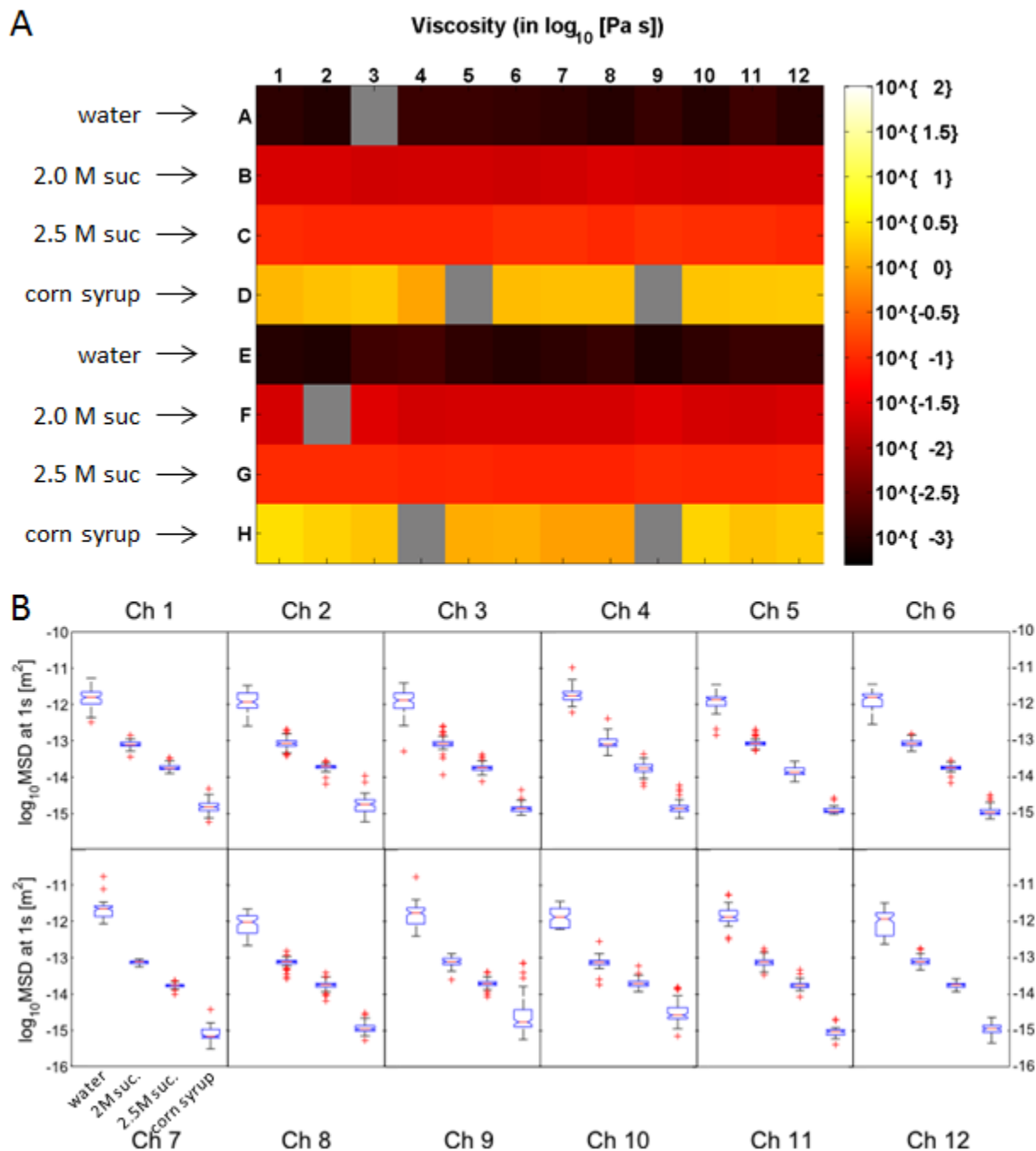


Figure 6.8: **Characterization of standard Newtonian fluids using the ATH microscope.** (A) Viscosity heatmap for alternating rows of water, 2.04M sucrose, 2.5M sucrose, and corn syrup. The materials were dosed with 1 μm fluorescent beads and a 50 μl volume was loaded into each well of a 96-well plate. Five 2 min videos at 34 fps were taken at different FOVs in each well. The viscosity at the 1 sec timescale is plotted (see workflow in Fig. 6.4; grey wells indicate positions where no data was collected). (B) Boxplots of the fluid-aggregated MSD distributions at the 1 sec timescale by channel (each channel was loaded with two wells of each fluid). Results indicated that each channel could statistically distinguish each fluid ($p < 0.0001$).

6.5 Conclusions

We have presented the novel technology of a high throughput microscopy system designed to characterize the mechanical properties of biomaterials. Our system generates parallel, high throughput capacity by integrating twelve imaging systems, automating video data acquisition, utilizing rapid and accurate particle tracking software, and automating rheological analysis. The end result is accurate biophysical measurements generated in 5% of the time required to manually execute these experiments. Fig. 6.9 itemizes the time saved using the AHT microscope to run the Newtonian fluid experiment described in the previous section. Done manually, this experiment would take over 90 continuous hours of assay time (480 total videos). Using the AHT system, this experiment took 2.2 hrs (all of which was unattended) and we obtained viscosity measurements for water, 2.0 M and 2.5 M sucrose within 5.5% error.

time per FOV	manual biomaterial/cell rheology expt	automated biomaterial rheology expt	automated cell rheology expt
find FOV find focus set parameters name file	2 min	15 sec	1 min 45 sec
collect video	1 min	1 min	1 min
set VST parameters track video tracking file aggr. analysis (MATLAB)	8 min 20 sec	2 min	2 min
96 specimens 5 FOV	90.6 hrs	2.2 hrs (98 % saved)	3.2 hrs (96 % saved)
	unattended for no longer than 2 min !	unattended	unattended

Figure 6.9: Time saved with the AHT Microscope.

Chapter 7: Towards High-Throughput Cancer Cell Mechanics

7.1 Overview

The central theme of this work has been in using and developing biophysical tools to investigate contexts in biology where the mechanical properties of cells play a role in cell behavior and disease progression. In Chapter 5, I described a biophysical and biochemical study of the epithelial to mesenchymal (EMT) that yielded a mechanistic link (RhoA activators LARG and GEF-H1) between the mechanical properties and invasive capacity of cells during the physical initiation of cancer. Like many of the efforts described in Chapters 3 and 4, the EMT study examined a hypothesis through a series of low throughput, manually performed experiments testing a small number of environmental conditions or genetic manipulations. In general, the technical overhead and time constraints of manual experiments ultimately limits the range of assayed conditions and the ability to test the generality of findings by examining additional cell lines and model systems (Fig. 7.1).

In Chapter 6, I described an array high throughput (AHT) microscope that addresses the challenge of automating and accelerating the collection of mechanical measurements of biomaterials. Passive microbead results from Chapters 4 and 5 demonstrate the potential for the AHT system to characterize cell mechanics. In this chapter,

I discuss the need for high throughput mechanical measurements in the cancer community and describe the key contributions I have made towards this effort, including:

- Execution of high throughput experiments studying the effect of oncogenic signaling pathways on the mechanical properties of cancer cells.
- Development of an automated MSD-based analysis procedure that applies published software to filter PBR data based on trajectory behavior.
- Propose a workflow for a high throughput screen that uses the mechanical properties of cancer cells as an assay for future drug development and selection for animal-based testing.

Note about MSD measurements and cell stiffness: The output of the AHT cell mechanics assay is MSD trajectories. While the ensemble average MSD for a condition can be converted into elastic moduli (G' , G'') using Eqn. 2.45, we choose not to perform this calculation in this chapter for reasons described in Sec. 2.7.2. Instead, we compare results across conditions in terms of MSD distributions at a timescale, and perform statistical tests on the median of these distributions. We will refer to changes in the MSD across conditions as changes in cell mechanics/stiffness (*e.g.* increased bead motion captured by increased MSD corresponds to a decrease in cell stiffness).

Pancreatic collaboration. In this project, I designed and performed the PBR experiments, developed the MSD analysis software, and analyzed the data. Jian Chen managed cell culture and construct transfections, prepared specimens for mechanical

experiments, and executed biochemical and invasion assays. The work was directed by Rich Superfine, Gerry Blobe, and Tim O'Brien.

model systems	environment and bead ligands	oncogenic signaling pathways	nature of substrate
EMT models MET models ovarian pancreatic breast lung melanoma bladder prostate other cancers normal cells epithelial endothelial fibroblast	fibronectin collagen I collagen IV laminin elastin hyaluronan glycocalyx <i>mixtures</i> α integrin α ICAM α PCAM internalized	TGF-β BMP Ras-MAPK PI3K-AKT-mTOR NF-kB Jak/Stat Wnt/b-catenin JNK ERK5 Notch P38 Hedgehog Hippo P53 Ral	glass substrate stiffness substrate forces time endpoint correlations full time-course bead sizes 4.5 , 2, 1 μ m 200, 500 nm

Figure 7.1: **Paramters from the EMT study investigated in Ch. 5 (circled) in the context of the larger parameter space.** A high throughput biophysical assay is needed to minimize the technical overhead and time constraints of manual cell mechanics experiments.

7.2 The Need for High Throughput Cell Mechanics in Cancer Community

Although clinical advances have been made in the treatment of the cancer, treatment of metastatic cancer and an effective diagnostic or prognostic assay for cancer progression remain elusive (Steeg, 2006)(Wirtz et al., 2011). Here, we discuss the the need for high throughput cell mechanics in cancer research.

1. Cell mechanics appears to be functionally linked to cancer invasion.

Cell mechanics is integral to understanding the disease. Several pathological phenotypes (as discussed in Ch. 2 nad 5) have been shown to regulate or correlate with cancer invasion (Fig. 7.2). These include the epithelial to mesenchymal transition (EMT) (Yilmaz and Christofori, 2009), matrix metalloproteinase (MMP) production (Deryugina and Quigley, 2006), and cell contractility (force generation) (Kraning-Rush et al., 2012). Across model systems and methodologies, we and others have found that decreased cell stiffness correlates with invasiveness (Swaminathan et al., 2011)(Xu et al., 2012a)(Plodinec et al., 2012). Recently, we found a mechanistic link (RhoA activators LARG and GEF-H1) between the mechanical properties and invasive capacity of cells during EMT, the physical initiation of cancer (Osborne et al., 2014). Functional connections such as these hold potential as therapeutic targets.

2. Fast and high volume measurements are needed.

Low throughput methodologies cannot effectively capture disease complexity. As argued in Sec. 6.2, a complete understanding of *all* ubiquitous, functional connections between biomechanics and

diseases will require technology and methods designed to accelerate data collection and provide parallelized high throughput testing. High throughput mechanical screening of cells will enable probing of oncogenic signaling pathways, examination of physiologically relevant conditions (substrate mechanics and biochemistry), and study of population and single cell mechanical heterogeneity.

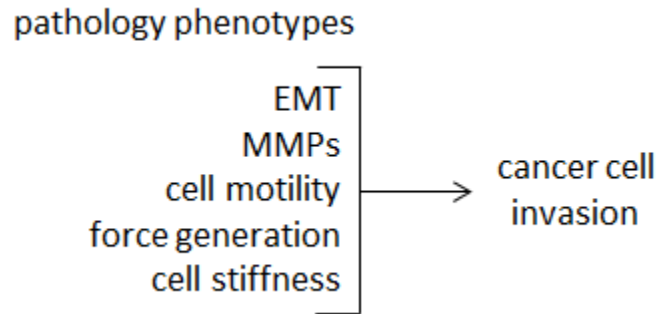


Figure 7.2: **A simplified list of the pathology phenotypes that “sum” and lead to cancer cell invasion.**

3. Why not study another cancer phenotype in high throughput? Current assays of metastatic potential involve *in vitro* assessment of migration or invasion which takes on the order of days, or *in vivo* assessment which takes weeks. Thus, significant effort has been placed in identifying genetic or biochemical biomarkers for metastatic likelihood; recent advances in genomics (Navin et al., 2011) and proteomics (Shi et al., 2012) have extended analysis to single cancer cells. To this point, however, results have not been generalizable across multiple types of cancer, nor do they directly describe the inherently physical behavior of invading cancer cells (Sidransky, 2002). Compared to individual genetic or biochemical mechanisms, alterations in cell mechanics hold promise as the most direct and accessible patholog-

ical indicator of invasion. As such, assays designed to examine biophysical properties may prove critical in the development of future cancer therapies by shortening the feedback loop between testing and readout of candidate targets, and by increasing the speed and efficiency of analyzing off-target effects.

7.3 The AHT System meets the Current Needs

Recent instrumentation advancements have increased the throughput of mechanical measurements compared to traditional cell mechanics techniques (magnetic and optical tweezers, AFM, etc.) which involve time-intensive manual and serial data collection (30 cells/hr; Fig. 2.7). Optical compression (Roth et al., 2013) and hydrodynamic stretching (Dudani et al., 2013) methods have successfully increased throughput of cell mechanical measurements reaching rates of 2000 cells/sec (Gossett et al., 2012). However, these techniques rely on using spherical, suspended cells, and despite holding promising as clinical tools, these methods only provide direct insight to the circulation step of the metastatic cascade.

As mentioned in Ch. 6, the success of particle tracking microrheology in characterizing biomaterials as well as adherent cells has long motivated high throughput implementation of passive microrheology (Breedveld and Pine, 2003). A recent ballistic injection protocol has taken steps to this end (Wu et al., 2012b), but requires time-intensive experimental overhead and remains relatively low-throughput (40 cells/hour).

Our AHT system provides a powerful solution for a high throughput (785 cells/hr), quickly enabled experiment (1.5 hr bead incubation) for testing adherent cells. We

have shown our PBR data is consistent with community findings (Fig. 2.4 D; (Hoffman et al., 2006)). Additionally, we have seen that PBR can identify the same correlations in biology conditions as magnetic tweezers: trends between cancer cells with varying metastatic potential (Fig. 4.8; (Hanna et al., 2013)) and cells before and after TGF- β induced EMT (Fig. 5.7; (Osborne et al., 2014)). Additional features and advantages of implementing PBR on our AHT system:

- As a result of using adherent cells, our system is capable of probing cell mechanics under physiologically relevant chemical and mechanical environments that mimic many aspects of the metastatic cascade, including: EMT, stromal cell invasion, and intra- and extravasation.
- In contrast to hydrodynamic stretching, PBR on AHT holds promise to test the effect of specific receptor-mediated signaling pathways by choosing various bead ligands.
- The 12 objectives of the AHT system enable both accelerated data collection and the ability to perform parallelized time-course cell mechanics experiments. For example, the time-dependent effect of an intervention can be tested by treating the cells within each channel (objective-viewable area) of a 96 well plate at a different point in time; therefore, 12 time points in the mechanical response to the intervention can be assessed on the same plate, simultaneously.

7.4 Oncogenic Gain of Function Library and Preliminary Results

Of the parameter space mentioned above (Fig. 7.1), we chose to examine the effect of oncogenic signaling pathways on human pancreatic cells through a gain of function library. Pancreatic cancer is the fourth most deadly form of cancer in the United States and has one of the poorest prognoses among all cancer types (Bardeesy and DePinho, 2002). Nearly all pancreatic cancer involves malignant transformation of pancreatic epithelial duct cells. In particular, mutations in the KRAS gene are present in over 90% of metastatic pancreatic cancers (Bardeesy and DePinho, 2002). For the model system in this study, we use human pancreatic duct epithelial (HPDE) cells. The cancer phenotype is captured by human pancreatic nestin-positive epithelial cells (HPNE), which are KRAS-transformed HPDE cells. Differences in cell morphology between HPDE (epithelial-like) and HPNE (mesenchymal-like) are observed in Fig. 7.3.

To determine the viability of our model system, we characterized invasion capacity and stiffness of the HPDE and HPNE cells. We assessed the invasiveness of the HPDE and HPNE cells using a standard invasion assay, and found the HPNE cells to be 25-fold more aggressive than the normal cells (Fig. 7.4). To test whether there was a difference in cell stiffness between the HPDE and HPNE cells, we assayed the cells on the AHT system. Mechanical testing showed a significant difference in stiffness between the HPDE and HPNE cells, as the median RMS displacement of beads increased from 32.29 nm to 40.25 nm ($p < 0.001$; Fig. 7.5). Together, these results are con-

sistent with community findings that report invasive cells having a decreased stiffness compared to non-invasive cells (Swaminathan et al., 2011)(Xu et al., 2012a)(Osborne et al., 2014). Moving forward, these results also provide a normal-to-pathology context for experiments where we manipulate specific signaling pathways in HPDE cells.

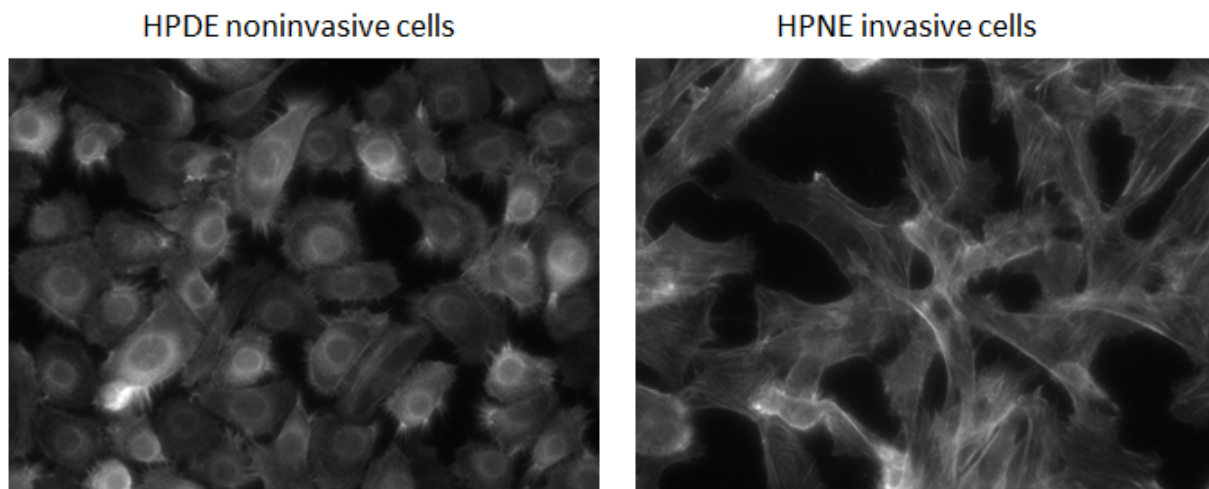


Figure 7.3: **HPNE cells are KRAS-transformed HPDE cells.** Morphology differences between HPDE and HPNE cells are observed by staining with phalloidin to show actin cytoskeleton organization (Images: Tim O’Brien). For all experiments in this chapter, HPDE and HPNE cells were cultured in one location to minimize genotypic and phenotypic drift over time.

To study oncogenic signaling in high throughput, we utilized a gain of function library (Kris Wood, Duke University). The Wood library targets 17 signaling pathways and modules that regulate cancer cell growth, differentiation, survival, and apoptosis, including: TGF- β , Wnt, Hippo, Hh, Notch, Ras, Ral, ERK, JNK, p38, PI3K/Akt, JAK/STAT, NF-kB, p53, ER, AR and caspases (Adjei and Hidalgo, 2005). For each pathway, specific genetic mutant constructs have been identified, so that when over-expressed, the signaling pathway is rendered constitutively active. The constructs are delivered to cells through a lentiviral vector that contains a selection marker, such that

in cell culture, cells expressing the construct can be specifically selected, thus forming a homogeneous population. A brief protocol describing construct preparation and selection can be found in the Appendix D.2.

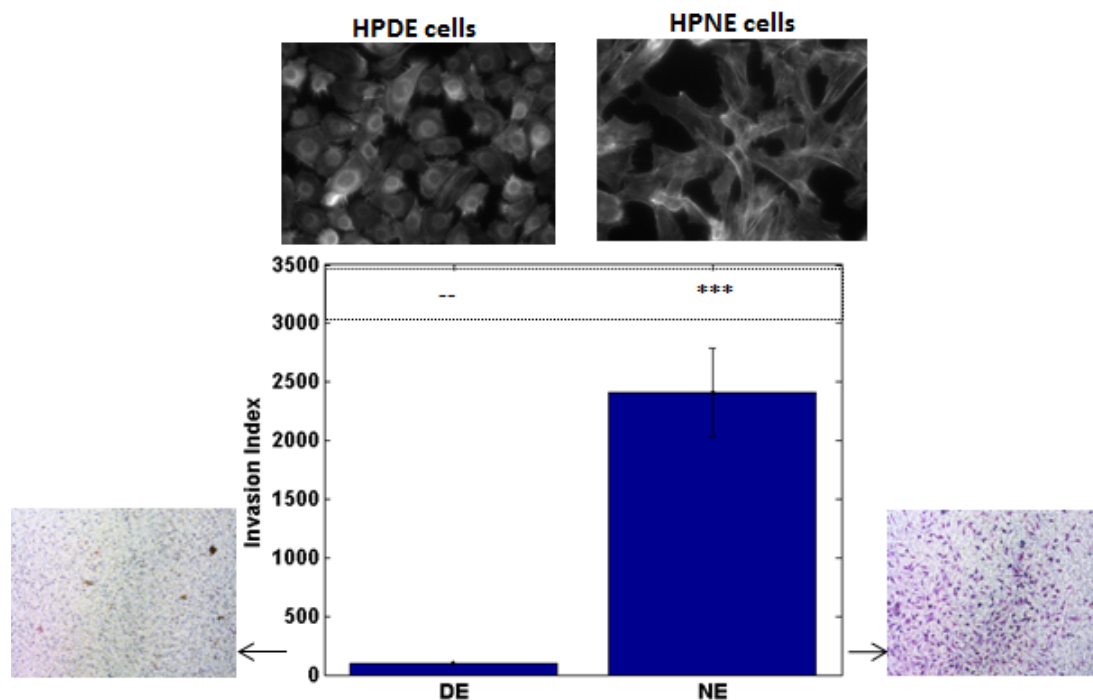


Figure 7.4: **Average invasiveness for HPDE (DE) and HPNE (NE) cells.** Cells were allowed to invade for 12 hr on Matrigel-coated transwell invasion filters according to protocol in Appendix D.3. Bottom images are representative cell densities (nuclei stained) for the two conditions. Data represent mean of 5 independent experiments; error bars represent SEM. (***) $p < 0.001$. Data collected by Jian Chen.

As an initial effort, we chose to test the effect of 4 genetic constructs, H-Ras, myristoylated Akt, type I TGF-beta receptor, and Bcl-2, expressed in HPDE cells, on cell invasion and stiffness. The H-Ras (Ras pathway) and type I TGF-beta receptor I (TGF- β pathway) constructs were chosen as positive controls (invasion-inducing) based on their known effects as an oncogene and in initiating TGF- β signaling, respectively. Myristoylated Akt (PI3K/AKT/mTOR pathway) and Bcl-2 (apoptosis pathway) were

chosen because of their known roles as oncogenes in reducing apoptosis during cancer progression. The effect of these genetic constructs was compared to a construct control (vector with no gene target transformed into HPDE cells), and for context we tested the HPDE and HPNE cells in parallel. Biochemical validation of construct expression and pathway activation was determined by western blot or immunofluorescence (Appendix F and Fig. F.1).

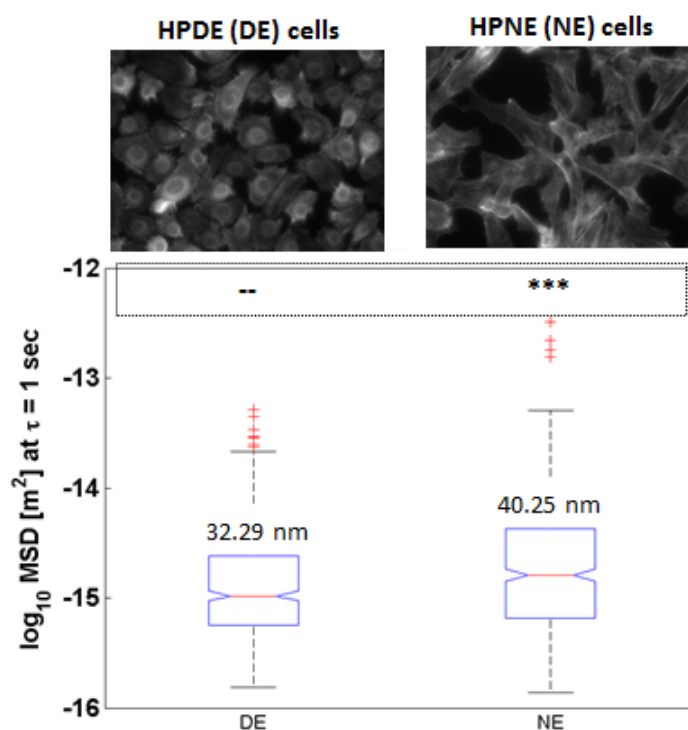


Figure 7.5: **Initial mechanical characterization of the HPDE and HPNE pancreatic cells using the AHT System.** MSD distributions (log scale) at the 1 sec timescale are shown for beads attached to HPDE (DE) and HPNE (NE) cells. Distributions include bead trajectories that exhibit anomolous diffusion (see Sec. 7.5.2 for analysis details; see Appendix D.1 for assay protocol). The median of each distribution is denoted by the red line and the median RMS displacement value is given above the box. Distributions compared using a Mann-Whitney test (***) $p < 0.001$.

The set of pancreatic cells were tested for invasiveness in a standard invasion assay.

Relative to the construct control cells, the H-Ras and TGF- β RI constructs showed a

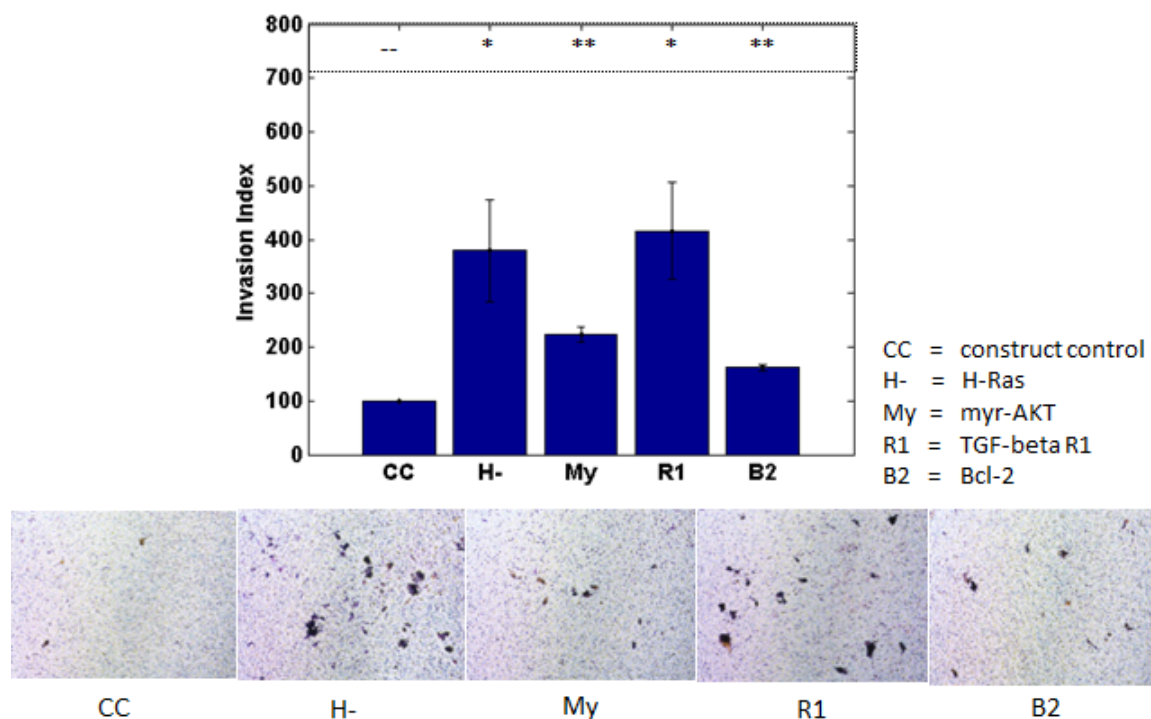


Figure 7.6: **Gain of function constructs increase invasion capacity of HPDE pancreatic cells.** Average invasion index for the following 5 cell conditions: construct control (HPDE with empty vector), H-Ras (H-), myr-AKT (My), TGF- β R1 (R1), and Bcl-2 (B2). Cells were allow to invade for 12 hr on Matrigel-coated transwell invasion filters according to protocol in Appendix D.3. Data represent mean of 2 independent experiments; error bars represent SEM. (* $p < 0.05$, ** $p < 0.01$). Data collected by Jian Chen.

3.8 and 4.2 fold increase in cell invasion, respectively (Fig. 7.6). Myristoylated Akt and Bcl-2 constructs increased invasion by 2.2 and 1.6 fold, respectively, relative to the construct controls (Fig. 7.6). While the increase in cell invasion for the 4 constructs is modest with respect to the invasion capacity of HPNE cancer cells, we conclude that the constructs promote the adoption of an invasive phenotype. Discussion of these results will continue in Sec. 7.6.

During development of our high throughput cell mechanics assay, we realized that our power to distinguish between conditions would require an advanced analysis procedure capable of identifying subpopulations within our data. In the next section, we will discuss the implementation of such a procedure, and in Sec. 7.6, we will discuss the results of 6 high throughput cell mechanics assays.

7.5 Automated Motion Model Selection for MSD Trajectories

A consequence of the passive microbead technique, including the implementation to external cell mechanics, is that no control over the location of the probe is provided. In the case of cell mechanics, washing steps are included in the assay protocol (Appendix D.1) to minimize beads that are in the specimen but are not attached to cells (*e.g.* bead diffusing in media or on the coverslip substrate). However, not all unwanted beads are eliminated, and it was hypothesized that the various diffusion behaviors of these unwanted beads resulted in the multiple populations typically observed in MSD data (Fig. 7.7). Thus, during optimization of our mechanical assay, we realized that our power to distinguish between conditions, as we increased data collection into high throughput, would require an advanced (and automated) analysis procedure capable of identifying subpopulations within our data.

To proceed, we sought to capture diverse behavior in passive microbead trajectories by fitting data to 5 MSD motion models:

$$\begin{aligned}
 \text{MSD}_N(\tau) &= N \\
 \text{MSD}_D(\tau) &= 4D\tau \\
 \text{MSD}_{DA}(\tau) &= 4D\tau^\alpha \\
 \text{MSD}_{DR}(\tau) &= R_C^2(1 - e^{-4D\tau/R_C^2}) \\
 \text{MSD}_V(\tau) &= v^2\tau^2
 \end{aligned} \tag{7.1}$$

corresponding to non-diffuion (N), free diffusion (D), anomalous diffusion (DA), con-

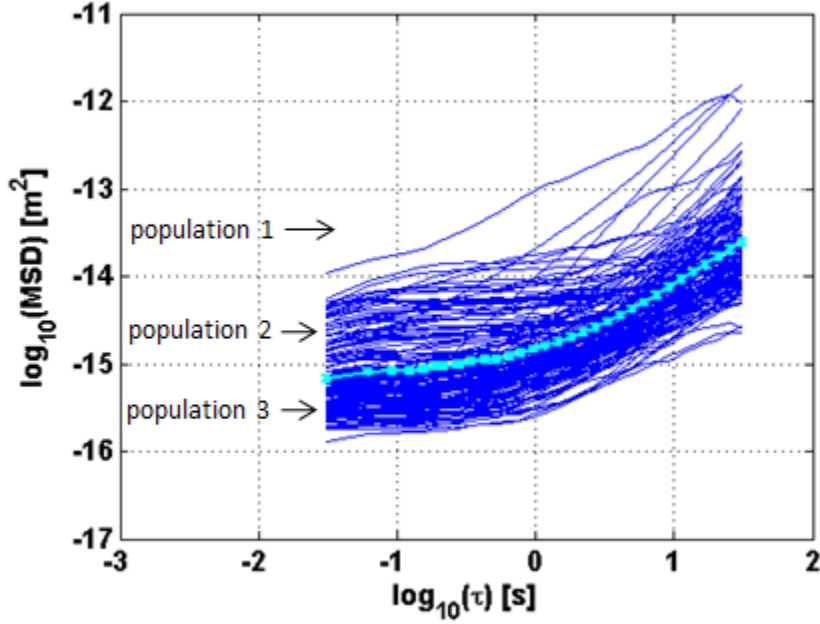
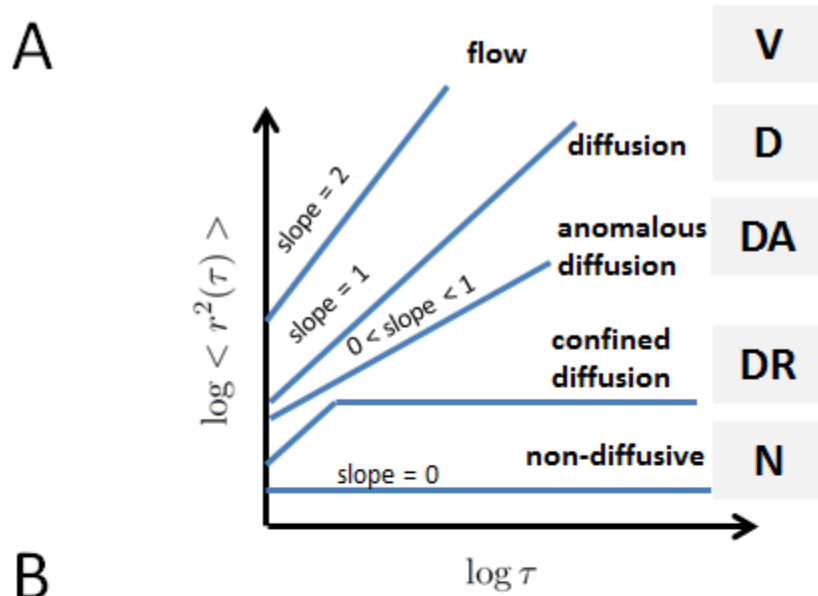


Figure 7.7: **Typical multiple population output of a passive cell microrheology experiment.** Towards assay optimization, our goal was to separate populations in order to facilitate like-model comparisons between cell conditions and maximize our power to distinguish condition-dependent differences.

finer diffusion (DR), and directed flow (V); schematic characteristic MSD curves for each model are given in Fig. 7.8. These models have previously been used with success to characterize the diffusive behavior of proteins in the cell membrane (Saxton and Jacobson, 1997). This basic set of diffusion models could, for instance, describe viscoelastic behavior predicted for beads attached to cells (DA and DR models), beads diffusing in media (D model), beads driven by drifts or molecular motors (V model), or beads diffusing below the sensitivity of the instrument (N model). Thus, the question became: “how do we use model selection to separate populations within our data?”

The classical approach to model selection involves fitting a model $\vec{f}(\vec{x}; \vec{\beta})$ at n sampled points $\vec{x} = [x_1, x_2, x_3, \dots, x_n]$



mathematical description	assay artifacts	biological hypotheses
$MSD_V(\tau) = v^2 \tau^2$	bead is being driven by flow in the media	bead is being driven by molecular motors
$MSD_D(\tau) = 4D\tau$	bead is diffusing in media and is not attached to cells	Over certain timescales, diffusion of the bead may be dominated by viscous properties of cell
$MSD_{DA}(\tau) = 4D\tau^\alpha$	bead is diffusing on substrate	1) bead is in membrane corral 2) bead is attached to actin cytoskeleton via focal adhesion
$MSD_{DR}(\tau) = R_C^2 \left(1 - e^{-4D\tau/R_C^2}\right)$	bead is diffusing on substrate	1) bead is in membrane corral 2) bead is attached to actin cytoskeleton via focal adhesion
$MSD_N(\tau) = N$	bead appears not to be diffusing because it is at the noise floor of the instrument	Over certain timescales, diffusion of the bead may be dominated by elastic properties of cell

Figure 7.8: **MSD motion models for diffusive behavior.** (A) Schematic representation of motion models in log-log MSD vs. τ space. Such models have previously been used to study the diffusive behavior of proteins in the cell membrane (Saxton and Jacobson, 1997). (B) Biological hypotheses for motion models.

$$\vec{f}(\vec{x}; \vec{\beta}) = [f(x_1; \vec{\beta}), f(x_2; \vec{\beta}), f(x_3; \vec{\beta}), \dots, f(x_n; \vec{\beta})] \quad (7.2)$$

to a data vector $\vec{y} = [y_1, y_2, y_3, \dots, y_n]$, where $\vec{\beta} = [\beta_1, \beta_2, \beta_3, \dots, \beta_p]$ is the p parameters of the model. For MSD data from passive microrheology experiments, the data vector is the calculated MSD for a vector of timescales $\vec{x} = [\tau_1, \tau_2, \tau_3, \dots, \tau_n]$. The data and the model differ at each sampled point by an error ϵ_i (*i.e.* residual from a model)

$$y_i = f(x_i; \vec{\beta}) + \epsilon_i \quad (7.3)$$

for $i = 1 \dots n$. In classical regression analysis, one attempts to minimize the sum of the squared errors for each sample point (programmatically, the method of least squares is an example), where the errors are assumed to be uncorrelated (independent of one another) and normally distributed around zero (He et al., 2012).

MSD curves, however, contain errors that are highly correlated with timescale, τ . Specifically, when data from a trajectory is used to calculate the MSD, the same time-series steps are simply grouped into different windows of time to compute the different $\text{MSD}(\tau)$ values. This procedure generates τ -dependent correlations in the deviation between individual MSD curves and the ensemble average (regardless of whether it is a bead-sampling weighted average) because error correlations from individual curves are not eliminated during the averaging. Subsequently, use of either a single MSD curve or an ensemble average as the data vector $\vec{y}$ will result in τ -correlated errors from the value of an analytical model (Eq. 7.3). The magnitude of

the τ -correlated errors increase with τ .

The result of MSD curves having τ -dependent error correlations is that classical regression analyses tend to fit the data with overly complex models. When the model for a given data set is unknown, classical approaches also fail to provide a direct ranking of several competing models based on their relative probabilities (He et al., 2012). Thus, an approach that penalizes model complexity and enables model competition is ideal for MSD analysis in our high throughput workflow. In the next section, we discuss a Bayesian statistical analysis that addresses these needs.

7.5.1 Bayesian Approach to MSD Model Analysis

Over the last decade, Bayesian analysis procedures have been increasingly implemented in many biophysical domains that deal with data issues of correlated error/noise, inherent heterogeneity, and uncertain model selection; these include data from: genetics (Friedman et al., 2000), fluorescence resonance energy transfer (FRET) (Bronson et al., 2009), and fluorescence correlation spectroscopy (TCS) (He et al., 2012)(Guo et al., 2012). Recently, the issue of correlated noise and a Bayesian approach to model selection was addressed for MSD-based microrheology data (Monnier et al., 2012). In this section and in Appendix E, the Monnier *et.al.* procedure is described; in subsequent sections, we implement their software (msd-bayes.org) to estimate model parameters and model probabilities for the 5 MSD motion models in Eqn. 7.1.

To account for τ -correlated error in a mean MSD curve, the residuals from the

mean, ϵ_{mean} , are calculated for each single curve at each time scale τ_i . For the j -th trajectory and the i -th timescale, ϵ_{mean} is given by

$$\epsilon_{\text{mean},i}^j = y_i^j - \bar{y}^j \quad (7.4)$$

To capture how error in a mean MSD curve varies with τ , the error covariance matrix $\mathbf{C}$ of the mean MSD is calculated using (Monnier et al., 2012)

$$C_{ik} = \frac{1}{J(J-1)} \sum_{j=1}^J \left(y_i^j - \bar{y}_i^j \right) \left(y_k^j - \bar{y}_k^j \right) = \frac{1}{J(J-1)} \sum_{j=1}^J (\epsilon_{\text{mean},i}^j) (\epsilon_{\text{mean},k}^j) \quad (7.5)$$

where J is the total number of trajectories. For our data, the covariance matrix is a n -by- n matrix where n is the total number of τ . The on-diagonal elements of $\mathbf{C}$ provide the variance of the mean MSD at each timescale. The off-diagonal elements of $\mathbf{C}$ provides the covariance of the mean MSD at different timescales. For example, C_{12} tells us how the error in single curves relative to the mean MSD at the first timescale varies with the error at the second timescale. In this way, the covariance matrix is a generalization of variance in multiple dimensions.

Factorization of the covariance matrix as $\mathbf{C} = \mathbf{A}\mathbf{A}^T$ allows us to apply the transformation matrix $\mathbf{A}$ to our model-data equation, Eqn. 7.3, to obtain an expression that has uncorrelated errors (Monnier et al., 2012):

$$\mathbf{A}\vec{y} = \mathbf{A}\vec{f}(\vec{x}; \vec{\beta}) + \mathbf{A}\vec{\epsilon} \quad (7.6)$$

Using this equation, the MSD motion models from Eqn. 7.1 can be fit using standard generalized least squares procedures (in MATLAB, for example) to estimate model parameters.

To determine the probabilities for competing models, Monnier *et.al.* implemented Bayes' theorem. In the Bayesian approach, for a set of K models ($M_1, \dots, M_K$), the probability of model k given the observed data $\vec{y}$ is

$$P(M_k|\vec{y}) = \frac{P(\vec{y}|M_k)P(M_k)}{P(\vec{y})} \quad (7.7)$$

where $P(\vec{y}|M_k)$ is the marginal likelihood of model k , $P(M_k)$ is the prior probability of model k , and $P(\vec{y}) = \sum_k P(\vec{y}|M_k)P(M_k)$ is the total marginal likelihood of the data given all models (can be thought of as a normalization factor). Consistent with new studies where no information is available to assign prior preferences to models, we assume $P(M_k)$ is equal for all k . Similarly, since we have no prior information about the probability distribution for the parameters in each model, we assume a uniform distribution, centered at the estimated model parameter, with a range of 200 times the standard error determined for that parameter (He et al., 2012). Calculation of $P(\vec{y}|M_k)$ depends on the covariance matrix $\mathbf{C}$, and therefore inherently accounts for τ -correlated errors in the MSD. For more details regarding the computation of model probabilities using Eqn. 7.7, see Appendix E.

7.5.2 Wrapper for Implementing MSD Model Selection

To implement the Bayesian approach to MSD-based model selection as a subpopulation filtering device, as opposed to a population characterization tool, an automated analysis wrapper was written around the publically available software from Monnier *et.al.*. The wrapper was designed to fit into the standard rheology analysis already implemented in the AHT system.

The workflow begins by taking a single MSD curve from an aggregated dataset (for cases discussed here, a dataset will correspond to all wells/FOVs for a condition from an AHT system experiment, such as a cell type) and breaking up the curve into a predetermined number of subtrajectories (Fig. 7.9 A,B,C). The set of single-curve subtrajectories and associated ensemble mean curve are sent into the Bayesian software from Monnier *et.al.* in order to determine model parameters, associated standard errors, and relative model probabilities for the 5 MSD models given in Eqn. 7.1 (Fig. 7.9 D). In order to separate subpopulations within an aggregated dataset, a motion model was assigned to a single if the relative probability of that model was greater than 50% (Fig.7.9 E). The wrapper repeats this procedure *for each trajectory* in the aggregated dataset (Fig. 7.9 F). Finally, the wrapper repeats over all aggregated datasets collected during the experiment, and then provides plotting and analysis metrics for the curves that belong to each model type – enabling filtering based on model type across experimental conditions.

To test the ability of the wrapper to separate subpopulations, an aggregated dataset

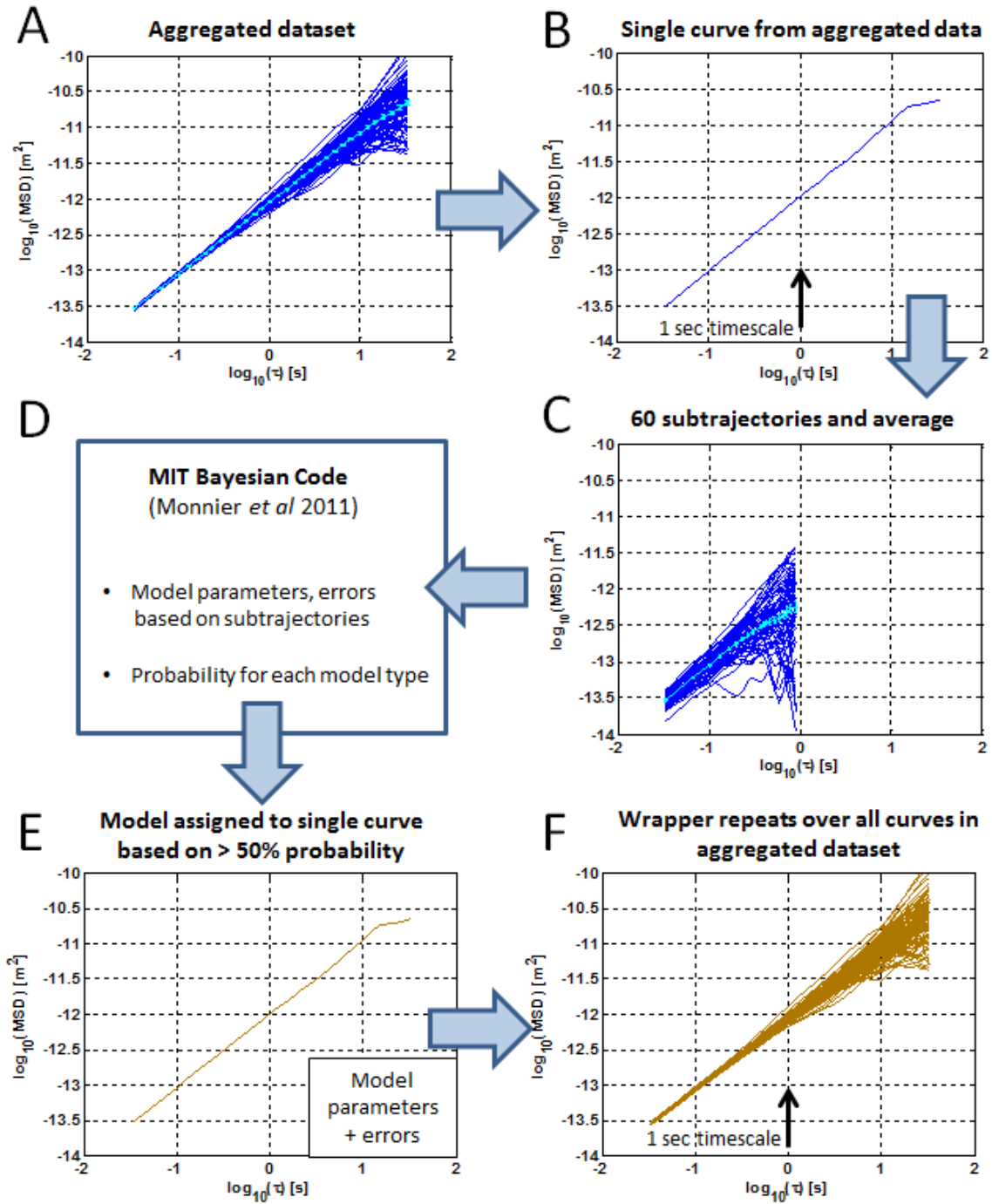


Figure 7.9: **Workflow for UNC wrapper to the MIT Bayesian MSD analysis.** (A) An aggregated dataset potentially containing multiple populations. (B,C) A single MSD curve calculated for 60 sec is broken up into 60 1-sec subtrajectories. (D) The subtrajectories and average are sent to the MIT Bayesian code for model parameter and probability estimation. (E) A model type is assigned to a single curve based on a 50% threshold. (F) Procedure repeats over all single curves in the aggregated dataset.

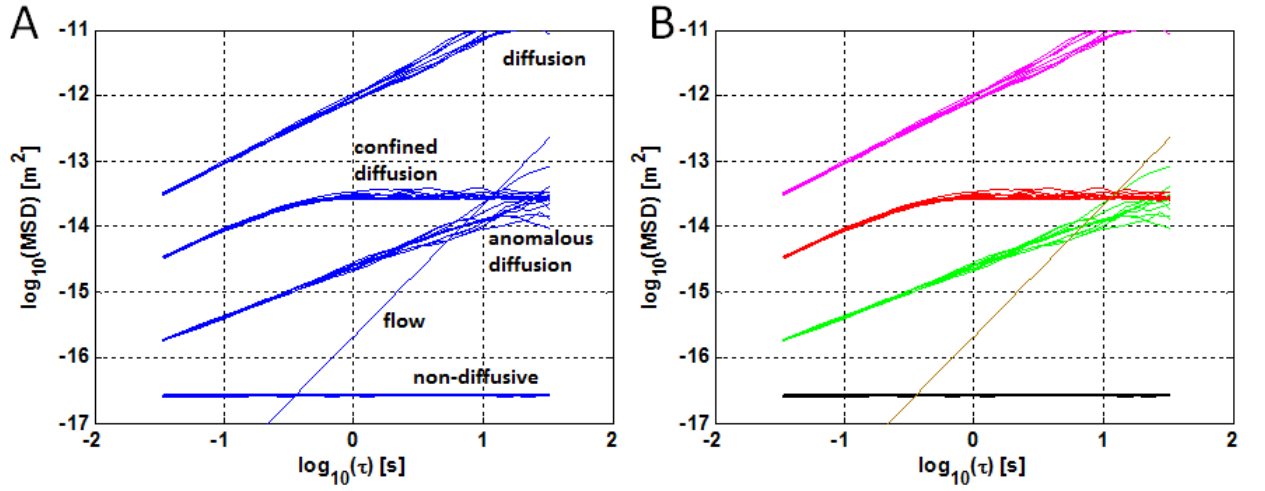


Figure 7.10: **Validation of the wrapper workflow for MSD-based Bayesian analysis.** (A) 10 simulated trajectories from the 5 MSD motion models from Eqn. 7.1 were combined and tested as an aggregated dataset with 5 inherent populations. (B) The wrapper workflow broke single trajectories into 1-sec subtrajectories and successfully sorted the free diffusion (pink), confined diffusion (red), anomalous diffusion (green), flow (gold), and non-diffusive (black) subpopulations.

of 50 trajectories, composed of 10 simulated trajectories for each of the 5 MSD models (Eqn.7.1), was analyzed. The wrapper workflow (Fig. 7.9) successfully separated all 5 subpopulations (Fig. 7.10 A,B).

An assumption of the Bayesian MSD analysis is that the motion model does not change during the trajectory (*i.e.* the particle behavior is static in time). During experiments, a mechanical drift (potentially due to the AHT XY-translation stage) begins to dominate at timescales past 1 sec; this instrumentation artifact is observed as a driven motion in MSD space, leading to trajectories having a slope greater than 1 (Fig. 7.11 A). From manual analysis of passive diffusion for bead attached to cells, we know that "good" cell mechanics data will suffer from this drift at timescales longer than 1 sec. In order to prevent this driven contribution from negatively impacting MSD model

selection, we break all single curves in experimental data into 1-sec subtrajectories (Fig. 7.11 B). Thus, from the perspective of the Bayesian MSD-analysis, the mechanical drift does not effect model selection. For cell mechanics experiments discussed in Sec. 7.6, typical video duration is 60 sec, so 60 1-sec subtrajectories are created, and results are visualized at the 1-sec timescale.

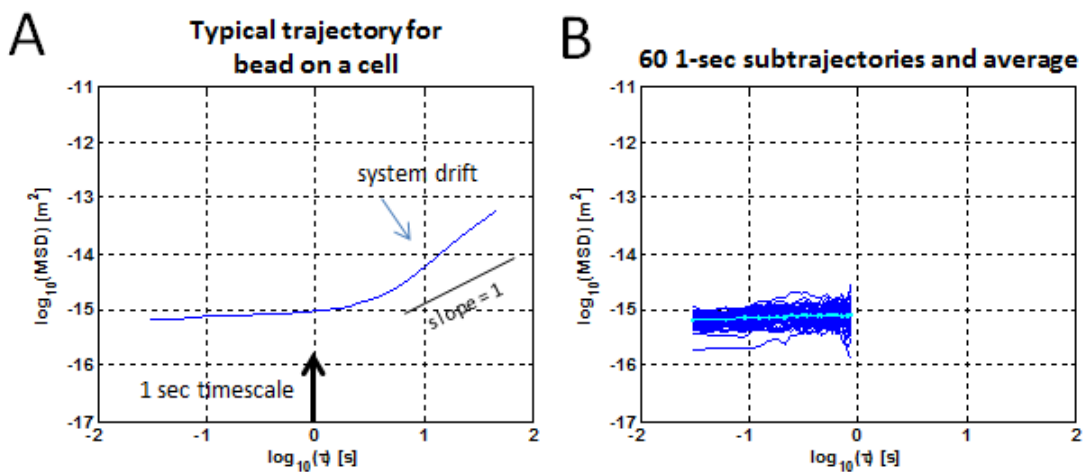


Figure 7.11: **Typical MSD trajectory for a bead attached to a cell.** These plots fit into the Bayesian analysis workflow in steps B and C of Fig. 7.9. Single curves are broken up into 1 sec subtrajectories because of a system drift that dominates for τ greater than 1 sec. The drift is observed as a driven process, indicated by a slope greater than 1.

7.5.3 Analysis of Cell and AHT Noise Floor Data

Next, we revisit the cell mechanics dataset described in Fig. 7.7 (reshown in Fig. 7.12 A) which served as the initial motivation to incorporate a model selection procedure into our MSD analysis. If we run this dataset through the analysis workflow, we find that the 3 initially observed populations can now be identified as free diffusion (D), confined diffusion (DR), and anomalous diffusion (DA) (Fig. 7.12 B). The dominate model type is anomalous diffusion (DA), which accounts for approximately 70% of the total dataset (Fig. 7.12 C). The DA-model population selected in Fig. 7.12 B is consistent with the motion of beads attached to cells from experience with manual data collection and analysis. While additional validation the DA-model reflecting should be pursued in the future, for the purposes of the experiments discussed here and in Sec. 7.6, we will assume the DA population reflects cell stiffness (technically, the viscoelastic response of the cells). Therefore, when we compare MSD data for different cell biology conditions, we remove trajectories that are identified as N, D, DR, and V-models.

To determine whether cell stiffness data collected with the AHT microscope is above the system noise floor, we compare the MSD of beads attached to cells (DA-model) to the MSD of beads dried to the substrate (noise floor data is collected in each experiment, in wells that do not contain cells or media) (Fig. 7.13 A). In this comparison, we plot the N-model and the DA-model distributions for the noise floor data. We expect “diffusion” of the noise floor beads to appear to the MSD-model analysis as an elastic solid, which is captured by the non-diffusive (N) model. This claim is supported by the

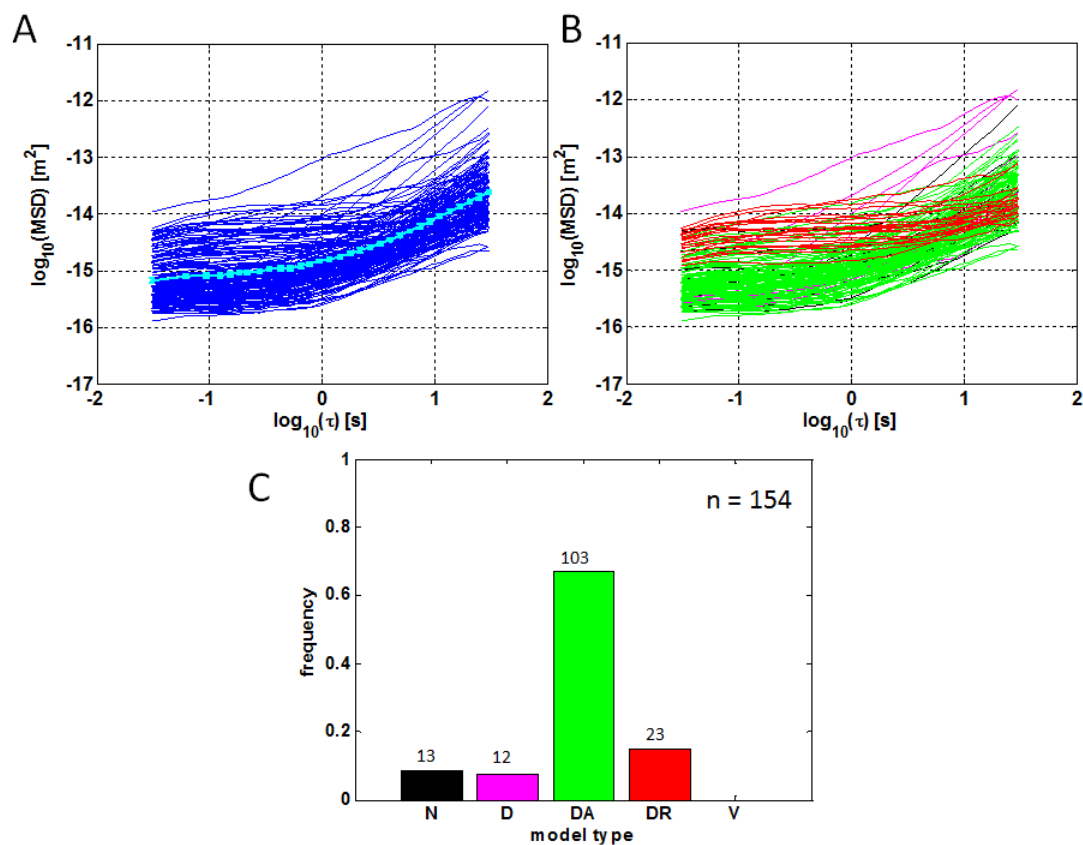


Figure 7.12: **MSD model selection applied to cell mechanics data.** (A) An aggregated dataset from a cell mechanics experiment is (B) separated by MSD motion model. (C) Frequency of MSD model type.

noise floor data showing that the N-model accounts for approximately 50% of the total dataset (Fig. 7.13 B, left). We find the distributions of beads bound to the HPDE and HPNE cells (median RMS displacements of 27.35 nm and 36.38 nm, respectively) to be significantly above the N-model and DA-model noise floor bead distributions (median RMS displacements of 17.01 nm and 16.44 nm, respectively) (Fig. 7.13 A). These data indicate that the diffusion of beads on cells is above the system noise.

In this section we have provided noteworthy steps towards HT mechanical assay optimization. With these results, we have accomplished our goal of separating populations from single particle tracking experiments in order to facilitate like-model comparisons between cells conditions and maximize our power to distinguish condition-dependent differences. In the next section, we perform HT assays using the AHT system to determine whether the gain of function constructs affect cell mechanics in addition to enhancing cell invasion.

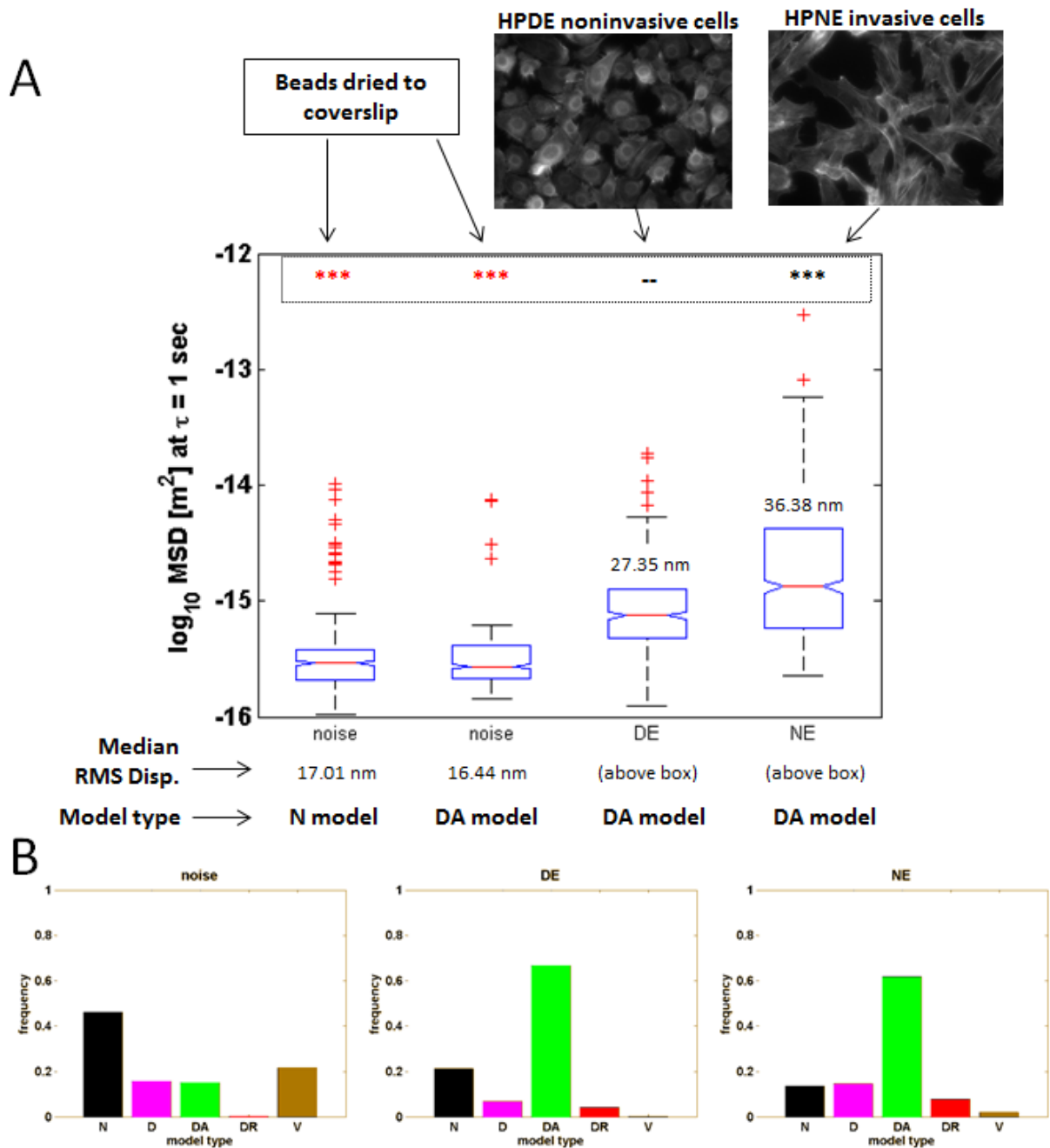


Figure 7.13: **Assessment of cell mechanics data in context of AHT noise floor.** (A) MSD distributions (log scale) at the 1 sec timescale are shown for beads dried to substrate (noise), and attached to HPDE (DE) and HPNE (NE) cells. The noise distribution is filtered for N- and DA-model behavior, and cell distributions are filtered for DA-model behavior. The median of each distribution is denoted by the red line and the median RMS displacement value is given below the plot or above the box. (B) The frequency of model types for each condition. MSD distributions compared using a Mann-Whitney test (***) $p < 0.001$.

7.6 HT Cell Mechanics Assays test Effect of Gain of Function Constructs

In Sec. 7.4, we tested the effect of 4 genetic constructs, H-Ras, myristoylated Akt (myr-AKT), type I TGF-beta receptor (TGF- β RI), and Bcl-2, expressed in HPDE pancreatic cells, on cell invasion. We found that that all 4 constructs enhanced the invasion capacity of HPDE cells. Relative to the construct control, we observed a 3.8 and 4.2 fold increase in cell invasion for the H-Ras and TGF- β RI constructs, respectively (Fig. 7.6). Additionally, myristoylated Akt and Bcl-2 constructs increased invasion by 2.2 and 1.6 fold, respectively, relative to the construct controls (Fig. 7.6).

Using the AHT microscope developed in Ch. 6 and the MSD-model analysis developed in Sec. 7.5.2, we executed and analyzed 6 cell mechanics assays testing the effect of the 4 gain of function constructs on cell stiffness (Fig. 7.14). Each assay was performed according to the protocol given in Appendix D.1. In the first 4 assays, our results show a significant mechanical change for the H-Ras construct in each assay relative to the construct control (Fig. 7.14). Myr-Akt and Bcl-2 constructs showed a significant mechanical change compared to the construct control in 3 of the first 4 assays (Fig. 7.14). In the first 4 assays, the effect of the TGF- β RI construct on cell stiffness was inconclusive as no change was observed in 2 of the assays, and in the other 2 assays, the MSD was observed to increase and decrease (Fig. 7.14).

In the final 2 assays, no construct showed a significant decrease in cell stiffness compared to the construct control (Fig. 7.15 A). In assay 5, the TGF- β RI construct appeared to increase cell stiffness (Fig. 7.15 A). In order to determine whether the

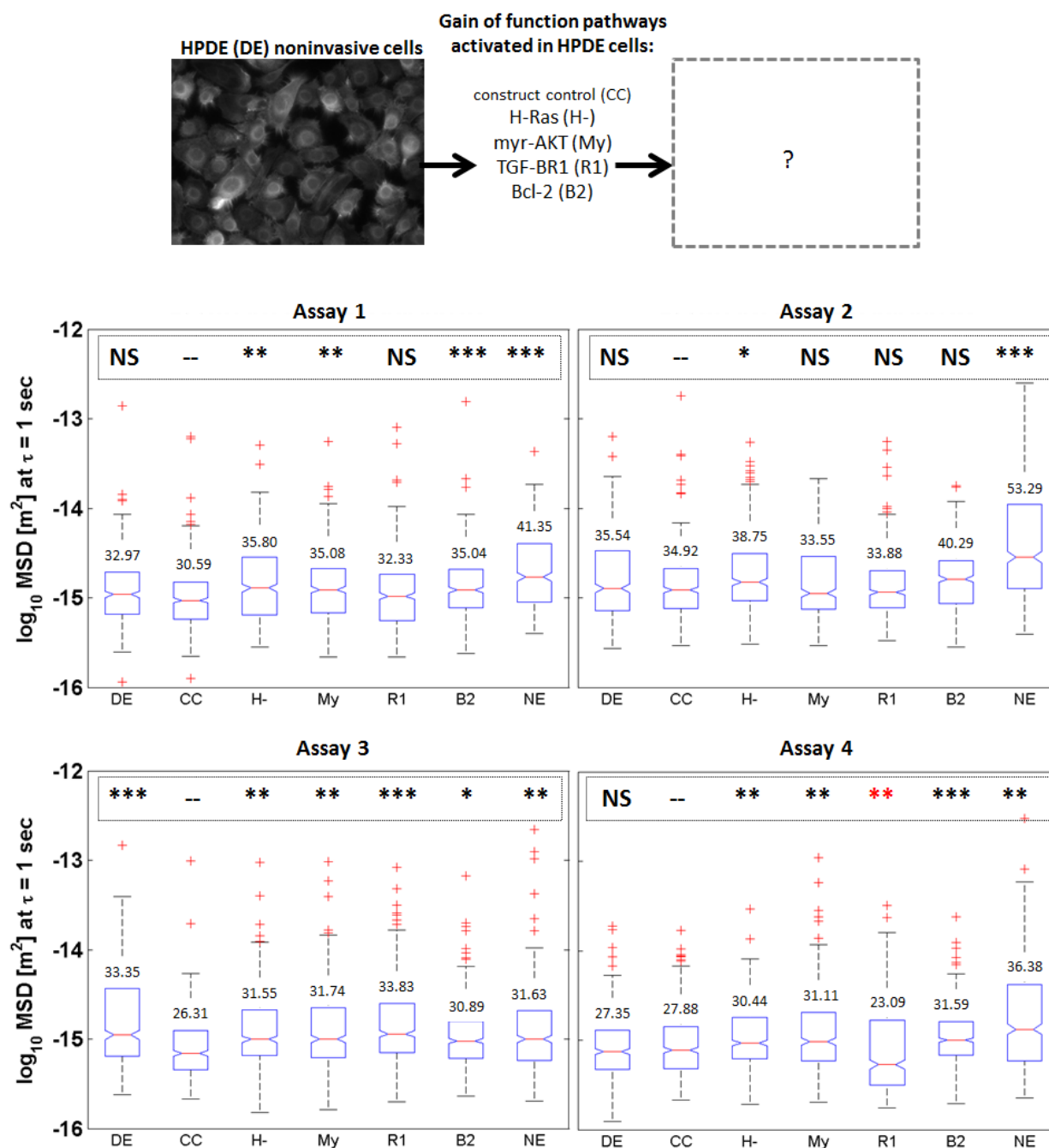


Figure 7.14: **Effect of gain of function signaling pathways on mechanics of pancreatic cells.** MSD distributions (log scale) at the 1 sec timescale are shown for beads attached to each cell condition. Distributions are filtered for anomolous diffusion (DA-model) behavior. The median of each distribution is denoted by the red line and the median RMS displacement value is given above the box. Distributions compared using a Mann-Whitney test (NS = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; red * indicates MSD decreased).

difference in cell stiffness observed in assays 1-4 compared to assays 5-6 was due to a loss of construct expression, we re-performed the biochemical validation of pathway activation. Western blot and immunofluorescence analysis (Appendix F and Fig. F.2) revealed that the expression of all 4 constructs was diminished after assays 5-6 (Fig. 7.15 B). While it is unknown whether the construct expression was lost suddenly or decayed slowly over time, these results suggest that the AHT mechanical assay was sensitive to the loss construct expression as changes were observed *before* the biochemical re-validation.

Taken together, we have demonstrated that constitutive activation of H-Ras, myr-Akt, and Bcl-2 increase invasion and decrease cell stiffness (Fig. 7.6, 7.14). These results suggest that H-Ras, myr-Akt, and Bcl-2 regulate the adoption of a cancer phenotype, defined by the K-RAS transformed HPNE cells (Fig. 7.6, 7.14). Despite having the largest effect on invasion, our results do not show a change in cell stiffness from constitutive activation of the TGF- β pathway using the TGF- β RI construct. A possible explanation is that activation of TGF- β RI may not phenocopy the total effect of the TGF- β ligand (which we explored in Ch. 5).

In conclusion, while the oncogene characteristics of H-Ras, myr-Akt, and Bcl-2 held promise to increase cell invasion, our results provide novel evidence that the Ras, PI3K/AKT/mTOR, and Bcl-2 apoptosis signaling pathways regulate decreases in cell stiffness. In Sec. 7.7, we will discuss how a AHT cell mechanics assay testing more Wood library constructs could fit inside a high throughput mechanical screen.

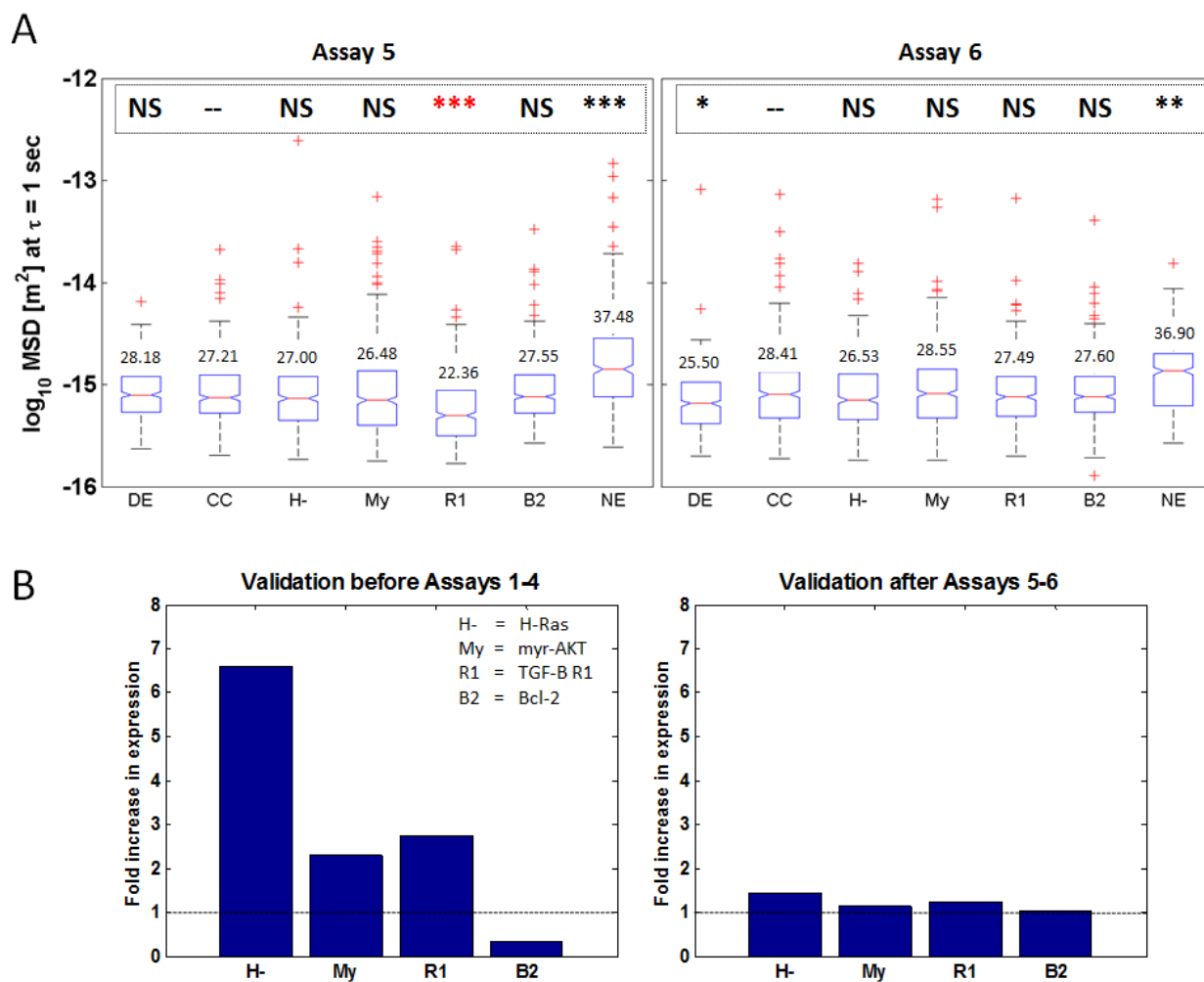


Figure 7.15: AHT mechanical assay predicts loss of construct expression. (A) MSD distributions (log scale) at the 1 sec timescale are shown for beads attached to each cell condition. Distributions are filtered for anomolous diffusion (DA-model) behavior. The median of each distribution is denoted by the red line and the median RMS displacement value is given above the box. Distributions compared using a Mann-Whitney test (NS = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; red * indicates MSD decreased). (B) Construct expression normalized to internal control set to 1 (dashed line) before assays 1-4 and after assays 5-6 (biochemical data in Appendix F). The H-Ras, myr-Akt, and TGF- β RI constructs activate their respective pathways through increased expression, as observed before assays 1-4. The Bcl-2 construct activates the apoptosis pathway by cleaving caspase3.

7.7 Future Directions

Looking forward, the mechanical assays performed here using the AHT microscope could be imagined inside a HT screening process for discovery of signaling pathways and constitute biochemical mechanisms that regulate cancer cell invasion. High throughput assays typically involve binning procedures to identify candidate “hits” (Birmingham et al., 2009). Our results from Fig. 7.14 could be summarized such that the extent of mechanical change across the 4 screens was binned according to a threshold significance level; for example, at the $p < 0.05$ level (Fig. 7.16). Thus, due to consistent mechanical change in at least 3 of the 4 screens, H-Ras, myr-Akt, and Bcl-2 could be categorized into a “high-confidence” bin, where further testing using additional assays is well motivated. Inconsistent mechanical testing of the TGF- β RI construct could result in this condition being placed into a “low-confidence” bin, where further testing would be motivated if time allowed or if complementary screens that activated TGF- β RI in another manner showed promising results.

A suggested workflow for a high throughput screen is provided in Fig. 7.17. This workflow could be, for example, applied to the entire Wood gain of function oncogenic library. A sample preparation step would involve validation of the genetic constructs, placing constraints on passage number in cell culture to minimize genetic and phenotypic drift, and plating into multiwell plates. After sample preparation, a primary cell mechanics screen would be performed using the AHT microscope system and construct conditions would be tested on 3-5 plate replicates. After the initial mechanical screens,

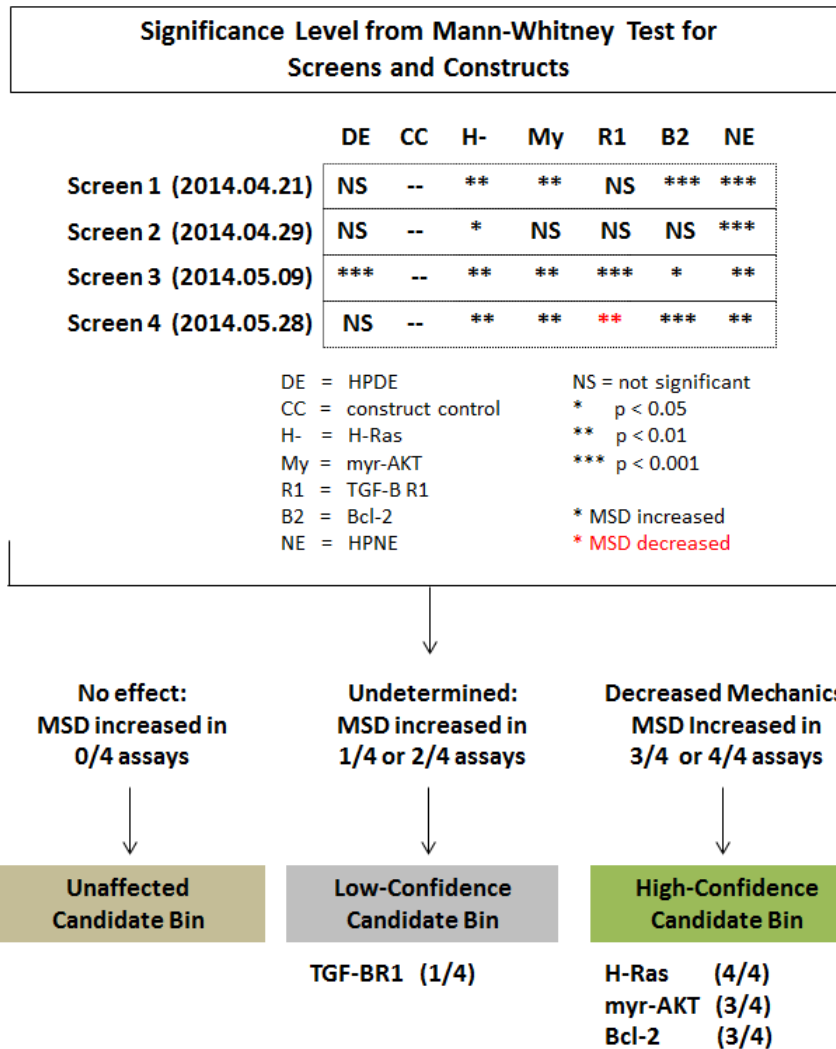


Figure 7.16: **Proposed procedure for binning HT mechanical assay results.** Gain of function genetic constructs are binned according to consistency of mechanical change as assessed by Mann-Whitney tests. Constructs that show consistent increases in MSD (decreased cell mechanics) are binned into a "high-confidence" pool that are well motivated for further study in other assays.

constructs would be sorted into “high-confidence”, “low-confidence”, or “unaffected” bins. “High-confidence” candidates would progress to be assayed in a complementary screen that activated candidate biochemical mechanisms by another method (3-5 replicate plates). To determine whether mechanisms have an impact on cell invasion, “High-confidence” candidates resulting from the complementary screen would be sent through a Matrigel invasion assay.

As “high-confidence” candidates from the invasion assays are discovered, a series of additional assays could be performed to further determine the fidelity of candidate mechanisms, including: 1) mechanical validation on higher precision instruments such as AFM; 2) secondary mechanical screens on the AHT microscope to assess the effect of candidates in additional cell lines, or to assess the synergistic effect of multiple candidates on cell mechanics. Ultimately, the candidates emerging from mechanical validation and secondary AHT assays are highly validated mechanisms that have been shown to effect mechanics and invasion.

These mechanisms become well-motivated targets for cancer therapies, with the final goal of testing in cancer progressed animals.

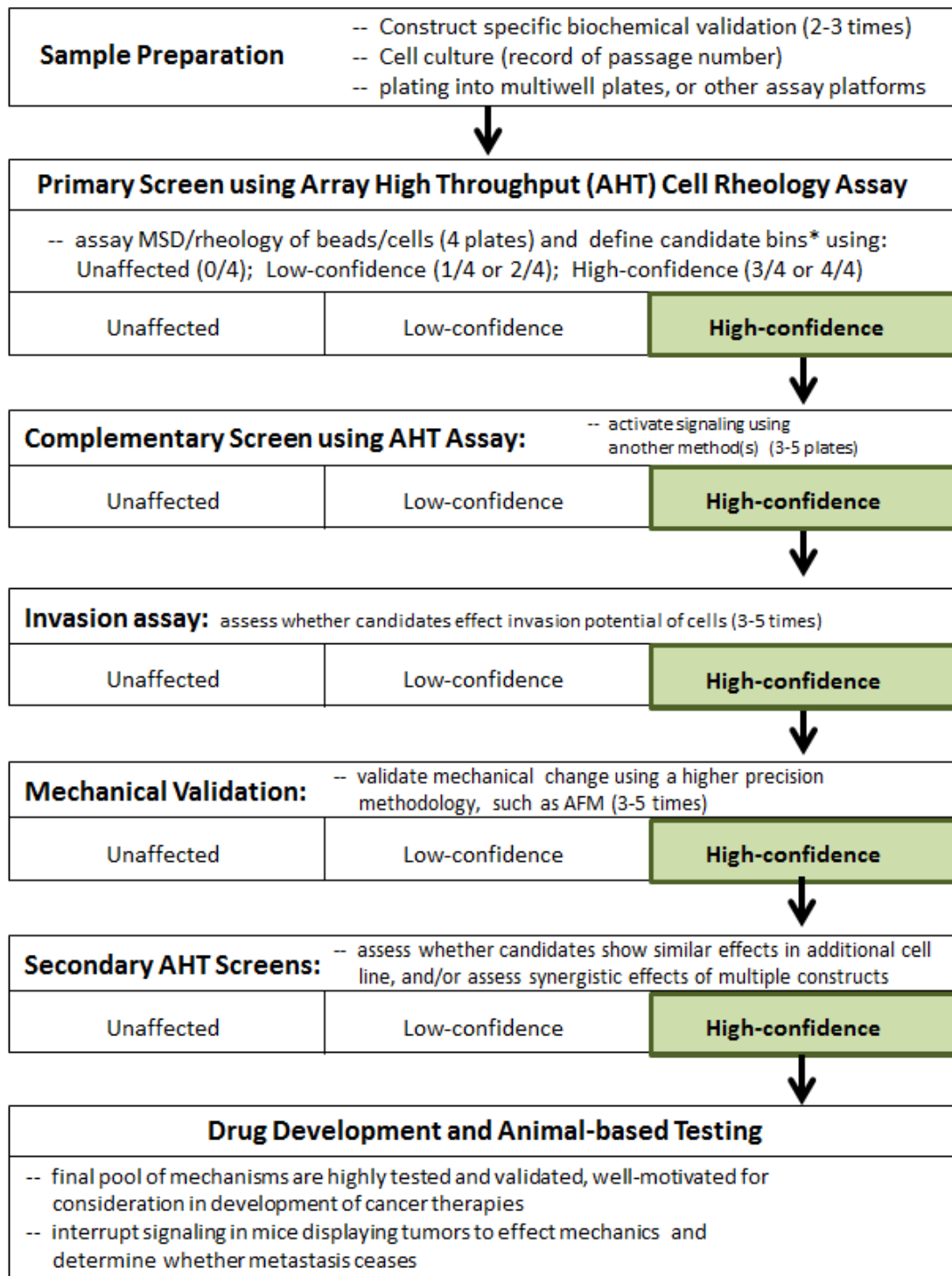


Figure 7.17: Schematic workflow for a high throughput mechanical screen.

* The metric is the p-value for the Mann-Whitney significance test. For example, if a construct is determined to increase MSD (decrease mechanics) for 3 out of 4 plate replicates, it is binned in the "high-confidence" bin.

Appendix A: Magnetic Tweezer Instrumentation and Calibration

A.1 Instrumentation and Force Calibration

The magnetic tweezer experiments in this work were conducted on the UNC 3-dimensional force microscope (3DFM) (Fisher et al., 2006). Although the 3DFM was designed to deliver forces in 3 dimensions, we implemented an instrument mode that enabled force application to cells in one direction – meeting the requirements of experiments described here.

The 3DFM is a user-controlled electromagnet. The experimenter defines a desired force regimen through a set of instructions (*e.g.* voltage amplitude, core geometry) which are sent to a data acquisition (DAQ) board through MATLAB software (Spero et al., 2008). The control signal (voltage/core for up to six cores, 3 possible force directions) is sent to the 3DFM Magnet Drive Amplifier (Vicci, 2005) where conversion into current occurs at a rate of 0.5 A/V. Based on the control signal, the 6-channel amplifier provides a desired current for each of the specified channels. Each channel is connected to its own coil wrapped iron core, and can be activated independently. When current is run through the wires of the coil, a magnetic flux density ($\vec{B}$ -field) is created. In the presence of the $\vec{B}$ -field, the core is magnetized (the once randomly oriented domains in the core are aligned), leading to enhancement of the magnetic flux

density.

The cores direct the $\vec{B}$ -field through a thin foil, called the pole, which extends into the specimen. The $\vec{B}$ -field travels through the specimen, across a 550 nm gap, and returns to another thin foil, called the flat. For cell mechanics experiments, the pole flat is lowered to a height of $30\mu\text{m}$ above the surface of cells that are labeled with magnetic beads.

In the presence of a magnetic field, with magnetic flux density $\vec{B}$, the force on a paramagnetic microbead is given by (Spero et al., 2008):

$$\mathbf{F} = \nabla(\mathbf{m} \cdot \mathbf{B}) = \frac{\pi d^3}{4\mu_0} \frac{\mu_r - 1}{\mu_r + 2} \nabla(B^2) \quad (\text{A.1})$$

where $\mathbf{m}$ is the magnetic moment of the microbead and d is the bead diameter. In order to achieve sufficient forces the gradient of the $\vec{B}$ -field is optimized by tapering the pole to a tip with a radius of $15 - 20\mu\text{m}$. The small-thickness pole-flat design is compatible with the use of high numerical aperture (NA) objectives, ultimately enabling high resolution imaging of force application (Fig. A.1).

Forces generated by the magnetic tweezer system are calibrated experimentally. Magnetic beads are loaded into a Newtonian fluid of a known viscosity, η , and pulled towards the pole tip. Recall that for a microbeads in a Newtonian fluid (no elastic effects) in the low Reynolds number regime, the total drag force is given by Stokes law $F_D = 6\pi a \eta v$ (Eqn. 2.14), where a is the bead radius and v is the velocity. Therefore, an applied external force is given by the drag force $F_{\text{external}} = F_D$. Ultimately, the

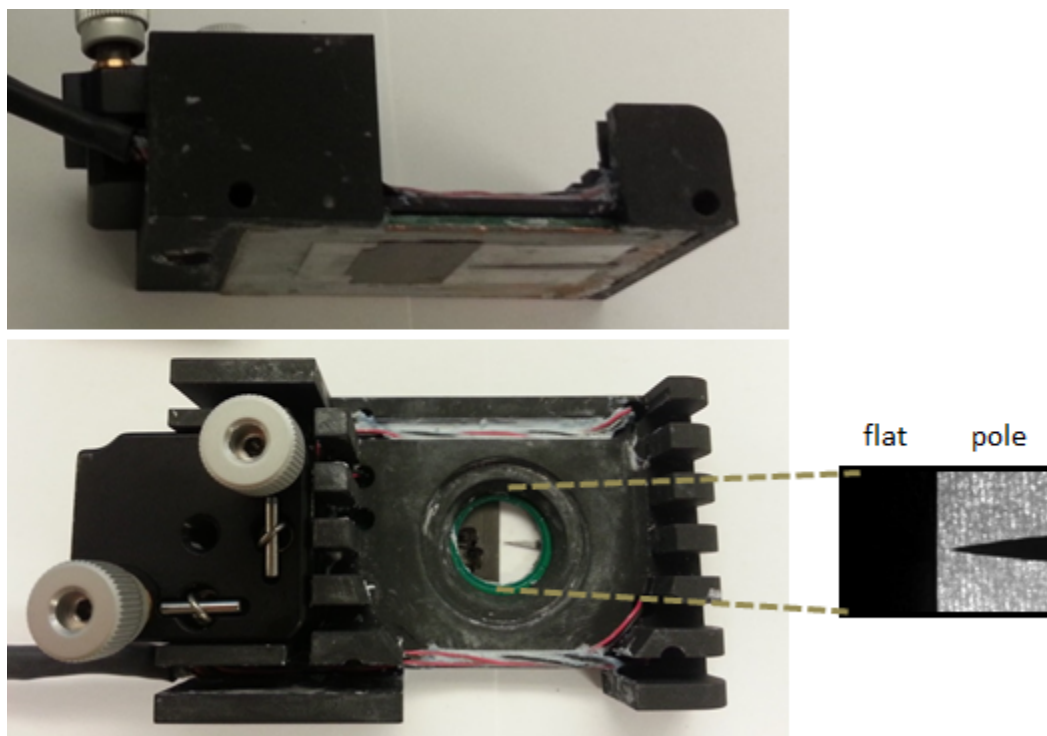


Figure A.1: **3DFM lid with pole-flat geometry.** The lid lowers into the specimen that is loaded on an inverted microscope stage.

goal of our calibration gets casted into determining the velocity of the bead $v(\vec{r}, I)$, as a function of radial displacement $\vec{r}(t)$ and the drive current I . Variability in the manufacturing of pole tips requires that each 3DFM lid is individually calibrated. In this work, 3 separate 3DFM lids were calibrated, corresponding to magnetic tweezer experiments investigating ICAM-1 (Sec. 4.4), nuclear (Sec. 4.3), and integrin/cancer (Sec. 5 mechanotransduction). Figure A.2 shows the force calibration curves for the 3DFM lid used in Sec. 5.

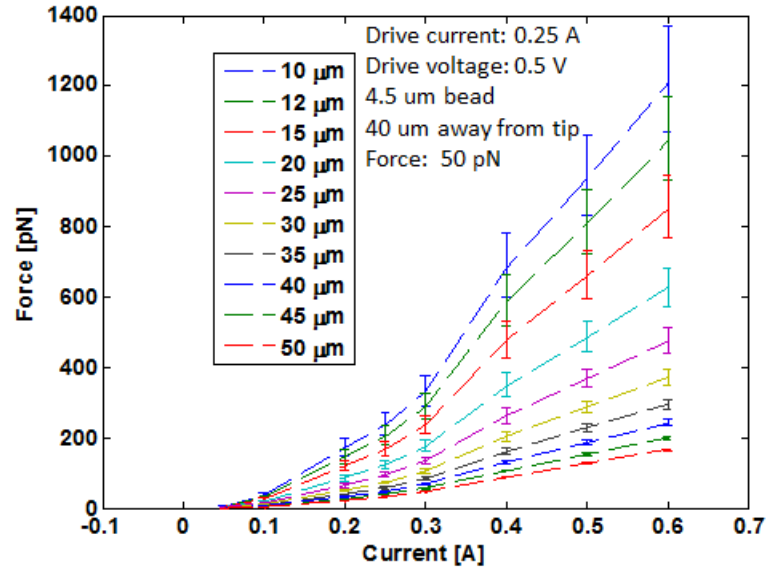


Figure A.2: Force calibration for different driving currents and pole-bead distances. A sequence of constant current pulses (over given periods of time) are applied to $4.5 \mu\text{m}$ beads in 2.5 M sucrose ($\eta = 1.42 \text{ Pa s}$) using a Netic material lid. A degauss routine (consisting of a high frequency decaying sinusoidal current), was used to remove remanence magnetization of the pole between current pulses used in the calibration. Video was taken at 120 fps using a 40X objective. To ensure sufficient sampling, each pull lasted at least 100 ms (10 data points $\times$ 8.6 ms exposure).

A.2 Data Analysis Recipe for Magnetic Tweezer Experiments

In raw form, magnetic tweezer data are position and time measurements recorded by video tracking software. The details of active microrheology experiments will be discussed in subsequent chapters, but for now we can assume a constant step force is applied to a cell in a 5 sec “on”, 10 sec “off” force regimen. Figure A.3 illustrates the following procedure for turning particle position time series data, $(x(t), y(t))$, into cell rheology measurements:

1. The radial displacement of the particle $r(t)$ relative to the origin of the measurement, $(x(t_0), y(t_0))$, is computed using:

$$r(t) = \sqrt{(x(t) - x(t_0))^2 + (y(t) - y(t_0))^2} \quad (\text{A.2})$$

2. The time-dependent compliance $J(t)$ is then computed using $r(t)$ and the following expression (Ziemann et al., 1994):

$$J(t) = \frac{6\pi a r(t)}{F(t)} \quad (\text{A.3})$$

where a is the bead radius and $F(t)$ is the applied force

3. The time-dependent compliance $J(t)$ is fit with a Jeffreys model viscoelastic model, discussed in Sec. 2.6.2, and given by the following expression

$$J(t) = \frac{1}{G} \left(1 - e^{-\frac{G}{\eta_1} t} \right) + \frac{1}{\eta_0} t \quad (\text{A.4})$$

4. Each pulse of force provides a measure of the cell rheology during the respective period of time:

$$\text{a modulus and a viscosity} \quad \rightarrow \quad G, \eta \quad (\text{A.5})$$

5. The dynamic state of the rheology of a cell in response to force application can be quantified by computing the relative material properties by normalizing parameters to the initial parameter value. For example, after the 2 minute experiment detailed in Figure A.3, the change in the elastic modulus is given as $\frac{G_8}{G_1}$.

The above procedure can be used to obtain material properties of various cells under many different conditions. Figure 1.4 details 5 different projects that use the active microrheology methodology that will be discussed in Chapters 4 and 5. Active microrheology is a powerful technique for studying cell mechanics because of the variations of experiments that can be done: varying cell types, substrate ligand, bead size and ligand, and the force function. As will be discussed in Chapter 3, the active microrheology assay can be coupled with a permanent magnet assay that applying a similar force regimen in order to investigate the biochemical responses of cells after force application.

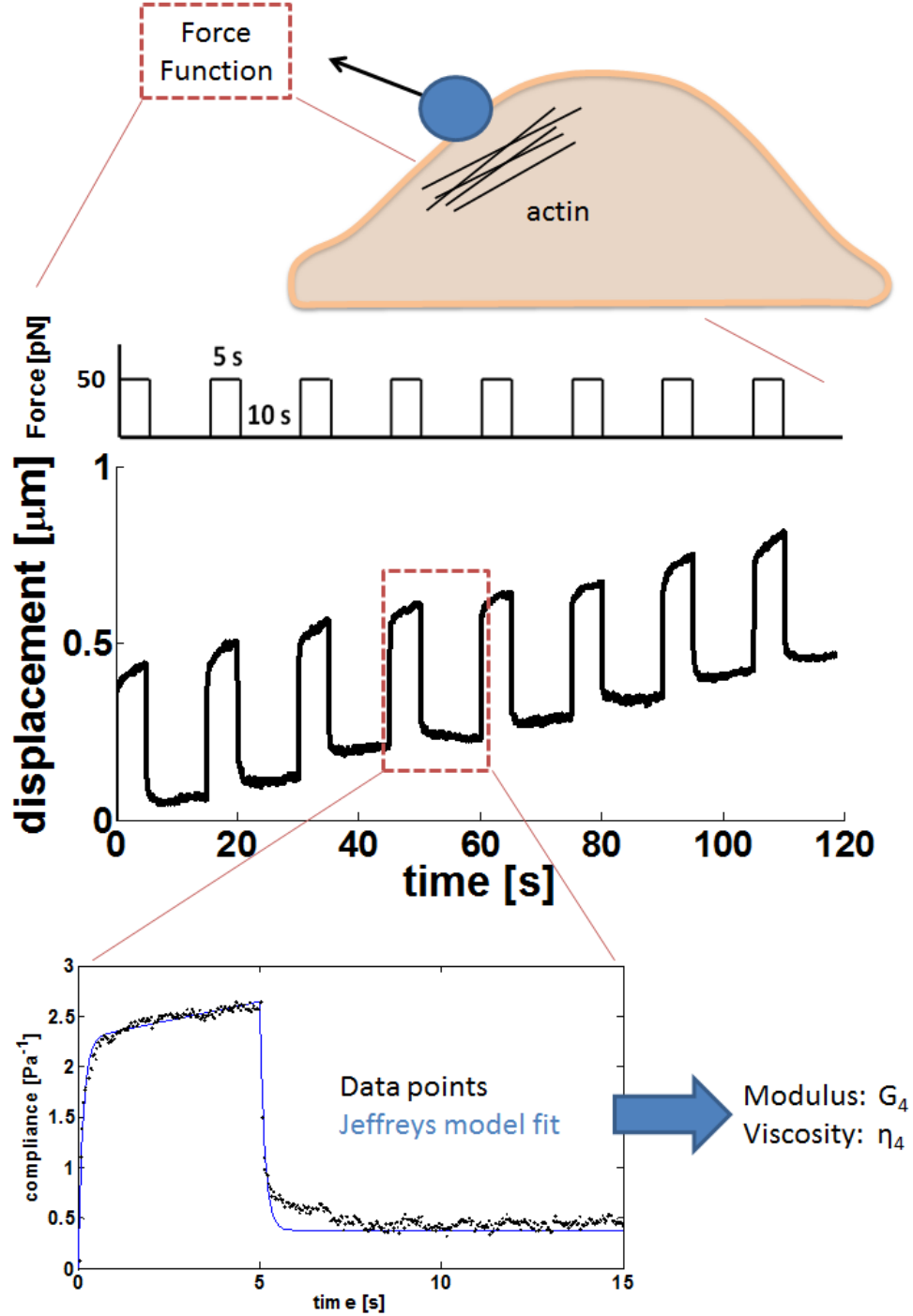


Figure A.3: **Active microrheology to cell mechanics workflow.** After force application, the raw measurement is a position time series data set. The radial displacement is transformed into the time-dependent compliance and fit to a viscoelastic Jeffreys model to obtain the elastic modulus and viscosity.

Appendix B: Improved Selection Criteria for 3DFM Experiments

As with the use of any technical device and methodology, implementing magnetic tweezers to study cell mechanics involves instrument and experiment overhead, as well as experiment execution and data analysis. In order to maintain consistency across experiments at the point of execution and data analysis, a selection criteria was established. The 3 parts of this procedure are discussed below: (1) Field of view (FOV) selection, (2) Pre- data-collection selection, (3) Post- data-collection selection.

	total # of FOVs in cloning ring sample		2400
2%	# of FOVs that pass FOV selection		48
50%	# of FOVs that pass Pre- data-collection selection		24
33%	# of beads that pass Post- data-collection selection		8

Figure B.1: **Selection criteria statistics.** After selection criteria, a typical magnetic tweezer experiment yields approximately 8 validated data points (bead trajectories in response to force).

B.1 Field of view (FOV) selection

This criteria specifies the FOVs that are appropriate for cell pulling video data collection; the FOV criteria contains the following 3 parts:

- (a) bead location on cell: a bead must be within a cell boundary (Fig. B.2 A) or in a region surrounded by cell 'texture' – distinguishable from the substrate background (Fig. B.2 B)
- (b) bead-to-bead distance: beads must be separated by at least 2 bead diameters
- (c) bead-to-pole tip distance: beads are in focus 30 μm below the focal plane of the pole tip, and approximately 55-70 μm away, in the xy plane, from the center of the pole tip

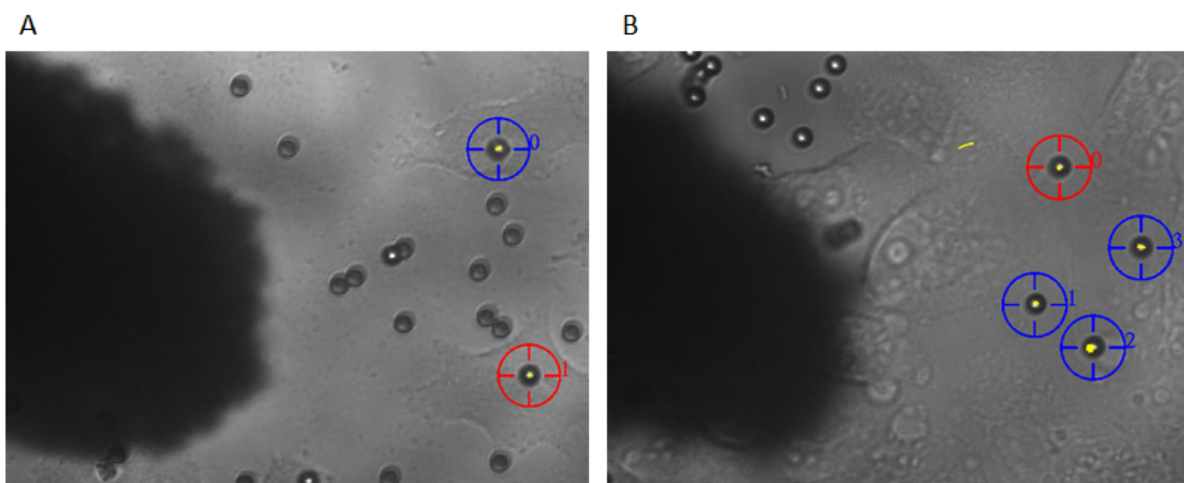


Figure B.2: **FOV selection.** (A) Beads on single cells. (B) Beads on a sheet of cells (identified by the cell 'texture'). The 2 and 4 beads in (A) and (B), respectively, pass the FOV selection.

B.2 Pre- data-collection selection.

Once a FOV is deemed acceptable, the next step is to estimate whether the bead-to-cell connection is established and appropriate for probing cell mechanics. This is an important step, since running a force protocol and collecting video at a "bad" FOV takes time away from collecting "good" video (After the bead incubation period (~ 20 min), there is a window of approximately 1 hour before beads become strongly attached and are unable to be displaced with the deliverable force from the magnetic tweezers instrument). A bead-to-cell linkage is considered established and appropriate for probing cell mechanics if the following 2 conditions are met:

- (a) passive motion selection: beads are observed for several seconds. Beads that are diffusing freely on the cell surface are not considered attached. If there are only freely diffusing beads on cells in the FOV, no data is collected. If there are beads that appear connected, they are subjected to the active motion selection.
- (b) active motion selection: a short (~ 0.5 sec), low force (~ 5 pN) single pulse of force is delivered to candidate beads. If the beads detach from the cells or the linkage is compromised, no data is collected. If the beads withstand the brief force pulse, the beads and FOV are considered acceptable for data collection.

B.3 Post- data-collection selection.

Once video is collected and bead responses are tracked, the trajectories are processed into time-dependent radial displacement $\mathbf{r}(t)$ and time-dependent compliance $\mathbf{J}(t)$. The criteria that $\mathbf{r}(t)$ and $\mathbf{J}(t)$ signatures are selected against is discussed below.

Beads are not included in further analyses if they exhibit the criteria described for: stuck beads, beads that detach from cells, directed membrane drift, broken bead-cell attachment, anchorage displacement, or broken bead-cell attachment and anchorage displacement. Beads are included in further analyses if they exhibit criteria for: viscoelastic response, viscoelastic cell stiffening response, or viscoelastic cell softening response.

For each criteria, there is: (1) a full-length response plot for the experiment either in displacement (green) or compliance (red) space, and (2) a displacement or compliance stacked plot that compares the responses for each force pulse. In each plot, time progression is encoded by increasing intensity of green (displacement) or red (compliance), such that black is the displacement or compliance during the first pulse of force.

Additionally, each criteria is accompanied by a schematic which attempts to describe the hypothesized physical picture of what is observed from the displacement or compliance signatures.

Note: These steps are for a constant-force pulsatile regimen (*e.g.* a 5 sec 'on', 10 sec 'off' 50 pN sequence); other force regimens (*e.g.* ramp sequence of increasing force or variable pulse duration) may require different selection steps.

B.3.1 Stuck beads

Stuck beads are beads that pass FOV and pre- data-collection selections, but are so strongly attached to the cells, that a viscoelastic response to the applied force regimen is not observed.

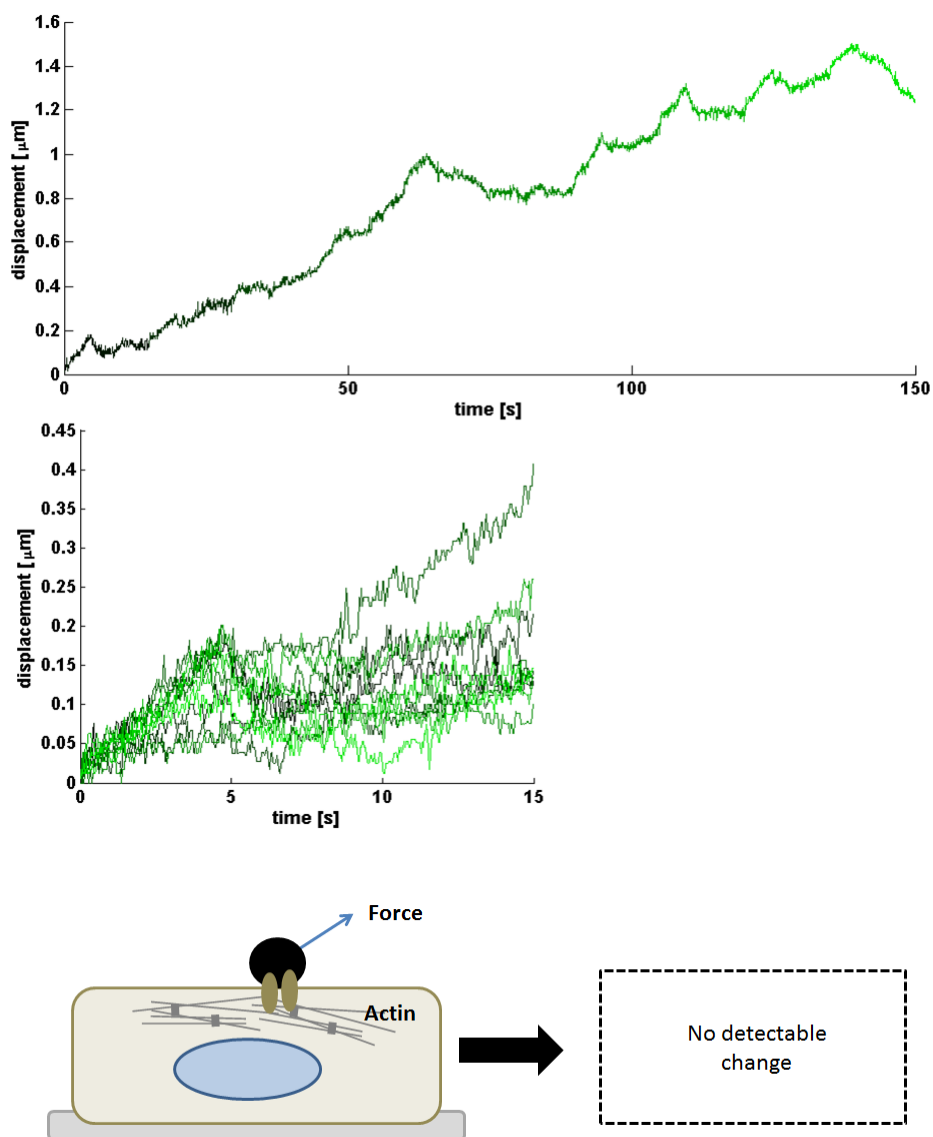


Figure B.3: **Stuck bead.** No detectable mechanical changes leads us to not be able to speculate about a physical picture.

B.3.2 Beads that detach from cells

Despite the FOV and pre- data-collection selections, some beads are not attached strongly enough to withstand the force regimen (or part of it). In general, these beads are not included in further analyses. If, for example, a bead exhibits a viscoelastic response for half of the force sequence and then is pulled off of the cell for some reason (*e.g.* another bead collides into the bead of interest), the pulses up to the point detachment are used in further analyses.

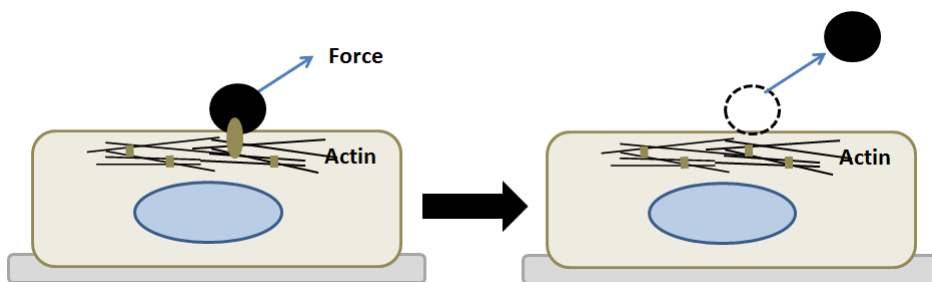


Figure B.4: **Beads that detach from cells.**

B.3.3 Directed membrane drift

Some beads are attached to the membrane, and under an applied force tend to drift along the membrane towards the force. The individual pulses of force cannot be discernible in the displacement. Little to no viscoelastic response is observed.

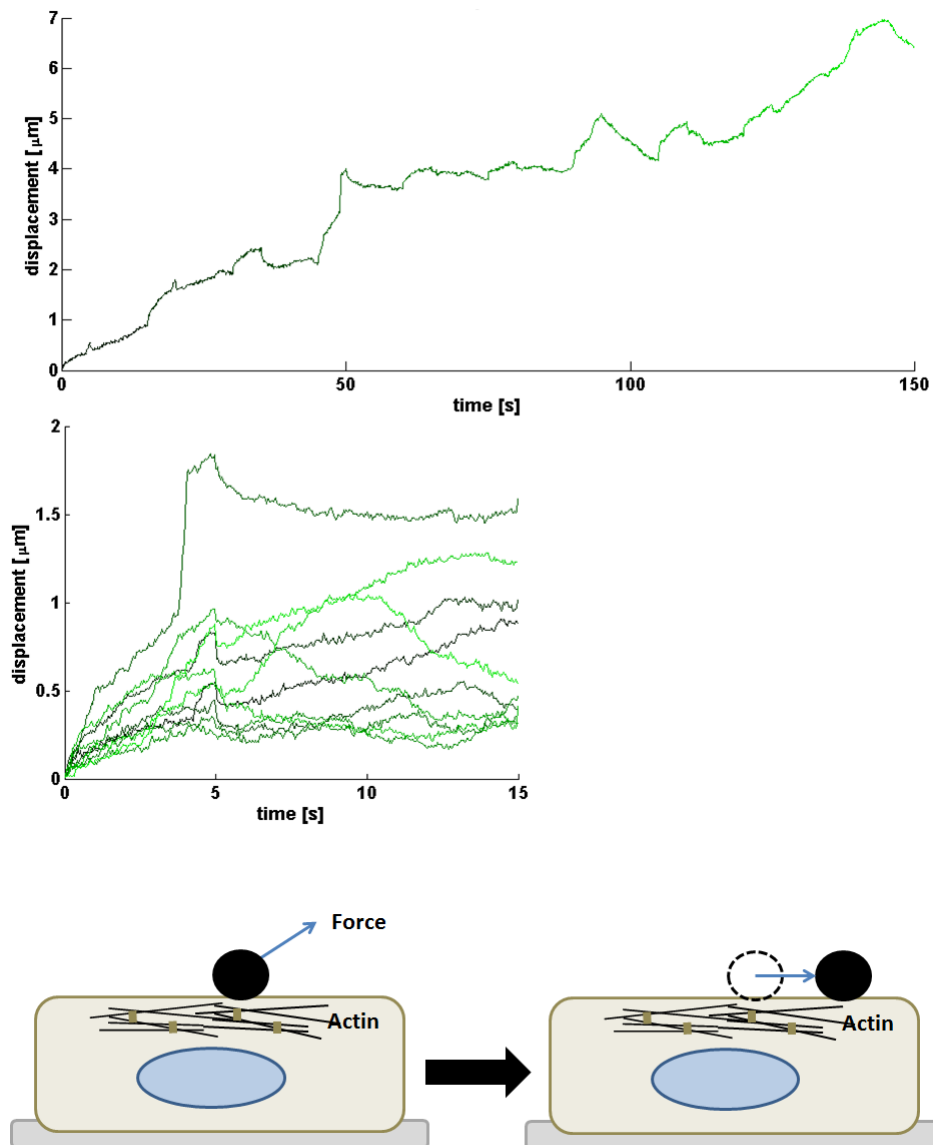


Figure B.5: **Directed membrane drift.** The bead is attached to the membrane, not linked to the actin cytoskeleton.

B.3.4 Broken bead-cell attachment

Some beads exhibit a disrupted response at the first pulse of force (shown here) or a subsequent pulse. It is hypothesized that these disruptions are due to a broken bead-cell attachment. The bead is not included in further analyses.

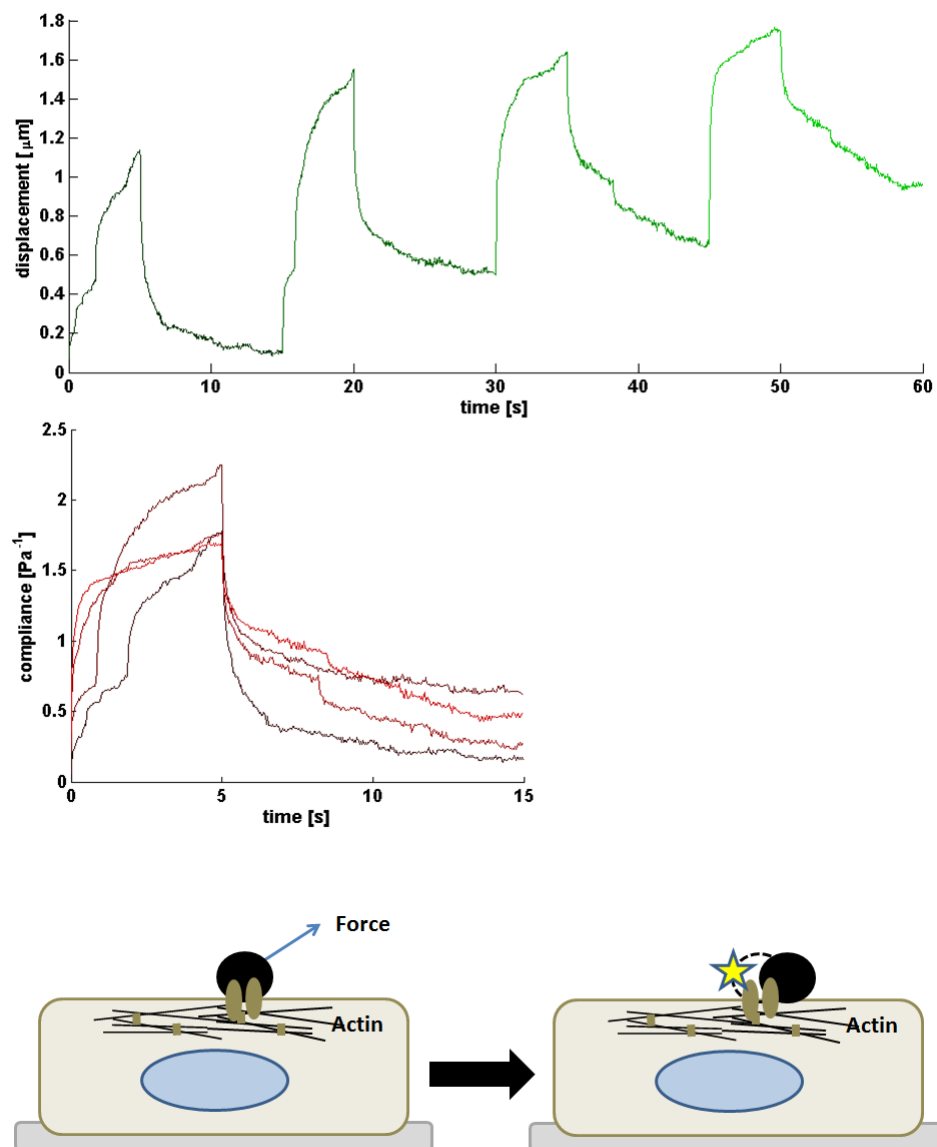


Figure B.6: **Broken bead-cell attachment.** Beads may exhibit viscoelastic response after disruption, but data is not included.

B.3.5 Anchorage displacement

Some beads exhibit an exaggerated initial displacement (typically $\gtrsim 0.5$ bead dia.) at the first pulse of force. It is hypothesized that this is due to a displacement of the bead-cell anchorage point. The bead is not included in further analyses.

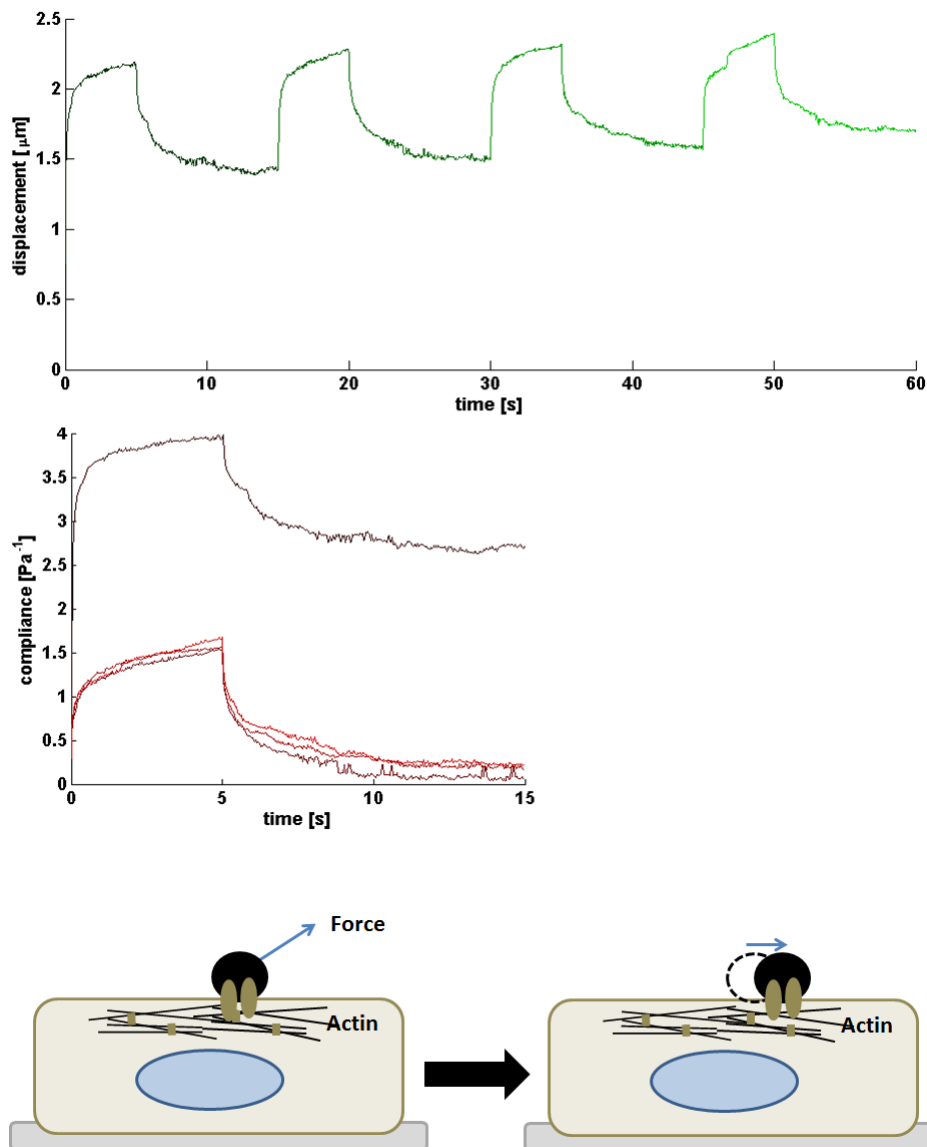


Figure B.7: **Anchorage displacement.** Beads may exhibit viscoelastic response after disruption, but data is not included.

B.3.6 Broken bead-cell attachment and anchorage displacement

Some beads exhibit both a broken bead-cell attachment and an exaggerated initial displacement at the first pulse of force or subsequent pulses. For reasons above, the bead is not included in further analyses.

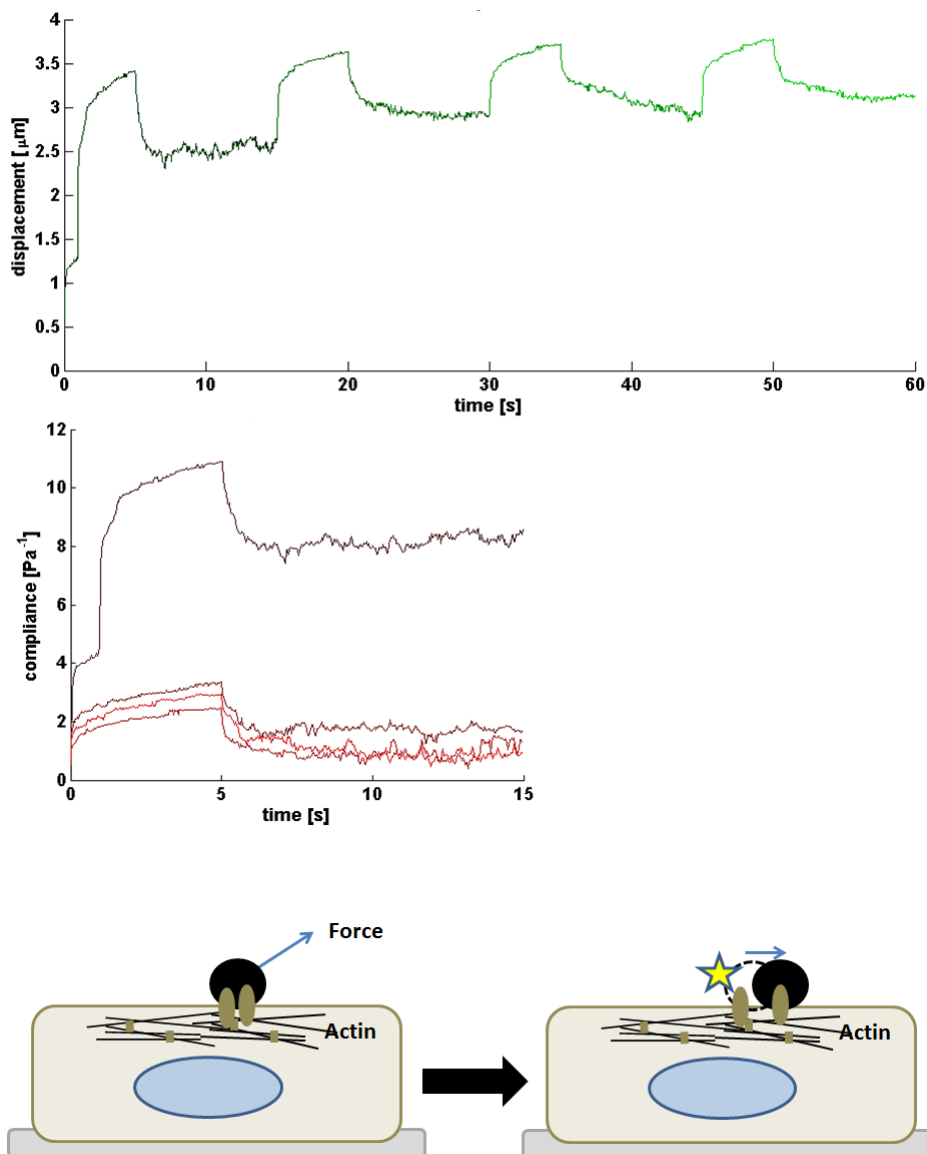


Figure B.8: **Broken bead-cell attachment and anchorage displacement.**

B.3.7 Viscoelastic response

It is hypothesized that beads exhibiting viscoelastic behavior under an applied force are probing the cortical actin cytoskeleton that lines the cytoplasmic side of the cell membrane. These beads are included in further analyses.

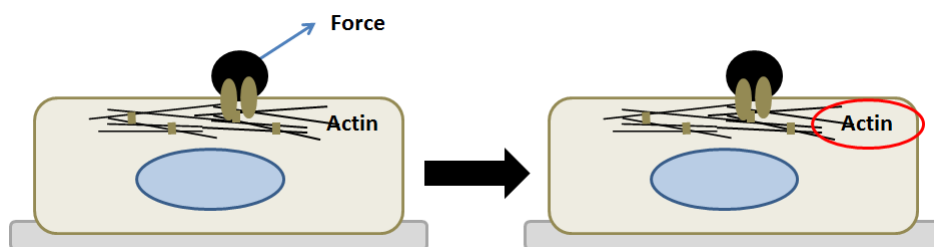
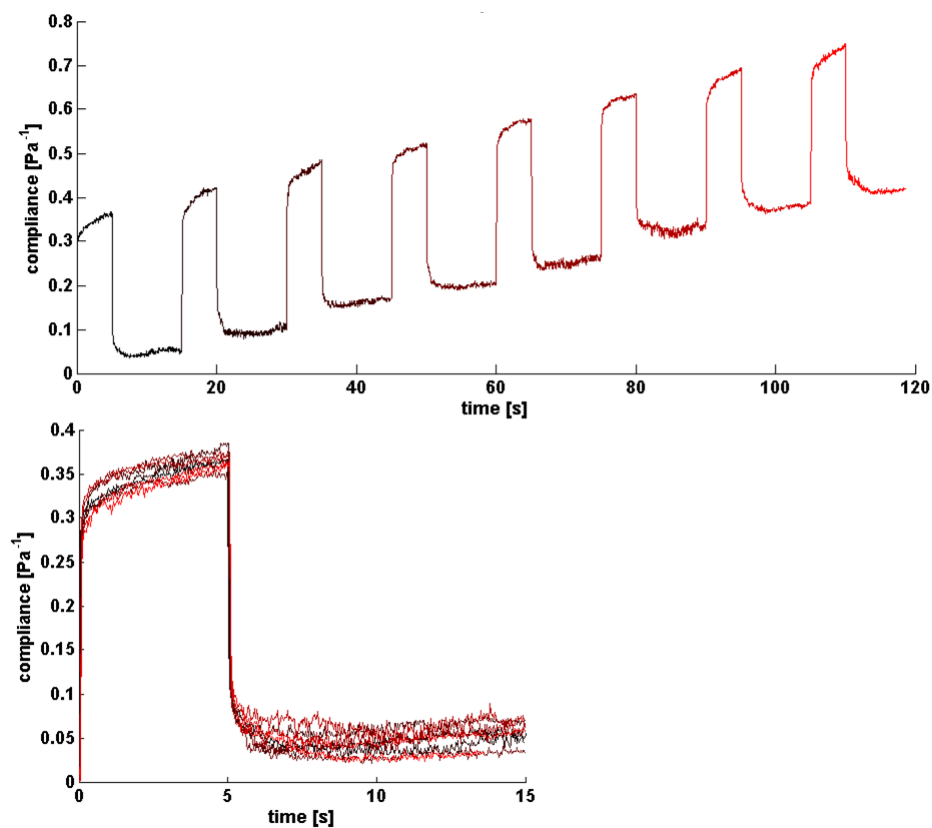


Figure B.9: **Viscoelastic response.** A viscoelastic response without a trend towards cell stiffening or softening.

B.3.8 Viscoelastic cell stiffening response

It is hypothesized that beads exhibiting a reduced displacement under an applied force are experiencing a stiffening of the the cortical actin cytoskeleton via actin polymerization and/or stabilization of filaments. These beads are included in further analyses.

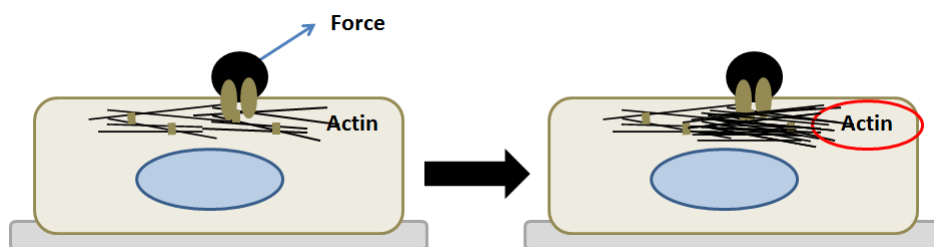
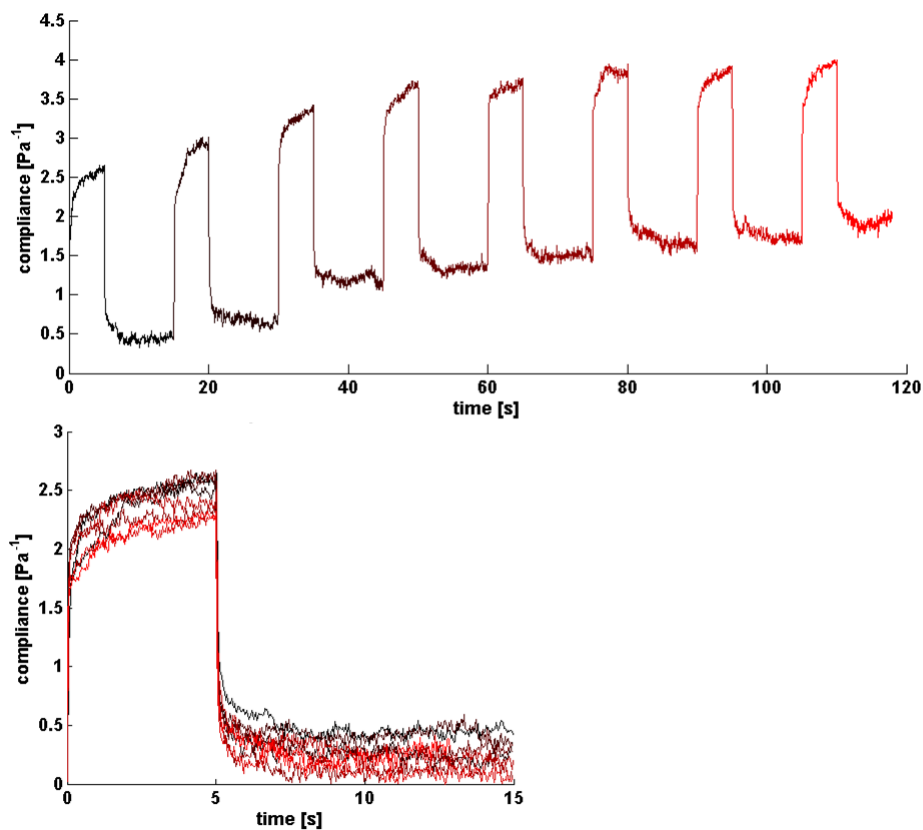


Figure B.10: **Cell stiffening response (ex. 1).** Beads exhibit less instantaneous displacement during a pulse of force when the cortical actin network is more mature.

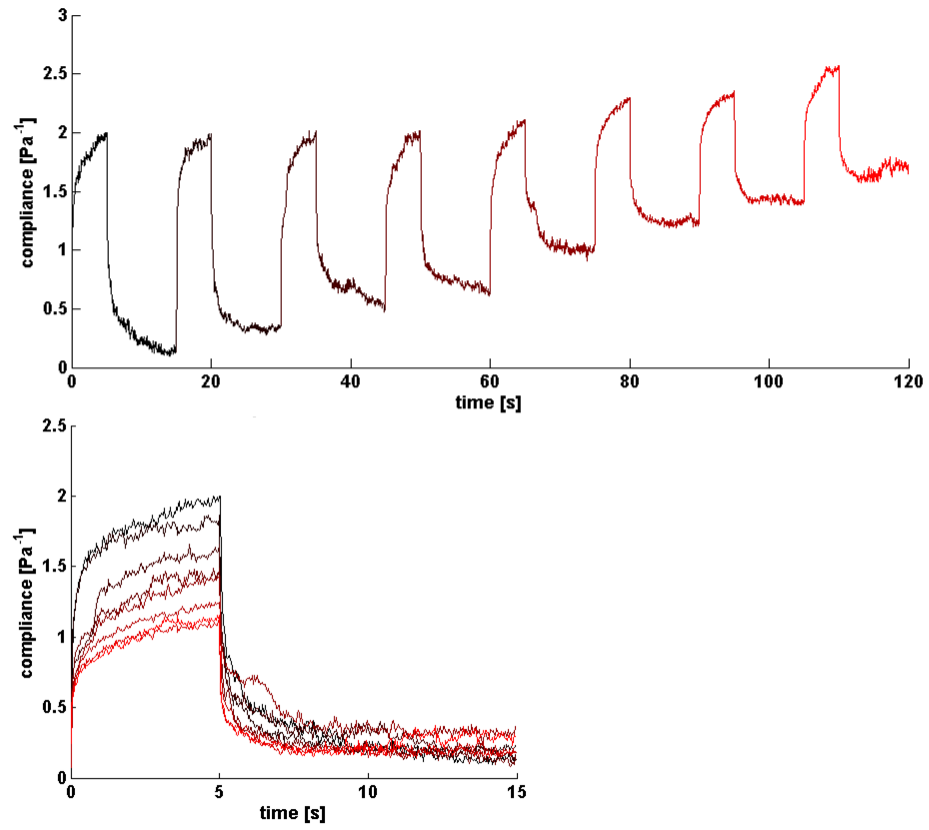


Figure B.11: Cell stiffening response ex. 2.

B.3.9 Viscoelastic cell softening response

It is hypothesized that beads exhibiting an increased displacement under an applied force are experiencing a softening of the the cortical actin cytoskeleton. These beads are included in further analyses.

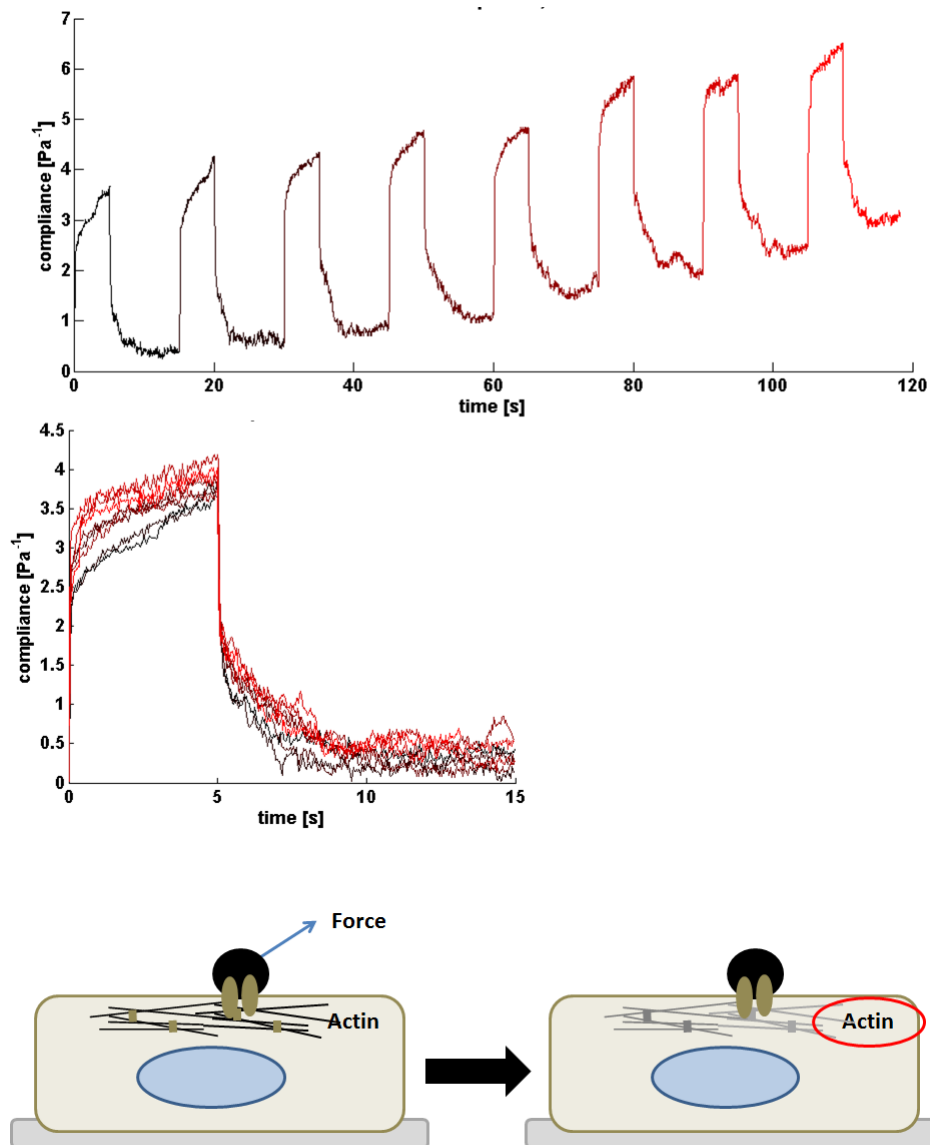


Figure B.12: **Cell softening response ex. 1.** Beads exhibit larger instantaneous displacement during a pulse of force when the cortical actin network is less mature.

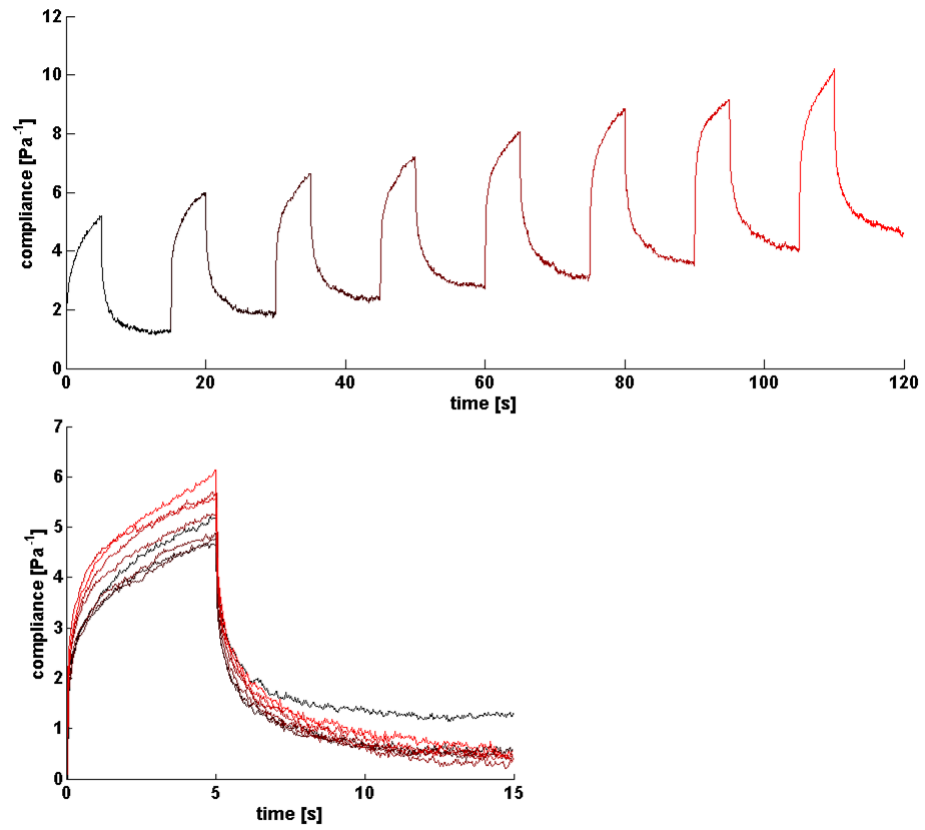


Figure B.13: Cell softening response ex. 2.

Appendix C: Scaling of Hoffmann 2006 PBR Data

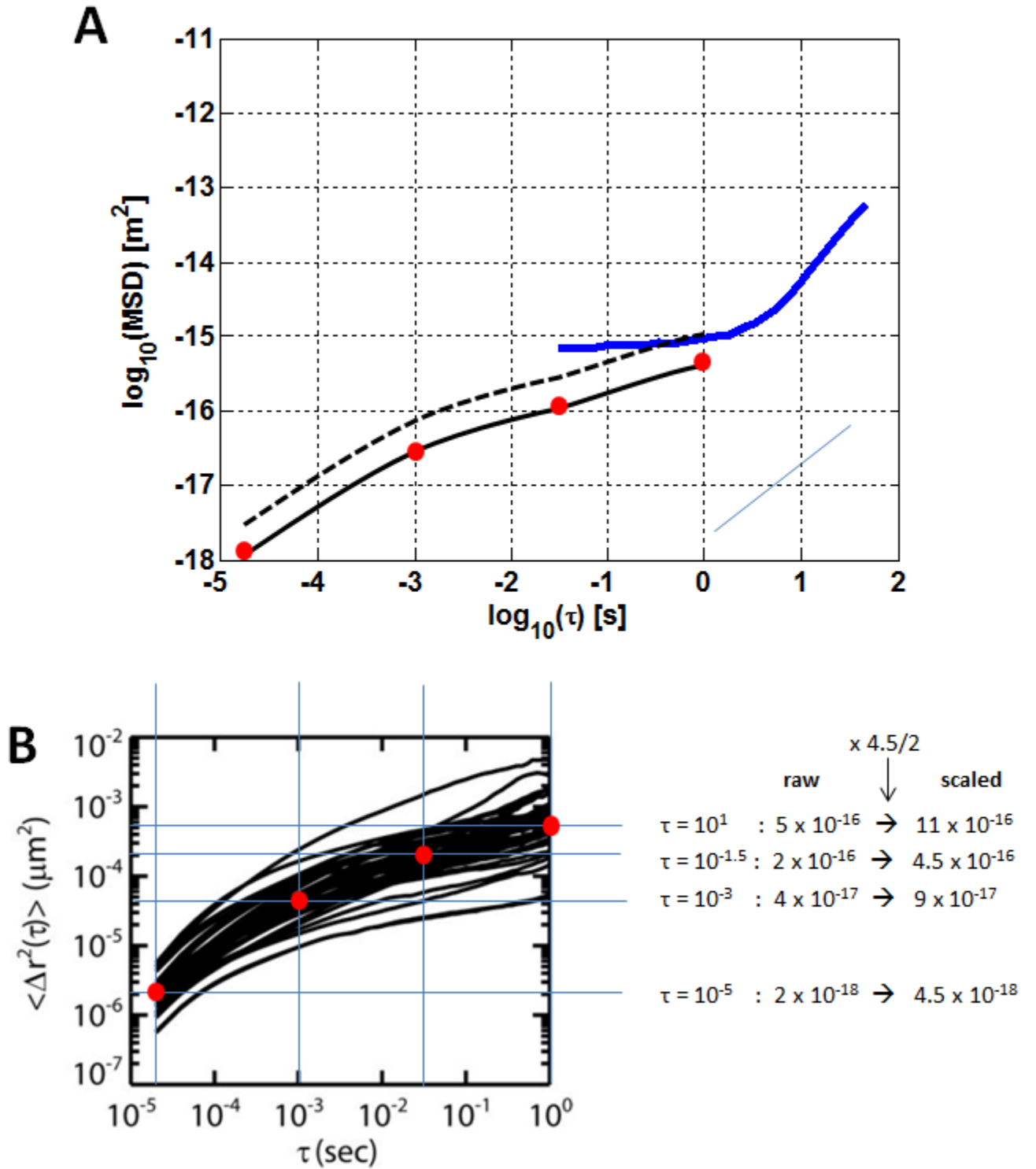


Figure C.1: **Scaling of Hoffmann *et. al* PBR data.** (A) Blue curve is our data. Red points are data from MSD data determined from (Hoffman et al., 2006). (B) 4.5 μm beads externally attached to epithelial cells through integrin receptors.

Appendix D: Protocols

D.1 High Throughput Cell Rheology Assay

1. Data collection. See instrument workflow for general details regarding data collection using the high throughput system. The current cell rheology protocol involves taking 48 1 min long videos at 32 fps videos per condition (8 FOVs in 6 replicate wells) at 4-5 beads per video. This yields 200-300 beads per condition.

2. Bayesian analysis of motion model fitting to MSD trajectories. Automated data collection requires a filtering routine to ensure quality of the data and to reduce the impact of data heterogeneity from variable bead to cell attachments. The filtering routine uses fitting procedures from (Monnier et. al. 2012) to look at 1 sec subtrajectories in the MSD to categorize the motion of beads. Currently, we filter out beads driven by flow (driven beads), beads that are unattached or weakly attached to cells (freely diffusing and confined diffusing), and beads that do not appear to move over 1 sec timescales. Beads that exhibit anomalous diffusion (DA) are hypothesized to be well attached to cells. Approximately 60 – 70% of beads exhibit anomalous diffusion, yielding 100-150 beads per condition.

3. DA-model filtered MSD distributions are visualized via box plots. In order to perform statistical hypothesis testing on our data, we need to look at the dis-

tributions in MSD space (as opposed to stiffness space). In order to easily compare distributions across conditions, we decided to use box plots to visualize the data. Briefly, the box is created by the 25th (Q1) and 75th (Q3) percentiles and the line represents the median of the distribution. The notch represents 95% confidence intervals, such that if intervals between two conditions do not overlap, the medians of the two distributions are significantly different at the 5% level. Whiskers extend to the most extreme non-outlier data points. Outliers are plotted individually using a (+) symbol, and are defined by data points greater than $Q3 + 1.5 * (Q3 - Q1)$ and less than $Q1 - 1.5 * (Q3 - Q1)$, where 1.5 corresponds approximately to ± 2.7 standard deviations. For context, for a normal distribution, ± 2.7 standard deviations account for over 99% of the data in the distribution. The median RMS displacement of the distribution is given above the box.

4. Mann-Whitney U-tests are performed to determine significance between conditions.

The distributions are, in general, not normal based on analysis of skewness, kurtosis, and the Shapiro-Wilk test (null hypothesis is that the distribution is normal). Thus, we used the non-parametric Mann-Whitney U-test that tests the null-hypothesis that the medians of two distributions are the same.

D.2 Gain of Function Construct Preparation Protocol

Procedure for preparing lentivirus from expression DNA for ORF overexpression or shRNA knockdown studies

Updated: February 2014

Wood Lab

*Note: This procedure starts with purified ORF/shRNA expression plasmids encoding the construct(s) of interest

Part I: Amplify and Mini-Prep DNA

Note: This work can be done on the bench top using sterile tips.

1. Transform 20 ng of DNA per construct (can do less if necessary). Add 50 uL of DH5alpha competent bacteria to 1 uL containing 20 ng/uL DNA in labeled, round bottom tubes on ice. (Thaw DH5alpha competent cells slowly on ice. Make sure that tubes are pre-chilled on ice prior to adding bacteria + DNA.) (Note: For pLEX plates containing ORF DNA, most constructs are at around 20 ng/uL, so 1-2 uL DNA is plenty.)
2. Gently mix by pipetting up and down carefully and swirling. Incubate each tube of bacteria + DNA on ice for 30 min.
3. Heat shock by immersing in 42° C water bath for 45 seconds.
4. Incubate on ice for 2 min.
5. Add 250 uL of SOC media to each tube. Transfer to warm shaker for 1h.
6. Add 50 uL of each mixture to individual, labeled, warm, AMP+ agar plates (always keep plates with agar on the top so that it doesn't dry out). Use a glass spreader, dipping in EtOH and flame sterilizing before use and between each construct. (Can also use sterile glass beads.) Try to spread the liquid evenly across each plate. Place plates in incubator overnight (agar up so that water doesn't evaporate!). Place the remaining liquid in each tube in the fridge overnight – you can try to re-innoculate plates the next day if no colonies have grown or if too many have grown. Also, if you don't want to add colonies to liquid culture the next day, you can refrigerate agar plates at 4C until you are ready to start the liquid cultures.
7. The next day, pick colonies (one per construct) with a 10 uL sterile pipette tip by simply touching the colony and then ejecting the tip into 5 mL of LB-Amp. Incubate in warm shaker overnight.
8. Next day, spin down and freeze bacterial pellets from each solution in 5 mL tubes.
9. Mini prep, purify, and quantify DNA for each construct. (Quantify using Nano Drop with pure TE buffer as blank.)

Figure D.1: Protocol for Wood library gain of function constructs. Obtained by Jian Chen.

D.3 Invasion Assay Protocol

Matrigel invasion and transwell migration assays Cells were trypsinized and seeded at a density of 50,000 cells per well on either Matrigel-coated (BD biosciences) or uncoated (Corning) transwell filters in a 24-well plate and allowed to invade for 12 hours toward 10% FBS in the lower chamber. Cells invading and migrating through the Matrigel-coated and uncoated filters, respectively, were stained with Three Step stain (Richard-Allan Scientific). Each filter was counted in its entirety with four 10x fields, and invasion or migration was quantified as fold relative to control.

Appendix E: Bayesian Approach to MSD Model Probability

To determine the probabilities for competing models, Monnier *et.al.* implemented Bayes' theorem. In the Bayesian approach, for a set of K models ($M_1, \dots, M_K$), the probability of model k given the observed data $\vec{y}$ is

$$P(M_k|\vec{y}) = \frac{P(\vec{y}|M_k)P(M_k)}{P(\vec{y})} \quad (\text{E.1})$$

where $P(\vec{y}|M_k)$ is the marginal likelihood of model k , $P(M_k)$ is the prior probability of the model k , and $P(\vec{y}) = \sum_k P(\vec{y}|M_k)P(M_k)$ is the total marginal likelihood of the data given all models (can be thought of as a normalization factor). Consistent with new studies where no information is available to assign prior preferences to models, we assume $P(M_k)$ is equal for all k . This assumption, in addition to constraining the total model probabilities to be equal to 1, $\sum_k P(M_k|\vec{y}) = 1$, allows for the following proportionality relation (Monnier et al., 2012):

$$P(M_k|\vec{y}) \propto P(\vec{y}|M_k) \quad (\text{E.2})$$

The likelihood of the data given model k , $P(\vec{y}|M_k)$, is calculated using the expression (Monnier et al., 2012)

$$P(\vec{y}|M_k) = \int \underbrace{P(\vec{y}|\vec{\beta}_k, M_k)}_{\text{depends on residuals}} \underbrace{P(\vec{\beta}_k|M_k)}_{\text{priors}} d\beta_k \quad (\text{E.3})$$

Since we have no prior information about the probability distribution for the parameters in each model, $P(\vec{\beta}_k|M_k)$, we assume a uniform distribution centered at the estimated model parameter, with a range of 200 times the standard error determined for that parameter (He et al., 2012); computationally, for a uniform distribution this is given by (Monnier et al., 2012)

$$P(\vec{\beta}_k|M_k) = \frac{1}{\prod_{\text{parameters}} (\beta_k^{\max} - \beta_k^{\min})} \quad (\text{E.4})$$

Therefore, models whose parameters have high uncertainty determined from fitting (Eqn. 7.6) have reduced the overall marginal likelihood compared to models whose parameters have smaller standard error. Additionally, this term inherently penalizes models that are overly complex. The probability of observing the data given a set of parameter values $\vec{\beta}_k$ in a model $\vec{f}_k(\vec{x}; \vec{\beta}_k)$ is given by (Monnier et al., 2012)

$$P(\vec{y}|\vec{\beta}_k, M_k) = \frac{1}{(2\pi)^{n/2} |\mathbf{C}|^{1/2}} \exp \left\{ -\frac{1}{2} [\vec{y} - \vec{f}_k(\vec{x}; \vec{\beta}_k)]^T \mathbf{C}^{-1} [\vec{y} - \vec{f}_k(\vec{x}; \vec{\beta}_k)] \right\} \quad (\text{E.5})$$

where τ -correlated errors ϵ_i in the data are addressed using the covariance matrix $\mathbf{C}$. Eq. E.5 shows explicitly that computation of model likelihoods (and hence probabilities) require calculation of the covariance matrix.

Programmatically, the model k likelihood $P(\vec{y}|M_k)$ is estimated using the Laplace approximation (He et al., 2012) of Eqn. E.5. After calculating the likelihood of each model, the probability of model k is then computed by normalizing to the total likelihood of all tested models.

Appendix F: Gain of Function Construct Validation

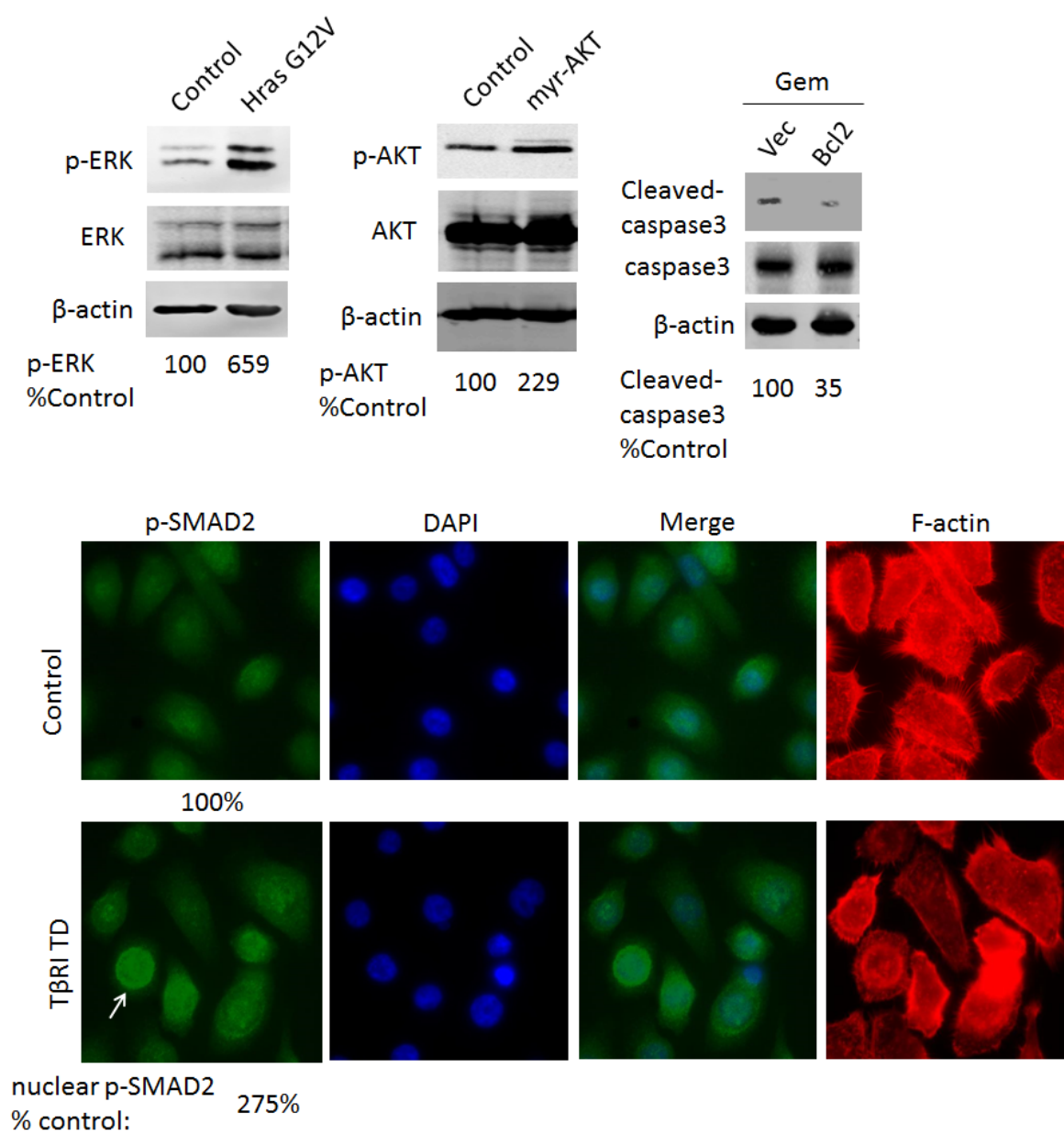


Figure F.1: Initial validation of the gain of function constructs.

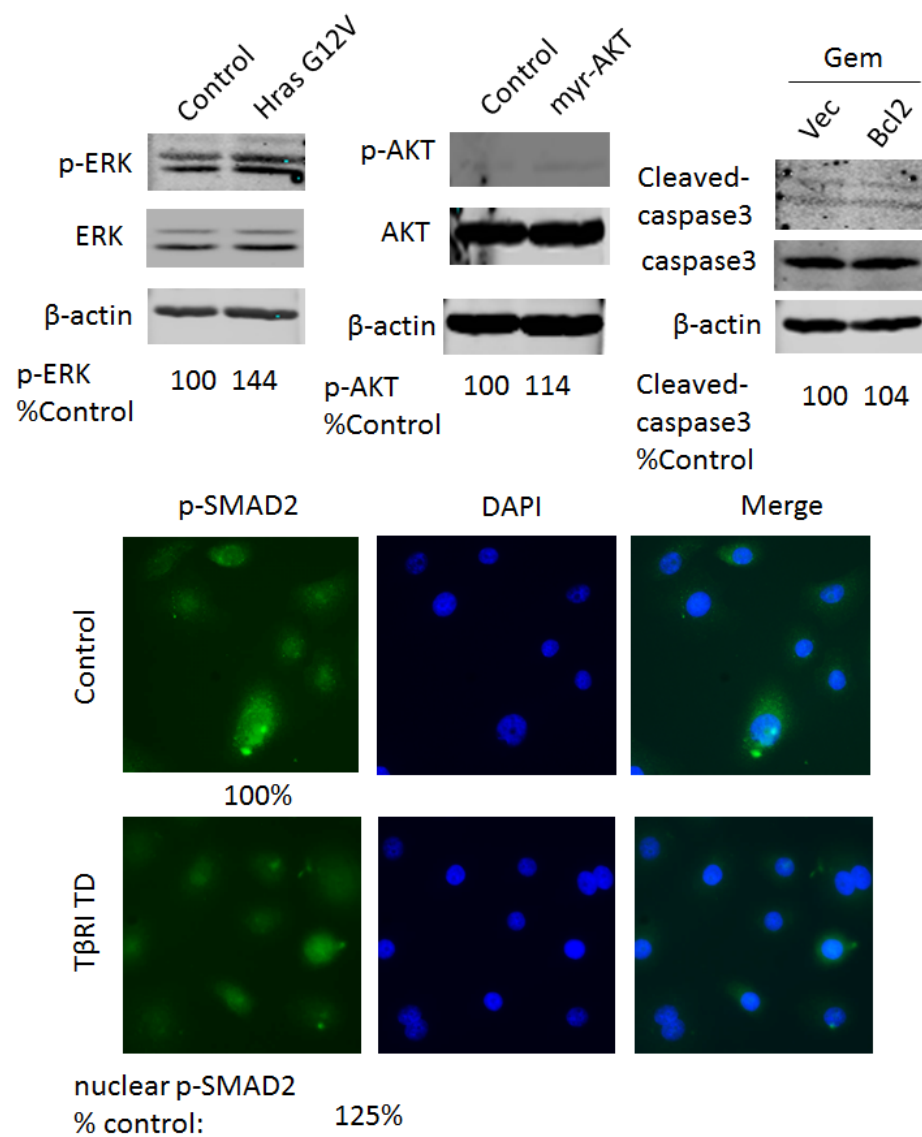


Figure F.2: Re-validation of the gain of function constructs shows loss of construct expression.

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