MEASURING PATTERNS, REGULATION, AND BIOLOGIC CONSEQUENCES OF CELLULAR TRACTION FORCES
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ABSTRACT

The exchange of mechanical signals between mammalian cells and their extracellular matrix microenvironments is a focus of keen interest for biologists in the diverse fields of development, vascular disease, tissue engineering, and oncology. The molecular machinery of cellular mechanical signal response includes at least three major components: transmembrane adhesion receptors for extracellular matrix; the microfilament and microtubule cytoskeletal systems; and the Rho family of GTP-binding proteins that modulate the density and contraction state of cytoskeletal elements. Complex signaling pathways link these three arms of mechanical signal response as they interact with the extracellular matrix microenvironment. Arrays of microfabricated silicon posts have provided a system for reporting the traction force at individual cell-matrix interaction sites. Volumetric imaging of posts with known spring constants allows the efficient analysis of maps of cell traction force vectors and their coincidence with both actin polymerization and signal molecule localization. The effects of cytoskeletal contraction on patterns of cell traction forces are reported here. We have also defined unique patterns of force generation that are specific for cells of fibroblast, endothelial, epithelial, and smooth muscle lineages. Cell-generated mechanical forces directly affect patterning of the matrix milieu and the mechanical cues that are stored in it. The contributions of signaling molecules including non-receptor tyrosine kinases and GTPases to the exchange of mechanical data between the cell and its environment are discussed. These investigations have important implications for understanding and optimizing cellular growth behaviors during tissue development and repair in normal and microgravity settings.

INTRODUCTION

Restrictions in cell size, shape, and volume are dictated by the spatial array and stiffness of the immediate microenvironment. These material properties thereby become mechanical input signals. The far-reaching consequences of these signals include the most basic determinations in the life of the cell and its organism. Protein synthesis may be completely suppressed in mouse fibroblasts by preventing cell adhesion (Ben-Ze'ev et al., 1980), and apoptosis is induced in endothelial cells whose cell attachment area is restricted to less than that of a 25 \mu m microbead (Chen et al., 1997). Human mesenchymal stem cell lineage commitment was limited to adipocyte differentiation by spatial restriction to surface areas of 1-2 x 10^2 \mu m^2 and was consistently altered to an osteogenesis program by the availability of a more generous attachment surface area of 1 x 10^4 \mu m^2 (McBeath et al., 2004). More complex developmental schemata apparently follow similar imperatives. Thus, \textit{Xenopus} notochord development is accomplished through patterns of cellular growth that optimize the balance between flexural stiffness and isometric force (Keller, 2006), and precise focusing of compressive and tensile forces may activate transcription factors, thereby marking localization signals for cartilage and bone formation during human craniofacial morphogenesis (Radlanski and Renz, 2006).

A number of recent studies have highlighted the significance of mechanical signaling in physiologic and pathologic events. Paszek et al. have demonstrated that cancerous tumors have a stiffer stroma and elevated Rho-dependent contractility (Paszek et al., 2005). They demonstrated that increasing substrate stiffness led to increased ERK activity, increased Rho activity, and increase focal adhesion formation, all of which contributed to a malignant phenotype. In addition, transformed cells reverted to a non-transformed phenotype upon Rho-Kinase or ERK inhibition. Baneyx et al. investigated the effects of traction forces on the matrix protein fibronectin, and have shown in vitro that cell traction forces are capable of stretching this protein (Baneyx et al., 2002). They have constructed a model in which excessive stretching of fibronectin by cell traction forces alters integrin-dependent angiogenic signals which originate from integrin-fibronectin ligation (Vogel and Baneyx, 2003).

Altered gravity (either microgravity or hypergravity) can potentially affect cell traction forces and mechanical signaling in different ways. First, it has been demonstrated that focal adhesions respond to applied forces, be it either internally applied via the cytoskeleton or externally applied by an outside force (Riveline et al., 2001). The combined mass of the cell and the attached extracellular matrix will exert a force at these adhesion sites that is dependent on the degree of gravitational pull and the orientation of the tissue. Second, it has been shown that the cytoskeletal architecture, which transmits cellular force to focal adhesions, is altered in response to gravity. Studies in primary osteoblasts have shown that microtubule network height is decreased in a dose-dependent manner to hyper-gravity (Searby et al., 2005). Rosner et al. showed that neuroblastoma cells had increased prosorative lamellae activity when subjected to...
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Figure 1 - Reciprocal exchange of mechanical data.
A: Mouse embryo fibroblasts were grown on the vulcanized surfaces of phenylmethyl-siloxane puddles that were prepared in Rappaport chambers as described in the Methods. Phase contrast microscopy at three hours revealed wrinkles in this thin silicon membrane.
B: Mouse embryo fibroblasts were cultivated on fibronectin (25 µg/ml) adsorbed to glass coverslips or poly-dimethylsiloxane with a base to curing agent ratio of 30 to 1 (“30:1 PDMS”), and processed for epifluorescence imaging of actin and vinculin. Surfaces were UV-oxidized prior to plating cells. Merged images show actin in red and vinculin in green. The stiffer glass surface enhances actin organization (see detail in the insets in the top two panels) and matrix adhesion formation. Scale bars = 20 µm.

Despite its profound effects, mechanical data exchange across the cell membrane is readily accessible with straightforward experiments. Mechanical force is transmitted and sensed at cell-matrix attachment sites. Cytoskeletal traction forces that are transmitted to the cell surroundings can be visualized as wrinkle patterns when cells are plated onto a deformable silicon sheet (Figure 1 A) (see also (Harris et al., 1980)). The size, number, and geometric complexity of these wrinkles is directed in part by the force of cytoskeletal contraction, and this relationship may change with adhesion maturation and from cell type to cell type (Beningo et al., 2001; Pletjushkina et al., 2001). Cells can also sense and respond to mechanical signals. Organization of the F-actin cytoskeleton and formation of focal adhesions in mammalian fibroblasts are significantly altered depending on the stiffness of the underlying substrate. Cells growing on a stiff glass surface, which has a Young’s Modulus of approximately 70 GPa, have well-developed vinculin-containing focal adhesions after only one hour of spreading, particularly at the cell periphery, and densely packed peripheral arrays of F-actin filaments (Figure 1 B). In contrast, cells adherent for one hour to soft polydimethyl-siloxane with a base to curing agent ratio of 30 to 1 (30:1 PDMS) and a Young’s Modulus of approximately 1 MPa (Carrillo et al., 2005), lack well-developed focal adhesions. F-actin fibers in the cells growing on the softer substrate are thinner and more uniformly distributed across the cell. The distinct patterns of F-actin organization that are manifested in these two very different mechanical environments are shown in detail in the insets at the upper right corners of the two top panels in Figure 1 B.

Quantification of forces on deformable substrates has been achieved by embedding fluorescent beads within the substrate and tracking bead location before and after contraction (Dembo and Wang, 1999). The displacement of each bead within the substrate was used to determine the traction forces applied to the substrate. This system was used to demonstrate that the magnitude and direction of force applied to an individual adhesion correlated with the direction and size of the adhesion and estimated the force applied to an individual adhesion to be approximately 5 nN/µm² (Balaban et al., 2001). The continuous nature of the substrate in this system makes force quantification computationally complex; the calculation of forces from the displacement matrix cannot be solved without making certain assumptions.

hypergravity (2 g) (Rosner et al., 2006). Finally, studies have shown that gene expression is altered in response to altered gravitational pull. Shimada et al. showed that expression of heat shock protein 70 was increased under both microgravity and hypergravity (Shimada and Moorman, 2006). Thus it is possible that downstream signaling pathway activity may be altered by specific changes in protein expression.
Figure 2 - Measuring cell traction force.
A and B: A mouse embryo fibroblast is shown on an mPAD array. Actin (labeled with phalloidin) appears red, antibody-labeled fibronectin is blue, and fluorophore-conjugated BSA is green. 3D reconstructions of z-stacks of images are shown following iterative deconvolution in top (A) and bottom (B) views. mPAD posts are 10 µm high and 3µm in diameter with a post-to-post spacing of 9µm.
C: Surrounding each post with fluorophore signal allows independent imaging of both the anchored (red) and free (blue) ends of each cantilever post. Superposition of these images provides a measurement of centroid displacement (white arrows).

Calculation of forces can be more directly measured using the microfabricated post-array detector (mPAD) system: this assay consists of a substrate of uniformly-spaced, deformable cantilevers, which deflect when cell traction forces are applied (Tan et al., 2003; Lemmon et al., 2005; Li et al., 2007). Cell traction force applied to an individual mPAD post is linearly proportional to the deflection and stiffness of that cantilever post. The design and surface chemistry of the mPAD limits cell attachment to distinct and discrete locations, which simplifies the analysis of traction forces. Thus, each cantilever acts as an individual mechanosensor for cell traction forces. Fluorescent-labeling of the mPAD post with fluorophore-conjugated BSA facilitates volumetric imaging of the entire post surface (Figure 2 A and B, green). Microcontact-printing of fibronectin onto the mPAD free ends (Figure 2 A, blue) limits cell attachment to the top surface. Deflections are calculated by analysis of the BSA fluorescence images at the anchored and free ends of the mPAD posts (Figure 2 C). Superposition of the free end (blue) and anchored end (red) images provides a measurement of the centroid displacement (white arrows).

A key element in the generation of cellular traction force is the activation of actomyosin contraction by myosin light chain phosphorylation (Tan et al., 2003) (also reviewed in (Romer et al., 2006)). This process is regulated by the GTP-binding protein Rho, and its target effector proteins Rho kinase (ROCK) and mDia-1 (Ridley and Hall, 1992; Nakano et al., 1999; Watanabe et al., 1999; Wozniak et al., 2003). ROCK increases the strength of actomyosin contraction by phosphorylating the myosin light chain, and maintains this increased contractility by inhibiting myosin light chain phosphatase. mDia1 then supports the incorporation of contractile filaments of F-actin and type II myosin into stress fibers – the organelles that exert traction force on the cytoplasmic face of cell-matrix adhesions. It is intriguing to note that complex cellular responses to mechanical stimuli, including the molecular rearrangements of matrix adhesions upon exposure to external centripetal pulling force and the lineage commitment of mesenchymal stem cells based upon cell adhesion surface area, are dependent upon mechanical and chemical signaling through mDia-1 and ROCK, respectively (Riveline et al., 2001; McBeath et al., 2004). Molecules that regulate the activation state of Rho family GTP-binding proteins provide an additional level of modulation of cytoskeletal organization, contraction, and contribution to cell traction forces. These are the guanine nucleotide exchange factors (GEFs) that activate Rho by facilitating the exchange of GDP for GTP, and GTPase activating proteins (GAPs) that inactivate Rho family members by accelerating their otherwise very slow GTPase activity. Both GEFs and GAPs are responsive to mechanical cues in the extracellular environment (Arthur et al., 2000; Arthur and Burridge, 2001; Russell et al., 2002; Hornstein et al., 2004; Tcherkezian and Lamarche-Vane, 2007).

In this communication a technique for quantitative study of cell traction forces at discrete cell-matrix interaction sites is described. Data on changing patterns of cell traction force due to cytoskeletal contraction and biological diversity are reported. The molecular basis of mechanical signal response at cell-matrix adhesion sites is then reviewed, and we propose a biophysical model for cellular mechanosensing. It is hoped that this presentation will further new interest and cross-disciplinary collaborations in the investigation of mechanical signaling.

METHODS

Cell Culture
Mouse embryo fibroblasts (MEF) and Swiss 3T3 cells (both obtained from ATCC, Rockville, MD) were cultivated in DMEM with 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVEC; VEC Technologies, Rochester, NY) were cultivated in standard medium from the same source. MCF10a cells (ATCC) were cultivated in DMEM with 5% horse serum as described previously (Lemmon et al., 2005). Bovine
aortic smooth muscle cells (SMC; gift from Donald Ingber, Harvard) were cultivated in DMEM with 10% calf serum.

**Extracellular Deformation by Cytoskeletal Tension**

To evaluate the deformation of extracellular matrix by cytoskeletal contraction (Figure 1), Rappaport chambers designed for the stage of an inverted microscope were assembled with modifications of published specifications (Burton et al., 1999). A glass ring 1 cm in height and 2.2 cm in outer diameter was attached to a number 0 cover glass (22 x 22 mm, Thomas) with paraffin, petroleum jelly, and Lanolin (1:1:1). The glass ring was heated to 60-70°C, and then used to melt the mountant and make a seal on the cover glass. Phenylmethyl siloxane (#710, viscosity = 500 centistokes; Dow Corning, Midland, MI) was spread evenly on the bottom of the chamber and crosslinked in a standard sputter coater (Polaron E5100) with a target to specimen distance of 60 mm. The pressure was reduced to 150 millitorr, the air was replaced with argon, and the top layer of the siloxane was vulcanized using gold palladium and a current of 20 mA for 16 sec. The silicon rubber was exposed to UV light for 4 min to uncouple the cross-linking of phenyl side chains and increase the deformability of the silicon rubber surface. 3 hours after plating on this surface, cells were viewed with a 60x phase contrast objective using the Nikon Eclipse TE200 microscope and the image capture apparatus detailed above.

**Microfabricated Post Array Detector (mPAD) Fabrication and Preparation**

mPADs were fabricated using previously described techniques (Tan et al., 2003). Briefly, an array of 3-micron diameter, 10-micron tall posts with a 9-micron by 9-micron grid spacing was fabricated by spin-coating a positive resist (SU-8) onto a silicon wafer and exposing the wafer to UV light to polymerize the SU-8. Polydimethylsiloxane (PDMS) (Dow Corning, Midland, MI) was then poured over the silicon wafer and cured overnight at 100°C. The silicon wafer was peeled away from the PDMS, leaving a negative image of the post array. The negative mold was then treated for 1 minute in oxygen plasma and coated with [tridecafluoro-1,1,2,2,-tetrahydrooctyl]-1-trichlorosilane vapor (United Chemical Technologies, Bristol, PA) under vacuum overnight to aid in subsequent removal of mPAD substrates. PDMS was subsequently poured into the negative mold, cured for 20 h at 100°C, and peeled away from the negative mold, generating a positive feature mPAD substrate.

Prior to cell plating, mPADs were microcontact-printed and prepared as previously described (Tan et al., 2003; Lemmon et al., 2005). Briefly, PDMS stamps with a base to curing agent ratio of 30 to 1 were coated with 50 µg/ml human plasma fibronectin in PBS (Gibco) and incubated for 1 h at room temperature. Stamps were then rinsed, dried, and brought into contact with mPAD substrates that had been UV-oxidized for 7 min. Stamps were peeled away from the mPAD, leaving fibronectin on the top surface of each mPAD post. mPADs were subsequently incubated in 2 µg/ml cy5-conjugated BSA at room temperature for 1 h to facilitate mPAD post volumetric imaging, rinsed in de-ionized water, and incubated in 0.2% Pluronics F-127 to prevent cell attachment to surfaces other than the top surface of the mPAD posts.

**Immunofluorescence labeling, imaging, and analysis**

Cells plated onto mPADs were fixed and permeabilized with 3% paraformaldehyde and 0.5 % Triton X-100 in PBS. Fixed samples were labeled for immunofluorescence imaging by incubation with fluorophore-conjugated phalloidin and polyclonal antisera against fibronectin (Abcam, Cambridge, MA) for 30 min at 37°C, and then incubated in fluorophore-conjugated, isotype-specific and affinity cross-adsorbed anti-IgG antibodies (Chemicon, Temecula, CA) for 30 min at 37°C. Vinculin was labeled in some cells using a monoclonal antibody (gift of Alexey Belkin, University of Maryland). Cells were viewed with a Nikon Eclipse TE200 microscope and an internally cooled 12-bit CCD camera (CoolSnapHQ, Photometrics, Tucson, AZ). Images were collected using OpenLab software (Improvision, Lexington, MA)

Acquired images were exported as 16-bit TIFF images and read into an original Matlab code designed to analyze mPAD post deflections, as previously described (Lemmon et al., 2005). Briefly, acquired images were imported, and a thresholding algorithm was used to determine cell area, detect cell edges, and define mPAD post centroids. Deflections were then calculated and a vector map of the resulting cell-generated forces was generated. This original Matlab code is available for downloading free of charge at: www.hopkinsmedicine.org/anesthesiology/research/mpad tools.

**Computational Analysis of Cell Strain**

Cell strain was calculated using finite element analysis. A grid of rectangular elements was created using the cell outline, as determined from immunofluorescence of the F-actin cytoskeleton. The shape and position of each element in the model was determined from the measured mPAD deflection maps: the strained position and shape of each element was obtained from the deflected mPAD post centroid grid, while the unstrained position and shape of each element was derived from the undeflected mPAD post centroid grid. Normal strains and shearing strain were calculated for each element, and maximum strain was calculated using Mohr’s Circle equation for 2-dimensional plane strain:

\[ \gamma_{\text{max}} = \sqrt{(\varepsilon_x - \varepsilon_y)^2 + \gamma_{xy}^2} \]

where \( \gamma_{\text{max}} \) is the maximum strain, \( \varepsilon_x \) and \( \varepsilon_y \) are the normal strains along each axis, and \( \gamma_{xy} \) is the shear strain. Element size was approximately 0.25 µm².
Figure 3 - Cytoskeletal contraction changes the magnitude and pattern of cell traction forces.

The addition of serum for one hour (data on left) to Swiss 3T3 cells that had been serum-starved for 22 hours induced rapid actin polymerization and actomyosin contraction as compared to controls without serum (data on right). Epifluorescence imaging of F-actin (A), mPAD analysis of cell traction force vectors (B), and strain maps (C) were analyzed; representative data are shown for each group. The blue arrows denote a 10 nN vector for each traction force map shown. Axis labels in C are in pixels, and maximal strain is plotted from -0.1 (tensile strain, blue) to 0.1 (compressive strain, red).

D: Total exterior force (defined as mPAD posts which are contacted by the cell outline) is summed for both the serum and no serum cases. The ratio of the average exterior force per mPAD post to the average interior force per mPAD post are shown for each condition (right).
RESULTS

Force Distribution Patterns within Swiss 3T3 Fibroblast Cells

Serum-starvation of cultured cells leads to decreased actin stress fiber formation and focal adhesion disassembly, and re-exposure of these cells to serum reverses these effects in a Rho-dependent fashion (Giuliano and Taylor, 1990; Ridley and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1994). We wished to study the alterations in patterns of cell traction forces caused by this serum-dependent cytoskeletal remodeling. Swiss 3T3 fibroblasts were plated onto mPADs in the presence of serum for 2h to allow sufficient spreading and attachment to the mPADs. Cells were then rinsed twice with serum-free DMEM, and incubated for an additional 22h in serum-free conditions. Cells were then either stimulated by addition of serum for 1h, or left in serum-free conditions for 1h. As shown in Figure 3A, serum-starved cells showed a predicted decrease in F-actin fibers while cells stimulated with serum developed dense F-actin fibers that crossed the cell. Additionally, serum-activated cells exhibited increased traction forces at the cell periphery compared to the control group (Figure 3B). Serum-starved cells also spread better than serum-activated cells (Giuliano and Taylor, 1990; Ridley and Hall, 1992; Chrzanowska-Wodnicka and Burridge, 1994). It is possible that the decreased traction forces at the periphery allow for more efficient spreading in these cells.

Finite element modeling was used to predict areas of high and low strain within the cell. Two grids of rectangular elements were generated based on mPAD deflection data: a grid of elements was generated based on the mPAD post base image, which represented the undeflected position of mPAD posts, and thus the relaxed element state of the model. A second grid of elements was generated based on the mPAD post free-end image, which represented the deflected position of mPAD posts, and thus the contracted element state of the model. The x-, y-, and maximum strains were then computed based on the shape change of each element from the relaxed to the contracted state. Maximum strains were plotted for serum-activated cells as well as control, unstimulated cells (Figure 3C). Maximal strain was plotted from -0.1 (tensile strain, blue) to 0.1 (compressive strain, red). Strain maps indicated that serum-activated cells had high levels of peripherally localized tensile strain, with reduced strain within the interior of the cell. In contrast, unstimulated cells showed a more uniform distribution of moderate compressive and tensile strain throughout the cell. Bar graphs in Figure 3D demonstrate the spatial segregation of cell traction forces seen in the serum-stimulated Swiss 3T3 fibroblasts. Traction forces were separated into two categories: “exterior” forces, which are applied by the cell at the cell periphery; and “interior” forces, which are applied by the cell beneath the perinuclear regions of the cell. The average exterior force per post in serum-stimulated cells was 10.44 +/- 0.76 nN (385 posts in 13 cells), while unstimulated cells had a significantly lower average exterior force per post of 7.99 +/-0.45 nN (296 posts in 10 cells) (p= 0.037). This spatial segregation of forces can also be seen in the ratio of average exterior force per mPAD post to average interior force per mPAD post. Serum-stimulated cells had a ratio of 1.74 +/- 0.14 (13 cells), and unstimulated cells had a ratio of 1.10 +/- 0.07 (10 cells) (p = 0.005). Thus, the ratio of exterior to interior traction force was nearly doubled by serum exposure.

Biological Diversity in Cell Traction Force

Four different cell types were plated onto microfabricated post array detectors (mPADs) to quantify differences in traction force and cell spreading between cells of different lineages as shown in Figure 4. MEF, HUVEC, SMC, and
MCF10a were plated onto mPADs for 24h in serum-containing media under standard culture conditions. Results indicated that SMC cells generated the strongest traction forces, HUVEC generated the weakest traction forces, and MEF and MCF10a cells generated similar moderate traction forces. Cell size was also quantified for each cell type (Figure 4B). Results indicated that HUVEC spread more efficiently than the other cell types, while MCF10a spread poorly. Interestingly, an inverse relationship seems to exist between the normalized traction force per post and cell size (Figure 4B). Cells with lower normalized traction forces, such as the HUVEC, have increased cell size, while cells with higher normalized forces, such as the SMC, are smaller in size. The average force per post for the SMC was higher than for any of the other cell types studied, including the smaller MCF10a cells.

**DISCUSSION**

Cells generate, sense, and respond to mechanical signals (also reviewed in Bershadsky, 2004; Bershadsky et al., 2006; Giannone and Sheetz, 2006; Romer et al., 2006; Vogel and Sheetz, 2006). Our data elucidate a new perspective on cellular mechanical responses – mapping cell traction forces to discrete points of cell-matrix contact. Focal adhesions are formed at points of cell attachment and act as a clutch between the actin cytoskeleton and the surrounding extracellular matrix; they are thought to act not only as the point of transmission of cytoskeletal tension to the surroundings, but also as the sensor capable of detecting mechanical stimulus. Mechanical stimuli sensed at focal adhesions can be internal, as in the application of cytoskeletal tension, or external, as in the application of environmental forces. Signaling proteins associated with focal adhesions transduce mechanical signals into chemical signals; these chemical signals drive downstream pathways that regulate cell functions including migration, apoptosis, and cell cycle progression. Although the transduction of mechanical signal into chemical signal is thought to be governed by proteins within transmembrane focal adhesions as shown in Figure 5A, the actual mechanical stimulus sensed by the focal adhesion proteins is modulated by surrounding elements of the system, including the organization and density of F-actin filaments, the density and rigidity of extracellular components, and the size and distribution of the focal adhesions.

We therefore propose the “3 spring” model depicted in Figure 5B for the biophysical performance of mechanical signal transduction complexes at sites of cell adhesion to extracellular matrix. The cytoskeleton, adhesion complex and matrix are the 3 component parts of this complex. Each is conceptualized as a spring that may be compressed or stretched by contiguous forces, and yet may reconstitute its initial shape when these extrinsic forces are removed. The “k” values in the figure denote independent and individual stiffness coefficients for each of the components. The model is configured with the 3 springs in series to indicate that differential stiffness values have a direct impact on neighboring parts of the compound spring: Increased stiffness in any single component will increase the deformation of the other components. Thus, stretch is enhanced in any one of the triumvirate by either decreasing its own stiffness or by increasing that of its neighbors. In other words, prestress or active pulling force applied at either the cytoskeletal or extracellular environmental ends of the compound structure will result in a net change in the shape of any given component that is dependent upon the relative stiffness of each member of the tripartite spring.
An exciting challenge to the student of mechanical signal transduction is the paucity of detail currently known about the molecular mechanisms that drive it, despite rapid expansion in the field. Here follows a discussion of candidate mechanosensors in each of the 3 components of the “3 spring” complex (please also see Table I). To begin, GEFs regulate Rho family activation states in the cytoskeletal portion of this complex. One GEF that is an attractive candidate mechanosensor molecule is the ~720 kDa, multidomain protein obscurin. Ankyrin-like repeats that could tie activation of its Dbll homology domain to membrane deformation and subcellular localization to sarcomeric z-lines in developing cardiac myocytes suggest that it may regulate tension, growth and development in the heart in response to mechanical stimuli (Russell et al., 2002; Borisov et al., 2003). Another protein that may sculpt the cytoskeleton in response to extracellular mechanics including plasma membrane stretch is the Rho effector mDia-1 (Riveline et al., 2001; Kozlov and Bershadsky, 2004). This form is a processive capper of growing actin filaments that may act as a molecular elastic bridge that translates the potential energy created by membrane stretch into an impetus for focused G-actin subunit addition to contiguous ends of F-actin.

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<thead>
<tr>
<th>Mechanical Subunits</th>
<th>Candidate Molecular Signals</th>
<th>Effector Systems</th>
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<tbody>
<tr>
<td>Cytoskeleton</td>
<td>RhoGEFs, Rho, Rac, ROCK, mDia1</td>
<td>actin polymerization &amp; crosslinking, actomyosin contraction, microtubule targeting</td>
</tr>
<tr>
<td>Adhesion Complex</td>
<td>integrins, Src, FAK, p130Cas</td>
<td>integrin engagement &amp; clustering, lateral mobility of membrane proteins, adhesion dynamics</td>
</tr>
<tr>
<td>Extracellular Matrix</td>
<td>fibronectin, laminin, collagen</td>
<td>fibrillogenesis, matrix density, matrix mechanics</td>
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Many studies have led to a focus upon multi-phosphocomponent signaling complexes at focal adhesions as potential substrates for mechanical signal response (Giannoni and Sheetz, 2006). Assembly of these complexes is enhanced by tyrosine phosphorylation on scaffolding units that associate with SH2 domains of signaling proteins including additional kinases (see Romer et al., 2006 for review). The cytoplasmic non-receptor tyrosine kinase FAK is also a substrate for Src, and is one of these phosphocomponent signaling proteins and has been implicated as a director of cell motility in response to environmental mechanics (Li et al., 2002). p130Cas is another Src substrate and has been the target of elegant analysis of its mechanical signaling potential (Sawada et al., 2006). The central substrate domain, positioned between the amino-terminal SH3 domain and the carboxy-terminal Src-binding domain, was shown to be specifically tyrosine-phosphorylated by Src family kinases and Abl in response to stretching to ~110% of baseline cell-matrix interface. This stretch-activated phosphorylation was not due to changes in the activities of the kinases studied, but reflected enhanced substrate susceptibility. This mechanically triggered molecular event was then followed by the activation of the Rap1 GTPase, indicating the next step in signal propagation.

A third focal adhesion component with remarkable responses to mechanical stimuli is zyxin. This LIM domain protein interacts with p130 Cas, and has been shown to mobilize from focal adhesions and enhance fibroblast stress fiber alignment after 15 minutes of cyclic stress (Yoshigi et al., 2005). Further, reduction in cytoskeletal tension also triggers alteration of baseline zyxin association with matrix adhesion sites in fibroblasts (Lele et al., 2006). In vascular smooth muscle cells, 30 minutes of cyclic stretch induced nuclear translocation of zyxin, where it was linked to the upregulation of endothelin B expression (Cattaruzza et al., 2004).

Transmembrane integrins respond to extracellular matrix ligand binding with long range allosteric rearrangements that increase ligand affinity. In addition, integrin clustering at sites of adhesion augments the adhesion strength or avidity (Hynes, 2002). Both of these mechanisms are apparently involved in the spatial rearrangement of new ligand formation and the downstream signaling through Shc that result from shear stress exposure in human endothelial cells (Jalali et al., 2001). These effects were abrogated by function-blocking antibodies, and this additional finding indicates an active role for matrix ligand presentation in the regulation of integrin function.

Studies on the third component of the “3 spring” model – the matrix – underline the active role that this partner has in mechanical signaling to the cytoskeleton and beyond. Fibronectin matrix density has been shown to modulate cytoskeletal tension. Thus, a five-fold increase in the density of fibronectin matrix presentation to bovine vascular smooth muscle cells (from .02 to .1 µg/cm2) induced an ~40% increment in myosin light chain phosphorylation and increased cytoskeletal prestress to 250% of levels measured at the lower matrix density (Polte et al., 2004).

The impact of Rho-modulated cytoskeletal contraction and subsequent cell traction force transmission on fibronectin fibrillogenesis provides a lucid application of the “3 spring” model. Transmembrane adhesion complexes transmit cell traction force to the fibronectin matrix causing unfolding of its complex quaternary structure and the exposure of fibronectin type III domain...
Loss of signal exchange between the donor and receptor unfolding by fluorescence resonance energy transfer. Repeats allowed direct measurement of this molecular magnitude. Based on this information and an estimate of adhesions can sense and respond to forces of this range of 1-5 nN per focal adhesion, and that focal mechanical traction from portions of the molecule that are dragged across the fibroblast surface to be incorporated into complex arrays that are essential mechanical templates for efficient wound repair or normal embryonic development (Kadler, 2004; Meshel et al., 2005).

Our data and those of others (Balaban et al., 2001; Lemmon et al., 2005) indicate that cells generate forces in the range of 1-5 nN per focal adhesion, and that focal adhesions can sense and respond to forces of this magnitude. Based on this information and an estimate of cell mass, we can predict whether or not an individual cell would sense a gravitational force resulting from its own mass. Assuming an average cell volume of 5000 µm³ and a cell density approximately equal to the density of water (1 g/ml), the mass of a cell would be approximately 5 ng. These assumptions are comparable to measured values of osteoblast cell size and density (Searby et al., 2005). This would result in a gravitational force of 50 pN. Assuming that this force is equally distributed across all focal adhesions, and that there is an average of 100 adhesions per cell, the force at each focal adhesion would be 0.5 pN. This would be 2000 times smaller than what could be sensed at an individual focal adhesion. However, based on this calculation, an inertial mass of approximately 5 µg applied over the surface of a cell could be sensed at individual focal adhesions – a value that is within range for mammalian cells subjected to normal locomotion (Lindemann, 2003).

In summary, mechanochemical coupling in cell-matrix adhesion involves 3 partners: the cytoskeleton, the transmembrane adhesion complex, and the extracellular matrix. The cytoskeletal contraction and details of cytoskeletal function that are cell-type specific regulate cell traction force. Finally, a dynamic reciprocal regulatory relationship exists between cell traction forces and the extracellular matrix microenvironment, and this may be modulated by inertial loads that are available in most multicellular organisms.

ACKNOWLEDGEMENTS
The authors thank Dr. Jack van Loon for the opportunity to learn from the ASGSB, the editors for their guidance, Dr. Elena Doutova for her work on the Rappaport chamber studies, and Dr. Fumin Chang for helpful discussions and careful reading of the manuscript. This work was supported by grants DE13079, HL058064, and AI061042 from the NIH, and the Funds for Medical Discovery from JHU to LR.

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