CHAPTER FIVE

Mechanical Cues Direct Focal Adhesion Dynamics

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Abstract

Focal adhesions play a fundamental role in force sensing, which influences a variety of cellular processes and functions, particularly migration and the cell cycle. They consist of large macromolecular assemblies of proteins that associate with integrins, in order to serve as anchor points between the cell and the extracellular matrix. These dynamic regions act as a hub for sensing and transmission of mechanical cues between cells and their surrounding microenvironments. A number of techniques have been used to study focal adhesions, including optical microscopy, substrate micropatterning techniques, and tools which can directly manipulate cells, such as the atomic force microscope. Mechanical stimulation of cells leads to changes in cell contractility, stress fiber remodeling, and focal adhesion position and size; several of the responses explored in this chapter.

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1. INTRODUCTION

In vivo, cells are exposed to a variety of mechanical forces, which are now well known to direct the fate and function of cells.[1–5] Not only do cells sense and respond to these physical forces, but also they too exert forces on their surrounding microenvironments.[6–9] Force transmission between cells and the underlying extracellular matrix (ECM) occurs at highly localized junctions, known as focal adhesions (Fig. 5.1). These protein-rich regions transmit and sense mechanical cues arising from both the cell's interior and exterior via integrins.[11] Moreover, these anchor points act as dynamic adhesion sites on the underlying matrix, as seen during cell migration,[12] and tension-mediated structural changes.[13]

Due to their complex composition, understanding the functional mechanisms behind focal adhesions remains challenging. With the use of fluorescent proteins and optical microscopy, a large number of studies have used visual means to examine and quantify the size, spacing, and turnover kinetics of focal adhesions at high levels of spatial and temporal resolution.[5,6,14–16] In tandem, the study of cellular-generated traction forces has also led to a significant depth of understanding on how cells employ adhesion and respond to varying microenvironmental conditions.[6,8,9,17]

The shape of adherent cells is directly affected by focal adhesion size and density, which depends on both the biochemical and physical properties of the ECM. As well, extracellular forces, such as mechanical tension or compression transmitted via the ECM, are also "sensed" by focal adhesions.[18] The dynamics of focal adhesion development can be monitored by exposing cells to a variety of mechanical cues *in vitro*. Recently, researchers have employed atomic force microscopy (AFM) to apply precise forces to cells apically.[5,6] Methods such as these, in tandem with optical techniques, have provided further insight into traction force and focal adhesion dynamics, in response to mechanical cues.

This chapter provides an overview of focal adhesions as sensors of mechanical cues. First, we briefly describe the form and function of focal adhesions, while providing a background on adhesion dynamics, and subsequently, how they act as mechanical sensors. Second, we will provide a brief introduction on techniques used to study focal adhesions. Specifically, we will focus on how AFM is used in conjunction with optical microscopy techniques in a combined effort to study focal adhesions. A comprehensive review of two leading examples of these combined techniques will follow. Finally, we discuss future directions of research in this field.

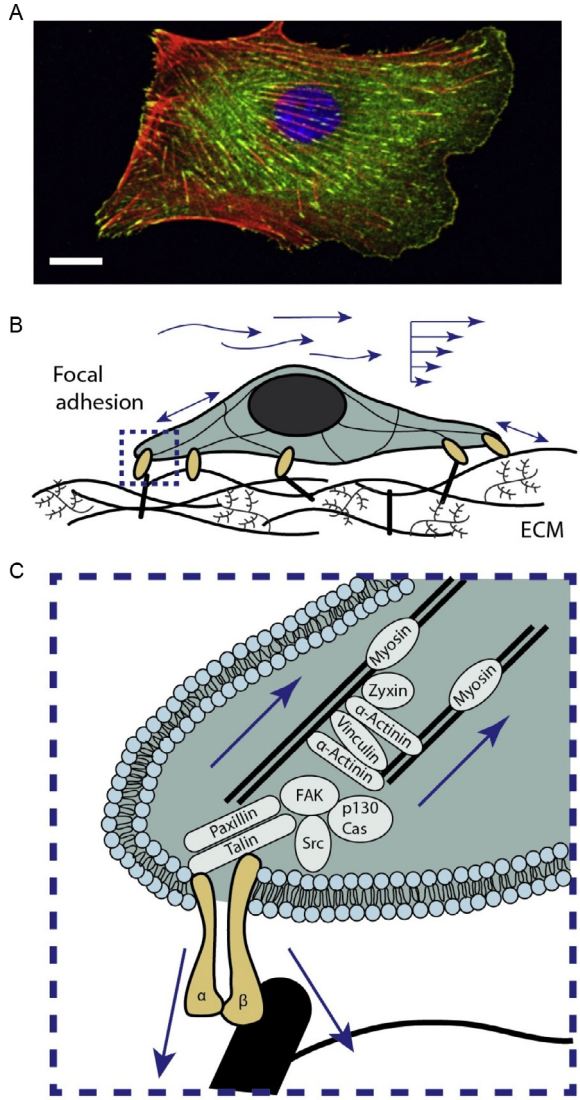

Figure 5.1 Form and function of focal adhesions. (A) Immunofluorescent image of a C2C12 myoblast cell cultured on a glass substrate coated with 0.1% gelatin. The cell was fixed and stained for DNA (blue), actin filaments (red) and a well-known focal adhesion protein, vinculin (green). Scale bar is 10 μm. (B) Schematic of a cell experiencing shear forces from fluid flow, and compression and tension between the cell's inner cytoskeleton and the underlying extracellular matrix. These forces are transmitted between the cell and ECM at focal adhesions. (C) Enlarged depiction of a focal adhesion. Integrin isoforms bind to talin and paxillin, linking them to the actin cytoskeleton. These proteins associate with focal adhesion kinase (FAK) and other adaptor proteins (Src and p130Cas). Actin-associated proteins such as α-actinin not only bind actin filaments together but also recruit other proteins, such as vinculin and zyxin, which are associated with mature focal adhesions. Tension generated in the contractile actomyosin network further activates stress sensitive proteins, such as zyxin. *(C) Adapted from Ref. 10.*

2. FORM AND FUNCTION OF FOCAL ADHESIONS

Proliferation,[19,20] migration,[7,21–23] differentiation,[1,24,25] and even apoptosis,[26,27] are dependent upon cellular adhesions to the ECM. These contacts, known as focal adhesions, are comprised of clusters of integrins linked to the actin cytoskeleton by associated proteins and by ligand–receptor connections to the ECM. Over the years, studies have provided valuable insight into the vast number of molecules associated with focal adhesions (Fig. 5.1C).[28,29] However, due to their complexity, how these macromolecular structures sense and respond to their surroundings are only partially understood. The physical interactions of adhesion sites are regulated by signaling and binding events[30–32]; however, mechanical cues generated either internally or from the substrate also influence their behavior and formation. In the following subsections, we describe how the ECM, integrins, and actin all contribute to the behavior and development of focal adhesions. Examples discussed herein use one of two main approaches of study: one method involves examining the molecular machinery, where the other involves changing the chemical and physical properties of the ECM, in an attempt to trigger a certain cellular response.[18]

2.1. Influence of the ECM

The form and function of focal adhesions are largely influenced by the composition of its underlying ECM. Focal adhesions have been observed to assemble/disassemble in response to variations in substrate elasticity,[15,25,27,31,33] geometry,[13,24,34,35] and ligand spacing,[21,36,37] thus, altering the form of the cytoskeleton and overall cell shape. Alterations in the cell's ability to sense these changes may be at the root of transformation into malignant phenotypes.[38] Indeed, transformed cells have been shown to generate weak traction forces,[9] and in some cases no longer require attachment to a substrate for growth.[27]

Systematic adjustment of substrate composition and geometry demonstrates the influence of the ECM on integrin-mediated adhesion. Researchers have used bioinert surfaces, such as polyethylene glycol (PEG), to test adhesion between certain cell–surface receptors and surface ligands.[39,40] Considering that the inclusion of ECM proteins, such as fibronectin, onto biomaterials can be complicated,[39] many studies employ biomimetic short peptides such as RGD (arginine–glycine–aspatate) onto these surfaces, as a means of studying known ECM signaling domains.[41] RGD, in

particular, has been used to functionalize glass,[42] PEG,[39] and hyaluronic acid substrates,[43] and, more recently, supported lipid bilayers (formed by adsorption/fusion of small lipid vesicles).[44,45] These techniques used in conjunction with microcontact printing (with micrometer resolution) have shown that cell adhesion and motility are directly related to ligand spacing,[21] size, and arrangement.[46]

Although microcontact printing has been successful at controlling cell shape and viability,[46] protein clustering *in vivo* is on the order of 5–200 nm.[37,47,48] Block copolymer micelle nanolithography provides an improvement on ligand spacing resolution.[49] This method involves functionalization of metallic nanoparticles, usually gold, inserted at tunable spacing (10–200 nm). One study employed hexagonally spaced gold nanodots (~8 nm in size) coated with an RGDfk peptide.[47] This small size restricted binding to only one integrin per bead. Based on their findings, a universal spacing for integrin clustering was proposed to be in the range of 58–73 nm, since spacing >73 nm resulted in reduced cell spreading and adhesion, and <58 nm resulted in erratic cell growth and migratory behavior.[47] Nevertheless, cells have been shown to be sensitive to nanometer spaced integrin ligands.[36]

Besides the arrangement of adhesive ligands, cells also sense the rigidity of their substrates (Fig. 5.2). A prominent example is that of Engler *et al.* who demonstrated that nonmuscle myosin II was necessary for sensing

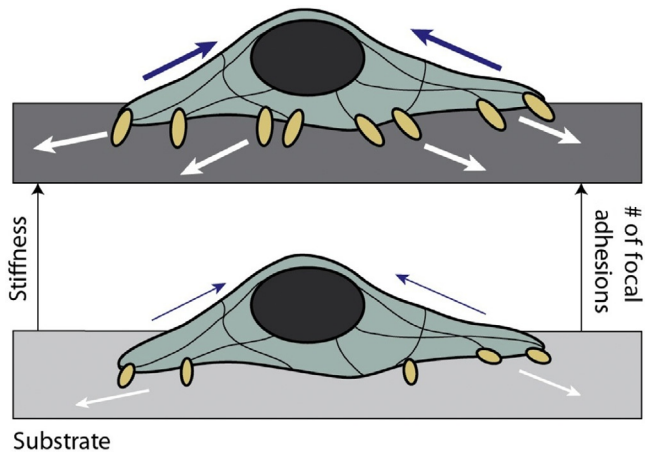

Figure 5.2 Mechanical cues, such as substrate stiffness, affect focal adhesion formation. Increasing substrate stiffness results in an increase in the number and size of focal adhesions. The reasoning behind this is that cells are able to pull with stronger traction forces on a less flexible substrate.

substrate stiffness, which was shown to direct the fate of stem cells.[1] When cultured on soft substrates mimicking brain tissue (0.1–1 kPa), mesenchymal stem cells differentiated into neurons, whereas when cultured on stiffer substrates mimicking muscle (8–17 kPa) or bone (25–40 kPa), they differentiated into myoblasts and osteoblasts, respectively.[1] Committed cells, however, are also influenced by substrate stiffness. For example, fibroblasts exposed to a stiffness gradient on polyacrylamide gels migrated from soft to stiff substrate regions.[25] Cells generated increased traction and cell spread area on their preferential stiff substrates. Moreover, when a strain was applied to the flexible substrate using a microneedle, the cells changed their polarization and migratory response, moving toward substrate tension and away from compression.[25] Application of controlled compression/tension transmitted through flexible substrates *in vitro* has also revealed that cells reorient themselves perpendicular to applied stretch.[50–52] It is clear from these examples that mechanical cues transmitted via the substrate influence adhesion, migration, along with cell polarization, and importantly cell fate.

Although substrate stiffness is important, geometric influences should also be considered. Geometric constraints on 2D substrates, limiting cell spreading and tension, have also been shown to direct the fate of stem cells.[13,24,34,35] More recently, cross-linked gels have been used to provide a 3D scaffold for cell culture. The differentiation of stem cells in these 3D environments appears to be controversial among researchers.[8,53] New evidence suggests that stem cell fate is regulated by different mechanisms that depend on the type of hydrogel employed, but that it may involve the degradation of ECM proteins in an effort to generate tension.[53]

Arrays of elastomeric pillars have also been used to examine cellular forces during adhesion. In a recent study,[33] microposts of varying stiffness and diameters were used to examine forces during the initial cell–ECM contact event. Micron-sized diameter posts resulted in global contractions toward the cell center, while submicron diameter pillars resulted in localized force balancing. The authors found that cells sensed substrate rigidity through periodic contractions. By adjusting their contractility, mouse embryonic fibroblasts maintained relatively stable deformations on the order of ~60 nm every minute. Moreover, the authors demonstrated that paxillin and phosphorylated myosin light chain are key components involved in this contractility, which occurs at a length scale of ~1 μm.[33] This particular example again demonstrates that cells respond to their substrate properties, by regulating cellular tension through focal adhesions.

2.2. Integrins are integral

As previously discussed, the ECM largely influences cellular adhesion; however, it does so by interacting with integrins (see Fig. 5.1B and C). Upon binding to the ECM, integrins form clusters resulting in the recruitment and grouping of scaffolding proteins, activation of tyrosine kinase and phosphatase signaling, and coupling of cell-generated forces.[31] The "integrin adhesome" (including its accessory proteins) are key regulators of physical phenomena such as cell motility, adhesion, matrix deformation, and remodeling.[29] Integrins also act as sensors, by responding to cues from the ECM, along with other signaling molecules such as kinases, phosphatases, and adaptor proteins.

Focal adhesion complexes are comprised of more than 150 proteins,[29] which all play a role in their formation and maintenance. Talins are particularly important, as they have been shown to bind β-integrins to the actin cytoskeleton (see Fig. 5.1C).[32] Interestingly, their recruitment is amplified by cytoskeletal tension. Some researchers hypothesize that the early formation of a nascent focal adhesion is reliant upon the interaction of two talin proteins connecting α and β integrins to actin.[54] Kindlins 2 and 3 are necessary along with talin to activate integrins.[55–58] Following this, many additional proteins must be recruited toward these sites in order to form the complex structures that comprise a fully mature focal adhesion site. Vinculin in particular, has been shown to play a role in the activation of integrin clustering following its attachment with talin.[59] Vinculin also attaches to actin, and so reinforces the focal adhesion site.

2.3. Formation of focal adhesions

Formation of nascent focal adhesions, or focal complexes, begins with force sensing. Protrusions at the cell edge, namely filopodia and lamellipodia, actively explore their surrounding microenvironment (Fig. 5.3). Filopodia are composed of actin bundles and are covered by a veil-like structure, known as lamellipodia, which are formed by Arp 2/3 dependent actin polymerization.[60] Nascent adhesion sites associated with these structures are ~100 nm in diameter, although there have been reports of smaller integrin-associated structures (~30–40 nm).[61,62] Focal adhesion formation occurs under these protruding structures, which is not surprising, since adhesion is strongly associated with motility.

Cells propel forward by actin polymerization in the direction of motion; however, there is an overall backward flow of actin, known as actin

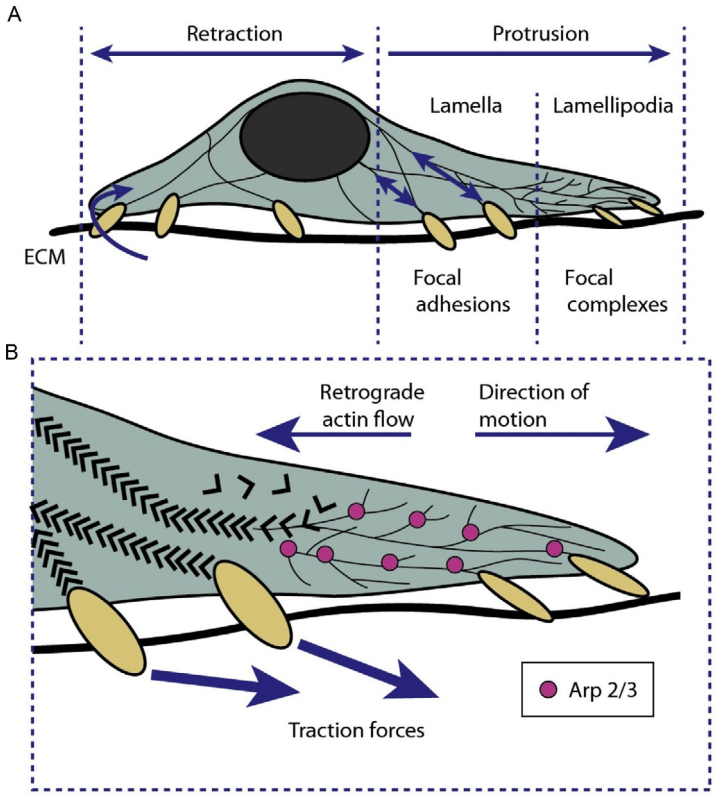

Figure 5.3 Cellular adhesion is a key process involved in motility. (A) Schematic representation of a motile cell demonstrating protrusion in the leading edge and retraction of the cell rear. Small nascent adhesions (also known as focal complexes) are formed in the lamellipodia, whereas mature focal adhesions form in the lamella. Tension generated in stress fibers is necessary for the promotion of focal complexes into mature focal adhesions. (B) Magnified view of the leading edge of a motile cell. Actin polymerization occurs in two forms: actin branching is promoted by Arp 2/3 which occurs in the lamellipodia, and actin polymerization of stress fibers occurs in the lamella. These regions are associated with the retrograde flow of actin, which occurs in the opposite direction to that of motion. Actin flow decreases dramatically upon the formation of a new focal adhesion (and so is slower in the lamella).

retrograde flow.[14] The rate of polymerization in the direction of motion is slower than that of retrograde flow, yet fibroblasts and epithelial cells are still able to migrate at rates on the order of a few millimeters per minute.[14,63] A closer look at the leading edge (Fig. 5.3B) reveals a decreasing gradient of actin, from the lamellipodia tip region to the lamella. The outer lamellipodia is a region of fast retrograde actin flow, whereas the inner lamella

is a distinctly slower flow region. Alexandrova *et al.*[14] demonstrated that flow drastically slows, within seconds, following the formation of a new focal adhesion site. Through its integration with the actomyosin network, the lamella is what drives the cell forward.[63] Components of actin branching (Arp 2/3 complex and ADF/cofilin) are no longer present in the lamella; however, components of actin bundles are (topomyosin and myosin II).[63,64] Myosin II contractility is responsible for the retrograde flow of actin in the lamellar region. These contractile forces occur above stationary focal complexes, and so there is a transmission of force to these elements.[65] Retrograde flow provides the forces necessary to develop these nascent sites into fully mature focal adhesions.[18]

As cells spread or migrate, focal complexes transition into mature focal adhesions under lamellar regions, not at the cell periphery. Mature adhesions are usually identified by their size, as they appear elongated and visibly connected to actin fibers (see Fig. 5.1A). Well-known proteins, such as zyxin, can also be used for identification, as they appear only upon maturation.[12] Interestingly, nascent adhesions have been shown to generate stronger traction than mature adhesions.[12] Although this seems counterintuitive, strong forces are necessary only for the initiation of focal adhesion growth. In a recent study, high-resolution time lapse traction force microscopy (TFM) demonstrated that traction forces are asymmetric within a single mature focal adhesion.[15] The distribution of traction indicated a peak near the center, usually directed toward the cell front. TFM performed on a number of mature focal adhesions resulted in the observation of two distinct modes of traction: a stable mode and a tugging mode. Moreover, loss of tension, either by a reduction in substrate stiffness or by inhibition of Rho-dependent kinase (ROCK) resulted in increased numbers of focal adhesions presenting tugging traction.[15]

It is clear from the above examples, that without actin polymerization or myosin II contractility focal adhesions cannot form.[66] These highly localized growth patterns require a range of force magnitudes, which must be sensed by the focal complex. Potential mechanosensors include integrins, mechanosensitive ion channels, and stretch sensitive proteins, such as p130Cas.[67] Other known force-sensitive proteins include the Src family kinase,[68] focal adhesion kinase (FAK) (reviewed in Ref. 69), Src homology 2 (SH2) domain-containing phosphatase,[70] and receptor-like protein tyrosine phosphatases (reviewed in Ref. 71). FAK/Src signaling in particular has been shown to regulate turnover of adhesions at the leading edge.[72–75]

Interestingly, although forces at the leading edge are what drive the formation of nascent adhesions, forces at the rear of the cell also cause their degradation.[76–78] Traction forces exerted by the cell onto its substrate are likely responsible for triggering their disassembly. In addition, the Rho/ROCK pathways have been implicated in cell retraction.[75] Two types of disassembly have been proposed: weakening of the integrin–actin cytoskeleton bonds or of the integrin–ECM bonds. Interestingly, FAK, Src, and SH2, which promote phosphorylation and assembly of focal adhesions, may also play a role in their disassembly.[79] Moreover, differences in molecular compositions, specifically the activation of myosin IIA in the rear of the cell, may influence cell polarity and direct migration.[80] A comprehensive review on nonmuscle myosin II and its role in adhesion can be found in Ref. 81.

It is quite evident that the progression of growth, from nascent to mature adhesion sites, is reliant upon mechanical cues. It is these mechanical cues that initiate integrin clustering, protein recruitment, and conformational changes of proteins, such as talin, allowing for binding of vinculin, and the growth of these sites. Vinculin is also an important regulator of mechanical forces within adhesion sites (reviewed in Ref. 82). Considering the large number of possible mechanosensors, it is difficult to understand the exact mechanisms behind their concerted response to mechanical force. Wozniak *et al.* have provided a thorough overview of the various effects of different focal adhesion molecules on integrin activity, mechanosensing, cell turnover, migration, and proliferation.[83] Focal adhesions are certainly sensitive to forces—whether generated externally (e.g., from the ECM) or internally (actin-generated forces). Moreover, actin polymerization is known to play a major role in the formation and maintenance of these sites.

2.4. Cytoskeletal interplay

Although actin polymerization influences the formation and growth of focal adhesions, these sites also affect the growth of actin (see Fig. 5.4). In fact, actin nucleation has been shown to occur mainly at the ends of stress fibers associated with focal adhesion sites.[84] Stress fibers have been categorized into three major classes: ventral, dorsal, and transverse arcs (a nice overview can be found in Ref. 85). The most commonly observed are ventral stress fibers, which appear in the basal region of the cell, wherein bundles of actin filaments are joined end to end by focal adhesions.[28] Studies have shown that dorsal fibers form by elongation at focal adhesions, resulting in short

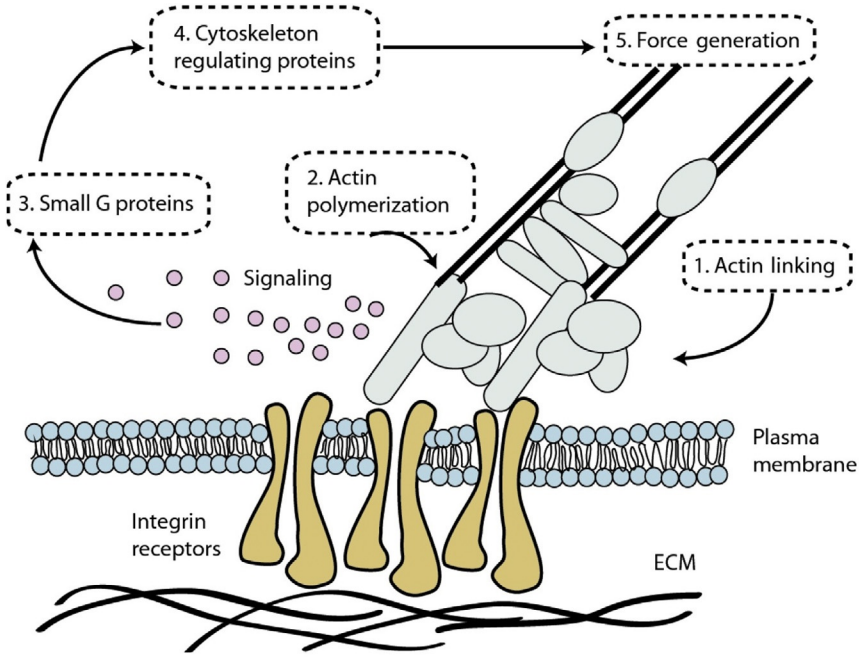

Figure 5.4 Both focal adhesions and actin regulate one another as part of a feedback loop. Integrins are transmembrane receptors, which cluster together and attach to various ECM ligands. These contacts attract actin-linking molecules and promote actin polymerization at the attachment site. However, the rate of polymerization is affected by the unified response of the entire focal adhesion system to interactions with the ECM (stiffness, composition, and ligand density all affect integrin adhesions). Actin polymerization further promotes signaling of GEFs and GTPase by FAK and Src, thus activating small G proteins (e.g., Rho and Rac). Subsequently, G proteins initiate cytoskeleton regulating proteins, and thus force generation of the actomyosin network. Furthermore, tension along stress fibers also activates mechanosensitive molecules, such as vinculin and zyxin. *Adapted from Ref. 18.*

filaments containing α-actinin, which then join to myosin clusters.[86] Ventral fibers then form by the connection of two dorsal fiber ends.[86] By culturing cells on specifically shaped micropatterned surfaces, Thery *et al.* have shown that stress fiber growth follows focal adhesion formation along the edges of these adhesive geometries.[87] Stress fibers are also associated with focal adhesion formation at the lamella/lamellipodia boundary.[14] Cells treated with Cytochalasin D, a well-known actin depolymerizer, results in a reduced actin flow rate, leading to inhibition of focal adhesion formation.[14] There is a clear interplay between tension in stress fibers, and focal adhesions, that is likely also influenced by signaling mechanisms.

The major regulators of the cytoskeleton are the small Rho family of GTPases: Rho and Rac. Rho is activated by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP.[88–91] Rac activation follows adhesion of the GEF known as the DOCK180–ELMO complex, which is activated by paxillin and p130CAS (known FA proteins).[18] Besides GEFs, integrin adhesion also negatively affects RhoA activation through GTPase-activating proteins (GAPs). FAKs are known to regulate the transduction of integrin signals to GEFs and GAPs through binding, phosphorylation, and activation alongside other proteins such as Src (reviewed in Refs. 10,69). It is clear that factors allowing for the transmission of integrin signals to GEFs and GAPs are important; however, many questions still surround their specificity and activation.[18]

Stress fibers are highly cross-linked structures and are associated with myosin II. These structures are under continuous tensile and compressive stresses and provide a constant source of mechanical force to mature focal adhesions. These stresses are transferred via integrins to the ECM. Cells have been shown to produce drastic forces upon their substrates, which has been shown *in vitro* by substrate wrinkling.[92] Moreover, as discussed earlier, traction within a particular focal adhesion can be modulated by ECM rigidity or actomyosin tension.[15] Cells appear to maintain a quasi-homeostatic tension with their substrate that was found to be dependent upon extracellular signal-related kinase and Rho.[38] These examples demonstrate the existence of a feedback mechanism associated with focal adhesions, wherein actomyosin tension is regulated in response to matrix stiffness.

Although we have only discussed actin, the role of microtubules is indispensible in the regulation of focal adhesions. In brief, microtubules have been shown to target and disassemble focal adhesions, a process dependent upon FAK (a known activator of adhesion turnover) and dynamin (a GTPase involved with endocytosis).[93] In this way, microtubules disrupt local adhesion components, likely resulting in local tension relief. Independently, activation of actomyosin contractility (using calyculin A),[94,95] or depolymerization of microtubules (using nocodazole) has been shown to result in increased cell spreading, morphological changes, and the formation of focal adhesions.[96,97] However, a recent study demonstrated that when employed consecutively, these treatments do not have an additive effect. In contrast, there were time dependent changes in cell cortical elasticity and long-term decreases in cell height and focal adhesion size.[97] Clearly, myosin II-dependent contractility and microtubules are intimately linked in regards to their influence on focal adhesion dynamics. Both

microtubules and actin appear to be key players in the maintenance of focal adhesion turnover.

3. AFM AS A TOOL TO STIMULATE A CELLULAR RESPONSE

As we have discussed in the preceding section, mechanical forces are necessary for the initiation, maturation, and disassembly of focal adhesions. Integrin-mediated adhesion sites allow for the transmission of internally generated forces to the ECM and vice versa. Both actomyosin generated forces and those arising from the substrate influence one another and focal adhesion formation. Considering that mechanical forces are well known to influence cellular fate and function, recent decades have given rise to numerous studies employing a variety of tools and techniques to mechanically manipulate cells. Tools such as micropipette aspiration,[98] magnetic bead twisting cytometry,[99] optical stretchers,[100] optical traps,[101–103] and AFM,[104] are just a few of the tools used to induce a cellular response. Although each technique has its own advantages and disadvantages; here, we focus on AFM as a unique tool for examining the mechanisms behind cellular adhesion.

The AFM is a versatile tool for studying the cellular response to force. Besides its capacity for high-resolution imaging, AFM can be used to apply precise pico- to nano-Newton forces in order to measure both the elastic and viscoelastic properties of cells.[104–106] Depending on the shape and size of the cantilever probe, the cell can be subjected to either whole-cell or localized forces. Changes in Young's modulus can be measured by fitting force–indentation curves to modified versions of the Hertz contact model.[107] Alternatively, one can employ the cantilever in a raster-scanning mode across the sample to generate force maps. Force maps of cells can be used to visualize variations in cell height, adhesion strength, and to measure variations in elasticity across the cell.[6,105,108] Measurements of the elastic properties of cells can lend information to the physical changes of the cell, as they are exposed to various chemical or mechanical cues. Considering that actomyosin contractility is directly related to focal adhesion dynamics, elasticity measurements are both relevant and important.

In the following subsections, we focus on the discussion of two studies which examine the behavior of cells following direct application of mechanical force using AFM. The first study by Guolla *et al.*[5] looks at how stress fibers deform in response to force, and how their strain leads to actin and focal adhesion remodeling. The second study by Al-Rekabi *et al.*[6] examines

changes in traction forces in response to an applied force and altered substrate rigidity. These examples demonstrate the efficacy of using the AFM as a tool to apply precise forces, and to measure cellular elastic properties, all while using visual methods to elucidate the mechanics and function of cells, with particular focus on stress fibers and focal adhesions here.

3.1. Cytoskeletal strain directs focal adhesion formation

In vitro application of mechanical forces applied directly to cells has resulted in direct stress fiber and focal adhesion remodeling, over both short and long durations.[109–111] Considering that tension plays a direct role in the formation and remodeling of focal adhesions,[15,66] measurements of deformation and strain of stress fibers may provide information relevant to the development of adhesion sites. In a recent study,[5] both lateral stress fiber deformations and longitudinal strains were measured following local cell perturbations using an AFM. In particular, nanoscale forces (0–20 nN) were applied to NIH3T3 fibroblasts transiently transfected with actin–EGFP (Fig. 5.5A). Laser scanning confocal microscopy (LSCM) was used to observe the deformation of actin fibers following several minutes of force application. Lateral displacement vectors were measured by tracking regularly spaced (~5 μm) points along the stress fibers over time (Fig. 5.5B).

Applying a force apically to cells resulted in nonisotropic deformations of stress fibers.[5] Although many stress fibers displayed no lateral motion; fibers that deformed did so proportionally to the amount load magnitude (Fig. 5.5C). While unperturbed cells also displayed stress fiber movements over time, an applied force resulted in a significant increase in displacements. For instance, stress fibers near the point of contact (within 5–10 μm) resulted in an average lateral motion bulging outwardly (~100–300 nm). This force-dependent displacement occurred within 60 s of force application and increased with increasing time. On the other hand, stress fibers distant from the point of loading (30–50 μm away) demonstrated a retraction of the cell edge toward the center of the cell, following 1 min of force application, indicative of actin remodeling (see Fig. 5.6).

Importantly, the remodeling and relaxation of the deformed stress fibers were shown to be directly correlated to focal adhesion movement. This was directly visualized by cells simultaneously expressing actin–EGFP and zyxin–mRFP (Fig. 5.7).[5] Zyxin, as was discussed in preceding sections, is a well-known focal adhesion protein that has been described as a tension sensor. Zyxin has been shown to appear with increasing tension, and

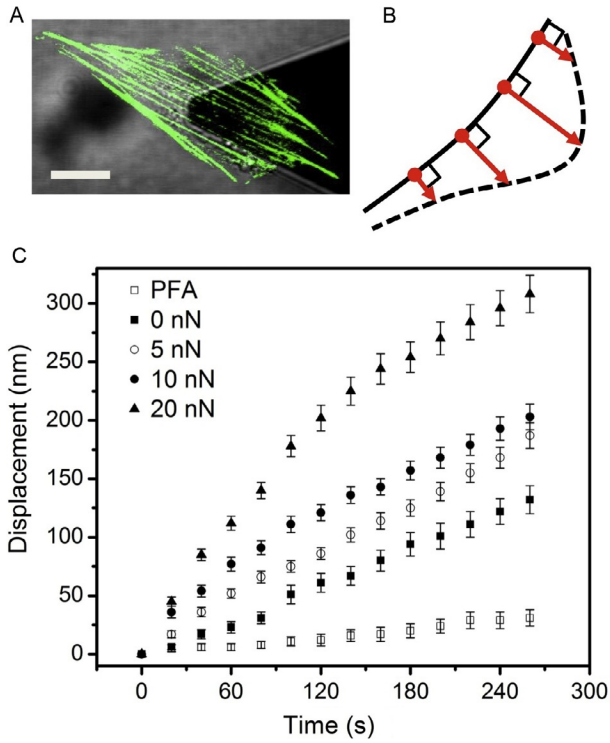

Figure 5.5 Lateral stress fiber deformations in response to an applied force. (A) AFM tip is used to apply a local force, centered over the nucleus, to NIH3T3 fibroblast expressing actin–EGFP. Scale bar is 15 μm. (B) Stress fiber deformations were tracked by mapping perpendicular displacement vectors (red) (gray in print) from an initial point on a filament at time zero (solid line) to a new point on the same filament at the next time step (dashed line). (C) Average displacement of stress fibers over time in response to locally applied forces. Stress fiber displacement is proportional to the applied force and occurs within 20 s. Displacement occurring in cells in the absence of force (0 nN) represents normal stress fiber remodeling dynamics. Displacements for cells treated with paraformaldehyde (PFA) can be attributed to microscope drift or error within the tracking method. Displacement is shown as mean + s.e.m. *Reproduced with permission from Ref. 5.*

conversely disappear with decreasing tension at both mature and nascent focal adhesion sites.[111] Guolla *et al.*[5] used kymographs to demonstrate that zyxin remained visibly linked with stationary stress fibers (in the presence of an applied force). Moreover, only large deformations of stress fibers resulted in the movement of focal adhesions. Consistent fluorescence intensity of zyxin–mRFP within the kymographs suggests that tension remains relatively stable among the stress fibers, as the concentration of this force-sensitive protein is neither increased nor decreased.

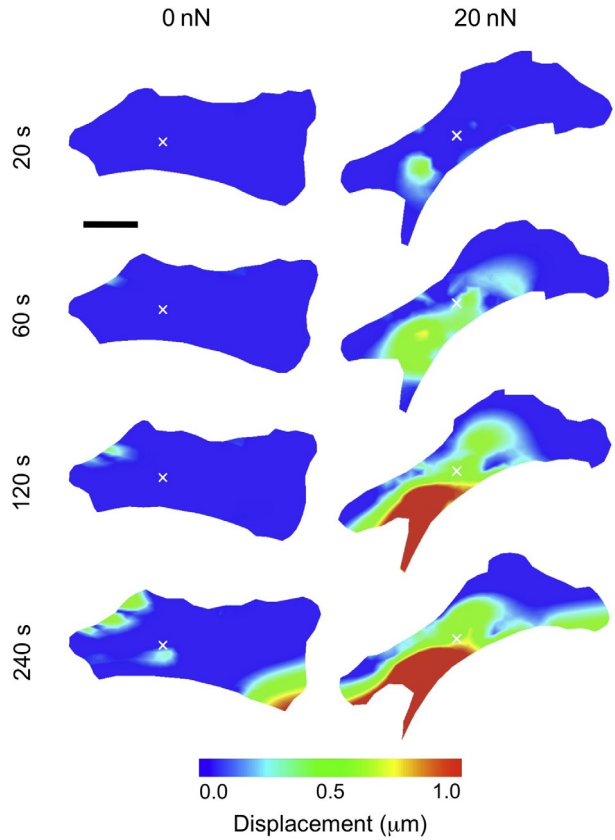

Figure 5.6 Spatial heat maps of stress fiber displacement in fibroblast cells experiencing 0 or 20 nN of applied force. The magnitude of the displacement vectors was plotted as spatial heat maps, demonstrating the increased motion of stress fibers in response to force. Stress fiber displacements occurred immediately (within 20 s) and near the point of force application (the contact point of the AFM cantilever is marked with a white "*x*" and is over the center of the nucleus). At later times, retraction sometimes takes place at distant cell edges (red areas at 120 and 240 s after the application of 20 nN). Scale bar: 15 μm. *Reproduced with permission from Ref. 5.*

Although lateral displacement vectors provided general information about stress fiber motion,[5] tension along the length of these fibers is what has been shown previously to contribute to remodeling of focal adhesion sites.[112] In order to measure strain, a regularly spaced (5 μm) grid was photobleached over images of the actin stress fibers. Deformations in the repeated bleached and nonbleached segments were measured over time, and measurable strains along the fibers. Basal measurements (0 nN load) showed a highly

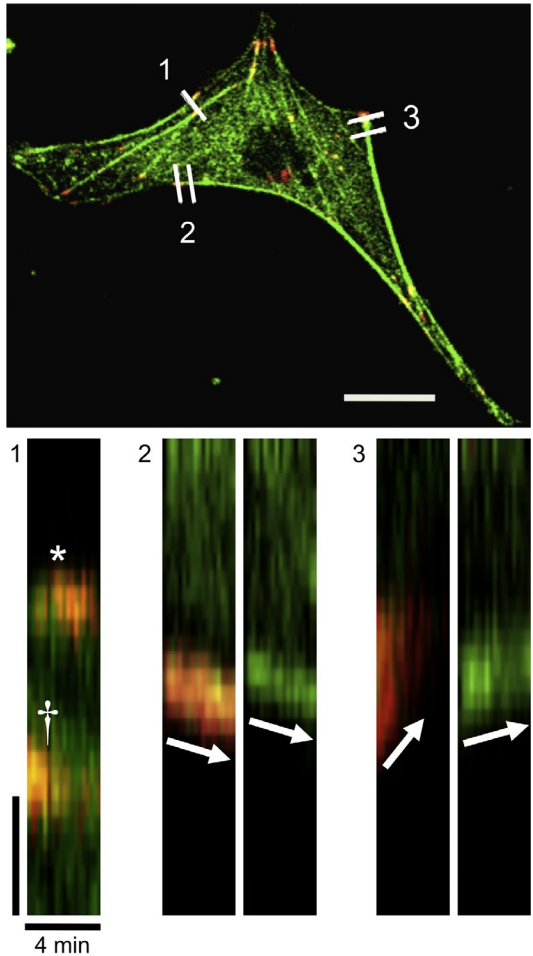

Figure 5.7 Correlated movement of focal adhesions and stress fibers in response to local forces. An LSCM image of an NIH3T3 cell expressing both actin–GFP and zyxin–mRFP (scale bar = 24 μm). Kymographs were generated for three separate locations on the cell (vertical scale bar = 2 μm). It can be seen in (1) that these fibers are sliding along their length (but not displacing laterally) which results in the movement of one focal adhesion into the image at ~120 s (*) and another out of the image at ~60 s (†) after force application. In (2) and (3), a correlated downward lateral displacement of F-actin filaments and focal adhesions are observed (upward and downward arrows). In (3), the F-actin filament begins to retract downward after force application, resulting in the movement of an associated focal adhesion. *Reproduced with permission from Ref. 5.*

heterogeneous distribution of tensile and compressive strains along the length of stress fibers (see Fig. 5.8). Moreover, upon force application (20 nN), strain variations increased by nearly 50% and resulted in more contractile strains. This was hypothesized to be the result of force-induced upregulation of myosin II.[5]

In order to examine their role in the transmission of force, the authors employed selective inhibitors of microtubule polymerization (nocodazole) and actin polymerization (Y27632—specifically inhibits ROCK). Treatment with nocodazole resulted in a loss of induced stress fiber remodeling and demonstrated the necessity of microtubules for any force transmission through the cell.[5] Alternately, treatment with Y27632 inhibited the formation of actin stress fibers and caused a reduction in the cell's elastic modulus (1 kPa, compared to 7 kPa for untreated). Despite the loss of intact stress fibers, forces were propagated throughout the cell, but were observed to be isotropic in nature, and noncontractile at the cell edge.[5]

The importance of this study is that it demonstrated heterogeneous displacement and strain among actin stress fibers, which was directly correlated to focal adhesion movements. Local forces applied to the cell were shown to result in immediate deformations within the vicinity of the load, and contractile movements occurring later on in more distal regions. These results clearly demonstrate that mechanical cues initiate long-term actin and focal adhesion remodeling.

3.2. Forces and substrate elasticity influence traction

Many studies, such as the example previously discussed,[5] have shown that mechanical cues influence cellular structure,[17,113,114] as well as their function (reviewed in Ref. 115). Moreover, both the mechanical and biochemical properties of the ECM are known to direct cell fate and function.[23,116,117] For example, cells actively probe their surrounding microenvironment, and can sense changes in matrix elasticity through a complex interplay of actomyosin dynamics and focal adhesion remodeling.[18] Through these processes, the cell applies dynamic traction forces on its underlying substrate.[118,119] We can naturally anticipate then, that both extracellular forces and the material properties of the cellular microenvironment should concurrently affect critical cellular processes, including the formation of focal adhesions and generation of traction forces.

In a recent study, Al-Rekabi and Pelling studied the response of cells to both external force and substrate elasticity using simultaneous AFM and

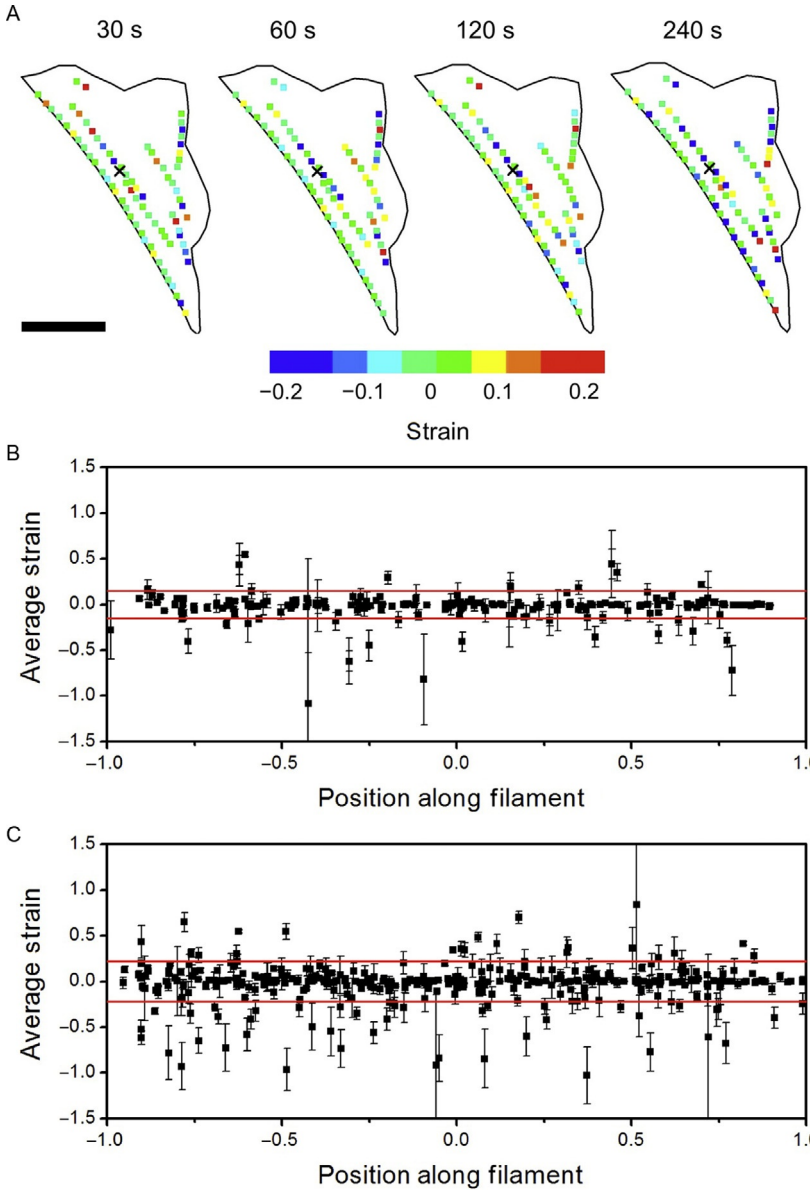

Figure 5.8 Heterogeneous strain occurs along the length of stress fibers. (A) A diagram of stress fiber strain for a cell undergoing mechanical stimulation (20 nN). Strain along each stress fiber is shown as a colored spot according to magnitude (the contact point of the AFM is marked with a black "*x*"). The strain magnitude and sign is heterogeneous along the length of any given filament and fluctuates in time, indicating rapid stretch/contraction dynamics. Scale bar: 20 μm. (B and C) Plots of normalized stress fiber strain as a function of position along the fiber after 240 s. The measured strain tended to fluctuate around zero. However, the standard deviation (red lines) for nonstimulated cells (B) is 0.15 and mechanically stimulated cells (C) is 0.22; a 50% increase. These strain fluctuations might reflect an increase in actomyosin activity in response to applied force. Error bars are s.e.m. *Reproduced with permission from Ref. 5.*

TFM on C2C12 mouse myoblasts.[6] Considering that muscles *in vivo* involve highly dynamic tensile and compressive forces and changes to the microenvironment, these muscle precursor cells are ideal for studying these two effects simultaneously. This is particularly true as they have been shown to provide traction forces while withstanding large substrate stretching and actin depolymerization.[120] The elasticity of muscle tissue has been shown to increase nearly eightfold during its transition from resting to active muscle tissue.[121–125] Muscle contractions promote changes in the microenvironment of myoblast cells, which are necessary for the formation of myotubes, a process known as myogenesis.[126–130] Numerous studies have shown that traction forces generated by C2C12 cells vary drastically and are dependent on the present stage of myogenesis.[121] Interestingly, the onset of myogenesis begins on stiffer substrates (~38–97 kPa) in muscle tissue.[121,122,129] Conversely, myotube formation has been shown to occur optimally on softer substrates (~12–39 kPa),[119] which can be linked to resting muscle tissue. With this in mind, the authors hypothesized that myoblast-generated traction forces would increase during the application of an externally applied force, however, only on substrates imitating active muscle tissue.

In order to test the hypothesis, C2C12s were grown on glutaraldehyde cross-linked gelatine (GXG) substrates of varying stiffness, ranging from soft to stiff (~16–89 kPa), mimicking resting and active tissue, respectively.[121–124] The stiffness of the substrates was increased by accumulative gelatine content, from 3% to 7% (w/v), and was verified by measuring force–indentation curves and fitting them to the modified Hertz model, as well as by AFM force-mapping in some cases. Force–indentation measurements of myoblasts revealed a relatively constant stiffness (~7 kPa) despite the cells having been cultured on substrates with increasing stiffness.[6]

Many researchers have used TFM techniques to quantify the level of force exerted by cells onto their substrates.[7–9,17] In order to perform TFM, fluorescent beads are embedded into a flexible, clear substrate, allowing for their movement to be tracked by optical microscopy. For the study by Al-Rekabi *et al.*, 200-nm red fluorescent beads were embedded within the cross-linked gelatine.[6] TFM requires the capture of two simultaneous images: a phase-contrast image of the cell, and a fluorescent image of the beads. The cross-linked gelatine substrates used in this particular study were ~500 μm thick and clear, allowing for both confocal and phase-contrast imaging (Fig. 5.9A). Images were captured every 30 s for 120 s using a CCD camera. Two methods of analysis were carried out:

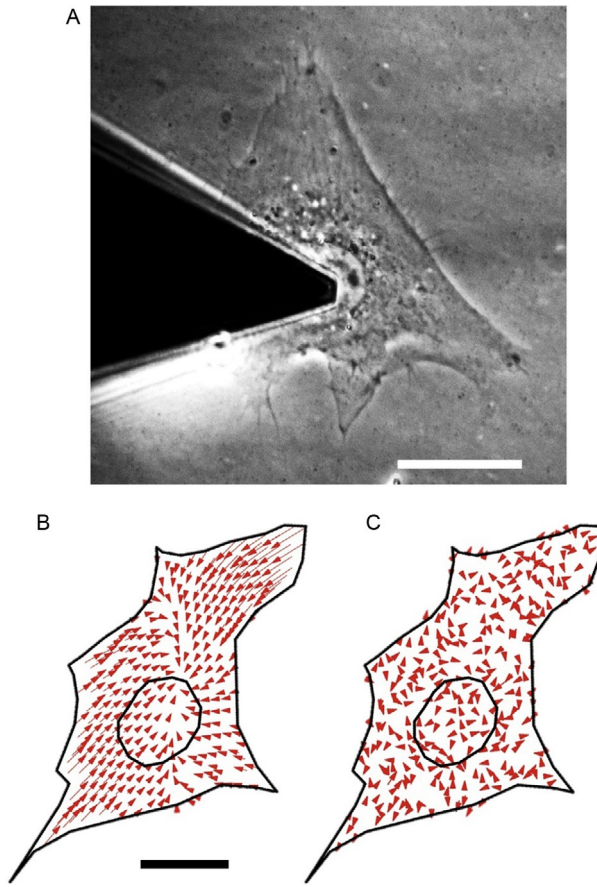

Figure 5.9 AFM and TFM measurements performed on C2C12 cells cultured on a tunable hydrogel. (A) A phase-contrast image of a C2C12 cell on a ~64 kPa glutaraldehyde-cross-linked gelatin substrate with an AFM tip pushing on the nucleus with a constant force (10 nN). (B and C) Two methods were used to measure the traction forces in this experiment: (B) absolute TFM reveals a smooth traction force field with force vectors pointing toward the cell center, indicative of cell contractility and (C) differential TFM illustrates that the change in traction forces between sequential times can be spatially heterogeneous. *Reproduced with permission from Ref. 6.*

absolute TFM and differential TFM. In the absolute method, bead displacements are tracked over time and then compared to the corresponding null image—wherein the cell is removed from the substrate (with EDTA in this case), thus relieving the substrate of elastic strain. In the differential method, TFM is first carried out on the cell prior to applying mechanical stimulation, and then while applying a continuous load. Thus, the

differential method allows for measurement of the change in traction forces between sequential images.

The absolute method resulted in traction vectors directed toward the centroid of the cell (Fig. 5.9B), indicating an overall contractile behavior.[6] Differential TFM, on the other hand, resulted in varied traction vectors, revealing the dynamic temporal fluctuations of locally generated traction forces (Fig. 5.9C).

By stimulating the cells with a local force (10 nN applied via AFM), absolute TFM revealed that myoblast cells responded as hypothesized; traction forces increased dramatically, however, only on stiff substrates (Fig. 5.10A and B).[6] Absolute traction forces remained heightened for long durations (120 s) of applied force. This increase was shown to be transient in nature when analyzed with the differential method, as traction forces increased within 30 s, and then diminished to basal levels following 60 s (Fig. 5.10C and D). Interestingly, stimulation of cells with mechanical force appeared to have no significant influence on traction force generation when cells were grown on soft substrates (16–51 kPa GXG substrates), as visualized by traction force heat maps (Fig. 5.11).[6] These results demonstrate the complex interplay between substrate stiffness and external force on cellular traction. This work also highlights the different findings presented by absolute and differential TFM analysis methods.

In order to examine the cytoskeleton's role in traction force generation, the authors employed Y27632 to selectively inhibit ROCK, and blebbistatin to inhibit myosin II, separately.[6] Both treatments totally impaired the contractility of myoblasts, even after 240 s of applied force. The once elongated actin stress fibers of untreated cells became drastically reduced or altogether absent, due to these drug treatments, regardless of substrate. Moreover, the focal adhesions became smaller in size and less well defined.[6] Basal levels of traction, however, were unaltered, which is inconsistent with others.[7,17] It is possible that the response is cell type or dose dependent, or could differ due to the range of substrate stiffness employed, as others tend to implicate much softer (<10 kPa) or stiffer (glass) substrates. Considering that there was no increase in traction on stiff substrates either, this finding may suggest an inability of the cell to transmit the locally applied force throughout its cytoskeleton on soft substrates, which also resulted in a 20% decrease in focal adhesion size. Considering that traction force magnitude is directly related to size,[131,132] the authors speculated that focal adhesions must have a threshold below which they cannot resist contractility.

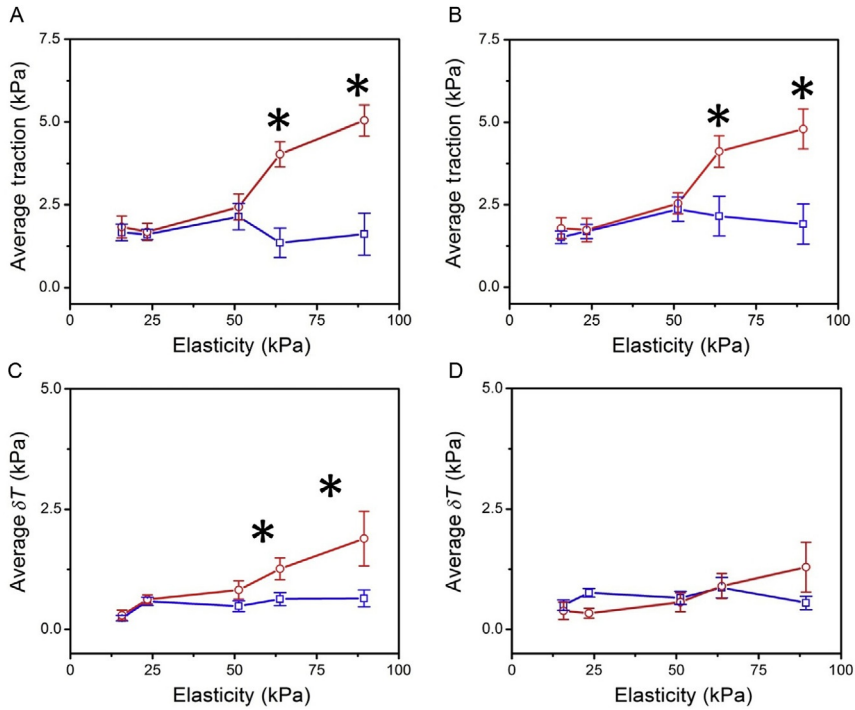

Figure 5.10 Absolute and differential traction force dynamics on substrates mimicking resting and active muscle tissue in response to a local force. (A) Average absolute traction as a function of substrate elasticity and force: control/0 nN (blue squares) (dark gray in print) and 10 nN (red circles) (light gray in print) after 30 s. C2C12s clearly show an increase in traction when subjected to a 10 nN force on substrates with an elasticity of ~64 kPa ($P = 0.003$) and ~89 kPa ($P = 0.004$). (B) Average absolute traction after 120 s (~64 kPa ($P = 0.019$) and ~89 kPa ($P = 0.004$)). (C) Differential traction reveals a statistically significant increase after 30 s of stimulation with a constant 10 nN force (in agreement with (A)) on ~64 kPa ($P = 0.032$) and ~89 kPa ($P = 0.022$) substrates: control (blue squares) (dark gray in print) and 10 nN (red circles) (gray in print). (D) The average change in traction forces dissipates to basal levels for 10 nN after 60 s. *Reproduced with permission from Ref. 6.*

In conclusion, this work demonstrated that mechanical cues (by AFM) and substrate material properties both affect the outcome of cellular traction forces of C2C12s, in a complex manner. In particular, myoblasts responded to mechanical force with a transient increase in traction forces, only apparent on substrates mimicking stiff tissue. Local traction hotspots (Fig. 5.11) appeared throughout the basal cell region, confirming that the localized force from the AFM tip results in remodeling far from the point of contact, as also seen in the previously described work in Section 3.1.[5] As well, both ROCK

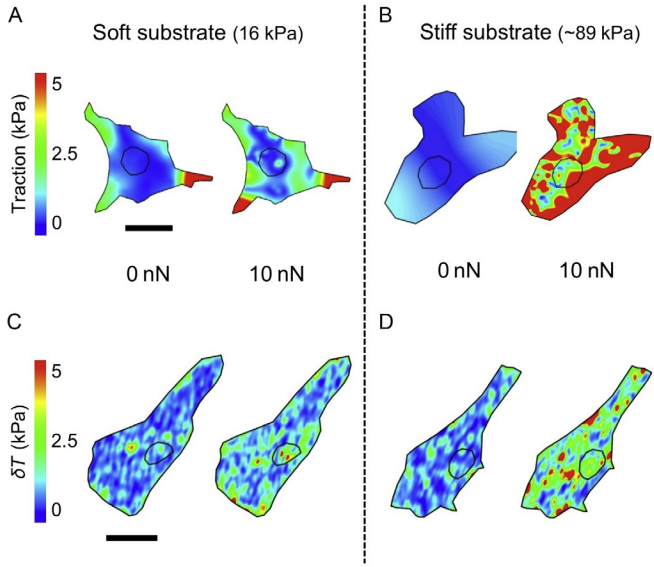

Figure 5.11 Heat maps for representative cells for the absolute and differential traction force measurements. (A) Absolute traction for a cell exposed to 0 and 10 nN of force on a soft (∼16 kPa) substrate. (B) Absolute traction for a cell exposed to 0 and 10 nN of force on a stiff (∼89 kPa) substrate. A significant increase in traction is observed alongside the appearance of local traction hotspots throughout the contact area. (C) Differential traction for a cell exposed to 0 and 10 nN of force on a soft (∼16 kPa) substrate. (D) Differential traction for a cell exposed to 0 and 10 nN of force on a stiff (∼89 kPa) substrate. A significant increase in traction fluctuations are observed as well as local hotspots throughout the contact area. These results demonstrate that both absolute and differential traction forces are profoundly affected when a mechanical force is applied to these cells, particularly on stiff substrates. *Reproduced with permission from Ref. 6.*

and myosin II were necessary mechanotransduction pathways for the initiation of increased traction force generation on stiff substrates. In the context of *in vivo* myogenesis, the notion stands that myotube formation occurs optimally on soft substrates,[125,133,134] yet follows primary initiation on stiff substrates.[135,136] Muscle contraction during stretching or exercise is known to produce many changes *in vivo* with regards to myogenesis.[136–139] The increase in traction forces on stiff substrates in response to applied force, as seen in this reviewed work, could implicate the combination of these two stimuli for the onset of myogenesis. Of course, the results are limited to the experimental conditions; however, it does highlight the necessity for research into the response of cells to multiple stimuli.

4. FUTURE DIRECTIONS

Although many focal adhesion-associated proteins have been identified over the years, the inner mechanics of this important cell feature remain poorly understood. It is apparent that both biochemical and physical mechanisms are at play and that both the cell and its microenvironment act in unison. The composition and mechanical properties of the ECM largely influence the formation and growth of focal adhesions. Rigidity sensing of focal complexes, along with other internal mechanical cues, play a large role in the formation and maturation of focal adhesions. Mechanical forces are at play during a number of processes including: conformational changes of proteins in order to expose hidden binding sites, actin-generated tension-initiating nascent adhesions, myosin II-dependent contractility promoting the maturation of adhesions, and recruitment of associated proteins. Moreover, examples discussed in this chapter demonstrate the existence of a feedback mechanism associated with focal adhesions, wherein actomyosin tension is regulated in response to matrix stiffness.

Through a detailed discussion of two recent studies, we have seen that the combined use of AFM and optical microscopy techniques, allows for the investigation of the mechanics behind focal adhesion formation and traction force generation. Local deformations on the apical regions of cells were shown to transfer through the cytoskeleton to basal cell regions, influencing stress fibers and focal adhesions. Mechanical forces increased stress fiber deformations, and initiated long-term remodeling of stress fibers, and focal adhesion sites, as was demonstrated visually.

Traction forces, on the other hand, provide a measurable indication of a mechanotransductive response. It was demonstrated herein that different modes of analysis can also provide different insight into cellular-generated traction forces. For instance, it was seen that absolute TFM lends to an overall picture of cellular behavior—one that is generally contractile in nature. On the other hand, differential TFM analysis provides insight into the temporal behavior of local regions, revealing traction force hot spots.

Moreover, it was seen that when a cell is exposed to multiple forms of stimuli, the response can be complex. In the preceding example, it was shown that external mechanical stimulation affects traction alongside substrate elasticity. In particular, myoblasts were only sensitive to the applied forces on stiff substrates, reacting with a transient increase in generated traction.[6] Although studying a single effect, such as substrate elasticity is useful in

providing certain insight—it does not provide the whole picture, as clearly multiple mechanical influences simultaneously affect cellular adhesion *in vivo*. Future work will be necessary to expose the inner complexities of focal adhesion formation and remodeling. It will be necessary to involve a multitude of stimuli, in order to understand the complex interplay of the response mechanisms associated with focal adhesions.

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