THE EFFECT OF BIOLOGICAL LENGTH SCALE TOPOGRAPHY ON CELL SUBSTRATE ADHESION IN HUMAN CORNEAL EPITHELIAL CELLS

by

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Chemical Engineering)

at the

UNIVERSITY OF WISCONSIN-MADISON

2005

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submitted to the Graduate School of the
University of Wisconsin-Madison
in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

by

Nancy Wangechi Karuri

Date of Final Oral Examination: December 3, 2004

Month & Year Degree to be awarded: **December** **May** **August**

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Acknowledgements

I would like to thank Profs. Paul Nealey and Christopher Murphy for their guidance and advice from the inception to the completion of this dissertation. I would like to thank the research groups of both professors for their camaraderie, assistance and advice during the course of this project. I consulted with numerous individuals from both groups on a number of wide ranging problems; from cell biology to fluid transport and I am grateful for the assistance they provided. I am especially thankful to Ana, George and Sean for helping me start the project, teaching me many cell culture techniques and being invaluable sources of advice.

I would like to acknowledge the significant contribution of the center for nanotechnology and in particular the efforts of Scott Dhuey in creating the substrates that were pivotal for this study and I am indebted to Prof. Amy Wendt and her group for their contribution in the etching process that was necessary to create the topographic features. I am grateful for the patience and skill of John Cannon that resulted in a fully functional flow chamber.

I would also like to thank Prof. Ralph Albrecht for his guidance during the microscopy sections of this study and his counsel in both the project and in life in general. I am grateful to Phil Oshel for training me in scanning electron microscopy techniques and for providing useful pointers that were of great help when nothing else seemed to work. I am thankful to the Albrecht group members for their willingness to help with their laboratory equipment and their generosity with their reagents.

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I am thankful to Yong Jing Li for her friendship, kindness and patience in teaching me transmission electron microscopy techniques. I am also grateful to Randall Massey and Ben August for training in the use of the transmission electron microscope.

I am indebted to the faculty and staff at the department of Chemical and Biological Engineering, UW-Madison for their help, training and more importantly for the opportunity to be a part of an outstanding institution.

I would like to thank Prof. Henderson, Kelly Burton and the GERS group for their willingness to listen to my difficulties, their advice, encouragement and moral support during my stay in Madison.

I am thankful to Wanjugu, Wangari and Githenya for sharing many of my burdens and for making my journey lighter. I am thankful to Guka and Cucus for their support and for sharing their history and life experience. Finally and most importantly, I am indebted to my parents for their love, advice, support and encouragement. I endeavored to follow the guidance they dispensed during my life span and I am convinced it has been instrumental in the successful completion of my graduate studies.

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1. INTRODUCTION

1.1. Summary

This chapter is divided in three sections; the first section examines literature on cell-substrate adhesion and the effect of topography on cell behavior, the section presents the motivation behind this study and the third section presents an outline of this thesis. Included in the first section are details of the main protein molecules involved in cell-substrate adhesion as well as processes leading to their activation during cell-substrate adhesion. This includes integrin receptors, matrix proteins and cytoplasmic proteins such as focal adhesion kinase and vinculin. After which, the elements of the cytoskeleton and prominent actin cytoskeleton structures such as filopodia, lamellipodia, stress fibers and ruffles are introduced. The role of the cytoskeleton in mechanotransduction is then reviewed. Subsequently, the techniques used to probe the interactions between integrins and their ligands are reviewed. The first section ends with a review of studies investigating the influence of topography on cell behavior. The second section presents the motivation and aims behind this study. In the second section I present the hypothesis tested; that substratum topography is a significant stimulus in modulating the adhesion of anchorage dependent cells. Subsequently, the objectives of the study are presented. In the final section I present an outline of the thesis

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1.2. Background

Integrins as mediators of cell-substrate adhesion and signal transduction

Four decades ago, researchers used electron microscopy and interference-reflection microscopy to identify specialized adhesion sites between cells and the extracellular matrix (ECM) (Abercrombie and Dunn, 1975; Abercrombie et al., 1970; Abercrombie et al., 1971; Izzard and Lochner, 1976). The ECM is a mixture of fibrous proteins and polysaccharides secreted by cells and used for anchorage. These studies revealed the existence of specific sites, known as focal adhesions that were tear shaped, had micron scale dimensions and were located approximately 10-15 nm away from the substrates. Since then, adhesive interactions between cells and the ECM have been shown to regulate cell morphology (reviewed by (Ingber, 2003)), migratory properties (reviewed by (Palecek et al., (1999),) and growth and differentiation (reviewed by (Watt, 2002)). Focal adhesions are now associated with a plethora of proteins organized at the cytoplasm and forming a signaling complex as well as contractile bundles of actin filaments known as stress fibers. Figure 1.1 shows an inventory of the molecules associated with focal adhesions (Zamir and Geiger, 2001). This list includes integrins, cytoskeletal proteins, proteases, protein kinases and phosphatases, signaling molecules and proteins with unknown functions. Also evident in Fig. 1.1, is the fact that most of the components present have multiple binding sites for other components. This is an indication that binding can give rise to a number of different supramolecular structures. The growth of this inventory with time serves to demonstrate the complexity of cell-ECM

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adhesion and to highlight the need for investigations into the mechanisms that influence cell-matrix adhesion.

A transmembrane group of glycoprotein receptors known as integrins is largely responsible for mediating the interactions between cells and the ECM (Buck and Horwitz, 1987; Hynes, 1992b). Integrin receptors consist of heterodimers of α and β subunits that are non-covalently linked (Hynes, 1992b) (Fig. 1.2). Integrins are classified according to the type of β sub unit and there are 16 or more α sub units and 8 or more β sun units. α and β subunits associate to form at least 25 distinct integrins. Integrins have been shown to exhibit 'promiscuous' binding with respect to matrix proteins. Therefore, although some integrins bind to specific ligands, for instance $\alpha_5\beta_1$ integrins binds specifically to the ECM protein fibronectin; most recognize and bind several different proteins (Fig. 1.3).

Heparin sulfate proteoglycan cell-surface receptors, known as syndecans, are another group of transmembrane proteins that perform an important role in cell-substrate adhesion. They interact with ECM molecules, growth factors and protease inhibitors and subsequently influence cell proliferation, cell-cell adhesion and cell-ECM adhesion (Woods, 2001). While different classes of syndecans have been shown to have different functions in different cell types, cumulatively they are considered to be modulators of ligand-dependent activation of primary signaling receptors at the cell surface (Carey, 1997).

Studies with chemical cross linking of ligands, monoclonal antibodies, mutations, molecular modeling and x-ray crystallography have revealed three important integrin

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domains in cell-ECM adhesion. The first region consists of a seven repeat sequence of 60 amino acids in the N-terminal portion of the α chain (Loftus and Liddington, 1997; Webb et al., 1997). This domain forms a β-propeller structure, capable of binding both ligand and calcium ions and has been linked to protein-protein interactions. The second domain consists of an inserted domain of 200 amino acids found in many α chains that can bind both nucleotide and divalent cations (Bankston et al., 1995; Qu and Leahy, 1995). This second domain has been associated with ligand coordination. The third domain is an inserted domain in the β sub unit (Loftus and Liddington, 1997). It has been inferred that this domain plays a role in ligand binding. The three domains probably enable integrins to undergo dynamic structural changes and that allow for various integrin affinity states.

Integrins exist in different affinity states with respect to their ligands. The high affinity state can be achieved through a favorable environment that influences the integrin conformation. For instance, the presence of divalent cations and antibodies can increase integrin affinity towards it ligands, a process known as outside-in signaling. In addition, a high ligand affinity state can be reached in response to events within the cytoplasmic domain, a process known as inside-out signaling (Humphries, 1996; Keely et al., 1998; O'Toole et al., 1994). Both activation pathways create bidirectional flow of information going in and out of the cell that contributes to changes in cell adhesion, changes in expression of integrins and/or ligands, induction of matrix-degrading proteinases and changes in gene expression (Boudreau and Jones, 1999).

Integrin activation is characterized by the formation of clusters of adhesion molecules at the binding site. It has been shown that on ligand binding $\alpha_5\beta_1$ integrins

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form clusters of at least three integrins that induce complexing of proteins on the cytoplasmic side (Coussen et al., 2002). After cluster formation, the actin cytoskeleton is reorganized into stress fibers (Miyamoto et al., 1995). Studies have shown that in the early stages of focal adhesion formation, cell-ECM binding contains both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins (Zamir et al., 2000). As time progresses, a second type of adhesion site are observed on fibronectin-coated surfaces known as fibrillar adhesions (Zamir et al., 2000). These sites are rich in $\alpha_5\beta_1$ integrins and are separated from focal adhesion sites through coupling to the cytoskeleton. Motor proteins known as myosins connect $\alpha_5\beta_1$ associated cytoplasmic protein complexes and pull them towards the cell nucleus along actin stress fibers whereas the $\alpha_v\beta_3$ integrins remain in focal adhesions. Focal adhesions have been found to be rich in paxillin, vinculin and focal adhesion kinase. Fibrillar adhesions, on the other hand, are mainly associated with tensin on the cytoplasmic side. The formation of fibrillar adhesions is inhibited when fibronectin is covalently linked to glass surfaces (Zamir et al., 2000). These findings are an indicator that the ECM is not a static scaffold. By adhering to the ECM and moving on it, cells exert a traction force on the ECM that distorts it. In their report, Gao and colleagues examine cell adhesion and organization on fibronectin coated surfaces (Gao et al., 2003). According to their report, the price paid by the cell when it uses energy to change its physical and biochemical environment enhances adhesion by exposing binding sites buried in the native folds of extracellular matrix proteins, changing the relative distance of synergy sites to the binding site or deforming or straightening loops that contain molecular recognition sites. This may

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explain why smaller analogues of fibronectin are weaker in eliciting a full cellular response as compared to the full matrix protein.

The dual role of integrins as adhesion receptors and signal transducers has been demonstrated by a number of investigators. Kornberg and colleagues demonstrated that the tyrosine phosphorylation levels of a 115-130 kDa protein associated with $\alpha_3\beta_1$ integrins was higher in human carcinoma cells than in normal cells (Kornberg et al., 1992). This same type of protein was phosphorylated in NIH3T3 and rat embryo fibroblasts spreading on fibronectin-coated surfaces (Burridge et al., 1992; Guan et al., 1991). When they repeated the same experiments but with the incorporation of antibodies targeting non-integrin surface molecules or with surfaces coated with the adhesive polymer poly-L-lysine they did not observe the phosphorylation of this molecule. These and other results lead to the conclusion that integrins are able to transduce specific chemical signals from their extracellular environment and that these signals result in cellular changes.

Interestingly, integrins have no intrinsic signaling ability but their short cytoplasmic domains are associated with adapter proteins that connect integrins to the actin cytoskeleton, kinases and transmembrane growth factor receptors. A number of cytoplasmic proteins such as talin, α-actinin and focal adhesion kinase (FAK) have been associated with the β sub unit of integrins and in integrin-actin cytoskeleton interactions (Aplin et al., 1998; Burridge and Chrzanowska-Wodnicka, 1996b; Yamada and Geiger, 1997). Studies using truncation, mutation or domains swaps of the cytoplasmic domain of the β sub unit have revealed that it is necessary for integrin recruitment into focal

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adhesions and activation of FAK (Aplin et al., 1998; Burridge and Chrzanowska-Wodnicka, 1996b; LaFlamme et al., 1992; Reszka et al., 1992), cross talk between different integrins (Blystone et al., 1995), cell motility (Pasqualini and Hemler, 1994) and assembly of fibronectin fibrils (Wu et al., 1995). Mutations and truncations in the cytoplasmic of the α sub unit reveal that it strongly influences cell motility (Bauer et al., 1993; Chan et al., 1992). One model suggests that the role of the α sub unit is to inhibit the function of the β sub unit, which is actively involved in integrin binding (Burridge and Chrzanowska-Wodnicka, 1996b; Hughes et al., 1996). This model suggests that the α and β sub units are connected by a hinge and ligand binding serves to relieve this inhibition by swinging the hinge open.

The formation of signaling complexes depends on specific protein-protein binding motifs that act as molecular glue and hold the complexes together. These domains maintain the fidelity and efficiency of the signal generated by coupling of integrin receptors to their down stream targets. The Src homology domains (SH2 and SH3 domains) are two important domains that act as lock and key mechanisms in the assembly of the signaling complex (see key in Fig. 1.1). SH2 domains recognize and bind phosphotyrosine residues and SH3 domains recognize and bind the sequences Pro-X-X-Pro. A third type of protein domain, the PDZ domain (Fanning and Anderson, 1999), helps to organize small local protein complexes used for signal transduction. Proteins with PDZ domains can mediate complex formation because they can form homo- and heteromeric complexes with themselves and with other proteins with PDZ domains (Fanning and Anderson, 1999; Mandai et al., 1999). Subsequently, certain protein motifs

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are characteristic of adhesion molecules and serve as molecular adhesives in cluster formation.

Integrin occupancy is accompanied by the lateral diffusion of integrins into clusters that are associated with a cytoskeletal signaling complex and actin filaments. Studies have demonstrated that some integrins and signaling molecules associate with the protein caveolin-1 (Wei et al., 1999). Caveolin-1 is able to associate into oligomers and may enhance the ability of integrins to cluster at the plasma membrane. Investigations into the interaction between integrins and caveolin-1 have demonstrated that inhibiting caveolin-1 suppresses the formation of focal adhesions and integrin signaling (Wary et al., 1996; Wary et al., 1998; Wei et al., 1999). Nonetheless, the relative significance of integrin occupancy and clustering is yet to be resolved. Clustering may trigger a series of auto and transphosphorylation events that are believed to lead to the assembly of the signaling complex and the formation of stress fibers. Investigators have demonstrated that integrin clustering by non-inhibitory antibodies stimulates the localization of focal adhesion components, tensin and FAK but not talin, vinculin, α-actinin or actin (Burridge and Chrzanowska-Wodnicka, 1996b; Miyamoto et al., 1995). Recruitment of the former group of proteins is thought to be independent of tyrosine kinase activity. Subsequently, one model proposed in the literature is that integrin clustering increases tyrosine kinase activity and serves to activate important signaling pathways (Burridge and Chrzanowska-Wodnicka, 1996b). While it is difficult to resolve the role of ligand occupancy experimentally, it is thought that both clustering and occupancy are necessary to induce the assembly of the signaling complex at the cytoplasm.

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Integrin clustering leads to the phosphorylation and subsequently activation of focal adhesion kinase (FAK, Fig. 1.4), a phosphotyrosine kinase (Schaller et al., 1992). Subsequent to its activation by integrin-ECM interactions, FAK is recruited into nascent focal adhesions. Investigators have revealed that FAK interacts directly or indirectly with integrins (Chen et al., 1995; Lewis and Schwartz, 1995; Miyamoto et al., 1995). The amino terminus of FAK contains a binding site for the cytoplasmic domain of the β sub unit of integrins. FAK interacts indirectly with integrins through the adapter proteins paxillin and talin, which bind to the β sub unit cytoplasmic tail and the carboxy terminal domain of FAK. Investigators have demonstrated that FAK localization to integrins leads to its activation via three routes (reviewed by (Schwartz et al., 1995), Fig. 1.5). The first route is through Protein Kinase C (PKC) pathways, which in turn lead to the phosphorylation of FAK. The second route is through the activation of Phosphatidylinositol 4-phosphate-5 kinase (PIP5 kinase) and the GTPase Rho, which in turn phosphorylate FAK. The third route is through the clustering of integrins, which leads to the autophosphorylation of FAK associated with integrin tails. Autophosphorylation of FAK on residue is Tyr^{397} creates an SH2 domain that binds the protein Src (Schaller et al., 1994; Schlaepfer et al., 1994). Although FAK can form complexes with other signaling molecules, the association of the Src/FAK complex is vital because it is the basis for the formation of the signaling complexes associated with focal adhesions through protein-protein binding motifs. The Src/FAK complex is thus one of the key components in integrin mediated signaling events.

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While the exact role of FAK is yet to be elucidated, growing evidence suggests that FAK is important in cell migration but not cell spreading. For instance, FAK null fibroblasts have been observed to have normal morphology but decreased ability to migrate (Ilic et al., 1995). Moreover, over expression of FAK fails to alter cell spreading but increases cell migration (Cary et al., 1996; Gates et al., 1994; Romer et al., 1994). The Src binding site Tyr^{397} has been implicated in this role since a mutation at this site impairs cell migration.

FAK activates the MAPK signaling pathway, which is classically assigned to mitogenic factors (Assoian and Zhu, 1997; Roovers et al., 1999; Zhu and Assoian, 1995) (Fig. 1.6). Cell adhesion has an essential role in regulating proliferation during the first growth phase of the cell cycle (G1 phase), and loss of this adhesion requirement is a classic feature of oncogenic transformation (reviewed by (Jeon et al., 2000)). Zhu and Assoian demonstrated that integrin mediated MAPK activation is specific since soluble RGD peptides were able to block MAPK activation (Zhu and Assoian, 1995). Furthermore, no MAPK activation was detected on surfaces coated with the adhesive polymer poly-L-lysine. Integrin activation of MAPK was also found to differ from mitogenic activated MAPK in that it persisted longer. However, the level of MAPK activity, as determined by the degree of phosphorylation of the downstream target Extracellular Signal-regulated Kinase-2 (ERK-2), was less in integrin activated MAPK pathways than in mitogenic activated MAPK pathways.

In addition to initiating signaling pathways usually triggered by growth factors, integrins can co-operate with growth factors to mediate signaling pathways. This has

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been shown to occur through a number of ways. First, integrins can act synergistically with growth factors to control the intracellular environment. For instance, the activation of Na^+/H^+ antiporter in cells is more efficient when the cells are adherent as opposed to when they are in suspension (Ingber et al., 1990; McNamee et al., 1993; Schwartz et al., 1990) (Fig. 1.7). This is caused by low levels of phosphatidyl inositol 4,5,bisphosphate (PIP2), which is part of the signaling mechanism used by growth factors, in non-adherent cells. Subsequently, integrin signaling activates PIP5 kinase, which increases the production of PIP2. Another way integrins co-operate with growth factors is through physical association. Integrins have been found to associate with PDGF and EGF receptors (Kozlowski et al., 1997; Miyamoto et al., 1995). This enhances growth factor signaling through the clustering of growth factors or through the activation of signaling molecules associated with growth factors.

Vinculin is the most prominent cytoplasmic molecule associated with focal adhesions. On biomaterials, the presence of vinculin is used as a test for the materials cytocompatibility (Kooten et al., 1997; Richards et al., 2001; van Kooten and von Recum, 1999; Zhang et al., 2004). Vinculin binds to integrins through the adapter proteins paxillin or α-catenin (Burridge and Chrzanowska-Wodnicka, 1996b; Burridge and Mangeat, 1984; Geiger, 1979). Vinculin appears to be a key regulator of the cell-matrix adhesion since it is highly regulated structurally (Gilmore and Burridge, 1996; Huttelmaier et al., 1998; Johnson et al., 1998; Winkler et al., 1996). The structure of vinculin is composed of three domains (Fig. 1.8A): the head, neck and tail domain. Although the tail region of the molecule is moderately flexible, the neck confers a region

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of extreme flexibility to the molecule. As a result, vinculin can adopt a number of conformations. The head-to-tail conformation is the inactive conformation (Fig. 1.8B). The inhibition of vinculin is removed by its interaction with PIP2 or acidic phospholipids which cause a conformational change in vinculin that disrupts the head-to-tail association thereby exposing actin and other focal adhesion molecule binding sites. Therefore, the active form of vinculin facilitates the assembly of focal adhesions through the recruitment and cross-linking of various focal adhesion components.

Cytoskeleton

The cytoskeleton is a dynamic system of fibers in the cell that provides structural support and regulates a number of important cell behaviors such as motility, apoptosis, cell proliferation, distribution of mechanical stresses and organelle transport. Three types of cytoskeleton of filaments have been identified: actin filaments, intermediate filaments and microtubules. The characteristic diameters of these fibers are 7-9 nm, 10 nm and 24 nm respectively. The cytoskeleton is organized into bundles and networks by a number of cross-linking proteins.

Cell spreading and locomotion is dependent on the co-ordination of different parts of the cell in terms of adhesion and the polymerization and organization of the elements within the actin cytoskeleton. The cell membrane is attached to a cortical actin network by bivalent membrane-microfilament binding proteins. Other actin cytoskeleton structures identified in cells and thought to play a key role in spreading and motility are filopodia, the lamellipodium and membrane ruffles. The lamellipodium is a large flat membrane protrusion in the direction of motion. It consists of a network of actin

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filaments. Filopodia are thin finger like protrusions that extend from the cell membrane. They are transient in nature and are used for exploration of the environment and for adhering to the substrate. In some motile cells there are areas where adhesions are transient and these areas form a thin veil like structure known as ruffles. Also important are the aforementioned actin based structures known as stress fibers which are radially oriented actin bundles. Stress fibers are associated with clusters of adhesion molecules and their presence indicates activation of significant signaling pathways. The role of the cytoskeleton in cell motility in the past focused on the actin cytoskeleton gut growing evidence indicates that microtubules and intermediate filaments are important in the adhesion, spreading and motility.

Mechanotransduction in cells

Cells in their natural environments face mechanical forces; for instance, it has been known for some time that fluid shear play an important role in the health and biology of vascular tissue. Furthermore, the different characteristics exhibited by in vivo and in vitro biological systems that have a chemically equivalent environment have been attributed to the absence of physical stimuli present in the cells natural environment such as substrate topography, rigidity and dimensionality. This is indicative of a significant role played by mechanical forces in vivo.

Mechanotransduction is the process by which physical forces are converted to biochemical signals and integrated into a cellular response. Two components are essential for mechanotransduction to occur: the element or structure that is altered by the applied force and the elements that transmit the force to the ultimate target which may be a

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transcription site or a cytoplasmic protein that remodels the cytoskeleton (Janmey and Weitz, 2004). Furthermore, two models of the cytoskeleton have been proposed to describe the stress distribution in cells. The first is the continuum model, which ignores the microstructure details, and therefore stresses and strains are averaged over distances much greater than the characteristic dimension of the microstructure. This method is useful because it uses more established methods of analysis to investigate the stress distribution of cells. The second model takes into account the microstructure of the cell. It is thus more realistic but more complex than the continuum model. The tensegrity model of the cytoskeleton (Ingber, 1993a), is a microstructural model, that treats the cell as a prestressed tensegrity structure and takes into account the physical properties of the elements of the cytoskeleton. Actin filaments are elastic in nature (Gardel et al., 2004b; Liu and Pollack, 2002a; Mogilner and Oster, 2003a) and tend to pull the cell membrane towards the nucleus. Microtubules which are compressive in nature (Kurachi et al., ; Stamenovic et al., 2002a) and cell-substrate contacts (Maniotis et al., 1997a; Wang et al., 2001b) balance the pull of the actin cytoskeleton. Intermediate filaments connect the actin cytoskeleton to the microtubules (reviewed by (Chang and Goldman, 2004b)) and their elastic nature contributes to the pull generated by actin filaments (Fudge et al., 2003a; Haga et al., 2000; Yamada et al., 2003a). According to the tensegrity model, all these elements are hard wired together and subsequently an applied force on the cell results in an overall reorganization of the cell as it resists distortion.

The process of mechanosensing, within the scope of the tensegrity model of the cytoskeleton, has yet to be elucidated. Nonetheless, Janmey and Weitz in their review

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